
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Finkley, Alvin, Characterization and Activity of Endothelin Converting Enzyme- 1 in Human Non-Pigmented Ciliary Epithelial Cells. Master of Science (Biomedical Sciences).

Endothelins (ETs) are potent vasoactive peptides, that are present in many ocular tissues including the ciliary epithelium where active ET- 1 is produced from the precursor Big ET- 1 by a membrane-bound metalloprotease, endothelin-converting enzyme (ECE). Although the role of ocular ET's are uncertain, ETs have been shown to lower the intraocular pressure. In the current study, ET- 1 and Big-ET- 1 were detected in SV-40 transformed human ciliary epithelial (HNPE) cells by immuofluorescence suggesting the presence of ECE activity. The presence of ECE was confirmed by Western blotting using polyclonal antibodies against ECE- 1 which detected a 124 KDa protein in the membrane fraction and not in the cytosol. Further characterization of the enzymatic activity of ECE (conversion of Big ET- 1 to ET-1) was performed using a novel assay involving ^{121}I-Big ET- 1 (substrate; 2 fmole) and polyclonal antibodies specific for Big ET- 1. Mean ECE-1 activity (expressed as the ratio of ^{121}1-ET-1 produced to the total ^{125}I-Big ET-1 incubated X 100) was measured and corresponded to: 26 % (0.5 3 ± 0.02 fmole, 1 hr), 63 % (1.26 ± 0.07 fmole, 3 hr) and 66 % (1.33 ± 0.11 fmole, 24 hr) compared to blank controls at 13 % (0.25 ± 0.03 fmole). Thiorphan (2 mM), an inhibitor of ECE, abolished ECE- 1 activity. These results suggest that ECE- 1 is localized in HNPE cells and is essential for the production of ET- 1. The physiological importance of the proteolytic processing by ECE-1 in ocular tissue may reflect on how ET regulates intraocular pressure.

Key Words: endothelin converting enzyme-1; endothelin-1; Big endothelin-1; ciliary epithelium; aqueous humor dynamics; intraocular pressure, Western blotting, ECE- 1

CHARACTERIZATION AND ACTIVITY OF ENDOTHELIN CONVERTING ENZYME-I IN HUMAN NON-PIGMENTED CILIARY EPITHELIAL CELLS

Alvin Finkley, B.S.

APPROVED:

Major Professor

Committee

Victoria J. Rudick

Committee

Committee

Chair, Department of Biomedical Sciences

Dean, Graduate School of Biomedical Sciences

Characterization and Activity of Endothelin Converting Enzyme-1 in Human Non-Pigmented Ciliary Epithelial Cells

Thesis

Presented to the Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Alvin E. Finkley, B.S.

Fort Worth, Texas

ACKNOWLEDGEMENTS

This work was supported by Texas Advanced Research Program Grant from the Texas Higher Education Coordinating Board (Grant number: 09768/008/018).

TABLE OF CONTENTS

Chapter I

Background

Endothelins (ETs) represent a family of 21 amino acid-containing peptides with potent vasoconstrictor properties. The discovery that endothelial cells could synthesize and release vasoactive-substances that caused vasorelaxation and vasoconstriction, opened a new area for scientists who were interested in regulating cardiovascular, pulmonary and other homeostatic mechanisms. The first endothelium derived vasoactive factor, prostacyclin (vasorelaxant), was isolated and identified in 1976 (Moncada et al 1976). In 1980 Furchgott and Zawadzki discovered a second potent vasorelaxant substance which produced an endothelium-dependent relaxation in porcine, bovine and canine coronary arteries. This substance was named endothelium-derived relaxing factor (EDR-F) (Hickey et al 1985). With an intent on determining the activity of EDRF in conditioned medium from cultured bovine aortic endothelial cells, Hickey et al (1985) found, that the addition of conditioned cultured medium suprisingly caused a slowly developing, long lasting vasoconstriction in porcine, bovine and canine coronary arteries. Further characterization of this substance, revealed that the observed effect was caused by a peptide similar to EDRF but was named endothelium-derived contracting factor (EDCF) (Hickey et al, 1985).

This newly discovered peptidergic EDCF, was subsequently isolated, purified and identified as a 21 amino acid peptide termed Endothelin (ET- 1) by Yanagisawa et al.,

(1988). The peptide contains two disulfide bonds and is homologous to sarafotoxin S6b, a venom of *atractaspis engaddensis* (Kloog, Y., et al 1989). From the corresponding cDNA that was isolated and sequenced, it was determined that the biologically active peptide was synthesized from a larger (203 amino acid) preproprotein precursor that requires biochemical processing (Yanagisawa et al., 1988). Three different human endothelin gene products have since been identified as; Endothelin- 1 (ET- 1), Endothelin-2 (ET-2) and Endothelin-3 (ET-3), (Hickey et al 1985; Yanagisawa et al., 1988) which make up the three isoforms of ET.

Since the initial observation by Yanagisawa et al., (1988) of porcine endothelin-1 (ET1), three human isoforms have been identified, endothelin-1 (ET-1)(Yanagisawa et al, 1988) endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue et al, 1989). All three isoforms share common structural features, each of which contains, 21 amino acids, two disulfide bridges (Cys^{1}-Cys^{15} and Cys^{2}-Cys^{11}), and a hydrophobic C-terminal moiety with a tryptophan in position 21. Nearly half of the amino acids in the three isoforms are conserved (1,3,8,10,11,15,16,18,20,2 1). Marked differences can also be observed among the sequences of the various isoforms of ETs through the processing sites, with Trp^{21} -Val^{22} demarcating the dipeptide for ET- 1 and ET-2 and Trp^{21} -Ile^{22} for ET-3. Although these three peptides present high degrees of similarities, differences were observed between the predicted amino acid sequence of pre-pro ET- 1, pre-pro ET-2 and pre-pro ET-3 (Inoue et al., 1989) The sequence of the Big ET-1 isoforms from the human gene when compared to the porcine gene (original isolation product), differs by only two amino acids. The porcine isoforms contain 39 amino acids whereas the human

isoforms contain 38 amino acids with one additional amino acid substitution within the 38 residues. The Big ET-isoforms from the human gene contain 37 residues with 8 amino acid substituitions compared to Big ET-1. The Big ET-3 isoform from the human gene is slightly larger than the other two isoforms as it contains 41 amino acids. In addition, there are 12 amino acids substitutions within the first 38 amino acids of Big ET-3 compared with Big ET-1.

Endothelins are synthesized constitutively by the vascular endothelial cells of the smooth muscle and appear to act as locally produced peptide hormones in a paracrine fashion (Kennedy, 1993). ET not only exerts direct vasoconstrictor effects but potentiates the contractile action of other vasoconstrictor substances, including norepinephrine (Yang. et al., 1990). ET evokes a dose-dependent (10pM-10nM) contraction of isolated vascular preparations in all species so far examined, veins being generally more sensitive to the effect of ET than arteries (De Nucci et al, 1988). ET-induced contraction is typically slow in onset, followed by a uniform and long lasting effect (Yanagisawa et al., 1988). Indeed, a bolus administration of ET results, after a transient depressor response (0.5-2min), in a prolonged rise of arterial pressure lasting up to 2 hours in the rat. ET- 1 and ET-2 are equally potent, whereas ET-3 is less potent (Chabrier and Braquet et al., 1990).

ET-1 is one of the most potent vasoconstrictors yet to be identified (Pang and Yorio, 1997). ETs have been localized in extracellular tissues including lung, pancreas, spleen, brain and the eye. MacCumber et al., (1989), identified a 3.7 Kb form of ET mRNA in the eye by Nothern blot analysis. In situ hybridization was used to detect the

message and this correlated with the localization of ET binding sites in various ocular tissues, including the iris, retina and ciliary body (MacCumber et al., 1989). ET-1 like immunoreactivity can be demonstrated in the iris, ciliary body, choroid and retina (MacCumber, Jampel and Snyder 1991; Chakravarthy et al 1994) aqueous humor (Chakravarthy et al., 1994) and ciliary epithelium (Eichorn and Lutjen-Drecoll, 1993). From these localization studies, the results suggest that ET may potentially be responsible for important functions in a variety of ocular tissues and that the peptide may be released in a paracrine fashion to carry out its action. The potential role of ET in the regulation of intraocular pressure (IOP), has also been postulated by several groups (Lepple-Weinhues et al., 1992, Eichhorn and Lutjen-Drecoll, 1993). ET has been shown to contract the ciliary muscle in bovine (Lepple-Weinhues et al., 1991), humans (Lepple-Weinhues et al., 1992), and monkey eyes (Erickson-Lamy et al., 1991; Millar et al., 1995), which could possibly lower IOP by increasing the facility for outflow through Schlemm's canal.

The precise mechanism of the IOP-lowering effect of endothelins is not yet known, however it has been suggested that it is primarily through its action on the ciliary muscle. There are two possible pathways that could account for ET's effect on the ciliary muscle: (1) the microvasculature in the ciliary process could release ET which would penetrate through the stroma and epithelial cells and act on the smooth muscle layer, or (2) the local synthesis and release of ET from the pigmented or non-pigmented ciliary epithelial cells (HPE and HNPE) and ciliary smooth muscle cells (HCM) could result in paracrine or autocrine stimulation of the ciliary muscle (Prasanna et al., 1998).

Receptors

Not long after the cloning and characterization of the three ET isoforms it became evident that high-affinity binding sites for these peptides exist on cell membranes. It was presumed that, like other bioactive peptides, ETs produce their physiological effects through binding to these putative receptor sites (Rubanyi and Polokoff et al., 1994). Further studies suggested that many diverse physiological and pharmacological effects of ETs could not be mediated through a single receptor subtype. Prior to the discovery of a specific ET receptor antagonists, receptor subtype classification was made on the basis of rank order of potencies of either binding or function (Rubanyi and Polokoff et al., 1994).

Two distinct ET receptors have been cloned and named $ET_A$and ET_B; ET_A is selective for ET- 1 whereas the ET_Breceptor is non-selective subtype (Kondah et al., 1991; Sakurai et al., 1992). The presence of a putative ET_c receptor, which appears to be selective for the ET_3 peptide, has been suggested from clones of amphibian melanophores (Kame et al 1993). ET_A receptors are thought to mediate vasoconstriction while the ET_Breceptor has been implicated in both contractile and dilator actions. ET receptors are found in the retina and choroid, particularly in the retinal blood vessels, suggesting that ETs may be involved in regulating retinal blood flow and could contribute to retinal ischernia. Such effects of ETs may lead to a number of retinal pathologies, including normal tension glaucoma (Pang and Yorio 1997). Through the use of autoradiography with [^{125}I]-labelled ET- 1 competition binding, ET_A-like binding

sites were localized to the human retina, iris and choroid, with specific labelling within the retinal neural tissue (Macumber and D'anna, 1994). Recently binding studies using [^{125}I]-ET have demonstrated that both ET_A and ET_B receptors are localized in the ciliary epithelium (Ripodas et al., 1998).

ET-1 is a potent contractor of the iris sphincter of different mammalian species (Geppeti et al., 1989; Abdel-Latif et al, 1991). The presense of ET_A and ET_Breceptors was demonstrated in rabbit iris sphincter by El-Mowafy et al., 1994. In this tissue the ET_A receptor is coupled to the stimulation of phosphoinositide hydrolysis and the ET_B receptor is coupled to the stimulation of cAMP accumulation and both may be involved in contractile responses.

Recently in our laboratory, using HCM, HNPE and trabecular meshwork (TM) cells, it was found that ET_A receptors mediate $[Ca^{2+}]_i$ Tao et al., 1997. This was accomplished by using cell-specific agonists and antagonists. Other recent studies in our laboratory by Prasanna et al., 1998, demonstrated the effects of tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, on the cellular mechanisms leading to ET- 1 synthesis and release in HNPE cells. The data demonstrated that TNF-α regulates ET- 1 synthesis and release in HNPE cells possibly by activating PKC either to stimulate protein synthesis and/or to enhance ET- 1 secretion. Together these studies have shown that (1); cytokines like TNF-α can enhance ET- 1 concentrations by promoting its synthesis and release in HNPE cells, suggesting that ET- 1 concentrations in the anterior chamber can be influenced by cellular regulators (Prasanna et al 1998), and (2); ET_A receptors are expressed and mediate signaling for calcium mobilization in HNPE and

HCM cells (Tao et al., 1997). These studies clearly suggest endothelin may facilitate the function of tissues involved in the regulation of aqueous humor dynamics. Additionally, the study proposes that ET-1 is synthesized in select eye tissues.

Production of mature ET- 1 from precursors

In earlier reports concerning the discovery of endothelin, Yanagisawa et al., (1988), predicted that the endothelin gene product would be processed in a manner similar to that of many other peptides i.e. involving the cleavage of mature peptides from precursors via proteolytic processing. Based on cDNA sequencing of the cloned porcine endothelin gene, they proposed that the postranslational. synthetic pathway of endothelin would result from the intracellular proteolytic cleavage of a 203 amino acid precursor peptide, preproendothelin (PPET). This hypothesis was based on the similarities that PPET shared with other peptides, which presented a hydrophobic N-terminal signal necessary for inserting portions into the endoplasmic reticulum, and pairs of basic amino acids cleaving the 39 amino acids containing the endothelin sequence in between. The peptide that resulted from the removal of the signal sequence was termed proendothelin and the 39 amino acid fragment resulting from the cleavage of dibasic amino acids was termed Big endothelin. In the original characterization of endothelin, Yanagisawa et al., (1988), identified 5 pairs of principal basic amino acids Lys-Arg or Arg-Arg as recognition sites for cleavage by the conventional dibasic proprotein endopeptidase. This would produce 6 fragments. From the 6 fragments, only two were significant sites defining Big ET- 1 (3 9 amino acids), flanked by Lys^{51} -Arg^{52}

and Arg^{92}- Arg^{93} which contained the 21 amino acid sequence. The processing of porcine PPET- 1 by dibasic endopeptidases was further supported by the detection of peptides presenting an identical sequence to the N-terminal fragment of Big ET- 1 (Nakagawa et al., 1991). Human prepro, ET-1 , prepro ET-2 and prepro ET-3 presented 3-4 possible cleavage sites for dibasic endopeptidases (Itoh et al., 1988).

The proposal that an enzyme activity must exist that specifically converts Big ET- 1 to a mature 21 amino acid ET- 1 was contained in the original report of the cloning of porcine ET- 1 (Yanagisawa et al., 1988). The physiologicl importance of the conversion of Big ET- 1 to ET- 1 was demonstrated by the observation that ET- 1 was 140-fold more more potent as a vasoconstrictor compared to the precursor peptide Big ET-1 (Kimura et al., 1989), while the prepropeptide had no activity at all (Cade et al., 1990). The conversion of Big ET-1 to ET-1 and its C-terminal fragment (CTF) occurs through a unique processing event that involves a cleavage at Trp^{22}-Val^{22} bond and is catalyzed by endothelin converting enzyme (ECE-1) (Yanagisawa et al., 1988). The conversion of Big ET-2 to ET-2 also involves the cleavage of Trp-Val bonds, but in the conversion of Big ET-3 to ET-3 the enzyme will cleave Trp-Ile. Recently, ECE-2 has been identified and has been suggested that it cleaves Big ET-2, however ECE- 1 is more efficient in converting activity among all other ECEs.

Studies on ECE using various cell lines of tissues have revealed some distinctive features about its specificity. Conversion by ECE is limited to the production of ETs, so no other biologically active peptides or their precursors have yet been identified as substrates for ECE. Marked differences have been reported in the ability of purified,

cloned and expressed ECE to process the three different Big ET isoforms (Turner and Murphy 1996). The ECE activity partially purified from EA.hy926 cells was reported to process human Big ET-1 much more efficiently than human Big ET-2 (V_{max}/Km =0.09, relative to Big ET-1) and there was no comprabable activity detectable towards Big ET-3 (Waxman et al., 1994). Most of the cloned and expressed ECE proteins show a similar specificity, converting Big ET-2 poorly and failing to convert Big ET-3 (Shimada et al., 1994). This suggests that additional forms of ECE remain to be isolated. Immunocytochemical studies indicate a predominant cell-surface location for ECE- 1 like E-24.11 (Turner and Murphy 1996). This is consistent with the conversion of exogenous Big ET-1 when administered in vivo and the inhibition of this event by phosphormaidon (PR), an inhibitor of ECE. However, mature ET-1 can be detected in intracellular vesicles in endothelial cells, suggesting that some processing occurs in the constituitive secretory pathway (Sawamura et al., 1989). These results are similar to the ones observered in our laboratory by Prasanna et al., 1998 who reported that ET- 1 synthesis in HNPE cells is under the regulation of TNF-α. Acute treatment with TNF-α caused an increase in intracellular immunofluorescence in ET- 1 while changes in extracellular ir-[ET-1] were not significantly increased. However, when HNPE cells were treated chronically with TNF-oc significant concentrations of ir-[ET-1] were observed while intracellular levels remained the same as seen during the acute treatment. These observations are suggestive of a secretory pathway for ET-1 during chronic exposure, which results in immediate processing. It is now known that there are two genes encoding two different endothelin-converting enzymes, ECE- 1 and ECE-2

(Warner et al, 1996). Of these, ECE- 1 appears to be involved in much of the physiologically relevant processing of Big ET-1 to ET-1.

Efforts to regulate ECE activity led to the testing of various protease inhibitors of ECEs ability to catalyze the conversion of Big ET- 1 to ET- 1. It has been established that the secretion of ET- 1 in cultured endothelial cells is not affected by the intracellular accumulation of pepstatin, an inhibitor of aspartyl proteases (Shields et al., 1991; Nichols et al., 1991). From these reports it is unlikely that an aspartyl ECE is responsible for cleaving Big ET-1 to ET-1. It is unclear, however, if the same holds true for the processing of Big ET-2 and Big ET-3. When challenged with Tosylphenylalanyl Chloromethyl Ketone, an irreversible inhibitor of a variety of serine and cysteine proteases and inactivator of chymotrypsin, the secretion of ET-1 by cultured endothelial cells is decreased (Ohlstein et al., 1990). This data suggests the involvement of chymotrypsin-like ECE but it is not known as to what direct effect this has in effectivly inhibiting ECE activity. It could reflect changes in secretion rather than synthesis.

One of the first reports of a endothelin converting activity optimal at neutral pH was made by Ohnaka et al., 1990, who identified such an enzyme in cultured bovine aortic endothelial cells. This activity was membrane-specific, inhibited by metal chelators, EDTA and EGTA, and insensitive to other classes of protease inhibitors. However, Okada et al., 1990, first described the presence in endothelial cells of a membrane-bound PR-sensitive, thiorphan insensitive metalloprotease, implicating an E-24.1 I -like activity that was distinct from E-24.11 itself. This activity was very specific at a pH around 7.0 (Okada et al., 1990).

Initial purification of ECE to homogeneity was reported using rat lung (Takahashi et al., 1993) and from porcine aortic endothelial cells (Ohnaka et al., 1993). The rat enzyme was purified 6300-fold from a lung preparation following solubilization with Triton-X (Takahashi et al., 1993). The highly glycosylated nature of ECE was assayed by chromatography, followed by a zinc chelating sepharose seperation. The purified protein contained an apparent M, of 13 0,000 whereas E-24.1 I migrated as a protein of 90,000-1 00,000 Da (Turner and Murphy 1996). The purified porcine aortic endothelium ECE exhibits characteristics similar to that of the rat lung enzyme with a purification of 12,000-fold being required to achieve homogeneity (Ohnaka et al., 1993). An apparent M_r of 120,000 was observed for the protein on a SDS-PAGE and 13 1,000 Da on a sucrose density gradient. The EA.hy926 cell line expresses ET- 1 mRNA and has been shown to secrete Big ET-1 and ET-1 into the culture medium, in a PR-sensitive manner (Saijomnaa, et al., 1991). This cell line contains a metallopeptidase ECE that, on subcellular fractionation appears to be associated mostly with a plasma membrane fraction as an ectoenzyme (Corder et al., 1995). More importantly, the partially purified ECE from EA.hy926 cells could be renatured and assayed after non-reducing SDS-PAGE, and was shown to migrate as a protein of M_r 280 kDa (Waxman, et al., 1994). In these cells it is now evident that ECE exists as a dimer and its full conformation is required for full processing activity.

Following the purification of ECE, molecular cloning and sequencing soon followed. Partial amino acid sequence data obtained for the rat lung enzyme was used to devise probes for screening a vascular endothelial cell cDNA library (Shimada et al.,

1994). Like E-24.11, ECE is a type 11 integral membrane protein with a short N-terminal cytoplasmic tail, and a transmembrane hydrophobic domain that represents the uncleaved signal peptide, and a large putative extracellular domain containing the catalytic site and the motiff HEXXH typical of many zinc-dependent peptidases, e.g. E-24.1 I (Jiang and Bond. 1992; Rawlings and Barrett., 1993). The ECE protein also contains a cluster of 4 conserved cysteine residues in a 32 amino acid sequence immediately following the transmembrane domain.

The key identification of ECE processing of ET-1 was confirmed by the use of phosphoramidon [N-a-rhamnopyeranosyl-oxyhydroxy-phophinyl)-Leu-Trp], originally identified as a potent inhibitor of bacterial zinc protease thermolysin (Suda et al., 1973), and a specific inhibitor mammalian plasma membrane zinc peptidase, E-24.11 (Kenny, 1977). Nevertheless, it may be misleading to extrapolate from the biological maturation of endogenous endothelin peptide based on the in vivo effects of exogenous Big ET and their sensitivity to phosphoramidon.

Current evidence suggest that a neutral, membrane bound, zinc-dependent, phophoramidon-sensitive metalloprotease is responsible for the conversion of extracellular Big ET-1 to ET- 1 (Turner and Murphy 1996; Xu et al., 1994). This is supported by data that showed in cultured endothelial cells treated with phosphoramidon, the secretion of ET- 1 was decreased and the conversion of exogenously added Big ET- 1 to ET- 1 was attenuated (Okada et al., 1990; Corder et al., 1993). However, little is known about how endothelin is synthesized in ocular tissues.

Rationale:

The regulation of intraocular pressure in normal individuals and patients suffering from glaucoma is not yet fully understood. The elevation of intraocular pressure is a contributing factor observed in primary open angle glaucoma (POAG) and occurs as a consequence of an impaired outflow pathway for aqueous humor. This decreased outflow appears to be a result of abnormalities within the drainage system in the anterior chamber of the eye. One method to treat glaucoma is to enhance the rate of aqueous humor outflow. Our laboratory has shown that ET- 1 is present in the ciliary epithelium and that receptors for ETs, are expressed and functional in ciliary muscle and trabecular meshwork (TM) cells. It is the general hypothesis that ET- 1 is synthesized and released by the ciliary epithelial cells and through a paracrine action on the ciliary muscle induces contraction which has a positive effect on the TM decreasing resistance and enhancing outflow. Since ET- 1 and Big ET- 1 appear to be present in ocular tissues, ECE activity must be responsible for the generation of active ET- 1.

Hypothesis:

ECE-1 is contained in the human non-pigmented epithelial (HNPE) cell membrane and is responsible for the conversion of big ET-1 to active ET-1. The formed ET- 1 is released into the aqueous humor to exert its action on the ciliary muscle and TM. ECE may be an important regulating site in ocular tissues that is responsible for generating ET- 1 under conditions in which this active compound is needed, as in situations where the IOP is elevated, such as in glaucoma.

Specific Aims:

1. Is exogenous Big ET –1 being converted to mature ET –1 in ocular epithelial cells?
2. Does ECE – 1 activity occur in the cytosol or in the membrane of HNPE cells?
3. Can the presence of ECE –1 be detected using Western blot analysis?

Chapter II: Manuscript

Identification of Endothelin Converting Enzyme-1 in Human Non-Pigmented Ciliary Epithelial Cells

Exp. Eye Res. (in Press)

Chapter II

Summary:

Endothelins (ETs) potent vasoactive peptides, are present in many ocular tissues including the ciliary epithelium where active ET-1 is produced from the precursor Big ET-1 by a membrane-bound metalloprotease, endothelin-converting enzyme (ECE). Although the role of ocular ET's are uncertain, they are elevated in the aqueous humor of normal as well as glaucomatous eyes and have been shown to lower the intraocular pressure for prolonged periods of time. In the current study, an endothelin-converting enzyme-1 (ECE-1) has been identified and its activity has been studied in SV-40 transformed human non-pigmented ciliary epithelial (HNPE) cells. Western blotting using polyclonal antibodies against ECE-1, detected a 124 kDa protein in the plasma membrane, but not in the cytosol. Further characterization of the enzymatic activity of ECE-1 (conversion of Big ET-1 to ET-1) using the plasma membrane fraction of HNPE cells was performed by a novel assay involving ^{125}I-Big ET-1 (substrate; 80 fmoles/mg protein) and polyclonal antibodies specific for Big ET-1. Mean ECE-1 activity (expressed as fmoles ^{125}I-ET-1 produced/mg protein/time) increased linearly with time and was similar to that observed in rat lung tissue. ECE-1 activity was enhanced with increasing concentrations of substrate (^{125}I-Big ET-1; 30-200 fmoles/mg protein/180 min) as well as with increasing concentrations of protein (5-20 μg proteins at the substrate concentration of 80 fmoles/mg protein/180 min). Treatments with CGS-26303 (100 μM), an inhibitor

of ECE-1 and thiorphan (2 mM; a metalloprotease inhibitor), significantly decreased ECE-1 activity. Furthermore, both acidification of the reaction buffer (from pH 7.4 to 6.4) and the addition of a metal chelator, EGTA (2 mM) decreased ECE-1 activity by nearly 60%. These results suggest that the ECE-1 localized in HNPE cells, is a neutral pH-sensitive metalloprotease which is similar in its activity to that observed in lung tissue and is essential for the production of ET-1 in HNPE cells. The physiological importance of the unusual proteolytic processing by ECE-1 in ocular tissue may reflect on how ET regulates intraocular pressure.

Key Words: ciliary epithelium; endothelin converting enzyme-1; endothelin-1; Big endothelin-1; endothelin converting enzyme-1 assay

Introduction

The 21 amino acid residue peptide, endothelin-1 (ET-1), first isolated from the supernatants of cultured endothelial cells, has proven to be the most potent vasoconstrictor known to date (Yanagisawa et al., 1988) . ET-1 is first produced as a precursor of approximately 200 residues, prepro ET-1 (Inoue et al., 1989). The intermediate form termed Big ET-1, is cleaved from prepro ET-1 by a dibasic pair-specific endopeptidase (Yanagisawa et al., 1988). Mature ET-1 is then generated by proteolytic cleavage between Trp^{21}- Val^{22} by an endothelin converting enzyme-1 (ECE-1). This converting enzyme is a neutral pH sensitive, Zn^{2+}-dependent metalloprotease that is localized in the plasma membrane (Ohnaka et. al., 1992; Xu et al., 1994). Recently, ECE has been shown to exist as two major isoforms, ECE-1 and ECE-2, with the acidic pH-optimum ECE-2, localized in the intracellular secretory compartments of the cell while ECE-1 is present as an ectoenzyme (Xu et al., 1994; Emoto and Yanagisawa, 1995). The biological activity of Big ET-1 is negligible, and the conversion from Big ET-1 to ET-1 by ECE-1 is essential for expression of full biological activity of the mature ET-1 peptide (Kimura et al., 1989). Thus, the regulation of ECE-1 may be important in controlling the levels of ET-1.

ET-1 is well distributed in ocular tissue and is localized in aqueous humor, iris, ciliary body, retina and choroid and has been found to cause the contraction of isolated ciliary muscle cells in rabbits and humans (Lepple-Weinhues et al., 1992; MacCumber, Jampel and Snyder, 1991; Chakravarthy et al., 1994; Weiderholt, Lepple-Weinhues and Stahl, 1993). Previously, intravitreally and intracamerally injected ET-1 has been shown

to lower intraocular pressure (IOP), possibly by increasing the outflow facility in bovine, monkey and rabbit eyes (Lepple-Weinhues et al., 1991; Erickson-Lamy et al., 1991; MacCumber, Jampel and Snyder, 1991). These reports suggest the potential participation of ET-1 in the local regulation of IOP by inducing the contraction of the ciliary muscle.

One potential site for ET-1 synthesis and release, is the human non-pigmented ciliary epithelium (HNPE) (Eicchorn and Ludjen-Drecoll, 1993). The human ciliary epithelium, composed of pigmented and non-pigmented cells, maintains the blood-aqueous barrier through formation of tight junctions and gap junctions (Caprioli, 1987). The blood-aqueous barrier prevents the free passage of plasma proteins from the stroma into the posterior chamber which contains the aqueous humor (Caprioli, 1987). Therefore, the potential movement of ET from the plasma to the aqueous is probably limited by the blood-aqueous barrier.

ET concentrations in aqueous humor (AH) have been found to be significantly higher (2-3 times greater) than plasma in normal (i.e. non-glaucomatous) human and bovine eyes (Lepple-Weinhues et al., 1992). Furthermore, ET-1 levels are further elevated in the aqueous humor of patients with primary open angle glaucoma (Noske, Hensen and Weiderholt, 1997; Tezel et al., 1997) and plasma of normotensive glaucoma subjects compared to AH and plasma of normal non-glaucomatous subjects (Moriya et al., 1992; Kaiser et al., 1995; Sugiyama et al., 1995). The factors responsible in the regulation of ET-1 synthesis and release in ocular tissue have not been fully characterized, however, a factor that may be key in regulating ET-1 levels in glaucomatous diseases, is the activity of ECE-1. Presently, we report for the first time

on the localization and activity of ECE-1 in transformed HNPE cells.

Materials and Methods

Materials

Nitrocellulose membranes (0.2 μm) were purchased from Schleicher and Schuell (Keene, NH). Protease inhibitors: aprotonin, soybean trypsin, leupeptin and phenylmethylsulfonyl fluoride (PMSF), detergents: (Triton-X 100 and NP-40) and thiorphan were purchased from Sigma Chemicals (St. Louis, MO). Polyclonal antibodies were raised in rabbits against the C-terminal 16 amino acids of bovine ECE-1 and were a gift from Dr. M. Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX. Polyclonal antibodies against Big ET-1 were purchased from Peptides International (Louisville, KY). Big ET-1 and ^{125}I Big ET-1 were purchased for Peninsula Laboratories (Belmont, CA). The ECE-1 inhibitor, CGS-26303 was a gift from Dr. Arco Jeng (Novartis Pharmaceuticals, Summit, NJ).

Cell Culture

SV-40 transformed human non-pigmented ciliary epithelial cells (HNPE; passages 12-20), a gift from Dr. Miguel Coca-Prados of Yale University, were maintained at 37°C in DMEM media (Gibco, Grand Island, NY) supplemented with 44 mM $NaHCO_3$, 10% fetal bovine serum and antibiotics and grown to confluency in a T-150cm^2 tissue culture flask (Corning Star, Cambridge, MA.).

Isolation of plasma membrane fractions from rat lung tissue

The isolation of plasma membrane was performed as previously described (Dibas, Mia and Yorio, 1996). A male Sprague-Dawley rat was sacrificed and its lung tissue was dissected out and placed on ice. The tissue was then minced and added to modified Krebs Hanks buffer (134 mM NaCl, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, 5 mM KCl, 1.2 mM $MgCl_2$ and 25 mM Hepes, pH 7.4). The tissue pieces were homogenized by 10-20 strokes of a Dounce Homogenizer and quickly spun down. The pellet was suspended in 10 mM Na-HEPES, 0.25 M sucrose buffer and sonicated in the presence of PMSF (10 μg/ml), leupeptin (10 μg/ml), aprotonin (10 μg/ml) and soybean trypsin inhibitor (20 μg/ml). The samples were centrifuged at 100,000 X g for 35 minutes at 4°C. The resultant pellet was resuspended in 50 mM Tris-HCl buffer along with protease inhibitors, 0.1% Triton-X and 0.1% NP-40. This suspension was centrifuged at 30,000 X g for 30 minutes at 4°C. Part of the supernatant containing the plasma membrane fraction was saved for Western blotting of ECE-1 while the remainder was used for ECE-1 activity assay after a Bradford assay for total protein content was done.

Isolation of plasma membrane fraction from HNPE cells

The isolation of plasma membrane was performed as previously described (Dibas, Mia and Yorio, 1996). HNPE cells grown to confluency in T-150 cm^2 flasks, were washed twice in KRB buffer (460 mM NaCl, 20 mM KCl, 95.9 mM $NaHCO_3$, 4 mM $MgCl_2$ 6H2O, 10 mM $CaCl_2$ $2H_2O$, and 0.5 M HEPES at pH 7.4), briefly centrifuged and resuspended in 25 ml of KRB. The pellet was resuspended in homogenization buffer (10

mM Na-HEPES with 0.25 M Sucrose, pH 7.4) along with protease inhibitors: 20 μg/ml leupeptin, 10 μg/ml aprotonin, 20 μg/ml soybean trypsin and 40 μg/ml PMSF. The sample was quickly sonicated and placed on ice. The homogenate was centrifuged briefly to remove unbroken cells and nuclei. The supernatant was centrifuged at 100,000 x g for 35 minutes at 4°C. Portions of the supernatant (cytosolic fraction (C)) were removed for a) SDS-PAGE where it was mixed with 1/4 volume of SDS-sample buffer (4X: 62.5 mM Tris-HCl, pH 6.8, 0.1% (v/v) glycerol, 2% SDS, 0.05% 2-β mercaptoethanol and 0.005% (w/v) bromophenol blue) and b) Bradford protein assay. The remaining pellet (stored on ice) was sonicated in fresh homogenization buffer containing Triton-X 100 (0.1%) and NP-40 (0.1%) after incubating on ice for 30 minutes. The suspension was further centrifuged at 30,000 x g for 30 minutes (4°C). The resulting supernatant (plasma membrane fraction) was removed and part of it was mixed with SDS-sample buffer for gel electrophoresis while the other was used for the ECE-1 activity assay. Protein determination was performed by the Bradford assay. To assay ECE-1 activity of cytosolic fraction, HNPE cells were homogenized in 50 mM Tris-HCl, pH 7.4 with protease inhibitors and the homogenate was centrifuged at 100,000 x g. The supernatant was used for assaying ECE-1 activity in cytosolic fraction.

Localization of ECE-1 by Western Blotting

Membrane protein samples (75 μg total protein for HNPE, 8 μg for rat lung tissue) were separated on a 7.5% SDS-PAGE gel as described before (Laemmli et al.,

1970). Gels were equilibrated in transfer buffer (192 mM Glycine, 20% methanol and 25mM Tris-HCl pH 8.3) for 30 minutes at room temperature and electroblotted on a nitrocellulose membrane for 1 hour at 110 Volts using a Bio-Rad electroblotting unit. The nitrocellulose membranes were dried at room temperature. Western-blotting was performed using a Tropix Chemiluminescent Kit (alkaline phosphatase) (Milford, MA). Membranes were incubated with anti ECE-1 antibody (1:500) for 1.5 hours. The membranes were then incubated with goat anti-rabbit secondary antibody (1:20,000) for 30 minutes. The membranes were exposed to a X-ray film for 30 minutes and later developed.

ECE-1 Activity

The principle of the assay was to measure the production of ^{125}I ET-1 in the presence of the enzyme fraction with ^{125}I-Big ET-1 as the substrate. The reaction mixture included BSA (10 mg/ml as a carrier), membrane fraction (20 μg total protein), ^{125}I-Big ET-1 (80 fmoles/mg protein; substrate), protease inhibitors (2 μl each), 10 μM $ZnCl_2$ (10 μ l of 50 μM stock), inhibitors (when necessary) and the total reaction volume was made up to 50 μl with distilled water. The reactions were started with the addition of ^{125}I-Big ET-1 (80 fmoles/mg protein; substrate). In case of treatments with inhibitors/antagonists, the membrane fraction was pre-incubated for 30 minutes prior to the addition of the substrate. The incubations were done with interval vortexing for the desired time course at 37°C. Reactions were stopped by the addition of 25 μl of anti-Big ET-1 (Peptides

International, Louisville, KY) and immediately placed on ice for 30 minutes. The antibody stock was reconstituted in 1 ml of deionized water and aliquoted. The antibody showed no cross-reactivity to ET-1 but 100% reactivity to Big ET-1 and C-terminal fragment 22-39 a.a, which is the cleaved product along with ET-1. Pansorbin A cells (25 µl) were added to the samples and incubated for an additional 30 minutes at 4°C. The samples were centrifuged for 5 minutes. The supernatant containing the converted ^{125}I ET-1 and the pellet containing unconverted ^{125}I Big ET-1 were counted separately in a gamma counter. The % ECE activity was measured as the ratio of cpm in the supernatant (^{125}I ET-1)/cpm in the pellet (^{125}I Big ET-1) + cpm of supernatant X 100. The amount of ^{125}I ET-1 produced from the reaction (in fmoles), in the presence of the enzyme fraction, was calculated from 1.6 fmoles of ^{125}I Big ET-1 (approx. 10,000 CPM), which was set at 100%. Blanks (reaction mixture contained everything except the enzyme fraction) were run for different time periods to determine the amount of radioactivity attributable to substrate degradation. The blank value was 13% of 1.6 fmoles ^{125}I Big ET-1 (0.2 ± 0.03 fmoles) which was subtracted from the treatments' results and then were expressed as fmoles ^{125}I ET-1 produced/mg protein/time. In experiments involving enzyme inhibitors, EGTA and acidification, % enzyme activity was calculated based on amount of ^{125}I ET-1 produced in the control condition which was then set at 100%. Statistical significance between control and treatment was determined by Student t-test at $p<0.05$ while statistical significance among various treatments was determined by One-way ANOVA and Student-Newman-Keuls test at $p<0.05$.

Results

Endothelin-converting enzyme-1 is present in the plasma membrane of HNPE cells

Having localized Big ET-1 and ET-1 in HNPE cells previously (Prasanna et al., 1998), it was important to establish that these cells produced ET-1 through the action of endothelin converting enzyme-1 (ECE-1). The presence of ECE-1 was investigated using a polyclonal antibody to ECE-1 followed by Western-blotting (Fig. 1). Plasma membrane fractions from rat lung tissue, used as a positive control, also contained ECE-1 although in greater abundance than that seen in HNPE (Fig. 1). As mentioned earlier, ECE exists as 2 major isoforms with ECE-1 localized in the plasma membrane and ECE-2 present in the secretory compartments of the cell. It was therefore necessary to determine the distribution of ECE-1 between plasma membrane and cytosol in HNPE cells. As shown in Figure 1, endothelin converting enzyme-1 (ECE-1), was identified as a 124 kDa protein in the plasma membrane fraction but not in the cytosol fraction of HNPE cells. Interestingly however, in the plasma membrane fraction of HNPE cells, there are 2 additional bands of lower MW (70 & 55 kDa) which are recognized by the antibody (Fig. 1).

ECE-1 activity in HNPE cells occurs in a dose-, time- and substrate-dependent manner

To confirm that ECE-1 was a functional enzyme in HNPE cells, ECE-1 activity was measured by the conversion of ^{125}I Big ET-1 to ^{125}I ET-1 in the presence of the plasma membrane fraction containing the enzyme. The activity of ECE-1 present in the plasma

membrane fraction of rat lung tissue was also tested as a positive control. A protein concentration of 20 μg from HNPE cells was used for further experiments to measure ECE-1 activity. This protein concentration was chosen based on results from a protein-

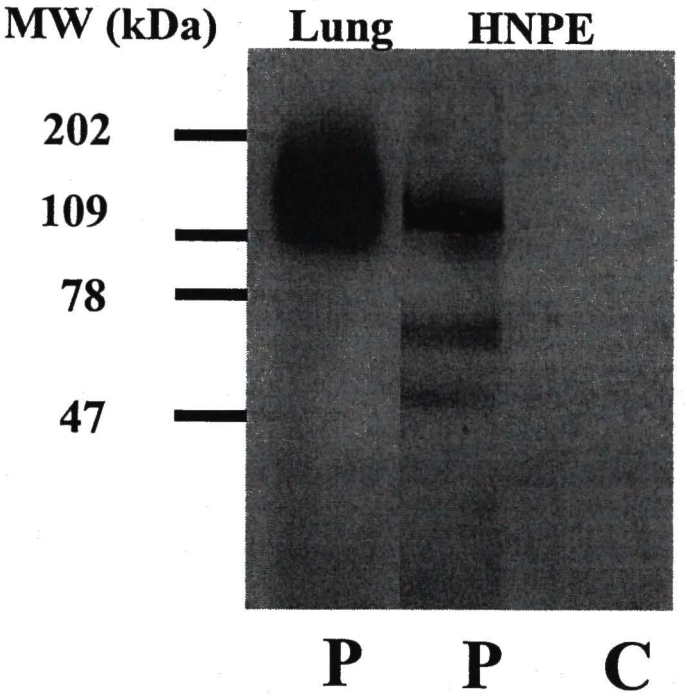

Fig. 1

Fig. 1 Legend

Detection of endothelin converting enzyme-1 (ECE-1) in HNPE cells and rat lung tissue. ECE-1 was detected as 124 kDa protein only in the plasma membrane fraction (arrowheads) of both samples (rat lung cytosol lane not shown). Cytosolic (C) and plasma membrane (P) protein fractions (75 μg total protein for HNPE and 8 μg for rat lung plasma membrane fraction) were separated by 7.5% SDS-PAGE under reducing conditions. They were electrotransferred on to a nitrocellulose membrane and Western blotting was performed using polyclonal anti-rabbit against ECE-1 (1:500) and secondary

goat anti-rabbit antibody (1:20,000). Following chemiluminescent treatment (alkaline phosphatase), the membranes were exposed to an X-ray film for 30 minutes.

dose response assay (5 μg protein: 0.33 ± 0.01 fmoles ^{125}I ET-1 produced/180 min; 10 μg protein: 0.40 ± 0.01 fmoles and 20 μg protein: 0.53 ± 0.02 fmoles) during which a constant substrate concentration (^{125}I Big ET-1: 80 fmoles/mg protein/180 min) was maintained. It should be noted that the fixed substrate concentration could become rate-limiting for ECE-1 activity even though protein concentrations (and consequently, the enzyme concentrations) are increased. Percent ECE-1 activity (measured as the ratio of cpm in the supernatant cpm in the pellet (i.e. 125I Big ET-1) + cpm of supernatant X 100) increased time-dependently and the production of ^{125}I ET-1 from ^{125}I Big ET-1 (expressed as fmoles ^{125}I-ET-1 produced/mg protein/time) was linear in plasma membrane fractions of both HNPE ($r^2 = 0.86$) and rat lung tissue ($r^2 = 0.94$) (Fig. 2). While figure 2 represents a bar graph of ^{125}I ET-1 produced over time, it should be noted that the regression analysis (r^2) and line equation ($y = mx + b$) for ^{125}I ET-1 production over time was initially calculated based on a line graph with equal scale on the X axis ranging from 0-1500 minutes (Figure not shown). In HNPE cells, ECE-1 activity was linear with increasing substrate concentrations (^{125}I Big ET-1; 30-200 fmoles/mg protein/180 min) and the amount of ^{125}I ET-1 produced/mg protein/180 min corresponded to: 5.0 ± 0.5 fmoles (30 fmoles substrate), 13 ± 0.6 fmoles (55 fmoles substrate), 27 ± 1 fmoles (80 fmoles substrate) and 67 ± 1.3 fmoles (200 fmoles substrate) respectively. This linear increase in ECE-1 activity did not reach saturation even at 200 fmoles of substrate (see data above). However, we chose to use the substrate concentration of 80

fmoles/mg protein/180 min for further experiments since it was well within the linear activity of the enzyme.

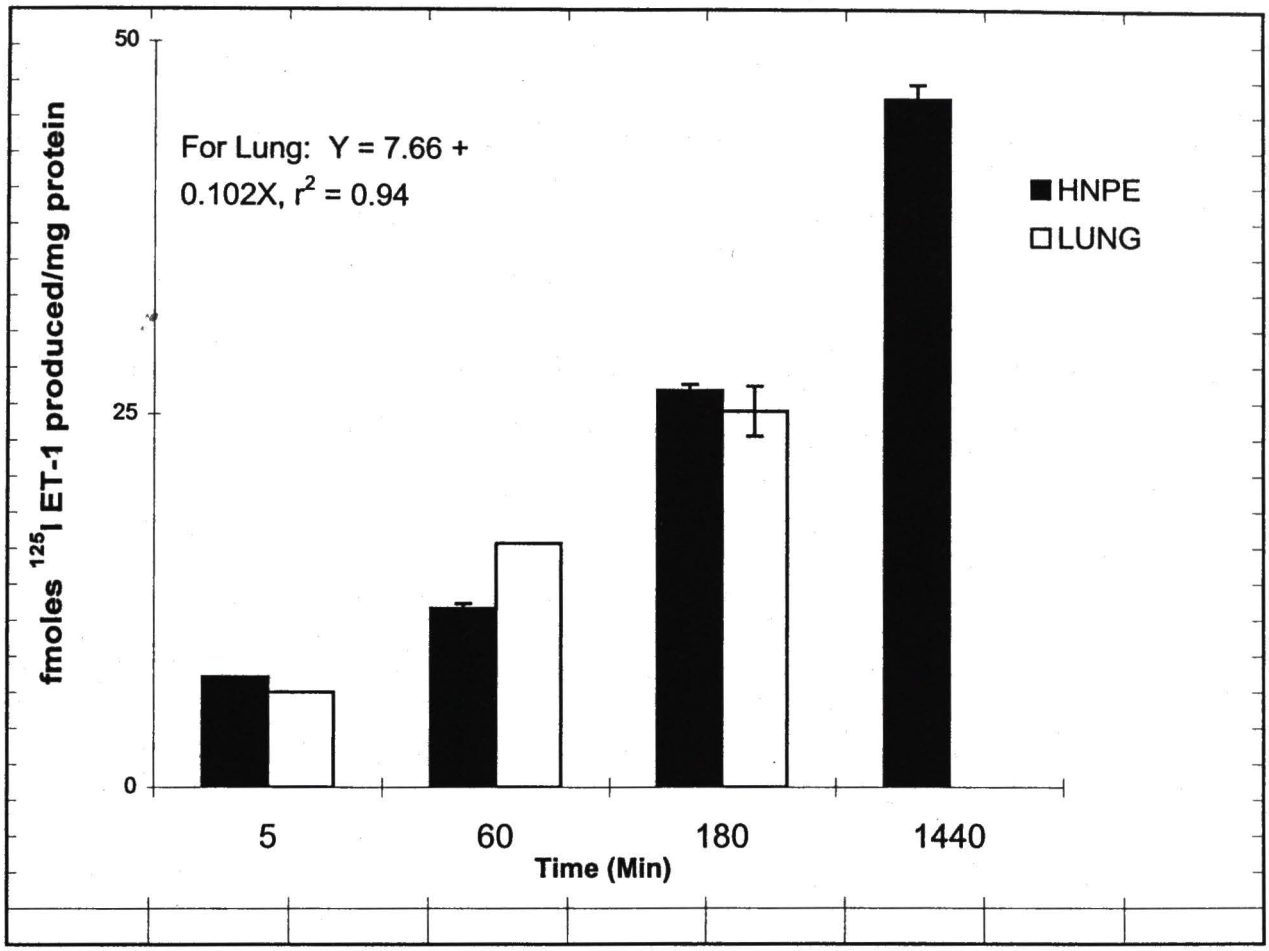

Fig. 2.

Fig. 2 Legend

Bar graph depicting the time-dependent endothelin converting enzyme-1 (ECE-1) activity in HNPE cells and rat lung tissue as measured by a novel assay in which the enzyme converts ^{125}I Big ET-1 to ^{125}I ET-1. Plasma membrane fraction of HNPE cells (20 μg total protein) was incubated with ^{125}I Big ET-1 (80 fmoles/mg protein; substrate) for 5 min, 60 min, 180 min and 24 hours at 37^{O}C while that for rat lung tissue was done for 5

min, 60 min, 180 min. Separation of the product in the supernatant (^{125}I ET-1) from the reaction mixture and determination of product concentration are described in the Materials and Methods section. * denotes statistical significance of mean [ET-1] among different time periods in HNPE cells as determined by Oneway ANOVA and Student-Newman-Keuls multiple comparison test at $p<0.05$ (5 min n=3; 10 min n=6; 180 min n=9; 24 hrs n=4). † denotes statistical significance of mean [ET-1] among different time periods in rat lung tissue as determined by Oneway ANOVA and Student-Newman-Keuls multiple comparison test at $p<0.05$ (n=6 for 180 minutes; n=4 for other time periods). It should be noted that the regression analysis (r^2) and line equation ($y = mx + b$) for percent ECE-1 activity were calculated based on a line graph with equal scale on the X axis ranging from 0-1500 minutes.

While no apparent ECE-1 was detected in the cytosol fraction by the Western blot analysis of HNPE cells, there was ^{125}I ET-1 production in this fraction which was unaffected by treatment with 100μM CGS-26303 (control (n=4): 13 ± 2 fmoles ^{125}I ET-1 produced/mg protein/180 min; CGS-26303 (n=3): 15 ± 0.3 fmoles). Furthermore, this protease activity was unaffected by acidification or EGTA treatments (data not shown). The substrate (^{125}I Big ET-1) concentration used in these experiments was 80 fmoles/mg protein/180 min.

Acidification of the reaction buffer significantly decreases ECE-1 activity in HNPE cells

Since ECE-1 is considered a neutral pH-sensitive enzyme, the effects of acidification on plasma membrane ECE-1 activity was assayed in HNPE cells. Acidification of the reaction buffer from pH 7.4 to 6.4 caused a 52% reduction in ECE-1 activity compared to that observed in neutral pH (at pH 7.4: 7.5 ± 0.3 fmoles ^{125}I ET-1 produced/mg protein/5 min; at pH 6.4: 3.6 ± 0.7 fmoles) (Fig. 3). This observation further confirmed that the majority of enzyme activity observed in HNPE cells was due to the action of neutral pH-sensitive ECE-1.

The metal chelator, EGTA significantly lowers ECE-1 activity in HNPE cell

Metal chelators have been widely used to inhibit the activity of metalloproteases including ECE-1 (Ashizawa et al., 1994). Hence, the effect of EGTA on ECE-1 activity in HNPE cells was tested in HNPE cells. EGTA (2 mM) significantly decreased ECE-1 activity by nearly 60% (5 ± 1 fmoles ^{125}I ET-1 produced/mg protein/60 min) compared to that observed in the untreated control (12 ± 0.5 fmoles ^{125}I ET-1 produced/mg protein/60

min) (Fig. 4).

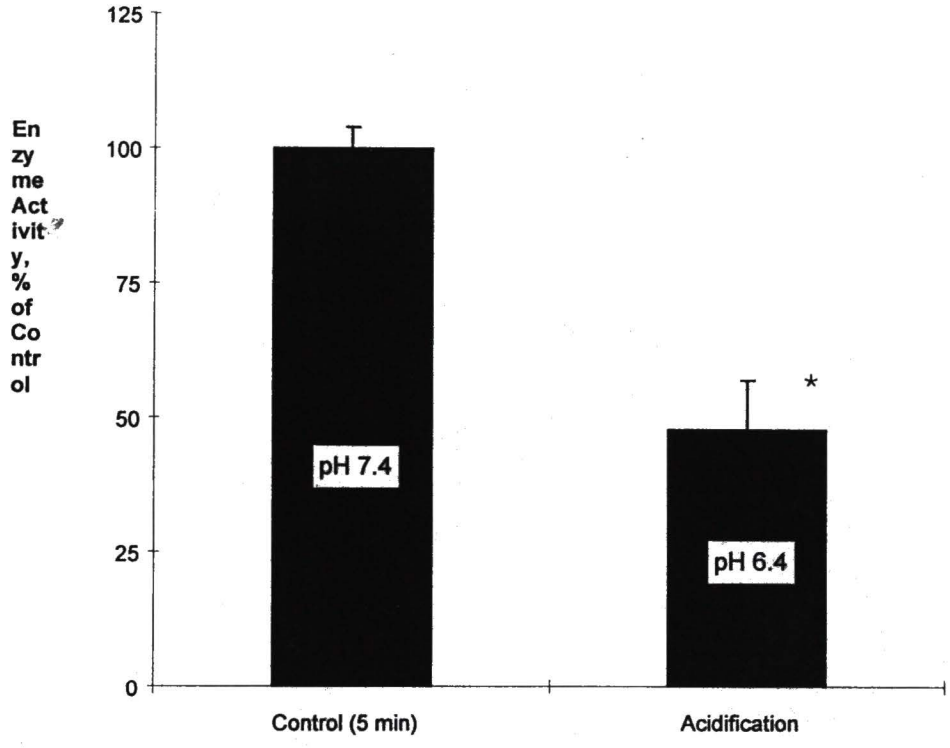

Fig. 3.

Fig. 3 Legend

Effect of acidification on ECE-1 activity in HNPE cells. Plasma membrane fractions of HNPE cells (20 μg total protein) were incubated with ^{125}I Big ET-1 (substrate: 80 fmoles/mg protein) for 180 minutes at 37^{O}C where the reaction mixture was acidified to pH 6.4 or maintained at pH 7.4. Percent enzyme activity in the treatments were compared to the control in which the amount of ^{125}I ET-1 produced was set at 100%. Separation of the product in the supernatant (^{125}I ET-1) from the reaction mixture and determination of product concentration are described in the Materials and Methods section. * denotes statistical significance of mean [ET-1] between neutral pH condition and acidic pH

condition as determined by Student t-test at $p<0.05$ (n=3).

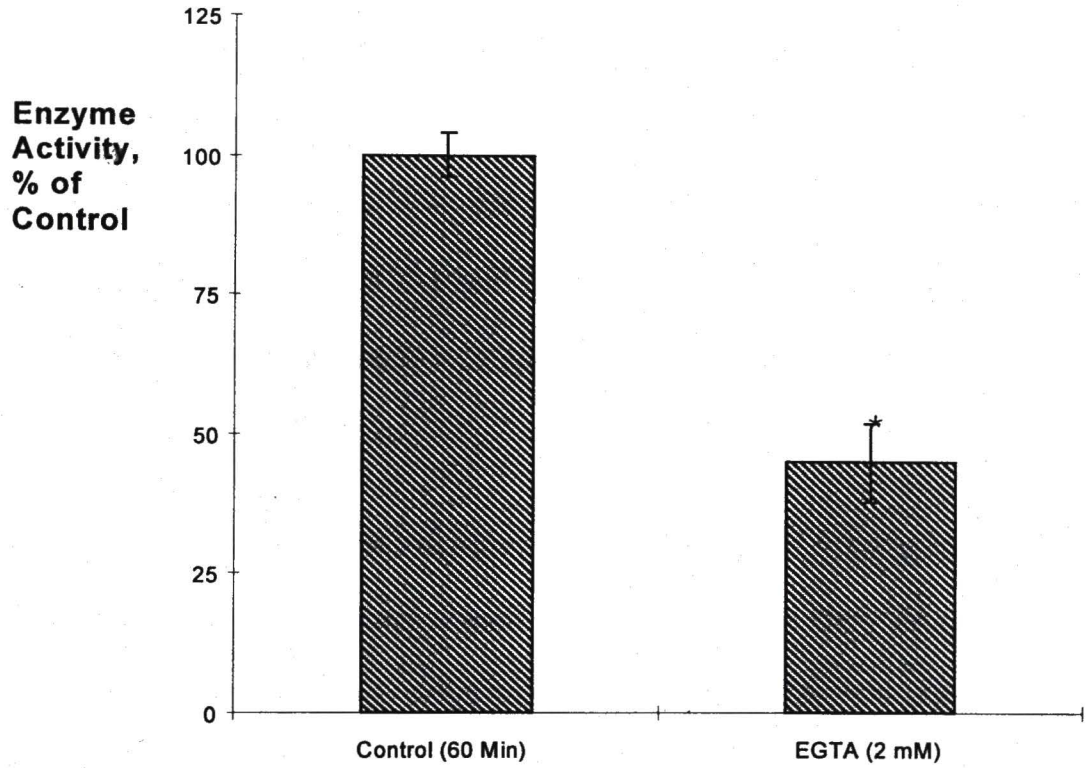

Fig. 4

Fig. 4. Legends

Effect of the metal chelator, EGTA on ECE-1 activity in HNPE cells. Plasma membrane fraction of HNPE cells (20 μg total protein) was pre-incubated with EGTA (2 mM) for 30 minutes following which ^{125}I Big ET-1 (80 fmole/mg protein; substrate) was added for 5 minutes at 37^{O}C while the control fraction did not contain EGTA. Percent enzyme activity in the treatments were compared to the control in which the amount of ^{125}I ET-1 produced was set at 100%. Separation of the product in the supernatant (^{125}I ET-1) from

the reaction mixture and determination of product concentration are described in the Materials and Methods section. * denotes statistical significance between control and EGTA treatment as determined by Student t-test at $p<0.05$ (n=5 control; n=3 EGTA).

ECE-1 activity is significantly inhibited by CGS-26303 in HNPE cells

CGS-26303 has recently been used as an inhibitor of ECE-1 in many different cells and tissues (De Lombaert et al., 1994; Pelletier et al., 1998). CGS-26303 (100 μM) caused nearly a 60% inhibition of ECE-1 activity in HNPE cells compared to the control (Table. 1). These values were similar to that observed in ECE-1 activity of rat lung tissue (Table. 1).

ECE-1 activity is inhibited only at high concentrations of thiorphan in HNPE cells

Thiorphan is an inhibitor of both neutral endopeptidase 24.11 (NEP 24.11), at low (nM) concentrations, and ECE-1, at higher (mM) concentrations (Malfroy and Schwartz, 1982; MacMahon et al., 1991; Turner and Murphy, 1996). ECE-1 activity was tested in the presence of the two concentrations of thiorphan (50 nM and 2 mM) since NEP 24.11 may also contribute to the conversion ^{125}I Big ET-1 to ^{125}I ET-1 in HNPE cells in addition to ECE-1. In HNPE cells, low concentrations of thiorphan (50 nM) did not inhibit ECE-1 activity compared to control, however, at higher concentrations (2 mM), ECE-1 activity was completely abolished (control: 27 ± 1 fmoles ^{125}I ET-1 produced/mg protein/180 min; low thiorphan: 29 ± 1 fmoles; high thiorphan: 1.1 ± 1 fmoles) (Fig. 5). Similar observations were found with the rat lung tissue (control: 32 ± 3 fmoles ^{125}I ET-1 produced/mg protein/180 min; low thiorphan: 29 ± 2 fmoles; high thiorphan: 0.45 ± 0.18

fmoles) (Fig. 5).

Treatment (*n*)	fmoles ^{125}I ET-1 produced/mg protein/180 min
HNPE Cells	
Control (9)	27 ± 1
100 μM CGS-26303 (5)	12 ± 2*
Rat Lung Tissue	
Control (6)	25 ± 1
100 μM CGS-26303 (4)	11 ± 4*

Each value represents mean ± S.D. of *n* samples. * $p<0.05$ vs. control

Table. 1. Effect of CGS-26303, an ECE-1 inhibitor on ECE-1 activity in the plasma membrane fractions of HNPE cells and rat lung tissue. Plasma membrane fraction of HNPE cells and rat lung tissue (20 μg total protein) were pre-incubated with CGS-26303 (100 μM) for 30 minutes following which ^{125}I Big ET-1 (80 fmole/mg protein; substrate) was added for 180 minutes at 37°C while the control fraction did not contain the inhibitor. Separation of the product in the supernatant (^{125}I ET-1) from the reaction mixture and determination of product concentration are described in the Materials and Methods section. Statistical significance between control and CGS-26303 treatment was determined by Student t-test at $p<0.05$.

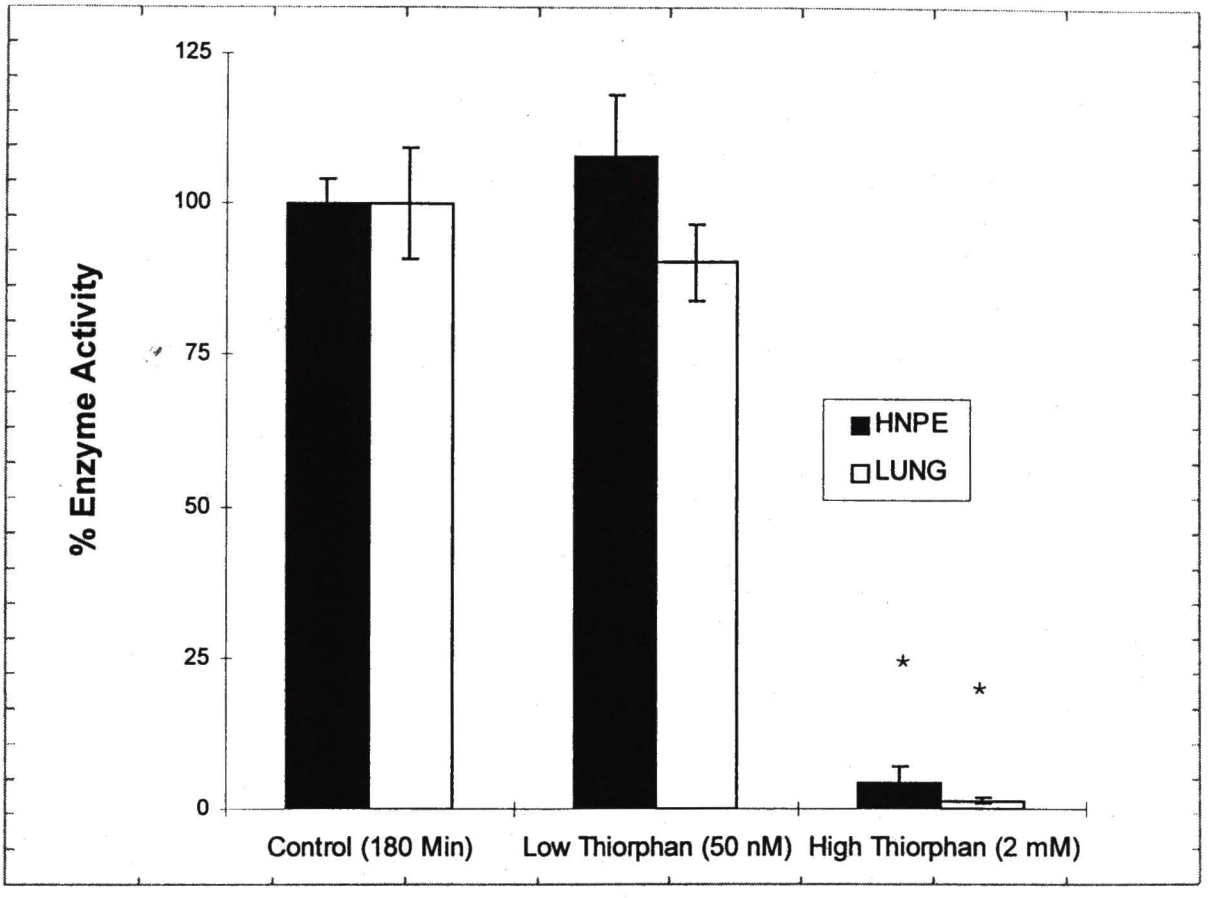

Fig. 5.

Fig. 5. Legend

Effects of low and high doses of thiorphan, a dual inhibitor of NEP 24.11 and ECE-1, on ECE-1 activity in HNPE cells and rat lung tissue. Plasma membrane fractions of HNPE cells and rat lung tissue (20 μg total protein) were pre-incubated with thiorphan either at low (50 nM) or high (2 mM) doses for 30 minutes following which ^{125}I Big ET-1 (80 fmole/mg protein; substrate) was added for 180 minutes at 37^{O}C while the control fraction did not contain the inhibitor. Percent enzyme activities in the treatments were compared to the control in which the amount of ^{125}I ET-1 produced was set at 100%. Separation of the product in the supernatant (^{125}I ET-1)

from the reaction mixture and determination of product concentration are described in the Materials and Methods section. * denotes statistical significance between control and high (2 mM) thiorphan treatment as determined by Oneway ANOVA and Student-Newman-Keuls multiple comparison test at $p<0.05$ (HNPE: n=9 control; n=3 for both doses of thiorphan; Lung: n=3 for all three conditions).

Discussion

In this study, we have identified for the first time, a functional endothelin converting enzyme, ECE-1, in human non-pigmented ciliary epithelial (HNPE) cells. Elevated endothelin (ET) levels in the aqueous humor (AH) of normal eyes (i.e. eyes of non-glaucomatous patients) with respect to plasma levels, could be contributed largely due to endogenous ET production from the anterior/posterior chamber tissues. Immunohistochemical and molecular techniques show an abundant distribution of ET in ocular tissues, including the ciliary epithelium, suggesting that the reason for elevated ET levels in AH could be due to endogenous ET production (MacCumber, Jampel and Snyder, 1991; Chakravarthy et al., 1994; Lepple-Weinhues et al., 1992). We have previously shown that HNPE cells secrete ET-1, constitutively and following cytokine stimulation, suggesting that many factors could indeed regulate ocular ET concentrations (Prasanna et al., 1998).

A major factor that regulates ET-1 levels, is the rate of conversion of Big ET-1 to ET-1 by an endothelin converting enzyme (ECE-1). Using a novel assay, we observed ECE-1 activity in HNPE cells to be linear for different concentrations of protein, substrate and time. Furthermore, ECE-1 activity in HNPE cells was comparable to that observed in rat lung tissue (Fig. 2 and Table. 1). ECE-1 activity has been well characterized in non-ocular tissue, including those of the lung and vascular endothelium, with maximal ECE-1 activity being observed in lung tissue since it is a major source of ECE-1 (Sawamura et al., 1993; Barnes et al., 1995; Takahashi et al., 1993). Takaoka et al., (1991) have shown the lung membrane fraction to exhibit 4-times more ECE activity

than that observed in the cytosolic fraction. In HNPE cells however, it was found that ECE-1 activity in plasma membrane was two times greater than that observed in the cytosolic fraction.

Results from additional experiments involving the effects of pH-sensitivity, metal chelators and enzyme inhibitors on ECE-1 activity in HNPE cells were comparable to that observed in earlier reports involving ECE-1 activity in different cells and tissues. ECE-1 has been shown to be a neutral pH-sensitive enzyme with an optimal pH of 6.8 (Xu et al., 1994). However, in transfected CHO cells, when the pH was altered to 6.5, ECE-1 activity decreased by nearly 50% but remained nearly unchanged at pH 7.4 (Xu et al., 1994). In porcine lung membranes, Sawamura et al. (1993) have observed that approximately 80% of ECE activity in membrane fractions occur at neutral pH ranges. In HNPE cells, a 50% reduction in ECE-1 activity occurred within 5 minutes of incubation in acidified buffer (Fig. 3).

In bovine aortic endothelial cells, ECE-1 has been shown to be a Zn^{2+}-metalloprotease whose activity is dose-dependently inhibited by the addition of metal chelators such as EDTA, 2,3 dimercapto-1-propanol and 8-mercaptoquinoline (Ashizawa et al., 1994; Xu et al., 1994). This inhibition could be reversed by the addition of increasing concentrations of Zn^{2+} but not with other divalent cations (including Mg^{2+}, Fe^{2+} and Ca^{2+}). The ability of EGTA (2 mM) in the absence of $ZnCl_2$, to produce a 60% inhibition of ECE-1 activity in HNPE cells further suggests that this enzyme is a metalloprotease. Since we observed no inhibition of ECE-1 in the absence of $ZnCl_2$ in HNPE and rat lung tissue (data not shown), it is probable that some residual zinc ions

could have been present in the membrane fraction to activate the enzyme. However, in the presence of EGTA, this activity was significantly reduced, suggestive of EGTA's chelation of the residual zinc ions in the membrane fraction.

To further determine if the observed endothelin conversion activity in HNPE and rat lung tissue was due to the metalloprotease ECE-1, experiments measuring ECE-1 activity were performed in the presence and absence of the metalloprotease inhibitors, CGS-26303, an ECE-1 inhibitor, and thiorphan. While CGS-26303 is considered as an inhibitor of ECE-1 (Lombaert et al., 1994; Caner et al., 1996; Pelletier et al., 1998), the role of thiorphan as an ECE-1 inhibitor has been considerably debated and appears to be effective only at micromolar-millimolar concentrations (MacMahon et al., 1991; Turner and Murphy, 1996). Furthermore, thiorphan (at nM concentrations) is widely used as an inhibitor of the neutral endopeptidase 24.11 (NEP 24.11), a enzyme capable of both degrading ET-1 and also performing ECE-like activity (conversion of Big ET-1 to ET-1) (Malfroy and Schwartz, 1982; McMahon et al., 1991; Rubanyi and Polokoff, 1994). The latter function of NEP 24.11 however, is thought to be minimal (Rubanyi and Polokoff, 1994). CGS-26303 (100 μM) and thiorphan (50 nM and 2 mM) were compared to determine the relative contributions of ECE-1 and NEP 24.11 on the observed ECE-activity in HNPE cells and rat lung tissue. At low concentrations of thiorphan (50 nM), sufficient to inhibit only NEP 24.11 activity, no inhibition on ECE-activity was observed both in HNPE cells or in rat lung tissue. However, at high thiorphan concentrations (2 mM), complete abolition of enzyme activity was observed, suggesting that ECE-1 was probably inhibited in both samples (Fig. 5). Furthermore, Western blot analysis using

monoclonal antibodies against NEP 24.11 failed to detect any proteins consistent with NEP 24.11 in the plasma membrane fraction and cytosol of HNPE cells (data not shown). These data strongly support the role of ECE-1 as the primary enzyme responsible for ET-1 production in HNPE cells. Western blot analysis confirmed that ECE-1 was detected only in the plasma membrane fractions of HNPE cells and rat lung tissue, as a membrane-bound protein with an approximate molecular mass of 120-130 kDa (Fig. 1). These observations are similar to previous studies on purified ECE found in other tissues (Ohnaka et al. 1993; Shimada, Takahashi and Tanzawa, 1994; Takahashi et al. 1995). In Figure 1, two additional smaller-MW bands were seen in the HNPE membrane fraction which could represent either the protein fragments that have undergone proteolytic degradation (during the isolation procedure of plasma membrane fraction) and are still recognized by anti-ECE-1 or these fragments could represent unique ECE-like enzymes which seem to cross-react with the antibody. Recently, Hasegawa et al., (1998) have purified a novel ECE in bovine iris microsomes which is specific for Big endothelin-3 and is quite different from ECE-1 and ECE-2.

Interestingly, ECE-like protease activity was also observed in the cytosol of HNPE cells which was not inhibited by acidification, EGTA or CGS-26303. Recently, ECE-1 has been shown to exist as three isoforms with distinct subcellular localization with ECE-1a and -1c localized at the cell surface while ECE-1b is found intracellularly, in the trans-golgi network (Shimada, Takahashi and Tanzawa, 1994; Schweitzer et al., 1997). This distinct subcellular localization of ECE-1 isoforms could explain the ECE-like activity observed in the cytosolic fraction of HNPE cells. Currently, there are no data

available in ocular cells, concerning the susceptibility of cytosolic ECE-like activity to inhibitors other than that reported here. In conclusion, we have identified an endothelin converting enzyme, ECE-1 and characterized its activity in HNPE cells based on a novel assay. ECE-1 activity in HNPE cells is similar to that observed in rat lung tissue and is the likely regulating enzyme responsible for aqueous humor ET-1. Therefore, ECE-1 activity could be considered as a potential target site for regulating ET-1 levels in ocular tissues.

Acknowledgments:

This work was supported by Texas Advanced Research Program Grant from the Texas Higher Education Coordinating Board (Grant number: 09768-008/018)

References

Ashizawa, N., Okumura, H., Kobayashi, F., Aotsuka, T., Takahashi, M., Asakura, R., Arai, K., and Matsuura, A. (1994). Inhibitory activities of metal chelators on endothelin converting enzyme. I. *In* Vitro studies. *Biol. Pharm. Bull.* 17 (2): 207-211.

Barnes, K., Murphy, L.J., Takahashi, M., Tanzawa, K. and Turner, A.J. (1995). localization and characterization of endothelin converting enzyme. *J. Cardiovasc. Pharm.* 26 (3). S37-S39.

Caner, H.K., Kwan, A.L., Arthur, A., Jeng, A.Y., Lappe, R.W., Kassell, N.F. and Lee, K.S. (1996). Systemic administration of an inhibitor of endothelin converting enzyme for attenuation of cerebral vasospasm following subarachnoid hemorrhage. *J. Neurosurg.* 85, 917-922.

Caprioli, J. (1987). The ciliary epithelia and aqueous humor. In: *Adler's physiology of the eye: clinical application* (Moses, R.A. and Hart, W.M. Eds.) pp 204-222. Mosby Press.

Chakaravarthy, U., Douglas, A. J., Bailey, J. R., McKibben, B. and Archer, D.B. (1994). Immunoreactive endothelin distribution in ocular tissue. *Invest. Ophthalmol. Vis. Sci.* 35, 2448-2454.

Corder, R., Harrison, V.J., Khan, N., Anggard, E.E. and Vane, J. (1993). Effects of phosphoramidon in endothelial cell cultures of endogenous synthesis of endothelin-1 and on the conversion of exogenous big ET-1 to ET-1. *J.Cardiovasc. Pharmacol.* 22 (8), S73-S76.

D

e Lombaert, S., Ghai, R.D., Jeng, A.Y., Trapani, A.J., and Webb, R.L. (1994). Pharmacological profile of a non-peptidic dual inhibitor of neutral endopeptidase 24.11 and endothelin converting enzyme. *Biochem. Biophys. Res. Commun.* 204, 407-412.

Dibas, A.I., Mia, A.J. and Yorio, T. (1996). Is protein kinase C alpha (PKC α) involved in vasopressin-induced effects on LLC-PK_1 pig kidney cells? *Biochem. Mol. Biol. Int.* 39, 581-588,

Eichorrn, M and Lutjen-Drecoll, E. (1993). Distribution of endothelin-likeimmunoreactivity in the human ciliary epithelium. *Curr. Eye. Res.* 12 (8), 753-757.

Emoto, N. and Yanagisawa, M. (1995). Endothelin-converting enzyme-2 is a membrane-bound phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J. Biol. Chem.* 270, 15262-15268.

Erickson-Lamy, K., Korbmacher, C., Schuman, J.S. and Nathanson, J.A. (1991). Effect on endothelin on outflow facility and accommodation in the monkey eye in vivo. *Invest.*

Ophthalmol. Vis. Sci. 32, 492-495.

Hasegawa, H., Hiki, K., Sawamura, T., Aoyama, T., Okamoto, Y., Miwa, S., Shimohama, S., Kimura, J. and Masaki, T. (1998). Purification of a novel endothelin-converting enzyme specific for big endothelin-3. *FEBS Lett.* 428, 304-308.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto., K. and Masaki, T. (1989). The human endothelin family: three structurally and harmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci.* USA. 86, 2863-67.

Kaiser, H.J., Flammer, J., Wenk, M. and Luscher,T. (1995). Endothelin-1 plasma levels in normal tension glaucoma: abnormal response to postural changes. *Graefes Arch. Clin. Exp. Opthalmol.* 233, 484-488.

Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989b). Conversion of big endothelin-1 to 21 residue endothelin-1 is essential for the expression of full vasoconstrictor activity: structure activity relationships of big endothelin-1. *J. Cardiovasc. Pharmacol.* 13 (Suppl 5). S5-S7.

Laemmli, U. K., (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature.* 227, 680-684.

Lepple-Weinheus, A., Becker, M., Stahl, F., Willner, U., Schäfer, R., and Wiederholt, M. (1991). Endothelin-evoked contractions in bovine ciliary muscle and trabecular meshwork: interaction with calcium, nifedipine and nickel. *Curr. Eye. Res.* 10, 983-989.

Lepple-Weinheus, A., Becker, M., Stahl, F., Berweck, S. and Hensen, J. (1992). Endothelin-like immuoreactivity in the aqueous humor and in conditioned medium from ciliary epithelial cells. *Curr. Eye. Res.* 11 (11): 1041-1046.

MacCumber, M. W., Jampel, H.D. and Snyder, S.H. (1991). Ocular effects of the endothelins: Abundant peptides in the eye. *Arch. Ophthalmol.* 109, 705-709.

MacMahon E.G., Palomo, M.A., Moore, W.M., MacDonald, J.F. and Stern, M.K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin-1 (1-39) *in vivo* and conversion of big endothelin-1 (1-39) to endothelin-1 (1-21) *in vitro. Proc. Nat. Acad. Sci. USA.* 88, 703-707.

Malfroy, B. and Schwartz, J. (1982). Properties of "enkephalinase" from rat kidney: Comparison of dipeptidyl-carboxypeptidase and endopeptidase activities. *Biochem. Biophys. Res. Commun.* 106, 276-285.

Moriya, S., Sugiyama, T., Shimizu, K., Hamada, J., Tokuoka, S. and Azuma, I. (1992). Low tension glaucoma and endothelin (ET-1). *Folia. Ophthalmol. Jpn.* 43, 554- 559.

Noske, W., Hensen, J. and Weiderholt, M. (1997). Endothelin-like immunoreactivity in aqueous humor of primary open-angle glaucoma and cataract patients. *Graefes Arch.Clin.Exp. Ophthalmol.* 235, 551-552.

Ohnaka, K., Takayangi, R., Yamauchi, T., Okazaki, H., Ohashi, M., Umeda, F. and Nawata, H. (1990). Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.* 168, 1128-1136

Pelletier,S., Battistini,B., Jeng, A.Y., and Sirios, P. (1998). Effects of dual endothelin converting enzyme/ neutral endopeptidase inhibitors CGS 26303 and CGS 26393, on lipopolysaccharide or interleukin-1b-stimulated release of endothelin-1 from guinea pig tracheal epithelial cells. *J. Cardiovasc. Pharm.* 31, S10-12.

Prasanna, G., Dibas, A., Tao, W., White, K. and Yorio, T. (1998). Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-?. *Exp. Eye Res.* 66, 9-18.

Rubanyi, G.M. and Polokoff, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, and pathophysiology. *Pharmacol. Rev.* 46, 325-415.

Sawamura, T., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T.and Kimura, S. (1993). Characterization of phosphoramidon-sensitive metallaproteinases with endothelin converting enzyme activity in porcine lung membranes. *Biochemica et Biophysicia Acta.* 1161, 295-302.

Schweitzer, A., Valdenaire, O., Nelbock, P., Deuschle, U., Dumas Milne Edwards, J-B., Stumpf, J.G. and Loffler, B.-M. (1997). Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localizations. *Biochem. J.* 328, 871-877.

Shimada, K., Takahashi, M. and Tanzawa, K., (1994). Cloning and functional expression of endothelin-converting enzyme form rat endothelial cells. *J. Biol. Chem.* 269, 18275-18278.

Sugiyama, T., Moriya, S., Oku, H. and Azuma, I. (1995). Association of endothelin-1 with normal tension glaucoma: clinical and fundamental studies. *Surv. Ophthalomol.* 39, S49-S56.

Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, H., Koike, Y., Yamamoto, Y. and Tanzawa, K. (1995). Localization of rat endothelin converting enzyme to vascular endothelial cells and some secretory cells. *Biochem. Journal.* 311, 657-665.

Takahashi, M., Matsushita, Y., Iijima, Y. and Tanzawa, K. (1993). Purification and characterization of endothelin-converting enzyme from rat lung. *J. Biol. Chem.* 268, 21394-98.

Takaoka, M., Shiragami, K., Fujino, K., Miki, K., Miyake, Y., Yasuda, M., Wang., G.F.,

Hisaki, K., Matsumura, Y. and Morimoto, S. (1991). Phosphoramidon sensitive endothelin converting enzyme in rat lung. *Biochem. Int.* 25, 697-704.

Tezel, G., Kass, M.A., Kolker, A.E., Becker, B. and Wax, M.B. (1997). Plasma and aqueous humor endothelin-1 levels in primary open-angle glaucoma. *J. Glaucoma.* 6, 83-89.

Turner, A.J. and Murphy, L.J. (1995). Molecular pharmacology of endothelin converting enzymes. *Biochem. Pharmacol.* 51, 91-102.

Weiderholt, M., Lepple-Weinhues, A. and Stahl, F. (1993). Contractile properties of trabecular meshwork and ciliary muscle. In: *Basic Aspects of Glaucoma Research III.* (Ed. Ludjen-Drecoll, E.) pp 287-306.

Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994). ECE-1: A membrane bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 78, 473-485.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 332, 411-15.

Chapter III: Discussion

Identification of Endothelin Converting Enzyme-1 in Human Non-Pigmented Ciliary Epithelial Cells

Chapter III

Discussion:

The role of endothelins as active ocular peptides involved in the regulation of IOP has gained increased interest, particularly in the regulation of aqueous humor dynamics (Erickson-Lamy et al., 1991, MacCumber et al., 1991). Support for this contention was enhanced with the finding that endothelins are widely localized in ocular tissues including the retina, choroid and ciliary body (Chakravarthy et al., 1994). Studies by MacCumber et al., 1989, have also shown that ET-1 binding sites and mRNA are highly concentrated in the iris. However, MacCumber et al., 1991, did not examine the distribution of ET- 1 binding in all ocular tissue compartments. It is the general hypothesis that ET-1 in the eye is synthesized and released by the ciliary epithelial cells and through a paracrine type action on the ciliary muscle induces contraction which has a positive effect on the TM by decreasing resistance and enhancing outflow. Since ET-1 is a mature and active regulatory peptide that is cleaved from an inactive precursor, ECE-1, the enzyme responsible for the conversion could possibly represent a candidate for regulation of ET-1 actions on the ciliary muscle and TM. As a result, this may offer insight into the regulation of aqueous humor dynamics and IOP by endothelin.

The current study demonstrated that ECE- 1 is contained in the HNPE cell membranes and is responsible for the conversion of the non-active Big ET-1 to the active ET-1 peptide. Confirmation of the presence of ECE-1 in HNPE cells was accomplished through Western blot analysis which resulted in the identification of a membrane bound

enzyme with an approximate molecular mass of 120-130 kDa, that was not localized in the cytosol. These findings confirm work performed by other groups using SDS/PAGE analysis with purified ECE indicating that ECE is a 120-130k Da protein (Takahashi et al.,1995; Ohnaka et al., 1993; Shimada et al., 1994; Turner and Murphy, 1996). Earlier studies predicted the molecular mass of ECE to be 86 kDa based on the cDNA sequence (Shimada et al., 1994; Xu et al., 1994). Others have suggested that the native molecular mass of ECE is between 250-350 kDa (Matsumura et al., 1992; Sawarnura et al., 1993; Waxman et al.,1994). It has been found that partially purified ECE from EA.hy926 cells could be renatured and assayed by SDS/PAGE and produces a distinct band that migrates as protein with a M_r of 280 kDa, which was consistent with a M, 250 kDa observed after gel filtration (Waxman et al., 1994; Corder et al., 1995). The EA.hy926 cell line contains a metallopeptidase ECE that on subcellular fractionation, appears to be associated predominantly with a plasma membrane fraction as a ectoenzyme (Waxman et al., 1994; Corder et al., 1995). Also, this particular cell line ECE is apparently twice the size of that reported in porcine aortic endotheliurn (Yanagisawa et al., 1988). Schmidt et al., 1994, have purified ECE from a membrane preparation of a permanent bovine endothelial cell line, FBHE. This represents the first demonstration that ECE may be an inducible enzyme. These and other authors have also established by SDS/PAGE, under reducing and non-reducing conditions, that bovine endothelial ECE exists as a disulfide-linked dimer, each subunit being 120 kDa (Schmidt et al., 1994; Takahashi et al., 1995). Therefore our current ocular ECE-1 enzyme appears to be identical to that reported in other tissues.

The cleavage site of Big ET- 1 to ET-1 by ECE is located between two hydrophobic residue (Trp21-Val^{22}) and qualifies as an "unusual " proteolytic cleavage (Yanagisawa et al., 1988).This was confirmed by researchers who observed no difference in common non-specific serine and aspartyl proteases that were able to perform ECE activity (Takaoka et al., 1990; Sawamura et al., 1990). Furthermore, Fabbrini et al., 1993, demonstrated that in Xenopus oocytes the conversion of Big ET-1 to ET-1 does not require the Trp-VaI sequence suggesting that conformation of the processing site rather than specific sequence defines cleavage by ECE. Therefore, a genuine ECE must be located in the correct cellular compartment with the appropriate topology and specificity to mediate ET production efficiently. Apparently the ocular ECE-1 identified is membrane-bound and probably mediates the extracellular conversion of Big ET- 1 . This may be important relative to its possible role as a paracrine regulator. The activity of ECE-1 is critical for reducing ET-1 since the enzyme appears to be responsible for the production of ET-1 in various tissues (Xu et al., 1995), including endothelium. and vascular endothelium with maximal activity being observed in the lung tissue (Sawamura et al., 1993; Barnes et al., 1995). Takaoka et al., 1991, have shown that the lung membrane exhibits four times more ECE activity than that observed in the cytosolic fraction. Corder et al., 1993, have demonstrated a concentration dependent conversion of Big ET-1 to ET-1 by ECE-1 after a I hour incubation. However, this conversion activity decreased with increasing substrate concentrations. In the current study using HNPE cells, ECE-1 activity (measured by the amount of ^{125} I ET-1 produced) increased with time following incubation with 2 femto mol of ^{125}I Big ET-1 (substrate) and 20~ig of

plasma membrane proteins. Maximal ECE- 1 activity was observed at 3 hours after incubation and was maintained through 24 hours of incubation with 63-66% turnover respectively, provided the substrate concentration was maintained at 2 fernto moles.

Such activity could account for the ET-I concentrations measured in aqueous humor. Recently it has been shown that POAG patients had increased aqueous humor ET-I concentrations as compared to matched non-glaucoma patients (Weiderholt et al., 1997).The ocular effects of ET have been well documented in a variety of species including, cats (Granstam et al., 1992), monkeys (Erickson-Lamy et al., 199 1), and rabbits (MacCumber et al., 199 1). ET produces a rapid and reversible contraction of the isolated retinal branches of short and posterior ciliary arteries (Nyborg et al., 1991) and is the most potent agonist in contracting bovine retinal pericytes (Ramachandran et al., 1993). Intravitreal injections of ET- 1 produces a dose-dependent and sustained decrease in optic-nerve head (ONH) blood flow without influencing blood flow in the ciliary body, iris and choroid. Intracameral injections of ET-1 into monkey eyes increased outflow facility from 22-77%, (EricksonLamy et al., 199 1). However, when ET- 1 was administered intracamerally a reduction in pupil size, and an increase in aqueous humor, and an increase in the concentration of PGE2 in the aqueous humor was recorded (Granstam et al., 1992). Sugiyama et al., I 995b, reported that intravitreal injections of ET-1 (0.05-.15~tg) caused a prolonged reduction in IOP without an initial elevation in IOP, whereas ET- 1 concentrations 0.5gg and higher caused the initial IOP rise followed by a prolonged reduction in IOP. This initial rise in IOP appears to be mediated by PGE_2 similar to the intracameral injection in cats (Granstam et al., 1992), and possibly other

cyclooxygenase products. Although the actions of endothelins are variable, it appears to have significant effects on aqueous dynamics and IOP. In the current study we have identified ECE-1 by immunofluorescence and Western blot analysis.and have characterized its activity in HNPE cells. The signal and production of the precursor peptide (Big ET-1) can be initiated from several sources including the ciliary arteries in the posterior chamber of the eye, where Big ET-1 can permeate through the pigmented and non-pigmented ciliary epithelial bilayer. The non-pigmented ciliary epithelial cells which contain ECE-1 may be responsible for the maturation of ET-1. Once Big ET is processed it can possibly cause a paracrine type stimulation of the ciliary muscle, that would aid aqueous humor outflow, not only in normal subjects but also in subjects with elevated IOP. However, if ET- 1 is over produced in POAG patients it could have detrimenal effects on the optic nerve head by causing retinal vascular vasoconstriction. Recently chronic administration of low doses of ET-1 intraviterally produced optic nerve head damage indistinguishable from glaucoma (Cioffi et al, 1995). In glaucoma, progressive optic nerve head neuropathy leads to blindness and has been associated with different risk factors such as elevated intraocular pressure (IOP) (as seen in primary open angle glaucoma) and blood flow dysregulations (ischemia or vasospasms; implicated in most normotensive glaucoma) (Flammer J. et al., 1997; Greve et al., 1997). Lepple-Wienhues et al., 199 1, clearly demonstrated ET mediated contractions on isolated TM and ciliary muscle strips. Lepple-Wienhues et al., 1992, demonstrated for the first time that ET- like immunoreactivity (ETIR) in aqueous humor in human and bovine eyes is 2-3 times greater than the corresponding plasma levels. Patients with primary open-angle

glaucoma (POAG) and cataract also had higher ET- 1 concentrations in AH than those with cataract alone (Noske et al., 1997). No significant differences in plasma ET- 1 concentrations have been observed in POAG and normal subjects (Noske et al., 1997; Tezel et al., 1997). These authors also demonstrated that non-pigmented ciliary epithelial cells express a high potential for releasing $ET\text{-}1_{ir}$. The current study confirms these observations and clearly demonstrates that ECE-1 is not only present but may also be responsible for producing immunoreactive ET- 1 in HNPE cells. These observations also suggest that the regulation of ET- 1 concentrations in the eye may be mediated locally i.e. within various ocular tissues (ciliary epithelium, ciliary muscle and iris) in a paracrine manner, rather than coming from the circulatory system. The non-pigmented ciliary epithelium has been suggested to be a major source of ET- 1 and may be a potential site for regulation of AH dynamics (Lepple-Weinhues et al., 1992; Eicchom and LudJen-Drecoll, 1993; Prasanna et al., 1998). These actions of ET -1 may be regulated by ECE-1 activity in HNPE cells. Evidence for ECE- 1 activity has been shown in vascular smooth muscle cells (Hioki et al., 1991; Woods et al., 1998). This suggests that ciliary smooth muscle could also be a potential site for ET- 1 production and could also participate in regulating AH outflow.

The pathophysiology of normal tension glaucoma (NTG) is currently not well defined, but vascular changes manifested by causes other than IOP, especially ocular vasospasms are thought to be important mediators normal tension glaucoma(NTG) Flammer et al.,1987)(Gasser. 1989). Clinical signs of NTG may include; optic disc hemorrhage (Drance et al., 1988), systemic disorders of the circulation (Flammer et al.,

1987) and impaired ocular blood flow (Takuoka et al., 1990). The potent vasoactive ET peptide thought by many to be implicated in the progression of vasospasms (Kurihara et al., 1989)(Miyauchi et al., 1989). Sugiyama et al., (1995) found that plasma ET levels in patients with NTG were significantly higher than in normal controls.

These results were reflective of NTG patients with early stages of visual field loss. This may suggest that elevated plasma ET- 1 is associated with NTG (Sugiyama et al., 1995) (Kaiser et al., 1995) Hollo, Lakatos and Farkas, 1997). It seems that ET-1 acts on the vessels of the optic nerve head more effectively than the peripheral choroid vessels (Sugiyama et al., 1995). This may also suggest that ET- 1 plays an important role in the autoregulation of optic nerve head circulation. If ET- 1 persistently acts on the eyes of patients with NTG, this can explain the fact that lesions in the optic nerve head progress although IOP remains low in NTG(Sugiyama et al., 1995)(Cioffi et al., 1995)(Orgul et al., 1996). Therefore efforts to control ET- 1 production by inhibition of ECE activity could be useful either to alleviate the effects of NTG and/or to regulate aqueous humor dynamics. Several groups have demonstrated ECE-1 inhibition using phosphoramidon (PHA; an inhibitor of ECE-1) in the nanomolar to micromolar range (Ikegawa et al., 1990; Takaoka et al., 1991; Corder et al., 1993; Sawamura et al., 1993; Laporte et al., 1996). Previously, our laboratory has shown that phosphoramidon (PHA) decreased immunoreactive ET-1 levels in intact HNPE cells, compared to untreated controls (Prasanna et al., 1998). For the current study we investigated the effects of PHA on ECE-1 activity in plasma membrane preparations of HNPE cells using the novel assay. Unexpectedly, we found no inhibition of ECE- 1 enzyme treated with varying

concentrations of PHA using the immunoprecipitation assay. Upon further examination, it became evident that PHA was unable to remain bound to the active site of ECE-1, when treated with antibody for Big ET-1, but bound irreversibly to the antibody instead. We also observed irreversible binding of PHA to the anti Big ET-1 under blank control conditions where no ECE-1 was present. It may also be misleading to extrapolate from the biological maturation of endogenous endothelin peptides based on the in vivo effects of exogenous big ET and their sensitivity to phosphoramidon (Bigaud and D' Orchymont, 1997). Phosphoramidon which is a natural compound, is a non-specific inhibitor of several metalloproteases such as thermolysin (EC 3.4.24.4), enkephalinase (EC 3.4.24.11), and angiotensin converting enzyme (EC 3.4.15. 1) and a host of others (Schwartz et al., 1985; Komiyarna et al., 1975 and Hudgin et al., 1981). Enkephalinase has been recognized as partially responsible for the degradation of endothelins (Abassi et al., 1993) and of other vasoactive neurohumoral peptides including atrial natriuretic peptide, bradykinin, enkephalins and substance P (Yamagushi et al., 1992; Erdos and Skidgel, 1989). Therefore inhibition of the in vivo effect of exogenous big ET- 1 by phosphoramidon might not necessarily imply only the inhibition of ECE but could result from a complex balance between maturation of ET s and inhibition of the degradation of several other vasoactive peptides, including ET-1. This type of event was reported by Modin et al., (1991), who showed that in anesthetized rats and pigs, phosphoramidon was able to inhibit the vasoactive effects of exogenously applied Big ET-1, but not the elevation of plasma ET-1. Whereas McMahon et al., (1991), Gardiner et al. (1992) and Trapani et al. (1993) reported that thiorphan was also able to diminish the vasoconstrictor

effects of exogenous big ET in both anesthetized and conscious rats. In the current study thiorphan was selected as the ECE inhibitor as it had also been found to be effective at concentrations in the micromolar to millimolar range (MacMahon et al., 1991; Turner and Murphy, 1996). Thiorphan did not compete with the Big ET-1 antibody and effectively inhibited the enzyme's ability to produce ET-1 If the findings by Tezel et al., 1997 of increased aqueous humor ET- 1 levels reflect an actual increase in POAG patients, what is a likely source of ET- 1 ? In one study carbachol has been reported to increase ETs in conditioned medium with a muscarinic stimulation of the nonpigmented ciliary epithelial cells by activating protein kinase C (Lepple-Weinhues et al., 1992). Our laboratory has also shown that TNF-α, through a PKC-dependent action, increases ET- 1 release from HNPE cells (Prasanna et al., 1998). The local ET synthesis of ET- 1 in the anterior segment is supported by the identification of ET mRNA in the anterior uveal tract, suggesting local synthesis by vascular endothelial cells (MacCumber et al., 1989) or vascular smooth muscle cells (Resink et al., 1990). It is clear that ET-1 plays an important role in IOP. A key to the regulation and production of ET-1 in the aqueous humor may be the responsibility of the non-pigmented ciliary epithelial cells since it is the major producer of ET- 1 in the eye. The current study demonstrates that ECE-1 is present in HNPE cells and ET-1 production is prevented with ECE inhibitors. Further studies should focus on understanding the regulation of ECE activity in HNPE cell under various conditions including elevation of IOP as occurs in POAG patients.

BIBLIOGRAPHY

Abassi, Z.A., Golomb, E. Bridenbaugh, R. and Keiser, H. R. (1993). Metabolism of endothelin- 1 by recombinant neutral endopeptidase EC.3.4.24.1 1. *Br. J Pharmacol.* 109, 1024-1028.

Abdel-Latif, A.A. and Zhang, Y. (1991) Species differences in the effects of endothelinI on myo-inositol triphosphate accumulation, cyclic AMP formation and contraction of isolated iris sphinchter of rabbit and other species. *Invest. Ophthamol. Vis.* Sci. 32,2432-2438.

Chakravarthy, U., Douglas, A.J., Bailie, J.R., Mckibben, B. And Archer, D.B. (1994) Immunoreactive endothelin distribution in ocular tissues. Invest. *Ophthalmol. Vis.* Sci. 35, 2448-2454.

Chabrier, P.E. and Braquet, P. (1990) Endothelin Horm. Res. 34, 169-174

Cioffi, G.A., Orgul, S., Onda, E., Bacon, D.R. and Van Buskirk, E.M. (1995). An *in* vivo model of chronic optic nerve ischemia: the dose-dependent effects of endothelin-I on the optic nerve microvasculature. *Curr. Eye Res.* 14, 1147-1153.

Corder, R., Khan, N. And Harrison, V.J. (1995) A simple method for isoolating human endothelin converting enzyme free from contamination by neutral endopeptidase*24.11.*

BiochemBiophysRessCommun207,355-362.

De Nucci, G., Thomas, R., D'Orleans, Juste, P., Antunes, E., Walder. C., Warner, T. and Vane, J. R. *(1988)* Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium. derived relaxing factor. *Proc. Natl. Acad Sci. USA. 85, 9797-9800.*

Drance, S.M., Douglas, G.R., Wijisman, K. et al., *(1989)* Response of blood flow to warm and cold in normal and low-tension glaucoma patients. *Am. J OphthalmoL 105,35-39.*

Eichhorn, M. And Lutjen-Drecoll, E. *(1993)* Distribution of endothelin-like immunoreactivity in the human ciliary epithelium. *Curr. Eye. Res. 12, 753757.*

EI-Mowafy, A.M. and Abdel-Latif, A.A. *(1994)* Characterization of iris sphincter smooth muscle endothelin receptor subtypes which are coupled to cyclic AMP formation and polyphosphoinosititde hydrolysis. *J PharmacoL Exp. Ther. 268, 13431351.*

Erdos, E.G. and Skidgel, R.A. (1989). Neutral endopeptidase 24.11 (enkephalinase) and 58 related regulators of peptides hormones. *FASEB J. 3,* 145-15 1.

Erickson-Lamy, K., Korbmacher, C., Schuman, J. S. and Nathanson, J.A. (199 1) Effects of endothelin on outflow facility and accomodation in the monkey eye in- vivo. Invest. Ophthalmol. *Vis. Sci. 32,* 492495.

Flammer, J. (1997). The concept of vascular dysregulation in glaucoma. In: *Ocular bloodflow. New insights into the pathogenesis of ocular diseases* (Kaiser, J.H., Flammer, J. and Hendrickson, P.H. Eds), Basel, Karger, pp 12-39.

Flammer, J., Guthauser, U. and Mahler, M. (1987). Do ocular vasospasms help cause low-tension glaucoma?. Doc *Ophthalmol Proc Ser. 49,* 397-399.

Furchgott, R.F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature. 288,* 373-376.

Gardiner, S.M., Kemp, P.A. and Bennett, T. (1992). Inhibition by phosphoramidon of the regional haemodynamic effects of pro-endothelin-2 and -3 in conscious rats. *Br. J Pharmacol., 107,* 5 84-590.

Gasser, P. (1989). Ocular vasospasm: a risk factor in the pothogenesis of low-tension glaucoma. *Int Ophathalmol.* 13, 281-290.

Gramstam, E., Wang, L. and Bill, A. (1992) Ocular effects of endothelin- 1 in the cat.

Curr. Eye. Res. 11, 325-332.

Geppeti, P., Patacchini, R., Meini, S. and Manzini, S. (1989) Contractile effect of endothelin on isolated iris spinchter muscle of pigs. *Eur. J Pharmacol. 168,* 119-121.

Greve, E.L., Duijm,H.F.A. and Geijssen, H.C. (1997). Risk factors in glaucoma. In: *Nitric oxide and endothelin in the pathogenesis ofg1aucoma* (Haefliger, 1.0. and Flammer, J. Eds) New York, Lippincott-Raven, pp 14-2 1.

Hickey, K.A., Rubayni, GM., Paul, R.J., Highsmith, R.R. (1985) Characterization of a coronary vasoconstrictor produced by endothelial cells in culture. *Am. J Physiol. 248,* C550-C556.

Hioki, Y., Okada, K.,rn Ito, H., Matsuyama, K. And Yano, M., (191) Endothelin cnverting enzyme of bovine carotid artery smooth muscle cells in culture. *Bichem Biophys Res. Commun.* 174, 446-45 1.

Hollo, G., Lakatos, P. and Farkas, K. (1997). Endothelins and glaucoma II. *Ophthalmologia. Hungarica.* 134,199-204.

Hudgin, R.L., Charleson, S.E., Zimmerman, M., Mumford, R. and Wood, P.L. (1981). Enkephalinase: selective peptide inhibitors. *Life Sci, 29,* 2593-2601.

Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. and Morimoto, S., (1990). Phosphoramidon, a metalloproteinase inhibitor, supresses the secretion of endothelin-1 from cultured endothelial cell by inhibiting a big endothelin-I converting enzyme. *Biochem. Biophys. Res. Commun. 171,* 669-675.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (19 8 9) The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc *Natl. Acad Sci.* USA. 86, 2863-2867.

Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., fujino, M., et al., (1988) Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett. 231, 440-444.*

Karne, S., Jayawickreme, C.K. and Lerner, M.R. (1993) Cloning and characterization of an endothelin-3 specific receptor (ET, receptor) from Xenopus *laevis* dermal melanophores. J *BioL Chem.* 268, 19126-19133.

Kimura S., Kasuya, Y., Sawamura, T., Shinimi, 0., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989). Conversion of big endothelin- 1 to 21 residue endothelin-I is essential for the expression of full vasoconstrictor activity: structure activity relationships

of big endothelin-1. J *Cardiovasc. Pharmacol.* 13 (Suppl 5). S5-S7.

Kloog, Y., Bousso-Mittler, D., Bdolah, A., and Sokolovsky, M., (1989) Three apparent receptor subtypes for the endothelin/sarafotoxin family. FEBS Letters. 253,199202.

Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T. and Umezawa, H., (1975) Studies on inhibitory effect of phosphoramidon and its analogs on thermolysin. Arch Biochem. Biophys., 171, 727-758.

Kurihara, H., Yoshizumi, M., Sugiyama, T., Takaku, F., Yanagisawa M., Masaki, T., Hamaoki, M., Kato. H. And Yazaki, Y. (1989) Transforming growth factor-beta stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 159,1435-1440.

Lepple-Wienhues, A., Becker, M., Stahl, F., Berweck, S., Hensen, J., Noske. W., Eichhorn, M. and Wiederholt, M. (1992) Endothelin-like immunoreactivity in the aqueous humor and in conditioned medium from cultured ciliary *62* epithelial cells. *Curr. Eye. Res. 11, 1041-1046.*

MacCumber, M.W. and D'Anna, S.A. *(1994)* Endothelin receptor-binding subtypes in the human retina and choroid. *Arch. Ophthalmol. 112, 1231-1235.*

MacCumber, M.W., Ross, C.A., Glaser, B.M. and Snyder, S.H. *(1991)* Endothelin:

Visualization of mRNAs by in *situ* hybridization provides evidence for local action. Proc. Natl. Acad. Sci. 86, *7285-7289.*

MacCumber, M.W., Jampel. H.D. and Snyder, S.H. *(1991)* Ocular effects of the endothelins. *Arch. QphthalmoL 109,705-709.*

Millar, C., Poyer, J.F., Gabelt, B.T. and Kaufmann. P.C. *(1995)* Endothelin subtypes: Effects on isolated Rhesus monkey ciliary muscle. J *PharmacoL Ex. Ther. 275,1143-1147.*

Miyauchi, T., Yanagisawa, M., Tomizawa, T., Sugishita, Y., Suzuki, N., Funino, M., Ajisaka, R., Goto, K. and Masaki, T. *(1989).* Increased plasma concentration on endothelin- 1 and big endothelin- 1 in acute myocardial infarction. *Lancet. ii, 53 –54.*

Modin, A., Pernow, J. and Lundberg, J.M. *(1991).* Phosphoramidon inhibits the vasoconstrictor effects evoked by eig endothelin- 1 but not the elevation of plasma endothelin- 1 *in-vivo. Life Sci. 49,* 1619-1625.

Nakagawa, 0., Nakao, K., Saito, Y., Shirakami, G., Jougasaki, M., Mukoyama, M., Hossada, K., Suga, S.I. Ogawa, Y., Kishimoto, I., Komatsu, Y., Hama, N. And Imura, H. (1991) Isolation and characterization of porcine endothelinlike peptide. J. Cardiovasc. *Pharmacol. 17(Suppl* 7) S13-SI69

Noske, W., Hensen, J. and Weiderholt, M. (1997). Endothelin-like immunoreactivity in aqueous humor of primary open-angle glaucoma and cataract patients. *Graefes Arch. Clin. Exp. OphthalmoL 235,* 551-552.

Nyborg, M.C.B., Prieto, D., Benedito, S., Reinach, P., Cranston, S.D. and Mita.S. (1991) Endothelin- 1 induced contraction of bovine retinal small arteries in reversible and abolished b nitrendipine. *Invest OphthalmoL Vis. Sci. 32,* 27-3 1.

Ohlstein EH. Storer B. Nambi P. Given M. Lippton H. 1990. Endothelin and platelet function. Thrombosis Research. 57(6):967-74.

Ohnaka. K., Takayanagi, R., Yamauchi, T., Okazaki, H., Ohashi, M., Umeda, F. and Nawata, H. (1990) Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.* 168,1128-1136.

Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M. And Nawata, H. (1993) in Purification and characterization of a phosphoramidon-sensitive endothelin converting enzyme in porcine aortic endothelium. J *Biol Chem. 268,* 2675926766.

Okada, K., Miyazaki, Y., Takada, J., Matsuyama, K., Yamaki, T. and Yano, M., (1990) conversion of big endothelin-1 by membrane bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem. Bioiphys. Res. Commun. 171,* 1192-1198.

Pang, I-H. and Yorio, T. (1997) Ocular actions of endothelins. *Societyfor Exp. Bio. and Med.* 21-34.

Prasanna, G., Dibas, A., Tao, W., White, K. and Yorio, T. (1998). Regulation of endothelin-1 in human non-pigmented ciliary epithelial cells by tumor necrosis factor-α. *Exp . Eye Res. 66,* 9-18.

Ramachandran, E., Frank, R.N. and Kenendy, A. (1993) Effects of endothelin on cultured bovine retinal microvascular pericytes. *Invest. OphthamoL Vis.* Sci. 34, 586-595.

Resink, T.J., Hahn, A.W.A., Scott-Burden, T., Powell, J., Weber, E. and Buhler, F.R. (1990). Inducible endothelin messenger RNA expression and peptide secretion in cultured human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun. 168, 1303-1310.*

Ripodas. A., DeJuan, J. A., Moya. J. and Durango. R. (1998). Exp. Eye. Res.

Rubanyi, G.M. and Polokoff, M.A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, and pathophysiology. *Pharmacol. Rev. 46,* 325-415.

Sawamura, T., Kimura, S., Shinmi, 0., Sugita, Y., Yanagisawa, M. And Masaki, T. (1989) Analysis of endothelin related peptides in culture supernatent of porcine aortic endothelial cells: evidence for biosynthetic pathway of endothelin-1, *Biochem. Biophys.*

Res. Commun. 162,1287-1294.

Sawarnura, T., Shinmi, 0., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T. and Kimura, S. (1993). Characterization of phosphoramidon-sensitive metallaproteinases with endothelin converting enzyme activity in porcine lung membranes. *Biochemica et Biophysicia Acta. 1161,* 295-302.

Schwartz, J.C., Costentin, I and Lecomte, J.M. (1985) Pharmacology of enkephalinase inhibitors. *TIPS, 6,* 472-47.

Shimada, K., Takahashi, M. And Tanzawa, K., (1994) Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J Biol Chem.* 269,18275-18278.

Sugiyarna. T., Moriya, S., Oku. H., and Azuma, 1. (1 995b) Association of endothelin- 1 with normal tension glaucoma: Clinical and fundamental studies. Surv. Ophthamol 39(Suppl 1) S49-S56.

Sugiyama, K., Haque, M.S.R., Okada, K., Taniguchi, T. and Kitazawa, Y. (1995a). Intraocular pressure response to intravitreal injection of endothelin-I and the mediatory role of ETAreceptor, and ETBreceptor, and cyclooxygenase products in rabbits. Curr. *Eye Res.* 14, 479-486.

Takahashi, M., Matsushita, Y., Iijima, Y. And Tanzawa, K. (1993) Purification and characterization of endothelin-converting enzyme from rat lung. *JBiol Chem.* 268, 21394-21398.

Takaoka, M., Shiragami, K., Fujino, K., Miki, K., Miyake, Y., Yasuda, M., Wang., G.F., Hisaki, K., Matsumura, Y. and Morimoto, S. (199 1). Phosphoramidon sensitive endothelin converting enzyme in rat lung. *Biochem. Int. 25,* 697-704.

Tao. W., Wu, X., Liou, G.I., Abney, T.O., Reinach, P.S. (1997) Endothelin receptor mediated Ca2+ signaling and isoform expression in bovine comeal epithelial cells. Invest. Ophthalmol Vis. Sci. 38, 130-141.

Tezel, G., Kass, M.A., Kolker, A.E., Becker, B. and Wax, M.B. (1997). Plasma and aqueous humor endothelin- 1 levels in primary open-angle glaucoma. J *Glaucoma. 6,* 83-89.

Trapani, A.J., Balwierczak, J.L., Lappe, R.W. , Stanton, J.L., Graybill, S. C., Hopkins, MR, Savage, P., Ssperbeck, D.M. and Jeng, A.Y. (1993). Effects of metalloprotease inhibitors on the conversion of proendothelin-1 to endothelin-1. *Biochem. Mol. Biol.* Int. 31, 861-867.

Turner, A.J. and Murphy, L.J. (1996) Molecular pharmacology of endothelin converting

enzyme. *Biochem Pharmacol. 51,* 91-102.

Waxman, L., Doshi, K.P., Gaul, S.L., Wang, S., Bednar, R.A. and Stem, A.M. (1994) Identification and characterization of endothelin converting activity from EAHY 926 cells: Evidence for the physiologically relevant human enzyme. *Arch Biochem Biophys.* 308,240-253.

Woods, M., Wood, E.G., Mitchell, J. A. and Warner, T. D. (1998). Evidence of intracellular endothelin converting enzyme in human vascular smooth muscle *cells.FASEBJ. 12*,A-384,2230.

Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994). ECE-1: A membrane bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell78,* 473-485.

Yagnagisawa, M., AKurihara, h., Kumura, S., Tomobe, Y., Kobayahi, M., Mitsui, Y., Goto. K. And Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (London)* 332, 411-415.

Yamagushi, T., Kido, H. and Katunuma, N. (1992). A membrane-bound metallo-endopeptidase from rat kidney. Characteristics of its hydrolysis of peptide hormones and neuropeptides. *Eur. J. Biochem. 204,* 547-552.

Yang, Z., Bauer, E., Von Segesser, L., Stulz, P., Turina, M. and Luscher, T.F. (1990) Different mobilization of calcium in endothelin- 1 induced contractions in human arteries and veins: effects of calcium antagonist. J. Cardiovasc. Pharmacol. 16, 654-660