Transcriptional Regulation of Endothelin

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Three isoforms of endothelin (ET) exist, ET-1, ET-2, and ET-3. Nucleotide sequences for the three human ET genes are highly conserved. ET-1 exactly matches the sequence of ET originally isolated from the conditioned medium of cultured bovine aortic endothelial cells (BAECs).[11] All three forms have been found in vascular, neural, adrenal, and kidney tissue, but are expressed in different proportions. Endothelial cells exclusively produce ET-1.[12] All three isoforms have different vasoconstrictive potencies but are otherwise qualitatively similar. ET-2 is the most potent vasoconstrictor, followed by ET-1 and ET-3.[13]

The amino acid sequence of ET-1 is unique among known peptides of mammalian origin. It contains four cysteine residues forming two disulfide bonds in the N-terminal half, and a cluster of hydrophobic amino acid residues at the carboxyl end of the peptide.[5] The N-terminal domain determines the affinity of binding to its receptor, while the C-terminal domain contains the binding site (Figure 1).

All three ETs are synthesized as large preproforms of approximately 200 amino acids which are cleaved at the basic-pair sites Arg52-Cys53 and Arg92-Ala93 by dibasic amino acid endopeptidases (Figure 2). Carboxypeptidases sequentially trim the Arg92 and Lys91 residues from the C-terminus to produce propeptides of 37 to 41 amino acids, the so-called "big ETs". An as yet poorly characterized endothelin-converting enzyme (ECE) has been identified that specifically cleaves between Trp73 and Val74 of big ET-1 to generate the mature 21-amino acid peptide.[5] This cleavage step is inefficient and/or incomplete both in vivo and in vitro, since big ETs have been identified in plasma[14] and in the medium of cultured cells[15] The conversion of big ET-1 to ET-1 is important for ET activity. It was observed that ET-1 was 140 times more potent as a vasoconstrictor than the precursor peptide,[16] and that the preproform has no vasoconstrictor activity at all.[17]

GENE AND PROMOTOR STRUCTURE

The human ET-1, ET-2, and ET-3 genes have been mapped to chromosome 6,[7,8,18,19] chromosome1,[18,20] and chromosome 20,[7,18,21] respectively. The human ET-1 gene contains four introns, 5'- and 3'- flanking regions, and spans approximately 6.8 kb of DNA (Figure 3).[9,11] The 2026 nucleotide mRNA for ET-1 is encoded in 5 exons.[7-9] Exon 1 encodes the first 21 amino acids of preproendothelin, exon 2 encodes the mature ET-1 peptide and the first four amino acids of "big endothelin", exon 3 encodes the "endothelin-like" peptide, exon 4 encodes residues 131-178 of preproendothelin, and exon 5 contains the sequence coding for the last 34 amino acids and the 3' untranslated region of preproendothelin.[8,9] The ET-1 transcription start site, mapped by S1 nuclease protection, is located 31 bp downstream from a TATA consensus sequence, and a CAAT box is located 65 bp upstream to the TATA box.[8,9]

Intron 1, intron 4 and exon 5 contain motifs of the binding sequence for the transcription factor nuclear factor-1 (NF-1).[7-9,11,22] While no functions have been identified for these sequences, NF-1-binding sites are known to be involved in the induction of the mouse type I collagen gene by TGF-ß.[23] Four sites containing the sequence for the acute phase regulatory element (APRE), CTGGGA, are found in the gene, two in the 5'-flanking region, one in intron 1, and the other in intron 4.[9,11] These elements may be responsible for the induction of ET-1 gene expression following surgery, myocardial infarction, and other stresses.[14] Consensus sequences

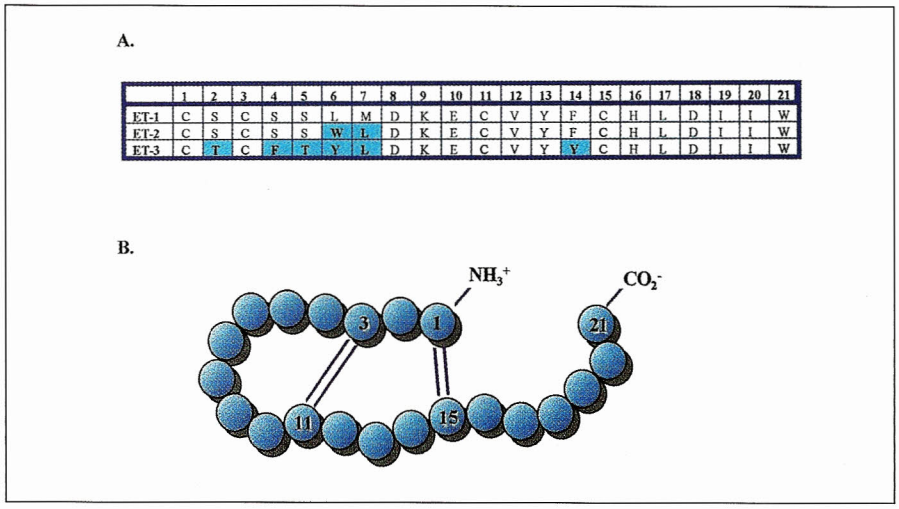

Figure 1. Structure of the ET family of peptides. (A) Primary structures of all three human Ets. Residues that differ from ET- 1 are shaded. (B) Secondary structure of Ets. All four cysteine residues (at positions 1, 3, 1 1, and 15) participate in disulfide bonding which defines the compact amino-terminal core region consisting of inner and outer loops. The carboxyl terminal is strongly hydrophobic, and forms a non-flexible b-sheet. The terminal tryptophan residue at position 21 is essential for bioactivity.[3]

to the AP-1/Jun-binding site occur at three positions in the 5'-flanking region, which might explain the rapid induction of ET-1 mRNA following treatment of human umbilical vein endothelial cells (HUVECs) with phorbol ester.[9,11]

Functional studies on the importance of these binding sites have recently been reported. In these promoter studies, a series of nested point deletion and cluster deletion mutant constructs spanning the -4.4 kb promoter range were coupled to a chloramphenicol acetyltransferase (CAT) reporter gene to identify those areas of the promoter that were necessary for downstream gene expression. BAECs were transfected with these CAT constructs in which expression was driven by the mutant human ET-1 promoter sequences.[24] It was found that a 143-bp region upsteam of the ET-1 coding region promoted CAT expression. This region contained two sites, both of which were required for full constitutive transcriptional activity in cultured BAECs. When a 119-bp ET-1 genomic fragment containing these two regions was joined to a heterologous promoter, this sequence was capable of transcription . Expression of this reporter construct was endothelial cell specific.[24]

Region A is located at bp -148 to -117 and Region B is located at bp -129 to -98 of the ET-1 gene. The functional significance of Region A was confirmed by reconstitution experiments and site-directed mutagenesis studies at position -109 of the ET-1 gene which resulted in a 30-fold reduction in promoter activity.[24,25] Sequence-specific protein binding to a GATA motif in this region was documented and was shown to be present in a large variety of cell types. This binding, however, was different from the similar and well-characterized erythroid-specific consensus Eryf-1 (GATA-1) binding motif.[26] A trans-acting factor which binds to this GATA-like region has been recently cloned. This factor likely represents the human transcription factor GATA-2 because of its homology with the chicken GATA-2 gene.[27] Transactivation experiments have demonstrated that GATA-2 interacts with the GATA-like region to increase transcription of the reporter gene. This occurs not only in endothelial cells but also in HeLa and NIH 3T3 cells. GATA-2 is down-regulated by the treatment of endothelial cells with retinoic acid, and it is this down-regulation that is apparently responsible for the inhibition of ET-1 expression in HUVECs by retinoic acid.[25,27]

Nuclear proteins have also been shown to bind to a GTGACTAA motif in Region B at bp -109 to -102, similar to the well-characterized AP1 site which mediates hormone and growth factor responsiveness in

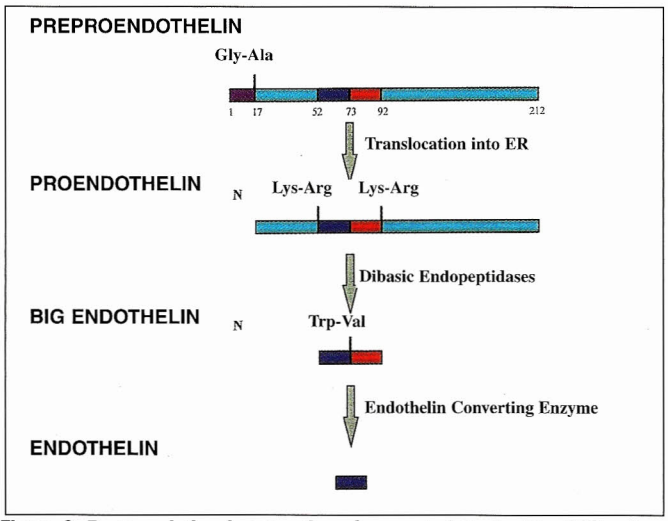

Figure 2. Posttranslational maturation of preproendothelin. The 212 amino acid human preproET-1 peptide contains a 17 amino acid signal peptide at the amino terminal that allows the precursor peptide to be translocated into the endoplasmic reticulum (ER). After the signal peptide is cleaved at Gly17-Ala18, the peptide is cleaved by dibasic amino peptidase action at two sites (followed by carboxypeptidase activity) to yield big ET- 1. This is then cleaved by endothelin converting enzyme (ECE) at Trp73-Val74 to yield the mature 21 -amino acid peptide, endothelin.

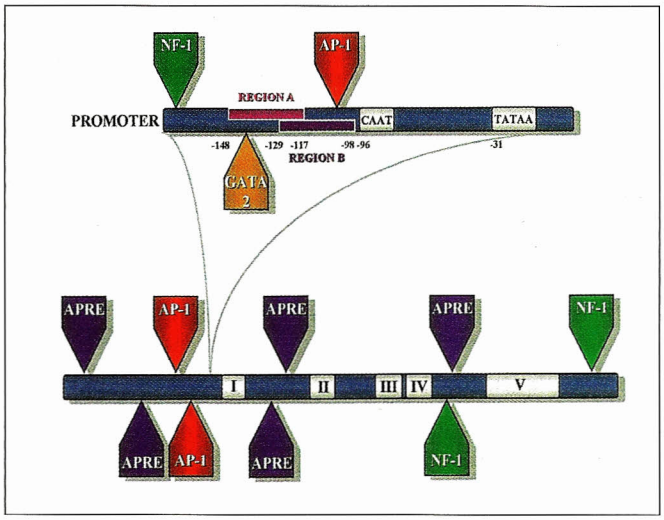

Figure 3. Map of the human ET-1 gene. The 6.8 kb coding region contains five exons (regions I, II, III, IV, V), four introns, and 5'- and 3'-flanking regions. Each of the exons encodes a portion of the preproET peptide. Approximate locations for several regulatory binding sites and sequences, including GATA-2, AP-1, nuclear factor-1 (NF-I), and acute phase reaction regulatory elements (APRE) are shown.[3] Malek has also demonstrated the existence of a cis-acting shear stress responsive element (SSRE) between -2.5 kb and -2.9 kb from the transcription initiation site.

association with other eukaryotic genes.[28,29] Site-directed mutagenesis of this sequence abolished binding to Region B and markedly decreased the transcriptional activity of the ET-1 promoter. The role of the ET-1 AP1 consensus sequence site as a functional sequence in Region B is thus firmly established. Electromobility shift assays using anti-Fos and anti-Jun antibodies have confirmed binding by members of the Fos and Jun nuclear protein families to the ET-1 AP1 sequence, and transactivation studies demonstrate that c-fos and c-jun are capable of stimulating expression through the AP1 sequence. These data support the thesis that the binding of Fos and Jun to the ET-1 promoter reflects a functional interaction.[25]

TRANSCRIPTIONAL REGULATION

There is much about the pathways involved in the synthesis and release of ET that is yet to be revealed. A direct correlation between preproET-1 mRNA levels and secretion of ET-1 exists, suggesting that ET-1 regulation occurs at the transcriptional level. A known exception is the modulation of ET-1 synthesis by atrial natriuretic peptide (ANP) which occurs at the posttranscriptional level by reducing the amount of preproET-1 available for processing and secretion.[30] A wide variety of hemodynamic conditions, growth factors, cytokines, and other vasoactive substances have been found to affect ET-1 production.

Hemodynamic Forces

Vascular endothelial cells are continuously subjected in vivo to the mechanical forces of shear stress and cyclic strain. Shear stress is a function of the tangential force applied by the resistance of flowing blood across the endothelial cell (EC) surface. Cyclic strain is a function of a pressure-induced mural distention resulting in a cyclic expansion and contraction of the blood vessel. Shear stress has been shown to activate a membrane potassium channel, increase tissue plasminogen activator production and mRNA levels, and to transiently modulate endothelin mRNA levels as well as the production of prostacyclin.[31-35] Morphologically, shear stress induces cell alignment parallel to the direction of flow, while cyclic strain causes alignment perpendicular to the principal axis of strain.[36-38] EC subjected to cyclic strain in vitro proliferate at a faster rate,[39] have altered morphologic features,[40,41] and secrete different amounts of vasoactive molecules compared with EC maintained in a stationary environment.[42]

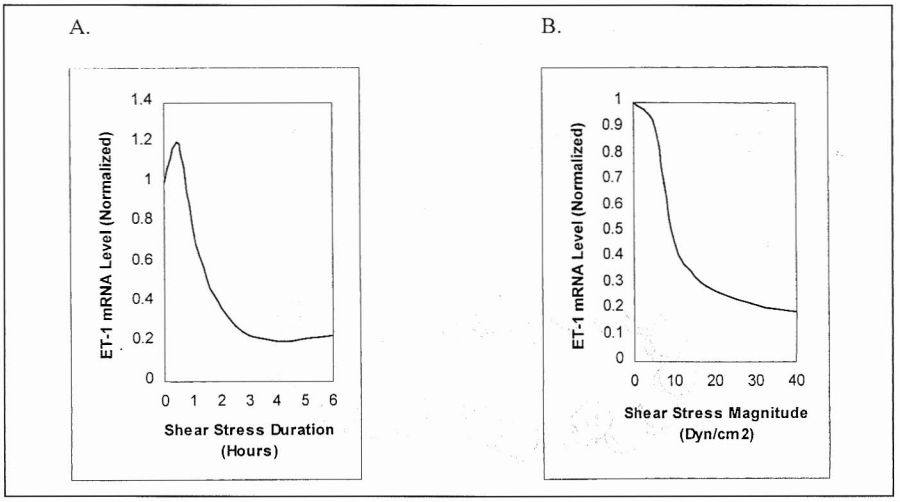

Figure 4. Regulation of ET-1 in response to shear stress. ET-1 mRNA regulation by shear stress is a time-dependent and magnitude-dependent process. The early transient upregulation is followed by a down-regulation that is mediated by a shear stress responsive element (SSRE) independent of protein kinase C (PKC).[78]

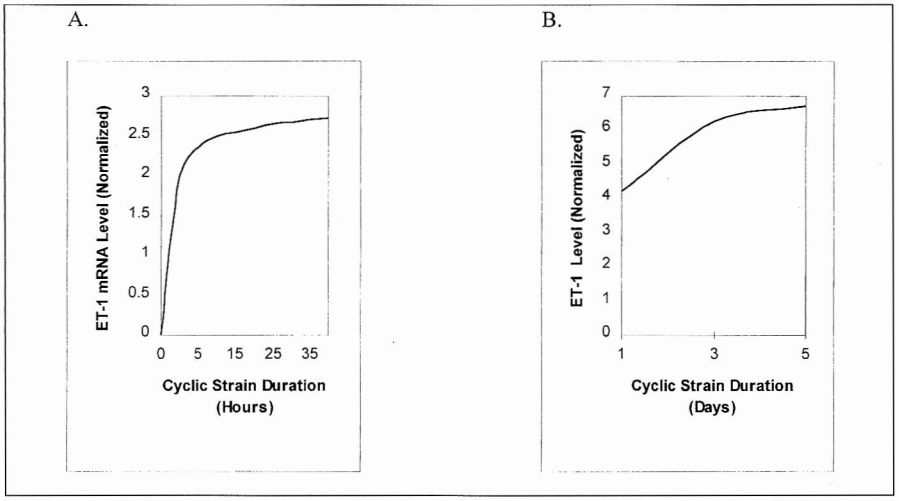

Figure 5. Regulation of ET-1 in response to cyclic strain. (A) ET-1 mRNA expression in HUVECs subjected to strain is increased.[84] (B) A rapid and prolonged increase of ET-1 secretion is also seen in BAECs subjected to a maximum strain of 24% (-20 kPa) at 60 cycles/min.[85]

ET-1 was the first vasoactive substance found to be subject to regulation by fluid flow. Yanagisawa et al. initially reported down-regulation of ET-1 gene expression in response to shear stress[5] with Yoshizumi et al. later reporting transient upregulation by shear stress of 5 dynes/cm^2 in porcine aortic endothelial cells.[34] Later, decreased ET-1 mRNA was described in both HUVECs and BAECs shortly after the onset of shear (< 1 h) at levels in excess of 15 dynes/cm^2 (Figure 4).[43-46] Kuchan and Frangos, in measuring ET-1 peptide levels rather than mRNA expression, have proposed two mechanisms for the shear stress regulation of ET-1. The early upregulation, which is prominent at low shear stress, is independent of magnitude and mediated by the activation of PKC; the late downregulation is dependent on nitric oxide (NO).[47] Malek et al., however, was unable to prevent downregulation with the NO-synthase inhibitor L-NMMA .[48] Noris et al. has recently suggested that ET-1 gene expression is downregulated independent of NO since it was observed under shear stress conditions where no concomitant change in NO production was seen.[44]

Morita et al. have suggested that the early transient upregulation of ET-1 is mediated by G-actin, which increases in response to shear in a $[Ca^{2+}]_i$ and PKC-dependent process, and is dependent on the integrity of microtubules.[49,50] Following disruption of F-actin into G-actin, ET-1 mRNA levels in endothelial cells increased, with a peak at 6 h. This increase in

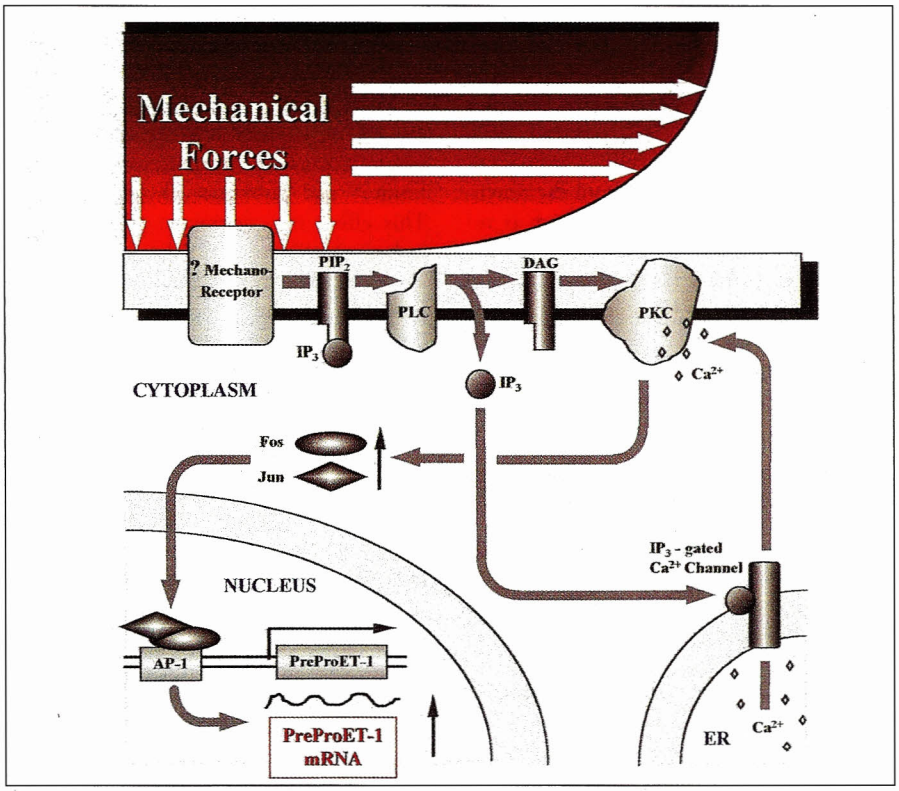

Figure 6. Proposed mechanism for ET regulation by mechanical strain. Acting through some type of mechanoreceptor, strain activates Phospholipase C(PLC) which catalyzes the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 diffuses through the cytoplasm and activates IP_3 -gated Ca^{2+} channels releasing intracellular Ca^{2+}. Protein kinase C (PKC) is activated by DAG and Ca^{2+}, and induces Fos and Jun which then translocate into the nucleus, bind at the AP- 1 site, and cause upregulation of preproET-1.

stressed cells was abolished by the F-actin stabilizer, phalloidin, which failed to inhibit increases in cells subject to other inducers. Malek et al. have demonstrated that the downregulation of ET-1 mRNA is independent of PKC, and is transcriptionally mediated by a cis-acting shear stress responsive element (SSRE) site between -2.5 kb and -2.9 kb of the 5' end of the ET-1 transcription initiation site (distinct from the AP-1 and GATA-2 DNA binding sites).[51] Downregulation required ongoing protein synthesis while the upregulation did not. Further, downregulation was dependent on the mean magnitude of shear rather than on small shear variations.

The effects of cyclic strain due to the pulsatile oscillation of systole and diastole, have not been as well characterized. Different lines of evidence suggest that the response of EC to shear stress and cyclic strain are different. It is well established that endothelin production is increased in EC exposed to cyclic strain. Wang et al. have demonstrated increased endothelin gene expression HUVECs exposed to cyclic strain.[52] Our laboratory has shown that stretching BAECs produces a rapid and prolonged increase in endothelin release fivefold to sixfold above basal rates (Figure 5).[53] This increase is dependent on the strain regimen to which the cells are subjected.[54,55]

The protooncogene c-fos has been induced in human endothelial cell subjected to shear stress,[56] but the mechanism in cells under strain is not clear. Stretched cardiac myocytes have induced protooncogene c-fos with a possible role for PKC.[57,58] PKC is known to be activated by diacylglycerol. Stretch can cause a sustained increase in cardiac myocyte diacylglycerol cellular content.[59] Our laboratory has previously shown that cyclic strain increases inositol triphosphate and diacylglycerol levels in endothelial cells and that phosphatidylcholine hydrolysis by phospholipase D can contribute to the sustained diacylglycerol formation in endothelial cells subjected to cyclic strain.[60,61] The sustained elevation in diacylglycerol can conceivably activate PKC which could then induce ET-1 mRNA expression in strained cells. Further, PKC activation leading to the induction of protooncogenes fos and jun, followed by binding to the AP-1 consensus sequence in the ET-1 promoter has been suggested to be essential for ET-1 gene expression.[25,62,63] Wang et al. have observed that treatment with the PKC inhibitor, calphostin C, abolishes ET-1 induction, and that cAMP and cGMP-dependent protein kinase inhibitors only partially inhibited inducton.[64] In whole, these findings suggest that ET-1 induction is mediated primarily through the PKC pathway (Figure 6).

Strain increases intracellular Ca^{2+} concentration.[65] This may be due to the increased levels of inositol triphosphate (IP3)or increased Ca^{2+} influx. Recently, it was shown that BAECs exposed to strain increase calcium release from intracellular stores, and mechanical stimulation may cause an alteration in cell membrane permeability to calcium.[66] This may involve stretch-activated ion channels.[65]

Other Modulators

Induction of ET-1 mRNA in endothelial cells has been observed with various growth factors and cytokines such as thrombin,[67] transforming growth factor-ß (TGF-ß),[68] tumor necrosis factor-α (TNF-α),[69] and insulin.[70]

Thrombin induces the ET-1 gene and ET-1 peptide synthesis by a PKC-independent PTK-dependent pathway in both macrovascular (HUVECs and bovine pulmonary artery endothelial cells (BPAECs)), and microvascular endothelial cells (human microvascular endothelial cell line (HMEC-1)).[71] Thrombin also induced ET-1 release in BAECs, possibly through activation of PKC and mobilization of intracellular Ca^{2+} resulting from receptor-mediated phosphoinositide breakdown.[67] An immediate and dose-dependent formation of IP3 with a concomitant increase in intracellular Ca^{2+} concentration was seen which was abolished by phospholipase C inhibitor, PKC inhibitor, and an intracellular Ca^{2+} chelator.

TGF-ß induces the expression of ET-1 mRNA in endothelial, smooth muscle, and mesangial cells in a process that is possibly mediated by NF-1[68,72,73] Rossi et al. had previously shown that NF-1 mediates the TGF-ß-induced transcriptional activation of a collagen promoter.[23]

Nuclear transcription runoff analysis showed that TNF-α increases ET-1 gene transcriptional rates in a process that was not dependent upon protein synthesis and that was not modified by thrombin cotreatment. This suggests that thrombin and TNF-α share common transcriptional activator mechanisms.[69] Gamma-interferon alone had no effect on ET-1 mRNA expression, but it potentiated the effects of

TNF-α when endothelial cell cultures were cotreated with the two, and antagonized the stimulatory effects of TNF-α when the cultures were pretreated with γ-interferon.

Oliver et al. suggested that insulin stimulates ET-1 expression in BAECs mostly through a PKC-independent pathway, and possibly exerted its regulatory effect at the transcriptional level by an insulin-responsive element in the ET-1 5'-flanking region.[70]

Additionally, angiotensin II, bradykinin, oxidized or acetylated low-densitry lipoprotein, and hypoxia have been shown to affect ET-1 gene expression.[3] In a series of experiments on cultured endothelial cells from rat hearts, angiotensin II was shown to induce ET-1 mRNA in a dose- and time-dependent manner. Calphostin C abolished this effect suggesting that this induction was mediated by the PKC pathway.[74] ET-1 secretion is significantly increased by bradykinin in cultured glomerular capillary endothelial cells in a biphasic manner that is regulated by phospholipase C.[75]

There have been conflicting reports as to the effect of hypoxia on ET-1 regulation. ET-1 mRNA levels in lung tissues in hypoxic anesthetised sheep increased in proportion to the decrease in pulmonary blood flow. ET-1 levels returned to baseline when the hypoxia was reversed.[76] Circulating levels of ET-1 and ET-1 gene expression was also found to be enhanced in rats exposed to hypoxia.[77] Recently, however, exposure of rat lung endothelial cells to hypoxia for 24h decreased ET-1 production by 50% compared to control.[78]

It has also been demonstrated that exposure of human endothelial cells to sickled erythrocytes resulted in a four to eight-fold transcriptional induction of the gene encoding endothelin.[79] Expression of the ET-1 gene is also up-regulated during in vitro cellular aging of human umbilical vein cells.[80]

The 3' untranslated region of the ET-1 mRNA, conserved among different species, contains 2 AUUUA sequences that are commonly thought to be involved in the highly specific, translation-dependent destabilization of mRNA, thus providing an additional regulatory mechanism by which the cellular level of ET-1 mRNA may be controlled by post-transcriptional message degadation.[9,11]

Further, ET-1 gene transcription can occur from one or more distal promoter elements. A second class of ET-1 mRNA has been discovered that is transcribed from a promoter region upstream from the original transcription start site identified in BAECs.[81] This upstream promoter was able to induce CAT reporter gene transcription in COS-1 cells, a kidney endothelial cell line, even without the downstream GATA-2-binding site which is required for efficient transcription of ET-1 mRNA in BAECs. Distinct mechanisms for the regulation of ET-1 gene expression thus appear to exist in these two cell types.[3] This may be an important consideration when comparing disparate data obtained from different cell lines.

CONCLUSIONS-CLINICAL IMPLICATIONS

The discovery in 1985 of a peptidergic EDCF which was produced by cultured endothelial cells created a genuine sense of excitement amongst the global research community and spurred a flurry of research activity in a number of diverse fields. Based on the data generated by the plethora of studies that resulted, several hypotheses have been proposed as to the role of endothelin in both health and disease.

It has been suggested that the balance of production between vasoconstricting and vasorelaxing factors maintains a homeostatic vascular tone. Any disturbance of this balance could lead to a pathologic increase or decrease in tone.[82] There is an increased release of endothelin from the neurohypophysis during water deprivation, and it has been demonstrated that centrally applied endothelin can inhibit water intake.[83,84] Endothelin may be involved in a compensatory homeostasis that controls water balance. Increased levels of circulating endothelin was seen in severe hypotension (cardiogenic, septic, and hemorrhagic shock). Recent reports suggest that endothelin may protect the organism from hemorrhagic conditions both locally and systemically since both thrombin and TGF-ß have been shown to be potent stimulators of ET synthesis and release.[3]

Endothelin is also speculated to play a role in atherogenesis. Atherosclerotic lesions more commonly occur at bifurcations and branch regions, where fluid shear stress is lower than in straight segments.[64] Cyclic strain is also higher at these sites than in straight segments.[85] The low shear and high strain may lead to local elevation of ET and thus contribute to the formation of atherosclerotic plaques by increasing smooth muscle cell proliferation. In this regard, elevated ET-1 mRNA expression in atherosclerotic lesions has been clearly demonstrated.[86]

High plasma levels of ET have been observed in certain hypertensive populations, especially in patients with systemic,[87] and pulmonary hypertension.[88,89] This effect may aggravate atherosclerosis in hypertensive patients due to the generalized increase in vascular tone. ET is also postulated to be a mediator of local increases in vascular tone, vasospasm. The long-lasting and potent vasoconstriction caused by ET make it ideal to mediate the initiation or maintenance of vasospasm.[3]

Despite these speculations, however, much work remains to be done in characterizing the regulation of ET and its role in physiologic and pathologic states. The recent discovery of ET-receptor antagonists have shed new light on ET's importance in the pathophysiology of multiple disease states. With novel models and new technology, we can expect further exciting breakthroughs in the coming years. **STI**

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