ENDOTHELIN IN THE HEALTHY AND ISCHAEMIC MYOCARDIUM

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ABSTRACT

The effects of intravenous infusion of endothelin-1 (ET-1) on the incidence and severity of arrhythmias induced by a period of myocardial ischaemia were assessed in anaesthetized rats. To determine the role of ET_A-receptor-mediated events during ischaemia the effects of the ET_A antagonist, BQ123, on the pro-arrhythmic actions of both exogenous and endogenous endothelin were examined. Infusion of ET-1 (0.05 and 0.1 $nmol.kg^{-1}.min^{-1}$) resulted in a significant increase in the total number of ventricular ectopic beats (VEBs), and in beats occurring as ventricular tachycardia (VT). There was a marked increase in the incidence of ventricular fibrillation (VF). Over a range of doses of BQ123 (5-100 $\mu g.kg^{-1}.min^{-1}$), only one dose (10 $\mu g.kg^{-1}.min^{-1}$) significantly attenuated the total number of VEBs by reducing all types of ischaemic arrhythmias. With the highest dose of BQ123, there was an increased incidence of irreversible VF. This may reflect the U-shaped dose-response curve which has been reported for the antagonism of the pressor effect of ET-1 by BQ123 (Bird et al., 1993). BQ123 (10 $\mu g.kg^{-1}.min^{-1}$) also significantly reduced the pro-arrhythmic effects of infused ET-1. These findings suggest that exogenous ET-1 can intensify ischaemia-induced arrhythmias through the activation of ET_A receptors. However, while endogenous ET-1 may make some contribution to the genesis of arrhythmias resulting from ischaemia by an action at ET_A receptors, the observed pro-arrhythmic effect of high doses of BQ123 suggests that this may unmask an effect of ET-1 at other receptors.

This study also examined the plasma and tissue concentrations of endothelin during coronary artery occlusion and reperfusion. Following 30 min of coronary artery occlusion and 30 min reperfusion the mean immunoreactive ET-1 (IR-ET-1) concentration in right atrial plasma was elevated 2.5-fold and 2.9-fold respectively (from 3.2 ± 0.5 $pg.ml^{-1}$ in sham-operated animals to 8.2 ± 0.9 and 9.1 ± 1.7 $pg.ml^{-1}$).

The myocardial tissue mean level of endothelin, unchanged during the ischaemia, increased from 11.2±1.5 $pg.g^{-1}$ tissue to 17.8±0.7 $pg.g^{-1}$ tissue only after 30 min reperfusion. Thus, it is unlikely that the ischaemic heart is the source of the endothelin released into the plasma. To evaluate the involvement of the myocardium in the conversion of big ET-1 to ET-1, the degradation of exogenous big ET-1 (the precursor of ET-1) in rat ventricular homogenates, was studied by using reversed phase HPLC and electrospray mass spectroscopy. Big ET-1 was degraded optimally at pH 7.2 and produced two detectable metabolites. Neither was mature ET-1. One of the fragments was ET-1 sequence 1-17, which was produced in only trace amounts by incubating ET-1 itself with the ventricular extract at pH 7.2. Thus, even if the myocardium produced endothelin, the level of myocardial ET-1 formation was so slow and small as to be unlikely to have a direct influence on circulating levels.

The present study also investigated the enzymic activity involved in ET-1 degradation by homogenized rat ventricle and compared it with that in the lung extract. ET-1 was degraded by both ventricular and lung extracts optimally at acid pH. Degradation of ET-1 by the lung and ventricular extracts at pH 5.2 produced respectively 4 and 2 detectable metabolite peaks on HPLC. The two ET-1 metabolites produced by ventricular extracts were identified as tryptophan and des-Trp-ET-1. The degrading activity was not inhibited by EDTA, aprotinin, pepstatin A or captopril. However, phosphoramidon and PMSF (methyl sulfonyl fluoride) produced significant inhibitions. These results indicate that myocardial degradation of ET-1 at pH 5.2 involves cleavage of the C-terminal tryptophan and that more than one enzyme may be involved.

CONTENTS

Chapter 1

INTRODUCTION

1 INTRODUCTION

1.1 History

Since 1980, when Furchgott and Zawadzki reported an endothelium-dependent vasodilation, vascular endothelium has been recognised as an essential unit in the regulation of vascular smooth muscle tone. It was suggested (Furchgott and Zawadzki, 1983) that acetylcholine- and bradykinin- induced vasodilation is caused by secretion of a short-lived endothelium-derived relaxing factor(s) (EDRF). Hickey et al (1985) attempted to bioassay EDRF by testing the biological activity of the culture medium obtained from bovine aortic endothelial cells on isolated pig coronary arteries. Instead of the expected vaso-relaxing effect of EDRF, the culture medium produced a slowly developing and long-lasting contraction of vascular smooth muscle. The contraction was not affected by inhibitors of cyclo-oxygenase and lipoxygenase or by antagonists to the α- or β-adrenoceptors, serotonin receptors, histamine receptors or cholinoceptors. However, treatment of the endothelial cell media with either sodium dodecyl sulfate, trypsin, alkali, or with acid hydrolysis completely abolished the vasoconstriction. The vasoconstricting substance was thus shown to be a peptide (Hickey et al., 1985). Thus the bioassay designed to identify EDRF led to the introduction of the peptide concept of an EDCF (endothelium derived constriction factor) or endotensin. Two years later EDRF was identified as nitric oxide or a closely related substance (Palmer et al., 1987) but the identity of EDCF was still unknown.

Masaki's group first isolated a potent vasoconstrictor peptide from the culture supernatant of porcine aortic endothelial cells (Yanagisawa et al., 1988a) and within a very short period determined its amino-acid sequence, and cloned the peptide precursor. They called the active peptide **Endothelin (ET)**. These novel 21-residue peptides, endothelins, did not belong to any previously known peptide family. Since

then a large amount of information has been generated about endothelins; three different isoforms of endothelin named ET-1, ET-2, ET-3 have been identified from the finding of three distinct human genes (Inoue et al., 1989). The endothelins were found to be very similar to the highly toxic sarafotoxins (STX), which are isolated from venom of the snake *atractaspis engaddensis* and which are potent agonists of endothelin receptors (Kloog et al., 1988). A putative enzyme (endothelin-converting enzyme) which proteolytically converts the ET-1 precursor, big ET-1, to mature ET-1 has been postulated (Yanagisawa and Masaki, 1989) and almost characterised. Two different ET-receptors (and possibly a third) have been cloned (Arai et al., 1990; Sakurai et al., 1990). Furthermore, selective and non-selective ET-receptor antagonists and endothelin-converting enzyme (ECE) inhibitors have been introduced. Several possible pharmacological and pathological roles of endothelins in animal and human tissues, in vivo and in vitro, have been studied, and several techniques have been developed to detect the ET levels in tissues and plasma, a large number of questions still remain to be answered.

1.2 Synthesis of Endothelins

Following the initial isolation of endothelin from culture media of porcine aortic endothelial cells, Yanagisawa et al (1988a), by cloning and sequencing porcine preproendothelin cDNA, showed that endothelin synthesis involved production firstly of a precursor of 203 amino acids, which was cleaved proteolytically at paired basic residues by specific endopeptidase(s) to produce the 39 amino acid intermediate big endothelin-1 (big ET-1). A cDNA encoding a human preproendothelin was also isolated from the human placenta cDNA library (Itoh et al., 1988). The human preproendothelin was shown to have 212 amino acid residues with high homology to porcine preproendothelin. In addition to the sequencing of both human and porcine cDNA, a preproendothelin-related gene from rats was also cloned and sequenced (Yanagisawa et al., 1988b). Further cloning and sequence analysis showed the

existence of three distinct genes in humans for the endothelin family (Inoue et al., 1989) and that one of these genes encode classical endothelin (ET-1). The other two genes encoded [Trp^6, Leu^7] endothelin (ET-2) and [Thr^2, Phe^4, Thr^5, Tyr^6, Lys^7, Tyr^{14}] endothelin (ET-3). These are similar to ET-1 but differ in the two and six amino acid positions, respectively. Inoue et al (1989) also showed three corresponding chromosomal loci in porcine and rat genomes. It is believed that many mammalian species produce three isopeptides from the endothelin family; these are identical with, or very similar to, human ET-1, ET-2 and ET-3. Porcine, dog and rat ET-1 is identical to human ET-1. However, unlike human big ET-1 which contains 38 amino acids, porcine and rat big ET-1 contain 39 amino acids (Yanagisawa et al., 1988a,b; Itoh et al., 1988).

The proposed proteolytic processing pathway for the conversion of preproendothelin to mature endothelin is illustrated in figure 1.1. Since the subsequent conversion of big ET-1 to mature ET-1 required an unusual cleavage between the Trp^{21}-Val^{22} peptide bond, it appeared that a novel "endothelin converting enzyme" (ECE) was responsible for this process. Big ET-2 and big ET-3 are probably cleaved by a different converting enzyme(s). The putative ECE could be present in one of three forms: a soluble intracellular enzyme; membrane-bound; or secreted and active outside the cells (Yanagisawa et al., 1988a). From the structural point of view the four cysteine residues of endothelins form two intra chain disulfide bonds (between positions 1-15 and 3-11). The molecular weight of ET-1 is estimated to be 2492 Da from the sequence data.

The vasoconstrictor activity of big ET-1 in rat pulmonary artery, and its ability to displace ET-1-receptor binding from cultured rat vascular smooth muscle cells, is at least 100-fold less than that of ET-1 (Hirata et al., 1990a). Therefore, ECE has attracted attention as a target for therapy. It is estimated to be two types of enzymes,

one of which is a neutral membrane-bound enzyme (Okada et al., 1990) and the other a cytosolic enzyme (Takada et al., 1991). They are metalloproteinases whose activities are inhibited by EDTA, o-phenanthroline and phosphoramidon but not by di-isopropylfluorophosphate (DFP) or pepstatin A and are most active at pH 6.6-7.6. However, some studies (Ikegawa et al., 1990b; Sawamura et al., 1990) have also demonstrated the conversion of big ET-1 to ET-1 at acid pH (3.5-4) by extracts or soluble fractions of endothelial cells. This conversion is inhibited by pepstatin A, an aspartic peptidase inhibitor, suggesting the involvement of a cathepsin D like enzyme in the processing of ET-1. This enzymic activity does not seem to be responsible for conversion of big ET-1 to ET-1, either in the circulation (Bird et al., 1992) or inside cells (Shield et al., 1991). The suggestion that phosphoramidon-sensitive ECE is the most relevant is supported by the finding that phosphoramidon decreases both the hypertensive responses to big ET-1 (but not to ET-1) in vivo (Matsumura et al., 1991; Bird et al., 1992) and the release of ET-1 from cultured endothelial cells (Ikegawa et al., 1990a; Shield et al., 1991). Shield et al (1991) demonstrated that when pepstatin accumulated in the intact cultured vascular endothelial cells to a concentration sufficient to inhibit aspartic acid proteases, it did not change the rate of secretion of ET-1 or big ET-1. However, phosphoramidon under identical conditions significantly reduced the secretion of ET-1 with a concurrent increase in the secretion of big ET-1. There are many similarities between the neutral endopeptidase 24.11 and ECE and it has therefore been suggested that the two enzymes are probably identical. However, some studies do not confirm this, since thiorphan, which is another inhibitor of neutral endopeptidase, had no effect on either the renal or cardiovascular response to big ET-1 in anaesthetised rats (Pollock and Opgenorth, 1991).

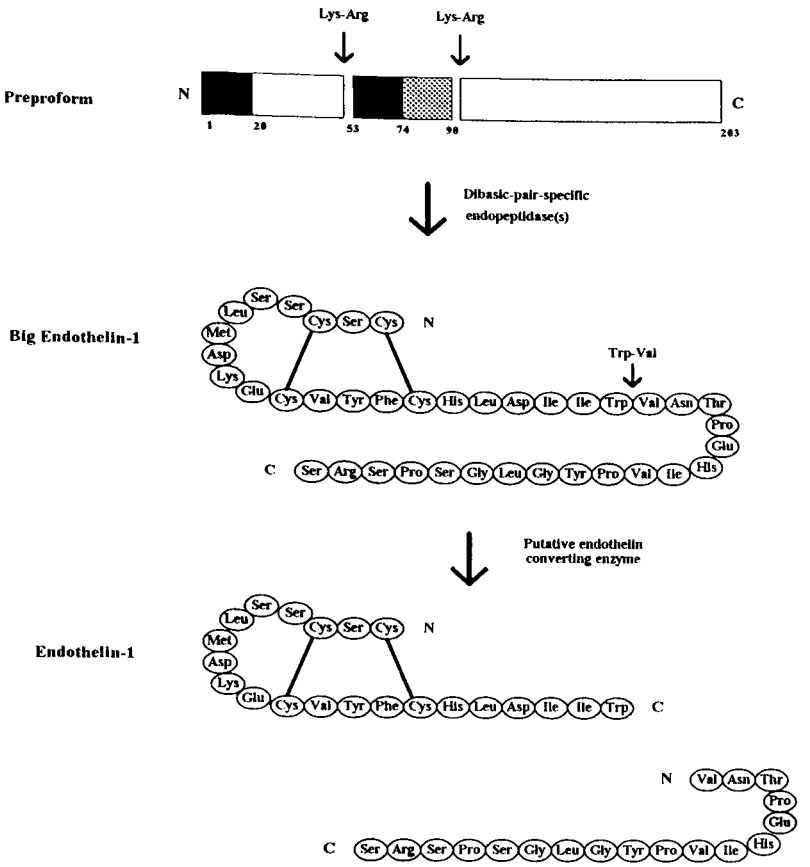


Figure 1.1 The proposed proteolytic process of formation of 39-amino-acid big endothelin-1 from preproendothelin-1 and further cleavage to mature endothelin-1 by endothelin converting enzyme (from Yanagisawa and Masaki, 1989).

1.3 Pharmacokinetics of Endothelin

The plasma concentrations of ET-1 are in the low picomolar range in various species. Botelho et al (1992) have reported results of 10.8, 0.1-7.5, 0.7-1.6, and 25 pM for pig, human, rat and dog respectively. In addition to circulating endothelin, immunoreactive endothelin has also been detected in human urine at levels (33 $pg.ml^{-1}$) that are significantly higher (about 6 times) than in plasma (5 $pg.ml^{-1}$) (Berbinschi and Ketelslegers, 1989). Endothelin has also been detected in cerebrospinal fluid (CSF) of patients with cerebrovascular disease (28 $pg.ml^{-1}$) which is much higher than in plasma (1.5 $pg.ml^{-1}$) (Hirata et al., 1990b). The study of Hirata

et al (1990b) suggested the possibility of secretion of ET-1 from neural tissue in human. Although endothelins are stable in blood and plasma (Pernow et al., 1989), pharmacokinetic studies show that intravenous bolus injection of ^{125}I-ET-1 is rapidly eliminated from the circulation with a half life of less than two minutes in rats (Shiba et al., 1989) and seven minutes in pigs (Pernow et al., 1989). In the anaesthetized rat more than 60% of ^{125}I-ET-1, injected into the left ventricle of the heart, was removed from the blood stream within the first minutes (Anggard et al., 1989) and the highest uptake of radioactivity was seen in lung, kidney and liver respectively. However, the mechanism of this clearance is not yet known. The rapid decay of endothelin could be due to degradation by peptidase, binding to receptors, or first pass elimination by organs. DeNucci et al (1988) demonstrated that about 60% of ET-1 infused through the pulmonary circulation of guinea pigs, in vitro, was removed by the lung after the first passage. In another study, following a bolus administration of labelled-ET-1 into the jugular vein, ^{125}I-ET-1 was taken up by the lung (82%) and to a lesser extent, by the kidneys (10%), heart (3.6%), liver (2.7%), and spleen (1.5%) in rats (Sirvio et al., 1990). The rapid clearance of ET-1 from the circulation may be mainly due to the trapping of the intact peptide by the organs. A local degradation by tissue peptidase could contribute to the elimination and inactivation of the peptide from the circulation. Several studies show that endothelin is degraded by two different types of enzyme, one that is a membrane bound metallo-endopeptidase and is active at physiological pH (Yamaguchi et al., 1992a; Vijaraghavan et al., 1990). The other is a soluble cytosolic enzyme which inactivates endothelin rapidly and effectively, optimally at acid pH, by removing the carboxyl terminal tryptophan (Deng et al., 1994; Jackman et al., 1993). Since a rapid and high degradation of endothelin has been shown by cytosolic enzymes, it appears that this mechanism of elimination is more dominant in pathophysiological conditions where the cytosolic enzymes are released. Another hypothesis could be that the clearance of ET-1 is achieved by binding to functional receptors. The extremely slow dissociation (with half time of 30

minutes to 40 hours) of ET-1 from its receptors (Frelin and Guedin, 1994) may at least partly account for the discrepancy between the time-course for elimination of the exogenously applied peptide from circulation and for the long lasting pressor effects of the peptide.

1.4 Endothelin Receptors

Following the cloning and identification of three isopeptides of endothelin (ET-1, ET-2, and ET-3; Inoue et al., 1989), endothelin receptor subtypes were characterized by the comparative activities of these agonists. Initially it was speculated that endothelin may be an endogenous dihydropyridine (DHP)-sensitive, voltage-dependent Ca^{2+} channel agonist, since ET-1-induced vasoconstriction was dependent on extracellular Ca^{2+} and decreased in the presence of the voltage-dependent Ca^{2+}-channel antagonist nicardipine (Yanagisawa et al., 1988a). To demonstrate that endothelin was not an endogenous agonist of the DHP-sensitive Ca^{2+} channels, Miyazaki et al (1989) showed that ^{125}I-ET-1 binding to chick heart cardiac membrane was not affected by Ca^{2+}-channel antagonists nicardipine, verapamil, and diltiazem, while unlabelled ET-1 inhibited the binding in a dose-dependent fashion. In another study by the same group (Miyazaki et al., 1990) using auto radiographic analysis of the binding of ^{125}I-ET-1 to chick cardiac membranes, the molecular weight of the binding protein (receptor) was calculated as 50 kDa. Soon after, many studies suggested that the various biological actions of endothelins could not be mediated by a single receptor subtype. Prior to the discovery of specific endothelin receptor antagonists, receptor subtypes were classified on the basis of rank order potencies of either binding or functions. Early studies of the effects of intravenous bolus injection of ET-1 into rats showed a biphasic systemic blood pressure response, comprising an initial transient depressor phase which was followed by a sustained pressor phase (Yanagisawa et al., 1988a). The structure-activity relationships of different ETs for these two phases were distinct. ET-1 was shown to have three times greater potency than ET-3 as a pressor

agent (Spokes et al., 1989). Similarly Warner et al (1989a) showed that ET-1 was 10 times more potent than ET-3 as a vasoconstrictor in the rat isolated perfused mesentery. Moreover, ET-3 was more selective than ET-1 as a vasodilator and also for the release of nitric oxide from vascular preparations (Warner et al., 1989b). Thus it appeared that the receptors mediating the constrictor effects of endothelins were selective for ET-1, while the vasodilator receptors were ET isotype-non selective. These data suggested the existence of multiple receptor subtypes for endothelin.

Binding studies indicated that the binding of ^{125}I-ET-1, ^{125}I-ET-2, and ^{125}I-ET-3 to chick cardiac membrane was competitively inhibited by increasing concentrations of unlabelled peptides (Watanabe et al., 1989a). The order of potency in displacing the binding of ^{125}I-labelled ET-1 and ET-2 was ET-1≥ET-2>ET-3>STX. In contrast, the order of potency in displacing ^{125}I-ET-3 binding was ET-3>ET-2≥ET-1>STX. These results indicate that there are at least two different types of endothelin receptors, one of which has high affinity for ET-1 and ET-2 and the other is ET-3 preferring. Similarly, data from further binding and pharmacological studies and development of novel endothelin receptor agonists and antagonists have confirmed the presence of these two types of endothelin receptor which have been named ET_A and ET_B (Masaki, 1991). ET_A receptors are distributed predominately on smooth muscle, are responsible for constrictor effects and have a high affinity for ET-1. The ET_B receptors, distributed mainly on endothelial cells, are responsible for nitric oxide release and has a equal affinity for ET-1 and ET-3 (Masaki, 1991). In addition to different ET receptor subtypes differing in their ligand selectivity, recent studies indicate that receptors with similar ligand selectivities but different ligand affinities, with high (kd=nM) or super high (kd=pM) affinities, may coexist within the same tissue (Sokolovsky et al., 1992). It is now clear that both ET_A- and ET_B-receptors can mediate vascular smooth muscle contraction (Harrison et al., 1992; Sumner et al., 1992; Clozel et al., 1992). There is also a suggestion that the ET_B receptor present on

the endothelium, which mediates the release of EDRF in response to the endothelins, is functionally different from the ET_B receptor mediating the vasoconstrictor effects of the endothelins (Warner et al., 1993a). In another study by the same group these two types of ET_B receptor were classified as ET_{B1} (present on the endothelium and mediate release of EDRF) and ET_{B2} (present on vascular and non-vascular smooth muscle and mediate contraction) (Warner et al., 1993b).

The existence of a third subtype of ET receptor (ET_C), with a rank order of affinity of ET-3>ET-1 (i.e. selective for ET-3), has also been proposed (Kloog and Sokolovsky, 1989). Indeed, detection of high concentrations of ET-3-like immunoreactivity at levels of ng per mg of protein in rat hypothalamus and pituitary gland extracts, but not ET-1, suggests the distribution of a third type of ET receptor (ET_C) in the neural tissue of rats (Samson et al., 1991). Emori et al (1990) showed that, in cultured bovine artery endothelial cells, ET-1 failed to displace ^{125}I-ET-3 from the cell surface or to evoke increased intracellular calcium, whereas ET-3 was effective in both cases. This suggested the presence of a specific receptor for ET-3. The study also suggested the presence of two distinct subclasses of ET-3 receptors with high and low affinities. Activation of low affinity receptors by ET-3 resulted in phosphoinositide breakdown and an increase in $[Ca^{2+}]i$.

The existence of different ET receptors was confirmed by the cloning and expression of cDNA encoding ET_A receptors (Arai et al., 1990) and ET_B receptors (Sakurai et al., 1990). Both receptors are expressed in lung, heart, brain and other tissues. The primary structures of these two receptors were cloned and characterized as single-chain peptides containing seven sequences of 20 to 27 hydrophobic amino acid residues, typical of G-protein-coupled receptors of the rhodopsin superfamily. The homology of the amino acid sequences of bovine ET_A and rat ET_B was 65%. The molecular weights of bovine ET_A and rat ET_B, calculated from their amino acid

sequences, are 48516 and 49317 Da respectively (Masaki and Yanagisawa, 1992). Recently a third ET receptor (probably ET_C) specific for ET-3, with 47 and 52% identity to ET_A and ET_B respectively, was cloned and characterized from *Xenopus Laevis* dermal melanophores (Karne et al., 1993).

1.5 Endothelin Receptor Antagonists

There is much evidence in support of a role for endothelin in cardiovascular, renal and cerebrovascular disease. Thus the generation of selective endothelin antagonists could be of benefit in these diseases. By now several endothelin receptor antagonists have been identified, some of which are endothelin or other peptide derivatives. For example, [Dpr^1, Asp^{15}]-ET-1 (where Dpr is diamino-propionic acid) is a full-length ET-1 derivative in which the outer disulfide is replaced by an amide bond and the inner one is left intact (Spinell et al., 1991), while [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-substance P is a non-endothelin-related peptide antagonist (Fabregat and Rozengurt, 1990). However many of these substances have not been studied extensively and are not very selective. Many of the currently available endothelin antagonists are natural products and/or library screening compounds.

The first ET antagonists were the cyclic pentapeptides, BE-18257A and BE-18257B, isolated from the fermentation products of *streptomyces misaukiensis* (Ihara et al., 1991) and their synthetic analogs, BQ-162, BQ-123 and BQ-153. These peptides bear a structural resemblance to the C-terminal sequence of endothelin. For instance, BQ-123 has the chemical structure of Cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-). BQ-123 is the most widely used of the endothelin antagonists. It is highly potent and selective for the ET_A-receptors. BQ-123 and BQ-153 have both been shown to antagonize contraction of the isolated porcine coronary artery induced by ET-1 and inhibit binding of ET-1 to endothelin receptors on vascular smooth muscle cells (Ihara et al., 1992). However, a small amount of the ET-1-induced vasoconstriction was

found to be resistant to these antagonists, parallel with an incomplete inhibition of labelled ET-1 binding to the aortic smooth muscle membrane. This suggested that the artery has both ET_A and ET_B receptors responsible for ET-1 induced vasoconstriction. Preliminary studies with BQ-123 and BQ-153 in conscious rats, showed a marked dose-dependent inhibition of the ET-1-induced sustained pressor effect, without affecting the ET-1-induced transient depressor action. This suggested that the pressor action of ET-1 was mediated by ET_A receptors, while the depressor effect was mediated by ET_B receptors (McMurdo et al., 1993a). However, further studies in rats in vivo have shown that BQ-123 is unable to block entirely the pressor effect of ET-1, again suggesting the presence of non-ET_A constrictor receptors within the rat circulation (Bigud et al., 1992). In addition to the cyclic pentapeptide natural product, a linear tripeptide, which is a potent and highly selective ET_A-receptor antagonist, FR 139317 was also developed (Aramori et al., 1993). FR 139317 was shown to inhibit ET_A mediated phosphatidyl inositol hydrolysis and arachidonic acid release in bovine ET_A expressing cells. A well characterized ET_B antagonist, IRL 1038, has been introduced (Urade et al., 1992). IRL 1038 has a structure of [Cys^{11}-Cys^{15}]-ET-1 (11-21), and has been shown to have a much higher affinity for ET_B receptors than for ET_A receptors. IRL 1038 antagonizes ET_B-mediated contraction of guinea pig ileal and tracheal smooth muscle induced by ET-3, but has no effect on the ET_A-mediated contraction of rat aortic smooth muscle.

Modification of the C-terminal hexapeptide fragment of ET-1, [ET(16-21)], led to identification of a new series of non-selective $ET_{A/B}$ receptor antagonists. ET(16-21) is known to interact with ET receptors. Cody et al (1992) reported the first functional $ET_{A/B}$ mixed antagonist in this series of agents. This mixed antagonist is called PD 142893 which in His^{16} of ET(16-21) is substituted with D-diphenylalanine (D-Dip-Leu-Asp-Ile-Ile-Trp). Substitution of His^{16} with D-Bhg (5H-dibenzo cycloheptene glycine) produced another ET non-selective $ET_{A/B}$ receptor antagonist

which was named PD 145065 (Doherty et al., 1993). In addition to completely inhibiting the pressor effects of ET-1, PD 142893 and PD 145065 also blocked the depressor actions of intravenously administrated ET-1 in rats (Cody et al., 1992; Doherty et al., 1993), whereas BQ-123 and FR 139317 did not affect this portion of the ET-1 response (Sogabe et al., 1993; McMurdo et al., 1993a). Therefore these experiments confirmed the hypothesis that, at least in rats, both ET_A and ET_B receptors mediate the vasoconstrictor response and ET_B receptors mediate the transient depressor action of endothelins.

[Thr^{18}, γ-methyl Leu^{19}]-ET-1 (TMET-1), another ET-1-derivative, has also been shown to bind to both ET_A and ET_B receptors with high affinities (Shimamoto et al., 1993). TMET-1 was shown to inhibit the ET-1-induced systemic blood pressure increase in rats more effectively than BQ-123. This once again suggests a contribution of ET_B-mediated contraction in resistance blood vessels. The most interesting compound, RO 46-2005 (Clozel et al., 1993), is the first synthetic orally active (30% bioavailability in rats) endothelin receptor antagonist which inhibits binding to both ET_A and ET_B receptors. This sulfonamide derivative was first synthetized as a part of an antidiabetic drug project.

In addition to studies of the effects of ET antagonists on the response to exogenous ET-1, there are some reports about the effectiveness of ET antagonists in pathophysiological animal models. In a rabbit model of myocardial ischaemia/reperfusion, FR139317 was shown not to affect infarct size (McMurdo et al., 1993b), while in a similar study a monoclonal antibody against ET-1 significantly reduced the infarct extension (Kusumoto et al., 1993). Interestingly, in a dog model of ischaemia and reperfusion BQ-123 decreased infarct size following 90 minutes ischaemia and 9 hours of reperfusion (Grover et al., 1993). This discrepancy may indicate the inter-species variation in the sensitivity to modulation of the

endothelin system, or could be due to the difference in the duration of these protocols. ET-1 antagonists have also been studied in animal models of hypertension. Nishikibe et al (1993) reported an antihypertensive effect of BQ-123 in stroke-prone spontaneously hypertensive but not in spontaneously hypertensive or normotensive rats. This was in contrast to the studies showing that BQ-123 would reduce blood pressure and peripheral resistance in spontaneously hypertensive rats (Ohlstein, 1993). Furthermore, it has been shown that the orally active $ET_{A/B}$ receptor antagonist RO 46-2005 reduces blood pressure in sodium depleted (high-renin) monkeys (Clozel et al., 1993).

1.6 Pharmacological Actions of Endothelin in the Cardiovascular System:

In addition to vasoconstriction, endothelin has been reported to produce many other biological actions. Endothelin can contract different types of smooth muscles, including vesicular and bronchial (Stewart, 1992). It appears to be a growth factor which stimulates proliferation of smooth muscle cells, fibroblasts and mesangial cells in culture. It increases the contractility of atrial and ventricular cardiac muscle and has a direct action on the renal glomerulus and tubules. Endothelin also induces the secretion of neurohumaoral agents such as atrial natriuretic peptide, renin, and aldosterone. Further it has been suggested that ET-1 and ET-3 may act as a neuropeptide and modify neurotransmission (Stewart, 1992).

1.6.1 Haemodynamic Effects

In their original report Yanagisawa et al (1988a) showed that bolus intravenous injection of ET-1 to anaesthetized rats caused a triphasic response: an initial transient depressor phase, followed by a transient pressor and then a sustained pressor phase. The biphasic pressor response and the sustained nature of the vasoconstriction distinguishes endothelin from other vasoconstrictor substances. The depressor effect of ET-1 is thought to be due to the production of prostacyclin and/or nitric oxide.

ET-1 has been shown to induce the release of cyclo-oxygenase products such as prostaglandin I_2 (PGI_2), prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2) in isolated perfused rabbit kidney, spleen (Rae et al., 1989), and isolated perfused rat lungs (DeNucci et al., 1988). However, pretreatment with indomethacin has no effect on the depressor action of ET-1 but potentiates the sustained pressor response (Rubanyi and Polokoff, 1994). The effect of ET-1 on the release of prostacyclin or TXA_2 may be due to the increase in arachidonic metabolism via the activation of phospholipase A_2 (Resink et al., 1989), since neomycin (phospholipase C inhibitor) had no effect on the ET-1-induced release of the arachidonic products, but quinacrine (an inhibitor of phospholipase A_2) had.

It is possible that a release of nitric oxide could be responsible for the vasodilator effect of ET-1 (Warner et al., 1988). ET-1-induced vasodilation by nitric oxide is supported by a study that shows that L-N^G-nitro-L-arginine methyl ester (L-NAME; an inhibitor of nitric oxide biosynthesis) in conscious normal rats attenuates the hypotensive and renal vasodilator effects of ET-1 (Gardiner et al., 1990). Similarly, N-monomethyl-L-arginine (L-NMMA) was shown to inhibit the vasodepressor effect of ET-1 in anaesthetized ganglion-blocked spontaneously hypertensive rats (Fozard and Part, 1992). However, some other studies have shown that L-NAME and L-NMMA had no effect on the vasodepressor action of ET-1 (Lerman et al., 1992). Webb and Haynes (1993) suggested that endothelial production of prostacyclin, but not nitric oxide, may modulate *in vivo* responses to ET-1 in human veins. They showed that ET-1-induced venoconstriction in humans was only partially prevented by glyceryl trinitrate (a nitric oxide donor), whereas it was blocked effectively by prostacyclin. Furthermore, this venoconstriction was potentiated by aspirin, but not by L-NMMA.

Endothelin is also a potent secretagogue for the vasorelaxant atrial natriuretic peptide (ANP) in cultured rat atrial myocytes (Fukada et al., 1988) and increases the plasma level of ANP in conscious dogs (Goet et al., 1988). However, Fozard and Part (1990) reported that a comparison of the haemodynamic effects of ANP and endothelin in spontaneously hypertensive rats showed a qualitative difference. ANP produced a slowly developing small fall in blood pressure, along with a weak but consistent vasoconstrictor response in the renal, mesenteric, hind quarters, and carotid vascular beds. In contrast, ET-1 induced a rapid fall in blood pressure associated with vasodilation in the carotid and hind quarter vascular beds. Therefore the role of these substances in mediating the vasodilator effects of ET-1 is still unclear.

The sustained phase of the pressor response to ET-1 lasts for up to 60-90 minutes (Yanagisawa et al., 1988a). This pressor effect in conscious and anaesthetized dogs has been associated with a reduction in cardiac output, reflecting extreme peripheral vasoconstriction and consequent activation of baroreflexes (Given et al., 1989). In normally ventilated pithed rats, in the absence of a functional sympathetic nervous system, endothelin-induced increases in peripheral resistance were associated with no change in heart rate or cardiac output, whereas in rats with moderate hypoxia, hypercapnia and acidosis, the cardiac output was increased (Maclean et al., 1989). An increase in cardiac contractility was also observed in hypoxic rats (Maclean et al., 1989).

In porcine coronary artery strips, the endothelin subtypes cause a contraction with the following order of potency: ET-1>ET-2>ET-3 (Inoue et al., 1989). Similarly, endothelins and sarafotoxins (STX) cause pressor responses in anaesthetized rats with a potency order of: STX_{6b}>ET-1≥STX_{6c}>ET-3 (Rubanyi and Polokoff, 1994). Both the pressor and depressor actions of ET-1 are greater when given by the intra-arterial route compared to intra-venous administration (DeNucci et al., 1988). This difference

may be due to endothelin uptake by the lung or other tissues when administered to the venous side of the circulation. Indeed, the short plasma half-life of infused endothelin (1-7 min) indicates that endothelin is extracted very rapidly by organs. Thus, the rapid disappearance of labelled-ET-1 from the blood stream, coupled with the long-lasting vasoconstrictor effect (Anggard et al., 1989) may reflect a persistent binding of endothelin to vascular smooth muscle cells. ET-1, in addition to a direct vasoconstrictor action, also potentiates, even at subthreshold levels, the contractile responses to vasoconstrictor agents such as norepinephrine and serotonin (Yang et al., 1990). Thus even low concentrations of locally produced endothelin may act as a regulator of vascular tone and reactivity in the circulation.

1.6.2 Effects of Endothelin on Heart

There is a growing body of evidence that shows that endothelins have a direct effect on cardiac tissue as well as other organs. Although ET-1 is produced and released mainly by endothelial cells, recent evidence has demonstrated the expression of prepro-ET-1 mRNA in and release of mature ET-1 from neonatal rat cardiac myocytes (Suzuki et al., 1993), suggesting a possible autocrine role for ET-1 in the heart. ET-specific, high affinity binding sites have been localized in human and porcine coronary vessels (Power et al., 1989), cardiac myocytes (Hirata et al., 1989a), and in the porcine conducting system (Yamasaki et al., 1989). Endothelin receptor subtypes (ET_A and ET_B) localized in the human atrioventricular conducting system and myocardium have also been observed (Molenaar et al., 1993). The atrial muscle has been shown to be about three times more sensitive to the positive inotropic effect of ET-1 than is ventricular muscle from humans and rats (Moravec et al., 1989). This difference may be related to the finding that atrial muscle has been shown to contain more receptors than ventricular muscle (Nayler and Gu, 1992).

The main electrophysiological and mechanical actions of ET-1 on the myocardium include both positive inotropic and chronotropic effects and prolongation of the action potential duration. Endothelin also affects heart function through marked coronary vasoconstriction. A positive chronotropic action has been reported for ET-1 (Ishikawa et al., 1988a) in isolated beating atrial preparations. However, in some studies it was shown that intravenous injection of ET-1 caused bradycardia, probably due to the activation of baroreflexes (Given et al., 1989). The positive inotropic effect of endothelin has been reported for human (Davenport et al., 1989; Moravec et al., 1989), guinea pig (Ishikawa et al., 1988b), and rat (Hu et al., 1988) atria, and rabbit ventricular muscle (Watanabe et al., 1989b). The inotropic effects of ET-1 are in the nanomolar range (Hu et al., 1988; Watanabe et al., 1989b) and are readily detected in isolated preparations. The inotropic actions are usually concealed in intact animals secondary to coronary and systemic vasoconstriction (Clozel and Clozel, 1989). It has been shown that the positive inotropic action of ET-1 is associated with prolongation of the cardiac action potential (Watanabe et al., 1989b). Yorikane and Koike (1990) reported that in isolated canine ventricular muscle ET-1 induced prolongation of action potential duration, which was followed by early after-depolarization, whereas in atria ET-1 shortened the action potential.

In open-chest anaesthetized dogs, ET-1 induced a reduction in coronary blood flow which was larger in the subepicardium than in subendocardium, demonstrating an ET-1-induced increase of endocardial-epicardial blood flow ratio (Clozel and Clozel, 1989). The vasoconstrictor effects of endothelin on coronary flow are also accompanied by some important electrophysiological consequences, such as elevation of S-T segment of the ECG, an increase in end-diastolic pressure and the release of lactate (Hom et al., 1992; Ezra et al., 1989). Intracoronary infusion of high doses of ET-1 have also been shown to cause ventricular fibrillation and death (Ezra et al., 1989).

1.7 Cellular Mechanism of Endothelin

1.7.1 Cellular Mechanisms of ET-1 Action on Vascular Smooth Muscle

Among the many biological actions of endothelins, the vasoconstrictor response of ET-1 was the first and most widely studied effect. Therefore the signal transduction mechanisms of ET-1 in vascular smooth muscle are the most extensively studied and analysed so far. Endothelin is a potent constrictor of both vascular and non vascular smooth muscles (Yanagisawa and Masaki, 1989). Compared to classical vasoconstrictor agents, such as angiotensin II or the α_1-receptor agonist phenylephrine, ET-1-induced contractions develop more slowly, are prolonged and are resistant to wash-out (Yanagisawa et al., 1988a). Further, this contraction is resistant to receptor blockers and enzyme inhibitors such as phentolamine (α-adrenoceptors blocker), diphenhydramine (H1-histamine receptors blocker), methysergide (serotonin receptors blocker), indomethacin (cyclooxygenase inhibitor) and nordihydroguiaretic acid (lipoxygenase inhibitor) (Masaki, 1989).

The ET-1-induced contractile pattern of isolated arteries consists of a rapid, initial phase and of a slowly developing tonic phase. It is proposed that the initial and sustained phases of contraction produced by vasoconstrictors are mediated by different cellular and molecular events (Rasmussen et al., 1987). It is now believed that rapid contractile responses are triggered by an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) leading to binding of Ca^{2+} to calmodulin (Cal) and subsequent binding of the Ca^{2+}-Cal complex to the catalytic subunit of myosin light chain kinase, followed by myosin light chain phosphorylation that permits coupling of myosin and actin (Somlyo and Himpens, 1989). This phase is associated with a rapid increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from an intracellular compartment. However the mechanisms of tonic contraction are not well understood and a plasma membrane-associated Ca^{2+}-sensitive form of protein kinase C may have an important role in the

maintenance of contraction (Rasmussen et al., 1987). It has been shown (Griendling et al., 1986) that angiotensin II-induced contraction in cultured vascular smooth muscle cell (VSMC) consists of a rapid phase and of a sustained phase similar to that seen with ET-1. The initial rapid phase was associated with a rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) and subsequent accumulation of diacylglycerol (DAG) and inositol triphosphate (IP_3). The long lasting phase was associated with a continued DAG production which was not necessarily coupled to IP_3 generation, suggesting a role for DAG from the phosphatidyl inositol (PI) pool in the induction of the sustained phase of contraction (Griendling et al., 1986). Furthermore, Jiang and Morgan (1987) demonstrated that phorbol esters (potent activators of protein kinase C) caused a prolonged contraction in rat aorta. This phase was associated with a sustained increase in $[Ca^{2+}]_i$ and was found to be dependent on external Ca^{2+} and a consequence of transmembrane Ca^{2+} influx. Goto et al (1989) showed that ET-1-induced increases in $[Ca^{2+}]_i$ in porcine coronary artery strip were largely dependent on extracellular Ca^{2+}. The vasoconstrictor action of ET-1 on the rat isolated aorta was also shown to be attenuated by dihydropyridine but not completely abolished (Renterghen et al., 1989). In a study on vascular smooth muscle cells using microspectrofluorimetry of Fura-2, it was shown that ET-1 caused an increase in $[Ca^{2+}]_i$ which was characterized by an immediate upstroke followed by a sustained phase (Iijima et al., 1991). Pretreatment with nifedipine and $NiCl_2$ (an inhibitor of receptor-operated calcium channels) and calcium-free medium significantly suppressed the sustained phase, while the initial $[Ca^{2+}]_i$ rise remained intact. These observations suggest that the ET-induced $[Ca^{2+}]_i$ increase is due to both Ca^{2+} release from an intracellular compartment, presumably the sarcoplasmic reticulum, and Ca^{2+} influx across the sarcolemma. Goto et al (1989), using the whole cell-attached patch-clamp technique, demonstrated that ET-1 stimulated an L-type Ca^{2+}-channel in porcine coronary artery vascular smooth muscle cells which was blocked by nifedipine. Silberberg et al (1989), using the same method

and preparation, showed that ET-1 increased Ca^{2+} channel activity with no effect on channel opening time or conductance. This indicated that the peptide possibly acts via a second messenger system. All of these observations led to the concept that ET-1, at least in part excerts its vasoconstriction by, either directly or indirectly, activating the voltage-dependent Ca^{2+} channels.

Binding studies have indicated that ET-1 failed to compete with or displace the binding of several L-type Ca^{2+} channel antagonists on rat cardiac membrane or rat brain (Gu et al., 1989b; Hamilton et al., 1989). The Ca^{2+} channel antagonists (nifedipine, nicardipine, diltiazem) were also shown not to displace the ET-1 binding on blood vessels or isolated smooth muscle cells (Clozel et al., 1989). Therefore, ET-1 does not appear to be a ligand of Ca^{2+} channels and the effect of ET-1 on Ca^{2+} influx via calcium channels must be the consequence of an indirect gating action of ET-1 on the channels. Interestingly it was demonstrated that levcromakalim (ATP sensitive K^+ channel opener), but not nifedipine, significantly inhibited labelled-ET-1 binding to rat cardiac membrane (Haynes et al., 1993). This suggested the presence of a stereospecific interaction between K^+-channel openers and ET-1 binding sites. This observation, along with an other finding by the same group where cromakalim inhibited the ET-1-induced venoconstriction (Haynes and Webb, 1993), suggests an additional mechanism of action for ET-1.

The binding of ET-1 to a receptor(s) results in the activation of phospholipase C (Danthuluri et al., 1989). Consequent hydrolysis of phosphatidyl inositol-4,5-biphosphate (PIP_2) yields two products, namely inositol triphosphate (IP_3) (Araki et al., 1989; Rapoport et al., 1990) and DAG (Lee et al., 1989), both of which could be involved in ET-1-induced smooth muscle contraction. In addition to the rapid release of calcium from intracellular stores (Somlyo et al., 1985), IP_3 is also able to stimulate inward calcium current through voltage-dependent channels (Penner et al.,

1988), or is able to be phosphorylated to inositol tetrakisphosphate (IP_4) which has been shown to facilitate extracellular calcium entry into the cell (Irvine and Moor, 1986; Morris et al., 1987). DAG, on the other hand, is believed to be the endogenous activator of protein kinase C, which in turn causes phosphorylation of myosin light chain and consequently contraction. Activation of protein kinase C by phorbol esters causes a contraction in various vascular smooth muscle cells which is slow in onset and difficult to washout, similar to that observed with ET-1. Furthermore, direct protein kinase C stimulation using phorbol esters has been shown to activate Ca^{2+} channels including L-type Ca^{2+} channels in vascular smooth muscle cells (Kaczmarek, 1987; Fish et al., 1988). Besides phosphatidyl inositol (PI) hydrolysis, the source of DAG may also be hydrolysis of phosphatidyl choline by phospholipase C (PLC) or phosphatidic acid degraded by phospholipase D. Indeed Resink et al (1990b) showed that ET-1, in [^{3}H]choline-pre labelled cultured human vascular smooth muscle cells, caused both activation of phospholipase C and phosphatidyl choline hydrolysis. Moreover, in [^{3}H]arachidonate-prelabelled human vascular smooth muscle cells, ET-1 was shown to promote arachidonic acid metabolism through the activation of phospholipase A_2 (Reynolds et al., 1989; Resink et al., 1990b). DeNucci et al (1988) first demonstrated that ET-1 caused release of PGI_2 and TXA_2 from isolated perfused rat lung.

Several studies suggest that the ET-1-induced activation of PLC and consequent hydrolysis of PI is mediated by a pertussis toxin-sensitive G-protein. Reynolds et al (1989) demonstrated that pretreatment of cultured vascular smooth muscle cells with *pertussis toxin* inhibited both inositol phosphate formation and arachidonic acid metabolism induced by ET-1. These studies suggest that G-proteins may be involved in ET-1-induced PLC and PLA_2 activation in different target cells. However some studies show that *pertussis toxin* had no effect on ET-1 induced PI hydrolysis in smooth muscle cells (Muldoon et al., 1989).

As mentioned above ET-1 initially stimulates a rapid and transient rise in $[Ca^{2+}]_i$ and $[IP_3]_i$ which is followed by a sustained and long lasting DAG and $[Ca^{2+}]_i$ accumulation (Danthuluri and Brock, 1990). The sustained elevation of $[Ca^{2+}]_i$ is blocked by calcium antagonists and calcium-free medium (Iijima et al., 1991), while external calcium removal and calcium antagonists do not prevent the initial phase (Danthuluri and Brock, 1990). However, when the caffeine-sensitive intracellular Ca^{2+} store is depleted (in calcium free medium) by repeated treatment with caffeine (Kai et al., 1989), or is blocked by ryanodine (a selective inhibitor of Ca^{2+} release from the sarcoplasmic reticulum) (Wagner and Sturek, 1991), the early increase in $[Ca^{2+}]_i$ in response to ET-1 is suppressed. This suggests that ET-1 induces rapid release of Ca^{2+} from caffeine- and ryanodin-sensitive intracellular stores by IP_3 or DAG, generated via PLC activation. Conversely, the sustained phase is dependent on extracellular calcium and is carried out by IP_3 and/or IP_4, and PKC stimulated Ca^{2+} influx.

Another consequence of activation of PLC by ET-1 and subsequent stimulation of protein kinase C through the DAG generation, is cytosolic alkalinization. It has been shown that ET-1 in vascular smooth muscle cells (Koh et al., 1990) and rat mesangial cells (Simonson et al., 1989) stimulates Na^+-H^+ exchange and leads to an increase in intracellular pH. A large number of studies show that a wide variety of vasoconstrictors increase intracellular pH via protein kinase C mediated through the activation of Na^+-H^+ exchange, by increasing the intracellular H^+ affinity for the antiporter (Grinstein and Rothstein, 1986). Indeed, the intracellular alkalinization time-course induced by ET-1 was similar to that of the ET-1-stimulated accumulation of DAG and was completely blocked by staurosporin, an inhibitor of protein kinase C (Brock and Danthuluri, 1992). However, intracellular pH was shown to be decreased when cultured vascular smooth muscle cells were exposed to ET-1 in a HCo_3^--Co_2 buffer (Brock and Danthuluri, 1992). The dominant mechanism appeared to be

Na^+-independent Cl^--HCo_3^- exchange. Moreover it has been shown that ET-1 produces a sustained membrane depolarization which is independent of Na^+ (Iijima et al., 1991). A Cl^- channel inhibitor, indanyloxyacetic acid, significantly suppressed the ET-1-induced membrane depolarization, suggesting that Cl^- efflux, rather than Na^+ influx, is involved in its generation. Membrane depolarization can activate voltage operating channels and cause Ca^{2+} influx. Thus ET-1-induced Ca^{2+} influx may in part be dependent on the activation of CL^--HCo_3^- exchanges. The proposed cellular mechanisms of action of endothelin is illustrated in figure 1.2 (from Rubanyi and Polokoff, 1994).

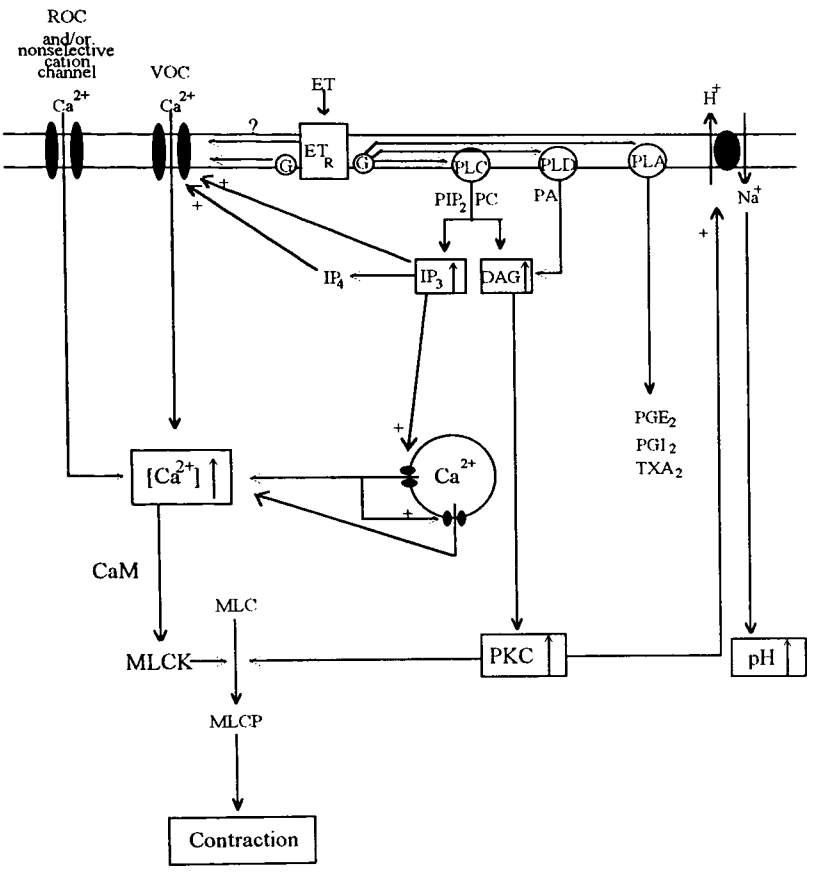


Figure 1.2 Proposed signal transduction mechanisms involved in ET-1 induced contraction of vascular smooth muscle. Abbreviations: CaM=calmodulin; ET_R=ET receptor; ?=link between ET_R and VOC opening is uncertain; VOC=voltage-operated calcium channel; ROC=receptor-operated calcium channel; G=G-protein; IP_4=inositol tetrakisphosphate; MLCK=myosin light chain kinase; PA=phosphatidic acid; PC=phosphatidylcholine; PGE_2=prostaglandin E_2; PIP_2=phosphatidylinositol phosphate; PLA_2=phospholipase A_2; PLD=phospholipase D (adapted from Rubanyi and Polokoff, 1994).

1.7.2 Mechanisms of Action of ET-1 on the Heart

The mechanisms involved in the positive inotropic effect of ET-1 are similar to those of vasoconstriction and are characterized by a slowly developing, sustained, and long lasting increase in the duration of the action potential (Watanabe et al., 1989b; Vigne et al., 1989). Similar to ET-1-induced contraction of vascular smooth muscle cells, the inotropic effect of ET-1 is associated with a rapid hydrolysis of phosphatidyl inositol in rat atrial cells (Vigne et al., 1989) and is relatively antagonized by ryanodin or caffeine. Furthermore, the ET-1-induced increase of action potential plateau in atrial cells (Ishikawa et al., 1988b) is accompanied by activation of the slow inward Ca^{2+} current which is sensitive to dihydropyridines. Thus it appears that the inotropic effect of ET-1 involves both Ca^{2+} entry through the voltage dependent Ca^{2+} channels and Ca^{2+} release from the sarcoplasmic reticulum. Fura-2 fluorescence measurements in isolated cultured neonatal myocytes show that ET-1-induced elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) is characterized by an initial rapidly developing phase (sensitive to ryanodine and caffeine), followed by a sustained plateau phase which is dependent on extracellular Ca^{2+} (Nayler and Gu, 1992). The positive inotropic effect of ET-1 is insensitive to α- and β-adrenergic blockade and to anticholinergic agents, but is partially suppressed by calcium antagonists including nicardipine (Ishikawa et al., 1988b). Although Naitoh et al (1990) observed that ET-1 did not activate isolated L-type Ca^{2+} channel activity of cardiac muscles, they suggested the involvement of receptor operated channels rather than voltage dependent channels. This observation, along with the fact that dihydropyridines (voltage-dependent Ca^{2+} channels blockers) can not block the response entirely, supports the hypothesis that the Ca^{2+} component of the positive inotropic effect of ET-1 may be divided into at least two parts: a voltage-dependent Ca^{2+} channel component and a voltage-independent Ca^{2+} channel component. Thus the effect of ET-1 on myocytes depends on both increased Ca^{2+} influx from the extracellular source and the mobilization of Ca^{2+} from internal stores. Kelly et al (1990)

demonstrated that in isolated rat ventricular myocytes, *pertusis toxin* completely blocked the positive inotropic action of ET-1. This suggested the involvement of G proteins in the inotropic effect of endothelin. However, the study of Vigne et al (1990) showed that *pertussis toxin* had no effect on ET-1-induced PI hydrolysis in rat atrial tissue. Stimulation of PLC resulting in PI hydrolysis has been demonstrated in rat ventricular myocytes (Galron et al., 1990) and it has also been shown that ET-1 stimulates PI turnover via ET_A receptors (Sugden et al., 1993).

Another possible explanation for the ET-1-induced increases in $[Ca^{2+}]_i$ and subsequent positive inotropic action, could be activation of the Na^+-Ca^{++} exchange mechanism due to the stimulation of the Na^+-H^+ exchanger by ET-1 (Simonson et al., 1989; Koh et al., 1990). Kramer et al (1991) demonstrated that in isolated rat myocytes a Na^+-H^+ exchanger inhibitor, amiloride, abolished ET-1 induced intracellular alkalinization and only partially attenuated the inotropic effect. Similarly, PKC inhibitors such as H-7 and sphingosin prevented the increase in intracellular pH, but did not completely prevent the positive inotropic response. Moreover, pretreatment with *pertussis toxin,* which blocks the positive inotropic effect of ET-1, only partially decreased the ET-1-induced intracellular alkalization. Thus, the positive inotropic action of ET-1 is due only in part to stimulation of the Na^+-H^+ exchanger by a protein kinase C-mediated pathway. In the studies of Kelly et al (1990) and Kramer et al (1991) it was shown that ET-1, at a concentration of 1 nM, increased the contractile amplitude of cardiac myocytes with little or no increase in cytosolic calcium. This suggests that an enhancement of the responsiveness of myofilaments to intracellular calcium via an induction of intracellular alkalosis is involved, since changes in intracellular pH are known affect to the calcium sensitivity of the contractile apparatus (Kramer et al., 1991).

1.8 The Role of Endothelin in Pathophysiological Conditions

Evidence from a great number of studies based on elevated plasma concentrations of endothelin, increased local endothelin gene expression and production, enhanced action and/or binding of endothelin, beneficial effects of anti-ET-antibodies or inhibitors of its production in animal models of the diseases have implicated a pathophysiological role for endothelin in a large number of diseases. To assess the pathophysiological significance of endothelin in disease states, to date, several sensitive methods including radioimmunoassays (RIAs) (Ando et al., 1989; Saito et al., 1989) and sandwich-enzyme immunoassays (EIAs) (Suzuki et al., 1989) have been developed to measure the plasma or tissue levels of ET-1-like immunoreactivity (IRET-1). The plasma IRET-1 levels in normal subjects have been reported in several studies and range from 0.26 to 19.9 $pg.ml^{-1}$. Many studies reported 3 or 4 fold elevations of plasma IRET-1 during the different pathophysiological conditions. The diseases which are most frequently reported to be accompanied by increased levels of endothelin in plasma are: Coronary vasospasm, systemic hypertension, pulmonary hypertension, shock (cardiogenic, septic, endotoxin), acute and chronic renal failure, myocardial infarction, severe congestive heart failure, and subarachnoid haemorrhage (Stewart, 1992).

1.8.1 Hypertension

Although many studies have examined the importance of ET-1 in hypertension, the role of endothelin in the aetiology of hypertension still is controversial. Hypertension is characterized by a general increase in peripheral vascular resistance mostly due to an increased vascular tone and also to structural alteration of vascular smooth muscle. Since ET-1 is the most potent vasoconstrictor both in vivo and in vitro, and its biological effects such as proliferation of smooth muscle cells or its effects on renal functions cause an increase in the vascular resistance, it has been implicated in the pathogenesis of hypertension. Increased plasma levels of endothelin have been

reported in patients with essential hypertension (Saito et al., 1992b). These patients had normal renal function and negative tests for urine protein. However other studies showed no elevation of plasma concentrations of endothelin in experimental models of hypertension (Vemulapalli et al., 1991) and in patients with untreated essential hypertension (Miyauchi et al., 1989b). In contrast to a moderate elevation of plasma levels of endothelin in essential hypertension without affected renal functions (Saito et al., 1992b), a marked increase was shown in patients with malignant hypertension associated with hemangioendothelioma (Yokokawa et al., 1991), renal failure (Widimsky et al., 1991), and pre-eclampsia (Florijn et al., 1991). These observations suggested that the elevated plasma level of the peptide was related to organ complications rather than to a high blood pressure itself.

McMahon et al (1991) demonstrated that intravenous infusion of phosphoramidon, an inhibitor of ET-1 production, lowered blood pressure in conscious spontaneously hypertensive rats (SHRs), suggesting that ET-1 production is involved in the rise in peripheral vascular resistance in SHR. BQ 123, a selective ET_A receptor antagonist which has no intrinsic ability to change vascular tone and blood pressure in normal subjects, did not affect blood pressure in SHR, but significantly reduced blood pressure in stroke-prone spontaneously hypertensive rats with malignant hypertension (Nishikibe et al., 1993). This suggested that ET_A receptors may at least partially contribute to the elevation of blood pressure in malignant forms of hypertension. Recently Clozel et al (1993) showed that an orally active mixed ET_A/ET_B receptor antagonist, RO 46-2005, significantly decreased arterial blood pressure in sodium depleted squirrel monkeys without altering heart rate, indicating that ETs may play an important role in hypertension and that both ET_A and ET_B receptors may be involved.

1.8.2 Coronary Vasospasm

Coronary vasospasm plays a main role as a cause of variant angina, myocardial infarction and a broad spectrum of clinical symptoms of atherosclerotic coronary artery disease (Maseri et al., 1979). Many factors such as smooth muscle hyper-sensitivity to vasoconstrictors (Kawauchi et al., 1984), coronary stenosis (MacCalpin et al., 1980) or platelet aggregation subsequent to stenosis (Folts et al., 1976), have been implicated in the pathogenesis of coronary artery spasm, but the real mechanism still is unknown. Potent and long lasting vasoconstriction produced by ET-1 makes it an ideal candidate for the pathogenesis of vasospasm. Elevated plasma levels of IR-ET-1 in the systemic and coronary circulation were shown during coronary spasm in patients with coronary spastic angina (Matsuyama et al., 1991; Toyo-oka et al., 1991). ET-1 injection into the coronary artery induced a profound and long lasting reduction in coronary blood flow with myocardial ischaemia in anaesthetized dogs (Kurihara et al., 1989a,b). It was also shown that coronary occlusion followed by reperfusion (Saito et al., 1992a) or low coronary perfusion pressure (Clozel and Sprecher, 1991), caused significant potentiation of ET-1-induced coronary artery vasospasm in dog hearts. Human coronary artery vasoconstriction induced by norepinephrine and serotonin was also shown to be potentiated by low concentrations (threshold) of ET-1 (Yang et al., 1990). These findings support a potential role for ET-1 in coronary vasospasm. In addition Matsuyama et al (1991) observed that the plasma level of ET-1 in the coronary circulation was elevated only in patients with increased lactate production in the coronary circulation (a sign of myocardial ischaemia), whereas in coronary vasospastic patients without increased lactate production there was no IR-ET-1 elevation. Therefore the ET-1 elevation may be secondary to myocardial ischaemia.

1.8.3 Myocardial Ischaemia, Reperfusion Injury and Acute Myocardial Infarction

A large number of studies have attributed a potential role for endothelin in the pathogenesis of myocardial damage during ischaemia and its consequences such as arrhythmias. These speculations are supported by findings of a profound elevation of circulating plasma levels of IR-ET-1 in patients with myocardial infarction (Stewart et al., 1991; Matsuyama et al., 1991; Miyauchi et al., 1989a). Supporting this, it has been shown that pretreatment with the endothelin converting enzyme inhibitor phosphoramidon (Grover et al., 1992) abolished the pre-ischaemic increase in coronary perfusion pressure induced by big ET-1 as well as its pro-ischaemic effect in the isolated rat heart. Furthermore, pretreatment with a monoclonal antibody against ET-1 significantly reduced myocardial infarction size in anaesthetized rats (Watanabe et al., 1991a). Thrombin and transforming growth factor β (TGF-β) were reported to increase the endothelin mRNA levels in cultured endothelial cells. This was accompanied by an increase in synthesis and release of endothelin (Kurihara et al., 1989b,c). It is possible therefore that ET-1 synthesis is increased in association with vascular intimal injury and subsequent thrombus formation. ^{125}I-ET-1 binding to cardiac membrane was also increased following ischaemia and reperfusion in rat hearts (Liu et al., 1990).

1.8.4 Congestive Heart Failure

Elevated plasma levels of endothelin in patients with heart failure (Stewart et al., 1992) and in experimental models of heart failure induced by ventricular pacing in dogs (Cavero et al., 1990) have been reported. However the source of the increased plasma concentrations of endothelin in congestive heart failure is not well known. Congestive heart failure is associated with an increase in plasma levels of angiotensin II and vasopressin (Rubanyi and Polokoff, 1994) while on the other hand, ET-1 secretion by endothelium was shown to be augmented in the presence of

angiotensin II or vasopressin (Resink et al., 1990a). It is therefore suggested that these agents may stimulate ET-1 production in heart failure. It is furthermore known that congestive heart failure is characterized by low cardiac output similar to that seen after haemorrhage. It is possible that endothelin levels may be elevated because of the decrease in intravascular volume. Alternatively, a reduction in cardiac output could result in decreased clearance of endothelin from the circulation and a subsequent elevation of plasma endothelin (Rubanyi and Polokoff, 1994). The vasoconstrictor effects of ET-1 may play a beneficial role in the early stage of heart failure. Increase of preload via venoconstriction increases systemic vascular resistance to maintain perfusion pressure, and increases plasma volume by fluid and water retention. However, the chronic effects of ET-1 appear to contribute to a continuing worsening of cardiac failure (Rubanyi and Polokoff, 1994).

1.9 Aims

Although far from complete, much information has accumulated since 1988 about the endothelins. This research aimed to examine the effects of ET-1 on the incidence and severity of arrhythmias induced by a period of myocardial ischaemia in open-chest anaesthetized rats. To attempt to determine the role of the ET_A receptor-mediated events during ischaemia, this study also aimed the effects of the ET_A antagonist, BQ-123, on arrhythmias and infarct size. HPLC and mass spectroscopy were also used to study the ability of rat myocardial extracts to convert big ET-1 to mature ET-1 and to degrade ET-1, and to perform a preliminary characterization of the enzymes involved in the ET-1 degradation. Furthermore, to evaluate the role of the myocardium in influencing circulating levels of ET-1, the last part of this study was performed to measure the plasma and tissue levels of endothelin during coronary artery occlusion and reperfusion in anaesthetized open-chest rats, using radio immunoassay.

Chapter 2

EXPERIMENTAL METHODS

2.1 HAEMODYNAMIC STUDIES IN ANAESTHETIZED RATS

In this thesis, the study of haemodynamic factors involved the measurement of systemic arterial pressure, heart rate and indices of ventricular contractility $LVdP.dt^{-1}_{max}$ and $LVdP.dt^{-1}.P^{-1}$.

2.1.1 Experimental Preparation

Male Sprague-Dawley rats (300-350g) were anaesthetized with sodium pentobarbitone (60 $mg.kg^{-1}$), given intraperitoneally. The trachea was cannulated and the animals were allowed to breath spontaneously; however, artificial ventilation was provided by a Harvard respirator (volume 1.5 ml $100g^{-1}$; rate 54 strokes min^{-1}) if necessary. A pre-calibrated steel thermostat probe was inserted into the rectum to measure temperature which was maintained at 37°-38°C with the aid of a heating lamp.

A polythene cannula (Portex; OD 0.98 mm, ID 0.58 mm) was inserted into the right femoral vein for the administration of anaesthetic when required, and drugs. The left femoral artery was cannulated for measurement of arterial blood pressure. A catheter was passed via the right carotid and down into the left ventricle for measurement of left ventricular pressure. A stabilisation period of 15 min was allowed following the surgical procedure before any drugs were administered. At the end of the experiment the animal was killed by an overdose of sodium pentobarbitone.

2.1.2 Parameters Measured

Systemic arterial blood pressure was recorded from the cannula inserted into the left femoral artery and left ventricular pressure was measured from the catheter placed in the left ventricle via the right carotid artery. The calibration of the transducer was checked daily against a mercury column. All measurements were made using Viggo-Spectramed pressure transducers and responses were continuously recorded on a

Buxco Electronic Haemodynamic Analyser. An on-line data analysis system digitised systolic, diastolic and mean arterial blood pressure (MAP, mmHg), heart rate (HR, beats.min^{-1}), systolic, diastolic and mean left ventricular pressure and calculated the derived indices of contractility $LVdP.dt^{-1}_{max}$ (mmHg.s^{-1}) and $LVdP.dt^{-1}.P^{-1}$ (1/sec). The expression $LVdP.dt^{-1}_{max}$ is the maximum rate of rise of ventricular pressure and can be used as an index of myocardial contractility. Although this index is preload and afterload dependent, it is widely used along with the related index $LVdP.dt^{-1}.P^{-1}$ which is the rate of pressure change at a fixed left ventricular pressure (in this case 50 mmHg). This pressure is relatively insensitive to afterload but still moderately sensitive to preload.

2.2 CORONARY ARTERY OCCLUSION IN ANAESTHETIZED RATS

The study of drug effects on acute myocardial infarction has been carried out using laboratory animals. In 1973 Kenedi and Losonci reported a technique of coronary artery ligation in anaesthetized rats to assess the effects of β-adrenergic blocking drugs on experimental cardiac arrhythmia. This model was later investigated and described in detail by Clark et al (1980). They confirmed and recommended it as a useful model for the study of early and late occlusion-induced arrhythmias and for the assessment of the extent of ischaemic myocardial injury. The model has since been widely employed for these purposes (Marshall et al., 1981; Wainwright, 1984). In this thesis, this model has been applied to the study of the effect of ET-1 and the ET_A receptor antagonist, BQ123, on ischaemia-induced arrhythmias in anaesthetized rats.

2.2.1 Anaesthesia

Male Sprague-Dawley rats, weighing 280-320g, were anaesthetized with sodium pentobarbitone (60 mg.kg^{-1}, i.p.) and maintained under anaesthesia by bolus injections of sodium pentobarbitone (6 mg, i.v.) as required.

2.2.2 Surgical Procedure

Following anaesthesia, the trachea was cannulated for artificial respiration and systemic arterial blood pressure was recorded from a catheter inserted into the left femoral artery. Femoral veins and the right jugular vein were cannulated for administration of anaesthetic or drugs as appropriate. A standard limb lead I electrocardiogram (ECG) was measured from subcutaneous electrodes and was monitored continuously over the experimental period on a Grass model 7D recorder (Grass Instrument CO, USA).

The chest was opened using a left thoracotomy at a point approximately 2 mm to the left of the sternum and this was followed by sectioning of ribs 4 and 5. Artificial respiration was immediately started using room air (volume, 1.5 ml.100 g^{-1}, rate, 54 strokes.min^{-1}). This is sufficient to maintain pCO_2, pO_2, and pH within normal limits (Clark et al., 1980). After incising the pericardium, the heart was exteriorised using gentle pressure on the rib cage, and a 6/0 braided silk suture (Mersilk; attached to a 10 mm micro point reverse cutting needle) was placed around the left coronary artery (figure 2.1). The heart was replaced in the chest and the animal allowed to stabilise for 15 min. During stabilization, any animal that had a mean arterial blood pressure of less than 70 mmHg or experienced persistent arrhythmias was discarded. A pre-calibrated steel thermostat probe was inserted into the rectum to measure temperature which was maintained at 37°C-38°C with the aid of a heating lamp.

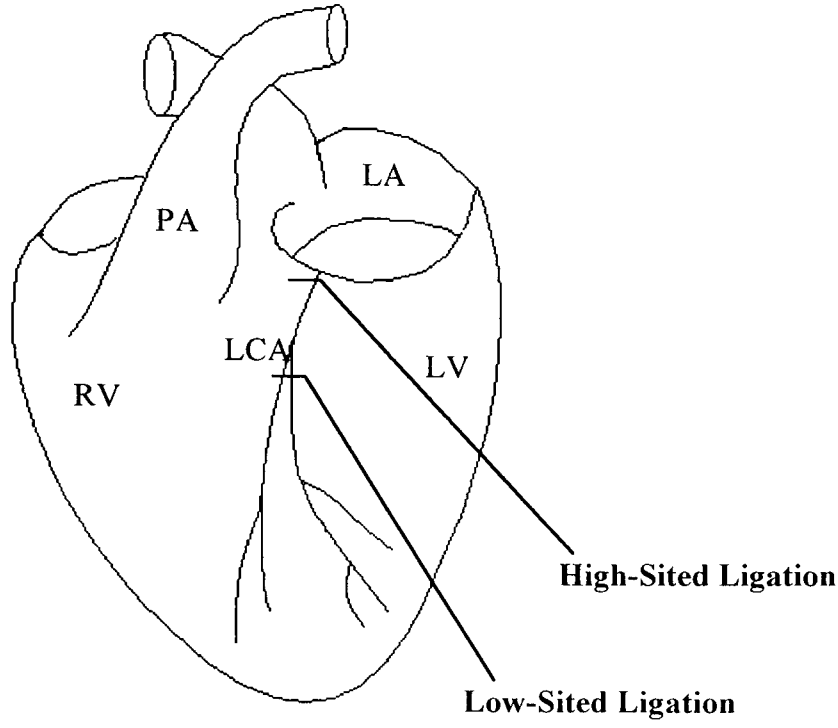


Figure 2.1 Schematic diagram of the rat heart showing the position of ligature around the left coronary artery. PA (pulmonary artery); LA (left atria); LCA (left coronary artery); RV (right ventricle) and LV (left ventricle)

2.2.3 Coronary Artery Occlusion

Coronary artery occlusion was induced by tightening the loose ends of the ligature in a knot. In animals which were ultimately subjected to measurement of myocardial infarct size, the ends of the ligature were threaded through a polyethylene occluder and clamped in place. Reperfusion to the previously ischaemic tissue was allowed by release of the clamp. Successful occlusion was identified by an immediate, temporary, and marked increase in the amplitude of the QRS complex of the ECG and a slight decrease in mean arterial blood pressure.

2.2.4 Parameters Measured

Systolic, diastolic and mean arterial blood pressures were measured from the arterial pressure trace. Mean arterial pressure (MAP) is not a simple arithmetic average of systolic and diastolic pressures, because the blood spends relatively longer near the diastolic than systolic level, therefore it was calculated as:

$$P_{MAP} = P_{diast} + (P_{sys} - P_{diast})/3$$

Where P_{MAP} = mean arterial blood pressure; P_{diast} = diastolic arterial blood pressure; P_{sys} = systolic arterial blood pressure.

Both the ECG and blood pressure were continuously recorded on a Grass polygraph (model 7D). Heart rate (beats/min) was calculated by counting the number of QRS peaks per minute every 5 minutes. The arrhythmias where occurring during the coronary artery occlusion were analysed from the electrocardiogram.

2.2.5 Classification of Post-Occlusion Ventricular Arrhythmias

Two distinct phases of post-occlusion ventricular arrhythmic activity have been shown to occur in anaesthetized rats (Parratt et al., 1981). The early phase of arrhythmias occurs during the first 30 min of occlusion (phase 1) and the second, later phase of variable ectopic activity, begins one and a half hours post occlusion and lasts for at least two and a half hours (phase 2). This study aimed to investigate the early phase of marked arrhythmias due to its clinical relevance to the early life-threatening arrhythmias associated with acute myocardial infarction. Ventricular arrhythmias were detected from both the ECG and blood pressure traces, and analysed under the guidelines of the Lambeth Conventions for analysis of experimental arrhythmias (Walker et al, 1988). These were identified as single ventricular ectopic beats, salvos, (sometimes present as bigeminal or trigeminal rhythm), ventricular tachycardia (VT)

and ventricular fibrillation (VF). The characteristics of the various arrhythmias can be defined as follows:

Normal sinus rhythm is identified in the ECG by three main deflections per cardiac cycle: the P wave, the QRS complex and the T wave. The normal sinus rate of anaesthetized rats is 350-500 beats.min^{-1}. Figure 2.2a shows an ECG and blood pressure trace from a non-operated anaesthetized rat and figure 2.2b presents normal sinus rhythm of anaesthetized rats immediately after the induction of myocardial ischaemia.

Acute ligation of the left main coronary artery in anaesthetized rats causes characteristic, reproducible electrocardiographic and haemodynamic changes, as illustrated in figure 2.2b. The most typical and marked change is a large increase in the amplitude of the QRS complex of the ECG. This change occurs almost immediately after ligation (usually within 2-3 seconds) and is usually sustained, lasting throughout the entire ischaemic period. The more immediate change in the ECG generally coincides with a slight fall in the mean arterial blood pressure. The fall is, however, usually transient and a gradual return towards pre-occlusion levels is usually in progress by the time of onset of arrhythmic activity.

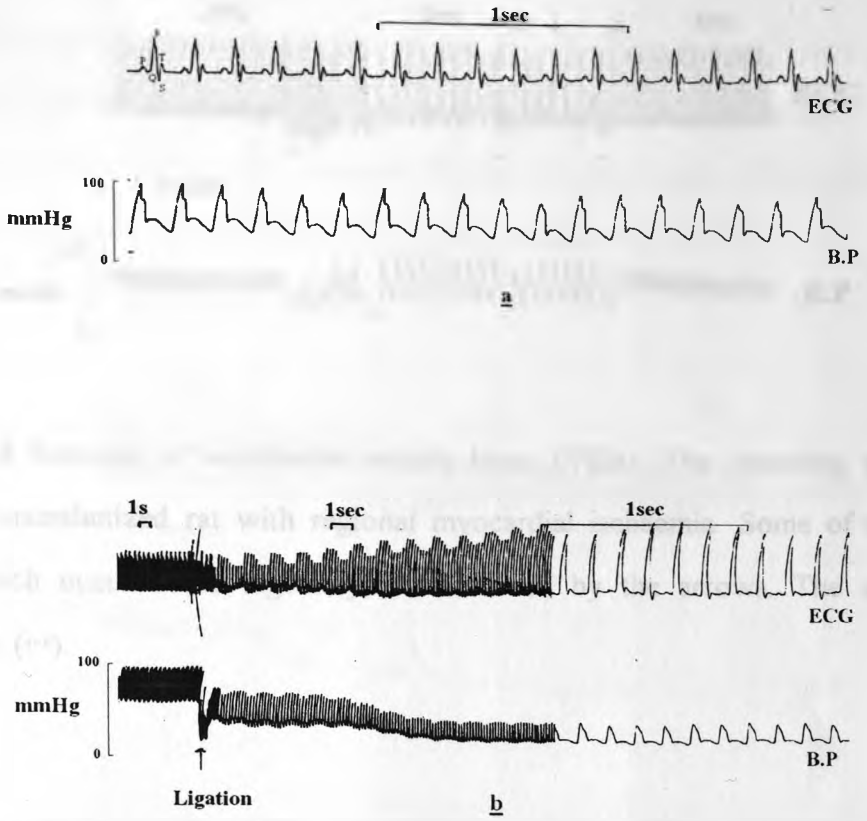


Figure 2.2 ECG and blood pressure traces from a non-operated anaesthetized rat recorded from Lead I (a) and a trace showing the characteristic ECG and blood pressure (B.P) responses to acute coronary artery occlusion in anaesthetized rats (b)

Single ventricular ectopic beats are defined as discrete and recognisable premature QRS complexes (premature in relation to the P wave), with a downward T wave (figure 2.3). In the rat coronary artery occlusion model single ventricular ectopic beats often appear as regularly repeating twin beats, which is termed **ventricular bigeminy**.

Salvos (couplets and triplets) are defined as two or three consecutive ventricular ectopic beats (figure 2.3). Repeated twin couplets or triplets are termed **trigeminy**. As shown in figure 2.3 single and salvo forms of ventricular ectopic beats are associated with a decrease in blood pressure, which forms a wider and deeper blood pressure wave.

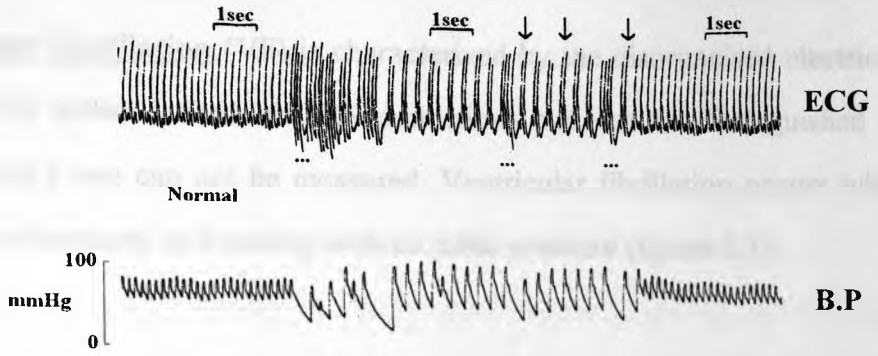


Figure 2.3 Example of ventricular ectopic beats (VEB). The recording was made from an anaesthetized rat with regional myocardial ischaemia. Some of the single VEBs which occurred as bigeminy are indicated by the arrows. The salvos are underlined (···).

Ventricular Tachycardia (VT) is determined as a run of four or more consecutive ventricular ectopic beats, occurring with clearly characterized R waves, at a rate faster than sinus rhythm (figure 2.4). In the rat model of coronary artery occlusion the heart rate during tachycardia is usually between 600-850 beats per minute. During the tachycardia blood pressure declines notably, with a very low pulse pressure.

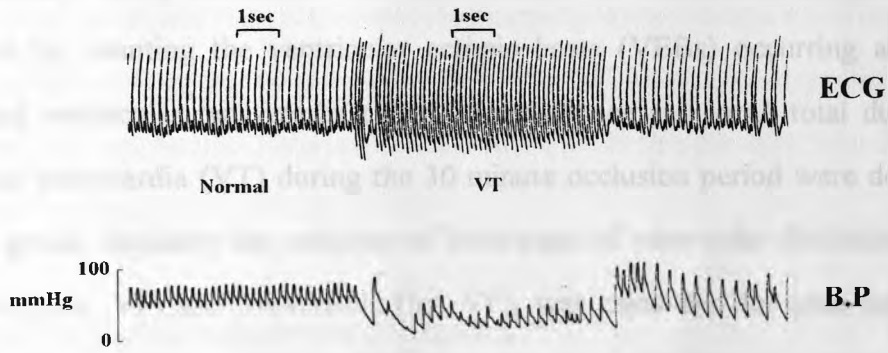


Figure 2.4 Example of ventricular tachycardia (VT). The ECG and blood pressure recording was made from an anaesthetized rat with regional myocardial ischaemia.

Ventricular Fibrillation (VF) is characterized by the disorganised electrical activity of the ECG where individual QRS deflections can not be distinguished from one another and a rate can not be measured. Ventricular fibrillation occurs with a sharp fall in blood pressure to 0 mmHg with no pulse pressure (figure 2.5).

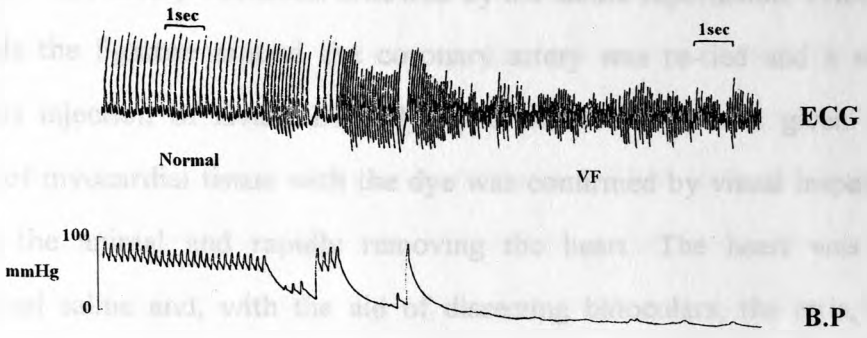


Figure 2.5 Example of ventricular fibrillation (VF). ECG and blood pressure recording was made from an anaesthetized rat with regional myocardial ischaemia.

2.2.6 Analysis of Ischaemic Arrhythmias

The period during which early arrhythmias were analyzed was the first 30 minutes after coronary artery occlusion. The total number of ventricular arrhythmias was calculated by counting the ventricular ectopic beats (VEBs) occurring as singles, salvos and ventricular tachycardia (VT). The incidence and mean total duration of ventricular tachycardia (VT) during the 30 minute occlusion period were determined for each group. Similarly the incidence of both types of ventricular fibrillation, that is reversible (Rev. VF) and irreversible (Irr. VF), was calculated for each group. The time spent in reversible ventricular fibrillation was also calculated as a mean value for each group. Mortality from irreversible ventricular fibrillation, bradycardia and A-V block was also recorded, and these animals were excluded from the calculation and

statistical analysis of the mean total number of ventricular ectopic beats and duration of VT and VF.

2.3 MEASUREMENT OF MYOCARDIAL INFARCT SIZE

In some experiments, myocardial infarct size was measured in rats subjected to 30 minutes coronary artery occlusion followed by 3.5 hours reperfusion. Prior to killing the animals the ligature around the coronary artery was re-tied and a slow bolus intravenous injection of Evans Blue dye (0.5 ml; 3% w/v) was given. Adequate perfusion of myocardial tissue with the dye was confirmed by visual inspection prior to killing the animal and rapidly removing the heart. The heart was rinsed in physiological saline and, with the aid of dissecting binoculars, the atria, aorta and pulmonary vessels were removed. The ventricle was cut into four equal transverse slices perpendicular to the apex-base axis. These were then placed in 1% triphenyltetrazolium chloride (TTZ) solution at 37°C for 10 minutes to dye the normal region (Watanabe et al. 1991a). TTZ stains the non-infarcted myocardium a brick red colour, indicating the presence of a formazin precipitate that results from the reduction of TTZ by dehydrogenase enzymes present in viable tissue. This procedure resulted in the normally-perfused tissue being stained blue, non-infarcted, non-perfused tissue stained brick red and infarcted tissue remaining unstained. The tissue free of Evans Blue was dissected from the normally perfused tissue and weighed as the area at risk. The infarcted tissue (unstained by TTZ) was then dissected from the stained tissue (dark red) of the area at risk and weighed to determine infarct size as a percentage (by weight) of the area at risk.

2.4 ENDOTHELIN RADIOIMMUNOASSAY

A radioimmunoassay is based on the competition between radio-labelled and unlabelled antigen for the binding site of the antigen-specific antibody. An antigen-antibody complex is formed. The concentration of the labelled antigen and the

concentration of the antibody are constant in all the tubes within one assay. Thus, the only variable parameter of the system is the concentration of unlabelled antigen in either the standard or the test samples. As the concentration of unlabelled antigen in the sample increases, the binding of the competing labelled molecules to the antibody is progressively reduced. Therefore, the radioactivity of the antigen-antibody complex is inversely proportional to the concentration of unlabelled antigen in the sample. In order to obtain a higher sensitivity, the antibody is pre incubated with the unlabelled antigen and then labelled antigen is added for competition. After the completion of the reaction the antigen-antibody complex (bound fraction B) is separated from the free antigen fraction (free labelled molecules included) by precipitation. The radioactivity of the bound fraction is counted. The concentration of antigen in test samples is determined by comparison of the radioactivity count with a standard concentration curve produced using pure synthetic antigen (ET-1) using a Fig P6 (Fig.P Software Corporation) curve fitting computer program. Radioimmunoassay of heart and plasma endothelin prior to and during coronary artery occlusion and after reperfusion was performed according to a protocol supplied by Biomedica with some modifications introduced in our laboratory.

2.4.1 Sample Preparation

Since the level of endogenous endothelin in biological tissue is low it was necessary to extract endothelin from plasma or myocardium in order to avoid interference of other components and to increase the sensitivity of the assay. Extraction was performed using a solid phase extraction with Waters Sep Pak C18 cartridges (Milipore Corp.).

Solutions required:

A: 100% Methanol

B: Methanol + Water = 10+90 (V/V)

C: Methanol + Water = 20+80 (V/V)

D: Methanol + Water = 85+15 (V/V)

E: Acetonitrile + Water = 1+2 (V/V)

The Sep Pak C18 cartridge had to be prewashed consecutively with 2 ml A; 2 ml B; 2 ml C; 2 ml D and 2x2 ml B.

Endothelin extraction from plasma: Blood samples (5 ml) were collected from anaesthetized rats via a catheter placed in the right atrium, through the right jugular vein directly into pre-chilled polypropylene test-tubes containing heparin (20 $IU.ml^{-1}$). Plasma was then separated by centrifuging for 20 min at 3000 g and 4°C. The supernatant was transferred into polypropylene tubes and analysed on the same day. For each fresh plasma sample (2.5-3 ml), 100 µl of solution E (acetonitrile:water = 1:2) was added, shaken, and any precipitate was removed by centrifugation. The supernatant plasma was loaded on the pre-washed cartridge and washed with 4 ml of solution B (10% methanol). Endothelin was eluted with 4x1 ml of solution D (85% methanol) into polypropylene tubes. At this stage samples were stable for at least 2 days at 4°C. The eluate was dried under a stream of nitrogen at 30°C. The dried samples were re-dissolved in 1.05 ml of RIA-Buffer (see section 2.4.2), left to stand for at least 1 hour at 4°C, and centrifuged if necessary. The supernatant solution was divided into two aliquots (500 µl) and used for assay.

Endothelin extraction from heart: At the end of the relevant experiments (Chapter 5) whole hearts were removed and placed in liquid nitrogen and stored frozen at -25° C for a period not exceeding 1 week. On the day of extraction the hearts were heated in boiling water for 5 min and homogenized (using an Ultra-Turrax® homogenizer) for 5 min at 4°C in 4 ml chloroform : methanol (2:1) (Watanabe et al., 1991a). Distilled water (200 µl) was added to the homogenate, which was then centrifuged for 20 min at 3000g. 3.5 ml from liquid phase was transferred onto the Sep Pak cartridge and taken through the protocol as described for plasma.

2.4.2 RIA-Buffer

Sodium phosphate buffer (0.07 M, pH=7.2-7.4), containing 0.01M EDTA and 0.3% bovine serum albumin was used as RIA-Buffer. The buffer also contained 0.09% sodium azide (NaN_3) as a preservative.

2.4.3 Standard Preparation

The 100µg of synthetic ET-1 lyophilisate was dissolved in 10 ml distilled water (10 $\mu g.ml^{-1}$), allowed to stand at room temperature for 15 min and then divided into 0.1 ml aliquots as stock solutions which were kept at -25°C until use. Before use each aliquot was thawed and allowed to stand at room temperature for 15 min and then diluted with RIA-Buffer as appropriate. The final volume of all standards was 100µl.

2.4.4 Antibody Preparation

Rabbit polyclonal antibody (Affiniti) to human endothelin-1 was used for this radioimmunoassay. The antibody in each vial was reconstituted with 100 µl distilled water and left to stand for 15 min. The reconstituted antibody was diluted to 25 ml with RIA-buffer. This was sufficient for 250 tests. The diluted antibody was stored at 2-4°C and used within 1 month.

2.4.5 Tracer Preparation

Methanol (625 µl) was added to 5 µci (185 kBq) labelled endothelin-1 (^{125}I ET-1) lyophilisate and was allowed to stand for 15 min at room temperature. RIA-buffer was then added to the reconstituted ^{125}I ET-1 (3.750 ml) and was allowed to stand for a further 15 minutes to dissolve. The solution was then divided into aliquots of 150 µl and kept at -25°C until used (tracer was stable for at least 2-3 weeks). On the day of the experiment the aliquots were thawed, 150 µl RIA-buffer added and allowed to stand for 15 min. After gentle vortex mixing, the aliquots were diluted to 1.65 ml

with RIA-buffer. This was sufficient for 30 assay tubes. all tubes were kept in ice (4°C) throughout the experiment.

2.4.6 Assay Protocol

The scheme below summarises the assay protocol:

	Total Total count	**NSB** Non specific binding	**Bo** Total binding	**Std** Calibration	**Samples** Assay
RIA-buffer µl	—	600	500	400	—
Standards µl	—	—	—	100	—
Samples µl	—	—	—	—	500
Antibody µl	—	—	100	100	100
Mixed well and incubated over night at 4°C					
Tracer µl	50	50	50	50	50
Mixed well and incubated for 6 hrs at 4°C					
Rabbit serum µl	—	20	20	20	20
Isopropanol µl	—	900	900	900	900
Mixed well and incubated for 15 min at 4°C					

In the final step the antibody-antigen complex was precipitated by addition of normal rabbit serum and cold isopropanol. The precipitates were centrifuged at 3000g for 20 min at 4°C and the supernatants were discarded. Then all tubes were counted for ^{125}I in a gamma counter (Packard Multi-Prias) for 2 min. Validation of assay and standard curve for ET-1 radioimmunoassay will be described in Chapter 5 (section 5.3).

2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN ENZYMATIC ANALYSIS

HPLC is a widely used technique. Some of the reasons for considering HPLC for the assay of enzymic activities are that it provides a method to enhance the separation of reaction components, allows extensive and complete analysis of the components in the reaction mixture during the reaction, employs sensitive detectors, and can be used for purification.

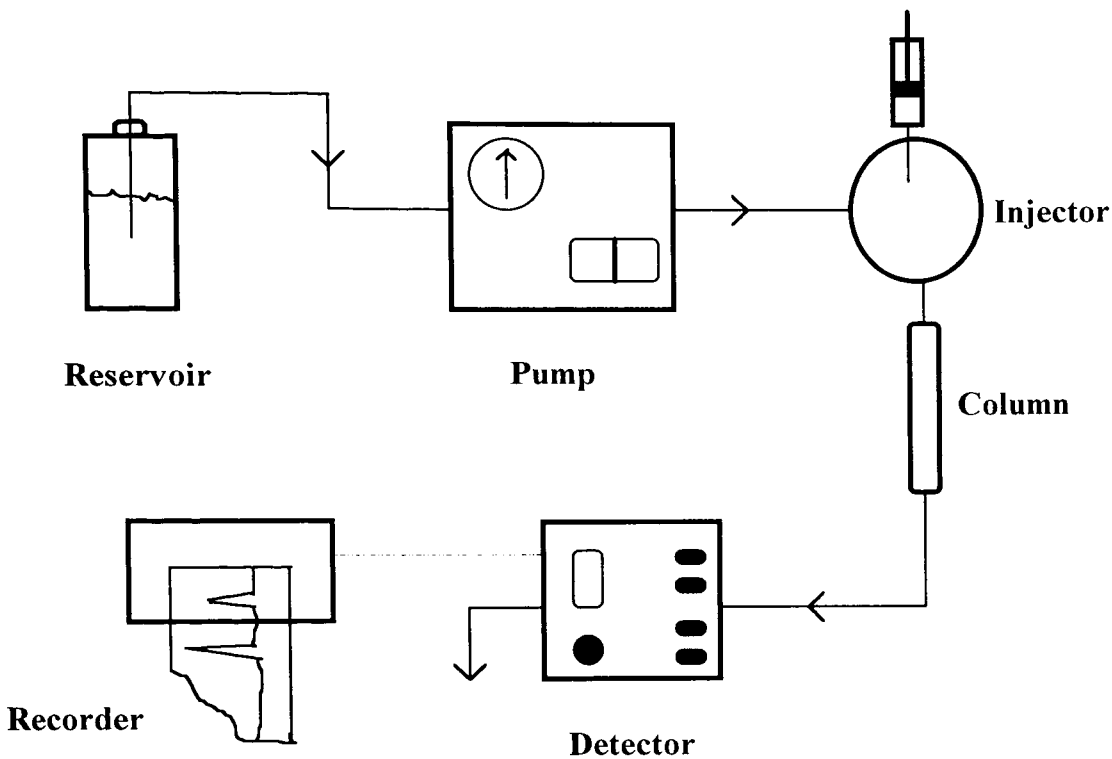


Figure 2.6 Schematic presentation of a simple HPLC system (from Dolan and Snyder, 1989).

2.5.1 Concepts and Principles of HPLC

An HPLC system, shown schematically in figure 2.6, contains (1) a *solvent reservoir* of eluent or mobile phase; (2) a *pump*, which is the solvent delivery system; (3) an

injector which injects the sample into the system without a fall in pressure or change in flow rate; (4) the *column*, which contains the solid or stationary phase; and (5) a *detector* to monitor the eluent (Dolan and Snyder, 1989). The sample is injected into the column and is then pushed by pumps, under high pressure, through the column by the mobile phase at constant flow rate. The mobile phase can be delivered in two ways: **isocratically**, that is, at constant composition, or in the form of a **gradient**, when the composition is varied. Following the output of the mobile phase from the other end of the column, the eluent is detected by a detector. Detectors operate on various principles. For example, some monitor the ultraviolet, visible, or fluorescent properties of molecules; others monitor radioactivity; and still others monitor differences in oxidation-reduction potential and refractive index (Rossomando, 1987). In this study an ultraviolet detector was used.

2.5.2 Reverse Phase Chromatography

Reverse phase HPLC (RP-HPLC) is a well known tool for the analysis and purification of peptides. High performance, large-pore separation media have been developed that allow rapid, and efficient separation of peptides and proteins (Pearson et al., 1982). As mentioned above, chromatography needs two phases: one solid and loaded into the column, the other mobile, the eluent or buffer that flows through the column. When the solid phase is made of polar material while the mobile phases are non polar organic solvents, this system is referred to as *normal-phase* liquid chromatography. When the situation is reversed and the columns are packed with a non polar solid phase and are eluted with a polar mobile phase, this type of chromatography is known as *reverse-phase*. Some times the term "hydrophobic" is also used (Rossomando, 1987).

2.5.3 Mobile Phases Used in the RP-HPLC of Proteins and Peptides

In peptide chromatography, to provide a high resolution separation, an increasing gradient of organic solvent in aqueous solvent containing an ion-pairing agent is usually used. Trifluoroacetic acid (TFA) is a widely used ion-pairing agent because it is volatile and easy to remove from peptide-containing fractions. The use of TFA in the gradient sometimes leads to an upward shift in the baseline when monitoring at 215-220 nm. As the dielectric constant of the solvent changes with increasing organic solvent concentration the adsorption spectrum of TFA shifts, resulting in an upward baseline deflection (Rossomando, 1987). Acetonitrile is the most commonly used organic solvent because of high UV transparency at low wavelengths, low viscosity, resulting in low column back-pressure and high column efficiency. It is also highly volatile, which allows easy removal of acetonitrile from peptide-containing fractions (Rossomando, 1987).

2.6 COLUMN SPECIFICATION

It is important to evaluate a column's performance before using it. The most commonly used solid phase for the chromatography of proteins and peptides is octadecylsilane (ODS), n-$C_{18}H_{37}$, a grouping which are abbreviated by C4, C8 and C18. The C4 phase is recommended for the chromatography of large peptides (greater than 20-30 residues) particularly for hydrophobic polypeptides. The C8 phase is effective for analysis of natural and synthetic peptides and small, hydrophilic proteins. The C18 phase is recommended for enzymatic studies and for analysis of small peptides. In this study a C18 column was used. Two parameters commonly used to test a column's efficiency are its theoretical plate number (N) and the asymmetry factor (As). The theoretical plate number of a column is a measure of the amount of "band broadening" a given solute undergoes as it travels through the column. The larger the N the narrower the elution bands, hence the better the column. Typical N values in LC are 2000 to 20,000 "plates". The other commonly used measure of

column efficiency is peak symmetry. If the column is not packed properly you may see peak "trailing" or "fronting". The ratio for a symmetrical peak is 1 and typical values for a good column are between 0.85 and 1.3. The aim of this experiment was to check the efficiency of the column by determining the theoretical plate number and the asymmetry factor of the C18 column (Phase Separation Ltd) which was used in this study.

2.6.1 Material and Procedure

The test mixture was made up of: acetone (300 mg); cresol (94 mg), xylenol (100 mg), and phenetol (125 mg); all were dissolved in 100 ml of the mobile phase (Methanol:Water; 60:40). The column was a Phase Separation Ltd C18 (S5 ODS2; 25 cm × 4.6 mm; particle size: 5 microns; part No: 839540). Reversed phase high performance liquid chromatography (RP-HPLC) was performed using a Beckman system Gold controller, model 166 variable wavelength U.V detector, two 110B pumps and a Beckman, model 507 autosampler injector. The column was equilibrated for 40 min at a flow rate of 1 $ml.min^{-1}$ with the detector set at 254 nm. The test mixture was injected (100 µl) through the autosampler and the system was allowed to run for 30 min. The last peak in the chromatogram (phenetol) was used to calculate the parameters.

2.6.2 Calculation of the Number of Theoretical Plates (N) and the Peak Symmetry (A_{10})

Chromatogram for the test mixture is shown in figure 2.7. The retention time of the last peak (t_R), the width of the peak at the 1/2 peak height ($W_{0.5}$), and the distance to the trailing (a) and front edges (b) at 10% of the last peak height were 20.99 min, 0.4 cm, 2.6 mm and 2.4 mm respectively.

The column plate number is calculated as:

$$\mathbf{N=5.54(t_R/W_{0.5})^2}$$

Where N is the column plate number, t_R is the retention time of last peak, $W_{0.5}$ (cm) is the width of the band at the 1/2 peak height. Therefore, N for the column = $5.54(20.99/0.4)^2$ = 15,255 plates/column and N = 15,255 × 4 = 61,020 plates/meter

Peak symmetry (A_{10}) is calculated at 10% of peak height by the equation:

$$\mathbf{As\ or\ A_{10} = a/b}$$

Where **a** is the distance to the trailing edge and **b** is the distance to the front edge at 10% of peak height. Therefore, A_{10} for the column = 2.6/2.4 = 1.08

The chromatogram fig 2.7 shows the column has a high selectivity in separating the components of the test mixture. The N value of 15,255/column also shows the column is good with regard to this parameter. Under ideal conditions a column should have a theoretical plate number close to Nmax (Dolan and Synder, 1989) given by the formula:

$$\mathbf{Nmax = 4000 \times L/dp}$$

L is column length (cm) and dp is particle size (μm) of column packing material, for the column under consideration, Nmax = 4000 × (25/5) = 20,000.

The band tailing as measured by the asymmetry factor was 1.08. It shows symmetrical peaks. Typical A10 values for a good column are between 0.85 and 1.3 (Dolan and Snyder, 1989). A significant change in either the A10 value or the theoretical plate number during the life of a column suggests a deterioration in the column's performance.

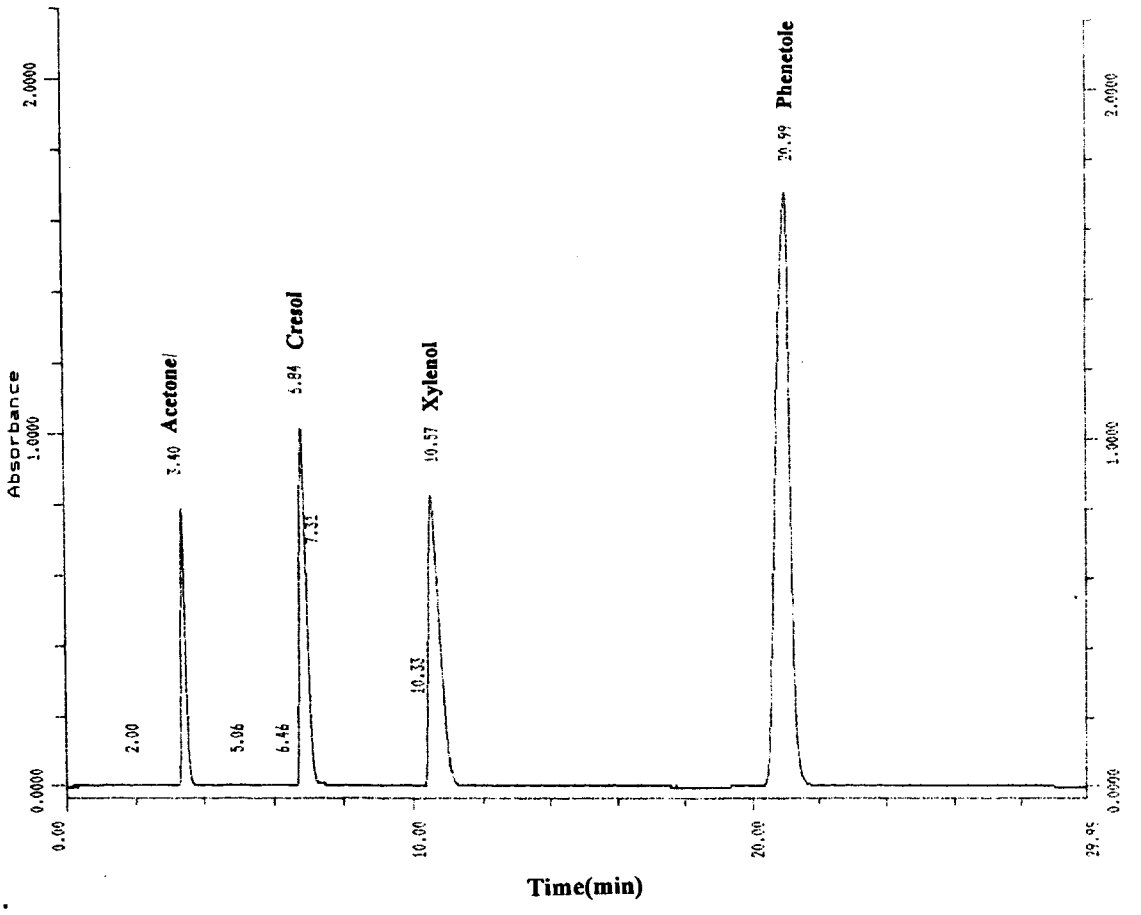


Figure 2.7 Separation of the components of the test mixture (composition: acetone, cresol, xylenol, and phenetol) on the C18 column (4.6 mm x 25 cm, particle size 5 microns). Mobile phase: methanol:water 60:40, flow rate: 1 $ml.min^{-1}$.

2.7 DETECTION, CHARACTERISATION AND CALIBRATION CURVE OF ET-1

The degradation of ET-1 was studied in rat ventricle and lung tissue extract at different pHs. Because of the presence of many interfering substances in biological fluids and ET-1 metabolites, it was essential to verify that the method used for analysis in the HPLC system could effectively separate out bands of interest. This

experiment was to determine the retention time and the relation between the concentrations of the standard sample of ET-1 and peak size.

2.7.1 Method

Reversed phase high performance liquid chromatography (RP-HPLC) was performed using a Beckman System Gold Controller, model 166 variable wavelength U.V detector and two 110B pumps. Various concentrations (0.1, 1, 5, and 10 $\mu g.ml^{-1}$) of the standard ET-1 were injected (100 µl) onto a C18 column (Octadecyl Silica, ODS2 Phase Sep., 5 µm, 0.45×25 cm) through an autosampler injector (Beckman, model 507) and eluted using the mobile phase. The mobile phase consisted of a gradient of 15-40% (over 10 min), 40-55% (over 30 min), and 55-70% (over 10 min) acetonitrile in water (V/V), containing 0.09% trifluoroacetic acid (TFA) and was delivered at a flow rate of 1.0 $ml.min^{-1}$. The detection was performed at 210 nm. The mobile phase was degassed continuously with helium sparging during the experiment. The system was washed by a mobile phase for 10 min from 70-100% and then for a further 15 min from 100-15% between each run and remained at 15% for a final 10 min for equilibration. This method was developed and established based on a method used by Hexum et al (1990). To identify background peaks due to the buffer and boiled or unboiled tissue extracts from those of ET-1 and its metabolites, blank samples of phosphate buffer, boiled and unboiled tissue extracts were passed through the column as described for standard ET-1 in this section.

2.7.2 Results

The background peaks produced by buffer and tissue extracts tested as blanks are shown in figure 2.8. In addition to the salt peaks which appeared during the first 4 minutes of all runs there was a strong peak at retention time of 23-23.5 min which was present in all chromatograms. This peak was independent of ET-1, Big ET-1 or their metabolites and is indicated as a buffer artefact (b.a.). The retention time for ET-1

was 26-27 min (figure 2.9). The peak heights for different concentrations of ET-1 are shown in table 2.1. A linear relationship (correlation coefficient: 0.9968) between the concentration of ET-1 and the peak height was observed (Figure 2.10). Minimum detection of ET-1 by this method was 20-40 pmole.

Table 2.1 Peak heights related to different concentrations of ET-1. Injection volume was 100 µl, data represents mean±sem, n=5.

No	**ET** µg/ml	**ET** µg/column	**ET** pmole/column	**Peak Height** $\times 10^3$
1	0.1	0.01	4	0.357±0.039
2	1	0.1	40	7.251±0.714
3	5	0.5	200	31.871±3.445
4	10	1	400	57.875±2.332

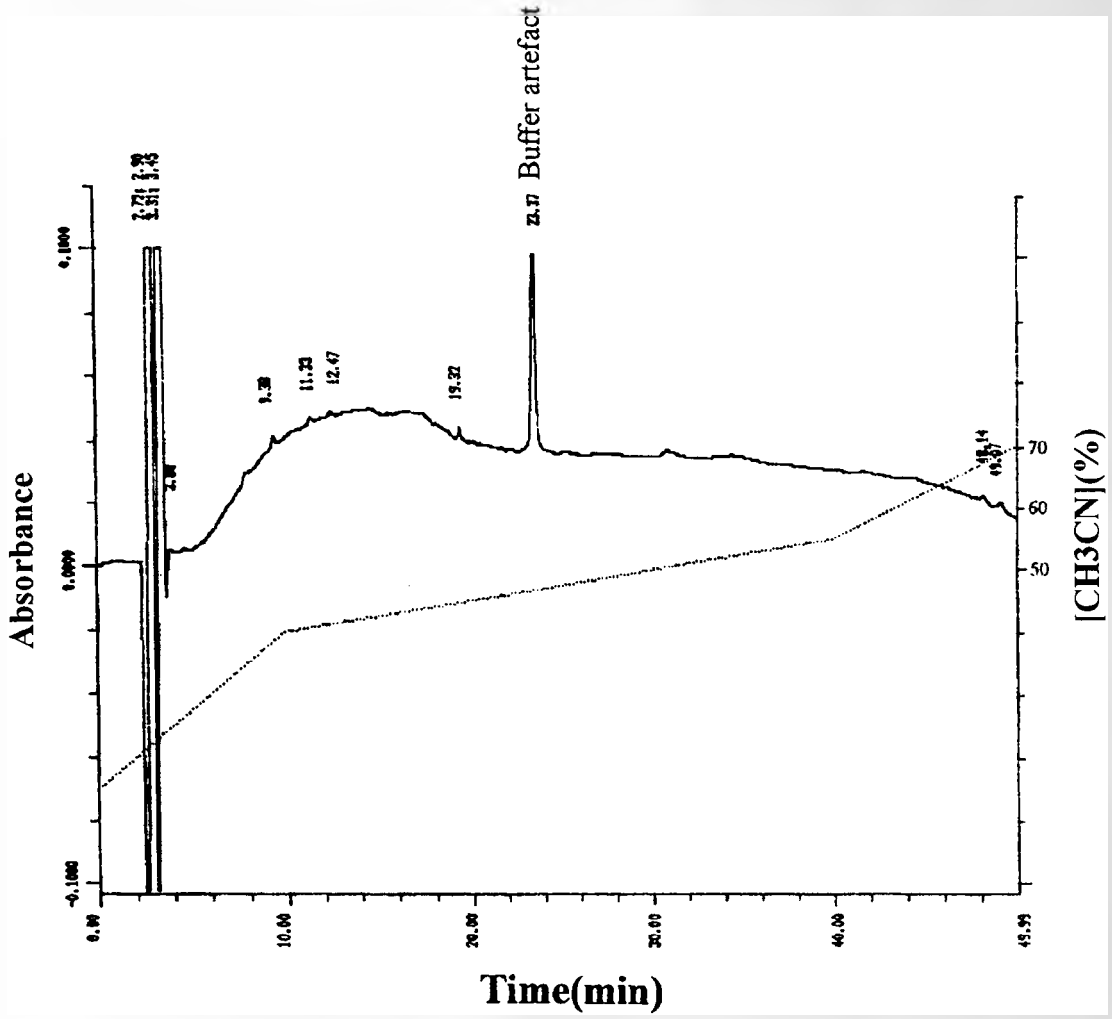


Figure 2.8 The background peaks of 20 times diluted unboiled myocardium (protein content of 412±4 $\mu g.ml^{-1}$) in phosphate buffer (0.1M; pH 5.2) as blank on a C18 column (0.46×25 cm, 5 mm). The column was eluted with a 15-70% acetonitrile gradient containing 0.09% TFA, over 50 min, at flow rate 1 $ml.min^{-1}$ (see the Method section 2.7.1 for details).

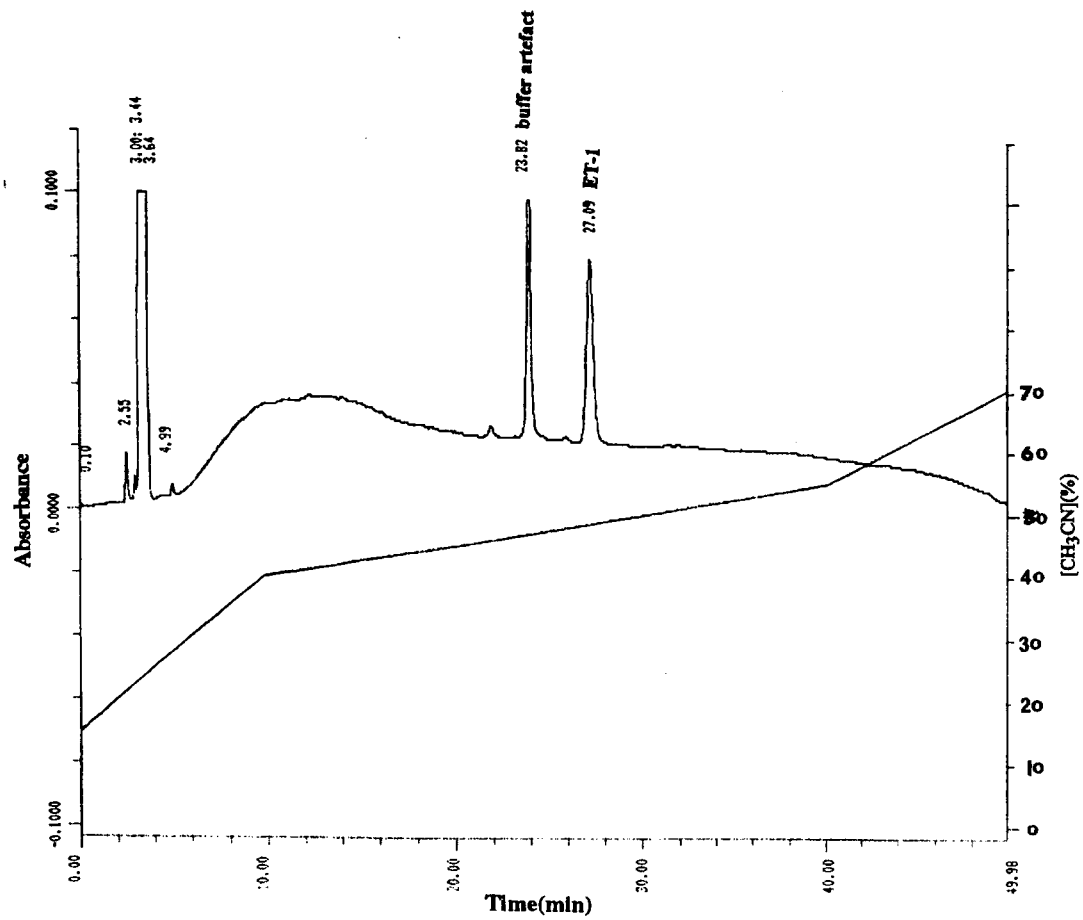


Figure 2.9 The separation of ET-1 (0.4 nmole) on a C18 column (0.46×25 cm, 5 mm) in a 15-70% acetonitrile gradient containing 0.09% TFA, over 50 min, at flow rate 1 ml.min^{-1} (see the Method section 2.7.1 for details).

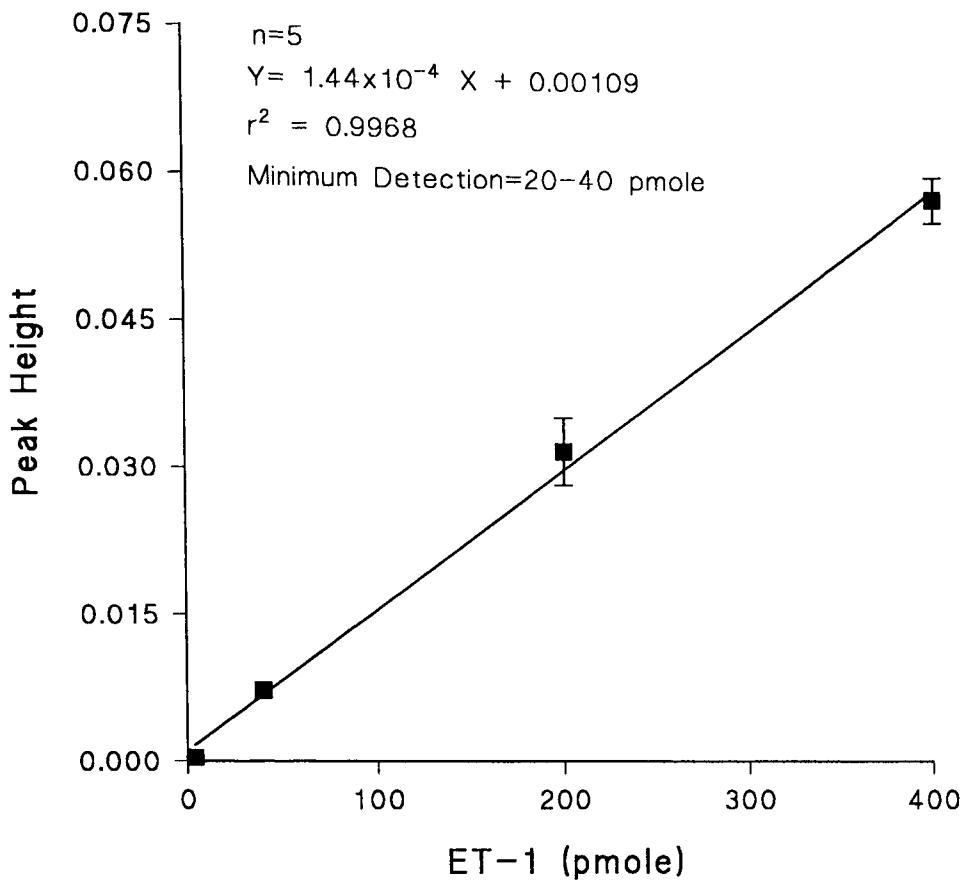


Figure 2.10 Calibration curve for ET-1 (4, 40, 200, 400 pmole) injected in 100 µl onto the HPLC system (C18 column, 0.46×25 cm, 5 µm) and eluted using 15-70% acetonitrile gradient containing 0.09% TFA, over 50 min, at a flow rate at 1 ml.min^{-1}. Points represent mean±s.e.m, n=5.

2.8 DEVELOPMENT OF A METHOD FOR ASSAY OF ET-1 DEGRADATION BY RAT VENTRICLE AND LUNG TISSUE EXTRACT

The assay of an enzymic activity in tissue extract is composed of several discrete steps. The first is preparation of the tissue homogenate, which usually contains such components as the buffer and a surfactant (such as Triton X-100) to dissolve cell membrane. The second step in the assay is the incubation, which starts with the addition of substrate to the reaction mixture. All subsequent time points are related to this time. At the final point, reactions require termination, which may be achieved in several ways, all of which usually involve inactivation of the enzyme. Termination is often followed by separation of the components in the tissue extract. Most often separation involves isolation of the substrate and reaction product from the extract components. Finally, detection refers to that process by which the amount of substrate destroyed or product formed by the enzyme is determined. Here the breakdown of ET-1 by tissue extracts of rat ventricle and lung was studied.

2.8.1 Preparation of Aqueous Tissue Extract

Male Sprague Dawley rats (300-350 g) were anaesthetized with sodium pentobarbitone (60 $mg.kg^{-1}$; i.p.). The abdomen and chest were opened along the midline and the heart and lungs removed. The lungs were washed with phosphate buffered saline (0.01M NaH_2PO_4, pH=7.2, containing 0.15M NaCl, PBS) until the tissues were cleared of blood. The hearts were cannulated via the aorta and perfused with heparinized saline (20 $IU.ml^{-1}$) and then saline alone to wash out any blood remaining in the coronary arteries. The ventricles and the lung were cut into small pieces with a pair of scissors and then homogenized (using an Ultra-Turrax® homogenizer) in phosphate buffer (0.01M, pH=7.2) containing Triton X-100 (0.1%). The homogenate was centrifuged (3000 g, 4°C, 30 min) and the supernatant was collected, divided into 0.1ml aliquots and stored frozen at -25°C until use.

2.8.2 Incubation

The supernatant was thawed immediately prior to use and diluted with phoshate buffer (ET-1 is stable in phosphate buffer) as appropriate. The diluted sample was then divided into two equal aliquots. One was boiled for 10 min in a water bath (100°C) to inactivate the enzymes and used as a blank, the other aliquot was used without boiling. Synthetic ET-1 (2 nmole) was incubated for various times with boiled and unboiled tissue extract prepared either from ventricle or lung at 25°C and varying pH. The final incubation volume was 250 µl.

2.8.3 Termination of Reaction

The degradation was stopped by boiling the incubate for 10 mins in a water bath (100°C). The boiled sample was centrifuged (3000 g, 25 min) and 200 µl of the supernatant was dried under a nitrogen stream. The residue was then dissolved in 400 µl of mobile phase and analyzed by reverse-phase HPLC.

2.9 ASSESSMENT OF ET-1 RECOVERY UNDER ASSAY CONDITIONS

It is essential that ET-1 must be stable through out the assay procedure and must be recovered in a relevant range. Since this method was to be used in this laboratory for the first time it was necessary to ascertain whether phosphate buffer, pH change, boiling and drying under nitrogen stream had any effect on ET-1 recovery from a RP-HPLC column.

2.9.1 Method

ET-1 solution (100 µl from 100 $\mu g.ml^{-1}$) was added to either 400 µl of phosphate buffer (0.1M; at either pH 8.2 or 5.2), or phosphate buffer in the presence of acetic acid (0.02M; to decrease the pH to 4.2) and incubated for 90 and 60 minutes respectively. After incubation the samples were divided into two equal volumes, one of which was diluted with 0.25 ml of mobile phase (30% CH_3CN/H_2O, containing

0.18% TFA) and then loaded onto the RP-Column (without processing). The other sample was boiled in a water bath (100°C) for 10 min and then dried under a nitrogen stream. The residue was redissolved in 0.5 ml of mobile phase (15% CH_3CN/H_2O + 0.09 TFA; with processing) and then loaded into the RP-Column and analysed as above (section 2.7.1). The samples which contained acetic acid were boiled, dried under a nitrogen stream and, before loading into the HPLC, the residue was redissolved in 1 ml of mobile phase (15% CH_3CN/H_2O + 0.09 TFA).

2.9.2 Results

The intact ET-1 and its recovery from the RP-HPLC C18 column after incubation under different conditions are shown in table 2.2. There was no significant difference between groups ($p>0.05$). Thus, incubation of ET-1 in phosphate buffer at pH 8.2 and 5.2 for 90 minutes had no effect on ET-1 stability. ET-1 was also completely stable in the presence of acetic acid and under boiling and drying conditions.

Table 2.2: Intact ET-1 recovered from C18 column after 90 minutes incubation with phosphate (0.1M) at pHs 8.2 and 5.2. The samples were assayed with and without processing. Recovered ET-1 after 60 minutes incubation with phosphate buffer (0.1M) in the presence of acetic acid (0.02M; pH 4.2) is also shown. Data represent mean±s.e.m and n=3-4.

Standard (μg)	**Intact recovered ET-1 after incubation with phosphate buffer (μg)**				
⇓	**without processing**		**with processing**		**acetic acid**
pH⇒	**5.2**	**8.2**	**5.2**	**8.2**	**4.2**
1 μg	0.96±0.02μg	0.83±0.05μg	0.84±0.07μg	0.84±0.08μg	0.96±0.01μg
Recovery⇒	96±2%	83±5%	84±7%	84±8%	96±1%

2.10 DETERMINATION OF PROTEIN CONTENT OF TISSUE EXTRACTS

This measurement was to determine the total protein content of extracts which were used for the study of in vitro degradation of rat ET-1 or big ET at different pH's. The method was based upon the dye binding reaction which was introduced by Bradford et al (1976). When dissolved in an acid-alcoholic medium, a brilliant blue G dye (Coomassie blue) reacts almost immediately with protein to form a blue-coloured protein dye complex. The complex causes a shift in the absorption maximum from 456 to 595 nm. The amount of colour produced is proportional to the protein concentration.

2.10.1 Method

1. To test tubes labelled Blank, Standard and Extract was added 2.5 ml protein assay solution (mixing 1 volume of Brilliant blue G, 35 $mg.ml^{-1}$, in phosphoric acid and methanol with 4 volumes deionized water).

2. To blank 0.05 ml phosphate buffer (0.1M with appropriate pH), to standard 0.05 ml protein standard solution (Human albumin, 30 $mg.dl^{-1}$, in saline with sodium azide, 0.1% as preservative), and to extract, 0.05 ml diluted (20 times) tissue extract were added.

3. After approximately 2 minutes the samples were transferred to cuvettes, and the absorbence [A] of standard and extract were recorded vs blank at 595 nm. Colour was stable for at least 30 minutes.

2.10.2 Calculations and Results

Protein concentration may be derived from a calibration curve or calculated directly as follows:

$$\text{protein } (\mu g.ml^{-1}) = [A]_{extract}/[A]_{standard} \times 300$$

The protein content of unboiled tissue extract is shown in table 2.3.

Table 2.3 The protein concentration of unboiled tissue extract (0.1 $g.ml^{-1}$), 20 times diluted by phosphate buffer (0.1M, pH= 5.2 and 7.2), n=5, mean±s.e.m

pH	Ventricle ($\mu g.ml^{-1}$)	Lung ($\mu g.ml^{-1}$)
5.2	412±4	400±10
7.2	480±18	427±19

2.11 IDENTIFICATION OF ET-1 AND BIG ET-1 METABOLITES PRODUCED BY RAT VENTRICULAR EXTRACT USING ELECTROSPRAY MASS SPECTROSCOPY

Big ET-1 and ET-1 (16 $nmol.ml^{-1}$) were incubated for 90 min with rat ventricular extract at pH 5.2 and 7.2 (see section 2.8 for method). The metabolite fragments were separated using HPLC, and individual peaks were collected and freeze dried. The dried peak extracts were submitted to electrospray mass spectroscopy (Fenn et al., 1989). Electrospray mass spectrometry was performed on a VG platform I mass spectrometer, with a quadrapole mass analyser, standard lens stack and electrospray source, using the VG Mass Lynx and MaxEnt software. The carrier solvent was 50/50 acetonitrile/water and the solvent flow was 10 $\mu l.min^{-1}$. Samples were dissolved in 50/50 acetonitrile/water containing 0.4% formic acid and injected directly into the carrier stream. Spectra were acquired every 4 seconds in the mass range 150-1200 atomic mass unit (amu). The masses quoted are for the peak heights for singly charged ions and the computer generated masses for multiply charged ion sets (the mass spectroscopy analysis of this study was kindly carried out by Dr. A.R. Pitt,

Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, Scotland, U.K.).

2.12 REAGENTS, STANDARDS AND DRUGS

ET-1 (rat), big ET (1-39, rat), ET (16-21), and phosphoramidon were purchased from Novabiochem and dissolved in distilled water, aliquoted as appropriate and kept at -25°C until used. BQ123 was a gift from Dr C Wilson, Zeneca Pharmaceuticals, Macclesfield, England and was dissolved in distilled water with a small amount of NaOH to aid solubility. Fresh solutions of BQ123 were prepared daily and kept on ice. Stock solutions of both ET-1 and BQ123 were diluted in physiological saline immediately prior to infusion. L-Tryptophan, aprotinin, and pepstatin A were purchased from Sigma. Pepstatin A was dissolved either in methanol or DMSO (Sigma) and kept at -25°C until used. Captopril was purchased from Squibb & Sons, INC.

2.13 STATISTICS

In vivo studies: Except for the incidence of ventricular tachycardia and ventricular fibrillation, all results are expressed as means±standard error of the mean (s.e.m), unless otherwise stated. To compare the number of ventricular ectopic beats (VEB) between groups, the Mann-Whitney non-parametric U test was employed and for incidence of ventricular tachycardia and ventricular fibrillation, the Fisher Irwin test (χ^2 with Yates Correction) was used. Mean arterial blood pressure (MAP) and heart rate (HR) data within group and between were analysed using a repeated measures ANOVA and one-way ANOVA respectively. Plasma and tissue IR-ET-1 data group were analysed using a one-way ANOVA and then significant differences were examined by the Newman-Keuls range test. Differences between groups were considered significant at a level of $p \leq 0.05$.

In vitro studies: All results are expressed as means±standard error of the mean (s.e.m), unless otherwise stated. To study the ET-1 and big ET-1 degradation and enzyme inhibitor effects, two tailed Mann-Whitney U test was used. Differences between groups were considered significant at a level of $p \leq 0.05$.

Chapter 3

THE EFFECTS OF ENDOTHELIN-1 AND THE ET_A-RECEPTOR ANTAGONIST, BQ-123, ON CARDIOVASCULAR FUNCTION AND ISCHAEMIC ARRHYTHMIAS IN ANAESTHETIZED RATS

3.1 INTRODUCTION

3.1.1 Haemodynamic Responses to Endothelin and ET_A Receptor Antagonist BQ-123

Endothelin-1 (ET-1) is a potent pressor agent in rats. Intravenous bolus administration of ET-1 produces a characteristic biphasic change in systemic blood pressure in anaesthetized rats. This response consists of an initial, transient depressor response followed by a long lasting increase in systemic arterial blood pressure (Yanagisawa et al., 1988a). Furthermore, both positive inotropic and chronotropic effects have been reported for endothelin (Hu et al., 1988; Moravec et al., 1989; Ishikawa et al., 1988a,b). There are at least two endothelin receptor subtypes which are named ET_A- and ET_B-receptors, both of which are widely distributed in cardiovascular and non-cardiovascular tissues (Masaki et al., 1991). It was originally suggested that ET_A receptors mediate vasoconstriction and ET_B receptors mediate vasodilation (Ihara et al., 1992), however vasoconstrictor effects which is possibly mediated through ET_B receptors have been reported (Clozel et al., 1992). BQ-123, (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) antagonises the ET-1 induced constriction of porcine isolated coronary artery strips and inhibits ET-1 binding to porcine aortic smooth muscle cells (Ihara et al., 1992) and has therefore been introduced as a putative ET_A receptor antagonist.

3.1.2 Endothelin in Ischaemic Heart Disease

An increasing body of evidence suggests that ET-1 plays an important role in a variety of cardiovascular diseases. Enhanced plasma levels of ET-1 have been reported in acute myocardial infarction (Miyauchi et al., 1989a), angina pectoris (Nakao et al., 1989), coronary artery vasospasm (Matsuyama et al., 1990), and congestive heart failure (Grenier et al., 1990). Furthermore several experimental observations indicate that ET-1 may be involved in the aetiology of myocardial ischaemia and the

consequences of reperfusion (Tsuji et al., 1991). Intravenous injection of ET-1 has been reported to enhance albumin extravasation in the rat coronary circulation (Filep et al., 1992), and therefore contributing to oedema formation, a characteristic feature of the inflammatory reaction associated with acute myocardial ischaemia (Entman et al., 1991). Recently Watanabe et al (1991a) reported that pre-treatment of rats with a monoclonal antibody against endothelin significantly reduced the size of the infarct caused by coronary artery ligation/reperfusion. Inhibition of the enzyme that converts pre-endothelin to endothelin by phosphoramidon was shown to have the same benefit (Grover et al., 1992). What is not clear at present, is whether ET-1 produces its effects on the coronary vasculature or whether the myocytes are the main target sites.

3.1.2.1 The Basis of Ventricular Arrhythmias Caused by Coronary Artery Occlusion

One of the important causes of death in cardiovascular disease is ventricular arrhythmias, especially ventricular tachycardia or fibrillation. Different clinical situations may cause these arrhythmias, the most important of which is ischaemic heart disease. Obviously study of drugs to reduce arrhythmias accompanying an acute myocardial infarction in patient is limited, due to unpredictability, difficulty in controlling the study, and patient safety. To overcome these limitations of clinical studies the development of animal models has a valuable role in the investigation of drug effects in acute myocardial infarction. Electrophysiological studies on ischaemic arrhythmias after a coronary occlusion have been performed on both anaesthetized and unanaesthetized experimental animals following coronary artery occlusion in a number of species. A rat model of coronary artery occlusion has been described and recommended by Clark et al (1980) as a useful model for the study of early and late occlusion-induced ventricular arrhythmias and for the study of the ischaemic myocardial injury.

When a coronary artery is ligated the ischaemic myocardial cells go through a process of reversible and irreversible injury with a myocardial infarction as the result. During the early phase of coronary artery occlusion (i.e. first 30 minute) the incidence of ventricular arrhythmias is high and clinically important, therefore this thesis aimed to study the early phase of arrhythmia associated with acute myocardial infarction. The severity of the acute ventricular arrhythmias that occur after experimental ligation of the coronary artery is related to the distance of the occlusion from the origin of the artery i.e the area at risk. Ligation of the left coronary artery near its origin causes the development of severe arrhythmias, whereas an occlusion sited below the first major branch distal to the origin, results in a reduced incidence of arrhythmias.

The mechanism underlying ventricular arrhythmias during myocardial ischaemia is not well understood. Cardiac muscle cells are electrically excitable and a temporary increase in membrane permeability to sodium ions due to depolarisation causes this excitability. In the normal heart the spontaneous and intrinsic rhythm is generated by the specialised cells of the sino-atrial node which produces action potentials which are propagated through the myocardium via the conducting system. The action potential of a cardiac muscle cell is divided into four phases: *Phase 0*, the rapid depolarisation, caused by inward current of sodium ions via sodium channels; *Phase 1*, the partial repolarisation, occurs as the Na^+ current is inactivated; *Phase 2*, the plateau, results from an inward calcium current; *Phase 3*, repolarisation, when the calcium current is inactivated and potassium permeability is increased; *Phase 4*, the pacemaker potential, is a gradual depolarisation during diastole (Rang and Dale, 1992; figure 3.1).

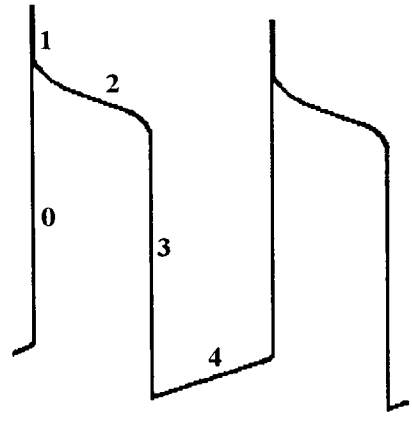


Figure 3.1 The cardiac action potential. 0 = rapid depolarization; 1 = partial repolarization; 2 = plateau; 3 = repolarization; 4 = pacemaker depolarization.

Ventricular arrhythmias may result from abnormalities of impulse initiation and/or impulse propagation through the heart. Hoffman and Cranefield (1964) suggested that three mechanisms may be responsible for cardiac arrhythmias: abnormal impulse generation, abnormal impulse conduction, or a combination of both. In the normal heart automatic rhythms have maximum diastolic potentials of approximately -65 to - 70 mv (Rang and Dale, 1992). In contrast to normal automaticity, **abnormal automaticity** is generated in fibres that are depolarised, to as low as -60 to -50 mv (Le Marec et al., 1985). This reduction in the resting potential can be caused by several conditions, such as ischaemia and high levels of catecholamine which are known to be released during ischaemia (Hirche et al., 1980). The relationship between ischaemia (and its consequences such as hypoxia and acidosis) and the reduction in resting potential has been studied extensively (Kleber et al., 1983; Dresdner et al., 1987). One of the early consequences of ischaemia is K^+ loss from the cell and a resultant extracellular K^+ accumulation. Because the ratio of $[K^+]_o/[K^+]_i$ is the major determinant of the resting potential, as $[K^+]_o$ rises the resting potential is displaced to more positive voltages (Binah et al., 1992). At low membrane potentials the fast

inward Na^+ current responsible for the normal action potential upstroke is inactivated and therefore causes a reduction in action potential amplitude, rate of rise of the action potential and a slowing of the conduction velocity of a propagating action potential.

During hypoxia and ischaemia a reduction in intracellular ATP activates ATP sensitive potassium efflux through the ATP-K^+ channels (Nichols et al., 1991), causing a $[K+]_o$ rise, and produces the above mentioned electrophysiological changes which may be responsible, at least in part, for the ischaemic arrhythmias. Furthermore, depletion of ATP due to hypoxia during ischaemia will attenuate Na^+-K^+ pump activity and consequently produce a low membrane potential. Moreover, during ischaemia intracellular pH decreases to acid pH (Khandoudi et al., 1990) and it has been shown that a decreased pH causes an elevation in $[K^+]_o$ and a decrease in resting potential (Skinner et al., 1976).

Another important cause of cardiac arrhythmias is abnormality in an impulse conduction and the main mechanism for this is **reentry**. In the intact heart, the term reentry refers to a certain area of the myocardium (or the whole heart) which is re-excited by a circulating impulse. Reentry may occur if conduction is changed in some way (for example due to a slowing of conduction or block in the conductive bundle) so that a propagating impulse can return to a recently excited area and find excitable tissue. In this case, re-excitation will occur and the impulse will be distributed throughout the heart. The possibility for a circulating impulse to be generated as a reentrant excitation depends upon a combination of the length of the damaged area, electrical activity such as a reduction in effective refractory period (ERP), and a low conduction velocity, which is in turn determined directly by the resting potential. In the ischaemic myocardium, in addition to reduction of conduction velocity, the conductive tissue is also damaged. Some studies show that electrical alternans during

early ischaemia does not immediately lead to onset of arrhythmias, but rather the appearance of arrhythmias coincides with recovery of action potential amplitude as well as conduction time and QRS width (Penny et al., 1983), while ventricular fibrillation is always associated with reduction in ERP. One important determinant of conduction velocity is the degree of cellular coupling. As the degree of intercellular uncoupling is higher the resistance to current flow is greater, thereby reducing conduction velocity. Experimentally, intercellular coupling can be attenuated by interventions that are associated with ischaemia, such as $[Ca^{2+}]_i$ overload and acidosis (De Mello, 1985).

A third cause of ventricular arrhythmias may be due to *triggered activity*. Triggered arrhythmias originate from oscillations in the membrane potential. These oscillations are called early after-depolarization (EADs) or delayed after-depolarizations (DADs). EADs occur during phase 2 or 3 of the action potential, whereas DADs occur after the termination of repolarization. If after-depolarizations reach a threshold value, an action potential may be produced. If this action potential results in the generation of another after-depolarization, sustained rhythmic activity may follow (Josephson and Gottlieb, 1990).

DADs have been observed under conditions which lead to an increase in $[Ca^{2+}]_i$ or to Ca^{2+} overload. Catecholamines which are released during myocardial ischaemia can lead to the appearance of, or to an increase, in amplitude of delayed after-depolarization. Catecholamine-induced changes in intracellular Ca^{2+} concentration are likely to be the mechanism that triggers the development of after-depolarizations (Schwartz et al., 1990). DADs also have been recorded from canine subendocardium after infarction (El-Sherif et al., 1983) and from hypertrophied ventricular myocardium (Aronson 1981) in all of which $[Ca^{2+}]_i$ is probably increased secondary to the disease state. EADs occur under a variety of experimental conditions, such as

hypoxia and high levels of catecholamines (Binah et al., 1992), both of which can be present in the ischaemic and infarcted myocardium. Moreover an increase in inward Ca^{2+} current may play a role in generating EADs (Coulombe et al., 1984).

3.1.2.2 The Arrhythmogenic Effects of Endothelin

The initial interest in endothelin focused on its biphasic effects on vascular smooth muscle (Yanagisawa et al., 1988a,b). However, evidence of a positive inotropic effect was soon reported for rat atria (Hu et al., 1988), pig atria (Ishikawa et al., 1988b), human atria (Davenport et al., 1989), and rabbit pupillary muscle (Watanabe et al., 1989b). A positive chronotropic effect has also been reported for ET-1 (Ishikawa et al., 1989a). At the same time, endothelin-specific high affinity binding sites were identified in cardiac myocytes (Hirata et al., 1989a), cardiac membranes (Gu et al., 1989a), sinus node (Yamasaki et al., 1989) and on the coronary arteries (Power et al., 1989). In addition, upregulation of cardiac ET-1 receptors is a well known phenomenon. Ischaemia and reperfusion of the rat heart causes an increase in ET-1 receptor binding sites (Liu et al., 1990), indicating that the action of ET-1 may be enhanced under ischaemic conditions. Furthermore, Clozel and Sprecher (1991) have demonstrated that in open-chest dogs, the coronary constriction and reduction of epicardial-to-endocardial blood flow induced by intracoronary administration of ET-1 is intensified under conditions of low (40mmHg) coronary perfusion pressure. This shows an increased sensitivity to ET-1 during low-flow states. It has also been reported that ischaemia and reperfusion of the isolated perfused heart enhances the coronary vasoconstriction elicited by ET-1 (Neubauer et al., 1990).

The vasoconstrictor action of ET-1 has been demonstrated in both isolated and in situ coronary arteries (Godfraind et al., 1989; Clozel and Clozel, 1989). Administration of ET-1 into the coronary artery of dogs (Nichols et al., 1990), pigs (Ezra et al., 1989), and rats (Yorikane et al., 1990), causes coronary artery constriction and ST segment

elevation of the electrocardiogram (ECG) similar to the clinical phenomena of variant angina was observed. Moreover, in a further study, the intracoronary administration of ET-1 induced not only ST segment elevation of the ECG, due to coronary artery constriction, but also arrhythmias involving atria-ventricular block (A-V block), ventricular premature contraction and ventricular fibrillation, and resulted in death in most animals (Harada et al., 1993). In addition to its vasoconstrictive effects, ET-1 could also contribute to the electrophysiological consequences of ischaemia and reperfusion, such as arrhythmias. A direct pro-ischaemic effect of ET-1, which was independent of ET-1 vasomotor actions, has been shown during global ischaemia (Grover et al., 1992). Yorikane et al. (1991) have demonstrated that ET-1 causes prolongation of the action potential duration (APD) in the right bundle branch of canine myocardial tissue, and that is followed by the development of early afterdepolarization (EADs), suggesting that arrhythmias caused by ET-1 are at least partly attributable to direct actions of ET-1 on myocardial cells.

3.1.3 The Effects of BQ-123, an Antagonist at ET_A-Receptors, on Ischaemic Arrhythmias

A variety of studies indicate that ET-1 is involved in the aggravation of the process of myocardial ischaemia, either through coronary constriction or through direct myocardial effects, but it is not clear which endothelin receptor subtype mediates these effects. Two well known receptor subtypes which have been cloned are ET_A and ET_B and mediate the biological actions of endothelin (Arai et al., 1990; Sakurai et al., 1990). The vasoconstrictor effects of ET-1 are mainly due to the action on the ET_A receptors, although ET_B receptors also mediate some vasoconstriction (Bigaud and Pelton, 1992; Moreland et al., 1992). In addition, binding studies recently described a new subtype of ET receptors in rat brain and atrium (Sokolovsky et al., 1992) which is different from ET_A receptors. They suggested that there are at least two subtypes of ET_B receptor, named ET_{B1} receptor (super-high affinity sites with

affinities in the picomolar range) and ET_{B2} receptor (high affinity sites with affinities in the nanomolar range). The ET_{B1} sites have been linked to the vasodilator property of endothelins, whereas the ET_{B2} sites participate in their vasoconstrictor action (Sokolovosky et al., 1992). Moreover, in that study it was shown that ET_{B1} receptors do not induce phosphoinositide hydrolysis whereas the ET_{B2} receptors do, demonstrating that they are linked to different intracellular signalling processes. Pharmacological studies have also suggested the existence of a third, non ET_A/ET_B receptor subtype in the pig coronary artery, which like the ET_A receptor, may mediate a vasoconstrictor action (Harrison et al., 1992).

The characterization of ET receptors prompted the development of selective ET receptor antagonists. BQ-123, which is a cyclic penta peptide, binds selectively to ET_A receptors (Ihara et al., 1992) and has therefore been classed as an ET_A antagonist. A subsequent study by Sokolovsky (1993), with BQ-123, characterized both pM and nM sites in rat myocytes and suggested the possible existence of a specific site for BQ-123 that interacts and/or interferes with the properties of ET-binding sites. Similar to their previous study of 1992, it was shown that in rat myocytes pM sites were not associated with phoshoinositide hydrolysis, whereas ET-1 at nanomolar or higher concentrations produced the accumulation of inositol phosphates through an action on the nM receptors. Furthermore, Teerlink et al (1994) showed in a study on canine coronary arteries, an ET_BH receptor (high affinity ET_B binding sites), with picomolar sensitivities to ET-1, which closely resembled the ET_{B1} receptors found in rat brain and atria (Sokolovsky et al., 1992). It has been reported that BQ-123 attenuates the pre- and post-ischaemic effects of endothelin-1 in isolated rat heart (Grover et al., 1992). Moreover, another study (McMurdo et al., 1994) has shown that blockade of ET_A receptors with FR139317 (a selective ET_A receptor antagonist) reduced the mortality due to ventricular fibrillation, 5-20 minute after

coronary artery occlusion, in rabbits. To date the possible involvement of endogenous endothelin and ET_A receptors in ischaemic arrhythmias has not been assessed.

3.1.4 The Effects of BQ-123 on Infarct Size in a Rat Model of Acute Myocardial Ischaemia and Reperfusion

Experimental studies have shown that restoration of coronary blood flow to the ischaemic area after coronary artery occlusion (reperfusion) causes injury and necrosis in the myocardium that was previously ischaemic. As described previously in this chapter, it has been speculated that the endogenous release of ET-1 may play a role in the extension of ischaemia/reperfusion injury. This idea is supported by the finding in anaesthetized rats (Watanabe et al., 1991a) and in anaesthetized rabbits (Kusumoto et al., 1993) that a monoclonal antibody against ET-1 reduced infarct size following coronary artery ligation and reperfusion. Likewise it has been shown that infusion of phosphoramidon, the endothelin converting enzyme inhibitor, resulted in a reduction in infarct size in rats (Grover et al., 1992). Studies with selective ET_A antagonists have shown these drugs to either decrease (Grover et al., 1993) or to have no effect (McMurdo et al., 1994) on infarct size, whereas a mixed ET_A/ET_B antagonist reduces infarct size (Richard et al., 1994).

3.1.5 Aims

To determine the degree of antagonism by BQ-123 of the in vivo haemodynamic responses to ET-1, a group of rats were prepared for measurement of heart rate, arterial blood pressure and left ventricular pressure for calculation of left ventricular $dP.dt^{-1}_{max}$ and $LVdP.dt^{-1}.P^{-1}$ as two indices of myocardial contractility.

In view of the very limited data on the role of ET-1 on arrhythmias during myocardial ischaemia, this study attempted to answer two questions -(i) does exogenous ET-1 exacerbate ischaemia-induced arrhythmias and, if so, is this mediated by

ET_A-receptors? and -(ii) does endogenous endothelin contribute to the severity of arrhythmias during an ischaemic insult by an action at ET_A-receptors? The aim of the first part of this study was therefore to determine whether or not ET-1, at doses which are not arrhythmogenic in the normal heart, enhances the severity of arrhythmias induced by acute myocardial ischaemia. The second part studied the effects of the ET_A-receptor antagonist, BQ-123, on ischaemic arrhythmias alone and its ability to reverse the proarrhythmic effects of endothelin during myocardial ischaemia. The last part of this study was performed to assess the effects of the ET_A receptor antagonist, BQ-123, on myocardial injury following a combined ischaemia-reperfusion insult in anaesthetised rats.

3.2 EXPERIMENTAL PROTOCOLS

3.2.1 To Assess the Effects of ET-1 on Haemodynamic Factors in Anaesthetized Rats and Their Antagonism by BQ-123

This protocol examined the haemodynamic responses to ET-1 in the presence and absence of BQ-123 infusion. For this purpose animals were anaesthetized and prepared for measurement of mean arterial blood pressure (MAP), heart rate (HR) and left ventricular pressure (LVP) as described in Chapter 2 (section 2.1.1). In the initial series of experiments, cumulative i.v. bolus doses of ET-1 (0.14, 0.42, 0.98 and 2.10 $nmol.kg^{-1}$) were administered to 8 rats, via the left femoral vein. The doses were separated by intervals of 10 minute. The parameters were measured when they reached maximum value. In the other two groups infusion of BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$, n=7; and 100 $\mu g.kg^{-1}.min^{-1}$, n=5) into the right femoral vein was commenced 10 minute prior to bolus injection of the first dose of ET-1, using the same ET-1 dosing schedule as above, and maintained during the experimental period.

3.2.2 To Assess the Effects of ET-1 on Ischaemic Arrhythmias

In these experiments male Sprague-Dawley rats (280-320 g) were anaesthetized and prepared for a low-sited ligation of the left coronary artery as described in Chapter 2 (section 2.2.3), thus the ligature around the coronary artery was placed below the first branch (low-sited ligature) to give an approximate area at risk of 25% (by weight) of the free left ventricular wall. This site of occlusion yields a model with mild ischaemia and less-severe arrhythmias and therefore low arrhythmia count which allows the detection of the proarrhythmic effect of drug intervention. Following 15 minute of stabilization, rats were allocated to one of three groups (n=9)- *i*) Saline infusion (control, 0.5ml); *ii*) ET-1 0.05 $nmol.kg^{-1}.min^{-1}$; *iii*) ET-1 0.1 $nmol.kg^{-1}.min^{-1}$. Infusions via the right femoral vein were commenced 5 minute prior to occlusion of the coronary artery and maintained during the period of occlusion (30 minute). Coronary artery occlusion was induced by tightening the loose ends of the ligature and maintained for a period of thirty minute. Mean arterial blood pressure and heart rate were calculated from the electrocardiogram. Arrhythmias were analysed to determine the total number of single ventricular ectopic beats, salvos and ventricular tachycardia (VT), and the incidence of VT, and reversible and irreversible ventricular fibrillation (VF) were determined for each group. The protocol is illustrated in figure 3.2.

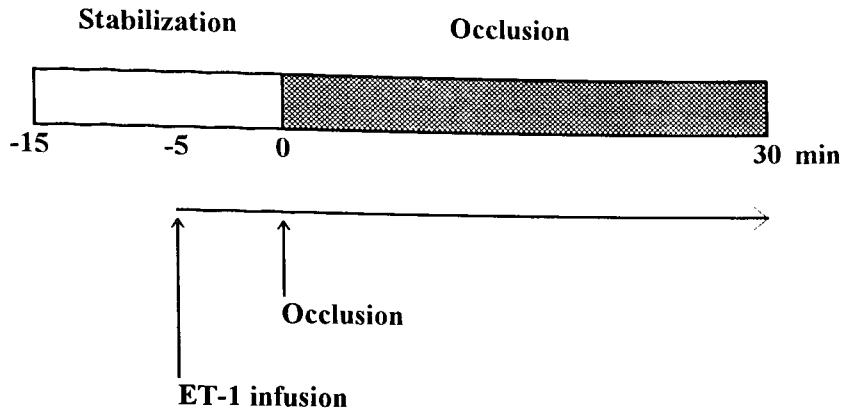


Figure 3.2 Experimental protocol. In this protocol following 15 minute stabilization anaesthetized rats were subjected to a 30 minute occlusion of the left coronary artery. Saline (as control) and ET-1 (0.05 and 0.1 nmol.kg^{-1}.min^{-1}) infusions were commenced 5 minutes before occlusion and maintained throughout the experimental period.

3.2.3 To Assess the Effects of BQ-123 on Ischaemic Arrhythmias

This Protocol examined the effects of the ET_A receptor antagonist, BQ-123, on ischaemic arrhythmias. For these experiments, coronary occlusion was performed at a higher position (just below the left atrial appendage) to produce an area at risk of approximately 50% of the free left ventricular wall and a higher yield of arrhythmias. This allowed assessment of the ability of BQ-123 to suppress ischaemia-induced arrhythmias. Following 15 minute of stabilization, rats were allocated to one of five groups- *i*) control (saline infusion; n=17); *ii*) BQ-123, 5 μg.kg^{-1}.min^{-1} (n=9); *iii*) BQ-123, 10 μg.kg^{-1}.min^{-1} (n=10); *IV*) BQ-123, 50 μg.kg^{-1}.min^{-1} (n=10) and *V*) BQ-123, 100 μg.kg^{-1}.min^{-1} (n=8). Infusions into the right femoral vein were commenced 5 minute prior to occlusion of the coronary artery and maintained during the experimental period. Coronary artery occlusion was achieved by threading the loose ends of the ligature through a polyethylene occluder and clamping in place and was maintained for a period of thirty minute, during which time haemodynamic

parameters and arrhythmias were recorded and analysed as described in Chapter 2 (section 2.2.6). The protocol is illustrated in figure 3.3.

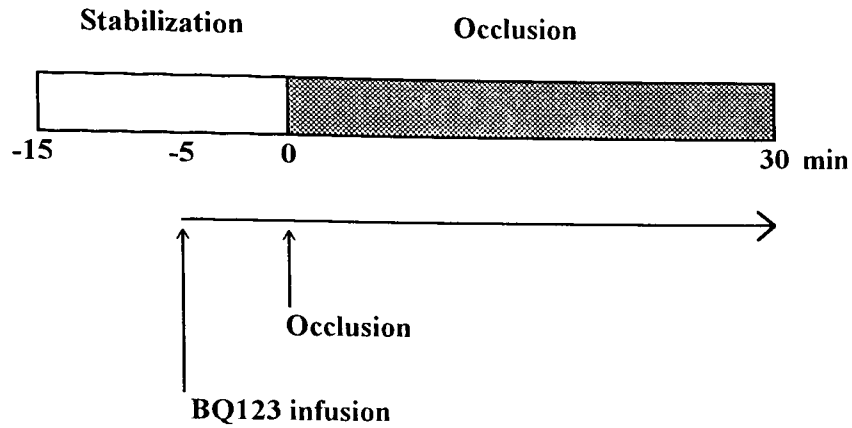


Figure 3.3 Experimental protocol. In this protocol following 15 minute stabilization anaesthetized rats were subjected to a 30 minute occlusion of the left coronary artery. Saline (as control) and BQ-123 (5, 10, 50, and 100 $\mu.kg^{-1}.min^{-1}$) infusions were commenced 5 minutes before occlusion and maintained throughout the experimental period.

3.2.4 To Assess the Effects of BQ-123 on Endothelin-Enhanced Arrhythmias

In these experiments rats were prepared for a low-sited ligation of the coronary artery. Following a 15 minute stabilization period saline (0.5ml, control; n=9) or ET-1 (0.05 $nmol.kg^{-1}.min^{-1}$, n=11) was infused from right jugular vein, commencing 5 minute before coronary artery occlusion and maintained for period of thirty minute. In the third group (n=10), BQ-123 (10 $\mu g.kg^{-1}.min^{-1}$) was infused into the right femoral vein, starting 10 minute before coronary artery occlusion, followed 5 minute later by an ET-1 infusion (0.05 $nmol.kg^{-1}.min^{-1}$) into the right jugular vein. The dose of BQ-123 was chosen on the basis of the results from the experiments performed in section 3.3.5 of this chapter. The protocol is illustrated in figure 3.4.

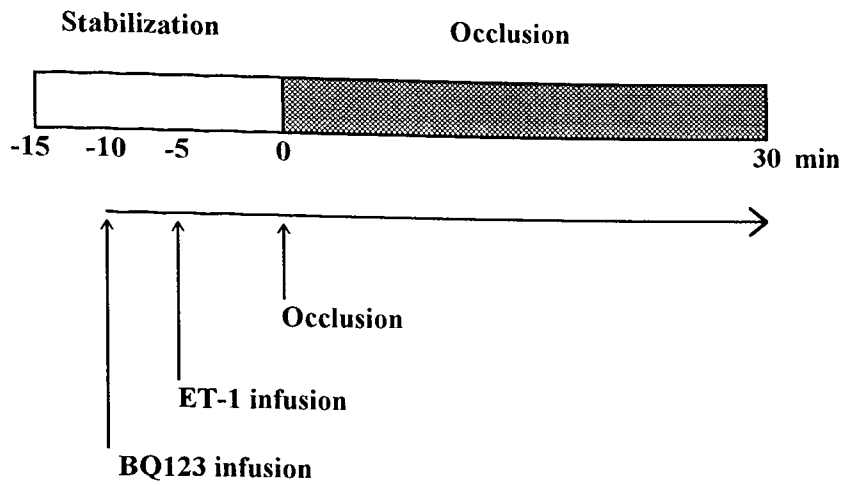


Figure 3.4 Experimental protocol. In this protocol following 15 minute stabilization anaesthetized rats were subjected to a 30 minute occlusion of the left coronary artery. BQ-123 (10 $\mu g.kg^{-1}.min^{-1}$) and ET-1 (0.05 $nmol.kg^{-1}.min^{-1}$) infusions were commenced 10 and 5 minutes before occlusion respectively and maintained throughout the experimental period.

3.2.5 To Assess the Effects of BQ-123 on Infarct Size

This protocol examined the effects of the ET_A receptor antagonist, BQ-123, on myocardial infarct size. In 6 of the saline-treated rat and 6 rats given BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) in protocol 3.2.3 the period of ischaemia was followed by 3.5 hours reperfusion for determination of the effects of BQ-123 on infarct size. Coronary occlusion was achieved by threading the loose ends of the ligature through a polyethylene occluder and clamping in place. Release of the clamp allowed reperfusion of the previously ischaemic tissue. Coronary occlusion was maintained for a period of thirty minute, this was followed by 3.5 hours of reperfusion, after which the animals were killed by an overdose of anaesthetic and the hearts removed for subsequent measurement of infarct size (percentage of area at risk) as described in Chapter 2 (section 2.3). The haemodynamic parameters were recorded throughout the experimental period. The protocol is illustrated in figure 3.5.

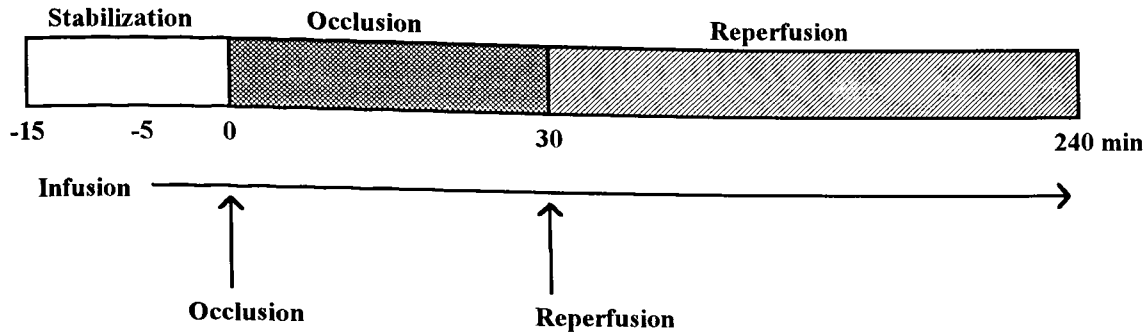


Figure 3.5 Experimental protocol. In this protocol following 15 minute stabilization anaesthetized rats were subjected to a 30 minute occlusion of the left coronary artery followed by 3.5 hours of reperfusion. BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) infusion was commenced 5 minute before occlusion and maintained through the experimental period.

3.3 RESULTS

3.3.1 Effects of Endothelin Receptor Antagonist, BQ-123, on Haemodynamic Responses Induced by ET-1 in Anaesthetized Rats

The typical haemodynamic responses to ET-1 in an anaesthetized rat are illustrated in figure 3.6 and the results for the three experimental experimental groups are summarised in table 3.1. In anaesthetized rats, i.v. cumulative bolus injections of ET-1 (from 0.14 $nmol.kg^{-1}$ to 2.1 $nmol.kg^{-1}$) induced a dose-dependent increase in the arterial blood pressure, which reached a maximum increase of 45±1.7 mmHg at 2.1 $nmol.kg^{-1}$ of ET-1. The pressor effect was long lasting and superseded the dose-independent initial transient (lasted about 2 min) depressor phase.

Infusion of saline (as control) or any of the tested doses of BQ-123 for 10 minutes before starting the bolus injections of ET-1 had no effect on the mean arterial blood, heart rate, $LVdP.dt^{-1}_{max}$, or $LVdP.dt^{-1}.P^{-1}$. Infusion of both doses of BQ-123

(50 and 100 $\mu g.kg^{-1}.min^{-1}$, which equate to 0.08 and 0.16 $\mu mol.kg^{-1}.min^{-1}$) shifted the cumulative dose-response curve of the effect of ET-1 on arterial blood pressure to the right (figure 3.7). In the presence of 50 $\mu g.kg^{-1}.min^{-1}$ of BQ-123, the pressor effect of ET-1 at the cumulative doses of 0.98 and 2.10 $nmol.kg^{-1}$ was inhibited by 58±3% and 60±4%, respectively and by 79±4% and 68±4% in the presence of 100 $\mu g.kg^{-1}.min^{-1}$ of BQ-123. BQ-123 had no effect on the initial depressor response induced by ET-1 (figure 3.8).

$LVdP.dt^{-1}_{max}$, was increased by ET-1 in saline-treated controls from 3341±164 ($mmHg.S^{-1}$) before injection of ET-1 to a maximum of 3884±200 ($mmHg.S^{-1}$) with 0.98 $nmol.kg^{-1}$ of ET-1 (figure 3.9). The lower dose of BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) completely abolished the ET-1-induced increase in $LVdP.dt^{-1}_{max}$, whereas in the presence of the higher dose of BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$), ET-1 produced a dose-dependent decrease in $LVdP.dt^{-1}_{max}$. $LVdP.dt^{-1}.P^{-1}$ was not affected significantly by i.v injection of ET-1 (figure 3.10), however there was a tendency for $LVdP.dt^{-1}.P^{-1}$ to be increased as $LVdP.dt^{-1}_{max}$ was increased by ET-1. In the presence of both doses of BQ-123, $LVdP.dt^{-1}.P^{-1}$ remained unchanged. At the doses studied, neither ET-1 nor BQ-123 altered heart rate significantly (figure 3.11).

Table 3.1: Mean arterial blood pressure (MAP, mmHg), heart rate (HR, beats.min^{-1}), LVdP.dt^{-1}max (mmHg.s^{-1}) and LVdP.dt^{-1}.P^{-1} (s^{-1}) in anaesthetised rats given cumulative doses of ET-1 in the presence of either saline infusion (control), BQ-123 (50 μg.kg^{-1}.min^{-1}) or BQ-123 (100 μg.kg^{-1}.min^{-1}).

Treatment		ET-1 Cumulative Concentration (nmol.kg^{-1})				
		0.00	**0.14**	**0.42**	**0.98**	**2.10**
Control (saline infusion) n=8	**MAP**	99±8	106±8**	114±8**	128±7***	144±8***
	HR	308±12	302±11	295±10	292±11	279±12
	LVdP.dt^{-1}	3341±164	3468±153	3633±164	3884±200*	3742±202
	LVdP.dt^{-1}.P^{-1}	21.51±0.5	22.05±0.6	22.36±0.6	22.06±.6	20.91±.8
BQ-123 (50 μg.kg^{-1}.min^{-1}) n=7	**MAP**	86±6	92±5	92±5	98±5♣	104±4♣
	HR	330±12	319±14	296±14	300±15	277±17
	LVdP.dt^{-1}	3319±163	3316±173	3270±174	3299±126♣	3482±133
	LVdP.dt^{-1}.P^{-1}	21.41±0.5	21.21±0.7	21.18±0.7	21.07±0.7	20.90±1.0
BQ-123 (100 μg.kg^{-1}.min^{-1}) n=5	**MAP**	82±3	85±3	84±2	88±2♣	96±2♣
	HR	323±18	320±16	313±15	304±15	292±15
	LVdP.dt^{-1}	3056±43	2985±26	2783±106*	2561±250**♣♣	2396±326**♣♣
	LVdP.dt^{-1}.P^{-1}	20.73±0.1	20.60±0.1	20.93±0.1	21.23±0.2	21.33±0.1

Data represents mean±s.e.m and n=number of rats; ***p<0.001, **p<0.01 and *p<0.05 denotes significant differences within groups, from pre-injection value (i.e at concentration zero); and ♣♣p<0.01, ♣p<0.05 shows significant differences between groups vs. same point in the controls.

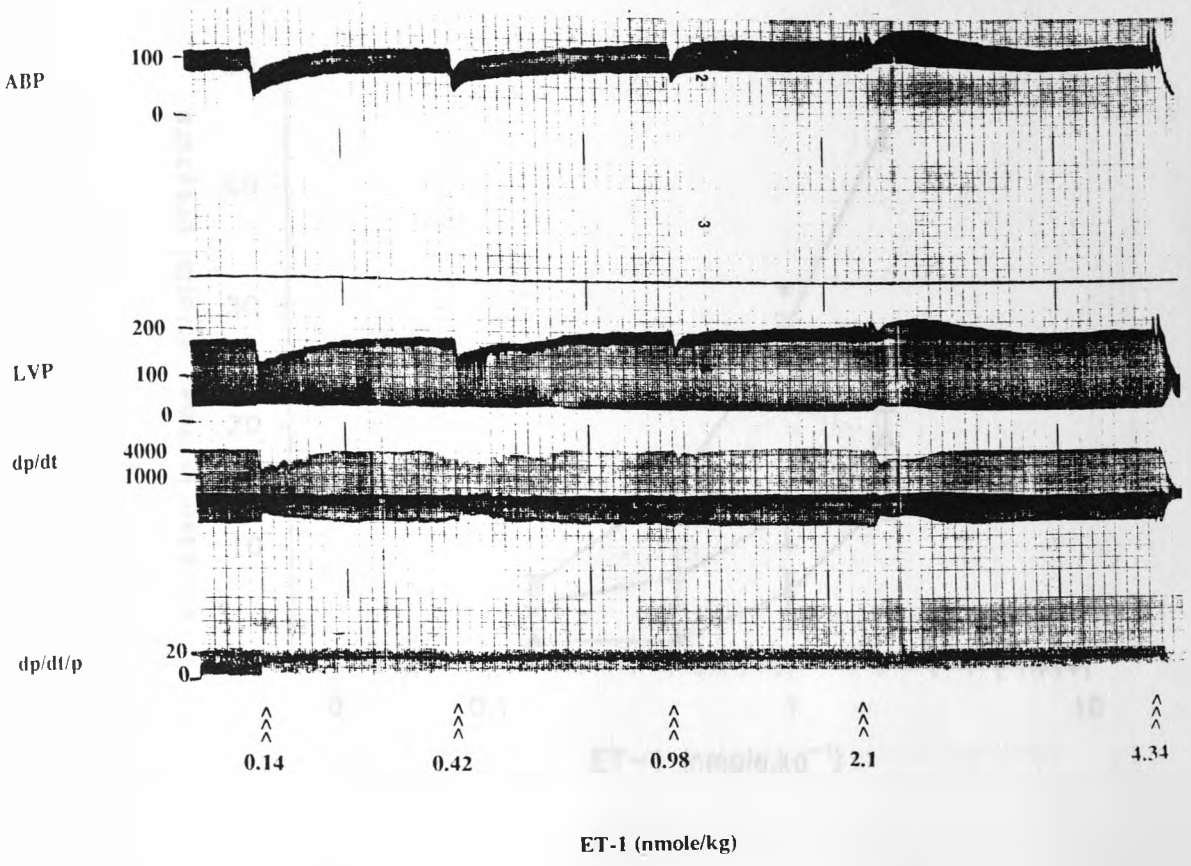


Figure 3.6 Typical experimental tracing of mean arterial pressure (MAP), left ventricular pressure (LVP), $LVdP.dt^{-1}_{max}$ and $LVdP.dt^{-1}.P^{-1}$ responses to i.v cumulative bolus injection of ET-1 in anaesthetized rats.

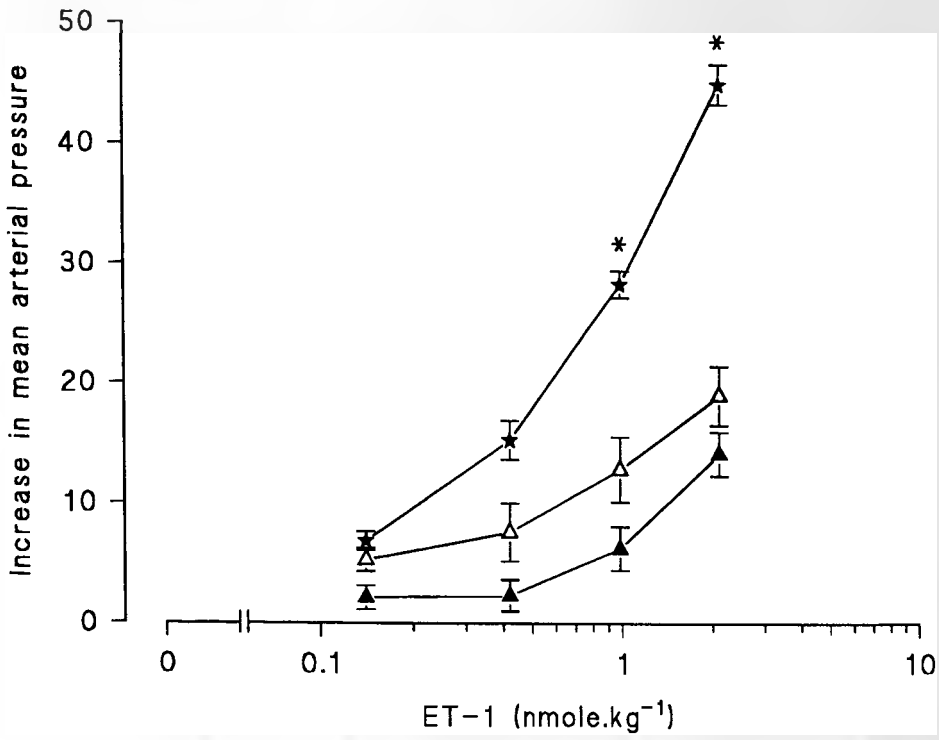


Figure 3.7 Cumulative dose-response curves for the pressor effects of ET-1 on mean arterial blood pressure in anaesthetized rats during infusion of saline (control, n=8, *), BQ-123 (50 μg.kg^{-1}.min^{-1}, n=7, Δ) or BQ-123 (100 μg.kg^{-1}.min^{-1}, n=5, ▲). Points represent the mean±s.e.m. Significant differences ($p<0.05$) between saline and BQ123 treated groups are denoted by *.

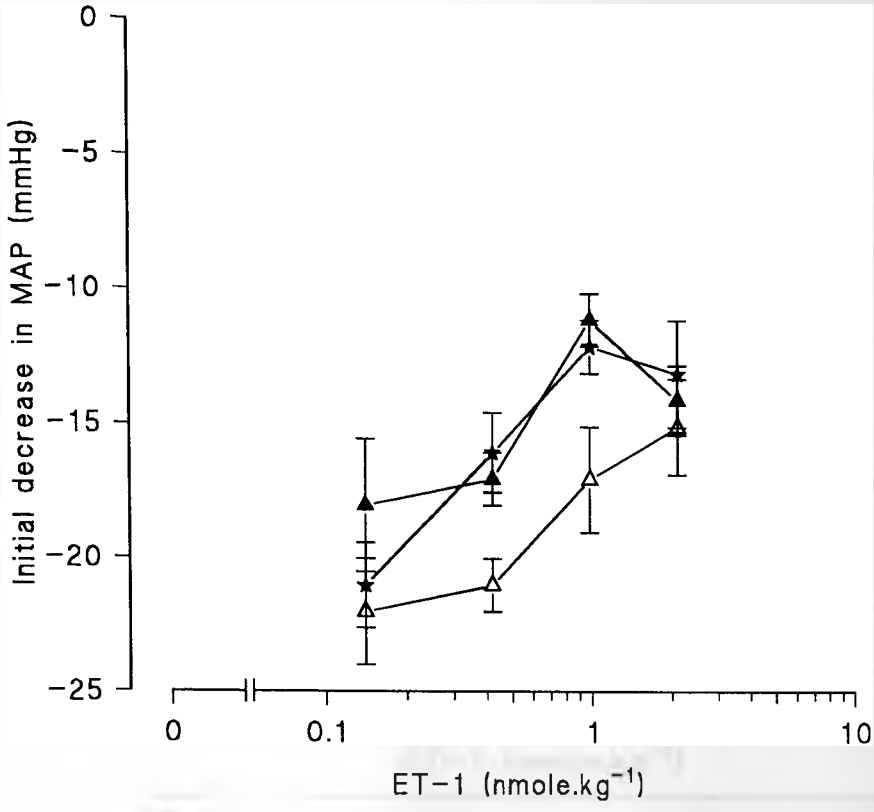


Figure 3.8 Dose response curves for the systemic depressor actions of ET-1 on mean arterial blood pressure in anaesthetized rats during infusion of saline (control, n=8, *), BQ-123 (50 μg.kg^{-1}.min^{-1}, n=7, Δ) or BQ-123 (100 μg.kg^{-1}.min^{-1}, n=5, ▲). Points represent the mean±s.e.m.

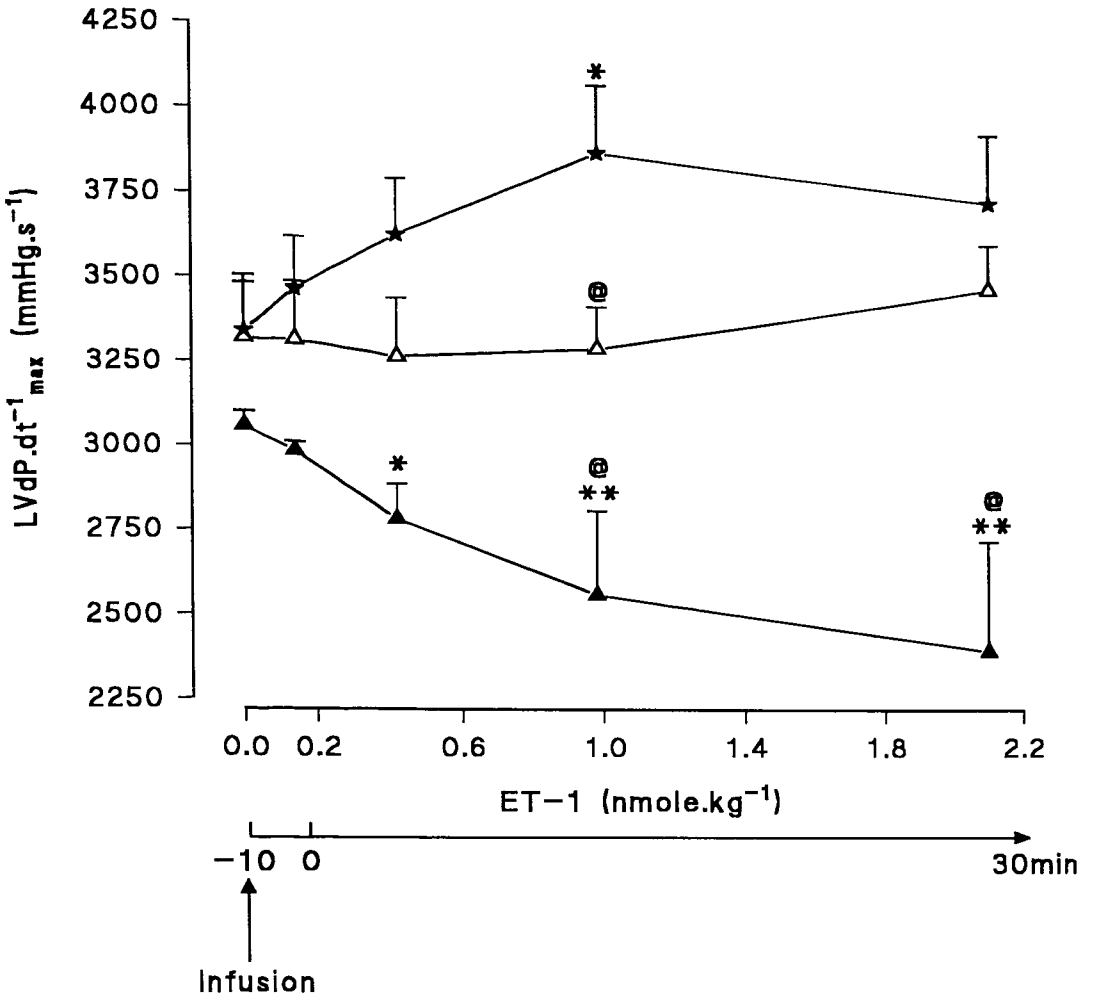


Figure 3.9 The change in contractile index $LVdP.dt^{-1}_{max}$ in response to cumulative doses of ET-1 (0.14, 0.42, 0.98 and 2.1 $nmol.kg^{-1}$) in the presence of an infusion of saline (control, n=8, *), BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$, n=7, Δ) or BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$, n=5, ▲). Infusion of saline or BQ-123 was started 10 min prior ET-1 bolus injection given at time 0. Points represent the mean±s.e.m. Significant differences ($p<0.05$ or 0.01) between BQ-123 and saline treated groups are denoted by @, and differences within groups from the pre-injection value (i.e at concentration zero) denoted by *.

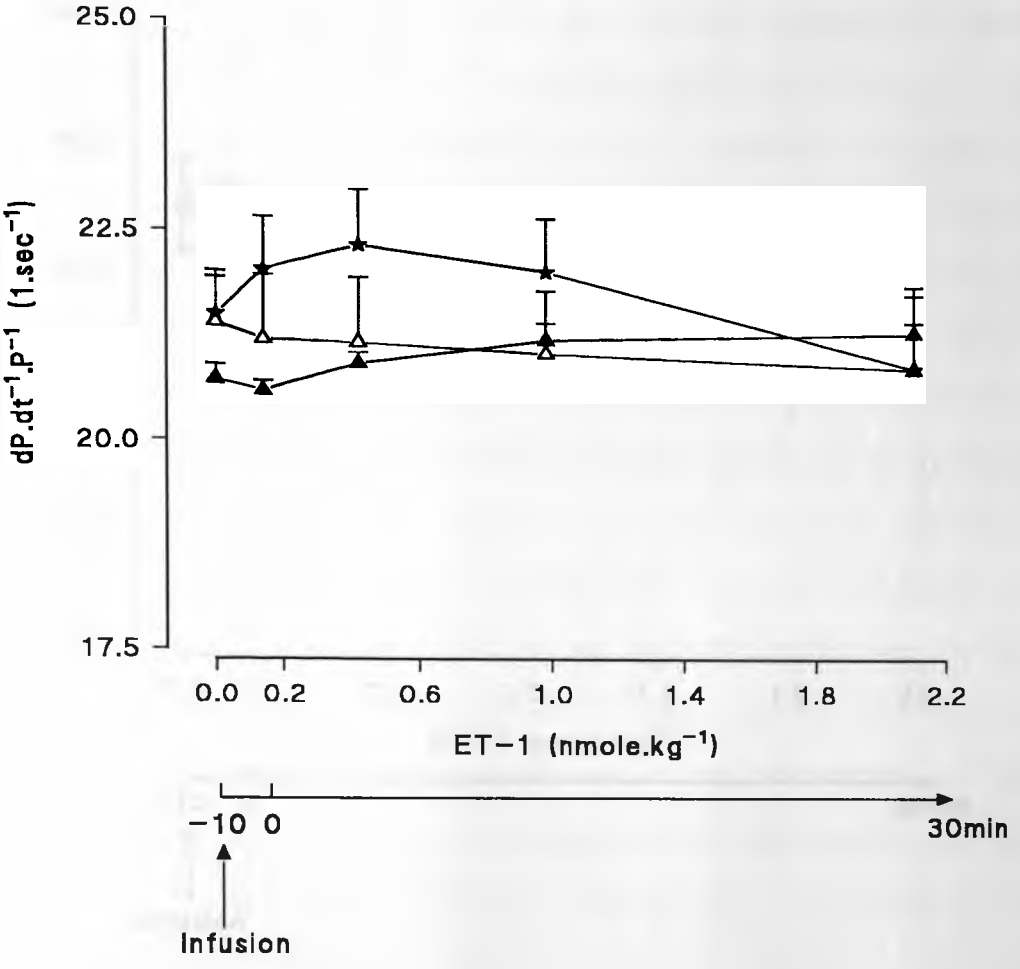


Figure 3.10 The change in contractile index $dP.dt^{-1}.P^{-1}$ in response to cumulative doses of ET-1 (0.14, 0.42, 0.98 and 2.1 nmol.kg^{-1}) in the presence of an infusion of saline (control, n=8, *), BQ-123 (50 μg.kg^{-1}.min^{-1}, n=7, Δ) or BQ-123 (100 μg.kg^{-1}.min^{-1}, n=5, ▲). Infusion of saline or BQ-123 was started 10 min prior to ET-1 bolus injection given at time 0. Points represent the mean±s.e.m.

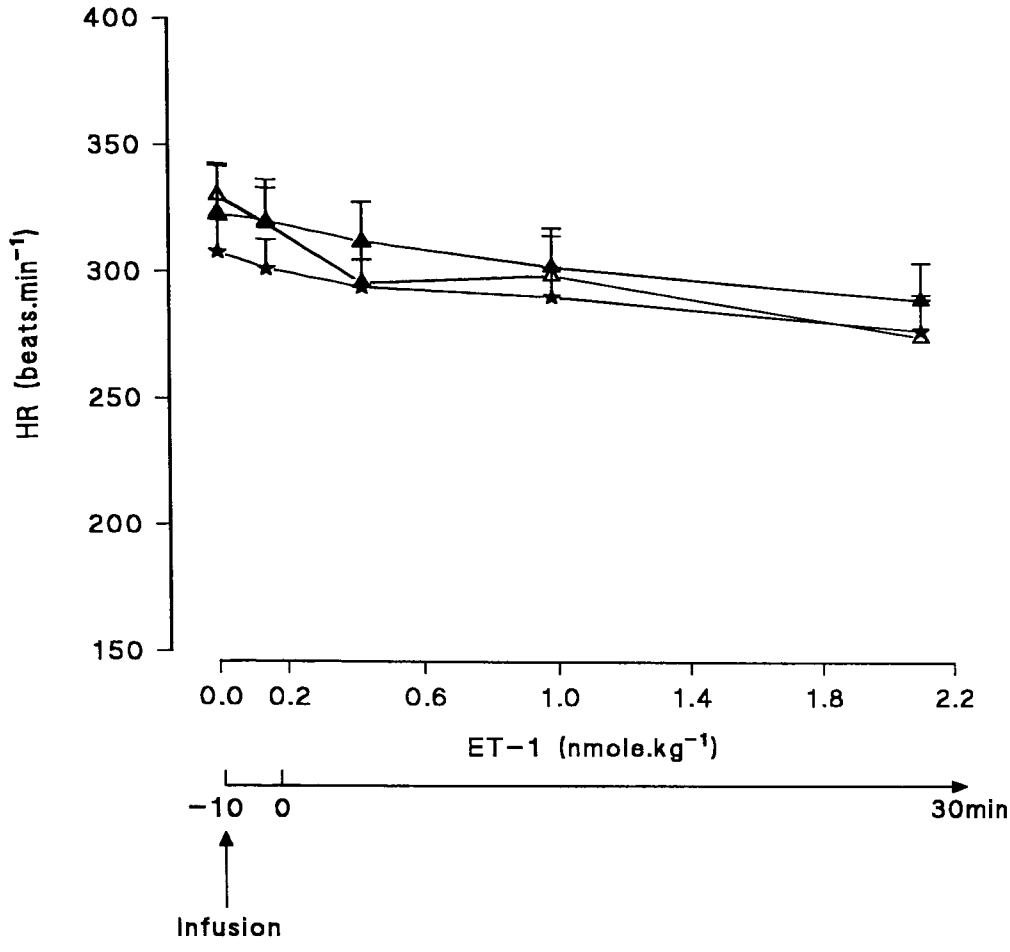


Figure 3.11 The change in heart rate (HR, beats.min^{-1}) in response to cumulative doses of ET-1 (0.14, 0.42, 0.98 and 2.1 nmol.kg^{-1}) in the presence of an infusion of saline (control, n=8, *), BQ-123 (50 μg.kg^{-1}.min^{-1}, n=7, Δ) or BQ-123 (100 μg.kg^{-1}.min^{-1}, n=5, ▲). Infusion of saline or BQ-123 was started 10 min prior to ET-1 bolus injection given at time 0. Points represent the mean±s.e.m.

3.3.2 The Effects of ET-1 on Ischaemic Arrhythmias

Coronary artery occlusion in rats resulted in immediate ST-segment changes and, after approximately 5-7 minutes, the development of severe ventricular arrhythmias with a characteristic temporal pattern of distribution as illustrated in figure 3.12. Thus, in most animals, ectopic beats commenced around 4-5 minutes post-occlusion and reached maximum activity around 9-12 minutes post-occlusion and then began to decline gradually. By 30 minutes post-occlusion all ventricular ectopic activity had ceased. Table 3.2 summarises the effects of ET-1 on the arrhythmias following coronary artery occlusion in anaesthetised rats. Coronary artery occlusion in control and treated rats resulted in ventricular ectopic beats with maximum activity between 5 and 12 minutes (figure 3.12). At a dose of 0.1 $nmol.kg^{-1}.min^{-1}$, ET-1 produced a significant increase in the total number of ectopic beats (table 3.2) which led to a peak around 8-9 minutes (figure 3.12). The increase ($p<0.02$) in the total number of ventricular ectopic beats seen with infusion of ET-1 (0.1 $nmol.kg^{-1}.min^{-1}$) occurred as a result of an increase in the number of beats occurring as ventricular tachycardia (figure 3.13). The number of single extrasystoles and salvos were unaffected by either dose of ET-1. The lower dose of ET-1 (0.05 $nmol.kg^{-1}.min^{-1}$) had no effect on the number of ventricular ectopic beats.

Both doses of ET-1 induced a marked increase in the total incidence of ventricular fibrillation (VF), although mortality due to irreversible VF was unaffected (figure 3.14). Furthermore, the time spent in both VT and reversible VF was significantly ($p<0.05$) increased by ET-1 (figure 3.15). The mean time to development of ventricular fibrillation in the group receiving low dose of ET-1 (0.05 $nmol.kg^{-1}.min^{-1}$), was significantly decreased from 7.7 ± 0.33 minutes in control to 6.1 ± 0.33 minutes post-occlusion ($p<0.05$; figure 3.15). In contrast, the higher dose of 0.1 $nmol.kg^{-1}.min^{-1}$ did not alter time to onset of VF (8.1 ± 1.03 min).

Table 3.2: The effects of ET-1 on ischaemic arrhythmia.

Arrhythmia	Control	ET-1(nmol.kg^{-1}.min^{-1})	
		0.05	0.1
n	9	9	9
Arrhythmia Counts			
Single VEB's	247±46	229±37	217±55
Salvos	54±12	50±15	44±9
VT	255±32	236±61	768±179*
Total VEB's	556±75	515±93	1029±182*
Duration (sec)			
VT	18±3	18±4	62±14**
Reversible VF	3±3	24±7*	22±11*
Time to VF (min)	7.7±0.33	6.1±0.33*	8.1±1.03
Incidence (%)			
VT	100	100	100
Reversible VF	11	56*	56*
Irreversible VF	22	44	33
Total VF	33	100*	88*

*P<0.05; **P<0.01 compared to controls. Total VEB's is the sum of arrhythmias occurring as single extrasystoles, salvos and ventricular tachycardia.

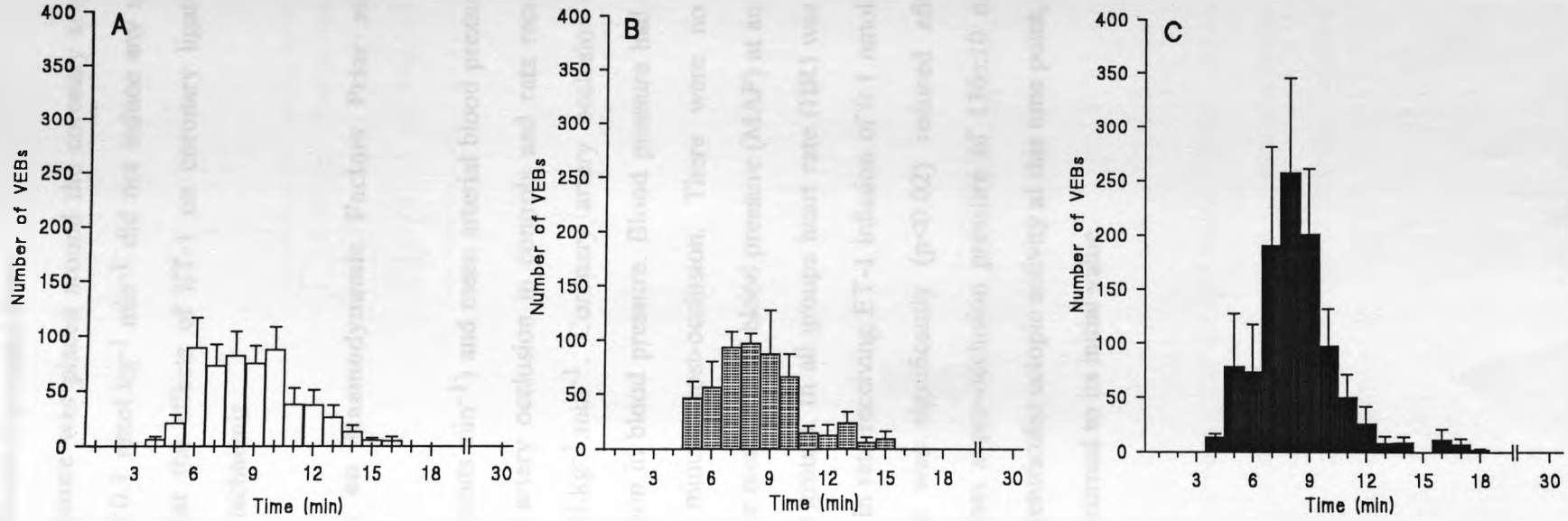


Figure 3.12 Distribution of ventricular ectopic beats (VEB's) over 1 min intervals for the 30 min occlusion of the left coronary artery, in rats receiving saline (Control, A), ET-1 (0.05 nmol.kg^{-1}.min^{-1}, B) and ET-1 (0.1 nmol.kg^{-1}.min^{-1}, C) infusion. Note the marked increase in the total number of VEBs in rats receiving high dose of ET-1 (C).

3.3.3 The Effects of ET-1 in Sham-Operated Rats

In sham-operated rats (i.e. ligature was placed around the coronary artery and left untied; n=5) ET-1 infusion of 0.1 nmol.kg^{-1}.min^{-1} did not induce any spontaneous arrhythmias. This confirms that the effects of ET-1 on coronary ligation-induced arrhythmias was related to the ischaemia.

3.3.4 The Effects of ET-1 on Haemodynamic Factors Prior and During Coronary Artery Occlusion

Figure 3.16 shows heart rate (beats.min^{-1}) and mean arterial blood pressure (mmHg) prior to and during coronary artery occlusion in controls and rats receiving ET-1 infusion of 0.05 or 0.1 nmol.kg^{-1}.min^{-1}. Coronary artery occlusion in controls resulted in an transient reduction in blood pressure. Blood pressure had returned to pre-occlusion levels by 15 minute post-occlusion. There were no significant differences in heart rate (HR) or mean arterial blood pressure (MAP) at any time point during ET-1 infusion between groups. In all groups heart rate (HR) was unchanged from the pre-occlusion value. In rats receiving ET-1 infusion of 0.1 nmol.kg^{-1}.min^{-1} mean arterial blood pressure was significantly ($p<0.02$) reduced after 10 min occlusion by 25±8 mmHg, from a pre-occlusion pressure of 136±10 mmHg. This reduction was due to marked ventricular ectopic activity at this time point, after which mean arterial blood pressure returned to its initial value.

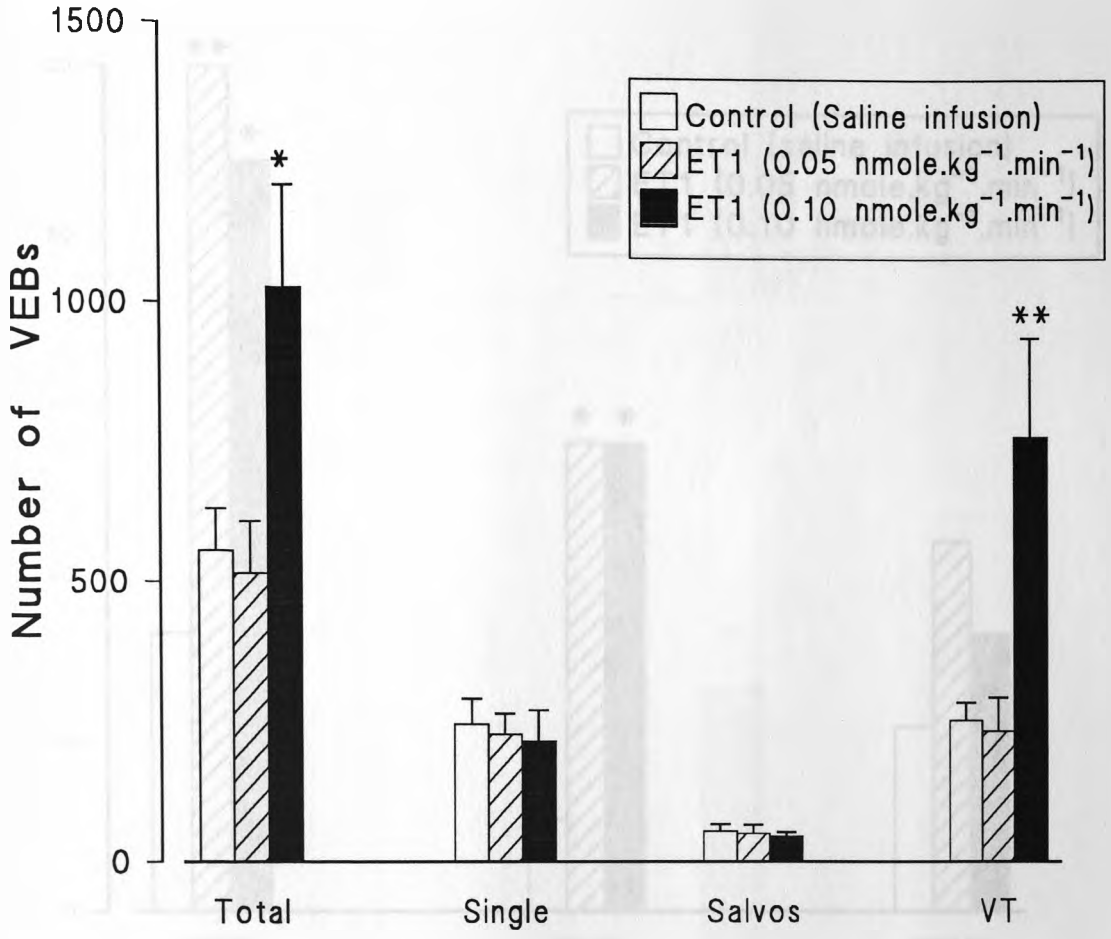


Figure 3.13 The total number of ventricular ectopic beats (VEBs), and the number of arrhythmias appearing as single extrasytoles, salvos and ventricular tachycardia (VT) in controls and rats receiving ET-1 infusion. *$p<0.05$, **$p<0.01$ compared to control.

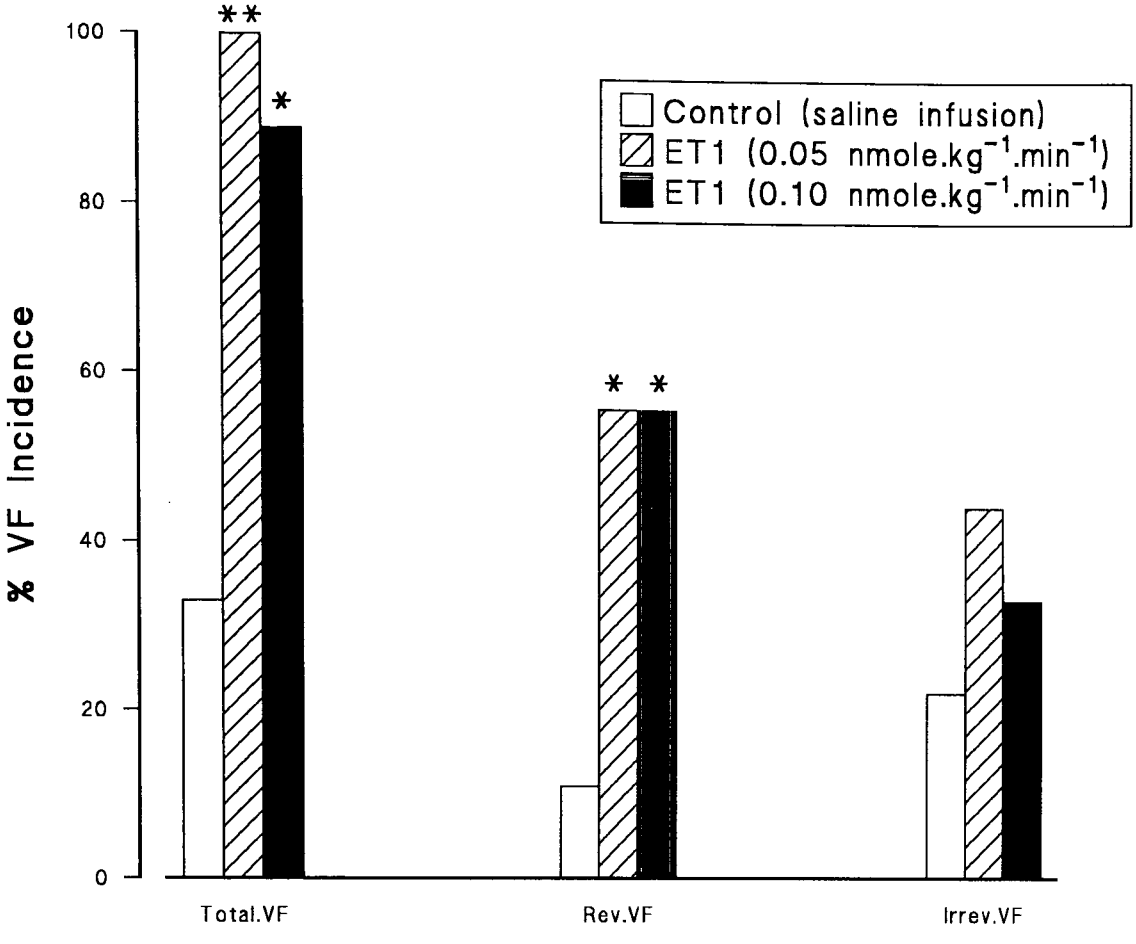


Figure 3.14 The incidences of total, reversible (Rev.) and irreversible (Irrev.) ventricular fibrillation (VF) during 30 min occlusion period of the left coronary artery in rats receiving saline (control) and ET-1 infusion. *$p<0.05$, **$p<0.01$ compared to control.

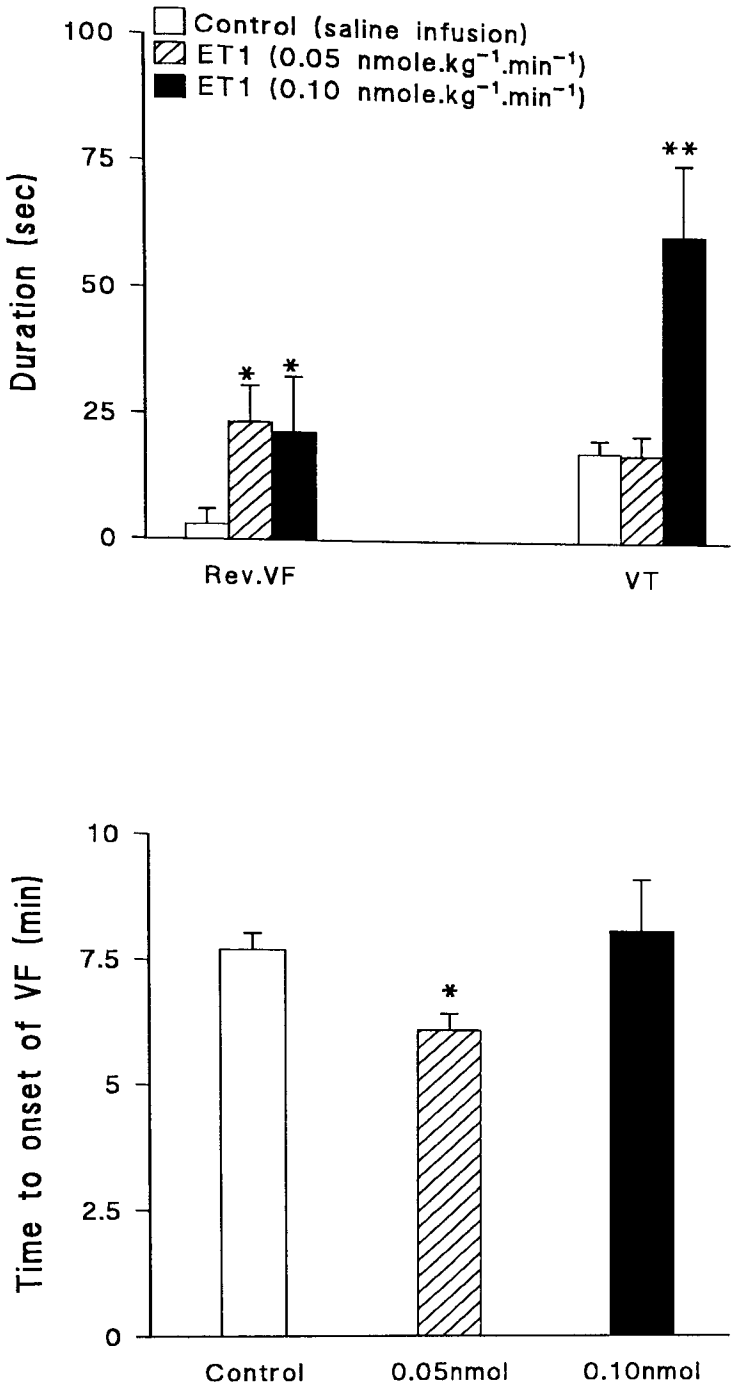


Figure 3.15 The effects of ET-1 infusion on the time (sec) spent in ventricular tachycardia (VT) and reversible ventricular fibrillation (Rev. VF; upper panel) and on the mean time (min) to onset of VF (lower panel). *$p<0.05$, **$p<0.01$ compared to control.

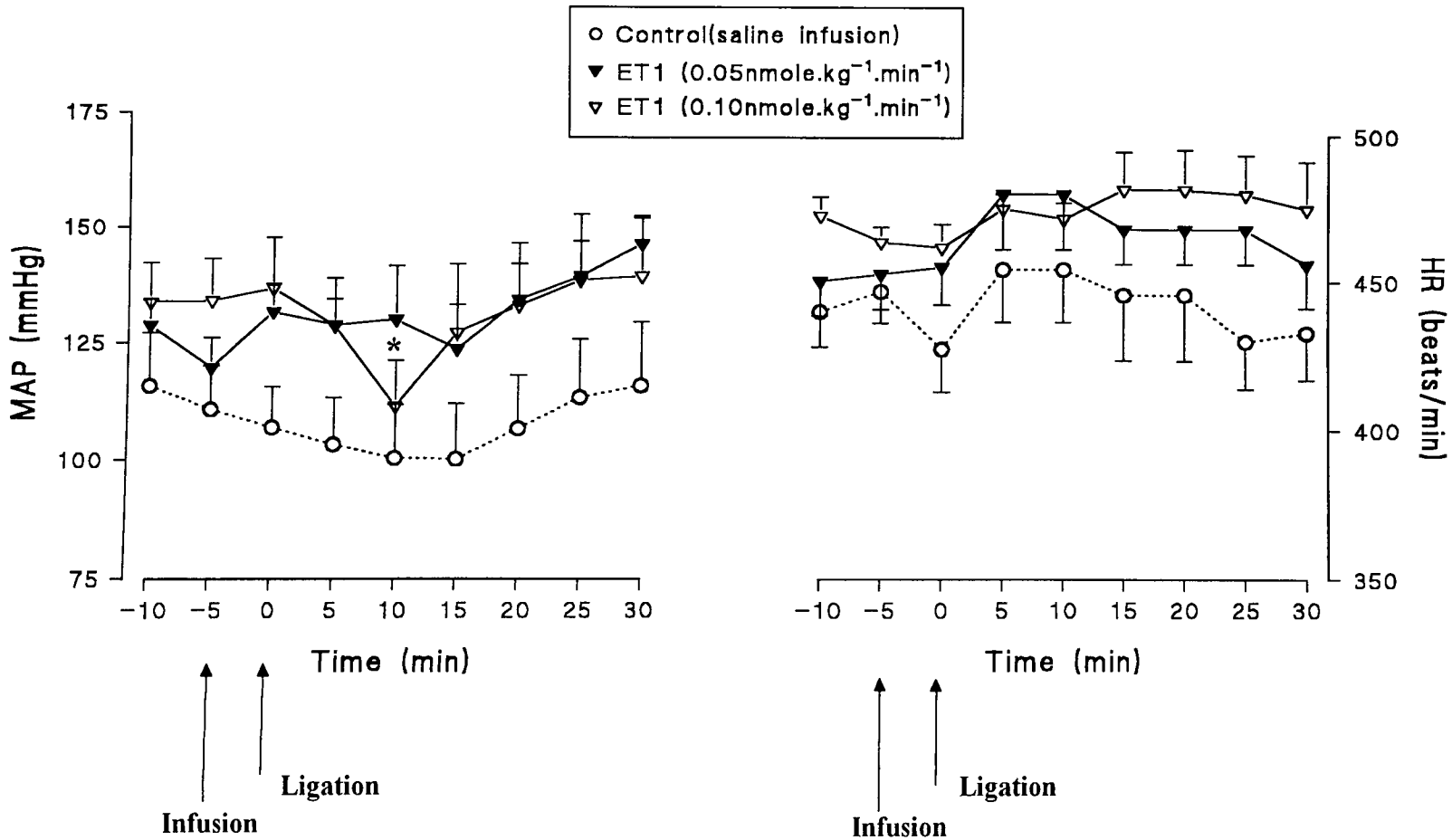


Figure 3.16 Heart rate (beats.min^{-1}) and mean arterial pressure (mmHg) changes prior to and during coronary artery occlusion in controls and rats receiving ET-1. In both treated groups ET-1 infusion was commenced 5 min before coronary artery occlusion (performed at time 0 min) and maintained for the duration of the experiment. *P<0.05 compared to preocclusion pressure.

3.3.5 The Effects of BQ-123 on Ischaemic Arrhythmias

Acute coronary artery ligation in these experiments also resulted in arrhythmias with a characteristic temporal pattern of distribution, as illustrated in figure 3.17. Similar to previous experiments, in most animals, ectopic beats commenced around 4-5 minutes post-occlusion and reached maximum activity around 9-12 minutes post-ligation and then began to decline gradually. BQ-123 suppressed the number of ventricular ectopic beats during the peak of activity (figure 3.17). Figure 3.18 and table 3.3 summarise the total arrhythmia counts and the number of arrhythmias appearing as the different types of arrhythmia seen over the 30 minute occlusion period during saline or BQ-123 infusion. Compared to control, BQ-123 in a dose of 10 $\mu g.kg^{-1}.min^{-1}$ significantly ($p<0.01$) reduced the total number of ventricular ectopic beats as a result of decreasing all types of arrhythmias. This dose of BQ-123 also reduced the duration of ventricular tachycardia (figure 3.20; $p<0.05$). At 5 and 50 $\mu g.kg^{-1}.min^{-1}$, BQ-123 infusion tended to decrease the total number of ventricular ectopic beats (as a result of reduction in the number of beats occurring as ventricular tachycardia) and the time spent in ventricular tachycardia, although these reductions did not attain statistical significance compared to control (figures 3.18, 3.20). With the highest dose of BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$) there was an increased incidence of irreversible ventricular fibrillation (see below), thus no arrhythmia counts could be performed.

Figure 3.19 summarises the effects of four doses of BQ-123 on the incidence of ventricular fibrillation. The groups receiving the doses of 5, 10, and 50 $\mu g.kg^{-1}.min^{-1}$ of BQ-123 had similar incidence of reversible ventricular fibrillation to that seen in controls (figure 3.19). The duration of the episodes of reversible fibrillation was also unchanged by drug treatment (figure 3.20). In the group of rats receiving the higher infusion dose of BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$), however, while a similar total incidence of fibrillation to controls was observed (6 out of 8), in all of these rats it was irreversible. Thus, in this group mortality due to irreversible ventricular

fibrillation was significantly higher than in controls. Figure 3.19 shows that the incidence of irreversible fibrillation increase as the dose of BQ-123 increases. Thus, as shown in figure 3.21 a significant positive correlation existed between the incidence of irreversible VF and the dose of BQ-123.

3.3.6 Effects of BQ-123 on Haemodynamic Variables Before and During Coronary Artery Occlusion

Mean arterial blood pressure (MAP) and heart rate (HR) from 5 minute before commencing BQ-123 infusion (5, 10 and 50 $\mu g.kg^{-1}.min^{-1}$), to the end of the 30 minute period of ischaemia are illustrated in figure 3.22. There was a reduction in MAP 10 minute after coronary artery occlusion in all groups which was due to the appearance of arrhythmias at this time. BQ-123 induced no significant changes in the mean arterial blood pressure and heart rate during the pre-occlusion infusion period. Since infusion of BQ-123 at the higher dose (100 $\mu g.kg^{-1}.min^{-1}$) resulted in a high mortality shortly after occlusion, haemodynamic data for this group is not available. None of the doses of BQ-123 had any significant effects on either blood pressure or heart rate at any time point when compared to controls receiving an infusion of saline at the same rate (500 $\mu l.hour^{-1}$).

Table 3.3: The effects of BQ-123 on ischaemic arrhythmias.

Arrhythmia	Control	BQ-123 ($\mu g.kg^{-1}.min^{-1}$)			
		5	10	50	100
n	17	9	10	10	8
Arrhythmia Counts					
Single VEB's	262±41	168±35	122±27*	287±51	–
Salvos	68±9	45±12	29±10*	55±16	–
VT	1092±143	618±282	525±142*	656±270	–
Total VEB's	1423±112	832±289	677±159**	998±301	–
Duration (sec)					
VT	98±13	53±25	50±12*	59±24	–
Reversible VF	22±11	32±26	20±14	53±28	–
Incidence (%)					
VT	100	100	100	100	–
Reversible VF	35	33	20	30	–
Irreversible VF	24	11	30	40	75*
Total VF	59	44	50	70	75*

*P<0.05; **P<0.01 compared to controls. Total VEB's is the sum of arrhythmias occurring as single extrasystoles, salvos and ventricular tachycardia.

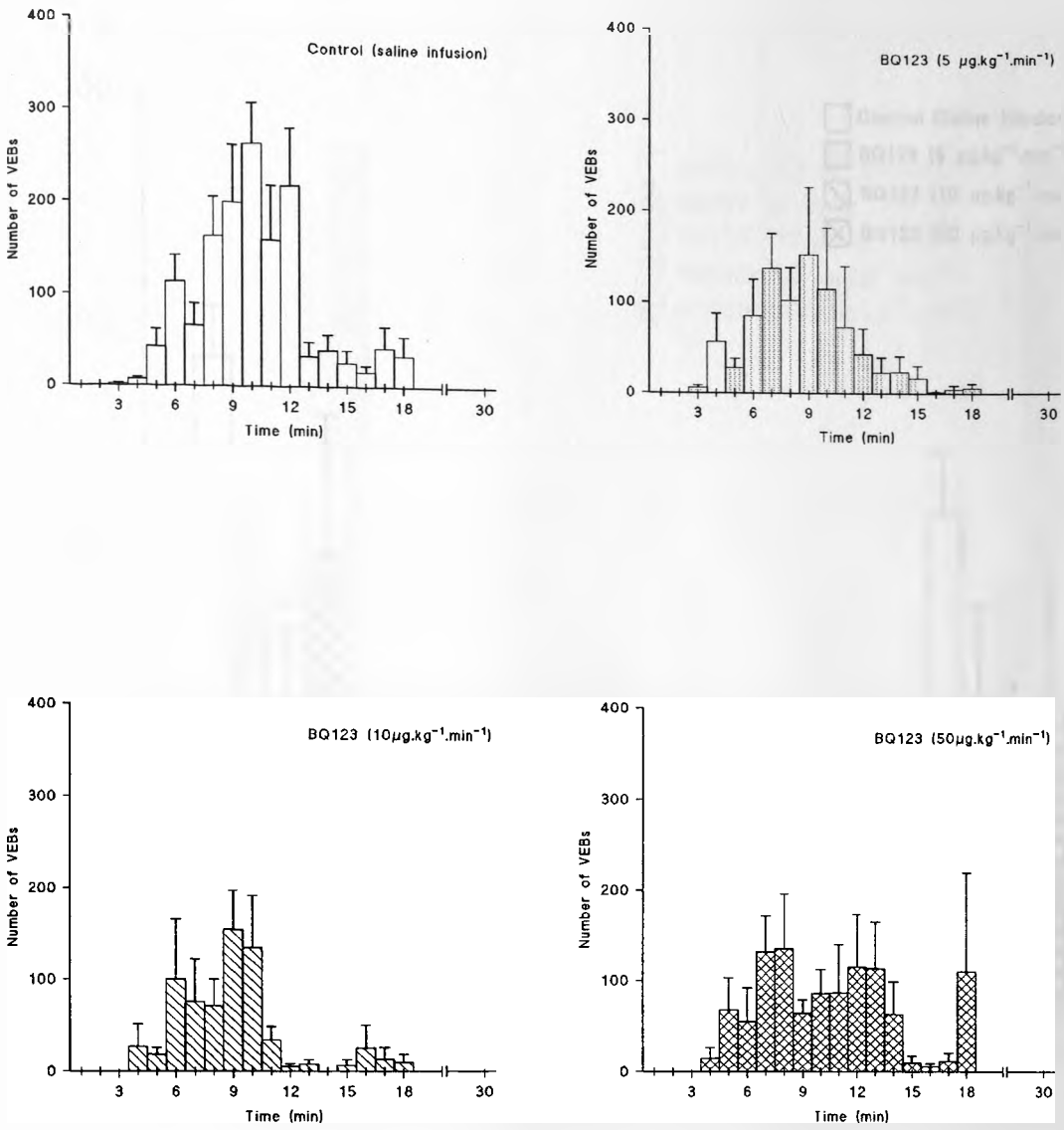


Figure 3.17 Distribution of ventricular ectopic beats (VEBs) over 1 min intervals for the 30 min occlusion of the left coronary artery, in rats receiving saline (Control) and BQ-123 (5, 10 and 50 $\mu g.kg^{-1}.min^{-1}$) infusion.

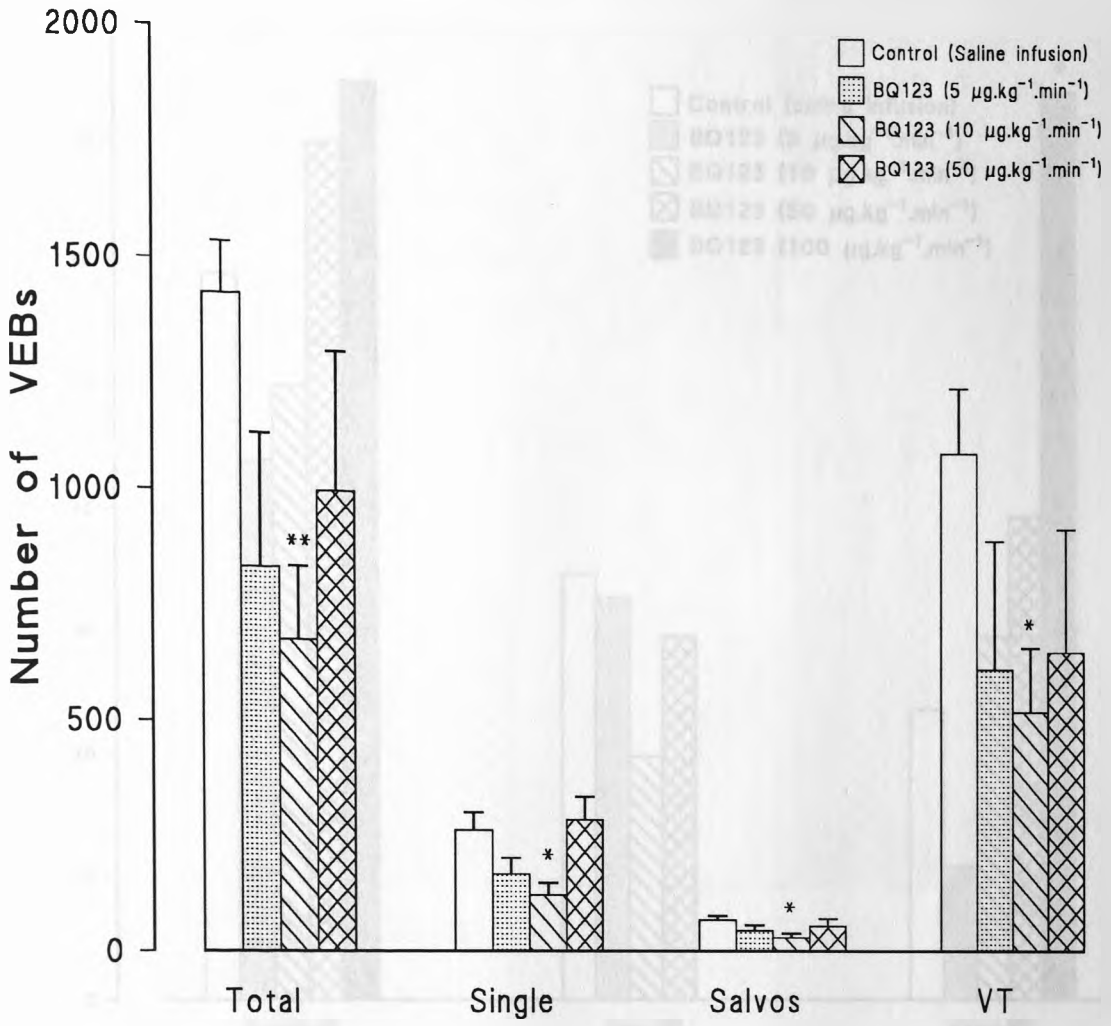


Figure 3.18 The total number of ventricular ectopic beats (VEBs), and the number of arrhythmias appearing as single extrasytoles, salvos and ventricular tachycardia (VT) in controls and rats receiving BQ-123 (5, 10 and 50 $\mu g.kg^{-1}.min^{-1}$) infusion. **p<0.01, *p<0.05 compared to control.

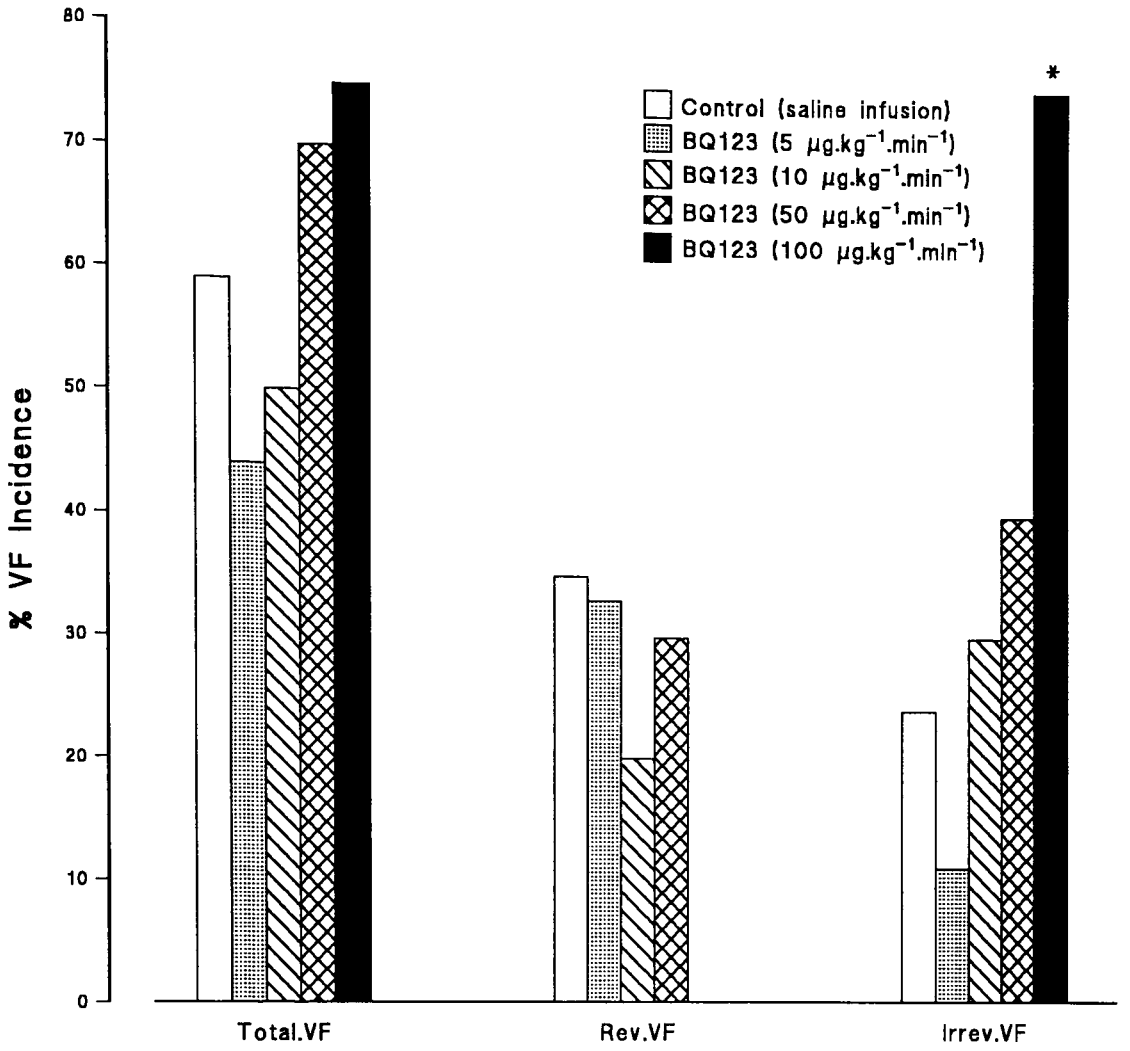


Figure 3.19 The incidences of total, reversible (Rev.) and irreversible (Irrev.) ventricular fibrillation (VF) during 30 min occlusion period of the left coronary artery in rats receiving saline (control) and BQ-123 infusion. *$p<0.05$ compared to control and BQ-123 (5 $\mu g.kg^{-1}.min^{-1}$).

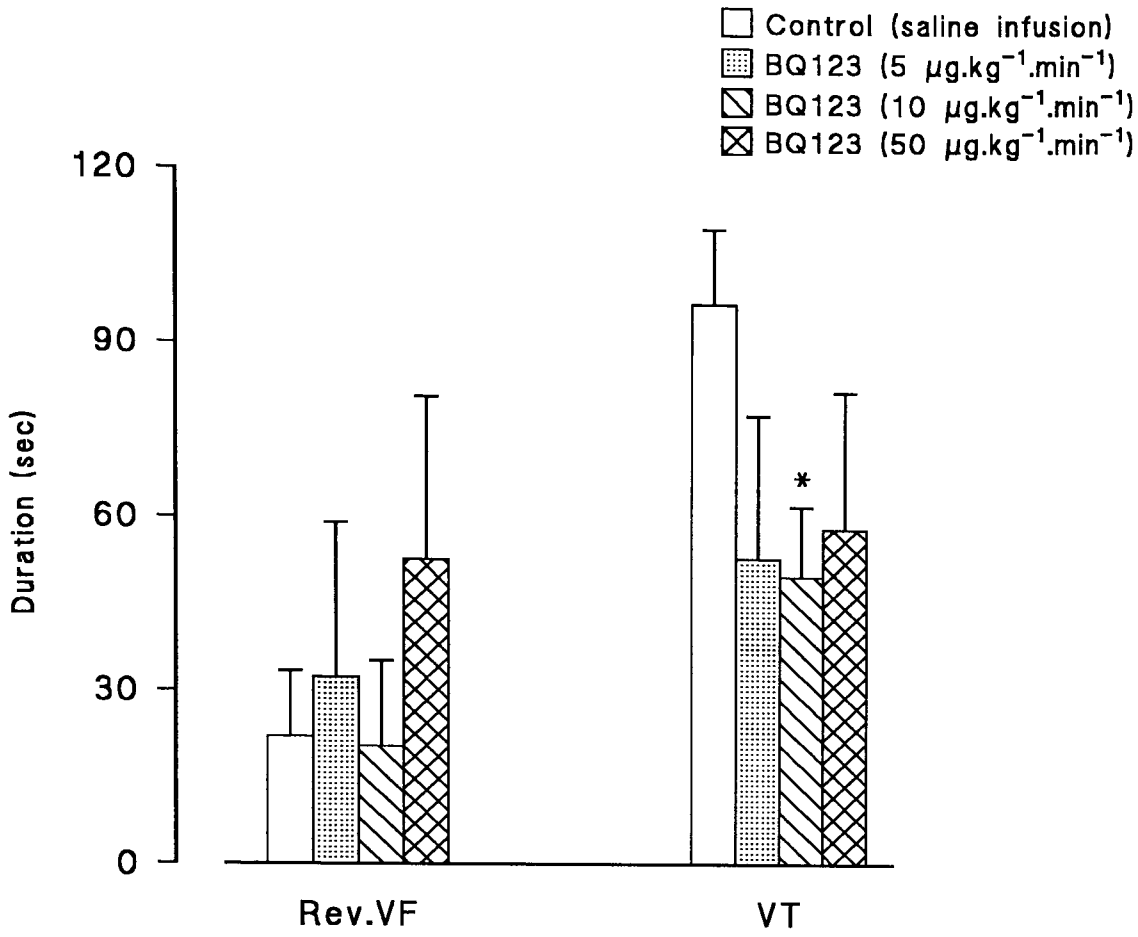


Figure 3.20 The effects of BQ-123 infusion on the time (sec) spent in ventricular tachycardia (VT) and reversible ventricular fibrillation (Rev. VF). *$p<0.02$ compared to control.

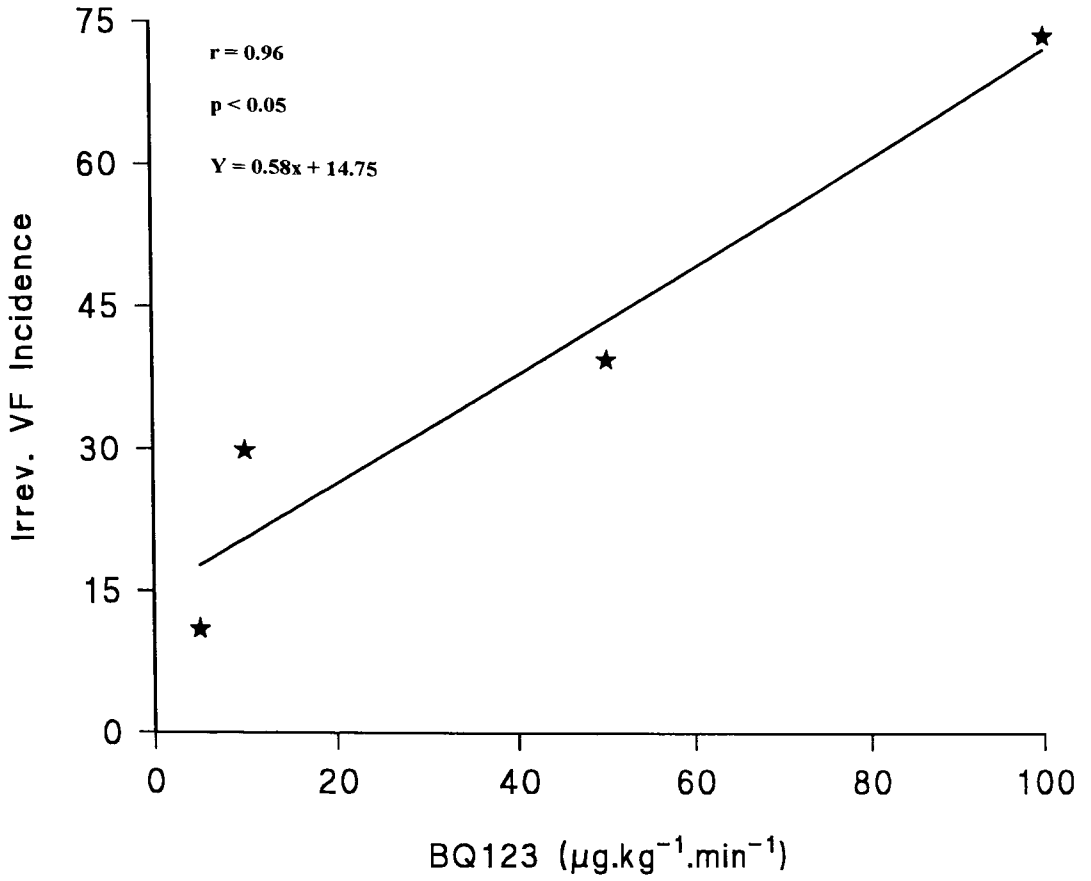


Figure 3.21 Relationship between incidence of ireversible ventricular fibrillation (Irrev. VF) and dose of BQ-123.

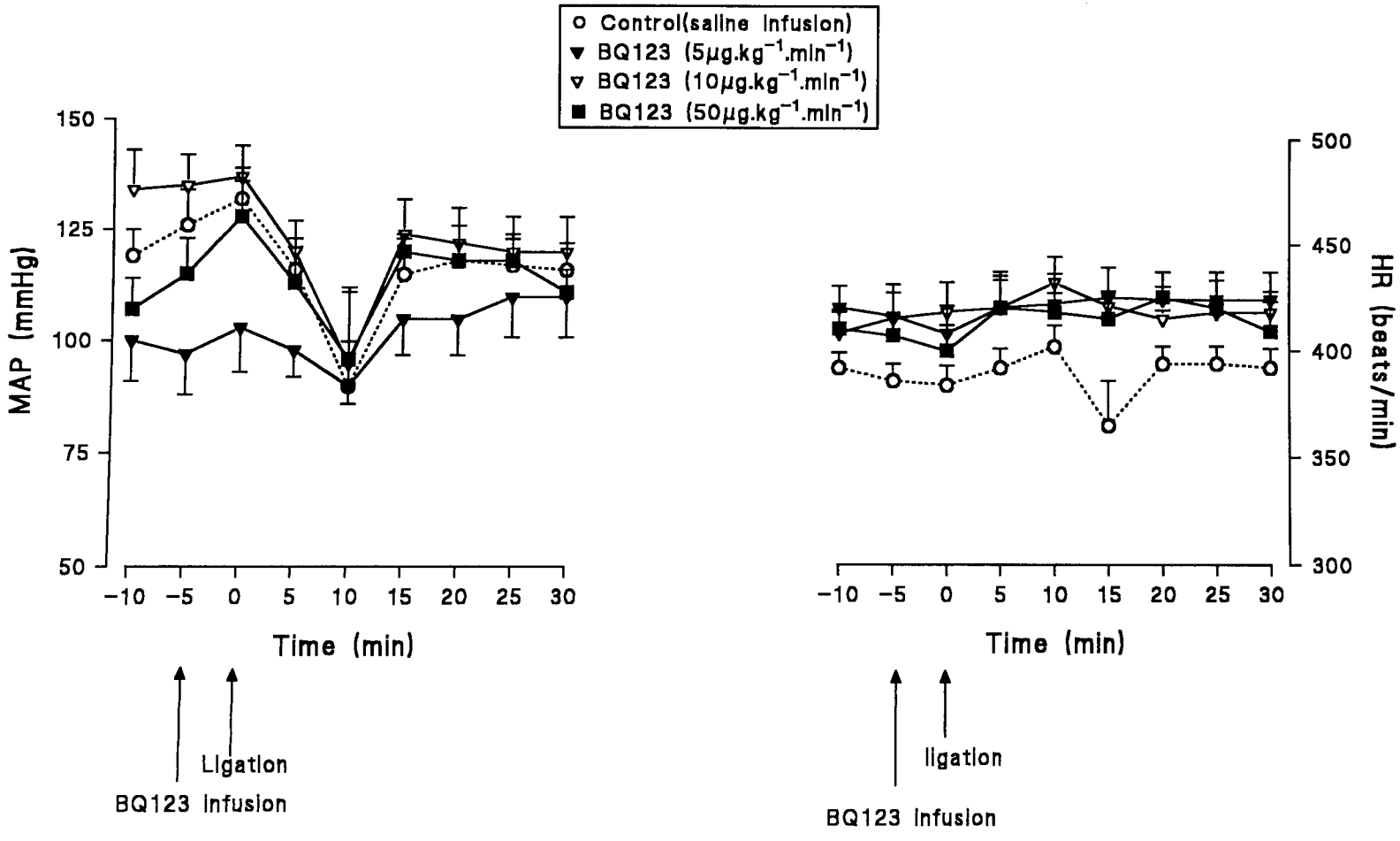


Figure 3.22 Heart rate (HR, beats.min^{-1}) and mean arterial pressure (MAP, mmHg) changes prior to and during coronary artery occlusion in controls, and rats receiving BQ-123. In control saline and in the treated groups BQ-123 infusion was commenced 5 min before coronary artery occlusion (performed at time 0 min) and maintained for the duration of the experiment.

3.3.7 The Effects of BQ-123 on the Endothelin-Enhanced Arrhythmias

In the rats which received an infusion of low dose ET-1 (0.05 nmol.kg^{-1}.min^{-1}) there was a significant increase ($p<0.02$) in the total number of ventricular ectopic beats compared to controls, as a result of increase in the beats occurring as ventricular tachycardia (table 3.4 and figure 3.24). These findings differ slightly from the first part of the study in which this low-dose of ET-1 increased the incidence of ventricular fibrillation, but not ventricular tachycardia or the total arrhythmia count. A possible explanation for this apparently increased sensitivity to endothelin is that the experiments were performed at a different time of year when there appears to be a greater susceptibility to arrhythmias. Alternatively a different batch of ET-1 was employed which may have had a different potency from the previous batch.

Figure 3.23 illustrates the arrhythmia distributions in the three groups of rats. In rats which received BQ-123 (10 μg.kg^{-1}.min^{-1}) co-infused with ET-1 (0.05 nmol.kg^{-1}.min^{-1}) the total number of ventricular ectopic beats, ventricular tachycardia and single extrasystoles decreased significantly compared to group which received ET-1 alone ($p<0.01$ and $p<0.05$ for singles). In this group the incidence of ventricular tachycardia was also decreased slightly from 100% in both control and ET-1 treated rats to 85% ($p>0.05$). In the rats receiving BQ-123 along with ET-1 the time spent in VT was significantly ($p<0.01$) less than that in the ET-1 treated group (figure 3.25). In comparison with control, ET-1 induced an increase in the total incidence of ventricular fibrillation (VF) from 33% to 73%, which was reduced to 40% by infusion of BQ-123 (figure 3.26).

3.3.8. The Effects of ET-1 on Haemodynamic Factors Prior to and During Coronary Artery Occlusion in the Absence and Presence of BQ-123 Infusion

Figure 3.27 shows mean arterial blood pressure (mmHg) and heart rate (beats.min^{-1}) prior to and during coronary artery occlusion in controls, and rats receiving ET-1

infusion (0.05 nmol.kg^{-1}.min^{-1}) in the absence and presence of BQ-123 (10 μg.kg^{-1}.min^{-1}). In both treated groups, ET-1 infusion induced no significant changes in either heart rate (HR) or mean arterial blood pressure (MAP).

Table 3.4: The effects of BQ-123 (10 μg.kg^{-1}.min^{-1}) on the pro-arrhythmic action of endothelin-1 (0.05 nmol.kg^{-1}.min^{-1}).

Arrhythmia	Control	ET-1	ET-1+BQ-123
n	9	11	10
Arrhythmia Counts			
Single VEB's	224±52	287±53*	134±31
Salvos	44±11	49±10	29±14
VT	367±86	993±185**♣	195±82
Total VEB's	636±135	1329±148**♣	358±112
Duration (sec)			
VT	31±7	81±16**♣	15±6
Reversible VF	1.6±1.5	11.5±8	5.6±5
Incidence (%)			
VT	100	100	85
Reversible VF	11	36	10
Irreversible VF	22	36	30
Total VF	33	73	40

*P<0.05; **P<0.01 compared to ET-1 + BQ-123 and ♣P<0.05 compared to controls. Total VEB's is the sum of arrhythmias occurring as single extrasystoles, salvos and ventricular tachycardia.

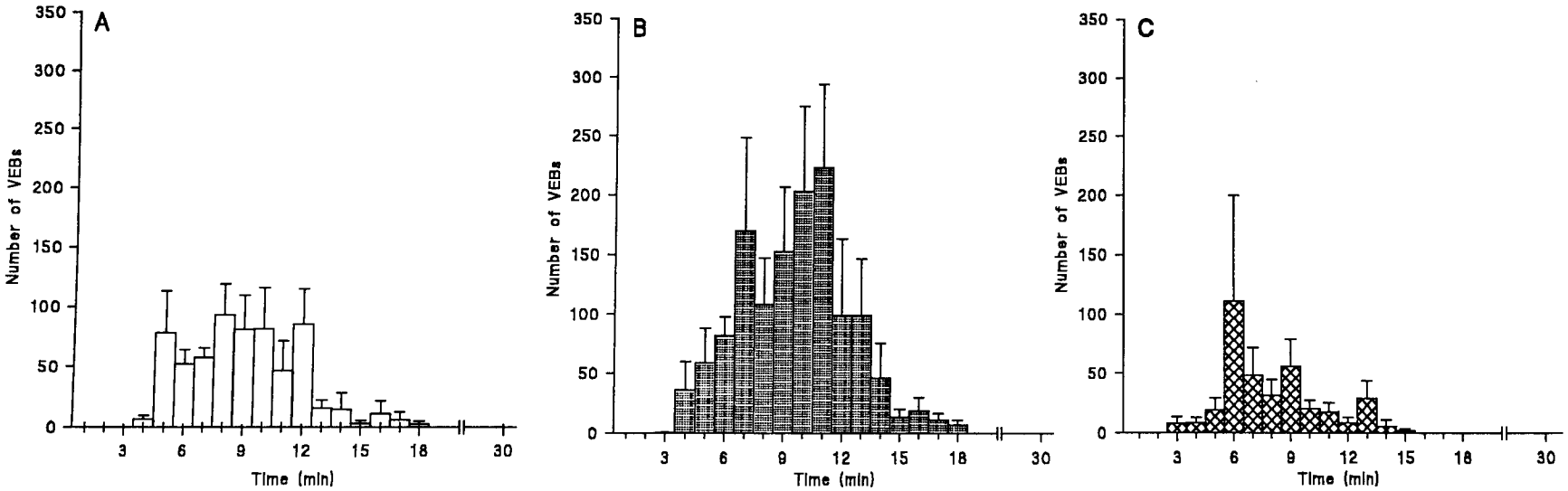


Figure 3.23 Distribution of ventricular ectopic beats (VEBs) over 1 min intervals during a 30 min occlusion of the left coronary artery, in rats receiving saline (Control, A), ET-1 (0.05 nmol.kg^{-1}.min^{-1}, B) and ET-1 (0.05 nmol.kg^{-1}.min^{-1}) coinfused with BQ-123 (10 μg.kg^{-1}.min^{-1}, C).

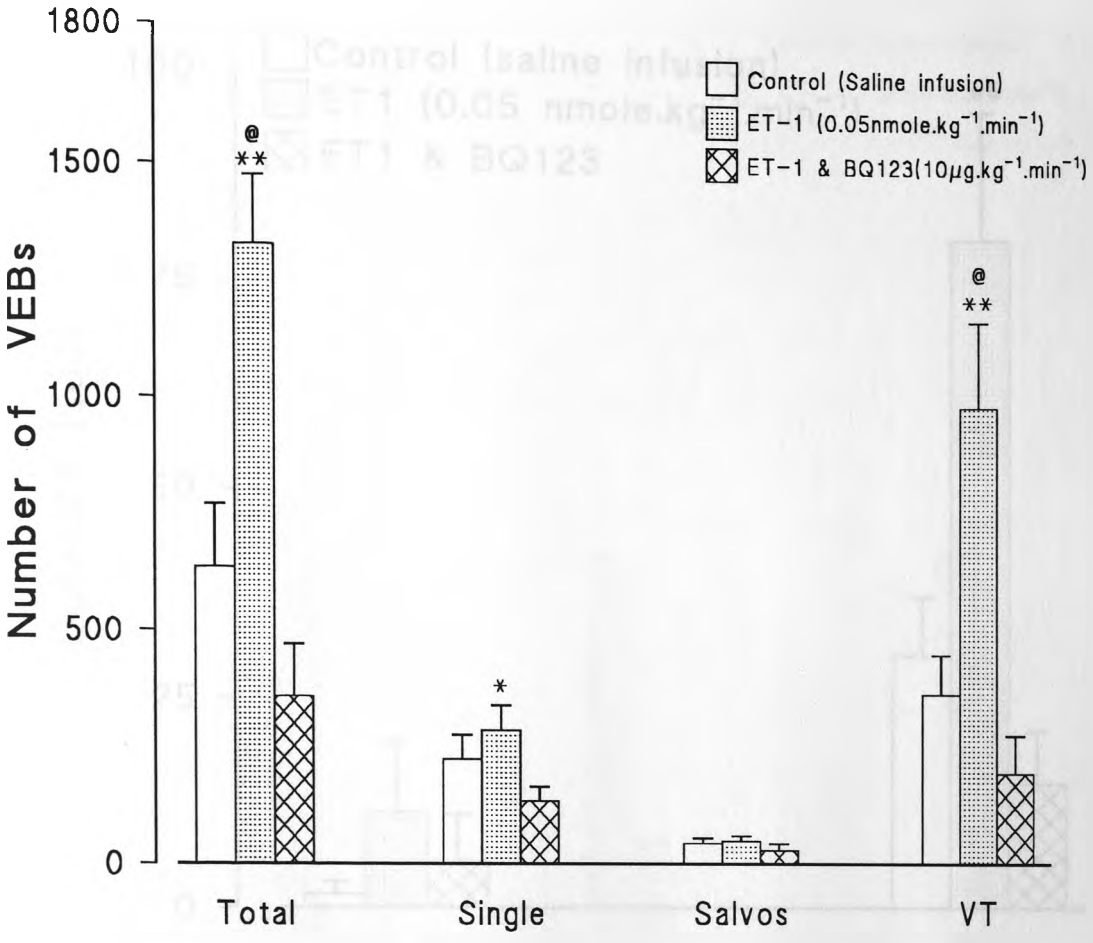


Figure 3.24 The total number of ventricular ectopic beats (VEBs), and the number appearing as single extrasytoles, salvos and ventricular tachycardia (VT) in controls and rats receiving ET-1 (0.05 nmol.kg^{-1}.min^{-1}) or ET-1 (0.05 nmol.kg^{-1}.min^{-1}) in the presence of BQ-123 (10 μg.kg^{-1}.min^{-1}). **$p<0.01$, *$p<0.05$ compared to ET-1 & BQ-123 and @$p<0.02$ compared to control.

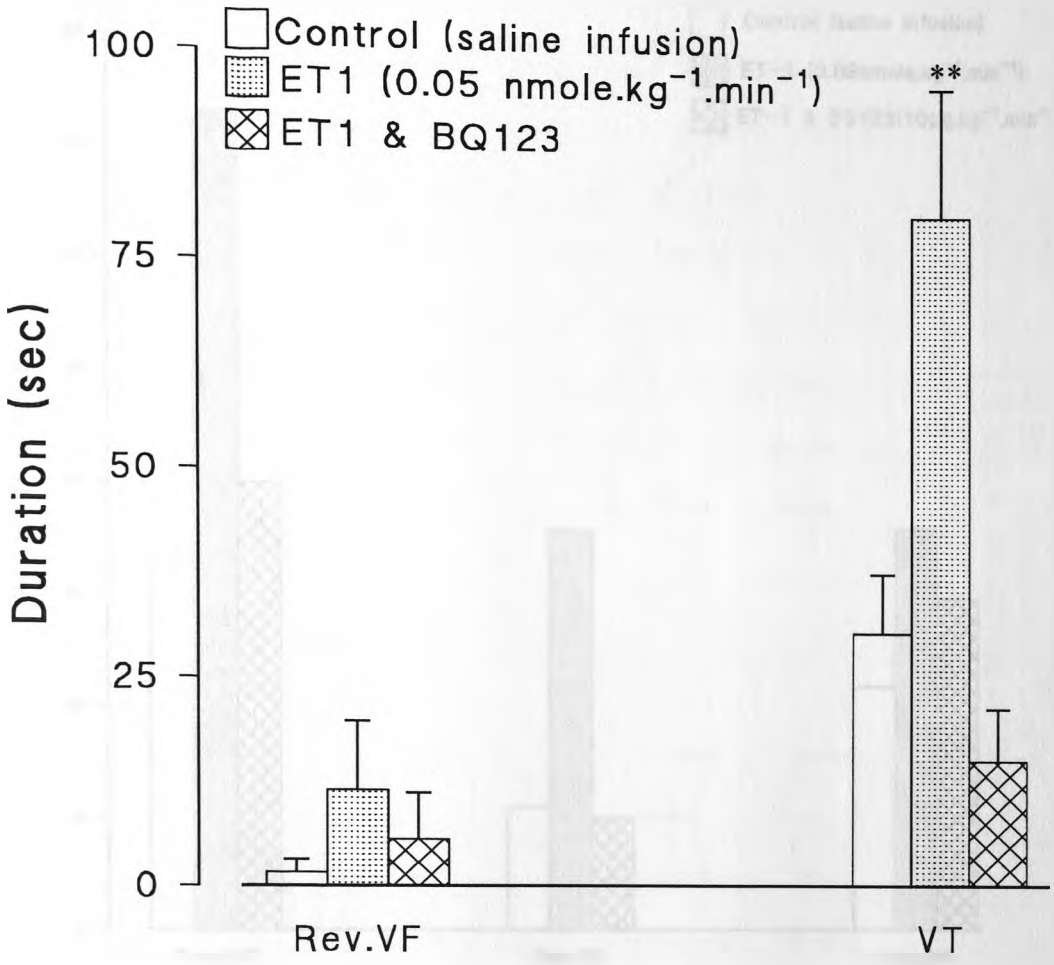


Figure 3.25 The effects of ET-1 infusion (0.05 nmol.kg^{-1}.min^{-1}) in the absence or presence of BQ-123 (10 μg.kg^{-1}.min^{-1}) on the time (sec) spent in ventricular tachycardia (VT) and reversible ventricular fibrillation (Rev. VF). **$p<0.01$ compared to ET & BQ-123.

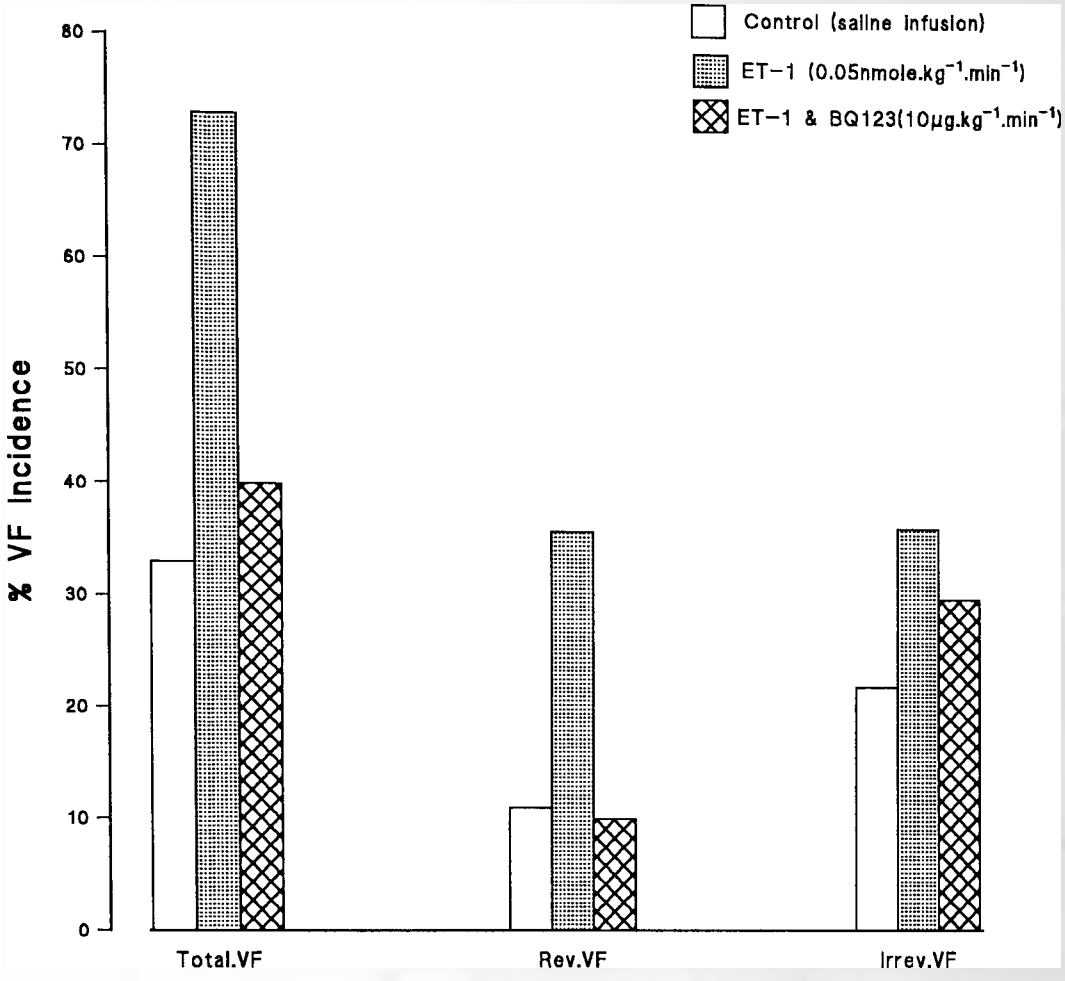


Figure 3.26 The incidence of total, reversible (Rev.) and irreversible (Irrev.) ventricular fibrillation (VF) during a 30 min occlusion period of the left coronary artery in rats receiving saline (control), ET-1 (0.05 nmol.kg^{-1}.min^{-1}) or ET-1 (0.05 nmol.kg^{-1}.min^{-1}) in the presence of BQ-123 (10 µg.kg^{-1}.min^{-1}).

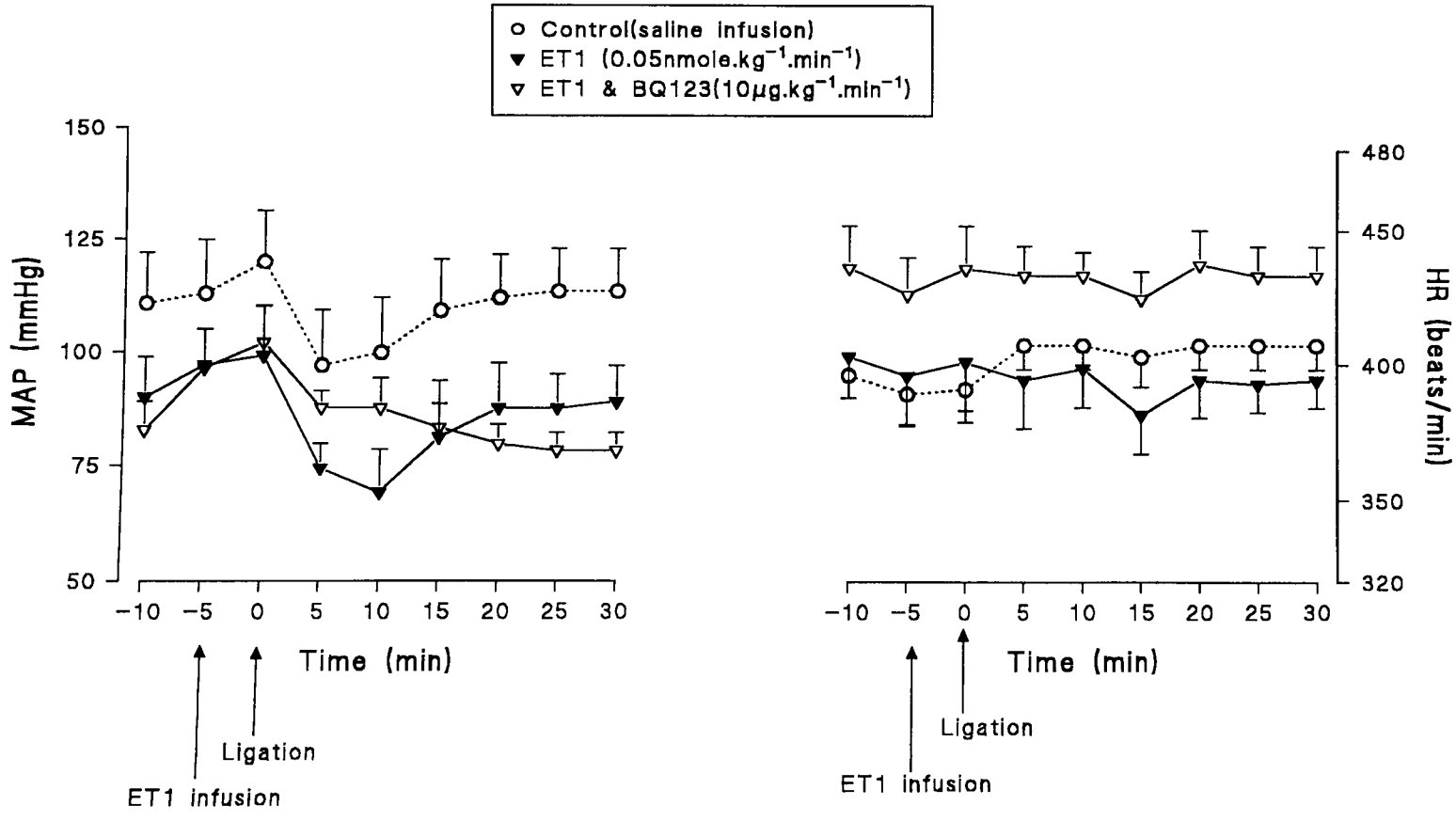


Figure 3.27 Heart rate (beats.min^{-1}) and mean arterial pressure (mmHg) changes prior to and during coronary artery occlusion in controls, and rats receiving ET-1 or ET-1 in the presence of BQ-123 infusion. In both treated groups ET-1 infusion was commenced 5 min before coronary artery occlusion (performed at time 0 min) and maintained for the duration of the experiment. BQ123 infusion was started 10 minutes before occlusion.

3.3.9. The Effects of BQ-123 on Myocardial Infarct Size

Table 3.5 summarises the measurements obtained from post-mortem analysis of heart tissue in controls and rats given BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) and subjected to 30 minutes occlusion followed by 3.5 hours reperfusion. Area at risk in both groups was similar. BQ-123 had no effect on either the absolute weight of the infarcted tissue, or on the infarct size expressed as a percentage (by weight) of the area at risk.

Table 3.5: The effect of BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) on myocardial infarct size after 30 minute coronary artery occlusion and 3.5 hours reperfusion.

Groups	n	Ventricles (mg)	Area at risk (mg)	Infarcted tissue (mg)	Infarct size (%)
Control	6	728±37	374±25	169±12	45.7±3.1
BQ-123	6	736±35	349±18	173±9	49.8±1.5

3.3.10 Effects of BQ-123 on Haemodynamic Variables During Reperfusion

Changes in heart rate and mean arterial blood pressure during the three and half hours of reperfusion are shown in figure 3.28. Both parameters showed a gradual decline in both groups of animals during reperfusion. In rats receiving BQ-123 (50 $\mu g.kg^{-1}.min^{-1}$) there was a significant ($p<0.001$ and $p<0.05$) fall in mean arterial blood pressure during the last 45 minute of reperfusion, whereas this reduction was not significant in rats receiving saline as control. The mean arterial blood pressure in the BQ-123 treated group was significantly lower than the controls at the end of the reperfusion. The decline in heart rate during reperfusion was similar in both groups.

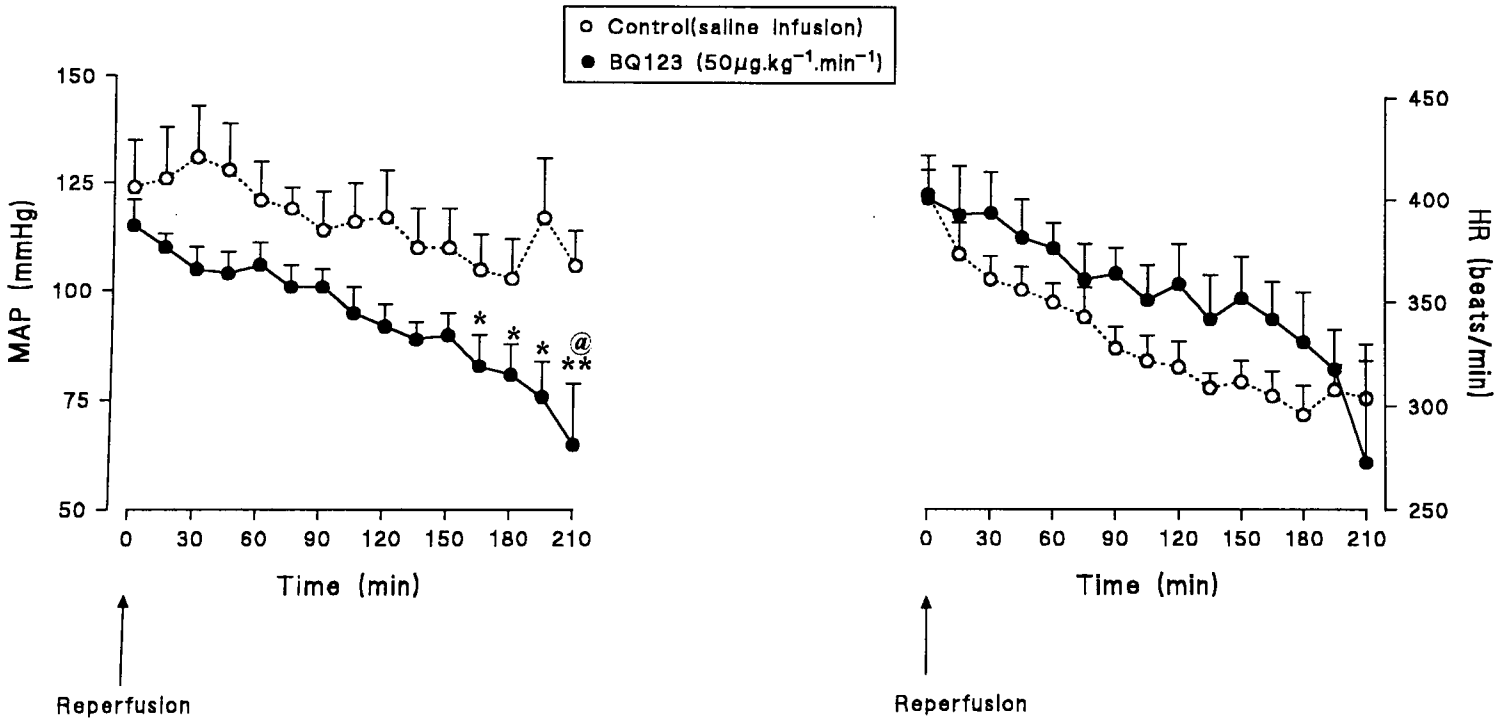


Figure 3.28 Heart rate (HR, beats.min^{-1}) and mean arterial pressure (MAP, mmHg) changes during 3.5 hours of reperfusion in controls, and rats receiving BQ-123 (50 μg.kg^{-1}·min^{-1}). Reperfusion was performed at time 0. **p<0.01, *p<0.05 (compared to the values at 0 and 15 minutes in the same group), @ p<0.05 (compared to control at the same point).

3.4 DISCUSSION

3.4.1 Haemodynamic Responses to Endothelin-1 and Their Antagonism by BQ-123

The haemodynamic response to intravenous injection of ET-1 is complex in that it produces a biphasic change in blood pressure (Wright et al., 1988; Le Monnier de Gouville et al., 1990). Following the early vasodepressor phase, cumulative bolus intravenous injections of ET-1 resulted in a rapid, sustained and dose-dependent rise in arterial blood pressure (maximum increase of 45±2 mmHg) associated with no significant change in hypotensive phase. The biphasic response, and the sustained nature of the vasoconstriction, distinguishes ET from other vasoactive substances. Over the last few years the nature of the receptors mediating the various components of this response have been identified. The pressor response to ET-1 was originally believed to be mediated via activation of ET_A receptors (Hosoda et al., 1991; Lin et al., 1991) and therefore it could be expected that a selective ET_A receptor antagonist, like BQ-123, would abolish it.

Our results show that BQ-123 at 50 and 100 $\mu g.kg^{-1}.min^{-1}$ (0.08 and 0.16 $\mu mol.kg^{-1}.min^{-1}$) inhibited the pressor responses to 2.1 $nmol.kg^{-1}$ of ET-1 by 60 and 68%, respectively. Bird et al., (1993) demonstrated that in conscious rats the pressor responses to ET-1 were blocked very effectively by BQ-123 (maximum 61%) in the dose range of 0.001-0.01 $\mu mol.kg^{-1}.min^{-1}$, but that this blockade declined with increasing dose up to 1 $\mu mol.kg^{-1}.min^{-1}$, where only 10% inhibition was observed. Furthermore, McMurdo et al., (1993a) were unable to achieve complete reversal of the pressor response to ET-1 (maximum inhibition 50%) with a dose of BQ-123 of 7 $\mu mol.kg^{-1}.min^{-1}$. Thus incomplete inhibition of the pressor responses of ET-1 in anaesthetised rats shown in the present study and by others, suggests that this effect is not mediated entirely via activation of ET_A receptors. Indeed ET_B or ET_B-like

receptors have also been reported to mediate vasoconstriction effects in in vivo studies in the rat systemic circulation (Clozel et al., 1992) and in in vitro studies in porcine coronary arteries (Fukuroda et al., 1992; Harrison et al., 1992). Furthermore a study by Warner et al. (1993b), using a mixed ET_A/ET_B-receptor antagonist, shows the existence of two types of ET_B receptors in rabbit pulmonary artery which mediate vasoconstriction and vasodilation. Thus, complete blockade of ET_A receptors by high doses of BQ-123 may leave the other receptors (ET_B or ET_B-like) in the systemic circulation free to produce vasoconstriction and therefore causes the incomplete inhibition of the pressor action of ET-1 by BQ-123.

The depressor response observed immediately after ET-1 injection has been reported to be mediated by ET_B receptors (Bigaud and Pelton, 1992). Subsequent studies have shown that the vasodepressor response to ET-1 may be due to release of nitric oxide (Filep et al., 1993) via endothelial ET_B receptors (Karaki et al., 1993). In the present study neither dose of BQ-123 affected the depressor responses to ET-1, confirming its selectivity for ET_A receptors.

The other haemodynamic measurements which were made in this part of study were $LVdP.dt^{-1}_{max}$, and $dP.dt^{-1}.P^{-1}$. $LVdP.dt^{-1}_{max}$ is the simplest index of myocardial contractility that can be derived from left ventricular pressure measurements during isovolumic contraction and is the maximal rate of pressure rise. However, this index is influenced by both preload and afterload. The preload is determined by the stretch of the left ventricle just before the onset of contraction and is determined mainly by the volume of blood returning into the left ventricle during diastole. The afterload refers to the aortic pressure during the period when the aortic valve is open and is determined by peripheral and pulmonary resistance. Dividing the ratio $LVdP.dt^{-1}_{max}$ continuously by a fixed pressure in the left ventricle (50 mmHg) yields a quotient,

$dP.dt^{-1}.P^{-1}$, which is relatively insensitive to afterload but still moderately sensitive to preload.

At a dose of 0.98 $nmol.kg^{-1}$ ET-1 increased $LVdP.dt^{-1}_{max}$ significantly ($P<0.05$) compared to the pre-injection value, whereas $dP.dt^{-1}.P^{-1}$ remained unchanged. The increased $LVdP.dt^{-1}_{max}$ could be due to the direct inotropic effects of ET-1 as previously described for rabbit papillary muscles (Watanabe et al., 1989b) or for the guinea pig atria (Ishikawa et al., 1988b) in vitro. Alternatively, at least some part of this effect could be a myogenic effect due to the increased afterload (i.e increase of peripheral resistance caused by ET-1), since $dP.dt^{-1}.P^{-1}$ was not affected. Whatever the mechanism underlying the rise in $LVdP.dt^{-1}_{max}$ is, this effect of ET-1 was completely abolished by BQ-123 at the dose of 50 $\mu g.kg^{-1}.min^{-1}$. Therefore it can be concluded that this effect of ET-1 is probably mediated by ET_A receptors either on the myocardium or peripheral arteries.

Surprisingly, in these experiments, ET-1 in the presence of the higher dose of BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$) induced a dose-dependent decrease in $LVdP.dt^{-1}_{max}$ compared to pre-injection value and to the same point in ET-1 alone treated group. This reduction in contractility could not have been due to a negative inotropic effects, since $dP.dt^{-1}.P^{-1}$ was not affected significantly. The reduction in $LVdP.dt^{-1}_{max}$ in the presence of high doses of BQ123 may be due to the blockage of the ET_A receptors and then subsequent reduction in peripheral resistance (or afterload).

3.4.2 The Effects of Endothelin-1 and the ET_A-Receptor Antagonist, BQ-123, on Ischaemic Arrhythmias in Anaesthetized Rats

Recent studies have shown that exogenous ET induces many pathological states, suggesting that endogenous ET may play a role in pathophysiological conditions. One

of the more interesting is the possibility that it may be involved in the pathogenesis of myocardial ischaemia.

The results of this study have demonstrated that infusion of ET-1 to anaesthetized rats during a period of acute regional myocardial ischaemia caused a significant increase in the total number of ventricular ectopic beats, and in beats occurring as ventricular tachycardia. Further, there was a marked increase in the incidence of ventricular fibrillation. These results illustrate a pro-arrhythmic effect of ET-1 under conditions of myocardial ischaemia. A variety of other studies have shown the pro-ischaemic and arrhythmogenic effects of exogenous ET-1 in different species (Nichols et al., 1990; Ezra et al., 1989; Yorikane et al., 1990), however, in contrast to our study which assessed ischaemic arrhythmias, these studies were carried out in animals with normal hearts.

A powerful constrictor activity of ET-1 has been demonstrated in both isolated and in situ coronary arteries (Godfraind et al., 1989; Clozel and Clozel, 1989), therefore it would be reasonable to conclude that the arrhythmias arose as a consequence of coronary artery constriction. However, ET-1 in a pathophysiological dose range in conscious dogs, has been shown to exert haemodynamic changes resulting from vasodilation and a positive inotropic effect (Donckier et al., 1991). Furthermore, a

potent coronary vasodilator action has been reported for ET-1 at low doses (Nichols et al., 1990). A subsequent study by Donckier et al. (1994) indicated that ET-1 at pathophysiological levels, in dogs subjected to coronary artery occlusion, increased collateral blood flow to the ischaemic myocardium, without affecting baseline coronary flow. Thus coronary constriction may not necessarily account for the pro-arrhythmic effects of ET-1. In the present study the coronary vasoconstriction hypothesis in the generation of ischaemic arrhythmias also seems implausible, since ET-1 infusion enhanced the arrhythmias which were caused by coronary artery occlusion (ischaemic arrhythmias) in doses which did not cause arrhythmias in the absence of ischaemia. Furthermore, in light of the fact that rats possess very little collateral flow (<6%; Winkler et al., 1984), it is improbable that a reduction in blood flow to the ischaemic area by ET-1, due to coronary vasoconstriction or collateral vessel constriction, plays an important role.

Hypoxic conditions have been shown to enhance the sensitivity to ET-1 in the hearts of pithed rats, including enhanced myocardial contractility (Maclean et al., 1989). Clozel and Sprecher (1991) demonstrated that in anaesthetized dogs at low coronary perfusion pressure (40 mmHg), the effects of intracoronary endothelin on coronary diameter and epicardial-to-endocardial blood flow were more pronounced than at normal perfusion pressure, showing an increased sensitivity to ET-1 during low-flow states. While this could suggest that ischaemia itself lowers the threshold for the proarrhythmic effects of ET-1, it does not support directly the concept that increased incidence of arrhythmias was due only to coronary artery constriction. Furthermore, in the study by Clozel and Sprecher (1991) endothelin was found to decrease subepicardial blood flow to a greater extent than subendocardial blood flow, whereas it is known that ischaemia decreases specifically coronary blood flow to the subendocardium (Bache et al., 1977; Griggs and Nakamura, 1968). Thus it could be that endothelin plays a role in maintaining the perfusion of the subendocardium by

constricting the vessels of the subepicardium. Once again this suggests that the effect of ET-1 on the coronary vessels is not predominant in its pro-arrhythmic effects and a direct effect on myocardium also should be considered. Harada et al. (1993) found that intracoronary administration of both ET-1 and methacoline in anaesthetized rats produced ST segment elevation due to constriction of the coronary artery. However, the duration of the ST segment elevation by ET-1 was greater than that produced by methacholine while both of them produced equivalent reductions in coronary artery flow. In addition, arrhythmias involving A-V block, ventricular ectopic beats and ventricular fibrillation (resulting in 67% mortality) were present only after administration of ET-1 but not methacholine. Consequently it was suggested that the occurrence of ST segment elevation and arrhythmias induced by ET-1 are due to both direct actions of ET-1 on coronary vascular smooth muscle and on the myocardium. Grover et al., (1992) showed that in isolated rat hearts ET-1 pretreatment reduced the time to contracture in globally ischaemic hearts, indicating that it may have a direct pro-ischaemic effect which is independent of its vasomotor activity. Accordingly, in spite of a coronary vasoconstrictor effect, ET-1 also appears to have a direct destabilizing effect on the myocardium. Intracoronary administration of ET in open chest dogs, has been shown to produce larger ischaemic foci and ST segment elevation than a coronary stenosis with an equivalent flow reduction (Inoue et al., 1993), indicating a direct action of ET-1 on the myocyte. Interestingly, in a study by Liu et al. (1990), it was verified that ischaemia can cause increase in ET-1 receptors in cardiac membranes, further suggesting that the effect of ET-1 on myocardium may be enhanced under ischaemic conditions. Furthermore, ET-1 and angiotensin II or cathecolamines act synergistically to induce many biological actions (Rubanyi and Polokoff, 1994). The arrhythmogenic effects of high levels of catecholamines, which are known to be released during ischaemia (Hirche et al., 1980), may be exacerbated by ET-1. The mechanism of ET-1 effects on the myocardium is far from being fully understood. Results from studies on rabbit ventricular myocytes (Lauer et al., 1989)

and rat cardiac cells (Xu et al., 1993) show that ET-1 increases intracellular Ca^{2+}. Hirata et al (1989a) indicated that rat cardiomyocytes have specific ET receptors which are responsible for an increase in intracellular Ca^{2+}. They showed that the binding sites for ET are apparently distinct from those of DHP-sensitive Ca^{2+}-channels. It is known that increases in Ca^{2+} play an important role in the development of ventricular arrhythmias (Billman et al., 1991). In fact, augmentation of increased $[Ca^{2+}]_i$ overload during ischaemia by ET-1 may attenuate intercellular coupling, which in turn leads to a reduction in the conduction velocity of the propagating action potential through the heart (De Mello et al., 1985), and therefore cause arrhythmias by a reentry mechanism. Yorikane et al (1991) have observed that ET-1 produces a prolongation of the action potential duration followed by the development of early after depolarizations (EAD) in canine myocardial preparations. As explained in the introduction of this chapter, EADs occur under conditions of hypoxia and increased levels of intracellular Ca^{2+}, both of which are present in the ischaemic myocardium and could therefore be aggravated by ET-1. Furthermore it has been reported that ET-1 in guinea pig ventricular myocytes enhances the delayed rectifier K^+ current (Habuchi et al., 1992) which could contribute to the development of arrhythmias. ET-1 may also be important to the ischaemic myocardium on the basis of its ability to block ATP-sensitive K^+ channels (Miyoshi et al., 1992). Following their demonstration that levcromakalim (an ATP-sensitive K^+ channel opener) inhibited ^{125}I-ET-1 binding to rat cardiac membranes, Haynes et al (1993) suggested a relationship between ET-1 receptor binding and the activity of ATP-sensitive K^+ channels. Harada et al (1993) showed that openers of ATP-sensitive K^+ channels reduced the appearance of ventricular fibrillation and death induced by ET-1 in anaesthetized rats. The effects of endothelin on the heart are therefore much more complex than simple induction of severe coronary vasoconstriction and the electrophysiological changes in the myocardium caused by ET-1 may contribute to the development of arrhythmias.

The effect of ET-1 on myocardium may be mediated through activation of one or more endothelin receptor subtypes. It remains to be determined which types of ET-receptors mediate the electrophysiological and arrhythmiogenic effects of ET-1. The second major finding from this study was that BQ-123 could reduce ventricular arrhythmias induced by coronary artery occlusion, but only within a very narrow dose range. Low doses of BQ-123 suppressed the ischaemic arrhythmias which reached a significant level at 10 $\mu g.kg^{-1}.min^{-1}$. With the highest dose there was an increased incidence of irreversible VF. Moreover a positive correlation existed between the incidence of irreversible VF and the dose of BQ-123. In addition, in another group of rats in which a low dose BQ-123 (10 $\mu g.kg^{-1}.min^{-1}$) was co-infused with ET-1, BQ123 also reversed the proarrhythmic effects of infused endothelin. Thus ET_A receptors may will be involved in the pro-arrhythmic action of exogenous ET-1 on the ischaemic myocardium.

These findings may reflect the U shaped dose-response curve which has recently been described for the antagonism of the pressor response to ET-1 by BQ-123 (Bird et al., 1993). Interestingly these results are also confirmed by the present finding that a dose of 50 $\mu g.kg^{-1}.min^{-1}$ of BQ-123 abolished the increased in myocardial contractility induced by ET-1, while with the higher dose of 100 $\mu g.kg^{-1}.min^{-1}$ there was a dose dependent reduction. In another study a different selective ET_A receptor antagonist, FR139317, suppressed the ST segment elevation induced by ET-1 but did not inhibit it completely (Filep et al., 1994b), whereas blockade of ET_A receptors with FR139317, (McMurdo et al., 1994) reduced the incidence of ischaemic ventricular fibrillation.

According to our results, endogenous ET-1 appears to contribute to ischaemic arrhythmias although it is not possible to say that this effect is due entirely to an action at ET_A-receptors alone. In a study on rat myocytes (Sokolovsky, 1993),

BQ-123 inhibited efficiently and completely the high affinity (pM) sites. However, at the lower affinity (nM) sites, which are associated with vasoconstrictor action of ET-1, BQ-123 did not affect ET-1 binding effectively. Therefore it is possible that with low doses of BQ-123 there are still some pM binding sites available for occupation by ET-1, while with higher doses of BQ-123, the pM sites are blocked completely while the nM sites remain open and are free to be activated by ET-1 to mediate the vasoconstrictor (and possibly other detrimental) effects of ET-1 which could increase the severity of ischaemic arrhythmias.

A large body of evidence indicates that endothelin stimulates phospholipase C in many systems (Yanagisawa and Masaki, 1989; Kloog et al., 1989) including rat atrial and myocytes (Galrón et al., 1989), leading to the formation of the second messenger, inositol triphosphate which in turn mobilises Ca^{2+} from intracellular storage sites. As described previously, overload of $[Ca^{2+}]_i$ is one of the main causes of arrhythmia induction. It has been suggested that pM sites are not coupled to the phosphoinositide hydrolysis pathway, whereas nM are (Sokolovosky et al., 1992; Sokolovosky, 1993). Consequently, it is possible that with high doses of BQ-123 the pM sites are blocked completely while nM sites are left free to mediate ET-1 induced intracellular Ca^{2+} overload via the activation of phosphatidyl inositol diphosphate (PIP_2).

Taking the above evidence together the following hypothesis could be proposed. The major ET-1 binding site on the myocardium is the pM receptor, which has a very high affinity for ET-1 and requires only low concentrations of ET-1 to be activated. These sites include ET_A receptors (since they are effectively blocked by BQ-123) and ET_{B1} (or ET_BH) receptors (which have a high affinity for ET-1 and are stimulated at low concentrations of ET-1 and mediate vasodilation). BQ-123 selectively inhibits the pM sites (Sokolovsky et al., 1993). Since the major part of pM sites are ET_A-receptors, at low doses of BQ-123 the prevention of the pro-arrhythmic effects of exogenous and

endogenous endothelin via the blockade of ET_A-receptors is more dominant than the blockade of ET_{B1} receptors which mediate vasodilation. In addition, at low concentrations of BQ-123 there are still open pM sites which can be activated to produce both vasodilation and vasoconstriction with a net effect of zero. In this study it was found that 30 minute coronary artery occlusion caused a marked increase in the plasma levels of endothelin (Chapter 5). Thus it is possible that the blockade of ET_A-receptors in the circulation and other tissues by high doses of BQ-123 increases the plasma levels of un-bound ET-1 and consequently increases the local concentrations of ET-1 within the myocardium to an extent which activates nM sites with low affinity. Thus it is possible that, in the presence of high doses of BQ-123, at which the pM sites are blocked entirely, nM sites (according to some studies ET_{B1}) are exposed to locally or systematically accumulated endothelin to produce arrhythmogenic effects, possibly by vasoconstriction and/or intracellular mobilization of Ca^{2+}. Alternatively BQ123 may be toxic at high doses and produce some non-specific effects.

3.4.3 The Effects of BQ-123 on Infarct Size

As a consequence of the effects of the higher dose of BQ-123 increasing mortality, it was only possible to determine the effects of the lower dose (50 $\mu g.kg^{-1}.min^{-1}$) of BQ-123 on infarct size, following 30 minutes of coronary artery ligation and 3.5 hours reperfusion. As can be seen from the data, area at risk in both in control and treated groups was similar, thus excluding the possibility of any marked difference between the groups. From the infarct size it can be seen that BQ-123 had no effect on the extent of myocardial ischaemia/reperfusion injury. The other main determinant of infarct size, heart rate, was not significantly different between the two groups. However, the mean arterial blood pressure in the BQ-123 treated rats declined significantly at the end of reperfusion time compared to the control group, suggesting that accumulation of BQ-123 at the end of infusion may have caused systemic vasodilation through an action of free endothelin at ET_B receptors.

These results are in agreement with another recent study in which the ET_A receptor antagonist, FR139317, had no effect on infarct size in a rabbit model of acute myocardial ischaemia and reperfusion (McMurdo et al., 1993b). However, the results are in contrast to previous studies in which an antibody to ET-1 (Watanabe et al., 1991) or the endothelin converting enzyme inhibitor, phosphoramidon (Grover et al., 1992) reduced infarct size in models of coronary artery occlusion and reperfusion in the anaesthetized rats. A reduction in infarct size in rats has also been shown in a recent study using the mixed ET_A/ET_B antagonist bosentan (Richard et al., 1994).

There are some differences between the present study and those studies reported previously in the literature. In both studies which showed a decrease in infarct size (Watanabe et al., 1991; Grover et al., 1992) the animals were subjected to a longer period of reperfusion (24 hours), as opposed to 3.5 hours in the present experiments. Since the tissue endothelin from the Watanabe study showed that high endothelin levels were still present 48 hours after reperfusion, this may implicate a later role for endothelin in the extension of damage, rather than during the early stages. Finally, we employed a selective ET_A receptor antagonist, while the other studies used an antibody to ET-1 or inhibited its formation. Thus in our study, only those actions of ET-1 mediated by ET_A receptors would be blocked, whereas with the antibody to endothelin or phosphoramidon, actions at all receptors could be prevented. In addition, ischaemia and reperfusion of the rat heart causes an increase in endothelin receptor binding sites (Liu et al., 1990), but it is not clear which subtype of endothelin receptors is upregulated. If other (non-ET_A) receptors are increased, then this could explain why BQ-123 is ineffective in reducing myocardial infarct size in this model.

Chapter 4

DEGRADATION OF ENDOTHELIN-1 IN MYOCARDIUM

4.1 DEGRADATION OF ET-1 IN RAT VENTRICULAR AQUEOUS EXTRACTS

The biosynthesis of ET-1 has been studied extensively but the degradation of ET-1 has received relatively little attention. Pharmacokinetic studies in rats (Shiba et al., 1989) and pigs (Pernow et al., 1989) indicate that intravenous injection of ^{125}I-labelled ET-1 is rapidly distributed from the blood to tissues, with a half life of less than 2 min in pigs and 7 min in rats. It remains to be determined whether there are specific peptidases inactivating and metabolizing endothelins in these tissues.

A soluble proteinase which effectively degraded ET-1 was purified from rat kidney by Deng et al (1994). It was shown that the enzyme has optimum activity at pH 5.6, and its activity was inhibited by PMSF (phenyl methylsulfonyl fluoride; inhibitor of serine proteinase), but not other proteinase inhibitors. The enzyme removed the carboxyl terminal tryptophan of ET-1 by cleaving the Ile^{20}-Trp^{21} peptide bond. In contrast proendothelin-1 (big ET-1) was not degraded by this enzyme. In a similar study by Janas et al. (1994) it was shown that a fraction from rat kidney cell membranes, but not heart, degraded ET-1 optimally at pH 5.5. Moreover Jackman et al (1993) showed that bovine vascular endothelial cells contain an enzyme which inactivates ET-1 by cleavage at the C-terminal (Ile^{20}-Trp^{21} bond) at pH 5.5. Degradation was blocked mainly by inhibitors of serine peptidase, including diisopropyl fluorophosphate (DFP), but not by inhibitors of some other peptidases. The metabolism of ET-1 by liver-derived cells has also been shown by Gandhi et al (1993). In this study phosphoramidon did not affect significantly the metabolism of ET-1 by hepatocytes or Kupffer cells, but an aminopeptidase inhibitor, bacitracin, strongly decreased the metabolism. Sessa et al (1991b) showed that activated human polymorphonuclear leukocytes (PMNs) metabolized ET-1. This was prevented by PMSF but not by phosphoramidon or pepstatin-A, indicating that a serine proteinase is responsible for destruction of ET-1 by PMNs. Likewise Fagny et al (1992) verified

that lysosomal proteinases released by activated PMNs cleaved ET-1 at the His^{16}-Leu^{17} site, leading to formation of ET fragment 1-16 and the C-terminal pentapeptide. This degradation was blocked by soya bean trypsin inhibitor, suggesting the involvement of a serine proteinase in ET-1 hydrolysis. However, several recent studies suggest that in the kidney, endopeptidase 24.11 is also involved in the metabolism of ET-1. Purified rat renal endopeptidase 24.11 (Vijayaraghavan et al., 1990) was shown to cleave ET-1 initially at the Ser^{5}-Leu^{6} bond, followed by cleavage at the Asp^{18}-Ile^{19} site. Furthermore, Yamaguchi et al (1992) reported that rat kidney membrane hydrolyses ET-1 into four major fragments and that the production of three of them was inhibited by phosphoramidon. They showed that the phosphoramidon-sensitive fragments were generated by cleavage at the Ser^{5}-Leu^{6} bond of ET-1 and the phosphoramidon-insensitive fragment was produced by cleavage at Leu^{17}-Asp^{18}. The degradation of ET-1 was also observed by a membrane fraction of rat vascular smooth muscle cells which was inhibited by neutral endopeptidase inhibitors (Edano et al., 1994a). It has also been shown that injection of SQ29,072 a neutral endopeptidase inhibitor into anaesthetized rats increased the urinary excretion of endothelin nearly 14-fold (Abassi et al., 1992a), indicating that neutral endopeptidase 24.11 may play a major role in the inactivation of endothelin. ET-1 degradation may thus be caused by various kinds of enzymes which are present both on the surface and in the intracellular compartments of cells.

Since no detailed reports have yet been published on ET-1 degradation in myocardium we examined the degradation of ET-1 by aqueous extracts of rat ventricle and compared it with that in the lung. Further, to perform a preliminary characterization of the enzymic activity involved in ET-1 degradation by rat myocardium, the effects of certain proteinase inhibitors were also determined.

4.1.1 Endothelin-1 Degradation by Rat Lung Extract

As with other peptides, ET-1 is extracted rapidly from the circulation. Shiba et al (1989) reported that a significant amount of arterial ET-1, when administered intravenously in rats, was removed by kidney, liver, spleen and lungs. Although the exact mechanism of this clearance of endothelin is not well understood, proteolytic degradation may be involved. The lung tissues are abundant in endothelins. The peptides are produced by endothelial cells and airway epithelial cells (Pernow et al., 1989). Furthermore, the pulmonary circulation may play an important role in the metabolism of endothelin as the lungs take up large quantities of the peptide during passage (Luscher et al., 1992). This tissue, which is rich in peptidase was chosen as a control to establish the method for the degradation study and to compare it with that in the myocardium.

4.1.1.1 Experimental Procedure

The rat lung extract (0.1 gram of wet tissue per ml) was prepared as described in the Chapter 2 (section 2.8.1) and the supernatant diluted 5, 10, 20, and 30 times with phosphate buffer (final concentration 0.1 M, pH=5.2). ET-1 (8 $nmol.ml^{-1}$) was incubated for 5 and 30 min, at 25°C, with tissue extract at various dilutions. The samples were analysed by reverse phase HPLC. The degradation of ET-1 incubated in tissue extracts containing active enzymes was calculated as the percentage of the same concentration of ET-1 in identical incubates containing boiled tissue extracts.

4.1.1.2 Results

Degradation of ET-1 in rat lung extract at pH 5.2 and at various dilutions for 5 and 30 minutes is shown in table 4.1 and figure 4.1. ET-1 degradation was concentration-dependent. Five times dilution of homogenate (equivalent to 20 mg of wet $tissue.ml^{-1}$ of extract) produced a high and rapid degradation (80±8%) after 30 min incubation. Even after only 5 min incubation the degradation was 69±7%. According to these

results a 5 times dilution gave too great an activity for comparative studies. For subsequent studies, a 20 times dilution (protein content 412±4 $\mu g.ml^{-1}$) of tissue extract was chosen. This contained approximately 50% of degradation activity after 30 min incubation.

Table 4.1: Degradation of incubated ET-1 with 5, 10, 20, and 30 times diluted lung extracts (equivalent to 0.1 g tissue.ml^{-1}; phosphate buffer, 0.1M, pH 5.2) during 5 and 30 min, at 25°C, n=3, data represent mean±SD.

Dilution→	Time (min) ↓	5	10	20	30
ET Conc. In boiled lung extracts (μg.ml^{-1})	**5 →**	4.5±0.5	5.0±0.3	5.9±0.1	6.5±0.4
	30→	6.3±0.5	7.4±0.7	8.4±0.2	8.8±0.5
ET Conc. In Unboiled lung extract (μg.ml^{-1})	**5 →**	1.4±0.4	3.1±0.2	4.4±0.2	4.8±0.3
	30→	1.2±0.5	1.9±0.6	4.0±0.6	5.4±1.0
Amount of degradation (μg.ml^{-1})	**5 →**	3.1±0.3	1.9±0.2	1.6±0.1	1.7±0.1
	30→	5.0±0.7	5.6±0.2	4.3±0.8	3.4±0.7
% Degradation (percentage of boiled)	**5 →**	69.2±7.1	38.5±3.8	26.8±2.2	25.8±0.9
	30→	80.2±8.8	75.3±6.0	51.7±8.2	39.3±9.6

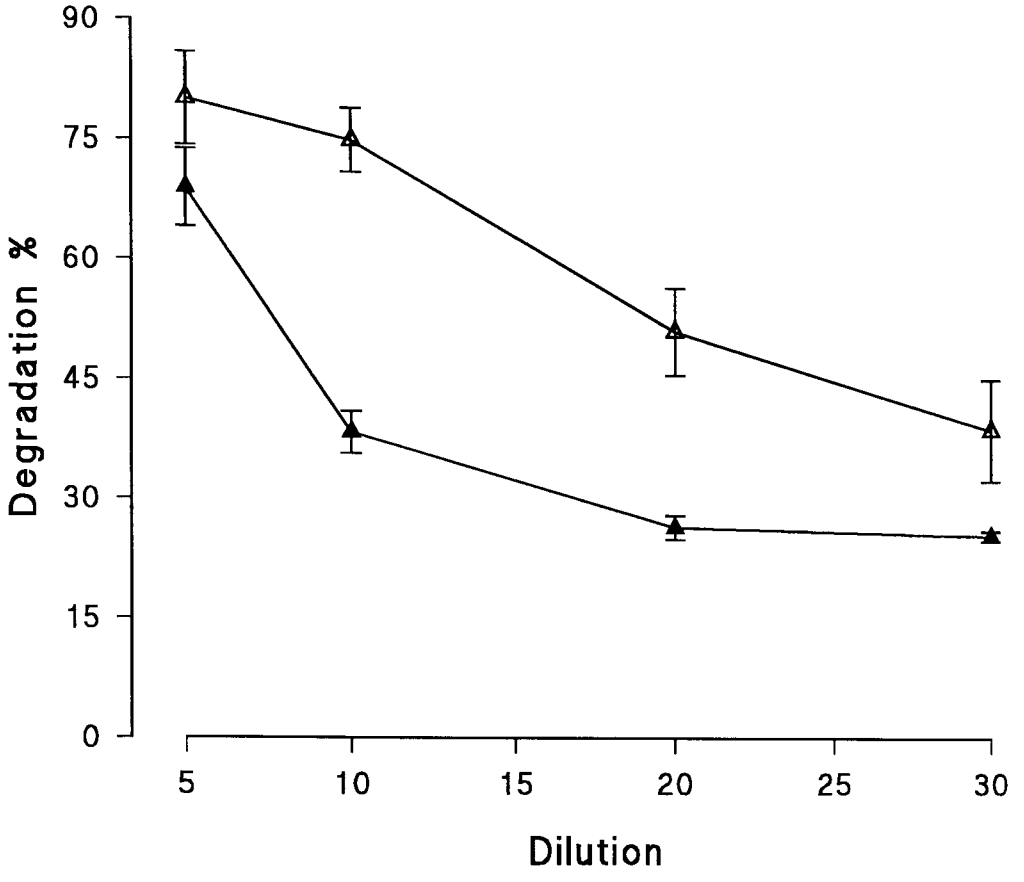


Figure 4.1 Degradation of ET-1 by 5, 10, 20, and 30 times diluted lung extracts (equivalent to 0.1g tissue.ml^{-1}; phosphate buffer, 0.1M, pH=5.2) at 25°C, incubated for 5 min (▲) and 30 min (Δ). Samples were analysed by HPLC as described in Chapter 2. Points represents mean±SD, n=3.

4.1.2 Time Course of ET-1 Degradation by Rat Ventricular and Lung Extract

Various studies have demonstrated a potential role for endothelin in modulating cardiovascular reflexes and functions (Yanagisawa et al., 1988b; Hu et al., 1988; Ishikawa et al., 1988a,b; Davenport et al., 1989; Ishikawa et al., 1989). Its involvement in the pathogenesis of cardiovascular disease has been strongly suggested (Miyauchi et al., 1989a,b; Nakao et al., 1989; Matsuyama et al., 1990; Grenier et al., 1990). Moreover, it has been shown that endothelin can be formed and metabolised in many tissues of the body (Pernow et al., 1989; Yamaguchi et al., 1992a; Vijaraghovan et al., 1990; Deng et al., 1994; Jackman et al., 1993). Since the inactivation of locally produced endothelin may be an important approach to inhibiting ET-1 mediated disorders, the degradation of ET-1 in rat ventricle homogenates was examined. The purpose of this experiment was to assess the time dependent degradation of ET-1 by rat ventricle and to compare it with that in lung extracts.

4.1.2.1 Experimental Procedure

For the time course study of ET-1 degradation by tissue extracts, ET-1 (final concentration 8 $nmol.ml^{-1}$) was incubated for 5, 30, 60, 90, and 180 min with 20 times diluted ventricular extract (protein concentration 412±14 $\mu g.ml^{-1}$) at pH=5.2 and 7.2, and for 5, 30, and 60 min with 20 times diluted lung homogenate (protein concentration 400±10 $\mu g.ml^{-1}$) at pH=5.2. The samples were analysed by RP-HPLC as described in Chapter 2 (section 2.7.1).

4.1.2.1 Results

The results of degradation of ET-1 (8 $nmol.ml^{-1}$) during various incubation periods in both rat ventricular and lung extract at pH 5.2 are reported in table 4.2. Incubation of ET-1 with lung extract at pH 5.2 produced approximately 100% degradation after 60 min incubation (0.169 $nmol.min^{-1}.mg^{-1}$ of protein content of extract), whereas this value for ventricular extract was 57±5.4% (0.084 $nmol.min^{-1}.mg^{-1}$). The degradation

of ET-1 by ventricular extract reached a maximum of 87±1.8% after 180 min incubation. Thus lung extract appears to degrade ET-1 more efficiently than that from ventricle at pH=5.2. The degradation of ET-1 by ventricular homogenate at both pH 5.2 and 7.2 reached a plateau after 90 min (figure 4.2) incubation. This time was therefore chosen to study the effects of enzyme inhibitors on endothelin degradation by the tissue extracts.

Table 4.2: Degradation of ET-1 (8 nmol.ml^{-1}) incubated for various times at 25°C with 20 times diluted lung and ventricular extract (protein content 412±14 μg.ml^{-1} and 400±10 μg.ml^{-1} respectively) in phosphate buffer(0.1M, pH=5.2). Data represents mean±S.D.

Duration of incubation (min)→		**5**	**30**	**60**	**90**	**180**
n⇒		***3***	***3***	***5***	***7***	***3***
ET Conc. (μg.ml^{-1}) In boiled extract	***Ventricle***	5.8±1.0	6.6±0.2	7.1±0.7	8.0±1.3	8.0±0.0
	Lung	5.9±0.1	8.4±0.2	8.4±0.6	—	—
ET Conc. (μg.ml^{-1}) In Unboiled extract	***Ventricle***	4.1±0.3	3.5±0.1	3.1±0.5	2.2±0.6	1.0±0.1
	Lung	4.4±0.2	4.0±0.6	0.3±0.3	—	—
Amount of degradation (μg.ml^{-1})	***Ventricle***	1.8±0.6	3.2±0.3	4.1±0.5	5.9±0.9	7.0±0.2
	Lung	1.6±0.1	4.3±0.8	8.1±0.5	—	—
% Degradation (percentage of boiled)	***Ventricle***	29.3±6.8	47.9±3.2	56.9±5.4	73.4±4.4	87.1±1.9
	Lung	26.8±2.2	51.6±8.2	96.7±3.5	—	—

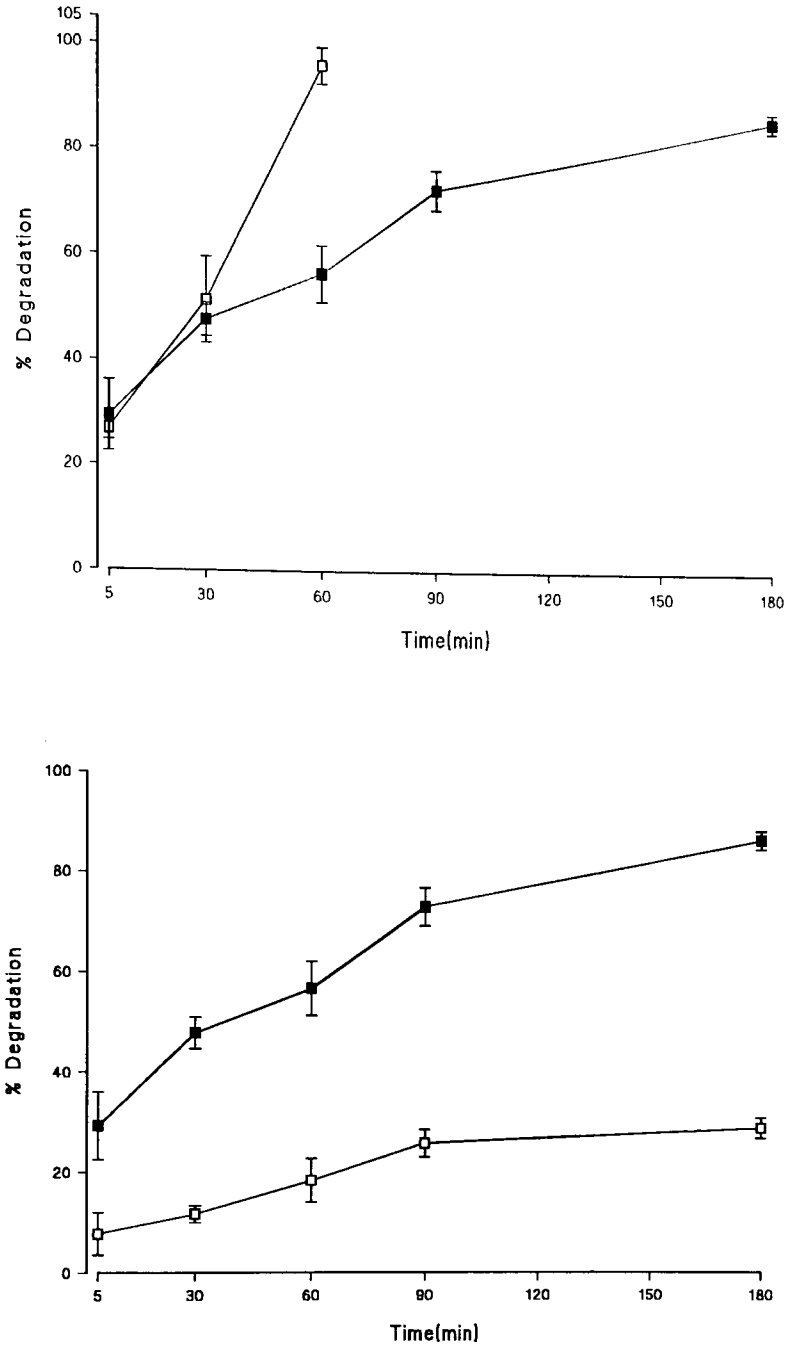


Figure 4.2 Time course of ET-1 (8 nmol.ml^{-1}) degradation by extracts of rat ventricle (■, protein content 412±14 μg.ml^{-1}) and lung (□, protein content 400±10 μg.ml^{-1}) at pH 5.2 (upper figure) and by ventricular extract at two different pH, 5.2 (■) and 7.2 (□) (lower figure). Points represent mean±S.D, and n=3-7.

4.1.3 Assessment of ET-1 Degradation in Sodium Barbital Buffer

The preliminary experiments to study the ET-1 degradation in this chapter were done in phosphate buffer (0.1M). However, the pH range of this buffer was 5-8.5, therefore, to provide a wider pH range to study the pH dependent degradation of ET-1, Michaelis's sodium barbital buffer (0.14 M; range 2.9-9.5) was examined.

4.1.3.1 Experimental Procedure

ET-1 (8 $nmol.ml^{-1}$) was incubated with 20 times diluted rat lung extract in sodium barbital buffer (0.14 M) for 60 min at 25°C and pH=5.2 or 8.2. The results were then compared with ET-1 degradation under the same conditions in phosphate buffer(0.1M).

4.1.3.2 Result

The HPLC chromatograms for degradation of ET-1 in Michaelis's barbital buffer (0.14 M) at pH 5.2 and 8.2 are shown in figures 4.3 and 4.4. ET-1 degradation in barbital buffer was poor and different from phosphate buffer. In phosphate buffer at pH=5.2 after 60 min incubation the degradation was 96.7±3.5% (table 4.2) , while in barbital buffer it was only 31.7±6.5%. There was no enzymic degradation of ET-1 at pH=8.2 in barbital buffer at all (figure 4.4). There is no obvious explanation for this difference. However, it was impossible to perform the experiments in two different buffers with distinct effects on enzymic activity. Since the enzymic activity in phosphate buffer was high, phosphate buffer was chosen for the pH studies.

4.1.4 Effects of Acetic Acid on ET-1 Degradation

To produce the pH range, phosphate buffer (0.1M, 5.2-8.2) was used, but incubating at the lowest pH (4.2) was carried out by adding acetic acid (0.02M) to phosphate buffer at pH=7.00. ET-1 proved to be absolutely stable in acetic acid solution for up to 90 min (Chapter 2, section 2.9). To determine whether acetate would affect the

enzymic activity, the degradation of ET-1 was studied at pH=5.2 in phosphate buffer (0.1M) in presence and absence of acetic acid (0.02M).

4.1.4.1 Experimental Procedure

The pH of phosphate buffer (pH=7.00, 0.1M) was reduced to 5.2 by adding acetic acid (0.02M). Rat lung and ventricular extract were diluted 20 times by using phosphate buffer (pH=5.2) alone and phosphate buffer containing acetic acid (pH=5.2). ET-1 was incubated in boiled and unboiled tissue extracts for 60 min at 25°C. In all experiments the pH of boiled and unboiled extracts was checked before incubation. To confirm the constancy of pH of tissue extracts in the presence of acetic acid, in some control experiments (n=6) the boiled and unboiled extracts were left for 60 min at 25°C and then the pH was checked. A pH variance of $\pm$0.04 units was accepted.

4.1.4.2 Results

In this pilot experiment no effect of acetate on ET-1 degradation was detected either in extracts of ventricle (61.86$\pm$5.30%, n=3, $p>0.25$) or lung (97.21$\pm$2.91%, n=3; $p>0.25$). The rate of degradation was the same as that in phosphate buffer without acetate (58.84$\pm$2.85% in ventricle and 98.91$\pm$1.88% in lung). Acetate did not affect the constancy of pH of tissue extracts during the incubation period.

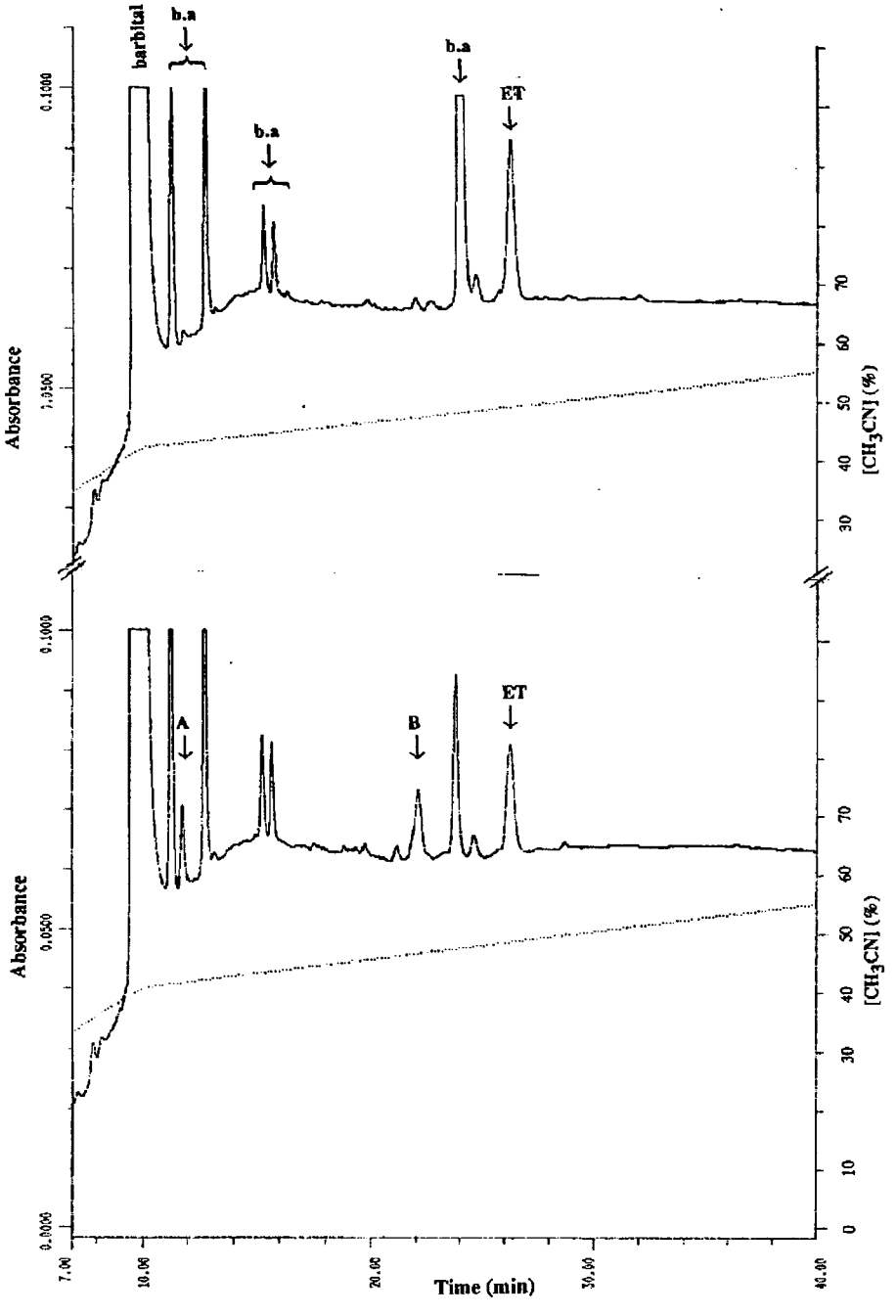


Figure 4.3 HPLC analysis of peptide products formed by incubation of ET-1 with rat lung extract diluted by Michaelis's barbital buffer. ET-1 (8 nmol.ml^{-1}) was incubated with boiled (upper trace) and unboiled extracts (lower trace), at pH 5.2, for 60 min at 25°C. The product peaks are indicated by A and B, and b.a indicates the buffer artefact.

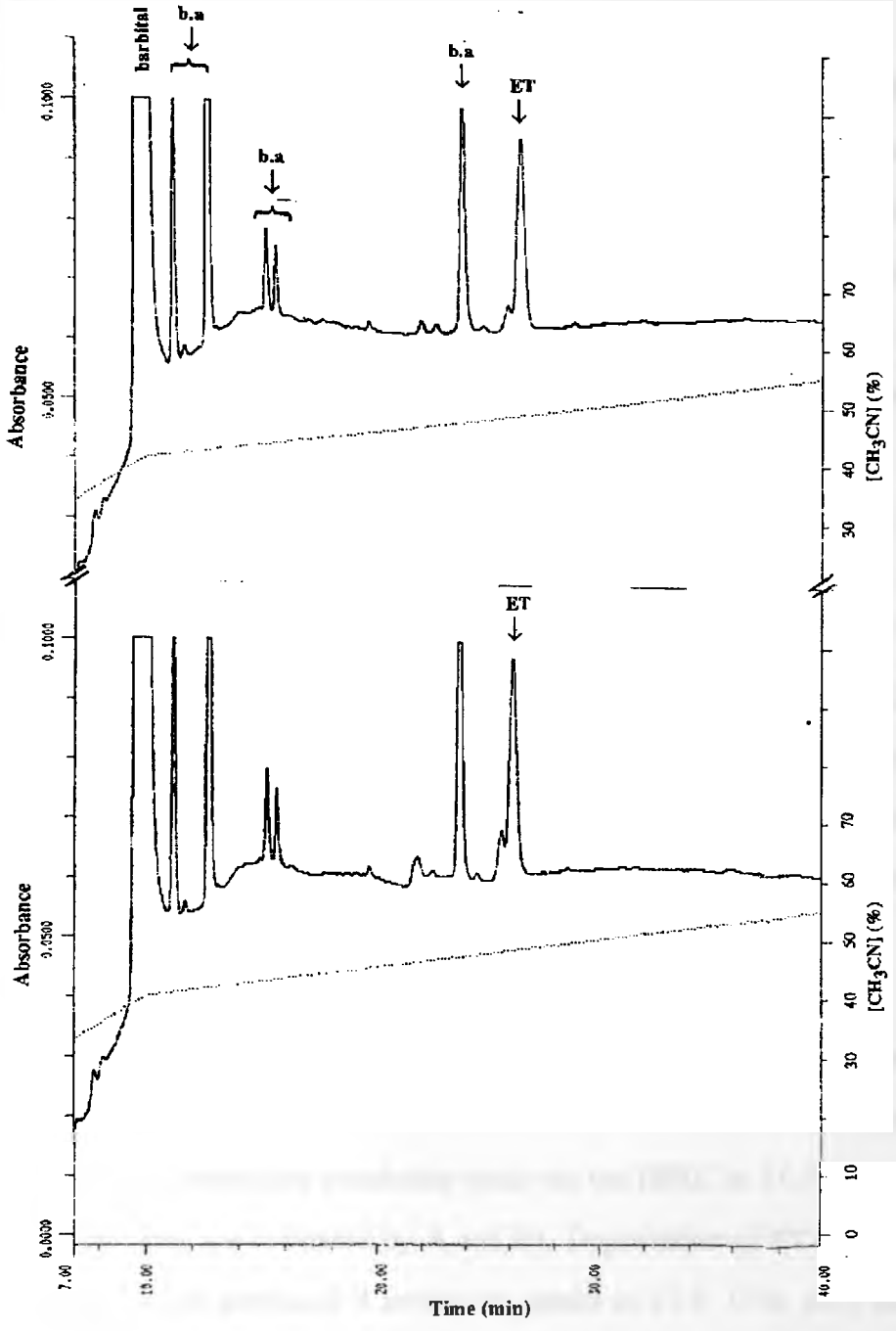


Figure 4.4 HPLC analysis of incubation of ET-1 with rat lung extract diluted by Michaelis's barbital buffer. ET-1 (8 nmol.ml^{-1}) was incubated with boiled (upper trace) and unboiled (lower trace), at pH 8.2, for 60 min at 25°C. No product peaks are present, and b.a indicates the buffer artefact.

4.1.5 pH Dependence of Degradation of ET-1

An enzyme characteristically has its peak activity in a relatively narrow pH range, which is the pH optimum for the enzyme. If the pH is changed from this optimum, the reaction rate decreases. This decreased enzymic activity is due to changes in the conformation of the enzyme and in the changes of the side chains (R) of the amino acids lining the active sites. The aim of this experiment was to determine the pH optimum for ET-1 enzymic degradation in aqueous tissue extracts of rat lung and ventricle.

4.1.5.1 Experimental Procedure

ET-1 at a final concentration of 8 $nmol.ml^{-1}$ was incubated for 60 min at 25°C with extracts of rat ventricle and lung, 20 times diluted with phosphate buffer (protein concentrations are shown in table 2.2), at pHs 4.2-8.2. The samples were analysed by reverse-phase HPLC as described in Chapter 2 (section 2.7.1). The degradation was calculated as percentage of ET-1 content remaining in a boiled extract incubate.

4.1.5.2 Results

The HPLC chromatograms of ET-1 degradation in ventricle and lung tissue extracts are illustrated in figures 4.5 and 4.6. Incubation of ET-1 with myocardial extracts at pH 5.2 produced two detectable metabolite peaks on the HPLC at 11.5 and 22.5 min (figure 4.6, metabolites are indicated by A and B). Degradation of ET-1 by the lung extracts at the same pH produced 4 metabolite peaks at 11.5, 17.6, 19.5, and 22.5 min (figure 4.5, metabolites are indicated by A, C, D, and B). Metabolites A and B were the same in both tissues but two other metabolites (C and D) were not detectably produced by ventricular extract. The results are shown in table 4.3. ET-1 was degraded by ventricular extracts optimally at acid pH and by lung extract optimally at pH 5.2 (figure 4.7). At pH 5.2, the myocardial extracts contained less ET-1 degrading activity (56.9±5.4% or 0.084 $nmol.min^{-1}.mg^{-1}$ of protein content of

extract) than those from the lung (96.7±3.5% or 0.169 $nmol.min^{-1}.mg^{-1}$ of protein content of extract, $p<0.01$). ET-1 degradation at pH 7.2 in both tissues, 18.4±4.4% (0.031 $nmol.min^{-1}.mg^{-1}$ of extract protein) in myocardium and 27.5±3.8% (0.052 $nmol.min^{-1}.mg^{-1}$ of extract protein) in lung, was not significantly different from each other but significantly less than at pH 5.2 ($p<0.02$; figure 4.7). The degradation of ET-1 at pH 7.2 produced no detectable metabolite peaks (figure 4.8). However mass spectroscopy of HPLC eluates of ET-1 degradation (at retention time of 17.5) by ventricular extract after 90 min incubation showed the presence of fragment 1-17 (figure 4.15). At pH 6.2, which is pathophysiologically important (the intracellular pH may decrease as low as 6.26-6.03 in rats after 10 min myocardial ischaemia; Khandoudi et al., 1990), the myocardial ET-1 degradation activity was greatly increased to 222% of that at the more physiological pH 7.2 ($p<0.02$).

Figure 4.5⇒

HPLC analysis of peptide products formed by incubation of ET-1 with extract of rat lung: ET-1 (8 $nmol.ml^{-1}$) was incubated (60 min, pH 5.2) with extract containing active enzyme (Unboiled) or identical extracts which had been inactivated by boiling (Boiled). Enzymic degradation of ET-1 by lung extracts produced 4 metabolite peaks (indicated by A, C, D, and B). A buffer artefact (b.a) was present in all runs.

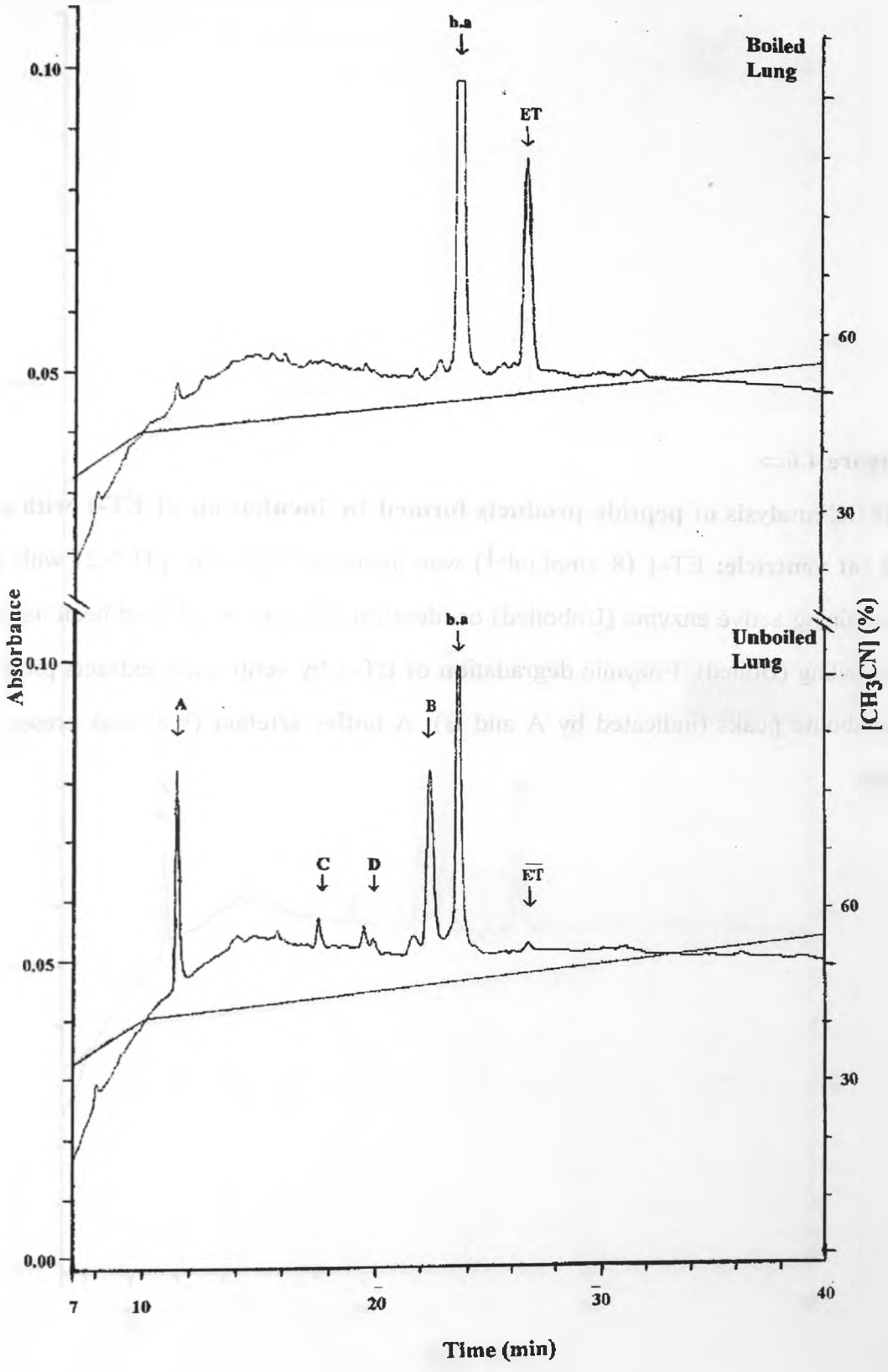

b.a
Boiled Lung
ET
0.10
0.05
60
30
Absorbance
b.a
Unboiled Lung
A
B
C
D
ET
0.10
0.05
0.00
60
30
[CH3CN] (%)
7
10
20
30
40
Time (min)

Figure 4.6⇒

HPLC analysis of peptide products formed by incubation of ET-1 with extract of rat ventricle: ET-1 (8 nmol.ml^{-1}) was incubated (60 min, pH 5.2) with extract containing active enzyme (Unboiled) or identical extracts which had been inactivated by boiling (Boiled). Enzymic degradation of ET-1 by ventricular extracts produced 2 metabolite peaks (indicated by A and B). A buffer artefact (b.a) was present in all runs.

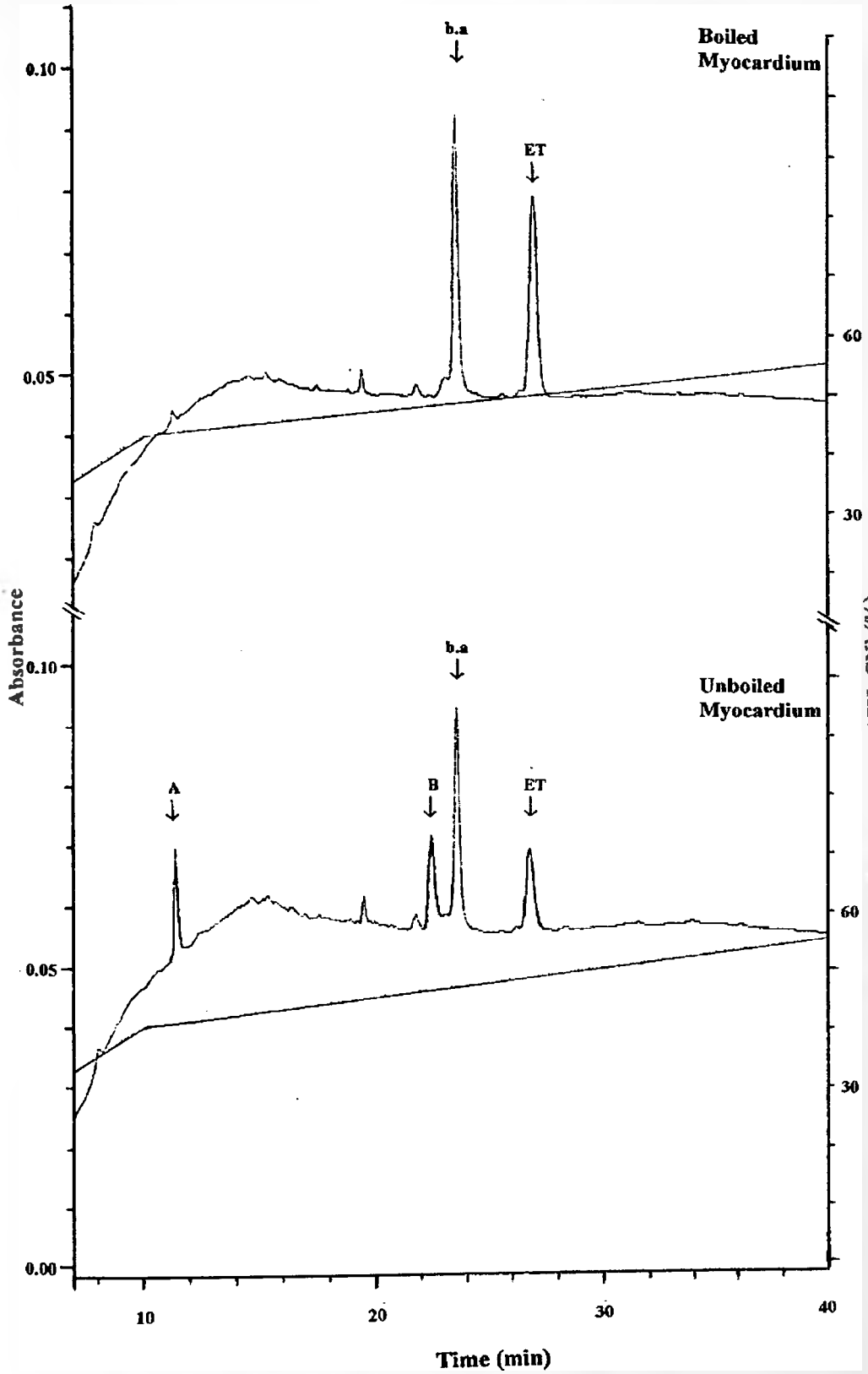

b.a
Boiled
Myocardium
ET
0.10
0.05
60
30
Absorbance
b.a
Unboiled
Myocardium
A
B
ET
0.10
0.05
0.00
60
30
[CH3CN] (%)
10
20
30
40
Time (min)

Table 4.3: Degradation of ET-1 (8 nmol.ml^{-1}) incubated for 60 min at 25°C with 20 times diluted lung and ventricular extract in phosphate buffer (0.1M), at various pHs. Data represents mean±SD.

pH⇒		**4.2**	**5.2**	**6.2**	**7.2**	**8.2**
n⇒		***3***	**5**	**3**	**5**	**3**
ET Conc. In boiled	***Ventricle***	7.5±0.5	7.1±0.7	7.6±0.3	8.5±1.6	8.0±0.7
extract (μg.ml^{-1})	***Lung***	8.6±1.2	8.4±0.6	7.5±1.0	9.3±0.6	7.5±0.1
ET Conc. In Unboiled	***Ventricle***	2.0±0.2	3.2±0.8	4.4±0.6	7.0±1.6	6.8±0.8
extract (μg.ml^{-1})	***Lung***	2.4±0.4	0.3±0.3	2.5±0.5	9.7±0.1	6.5±0.2
Amount of degradation	***Ventricle***	5.5±0.6	4.1±0.5	3.1±0.7	1.5±0.2	1.2±0.2
(μg.ml^{-1})	***Lung***	6.1±0.8	8.1±0.5	4.8±0.8	2.5±0.5	1.1±0.2
% Degradation	***Ventricle***	73.6±4.0	56.9±5.4	41.0±8.2	18.4±4.4	15.5±2.8
(percentage of boiled)	***Lung***	71.6±2.3	96.7±3.5	63.7±2.5	27.5±3.8	14.2±2.8

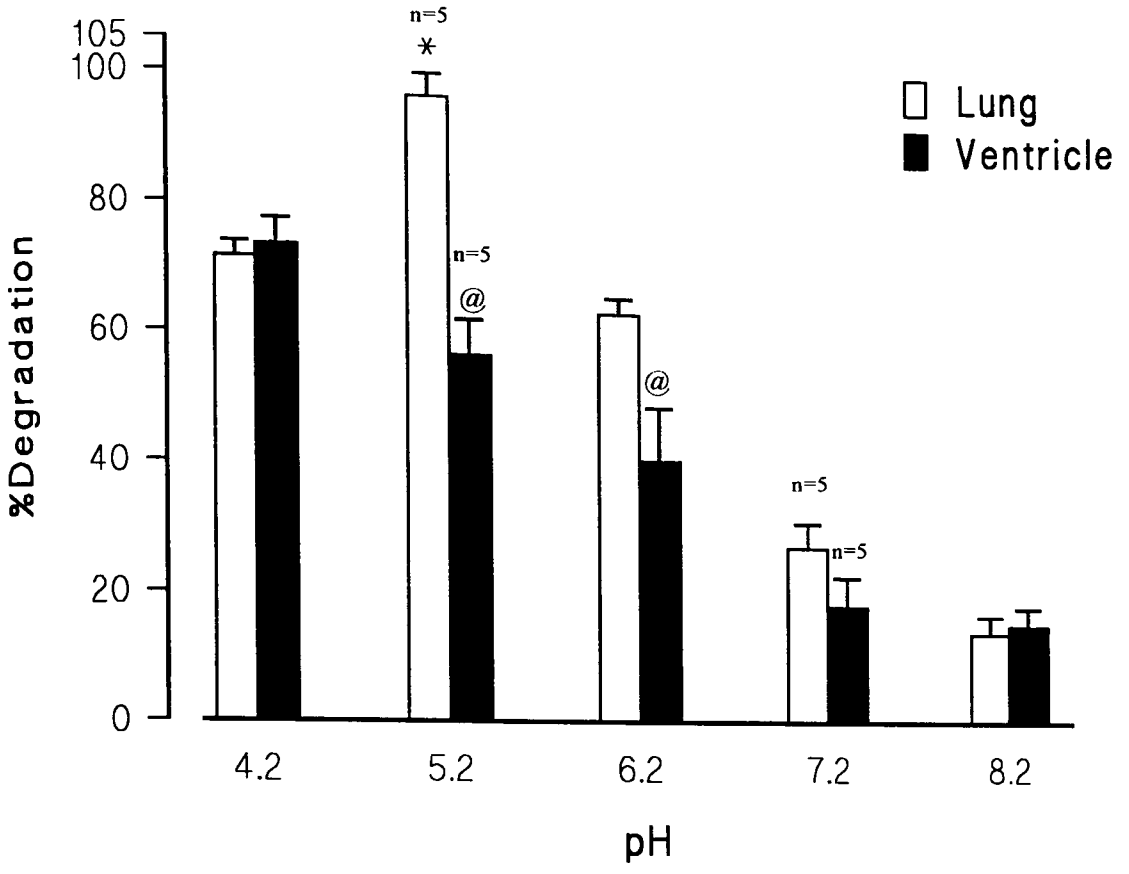
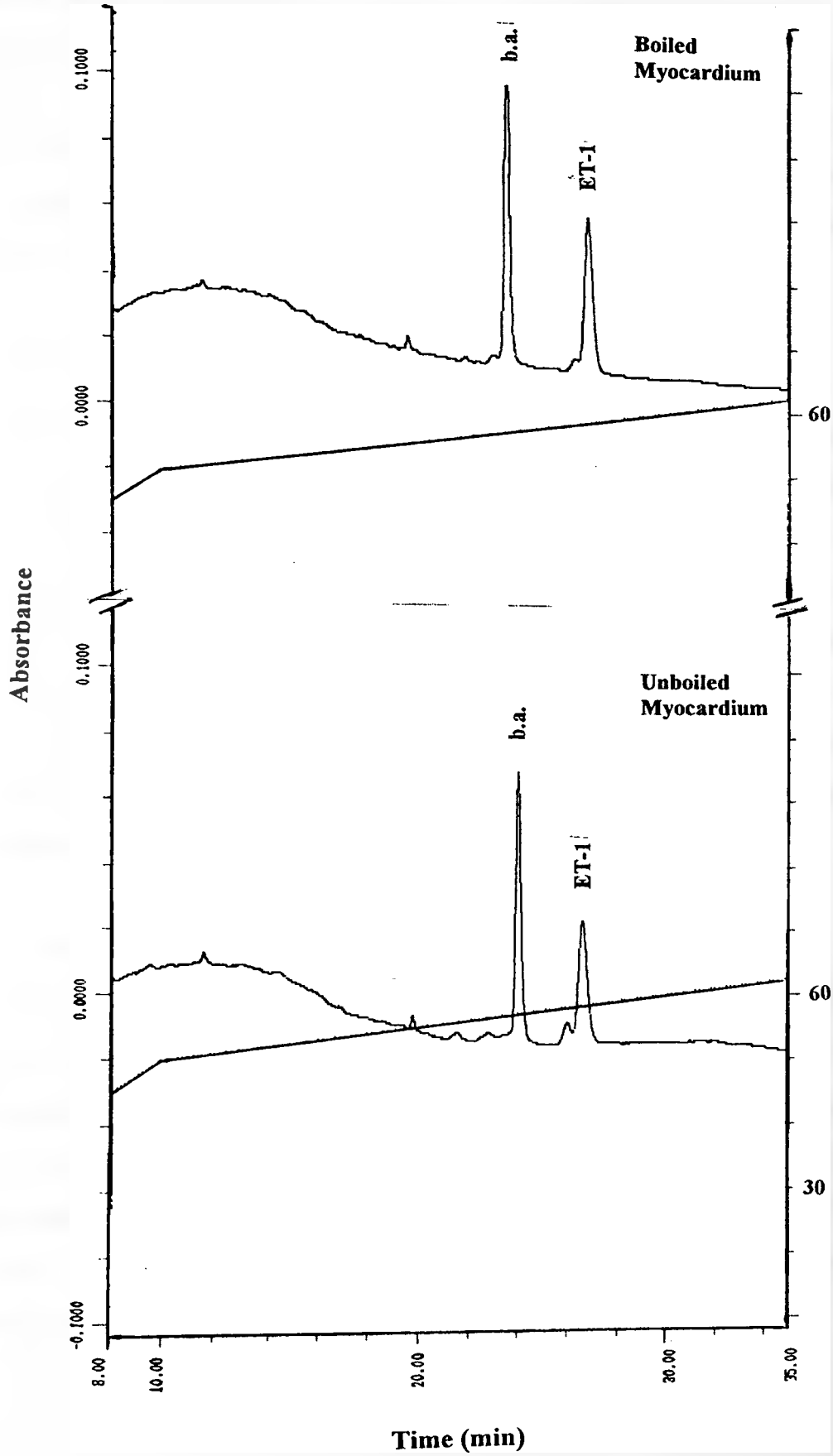


Figure 4.7 Degradation of ET-1 by rat lung and ventricular tissue extracts. ET-1 (8 nmol.ml^{-1}) was incubated with tissue extracts for 60 min, at pH 4.2-8.2. ET-1 was degraded by both ventricular and lung extracts optimally at acid pH. Points represent mean±S.D and n=3 or 5. *$p<0.02$ vs ventricle at pH 5.2; @$p<0.02$ vs ventricle at pH 7.2. Data for this figure is also shown in table 4.3.

Figure 4.8 ⇒

HPLC analysis of peptide products formed by incubation of ET-1 with extract of rat ventricle: ET-1 (8 nmol.ml^{-1}) was incubated (60 min, pH 7.2) with extract containing active enzyme (Unboiled) or identical extracts which had been inactivated by boiling (Boiled). No detectable product peaks are present. A buffer artefact (b.a.) was present in all runs.


Boiled
Myocardium
b.a.
ET-1
0.1000
0.0000
60
Unboiled
Myocardium
b.a.
ET-1
0.1000
0.0000
-0.1000
60
30
8.00
10.00
20.00
30.00
35.00
Absorbance
Time (min)
[CH3CN](%)

4.1.6 Possible Production of ET(16-21) as an ET-1 Metabolite

Fagny et al (1991) reported that endopeptidase 24.11 hydrolysed ET-1 in vitro at several sites, one of which was Cys^{15}-His^{16} leading to the generation of the C-terminal hexapeptide. Here, the aim was to determine whether the C-terminal hexapeptide of ET-1, His-Leu-Asp-Ile-Ile-Trp, is one of the ET-1 metabolites produced by myocardium at pH 5.2.

4.1.6.1 Experimental Procedure

ET(16-21) was dissolved in distilled water, diluted with mobile phase (8 $nmol.ml^{-1}$) and then chromatographed as described in Chapter 2 (section 2.7.1). To confirm that ET(16-21) is no a metabolite of ET-1 in another experiment ET-1 (8 $nmol.ml^{-1}$) was incubated with 20 times diluted rat ventricular extract (protein content 412±14 $\mu g.ml^{-1}$) for 90 min at pH=5.2 and 25°C, and processed as described in Chapter 2 (sections 2.8 and 2.7.1). At the final step, when the incubated ET-1 sample was ready to load into the RP-HPLC, to distinguish ET-1 metabolite peaks from the ET(16-21) peak, ET(16-21) was added to it at a final concentration of 8 $nmol.ml^{-1}$ and then chromatographed.

4.1.6.2 Results

ET(16-21) gave a peak at a retention time of 21.5 min. As the chromatogram (figure 4.9) shows, this peak was close to the ET-1 metabolite (B) at 22 min. ET(16-21) plus incubated ET-1 (containing ET-1 and its two metabolites A and B) gave peaks at 11.5, 21.5, 22, and 26.5 min which correspond to metabolite A, ET(16-21), metabolite B and ET-1, respectively. According to this chromatogram, ET(16-21) (the ET-1 C-terminal hexapeptide) is not an ET-1 metabolite which is produced by either rat ventricle or lung.

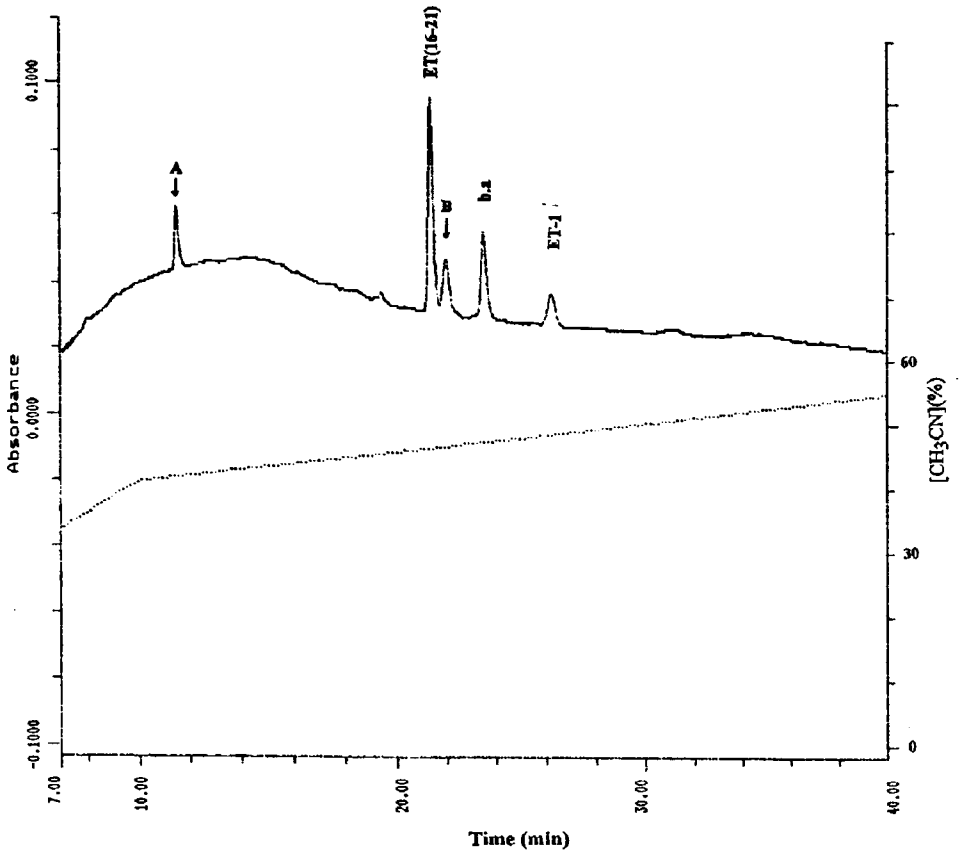


Figure 4.9 HPLC chromatogram of metabolites of ET-1, produced upon 90 min incubation at pH 5.2 with ventricular extract, containing ET(16-21). After incubation of ET-1 with tissue extract immediately before loading the sample into the HPLC, ET(16-21) was added to the sample.

4.1.7 ET(16-21) Degradation by Rat Ventricle

The aim of this experiment was to examine the enzymic degradation of ET-1 C-terminal hexapeptide [ET(16-21)] by rat ventricular extract, and to compare the produced metabolites with those produced by the degradation of ET-1.

4.1.7.1 Experimental Procedure

ET(16-21) (8 $nmol.ml^{-1}$) was incubated in phosphate buffer (0.1M, pH=5.2), for 90 min at 25°C, to determine whether ET(16-21) is stable under incubation conditions. In other experiments ET(16-21), at a final concentration of 8 $nmol.ml^{-1}$, was incubated with 20 times diluted, boiled (inactive) and unboiled (active; protein concentration 412±14 $\mu g.ml^{-1}$), rat ventricular extracts for 90 min at pH=5.2 and 25°C. The samples were analysed by RP-HPLC as described for ET-1 in Chapter 2 (section 2.7.1).

4.1.7.2 Results

The incubation conditions, in the absence of tissue extract, had no effect on ET(16-21) stability. Enzymic degradation of ET(16-21) produced 4 distinguishable metabolites (figure 4.10), one at 11.5 min (exactly same as the ET-1 first metabolite A), one at 17 min (probably the same as one of the ET-1 metabolites following incubation with lung extract) and two more at 18.34 and 18.60. The first metabolite of ET(16-21) at the retention time of 11.5 (figure 4.10) matches the first metabolite (tryptophan) of ET-1 produced by both rat ventricular and lung extracts (figures 4.5 and 4.6). The break down of ET(16-21) by rat ventricular extract at pH 5.2 was 45.9±5.5% (n=3, mean±SD) after 90 min incubation.

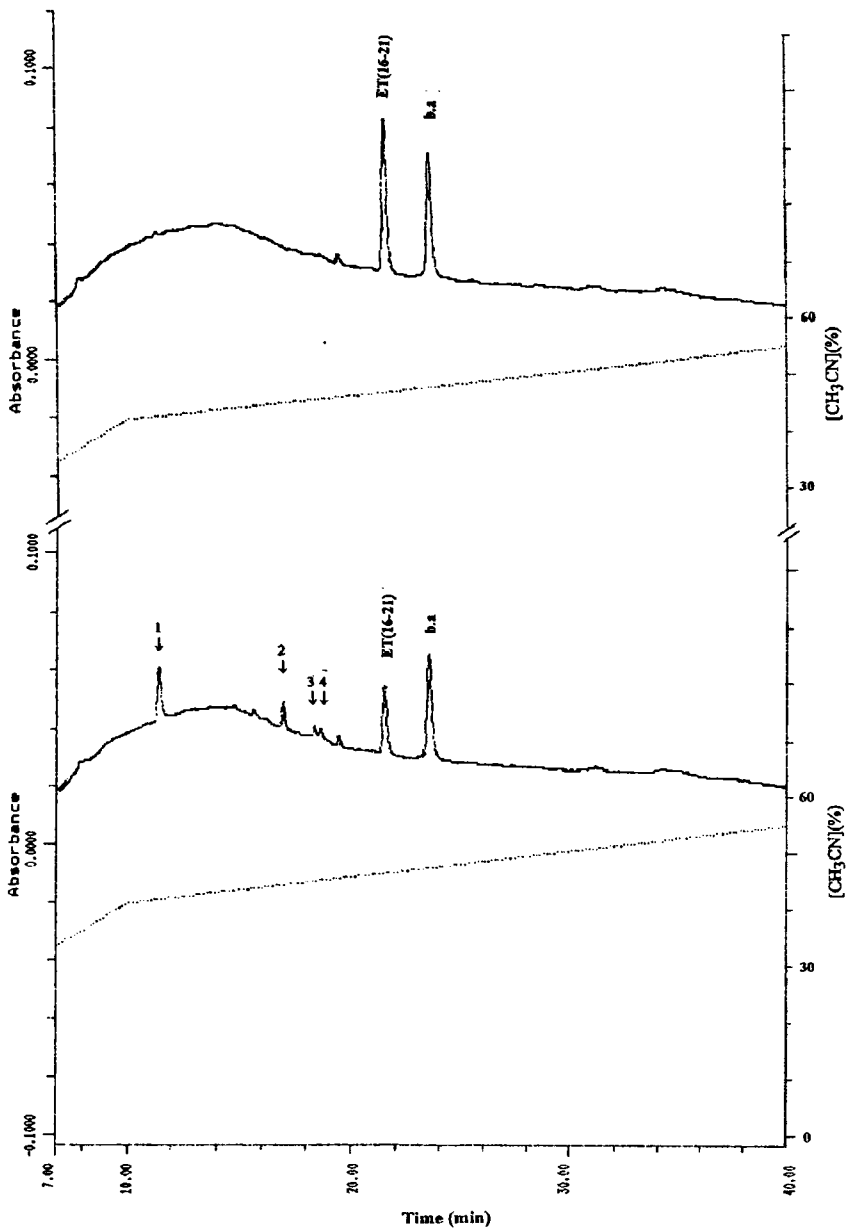


Figure 4.10 HPLC analysis of peptide products formed by incubation of ET(16-21) with extract of rat ventricle: ET(16-21) (8 $nmol.ml^{-1}$) was incubated (90 min, pH 5.2) with extract containing active enzyme (unboiled; lower trace) or identical extracts which had been inactivated by boiling (boiled; upper trace). Enzymic degradation of ET(16-21) by ventricular extracts produced 4 metabolite peaks (indicated by 1, 2, 3, and 4). A buffer artefact (b.a) was present in all runs.

4.1.8 Elution of Tryptophan in Reverse Phase HPLC

It has been shown that enzymes from rat kidney (Deng et al., 1994) and vascular endothelial cells (Jackman et al., 1993) remove the carboxyl terminal tryptophan of ET-1 by cleaving the Ile^{20}-Trp^{21} peptide bond. In our study, degradation of ET-1 by rat ventricular and lung extracts at pH 5.2 produced two and four detectable peaks, respectively. The aim of this experiment was to determine whether one of these peaks belongs to tryptophan.

4.1.8.1 Experimental procedure

L-Tryptophan was dissolved in distilled water and then diluted by mobile phase, making an 8 $nmol.ml^{-1}$ solution. The sample was loaded into the RP-HPLC column exactly in the same way that was described for incubated ET-1.

4.1.8.2 Results

Tryptophan produced a peak at retention time of 11.5, the same retention time at which the first metabolite of ET-1 and ET(16-21) was eluted. This indicates that tryptophan is one of the fragments cleaved enzymically from ET-1 (figure 4.11; the first one, indicated by A) by rat ventricular or lung extracts.

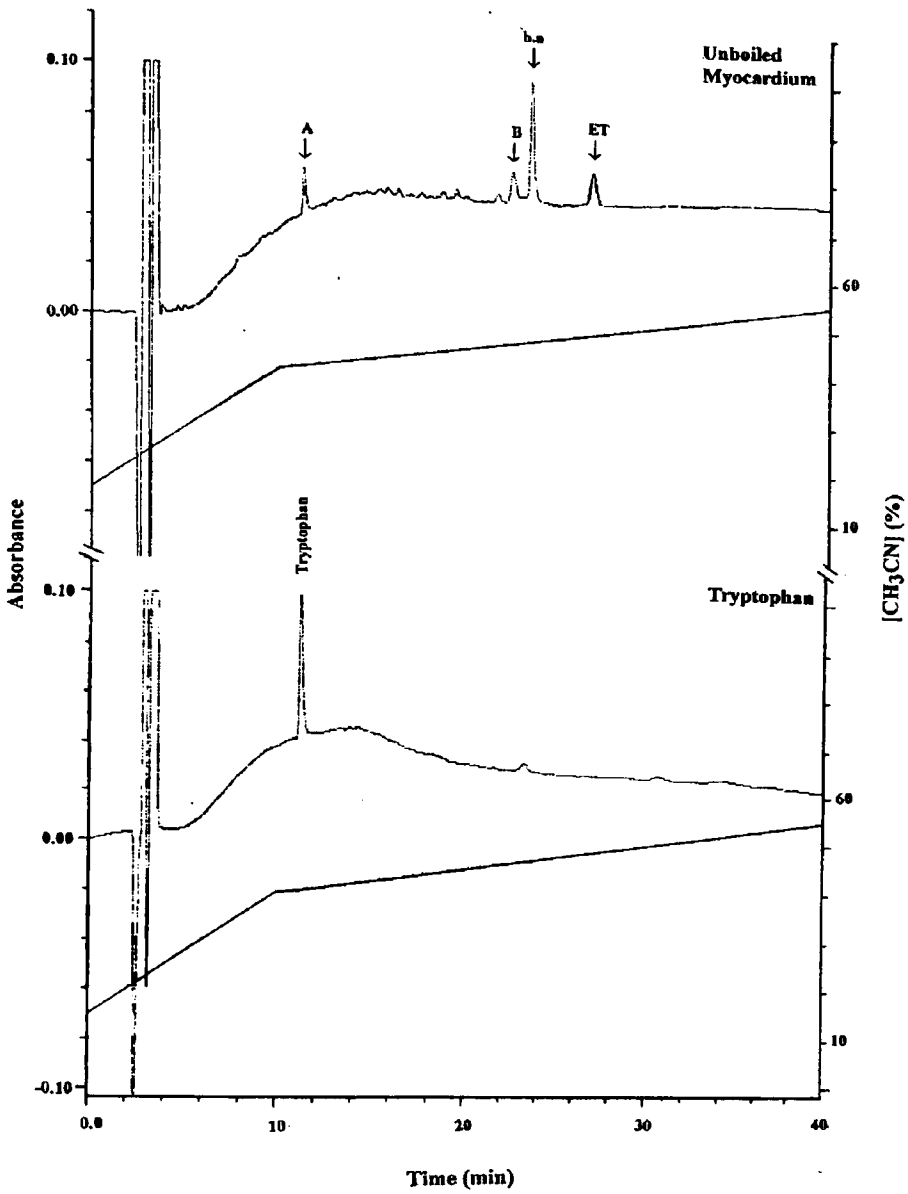


Figure 4.11 The chromatography of L-Tryptophan (8 nmol.ml^{-1}) on the RP-HPLC C18 column. The upper trace shows the elution of the degradation products produced by incubating ET-1 (8 nmol.ml^{-1}) with myocardial extracts. The lower trace shows the elution of tryptophan which has a retention time (11.50 min) identical to that of peak A. Peak A may thus be tryptophan, the C-terminal amino acid of ET-1.

4.2 IDENTIFICATION OF ET-1 METABOLITES PRODUCED BY RAT VENTRICULAR EXTRACT BY MASS SPECTROSCOPY

Degradation fragments of ET-1, produced by 90 min incubation with rat ventricular extract at pH 5.2, were separated using the HPLC and lyophilized. The molecular weights of the degradation fragments in the lyophilized extracts were determined by electrospray mass spectroscopy as described in Chapter 2 (section 2.11). The degradation of ET-1 by ventricular extract at pH 7.2 produced no detectable metabolite peaks on HPLC. Thus, the HPLC eluates of ET-1 degradation at pH 7.2 (after 90 min incubation) at retention times identical to the retention times of metabolites A and B of ET-1 degradation at pH 5.2 (10.5-11.5 and 21.5-22.5 min) and of 17.5 and 18 min (identical to metabolites of big ET-1 at pH 7.2; see section 5.4.1) were collected and analysed by mass spectroscopy.

4.2.1 Results

Mass spectroscopy showed the mean molecular weight of metabolite B (figure 4.13) to be 2306 Da. This molecular weight is identical to that of ET-1 (mean molecular weight of 2492 Da; figure 5.15) with the molecular weight of the carboxyl terminal tryptophan residue (186 Da) subtracted (i.e. des-Trp-ET-1). Figure 4.14 shows that the molecular weight of fraction A was 186 Da (after subtracting the molecular weight of water and proton) which is identical to that of tryptophan. These results strongly indicate that myocardial degradation of ET-1 at acid pH involves cleavage of the Ile^{20}-Trp^{21} peptide bond of endothelin-1 (figure 4.12). The HPLC fractions of ET-1 degradation at pH 7.2 at retention times of 17.5 and 18 min both contained a trace of fragment ET(1-17) with molecular weight of 1965 Da (figure 4.15, mass spectrum number 4). The ET-1 peak on HPLC was also identified as ET-1 (mean molecular weight of 2492) by mass spectroscopy. This confirmed that the major part of this peak corresponded to ET-1 and did not contain any other fragment (figure 4.15, mass spectrum number 1). Neither tryptophan (metabolite A of ET-1

degradation at pH 5.2, the mass spectrum was not shown) nor des-Trp-ET-1 (metabolite B of ET-1 degradation at pH 5.2) was detected at pH 7.2 by mass spectroscopy (figure 4.15, mass spectrum 2). Thus, the degradation of ET-1 at pH 7.2 does not produce the metabolites A and B of that at pH 5.2.

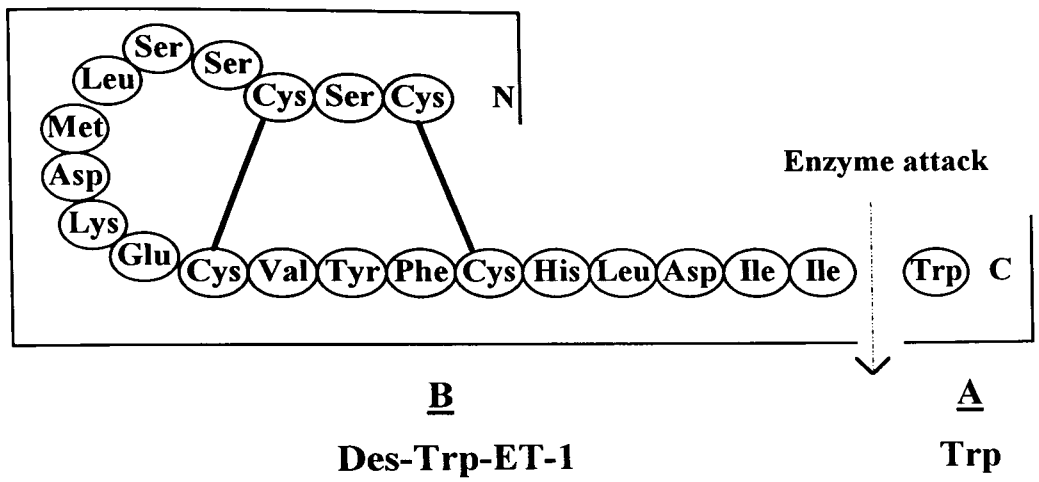


Figure 4.12 Schematic diagram of the sequence for ET-1. Cleavage at the arrow yields des-Trp-ET-1 and tryptophan.

Figure 4.13 ⇒

Mass spectrum of metabolite B produced by the degradation of ET-1 by rat ventricular extract at pH 5.2. The peaks 1153.74 (MW=2305.5) and 1164.57 (MW=2306.2) correspond to the des-Trp-ET-1 with mean molecular weight of 2306. Other peaks are background peaks due to solvent and tissue extracts and unrelated to any fragment of ET-1.

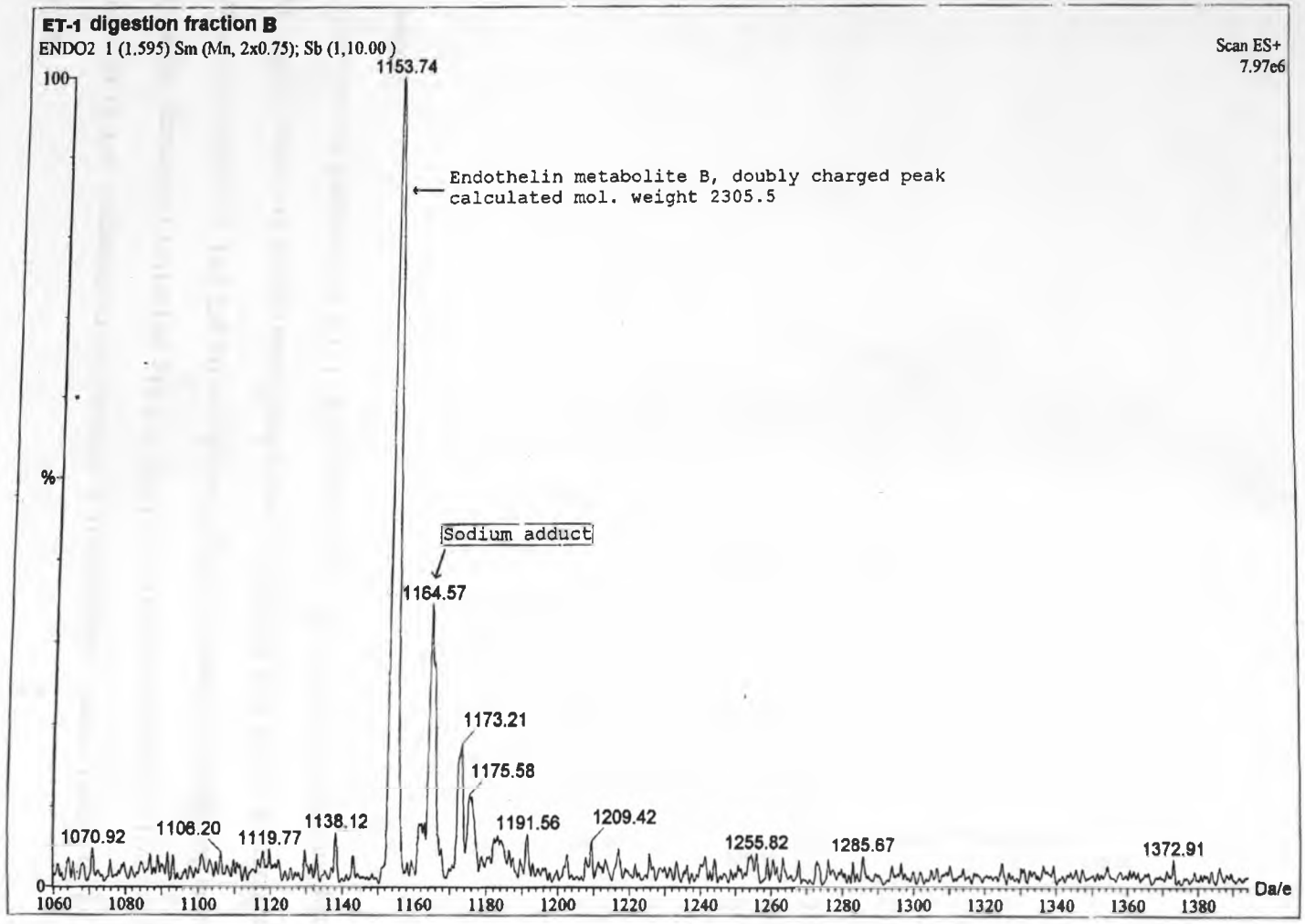

ET-1 digestion fraction B
ENDO2 1 (1.595) Sm (Mn, 2x0.75); Sb (1,10.00)
Scan ES+
7.97e6
100
%
0
1153.74
Endothelin metabolite B, doubly charged peak
calculated mol. weight 2305.5
Sodium adduct
1164.57
1173.21
1175.58
1070.92
1106.20
1119.77
1138.12
1191.56
1209.42
1255.82
1285.67
1372.91
1060
1080
1100
1120
1140
1160
1180
1200
1220
1240
1260
1280
1300
1320
1340
1360
1380
Da/e

Figure 4.14 ⇒

Mass spectrum of the degradation metabolite A (tryptophan, lower trace) of ET-1 produced by rat ventricular extract at pH 5.2. Peak 205.08 corresponds to tryptophan with a molecular weight of 186 Da after subtracting the molecular weight of proton and water. Other peaks are background peaks due to solvent and tissue extracts and are not related to any fragment of ET-1. The upper trace shows the background peaks (solvent peaks).

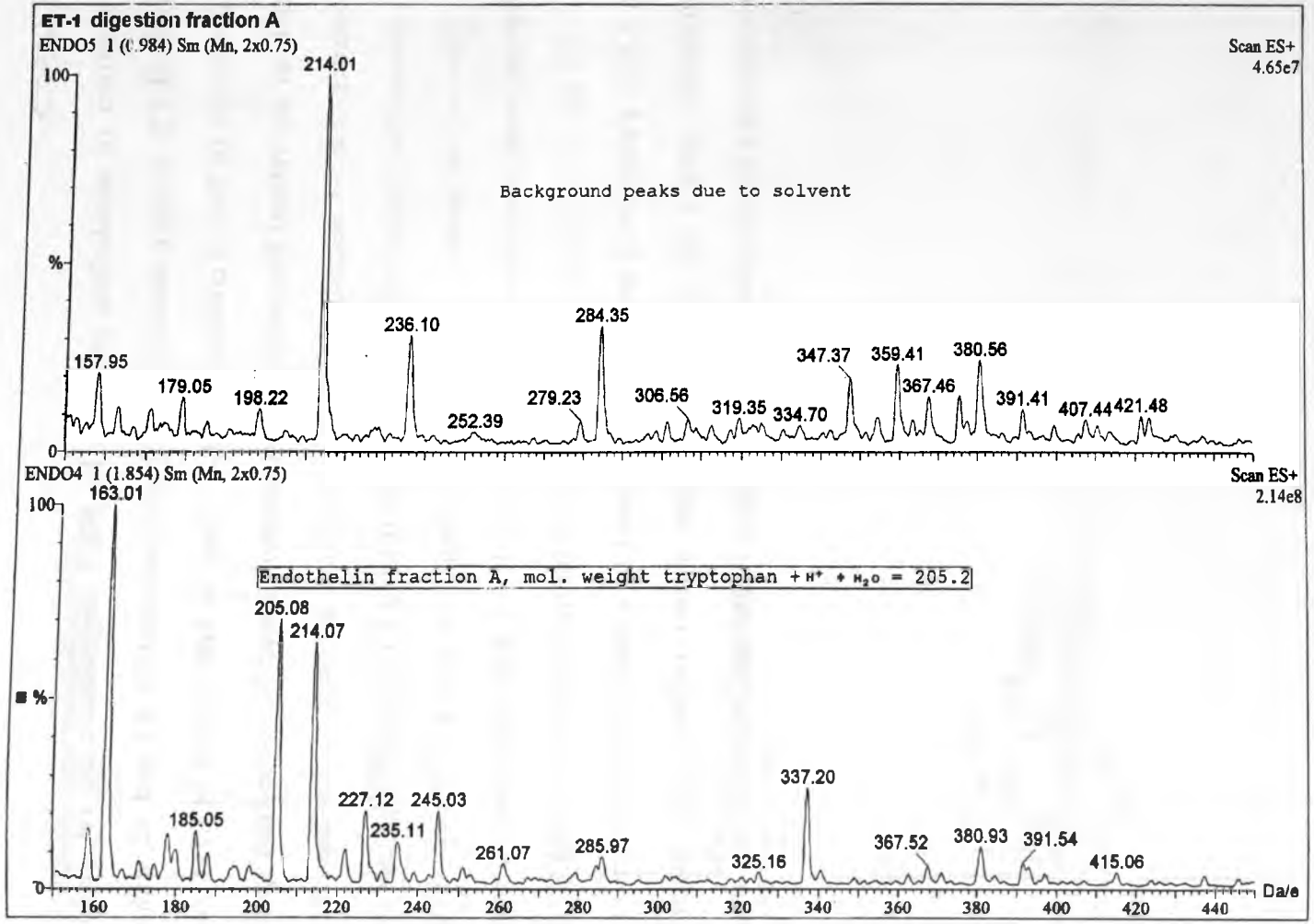
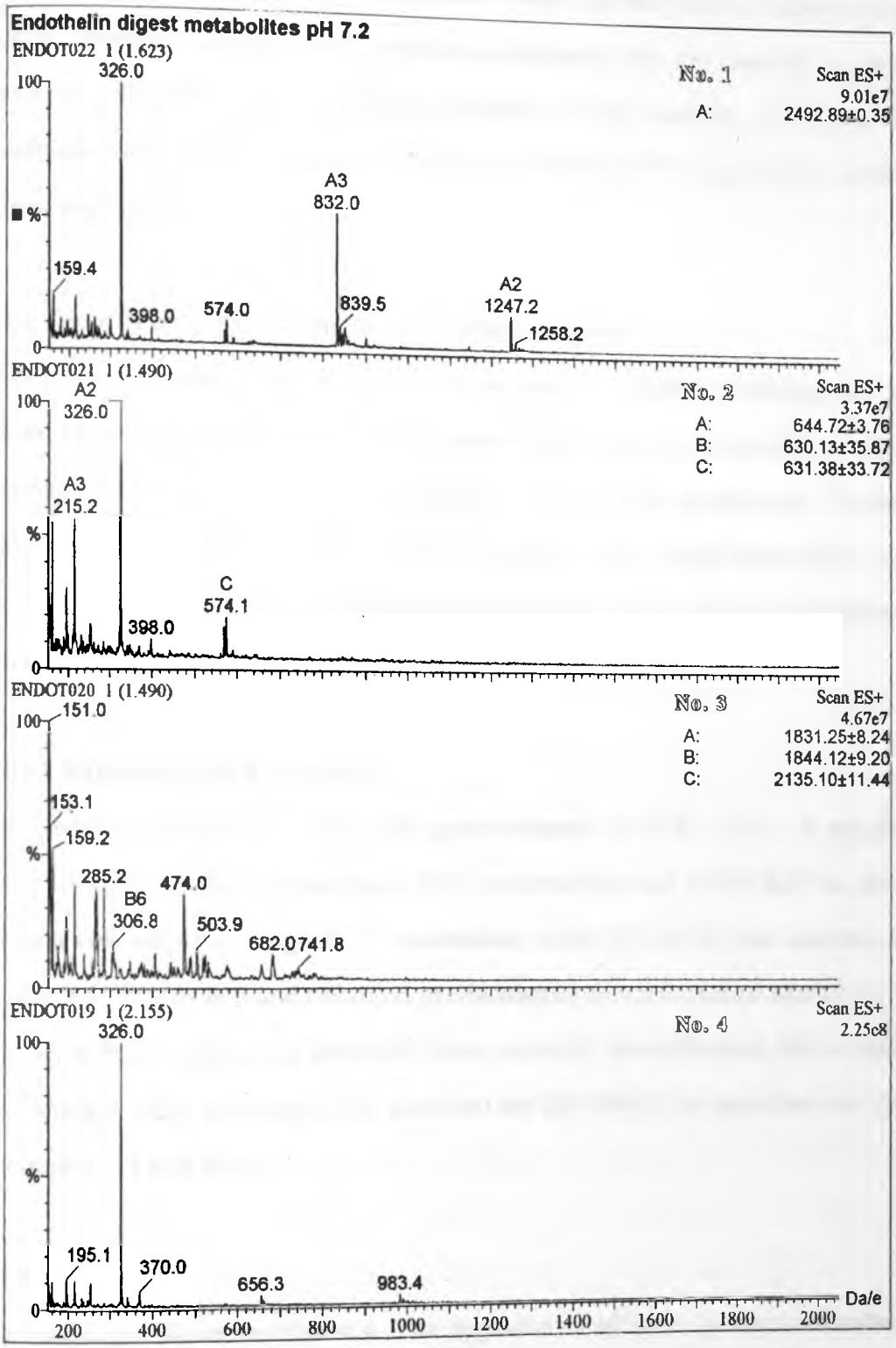

ET-1 digestion fraction A
ENDO5 1 (0.984) Sm (Mn, 2x0.75)
Scan ES+
4.65e7
214.01
Background peaks due to solvent
236.10
284.35
157.95
179.05
198.22
252.39
279.23
306.56
319.35
334.70
347.37
359.41
367.46
380.56
391.41
407.44
421.48
ENDO4 1 (1.854) Sm (Mn, 2x0.75)
Scan ES+
2.14e8
163.01
Endothelin fraction A, mol. weight tryptophan + H+ + H2O = 205.2
205.08
214.07
185.05
227.12
245.03
235.11
261.07
285.97
325.16
337.20
367.52
380.93
391.54
415.06
Da/e

Figure 4.15 ⟹

Mass spectrum of metabolites produced by ET-1 degradation by rat ventricular extract at pH 7.2. In *trace number 1,* the peaks indicated by A3 and A2 with mean molecular weight of 2492.5 correlate to ET-1. Thus the HPLC peak of ET-1 contains ET-1 and not any related fragments. *Traces numbers 2 and 3* are the mass spectra of HPLC eluates of ET-1 degradation at retention times identical to des-Trp-ET-1 and 18 min (identical to the retention time of one of the big ET-1 metabolites; see section 5.4.1). None of the peaks is related to any fragments of ET-1. *Trace number 4* indicates the mass spectrum of HPLC eluates of ET-1 degradation at the retention time of 17.5 min (identical to the retention time of another metabolite of big ET-1; see section 5.4.1). Peaks 656.3 and 983.4 correspond to a trace of ET-1 fragment 1-17 with molecular weight of 1965. Other peaks shown on the mass spectra are background peaks due to solvent and tissue extracts and not related to any fragment of ET-1.


Endothelin digest metabolites pH 7.2
ENDOT022 1 (1.623)
326.0
100
%
0
159.4
398.0
574.0
A3
832.0
839.5
A2
1247.2
1258.2
No. 1
Scan ES+
9.01e7
A: 2492.89±0.35
ENDOT021 1 (1.490)
A2
326.0
A3
215.2
C
574.1
398.0
No. 2
Scan ES+
3.37e7
A: 644.72±3.76
B: 630.13±35.87
C: 631.38±33.72
ENDOT020 1 (1.490)
151.0
153.1
159.2
285.2
B6
306.8
474.0
503.9
682.0
741.8
No. 3
Scan ES+
4.67e7
A: 1831.25±8.24
B: 1844.12±9.20
C: 2135.10±11.44
ENDOT019 1 (2.155)
326.0
195.1
370.0
656.3
983.4
No. 4
Scan ES+
2.25e8
200
400
600
800
1000
1200
1400
1600
1800
2000
Da/e

4.3 EFFECT OF ENZYME INHIBITORS ON THE DEGRADATION OF ET-1 BY RAT VENTRICULAR TISSUE EXTRACTS

An unknown peptidase may be assigned to one of the known classes of enzymes (serine, cysteine, aspartic and metallo-proteinases) by the use of group-specific inhibitors (Neurath, 1984). Peptidase inhibitors were used to provide preliminary characterization of the enzymes which are involved in ET-1 degradation at pH 5.2 by rat myocardium.

4.3.1 The Effect of EDTA on the ET-1 Degradation

EDTA is a chelator of the active site zinc ion in metallo-proteinases but can also inhibit other metal ion-dependent proteinases such as the calcium-dependent cysteine proteinases. EDTA may also de-stabilise some serine proteinases (Beynon and Salvesen, 1989). EDTA may interfere with other metal-dependent biological processes. Here the effect of EDTA has been studied on ET-1 degradation by rat ventricular extracts at pH=5.2 .

4.3.1.1 Experimental Procedure

Rat ventricular extract (0.1 ml) was pre-incubated at 25°C, with 1.9 ml phosphate buffer (0.1 M, pH 5.2) containing a final concentration of 5 mM EDTA, for 20 min (protein content 412±4 $\mu g.ml^{-1}$). Incubations were started by the addition of ET-1 (8 $nmol.ml^{-1}$) to boiled and unboiled pre-incubated extracts and continued for 90 min. The pH of both boiled and unboiled tissue extracts were checked before incubation. The samples were processed and analysed by RP-HPLC as described in Chapter 2 (sections 2.7.1 and 2.8).

4.3.1.2 Results

The results are shown in table 4.4. The degradation of ET-1 by rat ventricular extract in the presence of 5 mM EDTA was 73.3±4.2% and was comparable with

degradation of ET-1 under the same conditions without EDTA, as control (72.9±3.9%). ET-1 degradation in the presence of EDTA produced the same two metabolites which were produced in the absence of the EDTA. Thus, the degrading activity was not significantly ($p>0.93$) inhibited by an effective concentration of EDTA (5mM; Beynon and Salvesen, 1989) indicating that it was not metal dependent.

Table 4.4: Effects of proteinase inhibitors on ET-1 degradation by rat ventricular extract (protein content 412±4 $\mu g.ml^{-1}$), incubated for 90 min at pH 5.2. Data represent mean±S.D. *$p<0.05$ vs phosphate buffer as control. @$p<0.01$ vs phosphate buffer. **$p<0.02$ vs DMSO as control. *Control for this experiment was incubation of ET-1 in phosphate buffer alone.

Inhibitor	Final Conc.	n	Degradation (%)
Control(Phosphate buffer)	0.1 M	7	72.9±4
EDTA	5 mM	4	73.3±4
Phosphoramidon	10 μM	4	67.4±5
"	100 μM	6	51.6±6*
Captopril	1 mM	4	71.8±2
"	100 μM	4	67.2±3
Aprotinin	385 μM	4	74.4±4
Control (DMSO)	1%	4	37.8±2@
PMSF	200 μM	4	18.8±5**
Pepstatin A*	10 μM	4	68.4±5
Control (Methanol)	10%	5	53.4±2*
Pepstatin A	100 μM	6	47.0±6

4.3.2 The Effect of Phosphoramidon on ET-1 Degradation by Extracts of Rat Ventricles

Metallo-proteinases belong to one of the four classes of proteolytic enzymes. They are distributed throughout a variety of tissues and play key roles in numerous physiological processes. One of the most important families of this class which has received particular interest in recent years is that of membrane-bound metallo-peptidases. Some of these enzymes are believed to be essential for the production and degradation of many biologically active peptides. Furthermore, it is worth noting that membrane-bound peptidases are almost exclusively metallo-peptidases (Powers et al., 1986). One of the members of this family which is supposed to have an important role in production and degradation of ET-1 (Masaki et al., 1991; Fagny et al., 1991), is endopeptidase 24.11 (enkephalinase). Angiotensin converting enzyme (ACE) is another member of this group. Phosphoramidon (effective at concentrations of 1-10 μM) is an inhibitor of many metallo-endopeptidases and endopeptidase 24.11 is very susceptible to this inhibitor (Beynon and Salvesen, 1989). Phoshoramidon has been shown to suppress completely the production of most of the metabolites of ET-1 hydrolysis produced by kidney membranes (Yamaguchi et al., 1992a,b). In another study, phosphoramidon was observed to increase ET-1 concentration in human endothelial hybrid cells, suggesting that phosphoramidon sensitive proteinase(s) may be responsible for the ET-1 degradation (Saijonmaa et al., 1991). However, in another study by Sessa et al (1991b) it was shown that the metabolism of ET-1 by activated polymorphonuclear leukocytes was not prevented by phosphoramidon. It was decided to investigate the presence of a phosphoramidon sensitive component in ET-1 degrading activity in rat ventricular extract.

4.3.2.1 Experimental Procedure

The rat ventricular extract (0.1 ml) was pre-incubated, at 25°C, with 1.9 ml phosphate buffer (0.1M, pH=5.2; protein content 412±14 $\mu g.ml^{-1}$) containing phosphoramidon

at final concentrations of 10 and 100 μM for 20 min. Incubations were started by the addition of ET-1 (8 $nmol.ml^{-1}$) to boiled and unboiled pre-incubated extracts and continued for 90 min. The pH of both boiled and unboiled extracts were checked before incubation. The samples were processed and analysed by RP-HPLC as described in Chapter 2 (sections 2.7.1 and 2.8).

4.3.2.2 Results

Phosphoramidon at a concentration of 10 μM had no significant effect on the ET-1 degradation (67.4±4.9; $p>0.07$; Table 4.4). Increasing the concentration to 100 μM caused a 30% inhibition of ET-1 degradation (51.6±6.0%, $p<0.05$) compared to 72.9 ±3.9% degradation under control conditions (figure 4.16). These results indicate that a metallo-endopeptidase, possibly endopeptidase 24.11, at least in part is involved in the degradation of ET-1 by rat ventricular extract.

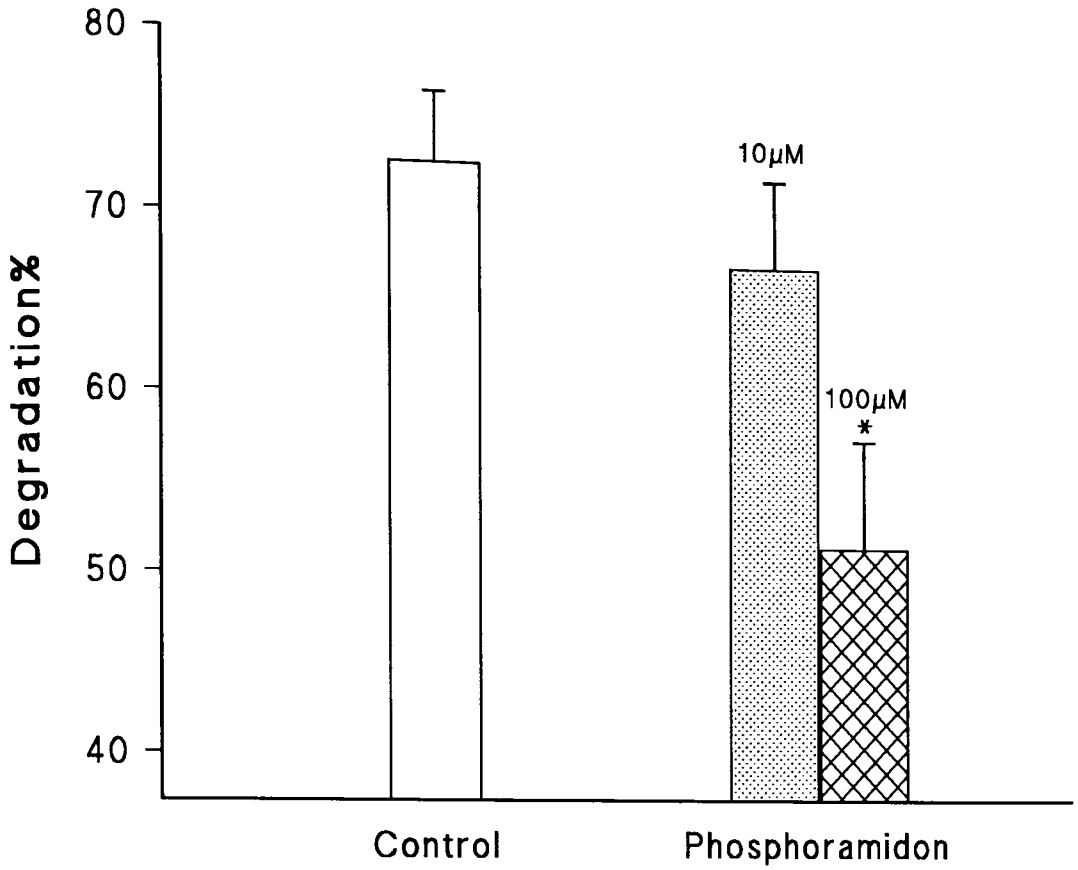


Figure 4.16 Degradation of incubated ET-1 by rat ventricular tissue extracts. The peptide was incubated for 90 min at pH 5.2 in the absence or presence of phosphoramidon. The samples were analysed by RP-HPLC as describe in Methods. Points represent mean±S.D, *$p<0.05$, n=4-7. Data for this figure is summarised in table 4.4.

4.3.3 The Effect of Captopril on ET-1 Degradation by Rat Ventricular Extract

Captopril is an angiotensin-converting enzyme (ACE) and kininase inhibitor (Beynon and Salvesen, 1989). The enzyme is a carboxypeptidase which splits off pairs of basic amino acids, and its active site is known to contain a zinc atom (Beynon and Salvesen, 1989). The aim of this experiment was to determine the effect of captopril on enzymic degradation of ET-1 by aqueous extracts of rat ventricle.

4.3.3.1 Experimental Procedure

Captopril solutions are acidic, therefore the pH of captopril solutions were adjusted to pH 5.2 using phosphate buffer (0.1M) with an appropriate pH. The rat ventricular extract, diluted 20 times with phosphate buffer (protein content 412±14 $\mu g.ml^{-1}$), contained captopril at concentrations of 5, 1, and 0.1 mM. The diluted extracts were pre-incubated at 25°C for 20 min. ET-1 at a final concentration of 8 $nmol.m^{-1}$ were incubated with the boiled and unboiled extracts for 90 min in a final volume of 250 µl. In other experiments to detect non-specific effects of captopril on ET-1 stability, ET-1 (8 $nmol.ml^{-1}$) was incubated for 90 min at pH 5.2 with phosphate buffer containing 5, 1, and 0.5 mM captopril. The samples were processed and analysed by RP-HPLC as described in Chapter 2 (sections 2.7.1 and 2.8).

4.3.3.2 Results

As shown in figure 4.17, the HPLC peak size of ET-1, after incubation with both boiled (without enzymic activity) and unboiled (with enzymic activity) extracts, was reduced (compared to standard in trace A of figure 4.18) very markedly in the presence of captopril at a concentration of 5 mM. The reduction of ET-1 content (80±4%; n=4) in boiled extract did not give rise to any detectable product peaks on the HPLC chromatogram. However, the chromatogram of the incubate of ET-1 in unboiled extract in the presence of captopril (5 mM) contained a weak peak (figure 4.17) identical to metabolite B of ET-1 degradation by ventricular extract at pH 5.2

without captopril. The marked reduction of ET-1 peak size without production of any detectable metabolite peak in boiled extract, and the approximate disappearance of the ET-1 peak in unboiled extract associated with a very weak metabolite peak both indicate that the reduction of ET-1 content is not due entirely to enzymic degradation and is induced non-specifically by captopril itself. Comparing the chromatograms of ET-1 incubated in phosphate buffer (0.1M; pH=5.2) in the presence of 0.5 mM (trace C of figure 4.18) and 5 mM (trace D of figure 4.18) of captopril with standard (trace A of figure 4.18) shows that captopril caused a reduction of the ET-1 peak height, dose-dependently, with no detectable product peak. However, as shown in figure 4.18 (trace D) captopril (5 mM) caused a tailing and a relative shift of the ET-1 peak, indicating a possible change of the chemical structure of ET-1. Incubation of ET-1 (8 $nmol.ml^{-1}$) with phosphate buffer containing captopril (pH 5.2) at concentrations of 5, 1, and 0.5 mM (n=4) caused a 59.7±4.5% , 34±1.4% and 29±4.6% reduction in ET-1 content respectively (figure 4.19). Captopril at 0.1 mM had no effect on the stability of ET-1.

Incubation of ET-1 with ventricular extracts in the presence of captopril at a concentration of 1 mM and 0.1 mM caused 71.8±2.22% ($p>0.78$) and 67.2±2.98% ($p>0.07$) enzymic degradation respectively neither of which were significantly different from control (figure 4.20). This degradation produced the same metabolites which were produced by extracts without captopril. These results show that captopril had no effect on enzymic degradation of ET-1 by rat myocardium extract.

Figure 4.17⇒

HPLC chromatogram of incubation of ET-1 with extract of rat ventricle in the presence of captopril (5mM): ET-1 (8 nmol.ml^{-1}) was incubated (90 min, pH 5.2) with extracts containing active enzyme (unboiled) or identical extracts which had been inactivated by boiling (boiled). Both the boiled and unboiled extracts contained 5 mM captopril. ET-1 peak height size reduced markedly in both active and inactive tissue extracts. The peak indicated by Met. B is identical to metabolite B of ET-1 degradation. A buffer artefact (b.a) was present in all runs.

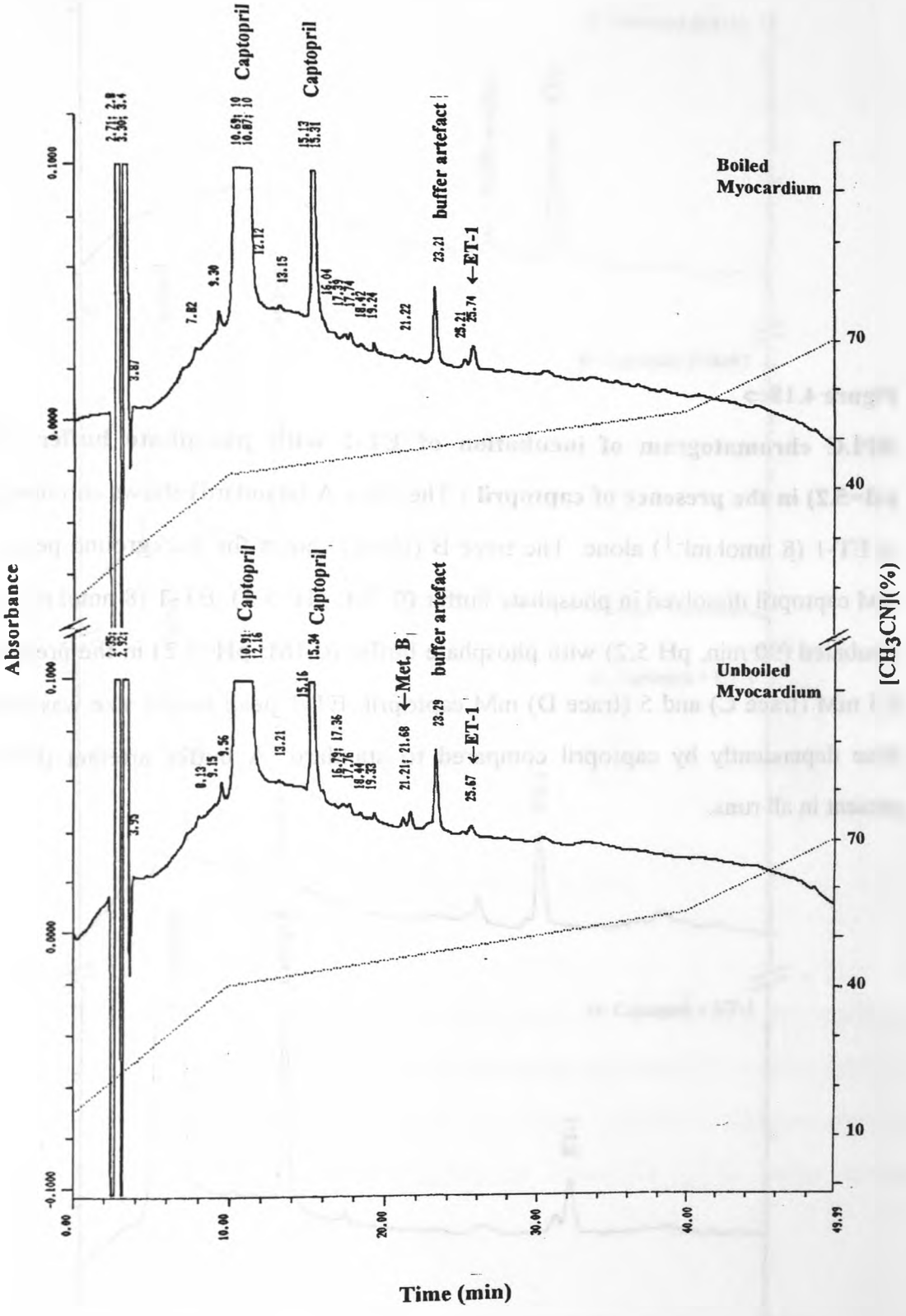
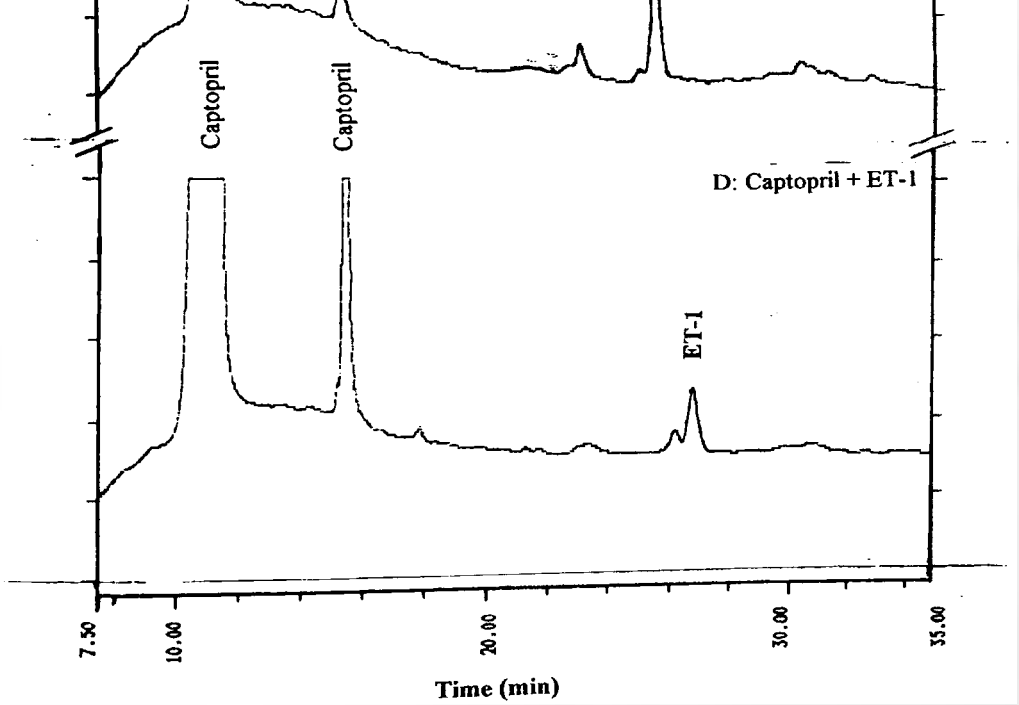

Absorbance
Time (min)
[CH3CN](%)
Boiled Myocardium
Unboiled Myocardium
Captopril
Captopril
buffer artefact
ET-1
Met. B
Captopril
Captopril
buffer artefact
ET-1
0.1000
0.0000
-0.1000
0.00
10.00
20.00
30.00
40.00
49.99
70
40
10

Figure 4.18⇒

HPLC chromatogram of incubation of ET-1 with phosphate buffer (0.1M; pH=5.2) in the presence of captopril : The trace A (standard) shows chromatogram of ET-1 (8 $nmol.ml^{-1}$) alone. The trace B (blank) shows the background peaks of 5 mM captopril dissolved in phosphate buffer (0.1M; pH=5.2). ET-1 (8 $nmol.ml^{-1}$) was incubated (90 min, pH 5.2) with phosphate buffer (0.1M; pH=5.2) in the presence of 0.5 mM (trace C) and 5 (trace D) mM captopril. ET-1 peak height size was reduced dose dependently by captopril compared to standard. A buffer artefact (b.a) was present in all runs.


Captopril
Captopril
D: Captopril + ET-1
ET-1
7.50
10.00
20.00
30.00
35.00
Time (min)

Absorbance
Captopril
Captopril
Captopril
Captopril
buffer artefact
ET-1
ET-1
C: Captopril + ET-1
B: Captopril (Blank)
A: Standard (ET-1)

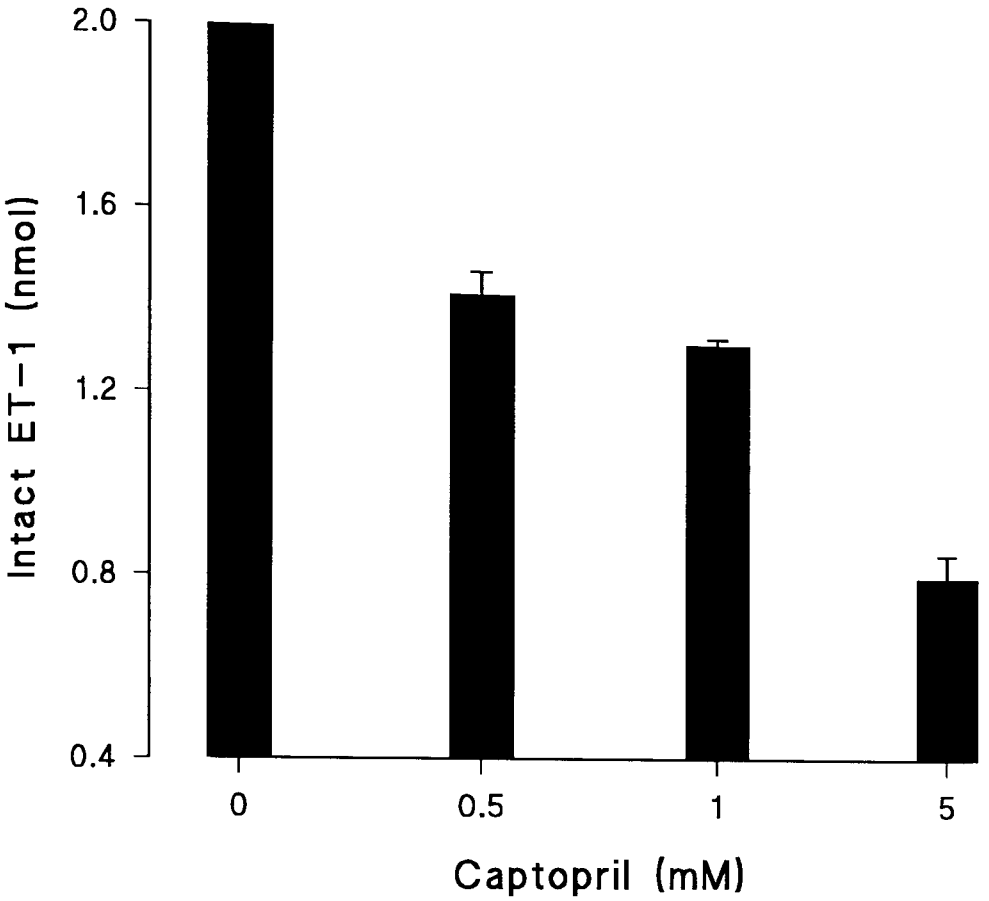


Figure 4.19 Non-specific reduction of content of ET-1 following incubation with phosphate buffer (0.1M, pH=5.2) in the presence of captopril (5, 1, and 0.5 mM). The peptide was incubated for 90 min in the presence of captopril. The samples were analysed by RP-HPLC as described in Chapter 2 (section 2.7.1). Points represent mean±S.D.; n=4.

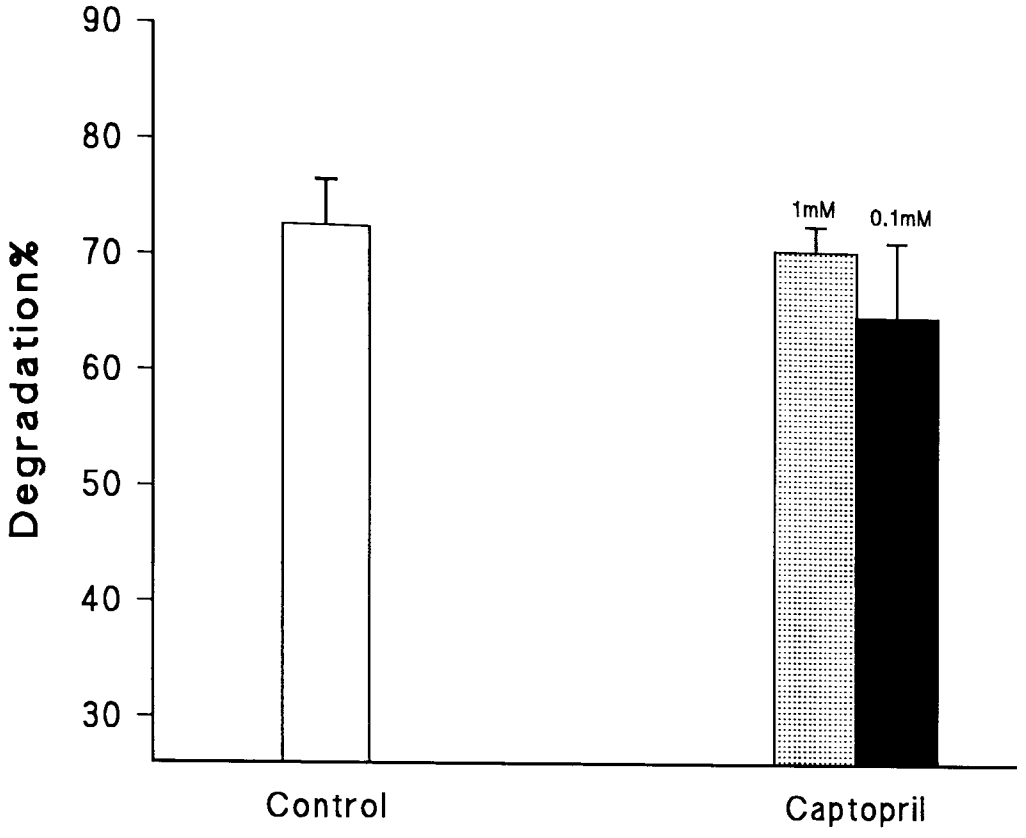


Figure 4.20 Enzymic degradation of incubated ET-1 by rat ventricular tissue extracts in the presence of captopril. The peptide was incubated for 90 min at pH 5.2 in the absence or presence of captopril. The samples were analysed by RP-HPLC as described in Chapter 2 (section 2.7.1). Points represent mean±S.D, n=4-7. Data for this figure is also shown in table 4.4.

4.3.4 The Effect of Aprotinin on ET-1 Degradation by Rat Ventricular Extracts

Serine proteinases comprise the largest and most understood group of proteolytic enzymes. They play critical roles in numerous physiological processes including digestion, blood coagulation, complement activation and development and the release of physiologically active peptides (Neurath et al., 1984). Aprotinin is a serine proteinase inhibitor which is also known as trypsin-kallikrein inhibitor (Gebhard et al., 1986). This experiment was done to determine its effect on ET-1 degradation activity in aqueous extract of rat ventricle.

4.3.4.1 Experimental Procedure

The rat ventricular extract (0.1 ml) was pre-incubated, at 25°C, with 1.9 ml phosphate buffer (0.1M, pH=5.2, protein content 412±14 $\mu g.ml^{-1}$) containing aprotinin at a final concentration of 385 μM (10 TIU or 10 Trypsin Inhibitor Units) for 20 min. Incubations were started by the addition of ET-1 (8 $nmol.ml^{-1}$) to boiled and unboiled pre-incubated extracts and continued for 90 min. The pH of both boiled and unboiled extracts were checked before incubation. The samples were processed and analysed by RP-HPLC as described in Chapter 2 (sections 2.7.1 and 2.8).

4.3.4.2 Results

The results are shown in table 4.4. Aprotinin at a concentration of 10 TIU (385 μM) had no effect on the ET-1 enzymic degradation. ET-1 breakdown (74.4±4.2%,) in the presence of aprotinin was comparable with ET-1 degradation in the same conditions without aprotinin (72.9±3.9; $p>0.4$). ET-1 degradation in presence of aprotinin produced two detectable metabolites, similar to those in the control incubate.

4.3.5 The Effect of PMSF on ET-1 Degradation by Rat Ventricular Extract

Phenyl methyl sulfonyl fluoride (PMSF) is a well-known irreversible inhibitor of general types of serine proteinases, such as trypsin and chymotrypsin, and of

mammalian acetylcholine esterase (Gold et al., 1964). Here the effect of PMSF on degradation of ET-1 by aqueous extract of rat ventricle was studied.

4.3.5.1 Experimental Procedure

PMSF decays in aqueous solutions with a half life of approximately 55 min (James et al., 1978) and this had to be taken into account when using this particular inhibitor. However, PMSF is stable in dimethyl sulfoxide (DMSO) and this was therefore used as the solvent. Consequently a control evaluation of ET-1 degradation was carried out in the presence of DMSO. Thus DMSO (3 μl) in the absence (as a vehicle control) and presence of PMSF (final concentration of 200 μM) was added to 300 μl of boiled and unboiled tissue extracts (protein content 412±14 $\mu g.ml^{-1}$) at pH 5.2 and pre-incubated for 10 min. ET-1 was then incubated with 200 μl of the pre-incubated extracts at a final concentration of 8 $nmol.ml^{-1}$ for 90 min. The samples were processed and analysed by HPLC as described in the Chapter 2 (section 2.7.1).

4.3.5.2 Results

The results are shown in table 4.4. In these experiments DMSO alone caused a considerable reduction of the degradation of ET-1 (48% inhibition, $p<0.01$; figure 4.21) by ventricular extract. This could be due to inhibition by DMSO of some of the enzymes which are involved in the degradation of ET-1. It has been shown that a dimethyl sulphonium salt inhibits cathepsin B, which is a lysosomal enzyme, in pig liver (Walker et al., 1985). In addition, DMSO plus PMSF reduced the degradation to 18.8±4.8% from a control value of 37.9±2.3% when DMSO was used alone ($p<0.02$; figure 4.21). In another word PMSF inhibited ET-1 degradation by rat ventricle by 50.5%($p<0.02$).

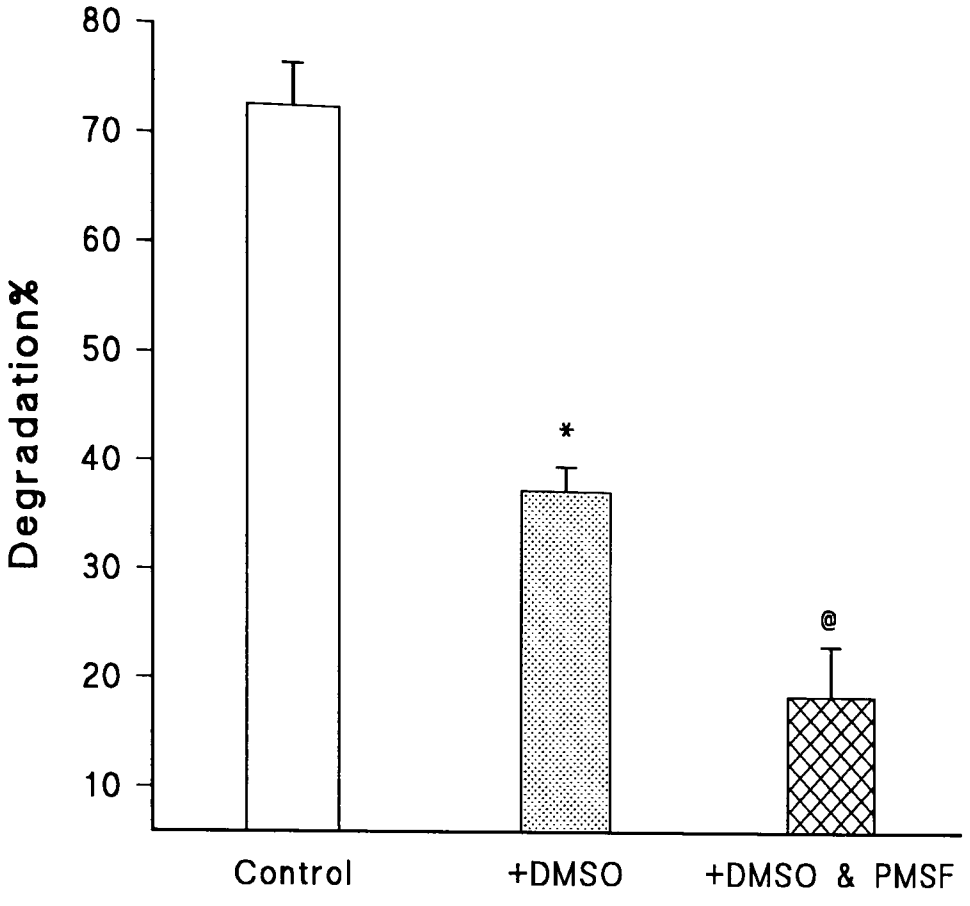


Figure 4.21 Degradation of ET-1 by rat ventricular tissue extracts (protein content 412±14 $\mu g.ml^{-1}$). The peptide was incubated for 90 min at pH 5.2 in the absence or presence of PMSF (200 μM). DMSO (1%) was used as vehicle. The samples were analysed by RP-HPLC as described in Chapter 2 (section 2.7.1). Points represent mean±S.D, n=4-7. *$p<0.01$ vs control (Phosphate buffer; 0.1M) and @$p<0.02$ vs DMSO. Data for this figure is also shown in table 4.4.

4.3.6 The Effect of Pepstatin A on ET-1 Degradation by Rat Ventricular Extract

Pepstatin is a potent inhibitor of cathepsin D, pepsin, renin and many aspartic proteinases. It has been shown to inhibit the acid optimum kininogenase in rat mesenteric arteries (Nolly et al., 1985), dog coronary arteries and ventricular myocardium (Zeitlin et al., 1989). Fagny and et al (1992) suggested a role for cathepsin G in ET-1 hydrolysis. They showed that incubation of ET-1 with purified cathepsin G led to the formation of ET-1 fragment (1-16) and the C-terminal pentapeptide. However, Sessa et al (1991b) showed the metabolism of ET-1 by PMNs (human polymorphonuclear leukocytes) or leukocyte cathepsin G was not inhibited by phosphoramidon or pepstatin A. This experiment was to evaluate the inhibitory effect of pepstatin on the acid optimum enzymic degradation of ET-1 by rat ventricular extract.

4.3.6.1 Experimental Procedure

Pepstatin is only sparingly soluble in water. Thus it was first dissolved in methanol (1mM) followed by dilution in phosphate buffer (0.1M; pH 5.2) to concentrations of 10 and 100 μM (containing 1% and 10% methanol respectively). The ventricular extracts were diluted 20 times (protein content 412±14 $\mu g.ml^{-1}$) by the above mentioned solutions (vehicle) and pre-incubated, at 25°C, for 20 min. ET-1 (8 $nmol.ml^{-1}$) was incubated with the boiled and unboiled extract for 90 min. To test the effect of methanol as vehicle on ET-1 degradation in rat myocardium, ET-1 was also incubated with boiled and unboiled extracts containing 10% methanol under the same conditions. Incubates were assayed using RP-HPLC as described in Chapter 2 (section 2.7.1). The pH was checked before the incubation (pH=5.2).

4.3.6.2 Results

Pepstatin A at a concentration of 10 μM had no effect on the ET-1 degradation (68.4±4.9%; $p>0.11$). Incubation of ET-1 with extracts containing 10% methanol

reduced the degradation from 72.9±3.9 (as control) to 53.4±2.5 (table 4.4, figure 4.22; $p<0.05$). This shows that methanol itself causes about 27% inhibition of the ET-1 degradation by the rat myocardium. Pepstatin at a concentration of 100 μM (containing 10% methanol) decreased the degradation to 47.0±6.0% (table 4.4). Thus, the main part of this inhibition (35.60%) by pepstatin solution was due to the methanol, presumably due to the enzyme destruction by methanol. Therefore, pepstatin A at the concentration of 100 μM produced no significant (11%; $p>0.08$) inhibition of the ET-1 degradation by rat ventricle (figure 4.22).

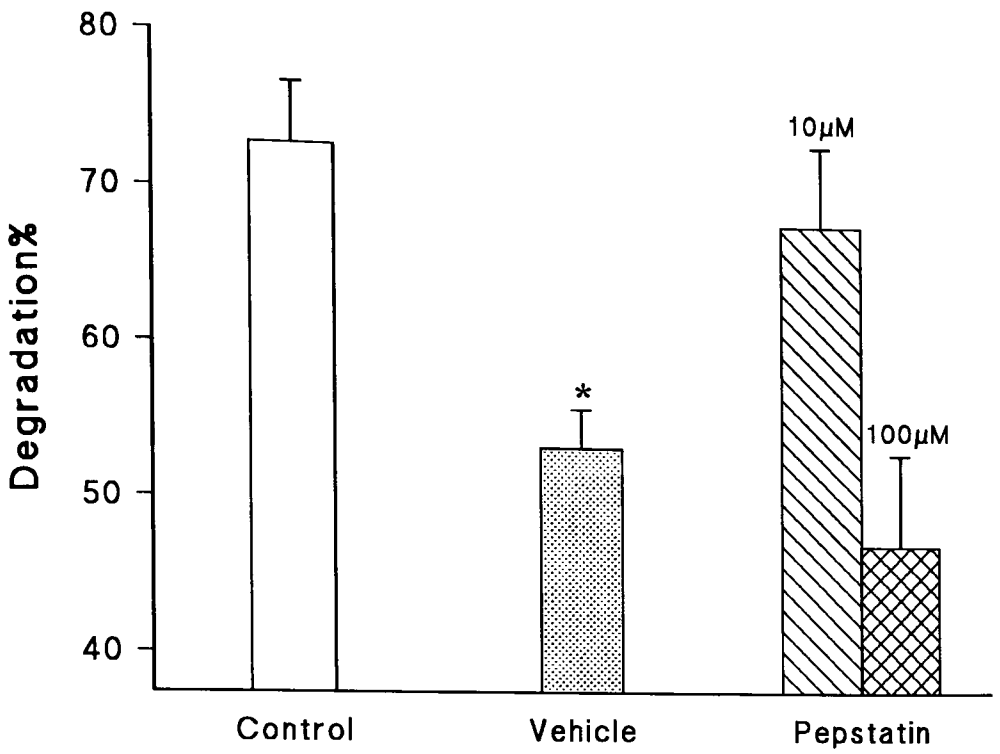


Figure 4.22 Degradation of ET-1 by rat ventricular tissue extracts. The peptide was incubated for 90 min at pH 5.2 in the absence or presence of pepstatin A or in the presence of 10% methanol (vehicle) alone. The samples were analysed by RP-HPLC. Points represent mean±S.D; n=4-7. *$p<0.05$ vs control. Data for this figure is also shown in table 4.4.

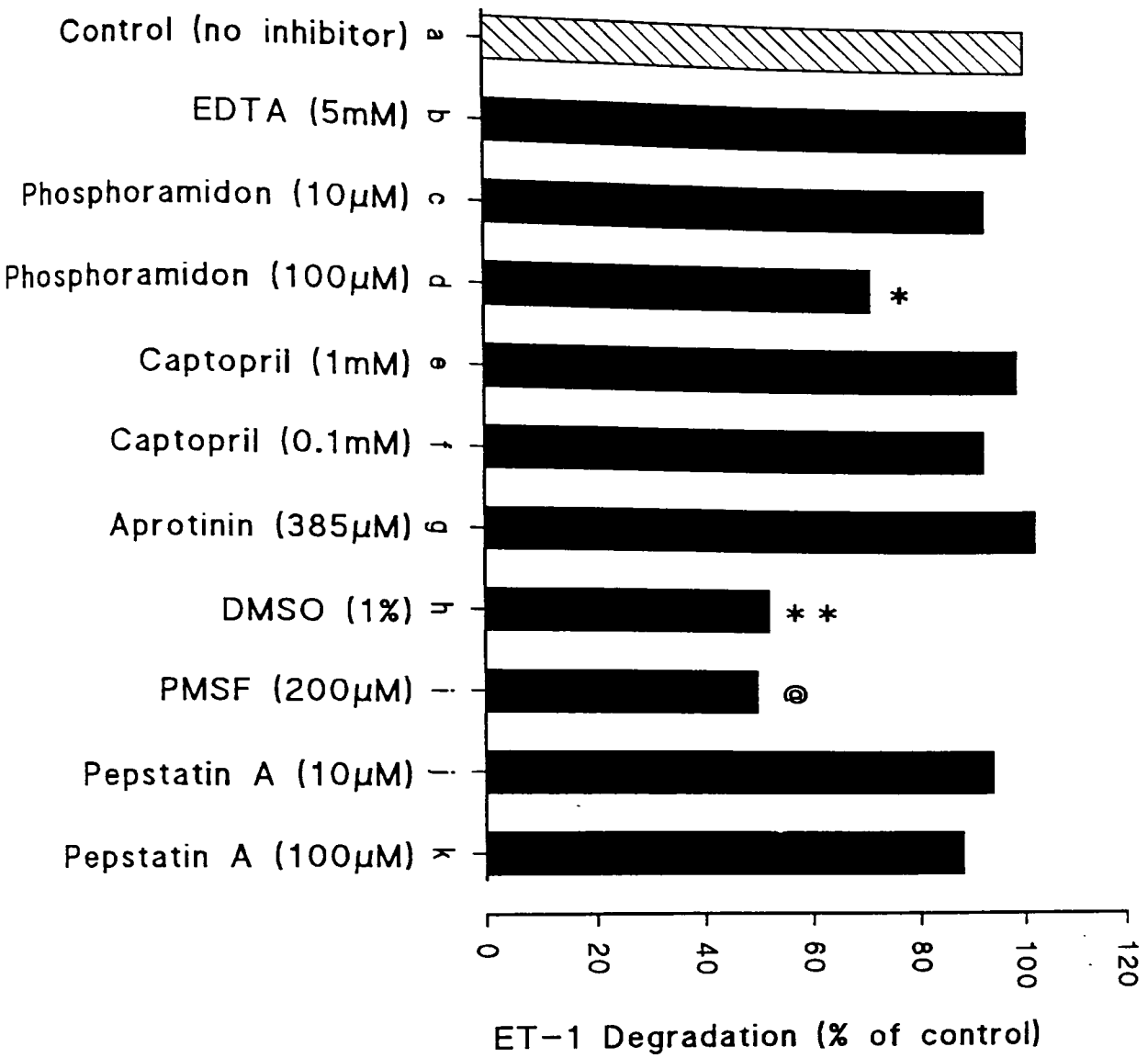


Figure 4.23 Effects of proteinase inhibitors on enzymic degradation of ET-1 by aqueous extracts of rat ventricle (protein concentration of 412±14 $\mu g.ml^{-1}$). ET-1 (8 $nmol.ml^{-1}$) was incubated (90 min, pH 5.2) in the absence and presence of enzyme inhibitors, DMSO is dimethyl sulfoxide; PMSF is phenyl methyl sulfonyl fluoride. Data for this figure is summarised in table 4.4. *$p<0.05$, **$p<0.01$, @$p<0.02$ vs control.

4.4 DISCUSSION

Big ET-1 has been found in human (Miyauchi et al., 1991; Suzuki et al., 1990) and rat (Watanabe et al., 1991a) plasma. Watanabe et al (1991a) showed that plasma levels of big ET-1 in rats subjected to myocardial ischaemia and reperfusion increase as much as the ET-1 levels increase. Furthermore, they found that normal rats have a plasma big ET-1 concentration of 4.38±0.44 $pg.ml^{-1}$, which is about 4 times higher than the ET-1 level (1.02±0.04 $pg.ml^{-1}$). Indeed, the conversion of intravenously injected big ET-1 to ET-1 has been shown in rats (Fukuroda et al., 1990; Matsumura et al., 1991) and it has also been demonstrated that the clearance of big ET-1 is significantly slower than that of ET-1 (Hemsen et al., 1991). One explanation for this discrepancy between the clearance rates and concentrations of ET-1 and of its precursor big ET-1 could be due to the existence of a specific enzyme which degrades ET-1 but not big ET-1. The difference in the plasma level of big ET-1 and ET-1 in some pathophysiological cases becomes more important and notable, especially when the situation is accompanied with decrease in tissue pH or the presence of activated polymorphonuclear leukocytes.

In a study by Miyauchi et al (1991) it was shown that in normal subjects the ratio of the plasma level of big ET-1 to ET-1 is about 2, while this ratio increases to 6.5 in patients on chronic haemodialysis. Similarly, in a study by Watanabe and colleagues (1991a), in normal rats the ratio of the plasma level of big ET-1 to ET-1 was shown to be about 4, which rose to 4.7 and 5.3 one and three hours after myocardial reperfusion respectively. The differences between plasma levels of big ET-1 and ET-1 may increase in pathophysiological conditions due to an increase in the degradation of ET-1, but not big ET-1. There is no degradation of ET-1 in plasma (Pernow et al., 1989). Therefore, the metabolism of endothelin in tissues can be as important as its production in the pathophysiology of cardiovascular disease. Indeed the higher breakdown of ET-1 by rat ventricle (84 $pmol.min^{-1}.mg^{-1}$) and lung

(169 pmol.min^{-1}.mg^{-1}) at low pH (5.2) compared to 31 pmol.min^{-1}.mg^{-1} in ventricle and 52 pmol.min^{-1}.mg^{-1} in lung at the physiological pH (7.2), has been demonstrated in this study. Deng et al (1994) also reported an endothelin-degrading enzyme in rat kidney which rapidly degrades ET-1, but not big ET-1, to an inactive fragment optimally at acid pH by removing the carboxyl terminal tryptophan. The presence of an acid optimum ET-1 degrading enzyme in the myocardium is therefore potentially of considerable pathophysiological significance. It has been shown that neutral endopeptidase 24.11 purified from rat kidney can degrade ET-1 at multiple cleavage sites, with the first step being the cleavage of the ser^5-Leu^6 peptide bond (Yamaguchi et al., 1992b; Vijayaraghavan et al., 1990). However, this cleavage does not readily yield a less active ET-1 fragment. Structure-activity relationship studies show that the C-terminal tryptophan of ET-1 is very important for its potent vasoconstrictor action (Kimura et al., 1988). Recently Deng et al (1994) indicated that removal of C-terminal tryptophan of ET-1 by the cytosolic fraction of rat kidney homogenates decreased the potency of ET-1 to cause contraction of smooth muscle by 3 order.

The results described in this thesis have shown that aqueous extracts of rat lung and ventricle degraded exogenous ET-1 optimally at acid pH. However, since a crude preparation of tissue extracts were used therefore other cells of myocardium rather than myocytes, for example fibroblasts, may also be involved in the acid optimum degradation of ET-1. Even at pH 6.2, the myocardial ET-1 degrading activity was much greater than that at the more physiological pH 7.2 (222%, $p<0.02$). At pH 5.2 lung extracts broke down ET-1 to give 4 detectable fragments on RP-HPLC, whereas ventricular extracts produced only 2 detectable peaks. The first metabolite, produced by both tissues, co-eluted with tryptophan, indicating that one of the metabolites was the C-terminal tryptophan of ET-1, since ET-1 has only one tryptophan in its structure. Furthermore, incubation of ET-1 C-terminal hexapeptide [ET(16-21)] with ventricular extract at pH 5.2 also led to the formation of the C-terminal tryptophan

which gave a peak identical to that of tryptophan on the HPLC chromatogram. In addition, ET(16-21) did not co-elute with any fragments of degraded ET-1, therefore the C-terminal hexapeptide of ET-1, which has been shown by Fagny et al (1991) to be a metabolite of ET-1 produced by endopeptidase 24.11, can not be a metabolite of ET-1 degradation by ventricular extract.

Finally using electrospray mass spectrometry, the molecular weight of metabolites (A and B) produced by degradation of ET-1 by rat ventricular extract at pH 5.2, were found to be identical to those of tryptophan and des-Trp-ET-1, indicating that myocardial degradation of ET-1 at acid pH involves cleavage of the Ile^{20}-Trp^{21} peptide bond of the ET-1. The ET-1 degradation at pH 7.2 also contained a trace of fragment ET(1-17) with a molecular weight of 1965 Da. Neither tryptophan nor des-Trp-ET-1 were detected by mass spectroscopy when the incubation was carried out at pH 7.2. Thus the degradation of ET-1 at pH 7.2 does not produce the metabolites tryptophan and des-Trp-ET-1 seen at pH 5.2. It appears that two different types of enzyme may be involved in degradation of ET-1, one of them acting effectively at acid pH and cleaving the Ile^{20}-Trp^{21} peptide bond and the other one acts at physiological pH with low efficiency and breaking the Leu^{17}-Asp^{18} peptide bond.

Our results in the ventricle are similar to those of Deng et al (1994 & 1992) in rat kidney and Jackman et al (1993) in vascular smooth muscle who showed that ET-1 to be cleaved at the Ile^{20}-Trp^{21} peptide bond and the optimum pH for maximum enzymic activity to be at acid pH (pH=5.2-5.6). In those studies the enzyme was purified from the cytosolic fraction of tissue or soluble tissue extracts were used. Several other studies also suggested different sites of cleavage for ET-1. For example, Fagny et al (1991) showed in vitro that purified endopeptidase 24.11 hydrolysed ET-1 mainly at the Cys^{11}-Val^{12}, Tyr^{13}-Phe^{14}, Cys^{15}-His^{16}, His^{16}-Leu^{17}, and Asp^{18}-Ile^{19} bonds and generated at least 10 degradation fragments. In this study they

incubated ET-1 at a high concentration (1200 $nmol.ml^{-1}$) with purified enzyme and for 24 hours. This does not seem to relate to physiological conditions. Vijayaraghavan et al (1990) reported that ET-1 was cleaved initially at the Ser^5-Leu^6 bond by purified rat kidney endopeptidase 24.11 and likewise Yamaguchi et al (1992a,b) showed that rat kidney membrane fraction degrades ET-1 into four fragments by cleavage at the Ser^5-Leu^6 and Leu^{17}-Asp^{18} bonds of ET-1. In all studies in which ET-1 has been shown to be degraded at different sites other than C-terminal tryptophan, purified endopeptidase 24.11 or membrane fractions of tissue were used and the pH of the incubation medium was neutral. ET-1 thus appears to be degraded by two different types of enzyme. One is located on the membrane of cells, and cleaves ET-1 non-specifically at multiple sites (usually at neutral pH) and degrades big ET-1 with similar efficiency (Yamaguchi et al., 1992b). The other enzyme is soluble and a cytosolic enzyme which degrades ET-1 rapidly (optimally at acid pH) by removing the carboxyl terminal tryptophan, while not degrading big ET-1 (Deng et al., 1994) or degrading it with very low efficiency.

The high ratio of big ET-1 to ET-1 in both normal and disease states is probably due to the higher metabolism of ET-1 compared to big ET-1. The degradation of ET-1, and consequently the increase in ratio of big ET-1 to ET-1, becomes more important in pathophysiological situations when the tissue is damaged, the pH falls to an acidic pH, or when the leukocytes are activated. From our studies and those of Deng et al (1994), and Jackman et al (1993), carried out in myocardium, kidney, and vascular smooth muscle respectively, it appears that soluble and cytosolic enzymes are responsible for rapid and high degradation of ET-1. During myocardial ischaemia and reperfusion, kidney insufficiencies, or vascular smooth muscle injury where the tissue is damaged, these enzymes may be released and degrade ET-1. Furthermore, Khandoudi et al (1990) have shown that the pH of myocardial cells falls to acid pH during ischaemia. Therefore during ischaemia the degradation of ET-1 becomes more

notable since endothelin degrading enzyme is active optimally at acid pH. As will be discussed in next chapter, blood components, especially during inflammatory processes, might have an important role in the production of ET-1 during ischaemia and reperfusion. Indeed this process can be as important in the degradation of ET-1 as its production. Thus there is a logical balance between generation and metabolism of ET-1. Incubation of ET-1 with activated polymorphonuclear (PMNs) leukocytes but not resting cells (Sessa et al., 1991a,b; Fagny et al., 1992) has been shown to degrade ET-1. This mechanism of ET-1 inactivation could play a role in acute inflammatory reactions where PMNs adhere to endothelial cells, or in any other reactions when the enzymes are released from leukocytes.

As was discussed above, both membrane-bound and cytosolic enzymes have been implicated in the degradation of ET-1. To characterise these enzymes further, the ET-1 degrading activity of aqueous extracts of ventricle was studied in the presence of peptidase inhibitors. ET-1 was incubated at pH 5.2 in the presence and absence of EDTA (a metal chelator), phosphoramidon (a potent inhibitor of endopeptidase 24.11 and other metalloproteinases), captopril (kininase/angiotensin converting enzyme inhibitor), pepstatin A (an inhibitor of aspartic proteinase), aprotinin, and phenyl methyl sulfonyl fluoride (PMSF; serine proteinase inhibitors). Table 4.4 and figure 4.23 summarise the effects of these peptidase inhibitors on the degradation of ET-1.

The ET-1 degrading activities associated with membrane fractions of porcine kidney (Edano et al., 1994b) and with rat kidney membrane-bound peptidase at pH 5.5 (Janas et al., 1994) have been reported to be inhibited by EDTA, implicating involvement of a metalloenzyme. Since in addition to EDTA, phosphoramidon and thiorphan also suppressed the degradation of ET-1 (Edano et al., 1994b), it was suggested that endopeptidase 24.11 is involved in ET-1 metabolism by membrane fraction. In another study by the same group (Edano et al., 1994a), the inhibition of ET-1

degradation in membrane fractions of smooth muscle cells by phosphoramidon has also been reported. Furthermore, Fujita et al (1994) suggested that neutral endopeptidase 24.11 may be responsible for the proteolytic degradation of ET-1 in perfused rat kidney. Similarly Yamuguchi et al (1992b) indicated that phosphoramidon almost completely suppressed the generation of some of the metabolites which were produced by rat kidney membrane, but production of one fragment was not affected by the inhibitor. This group showed that the phosphoramidon-insensitive fragment was produced by cleavage at Leu^{17}-Asp^{18} (the same cleavage site as in myocardium in our study at pH 7.2), and was distinct from the metabolites produced by endopeptidase 24.11. This suggested the involvement of more than one enzyme in ET-1 degradation by rat kidney membrane. A phosphoramidon sensitive degradation has also been shown in guinea pig airway tissues by Noguchi et al (1991). Likewise Fagny et al (1991) demonstrated that in vitro degradation of ET-1 by crude membrane preparations of human kidney and choroid plexus was inhibited by phosphoramidon.

Phosphoramidon-insensitive degradation has been shown in tissues or cells other than kidney or lung. Gandhi et al (1993) reported that phosphoramidon did not affect the metabolism of ET-1 by hepatocytes or Kupffer cells. It has also been demonstrated that phosphoramidon had no effect on the appearance of ET-1 metabolites when incubated with activated PMNs (when lysosomal proteinases are released into the medium) while soya bean trypsin inhibitor abolished the degradation completely. This indicated a role for serine peptidases in ET-1 hydrolysis. Similarly, Sessa et al (1991a,b) reported that the metabolism of ET-1 by activated PMNs or leukocyte cathepsin G (a serine peptidase) was prevented by phenyl methyl sulfonyl fluoride (PMSF), but not by phosphoramidon or pepstatin A. Deng et al (1994) showed that the endothelin-degrading enzyme from aqueous extracts of rat kidney (an acid optimum enzyme which removed the C-terminal tryptophan from ET-1) was inhibited

only 5% by EDTA. In the present study we demonstrated that EDTA had no effect on the ET-1 degradation by aqueous extracts of rat ventricle, but phosphoramidon, at a concentration of 100 μM, produced 30% inhibition ($p<0.05$). This shows that ET-1 degradation with rat ventricle extract, at least in part, is phosphoramidon sensitive. Captopril, which is another inhibitor of metallo-proteinases (especially angiotensin-converting enzyme and kininase) has been shown to have no effect on the in vitro degradation of ET-1 by human kidney, choroid plexus membrane, and purified endopeptidase 24.11 (Fangy et al., 1991), nor by either membrane fractions from porcine kidney (Edano et al., 1994b) or vascular smooth muscle cells (Edano et al., 1994a). This indicates that angiotensin-converting enzyme is not involved in this degradation. We demonstrated that captopril at concentrations of 0.1 and 1 mM, which had no non-specific effect, did not affect ET-1 degradation by rat myocardium. Interestingly, captopril, in the absence of any enzymic activity at a concentration of 5 mM, produced an efficient reduction in the apparent content of incubated ET-1 when measured on HPLC, but led to no detectable metabolites. The reduction of ET-1 peak size on the HPLC chromatogram following incubation with captopril in the absence of enzymic activity could be due to a change of ET-1 structure or to destruction by captopril. Since no degradation fragments were produced, non-specific destruction of ET-1 seems unlikely. However, production of metabolites with a maximum UV absorbance different from that used in the HPLC detector, or overlap of metabolite peaks with captopril peaks on HPLC chromatogram, is possible. Alternatively, captopril has a sulphydryl group which can reduce the two intrachain bonds of ET-1 and consequently break them. This modification in the structure of ET-1 possibly changes the UV absorbance property of the peptide or its retention time on RP-HPLC. This point should be considered when this enzyme inhibitor is used (as it is by many workers) in the study of enzymic activities.

Pepstatin A at a concentration of 100 μM produced only a non-significant ($p>0.08$) inhibition of 11% of ET-1 degradation by ventricular extracts. Other workers have shown that pepstatin A failed to prevent metabolism of ET-1 by the membrane fraction of smooth muscle cells (Edano et al., 1994a), activated PMNs (Sessa et al., 1991a,b), or by porcine lung membranes (Murphy et al., 1993). However, Edano et al (1994b) indicated that pepstatin A produced a 47% inhibition of the degradation of ET-1 by membrane fractions of porcine kidney. Our results suggest that aspartic proteinases do not appear to be involved in ET-1 digestion by rat ventricle.

As mentioned above cathepsin G (serine peptidase) from activated PMNs has been implicated in ET-1 degradation and was inhibited by an irreversible serine proteinase inhibitor, phenyl methyl sulfonyl fluoride (PMSF, Sessa et al., 1991b; Fangy et al., 1992). Furthermore, endothelin-degrading enzyme purified from rat kidney (Deng et al., 1994) was inhibited by PMSF. However in the study of Deng et al (1994), soya bean trypsin inhibitor and trypsin chymotrypsin inhibitor had no effect on endothelin-degrading enzyme activities. This suggested that the enzyme involved is likely to be a thiol proteinase, since PMSF has also been shown to inhibit cysteine proteinases by reduction of thiols (Beynon and Salvesen, 1989). Another serine peptidase inhibitor, diisopropyl fluorophosphate (similar to PMSF), was reported to block ET-1 degradation by an enzyme from vascular endothelial cells (Jackman et al., 1993). Similar to the present finding, this enzyme has been shown to cleave ET-1 at the Ile^{20}-Trp^{21} bond of the peptide. In this study, although the enzymic degradation is inhibited 50% by PMSF, it is not affected by aprotinin, which is also a serine proteinase inhibitor. Interestingly, dimethyl sulphonyl oxide (DMSO), which was used as a vehicle for PMSF, produced 48.5% inhibition by itself. It has been shown that dimethyl sulphonium salts can inhibit cathepsin B (Walker et al., 1985).

In summary, the data from this thesis on ET-1 hydrolysis by rat ventricular extracts suggest the presence in myocardium of an acid optimum, ET-1 metabolizing enzyme, which cleaves the C-terminal tryptophan to a much less active fragment (des-Trp-ET-1). This is potentially of considerable pathophysiological significance. The low ET-1 degrading activity at pH 7.2, and the production of a fragment (ET 1-17) different from that at pH 5.2 indicates the presence of at least two type of enzymes. Furthermore, the pattern of inhibition shown in our study does not correspond to any single enzymic activity and probably more than one enzyme is involved in ET-1 degradation in ventricular extracts at acid pH. One of these acts effectively and mainly is blocked by serine peptidase inhibitor (PMSF) and another one with less activity is phosphoramidon sensitive.

Chapter 5

FORMATION OF ENDOTHELIN-1 IN MYOCARDIUM

5.1 INTRODUCTION

5.1.1 Conversion of Big ET-1 to ET-1

Yanagisawa et al (1988a,b) proposed a biosynthetic pathway for ET-1 in endothelial cells in which the precursor big ET-1, containing 38- (in human) or 39-amino acid (in rats), is cleaved to ET-1 by a specific endothelin converting enzyme (ECE) at the Trp^{21}-Val^{22} bond. This enzymic activity has been found both in the cytosolic and membrane fractions of endothelial cells. Several studies indicate that the membrane-bound enzyme is a neutral phosphoramidon-sensitive metalloendopeptidase (Matsumura et al., 1990; Okada et al., 1990). Whereas, the cytosolic enzyme is an acid optimum phosphoramidon-insensitive enzyme which is inhibited by an aspartic proteinase inhibitor, pepstatin A (Matsumura et al., 1990; Ikegawa et al., 1990b). Since the vasoconstrictor activity of big ET-1 is much lower than that of ET-1 (Kimura et al., 1989; Kashiwabara et al., 1989) and since during ischaemia and reperfusion the level of ET-1 increases in parallel to big ET-1 (Watanabe et al., 1991a), the conversion from big ET-1 to ET-1 appears to be essential for the pathophysiological significance of ET-1.

In addition to endothelial cells, the conversion of big ET-1 to ET-1 has also been seen in other cells or tissues. Watanabe et al (1991b) demonstrated the conversion from big ET-1 to ET-1 in circulating blood by a blood cell-mediated process. Indeed leukocyte-derived enzymes have been shown to stimulate in vitro conversion of big endothelin-1 to endothelin-1 (Sessa et al., 1991a; Wypij et al., 1992). There is no evidence that myocardium effectively converts big ET-1 to ET-1 under physiological conditions. However, in hypoxic conditions cultured cardiac myocytes (Kagamu et al., 1994) have been reported to increase production of endothelin. The release of low but constant levels of endothelin has also been reported in isolated perfused rat hearts (Brunner et al., 1992). It is not clear if the peptide is released from endothelial cells or

from cardiac cells. Some studies have suggested that myocardium is the source of the increased plasma levels of ET-1 observed during myocardial ischaemia (Tsuji et al., 1991; Watanabe et al., 1991a). Interestingly in the study of Brunner et al (1992), it was found that endothelin release from isolated hearts was greatly reduced during ischaemia but during reperfusion was increased over the basal rate only in the group which had been subjected to ischaemia and reperfusion in the presence of foetal calf serum. This indicates a possible role for serum peptides in the stimulation of endothelin secretion.

5.1.2 Endothelin Release during Ischaemia and Reperfusion

The plasma level of endothelin is elevated in a variety of cardiovascular diseases, such as myocardial infarction (Stewart et al., 1991; Naruse et al., 1991) and vasospastic angina pectoris (Toyo-oka et al., 1991). Several studies have shown an increase in plasma endothelin in myocardial ischaemia and reperfusion under experimental conditions (Tsuji et al., 1991; Watanabe et al., 1991a). The mechanism whereby the release of endothelin is stimulated after myocardial ischaemia and reperfusion remains to be determined. Further, the exact role of endogenous endothelin under pathophysiological conditions is still unclear.

Coronary artery occlusion leads to regional hypoxia and ischaemia, while reperfusion increases coronary flow and shear stress. All of these factors could affect cardiac endothelin balance. In vitro studies (Hieda et al., 1990; Yoshizumi et al., 1989) have shown that hypoxia and shear stress can induce the release of endothelin from endothelial cells. A local increase in conversion of big ET to ET has also been proposed by Tonnessen (1993), since polymorphonuclear leukocytes (PMNs) have been shown to facilitate the rapid in vitro conversion of big ET-1 to ET-1 (Sessa et al., 1991a). Furthermore, Liu et al. (1990) have demonstrated that ischaemia causes an increase in ET-1 binding sites in rat cardiac membrane, thus increasing the

endothelin concentration in myocardium. There is growing evidence that endothelial cells are important targets in ischaemia and reperfusion disorders and play a fundamental role in the generation of mediators aggravating tissue injury. The majority of endothelin produced by endothelial cells has been shown to accumulate subendothelially (Masaki, 1989). Thus ischaemia and/or reperfusion induced injury of the endothelium may cause leakage of endothelin from the subendothelial space into the blood. The role of circulating leukocytes in myocardial infarction has also been studied. In dogs subjected to coronary artery ligation ^{111}Indium-labelled white blood cells accumulate in the myocardium corresponding to acute myocardial infarcts (Weiss et al., 1977). Experimental studies show that reperfusion following the ischaemia is potentially harmful to the myocardium. The possible causes of this damage include the production of oxygen free radicals and the accumulation of white blood cells. These inflammatory cells, the polymorphonuclear leukocytes (PMNs), are rich in proteinases and adhere and accumulate at particular sites within the circulation during ischaemia and reperfusion (Lucchesi et al., 1989; Lefer et al., 1993). PMNs are able to destroy the tissue by production of oxygen-derived free radicals. However, another harmful product derived from neutrophils are lysosomal enzymes, some of which are implicated in the conversion big ET-1 to ET-1 (Sessa et al., 1991a).

Yanagisawa et al (1988a) demonstrated that porcine preproendothelin mRNA is expressed in cultured porcine aortic endothelial cells and that this expression is enhanced by the clotting enzyme, thrombin. In addition, in vitro studies (Boulanger and Luscher 1990; Schini et al., 1989) have demonstrated that thrombin can induce the release of endothelin from endothelial cells. Indeed, the activation of the coagulation and fibrinolytic system in patients with acute myocardial ischaemia or infarction has been reported (Vaziri et al., 1992). The activated clotting cascade could stimulate systemic release of ET-1 during myocardial ischaemia.

5.2 AIM

In this study a radioimmunoassay assay was used to monitor the plasma and tissue concentrations of endothelin during coronary artery occlusion and reperfusion in anaesthetized open-chest rats. The period of occlusion (30 min) was chosen to determine whether any increase in ET-1 levels occurs in the same time-period as the appearance of serious post-occlusion arrhythmias. To further evaluate the involvement of myocardium in the conversion of big ET to E-1, the degradation of exogenous big ET in rat ventricular homogenates was studied using HPLC and mass spectroscopy.

5.3 EXPERIMENTAL PROCEDURE AND VALIDATION OF METHODS

5.3.1 Detection, Characterisation and Calibration Curve of Big ET-1

To differentiate the big ET-1 peak from ET-1 and other metabolites and homogenate peaks, it is essential to confirm that the method used for analysis in the HPLC system can effectively separate out bands of interest. This experiment was performed to determine the retention times and the relation between the concentrations of standard big endothelin-1 (2, 5, 10, and 20 $\mu g.ml^{-1}$) and peak size. Reversed phase high performance liquid chromatography (RP-HPLC) was used as described for the study of ET-1 degradation outlined in Chapter 2 (section 2.7.1). The retention time for big ET-1 was 21.5-22.55 min (figure 5.1). This retention time is completely separate from the retention time of 26-27 min for mature ET-1. The relationship between concentration and peak height is illustrated in table 5.1. A linear relation (correlation coefficient: 0.9988; $p=6.06\times10^{-4}$) between the concentration of big ET-1 and the peak height was observed (figure 5.2). Minimum detection of big ET-1 by this method was 45.6 pmol. This sensitivity is suitable for the assay of the degradation of 2 nmol big ET incubated in tissue extracts (250 μl) giving a final concentration of 8 $nmol.ml^{-1}$.

Table 5.1: Peak heights related to different concentration of big ET-1. Injection volume was 100µl. Data represents mean±s.e.m, n=4.

NO	big ET-1 µg/ml	big ET-1 µg/column	big ET-1 pmol/column	Peak height $\times 10^3$
1	2	0.2	45.6	5.80±0.61
2	5	0.5	114	15.51±1.01
3	10	1	228	28.90±1.78
4	20	2	456	61.08±3.32

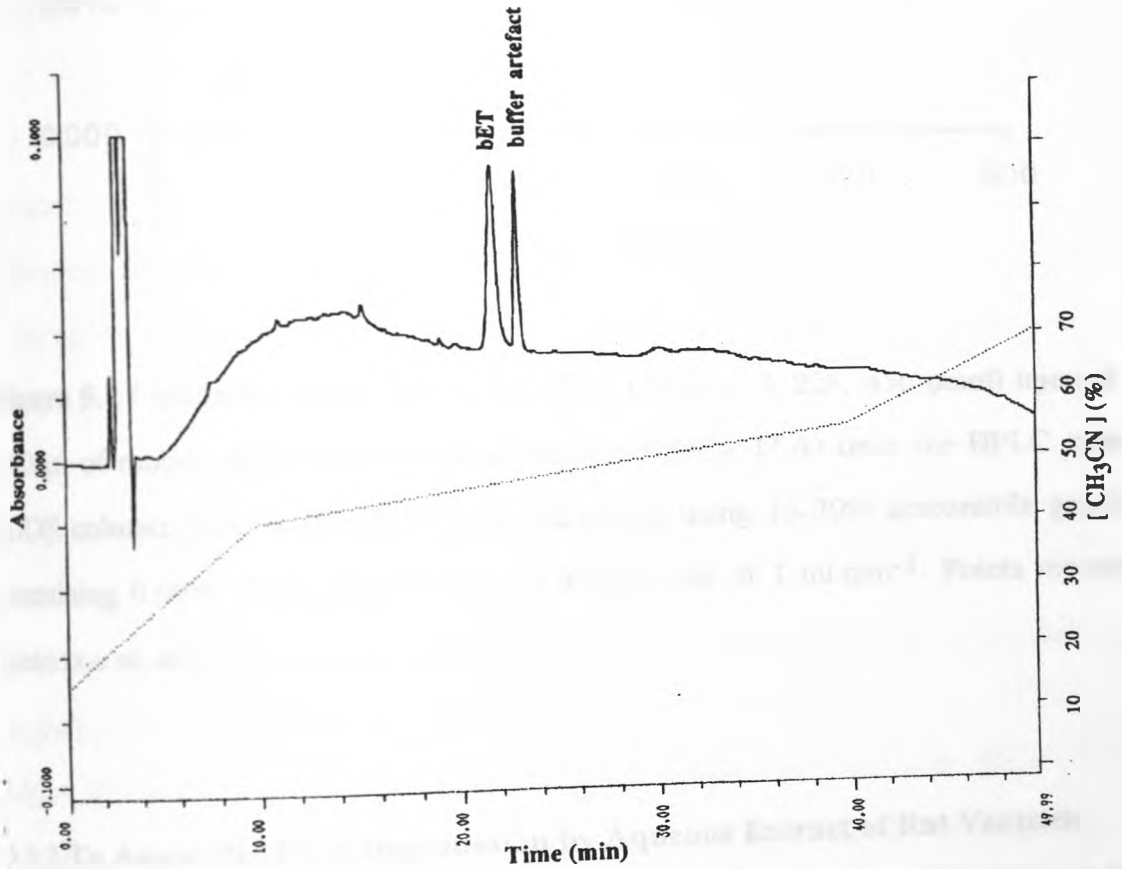


Figure 5.1 Elution of rat big ET-1 (2 µg) on an ODS column (0.46 X 25, 5 µm) in a 15-70% acetonitrile gradient in 0.1% TFA, over 50 min, at a flow rate of 1 ml.min^{-1}. See Chapter 2 (sections 2.7.1 and 2.8) for methodological details.

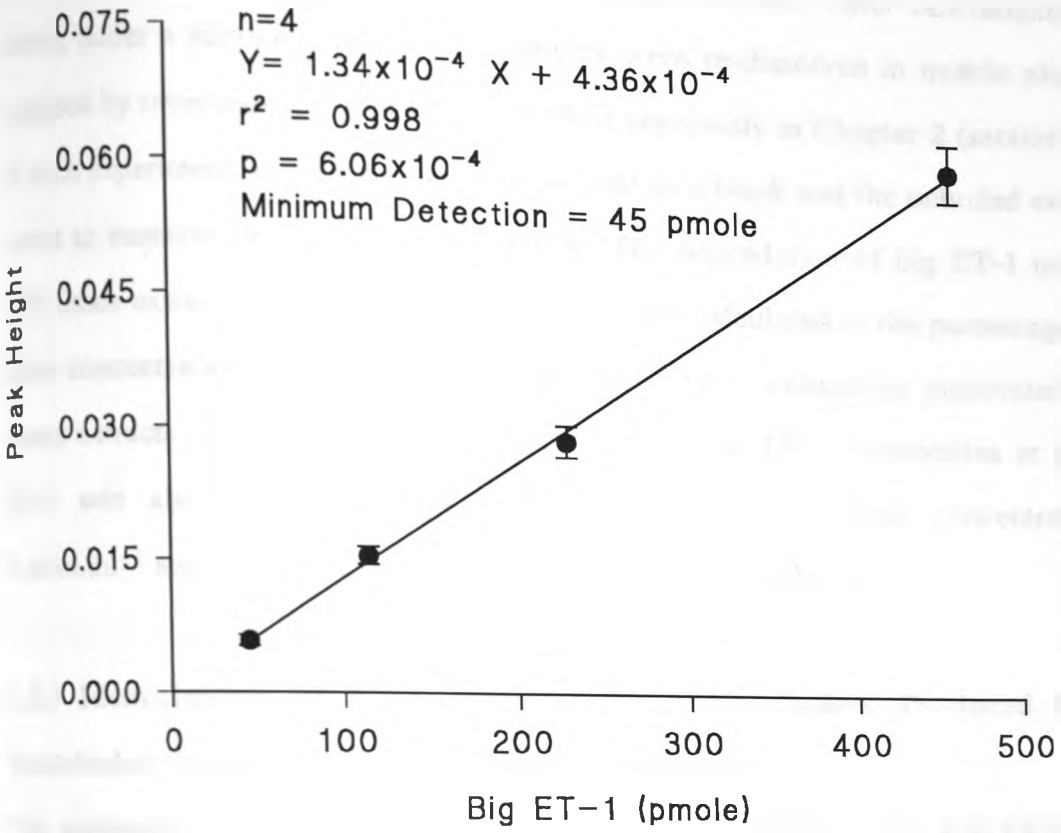


Figure 5.2 Calibration curve for rat big ET-1 (45.6, 114, 228, 456 pmol) injected in 100 µl of mobile phase (15% acetonitrile and 0.09% TFA) onto the HPLC system (ODS column, 0.46 x 25 cm, 5 µm) and eluted using 15-70% acetonitrile gradient containing 0.09% TFA, over 50 min, at a flow rate of 1 $ml.min^{-1}$. Points represent mean±s.e.m, n=4.

5.3.2 To Assess Big ET-1 Degradation by Aqueous Extract of Rat Ventricle

Rat ventricular homogenate was prepared as described in Chapter 2 (section 2.8.1). Rat big endothelin-1 (big ET-1) was incubated at a final concentration of 8 $nmol.ml^{-1}$ for 60 and 90 min at 25°C, with 20 times diluted rat ventricular extract (protein content 445 $\mu g.ml^{-1}$) in phosphate buffer (final concentration 0.1 M) at various pH (pH 5.2, 6.2, 7.2 and 8.2, n=4 for each). The final incubation volume was 250 µl. The

degradation was stopped by boiling at 100°C for 10 min. After centrifugation and drying under a nitrogen stream, the samples were re-dissolved in mobile phase and analysed by reverse-phase HPLC as described previously in Chapter 2 (section 2.7.1). In each experiment, the boiled extract was used as a blank and the unboiled extract as a test to measure the enzymic degradation. The degradation of big ET-1 incubated with tissue extracts containing active enzymes was calculated as the percentage of the same concentration of big ET-1 in identical incubates containing inactivated boiled tissue extracts. In another experiment to identify the ET-1 metabolites at pH 7.2, ET-1 was also incubated with ventricular extract at a final concentration of 8 $nmol.ml^{-1}$ and processed in exactly the same way as big ET-1.

5.3.3 Identification of ET-1 and Big ET-1 Metabolites Produced by Rat Ventricular Extract at pH 7.2 by Mass Spectroscopy

The molecular weights of the degradation products of big ET-1 and ET-1 by rat ventricular extracts at pH 7.2 were determined by electrospray mass spectroscopy as described in Chapter 2 (section 2.11). Before this analysis the degradation fragments of big ET-1 and ET-1 obtained following 90 min incubation with rat ventricular extract at pH 7.2 were separated using the HPLC and freeze dried.

5.3.4 To Assess the Plasma and Tissue Levels of ET-1 During Ischaemia and Reperfusion

Male Sprague-Dawley rats (280-320 g) were anaesthetised with sodium pentobarbitone and prepared for left coronary artery ligation (high-sited ligation) and reperfusion as described in the Chapter 2 (section 2.2). Rats were allocated into four groups. Five rats were anaesthetised and left without any further treatment or surgery for 45 minutes (non-operated group). Five animals in a sham-operated group were subjected to the surgical procedures and, following 15 minutes stabilization, the ligature was placed around the coronary artery and left untied for 30 minutes. Six rats

were prepared as above and underwent continuous ligation of the left coronary artery for 30 minutes (ligated group). Finally 7 rats were subjected to 30 minutes coronary artery occlusion followed by 30 minutes reperfusion (reperfused group). In all groups a blood sample (4-5 ml) was withdrawn at the end of the experimental period from a catheter (kept patent using heparinised saline, 10 $IU.ml^{-1}$) which had been placed in the right atrium via the right jugular vein. The animals were killed by an overdose of sodium pentobarbitone and the hearts were removed immediately, placed in liquid nitrogen and stored frozen at -25°C for a period not exceeding 1 week.

5.3.5 ET-1 Radioimmunoassay

Radioimmunoassay of plasma and myocardial ET-1 was performed according to the method described in Chapter 2 (section 2.4). Standard samples of synthetic ET-1 (100 µl) were incubated with 100 µl of rabbit polyclonal antibody to ET-1 and 400 µl RIA-buffer overnight at 4°C. Labelled ET-1 (^{125}I ET-1) was then added and incubation continued for 6 hours at 4°C. The antibody-antigen complex was precipitated by the addition of 20 µl of rabbit serum and 900 µl of cold isopropanol. The precipitates were centrifuged and the tubes were counted in a gamma counter for 2 minutes.

An example of a standard curve is shown in figure 5.3. A linear relationship (correlation coefficient $r^2 = 0.992$; $p=2.89\times10^{-6}$) between the concentrations of 0.5-10 fmol/tube of ET-1 and B/B_0 (count of bound ^{125}I-ET-1 over maximum bound count) was observed (figure 5.3). Therefore, the working range for the standard curve was fixed between 0.5-10 fmol/tube or 1.25-25 pg/tube. The detection limit of the radioimmunoassay was 0.5 fmol/tube of ET-1. Beyond these two points it is not certain if the correlation between the concentration and radioactive count was reliable. In each series of experiments a new standard curve was constructed.

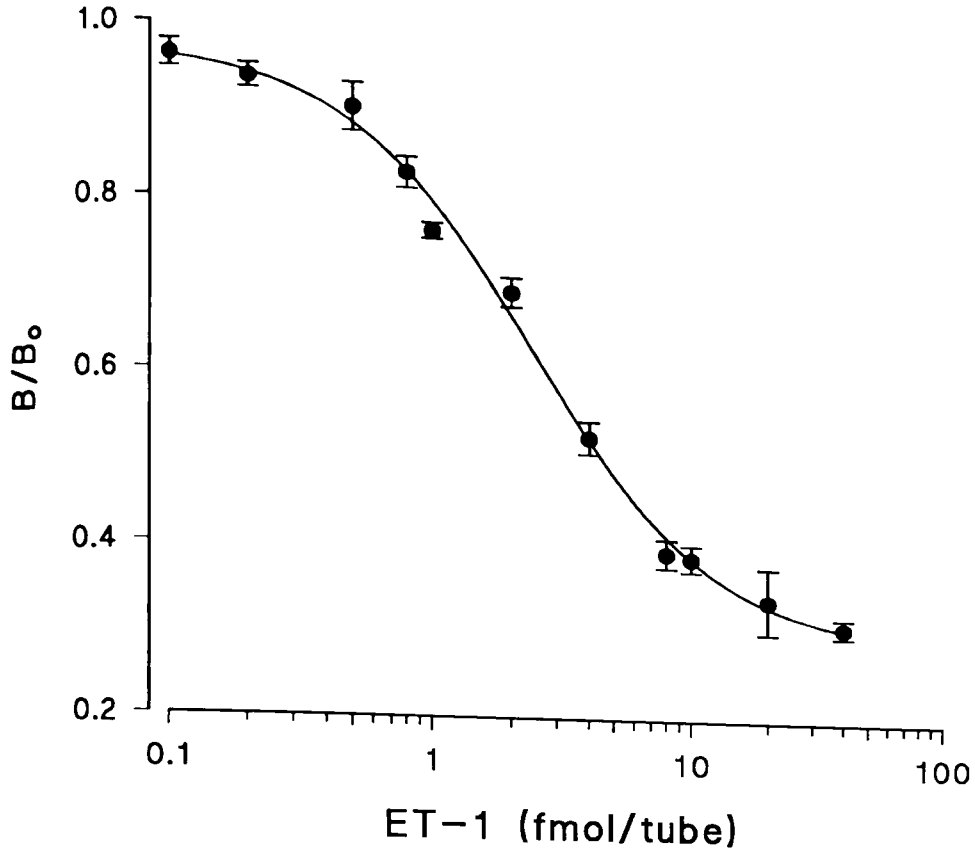


Figure 5.3 Example of a standard curve for the ET-1 radio immunoassay. Standard samples of 0.1-40 fmol were incubated with rabbit polyclonal antibody to ET-1 and the radioactivity of bound ^{125}I-ET-1 of each sample was counted in a gamma counter. B/B_0 was calculated as the count (cpm) of bound ^{125}I-ET-1 over the count of maximum binding. Points represent mean±s.e.m, n=5.

5.3.6 ET-1 Extraction from Plasma and Myocardium

Blood samples (4-5 ml) were collected via a catheter in the right atrium directly into pre-chilled test-tubes containing heparin (20 IU.ml^{-1}). Plasma was then separated by centrifuging for 20 min at 3000 g. The supernatant plasma (2.5 ml) was loaded on the pre-washed cartridge (C18, Sep Pak), the endothelin was eluted with 4×1 ml of 85% methanol into tubes and the eluate was dried under a stream of nitrogen at 30°C as described previously in this thesis (section 2.4.1). The dried eluate was re-dissolved in radioimmunoassay (RIA) buffer and was used for radioimmunoassay of endothelin.

To extract myocardial endothelin excised hearts were heated in boiling water for 5 min, chopped into small pieces with a pair of scissors and then homogenised (using an Uitra-Turrax® homogenizer) in 4 ml $CHCL_3$: CH_3OH (2:1) for 5 min at 4°C (Watanabe et al., 1991a). Distilled water, 200 µl, was added to the homogenate and centrifuged for 20 min at 3000g, then 3.5 ml aqueous phase was transferred onto the C18 Sep Pak cartridge and was treated the same as plasma extracts. Plasma and tissue endothelin-1-like immunoreactivity (IR-ET-1) was measured in duplicate or triplicate using the radio-immunoassay with a rabbit polyclonal antibody to endothelin-1 as described above. According to the information from the antibody supplier (Affiniti) the radioimmunoassay had no cross-reactivity with big endothelin, 60% cross reactivity with endothelin-2, and 70% cross reactivity with endothelin-3.

5.3.6.1 Validation of ET-1 Extraction

The recovery of the extraction was determined using standard samples of 1 and 4 fmol/tube (n=5) and radio labelled endothelin-1 (n=5). To measure the recovery using standard samples, 1 and 4 fmol of synthetic ET-1 were reconstituted in RIA-buffer to 1 ml and were extracted and assayed as for plasma samples. The recovery from the 1 fmol sample was 75.0±5.7% (mean±s.e.m) and from the 4 fmol sample was 71.30± 3.20% (mean±s.e.m). To determine the recovery using radio-labelled ET-1, ^{125}I-ET-1 (3193±89 cpm) in 1ml RIA-buffer was extracted on a Sep Pak cartridge and was counted in a gamma counter for 2 minutes (2025±59 cpm). In this case recovery averaged 63±2.65% (mean±s.e.m). The detection limit of the radioimmunoassay was 0.5 fmol/tube of endothelin-1.

5.4 RESULTS

5.4.1 Degradation of Big ET-1 by Aqueous Extracts of Rat Ventricle

Big ET-1 degradation activity in soluble extracts of rat ventricle was studied by incubation with rat big ET-1 for 60 minutes at 25°C. The results are given in table

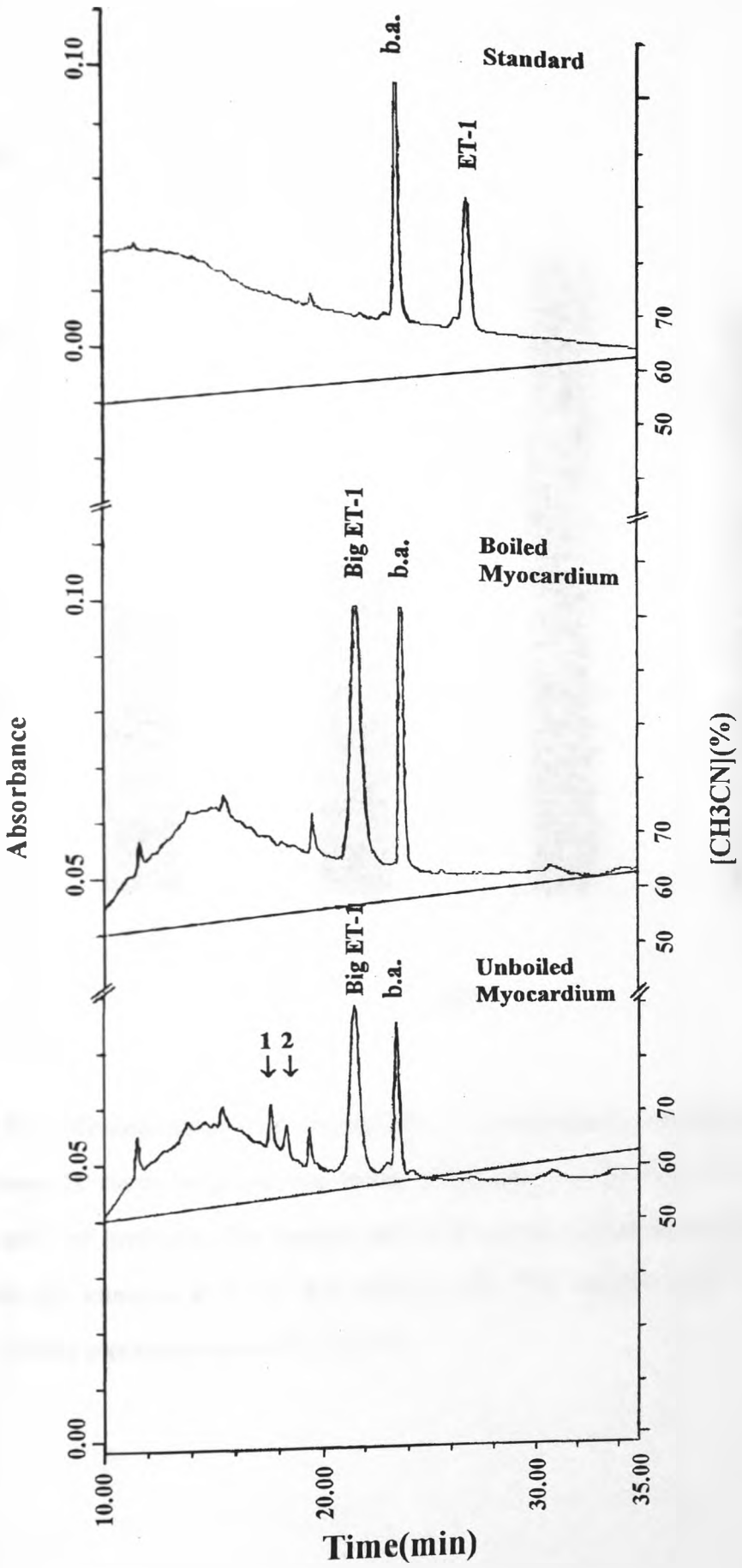
5.2. The degradation of the big ET-1 was measured at pH values between 5.2 and 8.2, and degradation was found to be greatest at pH 7.2 (figure 5.5). At this pH rat big ET-1 was degraded by 35.5±4.1%. Big ET-1 breakdown in rat ventricular extract at pH 7.2 produced two detectable metabolite peaks at 17.5 and 18 minutes on the HPLC chromatogram (figure 5.4). Neither HPLC peak was identical to the ET-1 peak. Big ET-1 degradation at pH 5.2 was 15.6±1.8% and produced no detectable metabolite peaks on the HPLC chromatogram. The degradation of ET-1 (results from chapter 4) and its precursor big ET-1 at various pH (5.2-8.2) are compared in figure 5.6. The degradation of big ET-1 by rat ventricular extracts at acid pH (pH=5.2) was about 3.7 times lower than ET-1 (57% for ET-1 compared to 15.5% for big ET-1) while this ratio was reversed at physiological pH (pH=7.2). The degradation of big ET-1 by rat myocardium was approximately 2 times higher than ET-1 at pH 7.2 (18.5% for ET-1 compared to 35.5% for big ET-1). Therefore, at physiological pH (7.2), big ET-1 was degraded more effectively than ET-1, while the degradation of ET-1 was more predominant than big ET-1 at acid pH. In addition to this difference, generally at optimum pH the degradation of ET-1 was higher than big ET-1 (57% compared to 35%).

Table 5.2: Degradation of incubated big ET-1 with 20 times diluted rat ventricular extracts (containing 445±80 $\mu g.ml^{-1}$ of protein) at various pH (phosphate buffer, 0.1M) over 60 min and at 25°C. Points represent mean±SD.

pH⇒	**5.2**	**6.2**	**7.2**	**8.2**
n ⇒	**4**	**4**	**4**	**4**
[big ET] in boiled extract ($\mu g.ml^{-1}$)	16.2±1.5	17.2±0.4	19.0±0.3	18.3±0.5
[big ET] in Unboiled extract ($\mu g.ml^{-1}$)	13.7±1.6	14.2±1.7	12.2±0.7	12.3±0.6
Amount of degradation ($\mu g.ml^{-1}$)	2.5±0.1	3.1±1.3	6.7±0.8	6.1±1.0
% Degradation (percentage of boiled)	15.6±1.8	18.1±8.1	35.5±4.1	33.0±4.6

Figure 5.4⇒

HPLC analysis of peptide products formed by incubation of rat big ET-1 with extracts of rat ventricle: Big ET-1 (8nmol.ml^{-1}) was incubated (90 min, pH 7.2) with extracts containing active enzyme ("unboiled") or identical inactivated "boiled" extracts. Incubation of big ET-1 with myocardium extracts produced two detectable metabolite peaks on the chromatogram (indicated by 1 and 2). A buffer artefact (b.a.) was present in all runs.


Absorbance
0.10
0.00
b.a.
ET-1
Standard
0.10
0.05
Big ET-1
b.a.
Boiled
Myocardium
0.05
0.00
1
2
Big ET-1
b.a.
Unboiled
Myocardium
50
60
70
[CH3CN](%)
10.00
20.00
30.00
35.00
Time(min)

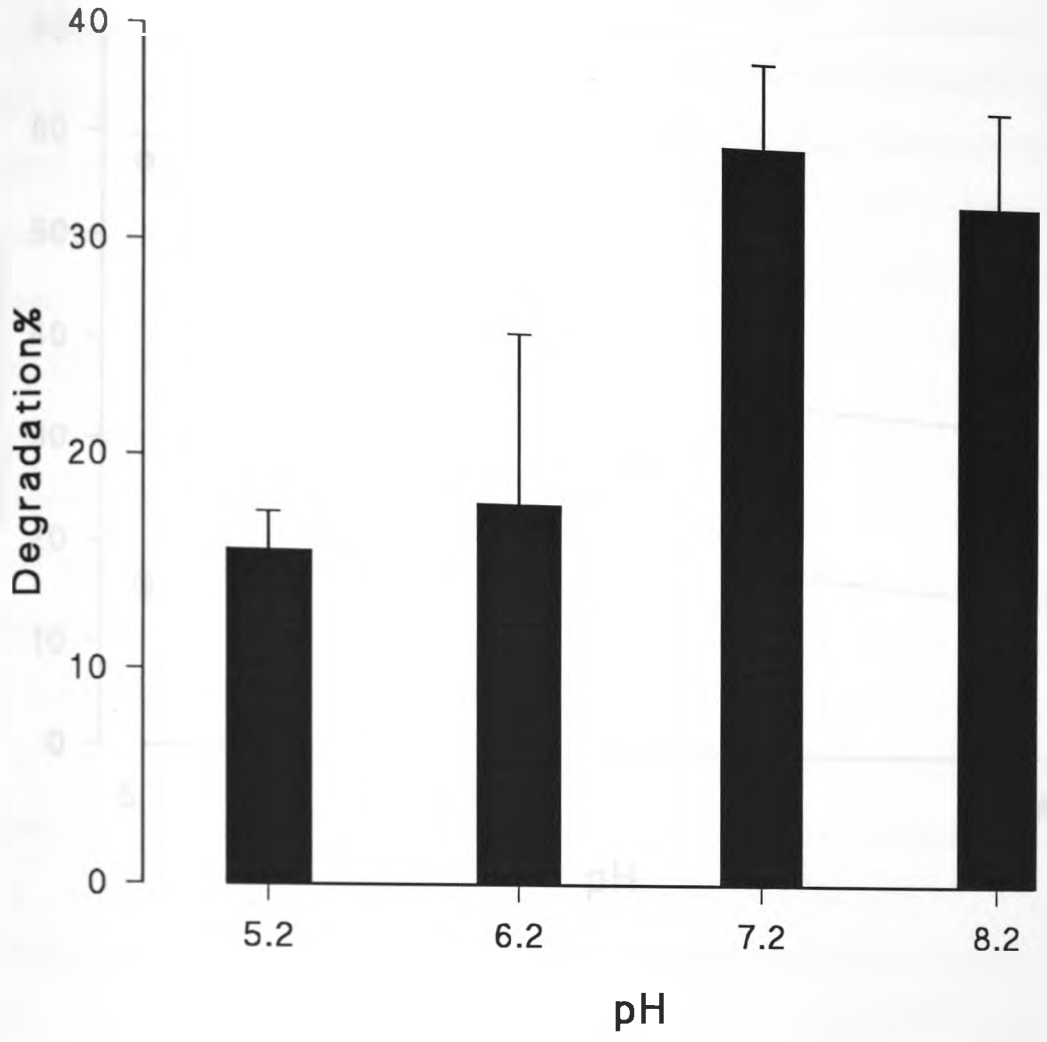


Figure 5.5 The pH dependence of rat big ET-1 degradation by rat ventricular tissue extracts, diluted 20 times by phosphate buffer (final concentration of 0.1M; containing 445±80 $\mu g.ml^{-1}$ of protein). The peptide at a final concentration of 8 $nmol.ml^{-1}$ was incubated for 60 minutes at 25°C and various pH. The samples were analysed by RP-HPLC. Points represent mean±S.D (n=4).

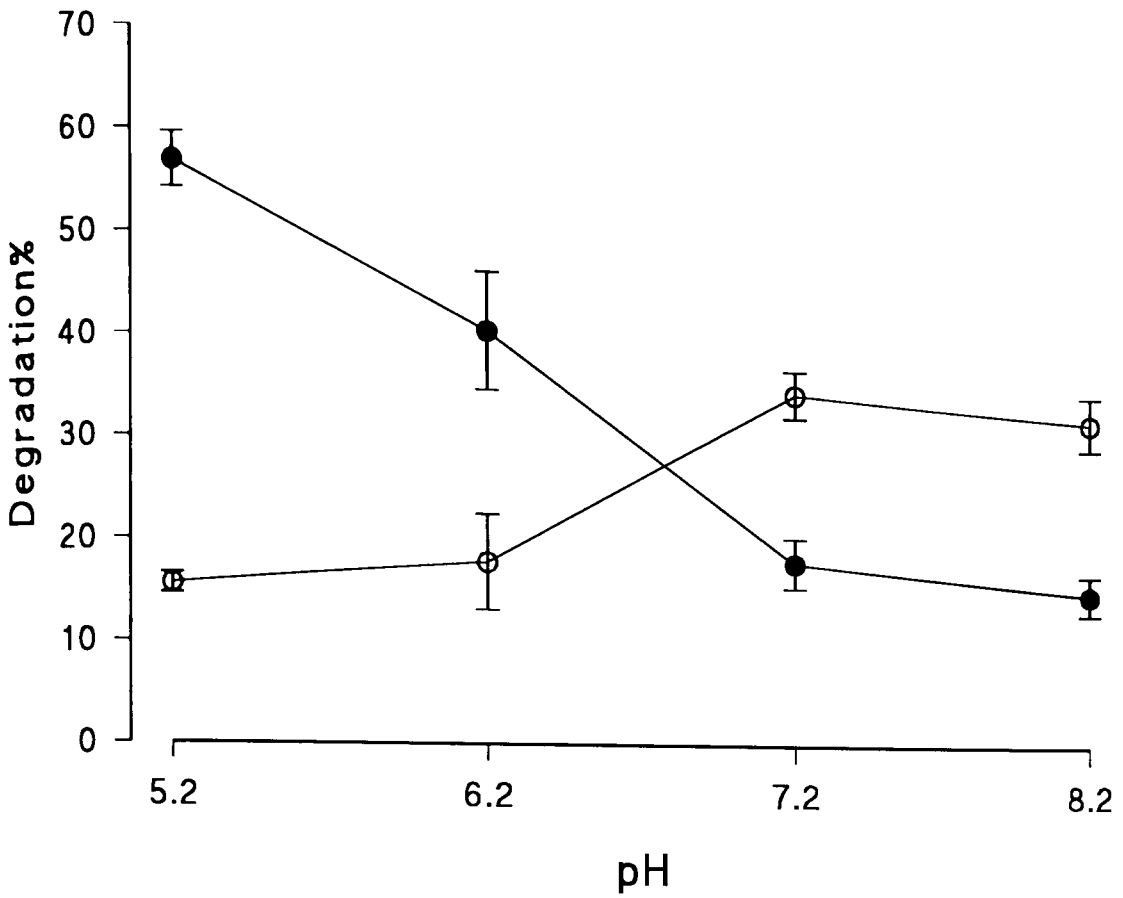


Figure 5.6 The comparison of pH dependent degradation of rat big ET-1 (open circles) and ET-1 (closed circles) by rat ventricular tissue extracts, diluted with phosphate buffer(0.1M). The peptide at a final concentration of 8 $nmol.ml^{-1}$ was incubated for 60 minutes at 25°C and various pH. The samples were analysed by RP-HPLC. Points represent mean±SD, n=4-7.

5.4.2 Identification of ET-1 and Big ET-1 Metabolites Produced by Rat Ventricular Extract at pH 7.2 by Mass Spectroscopy

The identity of the reaction product was verified by mass spectroscopy. Mass spectroscopic analysis showed that the peak which was indicated by number 1 in the HPLC chromatogram of big ET-1 degradation (figure 5.4) was identical to ET-1 or big ET-1 fragment 1-17 (MW=1965), i.e. the degradation of big ET-1 with ventricle extract at pH 7.2 involved cleavage at the Leu-17 and Asp-18 peptide bond (figure 5.7 and 5.8). In addition, the peak indicated by number 2 on the HPLC chromatogram of big ET-1 degradation also contained residue 1-17 of big ET-1 (figure 5.9, mass spectrum number 3). Big ET-1 and one of the ET-1 metabolites which was identified in Chapter 4 (section 4.2) as des-Trp-ET-1 gave peaks in the same area (retention time of 21.5-22.5) on the HPLC. Therefore, the HPLC fraction of big ET-1 peak was collected and identified as big ET-1 (MW=4390) by mass spectroscopy (figure 5.9, mass spectrum number 2). This confirmed that firstly the peak of 21.5-22.5 minutes on the HPLC chromatogram belonged to big ET-1 and secondly that this peak did not contain any other fragments of the peptide. HPLC eluates of the incubated big ET-1 at the retention time identical to retention time of tryptophan (10.5-11.5 minute; metabolite A of ET-1 at pH 5.2) was analysed by mass spectroscopy. None of the peaks corresponded to tryptophan (figure 5.9, mass spectrum number 5). There was no evidence of any endothelin appearing in the big endothelin degradation, since no ET-1 peak was detected by HPLC. Furthermore, the HPLC eluates of big ET-1 degradation at the retention time of 25-26 minutes, identical to the ET-1 peak, was analysed by mass spectroscopy and ET-1 was not detected (figure 5.9, mass spectrum number 1). These results show that big endothelin was not converted to endothelin-1, tryptophan or des-Trp-ET-1 by soluble extracts of rat ventricle. The degradation of ET-1 under the same conditions which were applied for big ET-1, produced no detectable metabolite peaks on HPLC. The HPLC eluates at the retention time points of metabolites number 1 and 2 of big ET-1 and at the retention times of 10.5-11.5 and

21.5-22.5 which belong to tryptophan and des-Trp-ET-1 respectively, and also the eluates at the time point of the ET-1 peak, were collected and analysed by mass spectroscopy. There was a trace of ET-1 fragment 1-17 (MW=1965) in the HPLC eluate of ET-1 degradation which coeluted with metabolite number 1 of big ET-1 (figure 5.10, mass spectrum number 4). The ET-1 peak on HPLC was also identified as ET-1 (MW=2493) by mass spectroscopy. This confirmed that the major part of this peak corresponded to ET-1 and did not contain any other fragment (figure 5.10, mass spectrum number 1). Neither tryptophan nor des-Trp-ET-1 was detected by mass spectroscopy (figure 5.10, mass spectrum 2). The degradation of ET-1 at pH 7.2 therefore does not produce the same metabolites as that at pH 5.2. The other peaks on mass spectrum of both ET-1 and big ET-1 degradation appear to be unrelated and can not be identified as part of either peptide.

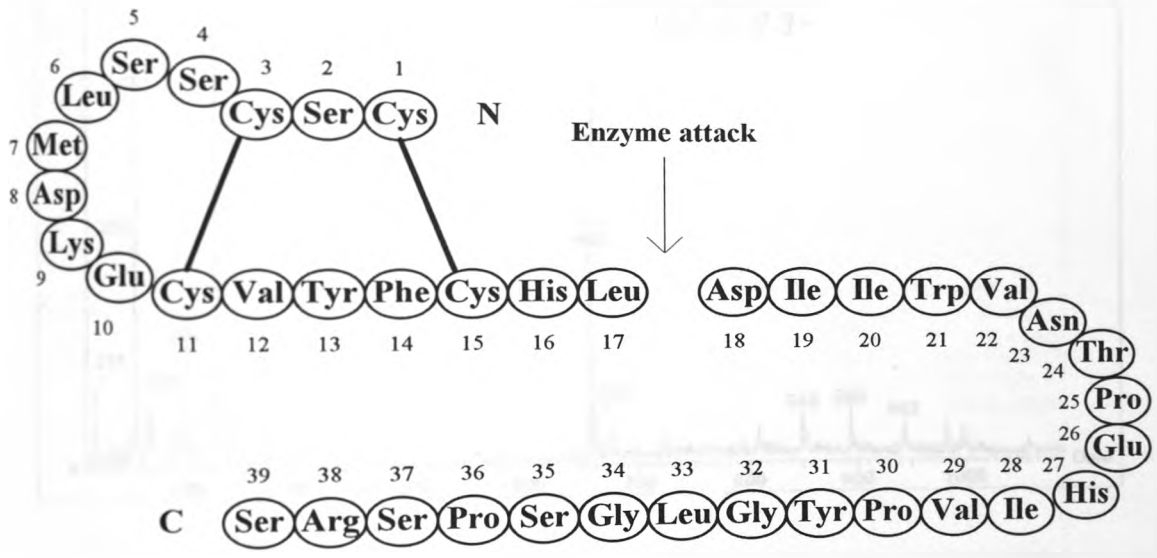


Figure 5.7 Schematic diagram of the sequence for rat big ET-1. Cleavage at the arrow yields fragment 1-17.

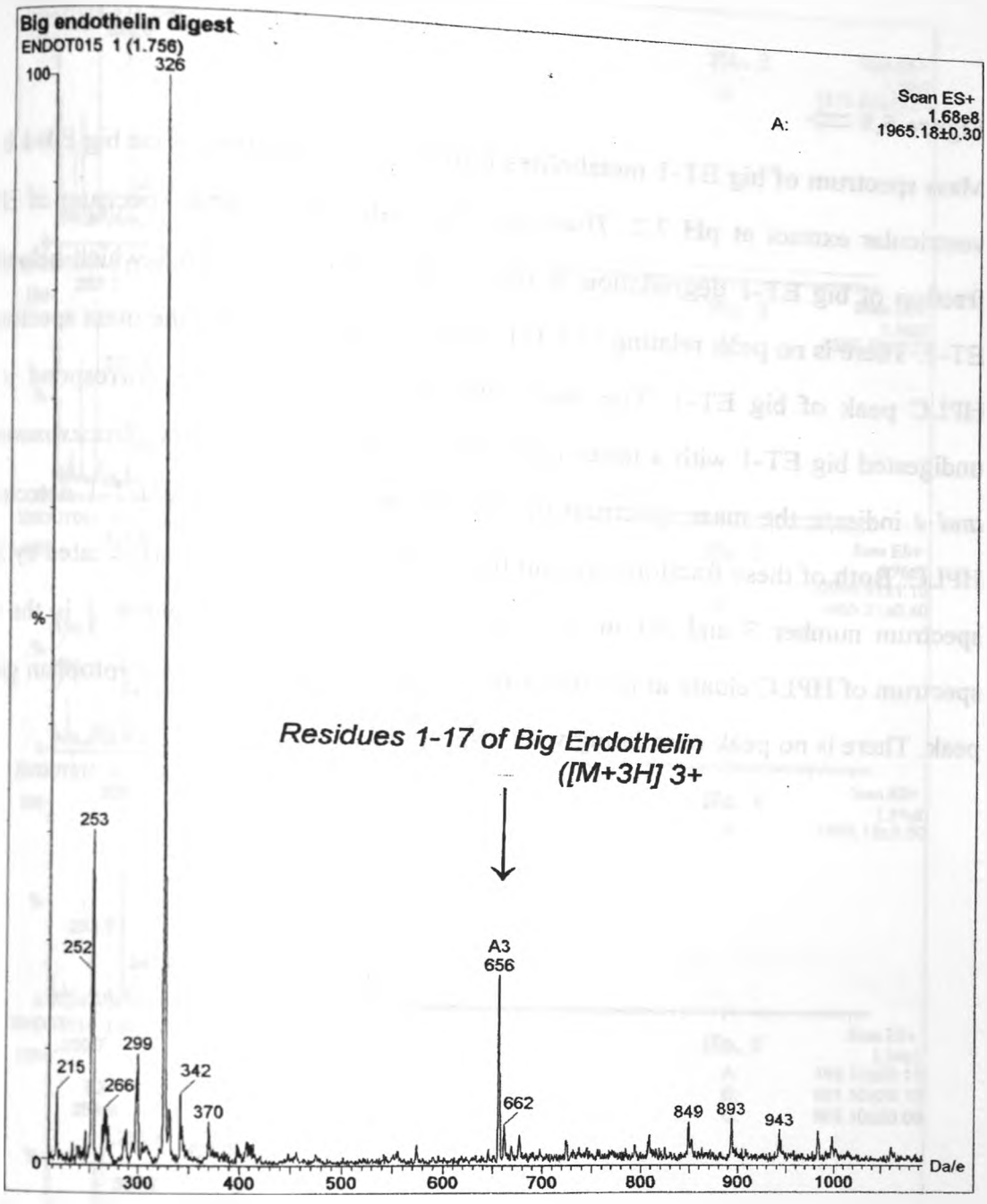


Figure 5.8 Mass spectrum of metabolite number 1 of big ET-1 degradation by rat ventricular extract at pH 7.2. The peak which is indicated by an arrow corresponds to fragment 1-17 of big ET-1 with a molecular weight of 1965 Da.

Figure 5.9 $\Rightarrow$

Mass spectrum of big ET-1 metabolites formed by degradation of rat big ET-1 by rat ventricular extract at pH 7.2. *Trace number 1* shows the mass spectrum of HPLC fraction of big ET-1 degradation at the retention time of 25-26.5 which belongs to ET-1. There is no peak relating to ET-1. *Trace number 2* shows the mass spectrum of HPLC peak of big ET-1. The peaks indicated by A4 and A5 correspond to the undigested big ET-1 with a mean molecular weight of 4390.6 Da. *Traces number 3 and 4* indicate the mass spectrum of metabolites 1 and 2 of big ET-1 detected by HPLC. Both of these fractions contain fragment 1-17 of big ET-1 (indicated by B3 in spectrum number 3 and A3 in spectrum number 4). *Trace number 5* is the mass spectrum of HPLC eluate at the retention time of 10.5-11.5 where tryptophan gives a peak. There is no peak correlating with tryptophan.

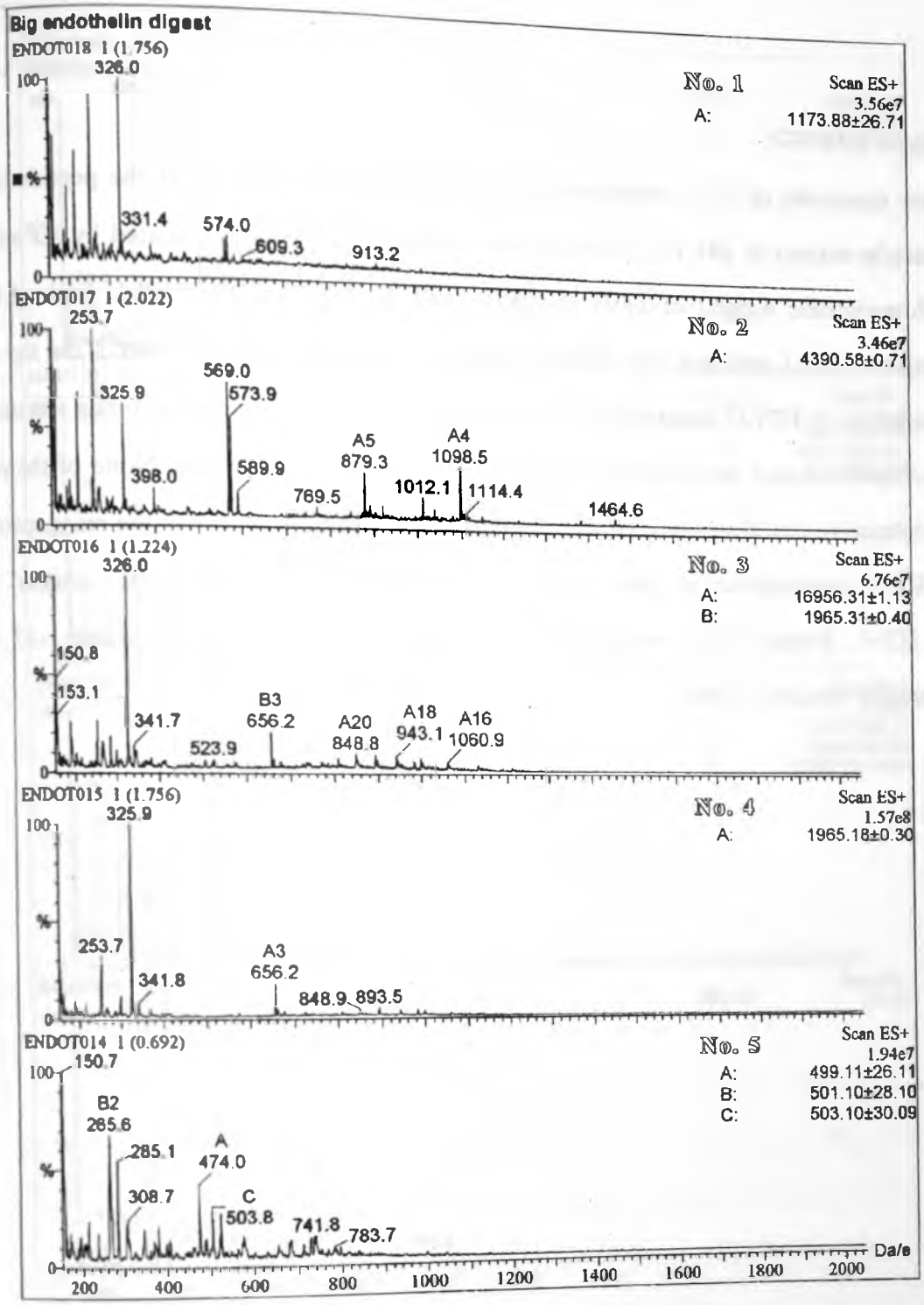
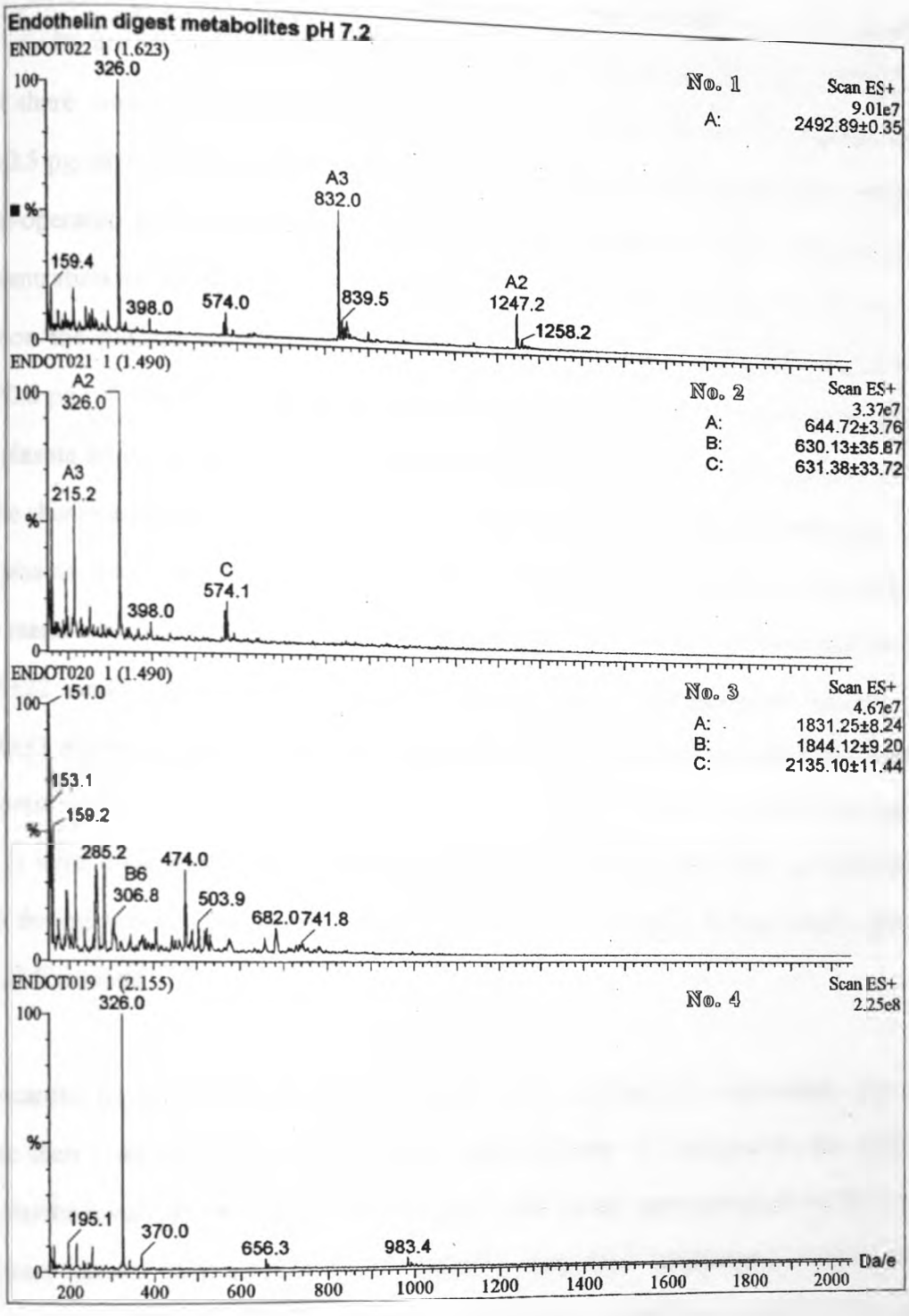

Big endothelin digest
ENDOT018 1 (1.756)
326.0
331.4
574.0
609.3
913.2
No. 1
Scan ES+
3.56e7
A:
1173.88±26.71
ENDOT017 1 (2.022)
253.7
325.9
569.0
573.9
398.0
589.9
769.5
A5
879.3
A4
1098.5
1012.1
1114.4
1464.6
No. 2
Scan ES+
3.46e7
A:
4390.58±0.71
ENDOT016 1 (1.224)
326.0
150.8
153.1
341.7
523.9
B3
656.2
A20
848.8
A18
943.1
A16
1060.9
No. 3
Scan ES+
6.76e7
A:
16956.31±1.13
B:
1965.31±0.40
ENDOT015 1 (1.756)
325.9
253.7
341.8
A3
656.2
848.9
893.5
No. 4
Scan ES+
1.57e8
A:
1965.18±0.30
ENDOT014 1 (0.692)
150.7
B2
265.6
285.1
A
474.0
308.7
C
503.8
741.8
783.7
No. 5
Scan ES+
1.94e7
A:
499.11±26.11
B:
501.10±28.10
C:
503.10±30.09
Da/e
200
400
600
800
1000
1200
1400
1600
1800
2000

Figure 5.10 ⟹

Mass spectrum of ET-1 metabolites produced by degradation of the peptide by rat ventricle extract at pH 7.2. In the *trace number 1* the peaks indicated by A3 and A2 with molecular weight of 2493 correlate with ET-1. Thus the HPLC peak of ET-1 contained ET-1 and not any related fragments. *Trace number 2 and 3* are the mass spectrums of HPLC eluates of ET-1 degradation at the retention times identical to des-Trp-ET-1 and metabolite number 2 of big ET-1 respectively. None of the peaks are related to any fragments of the peptide. *Trace number 4* shows the mass spectrum of ET-1 degradation at the retention time identical to metabolite number 1 of big ET-1. Peaks 656.3 and 983.4 correspond to traces of fragment 1-17 with molecular weight of 1965.


Endothelin digest metabolites pH 7.2
ENDOT022 1 (1.623)
326.0
No. 1
Scan ES+
9.01e7
A:
2492.89±0.35
A3
832.0
159.4
398.0
574.0
839.5
A2
1247.2
1258.2
ENDOT021 1 (1.490)
A2
326.0
No. 2
Scan ES+
3.37e7
A:
644.72±3.76
B:
630.13±35.87
C:
631.38±33.72
A3
215.2
C
574.1
398.0
ENDOT020 1 (1.490)
151.0
No. 3
Scan ES+
4.67e7
A:
1831.25±8.24
B:
1844.12±9.20
C:
2135.10±11.44
153.1
159.2
285.2
474.0
B6
306.8
503.9
682.0
741.8
ENDOT019 1 (2.155)
326.0
No. 4
Scan ES+
2.25e8
195.1
370.0
656.3
983.4
Da/e
200
400
600
800
1000
1200
1400
1600
1800
2000

5.4.3 Plasma and Myocardial Levels of ET-1 during Ischaemia and Reperfusion

The results are shown in table 5.3. In the non-operated group (non-thoracotomised rats) there was a plasma ET-1 like immunoreactivity (IR-ET-1) concentration of 1.9±0.5 $pg.ml^{-1}$, measured 45 minutes after anaesthesia. The surgical procedure in the sham-operated group produced a slight but not significant rise in the mean plasma concentration of IR-ET-1 (3.2±0.5 $pg.ml^{-1}$). After 30 minutes of coronary artery ligation the plasma concentration of IR-ET-1 increased significantly ($p<0.01$) from 3.2±0.5 $pg.ml^{-1}$ in sham-operated to 8.2±0.9 $pg.ml^{-1}$. After 30 minutes of reperfusion the plasma level of IR-ET-1 was elevated significantly to 9.1±1.7 $pg.ml^{-1}$ compared to the sham-operated group (figure 5.11). There was no significant difference between the plasma levels of ET-1 during coronary artery ligation and following reperfusion. The mean arterial pressure at the end of the ligation (102±8 mmHg) and reperfusion (125±22 mmHg) periods did not significantly change compared to prior to ligation (118±13 mmHg in ligated animals; 132±17 mmHg in reperfused animals). Ligation of the coronary artery for 30 minutes caused arrhythmias (Mean 30 minutes total = 1423 ±112) with a characteristic temporal pattern of distribution. Few arrhythmias were seen during reperfusion (Mean 30 minutes total = 66±42), being totally absent in 2 out of 5 animals.

Myocardial tissue contained about 4 times more endogenous endothelin per gram of tissue than 1 ml of plasma did in sham-operated rats. In contrast to the finding with the plasma levels, in the ligated group, the mean tissue concentration of IR-ET-1 after coronary artery ligation (11.2±0.9 $pg.g^{-1}$) remained unchanged compared to the sham-operated (11.2±1.5 pg.g-1) rats. However, reperfusion significantly ($p<0.01$) increased the mean tissue level of IR-ET-1 from 11.2±1.5 $pg.g^{-1}$ in the sham-operated group to 17.8±0.7 $pg.g^{-1}$ (figure 5.12).

Table 5.3: Plasma and myocardial tissue IR-ET-1 in non-thoracotomised (non-operated) rats, sham-operated and in rats following either 30 minutes coronary artery occlusion (ligated) or 30 minutes occlusion and 30 minutes reperfusion (reperfused). Data represent mean±s.e.m.

Experiment	No	Plasma IR-ET-1 ($pg.ml^{-1}$)	Myocardial IR-ET-1 ($pg.g^{-1}$)
Non-operated	5	1.9±0.5	—
Sham-operated	5	3.2±0.5	11.2±1.5
Ligated	6	8.2±0.9**	11.2.±0.9
Reperfused	7	9.1±1.7**	17.8±0.7***♣

**p<0.01 vs non-operated and sham-operated groups; ***p<0.001 vs sham-operated; ♣ p<0.01 vs ligated group.

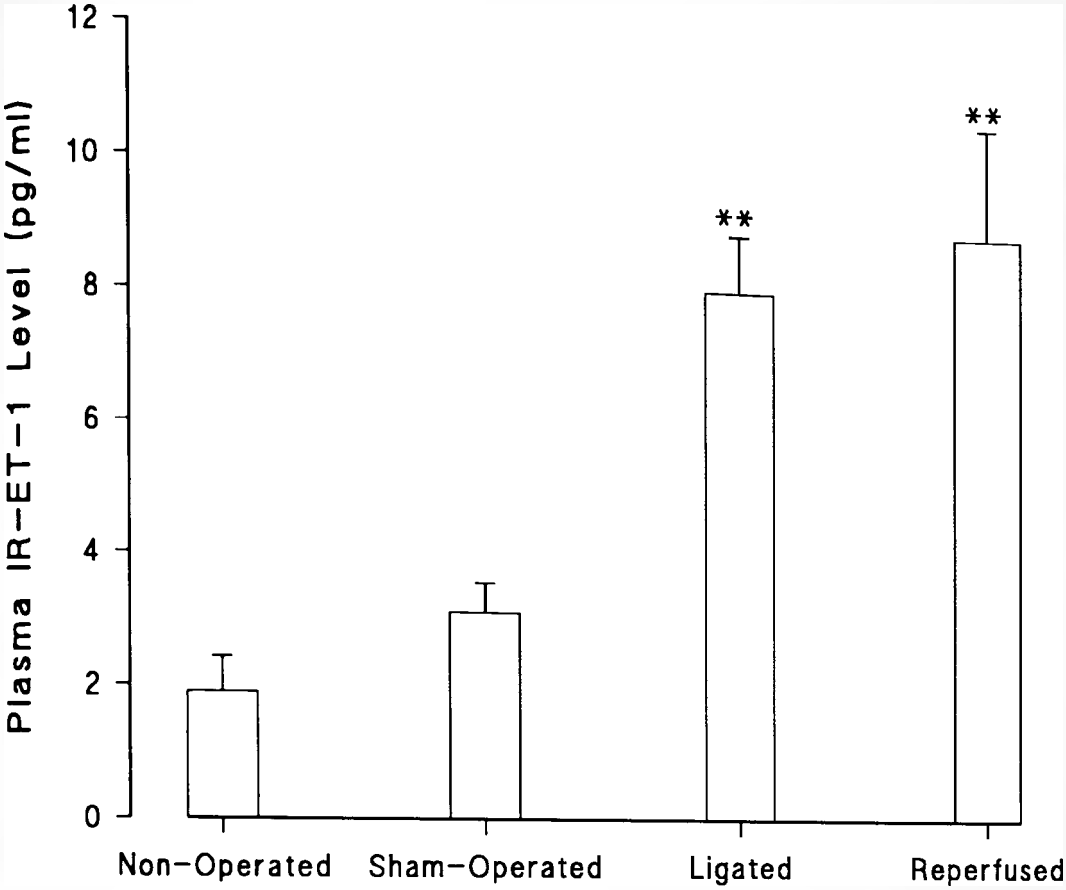


Figure 5.11 Plasma endothelin-1-like immunoreactivity (IR-ET-1) after 45 min anaesthesia (non-operated; n=5), 30 min after sham operation (Sham-Operated; n=5), 30 min after coronary artery ligation (Ligated; n=6), and 30 min after reperfusion (Reperfused; n=7). Significant differences from the non-operated (non-thoractomised) and sham-operated groups have been shown at $p<0.01$ (**). Points represent mean±s.e.m.

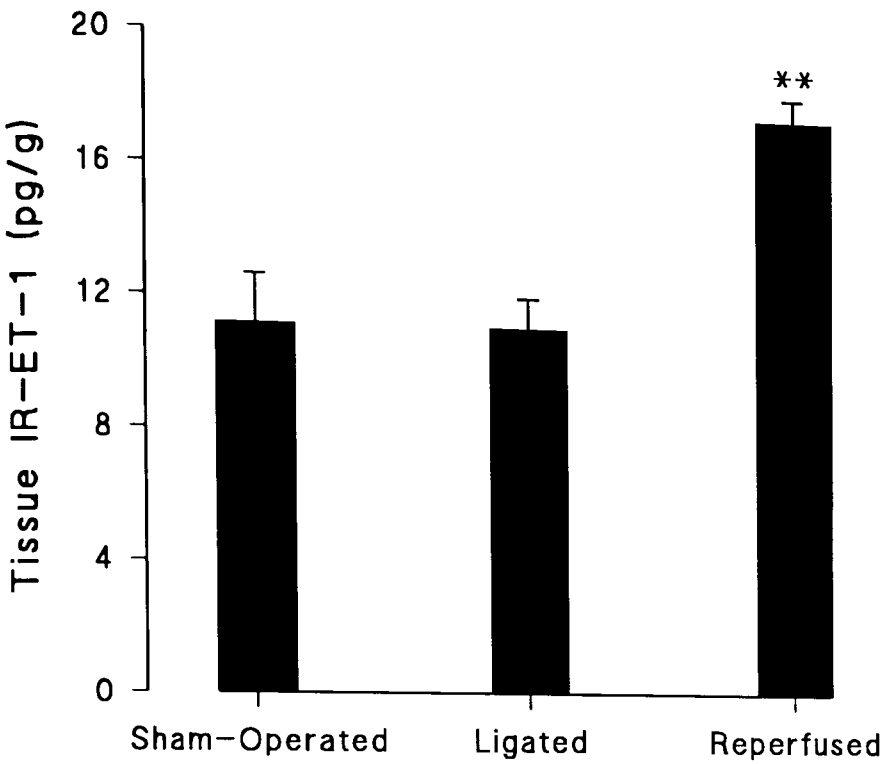


Figure 5.12 Myocardial tissue concentration of endothelin-1-like immunoreactivity 30 min after sham-operation (Sham-Operated, n=5), coronary artery ligation (Ligated, n=5), and reperfusion (Perfused, n=6). Significant differences from the sham-operated and ligated groups are shown at $p<0.01$ (**). Points represent mean±s.e.m.

5.5 DISCUSSION

Incubation of exogenous big ET-1 with rat ventricular aqueous extract at pH 7.2 produced two detectable metabolite peaks on HPLC. Both of them contained residue 1-17 of big ET-1. Thus, big ET-1 was cleaved at the Leu^{17}-Asp^{18} peptide bond by ventricular extract, yielding ET(1-17) as the amino terminal fragment. HPLC analysis of incubated big ET-1 with aqueous extract of rat ventricle at pH 7.2 did not show any detectable ET-1 peak. Moreover, there were no peaks corresponding to ET-1 on the mass spectrum of HPLC eluates of incubated big ET-1 at the retention time identical to that of ET-1. Thus, it appears that ventricular extract does not convert big ET-1 to ET-1. However, it could be argued that big ET-1 was converted to ET-1 and then the subsequent ET-1 was metabolised further by the tissue extract to produce the detected fragments. To test this, ET-1 was incubated with rat ventricular extract under exactly the same conditions which were applied to big ET-1. Only an unquantifiable trace of peptide fragment 1-17 common to ET-1 and big ET-1 was detected. The detection sensitivity of the mass spectroscope was in the femtomolar range, approximately 3 order below that of the HPLC UV detector. Thus, if the rat ventricular extracts were capable of converting big ET-1 to mature ET-1, it could only involve a minute proportion of the total big ET-1 metabolized and produce vanishingly small amounts of the free peptide. Further, residue 1-17 is the first 17-amino acid fragment of both peptides and the same enzyme which cleaves big ET-1 at the site of Leu^{17}-Asp^{18} peptide bond could therefore break down ET-1 to fragment 1-17. Thus, degradation of big ET-1 to residue 1-17 does not necessarily mean that the peptide was first metabolised to ET-1 and then further degraded to fragment 1-17. Furthermore, if big ET-1 was metabolised to ET-1 and then ET-1 was degraded to ET(1-17), there should have been at least 3 peaks on the HPLC chromatogram, whereas only two metabolite peaks were detected.

Another reason to suggest that big ET-1 was probably not degraded to residue 1-17 through the conversion to ET-1, is that the maximum degradation of big ET-1 happened at pH 7.2 (35% after 60 minutes incubation) whereas ET-1 was degraded optimally at acid pH (57-73.5%). The degradation of ET-1 at pH 7.2 was as low as 18.5% (after 60 min incubation) and reached a maximum of 22% (figure 4.2) following 90 minutes incubation. In addition, comparison of the peak size of fragment 1-17 on the mass spectrum of incubated big ET-1 and ET-1 (figures 5.9 and 5.10) shows that only a very weak peak of residue 1-17 was detected by mass spectroscopy of the ET-1 metabolites. The same molar concentrations (8 μM) of both ET-1 and big ET-1 were incubated under the same conditions. Therefore, the low degradation of ET-1 compared to big ET-1 makes it unlikely that big ET-1 was degraded first to ET-1 and then to fragment 1-17.

The mean plasma level of endothelin in normal healthy Sprague-Dawley rats was 1.9 ± 0.5 pg.ml^{-1}. The assay did not show cross reactivity with big ET-1 and it was sensitive enough to detect 0.5 pg/tube. In this study the surgical operation in itself caused a slight, but not significant, increase in the mean plasma concentration of endothelin, therefore the results in the ligated and reperfused groups were compared with those from the sham-operated group. Following 30 minutes coronary artery occlusion, the plasma concentration of endothelin increased by 156% ($p<0.01$), however, the cardiac tissue concentration remained unchanged during the course of this period of myocardial ischaemia. Reperfusion after coronary artery ligation elevated the plasma level of endothelin by 184% and the tissue concentration of endothelin also increased significantly ($p<0.01$) by about 60% during the 30 minutes reperfusion period.

In patients and in animal studies, elevated plasma concentrations of endothelin during coronary artery occlusion and reperfusion have been reported (Stewart et al., 1991;

Tsuji et al., 1991) and, in some studies, increase in ET-1 levels have also been demonstrated in cardiac tissue (Watanabe et al., 1991a). However, it is not clear how the release or production of endothelin is stimulated after myocardial ischaemia and reperfusion. This raises the question does myocardium possibly have a role in the production of endothelin from big endothelin during ischaemia? One study has demonstrated an increase in coronary sinus endothelin levels after coronary angioplasty (Tahara et al., 1991). This suggests that injury to coronary artery endothelium caused this increase, since endothelin was preferentially secreted and accumulated at the abluminal face of the endothelium (Masaki, 1989). Brief ischaemia has also been shown to cause endothelial injury and an increase in permeability (Dauber et al., 1990). It is therefore possible that leakage of endothelin from the subendothelial space may cause the appearance of increased levels of endothelin in the plasma or tissue. However, according to our study this mechanism appears improbable because the cardiac tissue concentration of endothelin did not increase during the coronary artery occlusion. The disagreement between the present study and that of Watanabe et al (1991a), who demonstrated an increase in the tissue level of endothelin during ischaemia, is perhaps due to the different procedure that was used. In this study we used a 30 minute ligation while Watanabe et al (1991a) applied a 50 minute coronary artery ligation where the ischaemic damage is certainly much greater than 30 minutes ligation.

Endothelin release has been demonstrated during early reperfusion after short and gentle constriction of the coronary artery, which did not cause significant endothelial damage (Tonnessen et al., 1993). Tonnessen and co-workers (1993) speculated that the increase in conversion of big endothelin to endothelin take places locally. They pointed out that simple washing out of the ischaemic area can not explain the rapid increase in cardiac venous endothelin levels after short term coronary artery ligation. Increased flow and shear stress can induce the release of endothelin from endothelial

cells (Hieda et al., 1990; Yoshizumi et al., 1989). This can only explain the increase in plasma and cardiac tissue concentrations of endothelin after reperfusion, but not the increase in plasma level of endothelin during coronary artery occlusion. Furthermore, if the increase in plasma and tissue endothelin during the reperfusion was due either to "washing out" of the peptide generated in myocardium during ischaemia, or to increased shear stress due to flow, the plasma concentration of endothelin during reperfusion would have to be higher than that during the period of ischaemia. In the present study, there was no significant difference between the plasma levels of the occlusion and occlusion/reperfusion groups. Therefore, the rise of endothelin level in plasma is unlikely to be due only to washing out of endothelin from the myocardial source or to shear stress.

The increased tissue concentration of endothelin during reperfusion, but not during coronary artery ligation, with no changes in plasma levels during reperfusion, suggests that the endothelin is not produced in the ischaemic myocardium. The low collateral blood flow in rats (Winkler et al., 1984) makes it unlikely that endothelin could pass from plasma into the ischaemic region or vice versa. Consequently, the observation that during the ligation the concentration of endothelin in the myocardium remained unchanged, while the plasma levels increased, may indicate that the increase in plasma endothelin originates at a site other than the ischaemic zone or even the myocardium. Furthermore, in this study we showed that the degradation of big ET-1 occurs optimally at neutral pH (35% degradation), in other words, as pH decreases, so does conversion of big ET-1 (18% degradation at pH 6.2). The degradation of ET-1, in contrast takes place optimally at acid pH. The myocardial tissue pH falls as low as 5.8 in normal rats after 30 minutes myocardial ischaemia (Khandoudi et al., 1990). Thus, in the ischaemic state, the probable conversion of big endothelin to endothelin will be low whereas the degradation of endothelin is high. It does not therefore appear that such a large elevation of circulating and tissue endothelin during ischaemia and

reperfusion could depend only on the myocardium. The systemic production of endothelin may be more predominant. It is possible that the stimulus for the increased release of endothelin from endothelial cells or intima of blood vessels during ischaemia and reperfusion depends on the presence of blood or some component of blood. However, the release of low levels of endothelin has been shown (Brunner et al., 1992) in isolated perfused rat hearts. It was not certain whether endothelin was released from endothelial cells or cardiac cells. In Brunner's study the hearts were subjected to coronary artery ligation and reperfusion in two groups. In one group hearts were perfused with the standard perfusion medium and there was no increase in the release of endothelin during the reperfusion. Endothelin release during reperfusion was increased over the basal rate only in the second group which had been subjected to reperfusion in the presence of foetal calf serum. In both groups the release of endothelin was greatly reduced during ischaemia. These results indicate the apparent importance of serum peptides in the stimulation of endothelin secretion. In fact, for a rapid and marked increase of plasma levels of endothelin during pathophysiological or experimental myocardial ischaemia and reperfusion the involvement of a widespread system such as blood components seems reasonable. Alternatively a reduction in the peptide clearance by lung and kidney during myocardial ischaemia (due to the reduction of cardiac out put) can elevate plasma levels of endothelin.

Coronary artery occlusion and its consequences, such as hypoxia, trigger the release and accumulation of leukocyte derived enzymes in the circulation and thus can stimulate the extra cellular conversion of big ET-1 to ET-1 (Sessa et al., 1991a). The role of circulating leukocytes in myocardial infarction has been confirmed by a study which showed that white blood cells accumulated in the myocardium corresponding to acute myocardial infarcts, in dogs subjected to coronary artery occlusion (Weiss et al., 1977). Experimental studies also show that reperfusion following the ischaemia causes accumulation and adherence of white blood cells at particular sites within the

circulation (Lucchesi et al., 1989; Lefer et al., 1993). These inflammatory cells, the polymorphonuclear leukocytes (PMNs), are rich in proteinases and some of these enzymes are implicated in the conversion of big ET-1 to ET-1 (Sessa et al., 1991a). Conversion of big ET-1 to ET-1 in the circulation by a blood cell-mediated process, has been reported (Watanabe et al., 1991b). It has also been observed (Yanagisawa et al., 1988a,b) that the expression of preproendothelin mRNA in the porcine aortic intima and in cultured endothelial cells is enhanced by thrombin, growth factor, angiotensine II and epinephrine (high levels of angiotensine II and catecholamines are released during ischaemia). Thrombin also evokes the release of endothelin from cultured porcine and bovine endothelial cells (Emori et al., 1989; Schini et al., 1989). Boulanger and Luscher (1990) demonstrated that endothelin release from the intimal layer of intact aorta of pig, was stimulated by thrombin. The role of thrombus formation in the pathogenesis of unstable angina and acute myocardial infarction is well known. Falk (1983) suggested that activation of the coagulation cascade and subsequently the thrombotic obstruction of an atherosclerotic coronary artery usually occurs at the site of a severe stenosis. In another study, thrombin generation and fibrin formation has also been demonstrated (Vaziri et al. 1992) in patients with acute myocardial ischaemia or infarction.

In the present study it has been shown that myocardial tissue contained about 4 times more endogenous endothelin per gram than did 1 ml of plasma in sham-operated rats. Since 1 g of myocardial tissue would contain only a fraction of a ml of plasma and interstitial fluid, it was clear that the tissue endothelin concentration was not due merely to contamination with blood. If endothelin is produced systemically by endothelial cells not locally in myocardium, why is the net concentration of endothelin in cardiac tissue much more than plasma? It is well known that endothelin is removed rapidly from the circulation by tissues, with more than 60% of the removal occurring in the first minutes (Anggard et al., 1989). One explanation is that the clearance of endothelin is achieved by binding to receptors (Frelin and Guedin, 1994). Evidence

for such a mechanism has been presented. Injections to rats of BQ-788, an ET_B receptor antagonist (Fukuroda et al., 1994), and Ro 46-2005, a mixed antagonist (Loffler et al., 1993), reduce the clearance of ET-1 and increase its plasma concentration. It is known that the binding of endothelin is very firm, with a dissociation half life of 40 to 200 hours (Frelin and Guedin, 1994; Waggoner et al., 1992). This finding can explain why in the study of Watanabe et al (1991a), the cardiac tissue levels of endothelin remained high several hours after reperfusion, while the plasma concentration declined to normal value. It has been demonstrated that ischaemia causes externalization of endothelin receptors in rat cardiac membrane (Liu et al., 1990), thus increasing the number of endothelin receptors available for the interaction with blood-born endothelin and subsequent elevation of tissue levels of endothelin.

It can therefore be postulated that ischaemia causes activation of inflammatory reactions and the coagulation cascade. This, in turn, may lead to increased systemic production of endothelin by endothelial cells or its release from the vessels' intima. Due to rapid clearance and increased binding sites of endothelin in myocardium during ischaemia, the elevated plasma level of peptide results in augmentation of the tissue concentration of endothelin during reperfusion, when the blood flow is restored to ischaemic area by releasing the ligation thus allowing access of circulating endothelin to the binding sites. During coronary artery occlusion, the cardiac content of endothelin remains unchanged, since due to the low collateral blood flow in rats the ischaemic zone has no access to plasma endothelin.

Chapter 6

CONCLUSION

6 CONCLUSION

Endothelin-1 (ET-1), a potent vasoconstrictor peptide, was first isolated from cultured porcine aortic endothelial cells. Subsequent studies have revealed the presence of two different ET-receptors (and possibly a third) which are named ET_A and ET_B receptors. Intravenous injection of ET-1 induced a biphasic haemodynamic response in anaesthetized Sprague-Dawley rats, characterized by a transient hypotension which was followed by a long-lasting hypertension. Cumulative doses of ET-1 resulted in a dose-dependent increase in arterial blood pressure associated with no significant change in the hypotensive phase. BQ-123, a selective ET_A receptor antagonist, caused an effective but incomplete inhibition of the ET-1-induced pressor response, suggesting that the pressor action of ET-1 is mediated mostly, but not entirely, via activation of ET_A receptors. Indeed, many in vitro and in vivo studies show the involvement of ET_B or ET_B-like receptors in the induction of vasoconstriction by ET-1 (McMurdo et al., 1993a; Harrison et al., 1992). BQ-123 did not affect the depressor responses to ET-1. This is not surprising since this effect has been reported to be mediated by ET_B receptors rather than ET_A receptors (Cody et al., 1992; Doherty et al., 1993).

$LVdP.dt^{-1}{}_{max}$, which is measured as an index of myocardial contractility, was increased significantly by ET-1, whereas $dP.dt^{-1}.P^{-1}$ remained unaffected with any doses of ET-1. The effect of ET-1 on myocardial contractility could be due to the direct inotropic action of the peptide which has been reported by many in vivo and in vitro studies (Davenport et al., 1989; Ishikawa et al., 1988b; Watanabe et al., 1989b). Since $dP.dt^{-1}.P^{-1}$ (an index of myocardial contractility, insensitive to afterload) was not changed by ET-1, the participation of the increased peripheral resistance should also be considered in the positive inotropic effect of ET-1. ET-1-induced increase in $LVdP.dt^{-1}{}_{max}$ was completely prevented by BQ123 (50 $\mu g.kg^{-1}.min^{-1}$), indicating that this effect was mediated by ET_A-receptors. However, a dose-dependent

reduction in $LVdP.dt^{-1}_{max}$, compared to the pre-injection value, was observed following injection of ET-1 in the presence of a higher dose of BQ-123 (100 $\mu g.kg^{-1}.min^{-1}$) whereas, $dp.dt^{-1}.p^{-1}$ which is insensitive to afterload remained unchanged. The reduction in $LVdp.dt^{-1}_{max}$ in the presence of high dose of BQ123 can be due to reduction in peripheral resistance where ETA receptors were blocked markedly.

ET-1 is assumed to play an important role in the pathophysiology of various diseases including myocardial infarction, vasospastic angina pectoris and heart failure. Pro-ischaemic and pro-arrhythmic effects of ET-1 have been reported from a variety of in vivo and in vitro studies. In the present study infusion of ET-1 into anaesthetized rats during a period of acute regional myocardial ischaemia caused a marked increase in the severity of ischaemia-induced arrhythmias. ET-1 increased very significantly the total number of ventricular ectopic beats (VEBs) as a result of an increase in the number of beats occurring as ventricular tachycardia (VT). There was also a marked increase in the incidence of ventricular fibrillation (VF). This pro-arrhythmic effect of ET-1 was also associated with a marked increase in the time spent for both VT and VF. Several mechanisms, such as coronary artery constriction, increased sensitivity to ET-1 during ischaemia, or a direct action of ET-1 on the myocytes such as an increase in intracellular Ca^{2+}, prolongation of the action potential or an enhancement of delayed rectifier K^+ current and blockade of ATP-sensitive K^+ channels, could all be suggested as explanations for this pro-arrhythmic effect. Thus, the effects of ET-1 on the heart appear to be much more complex than a simple effect via the one of the mentioned mechanisms. However, the results of this study implicate a direct action of ET-1 rather than coronary vasoconstriction for the pro-arrhythmic effect of ET-1.

The present study also demonstrated that BQ-123 significantly reduced both ventricular arrhythmias induced by coronary artery occlusion (effect of endogenous endothelin) and the pro-arrhythmic effects of infused ET-1 (effect of exogenous). This indicates the involvement of ET_A receptors both in the genesis of ischaemic-arrhythmias and arrhythmogenic action of exogenous ET-1 on the ischaemic myocardium. However, the antiarrhythmic effect of BQ-123 against ischaemic arrhythmias was observed only in a very narrow dose range. BQ-123 suppressed all types of ischaemic arrhythmias at a low dose, but with the highest dose there was an increased incidence of irreversible VF. These findings reflect the U-shaped dose-response curve which has been described for the antagonism of the pressor response to ET-1 by BQ-123 (Bird et al., 1993). The U-shape dose-response effect of BQ-123 on the ischaemic arrhythmias is consistent with the hypothesis that, in addition to ET_A receptors, other receptor subtypes may be involved in the arrhythmogenic action of endogenous ET-1. ET-1 binding sites with low (nM sites) and high (pM sites) affinities have been observed in the myocardium (Sokolovsky, 1993). It has also been shown that BQ-123 selectively inhibits the pM sites which mainly include ET_A receptors. With respect to the results of this study and evidence from other studies, it appears that BQ-123, at low doses, abolishes the pro-arrhythmic effects of exogenous and endogenous endothelin via blockade of pM sites. However, in the presence of a high dose of BQ-123, the pM sites are probably blocked entirely, leaving nM sites (or according to some studies ET_{B1} receptors) free to be activated by locally or systemically accumulated ET-1 to induce arrhythmogenic effects. It is proposed that nM sites are involved in the ET-1 induced vasoconstriction and mobilisation of Ca^{2+} from intracellular stores.

The present study also showed that infusion of BQ-123, at a dose (50 $\mu g.kg^{-1}.min^{-1}$), which had no effect on arrhythmias, did not alter infarct size in rats subjected to a 30 minutes coronary artery occlusion followed by 3.5 hours reperfusion. Considering the

results obtained from the arrhythmia studies, where only a dose of 10 $\mu g.kg^{-1}.min^{-1}$ was antiarrhythmic, it is possible that the higher dose used in the infarct size experiments was out with the effective dose-range for BQ123. However, the result is in agreement with another study which has shown FR 139317 (ET_A selective antagonist) to have no effect on infarct size (McMurdo et al., 1993b). This is in contrast to findings that show that a monoclonal antibody against ET-1, phosphoramidon and a mixed ET_A/ET_B receptors antagonist all reduce the infarct size (Watanabe et al., 1991a; Richard et al., 1994). This discrepancy may be due to the fact that only ET_A receptors are blocked by BQ-123, while an ET-1 antibody, an endothelin converting enzyme inhibitor, and mixed endothelin receptor antagonists would essentially prevent the action of ET-1 at all receptor subtypes.

ET-1 is cleared rapidly from the circulation with a half-life of 1-7 minutes by lungs, kidneys, spleen and heart. The exact mechanism of this tissue-specific clearance is not well understood, however, a proteolytic degradation may be involved. This study investigated the nature of ET-1 degradation in rat myocardium and compared it with that in the lung. A preliminary characterization of enzymes involved in ET-1 destruction was also studied using enzyme inhibitors. Exogenous ET-1 was degraded by both rat ventricular and lung aqueous extracts optimally at acid pH. At pH 5.2 lung and ventricular extracts produced considerable degradation of ET-1 to give 4 and 2 detectable product peaks respectively on RP-HPLC. Metabolites produced by ventricular extracts at pH 5.2 were identified by electrospray mass spectroscopy as tryptophan and des-Trp-ET-1, indicating the presence of an ET-1 metabolising enzyme in myocardium which cleaves the C-terminal tryptophan. Moreover, a trace of residue 1-17 of ET-1 was also identified as a metabolite of ET-1 produced by ventricular extract at pH 7.2. The fact that des-Trp-ET-1 is much less active than ET-1 (Deng et al., 1994) combined with the presence of an acid optimum ET-1 degrading enzyme in myocardium, may have considerable pathophysiological

significance especially during ischaemic conditions when myocardial tissue pH falls as low as 6 or less. Indeed, the results show that at pH 6.2 the myocardial ET-1-degrading activity was greatly increased to 222% of that at the more physiological pH 7.2.

In this thesis it was also found that exogenous big ET-1 was degraded by rat ventricular aqueous extract pH dependently, producing two detectable metabolite peaks on RP-HPLC at pH 7.2. Both metabolite peaks contained a fragment which was identified as ET(1-17) by electrospray mass spectroscopy. Neither of the fragments was mature ET-1. In contrast to ET-1 the degradation of its precursor, big ET-1, occurred optimally at pH 7.2. Thus, there was an inverse relationship between ET-1 and big ET-1 degradation in myocardium. This fact also has pathophysiological importance in ischaemic conditions associated with low pH where there is a notable degradation of ET-1 compared to relatively low degradation of big ET-1.

Phosphoramidon (an inhibitor of endopeptidase 24.11) and PMSF (a serine peptidase inhibitor) both inhibited the ET-1 degradation by ventricular extract at pH 5.2. EDTA (a metal chelator), captopril (kininase/angiotensin converting enzyme inhibitor), pepstatin A (an inhibitor of aspartic protease) and aprotinin (serine protease inhibitors) all had no effect on the ET-1 degrading activity. The results from this part of the study suggest the presence of at least two types of acid optimum ET-degrading enzymes in rat ventricular aqueous extracts. One of them, with high efficiency, is blocked by a serine peptidase inhibitor (PMSF) and another, with low efficiency, is phosphoramidon sensitive.

Elevated plasma levels of endothelin have been reported in patients with myocardial infarction and in experimental myocardial ischaemia and reperfusion. In this study, using radioimmunoassay, IR-ET-1 plasma levels of 1.9 ± 0.5 $pg.ml^{-1}$ and a mean tissue

concentration of 11.7±1.5 $pg.g^{-1}$ wet weight of myocardium were found in normal healthy and in sham-operated rats respectively. A period of 30 minutes of ischaemia induced by coronary artery occlusion resulted in a marked increase (156%) in the plasma concentration of IR-ET-1, but this was not associated with a parallel change in the tissue level of IR-ET-1. This indicates that elevation of plasma IR-ET-1 during ischaemia could not have originated from the myocardial tissue. Reperfusion (for 30 minutes) following the 30 minutes coronary artery occlusion elevated both plasma and tissue levels of endothelin significantly. Furthermore, since the previous results showed that big ET-1 degradation by aqueous extract of rat ventricle did not contain mature ET-1 as a product, and that at a low pH (similar to intracellular pH of myocytes during ischaemia) the degradation of ET-1 is high while that of big ET-1 is low, it is unlikely that elevation of circulating endothelin during ischaemia is merely due to the formation or release of ET-1 from the myocardium. Several studies have reported increased plasma levels of thrombin and activation of the coagulation process during myocardial ischaemia (Vaziri et al., 1992). Thus, blood components, especially thrombin and other factors which are involved in the clotting cascade and may be activated during ischaemia, may play an important role in the increased release or formation of endothelin from endothelial cells of blood vessels during ischaemia and reperfusion. However, reduction of renal function or reductin in blood input into the lung during ischaemia may cause an elevated plasma levels of endothelin due to a decrease in the endothelin clearance from plasma. The rapid clearance of ET-1 from the blood by tissues as well as the heart, the firm and long lasting binding of ET-1 to myocardial binding sites, and increased cardiac binding sites of endothelin during ischaemia may help to explain why the myocardial tissue levels of ET-1 are greater that those in plasma, without needing to attribute this to de novo synthesis in the myocardium.

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