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MINIREVIEW

The Role of Endogenous Lung Neuropeptides in Regulation of the Pulmonary Circulation

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Summary

Vascular resistance in the mammalian pulmonary circulation is affected by many endogenous agents that influence vascular smooth muscle, right ventricular myocardium, endothelial function, collagen and elastin deposition, and fluid balance. When the balance of these agents is disturbed, e.g. by airway hypoxia from high altitude or pulmonary obstructive disorders, pulmonary hypertension ensues, as characterized by elevated pulmonary artery pressure (P_{PA}). Among neuropeptides with local pulmonary artery pressor effects are endothelin-1 (ET-1), angiotensin II (AII), and substance P, and among mitigating peptides are calcitonin gene-related peptide (CGRP), adrenomedullin (ADM), atrial natriuretic peptide (ANP), vasoactive intestinal peptide (VIP) and ET-3. Moreover, somatostatin$_{28}$ (SOM_{28}) exacerbates, whereas SOM_{14} decreases P_{PA} in hypoxic rats, with lowering and increasing of lung CGRP levels, respectively. Pressure can also be modulated by increasing or decreasing plasma volume (VIP and ANP, respectively), or by induction or suppression of vascular tissue remodeling (ET-1 and CGRP, respectively). Peptide bioavailability and potency can be regulated through hypoxic up- and down- regulation of synthesis or release, activation by converting enzymes (ACE for AII and ECE for ET-1), inactivation by neutral endopeptidase and proteases, or by interaction with nitric oxide (NO). Moreover, altered receptor density and affinity can account for changed peptide efficacy. For example, upregulation of ET_A receptors and ET-1 synthesis occurs in the hypoxic lung concomitantly with reduced CGRP release. Also, receptor activity modifying protein 2 (RAMP2) has been shown to confer ADM affinity to the pulmonary calcitonin-receptor-like receptor (CRLR). We recently detected the mRNA encoding for RAMP2, CRLR, and the CGRP receptor RDC-1 in rat lung. The search for an effective, lung selective treatment of pulmonary hypertension will likely benefit from exploring the imbalance and restoring the balance between these native modulators of intrapulmonary pressure. For example, blocking of the ET-1 receptor ET_A and vasodilation by supplemental CGRP delivered i. v. or *via* airway gene transfer, have proven to be useful experimentally.

Key words

Pulmonary hypertension • Hypoxia • Monocrotaline • Vasoconstriction • Vasodilatation • Receptors • Neuropeptide interactions

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Introduction

Pulmonary hypertension

Vascular resistance in the mammalian pulmonary circulation is influenced by many endogenous agents that may directly or indirectly affect vascular smooth muscle, right ventricular myocardium, endothelial function, collagen and elastin deposition, and fluid balance. The pulmonary circulation is known to develop hypertension independent of systemic blood pressure. The best known stimulus for development of pulmonary hypertension (PH) is airway hypoxia, encountered at high altitude. Other causes for airway hypoxia, and subsequently hypoxia-induced PH (HPH), are hypoventilation due to sleep apnea or restrictive pulmonary disorders such as congenital diaphragmatic hernia (O'Toole *et al.* 1996, Cutz *et al.* 1997) and atelectasis (respiratory distress syndrome) in infants and chronic obstructive pulmonary disease (COPD) and adult respiratory distress syndrome among adults (Zapol and Hurford 1993) and also pneumonia or sepsis. Moreover, infants who have died from sudden infant death syndrome carry markers suggestive of airway hypoxia and PH (Valdes-Dapena 1992). Other known causes of PH are ingestion of the pyrrilizidine alkaloid monocrotaline (Kay *et al.* 1982) and serotonergic pharmaceuticals such as the weight loss agents Fen-phen, Redux, and Aminorex (Gurtner 1985, Gaul *et al.* 1992, Abenhaim *et al.* 1996, Weir *et al.* 1999). Primary PH constitutes yet another category of PH, in which the etiology is unknown. Because PH is usually associated with right ventricular hypertrophy, pulmonary vascular hypertrophy and muscularization, and lung edema (Hunter *et al.* 1974, Hultgren 1978), restoration of normal pulmonary artery pressure (P_{PA}) is clinically challenging.

Conventional pharmaceutics

Many conventional pharmacological agents have been used to reduce pulmonary hypertension, including the β-adrenoceptor antagonist metipranolol (Ošťádal *et al.* 1978), heparin sodium (platelet-derived growth factor inhibition) (Kentera *et al.* 1985), teprotide (inhibition of angiotensin converting enzyme) (McKenzie *et al.* 1984), bradykinin (pulmonary vasodilation) (Gavras and Gavras, 1988), and prostacyclin (Magnani and Galie 1996) among others. However, these agents have unwanted side effects and limited efficacy (Cuiper *et al.* 1996, Kulkarni *et al.* 1996, Kesten *et al.* 1999). Kneussl *et al.* (1996) stated that no selective vasodilator was yet available. However, endothelium-derived relaxing factor nitric oxide (NO) has been shown to act as a pulmonary vasodilator (Leeman *et al.* 1994). Thus, NO is currently used for inhalation treatment of PH in some intensive care units (Muller *et al.* 1996, Nakagawa *et al.* 1997), often with moderately beneficial results (Mariani *et al.* 1996, Nakagawa *et al.* 1997). Moreover, potentially serious side effects such as formation of methemoglobin (Iwamoto *et al.* 1994, Offner *et al.* 1996), DNA breakage, and endothelial and airway epithelial injury by its metabolite, peroxynitrite, have been reported (Beckman *et al.* 1990, Gow *et al.* 1998). There is thus a good reason to turn the attention to endogenous lung neuropeptides of which many have vasoactive effects on the pulmonary circulation. This is further supported by the notion that pulmonary vascular pressure is primarily regulated locally within the lung (Daly and Hebb 1966, Laros 1971).

Pulmonary vasoconstrictor and dilator peptides

A number of endogenous lung peptides have pressor effects on the pulmonary circulation. Among these are: endothelin-1 (ET-1), angiotensin II (AII), arginine vasopressin (AVP), substance P (SP), and peptide tyrosine Y (PYY). On the other hand, examples of peptides that reduce P_{PA} are calcitonin gene-related peptide (CGRP), adrenomedullin (ADM), atrial natriuretic peptide (ANP), vasoactive intestinal peptide (VIP), ET-3, and somatostatin$_{14}$. While other endogenous lung peptides may also have vasoactive properties, the ones named here are the most studied. Moreover, ET-1 is the most potent constrictor and CGRP and ADM are the most potent vasodilator peptides. Therefore, special attention will be given to these three endogenous, pulmonary regulatory peptides. Homeostasis of P_{PA} requires harmony in the balance between ET-1 and CGRP in particular, and also in the net balance between all pressor and depressor peptides within the pulmonary circulation (Fig. 1). The changes in this balance upon hypoxia and other agents, and its effects on the pulmonary circulation, are summarized below together with a selection of relevant references.

Pulmonary vasoconstrictor peptides

Endothelins (ETs)

ET-1 is a potent 21 amino acid vasoconstrictor peptide in the systemic and pulmonary circulation, and it also has mitogenic effects on vascular endothelium and smooth muscle (Yanagisawa *et al.* 1988). ETs are

produced by vascular endothelial cells (Yanagisawa *et al.* 1988) and also by alveolar type II pneumocytes (Markewitz *et al.* 1995), they are released to the pulmonary circulation, and also have paracrine function. Furthermore mRNAs encoding ETs have been detected in rat pulmonary nerves and ganglia using autoradiography (McKay *et al.* 1991) or *in situ* hybridization (Keith IM, unpublished data), and in airway neuroendocrine cells (Giaid *et al.* 1991). Mast cells have also been shown to synthesize ET-1, and release ET-1 independent of degranulation (Ehrenreich *et al.* 1992). Three structurally related isoforms ET-1, ET-2, and ET-3 have been described. Several G-protein-coupled ET receptor subtypes exist (Inoue *et al.* 1989, Sakurai *et al.* 1992) and have been reported in pulmonary arteries (Cardell *et al.* 1992, guinea pig). The ET_A receptor binds preferably to ET-1 and ET-2 rather than ET-3 (Arai *et al.* 1990, bovine lungs), with the highest affinity for ET-1 (100 times higher than that for ET-3). ET_B binds non-selectively to ET-1, ET-2 and ET-3 (Sakurai *et al.* 1990, rat lungs) and appears to mainly recognize the C-terminal structure. Pulmonary ET-1 binding sites are mostly located on alveolar capillary endothelial cells (Furuya *et al.* 1992), and ET-1 and ET-2 binding sites have been reported on the smooth muscle of human pulmonary artery sections (McKay *et al.* 1991). [^{125}I]ET-1 binding using BQ123 (ET_A specific blocker) and ^{4}Ala-ET-1 also showed pulmonary blood vessels rich in ET_A, whereas the lung parenchyma displayed ET_B receptors (Nakamichi *et al.* 1992). Moreover, the observation that tissue mast cells carry ET_A receptors suggests that ET-1 can autoregulate its own release from these cells (Ehrenreich *et al.* 1992).

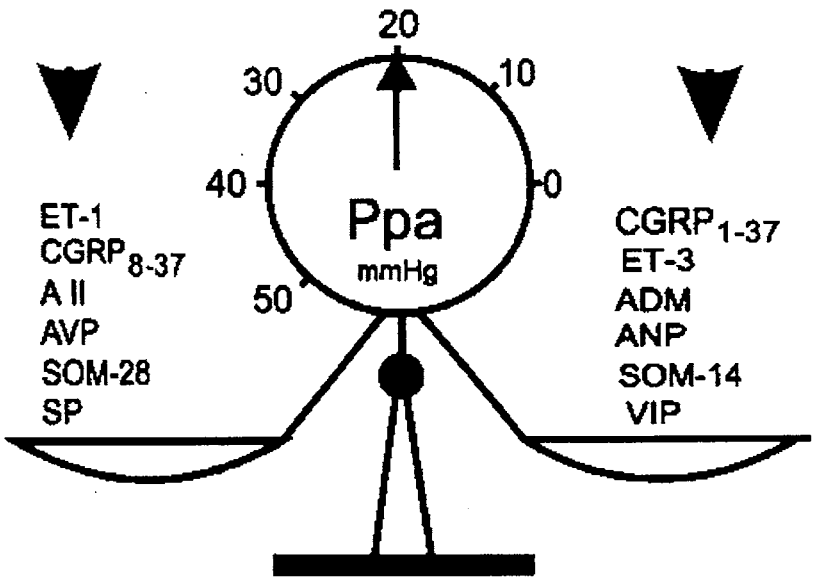

Fig. 1. *Peptidergic regulation of the pulmonary circulation can be likened by a scale tipping toward increase (left side) or decrease (right side) of the intrapulmonary vascular pressure, here exemplified in the rat. Upregulation of transcription and/or translation of ET-1 and the ET_A receptor, or downregulation or inhibited release of $CGRP_{1-37}$ or generation of inhibitory CGRP fragments, would shift the weight toward the left resulting in raised intrapulmonary pressure, perhaps pulmonary hypertension. On the other hand, overexpression of $CGRP_{1-37}$ or ADM, or blockade of the ET_A receptor, would shift the weight toward the right resulting in reduction in pulmonary pressure. Ultimately, the net effect of numerous pressor and depressor peptides, and their interactions, determines the pulmonary pressure. Homeostasis is achieved when these agents are in balance. See the list of abbreviations.*

Functional studies with ET-1 infusion demonstrated an initial transient vasodilation followed by long-lasting constriction. At resting pulmonary vasomotor tone *in vitro* rat lungs showed vasoconstriction (Lippton *et al.* 1991a), and vasodilation occurred while pulmonary vascular tone was enhanced with U-46619 and acute hypoxia (Lippton *et al.* 1991b, Eddahibi *et al.* 1991, Hasunuma *et al.* 1990). Also, infusion of low ET-1 concentrations is more likely to cause pulmonary vasodilation whereas higher doses induce dose-dependent vasoconstriction. Hence the ET-1 effect is sensitive to both pre-load and ET-1 concentration. However, ET-1's

vasodilatory effect was abolished by chronic hypoxia (Eddahibi *et al.* 1993). In fact, lungs from chronically hypoxic rats treated with ET-1 showed enhanced pressor effects compared to normoxic rats (Hasunuma *et al.* 1990, Tjen-A-Looi *et al.* 1996). In addition, Elton *et al.* (1992) showed increased ET-1 mRNA in lung tissue and right atrium after 48 h of hypoxia (10 % O_2), and Li and coworkers (1991, 1994a) implicated increased ET_A receptors in the pathogenesis of HPH. ET-1's role in HPH was further supported by Stelzner *et al.* (1992) who found doubled lung ET-1 levels and tripled prepro-ET-1 mRNA in normoxic fawn-hooded rats with idiopathic PH as compared with normotensive Sprague Dawley rats. Findings of concomitant increases in gene transcript levels for ET-1, and the ET_A (and also ET_B) receptors in lung tissue and pulmonary arteries during chronic hypoxia, but not in the great vessels or systemic circulation, indicate a selective pulmonary effect (Li *et al.* 1994b). Moreover, patients with PH have increased expression of endothelin-1 and its mRNA in endothelial cells of the pulmonary vasculature (Giaid *et al.* 1993, Cacoub *et al.* 1997), and also increased immunoreactivity for endothelin converting enzyme, which converts big ET-1 to the active ET-1 (Giaid 1998). Increased pulmonary artery ET-1 levels were also reported in COPD patients with PH, but not in those who did not develop PH (Celik and Karabiyikoglu 1998). Reduced lung clearance of ET-1, noted among patients with PH, may also contribute to the increased ET-1 levels and concomitant PH (Dupuis *et al.* 1998).

The role of endogenous ET-1 in HPH has also been demonstrated by prevention, reversal, or blunting of HPH development in rats treated with the selective ET_A receptor blockers BQ123 (Li *et al.* 1994a, Tjen-A-Looi *et al.* 1996), BMS-182874 and TBC112-51 (Holm 1997, Holm *et al.* 1998), and CI-1020 (Haleen *et al.* 1998), or intravenous ET antiserum (0.25 µl/rat/h, Tjen-A-Looi *et al.* 1996) during the hypoxic exposure. In addition to its vasoconstrictive function, ET-1 is also known to exert mitogenic effects on vascular smooth muscle (Komuro *et al.* 1988), which is antagonized by BQ123 (Ohlstein *et al.* 1992). Also, ET-1 mediates enhanced vascular permeability *via* the ET_A receptor as shown in the heart (Filep *et al.* 1992). On the other hand, ET-1's dilatory effect was shown to be mediated by ET_B receptors since the effect was abolished with the selective ET_B antagonist BQ-788 (Holm 1997). Furthermore, in a study of congenital diaphragmatic hernia in fetal, full-term rats, Okazaki and colleagues (1998) found that mRNA levels were increased for ET-1 and ET_A, but not ET_B, compared with normal controls, even though these rats were unborn and had not yet been breathing. These observations together clearly demonstrate a role of ET-1 in HPH, acting *via* ET_A receptors, and suggest that ET-1 is also involved in other etiologies of PH.

Angiotensin II (AII)

AII is a potent pressor peptide of the renin-angiotensin system, and is derived from conversion of AI to AII by angiotensin converting enzyme (ACE), located in caveolae of the pulmonary vascular endothelium. Most of AII's biological functions have been ascribed to the AT_1 receptor. For example, rats treated intravenously with the AT_1 receptor antagonist, GR138950C, during 7 days of exposure to hypoxia developed less HPH and remodeling compared with controls given saline (Zhao *et al.* 1996a), suggesting a role of AII in the early pathogenesis of HPH. Furthermore, in patients with HPH secondary to COPD, endogenous AII levels were lowered by ACE receptor blockade with captopril (Boschetti *et al.* 1985). In these patients, the expected vasodilatory effect was only obtained in conjunction with oxygen therapy, perhaps aided by ACE-induced increase in bioavailability of bradykinin, a potent vasodilator in the lung (Che and Bevan 1981). In another rat study, the ACE inhibitor, quinapril, reduced the development of HPH when given from onset of hypoxia, and partially reversed established HPH (Nong *et al.* 1996). It was concluded that AII's effects were primarily due to inhibition of vascular smooth muscle cell proliferation and/or growth. This is supported by the observation that AII stimulates proliferation of human pulmonary artery smooth muscle cells *via* the AT_1 receptors (Morrell *et al.* 1998).

Arginine vasopressin (AVP)

AVP is known to raise systemic blood pressure upon a sudden drop in pressure, and P_{PA} could also rise as a result. AVP's main pressor effect in the lung could be through its ability to dose dependently induce expression of prepro-ET-1 mRNA (Imai *et al.* 1992). However, in rats with HPH, AVP has also been found to lower P_{PA} indirectly by releasing ANP from the left atrium (Jin *et al.* 1989), and by reducing cardiac output (Nyhan *et al.* 1986).

Substance P (SP)

SP is a ubiquitous neuropeptide, also located in pulmonary perivascular sensory nerves (capsaicin-

sensitive C-fibers) together with CGRP (Cadieux *et al.* 1986, Ju *et al.* 1987). Lung SP levels in rats were elevated 1-3 weeks after monocrotaline administration (Lai *et al.* 1996). The SP elevation could have resulted from a documented reduction in levels of neutral endopeptidase 24.11 (NEP), the enzyme responsible for SP degradation (Lai *et al.* 1996). Elevated lung SP and reduced NEP were also noted in chronic intermittent hypoxia (Lai *et al.* 1995). However, SP is generally not considered a significant player in pulmonary pressure regulation, but it is essential in plasma protein extravasation (Gamse and Saria 1985) leading to perivascular pulmonary edema typical for HPH.

Somatostatin$_{28}$ (SOM_{28})

SOM is most known for its localization to pancreatic islet δ-cells, and has also been reported in pulmonary neuroendocrine cells and nerves (Dayer *et al.* 1985). Exogenous SOM_{28} was found to have an exacerbating effect on HPH in rats (Tjen-A-Looi *et al.* 1992). The mechanism for this action is not known, however, reduced lung tissue levels of the pulmonary vasodilator CGRP were associated with SOM_{28} infusion in the hypoxic rats. SOM exerts similar regulatory effects in other organ systems.

Peptide tyrosine Y (PYY)

PYY is a tyrosine-rich 36 amino acid member of the pancreatic polypeptide family. It has potent vasoconstrictive effects on some systemic vascular beds (Lundberg *et al.* 1982, Zukowska-Grojec *et al.* 1986), but has been little studied in the lung. Keith and Ekman (1990) demonstrated distinct PYY-like immunoreactivity in solitary neuroendocrine cells of the airway epithelium. Many of these cells were uniquely situated in alveolar ducts, i.e. in the gas-exchanging (respiratory) portion of the lung, suggesting local action restricted to the alveolar parenchyma and its capillaries. Immunoreactive PYY levels were doubled in lung tissue of rats with HPH, whereas blood levels were significantly reduced (Keith and Ekman 1992). Moreover, lung tissue and blood PYY levels among 50 normoxic and chronically hypoxic rats (17-21 days, 10 % O_2) correlated highly ($p<0.001$) with time in hypoxia and typical indicators of HPH, e.g. right ventricular pressure (reflects P_{PA}), lung weight, right ventricle to left ventricle plus septum weight ratio, percentage of capillaries with elastic lamina, and density of elasticized capillaries (Keith and Ekman 1992). Blood levels correlated inversely with these parameters, and also correlated highly with pulmonary artery medial thickness. These data suggest the possibility of an indirect or direct role for PYY associated with HPH.

Pulmonary vasodilator peptides

Calcitonin gene-related peptide (CGRP)

The 37 amino acid polypeptide CGRP is the most potent endogenous vasodilator peptide known to date (Brain *et al.* 1985, Wimalawansa 1996, van Rossum *et al.* 1997). It also counteracts hypoxia-induced tissue remodeling (e.g. right ventricular hypertrophy) associated with HPH (Tjen-A-Looi *et al.* 1992). There are two forms of CGRP, α and β, which differ in only 3 amino acids in humans and 2 in rats. αCGRP is derived from tissue specific, alternative mRNA splicing of the calcitonin gene (calcitonin being predominant in thyroidal C-cells) (Amara *et al.* 1982, Rosenfeld *et al.* 1983), and the β form is produced by a separate gene located on the same chromosome. αCGRP is prevalent in the lung and occurs in the sensory neural network, whereas βCGRP is common in intestinal neurons (Mulderry *et al.* 1988). CGRP-like immunoreactivity is localized in nerve fibers of the airway mucosa and around vascular smooth muscle (Cadieux *et al.* 1986, Tjen-A-Looi *et al.* 1998). Moreover, CGRP and its mRNA have been localized in the perikarya of intrapulmonary ganglia and in neuroendocrine cells of the airway epithelium (Keith *et al.* 1991). These neuroendocrine epithelial cells have been shown to function as airway oxygen sensors that respond to altered airway oxygen content (Lauweryns *et al.* 1977, Youngson *et al.* 1993) by modulating local pulmonary vascular tone. CGRP is therefore strategically localized, interconnecting neuroendocrine cells, airway epithelium and local vasculature in a local microcircuit (Tjen-A-Looi *et al.* 1998), and facilitating regional distribution of blood.

$CGRP_{1-37}$ belongs to a superfamily of closely related peptides (Wimalawansa 1997) of which both α and β forms, calcitonin$_{1-32}$, and ADM_{1-52} all derive from the human chromosome 11, whereas amylin$_{1-37}$ is generated from chromosome 12 (Christianson *et al.* 1990). Calcitonin$_{1-32}$, ADM_{13-52} and amylin$_{1-37}$ share both structural and functional homology with CGRP, although less potent, and are further related to the insulin superfamily of peptides which may all have diverged from an ancestral gene during evolution. The N-terminal end holds the agonistic properties, which depend upon an intact disulfide bridge between two cystein residues in positions 2 and 7 (Nuki *et al.* 1994), and ^{11}Arg is important for receptor interactions (Mimeault *et al.*

1993). The most homology among the members of the superfamily having vasodilator effects (e.g. CGRP, ADM and amylin) is within the sequence 1-13. On the other hand, the C terminal CGRP sequence 8-37 is a competitive antagonist with high affinity for the CGRP1 receptor (Chiba *et al.* 1989). Other, shorter C-terminal fragments were also found to have antagonistic properties (Rovero *et al.* 1992). All known members of the CGRP superfamily are believed to interact with seven-transmembrane domain G-protein receptors.

The effects of CGRP are mediated by binding to specific receptors that are positively coupled to adenylyl cyclase (Aiyar *et al.* 1997). Originally, two types of CGRP receptors were identified, namely the CGRP1 receptor, characterized by high affinity binding to the selective antagonist ligand, $CGRP_{8-37}$, and the CGRP2 receptor, characterized by binding to the selective, linear agonistic analog diacetoamidomethylcysteine CGRP (Cys(ACM2,7)CGRP) (Aiyar *et al.* 1996). To date, several receptors have been claimed to be CGRP1 receptors by cloning studies and functional assays. The canine orphan receptor RDC-1 was originally cloned from dog thyroid cDNA (Libert *et al.* 1989), and later identified as a CGRP1 receptor (Kapas and Clark 1995). The RDC-1 gene is expressed in normal tissues and transformed cells of neural origin (Collum *et al.* 1992), and may play a critical role in fetal development of neuronal tissues. In addition, a calcitonin-receptor-like receptor (CRLR) sequence was initially cloned in rat lung (Njuki *et al.* 1998). Stolarsky-Fredman and coworkers (1990) identified a tissue specific enhancer in the rat calcitonin/CGRP gene, which could improve receptor function in neurons and endocrine cells. Later, Muff and coworkers (1998) reported that certain proteins, named receptor activity modifying proteins (RAMPs), could modify the function of the CRLR. Cloning experiments were performed, and three biological functions for RAMPs were described. These functions involve transport of CRLR to the cellular plasma membrane, definition of the specific RAMP pharmacology, and regulating the CRLR's state of glycosylation (Fraser *et al.* 1999). In cell cultures, co-expression of RAMP1 with CRLR was found to result in novel CGRP1 receptors, while RAMP2 and RAMP3 presents the CRLR at the cell surface as an ADM receptor (McLatchie *et al.* 1998, Fraser *et al.* 1999). Using RT-PCR on extracted total mRNA from rat lung, Qing and Keith (2000) detected gene expression of RDC-1, CRLR, and RAMP2, suggesting that the pulmonary vasodilator ADM may also have a role in regulation of pulmonary vascular tone.

CGRP effectively dilates precontracted systemic and pulmonary arteries *in vitro* (McCormack *et al.* 1989, Martling *et al.* 1994) by acting on CGRP1 receptors (Tjen-A-Looi *et al.* 1992, Aiyar *et al.* 1996, Wimalawansa 1996, Han *et al.* 1997). CGRP has one endothelium-dependent mode of action (Chen and Guth 1995), but also dilates some systemic arteries, and the pulmonary circulation, independent of endothelial factors such as nitric oxide (McCormack *et al.* 1989, Samuelson and Jernbeck 1991, Tjen-A-Looi *et al.* 1992, Martling *et al.* 1994). Mannan and coworkers (1995) reported, that in hypoxic rats, CGRP's endothelium-dependent vasodilatory action was reduced and that CGRP binding sites were upregulated. This is consistent with the finding of elevated CGRP levels in airway neuroendocrine cells in hypoxic rats, which suggests reduced release from pulmonary sources (Springall *et al.* 1988).

Endogenous CGRP exerts a protective role in HPH, and circulating levels of immunoreactive CGRP are reduced in rats with HPH, correlating with the time-dependent rise in P_{PA} (Keith and Ekman 1992, Tjen-A-Looi *et al.* 1992), thus allowing constrictors such as ET-1 to act unopposed (Helset *et al.* 1995, Tjen-A-Looi *et al.* 1996). Moreover, HPH in rats can be ameliorated, prevented, and partially reversed with exogenous rat-αCGRP infusion depending on the timing of CGRP infusion (Tjen-A-Looi *et al.* 1992, Keith *et al.* 1995). Although *in vitro* work on guinea pig hearts failed to demonstrate agonistic effects by N-terminal CGRP fragments (Giuliani *et al.* 1992), the fragments CGRP 1-14, 1-13, and 1-8 were found to confer a degree of protection against HPH (Keith and Qing 1999). The protective role of endogenous, native CGRP was demonstrated by exacerbated HPH upon blocking of the CGRP1 receptor with $CGRP_{8-37}$ infusion, or immunoprecipitation of circulating endogenous CGRP by infusion of CGRP antiserum (Tjen-A-Looi *et al.* 1992). Precipitation of native CGRP further elevated P_{PA} in HPH rats, but was less effective in doing so compared with *in vivo* CGRP1 receptor blocking with $CGRP_{8-37}$, which elevated P_{PA} further by 18 % (Tjen-A-Looi *et al.* 1992). This suggests the presence of another pulmonary vasodilator not immunoreacting with the CGRP antiserum but acting on the same receptor, for example, ADM.

CGRP's protective effect was further emphasized by Champion and coworkers (1999), who

employed adenovirus-mediated transfer of the prepro-CGRP gene to the lungs of mice before exposure to chronic hypoxia (10 % O_2, 16 days) thus overexpressing CGRP. This resulted in increased CGRP and cAMP levels, reduced pulmonary vascular resistance, decreased right ventricular mass, and pulmonary vascular remodeling as compared with HPH controls. Of special interest is the finding that the elevated lung CGRP levels also attenuated P_{PA} responses to the pressor peptides ET-1 and AII.

The fact that both N- and C-terminal fragments bind to the CGRP receptor with distinct effects on the pulmonary circulation suggests that inhibitory fragments, generated by enzymatic cleavage, could compete with native CGRP and its agonistic fragments, thereby reducing CGRP's moderating effects on the pulmonary circulation. Moreover, using an isolated rat lung preparation, Janssen and Tucker (1994) showed that CGRP's attenuating effect on hypoxic pulmonary vasoconstriction could also involve suppression of the pressor response to AII.

Adrenomedullin (ADM)

Another member of this superfamily, ADM_{1-52}, is primarily produced by the adrenal medulla and also by vascular endothelium and the lung. Like CGRP, ADM has specific binding sites within the lung, and both increase cellular cAMP in vascular smooth muscle (Eguchi *et al.* 1994). ADM reduces systemic blood pressure and has a vasodilatory effect on the pulmonary vasculature (De Witt *et al.* 1994). In the fetal human lung, ADM-like immunoreactivity was localized to bronchial epithelial cells in which intensity increased with gestational age, and was also present in lung vascular endothelium, whereas bronchial immunoreactivity was absent after the onset of breathing and in adults (Marinoni *et al.* 1999). This pattern suggests a significant role of ADM in late fetal life, perhaps facilitating pulmonary vasodilation at the time of birth. Moreover, exogenous ADM causes dose-dependent increases in pulmonary blood flow in fetal sheep (De Vroomen *et al.* 1997), and reduces monocrotaline-induced PH in rats (Yoshihara *et al.* 1998). The mechanisms involved in ADM's effects in the fetal sheep lung depend largely on NO release and partly on activation of ATP-gated potassium channels (K_{ATP}), and do not involve a CGRP receptor (Takahashi *et al.* 1999). In human patients under 20 years of age with primary and secondary PH, plasma ADM-like immunoreactivity was significantly elevated with significant pulmonary uptake (Yoshibayashi *et al.* 1997). Chronic hypoxia also elevates ADM levels (Zhao *et al.* 1996b) and likewise, in adults with PH secondary to mitral stenosis, plasma ADM levels were proportional to the degree of pulmonary hypertension (P_{PA}, total vascular and total pulmonary resistance) (Nishikimi *et al.* 1997). These elevated ADM levels are taken as a compensatory rise to offset the increased P_{PA}, also supported by a net reduction of plasma ADM across the lung.

ADM and CGRP were found to interact with an abundant, seven transmembrane domain receptor related to the calcitonin receptor, resulting in the expected elevated intracellular cAMP (Han *et al.* 1997). Inhibition of binding by $CGRP_{8-37}$ suggests competition at a type CGRP1 receptor that is expressed in high levels in the pulmonary vascular endothelium (Eguchi *et al.* 1994).

Amylin (islet amyloid polypeptide, IAPP)

This is a 37-aminoacid peptide, co-synthesized and secreted with insulin from pancreatic islet β-cells (Nakazato *et al.* 1990). Beside its effects on insulin and glucose metabolism, amylin also has systemic vasodilatory properties (Brain *et al.* 1990). Amylin binding sites have been identified in rat lung membranes, where amylin was 100 times more effective in displacing ^{125}I-amylin binding compared with CGRP (Bhogal *et al.* 1992, Wang *et al.* 1991), suggesting a potential, independent role in the pulmonary circulation. The differential CGRP/amylin receptor binding in the lung suggests no competition between these two vasodilators (Aiyar *et al.* 1995).

Table 1. Pulmonary artery pressures (P_{PA}) after chronic infusion of amylin (10 μg/rat/h) in rats kept in hypobaric hypoxia (10 % O_2 for 8 days)

Treatment Group	P_{PA} (mm Hg)
Hypoxia+ rAmylin (n=2)	25.5 ± 2.1
Hypoxia Control (n=5)	31.5 ± 2.3
Normoxia Control (n=5)	20.3 ± 1.7

Data are means ± standard deviations, sample size is given in parentheses. All groups are significantly different from one another using the Student-Newman-Keuls test for multiple comparisons at $p<0.05$.

Preliminary work in collaboration with S.J. Wimalawansa suggests that intravenous rat amylin

infusion during chronic hypobaric hypoxia in rats mitigates the hypoxia-induced rise in P_{PA} (Table 1) compared with saline infused hypoxic controls, probably through vasodilation.

Atrial natriuretic peptides (ANP, BNP, CNP)

Natriuretic peptides can affect pulmonary vascular pressure directly by vasodilation (Thompson *et al.* 1994) and indirectly by lowering plasma volume through increased sodium excretion (Hirata *et al.* 1992). ANP elicits vasorelaxation and inhibits vascular smooth muscle proliferation, thereby partially reversing the cardiopulmonary changes associated with HPH (Thompson and Morice 1996). Part of ANP's effect is on pulmonary resistance vessels (Thompson and Morice 1995). Elevated ANP levels in the hypoxic lung have been reported (Thompson *et al.* 1994). Both ANP and BNP were elevated in plasma from patients with mitral stenosis (Nikishimi *et al.* 1997). These two peptides have pulmonary vasorelaxant activity in humans (Cargill and Lipworth 1995). CNP does not appear to have a significant role in the pulmonary vessels. Inhibition of the metabolic enzyme NEP may enhance the effects of ANP by further increasing lung ANP content, thus improving ANP binding which is reduced in hypoxic lung vessels. The protective effect of endogenous ANP against PH was illustrated in mice by Klinger and coworkers (1999) who found that gene-targeted disruption of the proANP gene caused pulmonary hypertension in both normoxia and hypoxia.

Somatostatin$_{14}$ (SOM_{14})

While SOM_{28} potently exacerbates HPH in rats, the isoform SOM_{14} has been shown to significantly ameliorate HPH in rats (Tjen-A-Looi *et al.* 1992). This disparity in action on the pulmonary circulation is not surprising, as the two isoforms have separate receptors, and opposite effects have been reported in other organ systems such as neurons (Wang *et al.* 1989). However, in a similar study using the SOM_{14} analog angiopeptin, an inhibitor of cellular proliferation in several vascular injury models, Sidney and colleagues (1996) did not find a P_{PA} effect in normoxic or chronically hypoxic rats. However, angiopeptin completely abolished the pressor responses to injected AII in isolated perfused lungs from chronically hypoxic rats, but not in those from normoxic rats. Although angiopeptin is a longer lasting analog, it was used at a much lower dose compared to the SOM_{14} in older rats, which could account for the difference in efficacy between SOM_{14} and angiopeptin.

Endothelin-3 (ET-3)

ET-3 has been shown to exert potent, dose-dependent vasodilatory effects in the pulmonary circulation of rats (Crawley *et al.* 1992), and completely reversed hypoxic vasoconstriction *in vitro* (isolated blood-perfused lungs). However, the response to ET-3 was biphasic, with sustained contraction at doses tenfold higher than those causing dilatation. The vasodilation was dependent upon NO, but not K_{ATP}, and ET-3 has been shown to actively release NO in bovine artery endothelial cells (Warner *et al.* 1992). Interestingly, NO release in turn depressed ET-1 release. ET_B-receptors have also been shown to mediate NO release in the adrenal medulla (Mathison and Israel 1998). Furthermore, ET-3's vasodilatory effect in the pulmonary circulation is abolished by chronic hypoxia, suggesting loss of another vasodilator mechanism in hypoxia (Eddahibi *et al.* 1993).

Wong and coworkers (1995) used the ET_B agonists ^{4}Ala-ET-1 and IRL 1620 to examine the effects of the ET_B receptor in the pulmonary circulation of newborn lambs. At rest, no hemodynamic effects were seen with ^{4}Ala-ET-1 and only limited decrease in P_{PA} occurred with high doses of IRL 1620. However, during U46619-induced PH, both agonists produced selective dose-dependent decreases in P_{PA} which were dependent upon endothelial NO release and activation of K_{ATP}. The ET_B receptor has also been implicated as a mediator of autocrine ADM secretion (Jougasaki *et al.* 1998), and could thereby exert vasodilation indirectly.

Vasoactive intestinal peptide (VIP)

VIP was first detected in the gastrointestinal tract, and has since been shown to exert smooth muscle relaxation throughout the body. In the lung, VIP is found in perivascular nerves, and VIP receptors were found in human lung membranes (Robberecht *et al.* 1988). This peptide has been shown to cause both bronchial and pulmonary artery vasodilation (Martling *et al.* 1990). Moreover, elevated VIP levels were reported in plasma of acutely hypoxic dogs (10 % O_2, 30 min) that showed decreased PaO_2 and increased P_{PA} (Li *et al.* 1990). Results from this study suggest that VIP was released both systemically and from the lung during hypoxia, perhaps as a compensatory response to elevated P_{PA}.

Peptide Interactions

While these peptides mainly exert direct effects on the pulmonary circulation, they may also act indirectly by interaction with one another, and with other agents. For example, ET-1 interacts with many agents, which could potentially result in indirect effects on the pulmonary circulation. Valentin and coworkers (1991) found that ET-1 infusion to nephrectomized rats (2 ng/kg/min, 45 min), increased significantly plasma levels of immunoreactive ANP. Interaction between ET and ANP has also been reported in cardiovascular and endocrine functions by Ota *et al.* (1992). ET-1-induced vascular contractility was found to be mediated by AII, while in turn, AII, and also AVP, induce endothelial prepro-ET-1 expression (Imai *et al.* 1992). Such interactions could propagate and amplify already detrimental effects of ET-1.

The ET-1 vasoconstrictor effect is potentiated by 5-hydroxytryptamine through a synergistic mechanism associated with thromboxane A_2 release (Yang *et al.* 1992). Several interactions are also documented between ET-1 and the vasodilatory NO. For example, ET-1's precapillary vasoconstriction in lungs from rats with chronic HPH was counteracted by endogenous NO (Muramatsu *et al.* 1997), and ET-1 enhanced NO-induced apoptosis of vascular smooth muscle cells in culture, after binding to ET_B receptors (Nakahashi *et al.* 1998). Morever, in proliferating endothelial cell monolayers, ET-1 mRNA transcripts and protein rose fourfold, whereas levels of endothelial constitutive NO synthase (ecNOS) mRNA transcripts and protein declined twofold, suggesting reciprocal regulation of these two agents (Flowers *et al.* 1995). Interaction between constrictor and dilator effects of ET is also suggested by increased pulmonary prepro-ET-1 and concomitant decrease in ET_B receptor mRNA associated with chronic intrauterine PH in fetal lambs (Ivy *et al.* 1998).

The ET_B receptor has wide ranging, beneficial interactions in that it mediates NO/cGMP formation in the adrenal medulla (Mathison and Israel 1998), and has an autocrine role in the secretion of adrenomedullin (Jougasaki et al 1998). Under certain conditions it also binds ET-1, resulting in constriction. Among other interactions in vasodilation are ADM-induced release of NO, K_{ATP} activation, and CGRP-induced NO activation in sensory neurons (Chen and Guth 1995). Also, the renin-angiotensin system interacts with natriuretic peptides.

ET-CGRP interaction

A functional interplay may also exist between ET-1 and CGRP. It was noted that acute alveolar hypoxia increased pulmonary ET-1 release but decreased release of CGRP (Helset *et al.* 1995). Tjen-A-Looi and coworkers (1996) further illustrated this relationship in a study on HPH in rats which showed that continuous infusion of ET-1 to the pulmonary circulation did not alter levels of immunoreactive lung tissue CGRP in normoxic rats, whereas ET antiserum and the ET_A antagonist BQ123 elevated lung CGRP. In this study, left ventricular blood CGRP levels were decreased in normoxia by ET-1 (14 days) associated with normoxic PH, and increased with ET antiserum infusion. In chronic hypoxia, ET-1 (2 pmol/kg/min) caused an increase in lung CGRP at 14 days, and ET antiserum elevated lung and blood CGRP levels after 3, 7, and 14 days concomitant with lessened P_{PA}.

Moreover, in a study on effects on the systemic circulation, infusion of exogenous ET-1 caused chronic hypertension, as in the pulmonary circulation, and the rise in systemic pressure was prevented by the ACE inhibitor captopril (Mortensen and Fink 1992). This suggests that ET-1-induced hypertension may involve the renin-angiotensin system.

SOM-CGRP interaction

Tjen-A-Looi and coworkers (1992) noted that chronic i.v. infusion of SOM_{14} to the pulmonary circulation of chronically hypoxic rats elevated lung tissue CGRP and SOM (10fold), but did not change blood CGRP levels. In contrast, SOM_{28} infusion reduced lung tissue CGRP, but did not change blood levels. In this study, hypoxia alone reduced blood CGRP and SOM compared with normoxic controls, and CGRP infusion restored normoxic blood SOM (and CGRP) levels. These findings suggest that a reciprocal interplay could be in effect between SOMs and CGRP.

It is clear that chronic hypoxia is a potent stimulus for changes in the bioavailability of lung neuropeptides. Whether airway hypoxia is caused by low FiO_2, restrictive lung disorders, hypoventilation, or monocrotaline, the results are similar. For example, in chronic hypoxia there is an increase in lung ET-1, AII, and SP, and a reduction of CGRP. Moreover, COPD and congenital diaphragmatic hernia are also associated with increased ET-1 levels, and monocrotaline treatment is associated with increased ET-1, tachykinins (e.g. SP), and ADM. Receptor density, binding affinity, and turnover,

and peptide half-life, are other important factors that affect regulation of the pulmonary circulation. For example, abnormal net balance between pulmonary release of ET-1 and its clearance was detected in subjects with primary pulmonary hypertension and this was improved by chronic infusions of epoprostenol (prostacyclin) (Langleben *et al.* 1999).

Peptide metabolism

Peptide metabolism is regulated by a variety of enzymes. For example, ET-1 is generated through cleavage of big ET-1 by endothelin converting enzyme (ECE-1 and ECE-2) and AII from AI by ACE. Moreover, catabolism of the active forms is accomplished by proteases such as NEP (ET-1, SP, ANP and enkephalin) (Turner and Murphy 1996, Thompson *et al.* 1994, Winter *et al.* 1991). Proteases also degrade airway VIP (Tam *et al.* 1990). Tryptase from SP-activated mast cells of human airways degrades CGRP (Walls *et al.* 1992), thus attenuating CGRP's vasodilatory activity. Because perivascular mast cells of the airways typically increase in numbers with hypoxic exposure (Tucker *et al.* 1977), the tryptase effect could potentially be amplified. Also, lower levels or absence of NEP in plexiform lesions of primary pulmonary hypertension (Cohen *et al.* 1998) results in elevated peptide levels, and could contribute to these lesions. Likewise, abundant expression of ECE-1 is present in diseased pulmonary vessels, which may contribute to higher ET-1 levels and the pathogenesis of arteriopathy and PH (Giaid 1998). Thompson and colleagues (1994) showed that short-term inhibition of NEP in rats with established HPH caused regression of established vascular remodeling, even though ANP levels did not rise significantly over those of hypoxic controls. This suggests that additional beneficial factors, also metabolized by NEP, could be in effect.

Conclusions

Impaired vasodilation has been postulated to play a key role in pulmonary hypertension (Weir 1978, McIntyre *et al.* 1995, Brett *et al.* 1996). Because vascular contractility is left intact (McIntyre *et al.* 1995), vasodilatory factors, some endothelium-dependent, may be amiss (Brett *et al.* 1996). Therefore, constrictive agents take over in lack of counteracting dilators. Moreover, considering the many documented interactions among particular peptides and between peptides and other agents, the pulmonary circulation is modulated at different levels, for example, directly by the balance between ET-1 and CGRP, and indirectly by the net effects from a web of additional, interacting factors. Although many reports presented here suggest causal relationships between peptides and measured effects, the possibility of such concomitant changes being merely incidental must be considered. It is apparent that a great deal of redundancy is in place in the intricate balance between vasoconstrictors and dilators, making clear results and analyses difficult. However, the increased collective knowledge of the interactions between lung peptides under various conditions, and their net effects, is taking us one step closer to understanding how the pulmonary circulation is regulated. The search for an effective, lung selective treatment of PH will likely benefit from exploring the imbalance and restoring balance between these native modulators of intrapulmonary pressure.

List of abbreviations

ACE	angiotensin converting enzyme
ADM	adrenomedullin
AVP	arginine vasopressin
AII	angiotensin II
ANP, BNP, CNP	atrial natriuretic peptides A, B, C
AVP	arginine vasopressin
CGRP	calcitonin gene-related peptide
COPD	chronic obstructive pulmonary disease
CRLR	calcitonin-receptor-like receptor
ECE	endothelin converting enzyme
ET	endothelin
HPH	hypoxia-induced pulmonary hypertension
K_{ATP}	ATP-gated potassium channels
NEP	neutral endopeptidase
NO	nitric oxide
PH	pulmonary hypertension
P_{PA}	pulmonary artery pressure
PYY	peptide tyrosine Y
RAMP	receptor activity modifying protein
SOM	somatostatin
SP	substance P
VIP	vasoactive intestinal peptide

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