Effects of Endothelin-1 on Components of Anterograde Axonal Transport in Optic Nerve

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PURPOSE. Increased levels of endothelins (ETs) are associated with glaucoma and have been said to contribute to the development of glaucomatous optic neuropathy. In glaucoma, movement of selected components of anterograde axonal transport essential in ganglion cell survival is impaired—specifically, the transport of mitochondria. This study evaluates the effect(s) of a single administration of intravitreous ET-1 on anterograde axonal transport in the rat optic nerve.

METHODS. Proteins for anterograde axonal transport were pulse labeled by intravitreous injection of [35S]methionine plus or minus ET-1 (2 nmol) in HEPES buffer (pH 7.4). At appropriate time intervals, optic nerves were dissected, sectioned while frozen, and homogenized in denaturing buffer, and transported protein was quantitated by liquid scintillation counting. Counts corrected for efficiency, quench, background, and decay were statistically evaluated (ANOVA, n = 7).

RESULTS. Effects of treatment with intravitreous ET-1 on anterograde axonal transport were significant, biphasic, and prolonged (4 hours to 21 days). The initial phase was a significant enhancement of transport at times normally associated with small, fast-moving tubulovesicles (4 and 24 hours), followed by significant impairments at times normally associated with transport of mitochondria (28–36 hours), cytoplasmic matrix (4 days), and cytoskeletal proteins (21 days). The most pronounced effect of ET-1 was decreased axonal transport at times associated with normal anterograde transport of mitochondrial proteins (28, 52, and 36 hours, P < 0.001, P < 0.015, and P < 0.001, respectively). This was mimicked by ET-3 at 28 hours.

CONCLUSIONS. Effects of intravitreous ET-1 are consistent with a receptor-mediated role for elevated ETs in pathologic misregulation(s) of anterograde axonal transport. (Invest Ophthalmol Vis Sci. 2002;43:3223–3230)

Glauc is a stereotypic optic neuropathy involving loss of retinal ganglion cells with axons that leave the retina to form the optic nerve.1 One characteristic of glaucoma is a dysfunction in the regulated delivery of mitochondria and uncharacterized tubulovesicles from their sites of synthesis and assembly in the cell body to sites of proper function that are situated along the axon and in terminals.2 The observed disruption in anterograde axonal transport may result from nerve compression, as a consequence of elevated intraocular pressure,3 or it may result from anoxic conditions4,5 during ischemia.6 The effect of direct compression on axonal transport appears to involve the loss of linear microtubule arrays in exposed axons,7 a mechanism also suggested to result from ischemia.8 However, experimental neuropathies known to act by this mechanism9 exhibit large proximal swellings in regions with unmyelinated axons, disorganized microtubules, and prominent accumulations of neurofilaments.10 In contrast, accumulations in glaucomatous human donor tissue appear to be predominantly mitochondrial and vesicular.2 This suggests that glaucomatous disease represents a selective misregulation of axonal transport, rather than an indiscriminate inhibition resulting from loss of microtubular arrays. Some other mechanism(s) could be involved in the full development of glaucomatous pathophysiology, which would contribute to glaucoma by subtly perturbing important physiological functions in the proximal optic nerve. One candidate agent to affect anterograde axonal transport is ET-1, a dually neuroactive10 and vasoactive11 peptide that is present in the normal eye12 and is reported to increase in experimental animal models of glaucoma with elevated intraocular pressure.13,14

ET-1, its cognate ET-3, and their receptors are normally present in the appropriate ocular regions to affect axonal compartments of retinal ganglion cells.1,2,15–17 ET-1, the most-studied member of the ET family of isopeptides,11,18 acts through the G-protein-coupled receptors ETα and ETβ with specificities of ET-1 > ET-3 and ET-1 ≈ ET-3, respectively.19,19 These receptors are located at the optic nerve head,20 as well as within the ganglion cell and nerve fiber layers of the retina.21 Because of its vasoconstrictive properties, ET-1 has been extensively used experimentally to model retinal ischemia.22–24 However, indications from other regions of the central nervous system (CNS) are that either ischemic events or mechanical injury can induce the secretion of ET-1 from resident astrocytes25,26 and alter the expression of ET receptors in CNS tissues.27,28–30 including the retina.31 In addition, nonselective ET receptor antagonists can block secondary axonal degeneration in long-fiber tracts,32 a mechanism believed to contribute to retinal ganglion cell loss in some experimental models of glaucoma.32 These data suggest additional roles for ETs that may include alterations in anterograde axonal transport in such CNS tissues as the optic nerve.

Anterograde axonal transport is a complex and tightly regulated process by which neurons supply the axons of their long-fiber tracts with protein elements required for maintenance and survival. Because axons do not have the machinery required for protein synthesis, the timely delivery of specific cargoes to their functional domains, represents a complex logistic burden for the neuron. Axonal transport comprises multiple distinct rate components that deliver specific types of cargo to axonal domains.33,34 Fast anterograde axonal transport delivers a variety of membrane-bound organelle (MBO) cargoes and may be divided into several subcomponents, including very fast, small tubulovesicles moving with synaptic vesicle precursor proteins, and slower-moving MBO cargoes.
with mitochondrial proteins. Regulation of fast anterograde axonal transport is only partially understood. In glaucoma, the anterograde axonal transport of mitochondria is seriously compromised as axons traverse the perilaminar region of the optic nerve head, presumably leading to bioenergetic perturbations as a consequence of inappropriate supply. The specific type(s) of tubulovesicles affected in glaucoma are unknown, making it difficult to assess their contribution(s) to development of the neuropathy. In the present study, elevated ET-1 induced a complex pattern of aberrations, affecting all the distinct rate components of anterograde axonal transport, with its most pronounced effect(s) on the mitochondrial subcomponent.

Methods

Rats

Young adult male Sprague-Dawley rats (n = 7 control and n = 7 experimental rats for each time point or injection–sacrifice interval, ISI), weighing 200 to 250 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and acclimatized to the animal facility for 2 weeks before use in this study. Rats were selected for use in because their retinal ganglion cell axons, as in humans, are unmyelinated before leaving the retina, and anterograde axonal transport in the optic nerve has been extensively characterized in this species in work previously published from this laboratory. All studies were conducted in accordance with NIH guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Intravitreous Injection of Radiolabeled Precursors, with or without ET

Newly synthesized proteins undergoing anterograde axonal transport in the optic nerve were pulse-labeled in the presence or absence of ET, as modified from previously published methods. Modifications were minimal and involved the replacement of a distilled water vehicle for resuspension of radiolabeled precursors with either HEPES-buffered ET-1 or HEPES vehicle buffer alone. 55-Methionine (EasyTag Express Protein Labeling Mix; DuPont-NEN Life Sciences, Boston, MA) was lyophilized and resuspended in either vehicle alone (10 mM HEPES [pH 7.4]; Sigma Chemical Co., St. Louis, MO) or in vehicle containing 500 μM ET-1 (Bachem, Belmont, CA). Rats were anesthetized by methoxyflurane inhalation, and 0.8 μCi (4 μL) of radiolabel in vehicle, with our without ET-1 (final dose 2 nmol), was injected into the vitreous of the left eye with a 30-gauge needle attached to a syringe (microliter 710, 22s gauge; Hamilton Co., Reno, NV) by polyethylene tubing (PE-20, Clay Adams Brand; BD Biosciences, Sparks, MD). In one experiment, ET-3 was substituted for ET-1, with the same methods used (2-nmol dose, 28-hour ISI, n = 7 control and n = 7 experimental animals). During intravitreous injections, retinas were observed through the pupil with a surgical microscope (model Stiftuss S; Carl Zeiss, Thornwood, NY). During introduction of the resuspended label into the vitreous, a transient blanching of the retina was observed in all animals, both control and experimental, that did not appear noticeably greater in the ET-1–treated animals and began to recover immediately after the injection was complete. One minute after injection, all retinas appeared normal in color. (Based on these initial observations, further observations of the retinas were not performed.) Information on the dose-related effect(s) of intravitreous ET-1 in this species (rat) were unavailable, and physiological-pathologic concentrations of ET in the optic nerve head’s microenvironment are generally unknown. Therefore, dose selection was made on the basis of a small pilot study, using these methods and measuring the total pulse-labeled protein axonally transported into the rat optic nerve. (Three rats in each group was used only for the pilot study, 4-hour ISI, data not shown.) The pilot study evaluated 0.3-, 0.4-, and 2-nmol doses of intravitreous ET-1 and the results showed a trend of increasingly enhanced axonal transport, compared with control, as the dose of ET-1 increased. However, significant effects on axonal transport (4-hour ISI) were seen only in the pilot study for the 2-nmol dose. The combination of a nonsignificant trend at lower doses with a large variance at the lowest significantly effective dose (2 nmol) was interpreted to mean that the 2-nmol dose was centrally located within the effective pharmacologic dose range, for anterograde axonal transport in rat optic nerve, at the 4-hour ISI. Possible effect(s) on nonassayed ocular tissues were not considered in dose selection, because data on these were unavailable for either acute or chronic intravitreous administration of ET-1 in rats.

Harvest and Preparation of Pulse-Labeled Optic Nerves

Animals were anesthetized with methoxyflurane at specified times after injection and then killed by decapitation. ISIs were selected based on the published characterizations of anterograde axonal transport in rat optic nerve for specific marker proteins associated with specific classes of axonally transported materials (Table 1). Animals were selected for use in because data on these were unavailable for either acute or chronic intravitreous administration of ET-1 in rats.

Table 1. Summary and Significance Effect of ET-1 on Anterograde Axonal Transport

<table>
<thead>
<tr>
<th>ISI</th>
<th>Component</th>
<th>CARGO/subtype</th>
<th>Effect of ET-1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>Fast</td>
<td>MBO/small tubulovesicles</td>
<td>Increased transport</td>
<td>0.010</td>
</tr>
<tr>
<td>24 h</td>
<td>Fast</td>
<td>MBO/small tubulovesicles</td>
<td>Increased transport</td>
<td>0.020</td>
</tr>
<tr>
<td>28 h</td>
<td>Fast</td>
<td>MBO/mitochondria</td>
<td>Decreased transport</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>32 h</td>
<td>Fast</td>
<td>MBO/mitochondria</td>
<td>Decreased transport</td>
<td>0.015</td>
</tr>
<tr>
<td>36 h</td>
<td>Fast</td>
<td>MBO/mitochondria</td>
<td>Decreased transport</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4 d</td>
<td>SCa</td>
<td>Cytoplasmic matrix proteins</td>
<td>Decreased transport</td>
<td>0.001</td>
</tr>
<tr>
<td>21 d</td>
<td>SCb</td>
<td>Cytoskeletal proteins</td>
<td>Decreased transport</td>
<td>0.010</td>
</tr>
</tbody>
</table>

ANOVA, n = 7, for every time and every treatment condition.

Statistical Analysis

Corrected disintegrations per minute (dpm) were analyzed by ANOVA (n = 7) performed on computer (Systat 5; SPSS Sciences, Inc., Chicago, IL). All statistical analyses included data from the whole optic nerve (the sum of all four segments from an individual optic nerve). For the comparison of the effect of ET-3 with ET-1 at the 28-hour ISI, corrected dpm for the ET-1 group (n = 7) were normalized to the group of control rats simultaneously treated (n = 7), and the same method was used in the ET-3 group (n = 7) and its simultaneously treated group of
Effects of ET-1 on Anterograde Axonal Transport

Intravitreous effects of ET-1 were most severe within a 28- to 36-hour window (Table 1; Figs. 1, 3, 4). In this interval, a large reduction in transport occurred. At these times, a large pulse (Fig. 1) of mitochondrial proteins normally moves through the rat optic nerve. A closely related ET, ET-3, was also tested for an effect on transport at 28 hours. ET-3 has 1000-fold less affinity than ET-1 for vasconstrictive ET<sub>B</sub> receptors, but has comparable affinity for ET<sub>A</sub> receptors. The ET<sub>A</sub>-selective agonist ET-3 had effects on axonal transport at 28 hours that were comparable to those of ET-1 (Fig. 5).

**Biphasic Effects of Intravitreous ET-1**

The effects of intravitreous treatment with ET-1 on anterograde axonal transport were biphasic (Table 1; Figs. 1, 3, 4). The initial, rapid effect of treatment with ET-1 was a significant enhancement of anterograde axonal transport into the optic nerve at 4 and 24 hours (Figs. 1, 4). The slower but more prolonged effect of ET-1 was a significant reduction of anterograde axonal transport into the optic nerve at 28, 32, and 36 hours and 4 and 21 days (Figs. 1, 4).

**Prolonged Effects of ET-1**

Intravitreous ET-1, administered as a single bolus, exerted significant effects (Table 1) on anterograde axonal transport as early as 4 hours after treatment (Figs. 1, 3) and as late as 21 days after treatment (Fig. 4), inducing an extended period of aberrant axonal transport within the retinal ganglion cell axons of the optic nerve (Fig. 4). Chronic administration was not required to achieve this effect.

**DISCUSSION**

A single intravitreous application of ET-1 had a complex and profound effect on anterograde fast axonal transport (Fig. 1).

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**RESULTS**

**ET-1: Alterations in All Components of Anterograde Axonal Transport**

The effects of intravitreous ET-1 treatment were significant, biphasic, and prolonged (Table 1, P < 0.05; Figs. 1, 2, 3, 4; n = 7 control and n = 7 experimental animals, at each time point). The most profound effect of ET-1 occurred at 28 hours. At the 28-hour ISI, this effect was mimicked by the ET<sub>A</sub>-receptor-selective agonist ET-3 (no significant difference between ET-1 and ET-3, P > 0.999, ANOVA, n = 7, Fig. 5) and suggests a receptor-mediated phenomenon, with similar effects for ET-1 and ET-3.

The direction and magnitude of the effects of ET-1 varied with time and the transported cargo (Table 1, Figs. 3, 4), suggesting a selective misregulation, as opposed to an indiscriminate inhibition, of anterograde axonal transport. Distributions of radiolabeled within optic nerves were monitored (Figs. 2, 3) but all statistical comparisons (Table 1; Fig. 4) were made on data from the whole optic nerve (n = 7 for each time point).

**Effect of ET-1 on Axonal Transport of Some Small, Fast Tubulovesicles**

There was a moderate but significant enhancement of axonal transport into the optic nerve at times normally associated with small, fast-moving tubulovesicles, but little or no mitochondrial marker proteins (4- and 24-hour ISIs; Table 1, Figs. 1, 2, 3, 4). The magnitude of enhancement of ET-1 was greater at the 4-hour ISI than the 24-hour ISI (Fig. 1). The 4- and 24-hour ISIs were selected for use in the study because they are normally associated with similar amounts of total anterogradely transported material, but the chemical compositions of transported material are different. Typically, a single form of the kinesin motor is associated with transport at the 4-hour ISI, whereas multiple isoforms of the motor are associated with the 24-hour ISI. The possibility of a differential regulation in the transport of various classes of tubulovesicles during this subcomponent was the basis for our use of the 4- and 24-hour ISIs. In this study, effects of ET-1 on anterograde transport at the 4- and 24-hour ISIs were consistent with a hypothesized differential misregulation in the transport of various classes of small, fast-transported tubulovesicles.
Changes occurred in both the very fastest moving material, thought to represent movement of small tubulovesicular structures including synaptic vesicle precursors, and the slower-moving subcomponents of fast anterograde transport, which contain mitochondrial markers. The most pronounced effect of intravitreous ET-1 was a sharp reduction in the mitochondrial subcomponent of anterograde transport (Figs. 1, 3). This sharp reduction in the mitochondrial subcomponent contrasted with a modest increase in material transported at the fastest rate (Figs. 1, 3).

Although, ischemia inhibits axonal transport, ETₐ-mediated vasoconstriction is apparently not a prerequisite for the effect of ET on axonal transport. The effects of the ETₐ receptor-selective agonist ET-3 on the mitochondrial subcomponent of axonal transport at 28 hours were comparable to the effects obtained with ET-1 suggesting that ET’s actions on axonal transport (Fig. 5) are an ETₐ-mediated effect (Fig. 5). This suggests the possibility that activation of ETₐ lies in a direct mechanistic line downstream from ischemia, perhaps shortening a pathologic ischemic circuit.

The complexity of the effects of ET-1, with early increases in transported material followed by later decreases in transport (Figs. 1, 3), appears to reflect a mechanism that perturbs the neuron’s ability to regulate the timely delivery of specific cargoes to their functional domains. The early increases, which were greater at the 4-hour than at the 24-hour ISI (Fig. 1), may

**Figure 2.** A single intravitreous ET-1 injection produced significant alterations in the distribution of radiolabel within the optic nerve at all times evaluated. The changes were biphasic and may reflect changes in both the amount of material transported (amount of radiolabel protein at each time point) and in the delivery of transported material to the nerve (changes in distribution at different times after labeling). This is a graphical representation of the raw data set (n = 7 for both control and experimental rats, at every time point), because the only corrections made were for radioactive decay and calibration of the liquid scintillation counting technique. Optic nerve segments, 2 mm in length, are numbered consecutively from immediately behind the eye (segment 1). Evaluation for statistical significance of effects of ET-1 on individual nerve segments was not considered appropriate because of their physical continuity at the time of treatment. Statistical comparisons for whole optic nerve are shown in Figure 4. Error bars: SEM.
reflect the markedly increased transport of a subset of the fastest tubulovesicles, highly enriched at the 4-hour ISI but less well represented at the 24-hour ISI. Markedly increased transport for a subset of vesicles, may partially obscure decreased transport for other types of vesicles that happen to be simultaneously transitioning through the optic nerve at the 24-hour ISI. This interpretation would not be inconsistent with the previously published neurochemical analyses on the composition of transported materials in the optic nerves of normal rats at these ISIs.35,39,40 Those studies have indicated that multiple distinct MBO cargoes move in fast axonal transport, with differences in protein content, motor isoforms, and destination.35,36,39,40 Targeted delivery of MBO cargoes to different destinations implies a different regulatory control. Such an explanation is compatible with a receptor-mediated event,36,56 and reconciles the early effects of ET-1 with observed glaucomatous disease.2

The effects of ET-1 and ET-3 on the subcomponent that contains mitochondrial proteins35,39 (Figs. 3, 4) appears to be consistent with evidence that a reduced percentage of mito-
chondria successfully transition through the perilaminar region in glaucoma.\(^2\) The perilaminar region is both adjacent to the vitreous site of ET-1 and ET-3 applications and within the primary site of glaucomatous disease—the optic nerve head.\(^{32}\) The results in this study suggest the hypothesis that increases in vitreous ETs could contribute to the development of glaucomatous optic neuropathy,\(^{13}\) through pathologic misregulation of anterograde axonal transport.

All components of anterograde axonal transport were significantly affected by treatment with ET-1, including the transport of cytoskeletal materials moved in slow component a (SCa; Table 1, Figs. 2, 4). This appears to be consistent with the occasionally observed fibrillary changes reported in human glaucomatous donor tissue.\(^2\) That intravitreal ET has lesser effects on the slow components of axonal transport than were seen in the earlier mitochondria-associated subcomponent may be explained by the continued reductions in axonal energy supply due to reduced mitochondrial transport, reductions in synthesis of cytoskeletal proteins, or a subcomponent-specific action.

The ability of a single intravitreal administration of ET-1 to elicit a biphasic response (Figs. 1, 3) may reflect activation of two separate signal transduction pathways. Activation of multiple ET receptors present on the retinal ganglion cell soma and/or axons\(^{15,21}\) or of a single class of receptors able to activate dual signaling pathways\(^{57,58}\) could produce a biphasic effect. Less-direct mechanism(s) may also contribute by activating ET receptors on retinal vasculature\(^{15}\) and resident glia,\(^{59,60}\) initiating processes that selectively alter either anterograde transport alone\(^{36,61}\) or the coordinated synthesis and anterograde transport of specific neuronal proteins.\(^{52,63}\) One type of resident glia, optic nerve head astrocytes, share intimate contact with retinal ganglion cell axons in the perilaminar region,\(^{59,60}\) express ET receptors,\(^{59}\) and undergo both morphologic and secretory changes in glaucoma.\(^{64}\)

These results demonstrate that a single exposure to elevated levels of ET-1 in the vitreous can produce an extended series of effects (Fig. 2) that may impinge on neuronal physiology and energy supply for retinal ganglion cell axons in the optic nerve. The simplest interpretive model suggests that ET-1 may act directly on receptors that may be located on retinal ganglion cell axons in the perilaminar region, locally affecting anterograde axonal transport, perhaps inducing cytoskeletal modification,\(^{65}\) and/or aberrant phosphorylation of proteins\(^{36,56,60,67}\) that are critical to transport. However, the effects of ETs in other regions of the CNS are generally not simple.

A simple but more probable model suggests that both direct and indirect effects of elevated vitreous ETs contribute to the prolonged, multiple-component dysfunction in anterograde transport observed in this study. In many regions of the CNS, the synthesis and secretion of ETs have a dynamic and complex interrelationship with both ischemia\(^{25,68}\) and mechanical injury.\(^{57,59}\) By analogy to other CNS regions, ET-1 synthesis and release from astrocytes may be stimulated by either mechanical injury, possibly from elevated intraocular pressure, or retinal ischemia. Elevated ET could then induce a pathologic dysregulation of the mitochondrial subcomponent of anterograde axonal transport, presumably resulting in energy perturbations within retinal ganglion cell axons. ET-1 could stimulate cytocytic ET\(_2\)-mediated responses, including increased ET synthesis and release,\(^{26}\) enhanced secretion of cytokines\(^{32}\) and efflux of glutamate.\(^2\) Vascular responses to elevated ET could include ET\(_2\)-mediated rapid vasoconstriction (with a possible subsequent ischemic event and reiterative astrocytic responses), and ET\(_1\)-induced vasodilation mediated by nitric oxide and possibly TNF-\(\alpha\).\(^{1,71,75}\) This series of events appears much like the vasospasms reported in some patients with glaucoma\(^7\) as elevated ETs and nitric oxide alternate with diminished retinal perfusion and glucose-oxygen deprivation.

The demonstration that elevated levels of ET-1 in the vitreous can produce an extended period of aberrant anterograde axonal transport (Table 1, Figs. 1, 4) within the optic nerve has a number of implications for the pathogenesis of glaucoma. ET-sensitive sites may be accessed by diffusion through the vitreous and/or local secretion of the peptide with similar pathologic consequences. Elevated ETs may exert direct receptor-mediated effects on retinal ganglion cells, resident glial cells, and retinal vasculature simultaneously or may affect a subset of these targets. In either case, increased activation of ET pathways initiates a shower of cascading events that interact to produce the stereotypic neuropathology of glaucoma. If accurate, this model predicts an important place for the regulation of ET and its receptors in glaucoma therapy.

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