Inhibition of Cultured Human RPE Cell Proliferation and Lysyl Hydroxylase Activity by Hydroxy Derivatives of Minoxidil

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Purpose. To examine the antiproliferative and lysyl hydroxylase suppressing effects of 3'-hydroxyminoxidil and 4'-hydroxyminoxidil, derivatives of minoxidil devoid of the antihypertensive effect, on human retinal pigment epithelial (RPE) cells in culture.

Methods. Subconfluent and confluent cultures of RPE cells, exposed to 0.01 to 5 mM 3'- or 4'-hydroxyminoxidil for 15 minutes to 7 days, were examined for proliferation, viability, and morphologic changes. Lysyl hydroxylase activity in confluent cultures exposed to 1 mM 3'- or 4'-hydroxyminoxidil was determined by measuring the amount of $\text{H}_2\text{O}$ released from L-(4,5-$\text{H}$)lysine-labeled unhydroxylated procollagen substrate after vacuum distillation.

Results. Both compounds inhibited the proliferation of subconfluent cultures of RPE cells in a dose-dependent fashion. The 50% effect occurred at 0.25 mM for 3'-hydroxyminoxidil and 0.5 mM for 4'-hydroxyminoxidil. The antiproliferative effect was detectable within 24 hours, required a minimum 1-hour exposure, and persisted even after the drug was removed from the culture medium. Cell viability experiments provided no evidence for toxicity. In contrast, the number of cells at confluent density was not affected. Both 3'-hydroxyminoxidil and 4'-hydroxyminoxidil suppressed lysyl hydroxylase activity by 72%.

Conclusions. The structure of minoxidil can be altered to reduce the antihypertensive activity while preserving the antiproliferative and lysyl hydroxylase suppressing effects. The hydroxy derivatives of minoxidil may be useful in the treatment of proliferative vitreoretinopathy, a disease with unwanted proliferation of RPE cells. Invest Ophthalmol Vis Sci. 1994;35:463-469.

Minoxidil, a piperidinopyrimidine derivative, is a potent antihypertensive drug that has been claimed to reverse male pattern baldness. We have previously demonstrated that minoxidil inhibits the proliferation of human retinal pigment epithelial (RPE) cells in culture. Minoxidil also suppresses the activity of lysyl hydroxylase, an enzyme essential for the formation of stable intermolecular collagen cross-links. Because RPE cells are involved in the pathophysiology of proliferative vitreoretinopathy (PVR), a disease characterized by unwanted cell proliferation and fibrous tissue formation, we have investigated the possibility of treating PVR with minoxidil. However, the hypotensive activity of minoxidil would be an undesirable side effect in its use as an antiproliferative agent in normotensive patients.

It is thought that a prolonged period of treatment, either by repeated intravitreal injections or systemic administration of an antiproliferative drug, will be necessary to inhibit the development of scar tissue in PVR. Successful therapy is contingent upon the ability of the drug to act with minimum side effects. In the case of minoxidil, the anticipated dose (millimolar range) for effective treatment of PVR is higher than the suggested systemic dose for treating hypertension (micromolar range), prompting the need for intravitreal administration. Hence, we have been interested in structural analogs of minoxidil with increased antiproliferative potency and a reduced incidence of side effects compared to the parent compound.
lation at the 3' or 4' position of the piperidine ring of minoxidil eliminates its antihypertensive activity. If found effective, the hydroxy derivatives of minoxidil could minimize unwanted hypotension from systemic absorption or potentially be administered systemically to treat PVR. The purpose of this study was to test the ability of these compounds to inhibit the proliferation of human RPE cells in culture.

MATERIALS AND METHODS

Cell Culture

RPE cells were explanted and maintained using a modification of a previously described technique. Briefly, human eyes derived from donors within 24 hours of death were obtained from the Carolina Organ Procurement Agency, an affiliate of the North Carolina Eye and Human Tissue Bank. The anterior segment, vitreous, and retina were removed. Eye cups were incubated with 0.3% trypsin in Ca²⁺-Mg²⁺-free balanced salt solution containing ethylenediamine tetraacetic acid; the incubation was carried out for 1 hour at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were gently dislodged from Bruch's membrane, centrifuged, and resuspended in Eagle's minimal essential media (MEM; Lineberger Cancer Research Center, Chapel Hill, NC) with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). The purity of RPE cultures was verified by immunohistochemical staining for cytokeratins as previously described. Medium was changed twice per week. All cells were grown to confluence in 75 cm² flasks (Costar Corp., Cambridge, MA). Establishing the effect of the hydroxy derivatives of minoxidil on confluent RPE cells would provide information on its potential toxicity to the pigment epithelium in vivo. Basic fibroblast growth factor (bFGF) induces a uniform and polygonal morphology on confluent cultured RPE cells, and therefore closely resembles the RPE cell monolayer in vivo than cells not treated with bFGF. In preliminary experiments, subconfluent cultures of RPE cells, seeded in quadruplicate wells at 40,000/well, were treated with 1 to 5 mM 3'-hydroxyminoxidil and 4'-hydroxyminoxidil (Upjohn, Kalamazoo, MI), with or without 1 ng/ml bFGF (Sigma Chemical, St. Louis, MO), for 7 days; bFGF supplementation did not alter the antiproliferative effect of these derivatives. To maintain identical experimental conditions, bFGF (1 ng/ml) was added directly, without a medium change, to subconfluent and confluent cultures every other day during proliferation assays. Only first through fifth passage cells were used.

Cell Proliferation

3'-Hydroxyminoxidil and 4'-hydroxyminoxidil (Upjohn) were dissolved in MEM + 10% FBS, and diluted to the proper concentration. To test the effect of these compounds on subconfluent cultures, RPE cells were seeded in quadruplicate on 24-well plates (Corning Glass Works, Corning, NY) at 40,000 cells/well. Twenty-four hours later, approximately 36,000 cells/well had attached to the wells. These cells were treated with a single dose of 3'- or 4'-hydroxyminoxidil (0.01 to 5 mM) in MEM + 10% FBS. Control cells were given MEM + 10% FBS without drug. To test the effect of these compounds on confluent cultures, RPE cells were grown until visual confluence, counted twice per week, and used in experiments when cell number no longer increased (approximately 3 weeks in culture). The duration of treatment for subconfluent and confluent cultures ranged from 15 minutes to 7 days. After the exposure period, all cells were rinsed twice with phosphate-buffered saline (PBS) to avoid drug carryover effects, and incubated in fresh MEM + 10% FBS until day 7. To determine whether the effect of the minoxidil derivatives was reversible, subconfluent and confluent cultures were treated with 3'- or 4'-hydroxyminoxidil (1 to 5 mM) for 7 days, washed twice with PBS, and incubated for an additional 7 days in fresh MEM + 10% FBS devoid of the drug. At the end of the incubation period, all cells were rinsed with PBS, trypsinized, and counted with an automated counter (Coulter Electronics, Hialeah, FL). Cell viability was determined by trypan-blue exclusion. Cell proliferation was evaluated by counting cells 7 days after replating. All cells were examined morphologically by phase-contrast microscopy. Prior to drug treatment, cells in the center of the wells had a more uniform appearance than cells in the periphery. Thus, drug-induced morphologic changes were more easily identified in cells from the center of the well. Accordingly, cells from this region were used for morphologic analysis. All experiments were repeated at least once with at least two different cell lines.

Prolyl and Lysyl Hydroxylase Assays

Enzyme studies were conducted with confluent cultures of RPE cells. The cells were grown on 60 × 15 mm dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C in a humidified atmosphere of 5% CO₂ in air. Minoxidil (Upjohn), 3'- or 4'-hydroxyminoxidil (1 mM) was added to the medium for 72 hours. Sodium ascorbate (100 μM) was added to cultures during the last 3 hours of treatment. Cells were detached by trypsinization, centrifuged at 1000 rpm for 5 minutes at 25°C, and washed twice with PBS, followed by centrifugation. The cell pellet was suspended in medium (5 × 10⁶ cells/1 ml) containing 0.2 M NaCl, 0.1 M glycine, 50 μM dithiothreitol, 20 mM Tris-HCl buffer, pH 7.5, 0.1% (w/v) Triton X-100, and 0.01% soybean trypsin inhibitor. The cell suspension was sonicated for 30 seconds. The cell lysate was centrifuged at 27,000g for 1
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hour at 4°C. The resulting supernatant was assayed for enzyme activities by a previously described technique, using L-(4,5-3H)lysine-labeled unhydroxylated procollagen substrate for lysyl hydroxylase and L-(4-3H)proline-labeled unhydroxylated procollagen substrate for prolyl hydroxylase. The amount of tritium released was quantitated as 3H2O after vacuum distillation. Protein was determined by a dye-binding technique using Bio-Rad (Richmond, CA) reagent and bovine gamma globulin standard. Experiments were repeated once. Enzyme activities are expressed as the average cpm/mg soluble cell protein.

Statistical Analysis

A two-tailed, nonpaired, Student’s t-test was used to compare differences in cell number between experimental and control groups. The dose, which gave a 50% antiproliferative effect, was determined from a natural log plot of cell number versus concentration.

RESULTS

Effect of Hydroxy Derivatives of Minoxidil on Cell Proliferation

A single 7-day treatment with hydroxy derivatives of minoxidil (0.1 to 5 mM) resulted in a dose-dependent reduction in the number of RPE cells in subconfluent cultures (P < 0.001 for all doses tested). The concentration required for 50% antiproliferative effect of a single 7-day treatment was 0.25 mM for 3'-hydroxyminoxidil and 0.5 mM for 4'-hydroxyminoxidil. In contrast, a similar treatment of confluent cultures had no effect on cell number (Fig. 1).

Under these conditions, hydroxy derivatives of minoxidil had minimal effect on cell viability, measured by trypan-blue exclusion. The viability of subconfluent cultures was 85% for control, and 87% to 90% each for 3'- and 4'-hydroxyminoxidil-treated groups. Similarly, confluent RPE cells exposed to hydroxy derivatives of minoxidil for 7 days had the same viability as untreated controls (85% to 90%).

Detectable antiproliferative effect occurred within 24 hours after addition of 5 mM (P < 0.001) and 48 hours after addition of 1 mM (P < 0.003) hydroxy derivatives of minoxidil. The difference between the number of treated and control cells became more obvious with time. Results of a representative experiment with 3'-hydroxyminoxidil are presented in Figure 2. Similar results were obtained with 4'-hydroxyminoxidil (data not shown).

To determine the duration of treatment needed to inhibit cell proliferation, subconfluent cultures were treated with each hydroxy derivative of minoxidil (1 and 5 mM) for intervals ranging from 15 minutes to 7 days and were counted on day 7. The magnitude of inhibition was dependent upon the duration of treatment (Fig. 3). The shortest period to achieve significant antiproliferative effect was 1 hour, resulting in a 57% inhibition (P = 0.005) at 1 mM and 63% inhibition (P = 0.003) at 5 mM 4'-hydroxyminoxidil. For the

FIGURE 1. A representative experiment showing the relative number of subconfluent (closed symbols) and confluent (open symbols) human RPE cells after a 7-day treatment with various concentrations of 3'-hydroxyminoxidil (circle) and 4'-hydroxyminoxidil (square). The initial number per well was 36,000 for subconfluent cells and 420,000 for confluent cells. Mean values for four replicate wells are shown. Standard deviations were less than 10%.

FIGURE 2. A representative experiment demonstrating the number of subconfluent RPE cells remaining at various time intervals after treatment with 0 mM (open square), 1 mM (closed square), and 5 mM (closed circle) 3'-hydroxyminoxidil. Results are presented as the mean (±SD) for four replicate wells.

FIGURE 3. A representative experiment showing the relative number of subconfluent (closed symbols) and confluent (open symbols) human RPE cells after a 7-day treatment with various concentrations of 3'-hydroxyminoxidil (circle) and 4'-hydroxyminoxidil (square). The initial number per well was 36,000 for subconfluent cells and 420,000 for confluent cells. Mean values for four replicate wells are shown. Standard deviations were less than 10%.
FIGURE 3. Relative number of subconfluent RPE cells remaining after treatment with 1 mM (open circle) and 5 mM (open square) 3'-hydroxyminoxidil and 1 mM (closed circle) and 5 mM (closed square) 4'-hydroxyminoxidil for various time intervals. Cells were seeded at 40,000/well and were counted after 7 days. Standard deviations were less than 10%.

same duration, treatment with 3'-hydroxyminoxidil resulted in a 22% inhibition (P = 0.0039) at 1 mM and 23% inhibition (P = 0.0076) at 5 mM.

The antiproliferative effect of these hydroxy derivatives of minoxidil continued in a dose-dependent manner even after the drug was removed from the culture medium. When cells were treated for 7 days with 1 to 5 mM 3'-hydroxyminoxidil, washed twice in PBS, and given fresh medium devoid of 3'-hydroxyminoxidil, the cell number failed to normalize after a 1-week recovery period. The viability of cells under these conditions was identical to controls (85% to 90%). Similar results were obtained with 4'-hydroxyminoxidil (data not shown). Results of a typical experiment are shown in Figure 4.

An ideal antiproliferative drug, though inhibiting cell division, would not damage nonproliferating cells. In this regard, the data, which show that the number and viability of confluent cells were not altered by drug treatment, are reassuring. However, in RPE cells, cell counting could underestimate cell viability because these cells are adherent and could remain attached to the wells even after cell death. In addition, trypan blue testing may overestimate viability because cells could exclude trypan blue but be sufficiently damaged to lose their proliferative ability. Conversely, viable, nondividing cells treated with antiproliferative agents should retain their proliferative potential once the antiproliferative agent is removed. Therefore, to assess further the health of confluent, nonproliferating cells after drug treatment, additional experiments were conducted. Cultures of confluent, nonproliferating cells were treated with hydroxyminoxidil derivatives for 7 days, washed twice with PBS, trypsinized, and replated in drug-free medium on 24-well plates at a density of 40,000 cells/well. Seven days later, the average number of cells—431,000 ± 35,000 SD, 440,000 ± 28,000 SD, and 434,000 ± 51,000 SD cells/well for cells pretreated with 3, 4, and 5 mM 4'-hydroxyminoxidil, respectively—did not differ from untreated controls (413,000 ± 21,000). Similar results were obtained with 3'-hydroxyminoxidil (data not shown). Thus, confluent, nondividing cells treated with hydroxyminoxidil derivatives retain their proliferative potential, further attesting to the health of these cells.

Effect of Hydroxy Derivatives of Minoxidil on Lysyl Hydroxylase Activity

The level of lysyl hydroxylase activity in RPE cells was reduced 72% after a 72-hour treatment of RPE cells with 1 mM 3'- or 4'-hydroxyminoxidil. In contrast, the level of prolyl hydroxylase activity was reduced only 13% by 3'-hydroxyminoxidil and 6% by 4'-hydroxyminoxidil. A similar treatment with 1 mM minoxidil reduced the level of lysyl hydroxylase activity by 71% and of prolyl hydroxylase activity by 4%.

Effect of Hydroxy Derivatives of Minoxidil on Cell Morphology

Dose-dependent morphologic changes were observed in RPE cells treated with the hydroxy derivatives of minoxidil. Immediately after seeding, untreated sub-
confluent RPE cells had spindle shapes with few cell-cell contacts in the center of the well. After 7 days of growth, untreated subconfluent RPE cells maintained a cuboidal morphology in the central zone with extensive cell-cell contact (Fig. 5A). In the peripheral zone, they were less cuboidal and had fewer cell-cell contacts. Subconfluent RPE cells treated with 1 to 5 mM of hydroxy derivatives of minoxidil for 1 to 24 hours appeared normal, but mild enlargement of the cell bodies developed in those treated for 7 days (Figs. 5B, 5C). The morphologic changes on subconfluent cells induced by 3'-hydroxyminoxidil and 4'-hydroxyminoxidil were identical. Untreated confluent RPE cells were smaller than subconfluent cells and were densely packed over the entire well. Treatment with the hydroxy derivatives of minoxidil did not cause a change in morphology of these cells.

DISCUSSION

Hydroxylation at the 3'- or 4'-position of the piperidine ring in minoxidil eliminates its antihypertensive activity. In the present study, 3'-hydroxyminoxidil and 4'-hydroxyminoxidil selectively inhibited the proliferation of subconfluent, but not confluent RPE cells in culture. These results indicate that hydroxylation of minoxidil does not interfere with its antiproliferative activity. In fact, the potency of these hydroxy derivatives of minoxidil may be enhanced compared to the parent compound. The concentration to produce a 50% antiproliferative effect was 0.25 mM for 3'-hydroxyminoxidil and 0.5 mM for 4'-hydroxyminoxidil, in contrast to 1.5 mM for minoxidil reported previously. With the reservation of extrapolating the in vitro studies to an in vivo situation, it can be predicted from these data that the hydroxy derivatives of minoxidil will be more effective than minoxidil in the treatment of PVR.

These hydroxy derivatives of minoxidil appear to act in a manner that is similar to minoxidil. As with minoxidil, the antiproliferative effect persisted after removing the drugs from the medium. Subconfluent cultures did not achieve the same density by the end of the recovery period, an effect that was related to the concentration of the inhibitor during pretreatment. Although the cell viability, measured by trypan-blue exclusion, remained normal under these conditions, it is possible that cells were sufficiently injured by the drug to prevent recovery. Alternatively, the reduced ability of hydroxyminoxidil-treated cells to recover from inhibition of proliferation could result from a subpopulation of cells that are rendered permanently incapable of dividing.

Again as with minoxidil, its hydroxy derivatives inhibited cell proliferation after a short exposure period but at a concentration lower than the 5 mM reported for minoxidil. With a 1-hour exposure, cell proliferation was inhibited 22% by 1 mM 3'-hydroxyminoxidil and 53% by 1 mM 4'-hydroxyminoxidil. The reason for the enhanced antiproliferative effect of the 4'-hydroxy derivative of minoxidil, compared to its 3'-hydroxy counterpart, is unknown. This finding is unexpected because the two compounds are indistinguishable with respect to the dose response, the time required for...
measurable antiproliferative effect, the continued antiproliferative effect after drug removal, the morphologic changes, and the cell viability measurements. In addition, the two compounds, because of similar solubility properties in aqueous medium, might be expected to internalize into cells at comparable rates. Regardless, the short duration of treatment would allow the drug to exert an antiproliferative effect before it is cleared from the pathologic, target tissue site.

The results of our studies do not support the possibility that the antiproliferative effect of the hydroxy derivatives of minoxidil is an indication of cytotoxicity. First, these compounds had no effect on the number of cells at confluent density. In contrast, a toxic drug would be expected to kill both proliferating and nonproliferating cells. Second, these derivatives induced morphologic changes on subconfluent cells that are similar to, though less severe, than the changes seen in several cell types after minoxidil treatment. How- ever, these drugs did not induce a morphologic alteration in cells at confluent density. Third, the viability of treated cells, measured by trypan-blue exclusion, was similar to controls. Finally, as a further test of viability, confluent cultures that had been pretreated with 3'-' or 4'-hydroxyminoxidil for 7 days, then trypsinized, replated, and grown for 7 days in medium devoid of the drug, proliferated similarly to untreated controls. Under normal conditions, unlike PVR, cellular activity is at a minimum in the eye. The goal of treatment in PVR is to eliminate proliferating pathologic cells and to leave nonproliferating normal cells intact. The ability of hydroxy derivatives of minoxidil to inhibit proliferation of RPE cells without killing nonproliferating ones could reduce the likelihood of toxicity in the treatment of PVR. Toxicity studies will ultimately determine potential harmful effects.

Lysyl hydroxylase, a post-translational modifying enzyme involved in collagen biosynthesis, catalyzes the synthesis of hydroxyllysyl residues that are essential for the formation of intermolecular cross-links in collagen. These cross-links stabilize the fibrillar structure of collagen and thereby increase the tensile strength of tissue. Prolyl hydroxylase catalyzes a mechanistically similar reaction resulting in the synthesis of hydroxyproline, which stabilizes the triple helical structure of collagen. In cultured human skin fibroblasts, minoxidil has been found to inhibit the synthesis of lysyl hydroxylase at a pretranslational level without affecting the expression of prolyl hydroxylase activity. A similar inhibitory effect of minoxidil on lysyl hydroxylase has been observed in cultured human RPE cells. In the present study with RPE cells, as with skin fibroblasts, the 3'-' or 4'-hydroxy derivatives of minoxidil were as effective as the parent compound in selectively suppressing lysyl hydroxylase activity. As with minoxidil, these hydroxy derivatives reduced the level of lysyl hydroxylase mRNA in skin fibroblast cultures. A diminished lysyl hydroxylase activity could produce unstable, and therefore easily degradable, collagen fibers and scar tissue with poor tensile strength, thus preventing the development of traction retinal detachment in PVR.

The evidence indicates that hydroxylation does not adversely alter the antiproliferative or lysyl hydroxylase suppressing effect of minoxidil. On the contrary, this structural alteration enhances the antiproliferative potency and reduces the cytotoxicity of minoxidil, as measured by trypan-blue exclusion and proliferation after reattachment. In addition, the structural change improved the effectiveness of minoxidil over a short exposure period and decreased the half-maximal antiproliferative dose compared to minoxidil. The antiproliferative effect of minoxidil appears to involve the conversion of minoxidil to minoxidil sulfate, the active metabolite, by minoxidil sulfotransferase. In preliminary studies, we demonstrated that minoxidil derivatives, which are better substrates for this enzyme, had more potent antiproliferative effects than the parent compound. Additionally, we showed that proliferating RPE cells, compared to confluent ones, convert more minoxidil to minoxidil sulfate by the sulfotransferase, which may explain the selectivity of minoxidil for proliferating cells. We speculate that the hydroxy derivatives are a better substrate for minoxidil sulfotransferase than minoxidil and, hence, are a more potent antiproliferative drug. However, the relatively high concentrations of minoxidil and its hydroxy derivatives needed to inhibit cell proliferation is a potential problem. Fluid retention and pulmonary hypertension have been reported with systemic administration of minoxidil. It is unknown if these effects occur with the hydroxy derivatives. Similarly, the effect of lysyl hydroxylase suppression from systemic administration or systemic absorption after intraocular injection is unknown. The safety of minoxidil or the hydroxy derivatives in the treatment of PVR is unknown. The selectivity of these compounds for proliferating cells could increase the therapeutic interval without compromising safety. The lack of hypertensive activity of the 3'-' and 4'-hydroxy derivatives of minoxidil would be an added benefit in their use for treating PVR. Testing these drugs with intravitreal or systemic administration, or both, in an animal model of PVR is necessary to determine the true efficacy and safety. The present study also provides an incentive to modify further the structure of minoxidil to increase its potency and safety.

**Key Words**

retinal pigment epithelial cells, minoxidil, hydroxy derivatives of minoxidil, lysyl hydroxylase, antiproliferative effect
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References


