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Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function

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Endothelin (ET), a peptide originally isolated from the supernatants of cultured endothelial cells, exerts a wide variety of biological effects in different tissues. Endothelial-cell-synthesized ET-1 has been proposed to act in a paracrine manner on adjacent smooth muscle cells (SMC) in vivo, with effects that include both vascular reactivity (vasodilation/vasoconstriction) and mitogenesis. This study, by the use of immunocytochemically characterized SMC (rVSMC) isolated from the aortas of spontaneously hypertensive rats, has investigated a possible autocrine role for ET in regulation of the vasculature. Although quiescent cultures of rVSMC apparently did not constitutively express prepro ET-1mRNA, ET-specific transcripts could be induced by a variety of growth factors (transforming growth factor β [TGF-β]; platelet-derived growth factor-AA homodimer [PDGF-A chain]) and vasoactive hormones (angiotensin II [Ang II], arginine-vasopressin, and ET-1 itself). The kinetics for prepro ET-1mRNA induction in rVSMC were characteristically rapid in onset and transient. Down-regulation of protein kinase C by 48 h pretreatment of rVSMC with phorbol ester markedly reduced the subsequent ability of rVSMC to express ET-1 transcripts and secrete ET-1 peptide in response to Ang II. Inducible prepro ET-1mRNA expression was accompanied by a cycloheximide-inhibitable release of ET-1 peptide into the medium of rVSMC. ET-1 peptide was determined by both radioreceptor- and radioimmunoassay. Stimulated rVSMC accumulated ET-1 (~200 pg · 10^6 cells^{-1} · 4 h^{-1}) at levels that attained biological relevance (~10^{-10} M). Sep-pak C_{18} extracts of medium from stimulated rVSMC elicited contraction of isolated endothelium-denuded rat mesenteric resistance vessels, and this response was characteristically protracted and difficult to "wash out." Synthetic (porcine) ET-1 promoted the expression of transcripts for PDGF-A chain, TGF-β, and thrombospondin in quiescent rVSMC. Such effects of ET-1 on gene expression may be relevant to the mitogenic potential of ET-1 on VSMC. Our findings imply a role for ET-1 in the control of vascular function via both paracrine and autocrine regulatory mechanisms. The expression of prepro ET-1mRNA and peptide biosynthesis by rVSMC may have both short-term (e.g., vasoconstriction) and long-term (e.g., structural remodeling) consequences. A sustained loop of autocrine stimulation by ET-1 in SMC could contribute toward the pathogenesis of vasospasm and/or atherosclerosis.

Introduction

Endothelial cell stimulation is accompanied by the synthesis and secretion of substances that can influence the underlying vascular smooth muscle cell (VSMC) layer in a paracrine manner (Furchgott, 1983; Ross, 1986; Vanhoutte *et al.*, 1986; Vane *et al.*, 1987). These events involve sensing/transduction of external signals and selective induction of genes within these cells (Dzau and Gibbons, 1988; Michell, 1989). Examples of endothelium-derived paracrine factors include endothelin (ET-1), angiotensin II (Ang II), transforming growth factor β (TGF-β), and platelet-derived growth factor-AA homodimer (PDGF-AA), all of which influence contractile properties (short-term effects) and/or growth control (long-term effects) of VSMC and thereby modulate vascular tone and structure (Langille and O'Donell, 1986; Dubin *et al.*, 1989).

VSMC themselves are capable of synthesizing and secreting a number of peptides (in addition to extracellular matrix molecules) (Majack *et al.*, 1985; Penttinen *et al.*, 1988) that generate the potential for autocrine regulation of VSMC structure/function (Naftilan *et al.*, 1989). Can-

didates for such autocrine regulation include PDGF-AA, thrombospondin (TS), and TGF-β (Majack *et al.*, 1986; Majesky *et al.*, 1988; Owens *et al.*, 1988; Penttinen *et al.*, 1988; Sarzani *et al.*, 1989) and also components of the kallikrein (Oza *et al.*, 1990) and renin-angiotensin (Campbell, 1987; Dzau and Safar, 1988) systems.

The peptide ET-1 was originally isolated from the supernatants of cultured porcine endothelial cells and proposed to function in vivo as a paracrine regulator of adjacent VSMC (Yanagisawa *et al.*, 1988). It is now evident that some non-endothelial cells can also synthesize and secrete ET-1 (e.g., epithelial [Baley *et al.*, 1990] and neuronal [Giaid *et al.*, 1989] cells, for which a local modulatory mechanism of action has also been proposed, because cells that express prepro ET-1mRNA are in close proximity to ET-1–binding sites [Yanagisawa *et al.*, 1988, 1989; Yanagisawa and Masaki, 1989; Baley *et al.*, 1990]). Expression of prepro ET-1 transcripts and peptide synthesis in endothelial and epithelial cells is increased in response to some growth factors (e.g., TGF-β) and/or vasoactive hormones (e.g., Ang II) (Emori *et al.*, 1989; Kurihara *et al.*, 1989; Baley *et al.*, 1990). The potential for autocrine regulation of VSMC by these same peptides prompted us to investigate whether prepro ET-1mRNA expression and peptide synthesis could be induced in cultured rat aortic smooth muscle cells (rVSMC) by growth factors and/or vasoconstrictor peptides and, furthermore, to determine the ability of ET-1 to induce expression of transcripts for relevant autocrine regulators in these cells.

Results

Inducible prepro ET-1mRNA expression in rVSMC by growth factors and vasoactive hormones

Prepro ET-1–specific mRNA is constitutively expressed in cultured endothelial cells, and levels of transcripts are increased by Ang II, TGF-β, and thrombin (Yanagisawa *et al.*, 1988; Emori *et al.*, 1989; Kurihara *et al.*, 1989). Using porcine endothelial cells, we confirmed the ability of 10^{-8} M Ang II, 2 ng/ml TGF-β, and 2 U/ml thrombin to elicit time-dependent increases in levels of prepro ET-1mRNA (Figure 1A). Additionally, constitutive expression of ET-1 transcripts in these cells was not altered after the cells' exposure to synthetic ET-1 (10^{-8} M) (Figure 1A), which is in agreement with the lack of specific receptors for, and response to, ET-1 in endothelial cells (unpublished observations).

Immunocytochemical characterization (Materials and Methods) of rVSMC cultures, by the use of monoclonal antibodies directed against smooth muscle α-actin and endothelial-cell-specific Von Willebrand factor (Factor VIII) verified both their smooth muscle cell (SMC) phenotype and the absence of endothelial cells. In contrast to endothelial cells, a constitutive expression of prepro ET-1mRNA was beyond the level of detection in confluent and quiescent rVSMC (Figure 1B). However, prepro ET-1 transcript expression in these cells could be rapidly (within 30 min) but transiently (return to zero-time levels within 3 h) induced by the growth factors PDGF-AA and TGF-β, by thrombin, and by the vasoconstrictor peptides Ang II and arginine-vasopressin (VP) (Figure 1B). ET-1 was also found to induce prepro ET-1mRNA expression in rVSMC (Figure 1B). Densitometric analyses of ET-1 transcript expression in rVSMC (normalized with respect to internal MHC controls) are presented in Figure 1C, and it is apparent that the various agonists might exert differential quantitative effects on prepro ET-1mRNA expression in rVSMC. The short transience of inducible ET-1 transcript expression in rVSMC by cell agonists (Figure 1, B and C) was notably different from the more sustained elevation of ET-1 transcripts in endothelial cells (Figure 1A and Yanagisawa *et al.*, 1988; Kurihara *et al.*, 1989). Prepro ET-1mRNA expression in rVSMC was not induced (data not shown) by insulin (15 μM), insulin-like growth factor-1 (IGF-1; 5 ng/ml) or low-density lipoprotein (LDL; 7.8–2000 μg/ml).

Synthesis and secretion of ET-1 peptide by rVSMC

The induction of prepro ET-1mRNA expression by growth factors and vasoactive peptides in rVSMC was accompanied by the release of immunoreactive ET-1 into medium (Figure 2A). The amounts of ET-1 peptide released by stimulated rVSMC ($\sim$120–300 pg $\cdot$ 10^6 cells^{-1} $\cdot$ 4 h^{-1}) are comparable to those described (Emori *et al.*, 1989; Yanagisawa *et al.*, 1989) for endothelial cells ($\sim$200–300 pg $\cdot$ 10^6 cells^{-1} $\cdot$ 4 h^{-1}). Furthermore, the ability of the different agonists to promote ET-1 peptide secretion correlated with their ability to increase prepro ET-1 transcript expression (compare Figures 1C and 2A) inasmuch as the effects of TGF-β, PDGF-AA, and VP were markedly greater than those of Ang II and thrombin. It was not possible to determine the effect of ET-1 stimulation on peptide release from rVSMC because of the excess concentra-

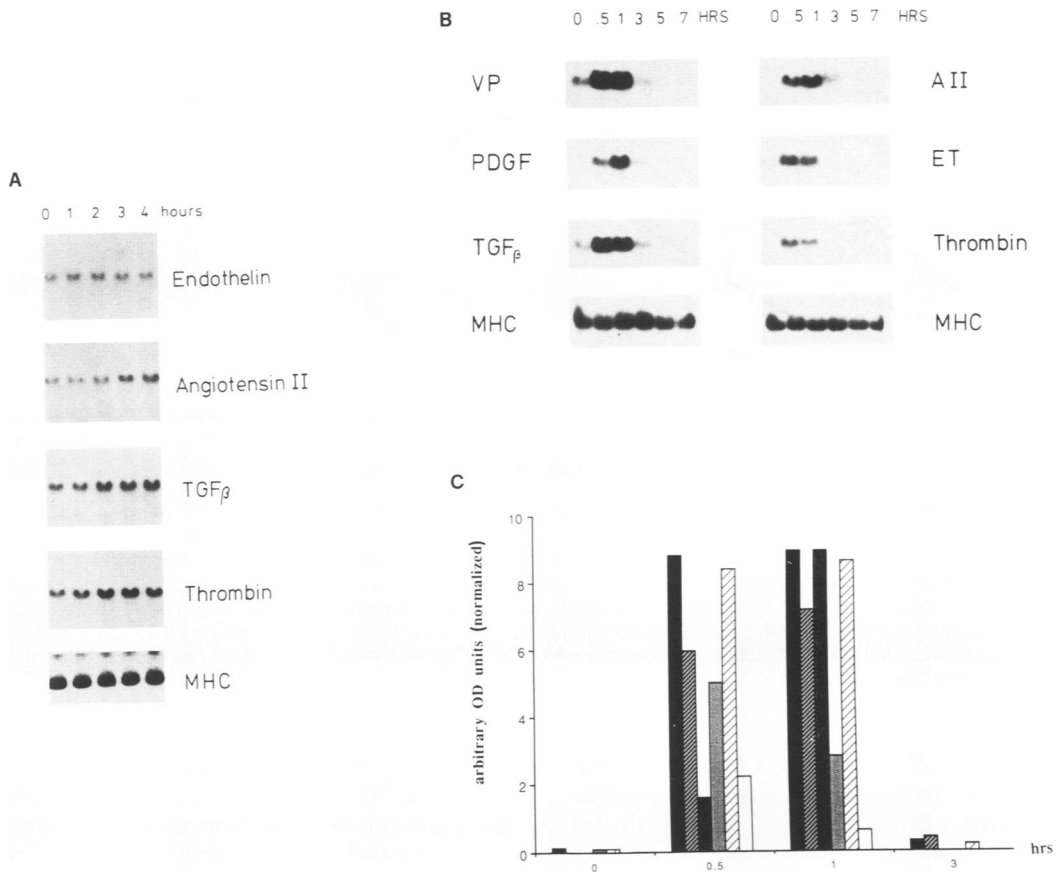

Figure 1. **Induction of prepro ET-1mRNA expression in rVSMC and endothelial cells.** (A) Quiescent confluent cultures of endothelial cells were exposed to ET-1 (10^{-8} M), Ang II (10^{-8} M), Thrombin (2 U/ml), or TGF-β (2 ng/ml) for the indicated periods. (B) Confluent quiescent rVSMC were stimulated for the indicated times with VP (10^{-8} M), Ang II (10^{-8} M), ET-1 (10^{-8} M), TGF-β (2 ng/ml), PDGF-AA (2 ng/ml), or thrombin (2 U/ml). ET-1– specific transcripts were visualized by hybridization to a random-primed cDNA probe, and rehybridization to a probe for MHC class I antigens reveals differences in amounts of total RNA blotted. (C) Densitometric analyses of Northern blot autoradiographs with normalization for control MHC hybridization. ■, VP; ▩, PDGF; ▧, A II; □, TGF-β; □, ET; and ▨, thrombin. Prepro ET-1mRNA expression was undetectable in vehicle(s)-treated rVSMC (data not shown, but not different from zero-time levels presented). All procedures are described in Materials and Methods.

tion (10^{-8} M) of exogenously added ET-1. In some experiments for Ang II exposed rVSMC, secretion of peptide into medium was measured using both radioimmuno- and radioreceptor-assay procedures (Figure 2B), and quantitatively ET-1-peptide concentrations tended to be lower (~15%) in radioreceptor assays. The appearance and accumulation of ET-1 peptide did not occur when rVSMC were exposed to agonists in the presence of 50 ng/ml cycloheximide (data not shown), thus implying a requirement for de novo synthesis rather than release of preformed/intracellularly stored peptide.

Although data from radioreceptor assays indicate that peptide secreted by rVSMC binds to ET-1–specific receptors (i.e., competitive displacement of [^{125}I]-ET-1), we considered it necessary to test the peptide for its biological activity. Sep-pak C_{18} extracts of medium from Ang II–stimulated (10^{-8} M, 24 h) rVSMC promoted contraction of isolated rat mesenteric resistance arteries even in the presence of 5×10^{-6} M [Sar^1 Ala^8]-Ang II (Figure 3C). Use of the Ang II antagonist at this concentration completely inhibited the contraction induced by 10^{-8} Ang II (Figure 3, A and B), and, furthermore, "wash out" procedures reversed contraction induced by Ang II (Figure 3A) but not that induced by extracts (Figure 3C).

The degree of contraction (~75% of contraction by 100 mM KCl, see Figure 3C) elicited by extracts (final perfusate concentration of immunoreactive ET-1 5.7×10^{-9} M) was comparable to that induced by ~5×10^{-9} M ET-1

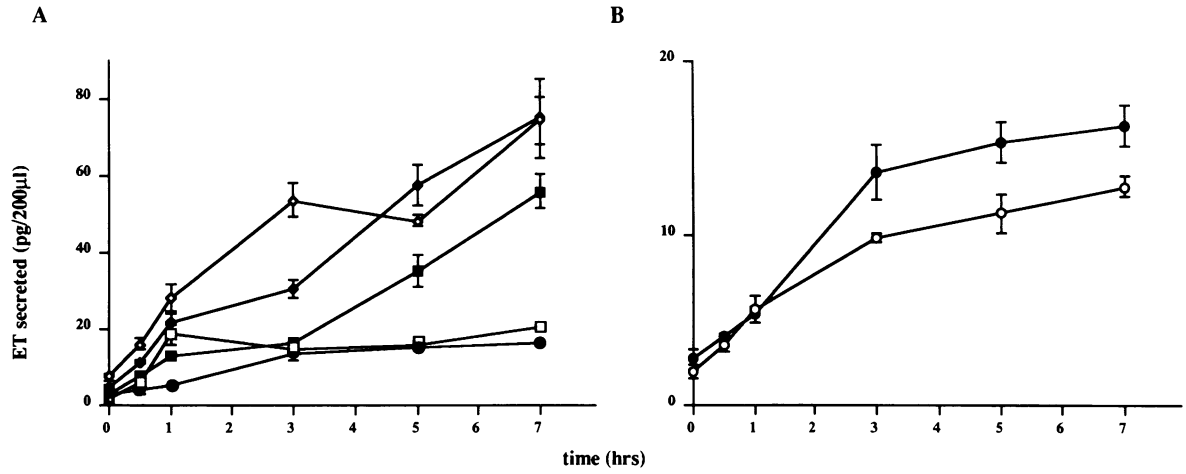

Figure 2. **Secretion of ET-1 peptide by rVSMC.** (A) Confluent, quiescent rVSMC were exposed to different agonists for the times indicated: □, thrombin (2 U/ml); ●, Ang II (10^{-8} M); ♦, PDGF-AA (2 ng/ml); ◇, VP (10^{-8} M); and ■, TGF-β (2 ng/ml). ET-1 peptide in medium overlay was determined by the use of a radioimmunoassay kit. (B) In a separate series of experiments, rVSMC were treated with 10^{-8} M Ang II and ET-1 in medium quantitated with the use of both radioimmuno- (●) and radioreceptor (○) assay procedures as described in Materials and Methods. Values are given as mean ± SD (n = 3) and express the amount of ET-1 in 200-μl aliquots of medium; the approximate cell/medium ratio in these experiments was 1.2–1.6 × 10^6 cells/2 ml medium. ET-1 peptide could not be detected in the medium from vehicle(s)-treated rVSMC (data not shown, but not different from zero-time values presented).

standard (70 ± 5 [SD], Dohi and Lüscher, 1990). Control medium extracts (i.e., cell-free incubation of medium containing 10^{-8} M Ang II) did not induce significant contraction of vessels (Figure 3D). Such a lack of response to control extracts, together with the data demonstrating saralasin-insensitive and protracted vasoconstriction to extracts from Ang II–exposed VSMC, excludes the possibility that the observed contractile response to rVSMC-medium overlay extracts might have been due to residual Ang II.

Dose-dependent induction of prepro ET-1mRNA expression in rVSMC: inhibition by protein kinase C–down regulation

The induction of prepro ET-1mRNA was also dose-dependent, as shown for Ang II (Figure 4A); an increase in ET-1 transcript levels was evident at a very low dose (10^{-11} M) of Ang II, and maximum levels were obtained between 10^{-9} and 10^{-8} M. After scanning of autoradiographs and normalization with respect to major histocompatibility complex (MHC) class I antigen hybridization (Figure 4B), we calculated the concentration of Ang II required to half-maximally stimulate prepro ET-1mRNA expression to be 0.6–1 × 10^{-10} M. As reported in the case of endothelial cells (Yanagisawa *et al.*, 1988; Emori *et al.*, 1989), acute exposure of rVSMC to phorbol ester (100 nM, 4 h) elicited the secretion of ET-1 peptide (340 ± 41 pg/10^6 cells, n = 5). However, chronic phorbol ester pretreatment (100 nM, 48 h) of rVSMC, and consequent down-regulation of protein kinase C, markedly reduced the ability of Ang II both to elevate prepro ET-1mRNA (Figure 4C) and to promote release of ET-1 peptide (22 ± 6 and 125 ± 8 pg · 10^6 cells^{-1} · 4 h [n = 4] in pretreated and control rVSMC, respectively).

Induction of growth factor transcript expression in rVSMC by ET

Recent studies have demonstrated that Ang II can increase levels of transcripts for TGF-β, PDGF-A chain, and the matrix glycoprotein TS (Naftilan *et al.*, 1989; Resink *et al.*, 1990). Furthermore, PDGF-AA and TGF-β can also increase growth-factor production (Wahl *et al.*, 1987; Soma and Grotendorst, 1989). The ability of ET-1 to promote expression of its own transcripts (Figure 1, A and B) prompted an investigation of its ability to influence expression of transcripts for other growth factors. Stimulation of quiescent rVSMC with ET-1 (10^{-8} M) resulted in elevation of transcripts for PDGF-A chain, TGF-β, and TS (Figure 5A). Densitometric analysis of Northern blots (with normalization for the internal control MHC class 1 antigen; Figure 5B) showed induction of TGF-β transcript expression to be a relatively weak response to ET-1 (i.e., at maximum, 20% of that for PDGF-A chain and TS). Differences between the ki-

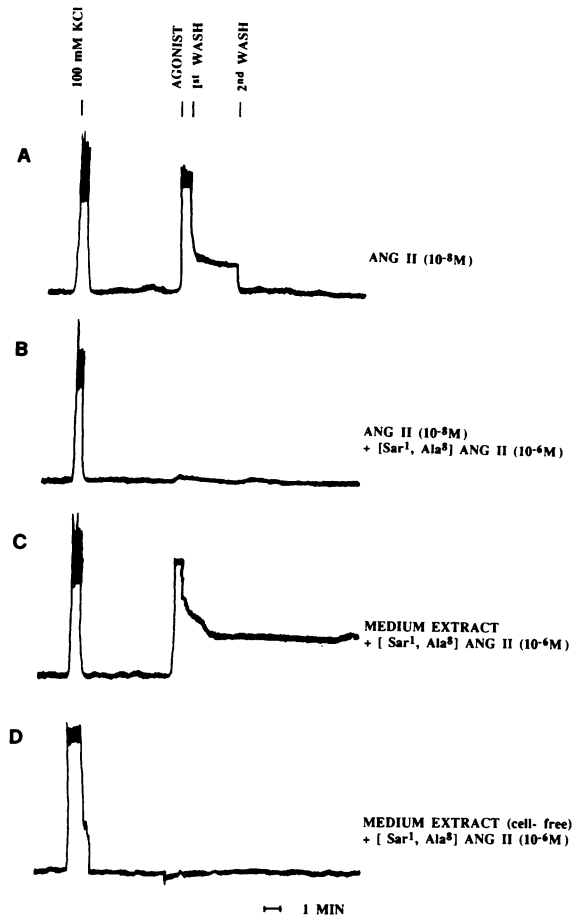

Figure 3. **Peptide secreted by rVSMC elicits contraction of isolated blood vessels.** Isolated, endothelium-denuded mesenteric resistance arteries from WKY rats were used to examine vasoconstrictive responses (described in Materials and Methods). Maximal contraction to 100 mM KCl was established before determination of the contractile response to 10^{-8} M Ang II (A); 10^{-8} M Ang II plus 10^{-6} M [Sar¹, Ala⁸]-Ang II (B); extract of medium (total of 500 ml extracted) from rVSMC exposed (36 h) to 10^{-8} M Ang II plus 10^{-6} M [Sar¹, Ala⁸]-Ang II (C); extract of control medium (500 ml containing 10^{-8} M Ang II without rVSMC) (D). Medium from Ang II–exposed rVSMC contained 15–20 pg immunoreactive ET-1/200 μl; recovery after extraction on Sep-pak C_{18} cartridges was 85% (~36 ng immunoreactive ET-1 from 500 ml). Extracts were reconstituted in 250-μl buffer (to give 0.057 μM ET-1) and diluted 10-fold for perfusion experiments. Cell-free medium samples (D) were similarly processed. All experimental details are given in Materials and Methods.

netics of increases in transcript levels for PDGF-A chain ($t_{1/2}$ ~ 4 h) and TS ($t_{1/2}$ ~ 8 h) were also evident (Figure 5B).

Discussion

Using cultured rVSMC for which both smooth muscle phenotype and absence of endothelial cell contamination was established, we have demonstrated their expression of prepro ET-1mRNA and secretion of biologically active ET-1 peptide after exposure to growth factors and vasoactive hormones. Induction of prepro ET-1mRNA/ET-1 peptide biosynthesis in rVSMC could be elicited by a spectrum of compounds similar to those (thrombin, TGF-β, Ang II, VP) that regulate ET-1 gene transcription/peptide secretion in endothelial cells (for review, see Emori *et al.*, 1989; Kurihara *et al.*, 1989; Yanagisawa and Masaki, 1989).

However, the two cell types differ with respect to stimulation by ET-1, which increased prepro ET-1mRNA in rVSMC but not in endothelial cells (Figures 1 and 2), a finding consistent with the absence of ET-1 receptor expression in endothelial cells (unpublished observation). Likewise, rVSMC responded to PDGF-AA (Figures 1 and 2), whereas endothelial cells do not (Kurihara *et al.*, 1989). Profiles for stimulated prepro ET-1 transcript expression were also markedly different between rVSMC (onset within 30 min and very transient; Figure 1, B and C) and endothelial cells (onset within 1–2 h and protracted elevation; Figure 1A and Kurihara *et al.*, 1989; Joshizumi *et al.*, 1990). Nevertheless, stimulated rVSMC secrete ET-1 peptide in amounts comparable to those produced by endothelial cells, and concentrations of peptide in medium attain levels (~$0.4–1.2 \times 10^{-10}$ M after 4 h) that are within the range of receptor K_D and response EC_{50} values for this peptide (Yanagisawa *et al.*, 1988, 1989; Yanagisawa and Masaki, 1989). The attenuation of Ang II–stimulated prepro ET-1 mRNA expression/ET-1 peptide release in rVSMC chronically pretreated with phorbol ester, as well as the release of ET-1 peptide by rVSMC after acute exposure to phorbol ester, provides strong evidence that protein kinase C mediates induction of the gene for prepro ET-1. These findings are in agreement with those for endothelial cells, whereby acute phorbol ester exposure stimulates both expression of ET-1 transcripts and release of immunoreactive ET-1 peptide (Itoh *et al.*, 1988; Yanagisawa *et al.*, 1988).

Both phorbol ester and calcium ionophore can independently elicit increases in ET-1 gene expression and peptide secretion in endothelial cells (Yanagisawa *et al.*, 1988, 1989; Emori *et al.*, 1989). It has thus been suggested that the production of ET-1 may be controlled by a transcriptional regulation that couples directly to the intracellular signals from the phosphoinositide pathway, i.e., activation of protein kinase C and increase in intracellular Ca^{2+} (Yanagisawa *et al.*, 1989). Because the phosphoinositide-phos-

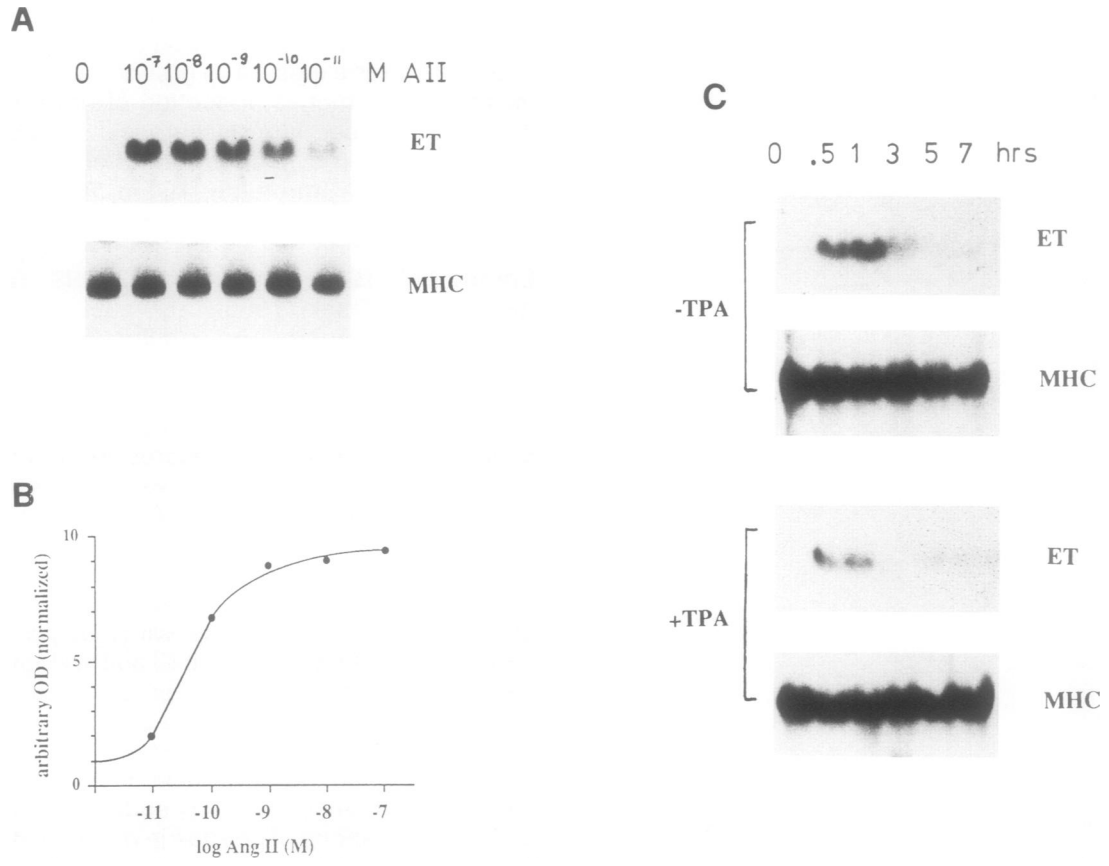

Figure 4. **Dose-dependent induction of ET-1mRNA by Ang II; requirement for protein kinase C.** (A and B) Quiescent rVSMC were treated for 1 h with various concentrations of Ang II; total RNA was isolated, blotted, and hybridized to ET-1– specific and MHC class I antigen–specific cDNA probes as described in Materials and Methods. Autoradiographs (A) of ET-1 and MHC signals were scanned and optical density recorded (arbitrary units). Absorbance for ET-1mRNA at each dose was normalized with respect to the corresponding MHC-signal (for control sample without exposure to Ang II an arbitrary unit = 1 was assigned) and a densitometric profile constructed (B). (C) Quiescent rVSMC were exposed to 10^{-8} M Ang II for the times indicated after a 48-h preincubation of cultures either in the absence (−TPA) or presence (+TPA) of 100 nM phorbol, 12-myristate, 13-acetate (TPA); total RNA was isolated and processed for hybridization to random primed cDNA probes for ET-1 and MHC class I antigens as above.

pholipase C signal transduction pathway is stimulated by most growth factors and vasoactive hormones (for reviews, see Exton, 1986; Rozengurt, 1986; Farese, 1988; Whitman and Cantley, 1988; Michell, 1989), the broad spectrum of compounds herein demonstrated to induce prepro ET-1mRNA expression and ET-1 peptide secretion in rVSMC raises the possibility that these effects may reflect a rather unselective/nonspecific response to elevations in cytosolic calcium and diacylglycerol. However, not all compounds (specifically insulin, IGF-1, and LDL: data not shown) that stimulate phosphatidylinositol-specific phospholipase(s) C and increase either diacylglycerol (insulin and IGF-1: Espinal, 1987; Farese, 1988) or both diacylglycerol and Ca^{2+} (LDL: Block *et al.,* 1988; Scott-Burden *et al.,* 1989b) are a priori promoters of ET-1 gene expression and peptide release in rVSMC. Therefore, although stimulation of these processes in both endothelial and SMC may require protein kinase C activation and/or an increase in intracellular Ca^{2+} as the mediatory signal(s), the occurrence per se of such signaling events need not necessarily lead to ET-1 gene expression and peptide release. Additional (presently unknown) mechanisms determining the selectivity/specificity of these responses are likely.

The induction of ET-1 transcript expression and peptide production in rVSMC by endothelial cell–synthesized compounds (e.g., PDGF-AA, TGF-β, and also ET-1) (Resink *et al.,* 1990) suggests a paracrine regulation of these processes. However, VSMC themselves are also capable of synthesizing PDGF-AA and TGF-β (e.g., in re-

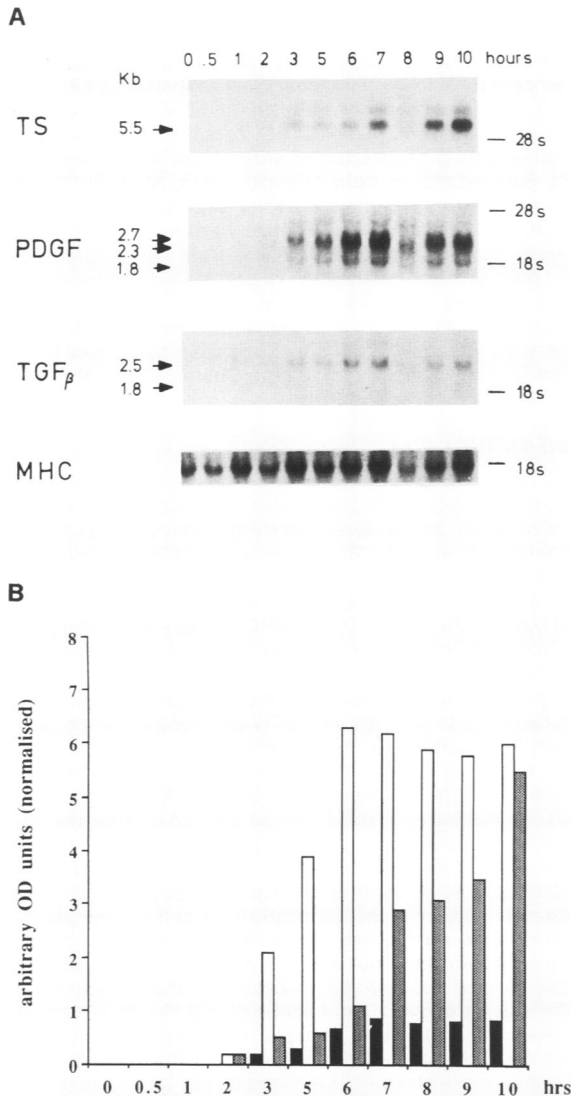

Figure 5. **ET-1 induces growth factor transcript expression in rVSMC.** Quiescent rVSMC were treated with 10^{-8} M ET-1 for the given times. (A) Total RNA was analyzed on Northern blots by sequential hybridization to random-primed cDNA probes for thrombospondin (TS), PDGF-A chain, and TGF-β. (B): Densitometric analyses of Northern blot autoradiographs with normalization for MHC hybridization. ▩, TS; □, PDGF (largest transcript); ■, TGF-β. Transcript expression in vehicle (PBS containing 0.1% BSA; 10 μl/ml medium)-treated rVSMC was not different (data not shown) from the zero-time levels presented in A. All procedures are given in Materials and Methods.

sponse to Ang II [Naftilan *et al.,* 1989; Resink *et al.,* 1990]), which gives rise to the potential for autocrine regulation of prepro ET-1mRNA expression and peptide biosynthesis in VSMC. Whatever the manner of induction, the ability of VSMC to express prepro ET-1 transcripts and secrete peptide may constitute a potentially important autocrine mechanism of action for ET-1 on the vasculature, with both short-term (e.g., vasoconstriction) and long-term (e.g., growth regulation) consequences.

The data presented in Figures 1 and 2 indicate quantitative differences in the ability of the various agonists (used at maximally effective concentrations) to induce ET-1 gene expression and peptide secretion. Although we propose a possible autocrine role for ET-1 in VSMC growth control, there does not appear to be an overtly defined relationship between the effects of agonists on endothelin expression and their efficacy as growth factors. For example, although PDGF-AA and arg-VP are both strong inducers of ET-1 gene expression and peptide secretion, they differ markedly in their mitogenic potency on VSMC (PDGF-AA, strong; arg-VP, weak; for review, see Owens, 1989). On the other hand, for TGF-β, also an effective inducer of ET-1 gene expression and peptide secretion, bimodal (stimulatory and inhibitory) control of cell growth has been reported (for review, see Lyons and Moses, 1990). Additionally, although LDL (Scott-Burden *et al.,* 1989b), insulin, and IGF-1 (Banskota *et al.,* 1989) are capable of potentiating VSMC proliferation, we found these substances to be incapable of stimulating ET-1 gene expression/peptide biosynthesis. It is pertinent, therefore, to reiterate (Rozengurt, 1986; Owens, 1989) that in vivo control of cell growth/proliferation is mediated by numerous growth factors/progression factors, each of which, in a finite temporal sequence, selectively stimulates a subcomponent of the events or signals required for the ultimate mitogenic response.

The question arises whether prepro ET-1mRNA expression and peptide synthesis/secretion by VSMC occur in vivo, or whether the observations presented herein represent a consequence of the phenotypic modulation processes undergone by rVSMC after their propagation in culture (Chamley-Campbell *et al.,* 1979; Chamley-Campbell and Campbell, 1981). Nevertheless, in vascular pathologies such as hypertension and atherosclerosis, SMC do undergo phenotypic modulation and exhibit/acquire abnormal growth/secretory characteristics (Schwartz *et al.,* 1986; Owens, 1989). Furthermore, although the present study utilized VSMC from the aortas of spontaneously hypertensive rats—which exhibit enhanced growth-factor responsiveness in culture compared with cells from the normotensive Wistar Kyoto counterparts (Scott-Burden *et al.,* 1989a)—we were unable to detect constitutive prepro ET-1mRNA expression in these rVSMC when they were

rendered fully quiescent. Therefore, in vivo expression of the gene for prepro ET-1 in SMC may depend on the existing and/or past integrity of the blood vessel wall (e.g., endothelial desquamation, phenotypic modulation of constituent SMC, and local production of growth factors).

In vitro experiments have shown that ET-1 stimulates mitogenesis in SMC and glomerular mesangial cells and in 3T3 fibroblasts (Makaki *et al.,* 1989; Simonson *et al.,* 1989; Takuwa *et al.,* 1989). Such mitogenic potential of ET-1 has been attributed to the ability of this peptide to stimulate expression of c-*fos* and c-*myc* protooncogenes (Komuro *et al.,* 1988). The present findings—that ET-1 can promote self-transcript expression as well as the expression of mRNA for PDGF-A chain; TS; and, to a lesser extent, TGF-β—offer additional explanations for the apparent growth-stimulatory properties of this peptide (for review, see Takuwa *et al.,* 1990). Both PDGF and TGF-β are capable of exerting a profound influence on the phenotypic behavior of VSMC by initiating modulation and proliferation (Majack *et al.,* 1988) and/or by stimulating their matrix biosynthesis in a manner that leads to the maintenance of a new (pathogenic) phenotype (Herman and Castellot, 1987; Scott-Burden and Bühler, 1988). The glycoprotein TS is essential for the proliferation of myocytes in culture and has been proposed to play an autocrine role in the control of SMC growth (Majack *et al.,* 1986, 1988).

Self- and cross-induction mediated by ET-1, TGF-β, and PDGF-AA (this study and Hahn, Scott-Burden, Resink, Baur, and Bühler, unpublished observations) underline the complex paracrine/autocrine regulation of VSMC function and modulation. Our observations support a paracrine role for ET-1 whereby endothelial cell–synthesized/secreted ET-1 may act on adjacent VSMC to induce not only contraction but also the expression of transcripts for prepro ET-1, PDGF-A chain, and TGF-β in VSMC. However, biosynthesis and secretion of these peptides by VSMC could subsequently generate loops of autocrine regulation and self-amplification. Sustained autocrine stimulation of VSMC by ET-1 would have profound effects on the contraction and growth control of VSMC. It remains to be determined whether expression and synthesis of ET-1 by VSMC represent a physiological and/or pathophysiological axis of regulation.

Materials and methods

Materials

All tissue culture material and chemicals were from GIBCO (Basel, Switzerland), with the exception of fetal calf serum (Fakola, Basel, Switzerland). Spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats were from Charles River (Sulzfeld, Federal Republic of Germany). With the exception of smooth muscle α-actin monoclonal antibodies (a gift from Dr. G. Gabbiani [Kocher *et al.,* 1984]), all immunoreagents were obtained from Dakopatts (IG, Zurich, Switzerland). Radiochemicals were purchased either from Amersham (Zurich, Switzerland) or from ACR (St. Louis, MO). PDGF-AA was provided by Hoffmann-La Roche (Basel, Switzerland); Ang II, arg^8-VP, and cycloheximide were from Calbiochem (Luzern, Switzerland); thrombin was from Sigma Chemical (St. Louis, MO) and synthetic ET-1 (porcine/human) was from Nova Biochem (Laufelfingen, Switzerland); TGF-β was obtained from Collaborative Research (Bedford, MA). Unless otherwise stated, all other chemicals were purchased from Fluka (Buchs, Switzerland), Sigma Chemical, and Bio-Rad (Glattbrugg, Switzerland).

Cell culture and stimulations

SMC from rat aortas were isolated, characterized, and propagated as described before (Scott-Burden *et al.,* 1989b). Porcine aortic endothelial cells were isolated and cultured as detailed previously (Boulanger *et al.,* 1989). Phenotypic characterization of rVSMC was performed with the use of the immunocytochemical procedures described by Petersen and Van Deurs (1988). The rVSMC isolates were negative for Von Willebrand factor (Factor VIII) and >95% positive for smooth muscle α-actin. Staining for vimentin was used as a positive control and cell bodies were revealed by counterstaining with Giemsa. rVSMC were used between passages 8–10, and, before experimentation, confluent cells were rendered quiescent by culture in serum-free medium containing 0.1% bovine serum albumin (SF/BSA) for 48 h with one medium (SF/BSA) change after 24 h. After the 48-h period of quiescence, medium was removed; cell layers were washed with SF/BSA; and then fresh SF/BSA was added to the cells. Cultures were stimulated (37°C) with the selected agonists at the concentrations and for periods indicated in the appropriate figures. Subsequent experimental protocols (Northern blot analysis and measurement of ET production) are described below. With the exception of PDGF-AA (stored in 10 mM acetic acid/0.1% BSA) agonist stocks were prepared in phosphate buffered saline (PBS)/0.1% BSA; dilutions of all agonists were made in SF/BSA. Neither prepro ET-1mRNA expression nor secretion of ET-1 peptide was detectable in rVSMC exposed (up to 18 h) to vehicle alone (i.e., SF/BSA containing PBS or acetic acid with appropriate dilutions as for stimulation protocols).

Northern blot analysis

After stimulation, medium was removed and cell layers were washed twice with PBS before lysis in guanidinium-isothiocyanate buffer (GT buffer), which was added directly onto the culture dishes. Total RNA was collected on $CsCl_2$-gradients as described before (Ullrich *et al.,* 1977). For Northern analysis, 20 μg of total RNA was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde at a constant voltage of 50 V for 6–8 h in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer. The gel was vacublotted onto HYbond N membranes (Amersham) with 20× sodium chloride, sodium citrate (SSC) as transfer buffer. Blotted RNA was fixed to the membranes by ultraviolet irradiation at 302 nm for 3 min. Blots were hybridized to random-primed cDNA probes (Feinberg and Vogelstein, 1983) according to the method of Church and Gilbert (1984), washed at high stringency (0.1× SSC/0.1% sodium dodecyl sulfate at 65°C) and then exposed to Kodak X-Omat films at −70°C overnight using one intensifying screen. To assess

for variabilities in sample RNA, blots were rehybridized to a 1.5-kb cDNA probe specific for MHC class I antigens (clone pMF 48) (Pohla *et al.,* 1989) and exposed for 6–10 h to X-ray film. Densitometric analysis of hybridization signals was performed by scanning (525 nm) autoradiographs; the given arbitrary optical density (OD) units were obtained by normalization of signals with respect to the OD values obtained for the MHC internal controls (highest MHC OD value within a given series was arbitrarily taken as 100%).

Probes used in this study were a 1.3-kb fragment (clone TS 33) for TS, generously provided by Dr. P. Bornstein (Penttinen *et al.,* 1988); a 1.2-kb cDNA (clone βc1) for TGF-β, provided by Dr. R. Derynck (Derynck *et al.,* 1985); a 1.2-kb cDNA (clone D3) for the PDGF-A chain; and a 1.3-kb cDNA for ET (both cloned by Dr. J. Powell, Hoffmann-La Roche; the identity of these clones was established by partial nucleotide sequencing). For rehybridization of blots to the different probes, membranes were stripped of their previous signal by incubation for 10 min in boiling hot tris(hydroxymethyl)aminomethane-HCl (Tris-HCl, pH 7.5)/10 mM EDTA. Before rehybridization, blots were exposed to Kodak film overnight to ensure complete removal of the previous signals.

Measurement of ET-1 production

After stimulation of quiescent rVSMC, medium was withdrawn and stored at −70°C. ET was measured in 200-μl aliquots of medium with the use of a commercial ET-1 radioimmunoassay kit according to procedures detailed by the manufacturer (Peninsula Laboratories, Belmont, CA). In some experiments ET was also measured in 200-μl aliquots of medium with the use of an ET-1 radioreceptor assay kit as detailed by the manufacturer (Anawa Laboratories, Wangen, Switzerland). Sep-pak C_{18} cartridges (Waters Associates, Milford, MA) were used to extract ET-1 from media; briefly, 20-ml aliquots of medium were applied to Sep-pak C_{18} cartridges that had been prewashed sequentially with methanol, acetonitrile/5 mM trifluoracetic acid, and H_2O/5 mM trifluoracetic acid; the cartridges were washed with H_2O/5 mM trifluoracetic acid and ET-1 eluted with methanol. Eluates were pooled, dried under vacuum, reconstituted in Krebs-Ringer bicarbonate solution, and stored at −70°C until use in functional studies. Recovery of ET-1 (determined by inclusion of tracer [^{125}I]-ET-1 during Sep-pak C_{18} extraction) was 80%. Immunoreactive ET-1 was also quantitated before and after extraction. For both prepro ET-1mRNA expression and peptide production experiments, zero-time samples represent those from rVSMC exposed to agonists for ≤5 min.

Contraction of isolated rat mesenteric resistance arteries

Mesenteric resistance arteries were removed from anaesthetized WKY rats and mounted into an arteriograph chamber as previously detailed (Dohi and Lüscher, 1990). Removal of the endothelium (after intraluminal perfusion with 0.5% CHAPS for 30 s) was controlled for by demonstrating a complete lack of relaxation to 10^{-8} M acetylcholine during a contraction to 10^{-6} M norepinephrine (Dohi and Lüscher, 1990). The control perfusion solution was Krebs-Ringer bicarbonate solution containing 1% BSA and gassed with 95% O_2-5% CO_2, and vessels were equilibrated for 1 h at 37°C before testing. Maximal contraction to 100 mM KCl was established and then the contractile response to Ang II or medium extracts (with or without inclusion of [Sar^1, Ala^8]-Ang II [Saralasin]) was determined. Control perfusion solution was used in "wash out" procedures (Dohi and Lüscher, 1990).

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