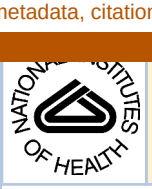

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Dexamethasone Increases Pigment Epithelium-Derived Factor in Perfused Human Eyes

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Abstract

Purpose—To investigate the effects of dexamethasone (DEX) on pigment epithelium-derived factor (PEDF) cDNA and secreted protein in human trabecular meshwork (TM).

Methods—Anterior segment organ cultures were perfused with 0.1 μM DEX (OD) and vehicle (OS). Primary human TM cells (HTM) were treated with DEX under similar conditions. PEDF mRNA and secreted PEDF protein were quantitated by RT-PCR and Western blot.

Results—PEDF mRNA and secreted PEDF protein levels were significantly higher in DEX over vehicle-treated cultures. In contrast, DEX decreased the activity of a 92-kDa gelatinolytic zymogen in organ culture effluents.

Conclusion—DEX action in the human TM might include a PEDF-mediating pathway.

Keywords

DEX; human; PEDF; perfused anterior segment; trabecular meshwork

INTRODUCTION

Glaucoma is a chronic eye disease that leads to irreversible blindness. It affects more than 70 million people worldwide, and it is most common among African Americans. Elevated intraocular pressure (IOP) is the major risk factor for the development of glaucoma.[1] This eye pressure is created by the resistance to aqueous humor flow exerted by the trabecular meshwork (TM), a tissue located at the anterior chamber angle formed by the iris and the cornea. A dysfunctional TM leads to an increase in resistance, which in turn translates into elevated IOP.

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The cells of the TM are endothelial-like, embedded in extracellular matrix (ECM), and arranged in a spongiform-like architecture.

Glucocorticoids are potent immunosuppressants and anti-inflammatory agents. Although dexamethasone (DEX) is frequently used to treat inflammation, its administration can adversely cause IOP elevation and glaucoma.[2] One mechanism by which DEX induces elevated IOP includes an increase in ECM deposition in the TM,[3,4] which would in part cause increased resistance to aqueous humor outflow. However, not all molecular mediators for this process have been elucidated. Interestingly, tissue differential microarray analysis of DEX induction of TM cells has revealed a number of candidate genes not currently associated with DEX and/or glaucoma.[5,6] One of these genes is encoded pigment epithelium-derived factor (PEDF).[5] PEDF is a potent antiangiogenic, neurotrophic, and antitumorigenic factor. This secreted glycoprotein of 50,000 Mr is found in the interphotoreceptor matrix and vitreous and aqueous humor of mammalian eyes.[7] It has binding affinity for ECM components, such as collagens and glycosaminoglycans.[7] PEDF acts on cells from the neural retina as well as on endothelial cells.[8] Its biological activities are associated with receptor interactions at cell surface interfaces and changes in protein expression.[8] However, little is known of the PEDF expression, distribution, and regulation in the TM.

Given that gene microarray analysis listed PEDF as a specifically DEX-induced gene in HTM cells in culture, it was of interest to examine in detail the regulation of PEDF in the human TM. Utilizing a perfused anterior segment model from post-mortem human donors[9] and primary human HTM cells, we investigated the PEDF mRNA and extracellular PEDF protein levels upon challenging both the organ and cell cultures with DEX.

MATERIALS AND METHODS

Perfusion of Human Anterior Segment Organ Culture

Eyes from non-glaucomatous donors were obtained from eye banks within 36 to 40 hr post-mortem following consent of the patients' families. Whole globes were dissected at the equator and mounted on perfusion chambers, as described previously.[9] Perfusion was conducted with serum-free high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA). DEX (Sigma, St. Louis, MO, USA, or MP Biomedicals, Solon, OH, USA) was prepared in absolute ethanol at 0.1 mM and diluted 1000× into fresh DMEM every other day. Pressure was continuously monitored with a pressure transducer connected to the dish cannula and recorded with a computerized system. Effluents were collected from the chambers just before the treatment with DEX and at different post-treatment times, and saved at -80°C for protein analyses. At the end of each experiment, a small wedge was fixed for immunohistochemistry, while the remaining anterior segments were immersed in RNAlater (Ambion, Austin, TX, USA) for subsequent TM dissection and RNA extraction.

RNA Extraction, Reverse Transcription (RT), and cDNA Quantification

TM tissue or cellular pellets were disrupted in RLT buffer and loaded onto a QIAshredder column (Qiagen, Valencia, CA, USA). RNA extraction continued using the RNeasy Kit with on-column, RNAse-free DNase digestion, according to manufacturer's recommendations (Qiagen).

For the relative quantitative PCR, RT reactions were performed using random primers (RETROscript, Ambion).[10] Determination of the exponential PCR cycle was conducted with 2.5 μl RT, 0.5 μl SuperTaq polymerase (Ambion) and intron spanning PEDF primers[5] (94°C, 4 min; and cycles of 94°C, 30 sec; 60°C, 30 sec; 72°C, 1 min; final extension 72°C, 4 min). Determination of internal 18S cDNA levels was conducted using classic primer-competimers

(Quantum RNA, Ambion) at the predetermined exponential cycle.[10] Multiplex PEDF and 18S amplifications were performed in triplicate at the predetermined linear cycle for all comparing conditions and electrophoresed on Tris-borate 1.5% SuperAcryl Agarose (DNA Technologies, Gaithersburg, MD, USA) containing 25 ng/ml ethidium bromide. The integrated optical densities of the PEDF and 18S bands (811 bp and 489 bp, respectively) were captured using the ChemiDoc System and LabWorks Software 4.0 (UVP, Upland, CA, USA).

Quantification of the differential expression of PEDF between treated and untreated samples was accomplished by first normalizing each PEDF and MYOC value to their internal 18S rRNA.

For the real-time *Taq*man PCR, RT reactions were conducted with 1 μg of total RNA in a total volume of 25 μl of proprietary RT buffer containing random primers, dNTPs, and 62.5 U of Multiscribe MuLV RT enzyme with RNAse inhibitor (High Capacity cDNA Kit, Applied Biosystems, Foster City, CA, USA) following manufacturer's recommendations (25°C for 10 min, 37°C for 2 hr). Fluorescently labeled *Taq*Man probe/primer sets for PEDF and MYOC genes were purchased from the Applied Biosystems *Taq*Man Gene Expression collection. The PEDF probe corresponded to sequences from exons 6 and 7 (Hs01106937 m1), the MYOC probe corresponded to sequences from exons 2 and 3 (Hs00165345 m1), and the 18S RNA probe corresponded to sequences surrounding position nucleotide 609 (Hs99999901 s1). Reactions were performed in 20-μl aliquots using *Taq*Man Universal PCR Master Mix on AmpErase UNG (Applied Biosystems), run on an Applied Biosystems 7500 Real-Time PCR System, and analyzed by 7500 System SDS software (Applied Biosystems). Fold-change values between treated and untreated samples were calculated by the formula $2^{-\Delta\Delta C_T}$, where C_T is the cycle at threshold (automatic measurement), ΔC_T is C_T of the assayed gene minus C_T of the endogenous control (18S), and $\Delta\Delta C_T$ is the ΔC_T of the normalized assayed gene in the treated sample minus the ΔC_T of the same gene in the untreated one (calibrator). Because of the high abundance of the 18S rRNA used as the endogenous control, and in order to get a linear amplification, RT reactions from control and experimental samples were diluted 10^4 times prior to their hybridization of the 18S *Taq* Man probe.

Immunohistochemistry

Wedge-shaped specimens containing the TM were fixed in 4% paraformaldehyde in 0.1 M PB, pH 7.4, for 48 hr at 4°C, embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA) and frozen at -80°C. 10-μm meridional sections were mounted on Superfrost/Plus microscope slides (Thermo Fisher, Waltham, MA, USA). Buffer for all incubation steps was 0.5% BSA, 0.2% Tween-20, 0.05% sodium azide in PBS. Blocking with 5% goat serum for 0.5 hr was followed by overnight incubation with monoclonal anti-human PEDF antibody (Chemicon, Temecula, CA, USA) (1:200). Secondary anti-mouse Cy5-conjugated (Jackson Immunoresearch, West Grove, PA, USA) was used at 1:400. Images were captured with a Leica SP2 laser scanning confocal microscope (Exton, PA, USA).

Protein Extraction

Proteins from serum-free effluents of perfused eye anterior segments were concentrated on a Centricon-30 (Millipore, Billerica, MA, USA). PEDF from serum containing cell culture medium was separated from most serum proteins by cation-exchange column chromatography[11] with SP Sepharose column (Amersham Biosciences, Piscataway, MA, USA) and 20 mM $NaHPO_4$ (pH 6.4), 1 mM DTT, 10% glycerol, and 50 mM NaCl as equilibration buffer. PEDF was eluted with 500 mM NaCl in equilibration buffer. Purified samples were used immediately or stored at -80°C.

SDS-PAGE, Western Blot Analysis and Quantification of PEDF Protein

Protein concentrations were determined using Protein Assay solution (BioRad, Hercules, CA, USA). Equivalent amounts of protein from control or treated conditions were loaded into each lane and resolved on 10-20% SDS-PAGE (Invitrogen). Western blot analysis was performed according to described methods.[11] Gel proteins were electrotransferred onto Protran membranes (0.2 μm, Schleicher & Schuell Bioscience, Keene, NH, USA). Blots were incubated with monoclonal human PEDF antibody (Chemicon) (1:1000) followed by incubation with biotinylated secondary goat anti-mouse IgG (Vector Laboratories, Burlingame, CA,USA) (1:1000). PEDF immunoreactive bands were visualized with an ABC Vectastain kit (Vector Laboratories) and HRP colorimetric substrate. The density of PEDF immunoreactive bands was measured with Scion Image software (Scion Corporation, Frederick, MD, USA). Additionally, levels of PEDF were determined by ELISA using the microtiter plates, standards, and antibodies provided in the PEDF kit (Bioproducts MD LLC, Middletown, MD, USA) and following the recommendations of the manufacturer. Relative amounts of PEDF protein were expressed as fold-change of the DEX-treated over control-treated samples.

Cell Culture of Primary Human TM (HTM) Cells

Post-mortem human eyes were obtained as described for the organ cultures. For isolation of HTM cells, the TMs from a single individual were dissected out from surrounding tissue, digested with collagenase type IV (Worthington, Lakewood, NJ, USA), and processed as previously described.[5] Cells were maintained in Improved Minimal Essential Medium (IMEM, Biofluids, Rockville, MD, USA), 10% FBS, and 50 μg/ml gentamicin (Invitrogen). These primary cells subsist for 9-10 passages, and experiments in this study were carried out with passages 4-6. The two primary cell lines used in this study originated from a 17-year-old African-American male and from a 2-year-old Caucasian female (HTM-46 and HTM-95, respectively).

Treatments with DEX were conducted two days postconfluent HTM cells in complete IMEM at final concentrations of 0.1 μM. DEX was prepared as above, diluted into fresh complete IMEM and added to the cells every other day. Control dishes received fresh medium containing drug vehicle. At designated time points, cells and supernatants were collected and analyzed for PEDF mRNA and protein.

Gelatinolytic Assays

Gelatinolytic activities were assayed by two methods: zymography using gelatin as substrate in polyacrylamide gels, and solution gelatinolytic activity assays using DQ-gelatin as substrate, both as described previously.[12] The reaction buffer for the solution assays was 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$.

RESULTS

DEX-Induced PEDF Gene Expression in Perfused Human Anterior Eye Segments

The effects of DEX on PEDF cDNA levels were investigated on perfused intact human TM tissue. Anterior segments from three Caucasian donors (between 56 and 78 years old) were perfused at constant flow (Ind #1, #2, and #3). The baseline outflow facility for the eyes of the three pairs averaged 0.43 ± 0.09 (n = 6), and the facility values for each eye at the times studied are included in Table 1. To avoid expression variations due to different genetic backgrounds between individuals, treatments were performed with paired eyes. One eye of each pair was perfused with 0.1 μM DEX, while its contralateral was perfused with vehicle (control). Choice of the DEX dose was based on the calculated clinical relevant dose and time course.[13]

Results from two individuals (Ind #1 and #2) showed that, after 7-8 days exposure to DEX, the average normalized PEDF cDNA levels were 6.5 ± 0.96-fold over the vehicle-treated control (Ind #1, $n = 3$, $p \leq 0.0029$) and 5.2 ± 0.27 (Ind #2, $n = 3$, $p \leq 0.007$) (Fig. 1A). The effect of DEX on PEDF cDNA levels was similar between the two individuals. In contrast to PEDF, the response of the glaucoma-linked and TM DEX-induced marker, the myocilin gene (MYOC) varied between these two individuals. It showed an induction of 1.9 ± 0.37-fold ($n = 3$, $p \leq 0.12$) in the first individual and 10.4 ± 0.75-fold ($n = 3$, $p \leq 0.02$) in the second one (Fig. 1B). This variation has been reported before for MYOC mRNA levels from different donors.[9]

PEDF Distribution in Human Trabecular Meshwork

In order to visualize the localization of the PEDF protein, wedges from the TM tissues from the above organ cultures were used for confocal evaluation. Immunohistochemistry showed localization of PEDF immunoreactivity in the human TM from perfused segments. In addition, a positive correlation between DEX treatment and PEDF immunoreactivity in the TM region was observed (Fig. 1C). DEX-perfused tissue reflected a marked increase in PEDF immunofluorescence compared to control-perfused tissues. Serial sections immunoreacted to secondary antibody only were negative. Our results also show that PEDF appears to be distributed throughout the human TM tissue, and that its induction is not limited to any particular TM region.

DEX Specifically Increases PEDF Protein in Effluents from Anterior Segments

To determine whether the DEX-induced PEDF mRNA expression was reflected on the translated product, PEDF protein was analyzed in the media flowing through the perfused anterior segments (effluents). Immunoblotting detected a PEDF protein of 50-kDa (Fig. 2A). Semi-quantitative analysis revealed that the relative PEDF protein levels increased significantly with time in effluents of DEX-perfused anterior segments over respective untreated controls (fold protein change). Averaged PEDF levels in the effluents of Ind #1 and #2 were 1.8 ± 0.07-, 2.2 ± 0.16-, and 6.5 ± 0.54-fold over controls at 4, 6, and 8 days of treatment, respectively (Fig. 2B). Protein determination assays revealed a decrease in total protein concentration over time, which was very similar in both control and DEX-treated effluents for two sets of individuals (Fig. 2C). To further confirm our results, we determined the induction of PEDF using a different technology. Although exposed to DEX for a shorter time period, we performed ELISA assays (in triplicate) on the effluents of a third individual. The effluents of the DEX-treated eye of Ind #3 showed changes in PEDF of 2.32 ± 0.27- and 3.70 ± 0.30-fold over the vehicle-treated contralateral eye at 4 and 6 days of treatment, respectively (not shown). Likewise, protein concentration determination assays showed a decrease in total protein concentration over time, which was very similar in both control and DEX-treated effluents to those observed in Ind #1 and #2 (not shown).

These results demonstrate that PEDF was secreted to the extracellular milieu of the TM and that its levels increased specifically and progressively over perfusion time with DEX.

Effects of DEX on PEDF cDNA in Primary HTM Cell Cultures

Total RNA from DEX-and vehicle-treated HTM cells was extracted and assayed for PEDF gene expression by semi-quantitative RT-PCR. On HTM-46, 1, 4, and 8 days after DEX exposure, normalized PEDF cDNA levels increased 1.5 ± 0.07-fold (n 6, $p \leq 0.02$), 3.0 ± 0.78-fold ($n = 5$, $p \leq 0.05$), and 1.9 ± 0.23-fold (n=6, $p \leq 0.01$), respectively (Fig. 3A and 3B).

In the same samples, the levels of MYOC cDNA also increased with time, as reported previously.[5] After 1, 4, and 8 days of DEX exposure, normalized MYOC cDNA was 2.3 ±

0.19-fold (n 6, $p \leq 0.005$), 4.3 ± 0.82-fold ($n = 5$, $p \leq 0.01$), and =6.9 ± 0.50-fold($n = 6$, $p \leq 0.01$), respectively (Fig. 3C and 3D).

On the HTM-95 cell line, derived from a different individual and analyzed using different technology (*Taq*Man PCR), the results had a similar trend than in cell line HTM-46. Upon normalization to 18S, the levels of PEDF cDNA in the DEX-treated cells, which increased with time over the untreated control, were 1.98 ± 0.12-fold (n 3) ($p \leq 0.0002$) at 1 day, 2.56 ± 0.02-fold $p \leq 0.001$) ($n = 3$) at 4 days, and 2.42 ± 0.03-fold ($p \leq 1 \times 10^{-6}$) ($n = 3$) at day 8 (not shown). In turn, the increased fold changes of MYOC cDNA were 10.14 ± 1.36-fold ($p \leq 0.0002$) (n 3) at 1 day, 61.72 ± 5.0-fold ($p \leq 1 \times 10^{-6}$) (n 3) at day 4, and 100.63 ± 7.67-fold ($p \leq 1 \times 10^{-7}$) ($n = 3$) at day 8 (not shown). The C_T values of PEDF and MYOC (before treatment) in this cell line were 25.7 and 35.05, respectively, indicating that the relative abundance of PEDF in cell-cultured TM cells at passage 4 is 750× higher than that of the glaucoma marker gene MYOC (not shown). These results also offer additional support to the hypothesis of an individual molecular response to DEX, and that the individual from whom the HTM-95 cells were obtained was a higher responder for MYOC and a slightly lower responder for PEDF.

Effects of DEX on PEDF Protein Levels in Primary HTM Cell Cultures

To determine the effects of DEX on PEDF protein levels, we collected the conditioned medium from the HTM-46 cells exposed to DEX. Western blots revealed the presence of PEDF protein of 50-kDa (Fig. 4A). Semiquantitative immunoblotting showed that the PEDF levels increased with treatments of 0.1 μM DEX for 1, 4, 7, and 8 days over untreated controls (Fig. 4B). No detectable differences in PEDF content were noted during the first 24 hr; however, extracellular PEDF was consistently higher in DEX-treated cell cultures for all subsequent time points, that is, 2.9 ± 0.003-fold, 2.5 ± 0.34-fold, and 4.0 ± 0.03-fold over controls by 4, 7, and 8 days, respectively. Analysis of secreted PEDF by ELISA in the serum-containing medium of the HTM-95 cells' DEX experiment showed a decrease for the first 24 hr. However, albeit at slightly lower ratio, extracellular PEDF was consistently higher in the DEX-treated cells with levels of 1.41 ± 0.27- and 1.56 ± 0.27-fold over the untreated controls at 4 days and 8 days, respectively (not shown). These results demonstrate that HTM cells secreted PEDF protein and that DEX increased PEDF levels in the extracellular milieu of these cells.

DEX Decreases Gelatinolytic Activities in Effluents from Anterior Segments

Because of the relevance of gelatinases/ metalloproteinases (MMPs) in the regulation of outflow facility,[14] and because PEDF has been shown to be a substrate for MMP-2 and MMP-9,[12] we investigated the activity of these secreted MMPs in the organ cultures treated with DEX above. Gelatin zymography of their effluents showed that DEX significantly and specifically decreased the 92-kDa gelatinase B zymogen (MMP9) activity with perfusion time at all time points (Fig. 5A). The 72-kDa gelatinase A zymogen (MMP2) showed only a slight decrease by day 2 of DEX treatment. Solution enzymatic assays of the same samples, run in triplicate, revealed a DEX-mediated decrease in the overall gelatinolytic activity at all time points of treatment (Fig. 5B and 5C). By 8 days, the gelatinolytic activity of the DEX-treated cultures was less than 50% of that in control, with a trend that paralleled the 92-kDa zymogen. Solution assays and zymograms performed in triplicate on the effluents of the perfused eyes from the third individual showed, however, a much lower response. The decrease of gelatinolytic activity reached statistical significance at only one time point. At this time, we think that this difference between individuals might be a sign of a DEX individual response.

These observations are in agreement with reported DEX-mediated decreases for MMP-2 and MMP-9 in ex-plant, non-perfused cultures using DEX concentrations five-fold over those used

here.[3] These results show that the PEDF increase was accompanied by a decrease in gelatinolytic activities (92-kDa zymogen).

DISCUSSION

In this study, we report that the synthetic glucocorticoid DEX specifically induces PEDF gene expression in the human TM and increases the extracellular PEDF protein levels produced by the intact perfused tissue and by the cultured HTM cells. Our results show that the effects of DEX on the levels of PEDF transcripts translate into increased levels of extracellular PEDF protein. These findings indicate that DEX has a specific and direct effect on the PEDF extracellular reservoir of the TM of the human eye. This PEDF increase may occur *in vivo* and play an important role in defining the molecular pathways by which DEX influences TM function, in particular, its effect on IOP.

The selected TM system is closer to *in vivo* conditions than cultured cells. In these *ex vivo* organ cultures, the TM maintains its natural architecture, and the perfused media flows in a manner that mimics the *in vivo* situation of aqueous humor exiting the eye by flowing through the tissue. The protein profile of the effluents can thus be considered a representation of the dynamic extracellular environment of the TM. In addition to PEDF, DEX perfusions induced the glaucoma-linked MYOC and inhibited 92-kDa gelatinolytic activities (MMP-9). The DEX-mediated induction of PEDF shows less variation among individual donors compared to MYOC, which displays a variation that is suggested to correlate with an individual response to stressful insults.[5] Although the perfused system does not allow one to determine whether the TM is the only tissue responsible for the increase seen in extracellular PEDF levels, similar induction obtained in isolated HTM cells indicates that the increase observed in the effluents is derived in great part from cells of the TM. Consistent with our results in HTM cells, we have also found that DEX significantly induced PEDF mRNA and extracellular PEDF protein levels in cultures of monkey retina pigment epithelial cells (unpublished observations).

Our results have biological implications for the response of the TM to DEX. The ECM of the TM plays a key role in the development of elevated IOP in the eye.[4] It is rich in collagens, glycosaminoglycans, and proteoglycans, and contains proteinases, including matrix metalloproteinases (MMPs).[3,15] MMPs, involved in ECM remodeling, are known to directly influence the resistance of the TM to aqueous humor outflow and subsequently affect IOP.[15] Of the MMPs MMP-2 and MMP-9 degrade PEDF and abolish its activities,[12] thus contributing to its likely turnover. Interestingly, DEX downregulates MMPs[3] (Fig. 5) and upregulates protease inhibitors (TIMPs),[3,6] eliciting an overall inhibition of proteolysis in the TM. Thus, the DEX-induced increase of PEDF seen here is not only the result of a transcriptional upregulation, but also of an upregulation at the post-translation level caused by the inhibition of its degradation enzymes.

The upregulation of PEDF could be a counteracting effect to the ECM DEX-mediated changes in ECM deposition and elevated IOP. PEDF has affinity for relevant TM collagens and glycosaminoglycans[7] and has been shown to specifically bind collagen type I,[7] which is highly abundant in the TM. These interactions could have positive implications on the conformation and quality of its ECM, which could influence aqueous humor outflow and mediate DEX-induced elevated IOP. To this effect, it is interesting to note that the levels of PEDF have been reported to be significantly lower in the aqueous humor of individuals of increasing age[16] and in patients with advanced glaucoma.[17] Furthermore, MMP-9, which degrades PEDF, has been found significantly upregulated in the leucocytes of normal tension glaucoma patients,[18] and, more recently, MMP-9 has been genetically linked to patients with acute primary angle closure glaucoma.[19] All together, these observations appear to support the notion that low levels of PEDF might be correlated with aging and glaucoma.

In conclusion, we provide evidence for an induction of PEDF gene expression and elevation of extracellular PEDF protein by DEX in human TM-perfused tissue and cultured cells. This DEX-mediated upregulation of PEDF in the TM may contribute to the conformational integrity of local ECM and influence IOP, or might perhaps exert a downstream systemic effect on other tissues. Although these potential effects need to be investigated, our results provide a new insight into DEX's influence in the human TM.

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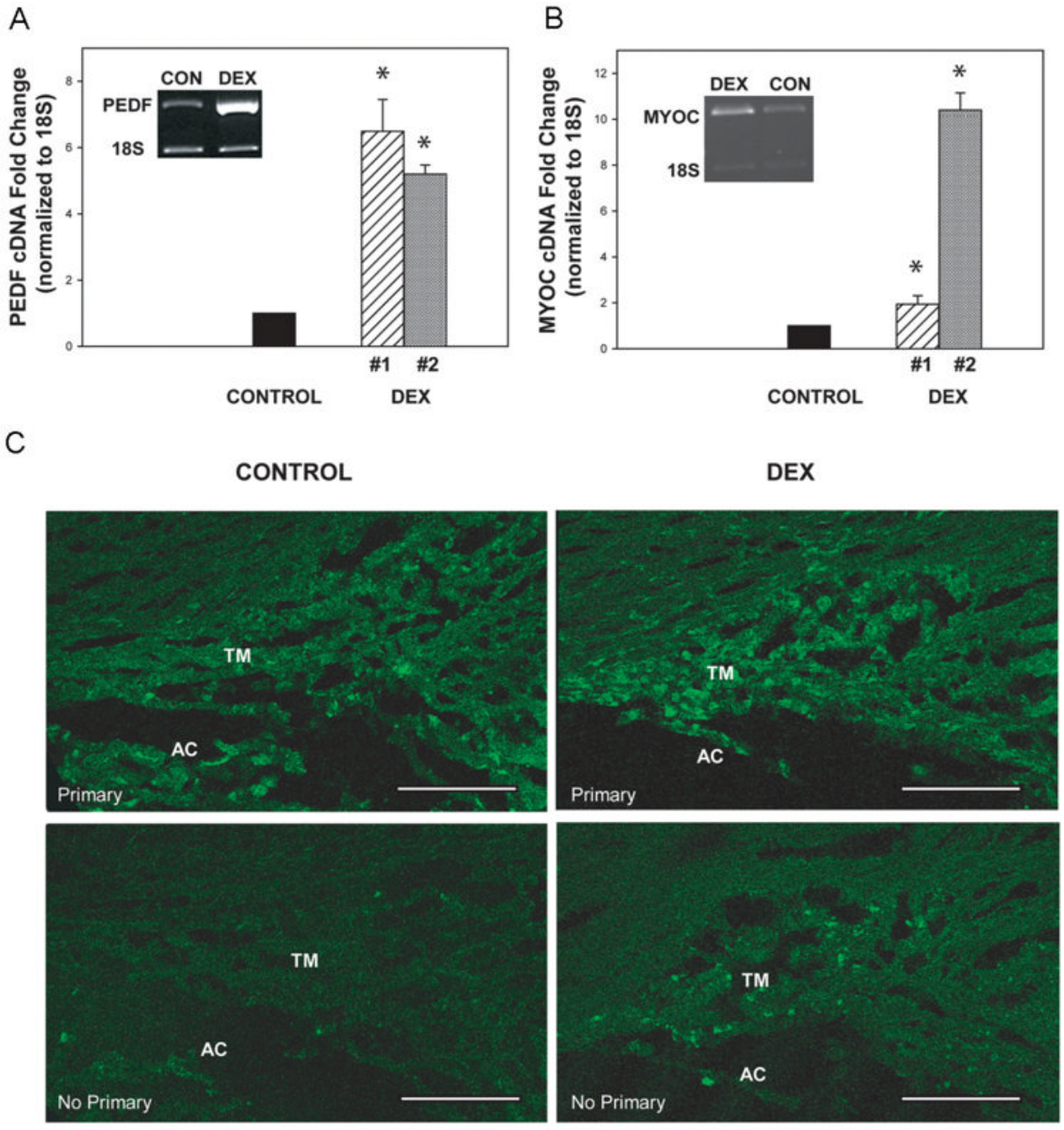

FIGURE 1.

Overproduction of PEDF in human perfused eyes. (A) Multiplex PEDF and 18S cDNAs at linear range amplifications in two individuals (n = 3 each) (31 and 29 cycles, respectively, and 18S primer:competimer ratio 1:15). The data are PEDF fold-change mean + SEM. Inset: Representative gel of PEDF of Ind #2 and 18S PCR products from untreated (CON) and treated (DEX) TMs. (B) Multiplex MYOC and 18S cDNAs at linear range amplifications in two individuals (n = 3 each) (27 cycles and 18S primer:competimer ratio 1:9). The data are MYOC fold-change mean + SEM. Inset: Representative gel of MYOC of Ind #2 and 18S PCR products from untreated (CON) and treated (DEX) TMs. (C) Confocal micrographs from untreated and DEX-treated tissues cross-reacted with anti-human PEDF antibody (1:200) and Cy5-

conjugated anti-mouse IgG (1:400). Control sections received no primary. AC: Anterior chamber. Scale bar: 150 μm.

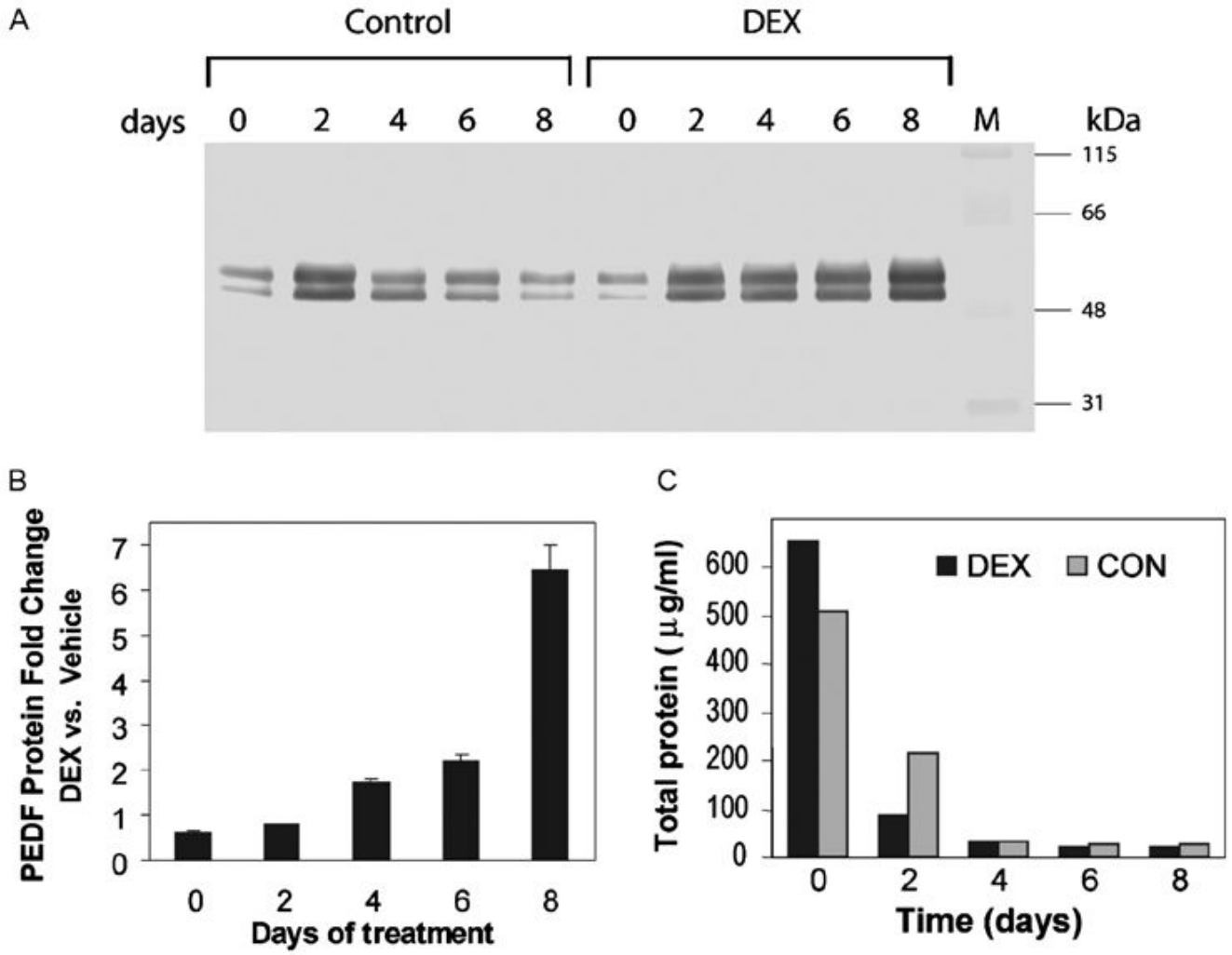

FIGURE 2.

PEDF protein secreted by DEX-treated perfused eyes. (A) Representative Western blot of DEX-treated and control effluents (10 µg/lane) and purified recombinant human PEDF protein (15 ng) cross-reacted with anti-human PEDF antibody. For each set of organ cultures, Westerns were performed twice. (B) Fold-change of PEDF immunoreactive bands over controls obtained after three different exposures of one blot (mean ± SEM). (C) Total protein concentration on effluents.

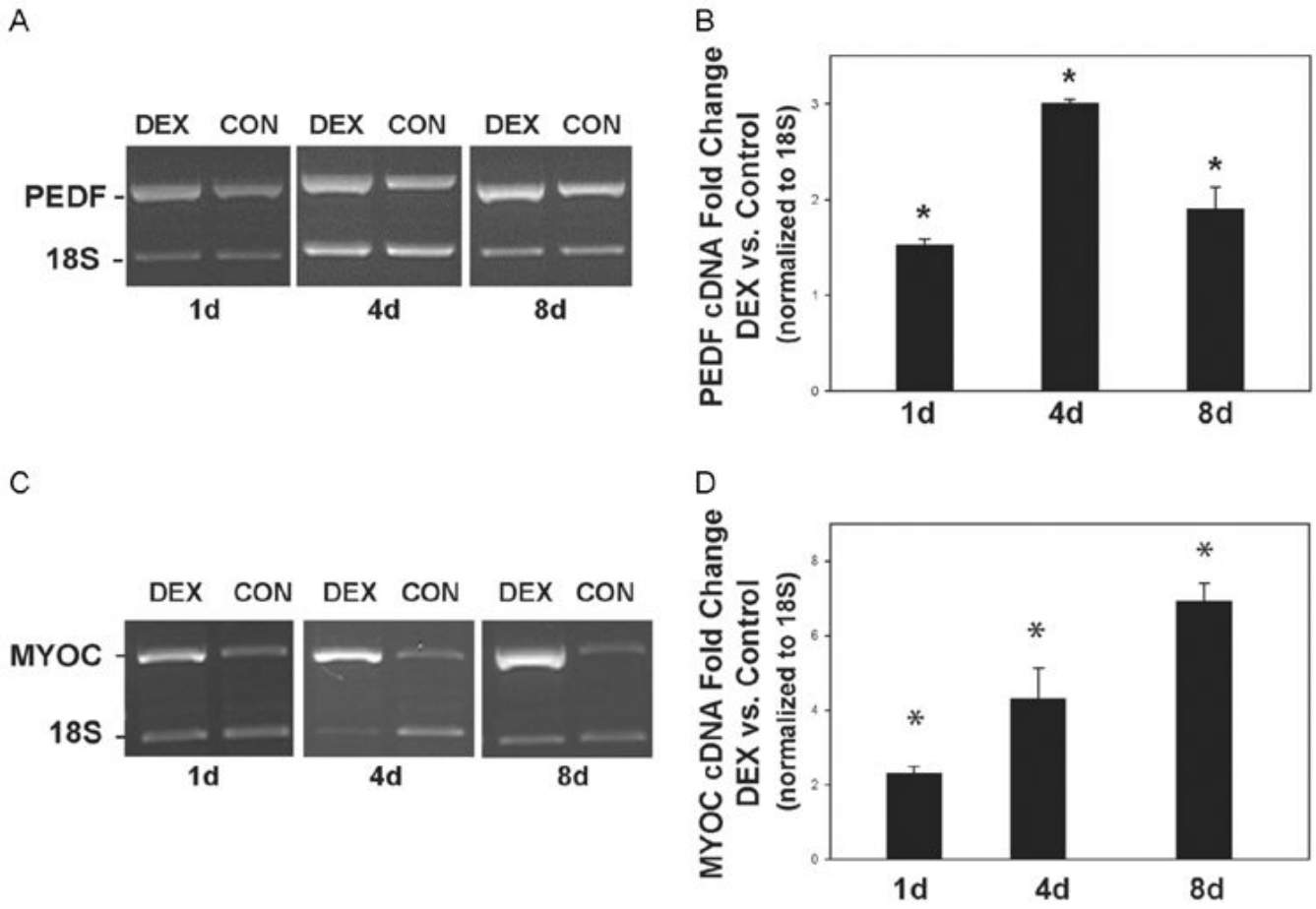

FIGURE 3.

Effect of DEX on PEDF from HTM cells. (A) Representative gels of multiplex PEDF/18S cDNAs linear amplifications at 1, 4, and 8 days. (B) Fold-change expression of PEDF on DEX-treated versus control, normalized to 18S (mean + SEM) (each $n = 6$). (C) Representative gels of multiplex MYOC/18S cDNAs linear amplifications at 1, 4, and 8 days. (D) Fold-change expression of MYOC on DEX-treated versus control, normalized to 18S (mean + SEM) (each $n = 6$).

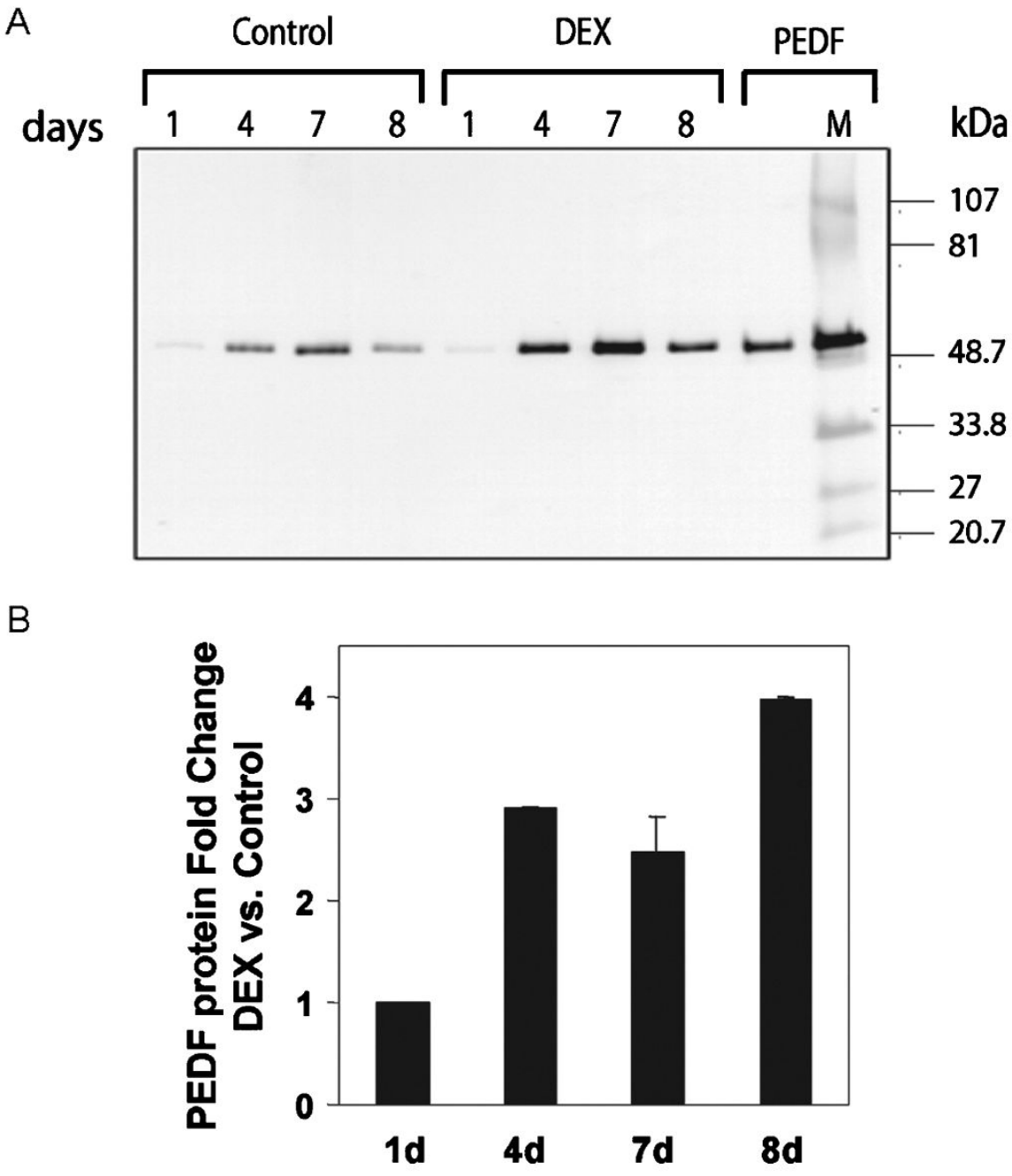

FIGURE 4.

Effect of DEX on PEDF from HTM cells. (A) Representative Western blots (15 μg/lane) of conditioned media by TM cells and purified recombinant human PEDF (15 ng) cross-reacted to human PEDF antibody. Westerns were repeated twice. (B) Fold-change of PEDF immunoreactive bands versus control (mean ± SEM).

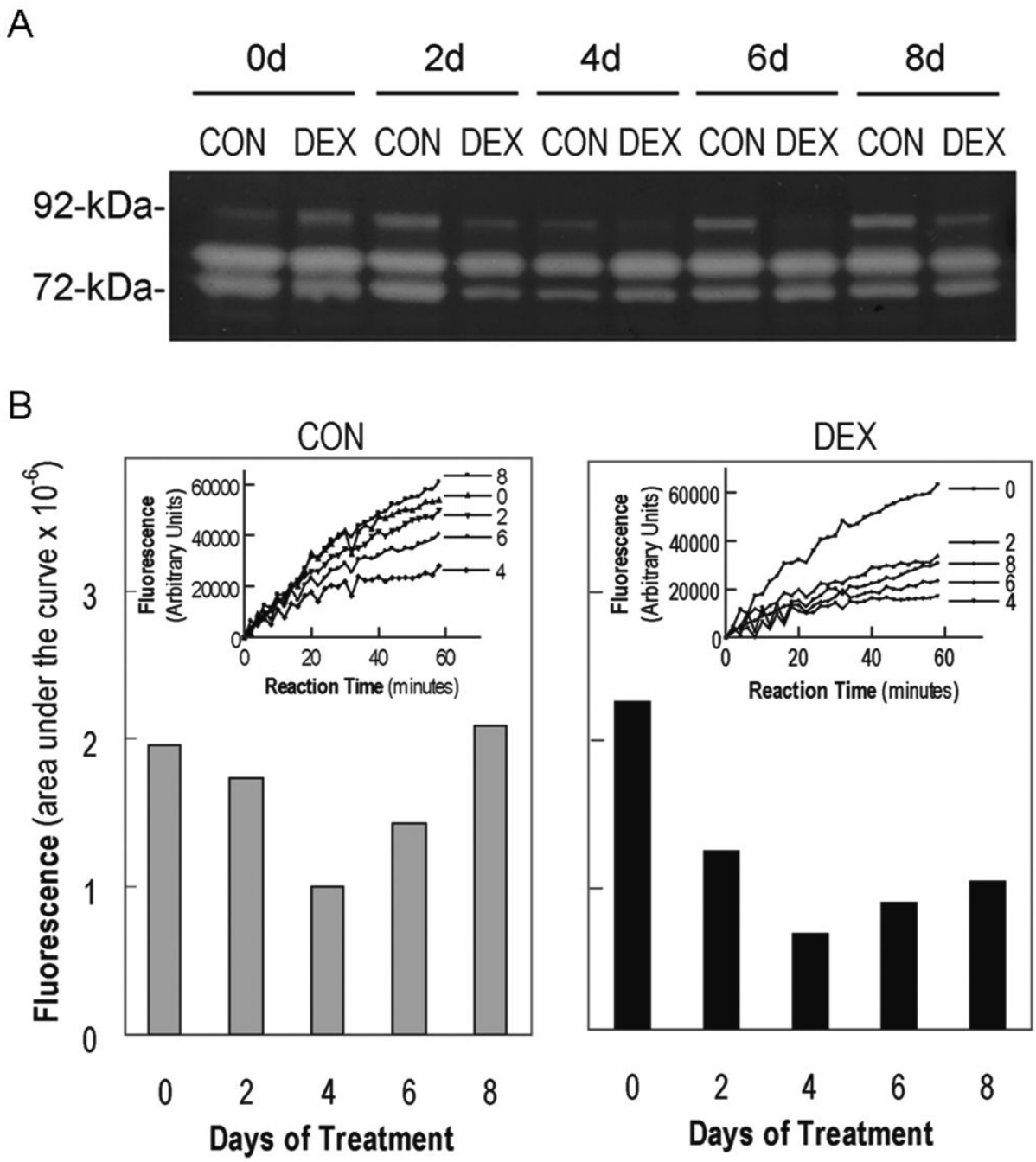

FIGURE 5.

Gelatinolytic activity of effluents from perfused eyes. (A) Zymogram of DEX and control (CON) effluents (10 μl/lane) on gelatin-containing gel, stained with coomassie blue. Migration positions of 92-kDa and 72-kDa would correspond to MMP-9 and MMP-2, respectively. (B) DEX and control (CON) effluents (10μl/well) incubated with DQ gelatin for the indicated times. Area under the curve values of the fluorescence of the degraded gelatin at each time point. Inset: Time course of fluorescence of the degraded gelatin for each time point.

TABLE 1

Outflow Facility Values of the Perfused Post-mortem Human Eyes

		Baseline	2 days	4 days	7 days
Ind #1	DEX	0.31	0.24	0.27	0.30
	vehicle	0.38	0.36	0.32	0.61
Ind #2	DEX	0.41	0.47	0.44	0.31
	vehicle	0.36	0.68	0.71	0.74
Ind #3	DEX	0.27	0.16	0.11	0.18
	vehicle	0.86	0.58	0.53	0.58
Average		0.43 ± 0.09			