THE UNIVERSITY OF CALGARY

Cellular Basis of Angiotensin II-induced Contraction in the Rat Tail Artery: The Role of Endothelium and Endothelin-1

By

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL

FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CARDIOVASCULAR AND
RESPIRATORY SCIENCES

CALGARY, ALBERTA

September, 1999

ABSTRACT

Angiotensin II (Ang II) mediates vasoconstriction via the activation of Ang II receptors (predominantly AT_1 subtypes) on vascular smooth muscle cells, however, a role for Ang II-mediated endothelin (ET-1) release from endothelial cells has been suggested from studies using the rat tail artery preparation. In present study I investigated the contractile effects of Ang II and ET-1 in endothelium-intact and denuded ring preparations from the rat tail artery as well as freshly dispersed single smooth muscle cells and endothelial cells from the same tissue. My data indicate that: 1) The contractile effects of Ang II and ET-1 are endothelium-independent and are antagonized by losartan (10nM) and BQ-123 (1μM), the AT_1 and ET_A selective antagonists, respectively. 2) The Ang II-mediated response is mainly dependent on intracellular Ca^{2+} release, whereas that of ET-1 is primarily dependent on extracellular Ca^{2+} entry. 3) AT_1 receptors mainly distribute on smooth muscle cells in the rat tail artery. These data indicate that the contractile responses to Ang II and ET-1 utilize distinct signaling pathways and that the response to Ang II does not involve ET-1 release from the endothelium in the rat tail artery.

ACKNOWLEDGEMENTS

I sincerely thank Dr. Chris Triggle for giving me the chance to study in his lab and for always being available to help me. I thank members of my supervisory committee, Dr. R. Loutzenhiser and Dr. G. Kargacin for their guidance and encouragements, as well as their involvements as committee members.

I would like to thank the Loutzenhiser, the Walsh and the Kargacin laboratories for providing resources to help in the isolation of vascular smooth muscle and endothelial cells, RT-PCR and immunocytochemistry, respectively. Thank you to all the members in these three laboratories. I would like to thank Dr. John McGuire for reading the thesis.

Finally, I want to thank my husband, Dr. Hui Dong, for his wonderful suggestions and support.

Dedicated to my great mom, Jianglan and two lovely sons, Xiao and Jack!

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Abbreviations

Ang II: angiotensin II

ACE: angiotensin converting enzyme

ACh: acetylcholine

AT_1: Angiotensin type 1 receptor

AT_2: Angiotensin type 2 receptor

BSA: bovine serum albumin

$[Ca^{2+}]_i$: Intracellular calcium ion concentration

$[Ca^{2+}]_o$: Extracellular calcium ion concentration

CPA: cyclopiazonic acid

CICR: Ca^{2+}-induced Ca^{2+} release

DAG: Diacylglycerol

DMEM: Dubecco's modified essential medium

DMSO: Dimethylsulphoxide

EC: Endothelial cell

EDHF: Endothelium-derived hyperpolarizing factor

EDRF: Endothelium-derived relaxing factor (or NO, nitric oxide)

EGTA: Ethylenglycol bis (β-aminoethyl ether)-N,N,N'-teraacetic acid

ET-1: endothelin-1

ET_A: Endothelin type A receptor

ET_B: Endothelin type B receptor

GFR: Glomerular filtration rate

HEPES: N-2HydroxyEthylPiperazine-N'2-EthaneSulphonic Acid

IP_3: Inositol 1,4,5-trisphosphate

IP_3R: IP_3 receptor

MLCK: Myosin light chain kinase

MLCP: Myosin light chain phosphatase

Nif.: Nifedipine (L-type VGCC antagonist)

PE: Phenylephrine (α-AR agonist)

PKC: Protein kinase C

PLC: Phospholipase C

PLA_2: Phospholipase A_2

PMT: Photomultiplier tube

PRA: Plasma renin activity

ΔP: Pressure gradient

RAS: Renin angiotensin system

RPF: Renal plasma flow

R_yR: Ryanodine receptor

S.E.M.: Standard error of the mean

SR: Sarcoplsamic reticulum

TG: Thapsigargin

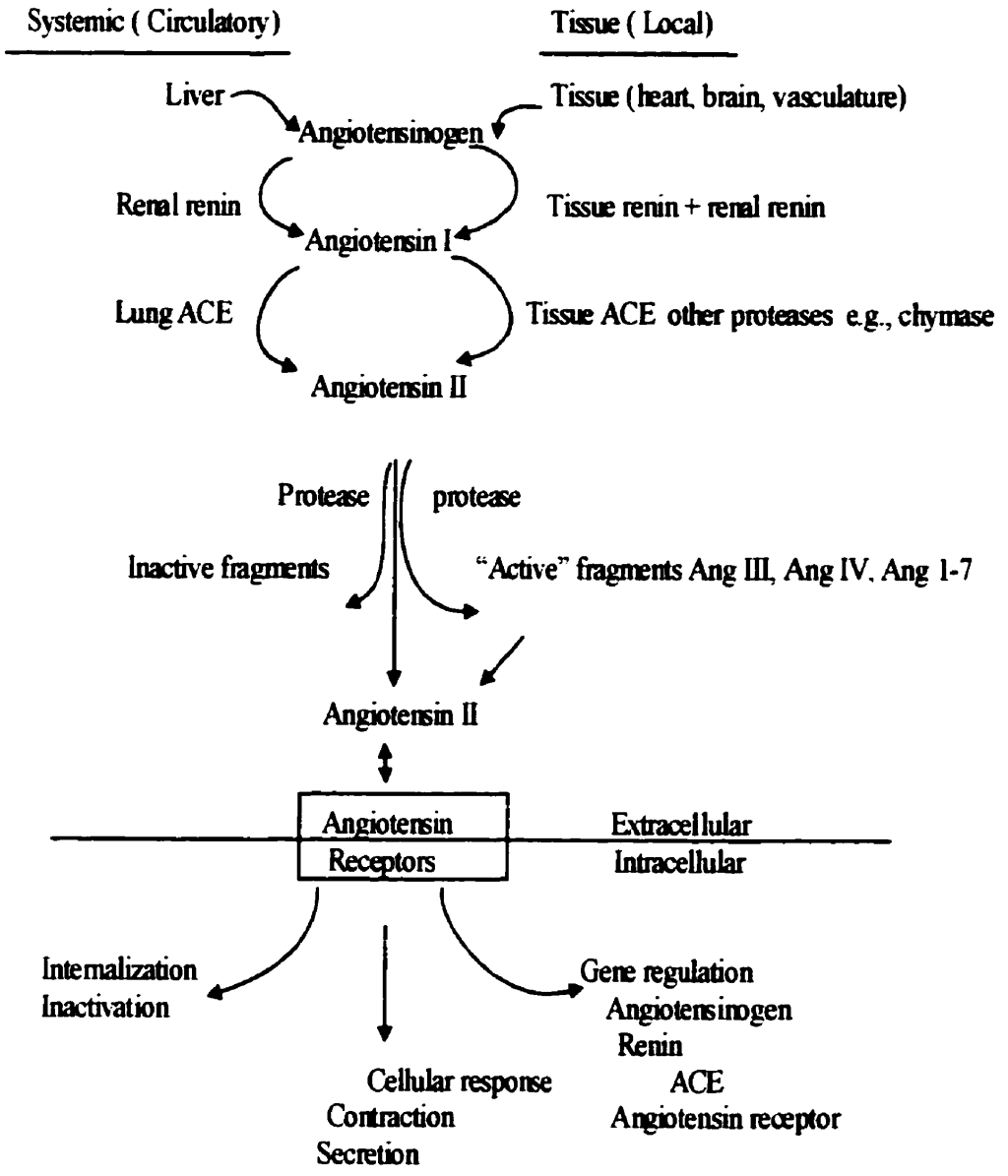
VSM: Vascular smooth muscle

Chapter One: Introduction

Part 1. Renin-angiotensin system

The renin angiotensin system (RAS) is a complex system that plays an important role in blood pressure maintenance and volume homeostasis (Peach, 1977; Inagami, 1994). This system includes the cleavage of angiotensinogen into angiotensin I (Ang I) by renin and the generation of angiotensin II (Ang II), the main effector of the system, by angiotensin converting enzyme (ACE) (Riordan, 1995). The generation of the Ang I and its cleavage into Ang II occurs in two compartments: the plasma-endothelium and the interstitium-tissue compartment (Admiraal *et al.*, 1990; Danser *et al.*, 1994). The octapeptide Ang II can be degraded into Ang III, Ang IV and Ang 1-7 (Fig1). All of them are biologically active, but debate continues over their relative importance compared to Ang II. Ang II, the dominant effector of RAS, regulates numerous physiological responses, including salt and water balance, blood pressure, and vascular tone. It also plays an important role in cardiovascular diseases, such as hypertension, atherosclerosis, and restenosis following interventional procedures (Berk and Corson, 1997). Here, I will focus on cardiovascular physiology and pathophysiology of Ang II.

Fig 1. The renin-angiotensin system. Ang II is generated by renin from angiotensinogen to angiotensin I and then by angiotensin converting enzyme from Ang I to Ang II. This diagram was derived from the one originally presented by Timmermans *et al.*, 1993.


Systemic (Circulatory)
Tissue (Local)
Liver
Tissue (heart, brain, vasculature)
Angiotensinogen
Renal renin
Tissue renin + renal renin
Angiotensin I
Lung ACE
Tissue ACE other proteases e.g., chymase
Angiotensin II
Protease
protease
Inactive fragments
"Active" fragments Ang III, Ang IV, Ang 1-7
Angiotensin II
Angiotensin
Receptors
Extracellular
Intracellular
Internalization
Inactivation
Gene regulation
Angiotensinogen
Renin
ACE
Angiotensin receptor
Cellular response
Contraction
Secretion

1.1. Physiological roles of angiotensin II (Ang II)

1.1.1. Ang II actions on vascular smooth muscle

One of the predominant physiological functions of Ang II is the regulation of blood pressure. Together with other vascular vasoactive hormones and mediators, Ang II participates in the dynamic modulation of vascular smooth muscle (VSM) tone. Ang II induces tonic contractions in isolated vascular strips, responses that are characterized by distinct periods of force development, force maintenance, and, eventually, relaxation (Morgan, 1987). Different cellular mechanisms of excitation-contraction coupling operate during these phases of the contractile cycle. Vascular tissues vary in their responses to Ang II, in that fibroelastic, conductive vessels such as the aorta exhibit weak contractile responses, whereas muscular, resistance-type vessels are more responsive (Marsden, *et al.*, 1990). Furthermore, VSM contraction in response to Ang II also differs from that elicited by other vasoconstrictors. Ang II-induced force development is generally rapid in onset, but force maintenance is not sustained (Deth & van Breemen, 1974 & 1977; Oshiro *et al.*, 1984). This is in contrast to the sustained tonic contractions produced by norepinephrine, histamine, or endothelin (St. Louis, et al., 1977; Yanagisawa et al., 1988; Takuwa & Rasmussen, 1987). Therefore, by mediating vasoconstriction, Ang II plays an important role in the control of VSM tone, and consequently in the control of human blood pressure. Ang II elicits cellular responses in all target tissues by binding to specific high-affinity cell-surface receptors. Ang II receptors and their signalling pathways will be discussed below.

1.1.2. Ang II actions in the heart

Ang II produces a positive inotropic effect in heart tissues, including those from human, dog,

rabbit, and chick (Baker & Aceto 1989; Moravec *et al.*, 1990; Ishihata & Endoh 1993). This myocardial effect has a marked age and species dependency, as Ang II evokes a negative inotropic response in cultured neonatal rat myocytes (Allen *et al.*, 1988a; Kem *et al.*, 1991). Work with isolated myocytes reveals that the inotropic response is not the result of indirect homodynamic or neural factors, but is mediated by specific high-affinity receptors on the myocyte surface (Baker *et al.*, 1984; Rogers *et al.* 1986). It has been shown that Ang II neither stimulates adenylyl cyclase nor elevates cAMP in cardiac calls (Allen *et al.*, 1988b). In fact, these studies have shown that Ang II inhibits this cascade through activation of the receptor-coupled G-protein, Gi (Allen *et al.*, 1988b). In addition, Ang II has direct effects on excitation-contraction coupling in cardiac myocytes, evoking positive inotropic and chronotropic responses in cardiac muscle (Ishihata & Endoh, 1993; Moravec *et al.*, 1990; Baker & Aceto, 1989). Ang II increases the entry of Ca^{2+} via voltage sensitive channels during the plateau phase of the action potential in atrial and ventricular myocytes. This action prolongs the plateau phase and increases the force of contraction (Garrison & Peach, 1990).

1.1.3. Ang II actions in the kidney

Not only does the kidney play an essential role in regulating the RAS, but it also serves as an important target tissue for Ang II. The physiological role of Ang II is to maintain extracellular fluid volume and blood pressure under conditions of volume loss or salt deprivation (Garrison & Peach, 1990). The contractile actions of Ang II on VSM serve to increase peripheral vascular resistance and thus, mean arterial pressure. The renal vasculature is highly sensitive to Ang II, although some heterogeneity of responses has been noted within

renal vascular beds (Arendshorst *et al.*, 1999). Intrarenal infusion of low dose Ang II elicits an increase in renal vascular resistance (Hsu, *et al.*, 1980) and consequently leads to a decrease in renal plasma flow (RPF) and, to a lesser degree, in glomerular filtration rate (GFR) (Carriere, *et al*, 1969; Fagard *et al.* 1976; Lohmeier *et al*, 1977). Since the decrease in RPF tends to be proportionately greater than the change in GFR, the filtration fraction (FF) increases (Fagard *et al.*, 1976; Lohmeier *et al*, 1977). Micropuncture studies have shown that Ang II has greater selectivity for efferent, rather than afferent glomerular arterioles (Myers *et al.*, 1975). The disproportionate increase in efferent arteriolar resistance leads to a marked increase in glomerular capillary hydraulic pressure and, therefore, an increase in the transcapillary hydraulic pressure gradient (ΔP) favouring filtration. The increase in ΔP, in turn, accounts for the increase in FF (Marsden *et al.*, 1990).

1.1.4. Ang II actions in regulation of blood pressure

As discussed above, Ang II has multiple actions in the cardiovascular system, including direct stimulation of vascular and cardiac muscle, indicating that Ang II plays an important role in regulation of blood pressure (Marsden *et al.*, 1990). Indeed, modest changes in the plasma concentration of Ang II increase blood pressure as a result of the direct cardiovascular actions of the peptide, these actions may account, at least in part, for the role of Ang II in hypertension (Garrison and Peach, 1990). On a molar basis Ang II is about 40 times more potent as a vasoconstrictor than norepinephrine. When a single moderate dose is injected intravenously, systemic blood pressure begins to rise within about 10 seconds, rapidly reaches a maximum, and then returns to control levels within a few minutes. When

the drug is infused continuously, blood pressure is maintained at an elevated level for hours or days. Ang II commonly causes a moderate rise in pulmonary arterial pressure that is due less, perhaps, to its feeble pulmonary vasoconstrictor action than to an increase of pressure in the pulmonary vein as end-diastolic pressure rises (Garrison and Peach, 1990).

1.2. Pathophysiological roles of angiotensin II

Ang II was isolated by virtue of its vasoconstrictior abilities and has long been thought to play a critical role in hypertension. Recent studies indicate that Ang II stimulates vascular smooth muscle cell (VSMC) growth, increases the expression of enzymes that produce mediators of inflammation, and activates gene transcription of proto-oncogenes, such as c-fos. These data suggest that Ang II plays an important role in various cardiovascular diseases associated with VSMC growth and vessel wall inflammation, such as hypertension, atherosclerosis, and congestive heart failure (Gibbons, 1998). Clinical trials with ACE inhibitors demonstrating survival benefits in hypertension, congestive heart failure (SOLVD investigators, 1991), and myocardial infarction (Pfeffer *et al.*, 1992) support the importance of Ang II in the pathogenesis of cardiovascular diseases.

1.2.1. Hypertension

The RAS contributes to hypertension of diverse etiologies. The role of Ang II in maintaining blood pressure is most clearly understood in patients with malignant hypertension or with hypertension resulting from stenosis of the renal artery, where plasma renin activity (PRA) is usually elevated (Garrison and Peach, 1990). Inhibitors of the RAS (either receptor antagonists or ACE inhibitors) commonly cause a marked fall in blood pressure in such individuals (Timmermans, *et al.*, 1993). However, there is no simple relationship between

excess secretion of renin (as reflected by PRA) and blood pressure in the large population of individuals with essential hypertension. Indeed, most patients with hypertension do not have high PRA, and this suggests that the RAS plays a limited role in the etiology of hypertension. The fact that ACE inhibitors lower blood pressure in a large fraction of hypertensive patients with normal PRA indicates that the RAS system is supporting the blood pressure in these individuals (Garrison & Peach, 1990).

1.2.2. Cardiac fibrosis

The RAS has been liked to the pathogenesis of cardiac fibrosis through the use of experimental animal models and by clinical studies (for details see review by Brecher, 1996). Cardiac fibrosis is characterized by fibroblast proliferation and concomitant deposition of extracellular matrix. These cellular events are frequently associated with myocyte hypertrophy or necrosis, inflammation, and microvascular changes. A relationship between cardiac fibrosis and Ang II was indicated 25 years ago when it was shown that cardiac fibrosis and renal damage were found in rabbits given relatively large amounts of Ang II (Gavras *et al.*, 1971). Subsequent studies (Giacomelli *et al.*, 1976) documenting the toxic effects in the microvasculature of the rat heart that occurred only 4 hours after the intravenous administration of a large dose of Ang II reinforced the concept that vascular changes provoked cardiac ischemic injury and repair, and that such effects could be produced by changes in the RAS. Direct interactions of Ang II with the cardiac fibroblast and myocyte have been described only recently. The presence of Ang II receptors on rat cardiac

fibroblasts is now established (Sadoshima & Izumo *et al.*, 1993, Crabos *et al.*, 1994). These receptors, predominately of the AT_1 type, mediate a mitogenic response, increase protein synthesis, and induce extracellular matrix gene expression. Ang II also has been shown to facilitate the contraction of collagen gels by cardiac fibroblasts, and influence integrin expression (Burgess *et al.*, 1994). Such effects provide evidence of an important role *in vivo* for Ang II in the modulation of fibroblast function during both normal and pathological conditions. Ang II also may act directly on cardiac myocytes to influence cell growth. Ang II increased spontaneous contractile frequency and stimulated calcium currents in cultured neonatal rat heart myocytes (Allen *et al.*, 1988a) and stimulated protein synthesis and cell growth in embryonic chick myocytes (Baker & Aceto, 1990).

1.2.3. Congestive heart failure

An activated RAS is a major risk factor for cardiovascular events, including congestive heart failure. Stimulation of AT_1 receptors is associated with endothelial dysfunction, mainly as the consequence of an increased vascular production of superoxide radicals, vasoconstriction, platelet activation, enhanced release of plasminogen activator inhibitor-1, activation of immediate early genes c-fos and c-jun, myocyte hypertrophy, connective tissue formation, endothelin-1 synthesis, and activation of growth factors like PDGF and TGF-β_1. Stimulation of AT_2 receptors can mitigate or abolish the growth promoting effects of AT_1 receptor stimulation (Dietz *et al.*, 1998). The contribution of these effects, single or in combination, on the progression of atherosclerotic lesions, the phenomenon of restenosis and the process

of remodelling in heart failure is being progressively elucidated (Dietz *et al.*, 1998). Although Ang II has been implicated in the pathophysiology of congestive heart failure, its temporal and regional changes during the development and progression of the disease are poorly defined. Luchner *et al.* (1996) has provided new insights into the temporal and regional alterations in Ang II during the progression of experimental congestive heart failure. They found that early increases in local renal, myocardial, and vascular Ang II do not occur in the experimental congestive heart failure of early left ventricular dysfunction and may even be suppressed. In contrast, increased myocardial and particularly renal Ang II in association with increased circulating Ang II are hallmarks of overt experimental congestive heart failure. ACE inhibitors have been shown to be effective in improving symptoms and survival in patients with systolic left ventricular dysfunction (Pinto *et al.*, 1999). Blockade of the effects of Ang II can, however, also be achieved with an AT_1 blocking agent such as losartan (Pitt, 1995). Taken together, all of these results indicate that RAS plays an important role in the pathophysiology of congestive heart failure.

Part 2. Angiotensin II receptors and distribution

Ang II elicits cellular responses in all targets tissues by binding to specific high affinity cell surface receptors (Timmermans *et al.*, 1993; Ardaillou, 1999). A major development in Ang II signalling studies has been the recent identification of multiple receptor subtypes for the hormone. Definitive evidence for receptor subtype heterogeneity is based principally on

ligand-binding studies with new nonpeptide Ang II antagonists (Timmermans *et al.*, 1993). It has been generally accepted that the actions of Ang II are mediated by two pharmacologically and biochemically distinct classes of receptors, AT_1 and AT_2 (de Gasparo *et al.*, 1995). Both receptor subtypes have been successfully cloned (Murphy *et al.*, 1991; Sasaki *et al*, 1991; Mukoyama, 1993). Losartan (DuP 735) has nanomolar affinity for AT_1 whereas antagonists such as PD 123319 and CGP 4221122A ($K_d > 10\ \mu M$) have much lower affinity. AT_2 is characterized by a reverse rank order of affinity for these antagonists (Timmermans *et al.*, 1993, de Gasparo *et al.*, 1995). The signal transduction pathway for AT_1 is mediated via an increase in intracellular Ca^{2+} and activation of protein kinase C, while that for the AT_2 is still unknown. There are two subtypes of AT_1, namely AT_{1A} and AT_{1B}. They are encoded by two different genes in the rat (Lewis *et al.*, 1993), but have similar amino acid sequences, pharmacological specificities and signal transduction pathways (Chiu *et al.*, 1993).

AT_1 and AT_2 exhibit a widespread central and peripheral distribution. AT_1 is responsible for most of the well known cardiovascular actions of Ang II, however, the function of the AT_2 is less well understood. Vasoconstriction is mediated mainly by the AT_1 on vascular smooth muscle cells. Nonetheless, a few vascular sites such as ovine mammary artery, rat cerebral artery, and rat uterine artery are also reported to contain AT_2 (Cox *et al.*, 1993; Burrell & Lumbers, 1997; Tsutsumi & Saavedra, 1991 a and b; Zwart *et al.*, 1998). More recently, cultured endothelial cells have been reported to contain Ang II receptors (Feener *et al.*, 1995; Patel *et al.*, 1989; Pueyo *et al.*, 1996; Stoll *et al.*, Vaughan *et al.*, 1995), but the nature of the subtypes of the receptor appear to be rather heterogenous. The presence of different types

of Ang II receptors on endothelial cells may also depend on the animal species.

2.1. AT_1 receptor

According to the Ang II receptor nomenclature proposed by the nomenclature committee established by the American Heart Association Council for High Blood Pressure Research (Bumpus *et. al.*, 1991), AT_1 would be designated for the Ang II receptor subtype inhibited by losartan and DTT that mediates the pressor effects of Ang II. The committee also recognised the subdivision of the recognized type into AT_{1A}-AT_{1B}. Although other nomenclatures have been proposed, almost all investigators have accepted the proposal (Table 1).

Physiological and pharmacological studies with receptor subtype selective blockers have revealed that the known biologic actions of Ang II are exclusively mediated by AT_1 receptors. At present, the AT_1 has been found to mediate all of the known actions of the hormone in vascular tissue, including pressor activity, Ca^{2+} mobilization, phospholipase C (PLC) activation, and VSMC contraction (Smith *et al.*, 1992, Timmermans et al., 1993). These results are consistent with the observation that AT_1 sites predominate (>80%) in VSMC (Viswanathan *et al.*, 1991).

The AT_1 gene (cDNA) encodes a 359-amino acid protein with a structure typical of seven transmembrane G protein-coupled receptors. There are two highly homologous subtypes of AT_1: termed the AT_{1A} and AT_{1B} (de Gasparo, 1995), respectively, in rodents. Both AT_1 isoforms share 94% identical amino acid sequence, with only 60% identical in the 5' and 3' untranslated regions. In the rat AT_1, the AT_{1A} gene is localized on chromosome 17 and the AT_{1B} gene on chromosome 2. However, in humans there is only one AT_1 gene that is located

Table 1. Distribution and pharmacology of angiotensin receptors AT_1 and AT_2

Receptor Type	Location	Potency Order	Selective Agonist	Selective antagonist
AT_1				
Subtype AT_{1A}	Mainly in lung, vascular smooth muscle cells, liver, brain, kidney	Ang II *Ki* ≤ 1 nmol/L Ang III *Ki*≈ 30nmol/L	None	*Ki* ≤ 50nmol/L for losartan, valsartan, and other related compounds *Ki* > 0.5 μmol/L for CGP 42112, PD 123177, PD 123319
Subtype AT_{1B}	Mainly in adrenals, pituitary gland	Same as AT_{1A}	None	Same as AT_{1A}
AT_2				
Subtype ?	Mainly in fetal tissues, brain, reproductive tissues	Ang II *Ki* ≤ 1 nmol/L Ang III *Ki* < 10 nmol/L	CGP 42112	*Ki* < 10 nmol/L for PD 123319 *Ki* > 10 μmol/L for losartan, valsartan, and other related compounds

Subtypes AT_{1A} and AT_{1B} have been described in rat and mouse but not in bovine and human. For both subtypes, suitable radioligands are [^{3}H]losartan, [^{125}I]EXP 985, and [^{3}H]SK&F 108566. [^{125}I]CGP 42112 is a suitable radioligand for AT_2. For more detail see de Gasparo, *et al.*, 1995.

on chromosome 3. AT_{1A} is expressed predominantly in the VSM, liver, lung, and kidney, whereas AT_{1B} occurs mainly in the adrenal and anterior pituitary (Allen *et al*, 1999). The major sites expressing AT_1 or AT_2 are shown in Table 2.

2.2. AT_2 receptor

The Ang II receptor which is inhibited by the nonpeptide PD123117 and its structural analog was designated as the AT_2 by the nomenclature committee (de Gasparo, 1995). The AT_2 is also inhibited by the peptidic analog CG42112A and potentiated by DTT. Although there have been reports of functional activity mediated through AT_2, its physiological role is yet to be defined. The function of the AT_2 remains of interest because of its differential distribution and enhanced expression in wound healing (Horiuchi *et al.*, 1999).

The AT_2 gene, initially cloned from rat fetus and rat pheochromocytoma cells, encodes a 363-amino acid protein, which has only 32 to 34% amino acid identity with the AT_{1A} protein. Although the AT_2 is also a seven transmembrane G protein-coupled receptor, its mode of signal transduction is uncertain. AT_2 activation results in growth inhibition and promotion of apoptosis associated with inhibition of MAP kinases, such as ERK ½, probably via activation of phosphotyrosine phosphatases (Horiuchi *et al.*, 1998). The AT_2 gene is located on chromosome X. Northern blot analysis shows that AT_2 receptors are expressed predominately in adrenal medulla, myometrium, and myocardium in the adult and in the fetal mesenchyme (Table 2).

AT_2 which has a widespread distribution and is predominant in fetal tissue, is believed to play an important role in cell proliferation and growth. However, mice with a disrupted AT_2 gene appear to develop and grow normally with no abnormal morphology observed in the

Table 2. Major sites expressing AT_1 or AT_2 in the adults mammal[a]

Site	AT_1	AT_2
Kidney		
Glomeruli	+	-
Proximal tubules	+	-
Vasculature	+	+
Medullary interstitial cells	+	-
Adrenal gland		
Cortex	+	-
Medullar	+	+
Heart		
Myocardium	+	+
Ganglia	+	-
Conduction system	+	-
Brain		
Circumventricular organs	+	-
Thalamus	+	+
Basal ganglia	+	-
Cerebellar cortex	+	+
Medulla oblongata	+	-

[a] The data are descriptive and no attempt has been made to indicate relative receptor densities.

For more information, please check Allen, *et al.*, 1999.

brain, blood vessels, heart, or kidney. However, AT_2 appears to have a functional role in blood pressure control, because deletion of this gene leads to a rise in basal arterial blood pressure and an enhanced pressor response to Ang II in vivo (Hein *et al.*, 1995; Ichiki *et al.*, 1995). Evidence accumulated thus far suggests that the major function of AT_2 receptors may involve functional antagonism of the vasoconstrictor action of AT_1 receptors by modulating the vascular sensitivity to Ang II, inhibiting the proliferative and growth-promoting effects of AT_1, or mediating programmed cell death.

2.3. Distribution of the AT_1 and AT_2 receptors

Tissue distribution of Ang II receptor subtypes in the adults was studied by autoradiography after administration of radiolabeled Ang II or by in situ hybridization. AT_1 is present in all of the organs in which physiological effects of Ang II have been demonstrated. Only the AT_1 is present in lungs, liver, and placenta. It is predominant in kidneys, adrenals, heart, and aorta.

AT_1 and AT_2 are equally distributed in the brain and uterus. The AT_2 is predominant in ovaries, medullary adrenals, and pancreas (Chiu *et al.*, 1989, de Gasparo *et al.*, 1990; Chang & Lotti, 1991). This distribution is characteristic of the rat but there are species differences. For example, the distribution of AT_1 and AT_2 in blood vessels is highly site- and species-specific. In vascular smooth muscle cells from the rat or rabbit aorta and mesenteric arteries AT_1 blockers completely inhibit binding of Ang II, and block Ang II-stimulated phosphoinositide turnover and proto-oncongene expression in vascular smooth muscle cells, whereas AT_2 blockers have no effect (Timmermans *et al.*, 1993). In contrast, in rat anterior cerebral arteries, the AT_2 appears to predominate (Tsutsumi & Saavedra, 1991a). The

distribution of AT_{1A} versus AT_{1B} also differs. Only the AT_{1A} is found in the liver. It is predominant in SMCs, kidney, and lungs, whereas the AT_{1B} receptor is mainly found in pituitary, adrenals, and uterus (Kakar *et al.*, 1992; Iwai & Inagami, 1992). In the rat kidney mesangial cells equal fractions of AT_{1A} and AT_{1B} are expressed (Chansel *et al.*, 1996).

Part 3. Ang II signal transduction pathway

The effects of Ang II are exerted through specific receptors on cell surfaces, which can be selectively blocked by peptide and nonpeptide antagonists. Ang II receptors are coupled to effector system via guanine nucleotide-binding regulatory protein (G protein). To date, the signal transduction pathway(s) activated by the AT_2 remains uncertain and thus the vast majority of literature focuses on AT_1 signalling. Recent findings demonstrate that AT_1 couples to many intracellular signal transduction events. The signal transduction events stimulated by Ang II are similar to those stimulated by growth factors and cytokines and include activation of PLC (Griendling, *et al.*, 1986), calcium mobilization (Brock, *et al.*, 1985), activation of PKC (Griendling, *et al.*, 1989), induction of proto-oncogenes (Taubman, *et al.*, 1989), protein tyrosine phosphorylation (Huckle, *et al.*, 1990; Molloy, *et al.*, 1993) and activation of the 42- and 44-kD MAP kinases (Duff, *et al.*, 1992; Tsuda, *et al.*, 1992).

3.1. G proteins

As mentioned above, Ang II receptors belong to the family of G protein-coupled receptors (GPCR). G proteins are made up of three polypeptides: termed α, β and γ subunits. The α subunit is able to bind GTP and possesses GTPase activity (Gilman, 1987). The β and γ

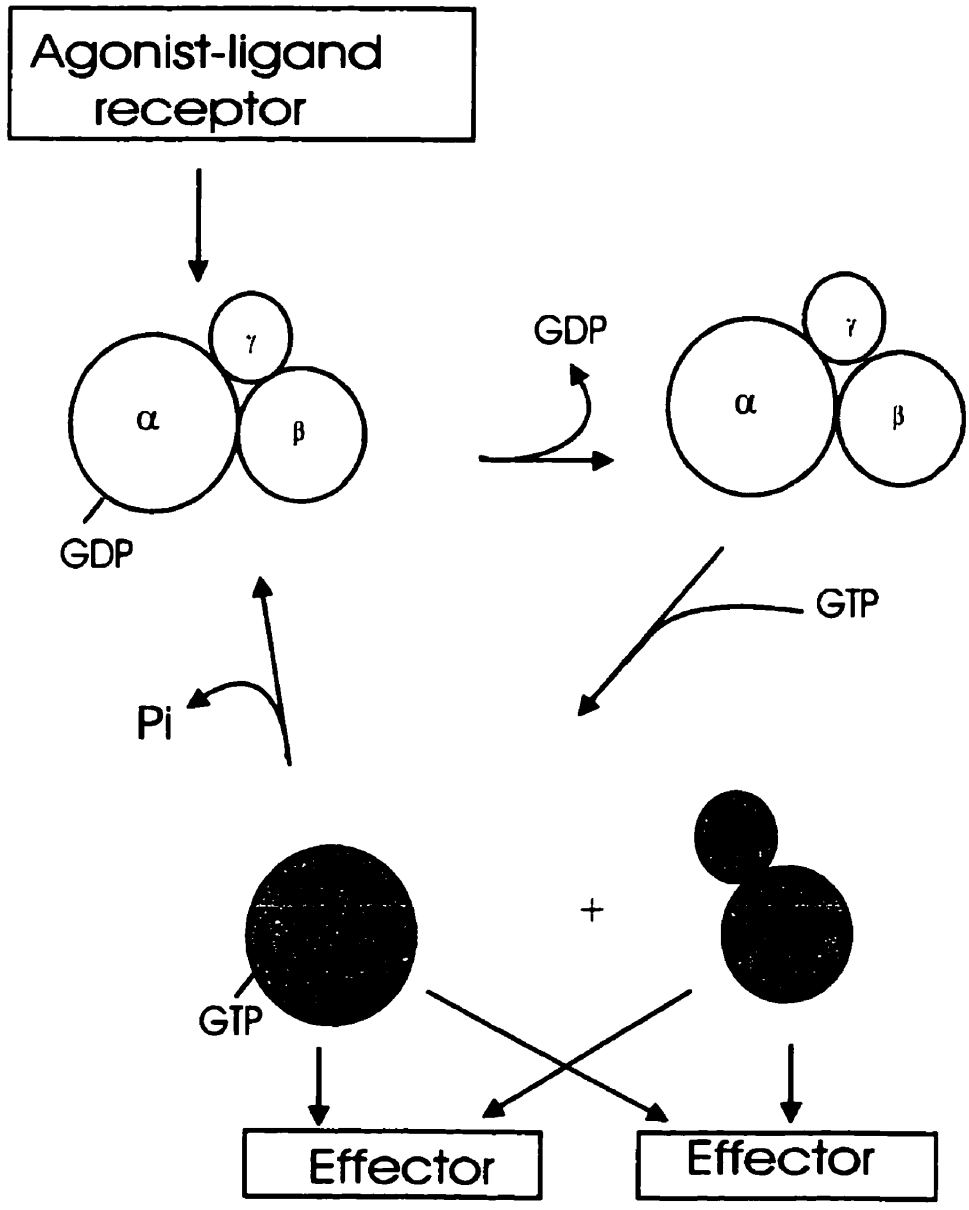
subunits form a dimer that only dissociates when it is denatured and is, therefore, functionally a monomer (Gilman, 1987; Clapham & Neer, 1993; Neer, 1994). Not only have about 20 isoforms of the α subunit been characterized (Hepler & Gilman, 1992), but also five isoforms of the β and twelve isoforms of the γ subunit have been characterized (Clapham & Neer, 1997). Figure 2 illustrates the cycle of G protein activation and deactivation that transmits the signal from receptor to effectors. In the presence of GDP-bound, heterotrimeric G proteins, Ang II receptors assume a high affinity state, which is dependent on divalent cations. Binding of Ang II to the high-affinity receptor initiates signal transduction by prompting the exchange of GDP for GTP on the α-subunit of G proteins. In turn, the association of GTP with the α-subunit of G protein lowers its affinity for the receptor-Ang II. Intrinsic α-subunit GTPase activity cleaves inorganic phosphate from GTP; this activity returns the G-protein to its inactive, GDP-associated state and returns the Ang II receptor to the high-affinity state. In different target cells, the AT_1 is coupled to different G proteins. Based on that whether the α subunit is able to be ADP-ribosylated by pertussis toxin (PTX), the G-proteins are divided into two groups, PTX-sensitive, e.g., G_i, G_o and G_t, and PTX-insensitive, e.g., G_q. The rat AT_{1A} has been shown to couple with three distinct G proteins: G_i, G_o and G_q (Gilman, 1987; Shirai, *et al.*, 1995).

3. 2. PLC/IP_3/Ca^{2+}

3.2.1. PLC

The phospholipase C (PLC) is the effector that directly produces the second messengers Ins (1,4,5)P_3 (IP_3) and diacyglycerol (DAG) though hydrolysis of phosphatidyl 4,5-bisphosphate (PIP_2). There are almost 20 isozymes of PLC, which are divided into three types, β, γ and

Fig 2. G-protein interaction with the Ang II receptor and effector. Binding of Ang II to receptor in the high-affinity state results in the exchange of bound GDP for GTP on the α-subunit. The activated α-GTP subunit dissociates from βγ-subunits, and one or both interact with an effector. When G-protein subunits are in the dissociated state, the Ang II receptor assumes a low-affinity state, and the bound Ang II released from the receptor. The cascade of activation ends with the hydrolysis of bound GTP to GDP, thereby freeing inorganic phosphate (Pi). GTPase activity is intrinsic to the α-subunit. The βγ-subunits reassociate with α-GDP subunits to form the inactive heterotrimer, and the Ang II receptor returns to the high-affinity state.


Agonist-ligand receptor
γ
α
β
GDP
GDP
γ
α
β
GTP
Pi
+
GTP
Effector
Effector

δ. Each type of PLC has its subtypes. All the isozymes of PLC have two regions with high homology, designated X and Y, which are the catalytic domains (Meldrum *et al.*, 1991: Lee and Rhee, 1995). They also have a pleckstrin homology (PH) domain in the N-terminal region. Pleckstrin homology (PH) domains are present in over one hundred signaling molecules, where they are thought to mediate membrane targeting by binding to phosphoinositides. All the isozymes are dependent on Ca^{2+}, and the Ca^{2+}-binding site is located in the C-terminal half of the Y region. Activation of PLC is the signalling pathway found in most cell types and results in many of the responses to Ang II in several tissues. It is one of the earliest events in AT_1-mediated signal transduction in vascular smooth muscle cells (VSMCs). However, the nature of the coupling mechanisms of AT_1 to PLC is controversial because both tyrosine phosphorylation of PLC-γ and G protein-dependent PLC-β activation pathways have been reported (Marrero, *et al.*, 1996). Ang II activates PLC-β related to early IP_3 formation (at 15'), but activation of PLC-γ has been related to later IP_3 formation (after 30') (Ushio-Fukai, *et al.*, 1998).

3.2.2. IP_3 and DAG

When Ang II binds to its receptor, PIP_2 is hydrolyzed to liberate IP_3 and DAG. Once IP_3 has been released from the membrane, it diffuses into the cytosol where it binds to specific receptors located on the intracellular stores. A family of IP_3 receptors has been identified. Upon binding IP_3, the IP_3 receptor undergoes a large conformational change leading to channel opening and Ca^{2+} release (Mignery & Südhof, 1990). Phosphoinositide hydrolysis is a critical component of Ang II-induced activation of VSM contraction. IP_3 formation is

detectable within 15 sec of Ang II exposure, but cytosolic levels fall rapidly after the initial stimulus. This PLC-catalyzed process releases water-soluble IP_3 from the membrane, which in turn, stimulates Ca^{2+} release from intracellular sarcoplasmic reticulum. IP_3 is a precursor for inositol 1,3,4,5-tetrakisphosphate, which may be involved in the slower process of Ca^{2+} entry across the plasma membrane. External Ca^{2+} is not required for the release of IP_3 (Alexander *et al.*, 1985), and the calcium ionophore A 23187 has no significant effect on IP_3 formation in VSMCs (Griendling *et al.*, 1986). IP_3 has been shown to stimulate Ca^{2+} release from non-mitochondrial organelles in permeabilized VSMCs (Suematsu et al., 1984). These findings strongly suggest that IP_3 serves as the second messenger that stimulates intracellular Ca^{2+} release in response to Ang II.

Another product of hormone-sensitive PLC, namely, *sn*-1,2-diacylglycerol (DAG), remains membrane-associated and, along with phosphatidylserine, activates membrane-bound PKC. DAG, the second important messenger generated in VSMC upon stimulation with Ang II, is detectable within 5 sec, but in contrast to IP_3, levels typically remain elevated for longer than 20 min. (Griendling *et al.*, 1989) The immediate rise in DAG is thought to reflect hydrolysis of PIP and PIP_2, whereas DAG released beyond 5 min thought to be derived from PI. Stimulation of PKC by phorbol esters is associated with inhibition of the Ang II-stimulated rise in intracellular Ca^{2+} as well as IP_3 formation and Ca^{2+} efflux. Since phorbol 12-myristate 13-acetate (PMA), which is a PKC activator, does not modify Ang II receptor density or affinity in intact cells, the data suggest that PKC may modulate Ang II modulate receptor coupling to PLC in VSMCs.

3.2.3. Calcium homeostasis

Intracellular Ca^{2+} is well known as the key second messenger regulating a variety of important cellular functions such as muscle contraction, cell motility, exocytotic secretion, electrical excitability, glucose metabolism, cell division and proliferation, and fertilization (Berridge, 1993). An increase in $[Ca^{2+}]_i$ is due to either Ca^{2+} entry from outside the cell across the plasma membrane (Ca^{2+} influx) or Ca^{2+} release from intracellular Ca^{2+} stores, which are generally accepted to be the endoplsamic reticulum (ER) or sarcoplasmic reticulum (SR). Intracellular Ca^{2+} is restored by means of Ca^{2+} pump and Na^{+}-Ca^{2+} exchanger in the plasma membrane and Ca^{2+} pump in the ER or SR membrane.

The two major Ca^{2+} release channels in the intracellular Ca^{2+} store (ER or SR) are the Ins (1,4,5)P_3 receptor (IP_3R)-channel which mediates IP_3-induced Ca^{2+} release (IICR) from the IP_3-sensitive store and the ryanodine receptor(RyR)-channel which mediates Ca^{2+}-induced Ca^{2+} release (CICR) from ryanodine-sensitive store. The two receptors have fragment homology in the amino acid sequence and have the similar macromolecular structure (Takeshima *et al.*, 1989). The distribution of IP_3- or ryanodine-sensitive Ca^{2+} stores varies with cell type. These two stores may interact with each other to generate Ca^{2+} signals.

Morgan and Morgan (Morgan & Morgan, 1982) first demonstrated concomitant VSM contraction and an increase in intracellular Ca^{2+} concentration in response to Ang II in aequorin-loaded *Amphiuma* aorta. Experiments with Quin-2 have confirmed findings of Ang II-induced Ca^{2+} transients in VSMCs (Capponi *et al.*, 1985), and have permitted microfluorometric measurements of individual cell responses. Such studies demonstrate

marked cell-to-cell heterogeneity of the intracellular Ca^{2+} response, with differences in the rate of rise and peak intracellular Ca^{2+} concentrations. Isotopic $^{45}Ca^{2+}$ flux studies have helped clarity the role for external Ca^{2+} in Ang II signalling. $^{45}Ca^{2+}$ efflux from VSMC resolves into fast and slow components, both of which are increased by Ang II, although the effect of Ang II on the initial rapid rate is most pronounced. The initial efflux rate is increased 10-fold above control in response to Ang II and is not altered by the removal of extracellular Ca^{2+}. In the presence of Ang II, the rate of the slow efflux component also remains elevated above control values for prolonged periods of time. Furthermore, $^{45}Ca^{2+}$ influx into cells is not stimulated during the first 60 sec of exposure to Ang II, whereas uptake rates at later time points are increased. Taken together with the data derived from cytosolic Ca^{2+} determinations with fluorescent probes, the finding suggest that late Ca^{2+} flux stimulated by Ang II is mediated, at least in part, by uptake of Ca^{2+} across the plasma membrane (Berk, *et al.*,1987; Smith & Smith, 1987; Brock, *et al.*, 1985).

The mechanisms whereby the cytosolic Ca^{2+} concentration is bought back to baseline following stimulation with Ang II depend on Ca^{2+} efflux pathways, intracellular buffering, and reuptake of Ca^{2+} into intracellular organelles. Movement of Ca^{2+} across membranes of intracellular organelles is mediated by sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPases (SERCA). Buffering of Ca^{2+} by intracellular proteins also reduces free cytosolic Ca^{2+} concentrations, as does extrusion of Ca^{2+} across the plasma membrane via the plasmic membrane Ca^{2+}-ATPase (PMCA) and via the Na^{+}-Ca^{2+} antiporter.

3.2.3.1. Calcium release

In canine mesenteric artery cells (Satoh *et al.*, 1987), Ang II induced a transient increase in $[Ca^{2+}]_i$. Contraction induced by Ang II was short-lasting. After initial exposure to Ang II, subsequently applied Ang II generated small contractions. In Ca^{2+}-free solution, Ang II also induced a transient contraction. Ang II-induced Ca^{2+} release accompanied IP_3 and DAG production as described above. The release of IP_3 mobilizes Ca^{2+} from intracellular stores, whereas DAG, in concert with cellular Ca^{2+}, activates protein kinase C (PKC). PKC may play a central role in phosphorylation of cellular proteins, including Ca^{2+} channels (Gutierrez *et al.*, 1994). However, Morel *et al.* (1996) reported that Ang II-evoked Ca^{2+} responses in the rat portal vein myocytes were abolished in the absence of external Ca^{2+} and in the presence of a L-type calcium channel blocker. Furthermore, intracellular applications of the IP_3 receptor antagonist, heparin or an anti-PdtIns antibody did not modify Ang II-induced Ca^{2+} responses. Therefore, their results indicate that Ang II releases Ca^{2+} from intracellular stores of the rat portal vein myocytes via a Ca^{2+} release mechanism activated by Ca^{2+} influx through L-type Ca^{2+} channels and that the IP_3 receptor is not involved in Ang II-mediated intracellular Ca^{2+} release.

3.2.3.2. Calcium entry

Ang II induced not only Ca^{2+} release but also Ca^{2+} influx. When injected into the renal artery of Wistar-Kyoto rats, Ang II produced a transient 30-50% decrease in renal blood flow without affecting arterial pressure. Nifedipine exerted maximum inhibition by blocking 50% of the peak Ang II response. This observation indicates that one-half of the Ang II-induced constriction of renal resistance vessels is mediated by voltage-dependent L-type calcium

channels (Ruan and Arendshorst, 1996). This is consistent with the finding that only in the afferent arteriole Ang II-induced response does contribute the activation of L-type, voltage-gated Ca^{2+} entry channels (Arendshorst, 1999).

In isolated rat renal arteries, Ang II caused sustained increase in $[Ca^{2+}]_i$. With diltiazem, a L-type calcium channel blocker in the bathing media, Ang II caused a transient increase in $[Ca^{2+}]_i$ in afferent arterioles but only a sustained increase in efferent arterioles. In low-Ca^{2+}-EGTA media, Ang II elicited a transient increase in $[Ca^{2+}]_i$ in both arterioles (Conger *et al.*, 1993). Seki *et al.* (1999) studied the effects of Ang II on the Ca^{2+}-channel currents of a cultured rat aortic smooth muscle cell line, A7r5, by using whole-cell voltage clamp recording. Application of Ang II significantly increased the amplitude of Ba^{2+} currents through voltage-gated Ca^{2+} channels (I_{Ba}). In the presence of lavendustin-A, a selective inhibitor of tyrosine protein kinase (Tyr-PK), Ang II failed to stimulate I_{Ba}. Ang II stimulation of I_{Ba} was also prevented by LY-294002, an inhibitor of phosphatidylinositol-3-kinase (PI-3-K) (Seki, *et al.*, 1999). These results suggest that Ang II may activate VSMC Ca^{2+} channels through Tyr-PK and PI-3-K. In human coronary smooth muscle, short-term treatment of nitrendipine had no significant effect on basal or stimulated $[Ca^{2+}]_i$, but after a 24 hr incubation decreased basal $[Ca^{2+}]_i$, attenuated the plateau phase of Ang II-evoked $[Ca^{2+}]_i$ transients, and reduced proliferative activity of these cells (Kruse, *et al.*, 1994; Hafizi, *et al.*, 1998). These findings indicate that Ang II elevates intracellular Ca^{2+} both through the L-type Ca^{2+} channels and Ca^{2+} release.

3.3. Role of tyrosine kinases

The signal transduction events stimulated by Ang II binding to the AT_1 are similar to those stimulated by growth factors and cytokines and include activation of PLC, calcium mobilization, activation of PKC, induction of protooncogenes, stimulation of protein tyrosine phosphorylation and activation of the 42- and 44-kD MAP kinases. However, unlike growth factor receptors such as the epidermal growth factor receptor (EGFR), the AT_1 does not have intrinsic tyrosine kinase activity (Berk & Corson, 1997). Furthermore, it has become clear that many Ang II effects require tyrosine phosphorylation as shown by studies with tyrosine kinase inhibitors. For example, genistein, a tyrosine kinase inhibitor, blocks Ang II-stimulated mitogen-activated protein (MAP) kinase (Liao, *et al.*, 1996) and c-Jun N-terminal kinase (JNK) activity (Zohn, *et al.*, 1995), vessel contraction (Hollenberg, 1994), and protein synthesis (Leduc, *et al.*, 1995).

Ang II binding to the AT_1 receptor results in stimulation of the phosphoinositide-specific PLC to hydrolyze PIP_2, thereby generating the second messengers IP_3 and DAG. As discussed above, PLC is a family of at least three related genes: PLC-β, PLC-γ, and PLC-δ (Rhee *et al.*, 1992). The PLC-γ isoforms are regulated by tyrosine phosphorylation (Kim *et al.*, 1991; Marrero *et al.*, 1994 & 1995; Bolen *et al.*, 1992). Those responses were completely inhibited by losartan, indicating that it is the AT_1 receptor that activates PLC-γ. G protein coupled receptors usually activate the PLC-β isoforms (Rhee & Bae, 1997). Thus the discovery that Ang II activates PLC-γ via tyrosine phosphorylation, just like a typical growth factor, appears paradoxical (Marrero *et al.*, 1994). A recent study by Ushio-Fukai *et al.*(1998) in cultured rat VSMCs demonstrates that Ang II couples sequentially, first to PLC-

β (0-30 seconds), though Gβγ as well as two different Gα (αq11 and α12) subunits; and second to PLC-γ (30s-minutes), the last involvement is PKC (minutes to hours). This alternative signalling pathway may regulate sustained vascular smooth muscle contraction (Epstein *et al.,* 1997). However, not every tissue possesses this alternative signalling pathway, like human VSMC, neither anti PLC-γ antibodies, nor incubation with genistein or herbimycin A had any effect on IP_3 formation or the calcium signal and PLC-β is the isoform that is critical for Ang II-regulated PLC signalling in these cells (Schelling *et al.,* 1997).

Two mechanisms for activation of tyrosine kinases by the AT_1 receptor have been proposed. The first mechanism involves binding motifs present in the AT_1 receptor that serve as scaffolds to recruit tyrosine kinases or proteins that activate tyrosine kinases (Pascal *et al.,* 1994; Fantl *et al.,* 1993). The second mechanism, termed as receptor transactivation, is defined by Ang II-mediated activation of a tyrosine kinase receptor such as the EGF receptor or PDGF receptor. This may occur in two ways. First, clustering of the EGF receptor or PDGF receptor may activate the intrinsic kinase activity of the receptor resulting in autophosphorylation and recruitment of signalling molecules. Second, a tyrosine kinase activated by the AT_1 receptor may phosphorylate the EGF receptor or PDGF receptor, and the receptor would then function as a scaffold for assembly of other signalling molecules. This may occur independently of receptor clustering and autophosphorylation (Berk, 1999). Five major tyrosine kinase-regulated pathways have been demonstrated based on activation of specific kinases shown to be regulated by Ang II in VSMC: Janus kinases (JAK and

TYK), $pp60^{c\text{-}src}$ kinase (c-Src), focal adhesion kinase (FAK), receptor tyrosine kinases (Axl, EGF, and PDGF), and calcium-dependent tyrosine kinases (Pyk2 or CADTK) that phosphorylate signal substrates such as Shc, Raf, and PLC-γ (for details see the review by Berk, 1999). The purpose of these multiple receptor-activated kinases is to provide an integrated series of regulated cellular events.

Exciting new findings point to functions of the AT_1 receptor related to growth, migration, and vasoconstriction mediated by multiple tyrosine kinases, including Src, Fyn, EGF-R, PDGF-R, Axl, JAK, TYK, and CADTK. The role of tyrosine kinases in Ang II-stimulated growth and migration seems well established. However, the role of tyrosine phosphorylation in Ang II-stimulated vasoconstriction remains uncertain. An important role for tyrosine kinases is suggested by the findings that activation of chloride and calcium channels by G protein-coupled receptors (including Ang II) is inhibited by tyrosine kinase inhibitors (Lev *et al.*, 1995; Marrero *et al.*, 1996). In particular, because c-Src has been shown to be involved in PDGF-mediated calcium activation and Ang II stimulates c-Src, it is likely that tyrosine kinases contribute to the contractile actions of Ang II.

3.4. Role of endothelium and endothelin-1

Vascular endothelium plays a critical role in the regulation of the functions of the underlying VSMC. It releases several important factors called endothelium-derived contracting factor(s) (EDCF) and endothelium-derived relaxing factors (EDRF) which mediate contraction and relaxation of vessels. These endothelium-dependent factors are elicited by physical and chemical stimuli and autacoids, local and circulating hormones. Since endothelial cells (ECs)

possess both AT_1 and AT_2 receptors (Pueyo & Michel 1997; Stoll *et al.*, 1995), these receptors are in direct contact with circulating Ang II. Recently it has been demonstrated that the endothelium is involved in the cardiovascular actions of Ang II by means of releasing endothelium-dependent contracting or relaxing factors. The influence of the endothelium in determining the response of the blood vessel to Ang II varies depending on the species and the vascular bed. For example, in some animals, the contractile responses induced by Ang II were not dependent on the presence of the endothelium. Examples of this include rabbit aorta (Saye *et al.*, 1984), canine carotid artery (d'Orlèans-Juste *et al.*, 1985) and bovine intrapulmonary artery and vein (Gruetter *et al.*, 1988). In contrast, results from other studies showed that destruction of the endothelium enhanced the Ang II-induced contraction in rabbit aortic, mesenteric and coeliac arteries (Oshiro *et al.*, 1985; Yilmaz *et al.*, 1987), rat aorta (Bullock *et al.*, 1986), and that removal of endothelium produced a much reduced contraction in response to Ang II in the canine cerebral artery (Yen *et al.*, 1990).

3.4.1. Endothelin

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that is generated via unique processing of a low activity precursor, big ET-1 by endothelin converting enzymes. The 21-amino acid peptide, ET-1, has a physiological role in the maintenance of basal vascular tone in humans, but may also have a role in the pathophysiology of cardiovascular diseases, including atherosclerosis, coronary vasospasm and congestive heart disease (Rubanyi & Polokoff, 1994). Since the discovery in 1985 and isolation in 1988 (Yanagisawa, 1988), significant progress has been made in ET research. Three different genes (ET-1, ET-2 and

ET-3 genes) have been identified in the human genome that have different chromosomal locations and are differentially regulated, the latter processes led to the expression of the different ET isopeptides (ET-1, -2, -3) in animals and humans. The putative enzyme converting the biologically inactive precursor, big ET, to the biologically active mature peptide (ECE) has been purified and characterized (Hasegawa *et al.*, 1998; Subkowski *et al.*, 1998). Two different ET receptors (ET_A and ET_B) have been cloned (Haendler *et al.*, 1992), and the biological and pharmacological activity of ETs have been tested in numerous animal and human tissues in vivo and in vitro. Signal transduction pathways, mediating both rapid and long-lasting changes in cell function after ligand-receptor interaction, have been identified (Rubanyi & Polokoff, 1994).

Endothelin may play a role in the Ang II action and signal transduction pathways. Ang II has been shown to stimulate the expression of ET-specific mRNA and release of ET from cultured endothelial cells (Dohi, *et al*, 1992). Chen *et al.* (1995a & b) have reported that there are endothelium-dependent regional variations in vascular tissue sensitive to Ang II. The responses to Ang II in the rat tail artery appear to be mediated by the indirect stimulation of vascular smooth muscle ET_A receptors subsequent to the activation of endothelial AT_1 receptors likely linked to the release of endothelins. A few studies have also shown that Ang II promotes the release of endothelin-1 (ET-1) in a time- and concentration-dependent manners from cultured bovine endothelial cells (Emori *et al.*, 1993), cultured rat mesangial cells (Kohno, *et al.*, 1992), and human endothelial cells (Ciafre *et al.*, 1993). These studies suggest that Ang II stimulates, via AT_1 receptor activation, the secretion of vasoconstrictor

agents such as endothelin, which accounts for a major part of the evoked vasoconstrictor response to Ang II in small vessels. However, the production of endothelin from stimulated endothelial cells requires de novo protein synthesis and thus is very slow, reaching a maximum after 4-5 hours of incubation (Yanagisawa *et al.*, 1988; Boulanger *et al.*, 1990). In intact mesenteric resistance arteries the endothelium-dependent potentiation was observed only after 5 hours but not after 1 hour of stimulation with Ang II (Dohi *et al.*, 1992).When experiments were performed with Ang II in combination with ET-1, there was no synergistic enhancement of the stimulation induced by either agonist alone (Scott-Burden *et al.*, 1991). Thus, it appears unlikely that the stimulatory activity of Ang II on the contractile, proliferative and synthetic activity of SMCs is mediated via the induction of ET-1 production.

3.4. 2. Nitric oxide (NO) and prostaglandin I_2 (PGI_2)

Activation of AT_1 receptors on endothelial cells also results in the production of vasodilatory products including NO (Pueyo *et al.*, 1998) and PGI_2, which counteract the direct vasoconstrictory effects of Ang II on adjacent VSMCs (Ardaillou, 1999). The signal transduction mechanism involves several steps. Thus binding to AT_1 receptors, Ang II stimulates PLC and PLA_2 activities, resulting in $[Ca^{2+}]_i$ increase and arachidonic acid release. An increase in cytosolic calcium stimulates constitutive nitric oxide synthase (NOS) activity and increases the local production of NO (Pueyo and Michel, 1997), which in turn stimulates guanylyl cyclase activity in the VSMC and increases the local production of cGMP. Arachidonic acid produced by the hydrolysis of the membrane phospholipids is transformed

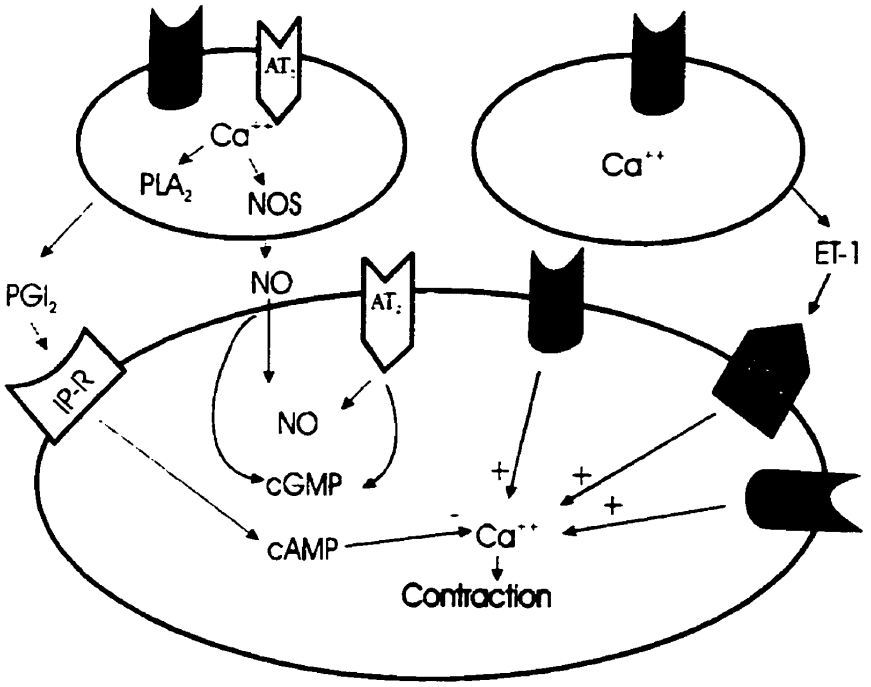
into prostaglandins, essentially PGI_2, which binds to specific receptors on the surface of VSMC producing activation of adenylyl cyclase and increased formation of cAMP. These two cyclic nucleotides are vasodilatory and growth-inhibitory factors. Thus, through endothelial cell AT_1 receptors, Ang II limits its direct effects on the VSMC (Fig 3; based on Arendshorst, *et al.* 1999).

Part 4. Objectives for current investigation

Ang II exerts a wide range of actions in the kidney, heart, vascular system and central nervous system via binding to AT_1 and/or AT_2 (Timmermans, *et al.*, 1993; de Gasparo *et al.*, 1995). In the vascular system, Ang II is a potent agonist of VSM contraction primarily via the activation of AT_1 (Timmermans, *et al.*, 1993). Although the important role for Ang II as an endogenous regulator of VSM tone is well established, the responses to Ang II vary in different vascular beds and among VSM preparations (Bottari, *et al.*, 1993). The heterogeneity in tissue response to Ang II may result from the modulatory effect of Ang II on the vascular endothelium (Toda, *et al.*, 1990; Cortes, *et al.*, 1996) or differences in the properties of Ang II receptors on the vascular endothelium and the underlying smooth muscles among the preparations and species studied (Toda, *et al.*, 1978; Boulanger, *et al.*,1995; Pueyo, *et al.*, 1997).

Chen *et al.*(1995a & b) have recently reported that there are endothelium-dependent regional variations in vascular tissue sensitive to Ang II and the vasoconstrictor responses to Ang II in the rat tail artery may be mediated by the indirect stimulation of vascular smooth muscle

Fig 3. Schematic diagram illustrating that Ang II can act on endothelial and vascular smooth muscle cells via AT_1 and AT_2. AT_1 and AT_2 on the vascular endothelium may be stimulated by Ang II and induce an increase in intracellular calcium. An increase in intracellular calcium results in: 1). NO synthase and release; 2). Stimulation of PLA_2 leading to the release of arachidonate and its metabolic products, e.g., PGI_2; 3). Endothelin-1 synthase and release. The release of NO and PGI_2 may counteract the Ang II-induced smooth muscle contraction though different pathways.



Endothelial Cells

Vascular Smooth Muscle Cell

ET_A subsequent to the activation of endothelial AT_1 receptors likely linked to the release of endothelins. A few studies have also shown that Ang II promotes the release of endothelin-1 (ET-1) in a time- and concentration-dependent manner from cultured bovine endothelial cells (Emori *et al.*, 1993), cultured rat mesangial cells (Kohno, *et al.*, 1992), and human endothelial cells (Ciafre *et al.*, 1993). Despite these elegant observations, the importance of ET-1 release in Ang II-mediated constriction of the rat tail artery remains unclear, since it is generally recognized that Ang II can directly bind to the AT_1 receptors on VSMC and directly exert vasoconstriction. My preliminary experiments with the rat tail artery have shown that:

1). Ang II produced vasoconstriction in the endothelium-denuded tail artery;

2). Ang II-mediated vasoconstriction of the endothelium-intact tail artery was not antagonized by BQ-123, a selective ET_A antagonist, but was blocked by losartan, a selective AT_1 antagonist;

3). The vasoconstrictor properties of Ang II were different compared to that of ET-1 in the endothelium-denuded tail artery;

4). Ang II directly produced increases in $[Ca^{2+}]_i$ with a simultaneous contraction of single SMC of the rat tail artery.

These data suggest that Ang II-mediated contraction of the rat tail artery is due primarily to a direct action on the VSMC. However, the cellular mechanisms for Ang II and ET-1-induced vasoconstriction and the synergistic interactions between these agonists are unknown. Therefore, the aim of the present study is to address these issues. Of particular

interest is a systematic comparison of the actions, and of the cellular mechanisms for Ang II- and ET-1-mediated vasoconstriction in the rat tail artery.

To address these objectives, the actions of Ang II and ET-1 in the rat tail artery were studied using ring preparations from whole vessels in organ bath to measure tension, single VSMC to measure $[Ca^{2+}]_i$, and reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemical and immunocytochemical methods were used to localize the AT_1 distribution. My thesis is composed of the following studies:

1. Determination of the role of the endothelium and ET-1 release in mediating the vasoconstrictor effects of Ang II in the rat tail artery;

2. Identification of pharmacological properties of the effects of Ang II and ET-1 in the rat tail artery.

3. Comparison of the cellular mechanisms and of the signal transduction for Ang II and ET-1-mediated vasoconstriction in the rat tail artery.

4. Effects of Ang II and ET-1 on $[Ca^{2+}]_i$ in single VSMC from the rat tail artery.

5. Study of synergistic interactions between Ang II- and ET-1-mediated vasoconstriction: It is well described that Ang II not only facilitates the release of norepinephrine from postganglionic sympathetic fibres, but can also amplify vascular responses to a variety of pressor substances. In organ bath studies with the rat tail artery ring preparation, I have investigated whether Ang II and ET-1 interact synergistically to enhance vasoconstriction.

6. Using RT-PCR, immunocytochemical and immnochemical methods the distribution of AT_1 receptors have been determined in rat tail arteries. Endothelium-intact and -denuded tail arteries, as well as isolated the VSMCs and ECs from the rat tail artery were assessed for

the expression of AT_1 receptor and the cellular distribution of AT_1 receptor determined in these preparations by immunocytochemistry and immunohistochemistry.

Chapter Two: Methods

Part 1. Tension measurement

Male Sprague Dawley (S.D.) rats (400-450g) were stunned and killed by cervical dislocation. The tail artery was quickly excised and placed in cold physiological salt solution (PSS, Krebs solution) of the following composition (millimolar): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 12.5; dextrose 11.1. The pH of the solution after saturation with 95% O_2 + 5% CO_2 gas mixture was 7.4. Adherent connective tissue was cleaned carefully from the artery which was then cut into 5 mm rings. Each ring was suspended between platinum hooks and mounted in 20 ml organ bath containing PSS maintained at 37°C and gassed continuously with 95% O_2 + 5% CO_2. Isometric tension was recorded with a force displacement transducer (Grass FT 03) coupled to a Grass polygraph model 7E (Fig 4). The rings were stretched in a stepwise fashion to a tension of 0.8 g, which condition produced a submaximum contraction. The tissues were equilibrated for 2 hours with repeated change of PSS solution every 20 min. In some experiments, the endothelial cell layer was removed by repeatedly passing a stainless steel wire through the vessel lumen. In the protocol followed by Chen, *et al.* (1995a,1995b) a 0.5% Triton x-100 was used to remove the endothelium. Comparing these two methods, it was found that the latter method not only removed endothelium, but also decreased smooth muscle contraction (Fig 5). The presence

Fig 4. Diagram of setup for measuring tension in intact tissues. This setup can be used to investigate what agents (agonists) contract or relax smooth muscle, and investigate the cellular mechanisms involved in mediating the tissue responses.

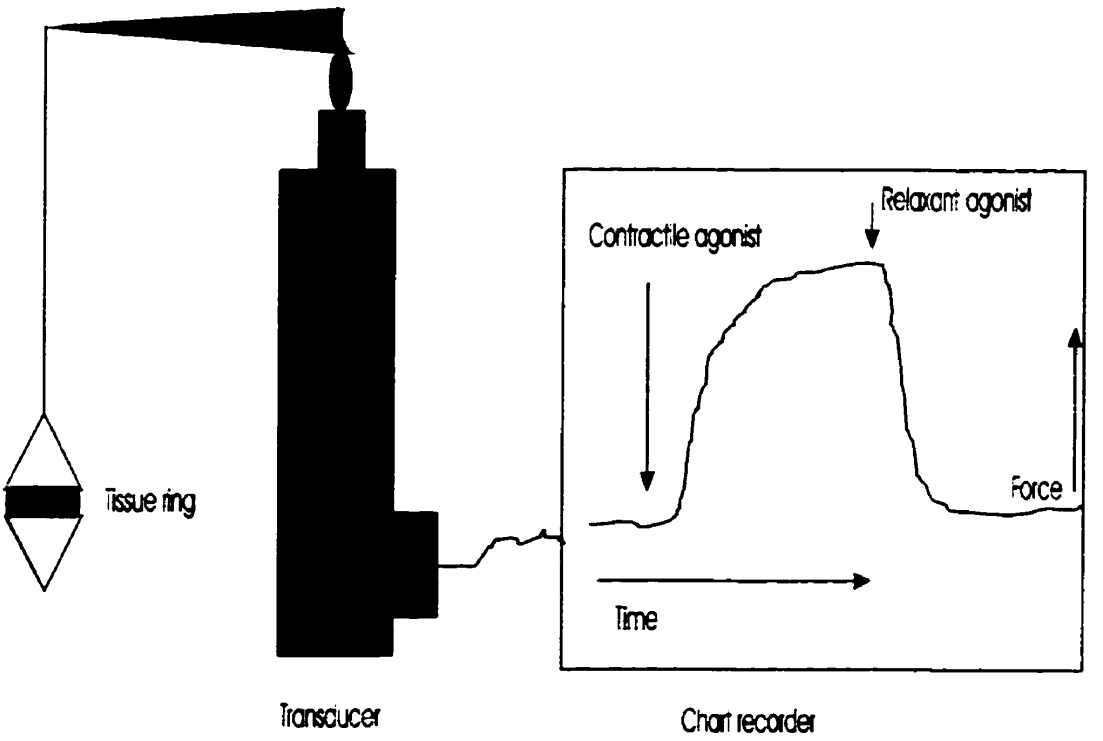
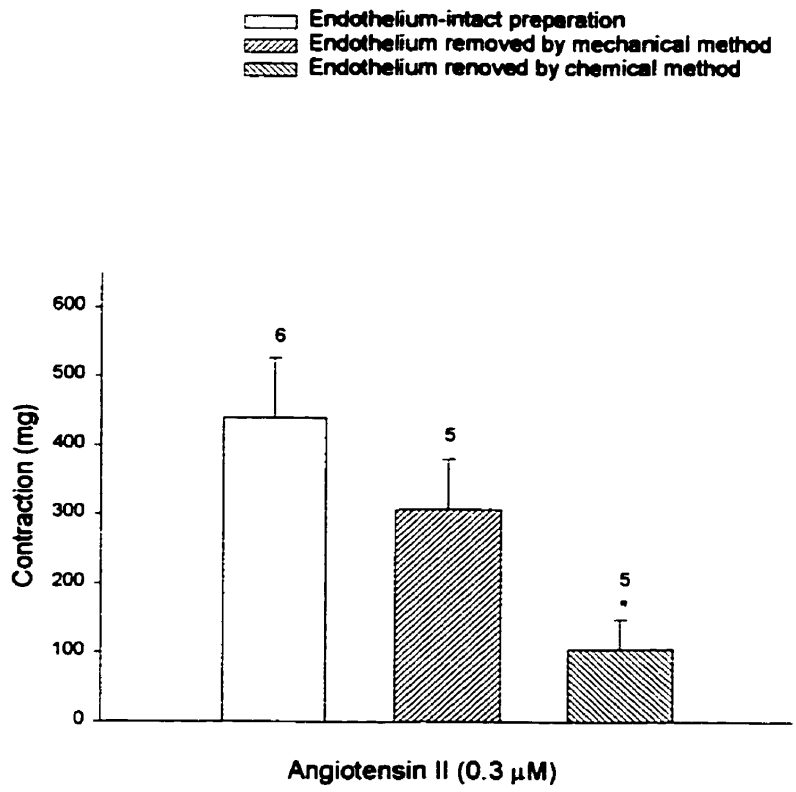

Tissue ring
Contractile agonist
Relaxant agonist
Force
Time
Transducer
Chart recorder

Fig 5. Comparison of the mechanical and chemical methods to remove the endothelium in Ang II-induced contraction of the rat tail artery. Mechanical removal by repeated rubbing of the lumen of the vessel with a stainless steel wire versus chemical removal by using 0.5 % Triton X-100 treatment for 3 seconds and then washing at least three times with PPS. The successful removal of the endothelium of the vessel was confirmed by loss of the relaxation response to ACh (10 μM) in vessels precontracted with PE (1 μM). Data are shown as means ± S.E.M. *, $P < 0.05$ *vs.* E (+) using ANOVA.


Endothelium-intact preparation
Endothelium removed by mechanical method
Endothelium renoved by chemical method
Contraction (mg)
600
500
400
300
200
100
0
6
5
5
*
Angiotensin II (0.3 μM)

of a functional endothelium was assessed by the ability of acetylcholine (ACh; 10 μM) to induce more than 20% relaxation of vessels pre-contracted with phenylephrine (PE) (1 μM). The absence of a relaxation response to ACh was taken as evidence that the vessel segment was functionally denuded of endothelium. After equilibration, 1 μM PE was added to elicit a standard contractile response, then tissues were washed two to three times with PPS, and one of the following experimental protocols was used:

1). A cumulative concentration-response curve for Ang II (10 nM-1 μM) was performed and only one curve was performed in each tissue;

2). To avoid Ang II-induced desensitization, parallel studies were performed in which one tissue was exposed to a single dose of Ang II and another tissue was treated to other drug protocols and then to Ang II, the results are represented as a percentage of the response to PE. In some experiments, after exposure to Ang II (control response), the tissues were treated to various drug protocols and it was determined whether these drug protocols influence desensitization of Ang II, the results are represented as to percentages of the control response;

3). Similar protocols as described for Ang II were used in order to investigate the effects of ET-1 on the rat tail artery.

All drugs were directly added to the organ bath.

Part 2. Cell preparation and intracellular Ca^{2+} measurement

2.1. Cell preparation

Male S.D. rats (400-450g) were anaesthetized with halothane, maintained with methoxyflurane and the lower abdomen was opened to expose the abdominal aorta. The common iliac arteries were located and tied off. The aorta was cannulated and 10 ml of Dubecco's modified essential medium (DMEM) was infused and the tail was immediately cut off. Following cleaning of connective tissue, the tail artery was cut into several pieces and incubated for 30~60 min (dependent on the type of collagenase IV used) in 1 ml DMEM containing collagenase IV (2mg/ml) , dispase IV (2mg/ml), elastase III (20 units/ml) and collagenolytic proteinase IV (0.085 units/ml) at 37°C. The enzymatic reactions were stopped by replacing the solution with DMEM supplemented with 2 mM EGTA and 0.1mg/ml soybean trypsin inhibitor (DMEM-EGTA-TI) for 5 min and moving the digestion dish to room temperature. Trituration of the vessels was commenced whilst still in the presence of the DMEM-EGTA-TI which was removed and replaced with DMEM plus 5%BSA (bovine serum albumin) once the cells started to separate from the vessels. The cells were kept on ice before using.

Smooth muscle dissection solution (SMDS) was also used as a digestion solution. SMDS contains (mM): NaCl 120; KCl 4.2; $CaCl_2$ 1.8; KH_2PO_4 1.2; $MgCl_2$ 1.2; $NaHCO_3$ 25; dextrose 11. The pH of the solution after saturation with 95% O_2 + 5% CO_2 gas mixture was 7.4. The cells isolated from the DMEM looked more relaxed than those from the SMDS, but the responses of individual cells demonstrated have no obvious difference. Using DMEM required sterilize conditions and/or 37°C , otherwise, the cells would not stay healthy.

2.2. Intracellular Ca^{2+} measurement

Cells were loaded by incubation in HEPES solution containing 5 μM fura-2/AM (fura-2-

acetoxymethylester) for 35 min at room temperature. HEPES solution contains (mM): NaCl 120; KCl 4.2; HEPES 10; $CaCl_2$ 1.8; KH_2PO_4 1.2; $MgCl_2$ 1.2; $NaHCO_3$ 3; dextrose 10; the pH of the solution was adjusted to 7.4 with NaOH. These cells were washed for 15 min to allow the conversion of fura-2/AM into active fura-2 by endogenous esterases. Cells that appeared to have smooth cell membranes and relaxed were regarded as healthy cells; healthy cells usually were viable for about 10 hours. Drugs were directly added to HEPES solution and superfused over the cell at 750 μl/min at room temperature. Most of the cells can only be stimulated by one agonist and then remained in a contracted state. For each cell one of the following experimental protocols was followed:

1). A single dose of Ang II was applied to a single smooth muscle cell after recording the background ratio for 1 min. and Ang II was then washed out for 10-15 min. If the cell did not relax, it was discarded; if it relaxed, a second agonist was applied;

2). The smooth muscle cell was incubated with inhibitors (i.e., losartan, BQ-123, genistein or L-NNA) for 20 min. and then a single dose of Ang II or ET-1 was applied;

3). The cell was incubated with caffeine or cyclopiazonic acid (CPA) for 5 min. and then a single dose of Ang II or ET-1 was applied;

4). A single dose of Ang II or ET-1 was applied to a sheet of endothelial cells, which had been isolated from the rat tail artery. After 5-10 minutes the cells were washed and ACh was applied. One sheet of endothelial cells could be stimulated repeatly.

Fura-2 is an example of an excitation-shifted or dual excitation indicator. When Ca^{2+} is bound to fura-2, the excitation maximum is found at a wavelength of 335 nm which shifts to 362 nm in the absence of Ca^{2+}. The fluorescence signals is obtained by exciting fura-2 at

340 nm and 380 nm and ratioing the fluorescence intensity detected at 510 nm.

The peak ratio of 340 nm to 380 nm which was subtracted from background ratio was used to indicate intracellular Ca^{2+} changes according to Grynkiewicz *et al.* (1985):

$$R=(F340_{cell}- F340_{b})/(F380_{cell}-F380_{b})$$

where $F340_{cell}$ and $F380_{cell}$ are the total measured fluorescence of the single cell at wavelength of 340 and 380 nm. $F340_{b}$ and $F380_{b}$ are the fluorescence background signals at the respective wavelength. The background signal in each experiment was obtained by measuring the empty field surrounding the cell. The area of empty field usually is as same as that of the cell field.

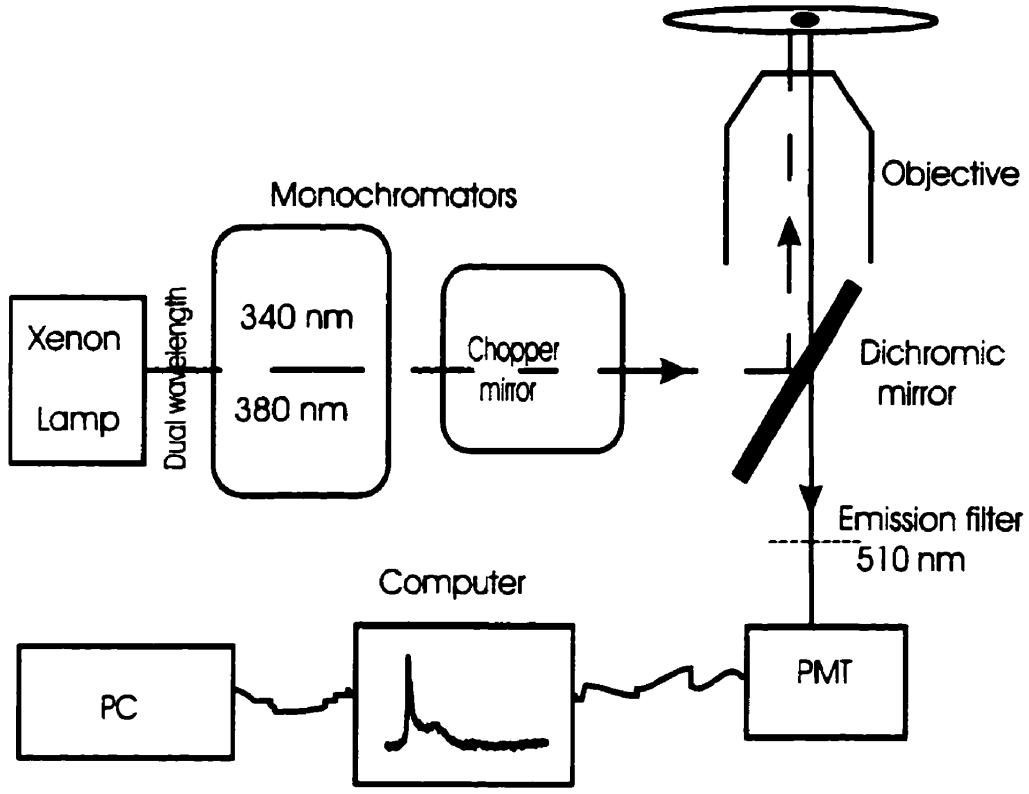
Fura-2 signals are reported as ratio values rather than converting into $[Ca^{2+}]_i$.

A photomultiplier tube (PMT) was used to measure fluorescence intensity. The high voltage applied to the PMT was maintained constant during the recording (700V, optimal range which varies for every PMT was 400-1000V). An oil immersion, x100 objective lens was used in all fura-2 studies. The signal from the the PMT was processed by PC and displayed digitally on the computer screen. Because fura-2 has a strong affinity for Ca^{2+}, most of the Ca^{2+} binds to fura-2 with 1:1 stoichiometry. An SLM-AMINCO 8000 spectrofluorometer (SLM Instruments Inc., USA) interfaced with a NIKON Diaphot inverted microscope was used in this study (Fig 6).

Part 3. RT-PCR

Rat tail arteries were dissected as described above. Endothelium-intact and -denuded arteries were frozen with liquid nitrogen. The mRNA was extracted from the arteries or isolated cells

Fig 6. Diagram illustrating the setup for measuring intracellularCa^{2+}. Light emitted from a xenon lamp was passed through a beam splitter and two monochromators set at 340 nm and 380 nm, respectively. A chopper was used to alternate the two wavelength light beams which passed through the fibre optic interfaced to an inverted microscope. The cell suspension loaded with fura-2/AM was placed in a chamber and was illuminated by the 340 and 380 nm wavelength light. The excitation light was directed toward the chamber with a dichromic mirror. The fura-2 fluoresence signal was passed through a 10 nm band pass filter centred at 510 nm and a shutter, and was then detected with a photomultiplier tube (PMT) which amplified the signal. The computer controls the monochromator wavelength, PMT voltage, chopper and data acquisition.


Monochromators
Objective
Xenon
Lamp
Dual wavelength
340 nm
380 nm
Chopper
mirror
Dichromic
mirror
Emission filter
510 nm
Computer
PC
PMT

using Poly (A) Pure™ MicroPoly (A) Pure™ *mRNA Isolation Kits* from Ambion.

The frozen tissue was ground into fine powder in liquid nitrogen with a motar and pestle and put into lysis buffer (0.1ml/10mg). Freshly dispersed SMCs and endothelial cells were centrifuged at room temperature for 3 minutes at high speed (12,000xg) and the pellet was added to 0.5~1 ml lysis buffer. An equal volume of dilution buffer was added, centrifuged for 15min at 12,000xg at 4°C, and the supernatant was transferred to 1.5 ml centrifuge tubes. The contents of one vial of oligo dT resin was added to the diluted lysate sample and mixed well to ensure that the oligo dT was dispersed. The oligo dT binding to mRNA was processed in Binding Buffer and the other RNA and DNA were removed by Washing Buffer. A spin column provided with the kit was used to bind mRNA, and mRNA was eluted with Elution Buffer containing 10 mM Tris pH 7.5, 1 mM EDTA. The mRNA was precipitated with 20 µl (0.1 volume) of 5 M NH_4OAc, 1 µl of glycogen and 2.5 volumes of 100% ethanol at -70°C overnight. After centrifugation at maximum speed (15,000 x g) for 20 minutes at 4°C, the ethanol was removed completely by using a pipettor and drying machine (DNASpeedVac®DNA110). The mRNA was resuspended in 20 µl of dH_2O/0.1 mM EDTA and vortexed vigorously to ensure the pellet was completely dissolved. The formula for calculating the concentration of RNA in µg/ml is A_{260} x dilution factor x 40. The ratio of A_{260} to A_{280} values (1.7 to 2.1) is a measure of RNA purity.

RT-PCR were performed using RETROscript™ *First-Strand Synthesis Kit for RT-PCR* from Ambion. Single-stranded cDNA was synthesized from a 20 µl reaction volume containing a mixture of 2 µg mRNA, 4 µl dNTP mix, 2 µl First-strand primers (provided in the kit), and 16 µl dH_2O (nuclease-free). The mixture was heated to 75°C for 3 minutes and then cooled

on ice.10x RT-PCR Buffer (2 µl), Placental RNase Inhibitor (1 µl), M-MLV Reverse Transcriptase (1 µl) were added, mixed (20 µl final volume) and then incubated at 42°C for one hour and at 92°C for 10 minutes to inactivate the reverse transcriptase. Each PCR sample (50 µl) contained 5 µl of RT reaction from above, 5 µl of 10x reaction buffer, 2.5 µl of 10x dNTP mix 1.25 µl each PCR primer, Super Taq (0.2 µl) and 34.8 µl dH_2O. Sequences were amplified using a Gene Amp PCR system 2400 for 30 cycles. Each cycle consisted of DNA dissociation at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and amplification at 72°C for 40 seconds, following by a final elongation at 72°C for 5 minutes. To visualize the RT-PCR reaction, 20 µl RT-PCR product was run on 1.5% agarose gel in the presence of ethidium bromide (4 µl), and the product was visualized under uv light. The remaining RT-PCR product can be stored at -70°C.

In this study, oligonucleotide primers were chosen from rat AT_1 genes; the antisense and sense primer sequences were 5'-GCA CAA TCG CCA TAA TTA TCC-3' (position 739-719) and 5'-CAC CTA TGT AAG ATC GCT TC-3' (position 295-314, Murphy, *et al.*, 1991), respectively, yielded a single fragment of 444 base pairs. Digestion with *Eco*RI (17000 u ul^{-1}) for three hours at 37°C was used to differentiate between the AT_1 receptor subtypes: this digestion does not affect the AT_{1B} product, but hydrolyses that of AT_{1A} into two fragments: 269 bp and 175 bp (Pueyo, *et al.*, 1998). From intact tissue the undigested PCR product was extracted from agarose gel using a GEL ECLIPSE™ DNA Purification Kit and sequenced with an ABI PRISM™ Kit.

Part 4. Immunohistochemistry and immunocytochemistry

4.1. Immunohistochemistry

The tail artery was quickly isolated and fixed in Modified Zamboni Fixative (100 ml containing the following compounds: 50 ml D.D.H_2O, 2 g paraformaldehyde, two pellets of NaOH or until solution is clear, 50 ml 0.2 M phosphate buffer, 0.5 ml 0.5% picric acid , pH 7.4). After washing 5x5 min with phosphate buffered saline (PBS), the tail artery was put into 20% sucrose in PBS at 4 ^{0}C, overnight, embedded in OCT compound, frozen, cut into 35 μM thick slices and mounted on slides treated with poly-D-lysine. The slices were skinned by 0.5% triton X-100 for 5 min. and incubated for one hour with rabbit serum to decrease nonspecific binding. The slices were divided three different treatments: 1). Incubated with polyclonal rabbit anti-human AT_1 IgG (diluted 1:200) in 3% bovine serum albumin (BSA) at 4^0 C overnight; 2). Incubated for one hour with AT_1 blocking peptide (100 times higher than rabbit anti-human AT_1 IgG) at room temperature, washed two times with PBS and one time with 3% BSA in PBS, and incubated with polyclonal rabbit anti-human AT_1 IgG (diluted 1:200) in 3% BSA at 4^0 C overnight; 3). Incubated with PBS. Excess antibodies were removed by washing for 2x5 minutes with PBS and 1x5 minute with 3% BSA in PBS. The treated slices were then incubated with CY3™-conjugated goat anti-rabbit IgG (diluted 1:1000)in 3% BSA for one hour in the dark at room temperature. After three washes with PBS, the slices were mounted in 90% glycerol, and photographs taken with a Nikon Microphoto-FXA fluorescent microscope.

4.2. Immunocytochemistry

ECs and SMCs were isolated from the rat tail artery as previously described. The cell

suspensions were centrifuged at 500xg for 5 min at room temperature. The cells were fixed with formalin (0.1%) for 15 min in G8, skinned by 0.5% Triton X-100 for 5 min. G8 contains (Millimolar): $Mg(OH)_2$ 1.03, EGTA 5 in KOH, PIPES 20, KOH 75.5. The cells were incubated with: 1). polyclonal rabbit anti-human AT_1 IgG (diluted 1:200) in 3% BSA for one hour at room temperature; 2). G8 in 3% BSA. Excess antibodies were removed by washing for 2x5 min with G8 and 1x5 min 3% BSA in G8. The cells were then incubated with CY3™-conjugated goat anti-rabbit IgG (diluted 1:1000) in 3% BSA for one hour in the dark at room temperature. After three washes with G8, the cells were mounted in 90% glycerol, and photographs taken with a Nikon Microphoto-FXA fluorescent microscope.

Part 5. Data analysis

The data are shown as means ±S.E.M. and the differences determined by Student's *t*-test (paired or unpaired) and One-way Analysis of Variance (ANOVA). A *P* value less than 0.05 was considered significant. The pEC_{50} values were determined as the negative logarithmic molar concentration of a drug which caused 50% of the maximal effect.

Chapter Three: Results

Part 1. Is Ang II-mediated vasoconstriction in the rat tail artery due to a direct action on SMC or indirectly via ET-1 released from the endothelium?

1.1. Ang II-induced contraction in the presence and absence of endothelium

In the presence of the endothelium, Ang II elicited concentration-dependent vasoconstriction of the rat tail artery. Similar levels of submaximal tension were obtained either by stepwise cumulative addition of Ang II or by the addition of a single submaximal concentration of Ang II (0.1 μM). Similarly, in endothelium-denuded preparations, Ang II evoked vasoconstriction of the rat tail artery in either a single concentration or in a concentration-dependent manner (Fig 7 & 8). Both pEC_{50} and maximal contraction response to Ang II were measured and compared in the presence and the absence of the endothelium. No significant differences were found in either pEC_{50}s (7.95 ± 0.06 *vs* 7.81 ± 0.02, $p>0.05$) or maximal contraction responses (0.46g ± 0.05 *vs* 0.41g ± 0.07, $p>0.05$) to Ang II in endothelium-intact (n=9) or denuded-endothelium(n=8), respectively.

1.2. Comparison of Ang II- and ET-1-induced vasoconstriction

As depicted in figure 9, the nature of the Ang II and ET-1 evoked contractile responses were quite different. Ang II (0.3 μM) induced a non-sustained contraction, which reached a peak

Fig 7. Tracings of Ang II-induced contraction of the rat tail artery. The tracing came from tissues obtained from the same rat in the presence (E +)and absence (E -) of the endothelium.

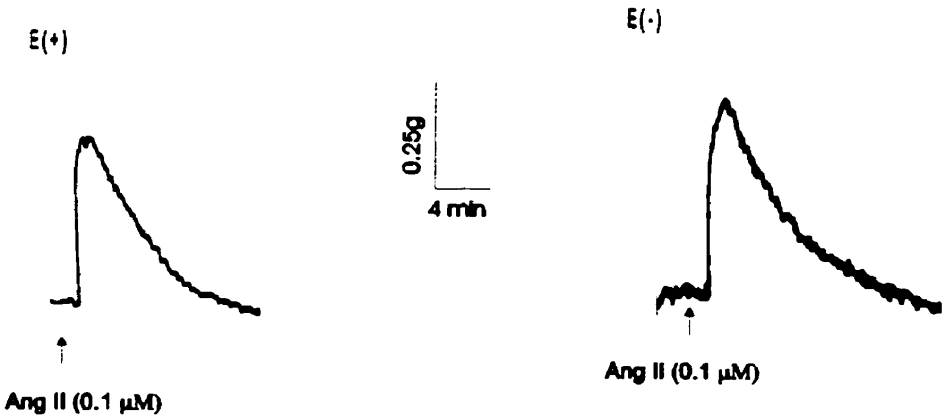
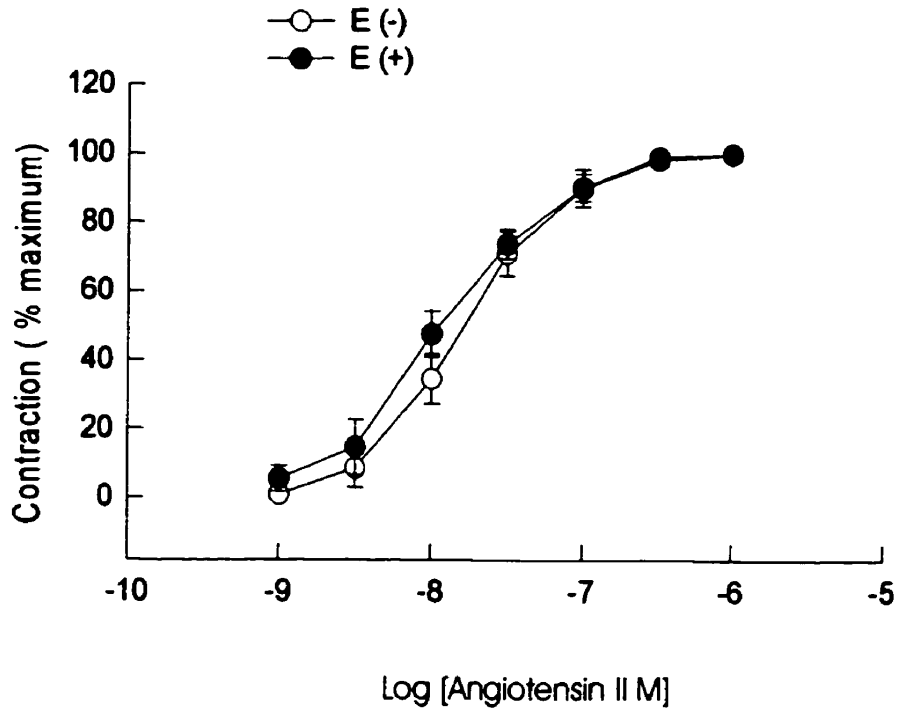

E(+)
E(-)
0.25g
4 min
Ang II (0.1 μM)
Ang II (0.1 μM)

Fig 8. The cumulative concentration-response curves to angiotensin II of rat tail artery. The results were expressed as a percentage of the maximal response induced by Ang II. Each point represents the mean ± S.E.M, n=9 (●:in the presence of endothelium, E+), n=8 (○: in the absence of endothelium, E-).


E (-)
E (+)
120
100
80
60
40
20
0
Contraction (% maximum)
-10
-9
-8
-7
-6
-5
Log [Angiotensin II M]

within 5-10 seconds and after 8-10 min returned to baseline. In contrast, ET-1 (10 nM) invoked a sustained contraction that slowly reached a plateau over 8-10 min was maintained for about 10-20 min and then very slowly (over 30-40 min) declined to baseline (Fig 9a & b). Ang II-induced contraction was not affected by BQ-123 (1 μM), a selective ET_A receptor antagonist, but was significantly inhibited by losartan (10 nM), a selective AT_1 receptor antagonist. In contrast, the ET-1-induced contraction was not inhibited by losartan, but was significantly inhibited by BQ-123 (Fig 10).

1.3. Different pathways for Ang II- and ET-1-induced vasoconstriction

To further investigate the signal transductions activated by Ang II and ET-1, I investigated the contractile action of Ang II in the rat tail artery rings maximally contracted with ET-1. As shown in Figure 11, ET-1 caused a concentration-dependent contraction that reached a maximum at 0.1 μM. When 0.1 μM Ang II was added on the top of the maximal response to ET-1, Ang II evoked an additional non-sustained contraction of the ring preparation of the rat tail artery (n=9).

1.4. Ang II-induced changes in $[Ca^{2+}]_i$ in endothelial cells and single smooth muscle cells

Measurement of intracellular Ca^{2+} concentration was made in freshly dispersed single endothelial and smooth muscle cells from the rat tail artery. Ang II induced an increase in $[Ca^{2+}]_i$ of smooth muscle cells in a concentration-dependent manner with a pEC_{50} value of 9.12 (Fig 12 a & b). The increase in $[Ca^{2+}]_i$ occurred with the simultaneous contraction of single smooth muscle cells that was observed as a shape change from spindle-like to a rounded-form (Fig 13 a & b). After incubation for 20 min. with losartan, Ang II-induced

Fig 9. Tracings illustrate the different properties of Ang II- and ET-1-mediated contractions. a. Ang II (0.3 μM) induced a phasic contraction, not inhibited by BQ-123 (1 μM), a selective ET_A antagonist. b. ET-1(10nM) induced a tonic contraction, (which was inhibited by BQ-123 (1 μM)- not shown in this figure).

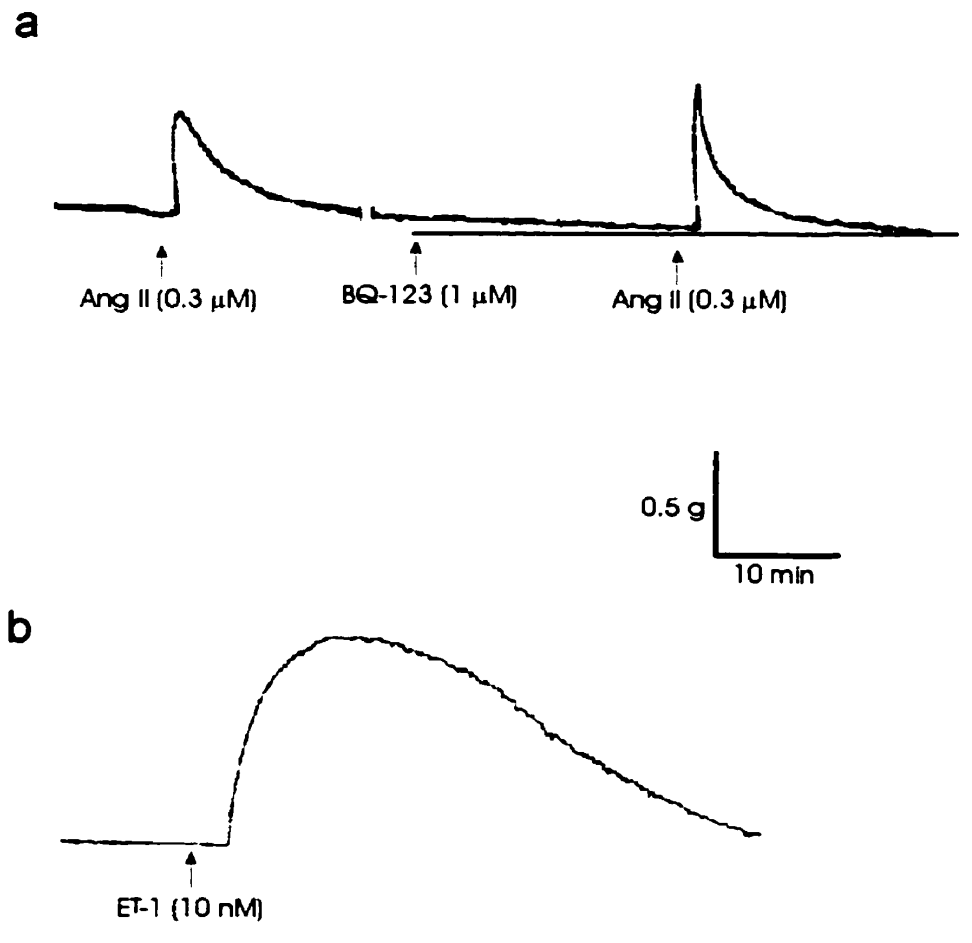

a
Ang II (0.3 μM)
BQ-123 (1 μM)
Ang II (0.3 μM)
0.5 g
10 min
b
ET-1 (10 nM)

Fig 10. Effects of BQ-123 and losartan on response to Ang II- and ET-1-induced contraction of rat tail artery. The rings were pretreatment with BQ-123 (1 μM) (an ET_A antagonist) or losartan (10 nM) (a selective AT_1 antagonist) for 20 minutes and then the response to Ang II (0.3 μM)- or ET-1 (10 nM) was measured. The results were compared with control, which represented either Ang II- or ET-1-induced contraction. **, $p<0.001$ in losartan- and BQ-123-treated *vs.* control groups. n=6-13, using ANOVA.

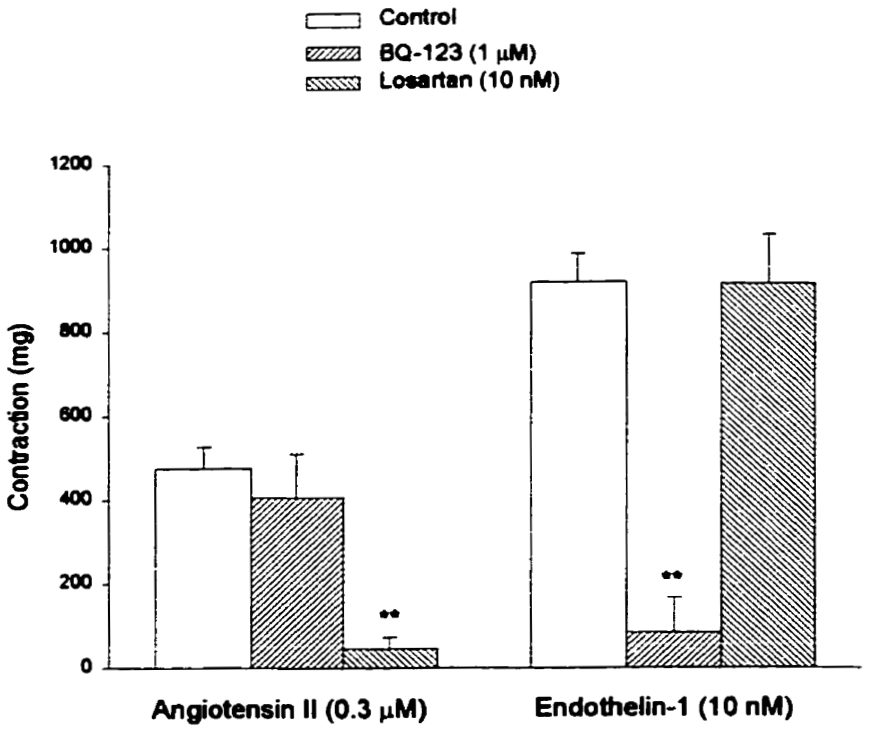
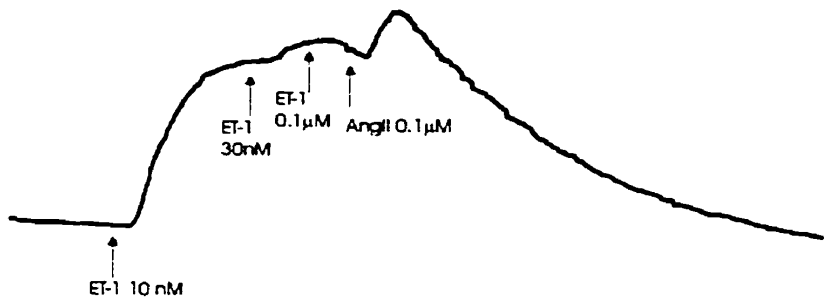

Control
BQ-123 (1 μM)
Losartan (10 nM)
Contraction (mg)
1200
1000
800
600
400
200
0
**
**
Angiotensin II (0.3 μM)
Endothelin-1 (10 nM)

Fig 11. Synergistic effect of Ang II (0.1 μM) on ET-1 (100 nM)-induced contraction. a. An original tracing of Ang II added on top of the maximal response to ET-1. b. Summary of nine experiments. ** P<0.01 Ang II added on top of the maximal response to ET-1 *vs.* ET-1 alone using Student's paired *t*-test.

a


ET-1
30nM
ET-1
0.1μM
AngII 0.1μM
ET-1 10 nM

b

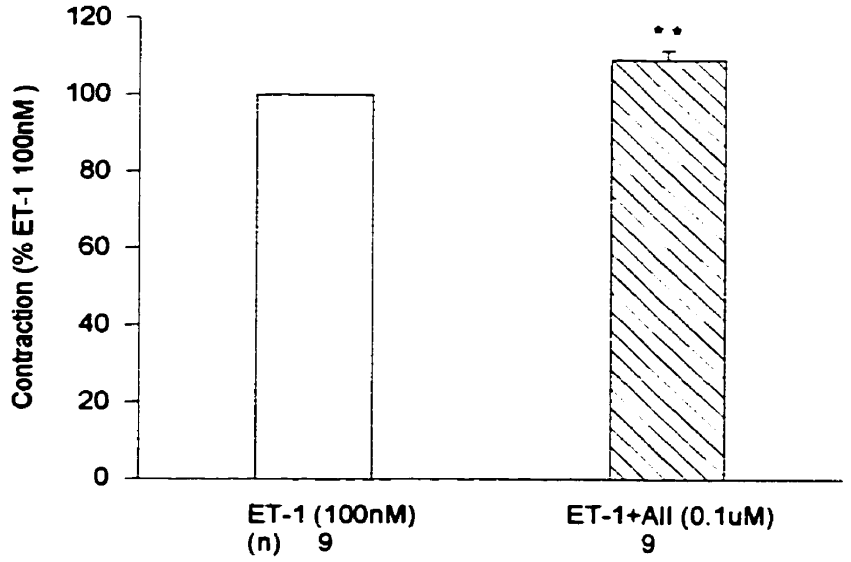

Contraction (% ET-1 100nM)
120
100
80
60
40
20
0
**
ET-1 (100nM)
(n) 9
ET-1+AII (0.1uM)
9

Fig 12. Ang II-induced increase in $[Ca^{2+}]_i$ of freshly dispersed single smooth muscle cells from rat tail artery. A. A typical time-course for Ang II (10 nM) elicited $[Ca^{2+}]_i$ response. B. Concentration response curve for Ang II composed of six different single doses from different cells. The results were expressed as change ratio of 340 to 380 nm (the peak ratio of 340 to 380 minors the background ratio of 340 to 380). Data represent the mean ± S.E.M. n=3-12.

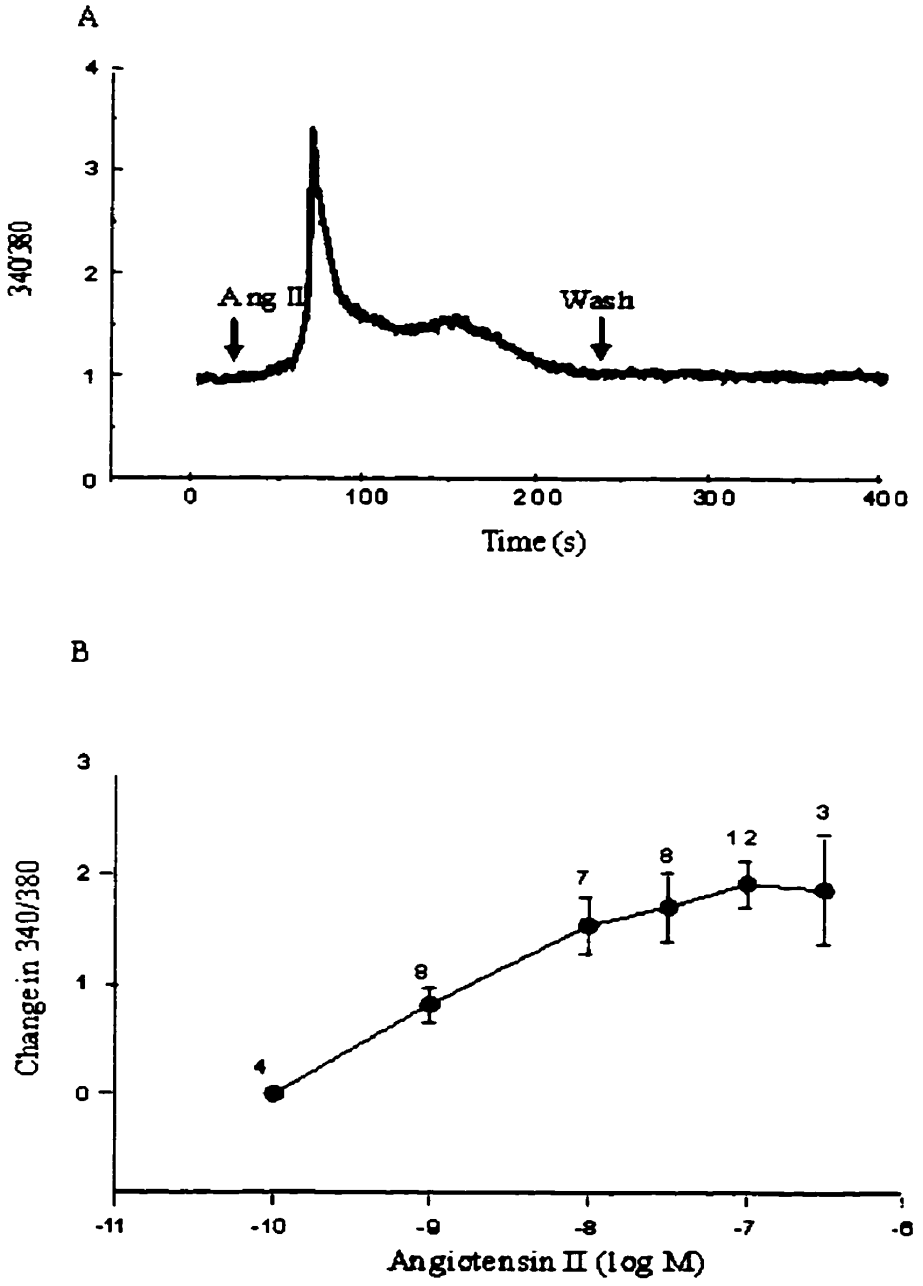
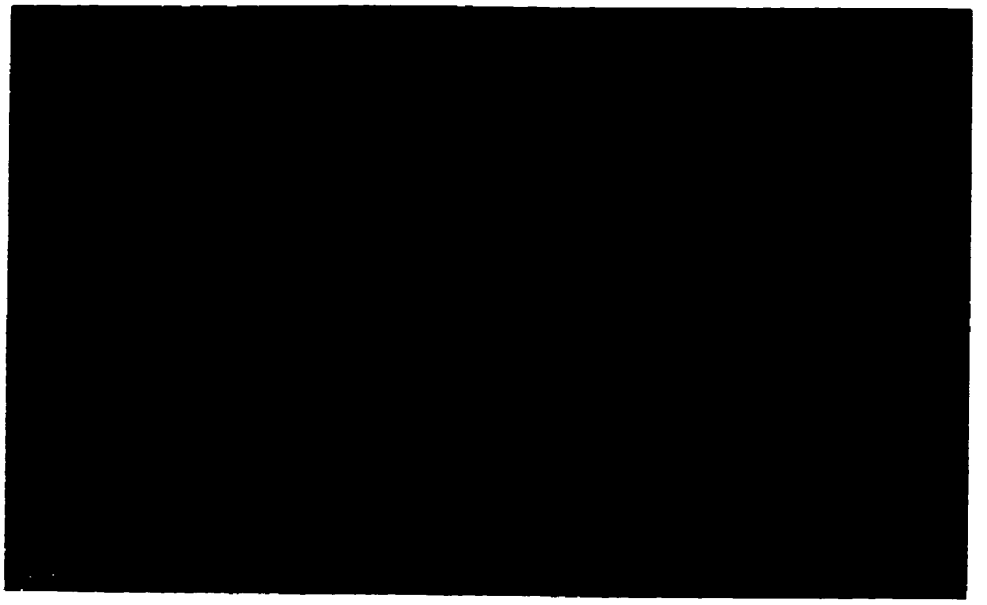
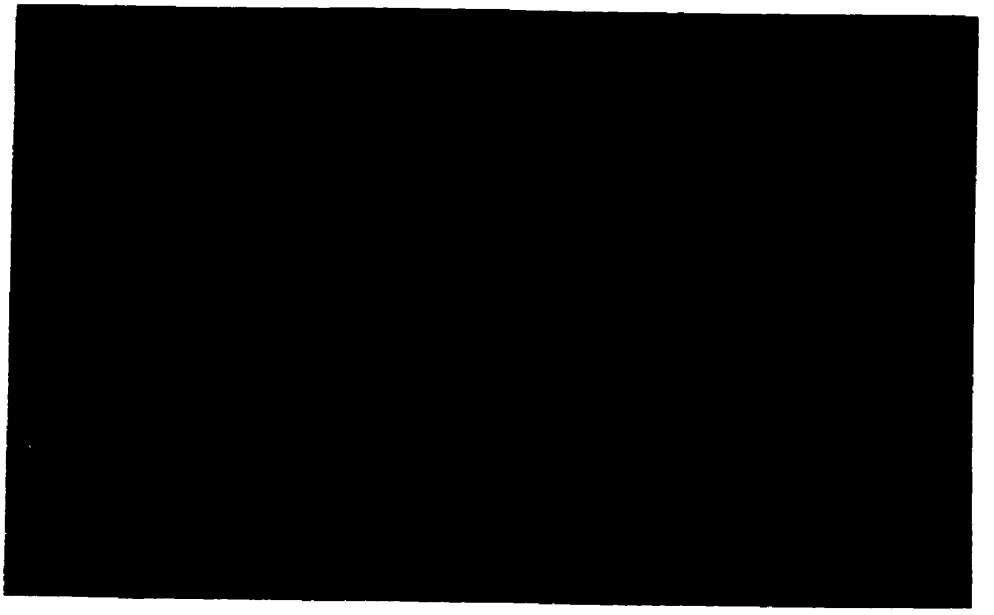

A
340/380
Ang II
Wash
Time (s)
B
Change in 340/380
Angiotensin II (log M)
4
8
7
8
12
3

Fig 13. Photographs of single smooth muscle cell: relaxed to contracted. A. Before adding Ang II; B. after adding Ang II. Scale bars: 30 μm.

A



B



increase in $[Ca^{2+}]_i$ was inhibited (Fig 14a & b). In contrast, a high concentration of 0.1 μM Ang II did not induce an increase in $[Ca^{2+}]_i$ in endothelial cells whereas in the same endothelial cells, acetylcholine (10 μM) induced a significant increase in $[Ca^{2+}]_i$ (Fig 15a & b).

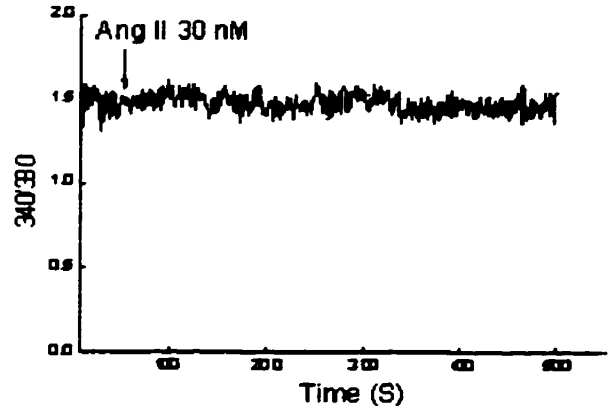
Part 2. Studies of Ang II- and ET-1-mediated signal transduction mechanisms in VSM

2.1. Comparison of the Ca^{2+} stores utilized by Ang II and ET-1 in ring preparations of the rat tail artery

Ang II (0.3 μM) induced a non-sustained contraction in Ca^{2+}-free PSS and the restoration of external Ca^{2+} (2 mM) did not produce contraction (Fig 16a). In normal PSS, pretreatment with nifedipine (1 μM), which is a selective inhibitor of L-type calcium channel, did not inhibit the response to Ang II (Fig 16b), but pretreatment with caffeine (2 mM), which binds to RyR to release calcium and depletes SR calcium, almost abolished the response (Fig 16c). Pretreatment with thapsigargin (TG, 1 μM), a selective inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), for one hour, then replacement with 0 Ca^{2+} plus 1 mM EGTA and TG for 5 minutes, also abolished the Ang II-induced contraction (Fig 16d). Conversely, ET-1 did not induce contraction in Ca^{2+}-free PSS but the restoration of external Ca^{2+} (2 mM) produced a measurable contraction (Fig 17a). Moreover, ET-1-mediated contraction was inhibited by Ni^{2+} (2 mM), a non-selective Ca^{2+} channel blocker or SK&F96365 (10 μM), a receptor operated Ca^{2+} channel blocker

Fig 14. Effect of losartan on Ang II-induced $[Ca^{2+}]_i$ increase in freshly dispersed single smooth muscle cells from rat tail artery. After incubation with losartan for 20 min., (a). 0.1 μM losartan totally abolished Ang II-induced increase in $[Ca^{2+}]_i$ (n=4 cells); (b). 0.01 μM losartan produces incomplete inhibition (n=3 cells). Each tracing represents individual tracing.

a. Losartan (0.1 μM) treated SMC



b. Losartan (10 nM) treated SMC

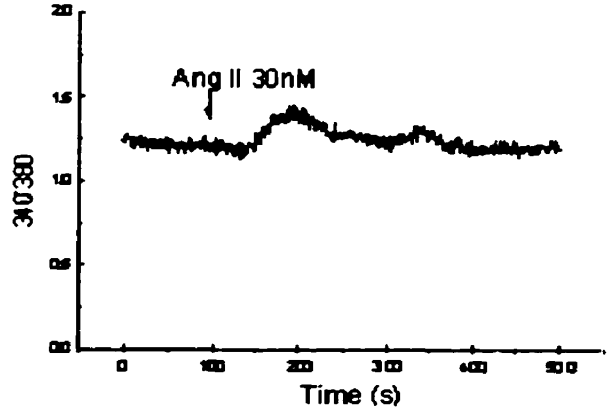


Fig 15. Effect of Ang II on $[Ca^{2+}]_i$ in endothelial cells. ECs were freshly isolated from the rat tail artery using the same process described for SMC isolation. Neither Ang II (0.1 μM) nor ET-1 (1 nM) induced an increase in $[Ca^{2+}]_i$ of endothelial cells, whereas in the same endothelial cells acetylcholine (10 μM) induced an increase in $[Ca^{2+}]_i$. The acetylcholine-induced increase in $[Ca^{2+}]_i$ of endothelial cell had two patterns: a. transient, observed in five of seven experiments; b. tonic which can be returned to baseline with PSS wash or reversed by atropine (10 μM), observed in two of seven experiments. au = arbitrary units (the ratio of fluorescence intensity at 340 nm to 380 nm).

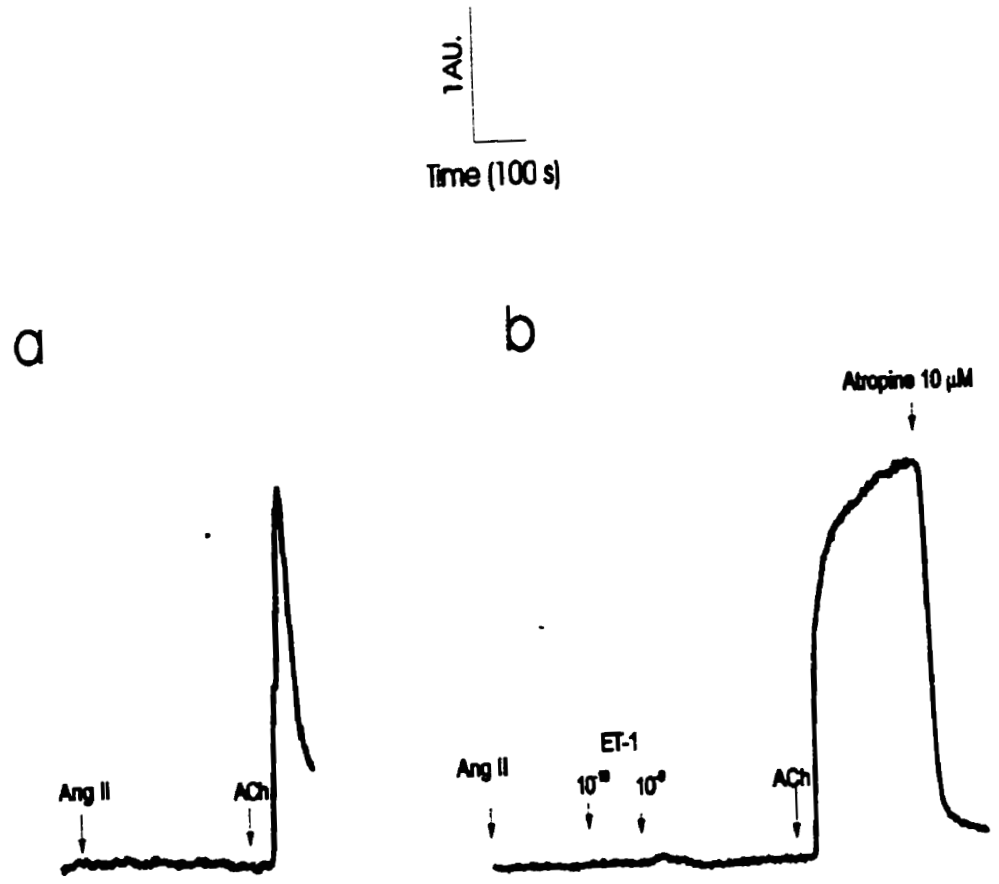
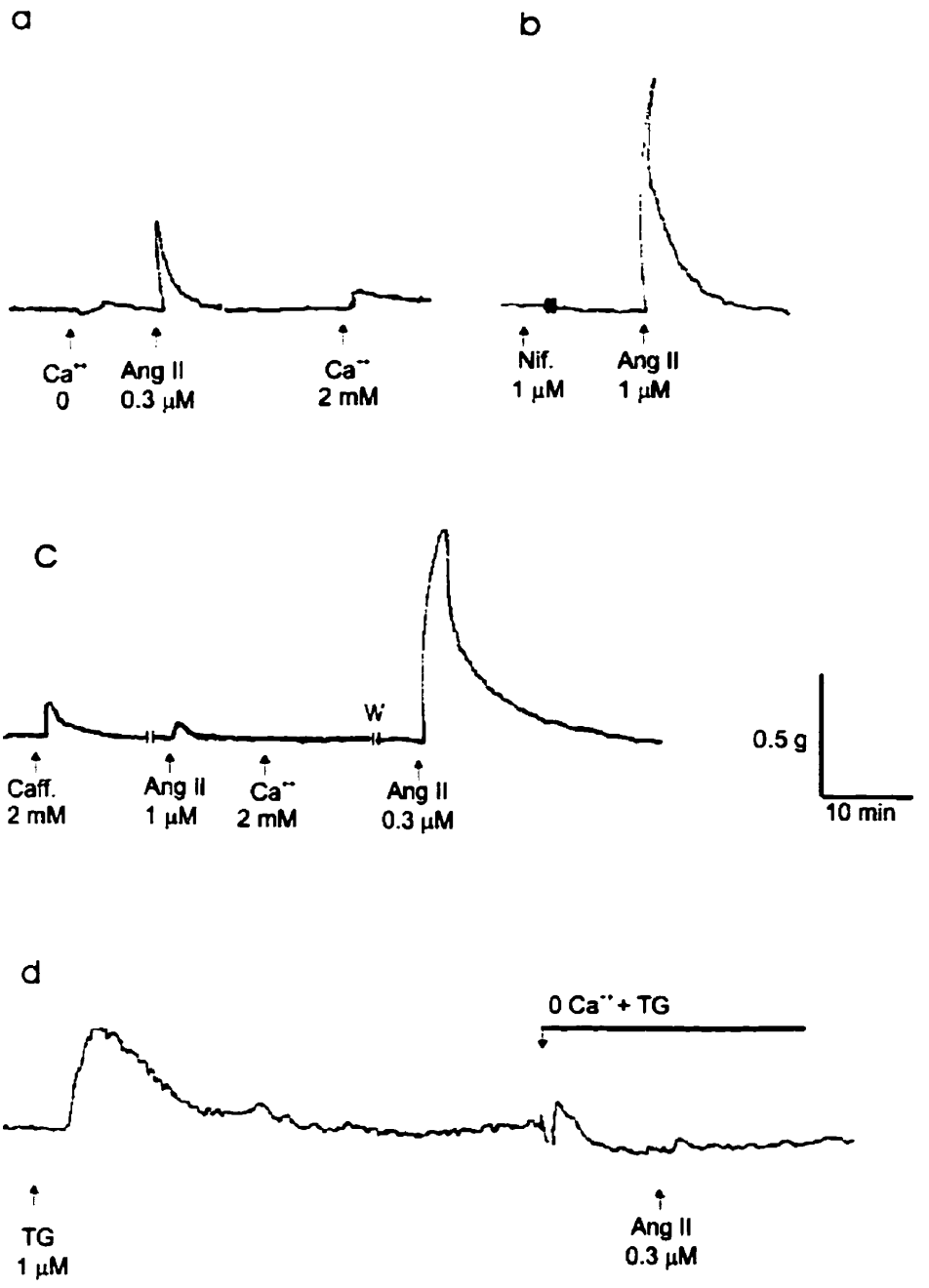

1AU.
Time (100 s)
a
Ang II
ACh
b
Atropine 10 µM
ET-1
Ang II
ACh

Fig 16. Sources of Ca^{2+} for Ang II-induced contraction of the rat tail artery. Tracings show the following conditions: a. Exposure to Ca^{2+}-free PSS and the restoration of external Ca^{2+} (2 mM) did not produce measurable contraction; b. In normal PSS after pretreatment with nifedipine (1 μM) Ang II induced a contraction; c. Pretreatment with caffeine (2mM) almost abolished the response to Ang II; d. Pretreatment with thapsigargin (TG, 1 μM) for one hour, then replacement by 0 Ca^{2+} plus 1 mM EGTA with TG for 5 minutes also abolished the Ang II-induced response.


a
Ca++
0
Ang II
0.3 μM
Ca++
2 mM
b
Nif.
1 μM
Ang II
1 μM
c
W
Caff.
2 mM
Ang II
1 μM
Ca++
2 mM
Ang II
0.3 μM
0.5 g
10 min
d
0 Ca++ + TG
TG
1 μM
Ang II
0.3 μM

(Fig 17b & c). Nifedipine (1 μM) or verapamil (10 μM) only partially inhibited ET-1-induced contraction (Fig 17d & e). Tissues were also incubated with SK&F96365 or nifedipine for 20 min. to compare their effects on Ang II- or ET-1-induced vasoconstriction. Neither nifedipine or SK&F96365, affected Ang II-induced contraction (Fig 18). Conversely, nifedipine partially reduce the ET-1-induced contraction, whereas SK&F96365 significantly inhibited the contraction (Fig 19).

2.2. Comparison of the Ca^{2+} sources utilized by Ang II and ET-1 in single rat tail artery cells

Figure 20 illustrates that in freshly dispersed smooth muscle cells from the rat tail artery Ang II (0.1 μM) increased $[Ca^{2+}]_i$ in normal PSS (Fig 20a) and Ca^{2+}-free PSS (Fig 20b) but did not induce an increase in $[Ca^{2+}]_i$ after pretreatment of the cell with 5 mM caffeine, which transiently increased $[Ca^{2+}]_i$ (Fig 20c). Similarly, after superfusion for 10 minutes with 10 μM cyclopiazonic acid (CPA), a SERCA inhibitor (as a competitive inhibitor of ATP binding), Ang II did not induce an increase in $[Ca^{2+}]_i$ (Fig 20d). In contrast, ET-1 (0.1 μM) increased $[Ca^{2+}]_i$ in normal PSS (Fig 21a), but not in Ca^{2+}-free PSS (Fig 21b). After superfusion of the single VSMC for 10 minutes with 10 μM CPA, ET-1 increased $[Ca^{2+}]_i$ (Fig 21c). Similarly, after the pretreatment of the VSMC with 5 mM caffeine, ET-1 increased $[Ca^{2+}]_i$ (Fig 21d).

2.3. Studies of the signal transduction pathway(s) for Ang II-induced contraction in rat tail artery

Figure 22 illustrates the effects of a variety of pharmacological agents on Ang II-induced

Fig 17. Effects of various treatments on ET-1-induced contraction of rat tail artery. Tracings illustrate the following conditions: a. In Ca^{2+}-free PSS but subsequent restoration of the external Ca^{2+} (2mM) produced a measurable contraction; b & c. ET-1-mediated contraction was inhibited by Ni^{2+} (2 mM) or SK&F96365 (10 μM); d & e. Nifedipine (1 μM) or verapamil (10 μM) only partially inhibited the ET-1-induced contraction.

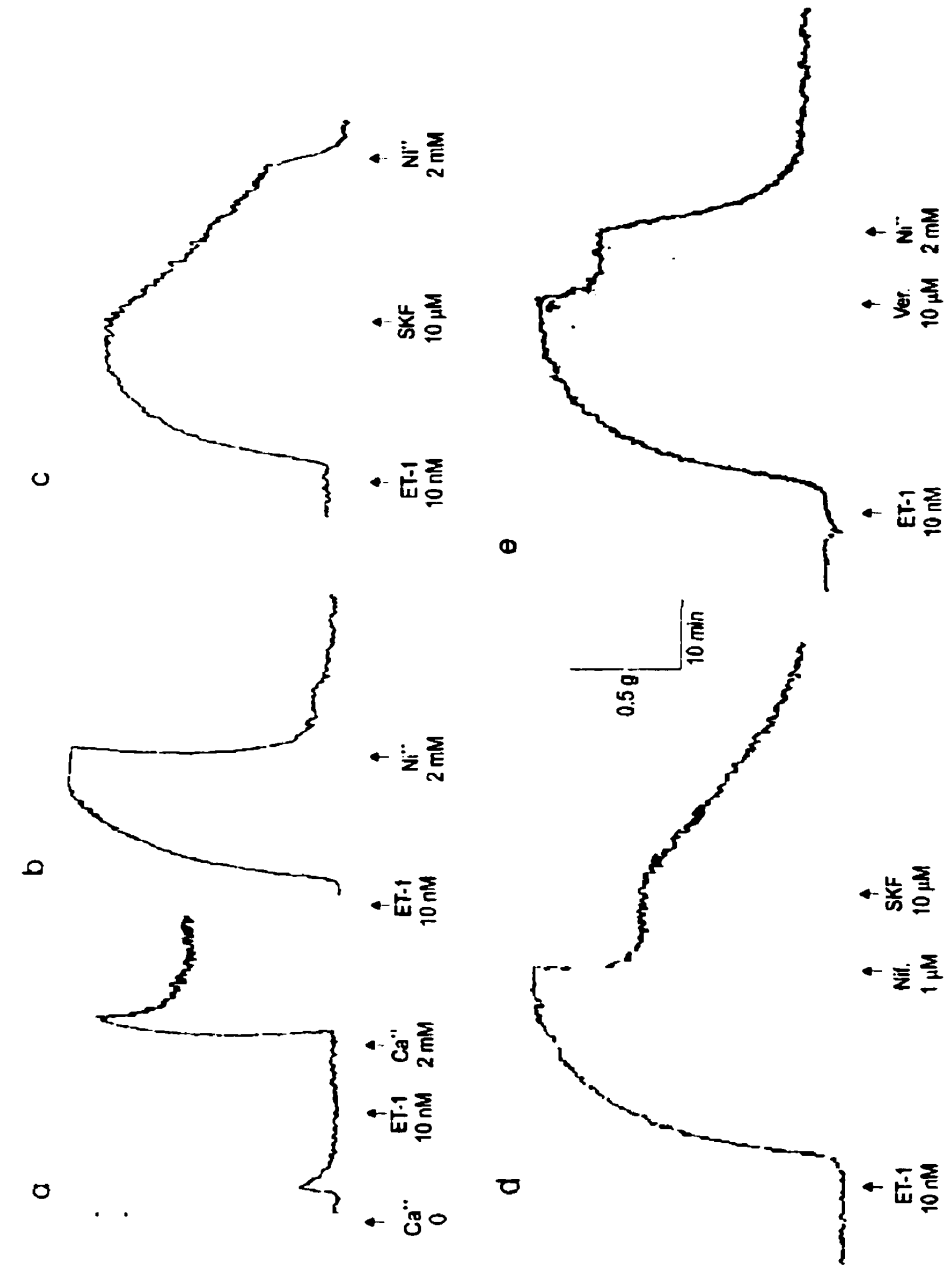

a
Ca++ 0
ET-1 10 nM
Ca++ 2 mM
b
ET-1 10 nM
Ni++ 2 mM
c
ET-1 10 nM
SKF 10 µM
Ni++ 2 mM
d
ET-1 10 nM
Nif. 1 µM
SKF 10 µM
e
ET-1 10 nM
Ver. 10 µM
Ni++ 2 mM
0.5 g
10 min

Fig 18. Effects of nifedipine and SK&F96365 on Ang II (0.1 μM)- induced contraction of rat tail artery rings. Ang II-induced contraction was affected by neither preincubation with nifedipine (1 μM), nor pretreatment with SK&F96365 (10 μM). n = 4, $P < 0.05$ *vs* control using ANOVA.

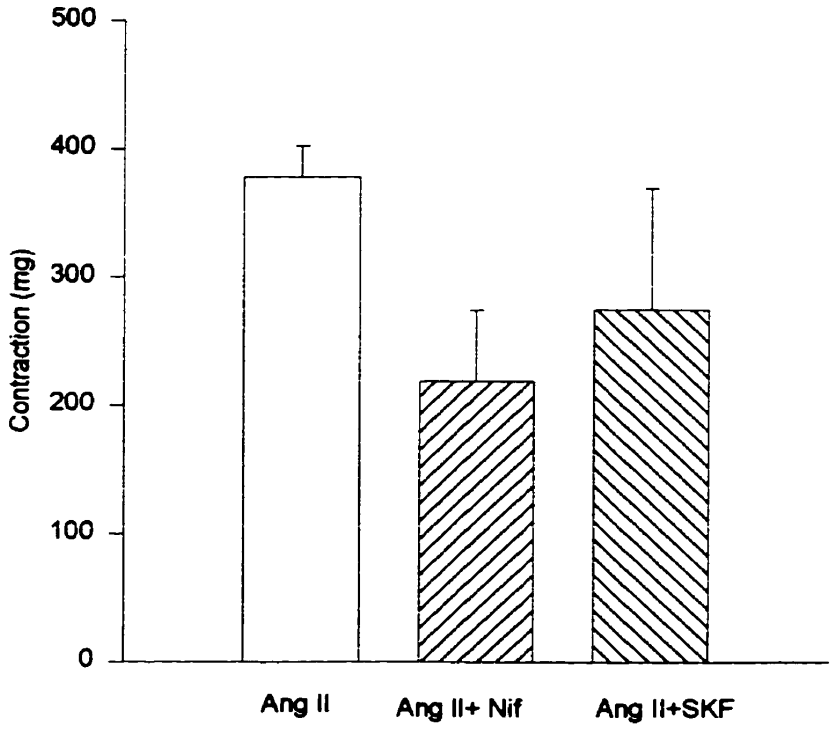

500
400
300
200
100
0
Contraction (mg)
Ang II
Ang II+ Nif
Ang II+SKF

Fig 19. Effects of nifedipine and SK&F96365 on ET-1 (10 nM)- induced contraction of rat tail artery. ET-1-induced contraction was significantly reduced by preincubation with SK&F96365 (10 μM), but was not affected by pretreatment with nifedipine (1 μM). n=4. **, $P< 0.01$ *vs* control using ANOVA.

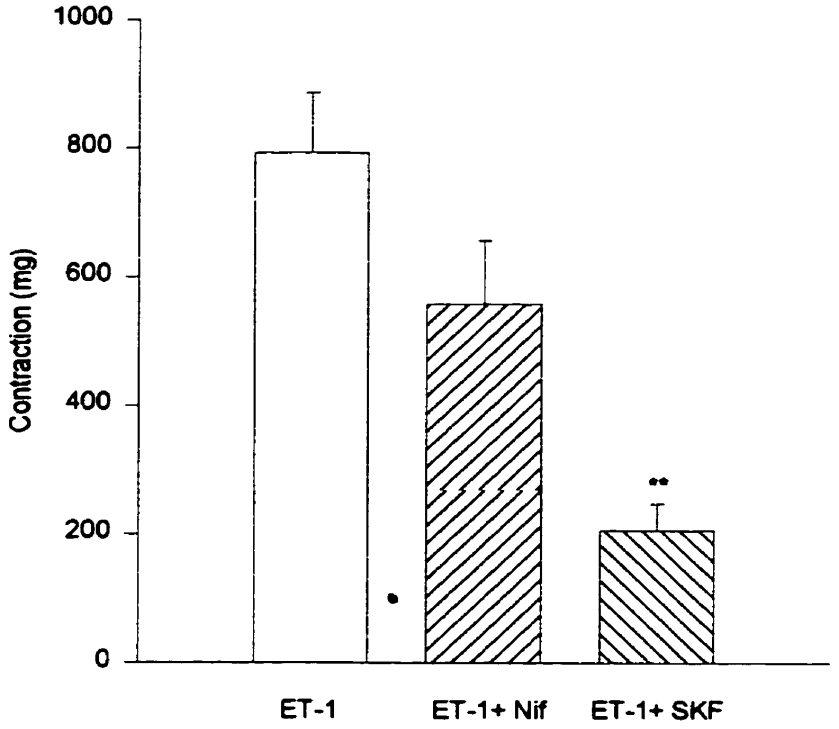

1000
800
600
400
200
0
Contraction (mg)
•
••
ET-1
ET-1+ Nif
ET-1+ SKF

Fig 20. Effects of different treatments on Ang II-mediated $[Ca^{2+}]_i$ increase in freshly dispersed single smooth muscle cells from rat tail artery. The original tracings illustrate the following conditions: a. in normal PSS; b. in Ca^{2+}-free PSS; c. in normal PSS after pretreatment with 5 mM caffeine; d. after superfusion with 10 μM cyclopiazonic acid (CPA). In each condition similar results were observed from 3 to 7 experiments.

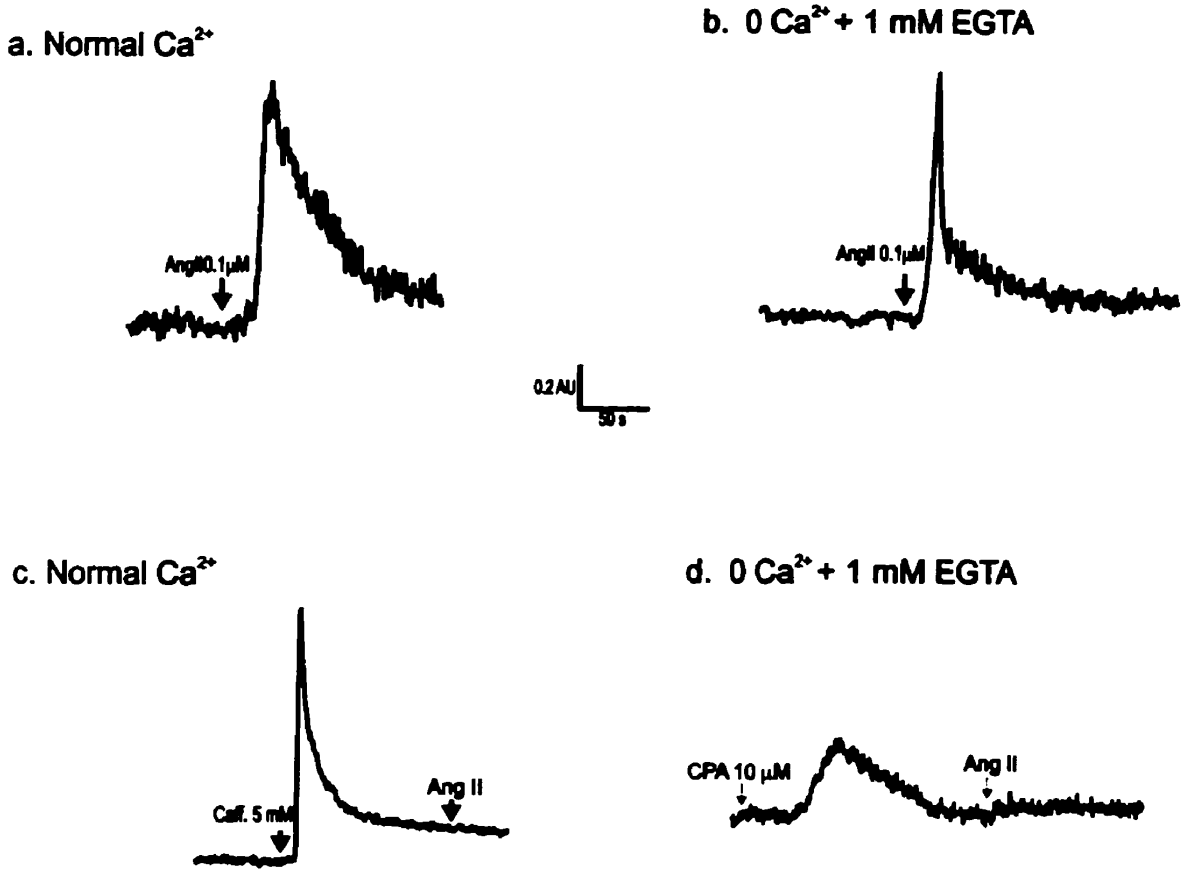

a. Normal Ca2+
AngII0.1μM
b. 0 Ca2+ + 1 mM EGTA
AngII 0.1μM
0.2 AU
50 s
c. Normal Ca2+
Calf. 5 mM
Ang II
d. 0 Ca2+ + 1 mM EGTA
CPA 10 μM
Ang II

Fig 21. Effects of cyclopiazonic acid and caffeine on ET-1-mediated $[Ca^{2+}]_i$ increase in freshly dispersed single smooth muscle cells from rat tail artery. Tracings illustrate the following conditions: a. in normal PSS; b. in Ca^{2+}-free PSS; c. after superfusion with 10 μM cyclopiazonic acid (CPA) 10 min., d. after pretreatment with 5 mM caffeine. In each condition similar results were observed from 3 to 5 experiments.

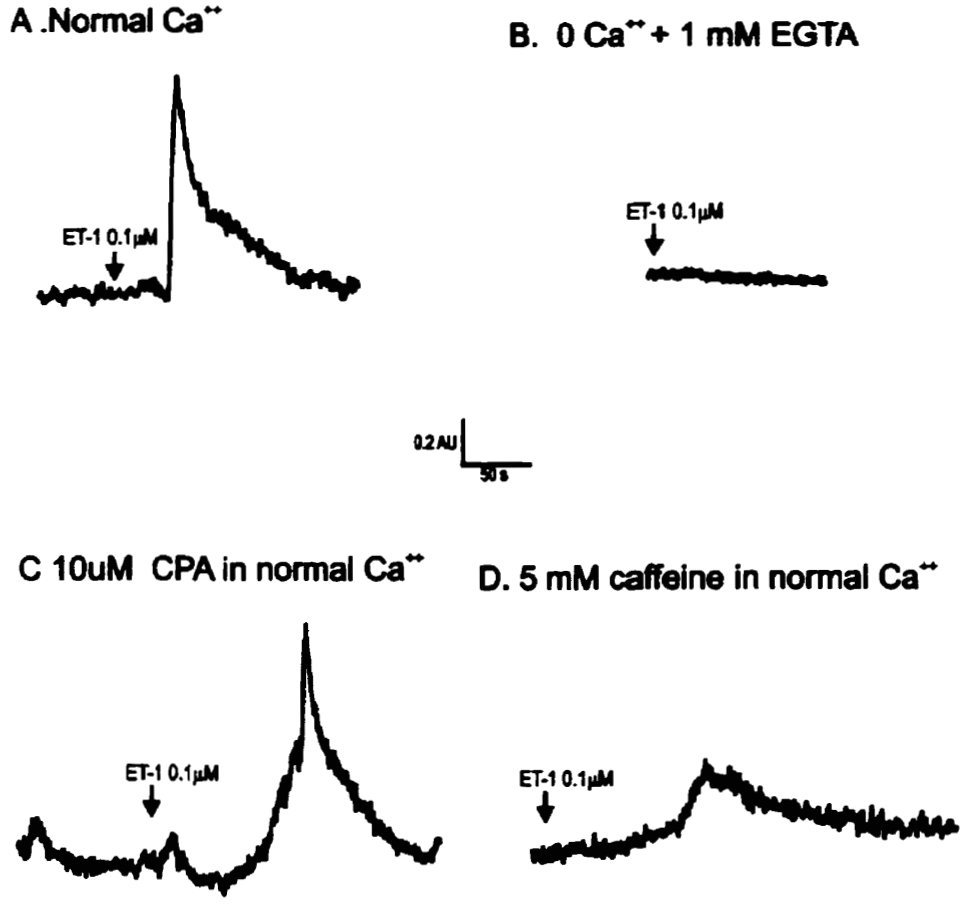
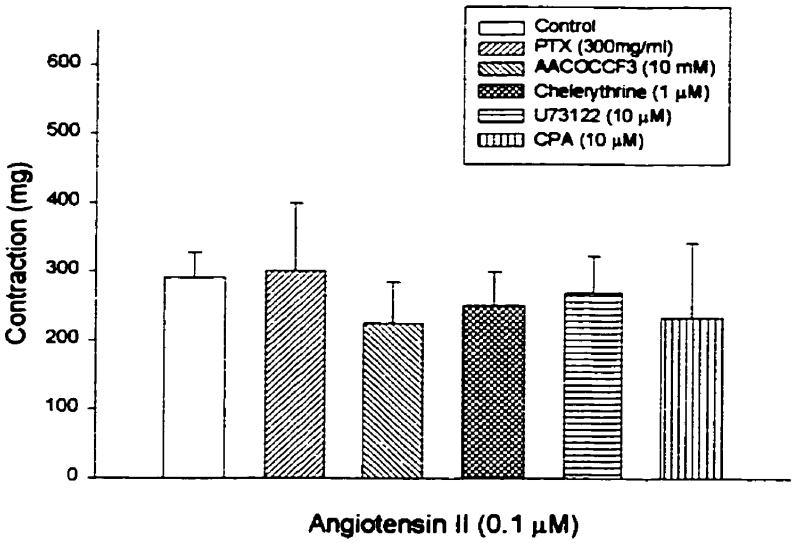
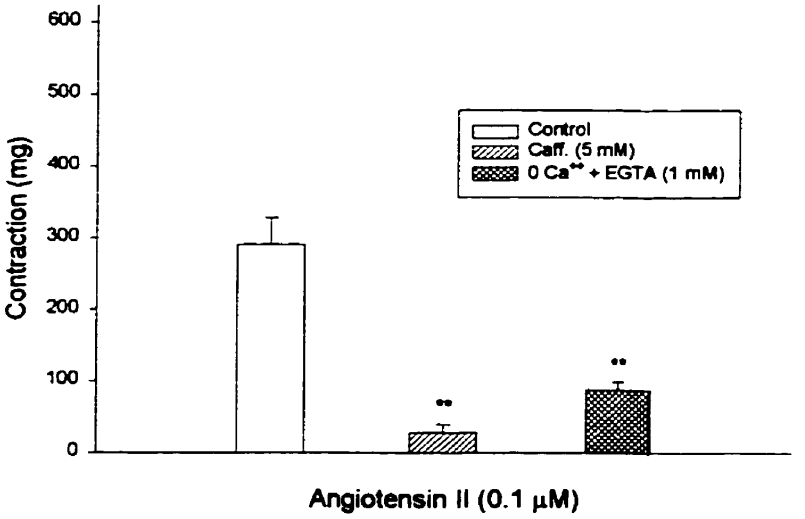

A .Normal Ca++
B. 0 Ca++ + 1 mM EGTA
ET-1 0.1μM
ET-1 0.1μM
0.2 AU
50 s
C 10uM CPA in normal Ca++
D. 5 mM caffeine in normal Ca++
ET-1 0.1μM
ET-1 0.1μM

Figure 22. Pharmacological analysis of Ang II-induced contraction pathway. Treatment illustrated in the text box. PTX: a Gi,Go,Gt-protein inhibitor; ACOCCF: a phospholipase A_2 inhibitor; U73122: a phospholipase C inhibitor; chelerythrine: a PKC inhibitor; CPA: a SERCA inhibitor. Data are shown as the means ± S.E.M. n = 3-20. **, $P < 0.01$ *vs* control using ANOVA.


Control
PTX (300mg/ml)
AACOCCF3 (10 mM)
Chelerythrine (1 μM)
U73122 (10 μM)
CPA (10 μM)
Contraction (mg)
600
500
400
300
200
100
0
Angiotensin II (0.1 μM)


Control
Caff. (5 mM)
0 Ca++ + EGTA (1 mM)
Contraction (mg)
600
500
400
300
200
100
0
**
**
Angiotensin II (0.1 μM)

contractions. Only preincubation with caffeine and/or treatment of the tissue with Ca^{2+} free PSS significantly altered the Ang II-induced contraction. The role of calcium-activated chloride channels in Ang II-induced contractions was studied using the calcium-activated chloride channel blocker, niflumic acid (30-100 μM) (Large & Wang, 1996). Ang II-induced contraction was not inhibited by either 30 or 50 μM (Fig 23), but was completely blocked by 100 μM niflumic acid (data not shown).

Part 3. Is there a synergistic interaction between Ang II- and ET-1-mediated vasoconstriction?

To address this question, the following protocols were used: 1). prior to addition of Ang II (0.1μM) or ET-1 (10 nM), the tissues were exposed to subcontractile concentration of ET-1 (0.1 nM) or Ang (10 nM), respectively; or 2). After Ang II (0.1μM)- or ET-1 (10 nM)-induced contraction returned to baseline, the tissue was exposed to ET-1 (10 nM) or Ang II (0.1μM). These data indicate that pre-incubation of the tissue with a contractile concentration ET-1 potentiates Ang II-induced vasoconstriction(Fig 24 A& B).

Part 4. Interactions of AngII with other vasoconstrictors

4.1. AngII and ET-1

The above data indicates that Ang II and ET-1 utilize different signalling pathway(s) to evoke contraction. Of interest is the observation that after an ET-1-induced contraction had returned to baseline, AngII evoked a much stronger contraction of the tissue (Fig 24A & B).

4.2. Ang II receptor activation and tyrosine kinases

Fig 25 shows the effects of different tyrosine kinase inhibitors on Ang II-induced contractions. 5μM genistein had no effect on AngII-induced contraction, although at this

Fig 23. Effects of niflumic acid on Ang II-induced contraction. Pretreatment of rat tail artery with niflumic acid (30 and 50 μM), a calcium-activated chloride blockor, for 20 minutes, did not block Ang II-induced contraction, but 100 μM completely abolished Ang II-induced contraction (not shown). Data are shown as the means ± S.E.M.. n = 5-7.

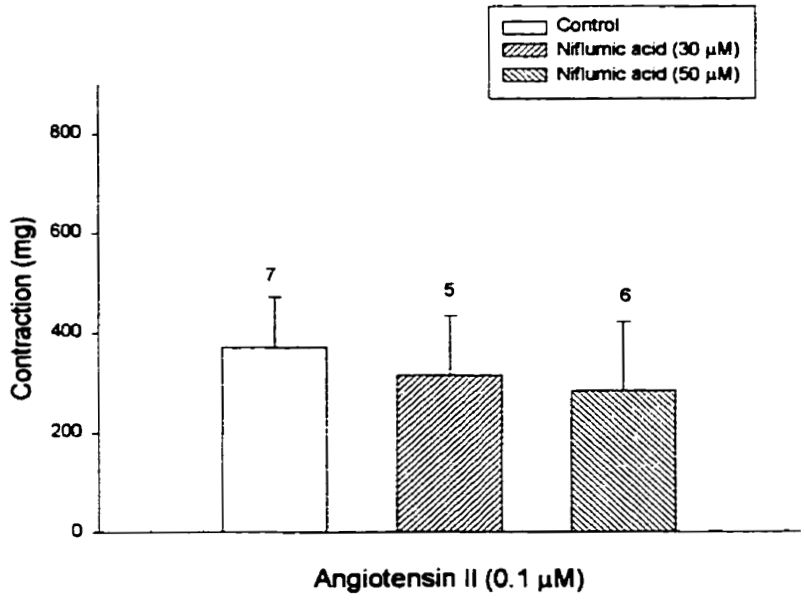
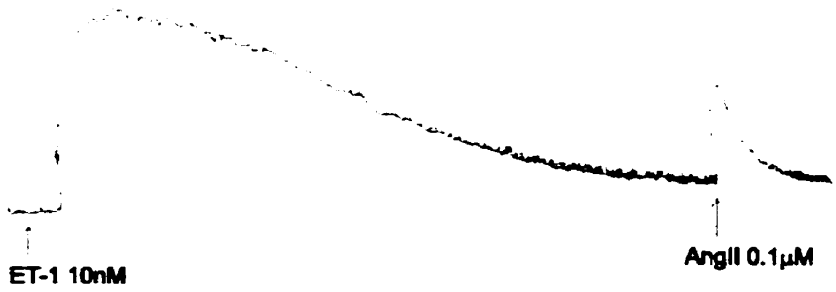

Control
Niflumic acid (30 μM)
Niflumic acid (50 μM)
800
600
400
200
0
Contraction (mg)
7
5
6
Angiotensin II (0.1 μM)

Fig 24. Interaction between Ang II- and ET-1-mediated contractions in rat tail artery. A. An original tracing shows Ang II-induced contraction subsequent to ET-1-induced contraction returning to baseline; B. Different protocols used to test interaction between Ang II- and ET-1-induced contraction: 1). Control: 2). After addition of subcontractile concentration of ET-1 (0.1 nM); 3). After ET-1 (10 nM)-induced contraction returned to baseline. n=5-18. The data are shown as the means ± S.E.M. **, $P < 0.01$ *vs* control using ANOVA.

A


ET-1 10nM
AngII 0.1μM

B

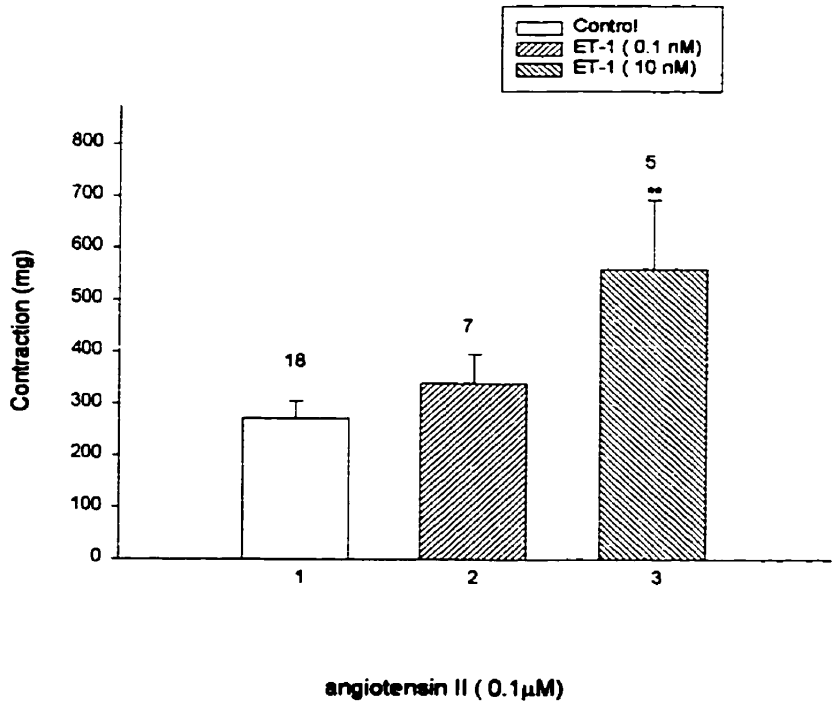
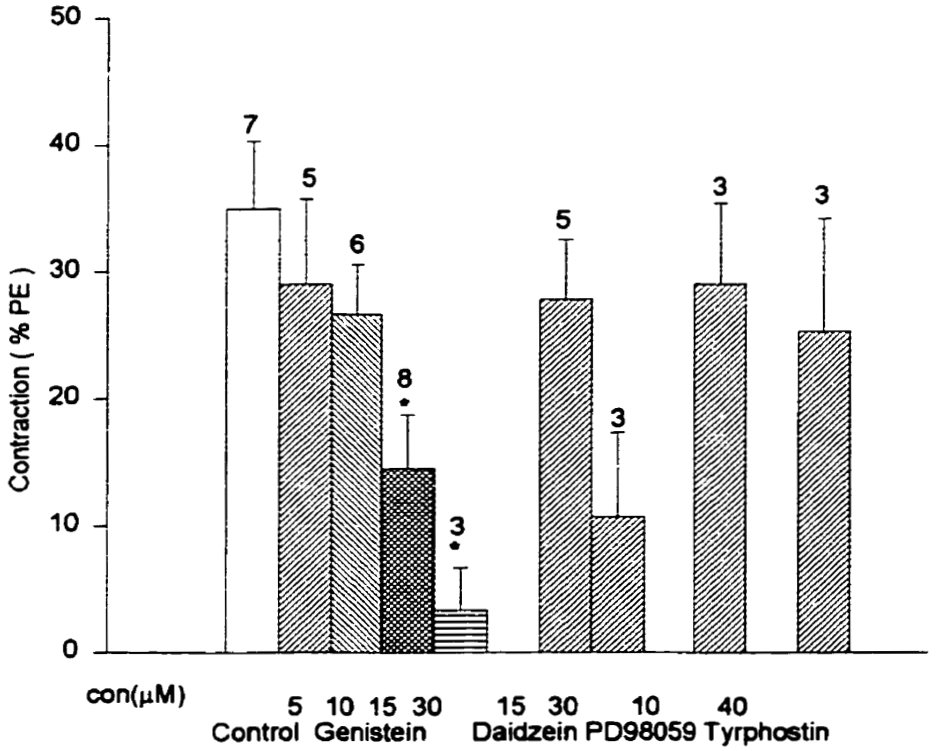
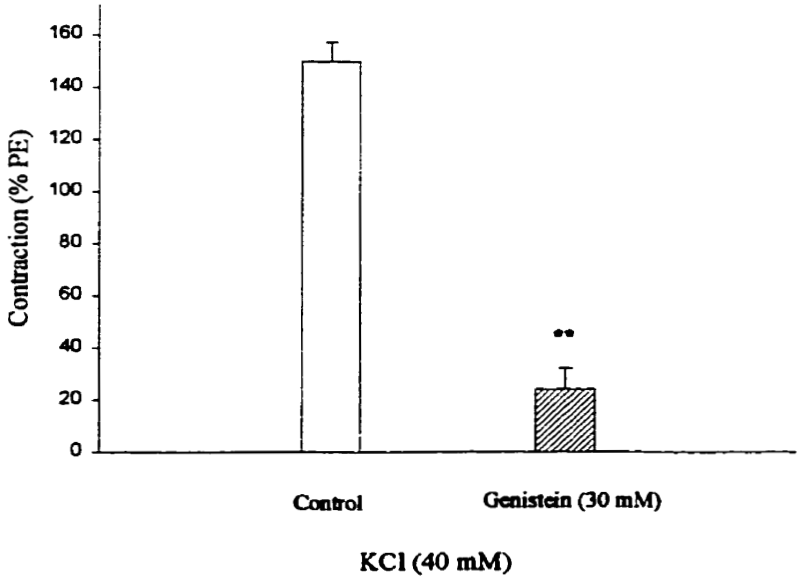

Control
ET-1 (0.1 nM)
ET-1 (10 nM)
Contraction (mg)
800
700
600
500
400
300
200
100
0
18
7
5
**
1
2
3
angiotensin II (0.1μM)

Fig 25. The role of tyrosine kinase activation in Ang II-induced contraction of the rat tail artery. A. Summary are shown the effects of different tyrosine kinases and MEK inhibitors on Ang II-induced contraction. B. The effects of genistein on KCl-induced contraction. The results were expressed as a percentage of tissue response to phenylephrine (1 μM). Each point represents the mean ± S.E.M. n = 3-8.*, $P<0.05$, **, $P<0.01$ *vs* control using ANOVA.

A


Contraction (% PE)
50
40
30
20
10
0
7
5
6
8
*
3
*
5
3
3
3
con(μM)
5 10 15 30
Control Genistein
15 30
Daidzein
10
PD98059
40
Tyrphostin

B


Contraction (% PE)
160
140
120
100
80
60
40
20
0
**
Control
Genistein (30 mM)
KCl (40 mM)

concentration tyrosine kinase has been reported to be inhibited (Laniyonu *et al.*, 1994). Furthermore, 10 μM genistein did not block the Ang II-induced contraction, nor did 40 μM tyrphostin, a tyrosine kinase inhibitor chemically distinct from the flavone-dirived genistein, nor the MEK kinase inhibitor, PD98059 (10 μM). However, 30 μM genistein did significantly reduce the AngII-induced contraction, but it also inhibited KCl-induced contraction of the rat tail artery and the inactive isoform of genistein, daidzein, at a concentration of 30 μM also partially inhibited the Ang II-induced contraction (Fig 25).

4.3 Ang II and NO

There was no significant difference between Ang II-induced contraction in the presence of and absence of N^G-nitro-L-arginine (L-NNA, 100μM) in endothelium-intact tissue (Fig 26). However, in endothelium-intact tissue, L-NNA diminished the Ang II-induced desensitization (Fig 27). A similar result, but enhanced sensitization induced by PE, was observed in PE-induced contraction (Fig 28).

Part 5. AT_1 in VSMC and EC

AT_1 mRNA was detected in both endothelial and smooth muscle cells (Fig 29a). The AT_1 subtypes were identified by RT-PCR analyses using *Eco*RI digestion and DNA sequencing. AT_{1A}, not AT_{1B}, can be digested into two fragments: 268 and 176 bp by *Eco*RI. AT_{1A} and AT_{1B} were detected on both endothelium-intact and -denuded tissues (Fig 29b). DNA sequence matched with rat AT_1 gene from gene bank. The localization of AT_1 in cross section and freshly dispersed SMCs and ECs from the rat tail artery were also examined using immunohistochemical (Fig 30) and immunocytochemical (Fig 31) methods. There are AT_1 receptors stained in Figure 30a, which was treated with rabbit anti-human AT_1 IgG; but

Fig 26. Effect of L-NNA on angiotensin II-induced contraction. After phenylephrine elicited a standard contractile response, the rings were washed two times, the one of the following protocols was used: 1). 200 µl distill water or 2). 200 µl 10^{-2} M L-NNA was added to 20 ml organ bath (100 µM L-NNA) and incubated for 20 min., then Ang II (0.1 µM) was added to the organ baths. Data are shown as the mean ± S.E.M. n = 3-6.

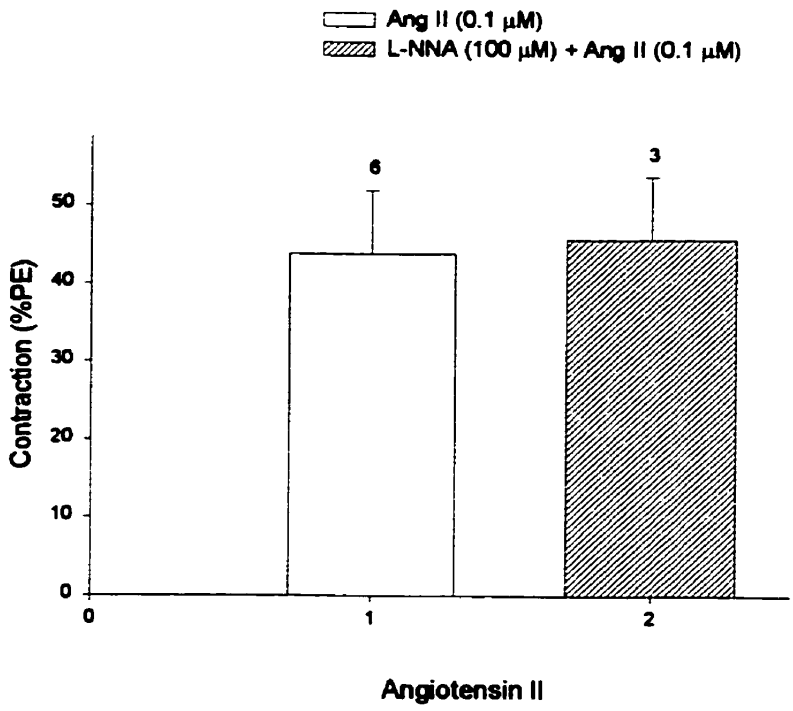

Ang II (0.1 μM)
L-NNA (100 μM) + Ang II (0.1 μM)
6
3
50
40
30
20
10
0
Contraction (%PE)
0
1
2
Angiotensin II

Fig 27. Effect of L-NNA on Ang II-induced desensitization of rat tail artery. 1). Control. After one hour first exposure to Ang II with washing every 20 min., 2). Second exposure to Ang II in the presence of endothelium (E+); 3). Second exposure to Ang II in the absence of endothelium (E-); 4). After incubation with L-NNA for 20 min. in E+; 5). After incubation with L-NNA for 20 min. in E-. Data are shown as percentage of control. n=3-16. *, $P < 0.05$ *vs* control using ANOVA.

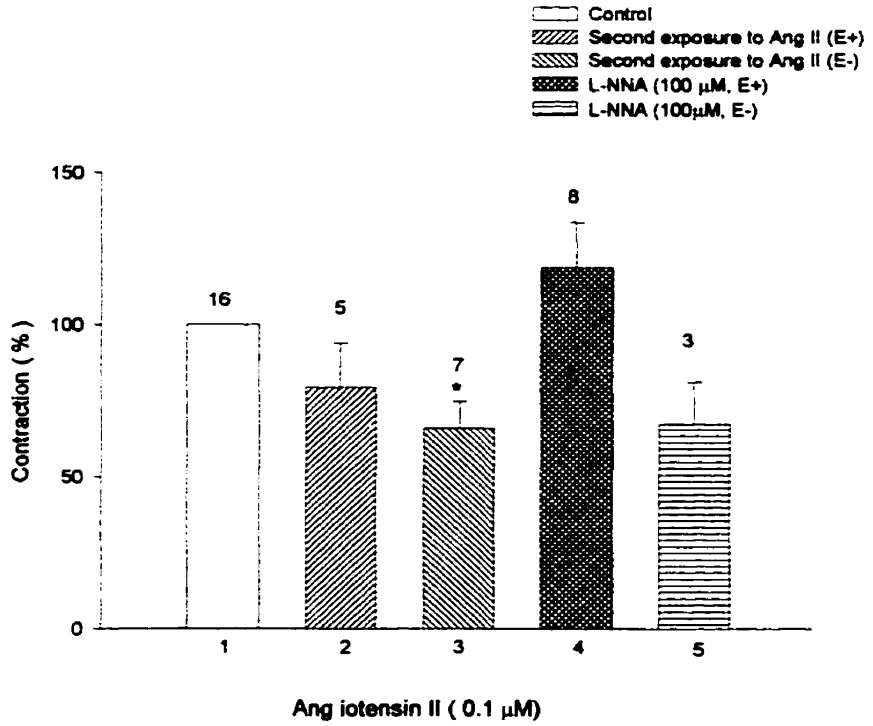

Control
Second exposure to Ang II (E+)
Second exposure to Ang II (E-)
L-NNA (100 μM, E+)
L-NNA (100μM, E-)
150
100
50
0
Contraction (%)
16
5
7
*
8
3
1
2
3
4
5
Ang iotensin II (0.1 μM)

Fig 28. Phenylephrine-induced contraction of rat tail artery. 1). Control; 2). The second exposure to PE; 3). Incubation with L-NNA for 20 min. in the absence of endothelium (E-); 4). Incubation with L-NNA for 20 min. in the presence of endothelium (E+). Data are shown as percentage of control. n=3-6. *, $P < 0.05$, ***, $P < 0.001$ *vs* control using ANOVA.

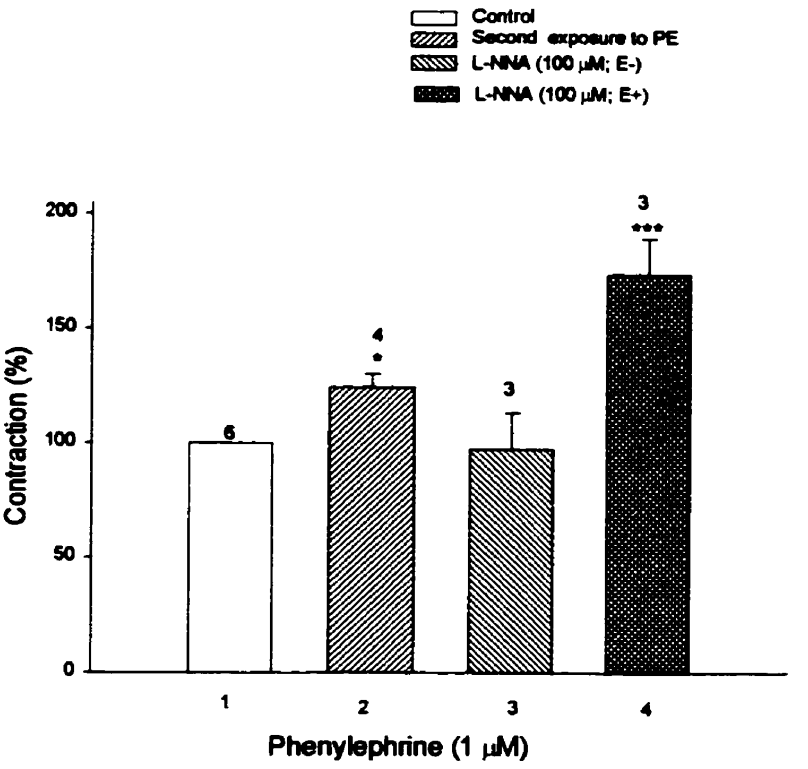
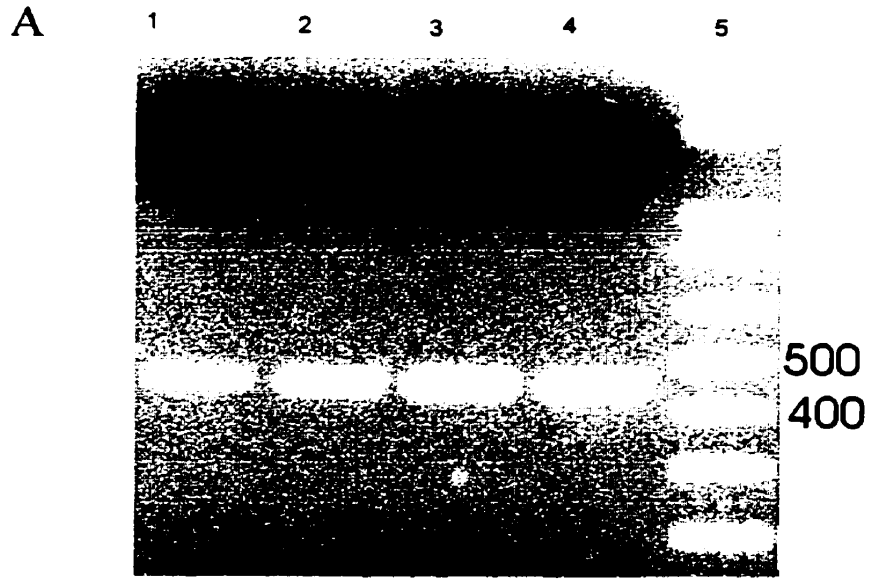

Control
Second exposure to PE
L-NNA (100 µM; E-)
L-NNA (100 µM; E+)
Contraction (%)
200
150
100
50
0
6
4
*
3
3
***
1
2
3
4
Phenylephrine (1 µM)

Fig 29. The expression of AT_1 mRNA in rat tail artery. mRNA was extracted from rat tail artery with and without endothelium, from freshly dispersed SMCs and ECs. RT-PCR products were electrophoresed on an agarose gel (1.5%) and visualized by adding ethidium bromide and photographing under u.v. light. a. Lane 1 denotes endothelium-denuded tail artery; Lane 2 endothelium-intact tail artery; Lane 3 SMCs; Lane 4 ECs and lane 5 1kb plus DNA ladder. b. After *Eco*RI digesting: lane 1 represents 1kb plus DNA ladder, lane 2 endothelium-denuded tail artery, lane 3 endothelium-intact tail artery, lane 4 SMCs. All lanes contain three fragments: 444, 268, and 176 bp.


A
1
2
3
4
5
500
400

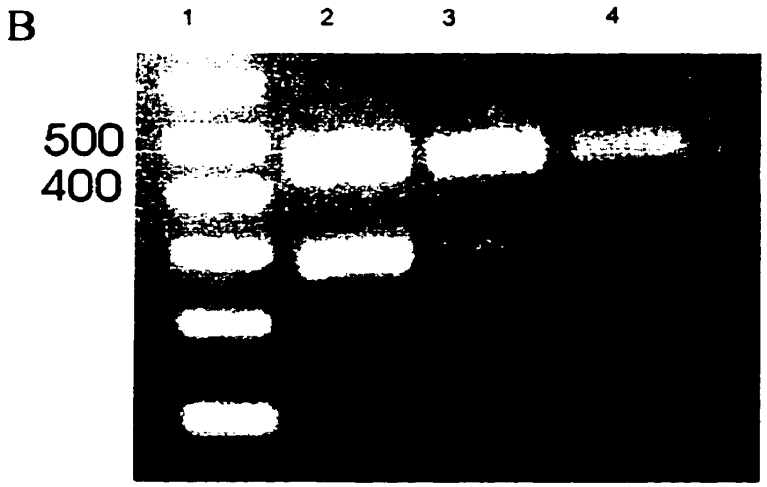

B
1
2
3
4
500
400

Fig 30. Immunohistochemical detection of AT_1 receptor in cross section of the rat tail artery. Cross sections of rat tail artery stained with rabbit anti-human AT_1 IgG and secondary antibody as described in the method. ← points vascular lumen. A. Treated with rabbit anti-human AT_1 IgG; B.Treated with rabbit anti-human AT_1 IgG and blocking peptide; C. Treated with secondary antibody only. Scale bars: 30 μm.

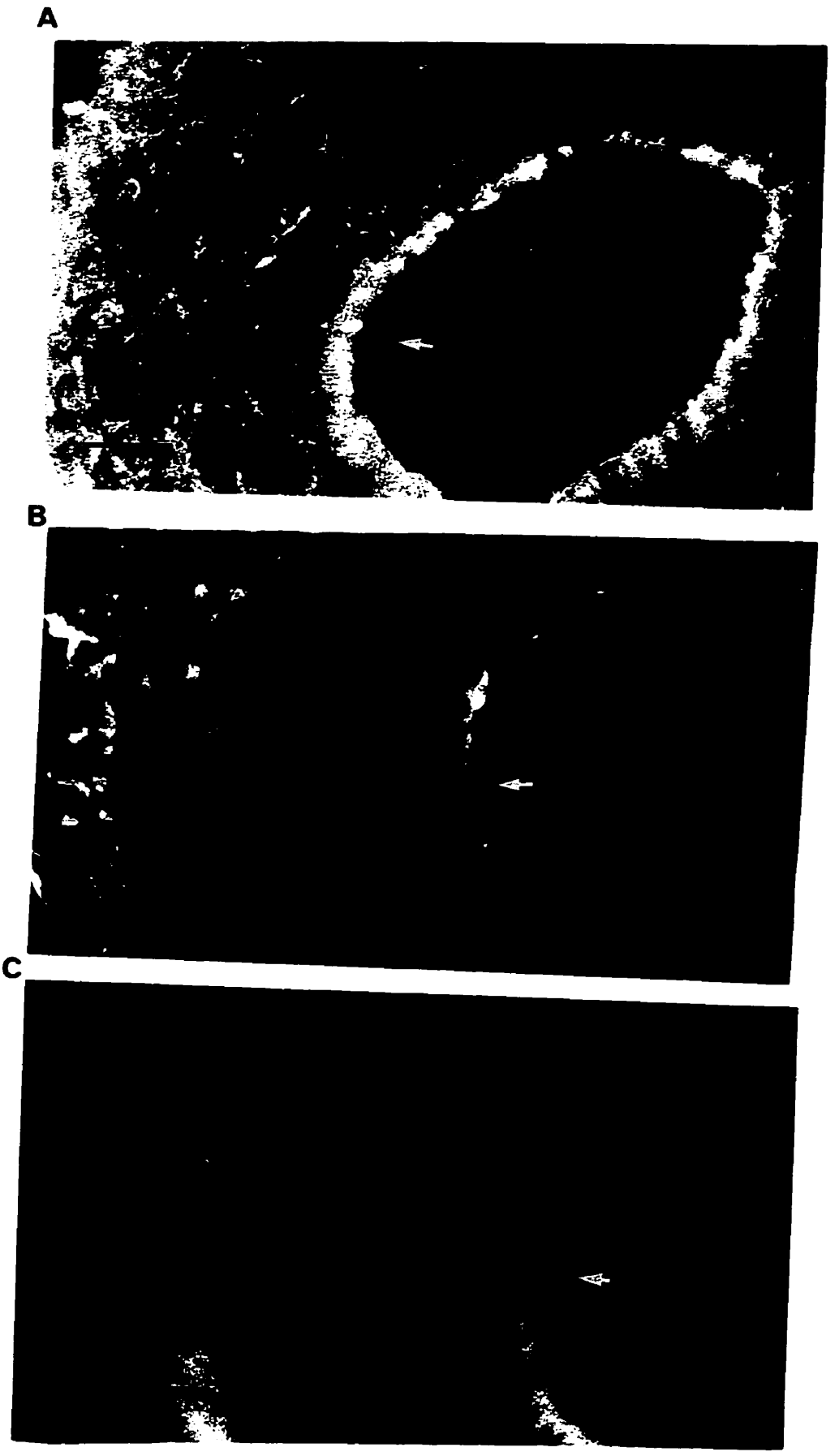
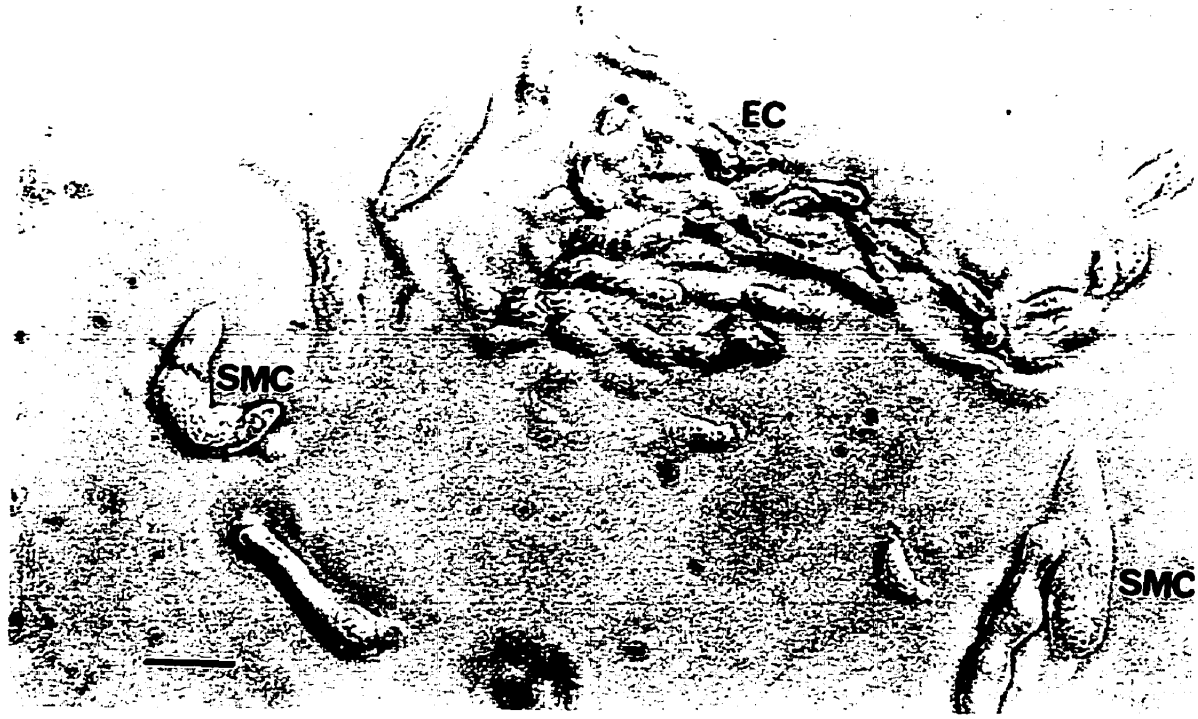

A
B
C

Fig 31. Fluorescent staining of AT_1 receptor on smooth muscle cells, but not on endothelial cells of rat tail artery. Smooth muscle cells and endothelial cells were isolated using the same method as described in the methods. Cells were stained with rabbit anti-human AT_1 IgG in A, B, and C; smooth muscle cell was stained with second antibody alone in D as described in the method 4. A. Photograph taken under bright field; B. Same field as A taken under flourescent light. Scale bars: 20 μm. C. SMC photograph taken under flourescent light. Scale bar: 8 μm. D. SMC photograph taken under flourescent light. Scale bar: 20 μm. SMC: smooth muscle cell; ECs: endothelial cells.

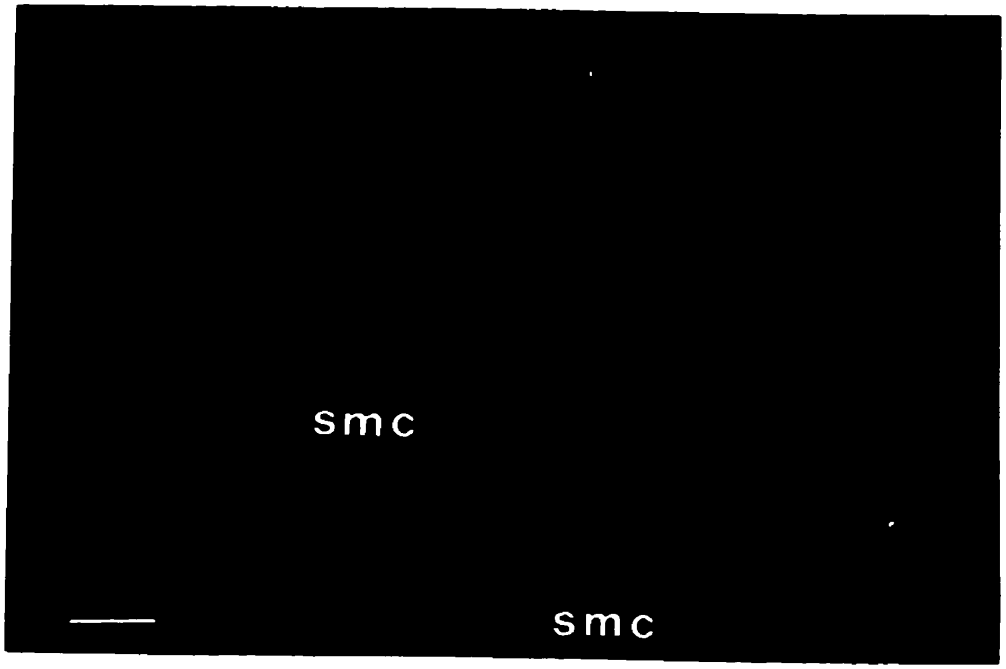
A



B

C

D



not in Figures 30b treated with rabbit anti-human AT_1 IgG and blocking peptide & c treated with secondary antibody only (n=3). In Figure 30 it is hard to tell whether there are any AT_1 receptors stained in the endothelium. However, in Figure 31 there are obviously AT_1 stained on smooth muscle cells but not on endothelial cells.

Chapter Four: Discussion

Part 1. Ang II-induced contraction of the rat tail artery is endothelium-independent

In the present study, I have demonstrated that Ang II-mediated contraction of the rat tail artery was endothelium-independent, and that ET-1 release from the endothelium did not contribute to the vasoconstrictor response of the tissue to Ang II. I have also demonstrated that Ang II- and ET-1-mediated contractions of the rat tail artery utilize distinct signalling pathways. Specifically, Ang II- and ET-1-mediated contractions were antagonized, respectively, by the AT_1 and ET_A selective antagonists, losartan and BQ-123. As well, the Ang II-mediated response was primarily dependent on intracellular Ca^{2+} release whereas that of ET-1 was primarily dependent on extracellular Ca^{2+} entry.

Ang II plays an important role in the regulation of blood pressure. It causes vasoconstriction by direct stimulation of AT_1 on smooth muscle cells (Peach, 1977; Schultz, *et al.*, 1981; de Gasparo *et al.*, 1995). However, experimental evidence suggests that endothelial cells may also be targets for circulating plasma angiotensins, and the presence of Ang II receptors on cultured endothelial cells has been reported by several investigators (Danser, *et al.*, 1995; Patel *et al.*, 1989; Vaughan *et al.*, 1995; Pueyo, *et al.*, 1997). Chen *et al.* (1995a & b) have also reported that there were endothelium-dependent regional variations in the sensitivity of vascular tissue to Ang II. Chen et al (1995a,b) noted that the vasoconstrictor response to

Ang II in the rat aorta involved activation solely of AT_1 located on VSMCs whereas the response in mesenteric artery involved activation of both vascular and endothelial AT_1 receptors. **In contrast, the response to Ang II in the tail artery was dependent on Ang II-mediated release of ET-1 from the endothelial cells and the subsequent activation of vascular smooth muscle ET_A.**

In the present study, I have shown that there were no significant differences in the pEC_{50} and maximal contraction responses to Ang II in endothelium-intact *vs* endothelium-denuded preparations. I have demonstrated that Ang II-mediated vasoconstriction of the endothelium-intact tail artery was insensitive to antagonism by BQ-123, a selective ET_A antagonist, but was blocked by losartan, a selective AT_1 antagonist. Furthermore, my studies have demonstrated that in freshly dispersed single SMC, Ang II produced a concentration-dependent increase in $[Ca^{2+}]_i$ simultaneously with the contraction of single SMC. In contrast, Ang II did not induce an increase in $[Ca^{2+}]_i$ in endothelial cells although in the same cells wherein Ang II failed to induce an increase in $[Ca^{2+}]_i$, acetylcholine did induce a significant increase in $[Ca^{2+}]_i$. **These data provide direct evidence that Ang II binds to AT_1 receptors on smooth muscle cells and elicits contraction via an increase in free $[Ca^{2+}]_i$ and Ang II-mediated contraction of the rat tail artery was independent of the release of endothelial cell-derived ET-1.** Nonetheless, evidence shows that long-term treatment with Ang II can stimulate ET-1 secretion from the endothelium or other cells in different tissues (Gray, *et al.*, 1998; Dussaule, *et al.*, 1998; d'Uscio, *et al.*, 1998; Barton, *et al.*, 1997; Moreau, *et al.*,1997; Dohi, *et al.*, 1992*)*, therefore, the effect of short-term (one hour) treatment with Ang II on the synthesis/release of ET-1 is still controversial.

My finding that ET-1 was not involved in the Ang II-mediated contraction of the rat tail artery is inconsistent with the findings reported by Chen *et al.* (1995 a & b). The reason for this discrepancy is unclear. In the present study, it was found that in some rat tail artery preparations no response to Ang II could be demonstrated, regardless of whether the tissues were endothelium-intact or -denuded. This suggests that there may be a heterogeneous distribution of Ang II receptors in the rat tail artery. I also used the same strain of rat (male Sprague Dewley rat) as Chen *et al* (1995a,b). However, Chen *et al.* (1995 a & b) used Triton X-100 to remove the endothelial cell layer from the rat tail artery preparation and in the present study it was found that this method also reduced the sensitivity of the underlying smooth muscle to Ang II. Therefore, I concluded that Triton X-100 treatment is not a reliable method to remove endothelial cells. In the present studies I used mechanical as the preferred method to eliminate the role of the endothelium (Monuszko, *et al.*, 1990).

Part 2. Signal transduction pathways for Ang II- and ET-1-induced vasoconstriction

In the present study, I have reported that Ang II- and ET-1-mediated contractions of the rat tail artery have diverse pharmacological properties: Ang II produced a non-sustained contraction whereas ET-1 produced a sustained contraction of the rat tail artery ring. Furthermore, Ang II evoked an additional contraction on the top of maximal response of the rat tail artery ring to ET-1. These findings imply that Ang II and ET-1 utilize distinct Ca^{2+} sources and signalling pathways to cause contractions. My data also indicated that the Ang II-mediated response was mainly dependent on intracellular Ca^{2+} release sensitive to caffeine (2 mM), whereas that of ET-1 was primarily dependent on extracellular Ca^{2+} entry sensitive

to Ni^{2+}.

2.1. The Ang II-induced contraction

The contractile response to Ang II has been studied in variety of isolated vessels from different species, and sensitivity of vascular smooth muscle to Ang II is reportedly quite different (reviewed by Bohr, 1974). Such regional and species differences in the response to Ang II may be due to heterogenous populations, sensitivity, and receptor sites of Ang II. Overall, however, Ang II activation of AT_1 on VSMCs leads to an increase in the influx and mobilization of Ca^{2+} (Toda and Miyazaki, 1978). In the present study, Ang II always induced a non-sustained contraction that was sensitive to caffeine, and attenuated by Ca^{2+}-free solution (Fig 16). In the freshly dispersed SMCs, Ang II-induced intracellular Ca^{2+} increase always has two phases: first transient and then plateau as shown in figure 12a. Ang II induced a transient increase in $[Ca^{2+}]_i$ that was only slightly affected by Ca^{2+}-free solution (Fig 20). These data indicate that in the rat tail artery Ang II-induced response mainly depends on release of Ca^{2+} from intracellular store, and partially on influx of Ca^{2+} from the extracellular medium. This pathway is consistent with the report of Roe *et al.* (1989) from the cultured porcine SMCs.

2.1.1. Contribution of the endothelium

2.1.1.1. AT_1 receptor on the endothelial cells

The role of the endothelium in Ang II-induced contraction is unclear. The presence of Ang II receptors in cultured endothelial cells has been reported by several investigators (Feener *et al.*, 1995; Guillot and Audus, 1991; Vaughan *et al.*, 1995; Patel *et al.*, 1989; Saito *et al.*, 1996; Ko *et al.*, 1997). The pharmacological characteristics of the Ang II receptors on

endothelial cells are similar to those on SMCs. The binding of Ang II to endothelial AT_1 leads to the activation of PLC and PLA_2, as in other cell types (Pueyo *et al.*, 1996). Ang II appears to release vasoconstrictor and dilator PGs from endothelial and subendothelial tissues of monkey and dog arteries (Toda and Miyazaki, 1981; Toda, 1981; Toda *et al.*, 1990). Boulanger et al. (1995) demonstrated that, in the rat carotid artery, stimulation of AT_1 caused the release of nitric oxide (but not PGI_2) which in turn inhibited the contractions to Ang II, also mediated by AT_1. However, the results of the current study indicate that the endothelium does not contribute to the vasoconstrictor response of the rat tail artery to Ang II. This is consistent with the results from rabbit aorta (Saye *et al.*, 1984), canine carotid artery (d'Orlèans-Juste *et al.*, 1985) and bovine intrapulmonary artery and vein (Gruetter *et al.*, 1988). Nonetheless, the RT-PCR data indicate that the endothelial cells possess AT_1 receptor mRNA, although the immunocytochemical data suggest there are no AT_1 receptors detected on endothelial cells. Furthermore, a selective ET_A antagonist BQ-123, a PLA_2 inhibitor $AACOCF_3$, and a nitric oxide synthase (NOS) inhibitor L-NNA had no effect on Ang II-induced contractions, but decreased the desensitization to Ang II in endothelium-intact vessels (fig 27) (this observation will be discussed below), and Ang II did not induce an increase in $[Ca^{2+}]_i$ in endothelial cells although in the same cells wherein Ang II failed to induce increase in $[Ca^{2+}]_i$, acetylcholine induced a significant increase in $[Ca^{2+}]_i$. My results indicating that Ang II did not induce an increase in $[Ca^{2+}]_i$ of endothelial cells are, however, inconsistent with those reported by Wang, *et al.*, (1995); Pueyo, *et al.*, (1996;1998); Saito, *et al.*, (1996); Ko, *et al.*, (1997); Boulanger, *et al.*, (1995).

Three possibilities can be suggested: 1). Different blood vessels have different histological

and anatomic properties: the endothelial function of conduit vessels differs from that of resistance vessels; 2). The function of cultured endothelial cells differs from that of freshly dispersed ones; 3) There are no functional AT_1 in ECs. With respect to the third point, the RT-PCR results indicate bands corresponding to AT_1 (444 bp) in endothelium-intact tissue and endothelium-denuded tissue, as well as freshly dispersed smooth muscle cells and endothelial cells. Does this mean that AT_1 receptors are associated with endothelial cells? Three explanations can be considered: 1). The bands observed in ECs were due to contamination with smooth muscle cells; 2). There might be a small amount of AT_1 receptor distribution, but no function in ECs; 3). There is mRNA expression, but no receptor distribution in the rat tail artery endothelial cells. Taken together, I believe that the absence of direct Ang II-induced response in endothelial cells of the rat tail artery is consistent with the lack of functional AT_1 receptors on endothelial cells of the rat tail artery. Nonetheless, a number of studies have found that endothelial cells from different origins do possess functional Ang II receptors (Wang, *et al.*, 1995; Pueyo, *et al.*, 1996 & 1998; Saito, *et al.*, 1996; Ko, *et al.*, 1997; Boulanger, *et al.*, 1995).

Although L-NNA had no direct effect on Ang II-induced contractions, it did attenuate the desensitization to subsequent Ang II-induced response in the endothelium-intact rat tail artery preparation. This would imply that NO might be involved in the development of desensitization to Ang II-induced contraction in the rat tail artery. Recently Boer *et al.* (1999) reported that phenylephrine induces vasoconstriction that is augmented by NOS inhibition and induces a concentration-dependent NO release that can be antagonized by an α_1-adrenoceptor antagonist and NOS inhibition. The pathway of α_1-adrenoceptor-induced

NO release is still unknown. Boer *et al.* (1999) suggest three possible mechanisms for α_1-adrenoceptor-induced NO release: 1). Stimulation of an α_1-adrenoceptor on pulmonary endothelial cells can result in activation of eNOS. But so far α_1-adrenoceptors have not been shown to be present on the endothelium. This is a questionable explanation. 2). Stimulation of receptors on SMCs can induce the release of substances, which stimulate the ECs to produce NO; 3). Vasoconstriction results in endothelial calcium influx, and therefore NO synthesis and release, via intercellular communication between endothelium and smooth muscle. Since my data suggests that there are no functional AT_1 receptors on endothelial cells, Ang II does not directly induce $[Ca^{2+}]$ increase in ECs. Thus, the last two possible mechanisms for PE-induced NO release could be the explanations for Ang II-induced NO release in the rat tail artery. Of interest, I report similar data for the role of the endothelium and NOS in PE-induced contraction of the rat tail artery, which enhanced PE-induced sensitization. (Fig 28).

However, that Ang II did not induce NO release in the rat pulmonary artery of male Wister rat (Boer *et al.,* 1999) is inconsistent with the report from Hill-Kapturezak *et al.* (1999) in which Ang II was shown to induce NO release from porcine pulmonary artery endothelium. These data raised a number of questions: 1). why does Ang II-induced NO release have no direct effect on Ang II-induced contraction in the rat tail artery? 2). If endothelium-derived NO is involved in Ang II-induced desensitization, why did not the endothelium-denuded preparation have less desensitization than that of endothelium-intact preparation? Possible explanations can be considered: 1). That removal of the endothelium damages smooth muscle cells and hence reduces vasoconstriction; 2). It is well known that endothelium can

release EDRF and EDCF. I hypothesis that EDRF and EDCF may antagonize (physiological antagonism) each other, and thus the inhibition of NO production will break this balance and enhance contraction. However, so far the mechanism of Ang II-induced desensitization is still unclear. Further investigation into the possible role of the endothelium in Ang II-induced desensitization is required.

2.1.1.2. Conversion of Ang I to Ang II

Angiotensin converting enzyme (ACE) is a membrane bound exopeptidase that is located on the luminal surface of pulmonary endothelial cells (Ryan *et al.*, 1975). In Wistar rat tail artery ACE is present at sites other than, or in addition to, the endothelium (Story and Ziogas, 1986), and this is consistent with the report of Saye *et al.*, (1984) using rabbit aorta. They reported that removal of the endothelium from rabbit aorta did not inhibit the contractile response to angiotensin I and Ang II, although it did slow the rate of conversion of angiotensin I to Ang II. It was established that the facilitation produced by Ang II was unchanged after removal of the endothelium (Story & Ziogas, 1986), thus, it is unlikely that in the rat tail artery substances released from the endothelium modify the effect of Ang II as has been shown to occur in other tissues (Webb, 1982; Lanier & Malik, 1982). In the present study, there were also no significant differences in response to Ang II between endothelium-intact and -denuded preparations, although, as noted, the endothelium does contribute to the rapid desensitization of the tissue to Ang II (Grutter, *et al.*,1987).

In conclusion, there are no functional AT_1 on the endothelium of the rat tail artery and these results eliminate endothelial ET-1, NO and PLA_2 involvement in Ang II-induced contraction of the rat tail artery (Fig 3).

2.1.2. Cellular basis of Ang II-induced contraction

It has been established that there are two sources of calcium that contribute to the increase in cytosolic Ca^{2+} concentration and trigger vasoconstriction: extracellular Ca^{2+} influx and intracellular Ca^{2+} release from SR (Karaki, *et al.,* 1997). Ang II stimulates Ca^{2+} entry through different Ca^{2+} plasma membrane channels and Ca^{2+} release from SR (Fig 32).

In the rat tail artery, Ang II increases $[Ca^{2+}]_i$ and induces vasoconstriction that is inhibited by losartan, an AT_1 antagonist, suggesting Ang II-induced contraction is mediated via the AT_1 receptor. Pretreatment of the tissue with pertussis toxin (PTX) has no effect on the Ang II-induced contraction suggesting the involvement of a PTX-insensitive GTP-binding protein. A phospholipase C (PLC) inhibitor, U73122, had no effect on Ang II-induced response, however, it should be noted that U73122 has effects other than the inhibition of PLC (Mogami *et al.,* 1997). In the present study I did not observe any effect of U73122. So the PLC involvement needs to further study using a specific inhibitor of PLC or directly measure the formation of IP_3. It is unlikely that protein kinase C (PKC) is involved in the Ang II-induced contraction of the rat tail artery, because a PKC inhibitor, chelerythrine, had no effect on the response to Ang II. In Ca^{2+}-free solution (omitted calcium and added 1 mM EGTA in Krebs solution), or in the presence of nifedipine (1 μM), Ang II produced a tension increase, but after incubation with caffeine (2 mM) or ryanodine (1 μM in Ca^{2+}-free solution) Ang II-induced contractions were almost abolished. These results are similar to the Ang II-induced $[Ca^{2+}]_i$ increase described in portal vein (Morel, *et al.,* 1996), the only difference is that Ang II increased $[Ca^{2+}]_i$ in the Ca^{2+}-free solution in the rat tail artery. Since I did not use direct methods to antagonize the IP_3 receptor (IP_3R), I do not know whether the

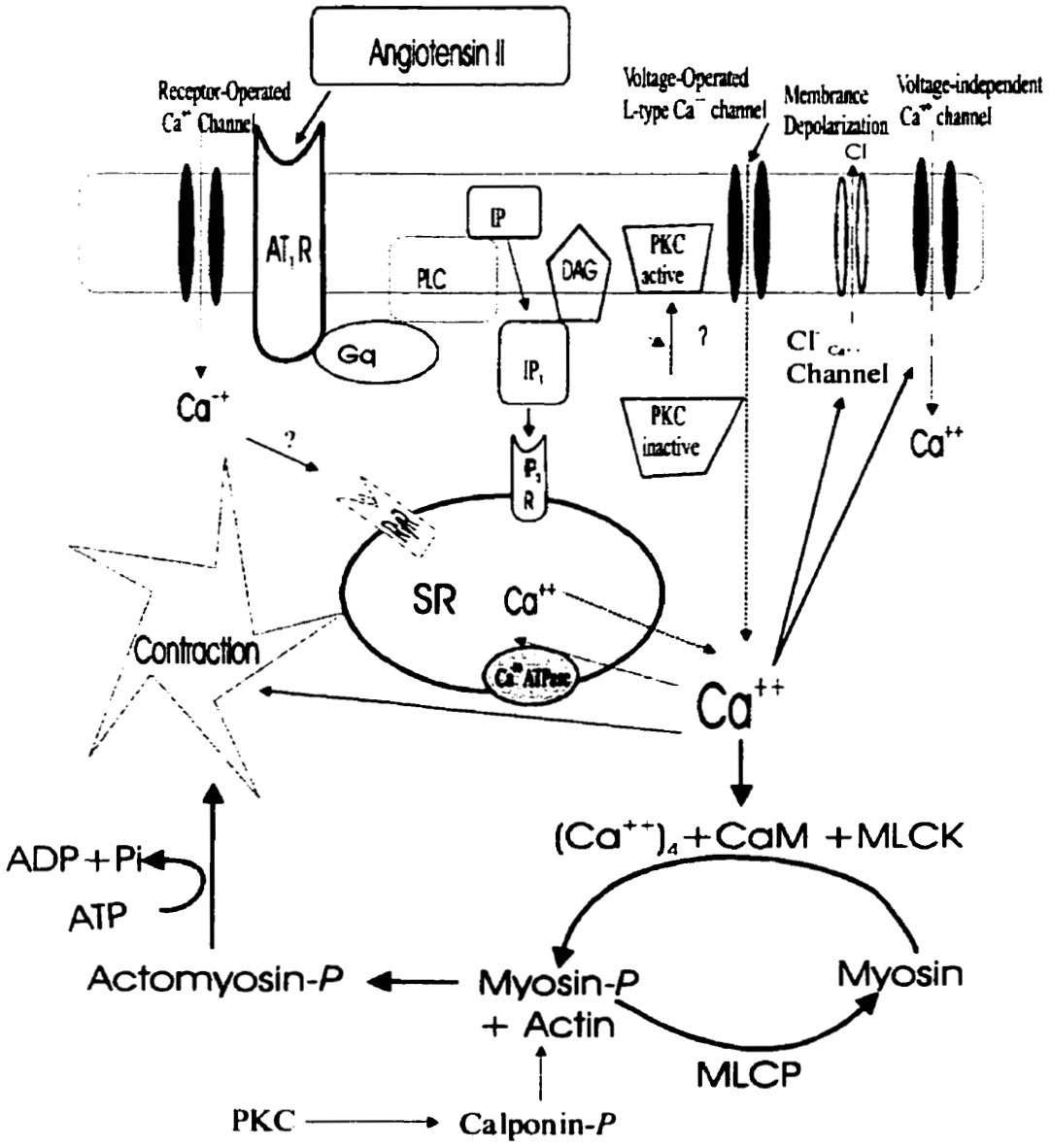


Fig 32. Schematic diagram of possible signalling pathways by which angiotensin II can elicit changes in intracellular calcium concentration And contraction of a vascular smooth muscle cell.

IP_3 pathway is involved with Ang II-induced contraction in the rat tail artery. Phenylephrine (PE) and caffeine stimulate intracellular Ca^{2+} release though the IP_3R pathway (Rembold, 1992) and the ryanodine receptor (RyR) pathway (Missiaen *et al.*, 1992), respectively. The effects of pretreatment with caffeine on PE- and Ang II-induced contractions were compared. Both PE- and Ang II-mediated contractions were significantly inhibited. These data indicate that either both PE and Ang II use a caffeine sensitive pathway or that there are certain overlaps between IP_3R and RyR-regulated Ca^{2+} pools. In comparing the signals for Ang II-induced $[Ca^{2+}]_i$ increases in the absence and presence of extracellular calcium (Fig 20), it was noted that the duration of the Ang II-induced $[Ca^{2+}]_i$ increase in the presence of calcium is longer than that in the absence of calcium suggesting that in the rat tail artery Ang II also uses both extracellular and intracellular calcium sources: Ca^{2+} influx from the extracellular medium and intracellular release from SR via a caffeine-sensitive pathway(s) to increase in $[Ca^{2+}]_i$.

I also investigated the role of the calcium-activated chloride channel in contributing to the Ang II-induced contraction. Niflumic acid in concentrations of 30 μM, 50 μM and 100 μM was used as it has been suggested that, within this concentration range, niflumic acid is a calcium-activated chloride channel blocker (Large & Wang, 1996). The Ang II-induced contraction was not affected by either 30 or 50 μM niflumic acid, but completely blocked by 100 μM. These data do not allow me to make a clear conclusion concerning the involvement of the calcium-activated chloride channel, because: 1). niflumic acid would be expected to inhibit contraction downstream of Ca^{2+} influx and intracellular Ca^{2+} release (Kato *et al.*, 1999); 2). There are different kinds of chloride channels which have different

sensitivities to different inhibitors (Jentsch & Guntter, 1997; Pollock *et al.*, 1998; Neson *et al.*, 1997). As yet no one has cloned the calcium-activated chloride channel. So before I can make a definitive conclusion I should try different chloride channel inhibitors, like DIDS (Guibert *et al.*, 1997), and IAA-94 (indanyloxyacetic acid) (Carmines, 1995).

Pretreatment of the tissue with SK&F96365, a receptor-operated Ca^{2+} channel blocker (Merritt *et al.*, 1990), did not significantly change the Ang II-induced contraction suggesting that receptor-operated Ca^{2+} channels (ROCCs) are not involved in Ang II-induced contraction of the rat tail artery.

2.1.3. Are tyrosine kinases involved in Ang II-induced contraction of the rat tail artery?

The ability of Ang II to activate tyrosine kinases in vascular smooth muscle cells is an indisputable fact (Berk and Corson, 1997; Griendling *et al.*, 1997; Berk 1999). However, the involvement of tyrosine kinase activation in Ang II-induced contractions is controversial (Laniyonu *et al.*, 1994; Hollenberg, 1994; Watts *et al.*, 1998). It seems that the involvement of tyrosine kinase activation in Ang II-induced contraction is dependent on tissue species, tissue preparations and the concentration of tyrosine kinase inhibitors employed (personal communication from Dr. Hollenberg). The endothelium may also contribute to the Ang II-mediated response via tyrosine kinase activation (Watts *et al.*, 1998). Is tyrosine kinase involved in Ang II-induced contraction in the rat tail artery? To answer this question, different tyrosine kinase inhibitors, including genistein, tyrphostin and the MEK kinase inhibitor, PD98059, were used. In the present study 5μM genistein had no affect on Ang II-induced contraction, although this concentration has been reported as high enough to inhibit

the tyrosine kinase involved contraction (Watts *et al.*, 1998). I also tested higher concentrations of genistein (≥ 10 μM), although at that concentration a clearcut selectivity in blocking agonist-mediated contractions may be absent (Laniyonu *et al.*, 1994). However, neither 10 μM genistein, nor 40 μM tyrphostin blocked Ang II-induced contraction. Furthermore, the MEK kinase inhibitor, PD98059 (10 μM) did not affect the Ang II-mediated response. In this study, 30 μM genistein did abolish the AngII-induced contraction, but it also inhibited KCl-induced contraction, and the inactive isoform of genistein, daidzein (30 μM) partially inhibited AngII-induced contraction. In conclusion, tyrosine kinases do not seem to be involved in Ang II-induced contractions of the rat tail artery, however, further studies may be wanted. In addition, and concerning concentrations of genistein above 10 μM, genistein cannot be used selectively as "there was no longer a clearcut selectivety in blocking agonist-mediated contraction" (Laniyonu *et al.*, 1994).

2.2. ET-1-induced contraction of the rat tail artery

In the present study, ET-1 induced a slowly developing, long-lasting contraction that could be blocked by BQ-123, a selective ET_A antagonist, but not blocked by B-788, a selective ET_B antagonist. This suggests that ET-1 induced contraction via activation of the ET_A receptor. After removal of extracellular calcium ($[Ca^{2+}]_o$), ET-1 did not cause contraction, although upon washing the agonist from the tissue in a Krebs solution containing 2.5 mM Ca^{2+}, an immediate contraction was observed. Washing with Ca^{2+}-free solution produced no contraction. These data indicating that ET-1 could not induce a contractile response in Ca^{2+}-free solution are consistent with data from a study using rat tail arteries from Wistar rats

(Huang *et al.*, 1991).

My studies have also demonstrated that ET-1-mediated contractions were less sensitive to the voltage-gated L-type Ca^{2+} channel (VGCC) antagonists, nifedipine or verapamil, but very sensitive to Ni^{2+}, a nonselective blocker of cation channels, and to SK&F96365, a putative ROCC blocker (Merritt *et al.*, 1990). These data suggest that VGCC only play a marginal role in ET-1-mediated contraction of the rat tail artery, which is consistent with reports of ET-1-induced vasoconstriction in rat mesenteric arteries (Wallnofer *et al.*, 1989), rat aorta and pig coronary arteries (Blackburn and Highsmith, 1990). Considerable evidence points to ET-1 stimulating Ca^{2+} influx into VSMC via non-dihydropyridine-sensitive Ca^{2+} channels (Rubanyi & Polokoff, 1994; Iwamuro, *et al.*, 1998). Ni^{2+}, which has no effect on L-type Ca^{2+} channels (Blackburn and Highsmith, 1990) and La^{3+} (nonspecific cation channel blockers) (Steffan & Russel, 1990), but not L-type Ca^{2+} channel antagonists, blocked ET-1-induced contraction or $[Ca^{2+}]_i$ increase, indicating that Ca^{2+} channels other than VGCC are involved in ET-1-induced increased Ca^{2+} influx. However, this is the first study to report that nonselective cation channels, and receptor-operated Ca^{2+} channels are also involved in ET-1-mediated contractions of the rat tail artery. Thus, in the rat tail artery ET-1-induced contraction is predominantly dependent on extracellular calcium infux. However, the Ca^{2+} signalling pathways activated by ET-1 is dependent on ET-1 concentration: higher concentrations of ET-1 ($\geq$ 10nM) induce activation of Ca^{2+} entry channels and increase the formation of IP_3, whereas lower concentrations of ET-1 ($\leq$ 1 nM) exclusively activate Ca^{2+} entry channels (Iwamuro *et al.*, 1998).

Part 3. Synergistic interaction between Ang II and ET-1

Ang II is one of the most potent stimulators of ET-1 synthesis and/or release (Gray, *et al.*, 1998; Dussaule, *et al.*, 1998; d'Uscio; *et al.*, 1998; Barton, *et al.*, 1997;Moreau, *et al., 1997;* Dohi, *et al.*, 1992; Scott-Burden *et al.*, 1991). ET-1 can augment the production of Ang II, by elevating plasma renin activity and/or by enhancing ACE activity (reviewed by Rubanyi & Polokoff, 1994). Ang II and ET-1 act synergistically to induce vasoconstriction, VSMC proliferation, and aldosterone secretion from the adrenal cortex (Cozza, *et al.*, 1992; 1993). However, the production of endothelin-1 from stimulated endothelial cells requires de novo protein synthesis, and therefore, is very slow, thus reaching a maximum after 4-5 hours of incubation (Yanagisawa *et al.*, 1988; Boulanger *et al.*, 1990). In intact mesenteric resistance arteries the endothelium-dependent potentiation was observed after 5 hours but not after 1 hour of stimulation with Ang II (Dohi *et al.*, 1992).When experiments were performed with Ang II in combination with ET-1, there was no synergistic enhancement of the stimulation induced by either agonist alone (Scott-Burden *et al.*, 1991). It appears unlikely that the stimulatory activity of Ang II on the proliferative and synthetic activity of SMCs is mediated via the induction of ET-1 production. In the present study, BQ-123 did not alter the contraction induced by Ang II, but after ET-1-induced contraction returned to baseline levels, a subsequent Ang II-induced contraction was enhanced (Fig 24). This is probably due to an ET-1-induced increase in intracellular calcium in VSMCs which subsequently enhances the response to Ang II. However, to confirm this point another series of experiments should be performed following different exposure times after ET-1-induced contractions had returned to baseline levels.

Part 4. Summary and future directions

4.1.Summary

The present study provides evidence against ET-1 involvement in Ang II-mediated vasoconstriction of the rat tail artery. Ang II induced an endothelium-independent contraction via a signalling pathway distinct from that utilized by ET-1 in this tissue. The Ang II-mediated response was mainly dependent on intracellular Ca^{2+} release whereas that to ET-1 was primarily dependent on extracellular Ca^{2+} entry. No functional AT_1 receptors could be detected on the endothelium in the rat tail artery and AT_1 were predominantly distributed on the smooth muscle cells. ET-1-induced intracellular calcium may enhance the response to Ang II in the rat tail artery.

4.2. Future directions

1). Further studies of the mechanism(s) of NO-induced attenuation of Ang II-induced desensitization in the rat tail artery:

NO has been reported to reduce the Ang II-induced tachyphylaxis calcium response in rabbit afferent arteries (Korfeld *et al.*, 1997), whereas the present study indicates otherwise.

2). A comparative study of the involvement(s) in the endothelium in Ang II-induced contraction of other vascular preparations such as the rat mesenteric artery.

3). The possible cross-talk between Ang II and vasodilators in small resistance vessels.

Recently, Brizzolara-Gourdie & Webb. (1996) found that exposure of rat aorta to Ang II enhanced the vasodilation response of these blood vessels to agonists that stimulate cAMP formation, and they suggested that such cross-talk between constrictor and dilator pathways

could represent an important mechanism in the modulation of vascular tone. However, it is still unclear whether Ang II enhances vasodilation of small resistance vessels. My preliminary experiments have found that Ang II enhances isoproterenol (ISO)-induced vasodilation of the rat mesenteric artery in a similar manner as Brizzolara-Gourdie & Webb (1996) reported for the rat aorta. Surprisingly, this Ang II enhancement of ISO-induced vasodilation of the rat mesenteric artery was not affected by SQ 22536 and 2,3-DDA, selective adenylyl cyclase inhibitors. My data suggest that there may be a cross-talk between Ang II and vasodilators not only in conduit vessels but also importantly in small resistance vessels. However, the cellular mechanism resposible for this cross-talk in small resistance vessels is unclear. It has been reported that Ang II activation of endothelial cell AT_1, increases $[Ca^{2+}]_i$ that presumably activates eNOS, and stimulates NO synthesis (Hennington, *et al.*, 1998). This Ang II-stimulated release of endothelium-derived NO may reduce the direct vasoconstriction actions of Ang II. To address whether a NO-cyclic GMP pathway is involved in the Ang II-mediated vascular effects in the mesenteric artery, the effects of NOS inhibitors (e.g., L-NNA) and soluble guanylyl cyclase inhibitors, e.g., 1H[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1-one (ODQ) on Ang II- mediated effects in the vasculature should be determined. In addition, the contribution of arachidonic acid products on Ang II-mediated effects in the vasculature should be tested using cyclooxygenase inhibitor (e.g., indomathecin) and PLA_2 inhibitors (e.g., $AACOCF_3$).

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