

Endothelins in Pulmonary Development

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Abstract

Endothelins (ETs), first discovered as potent vasoconstrictors, are a family of polypeptides with a wide range of activities in the airways and pulmonary vascular system. Synthesized by respiratory epithelium and by pulmonary vascular endothelium, ETs, as a potent bronchoconstrictors and mitogens, may play a role in the pathogenesis of pulmonary hypertension and bronchial asthma. ETs are a part of a complex homeostatic mechanism in mature airways however, a critical biological role of ETs have been implicated of modulating the development of the lung and vasculature, of Hirschsprung's disease, of asthma and potentially pulmonary hypertension. Lack of ET-1, ET_A receptor or endothelin-converting enzyme-1 (ECE-1), as result of complete knockout of the ET-1, ET_A receptor or ECE-1 genes, induces abnormalities of the pharyngeal-arch-derived craniofacial tissues and death by respiratory failure at birth. In humans and mice normal development of epidermal melanocytes and enteric ganglion neurons are affected by ET-3 and ET_B receptor genes. Mutations in the ET_B receptor gene mapped to chromosome 13 resulted in development of Hirschsprung disease in humans. Taken together, compelling evidence suggests that ET-1 may contribute to smooth muscle growth, vasculature, airways remodeling as well as modulate cardiopulmonary development.

Key words: growth, lung, respiratory, vascular and airway remodeling, signal transduction.

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Endothelins (ETs), a family of polypeptides with a wide range of activities in the airways and pulmonary vascular system, are synthesized by respiratory epithelium and by pulmonary vascular endothelium. As a component of a complex homeostatic mechanism, ETs may play a role in the pathogenesis of pulmonary hypertension and bronchial asthma. Developmental studies have also elucidated the importance of ET-1 for cardiopulmonary development.

Endothelins and their Receptors in the Lung

The endothelins, first discovered as potent vasoconstrictors, are produced by aortic and pulmonary artery endothelial cells [37, 69, 70]. In 1988, endothelin-1 (ET-1) was isolated, purified, sequenced, and cloned [106]. The existence of two other genes distinct from the ET-1 gene, which encode two distinct peptides, namely, ET-2 and ET-3, was also discovered [41, 42]. ETs comprise polypeptides of 21 amino acids with two disulfide bridges between cysteine residues, a hydrophobic tail and a terminal tryptophan residue [67, 106]. The biosynthesis of the endothelins (ETs) includes synthesis of a large mRNA-encoded precursor

preproendothelin; peptide cleavage yields a smaller proendothelin, the big ET, and endothelin-converting enzyme (ECE) then converts big ET to mature ET [71, 103]. ECE, a member of the zinc metalloprotease family, includes isoforms ECE-1a, ECE-1b and ECE-2. These enzymes convert big ETs to ETs and function with high substrate specificity [94]. ECE-1a and ECE-1b, products of the same gene, differ only in their N-terminal [85]. There exist differences in the transcriptional regulation of ECE-1a and ECE-1b: ECE-1a is present constitutively in some cells, whereas ECE-1b is inducibly expressed in response to specific stimuli [94]. ECE-2, a product of a separate gene, differs from ECE-1 in its optimum activity at acidic pH and in its sensitivity to inhibitors [18]. Recently, novel ECE was purified exerting high specificity to big ET-3 [32]. ET catabolism is regulated by neutral endopeptidase, a membrane metalloendopeptidase, which cleaves mature ETs and renders the peptides biologically inactive [88].

Airway epithelium and endothelium are also sites of ET synthesis in lung [8, 24, 81, 82]. *In situ* hybridization studies revealed the presence of large

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quantities of ET mRNA in human lung [68] and in bronchial epithelial cells of rat fetal lung [57]. Mattoli et al. demonstrated that ET-1, secreted by human bronchial epithelial cells, specifically binds to receptors on human bronchial smooth muscle cells in culture [61]. Growth factors and inflammatory cytokines modulate synthesis and release of ETs from epithelium. Endo et al. demonstrated that IL-8, TNF α and TGF β stimulated synthesis of ET-1, and IL-1 induced the synthesis of the ET-1 precursor big ET-1, whereas IL-2, IL-6 and IGF-1 stimulated the synthesis of big ET-1 and proliferation of guinea pig airway epithelial cells [19]. Thrombin induced release of ET from isolated rabbit tracheal epithelial cells [101]. Taken together, these data suggest that proinflammatory cytokines and growth factors may play a role in ET synthesis which, in turn, may modulate airway smooth muscle contractility and proliferation.

Pharmacological studies have suggested the presence of specific ET receptors. In 1990, two subtypes of ET receptors were cloned. The ET_A receptor, a 48 kDa protein, was characterized from bovine lung [4], and the ET_B receptor, a 47 kDa protein, was cloned from rat lung [83]. Both receptors have seven hydrophobic transmembrane domains and are similar to other G-protein-coupled receptors. The ET_A receptor subtype exhibits specific and high-affinity binding in rank order toward endothelin isopeptide ligands: ET-1 $\geq$ ET-2 $\gg$ ET-3 [22]. The ET_B receptor subtype equally associates with all ET isotypes.

A new subtype of the ET_B receptor, called the ET_{B2} receptor, was recently cloned by reverse transcriptase-PCR of an avian cDNA [52]. This receptor is abundantly expressed in melanoblasts and melanocytes, and is also present in liver and kidney. Pharmacological studies demonstrated that the ET_{B2} receptor binds with equal affinity to ET-1, ET-2, and ET-3 [52].

Recently, specific high-affinity binding sites for ET-1 were demonstrated in the airway of human, guinea pig, pig, rat and mouse smooth muscle, peripheral lung alveolar wall tissue and pulmonary arteries [25, 35, 36, 75]. Data suggest the existence of a predominant tissue-specific distribution of ET receptor subtypes [26, 34]. ET-1-induced ovine airway smooth muscle contraction was mediated through activation of the ET_A receptor isotype [26]. BQ-123, a selective ET_A receptor antagonist, abolished ET-1-induced contraction of human and guinea pig pulmonary artery, but did not affect contraction of human bronchus [34]. Sarafotoxin (S6c), a selective agonist of the ET_B receptor, induced contraction of guinea pig and human bronchus, but had no effect on pulmonary artery contraction. These findings suggest that in human and guinea pig bronchus, ET-1 predominantly stimulates the ET_B receptor subtype, and in human and guinea pig, pulmonary artery, ET-1 stimulates the ET_A receptor subtype [33,

34]. Other investigators demonstrated that BQ-123, an ET_A receptor selective antagonist, and BQ-788, an ET_B receptor selective antagonist, significantly antagonized ET-1-induced contraction of human isolated bronchi [21]. These data demonstrate that both ET_A and ET_B receptors are involved in ET-1-induced contraction in human bronchi.

Endothelins and Lung Development

The role ET-1 in cardiovascular and pulmonary development

Mutation and gene knockout studies have demonstrated the important role of ET-1 in cardiovascular and pulmonary development. Complete knockout of the ET-1 gene in mouse (ET-1/- homozygous mice) induced morphological abnormalities of the pharyngeal-arch-derived craniofacial tissues and newborn death due to respiratory failure [49]. ET-1/- homozygous mice developed multiple cardiovascular malformations that include interrupted aortic arch, tubular hypoplasia of the aortic arch, aberrant right subclavian artery, and ventricular septal defect with abnormalities of the outflow tract [48]. Respiratory failure in ET-1/- homozygous mice was due to mechanical obstruction of the upper airway and due to inability to open the mouth because of poor mandibular musculature [49]. It was demonstrated that ET-1 is expressed and appears to be important in respiratory control by the nervous system [23, 47, 50]; therefore, respiratory failure in ET-1/- homozygous mice could be due to an abnormality in central respiratory control. ET-1+/-heterozygous mice developed high blood pressure, suggesting the existence of an ET-1-mediated mechanism that may alter hemodynamic homeostasis. Also, a selective ET_A receptor antagonist BQ123 or neutralizing antibody to ET enhanced the frequency and the extent of these morphological abnormalities [48]. Targeted disruption of the ET_A receptor and ECE-1 genes revealed identical defects seen in ET-1-deficient embryos [12, 104, 105]. Together, these data, suggest that ET-1, ET_A receptor and ECE-1 are essential for normal cardiac and pulmonary development.

Analysis of microvascular endothelial cells from ET-1 knockout mice, however, revealed that lack of ET-1 expression did not alter endothelial cell morphology and growth [59]. Maemura et al. studied gene expression of ET-2, ET-3, ET_A and ET_B receptors in ET-1+/+ wild-type, ET1+/-heterozygous, and ET-1/-homozygous mice [58]. ET-1 gene knockout had little effect on endothelial cell mRNA levels of ET receptors. These data demonstrate that ET-1 expression is not essential for growth of endothelial cells.

Hoher et al. studied the effects of human ET-1 gene under the control of its natural promoter transferred into

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the germ line of mice [38]. This gene was predominantly expressed in brain, lung and kidney and was associated with development of glomerulosclerosis, interstitial fibrosis, and renal cysts. ET-1 gene expression had little effect on plasma and tissue levels of ET-1. Despite significant pathophysiological changes, ET-1 overexpression did not induce hypertension. These data suggest the existence of complex ET-mediated mechanisms involved in the development of such pathophysiological conditions.

ET-3 and ET_B receptor expression and development

Recent studies have demonstrated an important role of ET-3 and ET_B receptor genes in normal development of epidermal melanocytes and enteric ganglion neurons in humans and in mice. In humans, mutations in ET_B receptor gene mapped to chromosome 13 resulted in development of Hirschsprung disease (aganglionic megacolon) [2, 6, 39]. Heterozygous ET_B receptor gene mutations accounted for a frequent congenital malformation related to multigenic neurocristopathy. Homozygous ET_B receptor gene mutations in animals result in Hirschsprung disease-Waardenburg-Shah syndrome, aganglionic megacolon associated with pigment abnormalities [2, 22].

Hosoda et al. also studied the effects of targeted mutations of ET_B receptor gene [39]. Knockout of the murine ET_B receptor gene induced aganglionic megacolon and coat color spotting. These developmental defects were identical to those which occur in mice with spontaneously piebald-lethal mutation and were the result of complete deletion of the ET_B receptor gene. Mutations in the ET_B receptor locus resulted in lower levels of ET_B receptor mRNA and receptor expression, which was manifested as white coat spotting, but not aganglionic megacolon [39]. These data suggest that disruption of ET_B receptor gene may alter normal colonic development that appears to mimic hereditary Hirschsprung disease.

Other studies of ET_B receptor and ET-3 gene expression in animals with hereditary defects in the development of epidermal melanocytes and ganglion neurons also revealed the presence of null mutation in these genes. Analysis of the ET_B receptor gene of the spotting lethal rat, which exhibit aganglionic megacolon and pigment abnormalities, revealed a 301 base pair deletion in the exon 1-intron 1 junction [22]. This deletion produced an aberrant ET_B receptor lacking the first and second putative transmembrane domains of the G-protein coupled receptor, which completely abrogated functional activity of ET_B receptor. Baynash et al. found that interaction of ET-3, a ligand for ET_B receptor, was also necessary for development of epidermal melanocytes and enteric neurons [6]. These investigators demonstrated that the lethal spotting mice carry a

missense mutation in ET-3 gene. This mutation produces an inactive ET-3 intermediate with a substituted arginine residue with tryptophan in the C-terminal domain of big ET-3. This substitution makes it impossible to cleave big ET-3 by ECE-1 and produce mature ET-3. These data demonstrate the essential role of ET-3, the ET_B receptor subtype and their interactions in normal mouse, rat and human organogenesis.

Enteric neurons and epidermal melanocytes are derived from neural crest cells. During embryonic development, neural crest cells are formed from the dorsal neural tube. After neural crest development, pluripotent cells migrate to form diverse tissues. Nataf et al. studied spatiotemporal expression pattern of the ET_B receptor gene in neural crest cells of normal avian embryo [66]. *In vivo* and *in vitro* experiments revealed that ET_B receptor mRNA was abundantly expressed in neural crest cells as well as in migratory neural crest cells. ET-3 and activation of the ET_B receptor were necessary for growth and differentiation of neural crest cells *in vitro* [51]. ET-3 also induced neural crest cell growth and differentiation into melanocytes. These data clearly suggest involvement of ET-3 and ET_B receptor in tissue development derived from neural crest cells.

Endothelins in fetal and postnatal growth

Multiple studies have demonstrated differences in ET localization and expression in fetal, postnatal and adult lung [11, 24, 31]. Giaid et al., utilizing immunohistochemistry and *in situ* hybridization techniques, examined the localization of endothelin-producing cells in developing and adult lung [24]. Endothelin-like immunoreactivity and ET-1 mRNA were found predominantly in pulmonary endocrine and endothelial cells of fetal lung. The level of ET-1 mRNA expression and ET-1 immunoreactivity were highest in fetal lung, decreased before birth, and lowest in adult. Chan et al. studied preproET-1 expression in different tissues from embryonic mouse [11]. PreproET-1 expression was detected in the bronchial epithelium and the endothelial cells of dorsal aorta and aortic arches. In the late stages of embryonic development, the level of preproET-1 expression decreased in the bronchial epithelium. In contrary, in vascular and lung endothelium, high levels of preproET-1 expression were found at the late phase of development. Quantitative autoradiographic studies also revealed the presence of both ET_A and ET_B receptor subtypes in alveolar wall tissue of fetal pig lung, indicating their possible role in fetal lung development [25].

Immunohistochemical studies of the pulmonary artery and vein of postnatal and adult rats revealed localization of ET-1 within pulmonary vascular endothelium, suggesting that ET-1 may play a role in modulating pulmonary vascular function [56].

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ET-1 effects on tracheal smooth muscle contractility also revealed age-dependent differences [30]. The potency of ET-1-induced smooth muscle segment contraction of the two-week-old rabbit was significantly higher as compared with the airway smooth muscle of adult animal. This effect was associated with significantly higher ET-1-induced formation of inositol 1,4,5-trisphosphate in immature smooth muscle as compared with adult tissue. H-7, a protein kinase C (PKC) antagonist, and nifedipine, a Ca^{2+} channel antagonist, decreased ET-1-induced contraction in both smooth muscle segments, although to a greater extent in immature tissue than observed in adult tissue. Interestingly, at 10 nM, ET-1 induced relaxation of tracheal smooth muscle, although this effect was significantly greater in the adult. The relaxation of adult smooth muscle was associated with ET-1-induced release of prostaglandins (PG) E_2 and I_2 . These data demonstrate that ET-1 exerts a potent duality of action that is dependent on the stage of maturation of tracheal smooth muscle.

Endothelin and Airway and Pulmonary Vascular Remodeling

Effects of ETs in vivo

ETs, first discovered as vasoconstrictors, were also reported to act as a potent bronchoconstrictor [33, 60, 62, 95]. The schematic presentation of vasodilation and vasoconstriction induced by ET-1 or ET-3 is shown in Figure 1. Studies of ET-1 effects on airway smooth muscle in guinea pig revealed that intravenous administration of ET-1 induced immediate bronchoconstriction and an increase in airway resistance. A biphasic response was also observed using determinations of tracheal smooth muscle active tension: a short initial relaxation response was followed by a prolonged increase in force generation [101, 102]. Tracheal contraction and bronchoconstriction were attenuated by pretreatment of tracheal rings with indomethacin and the removal of epithelium, although initial tracheal relaxation was not effected. Phosphoramidon, a metalloprotease inhibitor, mimicked the effects of epithelium removal on smooth muscle contraction [33, 82]. These data suggest that contractile responses require synthesis of cyclooxygenase metabolites, and ET-1 effects *in vivo* may depend on the presence of an intact epithelium.

Endothelin-activated receptors coupled to G-proteins and phospholipase C activation

ETs signal by binding to receptors, which belong to the G-protein-coupled seven-transmembrane domain receptor superfamily. In most vascular smooth muscle preparations, ET-1 binds to the ET_A receptor, and, in airways, ET-1 preferentially interacts with the ET_B

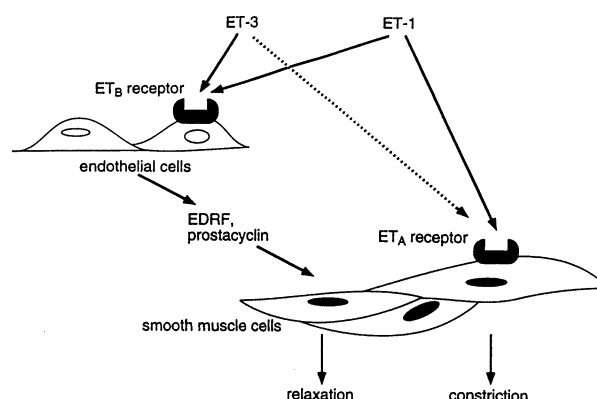


Figure 1. Endothelin-1 and endothelin-3 mediate vasodilation and vasoconstriction. ET_A receptor expression on smooth muscle manifests high affinity for ET-1 but low affinity for ET-3. Activation of ET_A receptor induces vasoconstriction. Expression of ET_B receptor on endothelium, which has equal affinity for both peptides, can release endothelium-derived relaxing factor (EDRF) or prostacyclin. The physiologic consequences of activation of a cell depend on the balance between expression of the ET_A and ET_B receptors (reproduced with permission from Goto et al. [27]).

receptor [33]. Activated receptors associate with G-proteins and activate phospholipase C (PLC) with hydrolysis of phosphatidylinositol-4,5-diphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Resink et al. demonstrated that ET-1 stimulated PLC activity in cultured vascular smooth muscle cells [76]. Studies also demonstrated that different G-proteins may be involved in ET signaling. Pertussis toxin, which ADP ribosylates Gi protein, significantly inhibited ET-1-induced phosphoinositide (PI) hydrolysis in cultured vascular smooth muscle cells [79]. In rat fibroblasts, however, pertussis toxin had no effect on ET-1-induced PI hydrolysis [63]. ET-1 stimulated adenosine 3',5'-cyclic monophosphate accumulation in canine tracheal epithelial cells [74] and in cultured rat vascular smooth muscle cells [17]. These data suggest that ETs can induce PLC and adenylate cyclase activation, which is cell specific and mediated through different G-proteins.

The second messengers of ET-induced PLC activation, IP_3 and DAG, also play an important role in intracellular signaling. IP_3 , through binding to its receptors on sarcoplasmic reticulum, induces release of Ca^{2+} [7, 45]. In response to ET-1 stimulation, rapid IP_3 accumulation was observed in isolated tracheal [30] and vascular smooth muscle cells [76, 96].

ET-induced activation of PLC and PI hydrolysis results in accumulation of DAG in vascular smooth muscle [28, 54], a second messenger involved in

activation of PKC. In addition to PLC-induced PI hydrolysis, DAG may also be a product of phosphatidylcholine hydrolysis by activated PLC or phosphatidic acid degradation by phospholipase D [82]. Resink et al. have shown in vascular smooth muscle cells that ET-1 stimulation induced hydrolysis of phosphatidylcholine and activation of phospholipase D [78].

Several investigators have demonstrated that PKC is involved in ET-1-induced signaling. Pretreatment of isolated rabbit tracheal smooth muscle with PKC antagonists, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, H-7 and staurosporine, inhibited ET-1-induced smooth muscle contractions [29, 30]. H-7 also blocked ET-1-induced contraction in rabbit pulmonary vein [89]. The specific PKC isoenzyme stimulated by ET remains unknown, and further studies are needed to define the role of PKC activation in mediating ET effects on smooth muscle cells.

ET-1 increases cytosolic calcium levels $[Ca^{2+}]_i$

ETs are potent spasmogens in trachea, bronchus and blood vessels isolated from a variety of species that include humans [1, 5, 29, 30, 60, 82]. Typically, ET-1-induced contraction develops slowly and is followed by a long sustained increase in force generation. Contractile effects of ET1, ET-2 and ET-3 are qualitatively similar but quantitatively different [1]. The potency of ETs for stimulation of human bronchial and pulmonary artery contraction are as follows: ET-1 > ET-2 > ET-3 [35].

ET-1-induced contraction appears to be mediated by activating PLC with a subsequent increase in cytosolic free calcium concentration. ET-activated PLC produces second messenger IP_3 which stimulates release of Ca^{2+} from intracellular stores (sarcoplasmic reticulum or calciosome) [55]. In isolated canine coronary artery, rapid PI hydrolysis was increased in parallel with stimulation of $^{45}Ca^{2+}$ uptake and contraction [73].

In most smooth muscle, ET-induced Ca^{2+} mobilization consists of two phases: a rapid transient phase and sustained phase. The rapid transient phase is induced by Ca^{2+} mobilization from sarcoplasmic reticulum; the sustained phase appears to be dependent on external cellular Ca^{2+} influx.

The ET-1-induced rapid increase in $[Ca^{2+}]_i$ in contrast to the subsequent sustained elevation of $[Ca^{2+}]_i$, is not sensitive to removal of extracellular Ca^{2+} [13, 45] or to pretreatment with L-type Ca^{2+} channel blockers [40, 45]. However, pretreatment of tissues or smooth muscle cells with caffeine in the absence of extracellular Ca^{2+} , [45, 97] or ryanodine, a selective inhibitor of Ca^{2+} release from the sarcoplasmic reticulum [97], reduced or completely inhibited the initial transient increase in $[Ca^{2+}]_i$. These data suggested that ET-1-induced

increases in $[Ca^{2+}]_i$ are due to Ca^{2+} mobilization from intracellular stores.

The sustained phase of ET-induced constriction appears to depend on activation of the voltage-operated L-type Ca^{2+} channels [106]. Studies have shown that the removal of Ca^{2+} from the medium or pretreatment of cells with antagonists of L-type Ca^{2+} channels (verapamil, diltiazem, nifedipine, nicardipine) attenuated ET-induced smooth muscle constriction of pig coronary arteries *in vivo* [16] and of isolated guinea pig, rabbit, human, or ovine bronchial smooth muscle [1, 26, 29, 60]. Using patch-clamp techniques to study single-channel Ca^{2+} currents, investigations have also shown that ET-1 does not directly activate L-type Ca^{2+} channels [64]. In cells clamped in the whole cell-attached mode, however, ET-1 did increase the activity of L-type Ca^{2+} channels [43]. These data suggested that ET induced activation of Ca^{2+} channels by stimulating selective second messenger systems.

Voltage operated Ca^{2+} channels (VOC) can also be activated by membrane depolarization. Studies have shown that ET-1 stimulation induced a sustained depolarization in bronchial, tracheal [53] and vascular smooth muscle [65, 98]. The mechanism of ET-induced membrane depolarization is poorly understood. Investigators have proposed involvement of Cl^- channels in mediating these effects. Takenaka et al. demonstrated that the Cl^- channel antagonist, indanyloxyacetic acid, inhibited ET-1-induced sustained phase of $[Ca^{2+}]_i$ increase and membrane depolarization in cultured vascular smooth muscle cells [40, 92]. Accordingly, ET-1 stimulated intracellular Ca^{2+} mobilization via generation of IP_3 , which activated Ca^{2+} -dependent Cl^- -permeable channels and induced an efflux of Cl^- and membrane depolarization which, in turn, activated VOCs and activated Ca^{2+} influx [82].

In addition to membrane depolarization, several second messengers were proposed to be involved in ET-1-induced stimulation of Ca^{2+} uptake into vascular smooth muscle cells. For example, in rat and rabbit vascular smooth muscle cells, platelet activating factor (PAF) antagonists (CV-6209 and WEB-2086) and inhibitors of arachidonic acid metabolism (e.g., indomethacin) significantly inhibited an ET-I-induced increase in $[Ca^{2+}]_i$ [91], suggesting that PAF and thromboxane (TX) A_2 (or other arachidonic acid metabolite) may also modulate Ca^{2+} uptake in some cell types [82].

ET-1 induces synthesis of prostaglandins (PG) and TXA_2

De Nucci et al. have determined that ET-1 induced release of PGI_2 and TXA_2 , products of arachidonic acid metabolism and phospholipase A_2 activation, in perfused isolated rat and guinea pig lung preparations [15]. ET-1 induced synthesis and release of PGI_2 and

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PGE₂ in rabbit tracheal epithelium and this effect was attenuated by pretreatment of cells with the cyclooxygenase inhibitor indomethacin [29, 30]. In vascular smooth muscle, ET-1 induced activation of phospholipase A₂ [77]. Schumacher et al. also reported that release of TXA₂ and activation of TXA₂ receptors are involved in ET-1-induced bronchoconstriction [84]. The consequence of ET-1 induced effects on arachidonic acid metabolism remains unknown. Clearly, blockade of cyclooxygenase abrogates the transient relaxation phase induced by ET in a variety of smooth muscle preparations but why a spasmogen induces relaxation and then sustained contraction remains unknown.

ETs and smooth muscle cell proliferation

ET-1 is a weak mitogen for cultured airway and vascular smooth muscle cells. Although the mitogenic effects of ET-1 are relatively weak in comparison with the effects of EGF and fetal calf serum, ET-1-induced mitogenesis is dependent on both pertussis toxin-insensitive and sensitive pathways in specific cell types [9, 93]. ET-1 does stimulate DNA and protein synthesis in pulmonary artery smooth muscle [44]; thus these data suggest that ET-1 may play a role in modulating the growth of pulmonary vascular smooth muscle.

ET-1 alone, however, does not induce significant human airway smooth muscle cell proliferation [72]. Interestingly, ET-1 potentiated mitogenesis induced by EGF, apparently via an ET_A receptor-mediated mechanism. ET_A receptor activation is involved in ET-1-induced mitogenic signaling in other mesangial cells [86]. Studies on lung organ cultures from dogs revealed that ET-1 increases positive staining for proliferation cell nuclear antigen in lung parenchyma [80]. BQ-123, an ET_A receptor antagonist, did not abolish this effect suggesting that in dog lung ET-1 signals through other ET receptor isotypes.

Takimoto et al. studied involvement of ET_A and ET_B receptors in ET-1-induced DNA synthesis in human lung cell line, CCD-18Lu, which expresses both receptor isotypes [93]. FR139317, a selective antagonist of ET_A receptor, completely inhibited ET-1-induced DNA synthesis. BQ-788 and IRL 2500, selective antagonists of ET_B receptor, partly inhibited ET-1-induced DNA synthesis suggesting that the ET_B receptor does not play a dominant role in ET-1-induced mitogenesis. In contrast to the mitogenic effect of ET-1, ET-3 did not reveal any stimulation of DNA synthesis [93].

Although ET-1-induced mitogenesis may require 12 to 18 hours to manifest, rapid desensitization of ET-1-stimulated ET_A and ET_B receptors occurs [20]. Receptor-specific, or homologous, desensitization was observed within four minutes in A10 cells transfected with human ET_A receptor, and in 239 cells expressing

either ET_A or ET_B receptor. Receptor desensitization coincided with ET-1-induced phosphorylation of each receptor. Freedman et al. demonstrated that inhibition of G-protein-coupled receptor kinases (GRKs) or dominant negative GRK2 mutant reduced ET-1-induced ET_A and ET_B receptor phosphorylation and abrogated receptor desensitization [20]. These data demonstrate that ET-1-induced signaling involves an activation of ET_A and ET_B receptors followed by rapid desensitization.

The activation of ET_A and ET_B receptors by ETs stimulates the mitogen-activated protein kinase (MAPK) cascade [10, 46, 99, 100]. A potential

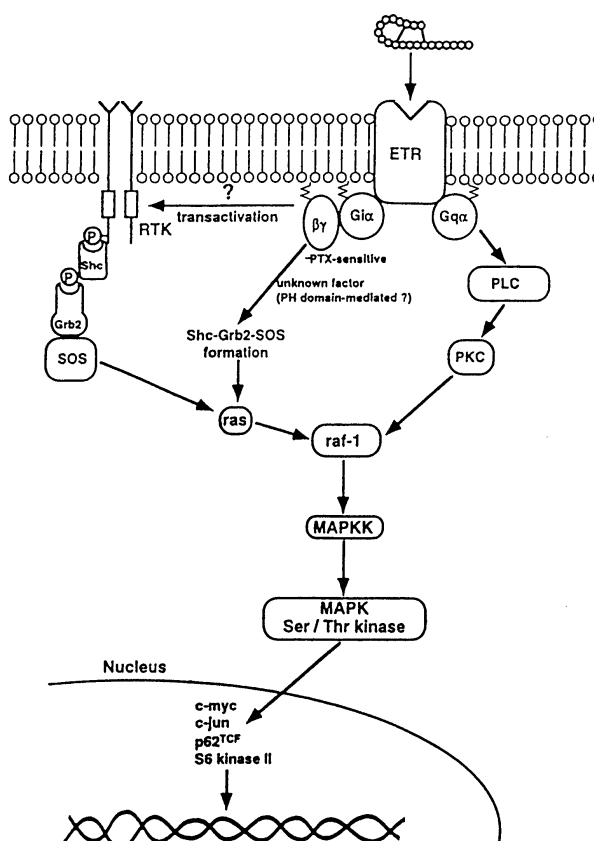


Figure 2. Endothelin-1 receptor activation which is coupled to G-proteins may induce activation of phospholipase C or possibly activate ras. The consequences of PKC and/or ras activation may lead to raf-dependent MAP kinase activation. Recent studies have suggested that ET receptor activation may alter responses generated from receptor tyrosine kinase (RTK) coupled pathways. ETR: endothelin receptor; RTK: receptor tyrosine kinase; G: GTP-binding protein; PLC: phospholipase C; PKC: protein kinase C; PTX: pertussis toxin; Shc: SH2-containing protein; Grb2: growth factor receptor bound 2; SOS: son of sevenless; MAPK: mitogen-activated protein kinase; MAPKK: MAPK kinase (reproduced with permission from Goto et al. [27]).

mechanism of MAPK kinase activation by ET-1 is shown in Figure 2. MAPK activation has been studied in order to determine the mechanisms underlying ET-induced mitogenesis. ET-1-stimulated MAPK activation was markedly inhibited by PKC downregulation [10, 100]. Investigators using PKC inhibitors, however, showed that PKC may be necessary but not sufficient for ET-1-induced mitogenesis, suggesting that alternative signaling pathways may be involved in ET-1-induced MAPK activation. Stimulation of MAPK by ET-1 is pertussis toxin-sensitive [46]. Daub et al. demonstrated that transphosphorylation of EGF receptor may also occur in response to ET-1 stimulation and is, dependent on G-protein-coupled receptors [14].

ET-1-induced signaling involves other members of the MAPK family. Aquilla et al. demonstrated that ET-1 activates the ET_B receptor and stimulates the extracellular regulated kinase (ERK) 2, c-Jun N-terminal kinase 1 (JNK) and p38 kinase [3]. Using a mutant of the ET_B receptor, they found that the C-terminal tail of the receptor is required for activation of these multiple MAPK signal transduction pathways.

ET-induced mitogenesis likely involves an activation of signaling pathways that alters gene transcription. In cultured rat vascular smooth muscle cells, ET-1 induced the expression of the immediate early response genes, *c-fos* and *c-myc* [27]. ET-1 stimulated tyrosine phosphorylation of the nonreceptor protein tyrosine kinase, pp60^{c-src}, which suggests a possible contribution of this tyrosine kinase in ET-1-induced mitogenic signaling [86, 90]. Simonson et al. also demonstrated that expression of dominant negative c-Src mutant and the COOH-terminal Src kinase (Csk), which both inhibit c-Src kinase activity, abrogated ET-1-stimulated *c-fos* transcription [87]. These data suggest that non-receptor tyrosine kinases play a role in ET-1-induced mitogenic signaling.

Summary

Current studies suggest a critical biological role of ETs in modulating the development of the lung and vasculature. Pathophysiologically, ETs may also play a role in Hirschsprung disease, asthma and potentially pulmonary hypertension. Mutation and gene knockout studies have also demonstrated an important role of ET-1 in cardiovascular and pulmonary function and development. Knockout experiments of the ET-1, ET_A receptor or ECE-1, as result of complete knockout of the ET-1, ET_A receptor or ECE-1 genes reveal that lack of ET-1, ET_A receptor or ECE-1 induces abnormalities of the pharyngeal-arch-derived craniofacial tissues and death by respiratory failure at birth. ET-3 and ET_B receptor genes may also play an important role in normal development of epidermal melanocytes and enteric ganglion neurons in humans and mice. In humans, mutations in the ET_B receptor gene mapped to

chromosome 13 resulted in development of Hirschsprung disease. ETs, first discovered as vasoconstrictors, were also reported to act as a potent bronchoconstrictor and mitogen. Taken together, compelling evidence suggests that ET-1 may contribute to smooth muscle growth, vasculature and airways remodeling as well as modulate cardiopulmonary development.

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