INSULINOMIMETICS AND THE VASCULAR ENDOTHELIN SYSTEM

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ABSTRACT

Insulin promotes vasodilatory, vasoconstrictor and atherogenic influences on vascular smooth muscle. Endothelin-1 (ET-1) is a potent, endothelium derived vasoconstrictor/mitogenic peptide whose production is stimulated by insulin both *in vitro* and *in vivo*. Consequently, ET-1 has been hypothesized to contribute to insulin evoked vasculopathy. The present study first characterized the effect of insulin and the insulinomimetic agent, vanadate, on ET receptor expression and action in rat aortic smooth muscle cells. Radioligand binding studies confirmed that insulin pretreatment selectively upregulated ET_A receptor expression in a concentration and time dependent manner, whereas vanadate upregulated both ET_A and ET_B. Upregulated ET receptors were coupled to increased $[Ca^{2+}]_i$. Upregulation by both compounds occurred at the mRNA level and required tyrosine kinase activation, active transcription, and new protein synthesis. In order to determine the *in vivo* relevance of these effects, both insulin deficient diabetic and non-diabetic rats were treated with insulin and vanadate for 2-weeks. Strikingly, the diabetic state *per se* was associated with both attenuated ET-1 plasma levels and exaggerated *ex vivo* aortic vasoconstrictor responses to ET-1. Two weeks of treatment with either insulin or vanadate did not further increase ET-1 evoked vasoconstriction as expected based on our cellular data, but in fact restored altered ET-1 release and action to normal in the diabetic rats. It is concluded that while insulin and vanadate might increase ET receptor expression and subsequent adverse vascular responses to ET-1, their profound beneficial effects on metabolic function and the resulting beneficial vascular actions clearly minimize the significance of these actions.

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LIST OF ABBREVIATIONS

Ang II	Angiotensin II
ASMC	Aortic smooth muscle cell
AVP	Arginine vasopressin
B_{max}	Maximal binding capacity
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Cytosolic free calcium
DAG	Diacylglycerol
DM	Diabetes Mellitus
DMEM	Dulbecco's modified essential medium
DMSO	Dimethylsulfoxide
EC	Endothelial cell
EC_{50}	Concentration of half-maximal response
ECE	Endothelin converting enzyme
EDCF	Endothelium derived contracting factor
EDRF	Endothelium derived relaxing factor
E_{max}	Maximal response
ET	Endothelin
ET-1	Endothelin-1
Fura-2/AM	Fura-2 acetoxymethyl ester
HBSS	Hank's balanced salt solution

IDDM	Insulin-dependent diabetes mellitus
IGF-1	Insulin like growth factor - 1
IP_3	Inositol 1,4,5-triphosphate
K_D	Equilibrium dissociation constant
LDL	Low-density lipoprotein
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
n_H	Hill coefficient
NIDDM	Non-insulin dependent diabetes mellitus
NOS	Nitric oxide synthase
PGI_2	Prostacyclin
PI-3K	Phosphatidyl inositol – 3 kinase
PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
SD	Sprague-Dawley
STZ	Streptozotocin
SR	Sarcoplasmic reticulum
RIA	Radioimmunoassay
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell

INSULINOMIMETICS AND THE VASCULAR ENDOTHELIN SYSTEM

1. INTRODUCTION

1.1. Endothelins

1.1.1. Discovery of a novel endothelium derived contracting factor (EDCF)

It was just 20 years ago that Furchgott and Zawadzki made the serendipitous, yet seminal discovery that the vascular endothelium mediates vascular relaxation by releasing a substance in response to stimulation with acetylcholine (ACh; Furchgott & Zawadzki, 1980). This substance was shown to be non-prostanoid in nature and was later identified as the gas, nitric oxide (Ignarro et al., 1987). Around the same time, endothelial cell (EC) cultures were also shown to produce a peptide that contracted both bovine pulmonary arteries (O'Brien and McMurtry, 1987), and isolated coronary arteries (Hickey et al., 1985). This substance was soon after characterized in the conditioned culture medium of porcine aortic EC and was determined to be the most potent and long-lasting vasoconstrictor agonist known to man (Yanagisawa et al. 1988). The substance was named endothelin-1 (ET-1).

1.1.2. Structural biology of endothelin-1 and its isoforms

ET-1 was determined to have a molecular weight of 2492 and contain 21 amino acids, with free amino and carboxy termini and two intrachain disulfide bonds. The human genome contains coding regions for two other structurally distinct endothelin (ET) isopeptides, ET-2 and ET-3 (Inoue et al., 1989). Each of these isoforms exhibit a high degree of sequence homology but different pharmacological profiles (D'Orleans-Juste et al., 1991a). The primary structure of ET-2 differs from ET-1 with respect to 2 amino acid residues, whereas ET-3 differs with respect to 6 (Figure

1). ET's are bicyclic peptides with a hairpin loop configuration with two disulphide bonds interlinked between Cys1 and Cys 15 and Cys 3 and Cys 11 (Rubanyi & Polokoff, 1994). The 3-D structure of ET-1 is presented in Figure 2. It is notable that both the hydrophobic C-terminus and hairpin-loop configuration is required for biological activity (Kimura et al. 1988).

1.1.3. Regulation of endothelin-1 production

Synthesis of ET-1 occurs in various tissues including EC (Yanagisawa et al. 1988), vascular smooth muscle cells (VSMC; Resink et al. 1990a), bronchial epithelial cells (Black et al. 1989), mesangial cells (Kohno et al., 1992), cardiac myocytes (Suzuki et al. 1993), and glial cells. EC produce almost exclusively ET-1 (Inoue et al., 1989). A pictorial of the synthetic pathway of ET production is represented in Figure 3. Sequence analysis of porcine ET-1 cDNA revealed the existence of a single copy gene encoding a precursor protein, preproET-1. This 203 amino acid precursor, formed upon translation of mRNA specific for ET-1, is acted upon by a dibasic-pair specific endopeptidase to form the 38-39 amino acid peptide, big-ET-1. big ET-1 is then acted upon by the protease, ET converting enzyme (ECE) found both within the cell of synthesis (in the golgi complex) and on the membrane of target cells. ECE cleaves the Trp-21-Val-22 bond to yield biologically active ET-1. The secretion of ET-1 is thought to occur immediately after its formation as there are no reports of storage granules for the peptide. ET gene transcription can be enhanced by various factors including angiotensin II (Ang II), insulin, thrombin, platelet derived growth factor, arginine vasopressin (AVP), transforming growth

factor-β, phorbol ester, oxidized low-density lipoprotein (LDL), hypoxia, and shear stress (Miyauchi & Masaki, 1999). Inhibitory stimuli can also modulate ET-1 production including endothelium derived relaxing factor (EDRF), atrial natriuretic factor, cyclic GMP, nitrosovasodilators, prostacyclin (PGI_2), and heparin. Accordingly, pathophysiological conditions whereby impaired formation of EDRF and/or PGI_2 is present may be associated with enhanced ET-1 synthesis.

1.1.4. Characteristics and function of endothelin receptors

To date, there have been two ET receptor subtypes sequenced and cloned, ET_A and ET_B. The IUPHAR Committee on Nomenclature and Drug Classification identified the ET_A receptor as having higher affinity for ET-1 than ET-2 compared to ET-3 and the ET_B receptor for having similar affinity for all three isoforms (Masaki et al., 1994). The human ET_A receptor is 427 amino acids and belongs to the seven-transmembrane spanning G-protein coupled receptor superfamily. The ET_B receptor is a 415 amino acid protein also belonging to the G-protein receptor family. There is 68% conserved amino acid identity between human ET_A and ET_B receptors. A high density of binding sites for ET-1 have been demonstrated in many tissues of the body including brain, blood vessels, heart, kidney, lungs, gastro-intestinal tract, spleen, liver, adrenal gland, and eye. Those tissues expressing predominantly ET_A include VSMC, human heart, and pituitary gland. Those expressing predominantly ET_B include EC's, renal cortex, brain, and lungs. Radioligand binding studies indicate that [^{125}I] ET-1 binding is specific, saturable, and of high affinity. K_D and

B_{max} values reported range from 0.01 – 1.0 nM and 0.1 to 1.0 pmol/mg protein, respectively. One unique aspect of ET-1 binding is its extremely slow dissociation from its receptor. This is the proposed reason for its long lasting, persisting vasoconstrictor effect. The ET_A receptor is accepted to be the main subtype modulating contractile and mitogenic responses in vascular, cardiac, lung, and renal tissues. It is also thought to modulate other functions such as stimulation of lipoprotein lipase activity in adipocytes, regulate urinary bladder tone, and participate in central cardiovascular regulation. The ET_B receptor expressed on EC's is involved in the regulation of EDRF and PGI_2, which results in a transient vasodilator and anti-aggregatory response in platelets (D'Orleans-Juste et al., 1991b). However, similar to the ET_A receptor, the ET_B receptor located on smooth muscle cells is coupled to contractile and mitogenic responses. Such tissues where this may be physiologically relevant include the pulmonary artery, bronchial smooth muscle, and kidney, coronary, and venous circulation. The contributions of ET_A and ET_B receptors to these responses are, however, widely species dependent. Other functions of the ET_B receptor include modulation of renal tubular ion transport, receptor mediated clearance of ET-1, and anti-aggregatory responses in platelets.

1.1.5. Physiological actions of endothelin-1

1.1.5.1. Autocrine, paracrine, and endocrine role

Another unique function of the ET system is its ability to act in an endocrine, paracrine, and autocrine manner (Rubanyi & Polokoff, 1994). By way of its autocrine effects, ET-1 elaborated from the vascular endothelium stimulates its own

receptors (ET_B) on EC promoting the release of PGI_2 and EDRF. Perhaps more importantly, ET-1 released from EC may act in a paracrine manner on underlying VSMC to promote contraction and mitogenesis. While ET-1 was initially considered a local hormone based on such autocrine and paracrine functions and because of the fact that it is rapidly cleared from the plasma via the lung and kidneys, some evidence supports the view that it may also act in an endocrine manner. In this regard, ET-1 has been shown to act as a glycogenolytic (Jouneaux et al., 1993), modulate the release of catecholamines from the adrenal medulla and sympathetic nervous system (Boarder & Marrit, 1991), and stimulate the pituitary to release follicle stimulating hormone and leutenizing hormone (Stojilkovic et al., 1992).

1.1.5.2. Signal transduction

Binding of ET-1 to its cell-surface receptors stimulates complex pathways of signal transduction resulting in characteristic short and long-term effects (Figure 4; Rubanyi & Polokoff, 1994) that mediate its profound vasoconstrictor effect in VSM. Binding of ET-1 to either ET_A or ET_B receptors results in coupling to phospholipase C (PLC) by both pertussis toxin sensitive and insensitive G-proteins. A rapid increase in inositol-trisphosphate (IP_3) levels results, which subsequently stimulates increased release of Ca^{2+} from the sarcoplasmic reticulum (SR) followed by a prolonged influx of extracellular Ca^{2+} through L-type channels. In the absence of extracellular Ca^{2+}, the sustained increase in $[Ca^{2+}]_i$ is abolished (Simonson et al., 1992). While the initial report by Yanagisawa et al. (1988) suggested that ET-1

directly interacts with dihydropyridine sensitive Ca^{2+} channels, subsequent reports indicate that ET-1 mediated activation of L-type channels occurs through diffusible second messengers (Rubanyi & Polokoff, 1994). The other second messenger liberated by PLC activation is diacylglycerol (DAG) which, along with Ca^{2+} and phosphatidylserine, activates protein kinase C (PKC). PKC regulates a variety of intracellular functions through phosphorylation of proteins (Nishizuka, 1988). ET-1 mediated vasoconstriction has been shown to occur even in the absence of changes in $[Ca^{2+}]_i$. This response is mediated by activation of "novel" Ca^{2+} independent isoforms of PKC. PKC activation has also been implicated in the promitogenic effect of ET-1. ET-1 also increases the phosphorylation of cellular proteins at tyrosine residues probably through activation of mitogen activated protein kinases (MAPK) (Wang et al., 1992). ET-1 also activates phospholipase D (PLD) resulting in the production of phosphatidic acid, choline, or ethanolamine from membrane bound phospholipids such as phosphatidylcholine and phosphatidylethanolamine. This occurs through both PKC dependent and direct G-protein mediated mechanisms (MacNaulty et al., 1990). ET-1 also activates PLA_2 leading to the formation of leukotrienes, thromboxanes, and prostaglandins (Resink et al., 1990b). This is thought to occur again through both G-protein coupled mechanisms and by increases in $[Ca^{2+}]_i$, and also possibly by parallel but separate pathways (Axelrod et al., 1988, Resink et al., 1990b). Finally, the mitogenic properties of ET-1 result from an effect on the expression of transcription factors such as c-*fos*, c-*jun*, and c-*myc* (Simonson et al., 1989). These events are thought to result from stimulation of protein tyrosine kinases, such as MAPK (Simonson et al., 1992).

1.1.5.3. Cardiovascular actions

ET-1 potently induces vasoconstriction in various vascular beds. ET-1 produces contractile responses in VSMC that are characteristically long lasting and difficult to wash out (Yanagisawa et al., 1988). This response is mediated by both ET_A and ET_B receptors present on VSMC, the degree to which each subtype contributes being dependent on the species and vascular bed under investigation (Gardiner et al., 1994). Subthreshold concentrations of ET-1 are also thought to potentiate vasoconstrictor responses to other agonists (MacLean and MacGrath, 1990). ET-1 also simultaneously promotes vasodilatation through stimulation of endothelial ET_B receptors and the subsequent release of EDRF. The simultaneous release of EDRF not only physiologically antagonizes contraction induced by ET_A and ET_B stimulation on VSMC, but may also promote dissociation of the ET-1-peptide receptor complex (Goligorsky et al., 1994). These opposing actions of ET-1 no doubt contribute to the characteristic biphasic change in arterial blood pressure (BP) elicited by the peptide, consisting of a transient depressor response followed rapidly by a sustained pressor response (Yanagisawa et al., 1988). In addition to modulating peripheral vascular function directly, ET-1 also modulates systemic hemodynamics through central mechanisms. Importantly, ET-1 is a basal regulator of cardiovascular tone (Haynes et al., 1996). ET-1 also acts as a potent mitogen of several cardiovascular cells by enhancing expression of protooncogenes, and stimulation of production of other growth factors including platelet derived growth factor, epidermal growth factor, transforming growth factor-β, fibroblast growth factor, insulin, and insulin like growth factor –1 (IGF-1). It may also act as a co-

mitogen with these factors (Brown & Littlewood, 1989; Frank et al., 1993). ET-1 also modulates hemostasis by indirectly modulating the *in vivo* and *ex vivo* aggregratory properties of platelets (Thiemermann et al., 1988). This response is mediated by ET_B stimulated PGI_2 and EDRF production that inhibits ADP-induced *ex vivo* platelet aggregation (de Nucci et. al., 1988; McMurdo et al., 1993). Through such indirect mechanisms, ET-1 also modulates vascular permeability (Filep et al., 1991). Finally, aside from expected growth and vasoconstrictor actions in the renal vasculature, ET-1 is also thought to have direct diuretic effects. These effects are mediated through inhibition of both AVP induced water transport (Tomita et al., 1992) and tubular sodium and bicarbonate transport (Perico et al., 1991). While most of the cardiovascular actions of ET-1 can be attributed to the above mentioned mechanisms, several other unique actions of ET-1 have been identified in various other organ systems of the body including the reproductive system, prostate, hypophyseal system, adrenal glands, thyroid, bone, and the gastrointestinal system.

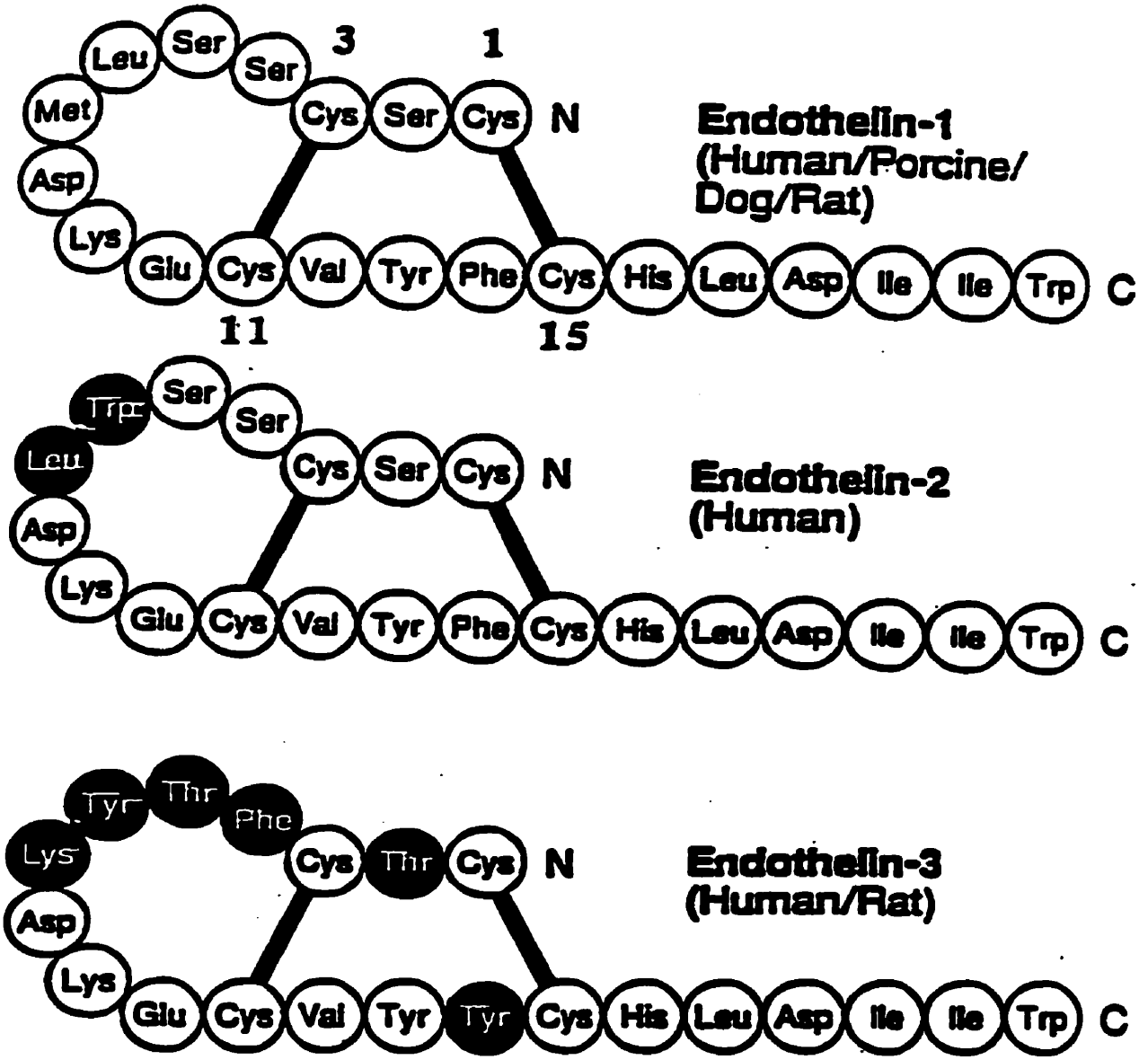


Figure 1. Primary structures of the three ET isopeptides. ET-2 differs from ET-1 with respect to 2 amino acid residues, whereas ET-3 differs with respect to 6 (adapted from Rubanyi & Polokoff, 1994).

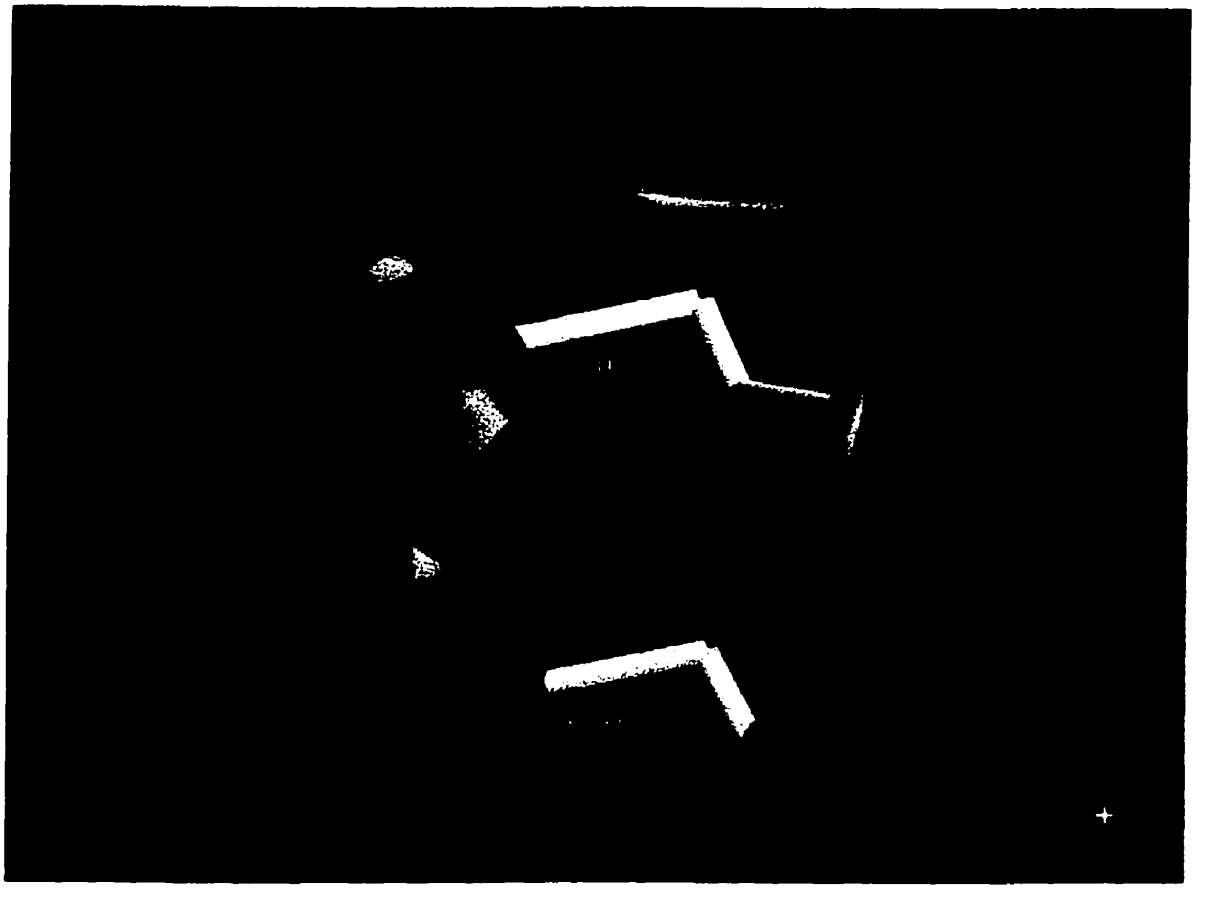


Figure 2. 3-Dimensional structure of ET-1. ET-1 is a bicyclic, 21 amino acid peptide with a hairpin loop configuratipon containing two disulphide bonds interlinked between Cys1 and Cys 15 and Cys 3 and Cys 11. Both the hydrophobic C-terminus and hairpin-loop configuration is required for biological activity (adapted from website: http://www.fmed.ulaval.ca/et6/map_e.htm).

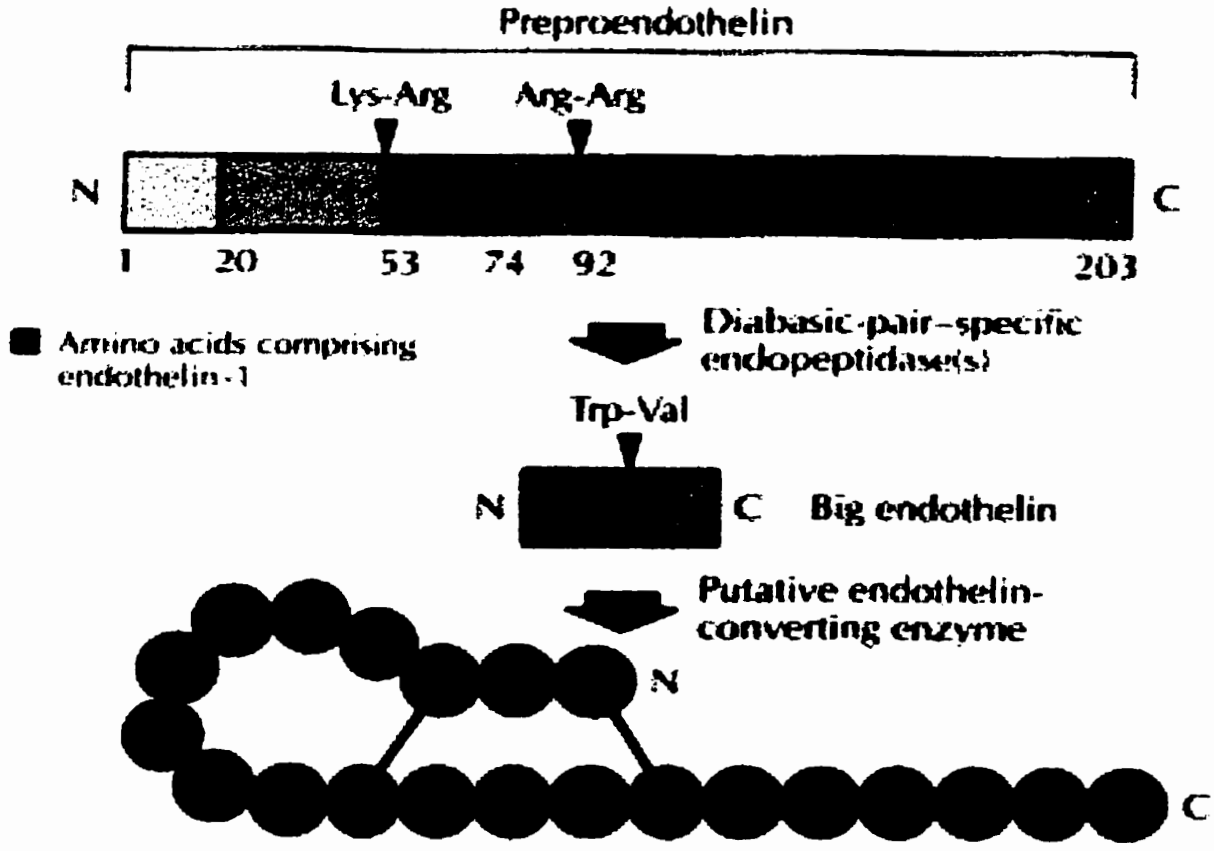


Figure 3. Pathways of formation of ET-1. Prepro-ET-1, a 203 amino acid precursor formed upon translation of mRNA specific for ET-1, is acted upon by a dibasic-pair specific endopeptidase to form the 38-39 amino acid peptide big-ET-1. big ET-1 is then acted upon by the protease, ECE that cleaves the Trp-21-Val-22 bond to yield biologically active ET-1. Secretion of ET-1 is regulated by various stimulatory and inhibitory factors and is thought to occur immediately after its formation as there are no reports of storage granules for the peptide (adapted from Rubanyi & Polokoff, 1994).

ECE – ET converting enzyme

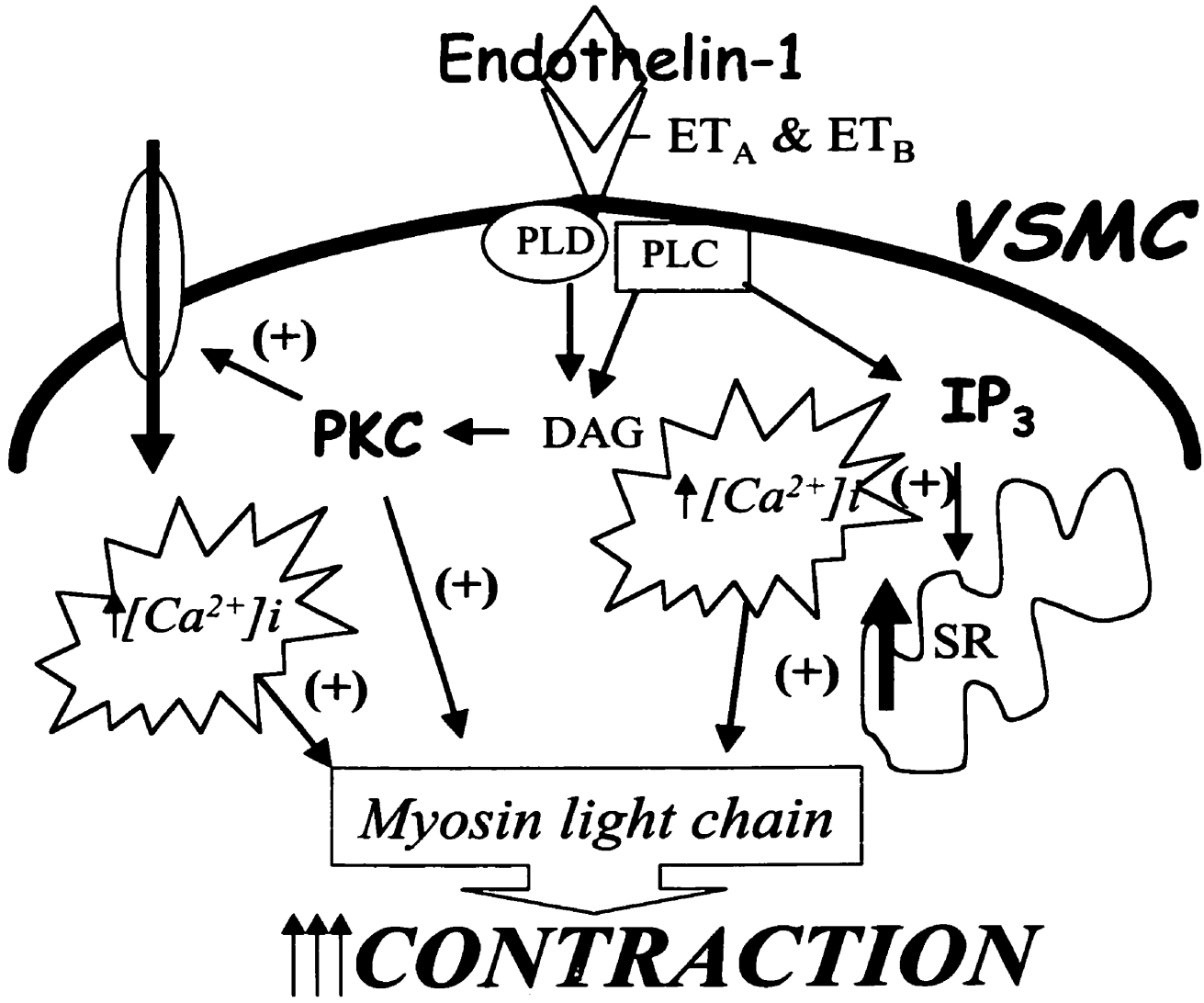


Figure 4. Signaling pathways for ET-1 evoked vasoconstriction. ET-1 activates ET_A and ET_B receptors on VSMC leading to PLC mediated production of IP_3 and DAG. The former increases release of Ca^{2+} from the SR and the latter activates PKC. ET-1 also directly activates PLD leading to PKC activation. Through these signaling pathways, $[Ca^{2+}]_i$ is also increased from the extracellular source through opening of Ca^{2+}channels on the plasma membrane. Both increases in $[Ca^{2+}]_i$ and activation of PKC contribute to the characteristic vasoconstrictor response to ET-1. PLC – phospholipase C; PLD – phospholipase D; DAG – diacylgycerol; PKC – protein kinase C; $[Ca^{2+}]_i$ – cytosolic free Ca^{2+}; SR – sarcoplasmic reticulum

1.2. Diabetes Mellitus

1.2.1. Diabetes, insulin, and vascular disease

Diabetes Mellitus (DM) is a common metabolic disorder with a worldwide prevalence estimated to be between 1 and 5%. After the discovery of insulin, the most common cause of mortality and morbidity in diabetic patients involved abnormalities of the vasculature occurring in both small and large vessels (King et al., 1994). VSMC hypertrophy, polyploidy, and hyperplasia have been demonstrated in several models of DM, but the mechanisms behind these abnormalities have not been fully elucidated. Enhanced platelet adhesion and aggregation (Hamet et al., 1985), lipoprotein abnormalities (Ramirez et al., 1992), endothelial dysfunction (Hsueh & Anderson, 1992), and VSMC abnormalities (Sowers et al., 1993) are all proposed contributors to diabetic vascular disease. Numerous reports implicate insulin resistance, hyperinsulinemia, and lipid disorders in linking DM to accelerated vascular disease (Modan et al., 1985).

Non insulin dependent diabetes (NIDDM) is characterized by not only hyperglycemia, but also hyperinsulinemia (Reaven, 1988). In conjunction with incompletely understood factors, obesity leads to resistance to the actions of insulin resulting in hyperinsulinemia. While many of the known actions of insulin have been implicated in the progression of vascular disease, significant evidence exists to disprove a role for insulin in pathological vascular changes. Not all hypertensives are hyperinsulinemic and many patients with hyperinsulinemia and insulin resistance are not hypertensive. Furthermore, insulin infusion has been shown to decrease BP

in dogs (Brands et al., 1991), and to cause vasodilatation in humans (Anderson et al., 1991). The functional significance of the vasodilator action of insulin is to redistribute blood flow to skeletal muscle tissue increasing delivery of glucose to these areas. Reports have suggested that insulin induces vasodilation via: a) a neural sympathetic effect whereby VSMC contraction to circulating catecholamines is reduced in the presence of insulin (Macleod & McNeill, 1985); b) extrusion of $[Ca^{2+}]_i$ in VSMC via enhanced cellular Ca^{2+} ATP'ase expression (Sowers et al., 1993); c) elevation of VSMC steady state membrane potential, via enhanced Na^+/K^+ ATP'ase activity (Tirapattur et al., 1993); d) enhanced β-adrenoceptor mediated vasodilatation (Gros et al., 1994); and e) attenuation of agonist evoked Ca^{2+} transients in VSMC's (Touyz et al., 1994).

Despite this evidence, it is widely thought that chronic hyperinsulinemia contributes to vascular pathology. Firstly, if the normal response to insulin is vasodilatation, in conditions of insulin resistance, this effect may be blunted. Indeed, the ability of insulin to reduce vascular resistance is reduced in obese, hypertensive, and NIDDM patients (Baron et al., 1993). Many early hypotheses attempting to address the role of insulin in hypertension focused on renal sodium handling, and sympathetic neural actions. An increase in plasma insulin level within or slightly above the physiological range greatly reduces fractional sodium excretion (Gans et al., 1991). Similarly, acute physiological increases of plasma insulin in healthy humans increases muscle sympathetic nerve activity and serum norepinephrine levels (Anderson et al., 1991).

Recent studies have focused on the cellular actions of insulin in explaining its role in vascular pathology. Insulin infusion promotes lipid accumulation (Sato et al., 1989) and VSMC hyperplasia (Stout, 1991). The mitogenic effects of insulin in VSMC require higher concentrations and longer incubation times than those needed to produce metabolic effects (King et al., 1980) and insulin likely mediates most of its VSMC proliferative effects through the IGF-1 receptor, which shows close structural homology to the insulin receptor. In addition, insulin may increase responses to other hypertrophic stimuli through enhancement of Na^+-H^+ exchange resulting in cellular alkalinization (Ferranini et al., 1989). Insulin also exerts atherogenic effects by: 1) increasing uptake and esterification of lipoprotein cholesterol by VSMC's and decreasing its deesterification and release (Sowers et al., 1993); 2) retarding the fibrinolytic process; and 3) stimulation of platelet activator inhibitor (PAI-1) and fibrinogen production (Vukovich et al., 1992).

In 1991, using cultured bovine aortic EC, it was shown that insulin (1.6 nM) increased prepro ET mRNA levels within 2 hours, with maximal effects at 17 nM (Oliver et al., 1990). Subsequently, insulin evoked increases in ET-1 production from human EC and VSMC were confirmed by others (Ferri et al., 1995, Anfossi et al., 1993). Insulin has also been reported to increase the expression of receptors for ET-1 *in vitro* (Frank et al., 1993), and levels of ET-1 rise during acute hyperinsulinemia (Wolpert et al., 1993). Thus, enhancement of ET-1 release and action may be another major mechanism by which insulin may alter vascular function, and the major thrust of this project is to examine this possibility.

1.2.2. The streptozotocin diabetic rat

STZ is an antibiotic derived from Streptomyces achromogenes; structurally it contains a methylnitrosourea group bound to the C2 position of 2-deoxy-d-glucose (Tomlinson et al., 1992). Rakieten et al. (1963) was the first to demonstrate that STZ, when given intravenously, produced DM in rats and dogs. Rats treated with STZ display many of the features seen in humans with uncontrolled DM including glycemia, polydipsia, polyuria, and weight loss. The STZ diabetic rat often develops diabetic complications including myocardial, cardiovascular, tracheal, kidney, urinary bladder, vas deferens, adrenal cortex, skeletal bone, connective tissue, and neuropathic dysfunctions (Ozturk et al., 1996). The present study utilizes this animal model for its lack of plasma insulin and profound changes in endothelial function that it exhibits, both of which could be relevant to ET-1 release and action.

1.2.2.1. Mechanism of STZ induced diabetes

As with other chemicals used to induce DM, STZ has a direct toxic action on pancreatic β-cells (Junod et al., 1969). This toxic effect was shown to begin within 1 hour after treatment and to lead to massive β-cell degranulation and necrosis followed by a transient increase in serum insulin and hypoglycemia. Within 1-2 days, prolonged hyperglyemia and hypoinsulinemia develop. The mechanism of STZ's effect is somewhat controversial, but might involve cellular permeability changes, depletion of NAD, alkylation of DNA, nitric oxide and hydroxyl radical formation, or release of interleukins and cytokines (Ozturk et al., 1996). The

depletion of pancreatic β-cells limits the ability of the rat to produce insulin and thus produces a model of insulin-deficient DM.

1.2.2.2. Dose of STZ

The severity of DM obtained in the STZ rat largely depends on the dose of the agent. Doses of 100 mg/kg severely deplete pancreatic β-cells rapidly causing death (within 2-3 days) due to a severe ketotic state. These rats require exogenous insulin to survive. Doses of 35 mg/kg cause only a mild diabetic state. Most cardiovascular studies use intermediate doses (in the range of 55-65 mg/kg) that promote 3-4 fold higher plasma glucose levels and cause the rats to fail to gain weight. Furthermore, these rats are able to survive for months without insulin supplementation. A dose of 55 mg/kg was chosen in the present study for these reasons. Exogenous supplementation with insulin is able to restore plasma glucose, body weight, and other metabolic parameters to normal. More importantly, exogenous insulin supplementation in these rats is representative of an insulin treated type I diabetic state, and such treatment would be expected to lead to fluctuations in levels of plasma insulin.

1.2.2.3. Vascular abnormalities

Of interest to this thesis is the vascular abnormalities produced by STZ DM. STZ is thought to have profound cardiovascular actions of its own (Tomlinson et al., 1992), however, STZ has a short half-life and is thought to be eliminated completely within 1-2 days. Most studies examine cardiovascular function not less than 2 weeks after

DM induction. Thus, the vascular complications in STZ diabetic rats are thought to arise from the diabetic state itself. Integrated hemodynamic studies are lacking in STZ diabetic rats. Due to experimental discrepancies and limitations to current methodologies, existing studies are inconclusive as to whether the STZ rat is hypotensive, normotensive, or hypertensive (Tomlinson et al., 1992). Similarly, isolated vascular tissue studies are completely inconclusive, as all of increased, decreased, and normal vascular responsiveness to vasoactive agonists has been observed in STZ rats (Tomlinson et al., 1992). Such disparities are thought to be due to inter-study discrepancies in the severity and duration of STZ DM as well as differences in methodologies for measuring vascular function. A relatively consistent finding in the vasculature of STZ rats of relevance to this thesis is the presence of marked endothelial dysfunction, manifesting as attenuated EDRF mediated vasorelaxation (Pieper, 1998). This effect appears to be dependent on the duration of exposure to the diabetic state (Hopfner et al., 1999a) and might involve free radical mediated oxidative damage to EC's (Chang et al., 1993), advanced glycation end product mediated EDRF quenching (Bucala et al., 1991), or a direct toxic effect of hyperglycemia on the vascular endothelium. Clearly, more studies are warranted to examine the status of vascular function in STZ rats, particularly integrated hemodynamic studies that will shed light on the global status of cardiovascular function in this model of insulin deficient DM. The present study focuses on characterizing responsiveness to ET-1 in isolated vascular tissue from these rats in an effort to elucidate the *in vivo* significance of insulinomimetic actions on ET-1 release and action treated diabetic states.

1.2.3. Endothelin: Emerging role in diabetic vascular complications

1.2.3.1. Endothelin-1 in diabetic plasma

Plasma concentrations of ET-1 in normal individuals range between 1 and 5 pM rarely exceeding 25 pM, even in pathological states (Battistini et al., 1993; Frelin & Guedin, 1994). This concentration is inadequate to elicit many of the pharmacological actions of the peptide. However, the increase (decrease) in ET-1 plasma levels is thought to reflect overproduction (underproduction) and spillover (or lack of) of ET-1 from EC (Lerman et al., 1991; Takeda et al., 1991; Wu & Tang, 1998). Indeed, the majority of endogenous ET-1 is released from EC abluminally towards the VSMC (Wagner et al., 1992). Thus, alterations in plasma levels of ET-1 might be of scientific interest in so far as plasma levels are representative of overall cardiovascular production of the peptide.

The status of ET-1 plasma levels in DM is controversial. The streptozotocin (STZ) diabetic rat, a widely used model of insulin deficiency characteristic of IDDM (Tomlinson et al., 1992), has been shown to exhibit elevated plasma ET-1 at 8 weeks (Tada et al., 1994), 10 weeks (Takeda et al., 1991; Makino & Kamata, 1998), and 14 weeks (Hopfner et al., 1999a) after induction of DM. Conversely, these rats have also been shown to exhibit either unchanged ET-1 levels at 2 days (Takeda et al., 1991) or attenuated levels at 4 days (Takahashi et al., 1991) and 2 weeks (Hopfner et al., 1999a; Frank et al., 1993) after DM induction. Thus, the status of ET-1 in the plasma of STZ rats appears to be dependent on the pre-existing duration of DM. In support of this, our laboratory has recently demonstrated that ET-1 plasma levels in

STZ rats are, in fact, differentially altered depending on the pre-existing duration of DM – with attenuated levels at 2 weeks, and elevated levels at 14 weeks after induction (Hopfner et al., 1999a). In patients with IDDM, both elevated (Collier et al., 1992; Haak et al., 1992) and decreased (Smulder et al., 1994; Malamitsi-Puchner et al., 1996) plasma ET-1 has been reported. Decreases in ET-1 production in early DM might be due to inhibitory effects of hyperglycemia on ET-1 release (Hattori et al., 1991), a lack of insulin mediated ET-1 production (Oliver et al., 1990), or to increased EDRF mediated suppression of ET-1 release (Boulanger and Luscher, 1990) from the vascular endothelium. Increases in late DM might be due to attenuated EDRF mediated suppression of ET-1 production (Boulanger and Luscher, 1990), progressive endothelial damage (Vermes et al., 1993), or to elevated lipoprotein mediated ET-1 production (Lerman et al., 1993). Other factors including severity and level of control of DM, presence of macrovascular complications (Collier et al., 1992), and methodology for measuring ET-1 (Baumgartner-Parzer et al., 1998) might also contribute to these discrepancies.

1.2.3.2. In vitro vascular reactivity studies

To date, *ex vivo* studies examining vascular responsiveness to ET-1 in models of DM have yielded equivocal results. Attenuated vasoconstrictor responses to ET-1 (but not other agonists) have been observed in vascular tissue from the STZ rat as early as 2 weeks after DM induction (Tada et al., 1994; Makino & Kamata, 1998; Fulton et al., 1991; Hodgson & King, 1992). Such selectively attenuated responses might be attributed to ET receptor downregulation resulting from either PKC

activation or elevated ET-1 production (Guillon et al., 1998; Awazu et al., 1991; Clozel et al., 1993) and can be reversed by either insulin treatment or restoration of high glucose in the bathing medium (Hodgson & King, 1992). On the contrary, we and others have actually demonstrated increased vasoconstrictor responses to ET-1 and other agonists in vascular tissue from STZ rats as early as 2 weeks after DM induction (Hopfner et al., 1999a; White & Carrier, 1990; Abebe & Macleod, 1990). Such non-specific increases in vasoconstriction might be attributed to modification of contraction resulting from either altered Ca^{2+} channel expression and action (White & Carrier, 1990) or PKC activation (Abebe & Macleod, 1990). Importantly, we have demonstrated that the trend towards exaggerated responses to ET-1 reverses and actually becomes slightly attenuated after long term exposure to DM (Hopfner et al., 1999a). This occurs in conjunction with the development of endothelial dysfunction and elevated plasma lipid levels - suggesting that attenuated responses to ET-1 may take time to develop and may result from other pathological processes occurring only after long-term exposure to DM. Thus, similar to ET-1 plasma levels, the status of vascular reactivity to ET-1 appears to be at least partly dependent on the pre-existing duration of DM.

We have also observed exaggerated vasoconstriction to ET-1 and upregulated ET_A receptors in aorta from obese Zucker rats (Hopfner et al., 1998a). These animals are hyperinsulinemic/insulin resistant, mildly hyperglycemic, and hypertensive (Kasiske et al., 1992). The Zucker rat is therefore considered an animal model resembling NIDDM. Exaggerated vasoconstrictor responses may, however, be non-specific in

this strain, since similar exaggerated responses were seen with other agonists (Ouchi et al., 1996; Hopfner et al., 1998b; Hopfner et al., 1999b). This abnormality has previously been attributed to alterations in Ca^{2+} channel activity in VSMC (Ouchi et al., 1996).

Most studies examining vascular responsiveness in DM use the aorta, a conduit vessel, as the model system. The hazard of correlating large artery tension response data with changes in vascular resistance in diabetic states has been outlined in a previous review (Tomlinson et al., 1992). Perfused arterial bed preparations have been developed which are thought to more adequately represent changes in vascular resistance (Christensen & Mulvany, 1993). As such, we (Misurski et al., 1999), and others (Makino & Kamata, 1998), have recently demonstrated that the perfused mesenteric arterial bed of STZ rats also exhibits altered vasoconstrictor responses to ET-1.

In summary, *in vitro* vascular reactivity studies suggest that alterations in responsiveness to ET-1 exist in both conduit and resistance type vessels in various models of DM. Similar to ET-1 plasma levels, the direction of these changes is controversial and might involve DM duration dependent effects as well as methodological and other study specific factors.

1.2.3.3. In vivo vascular reactivity studies

While several studies have examined *ex vivo* vascular responses to ET-1 in diabetic animals, few studies have assessed hemodynamic responses to the peptide *in vivo*. A few recent studies in STZ rats demonstrated that impaired *in vivo* pressor responses to ET-1 appear as early as 2 weeks after DM induction (Lawrence & Brain, 1992; Kiff et al., 1991; Guillon et al., 1998). Lawrence and Brain (1992) demonstrated attenuated reductions in cutaneous blood flow in response to ET-1 challenge in STZ rats - occurring in conjunction with attenuated EDRF action. In contrast, Kiff et. al (1991) demonstrated that renal and mesenteric vasoconstrictor responses to ET-1 were exaggerated in this model. However, initial hindquarter vasodilator responses to ET-1 were unchanged, while EDRF production was decreased. Lastly, Guillon et al. (1998) recently demonstrated that the *in vivo* pressor response to ET-1 was inhibited in STZ rats by 2 weeks after DM induction, while the response to norepinephrine remained unaltered until 5 weeks after induction. These data suggest that *in vivo* reactivity to ET-1 in STZ DM might be dependent on the duration of DM, the status of endothelial function and the vascular bed under consideration. Only one study to date has assessed *in vivo* vascular reactivity to ET-1 in human diabetic subjects. A lack of sensitivity to the vasoconstrictor effects of locally infused ET-1 was demonstrated in forearms of patients with NIDDM (Nugent et al., 1996).

1.2.3.4. Endothelin-1 in specific cardiovascular complications

1.2.3.4.1. Hypertension

Hypertension occurs in diabetics at a greater incidence than the general population and the combination of the two markedly accelerates the atherosclerotic process (Hsueh & Anderson, 1992). In IDDM, elevated arterial pressure usually develops after, and is thought to result from, nephropathy (Epstein & Sowers, 1992). However, in NIDDM, hypertension precedes renal damage but its cause is unknown (Epstein & Sowers, 1992). ET-1 is well known to promote sustained elevations in BP (Vierhapper et al., 1990) and bosentan, a non-selective ET_A/ET_B antagonist, has recently been shown to lower BP in essential hypertensives (Krum et al., 1998). Haak et. al (1992) first demonstrated that elevated plasma ET-1 acts as a marker of hypertension in IDDM patients. Studies in NIDDM do not support such a link. While plasma ET-1 was shown to be elevated in NIDDM, those with both hypertension and macrovascular disease, but not with hypertension alone, exhibited higher plasma ET-1 than those with uncomplicated NIDDM (Perfetto et al., 1997).

1.2.3.4.2. Atherosclerosis

Atherosclerotic vascular disease occurs in diabetics at a rate much higher than that of the general population and macrovascular complications resulting from atherosclerosis are responsible for the greatest number of deaths associated with the disease (Hsueh & Anderson, 1992). Altered endothelial function ultimately leads to VSMC proliferation - an integral feature of atherosclerosis (Hsueh & Anderson, 1992). ET-1, being a potent and highly efficacious VSMC growth promoting

peptide, might play an important role in this process. Atherosclerotic human arteries were shown to exhibit downregulated ET receptor mRNA, but upregulated ET-1 peptide mRNA (Winkles et al., 1993). A recent study demonstrated that the non-selective ET_A/ET_B receptor antagonist, bosentan, prevented neo-intima formation in a rabbit carotid artery model of atherosclerosis (Marano et al., 1998). Lerman et. al (1991) provided compelling evidence that ET-1 production and ET-1 plasma levels are elevated in patients with atherosclerosis. Later studies confirmed that elevated plasma ET-1 is indeed a marker of atherosclerotic macrovascular disease in patients both with and without NIDDM (Perfetto et al., 1997; Perfetto et al., 1998). Furthermore, those with the highest ET-1 levels had both atherosclerosis and NIDDM (Perfetto et al., 1998). Thus, considerable evidence indicates that ET-1 might be involved in the atherosclerotic process.

1.2.3.4.3. Neurovascular abnormalities

An early reduction in blood flow to peripheral nerves leading to endoneurial hypoxia contributes to nerve conduction deficits in DM (Tuck et al., 1984). Studies in STZ rats have established a possible role for ET-1 in the etiology of attenuated endoneurial blood flow in DM. Sciatic axons of STZ rats were shown to be selectively susceptible to ET-1 induced ischaemia (Zochodne et al., 1998). This was partly accounted for by more intense and prolonged vasoconstriction to the peptide – suggesting that ET-1 might contribute to nerve perfusion deficits in DM. In support of this, sciatic nerve motor conduction deficits in diabetic rats could be improved by 2 weeks of treatment with the ET_A antagonist, BQ123, in association with an

increase in sciatic nutritive endoneurial blood flow (Cameron et al., 1994). However, non-selective ET_A/ET_B blockade with bosentan failed to evoke similar actions (Stevens & Tomlinson, 1995), indicating that the addition of ET_B blockade might blunt the beneficial effects of ET_A antagonism. The addition of an angiotensin AT_1 receptor antagonist to diabetic rats already treated with an ET_A antagonist resulted in synergistic beneficial effects on nerve conduction (Cameron & Cotter, 1996). Thus, there is considerable evidence that an increased vasoconstrictive capacity to ET-1 is present in the neurovasculature of diabetic animals, and importantly, antagonists of the ET system appear to beneficially affect neurovascular function in these animals.

1.2.3.4.4. Retinopathy

Abnormal retinal hemodynamics in both human and animal models of DM contributes to the development of diabetic retinopathy (Kohner et al., 1995). Ocular tissue appears to be a rich source of ET-1 peptide expression. Production of the peptide occurs in both vascular and extravascular tissues in the retina, uveal tract, and optic nerve (MacCumber et al., 1991). Accordingly, alterations in ET-1 production and action have been hypothesized to contribute to diabetic retinopathy and other ocular vascular diseases (Pang & Yorio, 1997). Several *in vitro* studies have demonstrated that high glucose levels can alter both production and responses to ET-1 in pericytes - the contractile cells of the retinal microcirculation. Exposure to elevated glucose levels attenuated ET-1 evoked cellular signaling and contractile responses in rat retinal pericytes (de la Rubia et al., 1992; Chakravarthy et al., 1994).

Later studies showed that increases in retinal circulation time after intravitreal injection of ET-1 and ET-3 were attenuated (Bursell et al., 1995) in STZ rats and that altered retinal blood flow in these rats could be normalized by phosphoramidon, an ECE inhibitor (Takagi et al., 1996). PreproETmRNA was also increased 2-fold in the retina of diabetic rats compared to normal controls. These results implicate increases in the endogenous production of ET-1 as well as changes in sensitivity to ET-1 resulting in altered retinal blood flow in DM. There are few studies assessing the role of ET-1 in diabetic retinopathy in humans. One recent study demonstrated that ET-1 levels in the vitreous fluid of diabetic patients with proliferative retinopathy are actually decreased compared to non-diabetic controls (Ogata et al., 1998). Moreover, elevated plasma ET-1 has been observed in NIDDM patients with retinopathy (Kawamura et al., 1992; Laurenti et al., 1997) but without other DM related end-organ damage. Thus, there is considerable evidence indicating the presence of alterations in ET-1 peptide and receptor expression and action in the diabetic eye that could be relevant to the pathogenesis of diabetic retinopathy.

1.2.3.4.5. Nephropathy

The kidney is an important site of action for ET-1 (Laurenti et al., 1997). Glomerular EC, mesangial cells, and epithelial cells secrete ET-1. ET-1 constricts renal vessels, contracts mesangial cells, inhibits salt and water reabsorption, enhances glomerular proliferation, and stimulates extracellular matrix accumulation. While basal tone is only modestly maintained by ET-1, pathophysiological levels of

ET-1 cause profound renal vasoconstriction with little effect on systemic BP (Sorenson et al., 1994).

Alterations in urinary (Morabito et al., 1994; Turner et al., 1997) total renal (Shin et al., 1995; Vesci et al., 1995), glomerular (Fukui et al., 1993), and ureteral (Nakamura et al., 1997) ET-1 peptide, preproET mRNA, and ET receptor levels are present in animal models of DM. Studies with ET antagonists have also demonstrated a role for this peptide in renal dysfunction in DM. 24 weeks of treatment with the ET_A receptor antagonist, FR139317, attenuated the fall in creatinine clearance and reduced urinary protein excretion and mRNA expression for extracellular matrix components and growth factors in glomeruli of STZ rats (Nakamura et al., 1995). Another ET_A antagonist, LU135252, decreased elevated urinary ET-1 in STZ rats while having only modest effects on albumin excretion (Hocher et al., 1998). The non-selective ET_A/ET_B receptor antagonist, PD142893, normalized renal blood flow, and reduced urinary protein and albumin excretion in proteinuric STZ rats to a similar extent as the ACE inhibitor, enalapril (Benigni et al., 1998). Once again, there are few studies examining renal ET-1 in humans. Elevated 24 hr urinary ET-1 was present in NIDDM patients with albuminuria (Lee et al., 1994) and elevations in circulating ET-1 preceded the microalbuminuric phase of DM related renal damage (de Mattia et al., 1998). Thus, it is clear that alterations in urinary and renal ET-1 content are associated with diabetic nephropathy and this is another complication where ET antagonists have demonstrated protective effects.

1.2.3.4.6. Cardiac disease

The cardiac actions of ET-1 have been well characterized. Local administration of ET-1 to coronary vessels induces marked vasoconstriction resulting in signs of myocardial ischemia (Miyauchi & Masaki, 1999). It also confers positive chronotropic and inotropic actions, and may be arrythmogenic. STZ DM is associated with reductions in mechanical performance of the heart, and changes in responsiveness to various stimuli have been reported in this model (Tahiliani & McNeill, 1986). Radioligand binding studies revealed a decrease in cardiac ET-1 binding sites in STZ rats as early as 3 days after DM induction (Nayler et al., 1989). Positive chronotropic and inotropic actions of ET-1 in right and left atria are also reduced in STZ rats (Lieu & Reid, 1994). Plasma ET-1 levels were shown to be elevated in NIDDM, there was no further increase in those patients with concomitant coronary artery disease (Donatelli et al., 1994), indicating that elevated ET-1 levels might precede cardiac disease in NIDDM. Moreover, a recent study demonstrated that NIDDM patients exhibited elevated coronary sinus blood ET-1 levels during reperfusion subsequent to coronary artery bypass graft surgery (Fogelson et al., 1998). It was suggested that this alteration might contribute to the increased prevalence of cardiac morbidity and mortality observed in NIDDM patients after this procedure.

1.2.3.5. Metabolic variables influencing ET-1 production and action

From the above evidence, it is clear that the diabetic state is associated with changes in the release and action of ET-1 in nearly all of the target sites affected by the

disease. The question that remains is - what factors in DM are responsible for such changes? Due to the plethora of metabolic and cardiovascular abnormalities present in IDDM and NIDDM and differential effects of the various treatment options on these variables, isolating the causes of changes in ET-1 release and action assumes importance.

1.2.3.5.1. Hyperglycemia

Hyperglycemia, the primary metabolic disturbance of DM, has been shown to increase (Yamauchi et al., 1990), decrease (Hattori et al., 1991), and have no effect on (Baumgartner-Parzer et al., 1994; Metsarrine et al., 1994) the release of ET-1 from EC in culture. Species differences as well as the level and duration of incubation of glucose employed by these studies might account for these discrepancies. Studies on the effect of high glucose on receptors for ET-1 have also yielded somewhat equivocal results. Incubation of cultured aortic smooth muscle cells (ASMC) with high glucose has been shown to decrease the maximal binding capacity (but not affinity) for ET-1 (Frank et al., 1993; Sandirasegarane et al., 1994). In contrast, pericytes incubated with high glucose exhibited no significant changes in receptors for ET-1 (de la Rubia et al., 1992). Studies in both retinal pericytes (de la Rubia et al., 1992; Chakaravarthy et al., 1994) and ASMC (Sandirasegarane et al., 1994) have also revealed that contractile responses to ET-1 are blunted after high glucose incubation, apparently independent of effects on receptor expression. Thus, high glucose may regulate the release of ET-1 as well as responses to the peptide at both the receptor and post-receptor level (Figure 5).

Further studies using hyperglycemic clamps are warranted to determine the significance of these findings *in vivo*.

1.2.3.5.2. Insulin

NIDDM is often considered a hyperinsulinemic state and IDDM patients on insulin therapy exhibit fluctuations between insulinemia and glycemia. Since the seminal discovery that insulin increases gene expression for ET-1 in cultured EC (Oliver et al., 1990), intense research has focused on elucidating the relevance of this effect in pathological states characterized by elevated or fluctuating insulin levels. It was confirmed that physiological insulin concentrations could increase the release of ET-1 from both EC (Metsarrine et al., 1994; Ferri et al., 1995) and VSMC (Anfossi et al., 1993) in culture. Later studies went on to show that induction of hyperinsulinemia increased plasma levels of ET-1 in both humans (Ferri et al., 1995; Piatti et al., 1996) and animals (Frank et al., 1993). Other studies have, however, failed to show such an effect (Metsarrine et al., 1994; Leyva et al., 1997).

Studies have also demonstrated an effect of insulin on changes in tissue reactivity to ET-1, both at the receptor, and post receptor level. It was first demonstrated in 1993 that incubation of ASMC with high concentrations of insulin increased ET receptor expression, and that 2 weeks of *in vivo* insulin delivery to normal and STZ rats was associated with increases in kidney, brain, and ASMC ET receptors (Frank et al., 1993). The present study confirms these results demonstrating that long-term insulin incubation of rat ASMC selectively increases ET_A receptor expression and that

upregulated receptors are coupled to increases in ET-1 evoked $[Ca^{2+}]_i$ elevations. Furthermore, we demonstrated that aorta from the hyperinsulinemic/insulin resistant obese Zucker rat exhibit increased ET_A receptor expression, suggesting possible physiological relevance of these findings. This was supported by a recent study demonstrating that hyperinsulinemic fructose hypertensive rats exhibit increased tail artery ET_A receptor expression (Juan et al., 1998). In contrast, acute incubation of VSMC with insulin appears to have a suppressive effect on ET-1 (as well as other agonists) evoked increases in $[Ca^{2+}]_i$ levels. In the presence of supraphysiological insulin concentrations, ET-1 evoked contraction of cultured rat mesangial cells was attenuated (Inishi et al., 1994). In support of this, it was later demonstrated that porcine coronary artery $[Ca^{2+}]_i$ responses to ET-1 were attenuated in the presence of a physiological insulin concentration (Dick & Sturek, 1996). The functional relevance of these observations is evident in a recent study demonstrating that insulin attenuates ET_A receptor mediated coronary contraction through an endothelium independent mechanism (Hasdai et al., 1998). However, another recent study using endothelium intact rat aortic rings demonstrated that, in the presence of insulin, arterial contraction induced by KCl was exaggerated, and this was normalized in the presence of ET blockers (Nava et al., 1997). Thus, from the available evidence, it appears that the effect of insulin on VSM responsiveness to ET-1 *in vitro* depends on both the term of exposure to insulin, as well as on the presence or absence of intact endothelial function for the release of ET-1. In summary, insulin appears to be a modulator of ET-1 peptide, ET receptors and ET-1 mediated responses - both *in vitro* and *in vivo* (Figure 6).

1.2.3.5.3. Lipoproteins

Elevated plasma lipoproteins are present in both IDDM and NIDDM patients, particularly in those with poor metabolic control (Epstein & Sowers, 1992). A similar profile exists in diabetic animal models. The Zucker rat exhibits elevated plasma lipids (Kasiske et al., 1992), and the STZ rat develops hyperlipidemia and hypercholesterolemia after only a few weeks of exposure to DM (Hopfner et al., 1999a). Significant evidence indicates a profound ability of plasma lipoproteins to affect both the release and action of ET-1. Oxidized LDL was shown to evoke a concentration and time dependent increase in preproETmRNA expression in porcine and human EC (Boulanger et al., 1992). Subsequent studies confirmed that only high concentrations of native VLDL and LDL would have a similar effect (Horio et al., 1993). Several *in vivo* studies have substantiated these findings. Elevated plasma lipids were shown to elevate EC (Uyama et al., 1996), coronary (Lerman et al., 1993), and plasma (Lerman et al., 1993; Piatti et al., 1996), ET-1 levels *in vivo*. Furthermore, the effect of hyperlipidemia on plasma ET-1 was synergistic with hyperinsulinemia (Piatti et al., 1996). Accordingly, plasma ET-1 has been shown to be elevated in patients with elevated plasma lipoproteins (Haak et al., 1994) even before the development of atherosclerosis (Magniafico et al., 1996).

Similar to observations with insulin and glucose, elevated lipids also appear capable of altering VSM responsiveness to ET-1. It was demonstrated that hypercholesterolemic rabbits exhibit exaggerated vascular reactivity to vasoactive peptide agonists, including ET-1, before the development of endothelial dysfunction

(Merkel & Bilder, 1992). In addition, pigs fed a high cholesterol diet for 10 weeks exhibited accentuated coronary vasoconstrictor responses to ET-1 without exhibiting changes in epicardial coronary artery ET receptor density or affinity (Matthew et al., 1997). Thus, similar to both insulinemia and hyperglycemia, hyperlipidemia *per se* has the capability to increase plasma levels of ET-1 as well as receptors and tissue reactivity to the peptide (Figure 7).

1.2.3.5.4. Endothelial dysfunction and duration of disease.

The nature of endothelial dysfunction in DM is controversial. It has been hypothesized that elevated EDRF production occurs in DM due to hyperglycemia mediated nitric oxide synthase (NOS) activation (Wascher et al., 1994; Pieper, 1998). However, it is also well established that the metabolic dysregulation of DM is associated with inhibition of EDRF release and action through a multitude of direct and indirect actions (Figure 8; Pieper, 1998). Significant evidence implicates differences in the duration of exposure to DM in determining the nature and direction of these changes. While hyperglycemia might activate EDRF production acutely, as the duration of DM progresses, atherosclerotic (Hsueh & Anderson, 1992) and other events such as advanced glycation end product (Bucala et al., 1991) and superoxide radical (Chang et al., 1993) formation clearly promote pathological changes in endothelial function leading to suppressed EDRF action. In support of this concept, a recent study demonstrated that EDRF mediated vasodilatation is accentuated in 2-week STZ rats, but becomes paradoxically impaired as the duration of DM progresses (Pieper, 1999). Moreover, we have recently established that

EDRF mediated vasodilatation is attenuated in 14 week, but not 2 week, STZ rats (Hopfner et al., 1999a).

Alterations in EDRF in DM could significantly affect ET-1 activity. EDRF is well known to suppress ET-1 release (Boulanger and Luscher, 1990) and endothelial damage itself is thought to be associated with increased release of ET-1 (Lerman et al., 1991; Takeda et al., 1991). Moreover, ET receptor blockade in a model of atherosclerosis was recently shown to restore EDRF mediated endothelial function – suggesting that ET-1 may act to inhibit EDRF in pathological states (Barton et al., 1998). Such a link between changes in EDRF and endothelial function and the release and action of ET-1 likely has a profound effect on ET-1 activity in DM. Accordingly, as DM progresses from diagnosis to end stage, changes in ET-1 release and action may vary in parallel with changes in EDRF and endothelial function. Indeed, we have shown that changes in plasma levels of ET-1 and vascular responses to ET-1 in STZ rats are duration dependent and occur in parallel with changes in endothelial function (Hopfner et al., 1999a).

1.2.3.5.5. Summary – metabolic variables

In summary, it is clear that many of the well-known metabolic abnormalities encountered in DM contribute individually and synergistically to alterations in the release and action of ET-1 both *in vitro* and *in vivo*. Further studies examining how ET blockers affect the sequelae of each of these abnormalities are required to determine the physiological relevance of these actions. Furthermore, examining

how these factors interact *in vivo* and how current treatment options for the metabolic dysregulation of DM act to modulate ET-1 activity also assumes importance.

1.2.3.6. Conclusions

The diabetic state is associated with a multitude of metabolic abnormalities that are capable of directly and indirectly contributing to endothelial and VSM dysfunction. Alterations in both the release and action of ET-1 are clearly present in various models of DM. However, the nature and direction of many of these changes is controversial. Nonetheless, the important role of ET-1 as a modulator of vascular tone and growth indicate that any such changes might have important pathophysiological consequences in the development of diabetic vascular complications. Antagonists of the ET system are rapidly progressing to the clinic for the treatment of cardiovascular disorders including congestive heart failure and pulmonary hypertension. Groundbreaking recent studies in diabetic animal models indicate that blockers of the ET system might beneficially affect, and even ameliorate some of the cardiovascular complications of DM – notably nephropathy, and neuropathy. Future studies will determine the role of the ET system in other complications of DM and whether these actions are relevant to human DM. Such studies might provide rationale for the clinical use of ET antagonists in DM.

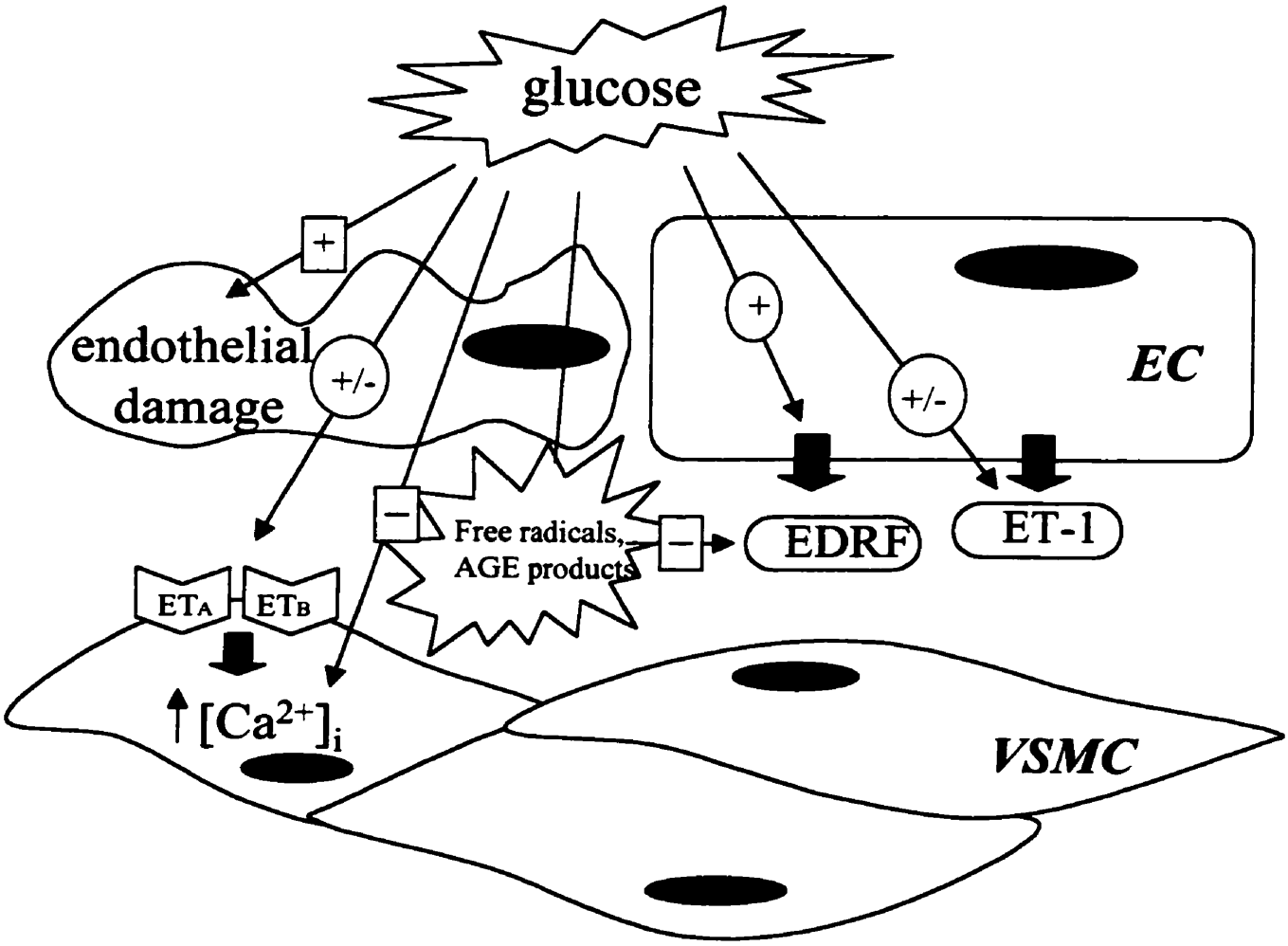


Figure 5. Effects of high glucose on factors related to ET-1 production and action. Glucose regulates ET-1 peptide and receptor expression as well as responses to the peptide *in vitro*. However, the direction of these changes is controversial. High glucose also increases EDRF production through activation of nitric oxide synthase. It also inhibits EDRF via endothelial damage and other actions such as advanced glycation end product and superoxide radical formation. Such actions on EDRF could also regulate ET-1 production and action.

EDRF – endothelium derived relaxing factor; EC - endothelial cell; VSMC – vascular smooth muscle cell.

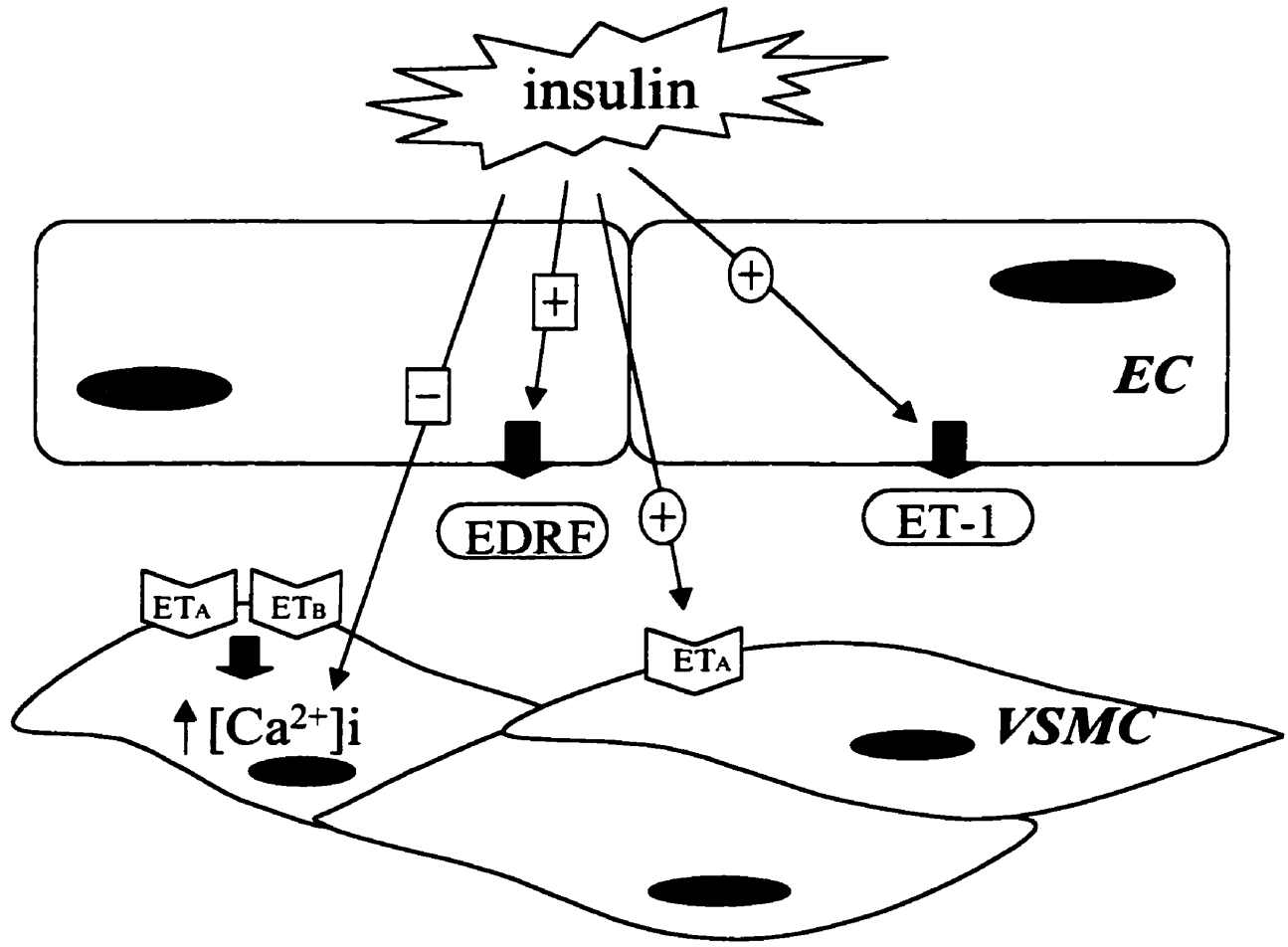


Figure 6. Effects of insulin on factors related to ET-1 production and action. Insulin increases ET-1 peptide production via gene activation of ET-1. Insulin also selectively upregulates ET_A receptors on VSMC after long-term incubation, and enhances subsequent $[Ca^{2+}]_i$ responses to ET-1. In contrast, short-term incubation of VSMC with insulin actually attenuates ET-1 evoked $[Ca^{2+}]_i$ responses. Insulin also increases EDRF formation via nitric oxide synthase activation.

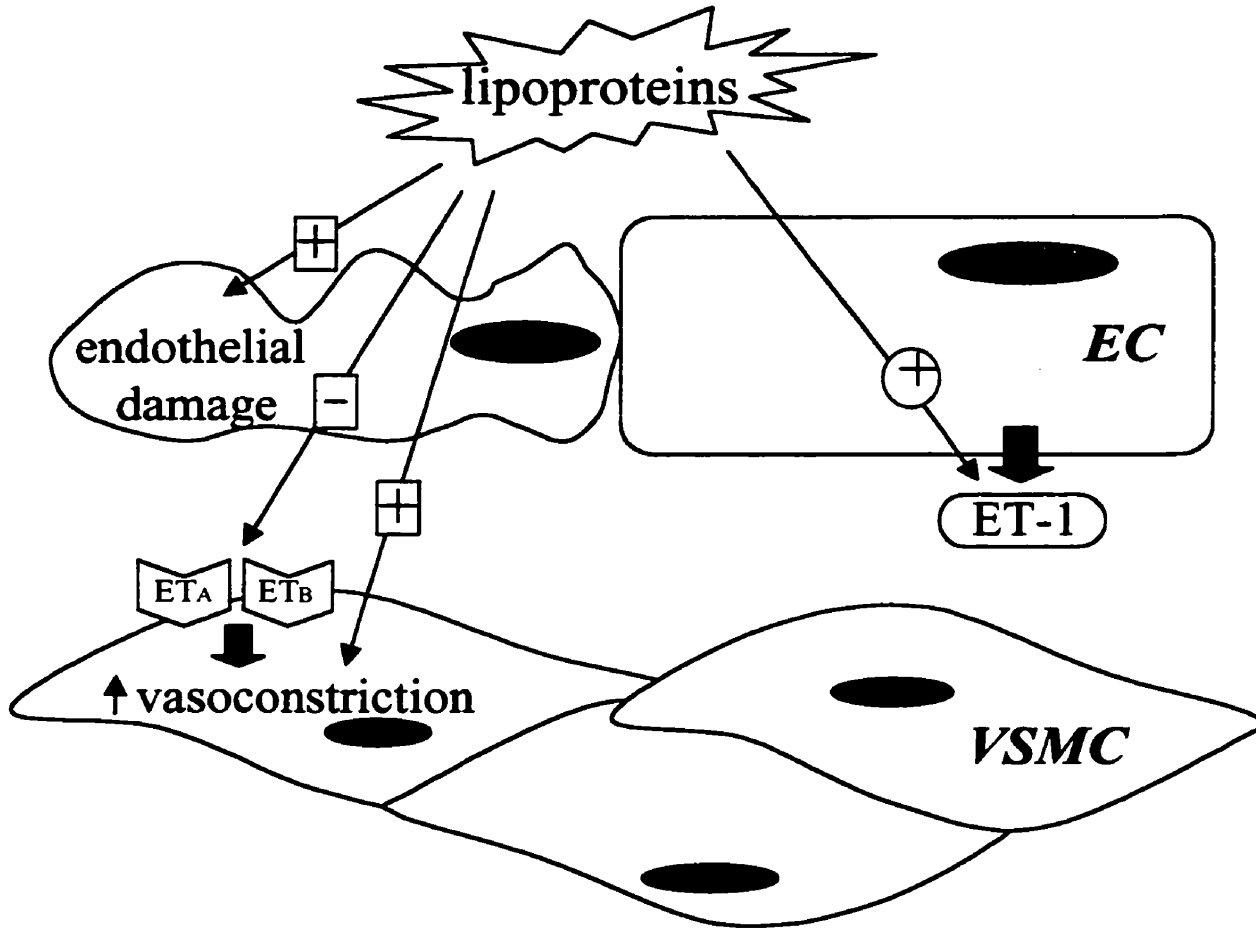


Figure 7. Effects of lipoproteins on factors related to ET-1 production and action. Oxidized low-density lipoproteins increase ET-1 gene expression and elevated lipoproteins *in vivo* accentuate vascular responses to agonists (including ET-1) before the onset of atherosclerosis. In atherosclerotic blood vessels, ET receptors are decreased and ET-1 peptide increased. Moreover, endothelial damage resulting from the cascade of atherosclerotic events resulting from dyslipidemia would be expected to modulate the release and action of ET-1.

LDL – low density lipoprotein

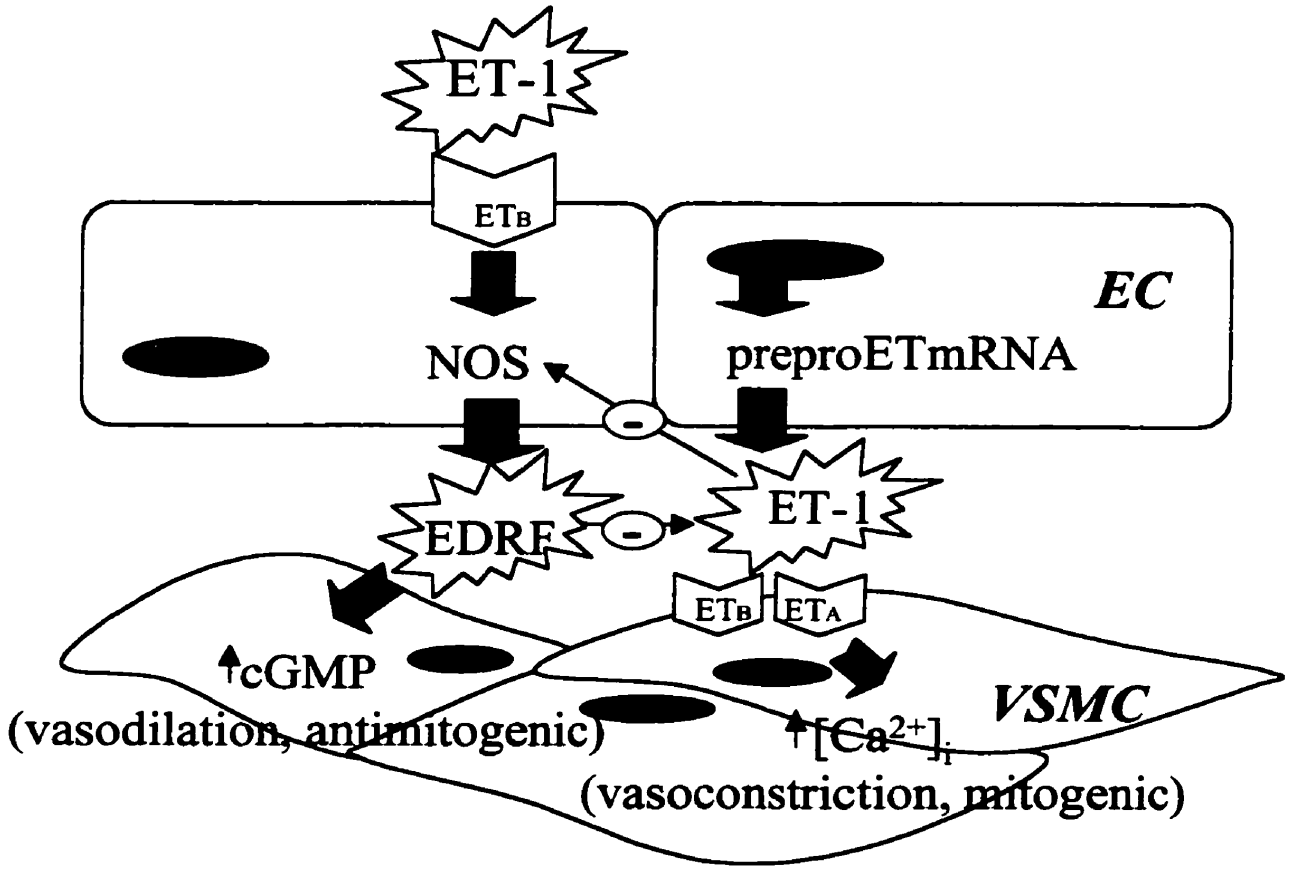


Figure 8. Inter-relationships between ET-1 and EDRF. ET-1 is the main vasoconstrictor/mitogenic mediator released by the vascular endothelium whereas EDRF modulates vasodilatation/anti-growth effects. EDRF inhibits ET-1 release at the gene level. Conversely, ET-1 might inhibit EDRF in pathological conditions. Thus, factors that regulate one system can be expected to affect the other. The metabolic dysregulation of DM has profound effects on both EDRF and ET-1 release and action. Such inter-dependence might be evident in early and late stage DM where changes in endothelial and EDRF function appear to coincide with changes in ET-1 release and action.

1.3. Vanadium Compounds

1.3.1. Insulinomimetic effects

1.3.1.1. In vitro

Vanadium is a transition metal found in abundance in nature (0.02% of earths crust). It has an extremely complex chemistry and can readily change its oxidation state and take an anionic or cationic form (Rehder, 1992). The first evidence that vanadium could influence a biological system came in 1977, when its *in vitro* inhibitory action on Na^+/K^+ ATP'ase was described (Cantley, 1977). The insulinomimetic effects of vanadium was first described in 1979, when vanadate was shown to increase glucose transport and oxidation in adipocytes, to stimulate glycogen synthesis in liver and diaphragm, and to inhibit gluconeogenesis in hepatocytes (Tolman et al., 1979). Since then, vanadium has been found to mimic most of the biological effects of insulin in various cell types. Vanadate, the V^{+5} oxidation state of vanadium, exhibits insulinomimetic effects in various tissues as follows - Vanadate: 1) accelerates glycolysis by inhibiting fructose 2,6 bisphosphatase, and inducing the L-type pyruvate kinase gene (Miralpeix et al., 1991); 2) decreases mRNA for phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme in gluconeogenesis (Bosch et al., 1990); 3) promotes glycogen deposition in hepatocytes; 4) recruits glucose transporters to the plasma membrane (Paquet et al., 1992); 5) activates lipogenesis, inhibits lipolysis and increases the release of lipoprotein lipase from adipose tissue (Ueki et al., 1989); and 6) enhances glucose uptake, glycolysis, and glycogen synthesis to a lesser extent than insulin, but causes a greater stimulation of lactate and glucose oxidation (Clark et al., 1985).

1.3.1.2. In vivo

The antidiabetic effects of vanadate *in vitro* prompted *in vivo* experiments. The demonstration of its efficacy by Heyliger et al. (1985) was the beginning of numerous studies in animal models of DM. The most marked effects of vanadate are seen in STZ rats, where oral administration of vanadate causes a fall in blood glucose levels within 2-5 days. Efficacy of treatment appears to be inversely related to the severity of DM and persists for up to one year. In addition, the antidiabetic effects of vanadate have been shown in genetically obese, hyperinsulinemic, and insulin resistant rats and mice (Meyerovitch et al., 1991; Brichard et al., 1989). Recently, two groups have done small scale clinical studies on the antidiabetic effects of vanadate (Goldfine et al., 1995, Cohen et al., 1995). The dose of vanadate used was much lower than previous studies in animals, and treatment periods were up to 3 weeks. In IDDM patients, no consistent influence on the glycemic control could be detected, but daily insulin requirements decreased significantly. In NIDDM, insulin sensitivity increased during vanadium treatment, via greater inhibition of hepatic glucose production by insulin and from a greater stimulation of peripheral glucose disposal. Most of these metabolic effects persisted for up to 2 weeks following cessation of treatment.

1.3.2. Cellular actions

The cellular actions of vanadate relevant to its insulinomimetic effects are depicted in Figure 9. Vanadate permeates the intestinal wall and cell membranes via either passive diffusion or by means of an anion carrier system (Cantley, 1977). Once

inside the cell, vanadate is converted primarily to the vanadyl (V^{+4} oxidation state) form. The insulin like properties of vanadate have been ascribed to increased phosphorylation of the insulin receptor by inhibition of phosphotyrosyl phosphatase (Swarup et al., 1982). Vanadate could also act at sites distal to the insulin receptor. This view is supported by several observations: 1) Insulin like effects of vanadate remain in adipocytes depleted of insulin receptors (Green, 1986); 2) Vanadate evoked phosphorylation of the insulin receptor is weak (Mooney et al., 1989); 3) Vanadate stimulation of MAP kinase is independent of insulin receptor phosphorylation (D'Onofrio et al., 1994); and 4) Vanadate activates a cytosolic insulin insensitive tyrosine kinase (Elberg et al., 1994). Taken together, these observations indicate that vanadate might be active in influencing glucose metabolism even when insulin signaling pathways are not functioning correctly. This could have obvious benefits in NIDDM, with its hallmark insulin resistance.

1.3.3. Toxicities

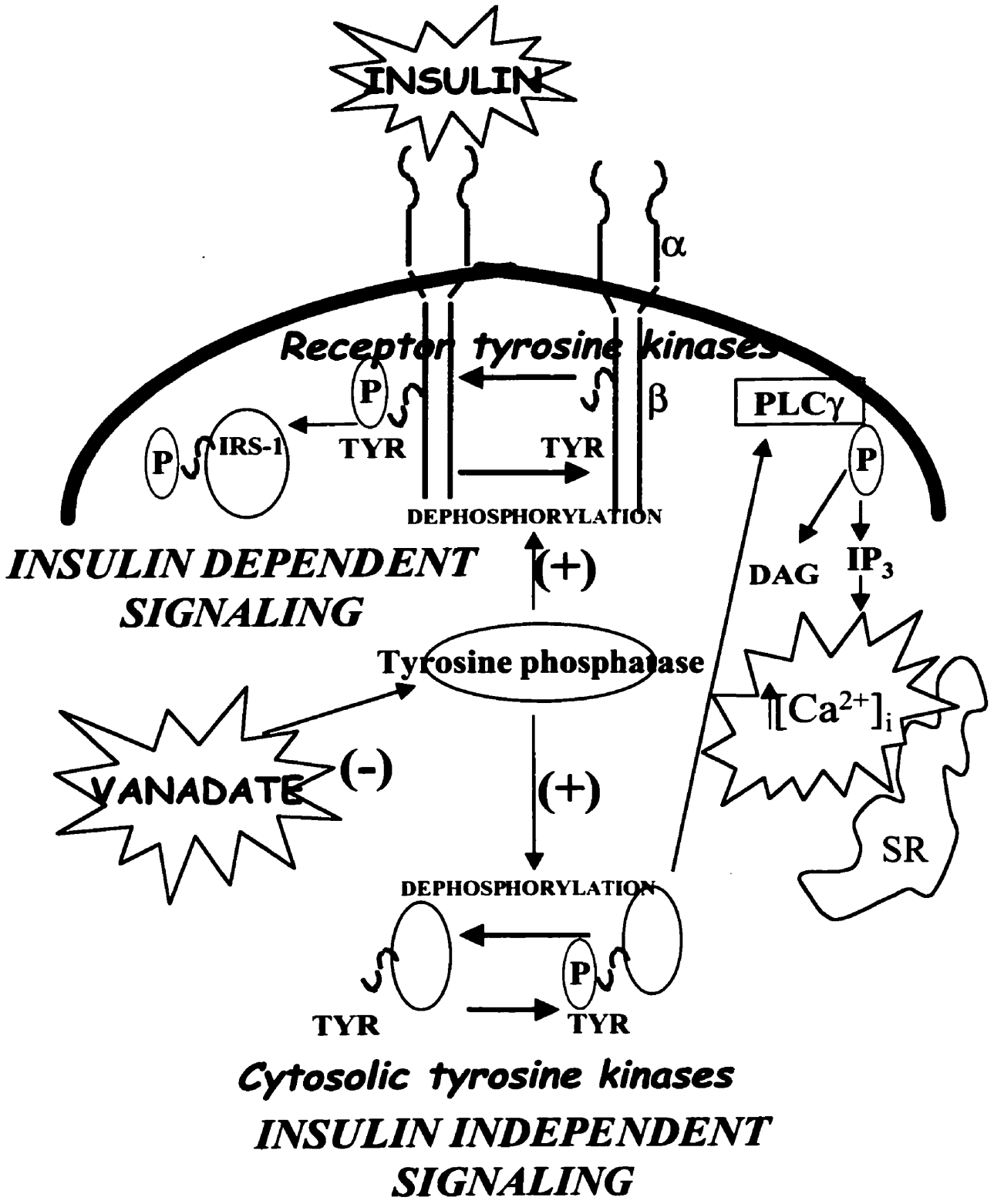
Concerns about toxicity have hampered research on the development of vanadium as a treatment for diabetics. Blood levels of vanadium average about 20 μM in diabetic animals chronically treated with oral vanadium, a level 100-fold greater than in untreated controls. Higher concentrations may be reached in bone and kidneys, where vanadium accumulates (Brichard & Henquin, 1995). The most prevalent side effect of vanadium is gastrointestinal intolerance and an anorectic effect. With some exceptions, long term oral vanadium treatment has not been shown to affect haematological indices or to alter renal or hepatic function. There is

little toxicological data regarding chronic vanadium treatment in humans. One of the major concerns of vanadate is its known contractile effect on both VSM and non-VSM tissue (Sunano et al., 1988; Laniyonu et al., 1994).

1.3.4. Cardiovascular actions

Dr. Hollenberg and associates have established that vanadate contracts rat aorta by promoting Ca^{2+} influx via activation of protein tyrosine kinase (Laniyonu et al., 1994). Our laboratory has shown that vanadate enhanced $[Ca^{2+}]_i$ levels in rat ASMC with an EC_{50} of 42 ± 11 μM; this is closely parallel to the EC_{50} value for its vasoconstrictor effect on aortic rings (Sandirasegarane & Gopalakrishnan., 1995). In agreement with *in vitro* reports, several studies have shown an increased vasopressor response to vanadate *in vivo* (Voelkel & Czartolomna, 1991; Steffen et al., 1981). In apparent contrast to these reports, Dr. John McNeill's laboratory reported that oral administration of vanadyl sulfate or an organic vanadyl compound reduced blood BP in both fructose induced hypertensive rat and spontaneously hypertensive rat models, but not in normotensive strains (Bhanot & McNeill, 1994; Bhanot et al., 1994). They hypothesized that correction of insulin resistance and subsequent hyperinsulinemia in these strains was responsible for this effect. It is one intent of this project to further explore the contractile effects of vanadate at the cellular and whole animal level in relation to ET-1 evoked alterations in vascular tone in an attempt to fully characterize the potential toxicity of this clinically useful insulin sensitizing agent.

Figure 9. Signaling pathways for insulin dependent and insulin independent cellular actions of vanadate. Vanadate gains access to cells in the VO^{3-} form via either passive diffusion or by means of an anion carrier system. Once inside the cell, vanadate is converted to the vanadyl form. Through inhibition of protein tyrosine phosphatases, vanadate can either enhance insulin mediated phosphorylation of the insulin receptor (insulin-dependent action), or activate cytosolic tyrosine kinases (insulin-independent action) coupled to PLCγ activation and the subsequent cascade of events leading to increases in $[Ca^{2+}]_i$.


INSULIN
α
Receptor tyrosine kinases
P
β
PLCγ
P
IRS-1
TYR
TYR
P
DEPHOSPHORYLATION
INSULIN DEPENDENT SIGNALING
(+)
DAG
IP3
Tyrosine phosphatase
[Ca2+]i
VANADATE
(-)
(+)
SR
DEPHOSPHORYLATION
P
TYR
TYR
Cytosolic tyrosine kinases
INSULIN INDEPENDENT SIGNALING

2. PRESENT INVESTIGATION

2.1. General

The diabetic state is associated with alterations in vascular function that contribute to the well-known cardiovascular complications of the disease. Recent studies have outlined a potential role for the ET system in contributing to these complications. It is also evident that some of the treatment options – including insulin and vanadate - for DM might not only correct the metabolic abnormalities responsible for this degradation in cardiovascular function, but may adversely affect cardiovascular function themselves. In light of the well-established interactions between insulin and ET-1, it is important to extensively examine the relationship between these two peptides. Moreover, while many studies have addressed the role of insulin in promoting the release of ET-1 both *in vivo* and *in vitro*, few studies have addressed the role of this important metabolic hormone in regulating receptors for, and responses to ET-1 at the VSM level. With these premises, this thesis addresses the role of both insulin, and the insulinomimetic agent, vanadate, in regulating ET receptors *in vitro*, and the consequences of these effects under treatment conditions in type I diabetic states.

2.2. Hypothesis

1. Long-term preincubation of ASMC in culture with both insulin and vanadate upregulates cell surface ET receptors. The subcellular mechanism initiating this response is tyrosine kinase phosphorylation, since both insulin and vanadate activate this process by direct coupling to cell surface tyrosine kinase linked receptors and inhibition of protein tyrosine phosphatases, respectively.

2. Insulin and vanadate induced upregulation of ET receptors on ASMC is coupled to enhanced ET agonist induced $[Ca^{2+}]_i$ responses as well as enhanced expression of ET_A and ET_B mRNA. This effect is specific to ET receptors, as responses to Ang II and AVP remain unchanged.

3. Vascular tissue isolated from STZ diabetic rats exhibits selectively attenuated responses to ET-1, since severe insulin deficiency would remove the upregulatory influence of this hormone on ET receptor expression *in vivo*.

4. Long-term *in vivo* treatment with insulin or vanadate will normalize attenuated ET-1 evoked vasoconstrictor responses in vascular tissue from STZ rats by restoring the insulinomimetic influence on ET receptor upregulation. Similarly, insulin and vanadate treatment will promote exaggerated responses to ET-1 in vascular tissue from non-diabetic controls.

2.3. Rationale and objectives

The major emphasis of this project was to provide insight into the role of insulinomimetics in modulating ET activity both *in vitro* and *in vivo*. Isolated ASMC in culture were used to examine the *in vitro* effect of insulinomimetic pretreatment on ET_A and ET_B receptor binding and mRNA expression, as well as on ET-1 induced $[Ca^{2+}]_i$ regulation. Interestingly, past studies have shown attenuated agonist evoked $[Ca^{2+}]_i$ responses in ASMC in the presence of insulin (Touyz et al. 1994; Standley et al. 1991). However, other studies have shown that long term preincubation with insulin actually increases ET receptor expression on VSMC (Frank et al., 1993). We proposed that after longer term preincubation with insulin, ET-1 evoked $[Ca^{2+}]_i$ would, in fact, rise due to upregulation of ASMC ET receptors. In addition, the effect of vanadate on these parameters was also measured since no studies have examined vanadate's effects on ET activity in any cell line.

The second major focus of this project was to determine the effect of insulin and vanadate on ET-1 release and action under treatment conditions in diabetic and non-diabetic states. If insulin and vanadate affected ET receptor expression *in vitro*, we were interested in determining whether similar effects could be seen *in vivo*. To this end, ET-1 evoked vasoconstrictor responses were determined in vascular tissues derived from insulin and vanadate treated rats. Most studies to date that have examined the effects of insulin on ET-1 production and action *in vivo* have been done using short-term insulin treatment (ie. hyperinsulinemic clamp). We deduced that the physiological situation would be more precisely mimicked if subjects were

pretreated with insulin for a longer time period. Thus, rats were treated for a period of 14 days using continuous delivery of insulin or oral delivery of vanadate after which vascular reactivity and plasma ET-1 measurements were made.

2.4. Basic Design

2.4.1. Specific Aims

2.4.1.1. Aim 1

The first aim of the study was to elucidate the effect of insulin pretreatment on ET receptor expression and ET evoked $[Ca^{2+}]_i$ changes in ASMC in culture. To these ends, three types of experiments were performed:

- Receptor binding studies
- Measurement of $[Ca^{2+}]_i$ changes in dispersed ASMC
- ET_A and ET_B mRNA measurements using northern blot methodology

It was important to ascertain whether other vasoactive peptides would elicit similar responses after insulin pretreatment. Therefore, in addition to the main focus on ET-1, additional $[Ca^{2+}]_i$ studies were carried out utilizing two other potent vasoactive peptides, Ang II and AVP.

2.4.1.2. Aim 2

The second aim of the study was to simply compare the *in vitro* effects of insulin with those of the well-known insulinomimetic, vanadate. It is important to determine whether insulinomimetics acting through different mechanisms (than insulin) would evoked similar actions as insulin on ET receptor expression and

action. Accordingly, all of the above experiments with insulin were carried out in parallel with vanadate.

2.4.1.3. Aim 3

The final, and most physiologically relevant aim of the study was to investigate the effects of longer-term *in vivo* insulin and vanadate treatment on ET-1 release and action in diabetic and non-diabetic animal models. To this end, both STZ diabetic and non-diabetic rats were treated with insulin and vanadate for a period of 2 weeks after which vasoconstrictor responses to ET-1 in vascular tissue *ex vivo* and plasma levels of the peptide were measured. Once again, since it was important to determine whether any of the observed effects of these compounds was specific to ET-1, we also measured vasoconstrictor responses to methoxamine (α_1-adrenergic agonist) and vasodilator responses to ACh, an endothelium dependent vasodilator.

2.4.2. Specific experimental objectives and design

2.4.2.1. Animal models

Towards the aforementioned receptor binding, $[Ca^{2+}]_i$, and mRNA measurements, ASMC derived from age/sex matched 10 to 12-week old male SD rats were employed. For *in vivo* studies, age and sex matched Sprague-Dawley (SD) control (non-diabetic) and STZ treated (diabetic) rats were employed.

2.4.2.2. In vitro studies

2.4.2.2.1. Receptor binding

It has previously been shown that insulin pretreatment upregulates ET_A receptors on VSMC's (Frank et al., 1993). However, there has been no attempt to differentiate the receptor subtype involved in insulin evoked ET receptor upregulation in rat ASMC's. In addition, vanadate's effects on ET receptor regulation has yet to be documented, which assumes importance in light of its known contractile effects on VSM (Sunano et al., 1988, Laniyonu et al., 1994). Thus, the effects of insulin and vanadate on ET receptor binding parameters in ASMC was first examined.

Saturation binding studies utilized radiolabeled ET-1 (ET_A/ET_B non-selective agonist), and IRL-1620 (ET_B agonist) to determine the density of maximal binding sites (B_{max}) and the equilibrium dissociation constant or affinity (K_D). The likely role of insulin and vanadate on ET receptor binding in ASMC was then assessed. Preliminary studies involved pretreatment of ASMC *in vitro* with insulin and vanadate for given time periods to determine the optimal time and concentration of pre-incubation which upregulates [^{125}I] ET-1 binding to the maximal extent. Once optimal conditions were established, saturation studies were performed and ET_A and ET_B sites per cell were ascertained. It is widely accepted that insulin exerts its effects through tyrosine kinase linked receptor activation promoting phosphorylation of several intracellular proteins involved in eliciting the actions of insulin. In addition, vanadate enhances insulin receptor kinase activation (Fantus et al., 1994) and phosphorylation of a cytosolic, non-insulin tyrosine kinase linked receptor

(Schechter et al., 1995) through inhibition of phosphotyrosine phosphatase. To determine whether changes in ET receptor regulation occur through tyrosine kinase mediated mechanisms, the effect of the tyrosine kinase inhibitor, genistein, on the observed ET receptor modulating effects of insulin and vanadate was determined.

2.4.2.2.2. mRNA measurements

If insulin and vanadate evoked changes in ET receptor binding characteristics, it was important to determine whether this effect was mediated at the gene level. Thus, subsequent to the binding studies, parallel experiments were carried out using northern blot analysis measuring ET_A and ET_B receptor mRNA expression in ASMC after various time periods of preincubation with these agents. In addition, further studies with genistein in this regard were performed to elucidate the importance of tyrosine kinase linked pathways in changes in ET receptor mRNA. Finally, the role of new protein synthesis and active transcription were investigated using cycloheximide and actinomycin-D, respectively.

2.4.2.2.3. Intracellular calcium ($[Ca^{2+}]_i$) measurements

It was of interest to determine whether upregulated ET receptors evoked by insulin and vanadate were coupled to a functional cellular response to ET-1. Under the optimal conditions established in receptor binding studies, the effect of insulin and vanadate pretreatment on ET agonist evoked increases in $[Ca^{2+}]_i$ was measured in isolated rat ASMC using fura-2 methodology. Changes in the $[Ca^{2+}]_i$ response to

ET agonists would add potential functional significance to ET receptor upregulation induced by insulin and vanadate.

Parallel experiments with AVP and Ang II were done where $[Ca^{2+}]_i$ responses to these two agonists were measured after the same pretreatment period with insulin and vanadate. This assumed importance in elucidating whether the actions of insulin and vanadate are specific to ET-1. To determine the ET_B response, IRL-1620, an ET_B selective agonist, was used in place of ET-1 under the same conditions. In addition, BQ123 (ET_A antagonist) and BQ788 (ET_B antagonist) were added to the cell suspension before ET agonist challenge in hopes of further differentiating the ET_A and ET_B contributions to agonist evoked $[Ca^{2+}]_i$ after insulin and vanadate treatment.

2.4.2.3. Ex vivo studies

2.4.2.3.1. Vascular reactivity

If our *in vitro* results were indicative of altered responses to ET-1 *in vivo* under treatment conditions, then ET-1 evoked vasoconstrictor responses might be exaggerated in vascular tissue from insulin and vanadate treated rats. Thus, to examine the functional relevance of the observed *in vitro* effects of insulin and vanadate, aortic rings were harvested from both non-diabetic SD and diabetic STZ rats treated with either insulin or vanadate. Towards this goal, rats were continuously treated with insulin via subcutaneous mini-osmotic infusion pumps for a period of 14 days, or with vanadate in drinking water. We deduced that long-term

maintenance of hyperinsulinemia with mini-osmotic pumps would more adequately mimic physiological hyperinsulinemia than most previous studies that use short-term (e.g. 3 hr) hyperinsulinemic clamps. After this period, we measured vasoconstrictor responses to ET-1 in aortic rings harvested from these rats. In order to determine whether changes in vascular responsiveness were selective to ET-1, we also measured responses to the α_1-adrenoceptor agonist, methoxamine. Finally, since endothelial damage often occurs in diabetic states, we also investigated responses to the endothelium dependent vasodilator, ACh.

2.4.2.3.2. Plasma endothelin-1

Insulin evoked increases in ET-1 production from human EC and cultured human VSMC has been well characterized (Ferri et al., 1995; Anfossi et al., 1993). Recently, *in vivo* studies have determined that this relationship also holds true in human hyperinsulinemia (Wolpert et al., 1993; Haak et al., 1992; Piatti et al., 1996). Thus, in addition to effects on vascular reactivity, parallel measurements of plasma ET-1 were made in insulin and vanadate treated rats. In addition to changes in responsiveness to ET-1, changes in the release of the peptide might also contribute to vasculopathy.

3. MATERIALS & METHODS

3.1. Animals

Six to nine-week old male SD rats were purchased from Charles River, Montreal, QC. They were housed in the animal facilities with due care under standardized conditions with a light-dark cycle of 12 hrs at a temperature of 22°C. Rats were fed ad-libitum with Purina Rat Chow and tap water (unless otherwise indicated). Depending on the experiment, rats were killed between 12 and 15 weeks of age.

3.2. Chemicals and Reagents

3.2.1. Radiolabeled substances

The radiolabeled substances, [^{125}I] ET-1 and [^{125}I] IRL-1620 (specific activity 2200 Ci/mmol) were from DuPont Canada (Mississauga, ON). The radioimmunoassay (RIA) kits for the measurement of plasma insulin and ET-1 were from Amersham Life Sciences (Oakville, ON).

3.2.2. Unlabeled peptides

The unlabelled peptide-agonists ET-1 (porcine/human) and IRL-1620 [Suc-(glu^{9},Ala^{11,15}) ET-1], as well as antagonists BQ123 and BQ788 were purchased from American Peptide Co Inc. (Sunnyvale, CA). Angiotensin II (Ang II) and arginine-vasopressin (AVP) were purchased from Sigma Chemical Co (St. Louis, MO).

3.2.3. ET_A and ET_B cDNA probes

Rat ET_A and ET_B cDNA probes were kindly provided as a gift from Dr. J. Winkles (American Red Cross, Rockville, MD) and Dr. P. Nambi (SmithKline Beecham Pharmaceuticals, King of Prussia, PA), and the β-actin probe from Dr. W. R. Roesler (University of Saskatchewan, Saskatoon, SK).

3.2.4. Other substances

Acetylcholine-chloride (ACh), α-actin, actinomycin D, arginine vasopressin (AVP), angiotensin II (Ang II), bovine insulin, collagenase (Type IV), cycloheximide, DMSO, elastase (type IV), insulin (human), methoxamine hydrochloride, phenylephrine hydrochloride, sodium orthovanadate (vanadate), soybean trypsin inhibitor, streptozotocin (STZ), were purchased from Sigma Chemical Co (St. Louis, MO). Fura-2 AM and pluronic acid F-127 were from Molecular Probes Inc. (Eugene, OR). Dulbecco's modified essential medium (DMEM), new born calf serum, horse serum, penicillin, and streptomycin were from GIBCO BRL (Burlington, ON). Phosphate buffered saline (PBS), 1% Hank's balanced salt solution (HBSS), fungizone, l-glutamine and trypsin were obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Genistein was obtained from Calbiochem (La Jolla, CA). Mini-osmotic infusion pumps [model 2002] were from Alza Corporation (Palo Alto, CA). Iletin II insulin and sterile diluent were from Eli Lilly (Indianapolis, IN). All other chemicals were of analytical grade purchased from either BDH (Dartmouth, Nova Scotia, Canada), Calbiochem (La Jolla, CA), GIBCO BRL (Burlington, ON), or ICN Biomedicals Inc. (Costa Mesa, CA).

3.3. Methods

3.3.1. Cultured rat aortic smooth muscle cells

3.3.1.1. Isolation and culture

Ten to twelve week old SD rats (150-200g) were used to isolate ASMC as described earlier (Gopalakrishnan et al., 1991). Before sacrifice, 3 or 4 rats were anesthetized by ether inhalation in a dessicator. Upon induction of anesthesia, rats were immediately killed by cervical dislocation and sterilized by immersion in 75% ethanol. All subsequent procedures of dissection, isolation and dispersion of ASMC were conducted under sterile condition (proviodine, 75% ethanol and heat were used for sterilizing surgical equipment). Aorta were carefully dissected out and cleared of fat and connective tissue by teasing with a teflon scraper. Aorta were then cut longitudinally and transferred to a plastic centrifuge tube containing 10 ml of Hank's balanced salt solution (HBSS) with 0.2% type IV collagenase, 0.1% type IV elastase, and 0.1% soybean trypsin inhibitor. This solution was prefiltered through a 0.2 μM filter to ensure sterility. The aorta in the digestion solution was incubated at 37°C in a circulatory water bath set at 80 rpm for 5-10 min. The tissues were then transferred to fresh HBSS and the vessels were cleaned further (with relative ease) to remove the remaining adventitia, fat, and EC's surrounding the vessels. The cleaned vessels were then cut into 1-2 mm pieces and digested in the fresh HBSS enzyme solution for a period of 40 min (80 rpm, 37°C). At the end of this incubation, the digestion solution was discarded and the remaining tissue was rinsed 3 times with culture medium [20 ml of DMEM containing 10% heat inactivated newborn calf

serum, 10% heat inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml fungizone, 5.5. mM glucose, and L-glutamine 2mM]. The tissue was then resuspended in fresh culture medium and triturated (40-60 times) with a sterile plastic transfer pipette until a single cell suspension was obtained. This suspension was then transferred to a sterile 75 mm^2 culture flask and kept at 37°C in a humidified 5% CO_2 / 95% O_2 atmosphere. After the cells adhered sufficiently to the bottom of the flask (3-4 days), the cell and tissue debris was removed and replaced with fresh culture medium.

3.3.1.2. Passage and maintenance

Confluent monolayers formed between 6 and 8 days after isolation. This was referred to as the primary culture. When confluent, the culture medium was removed and the cells were treated with 0.06% trypsin in DMEM for 2-3 min at 37°C until the cells started to show signs of detaching. This stage was identified morphologically under the microscope as when the spindle shaped attached cells became rounded. Trypsin was then quickly removed and the cells washed once with DMEM. Fresh culture medium was then added to the flask and the cells were detached by scraping with a teflon scraper and resuspended by trituration using a transfer pipette. The cells were then replated (in culture medium) at a split ratio of between 1:3 and 1:5 in either 24 well plates (~5 x 10^4 cells/well; for receptor binding studies) or 75 cm^2 culture flasks (for $[Ca^{2+}]_i$ and ET_A and ET_B mRNA studies). The culture medium was changed every 2-3 days (throughout the period of maintenance of ASMC) and the cells multiplied to reach confluency in 3-5 days for

each passage level. Sub-cultured cells were used between the 2nd and 5th passage level. We have previously ascertained that ET-1 binding characteristics as well as $[Ca^{2+}]_i$ responses to ET-1 were not significantly different between cells in primary culture and those maintained up to the fifth passage. Smooth muscle morphology was routinely assessed by microscope, and the level of contamination by fibroblasts was < 3% of the total population of cells. Moreover, occasional verification studies confirmed that >90% of cells showed positive immunoflourescent staining with α-actin antibody, confirming smooth muscle characteristics of adherent cells. Cell viability was routinely checked prior to experiments by trypan blue exclusion criteria. Dead or dying cells took up the blue dye while viable cells remained intact. Cell number was counted using a hemocytometer, Fisher counter and microscope. After ASMC attained confluency, the culture medium was removed and replaced with serum-free DMEM containing insulin and vanadate under various conditions.

3.3.2. [^{125}I] ET-1 and [^{125}I] IRL-1620 receptor binding studies

3.3.2.1. Treatment protocols

The optimal concentrations and duration of incubation with insulin and vanadate that elicited maximal changes in [^{125}I] ET-1 binding were first assessed. These were determined to be 100 nM for insulin and 25 μM for vanadate, and both after 24 hr incubation. These optimal concentrations and time periods were subsequently utilized for detailed characterization studies. In order to determine whether tyrosine kinase activation was required for the observed effects of insulin and vanadate on [^{125}I] ET-1 binding, the compounds were co-incubated with genistein (10 μg/ml), a

well known tyrosine kinase inhibitor. Finally, in order to fully characterize the effects of insulin and vanadate on subtypes of ET receptors recruited, saturation-binding studies were carried out. Such saturation studies enabled determination of the total population of ET_A and ET_B specific binding sites labeled by [^{125}I] ET-1 and ET_B sites labeled by [^{125}I] IRL-1620 in the same population of cells for all of the groups under identical conditions.

3.3.2.2. Receptor Binding Protocol

All binding assays were conducted in duplicate. At the end of incubation with insulin or vanadate under the prescribed conditions, confluent ASMC were carefully washed twice at room temperature with 2 ml of binding assay buffer with the following composition: Tris-HCL 10 nM, 0.1% BSA, 10 μg/ml bacitracin in HBSS (pH 7.4). For concentration and time dependency as well as genistein studies, a total volume of 400 μL of binding assay buffer containing a fixed concentration of 20 pM [^{125}I] ET-1 was then added to ASMC to the respective wells either in the presence or absence of unlabeled ET-1 (100 nM). For saturation studies, increasing concentrations of either [^{125}I] ET-1 or [^{125}I] IRL-1620 (2.5-150 pM; a minimum of 8 concentrations for each group) were used rather than the fixed concentration of radioligand. Assays were always performed on the same day using the same group of control and insulin or control and vanadate pretreated cells. Radioligand was incubated with ASMC at 37°C for 2 hr, when equilibrium binding was reached. At the end of this incubation period, binding was terminated by aspirating the supernatant and washing twice with ice-cold HBSS. The cells in each well were

then solubilized with 250 µl of 0.5% triton X-100 and transferred to a polystyrene tube (12 x 75 mm). Washings from the wells (2 x 250 µl of deionized, distilled water) were also added to the tube. Similar treatments were also applied to blank and total counts. Quantitation of radioactivity was measured by a gamma counter (Minaxi γ Auto-Gamma 500 series, United Technologies, Packard, Downers Grove, IL). Cpm (counts per minute) values were converted to dpm (disintegrations per minute) values based on the calculated efficiency of the counter determined with each assay. Specific binding was obtained by subtracting non-specific binding from total binding. The non-specific binding was characterized as the residual binding of radioligand obtained in the presence of incubation with a high concentration of unlabeled ET-1 (100 nM) maintained in parallel tubes.

3.3.2.3. Analysis of binding data

A combined computer program EBDA / LIGAND for the detection of single or multiple sites was used for analyses of the saturation binding data (McPherson, 1985; Munson & Rodbard, 1980). Briefly, the cpm values obtained in duplicate from saturation binding experiments were converted into the respective dpm values using the formula: dpm = cpm / % efficiency. The following values for each concentration of radioligand were then fed into the program: total dpm values, non-specific binding, total dpm added to each well, specific activity (4,840,000 dpm/mole) of radioligand and the volume of incubation medium (400 µl). The equilibrium dissociation constant (K_D), maximal binding capacity (B_{max}) and Hill slope (n_H) were obtained from Scatchard-plots. Since an estimate of number of cells

could be determined by counting the total number of cells in three wells for each batch of assay, based on Avagadro's number, it was possible to express the estimated B_{max} values as the maximal number of binding sites/cell.

3.3.3. Measurement of $[Ca^{2+}]_i$

3.3.3.1. Treatment protocols

Based on the optimal concentration and time of incubation conditions employed in the receptor binding studies, characterization of the $[Ca^{2+}]_i$ responses to ET agonists and other peptides was then undertaken. The aim of these studies was to determine the functional relevance of the observed effects of insulin and vanadate on ET receptor expression. Firstly, after a 24 hr incubation with either insulin (100 nM) or vanadate (25 μM), concentration–peak $[Ca^{2+}]_i$ responses to ET-1 and IRL-1620 were assessed in ASMC. Secondly, in order to determine the receptor subtypes recruited in the ET agonist evoked $[Ca^{2+}]_i$ response under the treatment conditions, the effect of the antagonists BQ123 (ET_A selective) and BQ788 (ET_B selective) on ET-1 and IRL-1620 evoked responses was determined. Since insulin is also known to activate the IGF-1 receptor in high concentrations (King et al., 1980), the effect of IGF-1 on ET-1 evoked $[Ca^{2+}]_i$ responses was also determined. Finally, in order to assess whether the observed effects of insulin and vanadate were selective to ET-1, $[Ca^{2+}]_i$ responses to the other peptide agonists, Ang II and AVP were examined after pre-incubation with these compounds.

3.3.3.2. Preparation of cell suspension and Fura-2 loading

After the prescribed treatments, confluent adherent ASMC maintained in 75 cm^2 flasks in serum free DMEM were trypsinized for 2-3 minutes and allowed to detach (as before). After washing twice with Krebs-HEPES buffer (145.0 mM NaCL, 5.0 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_20$, 1.2 mM $MgCl_2 \cdot 6H_20$, 10.0 mM glucose, 10.0 nM HEPES, and 0.2 % BSA pH 7.4), ASMC were detached from the flask with a teflon scraper and dispersed in the same buffer using a transfer pipette. The ASMC suspension was then transferred to a polypropylene tube. In the meantime, a stock solution of 1 mM acetoxymethyl ester of Fura-2 (Fura-2 AM) was prepared in dimethylsulfoxide (DMSO). 0.02% pluronic acid F-127 was added to fura-2 AM solution to enhance loading efficiency. An appropriate volume of this stock was added to the cell suspension (approximately 0.5 x 10^6 cells/ml) to give a final concentration of 5 μM fura-2 AM. The ASMC suspension with fura-2 AM was then incubated at 37 °C with a stirring speed of 120 rpm for 40 minutes. The suspension was subsequently subjected to centrifugation at 1100 g x 6 minutes at 4 °C and the pellet obtained was twice washed by resuspending with KREBS buffer, followed by trituration and centrifugation. This ensured removal of excess and unloaded dye. After the final wash, the cell pellet was resuspended in fresh buffer and diluted to achieve a concentration of ~ 1.5 x 10^6 cells/ml. The procedure for fura-2 AM loading has been described elsewhere (Gopalakrishnan et al., 1991; Sandirasegarane & Goplakrishnan, 1995; Sandirasegarane et al., 1994). Trypan blue exclusion test was used at this stage and also toward the end of the experiment (which takes 1-2 hr) to ensure that cell viability remained > 90%. Fura-2 loaded ASMC suspensions

were kept on ice in light-restricted flasks to ensure that viability remained high and that fura-2 did not become quenched.

3.3.3.3. Measurement of fura-2 fluorescence ratio

For each determination, 500 µl of fura-2 loaded ASMC suspension (0.15×10^6 cells) was added to the cuvette and maintained at 24 °C with a stirring speed of 800 rpm. The basal as well as agonist evoked changes in the fura-2 flourescence ratio were subsequently monitored by recording the signals R_{340}/R_{380} in a JASCO spectroflourometer, CAF-100 Ca^{2+} analyzer (Japan Spectroscopic, Tokyo). The cell suspension was challenged with agonist just once in order to avoid receptor desensitization, which is possible with repetitive additions of the agonist to the same cell suspension. Also, fura-2 leakage was minimal when the temperature was maintained at 25°C throughout the experiment. Fura-2 is excited at 2 different wavelengths (340 and 380 nm) with an emission fluorescence of 500 nm. Free fura-2 maximally excites at 380 nm, whereas the Ca^{2+} bound form is maximally excited at the lower wavelength (340 nm). Thus, the 340/380 fluorescence ratio is a measure of fura-2 chelation to Ca^{2+}, which is directly proportional to the rise in the levels of free Ca^{2+}. Since intracellular free fura-2 is essentially trapped in the cytosolic compartment and is available to chelate Ca^{2+} only in this compartment, free Ca^{2+} can therefore be considered cytosolic free Ca^{2+}, or $[Ca^{2+}]_i$. The basal $[Ca^{2+}]_i$ values and the maximal increase in response to the addition of each concentration of agonist (peak $[Ca^{2+}]_i$ values) were calculated from the respective flourescence ratio R according to the formula suggested by Grynkiewicz et al.

(1985): $[Ca^{2+}]_i = k_D \times (R-R_{min})/(R_{max}-R) \times Sf_2/Sb_2$. K_D (224 nM) is the dissociation constant of fura-2 for Ca^{2+}, R is the ratio of relative flourescence intensities (340/380) observed in mode II of the CAF-100 Ca^{2+} analyzer, Sb_2 and Sf_2 are the flourescence efficiencies of Ca^{2+} bound and Ca^{2+} free fura-2, respectively, excited at 380 nm in mode III. R_{max} and R_{min} represent the maximal and minimal ratio of flourescence intensities respectively. The determinations of firstly R_{max} and Sb_2 values were performed by the addition of 40 µl of 10% Triton X-100 to lyse the cells and expose the dye to the buffer concentration of Ca^{2+} (1.8 mM) which chelates to fura-2. R_{min} and Sf_2 were determined by subsequent addition of 20 µl of 150 mM EGTA solution containing 450 mM Tris, pH 8.5 to achieve a final concentration of 6mM EGTA.

3.3.4. Northern blot ET_A and ET_B mRNA measurements

3.3.4.1. Treatment protocol and optimization

In order to determine whether changes in ET receptor binding evoked by insulin and vanadate were due to changes in receptor expression, ET_A and ET_B mRNA measurements were undertaken using northern blot methodology. Firstly, using the optimal concentrations identified in concentration-dependency receptor binding studies, time-dependency studies were undertaken. Secondly, in order to determine whether tyrosine kinase activation was required for changes in ET receptor expression, insulin and vanadate treated ASMC were co-incubated with genistein (10 µg/ml) prior to mRNA studies. Finally, to determine whether new protein

synthesis and active translation were required for the effects of insulin and vanadate, ASMC were co-incubated with either cycloheximide (10 μg/ml) or actinomycin-D (1.5 μg/ml) prior to mRNA measurements.

3.3.4.2. Total RNA isolation

After the prescribed treatments, total RNA was isolated from confluent ASMC as previously described (Chomzynski & Sacchi, 1987). Serum-free DMEM was removed from the culture flasks and a denaturing solution was immediately applied (RNA STAT-60). Lysed ASMC were then homogenized on ice with a polytron using three, 15 second bursts. One-fifth volume of chloroform was then added in, vortexed, and kept on ice for 15 min. It was then centrifuged (10,000g 15 min, 4°C). The upper phase containing the RNA was carefully removed and RNA was precipitated out by the addition of an equal volume of isopropanol, and kept at –20°C overnight. The next day, the sample was again centrifuged at 10,000 g x 15 min at 4°C. The supernatant was removed and the sample washed twice. Washing entailed resuspending the pellet in 75% ethanol water followed by recentrifugation. After the second wash, the pellet was completely dried of ethanol then resuspended in Proteinase K buffer [0.1 M Tris (pH 7.5, 0.5 M NaCl, 1% SDS, 0.01 M EDTA (pH 7.5), 0.1 mg/ml Proteinase K] and incubated at 37 °C for 30 min. Phenol/chloroform/isoamyl alcohol (0.5 ml; 25:24:1) was then added, vortexed, and centrifuged (10,000 g for 5 min). Aqueous layer was added to an equal volume of chloroform, mixed, then centrifuged again. Finally, the aqueous phase was again removed and mixed with 2 volumes of 75% ethanol and 1/10 volume of 3M

NaAcetate (pH 5.2) and stored at –20 ^{o}C until the quantitation procedures. Before the subsequent procedures, the tubes were centrifuged (10,000 x g for 5 min) then washed once with 75% ethanol (as before) and finally suspended in DEPC.$H_2$0.

3.3.4.3. Quantitation of isolated total RNA

The amount and purity of RNA was measured by spectrophotometer. The concentration of RNA in solution was calculated based on an optical density of 1.0 being equal to 40 µg/ml RNA. The purity was assessed as the ratio of fluorescence at 260 nM to that at 280 nM.

3.3.4.4. RNA electrophoresis

Isolated total RNA was electrophoresed using the protocol developed by Pelle and Murphy (1993). RNA samples (20 – 30 µg/lane) and molecular weight standards were first combined with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 1.2% SDS, 60 mM sodium phosphate, pH 6.8). The mixture was incubated for 5 min at 65^{o}C to denature the RNA secondary structure followed by rapid transfer to ice. The sample was then loaded onto 1.2% agarose gel dissolved in 10 mM sodium phosphate (pH 6.8), with 0.1 µg/ml ethidium bromide. A charge of 5.5 V/cm of gel was applied allowing the RNA to traverse the gel. The gel was then visualized under U.V. light and photographed.

3.3.4.5. Transfer of RNA to nylon membrane

The standard blotting capillary elution technique adapted from Sambrook et al. (1989) was used to transfer the separated RNA to nylon membranes for further analysis. The gel was first rinsed in 10 x SSC (3 M NaCl, 300 mM sodium citrate-trisodium salt, pH 7.0). The RNA was then transferred from the gel to the membrane using a capillary action apparatus with 10 x SSC for 12 – 18 hr. After transfer, the membrane was covered with plastic wrap and fixed to the membrane using a U.V. Stratalinker (1,200 joules).

3.3.4.6. Preparation of probes

3.3.4.6.1. Bacterial transformation

100 ng of plasmid DNA containing either rat ET_A or ET_B cDNA fragments was added to 200 µl of competent E.Coli and DH5, respectively, mixed and stored on ice for 30 min. The mixture was then incubated at 42°C for 90 seconds and then rapidly chilled on ice for 1-2 min. 800 µl of LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0) was then added to each mixture. The competent cells were then incubated for 45 min at 37 °C to recover and express the antibiotic resistance marker encoded by the plasmid. The appropriate volume of transformed bacterial cells was spread over the surface of agar LB medium on 1.5% agar with 50 µg/ml tetracycline. The plates were incubated overnight at 37°C in an inverse position (Sambrook et al., 1989).

3.3.4.6.2. Small-Scale preparation of plasmid DNA

The procedure for both large and small-scale isolation of plasmid DNA was adapted from an earlier methodology (Sambrook et al., 1989). Different colonies picked up from each plate were cultured overnight at 37°C with vigorous shaking in 2 ml LB medium with 50 μg/ml tetracycline. 1.5 ml of the culture was then centrifuged at 12,000 g for 30 sec at 4 °C. The cell pellet was resuspended in 350 ml of STET [0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0), 5% triton X-100] containing 25 ml of freshly made lysozyme (10 mg/ml in 10 nM Tris.Cl, pH 8.0) and vortexed. The sample was then immersed in boiling water for 40 seconds and then centrifuged (12,000 g x 10 min, room temperature). The pellet was removed with a toothpick before the addition of 40 μl of 2.5 M sodium acetate (pH 5.2) and 420 μl of isopropanol. The mixed sample was kept for 5 min at room temperature before centrifugation (12,000 g x 5 min, 4°C). The pellet was washed with 70% ethanol and dried at room temperature. The isolated DNA was dissolved in TE [10 mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0)] and stored at –20 °C.

3.3.4.6.3. Large-Scale preparation of plasmid DNA

A large amount of cultured bacterial cells were collected by centrifugation at 4,000 g x 10 min and resuspended in 50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0) containing 1 mg/ml freshly made lysozyme, and then kept for 5 min at room temperature. After addition of 2 volumes of 0.2 N NaOH with 1% SDS, the sample was left at room temperature for 10 min. 1/3 volume of 5 M potassium acetate was then added and the sample was kept on ice for 10 min. The bacterial

debris was subsequently removed by centrifugation (12,000 g x 20 min) and rinsed with 70% ethanol. The dried DNA was dissolved in TE (pH 8.0) with 20 μg/ml RNA'se and kept at room temperature for 30 min. The DNA solution was extracted with an equal volume of phenol/chloroform (1:1 ratio) buffered with 0.1 M Tris.Cl (pH 8.0). The aqueous phase was separated by centrifugation and precipitated with 2 volumes of ethanol at 4°C. After being maintained at room temperature for several min, the DNA pellet was obtained by centrifugation, rinsed with 70% ethanol, and dissolved in TE buffer (pH 8.0).

3.3.4.6.4. Gel electrophoresis of DNA

Plasmid DNA and molecular weight marker were mixed with 1/5 volume of 6 x gel-loading buffer (0.25% bromophenol blue, 40% sucrose) before loading onto 1.2% agarose gels [dissolved in 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) containing 0.5 μg/ml ethidium bromide. A voltage of 1-5 V/cm was applied and the gel was run in 1 x TAE until the dye migrated to the appropriate position.

3.3.4.6.5. Recovery of DNA fragments

Plasmid DNA digested with restriction enzymes was separated by 1.2%, low melting point, agarose gel at 4°C according to the protocol in the preceding section. Bands of cDNA fragments of ET_A and ET_B receptors were cut under the long-wavelength U.V. lamp. 5 volumes of 20 mM Tris.Cl, 1 mM EDTA (pH 8.0) was added to the slice of agarose. The gel was incubated for 5 min at 65°C. Before the gel was melted, it was cooled to room temperature before the addition of an equal volume of

phenol, equilibrated to pH 8.0 with 0.1 M Tris.HCl. The sample was subjected to vigorous vortexing and then centrifuged at 4,000 g for 10 min at 20°C. The aqueous phase was re-extracted once with phenol/chloroform and once with chloroform, and precipitated with 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol at 4°C. After storage at room temperature, the DNA was recovered by centrifugation and rinsed with 70% ethanol. The dried DNA was dissolved in TE (pH 8.0) and the concentration measured by spectrophotometer following the procedure described earlier. An optical density of 1.0 corresponds to 50 µg/ml double stranded DNA.

3.3.4.6.6. Radiolabeling of DNA

Labeled nucleotides were incorporated in DNA synthesized by an oligo-labeling method (Feinberg & Vogelstein, 1983). The Prime-It-II random primer kit (Stratagene) was used. About 25-50 ng of cDNA fragment was heated in boiling water for about 2 min and then chilled on ice. 10 µl of labeling 5 x buffer [1 M HEPES (pH 6.6), 250 mM Tris.HCl (pH 8.0), 25 mM MgCl2, 10 mM DTT, 26A 260 u/ml random hexadeoxyribonucleotides], 2 µl of unlabeled dNTP's mixture (500 µM dATP, 500 µM dGTP, 500 µM dTTP), 50 µCi [α-32P] dCTP (3,000 Ci/mmol, 10 µCi/µl) and 5 units of Klenow fragment of E.Coli DNA polymerase I were then combined and adjusted to a total volume of 50 µl with water. After incubation for 60 min at room temperature, the reaction was terminated by 5 µl 0.2 M EDTA (pH 8.0). The radiolabeled probe was then separated from the incorporated dCTP through a Sephadex G-50 column.

3.3.4.7. Northern hybridization

The hybridization was performed using a QuikHyb (Stratagene) solution. A minimum of 33 µl of QuikHyb/cm^2 of blot was used during both prehybridization and hybridization procedures. The membrane was first prehybridized for 20 min at 68°C. 100 µl of 10 mg/ml salmon sperm DNA was added to the labeled probe (2.5 x 10^6 counts/2 ml hybridization solution) and boiled for 2 min followed by chilling on ice. The mixture was then added to the prehybridization solution and hybridization was done for 1 hr at 68°C. The membrane was then washed twice for 15 min with 6 x SSC with 0.1% SDS, twice with 2 x SSC, and then once with 0.2 x SSC for 5 min at 60°C. Autoradiography was done by first placing the membrane wrapped in plastic wrap in an exposing cassette overnight followed by visualizing and quantification using a PhosphorImager (Molecular Dynamics).

3.3.5. Ex vivo studies

3.5.5.1. Animals and treatment

Nine-week old SD rats (150-200g) were fasted for 24 hr before being given either a single dose of STZ (diabetic group) (55 mg/kg) in citrate buffer (pH 4.5) or citrate buffer alone (SD, non-diabetic group) via intraperitoneal injection. Hyperglycemia (>15 mmol/l) was confirmed in the treated rats after 1 week. At 3 weeks after STZ treatment, rats were divided into 8 groups of 8 rats each: SD-insulin control, SD-insulin treated, STZ-insulin control, STZ-insulin treated, SD-vanadate control, SD-vanadate treated, STZ-vanadate control, STZ-vanadate treated. Insulin treated rats

received Iletin II insulin (Eli Lilly, Indianapolis, Indiana, USA) via subcutaneous miniosmotic pump (ALZA Corp., USA) diluted with sterile diluent (Eli Lilly, Canada) to allow for a delivery rate of 12 mU/Kg/min for 2 weeks. Iletin II insulin is pork-derived regular insulin with just a 2 amino acid difference from human insulin. Iletin II is has previously been used in STZ diabetic rats and is detectable with the rat insulin RIA kit. The insulin pumps were inserted into rats under sodium pentobarbital anaesthesia (50 mg/kg i.p.). Insulin controls received sterile diluent only by the same delivery method. Vanadate treated rats received 0.5 mg/ml sodium orthovanadate (vanadate) in drinking water freshly prepared every second day for 2 weeks. Vanadate solution was buffered to pH 7.0 with ascorbic acid, as this has been suggested to decrease the toxicity of vanadate (Paulson et al., 1987). Vanadate doses were gradually increased over the week preceding the study to allow the rats to become accustomed to its inclusion in the water. Vanadate control rats were given tap water only. During the treatment period, all animals were housed in animal quarters with a 24 hr light-dark cycle and were fed standard rat chow ad libitum. Animals were weighed prior to and after treatment and food and water intake was monitored daily. Vanadate doses were gradually increased over the week preceding the study.

3.3.5.2. Metabolic variables

3.3.5.2.1 Blood and plasma sample collection

At the end of the 2-week treatment period, animals were killed by decapitation under ether anesthesia. Blood samples were subsequently collected from the decapitated

animals in tubes containing EDTA. 5 ml of combined arterial and venous blood was easily obtained by this method. These were then centrifuged at 2000 g x 10 minutes at 4°C. Two aliquots of the supernatant plasma was subsequently obtained (200 µl and 1 ml) and stored at –70 °C until plasma insulin and ET-1 measurements were undertaken. A drop of blood was also obtained from the decapitated animal at the time of sacrifice upon which plasma glucose was measured.

3.3.5.2.2. Plasma glucose measurements

Plasma glucose in the drop of blood was measured by a glucose oxidase method incorporated into an easy to use, hand-held blood glucose analyzer (One Touch Basic, Lifescan, Vancouver, BC). With this system, blood glucose levels were recorded accurately within 45 seconds.

3.3.5.2.3. Plasma insulin measurements

Plasma insulin concentrations were measured in the frozen 100 µl aliquots using a commercially available RIA kit (Amersham, Oakville, ON, Canada). The assay is based on the competition between unlabeled insulin and a fixed quantity of [^{125}I]-labeled human insulin for a limited number of binding sites on an insulin antibody. Using fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody is inversely proportional to the concentration of added non-radioactive ligand. Firstly, all reagents (standard, antiserum, and [^{125}I] insulin radiotracer) were diluted with assay buffer (0.052 M phosphate buffer pH 7.5 containing 0.1% sodium azide). Both the antiserum and radiotracer were then

combined with the insulin samples and standards, vortexed and allowed to incubate for 4 hr at room temperature. After this, the Amerlex-M second antibody reagent (which contains second antibody that is bound to polymer particles) was added to the antibody bound insulin, vortexed and allowed to incubate at room temperature for a further 10 min. Separation of the antibody bound fraction was then effected by centrifugation (1500 g x 10 min; 3°C) followed by decantation of the supernatant by inversion of the tubes in decantation racks and allowing to drain for 5 min. Radioactivity in the pellet from each sample was then counted for a period of 60 seconds in a gamma-counter. This enabled the amount of labeled insulin in the bound fraction to be calculated. Briefly, non-specific binding was first subtracted from all counts. The percent bound divided by zero standard was then calculated for each standard and sample and plotted against insulin concentration on a standard curve. The concentration of unlabeled insulin in the sample was then determined by interpolation from this standard curve. Further relevant information provided by the manufacturer is as follows: The assay was completely cross-reactive with insulin from a variety of species including rat and human. The sensitivity was 48 pg/ml, within-assay and between-assay precision was determined to be 5 and 13 % respectively, and recovery was between 110 and 117%.

3.3.5.3. Plasma endothelin measurements

Plasma ET-1 concentrations were also measured in frozen 1 ml plasma aliquots. Sample preparation was first completed by acidifying the plasma sample with 2 M HCl and centrifuging at 1000 x g for 5 min. The sample was then loaded onto

Amprep 500 mg C2 mini-columns (Amersham), washed with 0.1% trifluoroacetic acid (TFA), and then eluted with 80% acetonitrile and 0.1% TFA. The sample was then dried down under nitrogen and resuspended in assay buffer (20 mM sodium borate, 0.1% BSA, 0.1% tween 80, and 0.1% sodium azide). ET-1 levels in the samples were then measured using a commercially available RIA kit (Amersham, Oakville, ON, Canada). The assay is based on the competition between unlabeled ET-1 and a fixed quantity of [^{125}I]-labeled ET-3 for a limited number of binding sites on an ET 1-21 specific antibody. Similar to the insulin RIA, the amount of radioactive ligand bound by the antibody is inversely proportional to the concentration of added non-radioactive ligand. Briefly, all reagents (standard, antiserum, radiotracer) are first reconstituted with assay buffer. Antiserum is then added to the sample and standards, vortexed and allowed to incubate for 4 hr at room temperature. [^{125}I]-ET-3 (radiotracer) is then added to the tubes, vortexed and allowed to incubate for 16-24 hr at 2-8^{o}C. The Amerlex-M second antibody reagent is then added, vortexed and allowed to incubate for a further 10 min at room temperature. Separation of the antibody bound fraction is then achieved by centrifugation (1500 g x 10 min, 4^{o}C) followed by decantation. Radioactivity is then counted in a gamma-counter and ET-1 levels are subsequently determined in the same manner as with the insulin RIA. Cross reactivity of the kit was negligible with other unrelated vasoactive peptides as well as big-ET-1. However, cross reactivity was complete to other ET isopeptides including ET-2, ET-3, S6c, and vasoactive intestinal contractor. Recovery of ET-1 was determined to be 79%,

sensitivity of the assay is 0.2 pmol/l, and within-assay and between-assay precision was 3-6% and 9-17% respectively.

3.3.5.4. Ex vivo vascular reactivity studies

3.3.5.4.1. Isolation and preparation of aortic rings

Immediately after the rats were sacrificed, the thoracic aorta was dissected from each rat and transferred to a petri-dish containing modified Kreb's physiological salt solution (NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgCl_2 \cdot 6H_20$ 1.2 mM, $CaCl_2 \cdot 2H_2O$ 1.8 mM, $NaHCO_3$ 25.0 mM, and glucose 11.1 mM) continuously oxygenated with 95% O_2/5 % CO_2 at 37°C. It was then cleared of adhering fat and connective tissue using dissecting scissors and gentle teasing and subsequently cut into 5 mm ring segments. For experiments requiring endothelium denudation, the ring segment was gently rubbed between the thumb and forefinger. Denudation was verified by a lack of vasodilatory responses to ACh. Using this method, complete functional denudation of the endothelium was achievable with minimal effects on underlying VSM function. The rings were then suspended on ring supports in 10 ml organ baths containing modified Kreb's buffer continuously oxygenated with 95% O_2/5 % CO_2 at 37 °C. One end of the ring support was anchored to the base of the bath while the other was attached to an FTO.3 isometric force transducer connected to a Grass 7E polygraph. Baseline isometric tension was subsequently set at 2 g by adjusting the vertical level of the transducer apparatus. 2 g was chosen as baseline tension since this was the level at which force development to a maximal concentration of ET-1 (100 nM) was optimal.

3.3.5.4.2. Analysis of agonist evoked responses

The aortic segments suspended in the organ bath were allowed to equilibrate for 1 hr (with changes of the buffer every 20 min) before the tissue was precontracted with a single concentration of phenylephrine (30 μM) to stabilize the preparation. After the level of tension returned to basal levels (after ~ 15 minutes), agonist challenges were undertaken. The agonists were added in 50-100 μl quantities so as not to increase the required final bath concentrations. In one set of rings, ET-1 (100 pM – 100 nM) was added in a cumulative dose fashion in order to elicit a concentration-dependent vasoconstrictor response. In a separate set of rings, similar responses to methoxamine (100 nmol/L - 100 μmol/L) were asssessed. At the peak of methoxamine evoked contraction in these rings, cumulative dose relaxations to ACh (100 nmol/l - 100 μmol/l) were then undertaken. At the end of each experiment, tissues were allowed to dry overnight, weighed, and the cross sectional areas were calculated to allow for expression of tension development in g/mm^2. This method of representing tension-response data is advantageous in that it controls for differing amounts of smooth muscle in the vessel wall (Wyse, 1980).

3.3.6. Statistical analysis

The EC_{50} values for agonist induced increases in peak $[Ca^{2+}]_i$ levels and tension responses were derived from log concentration-response curves. All results were expressed as mean ± SEM. The comparison of data obtained for each group was subjected to analyses using ANOVA or student's t-test as appropriate. Simultaneous multiple comparisons were examined using Scheffe's F-test.

4. RESULTS

4.1. Aortic smooth muscle cells

After initial isolation, the time required for ASMC to reach confluency in the flask varied from 6 to 8 days. ASMC were allowed to proliferate due to the presence of L-glutamine, horse serum, and newborn calf serum in the incubation medium. ASMC grew in a characteristic 'Hill and Valley' pattern, with the valleys representing the monolayer of cells. Individual ASMC are spindle shaped and they differ from fibroblasts in that they have only one or two nucleoli present in the nucleus while fibroblasts have four or five. Although cobble-shaped EC's are common contaminants of the culture, care was taken to ensure the meticulous removal of these cells during the initial isolation as well as during the period of initial growth before the cells attained confluency. Although ASMC could be more adequately characterized with the routine use of anti-actin or anti-myosin antibodies, the above procedure was used to obtain ASMC cultures with high purity. The efficacy of this technique was verified with occasional antibody studies. Viability of cells was repeatedly monitored using the trypan blue exclusion test, whereby dead or dying cells take up the blue dye, while healthy cells remain intact. Viability of cells was consistently greater than 90%. ASMC viewed under a microscope on both low and high power are shown in Figure 10.

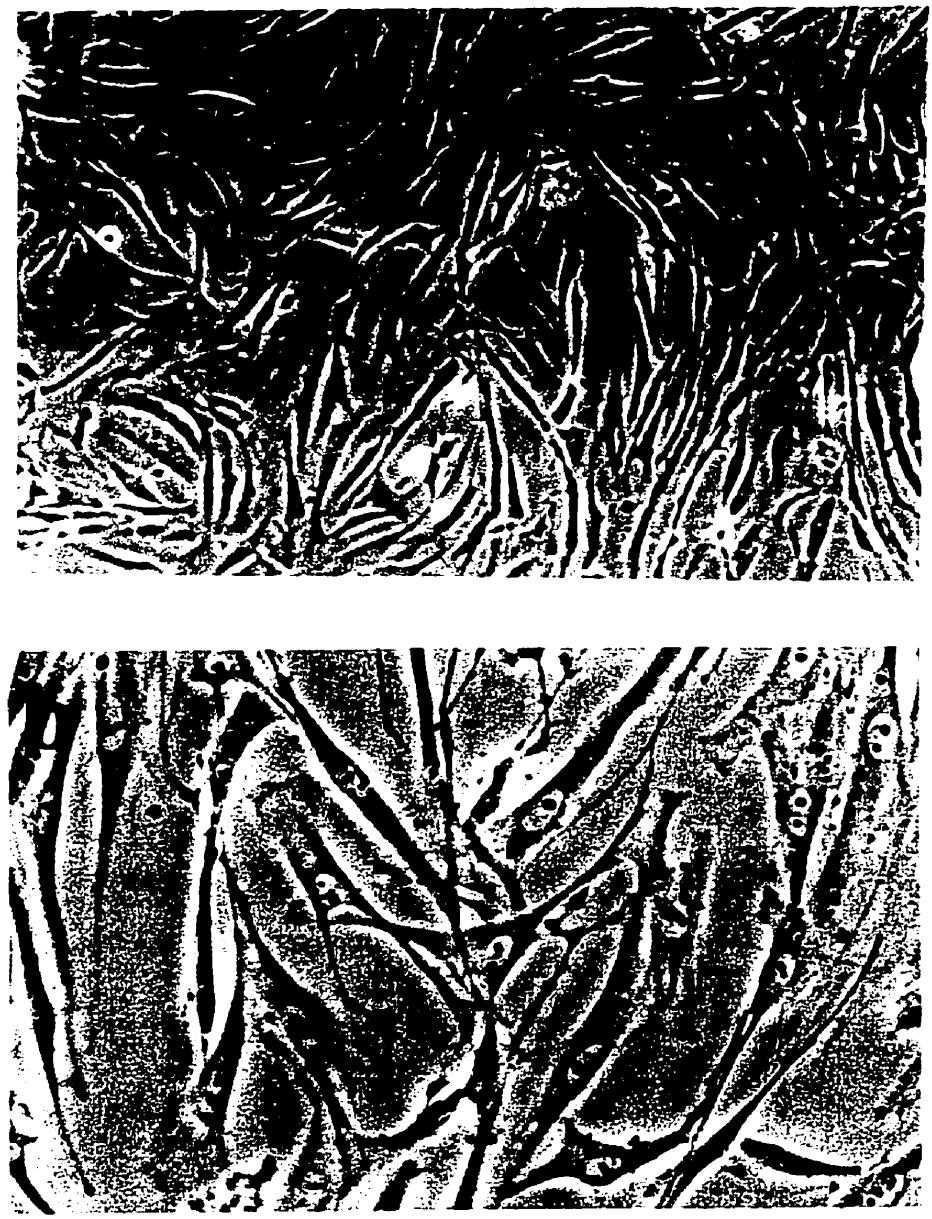


Figure 10. Aortic smooth muscle cells (ASMC) when viewed under a microscope at (a) x 10 and (b) x 40 magnification. Spindle shaped ASMC have one or two nucleoli - clearly observed at the higher magnifications.

4.2. Effects of insulin and vanadate on endothelin receptor characteristics and endothelin agonist mediated elevations of $[Ca^{2+}]_i$

4.2.1. $[^{125}I]$ ET-1 and $[^{125}I]$ IRL-1620 binding studies

4.2.1.1. Concentration and time dependent increase in $[^{125}I]$ ET-1 specific binding

4.2.1.1.1. Insulin pretreated cells

Pretreatment with insulin (0.1 – 1000 μM) for 24 hours led to a concentration dependent increase in $[^{125}I]$ ET-1 (20 pM) specific binding (Figure 11a). The minimum concentration of insulin needed to evoke a significant increase was 10 nM (144 ± 10 %; $p < 0.05$). Peak effect occurred at 100 nM insulin which was 2-fold higher (207 ± 14 %; $p < 0.01$) than specific binding in control cells. When ASMC were pretreated with a fixed concentration of insulin (100 nM) for varying time periods (6, 12, 18 and 24 hrs), a significant increase in $[^{125}I]$ ET-1 specific binding was evident by the 24 hr time point (Figure 11b). The maximal increase in specific binding at this point was 250 ± 45% of control levels ($p < 0.01$). Incubation for longer time periods elicited no further increases (30 hr time point; 211 ± 20% of control levels; $p < 0.01$). Therefore, in subsequent detailed characterization studies, a concentration of 100 nM and a time interval of 24 hr were chosen as the optimal conditions. Insulin was not maintained in the assay medium during the measurement of $[^{125}I]$ ET-1 specific binding. Pretreatment with insulin did not visibly affect cell morphology or viability (>90%).

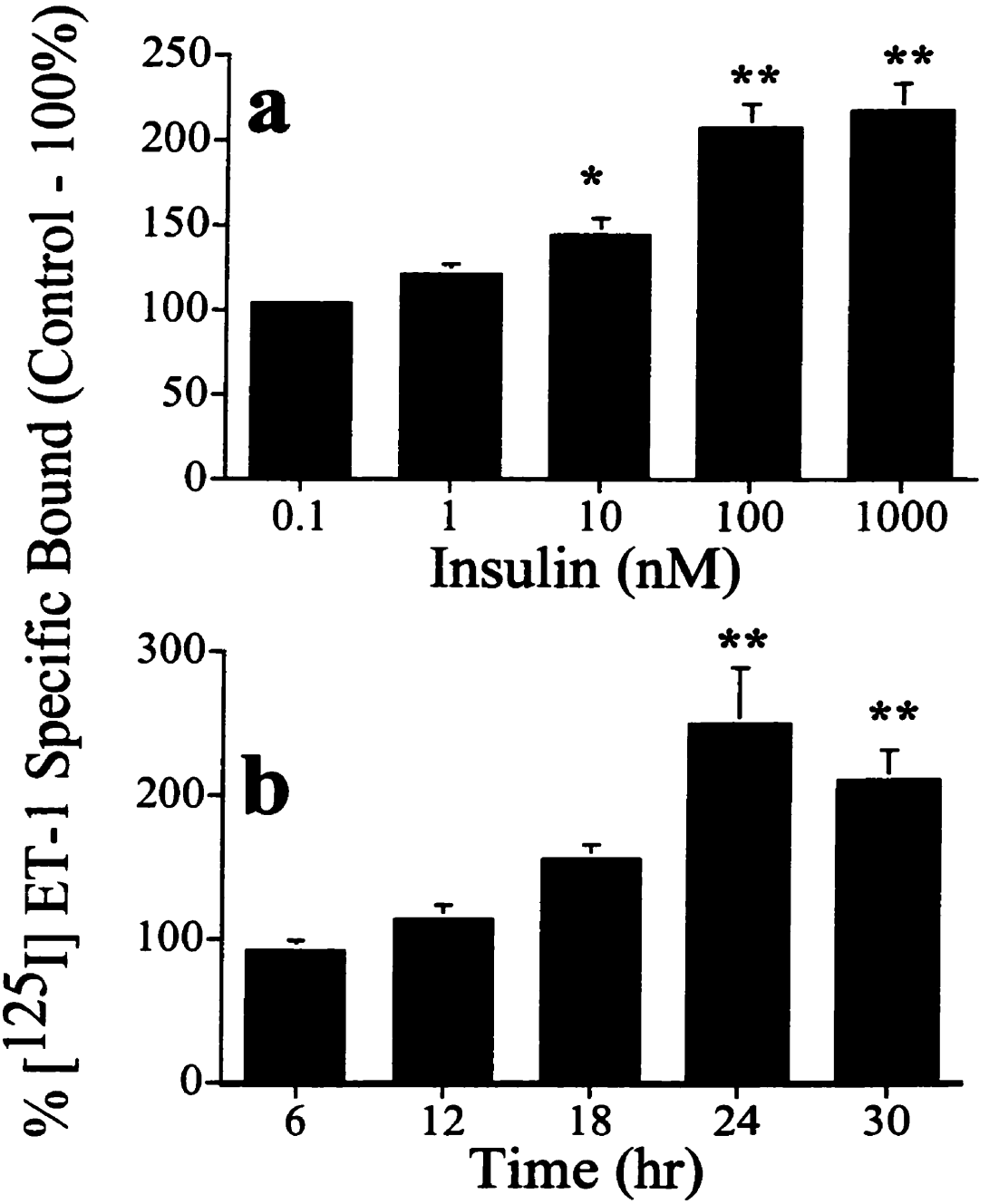


Figure 11. Concentration (**a**) and time (**b**) dependent increase in [^{125}I] ET-1 specific binding to rat ASMC after insulin pretreatment. Adherent cultured rat ASMC (~2 x 10^5 cells/well) were incubated with increasing concentrations of insulin for different time periods. Each data point is a mean ± SEM of 5 separate experiments performed in duplicate.

*$p < 0.05$; ** $p < 0.01$ vs. control group.

4.2.1.1.2. Vanadate pretreated cells

Pretreatment of rat ASMC with vanadate also led to a concentration and time dependent increase in specific binding of a fixed concentration of 20 pM [^{125}I] ET-1. The maximal effect was observed with a concentration of 25 μM vanadate which was statistically significant ($p < 0.01$) and was two fold higher (206 ± 16%) than the data from the control group of cells (Figure 12a). In time dependency experiments, a significant ($p < 0.01$) increase in [^{125}I] ET-1 specific binding was seen at 24 hr, and pretreatment with vanadate for longer durations (30 hr) did not result in further increases (Figure 12b).

4.2.1.2. Effect of genistein on [^{125}I] ET-1 specific binding in insulin and vanadate pretreated cells

To examine the role of protein tyrosine kinase in vanadate and insulin evoked increases in ET-1 binding, ASMC were incubated in the presence of a low concentration of genistein (10 μM), a known tyrosine kinase inhibitor. Pretreatment with genistein *per se* for 24 hr did not affect the specific binding (99 ± 9%) of a fixed concentration of 20 pM [^{125}I] ET-1 to ASMC (Figure 13). However, co-incubation of genistein with either insulin (100 nM) or vanadate (25 μM) for 24 hr abolished the increases in specific binding evoked by both compounds.

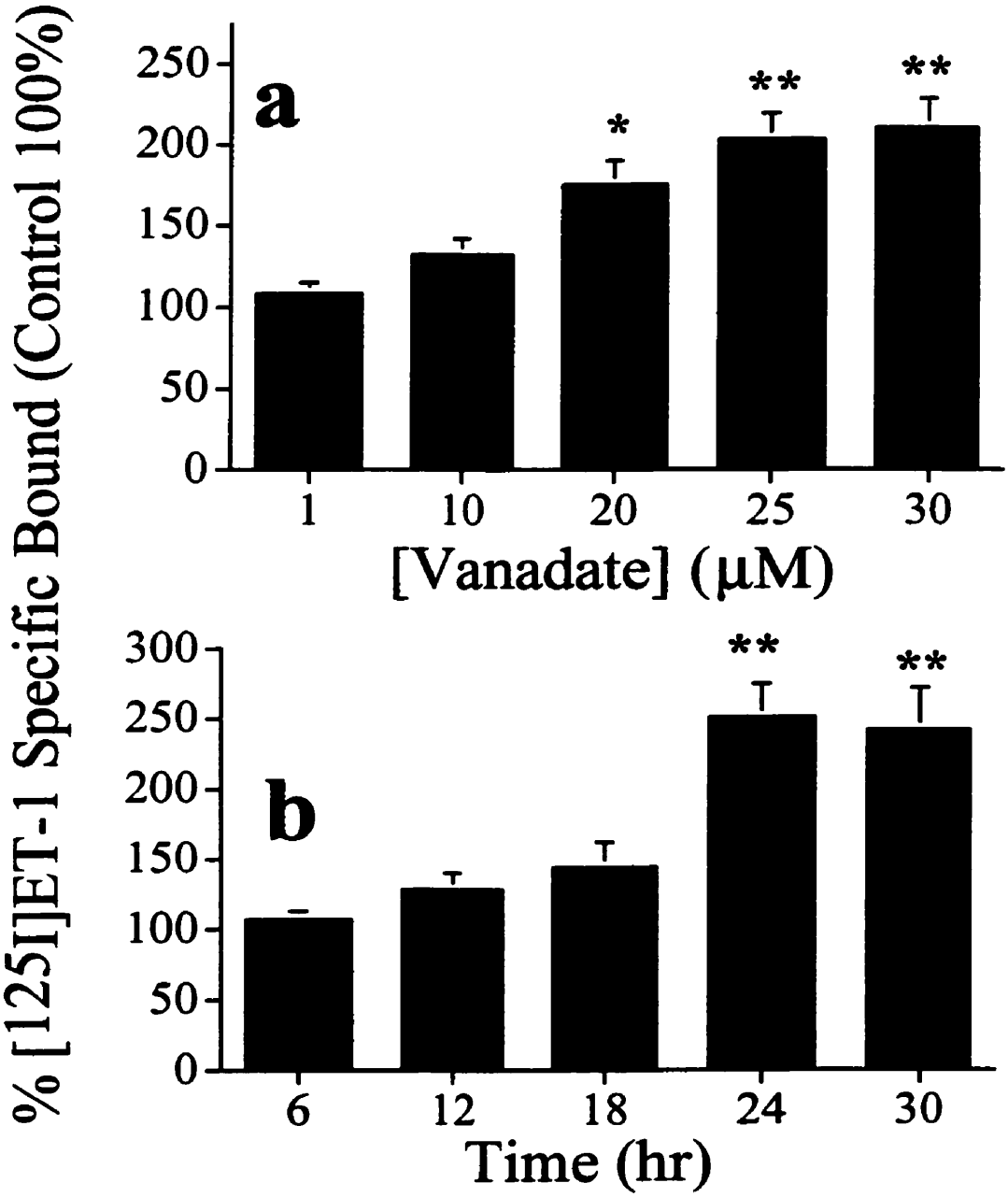


Figure 12. Vanadate evoked concentration **(a)** and time **(b)** dependent increases in [^{125}I] ET-1 specific binding to rat ASMC.

*$p < 0.05$ and ** $p < 0.01$ vs. control group ($n = 5$).

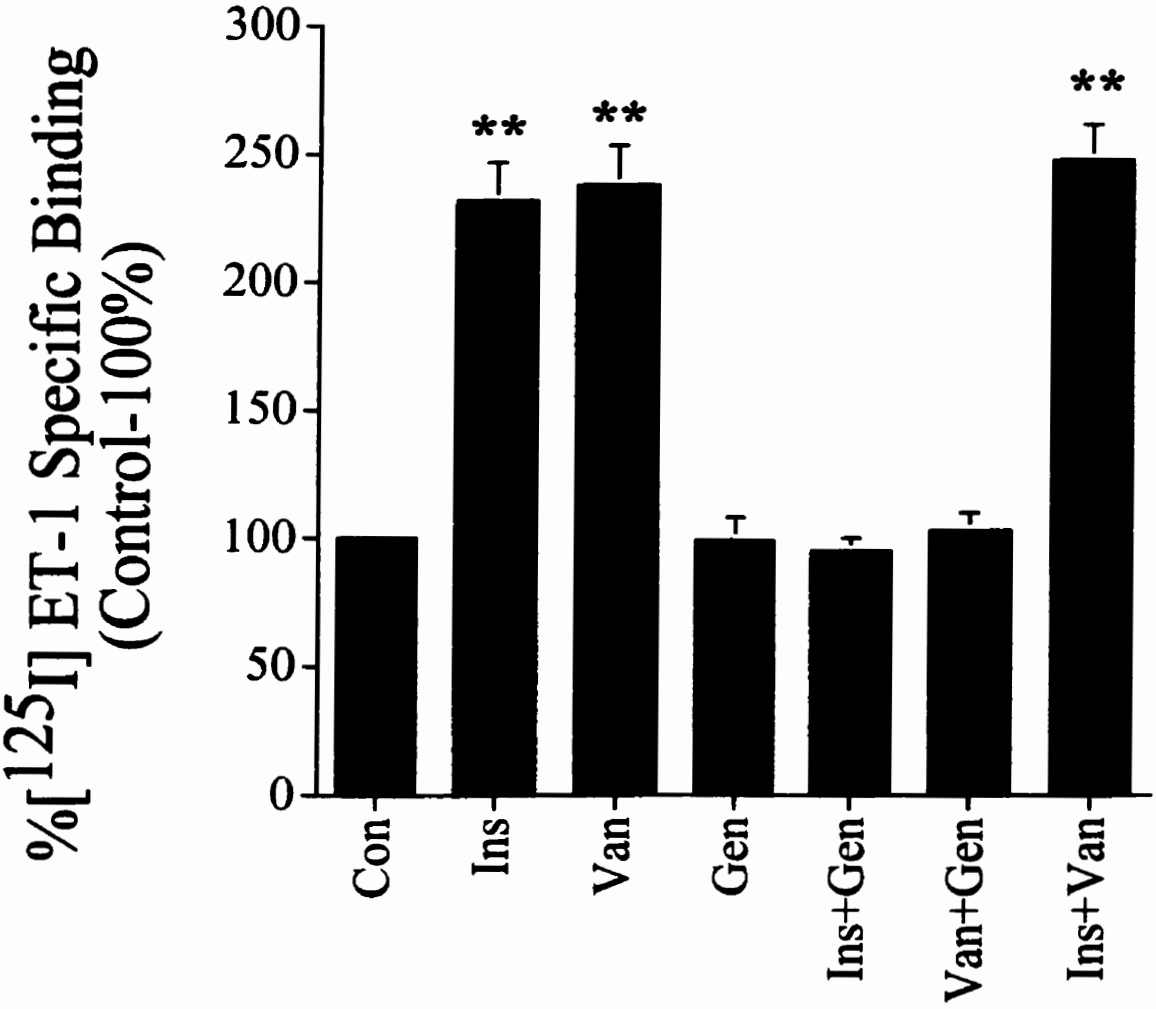


Figure 13. Effect of genistein on insulin and vanadate evoked increases in ET receptor binding.

** $p < 0.01$ vs. control group ($n = 5$).

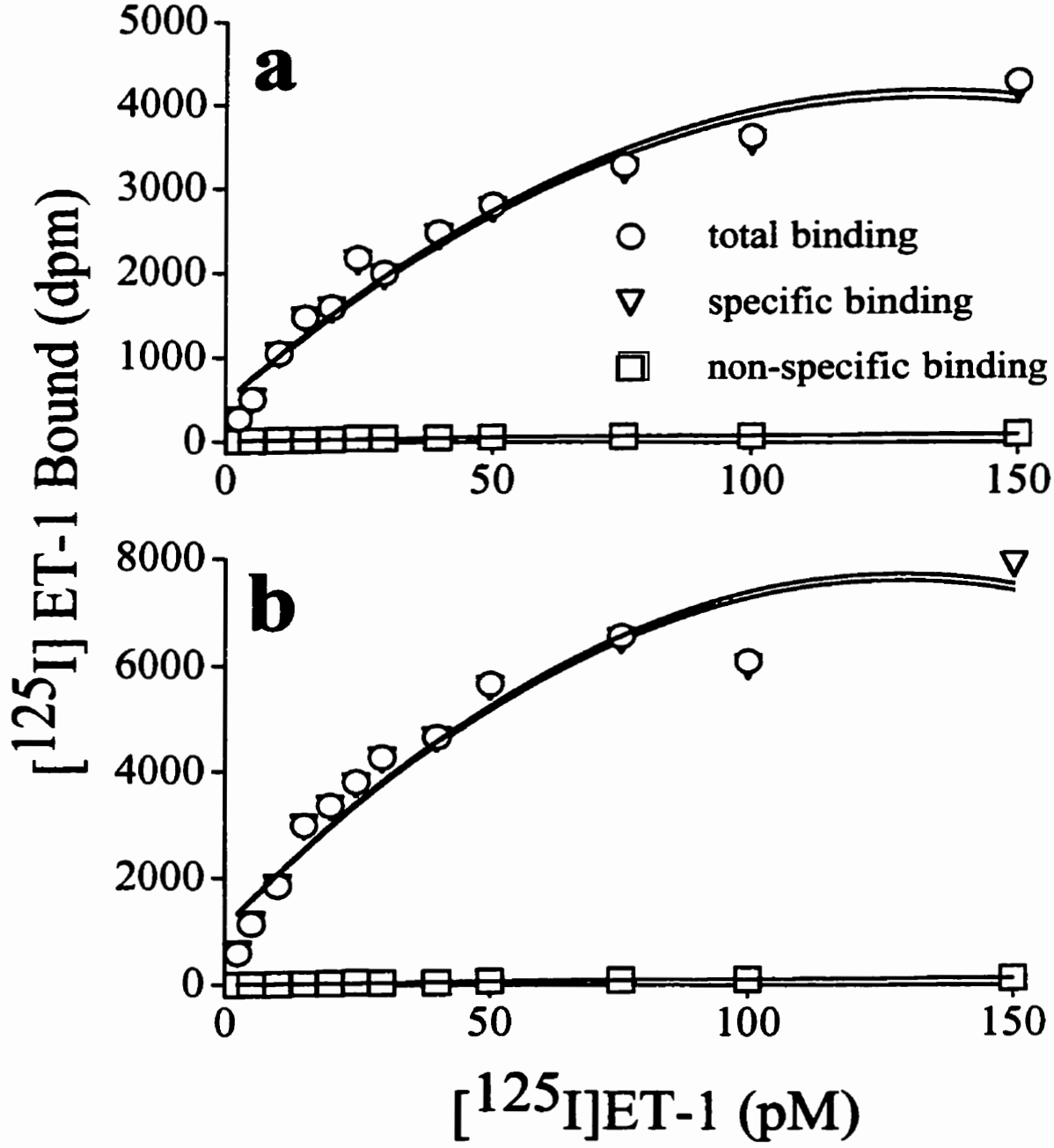
4.2.1.3. Saturation analysis of [^{125}I] ET-1 and [^{125}I] IRL-1620 specific binding

Saturation analysis of [^{125}I] ET-1 and [^{125}I] IRL-1620 was undertaken to determine the maximal number of binding sites per cell (B_{max}), and affinity constants (K_D) for each radioligand. The specific binding was 99% of total binding at low concentrations of [^{125}I] ET-1 and it decreased to 90% of total bound radioligand at saturating concentrations in the control ASMC. Moreover, bound radioligand amounted to 18 to 40% of the total ET-1 radioligand added to the medium. Interestingly, in insulin and vanadate pretreated cells, this effect was even more pronounced as the specific binding sites increased several fold and thus, the signal to noise ratio was much higher. Complete saturation of [^{125}I] ET-1 binding sites occurred at 300 pM. In contrast to [^{125}I] ET-1, the signal to noise ratio levels were relatively less in the case of [^{125}I] IRL 1620 (data not shown).

Saturation binding data from single experiments for both [^{125}I] ET-1 and [^{125}I] IRL 1620 performed on the same day using the same batch of cells under identical conditions are shown in figures 14 and 15 (for insulin groups), and Figures 17 and 19 (for vanadate groups). Scatchard analysis is shown in Figures 16 (for insulin groups) and 18 and 20 (for vanadate groups). Monophasic plots with Hill coefficient (n_H) values close to unity (between 0.90 and 1.00) were obtained for both radioligands. Vanadate pretreatment led to marked increases in B_{max} for both [^{125}I] ET-1 and [^{125}I] IRL 1620 saturation with no significant changes in slope or K_D values of the plots. Insulin pretreatment, however, led only to a significant increase in B_{max} for [^{125}I] ET-1, and a slight, but non-significant increase in that of [^{125}I]

IRL-1620. Moreover, with either radioligand, saturation data did not provide best fit for a two-site model. Summary data is presented in Table 1 (for insulin groups) and Table 2 (for vanadate groups). It is evident that [^{125}I] IRL-1620 labeled relatively fewer sites (685 - 945 sites/cell) denoting the existence of a small population of ET_B specific sites (12-14 % of the total sites labeled by [^{125}I] ET-1) in the control groups. Based on the data obtained with both radioligands, it is evident that rat ASMC possess predominantly ET_A sites (86-88%) with a very high ET_A / ET_B ratio of 6.05 - 7.3. Pretreatment of ASMC with insulin led to a 2.06 fold increase ($p < 0.01$) in sites labeled by [^{125}I] ET-1, but did not significantly affect [^{125}I] IRL-1620 labeled ET_B sites, indicating selective upregulation of ET_A specific binding sites. However, the overall, cell surface ET_A /ET_B ratio did not dramatically change (7.20 insulin vs. 6.05 control) (Table 2). Insulin had no affect on K_D values for either radioligand. Pretreatment with vanadate led to a 2.6 fold increase ($p < 0.01$) in the number of binding sites labeled by [^{125}I] ET-1 (14740 $\pm$ 2009) and a 7.8 fold increase ($p < 0.01$) in [^{125}I] IRL 1620 (5356 $\pm$ 393) labeled ET_B sites. Since the recruitment of ET_B specific sites was markedly higher, the overall, cell surface ET_A/ET_B ratio on ASMC was reduced from 7.3 to 1.8 by vanadate pretreatment. Pretreatment with vanadate also ($p < 0.05$) increased the K_D value for [^{125}I] ET-1 but not [^{125}I] IRL 1620 binding likely due to a very highly significant shift in the B_{max} for ET-1.

Figure 14. Saturation binding curves for [^{125}I] ET-1 in either untreated (**a**) or insulin (**b**; 100 nM; 24 hr) treated ASMC (~ 0.47 x 10^6 cells/well) under equilibrium conditions (37^{o}C for 3 hr) either in control or insulin (100 nM x 24 hr) pretreated ASMC. *Note*: Serum was deprived in DMEM medium 24 hr prior to initiating binding assays in both control and insulin pretreated cells; then the cells were washed in serum free DMEM thrice, and saturation binding studies were carried out in Krebs-HEPES buffer (pH 7.4). Increasing concentrations of [^{125}I] ET-1 were added to ASMC in duplicate in the absence (total binding) or presence (non-specific binding) of unlabeled ET-1 (100 nM) for a period of 3 hr (37^{o}C). Non-specific binding was never greater than 3% of total binding.


a
b
total binding
specific binding
non-specific binding
[125I] ET-1 Bound (dpm)
[125I]ET-1 (pM)
5000
4000
3000
2000
1000
0
8000
6000
4000
2000
0
50
100
150

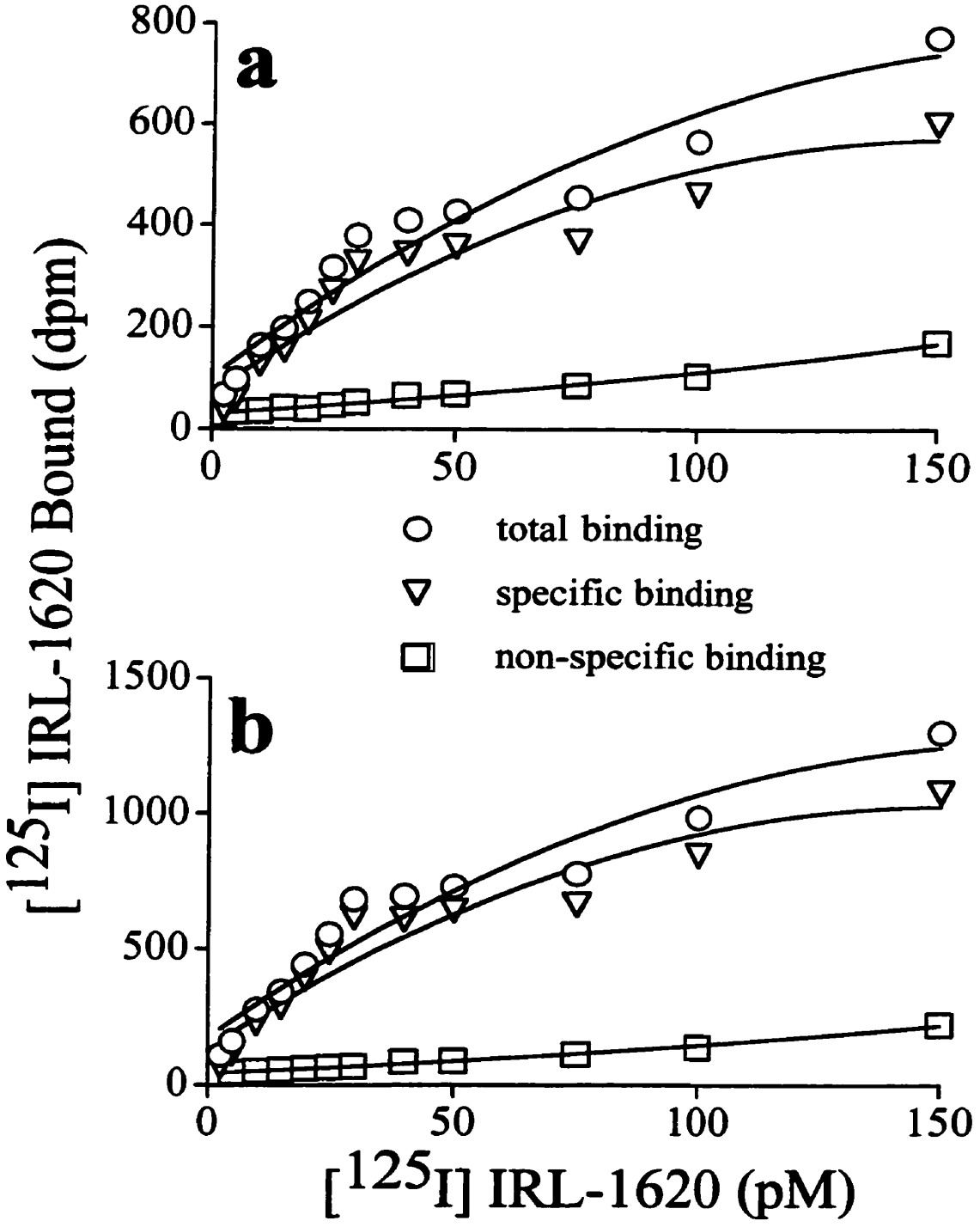


Figure 15. Saturation binding curves of [^{125}I] IRL-1620 in either untreated (**a**) or insulin treated (**b**; 100 nM; 24 hr) ASMC. Increasing concentrations of [^{125}I] IRL-1620 were added to ASMC in duplicate in the absence (total binding) or presence (non-specific binding) of unlabeled ET-1 (100 nM). Non-specific binding was never greater than 10% of total binding.

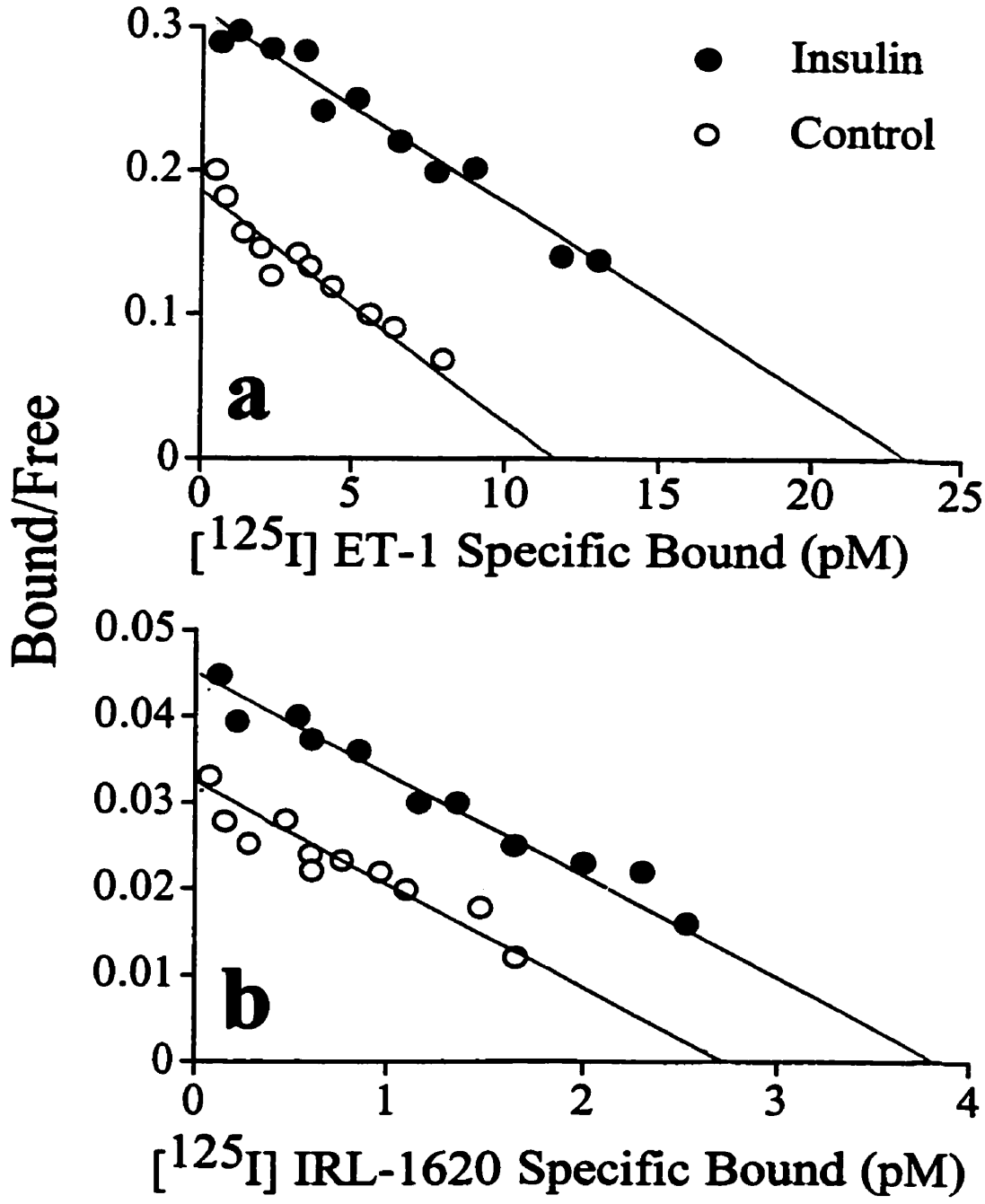


Figure 16. Scatchard plots of representative experiments performed under identical conditions examining [^{125}I] ET-1 (**a**) and [^{125}I] IRL-1620 (**b**) specific binding to insulin pretreated and control ASMC.

TABLE 1

Analyses of saturation of [^{125}I] ET-1 and [^{125}I] IRL-1620 specific binding in either insulin pretreated or control untreated aortic smooth muscle cells.

GROUP	ET-1		IRL-1620		ET_A/ET_B Ratio
	K_D (pM)	B_{max} (sites/cell)	K_D (pM)	B_{max} (sites/cells)	
Control N=6	33 ± 8	6,664 ± 543	36 ± 18	945 ± 317	**6.05** ET_A - 86% ET_B - 14%
Insulin 100 nM - 24 hr n=6	38 ± 13	13, 730 ± 1,528**	127 ± 56	1,675 ± 483	**7.20** ET_A - 88% ET_B - 12%

** $p < 0.01$ compared to respective control B_{max} values.

Note: After incubation of rat ASMC with insulin (100 nM for 24 hr), the cells were washed with serum free medium prior to binding assays.

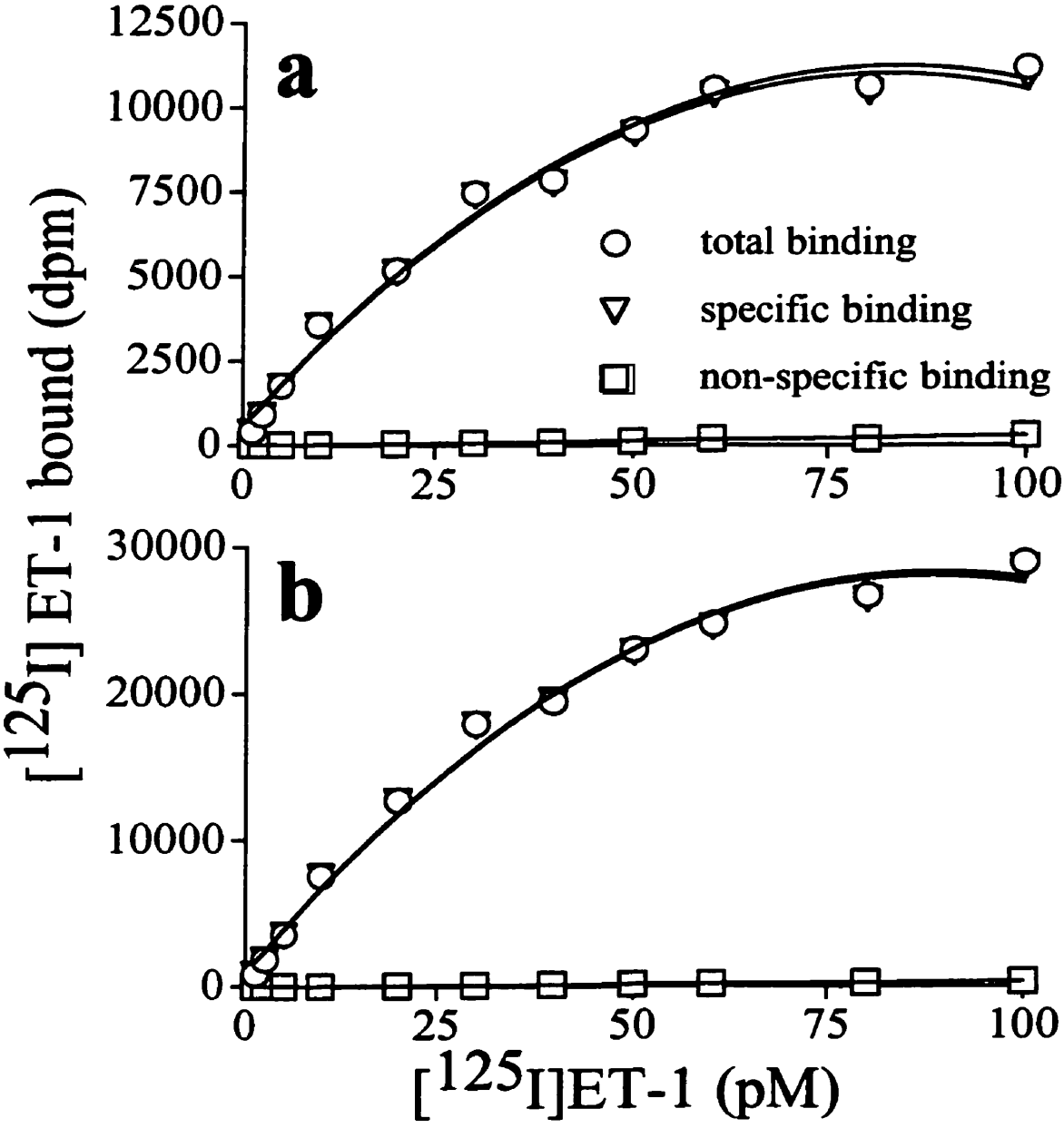


Figure 17. Saturation binding curves of [^{125}I] ET-1 in either untreated (**a**) or vanadate treated (**b**; 25 μM; 24 hr) ASMC. Increasing concentrations of [^{125}I] ET-1 were added to ASMC in duplicate in the absence (total binding) or presence (non-specific binding) of unlabeled ET-1 (100 nM) for a period of 3 hr (37^{o}C).

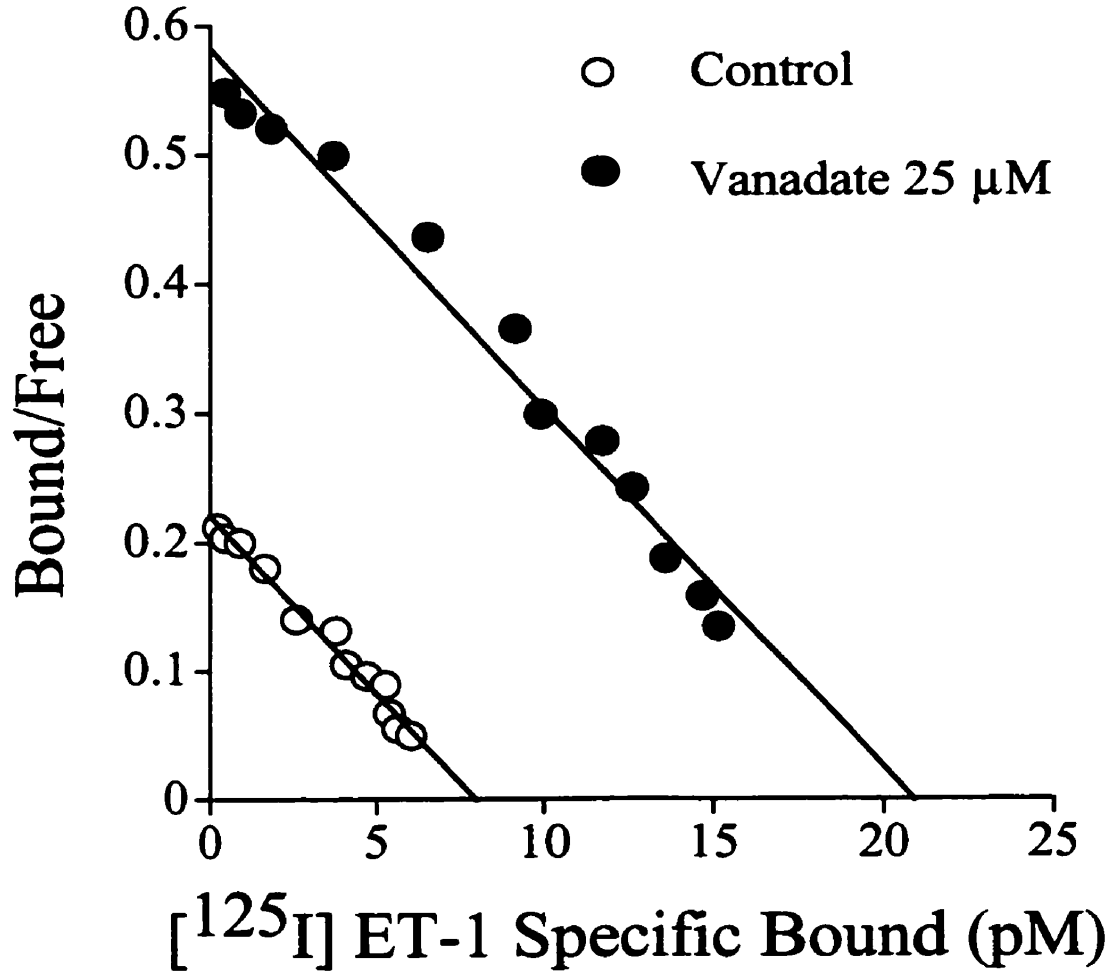


Figure 18. Scatchard plots of a representative experiment examining [^{125}I] ET-1 specific binding either in control or vanadate (25 µM x 24 hr) pretreated ASMC.

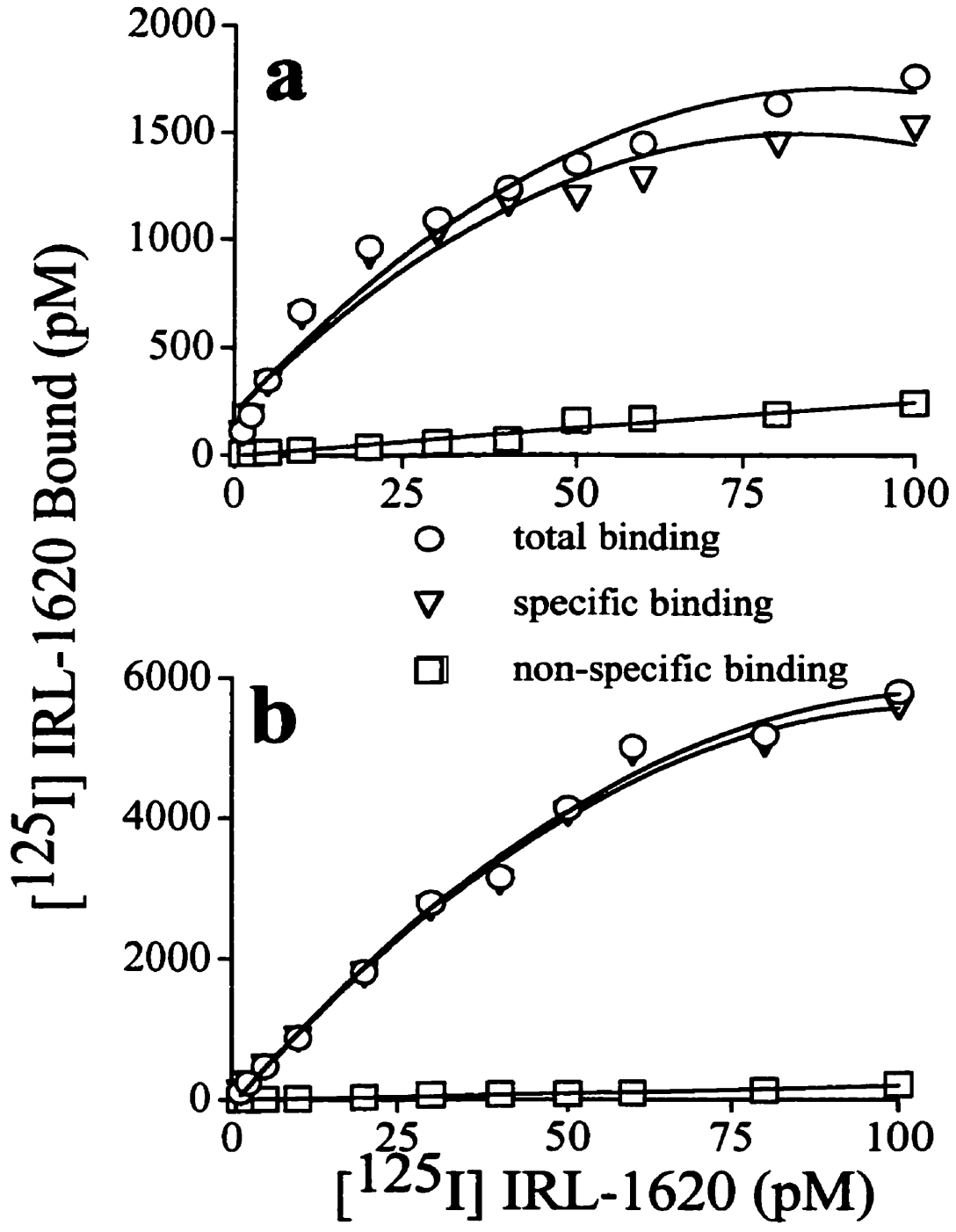


Figure 19. Saturation binding curves of [^{125}I] IRL-1620 in either untreated (**a**) or vanadate treated (**b**; 25 μM; 24 hr) ASMC. Increasing concentrations of [^{125}I] IRL-1620 were added to ASMC in duplicate in the absence (total binding) or presence (non-specific binding) of unlabeled ET-1 (100 nM).

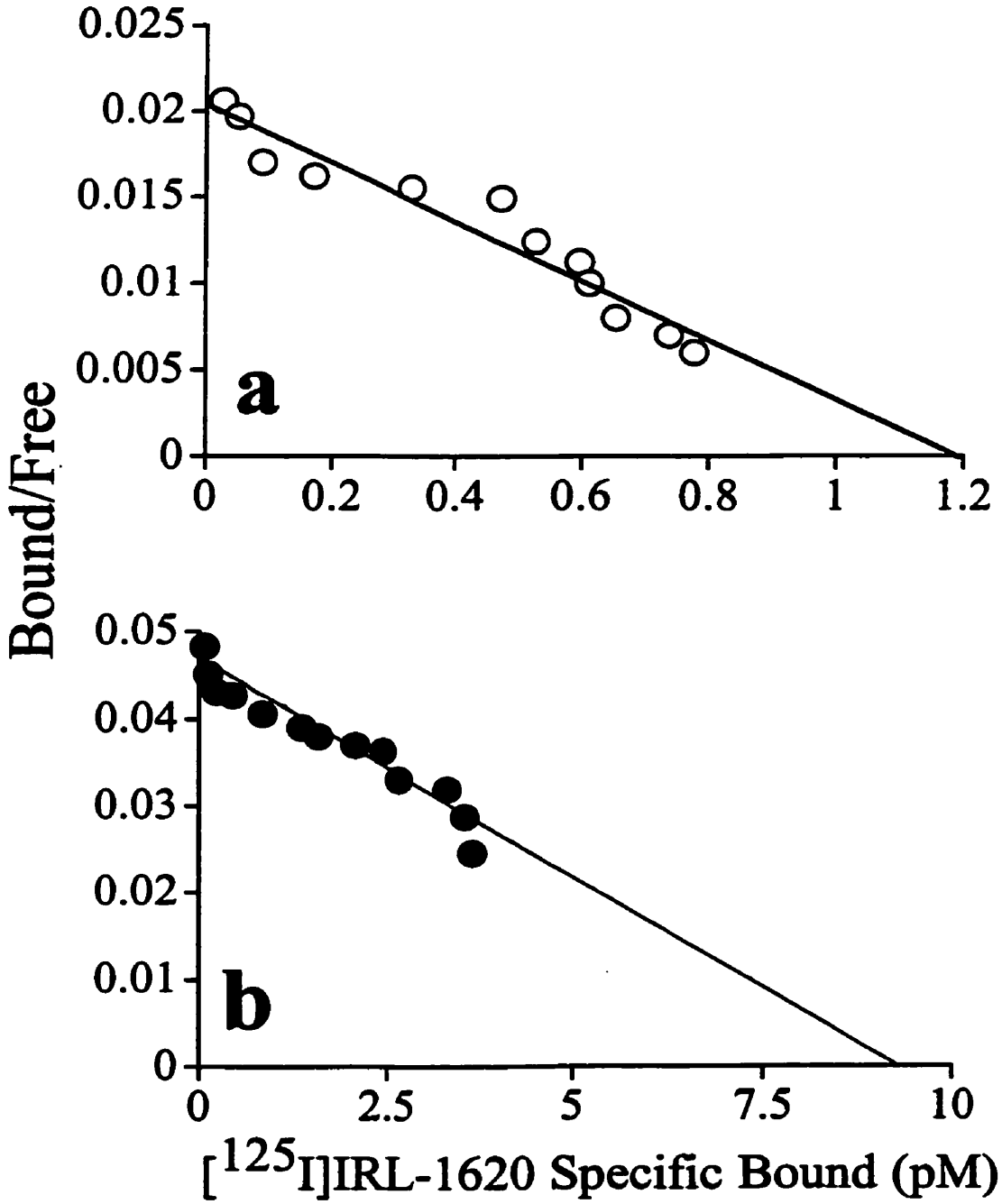


Figure 20. Scatchard plots of a representative ET_B binding assay examining [^{125}I] IRL-1620 specific binding to rat ASMC either in control **(a)** or vanadate (25 μM; 24 hr) pretreated ASMC **(b)**.

TABLE 2

Analyses of saturation of [^{125}I] ET-1 and [^{125}I] IRL-1620 specific binding in either vanadate pretreated or control untreated aortic smooth muscle cells.

Group	ET-1		IRL-1620		ET_A/ET_B Ratio
	K_D (pM)	B_{max} (sites/cell)	K_D (pM)	B_{max} (sites/cell)	
Control n=6	62 ± 18	5665 ± 1143	75 ± 20	685 ± 155	**7.3** ET_A - 88% ET_B - 12%
Vanadate 25 μM - 24 hr n=6	150 ± 50*	14,740 ± 2,009**	124 ± 23	5,356 ± 393**	**1.8** ET_A - 64% ET_B - 36%

* $p < 0.05$ compared to control K_D value for ET-1

** $p < 0.01$ compared to respective control B_{max} values.

Note: Vanadate incubation (25 μM for 24 hr) with rat ASMC was carried out in serum free DMEM; at the end of the incubation period, the cells were washed twice in serum free medium prior to initiating equilibrium binding assays.

4.2.2. Measurement of $[Ca^{2+}]_i$ in ASMC

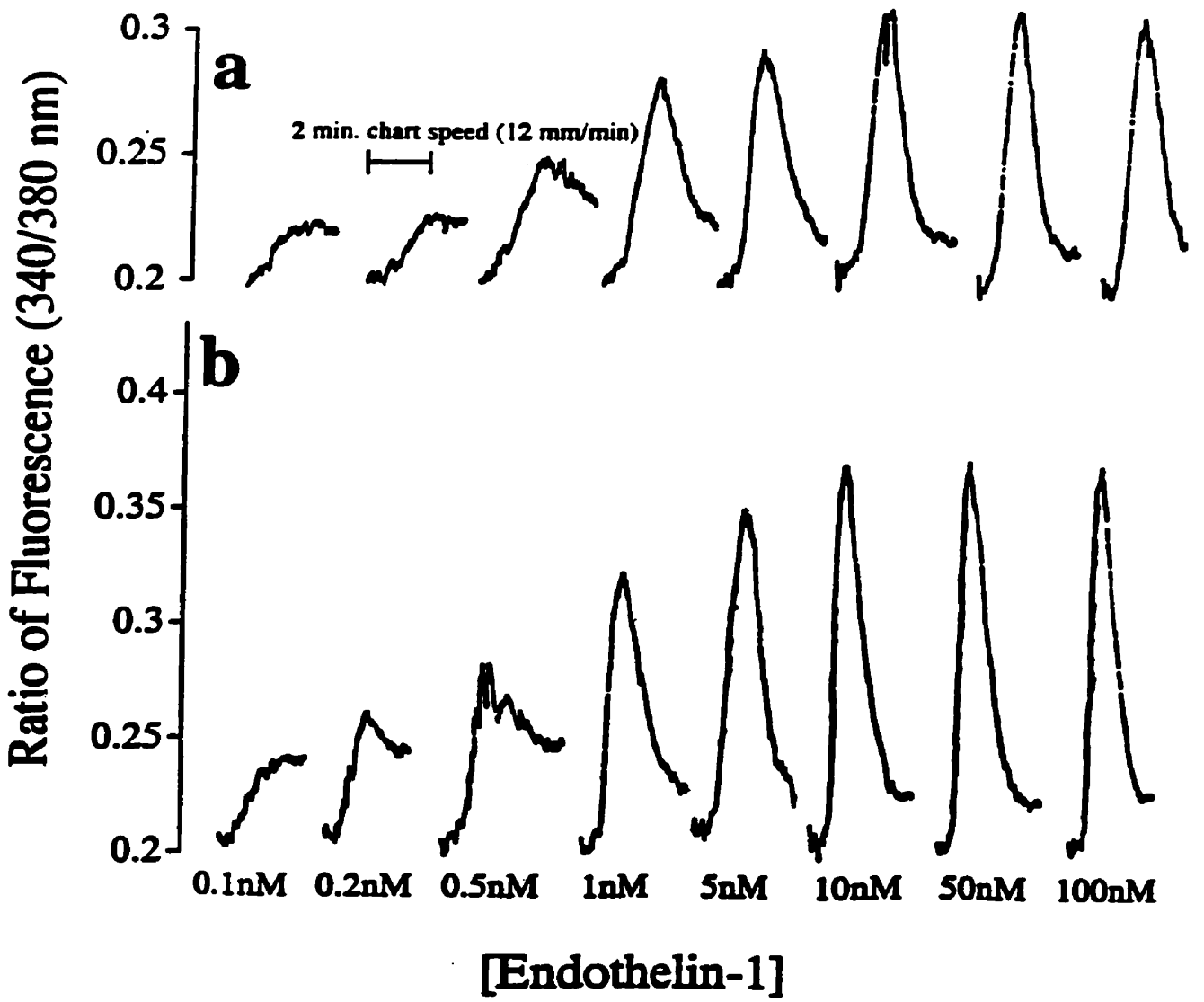
4.2.2.1. Effect of insulin and vanadate on concentration-peak $[Ca^{2+}]_i$ responses to ET-1 and IRL-1620

Incubation with vanadate (25 μM) or insulin (100 nM) for a period of 24 hr did not alter the morphological characteristics of the adherent ASMC prior to or after fura-2 loading. Dispersed cells from both treatment groups showed >90% viability prior to $[Ca^{2+}]_i$ measurements. Representative data for ET-1 evoked changes in fura-2 fluorescence from a single experiment performed on the same day using similar loading conditions from the same batch of ASMC for both control and insulin (Figure 21) and control and vanadate (Figure 22) pretreated groups is shown. There were no significant differences in basal $[Ca^{2+}]_i$ levels between either control (73 ± 12 nM) and vanadate (81 ± 18 nM) or control (80 ± 10 nM) and insulin (75 ± 11 nM) pretreated ASMC. ET-1 evoked a well-defined concentration dependent increase in the peak fura-2 fluorescence signal in all of controls, insulin, and vanadate pretreated groups. The peak $[Ca^{2+}]_i$ response to ET-1 was reached within 1 min and a steady state plateau phase was attained 3-4 min after agonist challenge in both control and insulin pretreated cells. The maximal response (ET-1 evoked peak $[Ca^{2+}]_i$) achieved was much higher in the insulin and vanadate pretreatment groups compared to untreated controls. Figures 23a and 24a represent pooled data of several ET-1 concentration-peak $[Ca^{2+}]_i$ response curves. At a maximal concentration of 100 nM ET-1, the maximal $[Ca^{2+}]_i$ increase (E_{max}) was 1.68 fold higher in the insulin (300 ± 20 nM) treated group compared to its respective control

group (179 ± 16 nM) and 1.7 fold higher (147 ± 13 nM; $p < 0.01$) in vanadate treated vs. its respective control group (87 ± 15 nM). There were no significant differences in calculated EC_{50} values between control (2.1 ± 0.4 nM) and vanadate (3.3 ± 0.6 nM) and control (7.8 ± 2.8 nM) and insulin treated (8.1 ± 2.1 nM) ASMC.

Pooled data for ET_B mediated IRL-1620 evoked changes in $[Ca^{2+}]_i$ are shown in Figures 23b and 24b. Although IRL-1620 also evoked a concentration dependent increase in peak $[Ca^{2+}]_i$ levels, the responses were much lower. In fact, a shift from basal values was marked only at concentration ranges above 10 nM IRL-1620. The maximal increase in $[Ca^{2+}]_i$ attained at a maximal concentration of 200 nM IRL 1620 was three-fold higher ($p < 0.01$) in vanadate treated cells (E_{max} 77 ± 4 nM) compared to its untreated control group (E_{max} 26 ± 7 nM). There were no significant differences in EC_{50} values between the two groups (vanadate: 8.5 ± 2.1 nM; control: 7.8 ± 0.8 nM). In contrast, insulin pretreatment had no significant effect on maximal $[Ca^{2+}]_i$ responses to IRL-1620 (control 31 ± 6 nM; insulin 24 ± 7 nM) and again, there were no significant differences in EC_{50} values between control (6.0 ± 3.3 nM) and insulin pretreated (7.1 ± 4.2 nM) ASMC.

Figure 21. Relationship between increasing concentrations of ET-1 and corresponding increases in fura-2 fluorescence (ratio of 340:380 excitation of fura-2) in a fixed aliquot of dispersed ASMC (0.15 x 10^6 cells / 0.5 ml Kreb's-HEPES buffer pH 7.4) in control (**a**) and insulin (**b**) pretreated ASMC. Initially, adherent ASMC maintained in culture flasks were first washed and then maintained in serum free medium (control) or treated with insulin (100 nM). At the end of 24 hr, ASMC were trypsinized, washed, and dispersed in Kreb's-HEPES buffer (pH 7.4). ASMC were loaded with fura-2 AM (5 μM), washed, and a fixed aliquot of cell suspension was utilized to determine $[Ca^{2+}]_i$ levels. Each aliquot was challenged with one concentration of ET-1 and then discarded. The tracings were recorded for a period of 4 min after the addition of ET-1 when a steady plateau phase had been reached.


a
b
2 min. chart speed (12 mm/min)
0.3
0.25
0.2
0.4
0.35
0.3
0.25
0.2
Ratio of Fluorescence (340/380 nm)
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0.2nM
0.5nM
1nM
5nM
10nM
50nM
100nM
[Endothelin-1]

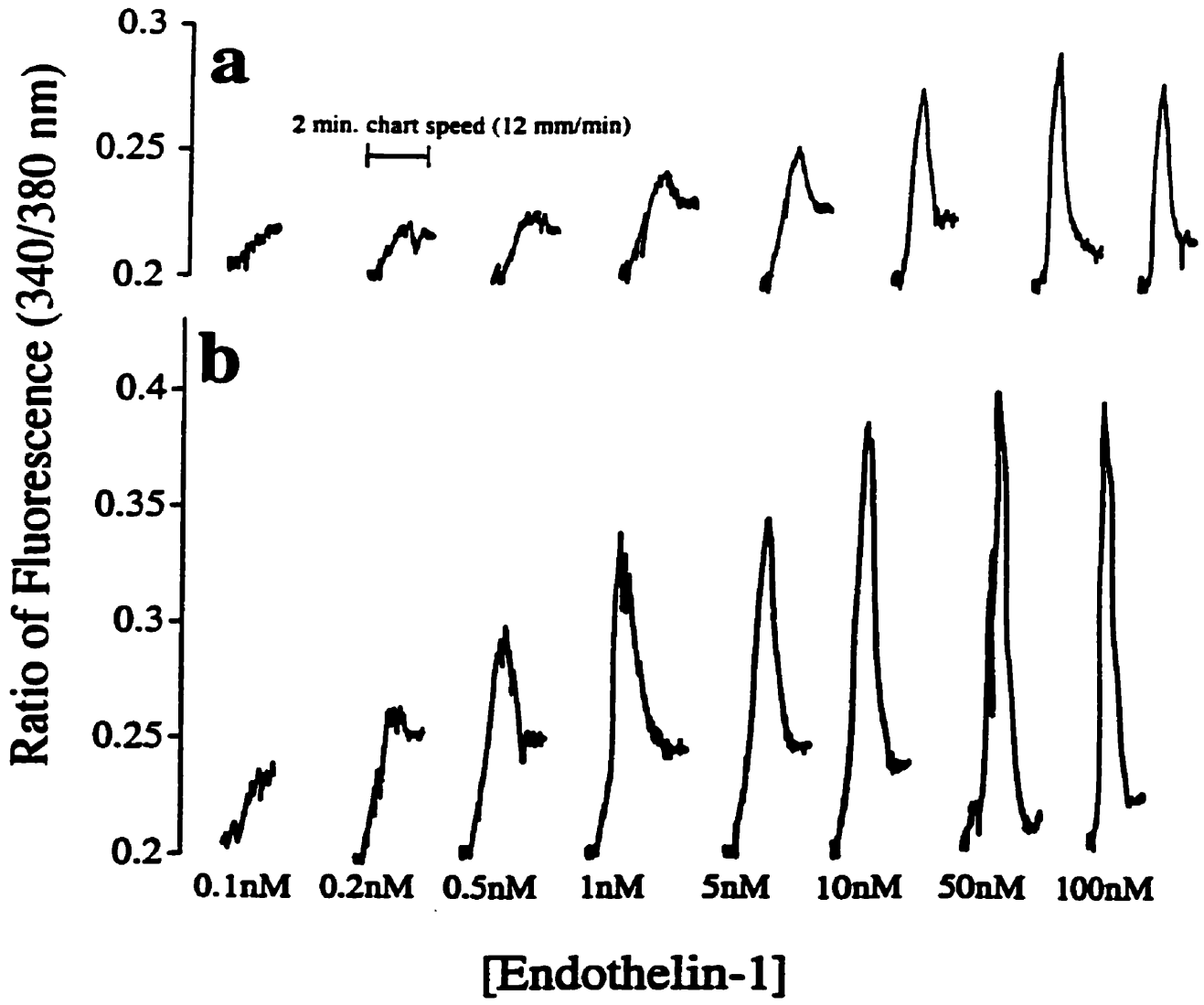


Figure 22. Representative concentration-dependent increase in $[Ca^{2+}]_i$ responses to ET-1 in control (**a**) and vanadate pretreated cells (**b**).

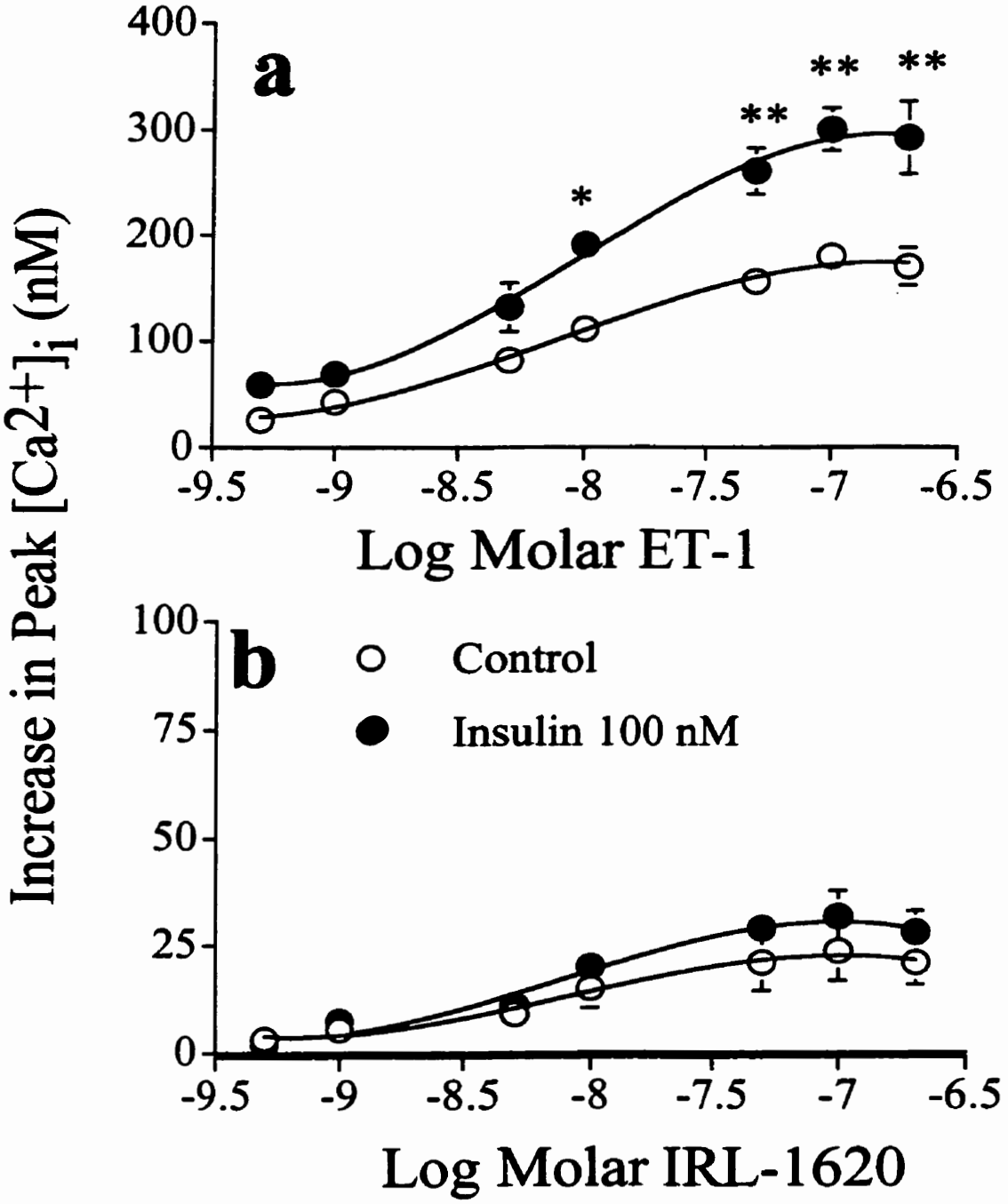


Figure 23. Relationship between concentration-peak $[Ca^{2+}]_i$ responses to ET-1 (**a**) and IRL-1620 (**b**) in control and insulin pretreated ASMC. Each data point is a mean ± SEM of six separate experiments using different batches of ASMC.
*$p < 0.05$ and * * $p < 0.01$ compared to the same data point for the control group.

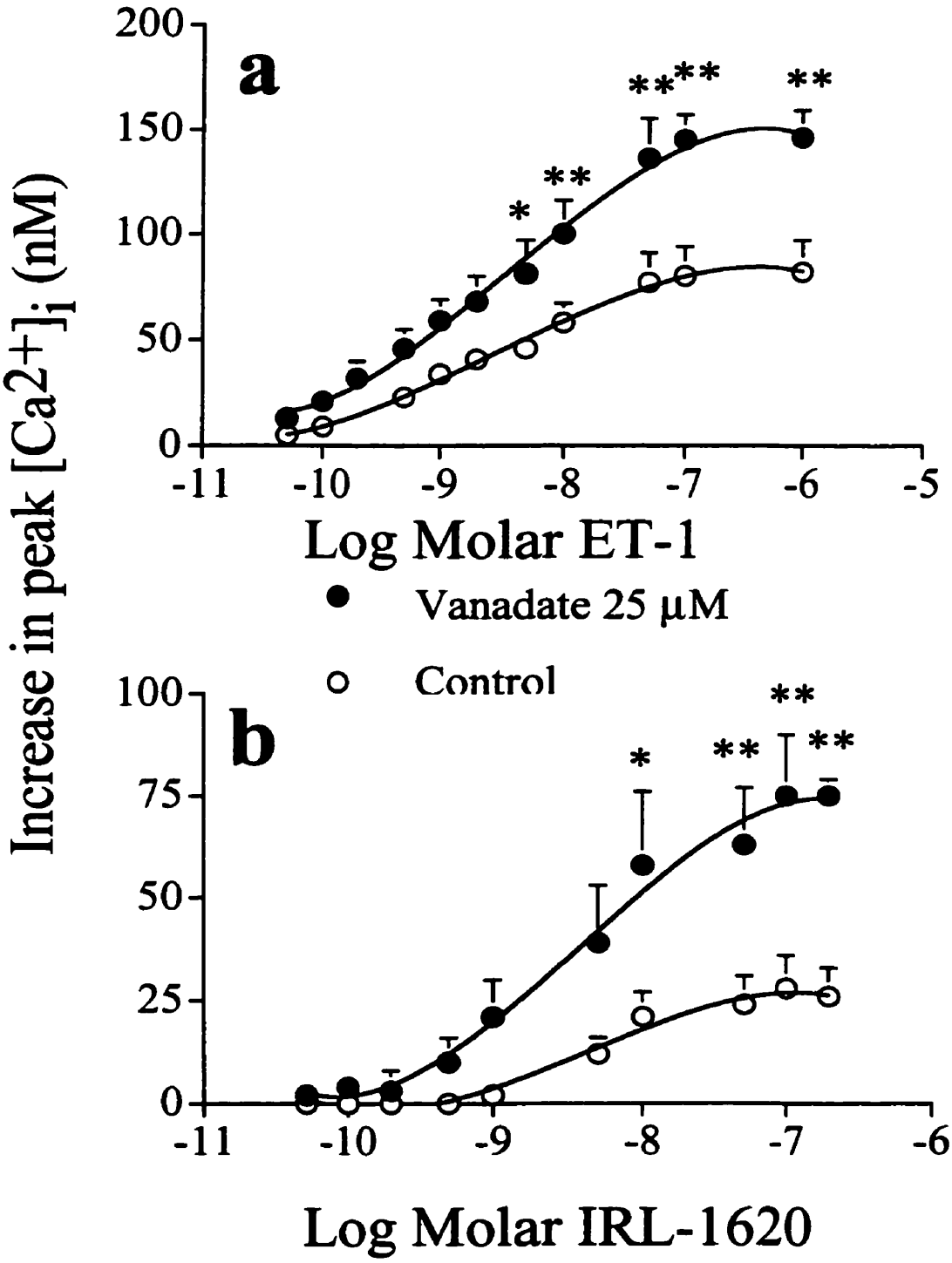


Figure 24. Relationship between concentration-peak $[Ca^{2+}]_i$ response curves to ET-1 **(a)** and IRL-1620 **(b)** in Vanadate pretreated (25 µM; 24 hr) and control ASMC. Each data point is a mean ● SEM of 4 separate experiments.

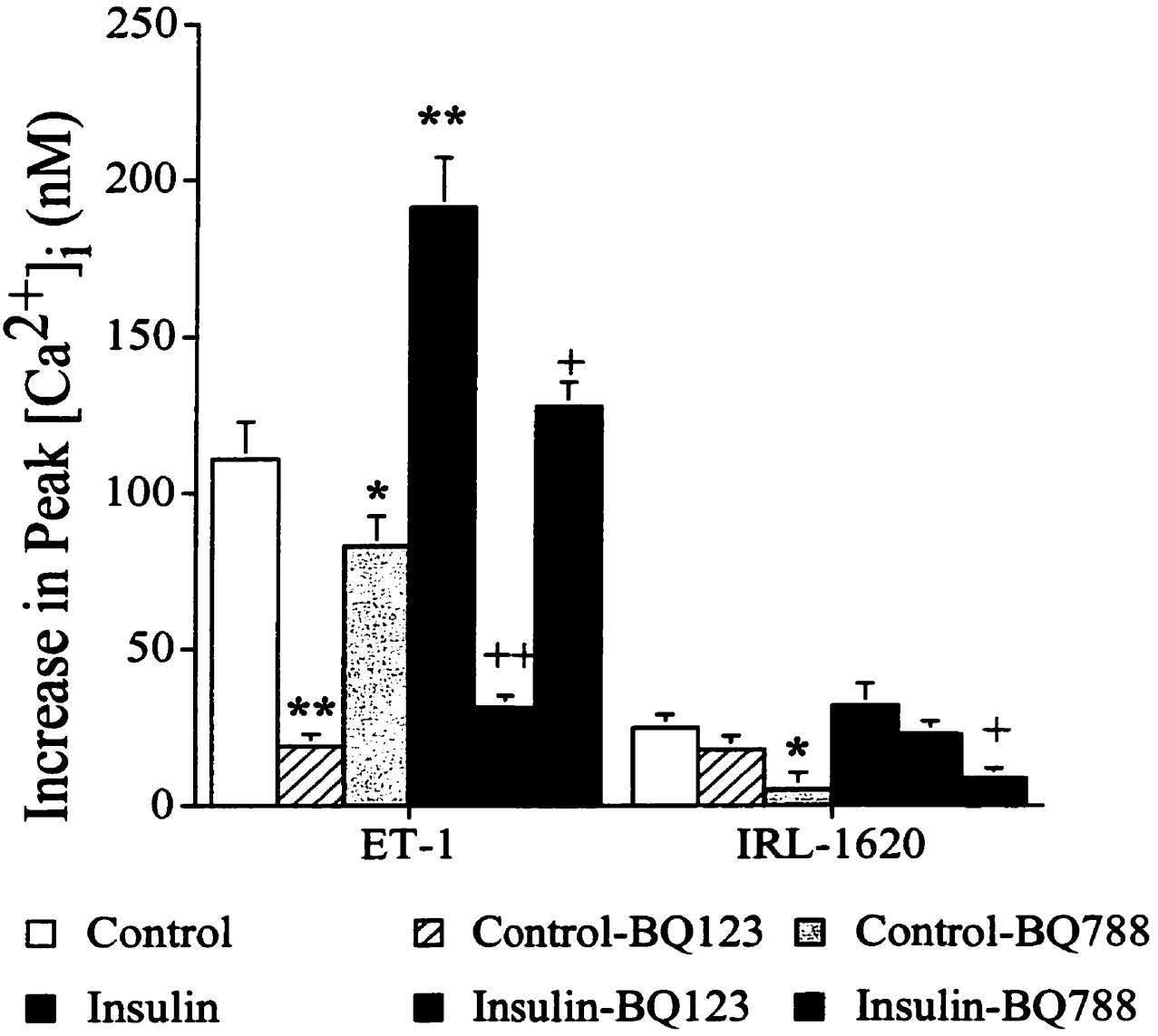
*p < 0.05 and ** p < 0.01 compared to control group.

4.2.2.2. Comparison of peak $[Ca^{2+}]_i$ elevations to ET-1 and IRL-1620 in the presence and absence of endothelin receptor subtype specific antagonists BQ123 and BQ788

The inclusion of a high concentration of BQ-123 (1 μM), an ET_A selective antagonist, to the ASMC suspension in the cuvette led to a significant ($p < 0.01$) abolition of $[Ca^{2+}]_i$ responses to a submaximal concentration of ET-1 (10 nM) in all of control, insulin, and vanadate pretreated cells (Figure 25 & 26). In the control group, the magnitude of blockade of ET-1 evoked $[Ca^{2+}]_i$ response was markedly higher in the presence of BQ-123, than in the presence of the ET_B selective antagonist, BQ-788 (1 μM). In both insulin and vanadate pretreated ASMC, as demonstrated earlier, ET-1 evoked $[Ca^{2+}]_i$ responses were exaggerated. In insulin pretreated ASMC, BQ-123 similarly blocked most of the increase in ET-1 evoked $[Ca^{2+}]_i$, while BQ788 had much less effect, suggesting greater recruitment of ET_A sites by insulin (Figure 25) – in line with the binding data. In contrast, in vanadate pretreated ASMC, both BQ-123 and BQ-788 abolished the increases in $[Ca^{2+}]_i$ responses to ET-1 to the same extent, suggesting enhanced recruitment of both ET_A and ET_B signaling (Figure 26). IRL-1620 evoked $[Ca^{2+}]_i$ responses in all groups were unaffected by BQ-123 but the responses were almost completely abolished by BQ-788, verifying the subtype selectivity of each of these agents. As demonstrated earlier, insulin pretreatment had no significant effect on IRL-1620 evoked $[Ca^{2+}]_i$ responses, whereas vanadate pretreatment greatly enhanced it, again suggesting enhanced recruitment of ET_B sites by vanadate.

Figure 25. Effects of the ET_A selective antagonist, BQ123, and the ET_B selective antagonist, BQ788, on peak $[Ca^{2+}]_i$ responses to ET-1 and IRL-1620 in control and insulin (100 nM; 24 hr) pretreated ASMC. Data shown are pooled values of mean $\pm$ SEM of 6 separate experiments for the maximal increase in $[Ca^{2+}]_i$ above resting levels attained after the addition of a fixed concentration of either ET-1 (10 nM) or IRL-1620 (10 nM) in both control and insulin pretreated ASMC. Subtype specific antagonists were incubated with ASMC for a period of 4 min prior to the addition of agonist (ET-1 or IRL-1620).

*$p < 0.05$; **$p < 0.01$ vs. respective control group; + $p < 0.05$; ++ $p < 0.01$ vs. respective insulin pretreatment group.


250
200
150
100
50
0
Increase in Peak $[Ca^{2+}]_i$ (nM)
**
*
**
++
+
*
+
ET-1
IRL-1620
Control
Control-BQ123
Control-BQ788
Insulin
Insulin-BQ123
Insulin-BQ788

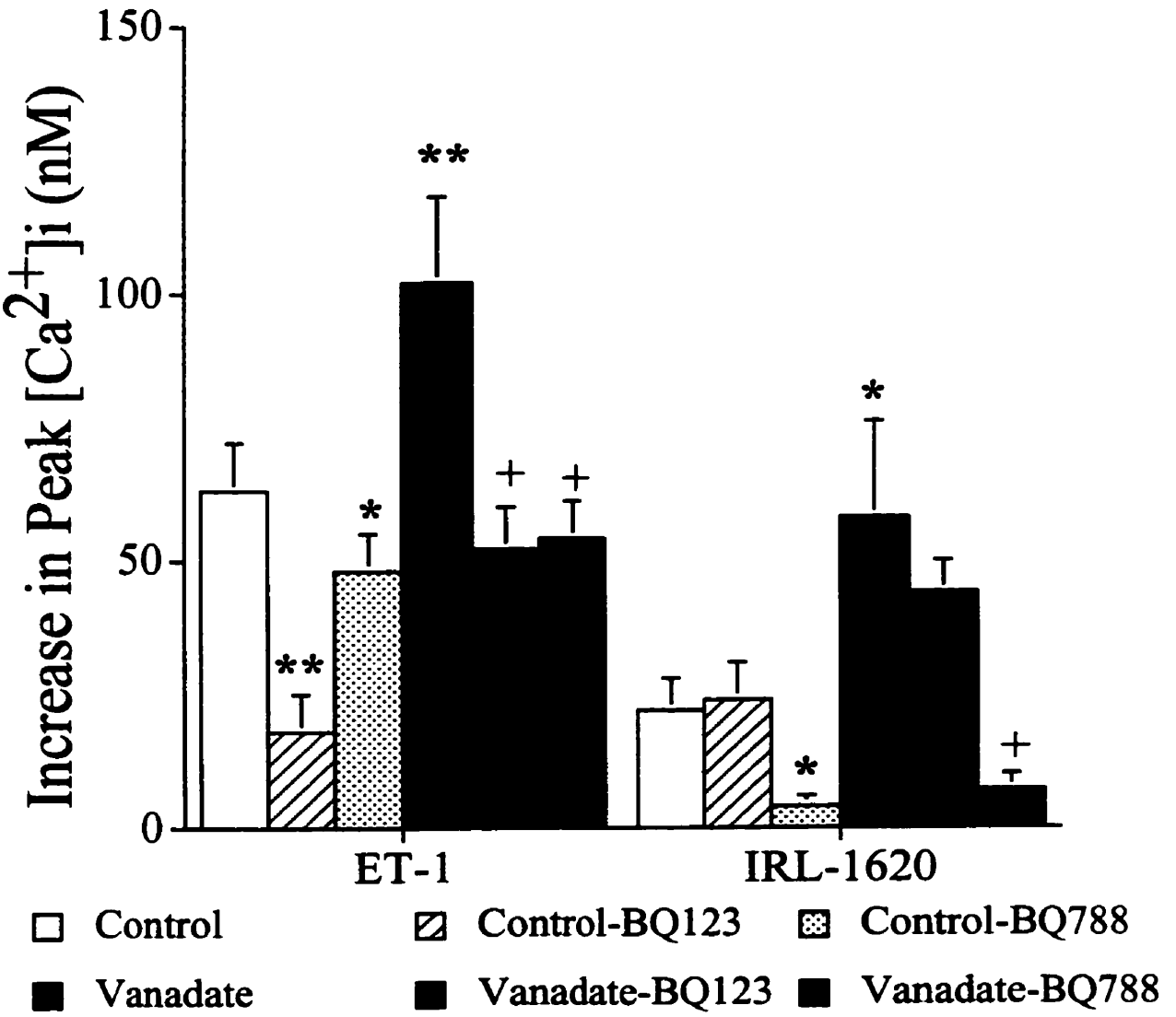


Figure 26. Effects of BQ123 and BQ788 on peak $[Ca^{2+}]_i$ responses to ET-1 and IRL-1620 in control and vanadate (25 μM; 24 hr) pretreated ASMC. Data shown are pooled values of mean ± SEM of 6 separate experiments for the maximal increase in $[Ca^{2+}]_i$ above basal levels.

*$p < 0.05$; ** $p < 0.01$ vs. respective control group; + $p < 0.05$; ++ $p < 0.01$ vs. respective vanadate pretreated group

4.2.2.3. Comparison of peak $[Ca^{2+}]_i$ responses to vasopressin, angiotensin II, and endothelin-1

In order to determine whether increases in ET receptor expression and action are selective to ET-1, experiments were completed whereby incubation with insulin (10 nM) and vanadate (20 μM) were tested for their ability to affect Ang II and AVP evoked $[Ca^{2+}]_i$ responses. Since high concentrations of insulin are known to activate IGF-1 receptors (King et al., 1980), IGF-1 (10 μg/ml) was also tested for its effects on ET-1, Ang II, and AVP evoked $[Ca^{2+}]_i$ responses. The effect of insulin on agonist evoked $[Ca^{2+}]_i$ signaling was selective to ET-1, since $[Ca^{2+}]_i$ responses to 100 nM Ang II and AVP were unchanged between control and insulin pretreated ASMC (Figure 27a). Similarly, IGF-1 selectively increased $[Ca^{2+}]_i$ responses to ET-1, leaving Ang II and AVP evoked responses intact (Figure 27b). However, in addition to promoting a 2-fold increase in ET-1 evoked $[Ca^{2+}]_i$ responses, surprisingly, vanadate actually dramatically attenuated Ang II evoked $[Ca^{2+}]_i$ responses (Figure 27c).

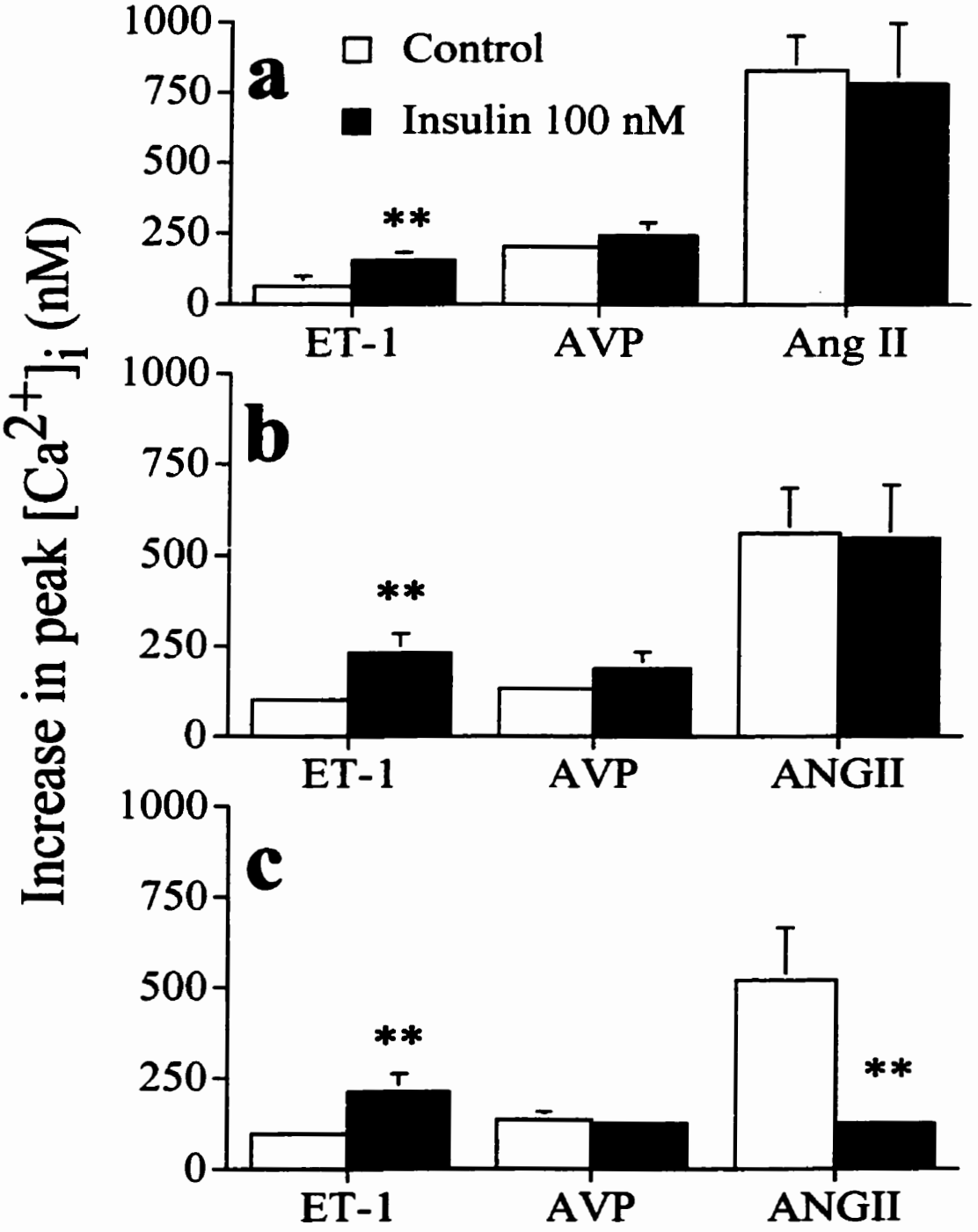


Figure 27. Effect of 10 nM insulin (**a**), 10 μg/ml IGF-1 (**b**), and 25 μM vanadate pretreatment on ET-1, Ang II and AVP (100 nM each) evoked peak $[Ca^{2+}]_i$ responses in rat ASMC. Data represents mean ± SEM of 4 separate determinations. **$p < 0.01$ vs. respective control.

4.2.3. Measurement of ET_A and ET_B mRNA

4.2.3.1. Time dependent changes in ET_A and ET_B mRNA expression evoked by insulin and vanadate

To examine whether increases in cell surface ET_A and ET_B binding sites evoked by insulin or vanadate pretreatment were due to enhanced ET receptor mRNA expression, northern analysis of ET_A and ET_B mRNA levels were carried out. Time dependency of this effect is presented in Figures 28 and 29. While only ET_A mRNA was upregulated in insulin (100 nM) pretreated ASMC (Figure 28), both ET_A and ET_B mRNA levels were upregulated in vanadate (25 μM) pretreated cells (Figure 29). These effects became apparent at 12 hours, and maximal at 20 hr.

4.2.3.2. Effect of Genistein on insulin and vanadate evoked changes in ET_A and ET_B mRNA expression

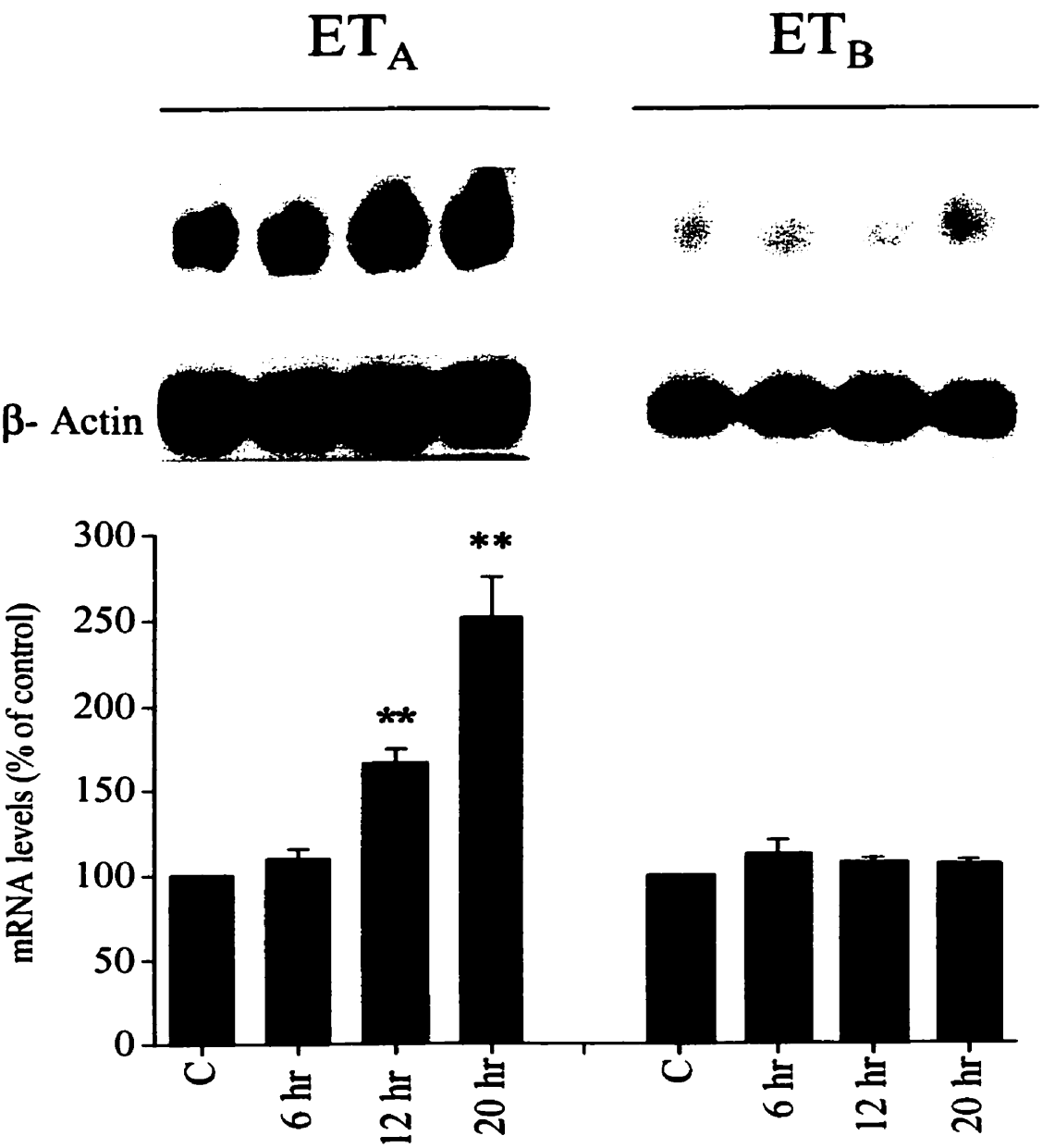
Incubation of ASMC with the tyrosine kinase inhibitor genistein (10 μM) *per se* for 20 hr failed to evoke appreciable changes in ET_A and ET_B mRNA levels. However, elevated ET_A mRNA levels evoked by insulin (Figure 30) and increased ET_A and ET_B mRNA levels evoked by vanadate (Figure 31) were abolished by inclusion of genistein in the incubation medium. These data indicate the requirement for tyrosine kinase activation in the effects of insulin and vanadate on ET receptor expression.

4.2.3.3. Effect of actinomycin-D and cycloheximide on insulin and vanadate evoked changes in ET_A and ET_B mRNA expression

Incubation of ASMC with the transcription inhibitor, actinomycin D (1.5 μg/ml), or the protein synthesis inhibitor, cycloheximide (10 μg/ml), *per se* for 20 hr did not evoke any appreciable changes in ET_A and ET_B mRNA levels. However, upregulation of ET receptor mRNA levels induced by insulin (Figure 32) and vanadate (Figure 33) was blocked by coincubation with either of these compounds, indicating the requirement of transcription and protein synthesis in the effects of insulin and vanadate on ET_A and ET_B mRNA expression.

Figure 28. Time course of increases in ET receptor mRNA levels induced by insulin. Confluent ASMC were incubated with serum-free DMEM containing 100 nM insulin for the indicated time intervals (6,12, & 20 hr). Control ASMC (C) were incubated with serum-free DMEM alone for 20 hr. After the 20 hr incubation, total RNA was isolated, fractionated (20 μg/lane) on 1% agarose formaldehyde gel, transferred to nylon membrane and hybridized with labeled ET_A or ET_B cDNA probe. The blots were rehybridized with labeled β-actin probe. The upper panel shows a representative blot of a single experiment, and the lower panel depicts the pooled mean ± SEM values after densitometric analysis of three separate blots with data expressed as % change compared to control (100%) value. Control value for 20 hr period is shown in the figure.

** $p < 0.01$ vs. untreated control


ET$_A$
ET$_B$
β- Actin
mRNA levels (% of control)
300
250
200
150
100
50
0
**
**
C
6 hr
12 hr
20 hr
C
6 hr
12 hr
20 hr

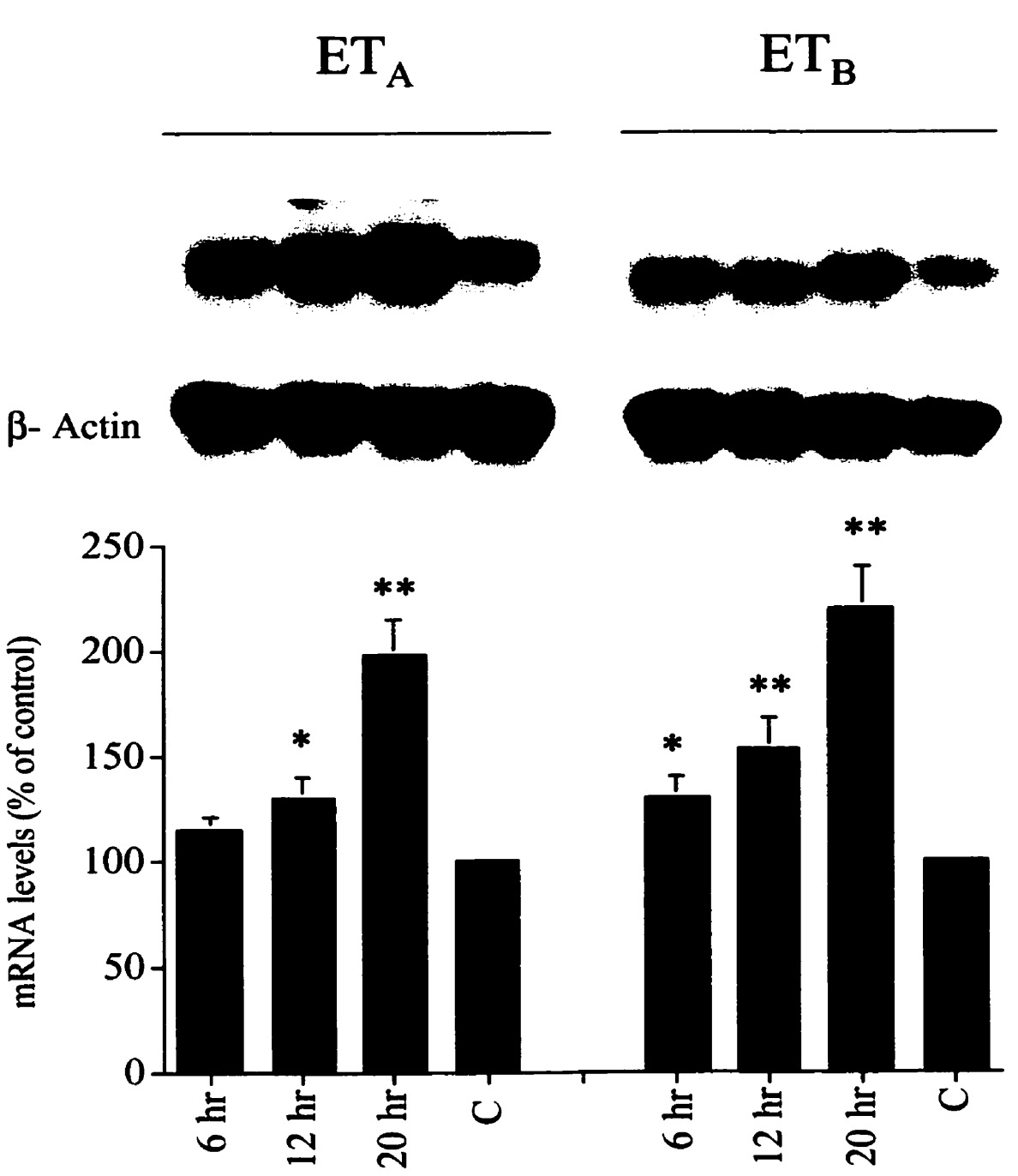


Figure 29. Time course of increases in ET receptor mRNA levels induced by vanadate. Upper panel is a blot from a representative experiment while the lower panel is a pooled mean ± SEM value of densitometric analysis for each time point from 4 different blots.

*$p < 0.05$; ** $p < 0.01$ vs. untreated control

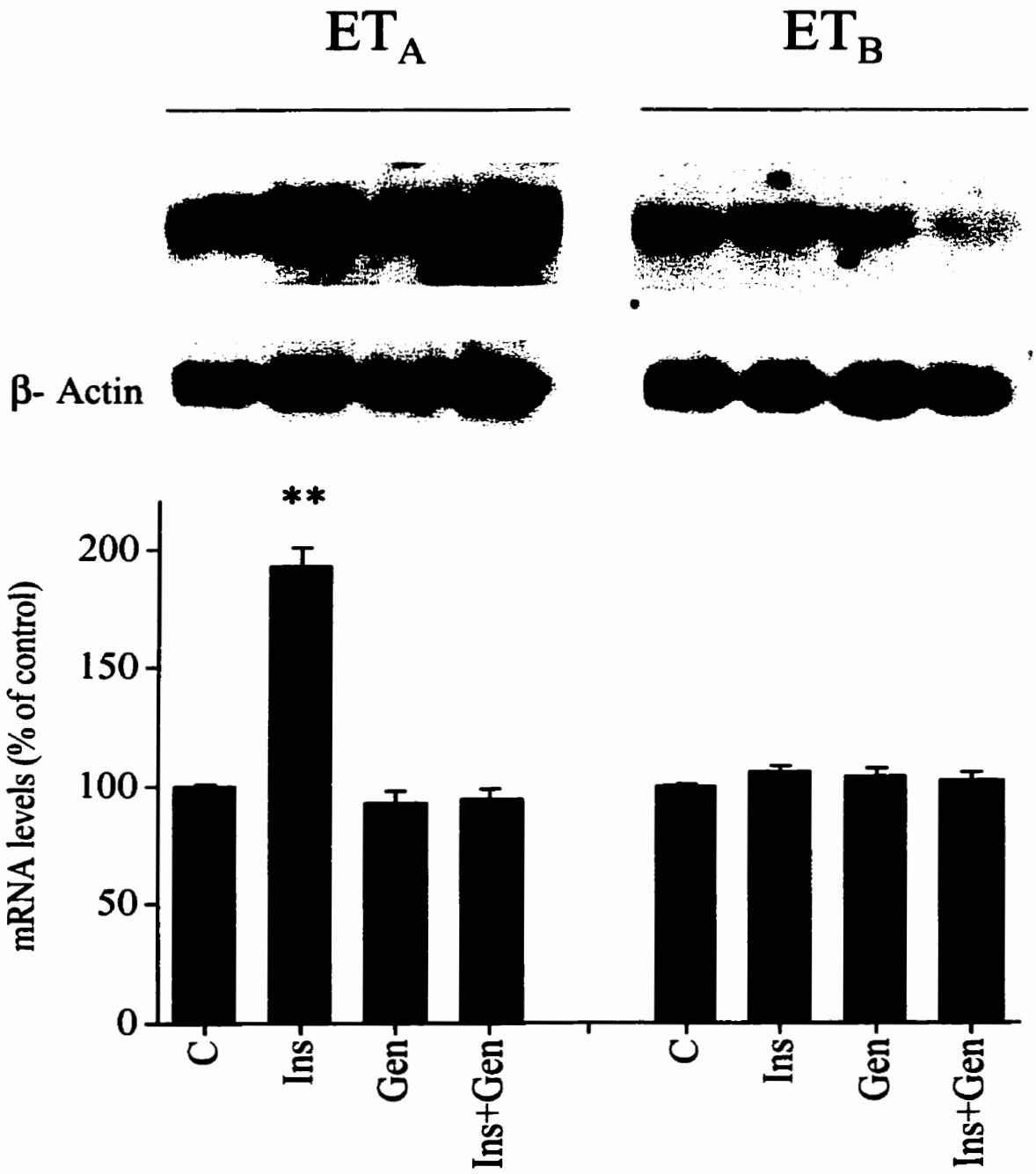


Figure 30. Effect of genistein on ET receptor mRNA changes evoked by insulin. The upper panel is representative of a single experiment while the lower panel shows mean ± SEM of 4 separate experiments.

** $p < 0.01$ vs. control group

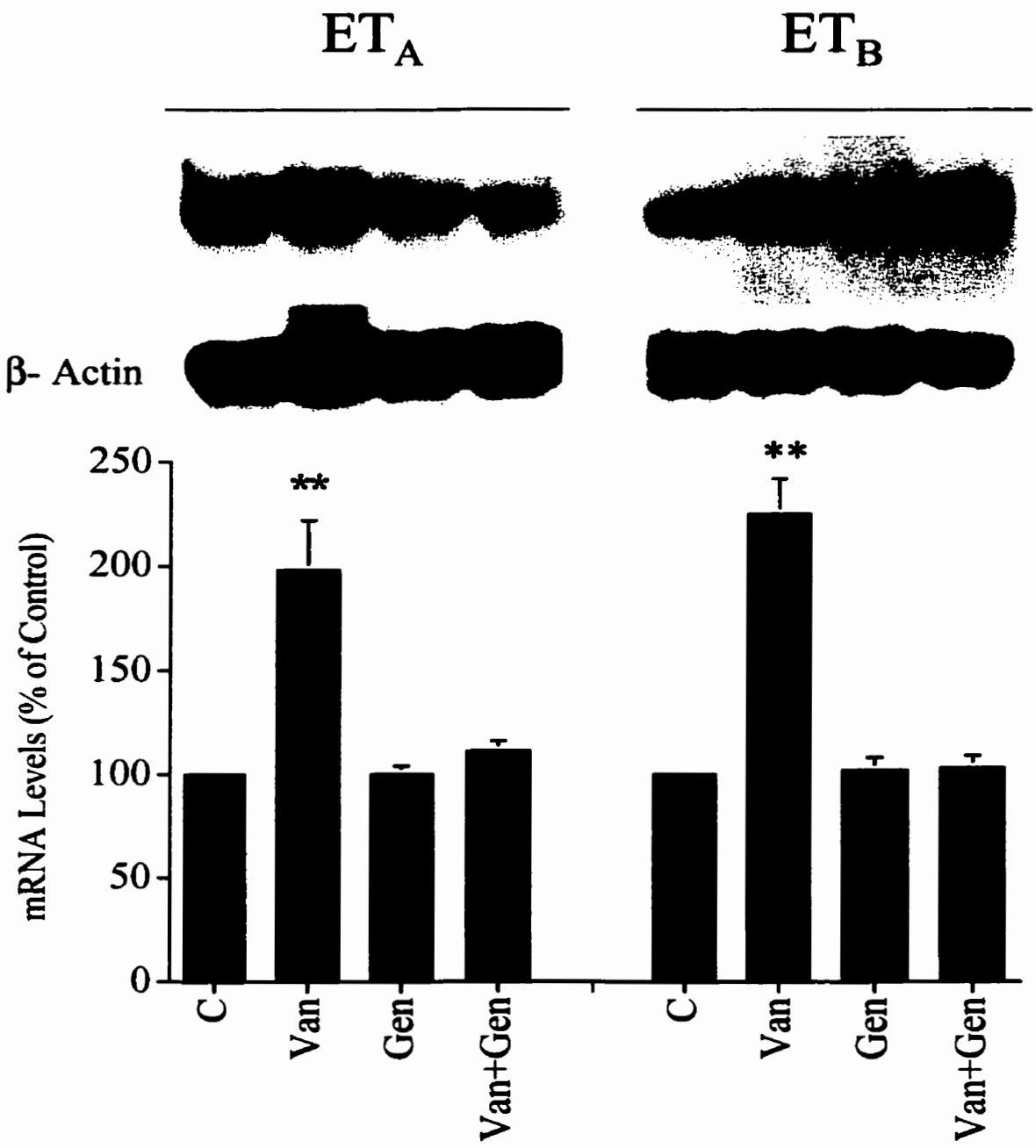


Figure 31. Effect of genistein on ET receptor mRNA changes evoked by vanadate. Upper panel is a representative experiment while the lower panel is pooled mean ± SEM value of densitometric analysis from 4 different blots.

** $p < 0.01$ vs. control group

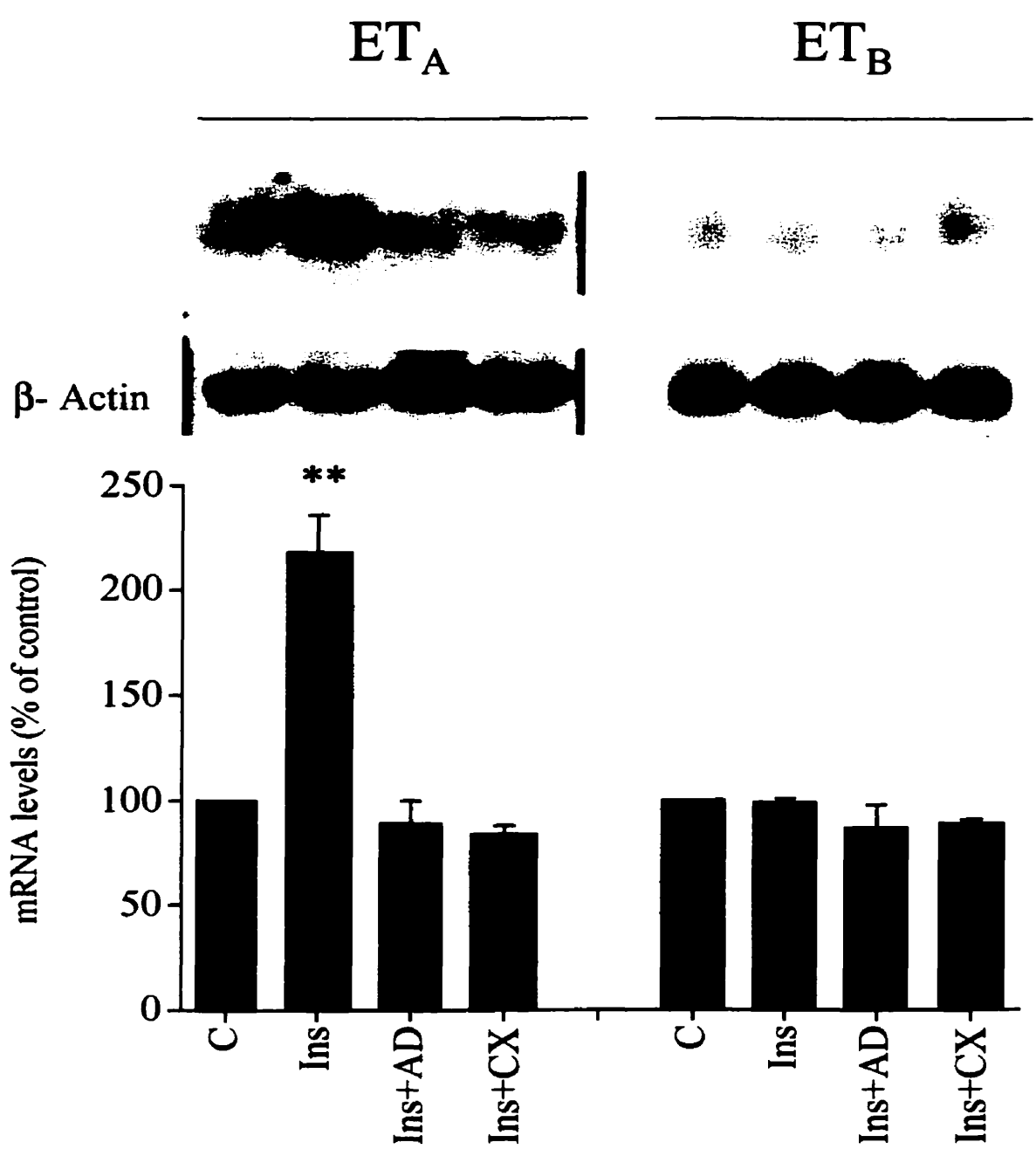


Figure 32. Effect of actinomycin D and cycloheximide on insulin evoked increases in ET receptor mRNA in ASMC. Actinomycin D (AD; 1.5 μg/ml) or cycloheximide (CX; 10 μg/ml) was added during insulin incubation as indicated. Upper panel is a representative experiment while the lower panel is pooled mean ± SEM value of densitometric analysis from 4 different blots. ** $p < 0.01$ vs. control group

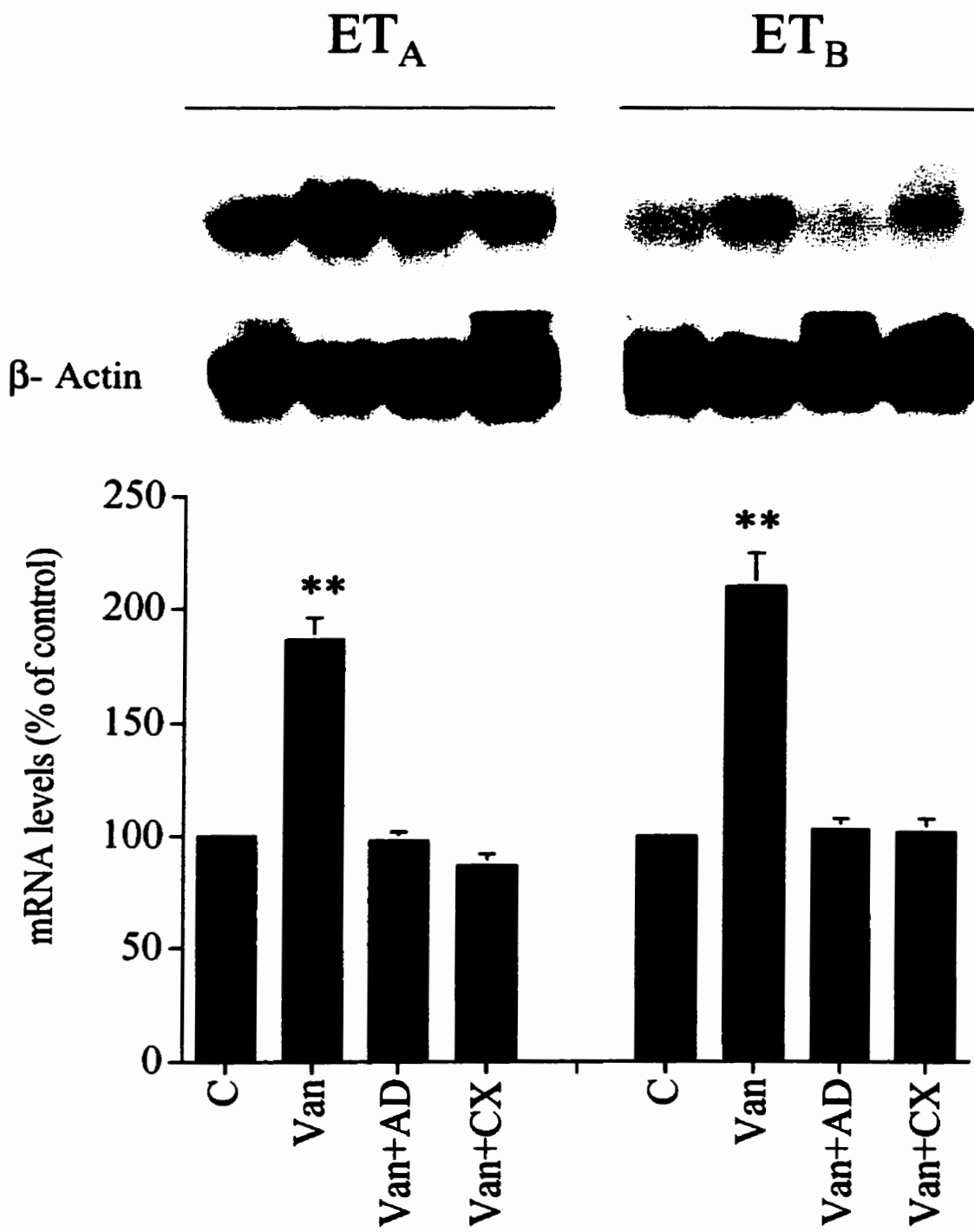


Figure 33. Effect of actinomycin D and cycloheximide on vanadate evoked changes in ET receptor mRNA. Upper panel is a representative experiment while the lower panel is pooled mean ± SEM value of densitometric tracing of 4 separate experiments.

** $p < 0.01$ vs. control group

4.3. In vivo effects of insulin and vanadate in streptozotocin diabetic and non-diabetic rats

4.3.1. Analysis of metabolic variables

Body weight and food intake for all groups before and after the 2-week treatment period is shown in Tables 3 and 4. The body weights of diabetic rats before treatment (3-weeks post-STZ) were lower ($p < 0.01$) than in normal rats in all groups. Over the 2-week treatment period, untreated diabetic rats failed to show a significant increase in body weight. Diabetic rats treated with vanadate gained less weight than their untreated counterparts. These observations are in accordance with other studies showing that, under short-term treatment (<20 days) with vanadate, diabetic rats tend to lose weight (Becker et al. 1994). Insulin treated diabetic rats, on the other hand, gained weight over the treatment period. Food intake was significantly greater in all untreated diabetic rats than in all non-diabetic control groups. Both vanadate and insulin treatment significantly lowered food intake in diabetic rats to near the level of untreated non-diabetic controls. Vanadate treatment also reduced food intake in non-diabetic rats. Vanadate intake, calculated by multiplying water intake by vanadate concentration, amounted to 30 ± 0.7 mg/rat/day in diabetic rats, and 22 ± 1.3 mg/rat/day in non-diabetic rats. This dose is in line with previous studies showing beneficial effects of vanadate on blood glucose concentrations (Brichard & Henquin, 1995; Becker et al. 1994).

Blood glucose and plasma insulin concentrations for all groups are also shown in Tables 3 and 4. Both untreated diabetic groups had higher fasting plasma glucose

and lower plasma insulin levels than their non-diabetic counterparts. Insulin and vanadate treatment dramatically lowered plasma glucose concentrations in the diabetic rats. As expected, insulin treatment raised plasma insulin levels in both diabetic and non-diabetic groups while vanadate treatment had no significant effect. Additionally, insulin treatment of diabetic rats raised plasma insulin levels above that of untreated, non-diabetic controls, but not above that of insulin treated non-diabetic controls.

4.3.2. Analysis of ex vivo vascular reactivity in aortic rings

Maximum evoked isometric tension (vasoconstriction) to ET-1 was greater in aorta from both untreated groups of diabetic rats than in their non-diabetic counterparts. This relationship was present in aortic rings both with (Figure 34) and without (Figure 35) intact endothelium. Insulin and vanadate treatment restored ET-1 evoked vasoconstriction in diabetic rats to control levels, but had no significant effect on ET-1 responses in non-diabetic rats. Once again, this relationship held true in both endothelium denuded and intact aortic rings. Also, there were no differences in EC_{50} values amongst any of the groups with the exception of endothelium removal which shifted the cumulative concentration-response curve to ET-1 in all groups to the left with no change in E_{max} (Table 5). Similarly, methoxamine evoked vasoconstriction was greater in diabetic rats compared to non-diabetic controls and both insulin and vanadate treatment appeared to correct this abnormality (Figures 36 & 37). Once again, these relationships held true in aortic rings both in the presence (Figure 36) and absence (Figure 37) of intact endothelium. Moreover, no

differences in EC_{50} values were observed amongst any of the groups with the exception of endothelium denuded groups exhibiting a shift to the left in the cumulative concentration-response curves to methoxamine with no significant differences in E_{max} values (Table 5). ACh evoked relaxations in methoxamine precontracted rings were unchanged in all groups (Figure 38).

4.3.3. Analysis of plasma endothelin-1 levels

Plasma ET-1 concentrations as shown in Figure 40 were lower in untreated diabetic groups than in untreated non-diabetic groups (in pmol/L; untreated insulin group: SD, 1.04 ± 0.20; STZ, 0.46 ± 0.08, $p < 0.05$; untreated vanadate group: SD, 1.05 ± 0.18; STZ 0.39 ± 0.06, $p < 0.05$). Plasma ET-1 concentrations were significantly higher in insulin and vanadate treated diabetic rats than in untreated animals (in pmol/L; insulin group: untreated, 0.46 ± 0.08; treated, 1.09 ± 0.19, $p < 0.05$; vanadate group: untreated, 0.39 ± 0.06; treated, 0.88 ± 0.12, $p < 0.05$). Non-diabetic rats treated with either vanadate or insulin also exhibited increased plasma ET-1 concentrations compared with untreated groups (insulin group: untreated, 1.04 ± 0.20; treated, 1.94 ± 0.12, $p < 0.05$; vanadate group: untreated, 1.05 ± 0.18; treated, 1.85 ± 0.22, $p < 0.05$).

TABLE 3

Metabolic parameters in diabetic (STZ) and non-diabetic (SD) rats treated with insulin (12 mU/Kg/min; s.c.) for 2 weeks.

Group	Body weight before (g)	Body weight after (g)	Food intake ($g \cdot rat^{-1} \cdot day^{-1}$)	Blood glucose (mmol/L)	Plasma insulin (pmol/L)
SD-control	380 ± 10	447 ± 5	31 ± 1.2	7.0 ± 0.8	678 ± 130
SD-insulin	390 ± 15	469 ± 11	29 ± 1.6	5.4 ± 1.2	1835 ± 266^{c}
STZ-control	271 ± 11^{a}	256 ± 16^{a}	49 ± 1.0^{a}	31.4 ± 1.4^{a}	104 ± 16^{a}
STZ-insulin	285 ± 14^{a}	361 ± 15^{b}	33 ± 1.5^{c}	8.4 ± 0.9^{c}	1560 ± 127^{c}

Data are mean ± SEM from 8 separate rats

[a] $p < 0.001$ vs control-SD group

[b] $p < 0.01$ vs respective untreated group

[c] $p < 0.001$ vs respective untreated group

TABLE 4

Metabolic parameters in diabetic and non-diabetic rats treated with vanadate (0.5 mg/ml; p.o.) for 2 weeks.

Group	Body weight before (g)	Body weight after (g)	Food intake ($g \cdot rat^{-1} \cdot day^{-1}$)	Blood glucose (mmol/L)	Plasma insulin (pmol/L)
SD-control	401 ± 15	490 ± 12	33 ± 1.0	7.6 ± 0.6	799 ± 109
SD-vanadate	390 ± 15	435 ± 11	25 ± 1.0[c]	7.2 ± 0.4	1036 ± 73
STZ-control	254 ± 15[a]	311 ± 21[a]	47 ± 1.0[a]	28.3 ± 1.0[a]	109 ± 18[a]
STZ-vanadate	268 ± 11[a]	274 ± 14[b]	26 ± 0.6[c]	9.0 ± 1.2[c]	145± 18[a]

Data are mean ± SEM from 8 separate rats

[a] $p < 0.001$ vs. control-SD group

[b] $p < 0.01$ vs. respective untreated group

[c] $p < 0.001$ vs. respective untreated group

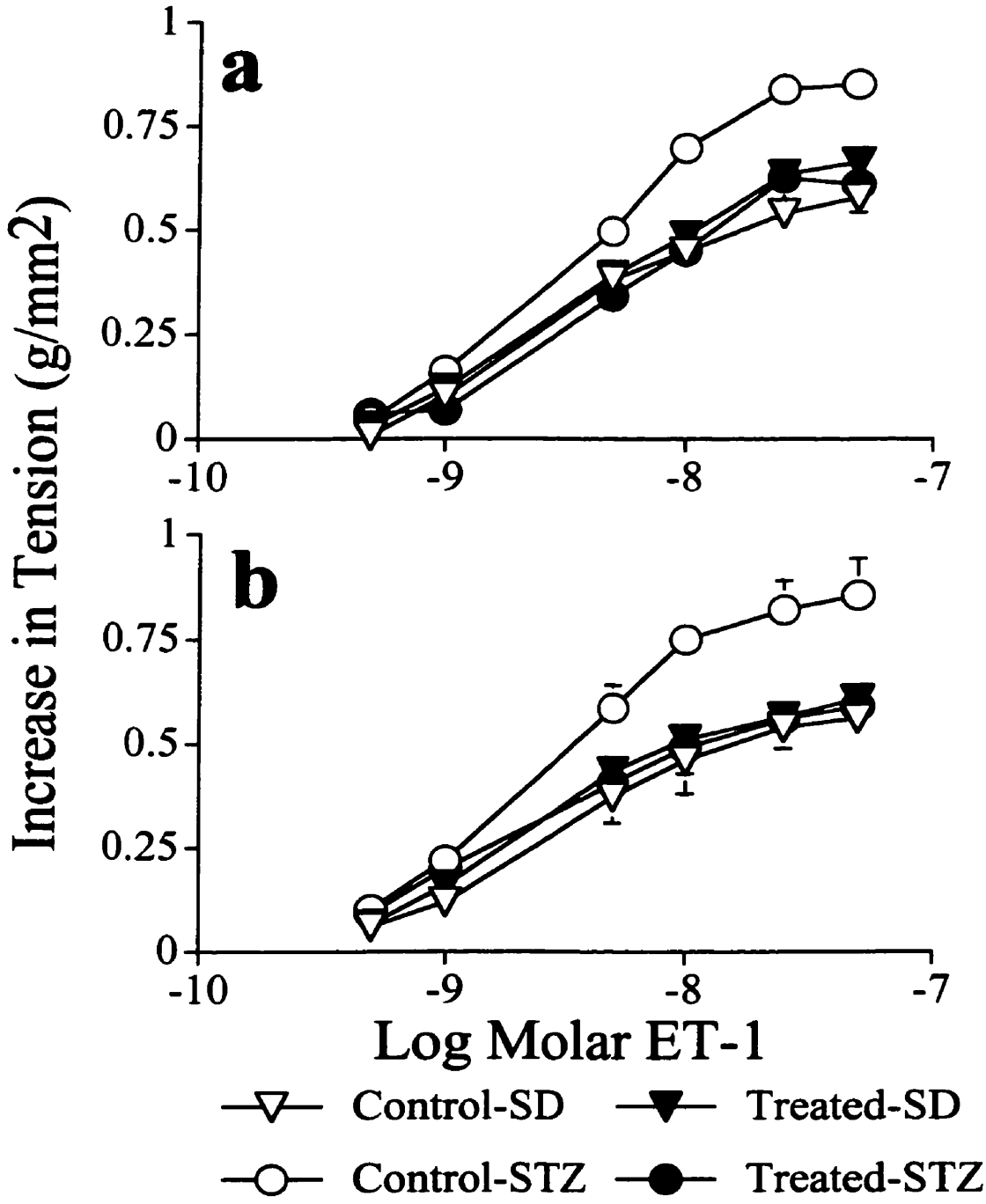


Figure 34. Cumulative-concentration response curves to ET-1 (100 pmol/L - 50 nmol/L) in aortic rings with intact endothelium from 5-week STZ diabetic rats and non-diabetic controls. Each group was either untreated or treated with insulin (**a**; s.c. 12 mU/Kg/min) or vanadate (**b**; p.o. 0.5 mg/ml) for 2-weeks. Each data point represents the mean ± SEM of 8 determinations using tissues from 8 different rats.

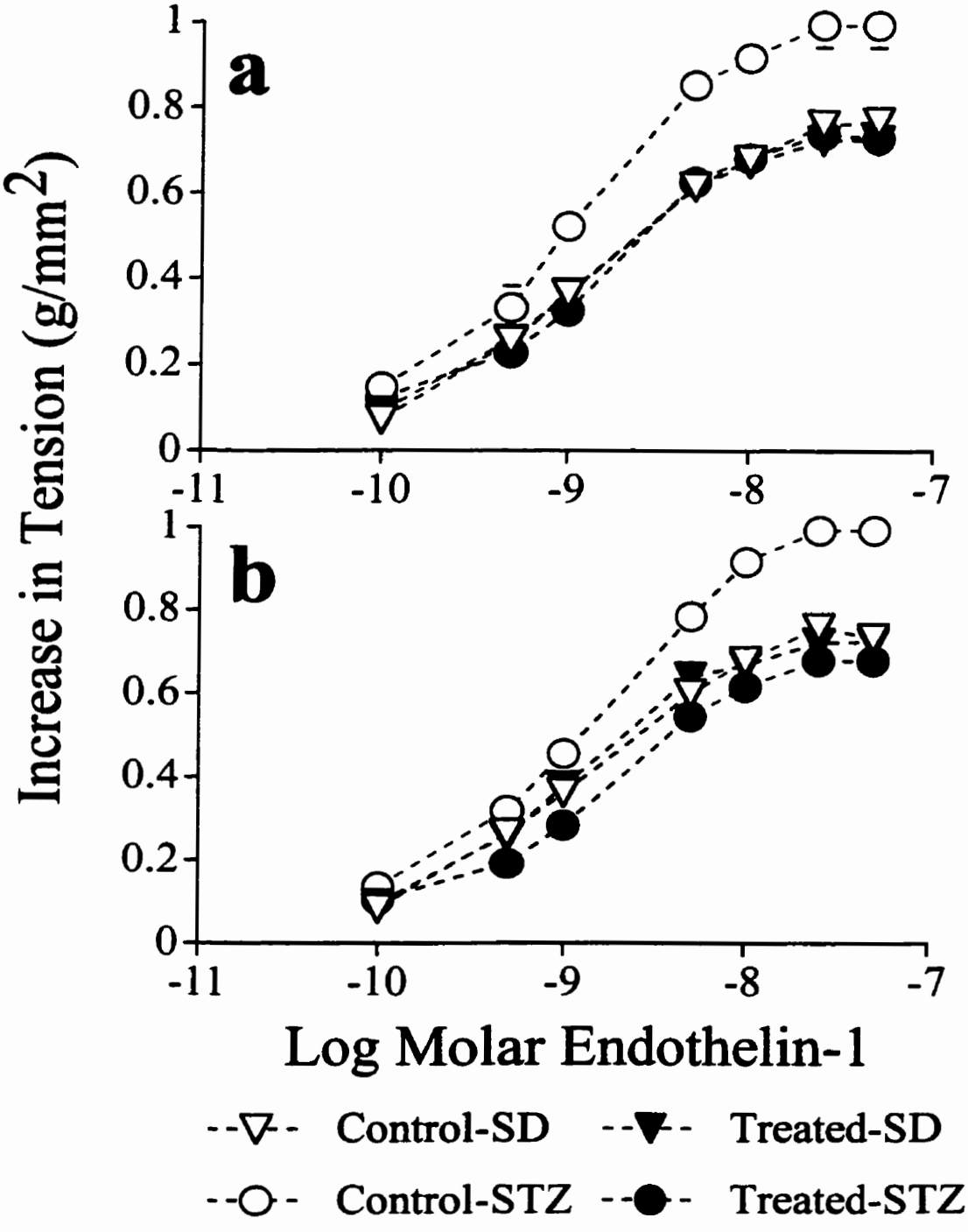


Figure 35. Cumulative-concentration response curves to ET-1 (100 pmol/L - 50 nmol/L) in aortic rings with denuded endothelium from diabetic (STZ) and non-diabetic (SD) control rats. Each group was either untreated or treated with insulin (**a**; s.c. 12 mU/Kg/min) or vanadate (**b**; p.o. 0.5 mg/ml) for 2-weeks. Each data point represents the mean ± SEM of 8 determinations using tissues from 8 different rats.

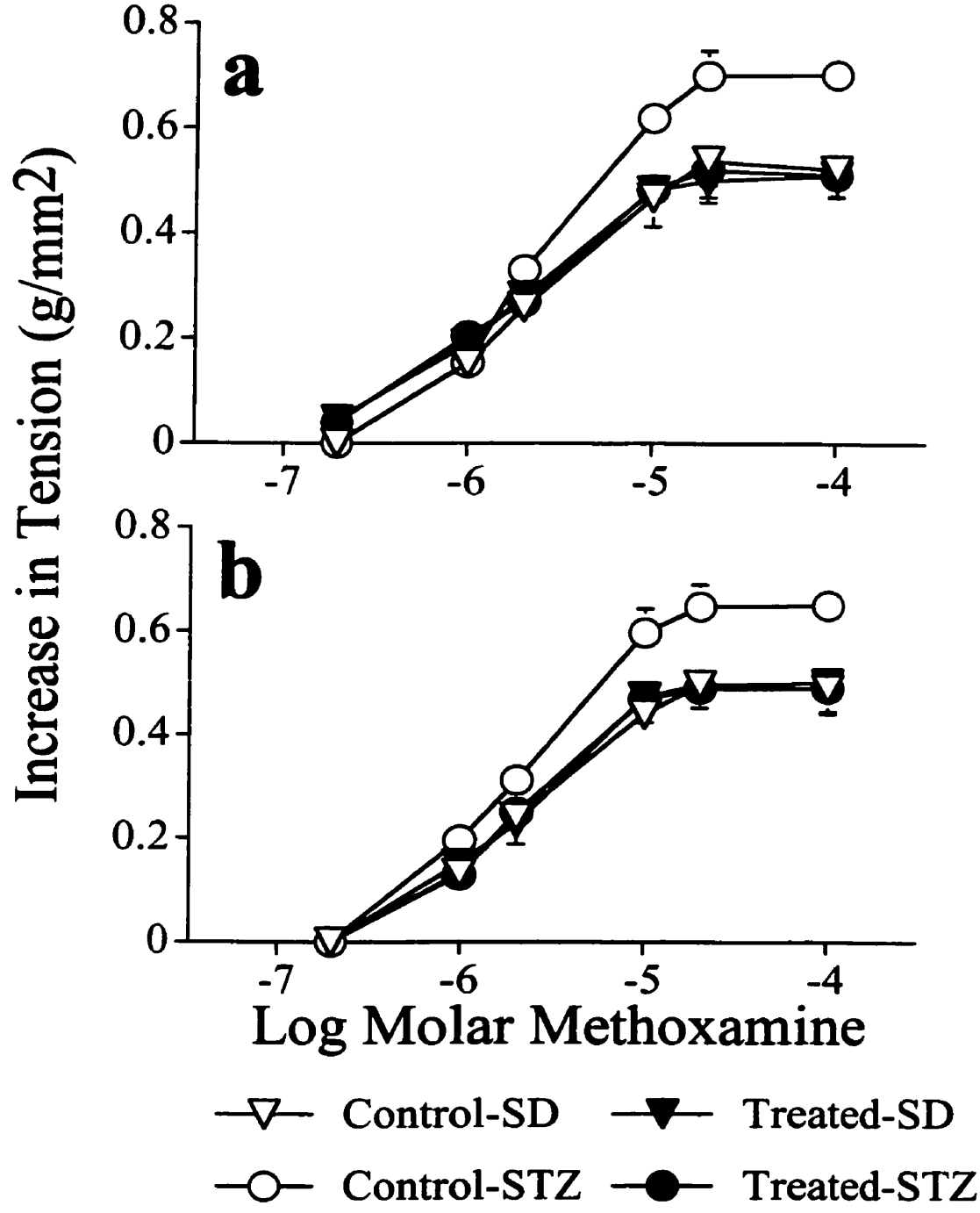


Figure 36. Cumulative-concentration response curves to methoxamine (100 nmol/L - 100 µmol/L) in aortic rings with intact endothelium from diabetic (STZ) and non-diabetic (SD) control rats. Each group was either untreated or treated with insulin (**a**; s.c. 12 mU/Kg/min) or vanadate (**b**; p.o. 0.5 mg/ml) for 2-weeks. Each data point represents the mean ± SEM of 8 determinations using tissues from 8 different rats.

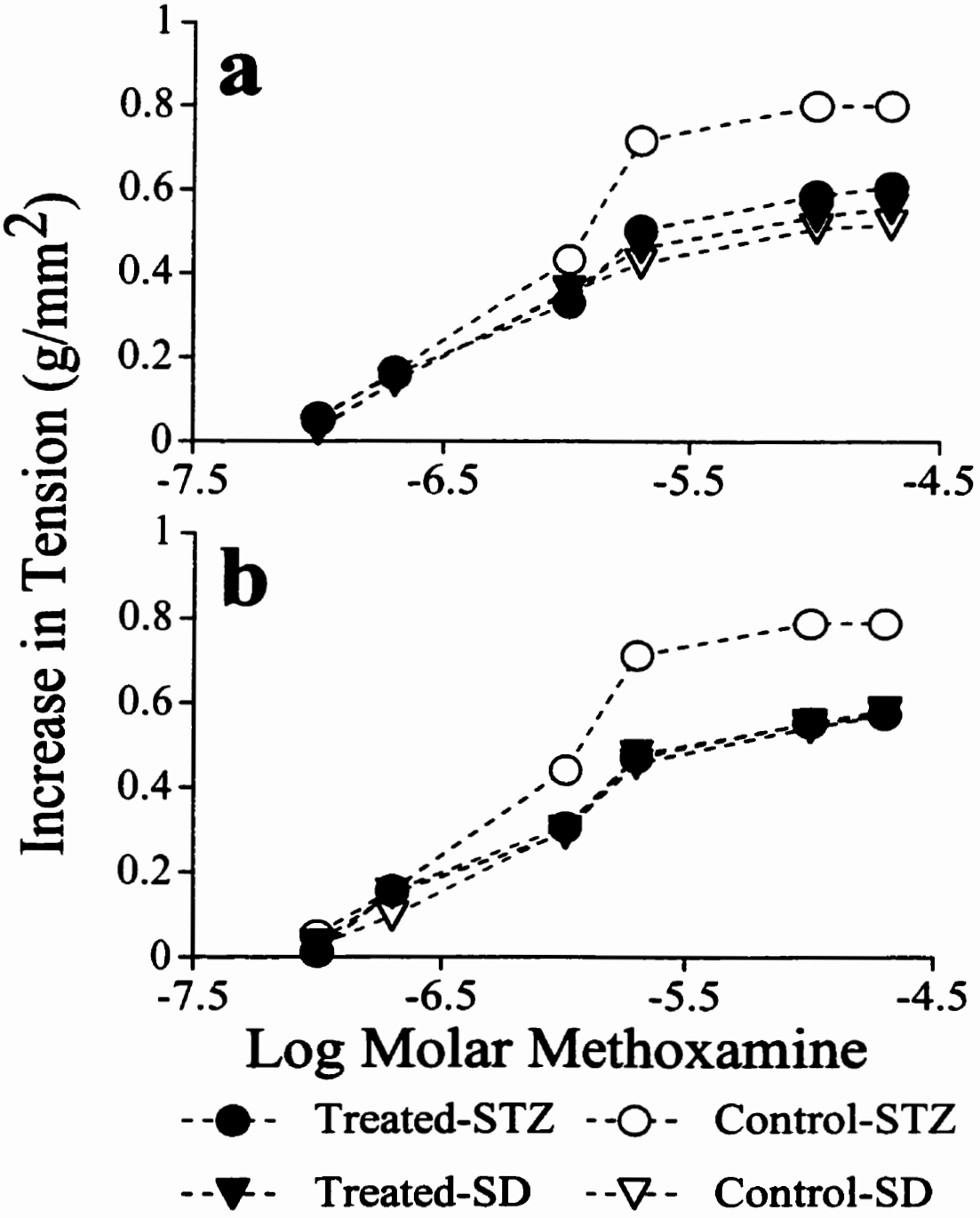


Figure 37. Cumulative-concentration response curves to methoxamine (100 nmol/L - 100 μmol/L) in aortic rings with denuded endothelium from diabetic (STZ) and non-diabetic (SD) control rats. Each group was either untreated or treated with insulin (**a**; s.c. 12 mU/Kg/min) or vanadate (**b**; p.o. 0.5 mg/ml) for 2-weeks. Each point represents mean ± SEM of 8 determinations using tissues from 8 different rats.

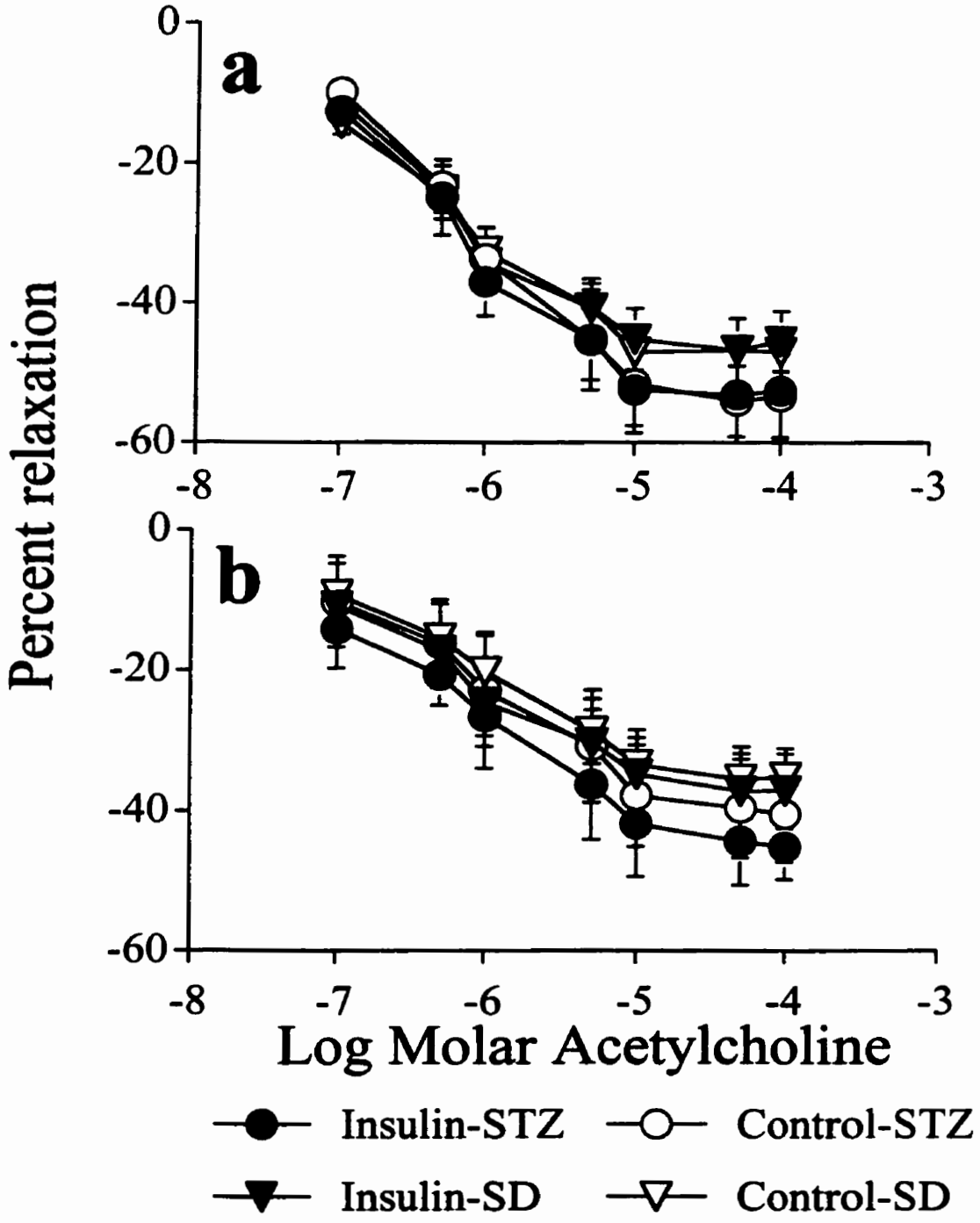


Figure 38. Cumulative-concentration response curves to acetylcholine (ACh) evoked relaxation (100 nmol/l - 100 µmol/l) in rat aortic ring preparations precontracted with methoxamine (100 µmol/l) from diabetic (STZ) and non-diabetic (SD) control rats. Upper panel - **(a)** insulin group; lower panel - **(b)** vanadate group. Each point is mean ± SEM of 8 determinations using tissues from 8 different rats.

TABLE 5

Sensitivity (EC_{50}) and maximal response (E_{max}) parameters in isolated aortic rings of treated and untreated STZ and SD rats.

Group	Endothelium intact				Endothelium denuded			
	Methoxamine		ET-1		Methoxamine		ET-1	
	EC_{50} (μmol/L)	E_{max} (g/mm^2)	EC_{50} (nmol/L)	E_{max} (g/mm^2)	EC_{50} (μmol/L)	E_{max} (g/mm^2)	EC_{50} (nmol/L)	E_{max} (g/mm^2)
Insulin (12 $mU \cdot Kg^{-1} \cdot min^{-1}$) s.c. x 2 weeks								
SD-control	2.00±0.06	0.54±0.05	2.90±0.03	0.58±0.05	0.72±0.04[c]	0.52±0.04	1.20±0.08[c]	0.77±0.04
SD-treated	1.70±0.02	0.51±0.04	3.50±0.05	0.66±0.07	0.83±0.05[c]	0.56±0.03	1.20±0.07[c]	0.73±0.03
STZ-control	2.30±0.04	0.70±0.04[a]	4.00±0.06	0.85±0.04[a]	0.83±0.04[c]	0.80±0.04[a]	1.00±0.05[c]	0.99±0.05[a]
STZ-treated	1.80±0.04	0.52±0.05[b]	5.50±0.07	0.63±0.07[b]	0.83±0.07[c]	0.60±0.03[b]	1.20±0.06[c]	0.73±0.04[b]
Vanadate (0.5 mg/ml) p.o. x 2 weeks								
SD-control	2.50±0.03	0.50±0.04	2.40±0.03	0.56 ± 0.05	0.90±0.06[c]	0.57±0.04	1.00±0.05[c]	0.73±0.04
SD-treated	2.30±0.02	0.50±0.04	2.50±0.04	0.61 ± 0.02	0.90±0.05[c]	0.58±0.04	1.00±0.04[c]	0.73±0.03
STZ-control	2.20±0.05	0.65±0.04[a]	2.60±0.03	0.86 ± 0.09[a]	0.75±0.08[c]	0.79±0.04[a]	1.15±0.08[c]	0.99±0.04[a]
STZ-treated	2.10±0.02	0.49±0.05[b]	2.10±0.04	0.59 ± 0.04[b]	0.90±0.07[c]	0.57±0.04[b]	1.15±0.07[c]	0.68±0.03[b]

Data are mean ± SEM performed using tissues obtained from 8 rats for each group.

[a] $p < 0.01$ vs untreated SD group, [b] $p < 0.01$ vs untreated STZ group, [c] $p < 0.01$ vs same point with endothelium intact.

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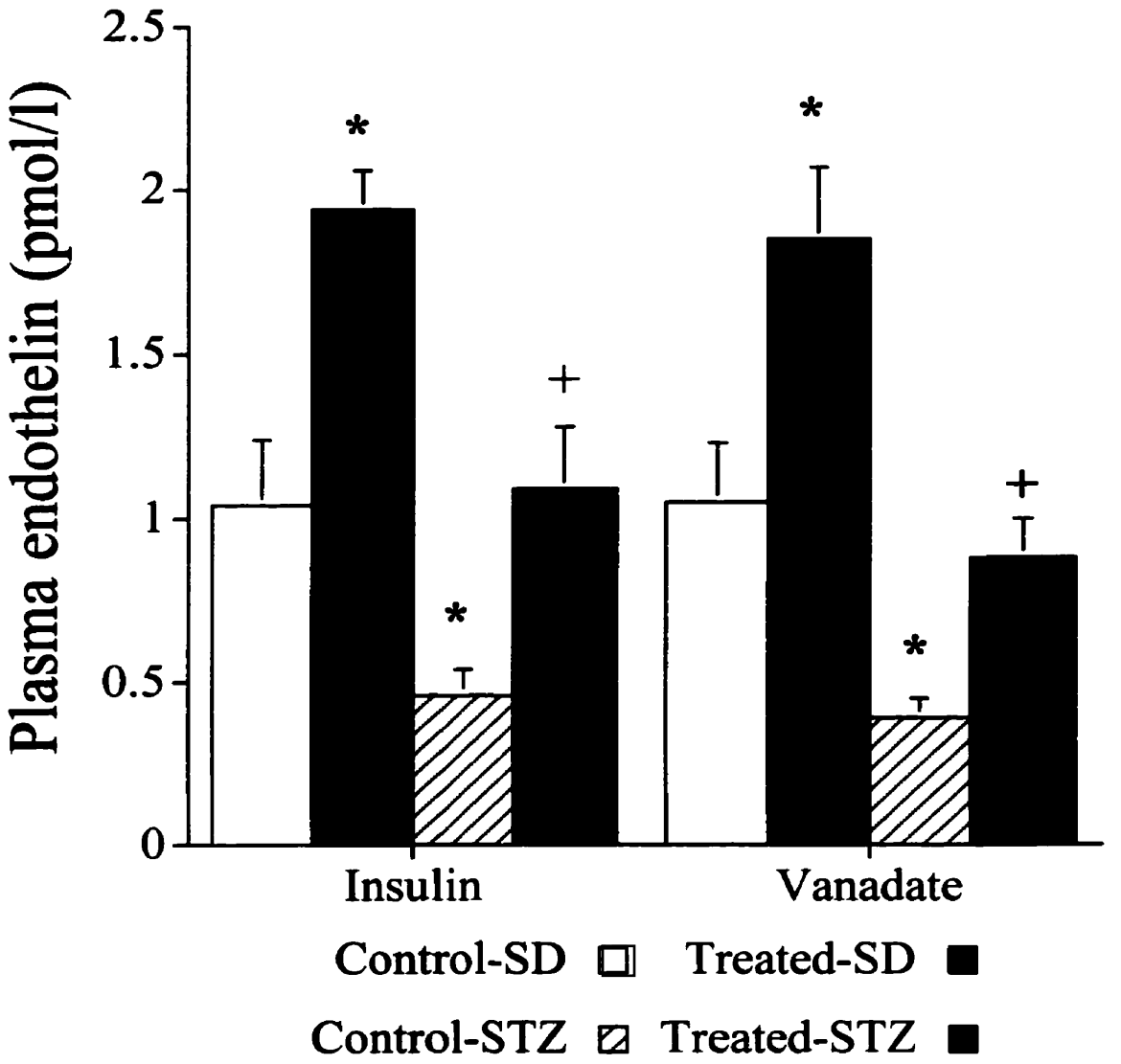

Figure 39. Plasma ET-1 levels in Sprague-Dawley (SD) control and streptozotocin (STZ) diabetic rats either treated with insulin or vanadate or untreated. Plasma ET-1 was measured with a RIA kit capable of distinguishing between ET and big-ET, but not between the different isoforms of ET. Results represent mean ± SEM of 8 separate determinations using plasma samples from 8 different rats.

*$p < 0.05$ vs. respective non-diabetic control; and +$p < 0.05$ vs. respective diabetic control.

5. DISCUSSION

5.1. Insulin effects on ET receptor expression and action

5.1.1. Summary

Long-term (24 hr) pretreatment of rat ASMC with a high concentration of insulin enhances ET-1 evoked peak $[Ca^{2+}]_i$ responses. This is primarily due to enhanced ET_A receptor signaling, as the ET_A selective antagonist BQ123 significantly attenuated the enhanced $[Ca^{2+}]_i$ response to ET-1 to similar levels in both treated and untreated ASMC, whereas BQ788 (ET_B selective anatagonist) attenuated the response to a lesser extent in both groups. The ET_B selective agonist, IRL-1620, evoked a concentration dependent increase in peak $[Ca^{2+}]_i$ levels in both groups of ASMC, albeit to a much lower extent than to ET-1, and failed to elicit greater responses in insulin pretreated ASMC.

5.1.2. Mechanistic considerations

While other subcellular mechanisms cannot be ruled out, it is probable that insulin evoked enhancement of ET-1 evoked $[Ca^{2+}]_i$ signaling is due to ET_A (but not ET_B) receptor upregulation since ET_B-evoked $[Ca^{2+}]_i$ responses remained unaltered. This is consistent with the observation that insulin pretreatment significantly and maximally upregulated only ET_A binding sites in a concentration and time dependent manner. Also, insulin significantly increased ET-1 evoked peak $[Ca^{2+}]_i$ responses at a concentration as low as 10 nM. This effect is selective to ET-1 since

$[Ca^{2+}]_i$ responses to both AVP and Ang II remained unaffected. These data suggest that insulin selectively enhances ET_A gene expression leaving VSMC AT_1 and V_1 receptor expression unaltered. In fact, it has been previously shown that insulin pretreatment of rat mesenteric VSMC has no effect on [^{3}H] AVP binding (Standley et al., 1991). Moreover, the observation that IGF-1 also selectively increased ET-1 evoked $[Ca^{2+}]_i$ responses indicates that this effect of insulin might be mediated through the IGF-1 receptor, which is known to be activated by high insulin concentrations (King et al., 1980).

The possibility that insulin could enhance ET_A expression in ASMC was suggested earlier (Frank et al., 1993). It was demonstrated that incubation of either bovine ASMC, rat brain capillary pericytes or kidney afferent arterioles led to a two-fold increase in ET-1 binding, increased [^{3}H] thymidine incorporation (evidence in support of DNA synthesis and mitogenesis) and increased ET_A mRNA levels. However, this study failed to characterize cell surface ET_A and ET_B binding sites in ASMC, the mechanism underlying changes in ET_A expression, or the effect of insulin on $[Ca^{2+}]_i$ responses to ET agonists. Indeed, most of these smooth muscle cells are known to possess both ET_A and ET_B receptors. This thesis has addressed these issues and also demonstrates that exaggerated $[Ca^{2+}]_i$ responses evoked by long term insulin incubation are selective to ET-1 and not other vasoactive peptide agonists. The *in vitro* data in this thesis with ASMC preincubated with high insulin

were supported by direct saturation binding studies in hyperinsulinemic obese-Zucker rats from our laboratory. These studies demonstrated that aortic microsomal preparations from hyperinsulinemic Zucker rats contain a greater number of ET_A specific binding sites than normoinsulinemic control lean rats (Hopfner et al., 1998a). Soon after, another group demonstrated that vascular ET_A receptor levels were also increased in hyperinsulinemic/insulin resistant fructose-hypertensive rats (Juan et al., 1998). These data support possible physiological relevance to our observations.

Enhanced cell surface expression of ET_A sites evoked by insulin is preceded by enhanced ET_A mRNA expression, which becomes apparent after 12 hr of insulin incubation. The increase in ET_A mRNA was abolished by inclusion in the incubation medium of the transcription inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide, indicating the requirement for *de novo* protein synthesis in insulin mediated enhancement of ET_A expression. The molecular mechanism by which insulin selectively promotes ET_A expression is presently unknown, but the present observations suggest that it may be due to induction of a factor that can bind to one of several regulatory sequences on the ET_A gene promoter. The signal transduction pathways recruited by insulin in the upregulation of ET_A expression were not investigated; however, the blockade of ET_A upregulation by genistein, a tyrosine kinase inhibitor, at both the receptor binding and mRNA expression levels indicates the requirement of tyrosine kinase activation.

Insulin is known to activate several cytosolic proteins in eliciting its cellular response. Phosphatidylinositol-3-kinase (PI-3K) has previously been shown to be important in insulin mediated enhancement of α_1-adrenoceptor expression in rat ASMC (Hu et al., 1996). Further work using intracellular enzyme inhibitors is needed to clarify this issue.

5.1.3. Reconciliation with previous discrepant results

Several studies provide evidence of a link between insulin and altered vascular reactivity. Insulin has been shown to attenuate agonist(s)-evoked $[Ca^{2+}]_i$ and/or contractile responses to agonists including ET-1 in different VSM preparations (Kim & Zemel, 1993; Dick & Sturek, 1996; Standley et al., 1991; Saito et al., 1993; Tirapattur et al., 1993; Touyz et al., 1994). This attenuation could be due to insulin evoked enhancement of Na^+/K^+ ATP'ase activity (Tirapattur et al., 1993), decreased influx of extracellular calcium in response to calcium mobilizing agonist stimulation via receptor or voltage gated calcium channels (Standley et al., 1991; Saito et al., 1993), inhibition of $[Ca^{2+}]_i$ release (Standley et al., 1991), or to stimulation of sarcoplasmic Ca^{2+} ATP'ases (Kim & Zemel, 1993). It is hypothesized that modulation of agonist evoked $[Ca^{2+}]_i$ is a major mechanism by which insulin promotes vasodilatation, and in cases of insulin resistance, this effect may be lost promoting enhanced vasoconstriction (Sowers et al., 1993). The present study shows that longer term exposure to insulin actually increases ET-1 evoked $[Ca^{2+}]_i$ responses in isolated VSMC which may translate into enhanced contractile responses to ET-1 in hyperinsulinemic states. The obvious discrepancy between

these results and the aforementioned studies could be due to several factors. *Firstly,* previous studies showing that insulin attenuates agonist evoked $[Ca^{2+}]_i$ responses were performed using insulin incubation of VSMC *in vitro* for a period not exceeding 120 min. The present study uses a 24 hr preincubation period after which agonist evoked $[Ca^{2+}]_i$ responses were monitored. Northern blot analysis and receptor binding studies confirmed that this effect may be secondary to ET_A receptor upregulation. It is possible therefore that any previous studies showing attenuated ET-1 evoked $[Ca^{2+}]_i$ responses with acute insulin incubation did not allow adequate time for enhanced ET_A gene expression to occur, hence the short term cellular response predominated. Indeed, the argument can be made that physiological hyperinsulinemia is more adequately mimicked with long term insulin preincubation. *Secondly,* unlike other acute studies, the present study did not include insulin in the medium when receptor binding, $[Ca^{2+}]_i$ or tension measurement studies were performed. However, it is unlikely that this would have a significant effect as upregulated ET_A receptors would be present regardless of whether insulin is included in the cell suspension or not. *Thirdly,* desensitization to the short-term cellular effects of insulin on agonist evoked $[Ca^{2+}]_i$ changes may occur after a certain time period. *Finally, in vivo* data from our laboratory revealing that an increased number of ET_A specific binding sites and exaggerated vasoconstrictor responses to ET-1 exist in aorta from hyperinsulinemic obese Zucker rats support our *in vitro* observations that long-term exposure to higher than normal concentrations of insulin may *in fact* be associated with enhanced ET-1-evoked responses in *in vivo* hyperinsulinemic states (Hopfner et al., 1998a).

5.1.4. Physiological Relevance

Since the concentrations of insulin (10 - 100 nM) used in this study were ten to one-hundred fold higher than the physiological range, it could be argued that the present results may not reflect the situation *in vivo* in hyperinsulinemic states. However, it must be noted that it cannot be assumed that the physiologically effective concentration of any substance in cell culture medium will be the same concentration needed to evoke the same response *in vivo*. Indeed, our recent observation of increased ET_A specific binding sites and increased vasoconstrictor responses to ET-1 in obese Zucker rats suggest that this phenomenon may occur *in vivo* as well in hyperinsulinemic states (Hopfner et al., 1998a). It must be noted that the this obese rat model also exhibits altered plasma lipid profiles, hyperglycemia, insulin resistance, obesity and hypertension, factors all of which could contribute to the observed altered reactivity to ET-1 (Kasiske et al., 1992). However, the *in vitro* results from this thesis as well another recent *in vivo* study demonstrating enhanced ET_A receptors in hyperinsulinemic fructose-hypertensive rats (Juan et al., 1998) support the view that hyperinsulinemia could indeed contribute to increased ET_A specific binding and exaggerated vascular responses to ET-1 in *vivo*.

5.1.5. Conclusion

Incubation of ASMC for a minimum of 24 hr enhances ET-1 evoked peak $[Ca^{2+}]_i$ responses. This effect of insulin is mediated through a concentration and time dependent enhancement of ET_A expression secondary to tyrosine kinase phosphorylation. High plasma ET-1 levels have been characterized in diverse

cardiovascular disorders that are often accompanied by hyperinsulinemia (Saito et al., 1990; Lerman et al., 1991; Naruse et al., 1991). Parallel increases in plasma ET-1 levels in concert with upregulated ET_A receptor expression and Ca^{2+} signaling on the VSMC, as well as the comitogenic effects of insulin and ET-1 further compound the potential for vasculopathy evoked by insulin induced enhancement of ET-1 action at the VSM level.

5.2. Vanadate effects on ET_A and ET_B receptor expression and action

5.2.1. Summary

In contrast to the selective effects of insulin on ET_A expression, vanadate (25 μM) upregulates both ET_A and ET_B binding sites on ASMC. The effect of vanadate on ET_B sites was more striking, as the ratio of ET_A to ET_B sites dropped from 7.3 in control ASMC to 1.8 in vanadate pretreated cells. In support of this observation, vanadate also increased both ET_A and ET_B mRNA levels, an effect that was dependent on both new protein synthesis and active transcription. This is the first report that provides evidence at the receptor and mRNA levels of a significant shift in ET receptor subtype expression in any cell population by vanadate pretreatment. In line with this data, the concentration- peak $[Ca^{2+}]_i$ responses to ET-1 (non-selective ET_A/ ET_B agonist) and IRL 1620 (selective ET_B agonist) were significantly higher in vanadate pretreated ASMC, and the weak $[Ca^{2+}]_i$ responses to IRL 1620 observed in the control group of cells became prominent in vanadate pretreated cells. Moreover, control ET-1-evoked $[Ca^{2+}]_i$ responses could not be completely abolished by a high concentration of BQ123 (ET_A selective antagonist),

whereas BQ 788 (ET_B selective antagonist) attenuated the response to a lesser extent. These data suggest the existence of a small ET_B mediated response in addition to the predominant ET_A response. However, in vanadate pretreated cells, the exaggerated $[Ca^{2+}]_i$ response attained with the same concentration of ET-1 was highly attenuated in the presence of either BQ123 or BQ788. These data confirm that vanadate induced ET_A and ET_B receptor upregulation is functionally coupled to $[Ca^{2+}]_i$ mobilization in a manner predictive of the changes in cell surface expression of each receptor subtype. This is clearly in contrast to the observed effects of insulin, which appear to be selective to the ET_A receptor.

5.2.2. ET_B receptor upregulation

Earlier, based on receptor binding and functional studies it was proposed that ASMC possessed a single class of ET binding sites (Martin et al., 1991). Subsequently, using [^{125}I] labeled ET-1, ET-2 and ET-3, it was shown that rat ASMC possess two classes of binding sites, an ET-1 and ET-2 preferring high affinity irreversible site (85%) and another site (15-20%) that binds all three isoforms with equal affinity in a reversible manner (Roubert et al., 1991). Based on our laboratories observation that sarafotoxin 6c, an ET_B selective agonist, evoked much larger increases in $[Ca^{2+}]_i$ in cultured ASMC derived from spontaneously hypertensive rats, it was suggested that there may be ET_B sites on ASMC (Batra et al., 1993). Consistent with this notion, the presence of cell surface ET_A and ET_B specific binding sites (using competitive inhibition of [^{125}I] ET-1 radioligand) and ET_A and ET_B mRNA transcripts in tissue homogenates from human aorta and rat ASMC were established by others (Eguchi et

al., 1994; Davenport et al., 1995; Winkles et al., 1993). In the present study, saturation binding data for the non-selective radioligand, [^{125}I] ET-1, showed the best fit for a one site model since the K_D values for ligand interaction at both ET_A and ET_B were not significantly different. In contrast, [^{125}I] IRL-1620 labeled only ET_B sites; from this, we could estimate the relative distribution of ET_A (88%) and ET_B (12%) sites by subtracting the value of total sites labeled by [^{125}I] ET-1. Although vanadate pretreatment led to significant increases in the number of ET_A and ET_B sites, its effect on the upregulation of ET_B sites was much more pronounced. Again, this is contrast to insulin, which upregulated only ET_A sites significantly. Interestingly, glycerol administration (at a dose of 10 ml/Kg that led to renal damage) increased the density of both ET_A and ET_B binding sites in the renal medulla of normal rats; in fact, renal ET_B up-regulation induced by glycerol was much more pronounced than ET_A (Roubert et al., 1994). A recent report has characterized the existence of a novel ET_B receptor that shows similar high affinity characteristics for ET_B selective ligands in membrane fractions of canine, monkey and human spleen (Nambi et al., 1997). Data from the present study suggest that vascular ET_B receptors present on rat ASMC also express such high affinity characteristics.

5.2.3. Comparison with effects of insulin

As mentioned earlier, in contrast to the well-established vasoconstrictor effect of vanadate (Bianchin et al., 1993; DiSalvo et al., 1993; Laniyonu et al., 1994), insulin is a vasodilator under normal physiological conditions. However, several

laboratories have now confirmed using both *in vitro* and *in vivo* models that insulin promotes the release of ET-1, a powerful vasoconstrictor/mitogenic peptide (Oliver et al., 1990; Ferri et al., 1995; Wolpert et al., 1993). In addition, ET-1 levels have been shown to be higher in NIDDM, atherosclerosis and hypertension which are conditions often accompanied by hyperinsulinemia (Poch et al., 1991; Takahashi et al., 1990; Haak et al., 1992). The observation that insulin selectively upregulates ET_A binding sites and action in rat ASMC *in vitro* is in contrast to the effects of vanadate on both ET_A and ET_B receptors with a more pronounced effect on ET_B. Vanadate's effects were also blocked by a very low concentration of genistein, a tyrosine kinase inhibitor, suggesting that protein tyrosine phosphorylation mediated events could contribute to its observed effects. It is accepted that vanadium compounds inhibit phosphotyrosine phosphatase, enhance tyrosine phosphorylation of the insulin receptor, and also act at sites distal to the insulin receptor (Brichard & Henquin, 1995). Vanadate, via blockade of phosphotyrosine phosphatase, could promote the activation of insulin-independent (cytosolic) tyrosine kinases, thus promoting events unrelated to insulin signaling which could account for the activation of the ET_B receptor gene at the level of transcription. While it is doubtful that insulin signaling involves phosphorylation and stimulation of PLCγ, vanadate activates this enzyme (Figure 9) and consequently enhances IP_3 production, elevates $[Ca^{2+}]_i$ levels and activates PKC (Bianchin et al., 1993; Jonas & Henquin, 1996; Wenzel et al., 1995). Thus, vanadate evoked increases in ET_B gene expression may involve insulin-independent but Ca^{2+} and/or PKC dependent mechanism(s). An interesting observation was that vanadate, in addition to enhancing ET-1 evoked

$[Ca^{2+}]_i$ responses, dramatically attenuated Ang II evoked $[Ca^{2+}]_i$ responses. This effect was not observed with insulin or IGF-1. Further studies are required to determine the mechanism of this agonist-specific effect.

5.2.4. Physiological Relevance

The optimal concentration of vanadate (25 μM) used in this study closely corresponds to the blood level achieved in rodent studies assessing the antidiabetic efficacy of vanadate, which varied between 10 and 20 μM (Brichard & Henquin, 1995). Increased ET_B mRNA transcripts are present in late passage ASMC (Eguchi et al., 1994). This increase is accompanied by increased incorporation of cell surface ET_B receptors as well as exaggerated IP_3 formation and ASMC mitogenesis to an ET_B selective agonist. It was suggested that such phenotypic alterations contribute to *in vivo* changes of increased ET-1 evoked VSMC proliferation and vascular remodeling encountered in conditions such as atherosclerosis and hypertension. Thus, alterations in ET_B receptor expression and action may be present in different pathological states. As previously discussed, circulating as well as tissue ET-1 levels were found to be elevated in atherosclerosis and elevated preproET mRNA levels have been encountered in atherosclerotic human aorta (Poch et al., 1991; Winkles et al., 1993). Accordingly, ET-1 is recognized as one of the contributing factors to the development of atherosclerosis (Hasdai & Lerman, 1995). In the present study, the vanadate-evoked shift in ET receptor expression and increased Ca^{2+} signaling to ET_B agonists seen in ASMC in early passages may

mirror that seen in untreated ASMC maintained in late passages. Thus, it is possible that vanadate treatment could alter vascular responses to ET-1.

5.2.5. Conclusion

The present study demonstrates that pretreatment of rat ASMC with vanadate, an insulinomimetic agent, leads to increased ET_A and ET_B mRNA levels, increased cell surface ET_A and ET_B binding sites, and exaggerated peak $[Ca^{2+}]_i$ responses to both ET-1 and the ET_B selective agonist, IRL 1620. Strikingly, while only a 1.7 fold increase in ET_A sites was detected, ET_B sites were upregulated by an order of 7.8 fold. This is in contrast to insulin, which only upregulated ET_A sites significantly. In addition, ET_B mediated $[Ca^{2+}]_i$ signaling becomes markedly more pronounced after vanadate pretreatment, since IRL 1620-evoked very low Ca^{2+} signaling in the control group of ASMC but showed a concentration-dependent increase in $[Ca^{2+}]_i$ response in the vanadate pretreatment group. These changes in ASMC ET receptor expression could mimic those seen in altered vascular states. Although the medicinal value of vanadium and its benefit in nutrition, DM and metabolism of lipids has been advocated (Brichard & Henquin, 1995), the observation that lower concentrations of vanadate enhance ET receptor expression in ASMC warrants a closer evaluation of its vascular effects.

5.3. In vivo effects in diabetic and non-diabetic rats

5.3.1. Summary

The physiological relevance of the aforementioned *in vitro* observations can be deduced from results in STZ diabetic and SD non-diabetic rats treated with insulin and vanadate. Strikingly, it is apparent that the STZ diabetic rat itself exhibits decreased plasma ET-1 levels and exaggerated ET-1 evoked vasoconstrictor responses. Administration of vanadate orally and continuous subcutaneous delivery of insulin for 2 weeks led to:

a) a decrease in fasting plasma glucose to non-diabetic levels in STZ rats,

b) restoration of decreased plasma ET-1 in STZ diabetic rats to control levels,

c) elevation of plasma ET-1 (~2-fold) in normal SD rats, and

d) normalization of increased E_{max} to vasoconstrictor agonists in STZ rats.

Thus, the present study demonstrates, for the first time, that short term insulin and vanadate treatment elevates plasma ET-1 levels, and prevents or reverses the exaggerated vasoconstrictor responses to ET-1 encountered in STZ diabetic rats. Thus, while our *in vitro* observations might have indicated that vasoconstrictor responses to ET-1 would be *attenuated* in hypoinsulinemic STZ rats and *exaggerated* in insulin and vanadate treated animals (due to the changes in levels of insulin), in fact, precisely the opposite was the case, in that *exaggerated* responses to ET-1 were present in STZ rats which were *decreased* to normal levels by insulin and vanadate treatment.

5.3.2. Altered endothelin-1 plasma levels in diabetes

The role of ET-1 in the diabetic state is controversial. As previously discussed, hyperlipidemia, hyperglycemia, and hyperinsulinemia are all thought to alter plasma levels of ET-1 in the diabetic state, but the direction of these changes is uncertain. In contrast to our observation of decreased plasma ET-1 levels in STZ rats, some studies have shown increased plasma ET-1 in STZ rats and IDDM patients (Haak et al., 1992; Tada et al., 1994; Makino & Kamata, 1998; Takahashi et al., 1990; Takeda et al., 1991). On the other hand, in support of our observations, significantly lower plasma ET-1 levels in IDDM patients and undetectable ET-1 immunoreactivity in plasma of STZ rats has also been reported (Smulders et al., 1994; Malmitsi-Puchner et al., 1996; Takahashi et al., 1991). Similarly, *in vitro* studies with cultured bovine or porcine aortic EC have shown both elevated and decreased levels of ET-1 production by the addition of high glucose to the culture medium (Hattori et al., 1991; Yamauchi et al., 1990). As previously discussed, the reasons for these discrepancies *in vivo* may be due to the degree and duration of hyperglycemia, presence of diabetic complications, and possibly to the state of endothelial function in the model studied. An interesting possibility is that differences in EDRF production might contribute to these variations since EDRF function declines in proportion to the duration of hyperglycemia in STZ DM, and EDRF is known to inhibit ET-1 release (Boulanger and Luscher, 1990; Chang & Stevens, 1992). Acetylcholine-evoked relaxation of aortic rings was intact in the present study, suggesting that EDRF production remained unaffected by STZ DM in this VSM preparation. Thus, it may be that unaltered EDRF production along with diminished

plasma insulin levels in the untreated STZ group resulted in decreased ET-1 production - since insulin stimulates ET-1 release from vascular EC (Oliver et al., 1990). Longer-term STZ rats exhibit augmented ET-1 levels secondary to endothelial dysfunction and attenuated EDRF production (Hopfner et al., 1999a).

5.3.3. Insulin and vanadate increase ET-1 plasma levels

The observation that insulin treatment restores plasma ET-1 to normal in STZ rats, and increases it 2-fold in normal control rats might be explained by either its direct actions on EC or by indirect effects on other metabolic variables. These include:

a) normalization of hyperglycemia and hyperlipidemia resulting in normalization of ET production from EC,
b) normalization of hyperglycemia leading to decreased glucose mediated EDRF production (Wascher et al., 1994) and consequently, decreased EDRF mediated inhibition of ET-1 release (Boulanger and Luscher, 1990) and/or
c) the direct effect of insulin on endothelial ET-1 production (Oliver et al., 1990).

The observation that vanadate increases plasma ET-1 in a manner similar to insulin in both STZ and normal rats is novel; however, as noted earlier, vanadate is well known to mimic most of the cellular and metabolic actions of insulin via inhibition of protein tyrosine phosphatases, leading to enhanced insulin sensitive and non-sensitive tyrosine kinase activation. Thus, vanadate may enhance plasma ET-1 in a manner similar to insulin.

5.3.4. Vanadate effects on food-intake

In the present study, vanadate treatment reduced food intake in both normal and diabetic rats. Earlier it has been shown that the hypophagic effect of vanadate is only transient (Becker et al., 1994). No studies to date have assessed the effect of hypophagia on plasma ET-1 in normal or diabetic states. Three points deserve mention here:

a) vanadium compounds both *in vivo* and *in vitro* mimic most, if not all of the metabolic actions of insulin,

b) both insulin and vanadate evoke similar effects on ET-1 action and,

c) it is now well established that the effects of vanadate on metabolic parameters are separate from its effects on hypoghagia (Brichard & Henquin, 1995).

It is thus improbable that reduced food intake contributed to the alterations in plasma ET levels in this study.

5.3.5. Alterations in ET-1 reactivity in diabetes

While our study suggests that STZ DM is associated with increases in vasoconstrictor responses to both ET-1 and methoxamine, other studies have shown both decreased (Fulton et al., 1991; Tada et al., 1994; Makino & Kamata, 1998), and increased (Kiff et al., 1991; Tammesild et al., 1992), vasoconstrictor and vasodilator responses to ET-1, and both decreased (Pfaffman et al., 1982; Cameron & Cotter, 1992), and increased (White & Carrier, 1990; Chang & Stevens, 1992; Ozcelikay et al., 1994), responses to α_1-adrenergic stimulation in STZ rats. As noted before, this inter-study variation may be due to differences in vascular bed studied, the severity

and duration of hyperglycemia, and differences in experimental procedures for measuring vasoconstriction (Tomlinson et al., 1992). Some studies showing increased vasoconstrictor responses in STZ diabetic rats to various agonists have demonstrated the increase to be of a non-specific nature leading to enhanced vasoconstriction regardless of the agonist used (White & Carrier, 1990; Chang & Stevens, 1992). Our observation that plasma ET-1 is decreased in STZ rats suggests ET receptor upregulation as a potential mechanism whereby responses to ET-1 *ex vivo* would be enhanced. A recent study has demonstrated desensitized ET_A mediated vasoconstrictor and ET_B mediated vasodilator responses in the perfused mesenteric vascular bed along with elevated plasma ET-1 levels in 10 week STZ diabetic rats (Makino & Kamata, 1998). Their results suggest that elevated ET-1 induced receptor down regulation may have contributed to desensitized vascular responses. In the present study, while we cannot rule out receptor changes due to attenuated ET-1 levels, it is probable that another common mechanism is responsible for elevated reactivity to ET-1. This is supported by the fact that both methoxamine and ET-1 evoked vasoconstrictor responses are enhanced (non-specific effect), both in the presence and absence of intact endothelium (endothelium independent). Alterations in metabolic parameters such as plasma lipids, glucose, and insulin levels could all potentially contribute to alterations in VSM function in STZ DM (Tomlinson et al., 1992), leading to a generalized enhancement of the contractile state of the vasculature. Importantly, it is apparent that insulin deficiency, at least under the conditions in the STZ rat, does not lead to attenuated ET-1 evoked responses as might have been expected from the *in vitro* results.

5.3.6. Insulin and vanadate correction of endothelin-1 reactivity

Insulin treatment has previously been shown to normalize altered contractile responses in the STZ rat (Pfaffman et al., 1982; Heygate et al., 1996). Furthermore, in addition to its well established insulinomimetic effects on metabolic abnormalities in the STZ rat (Brichard & Henquin, 1995), at least one group has shown oral vanadate therapy to improve vascular reactivity in this model (Ozcelikay et al., 1994). In this study, however, the effect of long-term (10-weeks) treatment was assessed and neither responses to ET-1 nor comparisons to insulin were undertaken. Given increasing evidence that metabolic abnormalities are a major causative factor in diabetic vascular complications - and the fact that they are reversible by insulin treatment and restoration of metabolic control, it is likely that the beneficial effects of these compounds are mediated by correction of metabolic parameters in the STZ rat (Tomlinson et al., 1992; Pfaffman et al., 1982; Heygate et al., 1996). Furthermore, since exaggerated responses are corrected in both endothelium denuded and intact aorta, it is likely that correction of abnormal vascular responses in the STZ rat occurs at the VSM level.

5.3.7. Physiological Relevance

As previously discussed, ET-1 is a potent endothelial derived vasoconstrictor and mitogenic peptide. Its potency and long lasting effects suggest that it is an integral component in local control of blood flow. Altered ET-1 action, and elevated plasma ET-1 levels have been observed in several cardiovascular disease states. Despite the fact that ET-1 is known mainly as a paracrine hormone, plasma levels of ET-1 might

parallel that released in a paracrine manner by the vascular endothelium (Lerman et al., 1991). The observation that STZ DM leads to alterations in plasma ET-1 levels and VSM action implicates this peptide as a contributor to the vascular complications observed in this animal model of IDDM. Moreover, normalization of these indices of ET-1 activity by insulin and vanadate treatment of provides further evidence of beneficial vascular effects of these compounds. Specifically, changes in aortic reactivity to ET-1 (as demonstrated in this study) might contribute to changes in systolic blood pressure or to changes in susceptibility to large vessel atherosclerotis in diabetes.

5.3.8. Reconciliation of in vivo with in vitro results

Our *in vitro* results have shown that insulin *per se* is able to upregulate ET_A receptors, and vanadate to upregulate ET_A and ET_B receptors, in cultured ASMC. Furthermore, we have recently observed that aortic tissue from hyperinsulinemic / insulin resistant obese Zucker rats exhibit increased ET_A receptor levels (Hopfner et al., 1998a). Thus, it is plausible that insulin has the capability to regulate not only ET-1 peptide expression, but also, at higher concentrations, regulate ET receptor expression *in vivo*. Thus, along with VSM mitogenic and other hypertension-promoting effects (Scherrer & Sartori, 1997), high plasma insulin may promote adverse cardiovascular events via augmented ET-1 action at the VSM level. Indeed, the observation that vascular complications still arise in diabetic patients despite adequate glucose control lends credence to the concept that insulin treatment may promote adverse vascular complications (Kroc Collaborative Study Group, 1984).

However, as demonstrated by the present study, poor metabolic control associated with hypoinsulinemia may also promote a general enhancement of vascular reactivity in addition to various other well-known adverse cardiovascular effects (Tomlinson et al., 1992). These observations underscore the importance of achieving tight metabolic control of IDDM (minimizing fluctuations between insulinemia and glycemia/lipidemia) in order to prevent potential ET-1 mediated cardiovascular complications in this metabolic disorder.

It is notable that a minimum concentration of 10 nM of insulin was required to upregulate ET_A receptors in the *in vitro* arm of this study while under treatment conditions, STZ and non-diabetic rats under insulin treatment never achieved greater than a 2 nM concentration of insulin in the plasma. Thus, it is plausible that the plasma concentration of insulin never reached the threshold level at which it would be capable of upregulating ET_A receptors *in vivo* – explaining why induction of hyperinsulinemia with exogenous insulin supplementation did not promote exaggerated responses to ET-1 in vascular tissue *ex vivo* in the present study. On the other hand, one cannot assume that the concentration of an agonist required to induce an effect *in vitro* would be the same as that required to elicit similar effects *in vivo*. This is particularly relevant in the present study since the *in vitro* arm of this study simply used VSMC in culture, while the *in vivo* situation obviously would have been influenced by the vascular endothelium as well as interactions with other circulating factors. Moreover, while plasma vanadate concentrations were not measured in this study, the chosen dose was previously shown to result in plasma

concentrations of ~ 20 μM, a concentration adequate to promote changes in ET receptor expression *in vitro*. Thus, drawing direct comparisons between the *in vitro* and *in vivo* effects of insulin and vanadate in this study is quite difficult.

5.3.9. Conclusion

The present results demonstrate that 5-week STZ diabetic rats exhibit enhanced agonist-evoked vasoconstrictor responses and decreased plasma ET-1 levels relative to non-diabetic controls. Both insulin and vanadate treatment of STZ rats for 2 weeks normalized these alterations. Since recent research has focused on metabolic abnormalities as a major causative factor in diabetic vascular complications (DCCT, 1993; Kroc Collaborative Study Group, 1984), the beneficial effects of insulin and vanadate on metabolic control suggests a beneficial effect on adverse cardiovascular alterations in STZ DM treated with these compounds. Despite potential atherogenic and hypertensiogenic effects, including upregulation of ET receptors at the VSM level, insulin and vanadate therapy in IDDM may beneficially affect alterations in vascular ET activity present in the STZ diabetic rat (Figure 40).

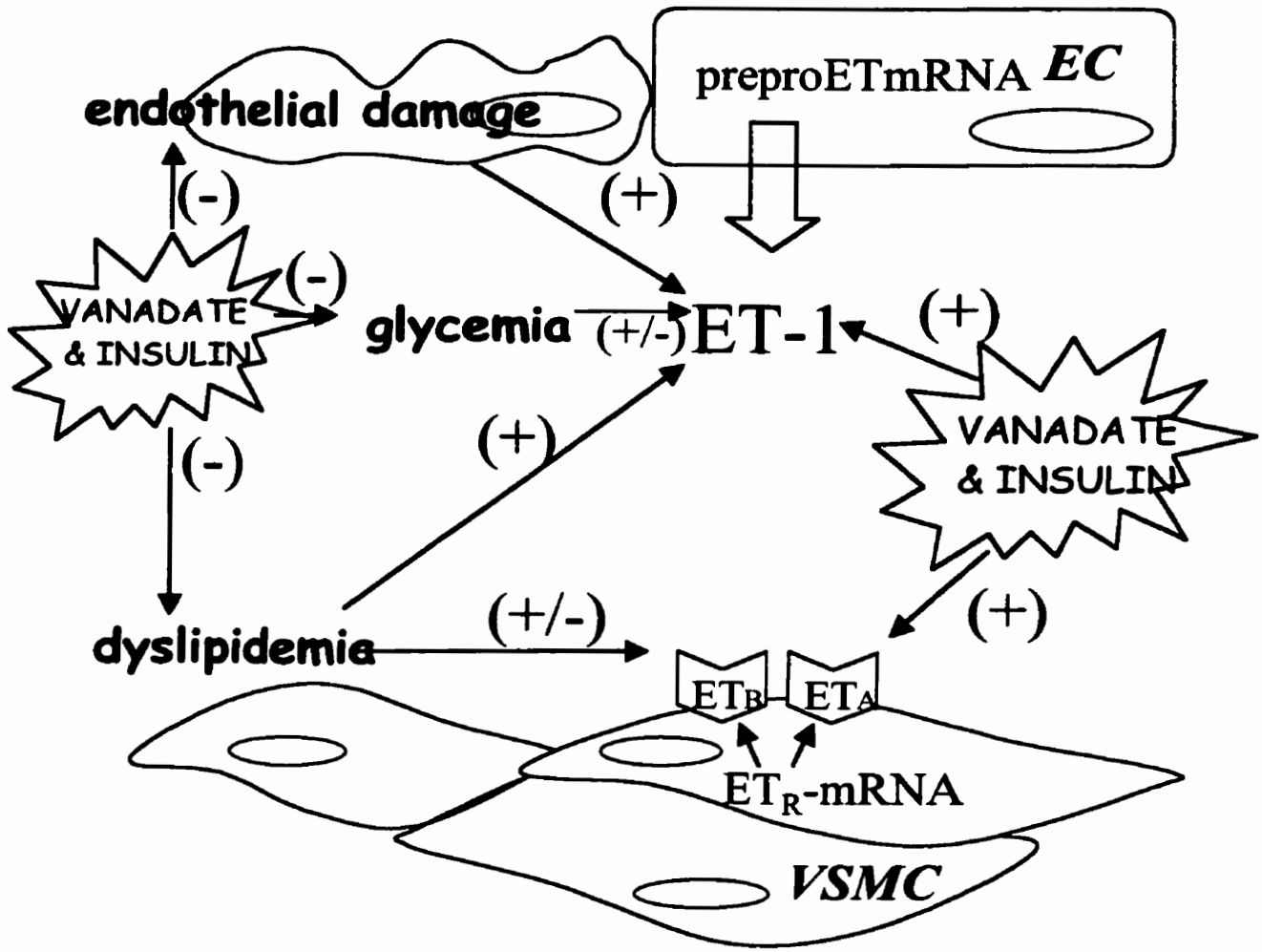

Figure 40. Summary of effects of diabetes, insulin, and vanadate on vascular ET activity. Factors associated with the diabetic state *per se* including hyperglycemia, hyperlipidemia, and endothelial dysfunction modulate both the release and responses to ET-1. Insulin and vanadate, while also having direct effects in promoting ET-1 release and action, may actually correct abnormalities in ET-1 activity in the diabetic state through restoration metabolic control.

6. SUMMARY & CONCLUSIONS

6.1. Insulin upregulates ET_A receptor expression and $[Ca^{2+}]_i$ responses in rat aortic smooth muscle cells

While insulin is known to promote VSM relaxation, it also enhances ET-1 secretion and action in conditions such as NIDDM and hypertension. In an effort to elucidate the effect of insulin on *responses* to ET-1, this thesis first examined the effect of insulin pretreatment on $[Ca^{2+}]_i$ responses to ET-1 in cultured ASMC. Pretreatment of rat ASMC with insulin (10 nM for 24 hr) failed to affect basal $[Ca^{2+}]_i$ levels but led to a significant increase in peak $[Ca^{2+}]_i$ responses (1.7 fold; $p < 0.01$) to ET-1. The responses to IRL-1620 (an ET_B selective agonist), Ang II and AVP remained unaffected. Since IGF-1 evoked similar effects, it was concluded that these effects of insulin might be mediated by stimulation of the IGF-1 receptor. ET-1-evoked peak $[Ca^{2+}]_i$ responses were significantly attenuated by the inclusion of the ET_A antagonist, BQ123. The ET_B antagonist, BQ788, abolished $[Ca^{2+}]_i$ responses to IRL-1620, but failed to affect the exaggerated $[Ca^{2+}]_i$ responses to ET-1. Saturation binding studies revealed a 2-fold increase ($p < 0.01$) in the maximal number of binding sites labeled by [^{125}I] ET-1 in insulin pretreated cells and no significant differences in sites labeled by [^{125}I] IRL1620 between control and treatment groups. Northern blot analysis revealed an increase in ET_A mRNA levels after insulin pretreatment for 20 hr, an effect that was blocked by genistein, actinomycin D, and cycloheximide. Parallel studies in our laboratory revealed a two-fold higher number of ($p < 0.01$) ET_A specific binding sites in aorta from obese Zucker rats compared to

lean controls (Hopfner et al., 1998a), and another study has recently demonstrated similar results in hyperinsulinemic fructose-hypertensive rats (Juan et al., 1998). These data suggest that insulin exaggerates ET-1 evoked peak $[Ca^{2+}]_i$ responses via increased vascular ET_A receptor expression. This may represent a potential mechanism whereby insulin contributes to enhanced vasoconstriction observed in hyperinsulinemic or insulin treated conditions.

6.2. Vanadate upregulates ET_A and ET_B receptor expression and $[Ca^{2+}]_i$ responses in rat aortic smooth muscle cells

The insulinomimetic agent, vanadate, has been considered recently for clinical use in the management of both IDDM and NIDDM. In parallel with insulin, the present study characterized the effect of vanadate pretreatment on changes in ET-1 binding, ET_A and ET_B mRNA expression, and ET-1 evoked $[Ca^{2+}]_i$ levels in rat ASMC. Similar to insulin, preincubation with vanadate also induced a concentration and time-dependent increase in specific binding of $[^{125}I]$ ET-1. Maximal increases were seen with 25 μM vanadate pretreatment for a period of 24 hr, and this effect was also abolished by the tyrosine kinase inhibitor, genistein. Vanadate also evoked an increase in the maximal number of specific binding sites for the non-selective $[^{125}I]$ ET-1 radioligand and, in contrast to insulin, for the ET_B selective radioligand, $[^{125}I]$ IRL 1620 as well - with the latter being increased to a greater extent. In line with this binding data, vanadate also induced an increase in both ET_A and ET_B mRNA that was abolished by both cycloheximide and actinomycin-D. Similarly, increased receptor expression is coupled to enhanced ET_A and ET_B signaling, as vanadate

pretreatment led to exaggerated peak $[Ca^{2+}]_i$ responses to either ET-1 or IRL-1620 in a manner reflective of receptor expression characteristics. Thus, vanadate, unlike insulin which upregulates only ET_A expression, upregulates both ET_A and ET_B receptor expression in rat ASMC. Despite beneficial insulinomimetic effects, vanadate may also influence diabetic vascular alterations via exaggerated VSMC responses to ET-1.

6.3. Insulin and vanadate normalize decreased plasma ET-1 and exaggerated vascular responses in the streptozotocin diabetic rat

Since our *in vitro* results indicated a possible mechanism whereby insulin and vanadate might promote exaggerated vasoconstriction to ET-1 under treatment conditions, the last arm of the study examined the effects of oral vanadate treatment (0.5 mg/ml; p.o.) and insulin infusion (12 mU. $kg.^{-1}min.^{-1}$ s.c.) for 2-weeks on plasma ET-1 and vascular responses to the peptide as well as the α_1-adrenoceptor agonist, methoxamine, in aortic ring preparations from STZ induced diabetic and non-diabetic rats. Plasma ET-1 was lower ($p < 0.01$) in diabetic rats compared to normal controls. Insulin and vanadate restored this abnormality to control levels ($p < 0.01$) and significantly increased plasma ET-1 in the control ($p < 0.05$) group. Surprisingly, higher maximal tension responses to ET-1 ($p < 0.01$) as well as methoxamine ($p < 0.05$) were present in aorta from STZ rats *per se* in both endothelium intact and denuded aortic preparations compared to control group. This was counter to our hypothesis that hypoinsulinemia would be associated with selectively attenuated vasoconstrictor responses to ET-1 in STZ rats due to the lack

of upregulatory influence of insulin on ET_A receptors. In addition, rather than further increasing responses to ET-1 *in vivo*, as our *in vitro* data might have indicated, both insulin and vanadate treatment actually normalized (attenuated) these exaggerated responses. It is concluded the profound metabolic dysregulation of STZ DM is associated with attenuated plasma ET-1 levels and a non-specific increase in vascular reactivity to vasoconstrictor agonists. Insulin and vanadate treatment restores metabolic variables as well as diminished plasma ET-1 levels to control levels and actually attenuates exaggerated ET-1 (and other agonist) evoked VSM responses in diabetic rats. Thus, in addition to well-known beneficial metabolic effects, insulin and vanadate may actually beneficially affect cardiovascular regulation in the STZ diabetic rat via normalization of abnormal ET-1 activity.

7. FUTURE DIRECTIONS

- An examination of the cellular mechanisms through which insulin and vanadate increase ET receptor expression assumes importance. Earlier evidence indicates that insulin increases α_1-adrenergic receptor expression through a PI-3K linked pathway. Further studies with enzyme inhibitors would clarify this issue. Moreover, since vanadate primarily mediates its insulinomimetic effects through phosphotyrosine phosphatase inhibition leading to activation of both insulin sensitive (receptor tyrosine kinase) and insulin insensitive (cytosolic tyrosine kinase) pathways, it is likely that pathways linked to the latter are responsible for ET_B receptor upregulation. Earlier evidence indicates that vanadate activates PLCγ leading to increases in $[Ca^{2+}]_I$ and activation of PKC. Such pathways may be linked to ET_B upregulation.

- Since ET-1 is thought to be primarily a paracrine hormone, determinations of tissue ET-1 peptide as well as ET receptor peptide and mRNA expression in untreated and treated type I and type II diabetic rats assume importance. One of the most important observations of this thesis is the profound dysregulation of ET-1 plasma levels and vascular responses in the diabetic state, and its normalization by restoration of metabolic control. Vascular tissue level measurements of ET-1 and ET receptor levels would determine the full extent of these alterations and add more physiologically meaningful data to these observations.

- Determination of the factors responsible for changes in ET-1 release and action in diabetic states. While all of hyperglycemia, insulinemia, lipoproteinemia, and endothelial dysfunction have been shown to alter ET-1 release and action, determining the relative contribution of each assumes importance. Many of the treatment options in existence for use in DM differentially alter these variables and targeting those treatments that promote changes in ET regulation might be of additional cardiovascular benefit.

- Finally, and most importantly, determining the role of alterations in ET-1 release and action in DM and under treatment conditions assumes importance. The role of ET-1 in DM is still controversial due to disparate results between different laboratories and a lack of definite proof that ET-1 causes diabetic vascular complications. If alterations in ET-1 activity *in vivo* indeed contribute to the cardiovascular complications of DM, then ET antagonists should have beneficial effects in alleviating some or all of these abnormalities. Thus, it assumes paramount importance that studies with ET antagonists in different models of DM be pursued to further examine this issue.

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