Regulation of Endothelin-1–Induced Trabecular Meshwork Cell Contractility by Latanoprostene Bunod

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Purpose. Previous in vivo studies demonstrated that latanoprostene bunod (LBN), a nitric oxide (NO)-donating prostaglandin F2α receptor agonist, results in greater intraocular pressure (IOP) lowering than latanoprost. The present series of investigations compared the effects of LBN and latanoprost on primary human trabecular meshwork cell (HTMC) contractility and underlying signaling pathways to determine whether LBN might mediate this additional IOP lowering via the conventional outflow pathway.

Methods. The effect of LBN (1–100 μM) on HTMC cGMP levels was determined by ELISA with or without the soluble guanylate cyclase (sGC) inhibitor ^{1}H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Endothelin-1 (ET-1) was used to induce HTMC contractility. To determine the effect of LBN on myosin light chain-2 (MLC-2) phosphorylation, HTMCs were pretreated with 10 to 60 μM LBN for 1 hour and then ET-1 for 5 minutes. MLC-2 phosphorylation was determined by Western blotting. Effects of LBN (30 and 45 μM) on ET-1–induced filamentous (F)-actin cytoskeletal stress fibers and the focal adhesion associated protein vinculin were determined by confocal microscopy. ET-1–induced HTMC monolayer resistance in the presence of LBN (45 μM) was determined by electrical cell substrate impedance sensing, as an indicator of cell contractility. Latanoprost and SE 175 (an NO donor which releases NO on reductive transformation within the cells) were used as comparators in all studies.

Results. LBN (1–100 μM) significantly increased cGMP levels in a dose-dependent manner, with a half maximal effective concentration (EC_{50}) of 1.5 ± 1.3 μM, and with maximal effect similar to that of 100 μM SE 175. In contrast, latanoprost caused a minimal increase in cGMP levels at 100 μM only. The cGMP elevation induced by LBN or SE 175 was abolished by ODQ and was therefore sGC-dependent. The two NO donors SE 175 and LBN elicited a reduction in ET-1–induced MLC-2 phosphorylation that was significantly greater than that mediated by latanoprost in HTMCs. SE 175 (100 μM) and LBN (30 or 45 μM) caused a dramatic reduction in ET-1–induced actin stress fibers and vinculin localization at focal adhesions, whereas 45 μM latanoprost was without observable effect. SE 175 reduced ET-1–induced increases in HTMC resistance in a dose-dependent manner. A synergistic effect on reduction of HTMC resistance was observed when latanoprost and SE 175 doses were given together. LBN significantly reduced ET-1–induced HTMC monolayer resistance increases to a greater extent than latanoprost, indicating a greater reduction in cell contractility with LBN.

Conclusions. LBN, SE 175, and latanoprost caused relaxation of ET-1–contracted HTMCs. The effect on HTMC relaxation observed with LBN was significantly greater in magnitude than that observed with latanoprost or SE 175. Data indicate that the NO-donating moiety of LBN mediates HTMC relaxation through activation of the cGMP signaling pathway and a subsequent reduction in MLC-2 phosphorylation. These findings suggest that increased conventional outflow facility may mediate the additional IOP-lowering effects of LBN over that of latanoprost observed in in vivo studies.

Keywords: contractility, glaucoma, nitric oxide, prostaglandin, trabecular meshwork

Latanoprostene bunod (LBN) is a nitric oxide (NO)-donating prostaglandin F2α (FP) receptor agonist under investigation for its ability to reduce elevated intraocular pressure (IOP) in patients with open-angle glaucoma or ocular hypertension. After ocular topical instillation, LBN is hydrolyzed by cellular esterases into latanoprost acid and 4-hydroxybutyl nitrate (BDMN), which is then reduced to 1,4 butanediol and NO.[1] Both nonclinical and clinical studies demonstrate LBN provides additional ocular hypotensive activity to that of latanoprost (Xalatan; Pfizer, Andover, MA, USA) when the active moiety latanoprost acid and NO-donating BDMN are combined (Saeki T, et al. *IOVS* 2009;50:ARVO E-Abstract 4064 and Refs. 1, 2).

In glaucoma, the conventional pathway, consisting of the trabecular meshwork and Schlemm's canal, is the limiting factor in aqueous humor outflow.[3] Evidence points to the role of vasoconstricting agents such as endothelin-1 (ET-1) and transforming growth factor (TGF) in the pathogenesis of glaucoma.[4–6] These and other signaling proteins cause multiple

pathological changes to tissues of the conventional pathway in glaucoma such as aberrant cell contraction and increased extracellular matrix secretion that leads to a reduction in conventional outflow.

Latanoprost appears to mediate its ocular hypotensive activity primarily through the uveoscleral (nonconventional) outflow pathway, mediated by a remodeling of the ciliary muscle, resulting in increased spaces for outflow.[7,8] In contrast, studies have shown that the trabecular meshwork is a major site of action for NO donors.[9,10] In vivo studies have demonstrated that NO donors including nitroglycerin and sodium nitroprusside (SNP) increase conventional outflow.[11–13] The role of NO in modifying cGMP-dependent signaling pathways, including inhibition of Rho-kinase (ROCK) and induction of myosin light chain phosphatase with subsequent dephosphorylation of myosin light chain-2 (MLC-2) and smooth muscle cell relaxation, is well established in vascular smooth muscle cells.[14] Trabecular meshwork cells are highly contractile in nature, analogous to vascular smooth muscle cells,[15] and in trabecular meshwork cells, NO donors increase outflow facility in isolated anterior segments, mediated by elevation of cyclic GMP (cGMP), activation of large conductance Ca^{2+}-activated K^+ (BK) channels, and a decrease in cell volume.[16,17] Furthermore, NO donors have been shown to elicit a dose-dependent biphasic effect on trabecular meshwork relaxation and a reduction in MLC-2 phosphorylation.[18] Therefore, NO likely induces alterations in trabecular meshwork signaling pathways, thus altering cytoskeletal dynamics and resulting in trabecular meshwork relaxation and ultimately an increase in conventional outflow facility.

The goal of the present study was to investigate the possible mechanistic basis of the increased efficacy of LBN in IOP lowering compared to that of latanoprost, observed in clinical and animal studies. Effects of the two agents and an NO donor, SE 175, which is reductively transformed to release NO, were determined in primary human trabecular meshwork cells (HTMCs) induced to contract with ET-1 as an in vitro model of glaucomatous pathophysiology. The NO-donating ability of LBN was confirmed by demonstrating cGMP elevation in HTMCs. The effects of LBN relative to those of latanoprost on HTMC ET-1–induced contraction were determined using multiple assays (MLC-2 phosphorylation, actin stress fiber, and focal adhesion [vinculin] localization, and monolayer resistance measurements).

Materials and Methods

Reagents

LBN was synthesized by Bausch & Lomb Inc. (Rochester, NY, USA). Latanoprost, ^{1}H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and ET-1 were obtained from Sigma-Aldrich (St. Louis, MO, USA). SE 175 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Latanoprost, LBN, SE 175, and ODQ were dissolved in anhydrous dimethyl sulfoxide (DMSO; Invitrogen, Carlsbad, CA, USA), and the final concentration of DMSO was 0.1%. Vinculin antibody was purchased from Millipore (Temecula, CA, USA), and Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Vector (Burlingame, CA, USA). Tissue culture medium, Alexa Fluor 488 phalloidin, and Alexa Fluor 568 goat anti-mouse antibody were from Invitrogen. Antibodies specifically against phosphorylated MLC-2 (Thr18/Ser19) and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling (Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Invitrogen. HRP-conjugated goat anti-mouse secondary antibody and Western blotting reagents were purchased from Bio-Rad (Hercules, CA, USA) or Pierce (Rockford, IL, USA). Gold electrodes (8W10E+) were from Applied Biophysics (Troy, NY, USA).

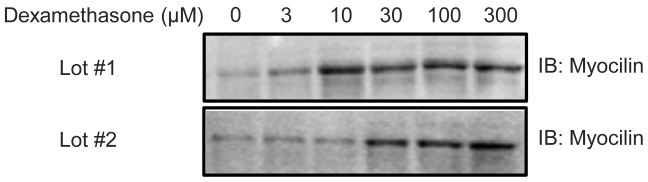

Figure 1. HTMCs express myocilin after long-term dexamethasone treatment. Western blots for the two lots of ScienCell primary HTMCs used in the study are shown. HTMCs were cultured in dexamethasone containing culture medium plus 10% FBS for 5 to 7 days. Cells were exposed to dexamethasone in basal culture medium for the last 24 hours, and then conditioned medium was harvested and concentrated 20-fold. Myocilin was detected using standard Western blotting techniques and anti-myocilin antibody.

Cell Culture

HTMCs were purchased from ScienCell (catalog no. 6590; Carlsbad, CA, USA). Cells were isolated from juxtacanalicular and corneoscleral regions. Expression of a known marker for HTMC, dexamethasone-induced myocilin, was verified by Western blotting as previously described,[19–21] using human myocilin antibody (R&D Systems, Minneapolis, MN, USA). Figure 1 shows induction of myocilin by dexamethasone in HTMC-conditioned medium for the two lots of cells used for these studies. HTMCs were cultured in complete medium (low-glucose Dulbecco modified Eagle medium [DMEM] plus 2 mM glutamine plus 10% fetal bovine serum [FBS] plus penicillin/streptomycin) until 100% confluent in an incubator at 37°C, 5% CO_2, and 95% humidity. When confluent, medium was aspirated, and cells were incubated in low-serum medium (DMEM plus 0.5% charcoal-stripped FBS) overnight.

cGMP ELISA

HTMCs were seeded in 6-well plates at 2×10^5 cells/well in complete medium. When cells were confluent, they were incubated in low-serum medium overnight. Cells were pretreated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) for 30 minutes and then exposed to LBN or latanoprost for 30 minutes. The NO donor SE 175 (100 μM) was used as a positive control. Cells were treated with IBMX (100 μM) plus the NO-independent activator of soluble guanylyl cyclase (sGC) 3-(5-hydroxymethyl-2-furyl)-benzylindazole (YC-1; 10 μM) in conjunction with the drug and/or NO donor treatments. To determine the role of sGC in NO-induced cGMP elevation, cells were also preincubated with the sGC inhibitor ODQ (1 μM) for 30 minutes. After the incubation period, cells were harvested. A cGMP enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY, USA) was performed with the cell lysates according to the manufacturer's protocol.

Myosin Light Chain-2 Western Blotting

HTMCs were seeded in 6-well plates in complete medium at a density of 2×10^5 cells/well. When approximately 75% to 85% confluent, cells were incubated with low-serum medium overnight. Cells were incubated with SE 175, LBN, or latanoprost for 60 minutes in basal DMEM. Following preincubation, cells were stimulated with 100 nM ET-1 alone or in combination with various concentrations of SE 175, LBN, or latanoprost for 5 minutes. Cell lysates were assayed for

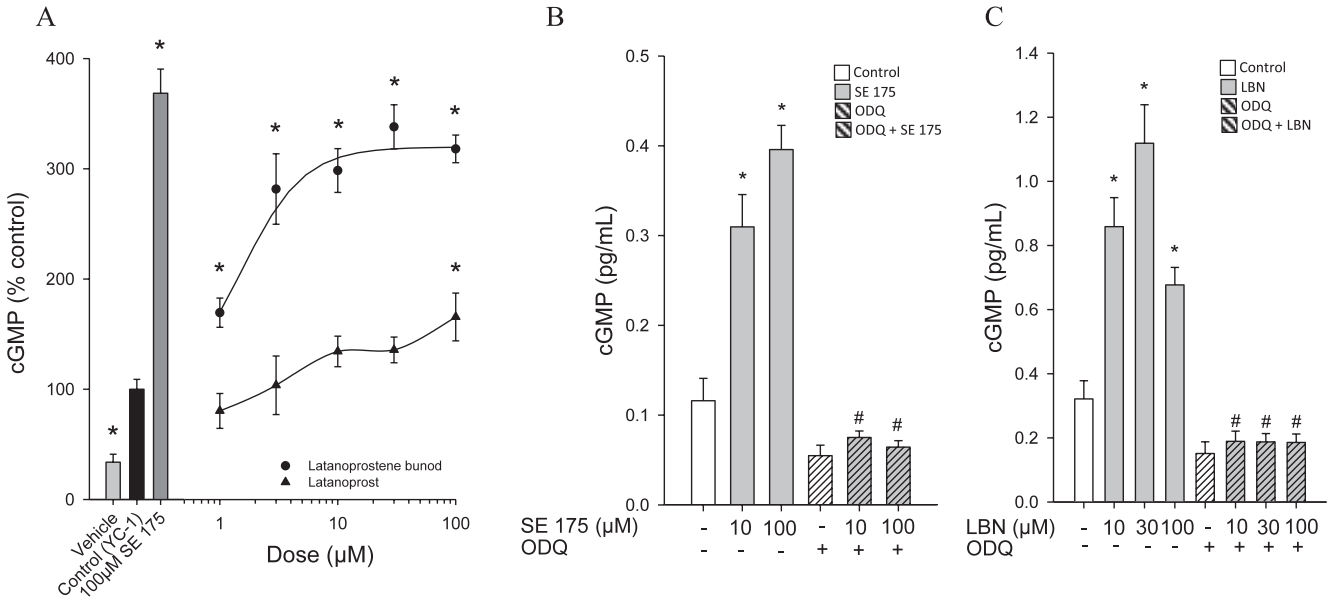

Figure 2. Latanoprostene bunod and SE 175 increase cGMP in primary human trabecular meshwork cells. (**A**) HTMCs were treated with IBMX plus the NO-independent soluble guanylate cyclase activator YC-1 (10 μM) plus increasing doses of LBN, latanoprost, or SE 175 (100 μM) for 30 minutes. Cell lysates were assayed for cGMP levels by using an enzyme immunoassay. $n = 6$–24 from 4 independent experiments; LBN line data were fitted to a four-parameter logistic equation; the line for latanoprost is an interpolation between data points. Data are means ± SEM. $^*P < 0.05$ versus control. (**B** and **C**) HTMCs were pretreated with ODQ (1 μM) for 30 minutes prior to incubation with the test agents as indicated. $n = 6$. Data are means ± SEM. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus SE 175 or LBN alone.

protein concentration and analyzed by Western blotting to assess the effects of test agents on MLC-2 phosphorylation, using antibody targeted at a dual phosphorylation site (Thr 18 and Ser 19). After being probed for phosphorylated MLC-2, blots were stripped and reprobed for GAPDH as a loading control. Western blots were imaged and quantitated via chemiluminescent detection with a Versa Doc 4000 MP imager (Bio-Rad) and the manufacturer's software. MLC-2 phosphorylation was normalized to that of GAPDH and expressed as relative to that of ET-1 alone.

Immunostaining

HTMCs were seeded at 6×10^4 cells/well in four-well tissue culture-treated chamber slides coated with collagen and fibronectin in complete medium. After reaching confluency, HTMCs were incubated for 18 hours in low-serum medium. Cells were then treated with ET-1 and LBN, latanoprost, or SE 175 for 60 minutes. After treatment, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes followed by 20 mM glycine in PBS for 10 minutes. Fixed cells were permeabilized in PBS/0.2% Triton X-100 for 10 minutes and then blocked with 1% bovine serum albumin plus 10% normal goat serum in PBS for 30 minutes. After blocking, cells were incubated with monoclonal vinculin antibody at 1:200 dilution in PBS plus 1% goat serum for 1 hour. After being washed, cells were incubated with goat anti-mouse Alexa-fluor 568 at 1:2000 dilution and phalloidin 488 Alexa-fluor at 1:50 dilution in PBS plus 1% goat serum for 1 hour. Cells were washed and mounted in Vectashield with DAPI. Cells were viewed using a Fluoview confocal microscope (Olympus, Tokyo, Japan) at ×40 magnification.

Electric Cell Substrate Impedance Sensing (ECIS)

The ECIS system (model 1600Z; Applied BioPhysics, Troy, NY, USA) was used to determine HTMC impedance in real time. In ECIS, cells are grown on a small gold film electrode deposited on the bottom of a tissue culture well, and a much larger counter electrode completes the circuit by using standard tissue culture medium as an electrolyte. A weak (<1 μA) alternating current signal (usually in the frequency range of 1 to 40 kHz) is applied to the system. At lower frequencies, flow of current between and under the cells is measured as impedance, and these impedance values can be converted to resistance values and capacitance values.[22,23]

HTMCs were seeded on ECIS 8-well electrode arrays (8W10E+) coated with collagen and fibronectin in complete medium, at a density of 2×10^4 cells/well and cultured until they reached confluence. Culture medium was removed by aspiration, and cells were incubated with basal DMEM. Cells were cultured under these conditions for 20 minutes, and the change in electrical resistance was monitored by ECIS every 5 minutes to establish a stable baseline. Test agents (100 nM ET-1 alone or in combination with various concentrations of LBN, latanoprost, or SE 175) were added, and resistance was monitored at different frequencies (0.625–64 kHz) in real time for 2 hours at 5-minute intervals. The most sensitive frequency at which change in impedance was highest was selected for analysis (4 kHz). For data analysis, each cell resistance for each individual treatment was normalized to the baseline resistance prior to agonist addition. Data were then normalized to the resistance of control (untreated) cells at each time point.

Data Analysis and Statistics

Data are means ± SEM. Number (n) refers to number of experimental replicates (each experiment was performed at least three times, unless otherwise stated). Statistical analysis was performed using ANOVA with either a Dunnett or Tukey-Kramer posttest for comparison to control or comparison among multiple groups respectively (JMP 8 software; SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant. For cGMP levels, concentration-versus-effect curves were plotted using averaged data from all samples. Curve fitting was conducted to estimate the half

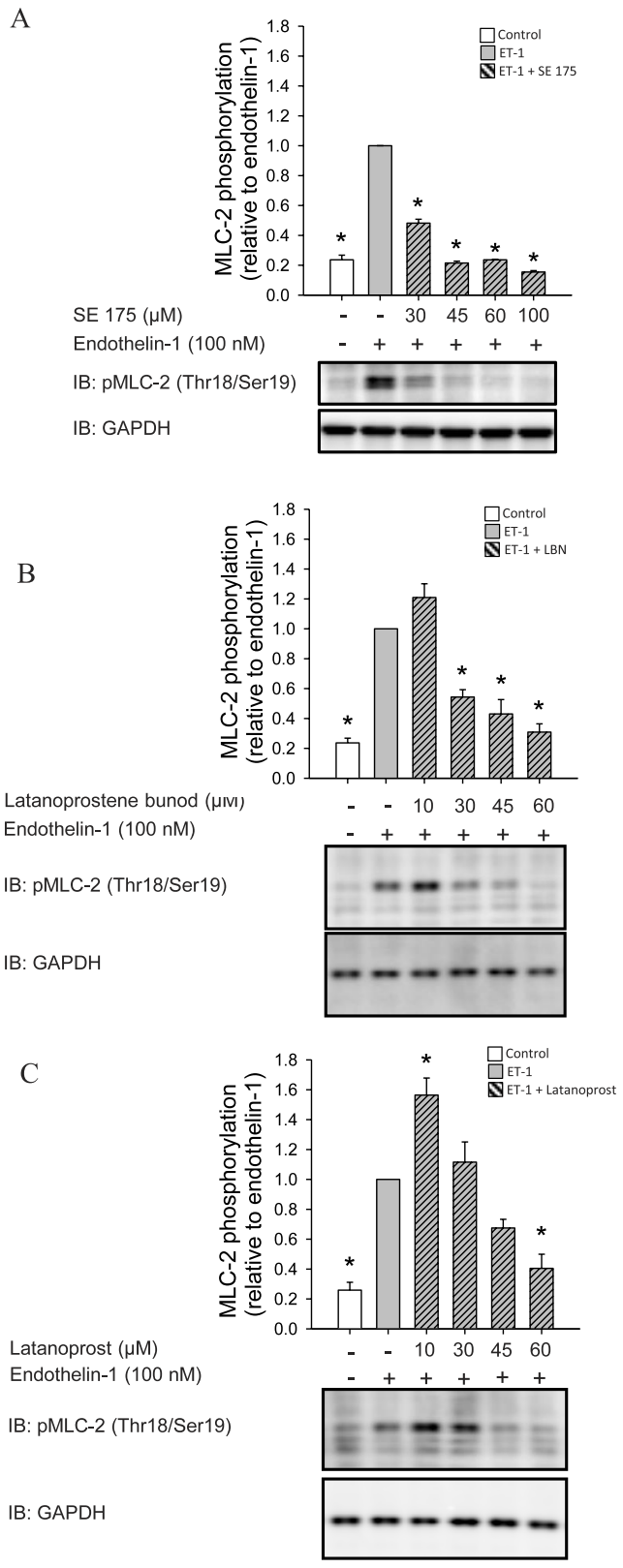

Figure 3. SE 175, latanoprostene bunod, and latanoprost dephosphorylate MLC-2 in HTMCs. Cells were preincubated with SE 175 (**A**), LBN (**B**), or latanoprost (**C**) at increasing concentrations for 60 minutes, followed by treatment with 100 nM ET-1 for 5 minutes. Following completion of treatments, cell lysates were collected and evaluated by Western blot analysis using chemiluminescent detection. Densitometry data are means ± SEM as the phosphorylated MLC-2 normalized to GAPDH, relative to ET-1 alone for respective experiments; $n = 3$ for SE 175; $n = 3$ to 6 for LBN and latanoprost. Data were analyzed using one-way ANOVA Dunnett's test. *$P < 0.05$ versus ET-1.

maximal effective concentration (EC_{50}) and E_{max} for LBN induction of cGMP levels. Data were fitted to a reparameterized four-parameter logistic equation using the JMP statistical analysis software.

Results

NO Donors LBN and SE 175 Increase HTMC cGMP Levels

NO donors have previously been demonstrated to elevate cGMP levels in HTMCs via activation of sGC[16,17]; therefore, the effects of LBN and SE 175 on cGMP were determined in HTMC. HTMCs were exposed to LBN, SE 175 (which releases NO following reductive transformation of its nitrate group), or latanoprost in the presence of the phosphodiesterase inhibitor IBMX (100 μM) and the NO-independent sGC activator YC-1 (10 μM). There was a significant increase in cGMP levels with 1, 3, 10, 30, and 100 μM LBN (Figs. 2A, 2C) and also with the NO donor SE 175 at both 10 and 100 μM (Figs. 2A, 2B). In contrast, there was no statistically significant effect of latanoprost on cGMP levels, except at 100 μM, when a small (approximately 60%) yet significant increase was observed (Fig. 2A). The EC_{50} for LBN for induction of HTMC cGMP levels was 1.5 ± 0.3 μM, and maximal efficacy was 320 ± 13%, whereas the EC_{50} for latanoprost could not be determined for the concentrations evaluated. To confirm the involvement of sGC in the elevation of cGMP levels by the two NO donors, the effect of the specific sGC inhibitor ODQ was determined. Incubation of HTMCs with ODQ (1 μM) prevented SE 175 and LBN induced cGMP generation (Figs. 2B, 2C).

LBN, SE 175, and Latanoprost Mediate Myosin Light Chain-2 Dephosphorylation

ET-1 significantly increased MLC-2 phosphorylation after 5 minutes whereas the NO donor SE 175 (30–100 μM) inhibited ET-1–induced MLC-2 phosphorylation, as detected with phospho-Thr18/Ser19 MLC-2 antibody (Fig. 3A). In contrast, there was no effect of SE 175 at 10 μM on MLC-2 phosphorylation in HTMCs (data not shown). Similarly, LBN significantly reduced ET-1–induced MLC-2 (Thr18/Ser19) phosphorylation at doses of 30, 45, and 60 μM (Fig. 3B). In contrast, latanoprost at 10 μM significantly increased MLC-2 phosphorylation. Sixty micromolar latanoprost was the only dose that significantly reduced ET-1–induced MLC-2 phosphorylation relative to that with ET-1 alone (Fig. 3C). Therefore, compared to latanoprost, equimolar LBN provides additional effects in reducing HTMC ET-1–induced MLC-2 phosphorylation, presumably through its NO-donating moiety.

LBN and SE 175 Alter Cytoskeletal Contractility and Cell Attachment

Distribution of F-actin was assessed as an indicator of cytoskeletal contractility, whereas the focal adhesion-associated protein vinculin was used as an indication of cell attachment to the underlying substrate. Treatment of HTMCs with ET-1 (100 nM) increased F-actin stress fibers and vinculin localization at focal adhesions compared to that of control, indicating increased cell contractility and cell attachment (Fig. 4). The effect of the NO donor SE 175 (100 μM) on ET-1–

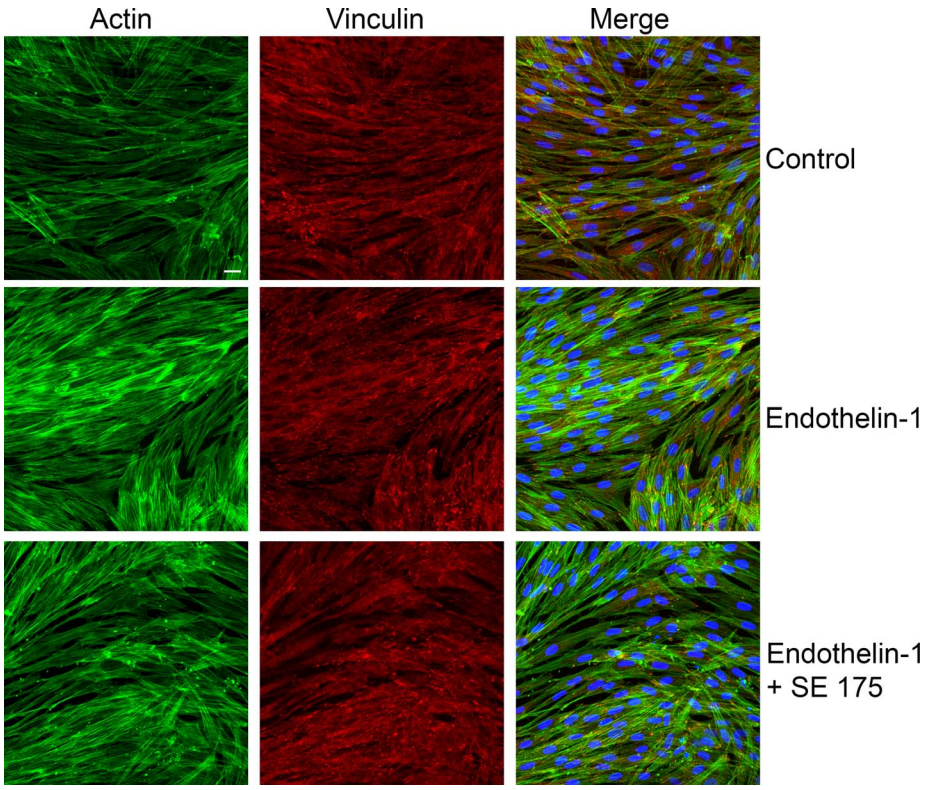

FIGURE 4. SE 175 alters ET-1–induced actin stress fiber formation and vinculin distribution in HTMCs. HTMCs were treated with ET-1 (100 nM) alone or in combination with SE 175 (100 μM) for 1 hour. Cells were fixed and stained for filamentous actin (*green* [using phalloidin]) and focal adhesions (*red* [using vinculin monoclonal antibody]). Nuclei were stained with DAPI (*blue*). Cells were then imaged by confocal microscopy at ×40 magnification. Treatment results with F-actin (*left*), vinculin (*middle*), and merged images (*right*) are shown. Images are representative of four individual experiments. ET-1–induced F-actin filaments and vinculin localization at focal adhesions were reduced with 100 μM SE 175 treatment. *Scale bar*: 20 μM (all images).

induced F-actin and vinculin distribution was then studied. Exposure of HTMC to ET-1 and SE 175 for 1 hour reduced stress fiber formation and vinculin localization at focal adhesions compared to ET-1 alone (Fig. 4). Pretreatment of cells with 45 μM LBN for 1 hour caused an alteration in endothelin-induced cytoskeletal architecture. There was a reduction in ET-1–induced stress fibers and vinculin localization at focal adhesions in HTMC cotreated with ET-1 and 30 or 45 μM LBN for 1 hour (Fig. 5). In contrast, there was minimal effect of latanoprost on the ET-1–induced actin stress fibers and vinculin under identical conditions (Fig. 5).

Latanoprostene Bunod, SE 175, and Latanoprost Reduce ET-1–Induced Electrical Cell Substrate Resistance

Previous work has shown that induction of cell contraction and/or increased cell adhesions with agents such as thrombin or ET-1 correlates with an increase in HTMC resistance. On the other hand, agents that decrease cell contractility elicit a decrease in HTMC resistance.[24] Therefore, the effects of LBN, SE 175, and latanoprost on ET-1–induced HTMC resistance were determined using ECIS as a quantitative measure of the effect of these agents on cell contractility. ET-1 caused a rapid increase in resistance at doses of 10 and 100 nM, with maximal increases observed after 60 minutes (Fig. 6A). HTMCs were then cotreated with 100 nM ET-1 plus the NO donor SE 175 (at 30, 45, 60, and 100 μM), and HTMC resistance was monitored for 2 hours. SE 175 had no significant effect on HTMC resistance at 30 and 45 μM but caused a significant decrease at 60 and 100 μM (Fig. 6B). The effect of SE 175 at the two lower doses in conjunction with latanoprost was determined. Latanoprost (45 μM) caused significant reduction in HTMC resistance, whereas SE 175 at 45 μM was again without significant effect. When combined, latanoprost (45 μM) and SE 175 (45 μM) resulted in significantly greater reduction of HTMC resistance than latanoprost alone, indicating a synergistic interaction (Fig. 6C). The effect of LBN on ET-1–induced HTMC contractility was then compared to that with latanoprost, both of which were administered at 45 μM (Fig. 6D). Although both of the compounds significantly decreased HTMC resistance, the effect of LBN was significantly greater than that of latanoprost at multiple time points, and the effect was evident over the 2-hour time course. After 1 hour of treatment, LBN inhibited ET-1–induced contractility to baseline (control) levels with an efficacy of 99%, whereas the efficacy of latanoprost at the same time point was 54%.

CONCLUSIONS

LBN is a new chemical entity which is metabolized to the NO-donating moiety BDMN and the FP receptor agonist latanoprost acid when applied topically to the ocular surface. LBN has been shown to lower IOP more effectively than latanoprost in nonclinical and clinical studies (Saeki T, et al. *IOVS* 2009;50:ARVO E-Abstract 4064 and Refs. 1, 2). In the present study, multiple complementary assays were performed to assess the action of LBN and the commercially available NO donor SE 175 in mediating HTMC relaxation in cells stimulated to contract by

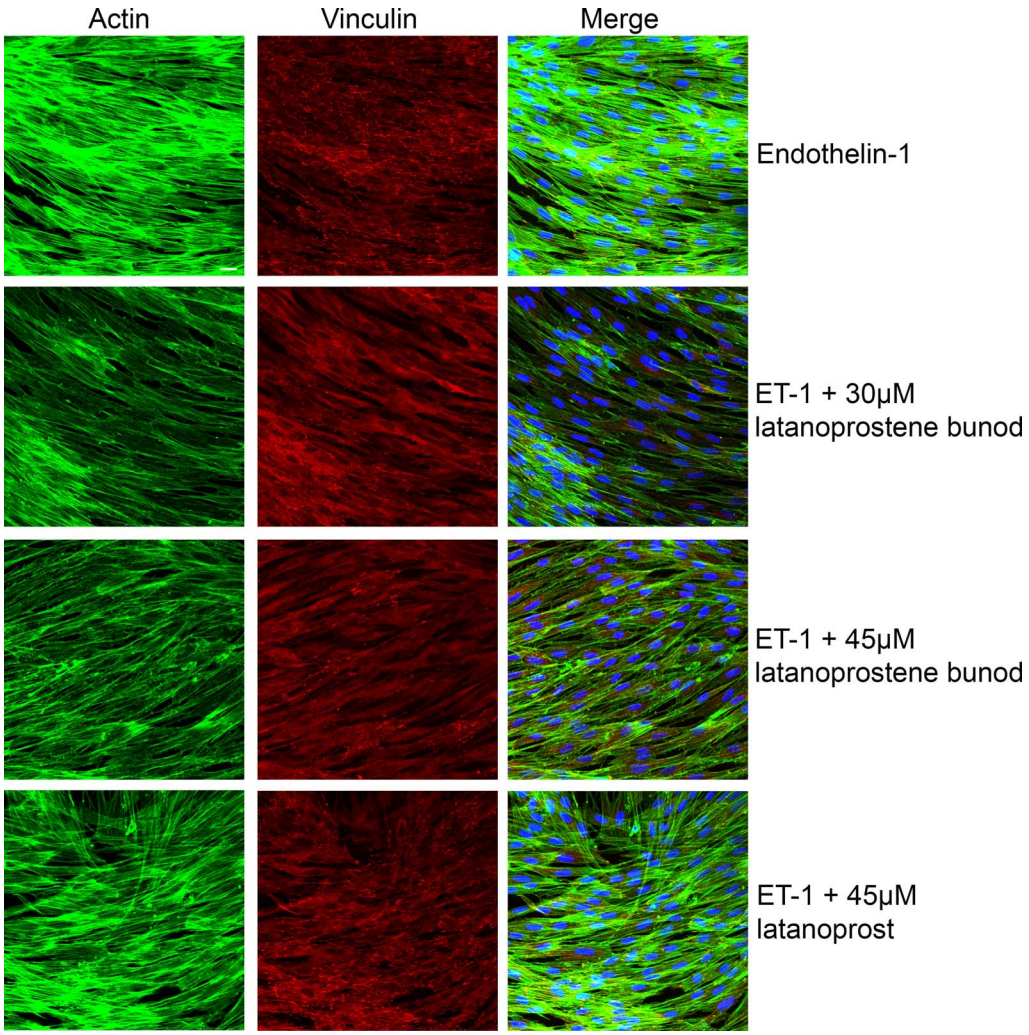

FIGURE 5. Latanoprostene bunod alters ET-1–induced actin stress fibers and vinculin localization in HTMCs. HTMCs were treated with ET-1 (100 nM) alone or in combination with LBN (30 or 45 μM), or latanoprost (45 μM) for 1 hour. Cells were fixed and stained for actin (*green* [using phalloidin]) and focal adhesions (*red* [using vinculin monoclonal antibody]). Nuclei were stained with DAPI (*blue*). Cells were then imaged by confocal microscopy at ×40. Treatment results with F-actin (*left*), vinculin (*middle*), and merged images (*right*) are shown. Images are representative of four individual experiments. ET-1–induced F-actin filaments and vinculin localization at focal adhesions (*top panels*) were reduced with 30 and 45 μM LBN (*middle panels*), whereas 45 μM latanoprost had a negligible effect (*lower panel*). *Scale bar*: 20 μM (all images).

using a pathogenic mediator of glaucoma, ET-1.[5,25] LBN and SE 175 dephosphorylated MLC-2, reversed ET-1–induced cytoskeletal contractility and focal adhesion formation, and mediated a reduction in HTMC resistance, indicating that these two NO donors mediate HTMC relaxation and a reduction in cell substratum interactions. The effects of LBN on the trabecular meshwork were compared directly with those of equimolar latanoprost, and in all parameters tested, LBN showed effects that were increased compared to those of latanoprost, providing a cellular mechanism for the ability of LBN to elicit greater IOP-lowering in the clinical setting.[2] In addition, the effect of LBN on cell relaxation was larger than that observed with the NO donor SE 175. The fact that the effect of LBN on HTMC relaxation was greater in magnitude than either SE 175 or latanoprost suggests a contribution of both the NO-donating moiety and the latanoprost acid moiety to the HTMC relaxation effect of LBN in vitro.

Dysfunction of the conventional outflow pathway is primarily responsible for ocular hypertension and primary open-angle glaucoma, although the causes of the pathogenic changes responsible for this dysfunction are not yet fully understood.[3] In the trabecular meshwork, cell shape, cytoskeletal contractility, and attachment to neighboring cells and extracellular matrix are keys to the regulation of aqueous humor outflow.[26] In glaucoma patients, ET-1 in the aqueous humor[4,27] and plasma[28,29] is increased compared to that in healthy subjects. Both the ET receptor A and the ET receptor B are expressed in trabecular meshwork cells,[30–32] and ET-1 mediates contraction of trabecular meshwork cells.[18,33,34] In agreement with current findings, previous in vitro studies in trabecular meshwork cells demonstrate that ET-1 induces reorganization of F-actin cytoskeleton and altered cell-to-extracellular matrix attachment.[35]

Previous studies have shown that NO-donating compounds target the conventional outflow pathway to mediate their IOP-lowering effects. Intracameral administration of SNP increased outflow facility in nonhuman primates[12] and rabbits,[36] whereas topical administration of nitroglycerin increased outflow facility in rabbits.[13] In cultured trabecular meshwork cells, the NO donor DETA-NO–induced a decrease in cell volume, indicating a reduction in cell contractility, which correlated with an increase

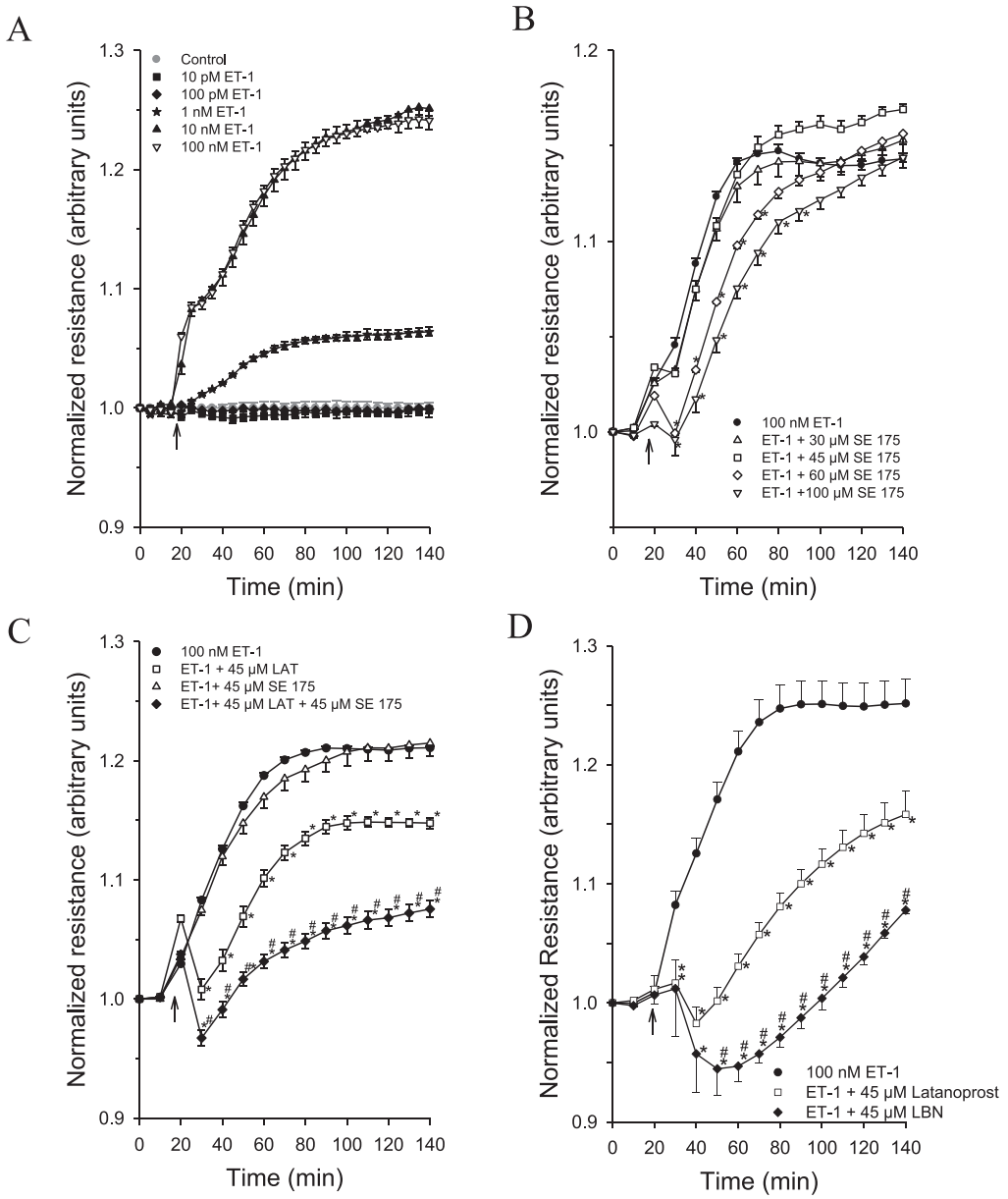

Figure 6. Effects of latanoprostene bunod, SE 175, and latanoprost on ET-1–induced HTMC resistance. HTMCs were grown to confluence and then incubated in basal growth medium plus 0.5% fetal bovine serum overnight. HTMCs were then placed in basal growth medium 2 hours prior to the experiment. HTMCs were equilibrated in the ECIS machine for 20 minutes to obtain a baseline reading. HTMCs were then treated with vehicle or (**A**) increasing concentrations of ET-1, or (**B**) 100 nM ET-1 plus increasing concentrations of SE 175, or (**C**) 100 nM ET-1 plus latanoprost and/or 45 μM SE 175 for 2 hours. (**D**) Effect of 100 nM ET-1 plus LBN or latanoprost at 45 μM for 2 hours is shown. HTMCs were monitored for change in resistance using a multiple scanning frequency mode every 10 minutes. Data at 4 kHz were analyzed because measured changes in resistance were maximal at this frequency. *Black arrow* indicates addition of ET-1 plus latanoprost/SE 175/LBN. Data are means ± SEM; $n = 3$ to 4 (**A–C**), and $n = 7$ (**D**). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus latanoprost at the same time point.

in outflow facility in perfused anterior segments.[16] Recently, the NO donors DETA-NO and SNP were demonstrated to decrease cell contraction in a cGMP-dependent manner.[18] Present data demonstrate that the NO donors SE 175 and LBN elicit cell relaxation after challenge with the contracting agent ET-1. Data presented here support the concept that NO donors and LBN in particular elicit IOP lowering via trabecular meshwork cytoskeletal relaxation and focal adhesion alterations, which increases the intercellular space between the cells and thus aqueous humor outflow in vivo.

Mechanistically, we demonstrated that LBN increased cGMP levels via soluble guanylate cyclase in HTMC. The efficacy and potency of LBN in elevating cGMP levels were similar to that previously reported in rat pheochromocytoma cells and human embryonic kidney cells.[1] Because latanoprost at equivalent doses showed negligible effect on cGMP, presumably this effect is mediated via the NO-donating moiety BDMN. Further studies are required to more fully investigate the downstream signaling elicited by the LBN-induced cGMP elevation. In vascular smooth muscle cells, activation of sGC by NO mediates cell relaxation via multiple mechanisms including BK channel activation with subsequent membrane hyperpolarization leading to L-type Ca channel blockade, lowering of intracellular calcium, and inhibition of the Rho kinase pathway.[14] Studies

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demonstrate that other NO donors mediate a reduction in HTMC volume and an increase in outflow by activation of BK channels in HTMC.[16] Therefore, these mechanisms are likely to be involved in the cell relaxation mediated by the NO-donating moiety of LBN and SE 175. S-nitrosylation is another possible mechanism mediating the effect of LBN and SE 175 on cell relaxation, S-nitrosylation is a reversible posttranslational modification in which an –NO group is attached to cysteine residues of target proteins[37] and has been shown to impact cell contraction/relaxation dynamics in other nonocular cell types. For example, in aortic smooth muscle cells, NO donors mediate a reduction in contractility by S-nitrosylation of protein kinase C, thus preventing protein kinase C-induced inhibition of MLC phosphatase.[38] The NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) elicits actin rearrangement in neuronal cells by a mechanism involving S-nitrosylation of F-actin.[39] Furthermore, vasodilation by NO is regulated by s-nitrosylation dependent mechanisms in addition to cGMP-dependent mechanisms depending on animal species and vessel type.[37]

The current consensus is that latanoprost acid lowers IOP by increasing outflow primarily via the uveoscleral route, through extracellular matrix remodeling.[7] However, nonclinical and clinical publications indicate a role for trabecular meshwork/Schlemm's canal (conventional) outflow. Latanoprost increases outflow facility in cultured anterior segments[40] and induces actin cytoskeleton rearrangement in HTMCs.[41] In addition, FP receptor agonists have been shown to inhibit ET-1–induced contractions of isolated bovine trabecular meshwork strips.[42] In humans, latanoprost significantly increases conventional outflow facility in multiple studies.[7] In the present study, latanoprost reduced both ET-1–induced MLC-2 phosphorylation and HTMC resistance, although to a lesser degree than LBN. Present data support those of previous studies that suggest that latanoprost may mediate a portion of its IOP-lowering effects by targeting the conventional outflow pathway in vivo. The underlying signaling that mediates the effect of latanoprost on HTMC relaxation requires investigation. FP receptor agonists have been shown to activate BK channels and inhibit L-type Ca channels in trabecular meshwork.[42,43] Activation of BK channels has been demonstrated to decrease trabecular meshwork and Schlemm's canal cell volume and thus to increase outflow facility.[16] On the other hand, FP receptor agonists activate their effector phospholipase Cβ, which via multiple signaling cascades can activate Rho kinase and the mitogen-activated protein kinase (MAPK) pathway, leading to cell contraction.[8,44]

We observed a synergistic effect of cotreatment with equimolar doses of the NO donor SE 175 and latanoprost in reduction of ET-1-induced HTMC resistance in vitro. This suggests that NO and latanoprost acid may affect multiple and unique targets which converge on the end point of cell relaxation. Another possibility is that NO can counter some of the effects of latanoprost acid which may actually increase cell contractility, such as increased inositol triphosphate signaling and increased MLC phosphorylation.[8,44] A synergistic effect of the two active moieties of LBN, NO, and latanoprost acid on conventional outflow in vivo could contribute to the additional IOP-lowering effects observed with this compound. Interestingly, in a transient ocular hypertensive rabbit model a synergistic effect of the NO donor SNP and latanoprost in lowering IOP was observed.[45] The authors hypothesized that both NO and latanoprost acid may act to increase endogenous PGE_2 generation leading to a greater IOP reduction because the effect was abolished in the presence of the cyclooxygenase inhibitor indomethacin.[45] In the present study, although the HTMC relaxation effect of a combination of SE 175 and latanoprost appears to be similar in magnitude to that of LBN, the in vitro model used does not address factors such as tissue penetration or stabilization of NO donation, which may impact IOP-lowering efficacy in vivo.

The most common type of glaucoma, primary open-angle glaucoma, is caused by increased resistance to aqueous outflow through the juxtacannalicular tissue, resulting in elevation of IOP, optic nerve damage, and visual field loss[3]; however, current therapies principally target aqueous humor production or the uveoscleral outflow pathway. In humans and in nonhuman primates, latanoprost primarily targets the uveoscleral pathway.[7,46] Both clinical and nonclinical studies have revealed that NO targets the conventional outflow pathway and also may decrease aqueous secretion.[10] The present study demonstrated that LBN increases HTMC cGMP signaling and mediates trabecular meshwork relaxation, suggesting that LBN may facilitate an increase in conventional outflow in vivo. The effect of LBN on trabecular meshwork relaxation was significantly greater than that of latanoprost, demonstrating the activity of the NO-donating moiety. These data support the concept that LBN has a dual-mode action in that it targets both aqueous outflow pathways in vivo to treat ocular hypertension in patients with glaucoma.

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The information presented on latanoprostene bunod concerns a use that has not been approved by the US Food and Drug Administration. The authors are currently or were employees of Bausch + Lomb, a division of Valeant Pharmaceuticals International, Inc., when this work was performed.

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