MINIREVIEW

Ocular Actions of Endothelins (44110)

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Endothelins (ETs) represent a group of 21 amino acid–containing peptides that include two disulfide bridges. Endothelin was first isolated as the potent vasoconstrictor from supernatants of cultured endothelial cells (1) and appears to be evolutionary related to a group of snake venoms that includes sarafotoxin S6c. Endothelin's vasoconstrictor activity is 10 times that of angiotensin II (2). These potent vasoconstrictors are important endogenous vascular mediators that are released in response to certain factors or during cell injury. It appears that the structures of ET are conserved among the different species, such that ET-1 in the human is identical to that in the rat. This conservation may be reflective of the critical role ET plays in vascular homeostasis. Three structurally and pharmacologically different peptides have been prepared based on genetic sequence information determined by molecular cloning techniques (3). Each of these peptides have different pharmacological profiles, tissue localization, and cellular actions (Table I). Endothelin-1 appears to have the greatest distribution in tissues and is a constituent of plasma. The concentration of ET in plasma varies between 0.26 and 5 pg/ml (4, 5) and may reach 35 pg/ml during some cardiovascular pathologies. The source of this ET is thought to be the vascular endothelial cells. However, the identification of ET and its precursor peptide in other tissues suggests that it may come from more than one source.

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Endothelin effects have been considered to be either autocrine, paracrine, or endocrine, depending on the tissue responses measured. It is clear, however, that the discovery of this potent peptide has led to many speculations concerning its endogenous role and function. For this review we have concentrated on ET's actions in ocular tissues. Because of the limited scope of this review, we have focused on the most recent findings in this area. We apologize in advance for having restricted our references and may have inadvertently failed to recognize everyone's contribution. For further detailed reviews of ETs vascular and nonvascular pharmacological actions, see Refs. 6 and 7.

Synthesis and Distribution of Endothelin

Endothelin-1 is synthesized from preproET, a 203–amino acid peptide, through the action of an endopeptidase and subsequently through ET-converting enzyme (ECE), which converts an inactive 39–amino acid precursor, big ET-1, into ET-1. Similarly, ET-2 and ET-3 are derived from their precursor peptides, big ET-2 and big ET-3, respectively. There are several isoforms of ECE, which differ in their cellular localization and biochemical activities. ECE-1a is the enzyme responsible for the formation of ET-1, however, big ET-1 is the preferential substrate for all the isoenzymes compared with big ET-2 or big ET-3 (8). ECE activity could be inhibited with the metalloprotease inhibitor, phosphoramidon, which inhibits both *in vitro* and *de novo* synthesis of ET-1 (9). Exogenously administered big ET-1 is converted into active ET-1 in whole animals, isolated perfused organs, isolated vascular and nonvascular preparations, and cultured cells, suggesting that ECE isoforms are ubiquitous. Phosphoramidon analogs have been developed, as have several other agents with selectivity for ECE. These drugs inhibit the *in vivo* responses to exogenously administered big ET-1 and are potentially clinically useful agents in preventing ET-1

Table I. Endothelin Receptors

Receptor	ET_A	ET_B	ET_C
Affinity	ET-1 = ET-2 > > ET-3 > S6C	ET-1 = ET-2 = ET-3	ET-3 > > ET-2 = ET-1
Agonist	ET-1	Sarafotoxin S6c	ET-3
Antagonist	BQ-123 Others	BQ-788 Others	None known
Second messengers	IP_3/DAG, Ca^{2+}	IP_3/DAG, Ca^{2+}	IP_3/DAG, Ca^{2+}
General actions	Vasoconstriction Mitogenesis	NO release Bronchoconstriction Neurotransmission Renal vasoconstriction	
Ocular actions	↓ Retinal blood flow ↓ IOP ↓ Aqueous humor formation ↑ Outflow of aqueous humor	↑ Outflow of aqueous humor	

vascular effects. The synthesis and release of ETs are regulated by a variety of factors depending on the tissue source of ET and the stimulating factor. These factors include thrombin, tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β) and transforming growth factor–β1 (TGFβ1) (10–12). Other agents effecting release include phorbol esters (13), endotoxin (14), and angiotensin II (15). In addition, those agents that elevate cyclic AMP, or downregulate or inhibit protein kinase C, inhibit ET secretion, suggesting that these protein kinases may play opposite roles in regulating the release of ET. Circulating ET levels rise or fall rapidly with changes in posture and plasma volume, reflecting the sensitive regulatory ET release mechanisms that are present in the circulation. Collectively, these release mechanisms suggest a key role for ETs in modulating vascular homeostasis.

Endothelin Receptors

There are two major pharmacological subclasses of ET receptors, the ET_A receptor, selective for ET-1 (ET-1 = ET-2 > ET-3), and the ET_B receptor, which does not distinguish among the three ET peptides (Table I). These receptors have been cloned in mammals and have been extensively characterized as to their physiological and pharmacological profiles in a number of species and tissues. Two additional novel RT-PCR transcripts have been identified in human lung and other tissues, which suggest the presence of additional ET receptors. The presence of a putative ET_C receptor, which appears to be selective for the ET-3 isopeptide, has been suggested from clones of amphibian melanophores (16), while a subclass of the ET_A receptor has also been cloned from the frog heart (17). These nonmammalian ET receptors may have counterparts in the human, and, indeed, pharmacological evidence has suggested the existence of subtypes of the ET_B receptor, ET_{B1} and ET_{B2} (18). Additional molecular approaches using site-directed mutagenesis, RT-PCR, and transgenics may identify additional subtypes that may be tissue or species specific. Currently, several pharmaceutical companies are developing non–peptide-selective antagonists for the ET_A and ET_B receptors. Using these compounds, receptor binding techniques coupled to physiological responses could yield additional information regarding ET receptor subtypes.

Pharmacology

Perhaps the best characterized pharmacological effects of ET concern its hemodynamic actions. Endothelin is one of the most potent vasoconstrictors yet to be identified. It not only exerts direct vasoconstrictor effects but potentiates the contractile action of other vasoconstrictor substances, including norepinephrine (19, 20). The pressor responses to ET appear to be mediated by the ET_A receptor subtype, although in some vascular beds ET can also cause vasodilation (21). In the eye, ETs produce a rapid and reversible contraction of the isolated retinal branches of short posterior ciliary arteries (22) and are the most potent agonist in contracting bovine retinal pericytes (23). Intravitreal injection of ET-1 produces a dose-dependent and sustained decrease in optic nerve head (ONH) blood flow; however, if ET is administered systemically by an i.v. injection, the ONH blood flow increases (24). The latter effect appears to be mediated through NO release, since L-NAME abolished such increase (24). Recently, ET-1 has been used to develop a model of optic nerve ischemia (25) that provides a mechanism to evaluate optic neuropathies, including normal tension glaucoma, which has been postulated as being associated with chronic ischemia (26, 27). In addition to its hemodynamic effects, ET has been shown to have positive inotropic and chronotropic actions, to stimulate endothelial cell mitogenesis, and to regulate a number of endocrine functions. Evidence also suggests that ETs may be considered neuropeptides, as they are localized within the CNS, receptors for ETs are present in the brain, and ETs modulate CNS functions (7).

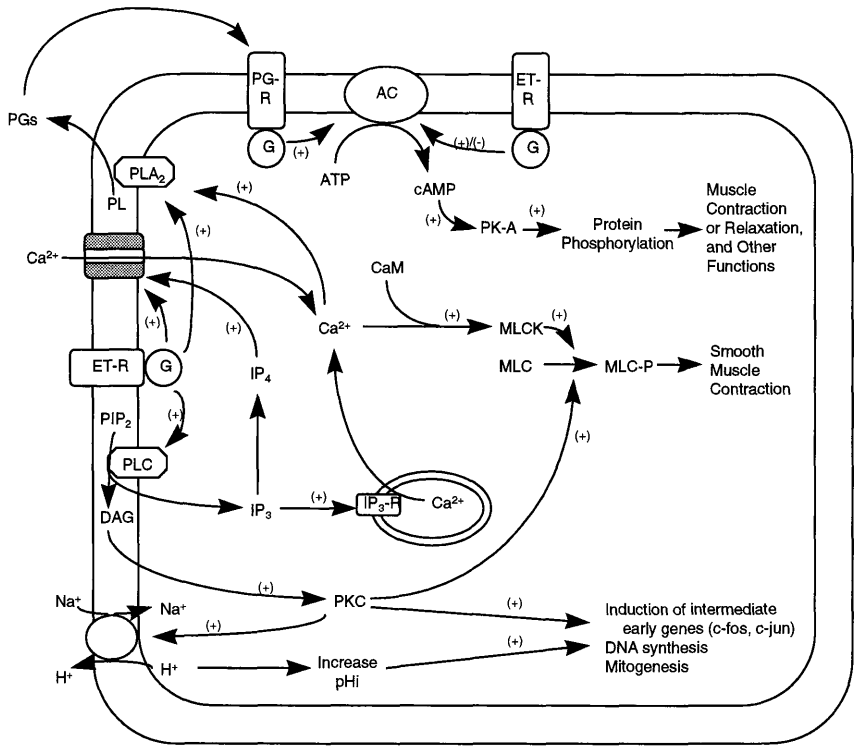

Figure 1. Endothelin receptor-mediated signal transduction systems. (+), activation; (−), inhibition; AC, adenylyl cyclase; CaM, calmodulin; cAMP, cyclic AMP; DAG, diacylglycerol; ET-R, endothelin receptor; G, G protein; IP_3, inositol trisphosphate; IP_3-R, inositol trisphosphate receptor; IP_4, inositol tetrakisphosphate; MLC, myosin light chains; MLCK, myosin light chain kinase; MLC-P, phosphorylated myosin light chains; PGs, prostaglandins; PG-R, prostaglandin receptor; pHi, intracellular pH; PIP_2, phosphatidylinositol diphosphate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PL, phospholipid; PLA_2, phospholipase A_2; PLC, phospholipase C.

Effects on Second Messengers

Endothelin receptors are involved in many functional changes of the cells. When activated, they affect cellular signaling systems, such as phospholipase C (PLC), calcium mobilization, phospholipase A_2 (PLA_2), phospholipase D (PLD), adenylyl cyclase, and protein kinases. Changes in these systems ultimately result in changes of cell contractility, production of autacoids, intracellular pH, cell growth, or proliferation. Reference 7 is an excellent review on this subject. A brief description of the major signal transduction pathways mediated by ET receptor is presented below (Fig. 1).

Phospholipase C, Inositol Phosphates, and Calcium Mobilization. In many vascular smooth muscles and cultured cells, ET activates PLC in a concentration-dependent manner, which leads to the hydrolysis of phosphoinositides and generation of diacylglycerol (DAG) as well as inositol phosphates, including inositol trisphosphate (IP_3). IP_3 activates IP_3 receptors that release calcium from intracellular calcium ($[Ca^{2+}]_i$) stores and elevate the intracellular concentration of calcium. In addition to the release of calcium from intracellular sequester sites, ET also affects the influx of extracellular calcium in some smooth muscle cells (28–31). The mechanism involved in the influx of calcium is not clear. It is speculated that ET receptor activation may increase the opening of certain plasma membrane calcium channels *via* inositol phosphates, such as IP_4. Alternatively, a G protein activated by ET may directly open the channels. Mobilization of calcium in turn activates calmodulin, which in smooth muscle cells causes contraction and in endothelial cells stimulates nitric oxide synthase and increases NO production.

Diacylglycerol and Protein Kinase C. Activation of PLC also increases the production of DAG, which is known to stimulate protein kinase C (PKC). Thus, ET-1 was shown to facilitate the translocation of PKC from cytoplasm to membrane and its subsequent activation (32). Activation of PKC leads to phosphorylation of many proteins. Phosphorylation of myosin light chains induces smooth muscle contraction. Inhibition of PKC by staurosporin inhibits ET-1–induced vasculature contraction (33). Protein kinase C–facilitated phosphorylation of the Na-H exchanger of the plasma membrane activates ion exchange, which in turn increases intracellular pH (34, 35). Hence, inhibitors of PKC or Na-H antiporter block the ET-1–induced alkalinization (36). Furthermore, both PKC activation and cell alkalinization can induce mitogenesis and proliferation. ET-1 is a weak mitogen for various cultured cells. It stimulates the expression of the immediate early response genes (37). These effects were partially or completely prevented by inhibitors of PKC (37, 38).

Phospholipase A_2 and the Synthesis of Prostaglandins. Endothelin-1 stimulates PLA_2 in smooth muscle cells (39, 40). It is not clear if the stimulation is mediated directly by an activated G protein or indirectly by the ET-induced calcium mobilization. Activation of PLA_2 increases the synthesis of various prostanoids (41).

Adenylyl Cyclase. Endothelin produces tissue-specific stimulation or inhibition of adenylyl cyclase. For

example, in cultured rat vascular smooth muscle cells or in embryonic bovine trachea cells, ET-1, *via* the activation of the ET_A receptor, increases cyclic AMP formation (42, 43), whereas, in rat brain capillary endothelial cells and human atrial slices, activation of the ET_A receptor inhibits cyclic AMP production (44, 45). Activation of adenylyl cyclase by the peptides may be a result of ET-induced prostaglandin production, since some prostaglandins, such as PGD_2 and PGE_2, can stimulate adenylyl cyclase. However, in other cases, such as in cultured rat vascular smooth muscle cells, the activation of adenylyl cyclase by ET was shown not to involve prostaglandins (42).

Ocular Endothelins

Distribution and Production in Ocular Tissues. Interest in the actions of ETs in the eye have come from initial studies in which ET binding sites were detected in the iris, retina, and choroid (46), and from reports of intraocular pressure (IOP)–lowering effects of intravitreal administration of ETs in rabbits and monkeys (47, 48). Immunolocalization studies have identified ET-like immunoreactivity in all ocular tissues; however, the iris, ciliary body, and choroid seem to have the highest concentrations of this peptide (48). Most studies have identified either ET-1 or ET-3 in ocular tissues, with apparently no ET-2 peptide immunoreactivity detected (48–50). ET-3 concentrations are higher than those of ET-1 in ocular tissues. Such differences in concentrations may be indicative of their relative function, but this remains unclear at this time. The pathway for the synthesis of ET-1 is through the activity of ECE acting on big ET-1. Previous studies using radioimmunoassays have detected minimal amounts of big ET-like immunoreactivity in human ocular tissue extracts, except the vitreous (49). Recently, our laboratory, using immunofluorescent techniques, identified big ET-1 in cultured human ciliary epithelial cells and human ciliary muscle cells, and its immunofluorescence was found to be enhanced by TNF-α (Fig. 2A–F). This differs from the previous radioimmunoassay observations. The reasons for this difference in findings are not clear. However, the earlier studies measured extracted big ET-1, which could have resulted in peptide degradation and decreases in concentrations below that detected by the radioimmunoassay. In contrast, the immunofluorescent studies are on intact cultured cells. If big ET was not present in ocular tissues, then ET would have to come from some other source, either from neurons, which contain both messenger RNA and ET peptide (51, 52), or it would be transported into the eye from the plasma. Endothelin-like immunoreactivity has been measured in human and bovine aqueous humor and found to be two to three times higher than in plasma (53). This higher concentration could reflect a specific transport system for ET, or differences in ET degradation in aqueous humor versus plasma. Since systematically administered ET does not cross the blood-brain barrier (54), it is highly unlikely that it would cross the blood-aqueous barrier under normal conditions. Therefore, it is most probable that the synthesis of ET is present in ocular tissues or that the peptide comes from neuronal ganglia and is transported to ocular nerve endings. As abundant levels of ET-mRNA have been found in the rat and rabbit iris (46, 55), some ocular tissues may serve as the source for aqueous humor ET. It has also been shown that preproET-1 mRNA was present in the optic vesicle, lens epithelium, and cornea of the developing mouse eye (56), adding further support for local synthesis of ETs. There are yet no specific reports on the isolation of any isoforms of the ECE in the eye. However, cultured human nonpigmented ciliary epithelial cells release ET-like immunoreactivity in response to fetal calf serum, thrombin, carbachol, and phorbol esters (53). The enhanced release of ET-like immunoreactivity was prevented with pretreatment with cyclohexamide (53). This finding provides evidence that ET production requires a translational event. Traditionally for ET-1, this involves the formation of the proET or big ET followed by its conversion to ET-1 (7). Our laboratory has recently found that, in addition to thrombin, TNF-α stimulates ET and big ET release from human ciliary epithelial cells and ciliary muscle (Fig. 2). Thus, the production and release of ET may be under the control of a number of factors, including cytokines.

There is also evidence that rabbit tear glands (lacrimal and Harderian gland) contain the gene transcripts for preproET-1, and that ET-1–like immunoreactivity is present in tears and lacrimal gland fluid (57). The concentrations of ET-1–like immunoreactivity in the tears and lacrimal gland are higher than those in plasma, which suggests that ET may either play a role in tear gland physiology as an autocrine or perhaps affect corneal epithelial renewal in a paracrine role (57–59).

Ocular Endothelin Receptors. Following the isolation and identification of ET isoforms, ligand binding studies demonstrated that there are high-affinity binding sites for ETs on many tissues. Pharmacological profiles of the activities of the different ET isoforms demonstrate that there are subtypes of ET receptors (see above). In the eye, ET receptor binding sites have been described using radioligand binding and have been inferred from pharmacological antagonism. Through the use of autoradiography of [^{125}I]-labeled ET-1 and competition binding with ET-3, ET_A-like binding sites were localized to the human retina and choroid, with specific labeling within the retinal neural tissue (60). Labeling was highest in the photoreceptor inner segment layer, and the outer and inner plexiform layers. There was no significant labeling in the photoreceptor outer segments or sclera (60). Although little labeling was seen in the

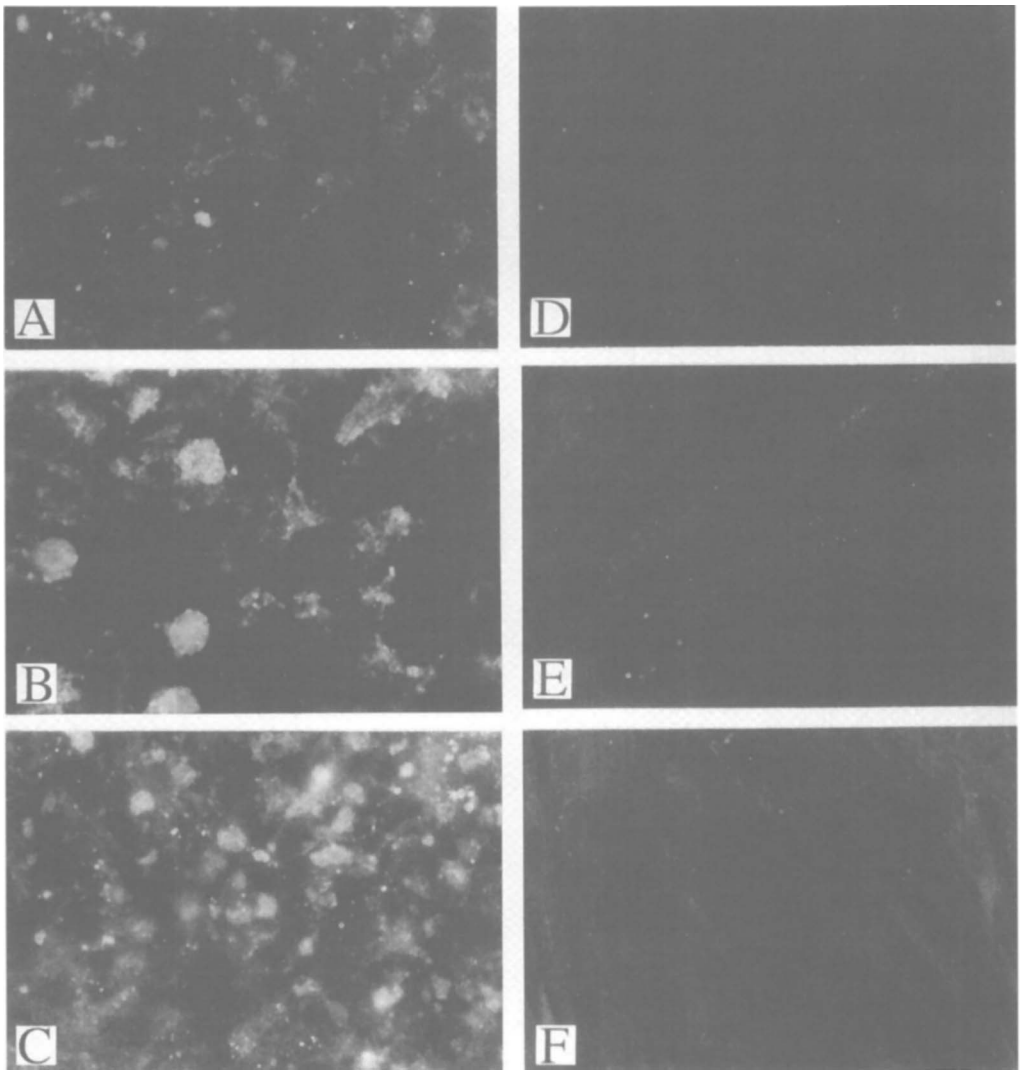

Figure 2. Immunocytochemistry of distribution of big ET-1 human ciliary epithelial cells (HCE) and human ciliary muscle cells (HCM). (A and D) Background immunofluorescence obtained from HCE (A) and HCM (D) cells seeded onto coverslips and serum-starved overnight in DMEM. Cells were washed in TBS (0.05 *M* Tris, 0.15 *M* NaCl, and 2% BSA), fixed with pre-cooled 1 : 1 mixture of methanol : acetone at −20°C and rapidly rinsed with TBS. Cells were incubated only with secondary antibody (goat anti-rabbit IgG-FITC; 1/200) at 37°C for 30 min, rinsed with TBS, deionized water, and mounted on a slide for viewing and photographing with a fluorescent microscope. (Magnification: ×560.) (B and E) Immunofluorescence labeling of basal big ET in HCE (B) and HCM (E). Cells were treated as above except primary antibody (rabbit anti–big ET-1; 1 : 500) incubation was done at 4°C for 15 hr. Cells were then incubated with secondary antibody (goat anti-rabbit IgG-FITC; 1 : 200) at 37°C for 30 min and treated and viewed as described above. (Magnification: ×660.) (C and F) Immunofluorescence labeling of big ET in HCE (C) and HCM (F) treated with TNF-α (10 n*M*: 30 min). Cells were handled as above in Panels B and E.

retinal pigment epithelium, the authors attributed this to a possible interference of the pigment with identification of silver grains. Since most of the label was blocked with low concentrations of ET-3, the authors concluded that the primary binding site was an ET_B-like receptor within the retina whereas the site in the choroid and retinal vessels was similar to the ET_A receptor. Endothelin receptor subtype distribution in rabbit eyes is similar to that in humans (60). Bovine retinal pericytes in culture also contain both ET_A and ET_B receptors (61). ET_A receptors are thought to mediate vasoconstriction, while the ET_B receptor has been implicated in both contractile and dilator actions. ET_A receptors appear to predominate in retinal pericytes (62). The presence of ET receptors in the retina and choroid, particularly in retinal blood vessels, suggests that ETs may be involved in regulating retinal blood flow and could contribute to retinal ischemia, which may lead to a number of retinal pathologies, including normal tension glaucoma.

The cornea also has abundant binding sites for ETs (48), and ETs have a number of actions on corneal epithelial cells suggesting functional receptors are present. Both ET_A and ET_B receptor genes are expressed in bovine corneal epithelium (59), and ET_B agonists enhance gene transcription for preproET-1 and increase ET-1 secretion in bovine corneal epithelial cells in culture (63). Furthermore, stimulation of the ET_B receptor enhances the effectiveness of epidermal growth factor

to promote wound closure in the corneal epithelium (59), suggesting that ETs may play a role as a growth factor in the corneal epithelium. A similar role for ET was also reported for the corneal endothelium, where a single class of receptors were identified by radioligand binding techniques (64). ET-like immunoreactivity and mRNA for preproET-1 were also reported for rabbit corneal epithelial cells, and ET_A receptor activation increased cell proliferation (58). Collectively, these results suggest that ETs may play an important role in corneal wound healing.

In cultured cells of the human ciliary muscle, our laboratory has shown that this tissue contains an ET_A receptor coupled to PLC and calcium mobilization (65). It was also shown that a separate ET_A receptor pathway was present that was coupled to the activation of PLA_2 and an increase in PGE_2 (65, 66). The increase in PGE_2 formation resulted in the activation of adenylyl cyclase and increases in cyclic AMP. It is unclear if this involves subtypes of ET_A receptors or the same receptor coupled to two different G proteins. ET-1 has been shown to induce contractions of the isolated human ciliary muscle (53), and this is presumably through the ET_A receptor subtype. In isolated bovine ciliary muscle strips, ET-1 caused a dose-dependent contraction and relaxation of the ciliary muscle (67). The contraction was mediated through the ET_A receptor, whereas relaxation was a result of ET_B receptor activation (67).

One of the highest density of binding sites for radiolabeled ET-1 has been in the iris (46, 54). In the iris sphincter muscle, ET-1 has been shown to enhance IP_3 and DAG formation in conjunction with contraction in a number of species, though not in monkey or humans (68). This response was also mediated by an ET_A receptor. By using radiolabeled ETs, it was determined that rabbit sphincter muscle contained both ET_A and ET_B receptors, but that ET_A receptors represented 80% of the total and were linked to IP_3 formation and muscle contraction (69). Whereas, in the bovine sphincter muscle ET_B receptors predominated, with 72% of the total, and were linked to increases in cyclic AMP formation and not to muscle contraction (69). In fact, the ET_B receptor is thought to counteract the contractile actions of the ET_A receptor activation by elevating cyclic AMP and to induce relaxation as in other smooth muscles. In the iris, ET-1 effects have also been linked to activation of phospholipase D (PLD) through ET actions on the ET_A receptor (70). This implies that the ET_A receptor in iris is linked to three phospholipase enzymes that are actively involved in signal transduction mechanisms. Most likely, this will involve coupling through different G proteins.

Effects on Signal Transduction Pathways in Ocular Tissues. Endothelin affects signal transduction pathways in a variety of ocular tissues, from the cornea at the very front of the eye to blood vessels of the retina. Table II provides a summary of its actions.

Cornea. In rat and rabbit cornea pieces, ET-1 activates the turnover of phosphoinositides in a time-dependent and concentration-dependent manner (58, 71). This effect is presumed to be mediated by the epithelial cells, since ET-1 does not significantly increase the production of inositol phosphates in the de-epithelialized rabbit cornea. In cultured rabbit cornea epithelial cells, ET-1 increases intracellular calcium concentration. Among the various iso-peptides, ET-1 is the most potent and efficacious, followed by ET-2 and ET-3, a pharmacological profile suggesting that the ET_A receptor is essential for this ET action (58). In the rabbit cornea, ET-1 also causes a slight increase in the accumulation of cyclic AMP that is dependent on the presence of extracellular calcium (58). It is not known if a direct ET effect on adenylyl cyclase or an indirect effect secondary to the ET stimulation of PLC and calcium mobilization is responsible for the increased cyclic AMP accumulation. The functional significance of the second messenger changes induced by ET in the cornea is not clear. However, they may be involved in the proliferative and mitogenic actions of ET. Such actions in the cornea may be important for wound healing responses.

Iris. In the iris sphincter of the rabbit, cat, dog, pig, and bovine, but not Rhesus monkey or human, ET-1 activates PLC and induces contraction of the smooth muscle (68–71, 74–78, 94). In rabbit and bovine sphincters (69), stimulation of PLC by ET-1 was shown to be mediated by the ET_A receptor subtype. Similarly, the ET_A receptor is involved in the ET-1–induced contraction of iris sphincters of the rat (78), bovine, and rabbit (69). However, Ishikawa *et al.* (76), based on potency profiles of agonists and antagonists, suggested that a novel or perhaps atypical ET receptor subtype, together with the ET_A receptor, contributes to the ET-1–stimulated contraction in rabbit iris.

In contrast to PLC, ET-1 stimulates adenylyl cyclase in the iris sphincter of all mammalian species studied, including primates (68, 69, 73). In the rabbit sphincter, the effect of ET-1 was almost completely blocked by the ET_A-selective antagonist BQ-123. Interestingly, in addition to ET-1, ET_B-selective agonists, such as ET-3 and sarafotoxin S6c, can also increase the accumulation of cyclic AMP. Their stimulative effects are not antagonized by BQ-123 (69). Thus, these results taken together indicate that activation of either the ET_A or ET_B receptor subtype activates adenylyl cyclase in the rabbit sphincter. Contrarily, only the ET_B subtype appears to mediate the ET activation of adenylyl cyclase in the bovine iris sphincter, since in this tissue the ET_A antagonist BQ-123 is not effective in preventing the actions of ET-1, ET-2, ET-3, or sarafotoxin S6c (69). At this time, whether ET_A or ET_B or both receptors are involved in the modulation of cyclic AMP production in the iris sphincter of other animal species is still not clear.

Table II. Effects of Endothelins on Signal Transduction Pathways in Ocular Tissues

Structure	Species	Tissue or cell	PLC	$[Ca^{2+}]_i$	Contraction	PLA_2/PG production	AC	Other effects	References
Cornea epithelium	Rabbit	Cell		+					58
		Tissue	+				+		58
Cornea endothelium	Rabbit	Tissue	+				0		71
Iris sphincter	Bovine	Tissue	+		+	+	+		68, 69, 72
	Cat	Tissue	+		+	+	+		68, 73
	Dog	Tissue	+		+		+		68
	Human	Tissue	0		+[a]		+		68
	Monkey	Tissue	0		0		+		68
	Pig	Tissue	+		+		+		68, 74
	Rabbit	Tissue	+		+	+	+	PLD(+)	68–71, 75–77
	Rat	Tissue			+				78
Iris dilator	Rabbit	Tissue	+		+				68, 75
Ciliary processes	Human	Tissue					−		79
	Rabbit	Tissue					−		71
Ciliary muscle	Bovine	Tissue	+		+	+	+		67, 80, 81
	Cat	Tissue	0		0	+	+		80
	Dog	Tissue	0		0	+	+		80
	Human	Cell	+	+	+	+	+		65, 82, 83
		Tissue	0/+		0/+	+	+		53, 80
	Monkey	Tissue			+				84
Trabecular meshwork	Bovine	Cell		+				Depolarization, alkalinization	85, 86
		Tissue			+				81
	Human	Cell						Deplolarization	87
Retinal vessel	Bovine	Pericyte	+	+	+			Proliferation PKC (+)	23, 88–90
		Tissue			+				22
	Human	Tissue			+				91
	Pig	Tissue			+				92, 93

Note. PG, prostaglandins, AC, adenylyl cyclase, +, stimulation, −, inhibition, 0, no effect.
[a]In longitudinal muscle.

In cat, rabbit, and bovine iris sphincters, ET-1 also stimulates PLA_2 and raises the production of prostaglandins and related compounds (72, 73, 94). In the rabbit, treatment with ET-1 increases the release of arachidonic acid, its cyclooxygenase metabolites, including PGD_2, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, as well as lipoxygenase products such as 12-HETE and 15-HETE (94). The physiological and pathological significance of the release of these eicosanoids in ocular tissues could be very important and remains to be investigated.

Unique to the cat iris sphincter, the effects of ET-1 on PLC, adenylyl cyclase, and contraction may be mediated by the activation of PLA_2 and subsequent elevated release of prostaglandins. Yousufzai *et al.* (73) demonstrated that ET-1 increased PGE_2 production in the cat iris sphincter tissue and that all actions of ET-1 were blocked by the pretreatment of indomethacin, a cyclooxygenase inhibitor. The authors suggested that the ET-1–induced release of PGE_2 increases cyclic AMP production *via* the activation of the prostaglandin EP_2 receptor and stimulates PLC, calcium mobilization, and resulting contraction *via* the EP_1 receptor. However, it should be noted that indomethacin is not specific in its actions. In addition to cyclooxygenase inhibition, it can inhibit protein kinases and phosphodiesterases as well as interfere with bindings of various prostanoids to their receptors. Consequently, whether prostaglandins mediate all functional responses of the cat iris sphincter to ET-1 requires additional investigation.

By measuring the formation of radiolabeled phosphatidylethanol, Zhang and Abdel-Latif (70) revealed that ET-1 is also a potent activator of PLD in rabbit iris sphincter muscle. They showed that the ET-1's effect on PLD is independent of the activation of PLC, increase in $[Ca^{2+}]_i$, or activation of PLA_2. Recent evidence indicates that PLD may elicit mitogenesis (95). Hence, a potential mitogenic action of ET-1 in this tissue cannot be ignored.

Despite the relative large number of studies on the iris sphincter, very few reports are available on ET's effects on the iris dilator muscle. Abdel-Latif and Zhang (68) as well as Ishikawa *et al.* (75) have shown that ET increases accumulation of inositol phosphates and causes contraction of the dilator of rabbits.

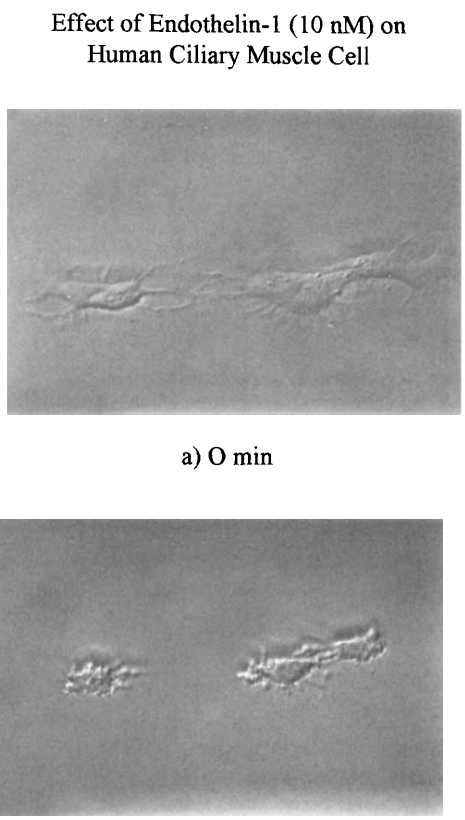

Figure 3. Photomicrographs of human ciliary muscle cells before (A) and 15 min after (B) the addition of 10 n*M* ET-1. Cultured human ciliary muscle cells were partially detached from the culture dish by incubation with a nonenzymatic dissociation buffer.

Ciliary processes. Bauscher and Horio (79) discovered that ET-2 suppresses forskolin-activated adenylyl cyclase in adult human ciliary processes without affecting the basal level. Similar results have also been observed in rabbit iris ciliary body (71). Since cyclic AMP is believed to regulate aqueous humor formation in the ciliary epithelium, these findings suggest a possible functional role for ET in the regulation of aqueous inflow.

Ciliary muscle. In cultured human ciliary muscle cells, ET-1 stimulates PLC, increases $[Ca^{2+}]_i$, induces membrane depolarization, triggers prostaglandin release, and activates adenylyl cyclase (65, 82, 83). All of these effects are mediated by the ET_A receptor subtype. Matsumoto and co-workers (65) further showed that the activation of adenylyl cyclase by ET-1 in these cells is mediated by the stimulated production and release of PGE_2. Endothelin-1 also appears to cause the muscle cells to contract (Fig. 3). This agrees with the findings that isolated ciliary muscle from human (53), Rhesus monkey (84) or bovine (67, 80, 81) contracts when treated with ET-1. Nevertheless, Abdel-Latif *et al.* (80) observed that whole human ciliary muscle obtained within 7 to 14 hr after death did not respond to ET-1 with regard to phosphoinositide turnover or contraction. However, longitudinal human ciliary muscle did respond to ET-1 with contraction. Abdel-Latif *et al.* (80) further indicated that ET-1 stimulates PLC and contraction in ciliary muscle isolated from the bovine but not the cat or dog. However, in tissues from different mammals that they examined, ET-1 always induces prostaglandin production and activates adenylyl cyclase. Contractility of the ciliary muscle is believed to affect aqueous outflow facility. In fact, ET-1 has been shown to enhance outflow facility *in vivo* in rabbits (96) and monkeys (47). Consequently, the actions of ET on the ciliary muscle indicate that these peptides may contribute significantly in controlling IOP.

Trabecular meshwork. In cultured human and bovine trabecular meshwork cells, ET-1 causes depolarization of the plasma membrane and elicits calcium mobilization (85–87). Lepple-Wienhues *et al.* (81) also showed that ET-1 induces contraction of bovine trabecular meshwork strips in a dose-dependent and calcium-dependent manner. Interestingly, ET-1 increases intracellular pH in the bovine cells (85). This effect is apparently mediated by the activation of Na-H exchange, since inhibitor of the antiporter prevents the alkalinization. Increase in cytosolic pH can affect gene transcription and cell proliferation (97). Currently, no information is available regarding whether ET induces cell growth in the trabecular meshwork. Dysfunction of this tissue is generally attributed as the etiology of most primary open-angle glaucoma cases. Therefore, ET-induced contraction and cellular alkalinization of this tissue may be implicated in the pathology or treatment of ocular hypertension.

Ocular blood vessels. Endothelin activates PLC, calcium mobilization, and contraction in many vascular smooth muscles. The same is true for the ocular vasculature. In bovine retinal microvascular pericytes, ET-1 contracts the cells, likely due to its activation of PLC and increase in $[Ca^{2+}]_i$ (23, 88, 89), suggesting that ET-1 may be involved in the autoregulation of retinal blood flow. Indeed, when treated with ET-1, bovine retinal small arteries, porcine ophthalmic, and ciliary arteries, and human ophthalmic artery contract promptly and reversibly (22, 91–93). In addition to inducing the contraction of ocular blood vessels, ET also affects the growth of vascular cells. The *in vitro* growth of cultured bovine retinal pericytes is increased by secreted factors from endothelial cells. Yamagishi and co-workers (90) showed that this factor is ET-1, and it likely serves as the general paracrine mitogen for pericytes.

Ocular Effects of Endothelins. *Cornea.* Topical administration of ET-1 (10 n*M* or μ*M* in a volume of 50 μl, five times a day for 3 days) increases by 25%–30% the closure rate of rabbit corneal epithelial wound induced by exposure of the epithelium to *n*-hexanol. The

healing effect of ET-1 is not associated with any epithelial hyperplasia, neovascularization, conjunctival hyperemia, vasoconstriction, or corneal opacification (98). This *in vivo* effect agrees well with *in vitro* actions of ET-1. The peptide dose-dependently increases $[Ca^{2+}]_i$ and proliferation of primary cultures of rabbit corneal epithelial cells as assayed by thymidine incorporation (58). The rank order of potencies of peptides tested suggests that the ET_A receptor is the primary receptor subtype involved. In this study, ET-1 was more potent in increasing thymidine uptake when 0.5% fetal bovine serum was also present, suggesting that synergism probably exists between ET-1 with other growth factors in the serum. A similar phenomenon was observed in bovine corneal endothelial cells. In the presence of 10% calf serum, ET-1 increases cell density by proliferation in culture and enhances the cell migration rate in an *in vitro* wound healing assay (64). Furthermore, Tao and co-workers (59) showed that ET-1, in the absence of added fetal calf serum, does not affect proliferation in bovine corneal epithelial cells in culture, but potentiates the effectiveness of epidermal growth factor to stimulate migration of the cells. The potentiating effect of ET-1 is apparently mediated by the ET_B receptor, since BQ-123 is ineffective as an antagonist and sarafotoxin S6c is an effective agonist equal to ET-1.

These studies demonstrate that ET stimulates the proliferation and migration of corneal epithelial and endothelial cells. Either alone or in conjunction with other growth factors, these peptides are likely involved in the wound healing process of the cornea. Clinically, they should be useful in shortening the recovery period after corneal damage produced by accident or surgery.

Iris and pupil. Similar to the actions of ET on second messenger pathways, *in vivo* actions of ET on the iris also vary among various animal species. In the rabbit, intravitreal injection of ET-1 (approximately 1 nmol) causes blockade of light reflex with an initial (2–3 days) mydriasis (48) then prolonged miosis lasting 7–10 days (99), whereas injection of ET-3 causes a slight miosis without blockade of light reflex. These results are likely due to the net difference of tensions generated by the ET-induced contraction of both the iris sphincter and dilator muscles. Apparently, prostaglandins are not involved in these actions, since indomethacin pretreatment does not modify the pupil effects of ET. Six hours after injection of either ET-1 or ET-3, the rabbit iris characteristically shows a central or crescent-shaped sector of blanching (48), probably a result of vasoconstriction (100). The remainder of the iris in ET-1–treated eyes is hyperemic. At 1 day after injection, the iris and conjunctiva become markedly hyperemic in both the ET-1– and ET-3–treated eyes (48). The hyperemia can be reduced by indomethacin pretreatment, indicating that ET-triggered prostanoid production plays an important role.

Intracameral administration of ET-1 causes marked miosis in the cat (101). Within minutes of injection of 0.04 pmol ET-1, the pupil size decreases to less than 50%. Addition of 0.4 and 4 pmol ET-1 further constricts the pupil. The pupil size is about 10% of the original size after the 4-pmol treatment. After indomethacin pretreatment, ET-1 is no longer miotic. Indeed, the ET-1 injection also raises concentration of PGE_2 in aqueous humor by almost 500-fold. These results agree very well with *in vitro* studies in cat iris sphincter, in which prostaglandins seem to mediate ET's effects on PLC activation and contraction (73).

In contrast to the rabbit or cat, ET does not affect PLC or contraction of the iris sphincter of primates. Consequently, perfusion of ET-1 (up to 100 n*M*) into the anterior chamber of anesthetized cynomolgus monkeys has no effect on the pupil diameter (47). These results predict that ET probably does not affect the pupil size in humans either.

Intraocular pressure. Although Sato *et al.* (102) could not detect a significant IOP effect following intravitreal injection of ET-1, many laboratories have demonstrated that ET peptides induce long-lasting ocular hypotension. MacCumber and co-workers (48) observed that intravitreal injection of ET-1 or ET-3 (1.2 nmol) significantly lowered IOP, by 30%–40%, in normotensive rabbits. The IOP lowering of ET-1 lasted for more than 7 days, whereas that of ET-3 had a slightly shorter duration, lasting between 5 and 7 days. In this study, aqueous protein was elevated in six of eight eyes 48 hr after ET-1 injection and two of five eyes after ET-3 injection. Pretreatment of the animals with Indomethacin reduced the increase in aqueous protein without affecting the ocular hypotensive effect of the ET peptides, suggesting that prostanoids were not involved in mediating the ET's IOP action. Sugiyama *et al.* (26) described a similar finding that intravitreal injection of ET-1 (0.1 nmol) decreased IOP by 40%–50% for at least 24 hr. Fourteen days after injection, the IOP of the treated eye was still statistically significantly lower than that of the control. They further showed that intravenous administration of ET-1 (0.1 nmol/kg) also lowers IOP.

In addition to ocular hypotension, ET-1 may even increase IOP *via* the stimulation of prostaglandin release. For example, Granstam and colleagues (103) demonstrated that intracameral injection of ET-1, ET-2, or ET-3 (0.8 or 4 pmol) caused a dose-dependent increase in IOP with an increase in protein and PGE_2 concentrations in the aqueous humor. The ET-1 effects on both ocular hypertension and aqueous protein increase were blocked by indomethacin pretreatment. Moreover, Sugiyama and colleagues (104) found that intravitreal injection of 0.05 or 0.15 μg ET-1 produced prolonged (>3 days) IOP lowering. At higher concentrations (0.5 and 5 μg), the IOP lowering became more

pronounced and prolonged. However, there was an initial (0.5–1 hr) significant increase in IOP when 0.5 μg (but not 5 μg) of ET-1 was administered. Levels of PGE_2 in aqueous humor was increased by approximately 300-fold at 1 and 24 hr after the 0.5 μg ET-1 treatment. Pretreatment with indomethacin blocked such increase in PGE_2 and the initial hypertension without affecting the subsequent IOP-lowering effect.

The IOP effects of ET-1 are likely mediated by both the ET_A and ET_B receptor subtypes, since intravitreal injection of ET_A-selective antagonists 97–139 (155 μg) or BQ-123 (126.5 μg) prior to ET-1 (0.5 μg) significantly reduces but does not completely eliminate hypotensive actions of ET-1 (104, 105). Moreover, the ET_B agonist Sarafotoxin-S6c also lowers IOP in rabbits after intravitreal injection. A 40% reduction of IOP is still evident 6 days after 2-nmol treatment (96, 105). Indomethacin does not block its effect. Thus, both the ET_A and ET_B receptors appear to be able to lower IOP directly.

The mechanism of the ET ocular hypotension is unclear. Both MacCumber *et al.* (48) and Azuma (107) reported that the outflow facility in rabbits was not changed after ET-1 injection. However, Taniguchi *et al.* (96) suggested that activation of the ET_A receptor can lower aqueous formation in the rabbit, whereas ET_B receptor activation increases outflow facility. In anesthetized cynomolgus monkeys, bolus injection of ET-1 (0.01–10 pmol) into the anterior chamber increases outflow facility by 22%–71% in a dose-dependent fashion (47). This finding is consistent with *in vitro* studies showing that ET stimulates the ciliary muscle and induces contraction. Contraction of ciliary muscle should increase aqueous outflow and induce accommodation. Indeed, the outflow effect of ET-1 in monkeys correlates very well with its accommodative action. However, the demonstration of increase in outflow facility does not exclude other possible mechanisms of ET on aqueous humor hydrodynamics. Its effects both on adenylyl cyclase in the ciliary processes and on vascular contractility may modulate the production of aqueous humor and contribute to ocular hypotension. Moreover, in perfused anterior segment of the bovine eye where the ciliary body including the ciliary muscle was removed, 2 or 20 n*M* of ET-1 reduces outflow facility (108), as does pilocarpine. Wiederholt and co-workers concluded from these data that contraction of trabecular meshwork induced by ET-1 or muscarinic agonists physically changes the outflow pathway and causes an increase of outflow resistance that leads to a reduction in outflow rate. Even though there may be relevant differences between the bovine and human eye, the authors speculated that contraction of the trabecular meshwork induced by ET or muscarinics is functionally antagonistic to the direct contractile effect on the ciliary muscle.

Ocular blood flow. The role of optic nerve blood flow in optic nerve neuropathies has received increased attention, particularly with respect to glaucomatous optic nerve damage. There are some schools of thought that suggest optic nerve damage in glaucoma may be initiated by abnormal local autoregulatory mechanisms of the optic nerve microvasculature (27, 109). Local vascular tone can be modulated by the release of relaxing and contracting factors by the vascular endothelium (110). The most potent vasoconstrictor peptide released is ET.

ET peptides produce vasoconstriction in almost all vasculatures tested, including ocular blood vessels. In the human ophthalmic artery, ET-1 produces marked contractions (94). However, repeated administration of ET-1 results in a decreased contractile response typical of tachyphylaxis. The authors suggest that this decreased responsiveness is indicative of downregulation of ET receptors and represents a preventive mechanism by which the vascular bed is protected from repeated and prolong exposure to ETs. It is tempting to speculate that a dysfunction in the ability of the vascular beds to modulate their responsiveness to ETs can lead to pathophysiology of chronic ischemia or alterations in ophthalmic blood flow.

In the cat, intravitreal injection of 0.4 nmol of ET-1 induces a 34% reduction in retinal blood flow but does not affect blood flow in the ciliary body, iris, or choroid (101). A dose-related sustained decrease in optic nerve blood flow after intravitreal injection of ET has also been observed (24). In rabbits, intravitreal injection of 0.1–5 nmol of ET-1 causes concentration-dependent constriction of retinal vasculatures (48, 111–115). ET-3 is less effective than ET-1 in causing the constriction (111, 116). The ET-1–induced vasoconstriction is so severe that, 1 min after injection of 1 nmol of the peptide, blood flow in the retinal arteries and veins surrounding the optic disc ceases completely, as determined by fluorescein angiography. The complete obstruction of flow persists for almost an hour (115). Pallor of the optic nerve head lasting for 10 days after injection has also been reported (99). In addition to retinal flow, ET-1 also decreases choroidal blood flow, but to a lesser extent (26, 107). In some incidences, it can actually increase choroidal blood flow, possibly because of the shunting of blood to the choroid by local autoregulatory mechanisms when blood flow to the optic nerve head or the retina is lowered (102). In the rat, intravitreal injection of ET-1 apparently does not affect calibers of retinal capillaries, even though it constricts the retinal arterioles, suggesting that rat capillary pericytes does not have contractile function in response to ET-1 (112).

Ischemia of the retina affects the health of retinal cells and produces abnormalities of electroretinographs. Takei *et al.* (115) showed that 110–150 min after intravitreal injection of ET-1 the amplitude of the scotopic b-wave is significantly elevated and the amplitudes of oscillatory potentials are significantly reduced, while the

a-wave is not affected. These findings suggest that the ET-1–induced damage is more pronounced in the retina than in the choroid. A similar study by Oku and co-workers (99) provides a slightly different conclusion. They demonstrated that the amplitudes of a- and b-waves and the latencies of the oscillatory potentials in the ERG are not changed by ET-1. Yet, prolongation of the N1 latency of visual evoked potential by 2–3 msec was observed from 1 hr to 14 days after the injection. The investigators concluded that ET acts on the circulation of the optic nerve head rather than that of the retina or the choroid. The differences in the two studies probably resulted from the extent of ET-induced ischemia. However, both studies do point out that severe ischemia generated by local injection of ET can be damaging to visual functions and that the peptide's effect is more significant in the optic nerve head and retina than in the choroid.

Other administration routes of ET also cause vasoconstriction in ocular structures. Intravenous injection of ET-1 (1 μM, 0.1 ml/kg) lowers capillary flow in optic nerve head for at least 3 hr without modifying choroid flow (26). Chronic perineural injection *via* an osmotic minipump to the area adjacent to the optic nerve of the rabbit causes vasoconstriction of the anterior optic nerve vasculature (113). Intraluminal perfusion of 1 pM of ET-1 through the common ophthalmic artery in isolated porcine eyes unexpectedly increases ophthalmic microcirculation by 20%, which returned to basal level within 5 min (116). This vasodilatory effect is mimicked by ET-3 and blocked by pretreatment of indomethacin, suggesting that production of prostaglandins *via* the activation of the ET_B receptor is critical. Higher concentrations of ET-1 decrease flow. At 100 pM, flow is reduced by 60% 5–10 min after administration and remains low for more than 30 min. This effect is blocked by FR139317, an ET_A receptor–specific antagonist. Thus, ET when administered *via* ophthalmic artery can induce vasoconstriction or vasodilation, depending on its concentration and the exact receptor subtype activated.

While intravitreous injection of ET does not affect the blood flow of the anterior of the eye, intracameral injection of approximately 1 nmol of ET-1 or ET-3 produces a marked blanching of the rabbit iris lasting for several hours (48).

Interestingly, retinal blood flow of streptozocin-induced diabetic rats has a blunted response to ET-1 (111). Exposure of retinal pericytes to 25 mM glucose also blunts the ET-induced activation of PLC and PKC without significantly affecting ET receptor binding (89). This may explain why hyperglycemia in diabetes impairs the retinal autoregulatory response to hyperoxia.

Normotension glaucoma. As mentioned above, recent studies have focused attention on optic ischemia as a potential impetus for the development of optic nerve damage in optic neuropathies like glaucoma. Although glaucoma has been thought of as principally involving a pathology of the normal pathway for aqueous humor outflow and damage dependent on an elevated IOP, recent studies of normotensive glaucoma patients suggest that other mechanisms may be involved (27). It has been suggested that prolonged vasoconstriction or vasospasm arising from an abnormal autoregulatory mechanism contributes to an insufficiency of blood flow to the optic nerve and produces a state of optic nerve ischemia (25). Endothelins, with their ocular vasoconstrictor effect, may be involved. Indeed, in preliminary reports, chronic administration of low-dose ET-1 in primates produces damage to the optic nerve head reminiscent of glaucomatous damage (117). These results suggest that contraction of the microvasculature may contribute to the development of optic nerve damage by producing optic nerve ischemia. Recently, ET-1 concentration in plasma was shown to be significantly higher (35%) in normal tension glaucoma patients than in normal age-matched subjects, while the plasma ET-1 concentration in primary open-angle glaucoma patients was greater than in normal tension glaucoma patients and less than in normal patients, but not significantly different from either (26). The clinical significance of this finding is still not certain. What is clear is that optic nerve blood flow can be influenced by autoregulatory mechanism and any changes in the functioning of these mechanisms could impact the ability of the microvasculature to respond to local demands, including ischemia. Whether or not ETs play a role in the development of optic nerve damage in glaucoma still needs further investigation. However, there is strong observational evidence to suggest that this vasoactive peptide plays an important role in ocular physiology and pathophysiology.

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