Triamcinolone Acetonide Destabilizes VEGF mRNA in Müller Cells under Continuous Cobalt Stimulation

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PURPOSE. To identify the molecular mechanism of steroid-induced downregulation of vascular endothelial growth factor (VEGF) synthesis in Müller cells.

METHODS. Confluent cultures of human Müller cells (M10-M1) were treated with 100 μM CoCl₂, 1 μg/mL triamcinolone acetonide (TA), or both. VEGF secretion was measured with respect to time by ELISA. VEGF mRNA quantity and stability were analyzed by reverse transcriptase-polymerase chain reaction. The activity of hypoxia-inducible factor (HIF)-1 was measured by the relative binding of HIF-1 protein to the hypoxia response element (HRE), by gel shift and ELISA. The HIF-1α protein level was determined with Western blot.

RESULTS. TA decreased VEGF secretion by at least 50% in the presence of continuous cobalt stimulus. VEGF mRNA decreased 50- to 100-fold 6 hours after treatment with TA and cobalt compared with cobalt alone. VEGF mRNA stability was decreased in cobalt-stimulated, TA-treated cells compared with cobalt alone in cells synchronized by exposure to actinomycin D. HIF-1α protein level was sustained for the entire 24-hour treatment period and partitioned into nuclear, non-cytosolic, fractions. HIF-1 activity was decreased by 20% to 30% in the presence of TA and cobalt compared with cobalt alone.

CONCLUSIONS. TA may decrease VEGF synthesis by nongenomic destabilization of VEGF mRNA in cobalt-stimulated Müller cells. There was little effect on the total HIF-1α protein level, HIF-1 partitioning, and HIF-1 activity.

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Posttranscriptional regulation of gene expression enables cells to control the contents of their proteomes by either rapid degradation of messenger RNA (mRNA) or translational silencing. In the case of vascular endothelial growth factor (VEGF), specific destabilization of VEGF mRNA is reported to be a function of the 3’ untranslated region (UTR). The VEGF 3’UTR contains nine copies of the nonamer consensus sequence for adenylate/uridate-rich (ARE) elements. Three proteins have been identified that bind and stabilize VEGF transcripts with specific affinity for the 3’UTR. HuC, a member of the ELAV family, the ribonucleoprotein hnrNPL, and poly(A)-binding protein-interacting protein 2 stabilize the destabilization of mRNAs that are essential in the inflammatory response, such as interleukin (IL)-1, -6, and -8; tumor necrosis factor-α; and monocyte chemoattractant protein (CCL2). In addition, glucocorticoids destabilize collagenase and stromelysin. Dexamethasone has been reported to destabilize VEGF mRNA in keratinocytes.

The recent success of intravitreal triamcinolone acetonide (TA) as a monotherapy for diabetic macular edema may be explained in part by a reduced synthesis of a common inflammatory mediator such as VEGF. We are interested in identifying the molecular mechanism of steroid induced downregulation of VEGF synthesis to develop specific therapies that lack the complications associated with chronic steroid use. We measured the effect of TA on cobalt-stimulated VEGF synthesis in Müller cells and analyzed the hypoxia inducible factor (HIF)-1 protein levels and activity to uncover whether the decrease in VEGF mRNA and protein we found after TA treatment was related to changes in HIF-1. We demonstrate a decrease in VEGF expression that parallels a decrease in VEGF mRNA but not HIF-1 activity, protein levels, or the nucleocytoplasmic distribution of HIF-1. Instead, we noted a rapid decrease in VEGF mRNA, even after simultaneous treatment with actinomycin D, suggesting a nongenomic signaling pathway that defines one mechanism of TA downregulation of VEGF synthesis.

METHODS

Müller Cell Culture

Müller cells (M10-M1) were kindly provided by G. Astrid Limb (Institute of Ophthalmology, London, UK) and plated at a density of 10⁵ cells per 100-mm plate in DMEM supplemented with 400 mM glutamine and 10% FCS. Before stimulation, cells were placed in serum-free medium for 24 hours. Cobalt chloride was added at 1:1000 from a 100-mM stock solution in DMEM, to provide a final concentration of 100 μM. TA was dissolved in ethanol at a concentration of 1 mg/mL and diluted 1:1000 into cell cultures, to provide a final concentration of 1 μg/mL—perhaps 1000-fold less than administered as an intravitreal injection in humans. Cells were treated with medium alone, cobalt chloride, TA, or both for various time points, as described in legends of the Results section.

VEGF ELISA

Supernatants were harvested and 50 μL added directly to sample diluent provided by the manufacturer of human VEGF colorimetric ELISA (Pierce Endogen, Rockford, IL), and VEGF measurements were performed according to the manufacturer’s instructions.

Reverse-Transcriptase–Polymerase Chain Reaction

Cells were harvested in each particular experiment by scraping them into 2 mL chilled PBS and pelleting them in a 15-mL conical tube at 500 rpm for 5 minutes. Total RNA was isolated according to the manufacturer’s instructions (RNaqueous 4PCR; Ambion, Austin, TX). RNA concentration was calculated by spectrophotometry.

Two-tube RT-PCR was performed according to standard techniques (Qiagen, Valencia, CA) with 1 μg RNA as a template with oligo dT primer.
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HIF-1 Binding Activity

An HIF-1 transcription factor assay (TransAM; Active Motif, Carlsbad, CA) was used to measure HIF-1 binding activity. The hypoxia response element (HRE) 5'-TAC GTG CT 3' is immobilized in this assay and a colorimetric ELISA with anti-HIF-1α antibody as a probe used to quantify the level of HIF-1 activity by binding of HIF-1 to the specific DNA sequence. Wells were treated with 40 μL of binding buffer containing 1 mM DTT to which 10 μL of nuclear extract was added, comprising 5 μg protein of nuclear extract, each according to the manufacturer’s protocol. An electrophoretic mobility shift assay (EMSA) was performed with a kit and a biotin-labeled probe 5'-AGC TTG CCC TAC-3' reverse primer.19 RT-PCR products were separated on 1.5% agarose gel with ethidium bromide. The number of cycles was varied for a rough determination of the difference in product.

RNA Stability Assay

Near-confluent cultures of Müller cells were stimulated with 100 μM cobalt chloride for 12 hours. Actinomycin D was added to each culture at a concentration of 6 μg/mL, and 1 μg/mL TA was added to half the cultures. RNA was extracted immediately (time 0), and at 1.5, 3.0, and 4.5 hours after addition of actinomycin D, with and without TA.

Western Blot for HIF-1α Protein Analysis

Cells were harvested in each particular experiment by scraping them into 2 mL chilled PBS and pelleting them in a 15 mL conical tube at 500 rpm for 5 minutes. Cell lysate was made by directly resuspending cells in 2× sample buffer with 1 mM dithiothreitol (DTT). Lysate was subjected to 4% to 20% PAGE and electroblotted to polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. Membranes were blocked with 5% nonfat dried milk in TBS and hybridized with anti-HIF-1 antibody (BD-Transduction Laboratories, San Diego, CA) and after washing and secondary antibody hybridization, exposed by chemiluminescence (Western Lightning; PerkinElmer, Boston, MA). Nuclear and cytosolic fractions were prepared with a nuclear extraction kit, according to the manufacturer’s instructions. (Actif Motif, Carlsbad, CA).

HIF-1-Binding Activity

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Statistical Analysis

The size of the difference between the means of gel densitometry were compared with the SE of that difference by using the t-test. The probability of error associated with rejecting the hypothesis of no...
difference between treated and untreated groups as a two-tailed probability was calculated and is supplied in the figures when relevant and useful. Gel experiments by nature are qualitative, and so the densitometry was designed to make the analysis of trends easier but cannot be considered a quantitative measurement, despite the presence of error bars and probabilities.

RESULTS

TA is usually administered at a dose of 4 mg, or roughly 1 mg/mL, in an intravitreal injection in humans. We initially noted an effect in RPE and bovine retinal endothelial cells after IL-6 stimulus at 1000 μM lower concentration (Ebrahim et al., manuscript in preparation). We continued these experiments with a concentration of 1 μg/mL by diluting a stock solution of TA dissolved in ethanol 1:1000 to eliminate any toxicity from the ethanol solvent. There was no cell loss noted in cultures treated with TA alone or with cobalt. We tested the effect of ethanol on VEGF secretion, with and without TA, to establish that the effect of TA on VEGF secretion was due to the solute and not the solvent (Fig. 1). We next tested a range of cobalt concentrations between 1 and 100 μM and established that 100 μM CoCl₂, the standard concentration generally used by others, was optimal for VEGF induction (Fig. 2). Figure 3 demonstrates that even in the sustained stimulus of 1000 μM cobalt, 1 μg/mL TA decreases VEGF secretion into the Müller cell culture medium by 50%. We were curious to determine whether this inhibition of cobalt-stimulated VEGF secretion results from a decrease in VEGF mRNA. Figure 4a demonstrates a reduction in VEGF transcript to baseline levels after 4 hours

**FIGURE 4.** TA decreased VEGF mRNA. (a) Top: RT-PCR demonstrated a reduction in VEGF mRNA to baseline levels after TA treatment in the presence of 100 μM cobalt. Bottom: control RT-PCR for GAPDH showing equal amounts of RNA template. The gel is a representative experiment, with identical results obtained in three separate experiments. (b) RT-PCR was interrupted at different cycle numbers, to obtain a rough determination of the difference in mRNA created by TA treatment. TA decreased VEGF mRNA by at least 50-fold, demonstrated by the roughly equivalent PCR product seen at cycle number 30 for cobalt and cycle number 35 for both TA- and cobalt-treated cells.

**FIGURE 5.** Triamcinolone did not affect levels of HIF-1α protein. To determine whether HIF-1, the transacting transcription factor that regulates VEGF expression during cobalt treatment, is affected by TA, Müller cells were grown to near confluence and serum starved for 48 hours before addition of cobalt (100 μM), with and without TA (1 μg/mL). Supernatants were tested for VEGF concentration before harvesting cells for Western blot experiments. (a) VEGF concentration in Müller cell supernatants after challenge with cobalt, TA, or both over a 24-hour period. TA decreased VEGF secretion into the supernatant by 50%. (b) Representative anti-HIF-1α Western blot of Müller cell lysates from Figure 3a probed with anti-HIF-1α antibody. The experiment was repeated six times. (c) Optical density (OD) of HIF-1α over a 24-hour period shows sustained increase in HIF-1α during constant cobalt stimulus. There is a slight reduction in samples from TA + CoCl₂-treated cells. HIF-1α is increased in cells treated with cobalt and with TA + CoCl₂ compared with untreated cells. TA therefore minimally reduces HIF levels, suggesting that the TA-induced decrease in VEGF mRNA and protein is either HIF-independent or that TA creates a change in the activity of HIF-1 without drastically reducing HIF-1 protein levels in cobalt-stimulated cells.
An estimate of difference in RT-PCR product can be seen in Figure 4b. RT-PCR product was equal in 25 versus 30 cycles when cobalt was used versus TA and cobalt-treated Müller cell mRNA template, respectively, suggesting that TA decreases VEGF mRNA by at least 50-fold under a continuous cobalt stimulus.

We hypothesized that the change in mRNA was secondary to a change in HIF-1, an early hypoxic response gene that is selectively stabilized by the hypoxia mimic cobalt. However, Western blot clearly shows the sustained level of HIF-1 protein beginning at 6 hours and maintenance of HIF-1 through a 24-hour period, despite a reduction in VEGF expression with TA (Fig. 5). We next considered whether the reduction in VEGF mRNA follows a reduction in HIF-1 activity because of a reduction in translocation of HIF-1 to the nucleus. Cytosolic and nuclear fractions were prepared and analyzed by Coomassie staining and Western blot analysis (Fig. 6). There was no indication that HIF-1 was sequestered in the cytoplasm outside the nucleus when analyzed by Western blot and an HIF-1 activity assay. We further tested the activity of nuclear extracts from Müller cells simultaneously treated with cobalt and TA and measured a definite but small decrease of HIF-1 binding to immobilized HRE, reduced by one quarter (Fig. 7a) in nuclear extract from these cells. This effect was not statistically significant. In concordance with the analysis of HIF-1 distribution (Fig. 4), no HIF-1 activity was observed in the cytosol (Fig. 7b). We confirmed a minimal change in HIF-1 activity by using a gel shift assay (Fig. 8).

The paradoxical decrease in VEGF mRNA while robust levels of active HIF-1 were sustained suggests that destabilization, not synthesis of VEGF mRNA was the primary action of TA. To test this hypothesis, Müller cell cultures were stimulated with cobalt for 12 hours and then treated with actinomycin D to halt transcription, to observe the degradation of VEGF mRNA over time in Müller cells treated and not treated with TA. We noted a steady decrease in VEGF mRNA beginning at 1.5, 3.0, and 4.5 hours, with statistically significant degradation at 5.0 hours, calculated with a two-tailed t-test (Fig. 9).

**DISCUSSION**

The antiangiogenic effect of steroids was first reported by Shubik et al., who noted that parenterally administered methylprednisolone inhibits tumor-induced angiogenesis in a hamster's cheek pouch. Gross et al. further reported that medroxyprogesterone, dexamethasone, and cortisone blocks tumor-induced limbal angiogenesis in a cornea micropocket. The practical utility of these observations was limited by the extraordinary complications of parenteral steroids, perhaps...

**FIGURE 6.** TA did not affect nucleocytoplasmic distribution of HIF-1α. HIF-1 is a heterodimer composed of a constitutive β-subunit and a tightly regulated α-subunit that assemble in the cytoplasm and are transported to the nucleus. To determine whether TA changes the nucleocytoplasmic distribution of HIF-1, cytosolic and nuclear fractions were prepared and individually tested for HIF-1α by Western blot (top). Representative Western blot and densitometry (middle) with an HIF-1α antibody demonstrated no appreciable effect of nucleocytoplasmic partitioning of HIF-1 by TA in samples treated with cobalt stimulus. Protein lysates from cytosolic and nuclear fractions were stained with Coomassie blue (bottom) and separated by 4% to 20% PAGE. Each experiment was repeated six times.

**FIGURE 7.** TA minimally reduced HIF-1 activity. To determine whether TA affects HIF-1 binding to the HRE, an ELISA-based analysis of HIF-1 binding to HRE was tested with lysates prepared from near confluent, serum-starved Müller cells. (a) HIF-1 activity measured by ELISA using immobilized HRE showed a small but definite decrease (30%) in TA-treated samples under continuous cobalt stimulus. Although this effect correlated with a decrease in VEGF mRNA, the decrease in activity may only partly explain the drastic decrease in VEGF mRNA and is not statistically significant. (b) HIF-1 activity measured by ELISA with immobilized HRE showed that nuclear versus cytoplasmic activity of HIF-1 followed nuclear distribution of HIF-1 and correlated with the nuclear targeting of HIF-1 protein demonstrated in Figure 6. Each experiment was repeated six times.
until the application of intravitreal TA (IVTA). Brooks et al. have demonstrated that IVTA reduces the vitreous cavity concentration of VEGF in humans. Our findings are in agreement with reports demonstrating an inhibitory action of TA on VEGF synthesis and specifically demonstrate that this effect can be reproduced in Müller cells, implying that a decrease in VEGF secretion may be one of the mechanisms responsible for the dramatic effect observed in the reduction of retinal thickness in patients treated with IVTA. Our model demonstrates that TA does not decrease the basal expression of VEGF, in accordance with other reports.

TA acts to reduce cobalt-stimulated VEGF expression, even in the presence of continuous cobalt stimulation. Cobalt increases VEGF expression by stabilizing HIF-1α protein by displacing Fe2+ from the O2-dependent prolyl hydroxylase, which has HIF-1α as a substrate. Hydroxylation of HIF-1α on two proline residues by prolyl-4-hydroxylases enhances the interaction of E3 ubiquitin ligase, or specifically the von Hippel-Lindau protein (pVHL), and subsequent targeting to the 26s proteasome. Although the use of cobalt is a well-established model that acts as a hypoxia mimetic, it does not represent the hypoxic state other than through the chronic activation of HIF-1. In this regard, cobalt investigations do not truly represent physiologic hypoxia, at least upstream of HIF-1. Further studies of HIF-1 and TA are under way in the mouse retinopathy of prematurity model in our laboratory.

TA does not decrease the amount of HIF-1α in our model and does not alter the nucleocytoplasmic partitioning of HIF-1 that is reported to occur after COX-2 pretreatment in prostate cancer cells, nor does it significantly decrease the activity of HIF-1. HIF-1α, an 826-amino-acid protein, is one of the first genes upregulated by hypoxia in a variety of models of ischemia. It is a heterodimeric protein composed of the inducible HIF-1α and constitutive HIF-1β subunits that are members of the basic helix-loop-helix-PerArntSim (PAS) family of proteins.

HIF-1 is a transacting transcriptional activator of VEGF, inducible nitric oxide synthase, lactate dehydrogenase, erythropoietin, and glycolytic enzymes. HIF-1α is maximally sensitive to cellular hypoxia at a concentration of 0.5% O2. Our investigation demonstrated a small decrease in the ability of HIF-1 to bind immobilized and soluble HRE using ELISA and gel-shift, respectively. These findings may not represent true HIF-1 activity, inasmuch as it is conceivable that HIF-1 could bind these DNA elements while being unable to enhance transcription.

The paradoxical decrease in VEGF mRNA in the presence of active HIF-1 may be explained by mRNA instability. At present, it is not known which protein cofactors or which signal transduction pathway modulates the TA-induced instability. Moreover, because the experiments were run in the presence of actinomycin D, the data imply that TA does not act through the

FIGURE 8. TA minimally affected HIF-1 binding to HRE. A gel shift assay (a) demonstrated the ability of nuclear extracts to bind biotinylated HRE. Samples from cobalt and TA-treated cells showed negligible reduction in the amount of gel-shifted probe compared with cobalt alone (b), in accordance with the ELISA in Figure 7.

FIGURE 9. TA induced destabilization of VEGF mRNA. To explain the paradox that TA decreases VEGF levels in cobalt-stimulated cells but did not appear to change either the protein level of HIF-1 or its activity drastically, qualitative RNA destabilization experiments were performed on Müller cells. All Müller cells were treated with 100 μM cobalt chloride for 12 hours and then treated with 6 μg/mL actinomycin D to synchronize levels of mRNA between all cultures. TA (1 μg/mL) was added to half the plates. (a) RNA was isolated at time 0 and then at 1.5, 3.0, and 4.5 hours and analyzed for VEGF transcript using RT-PCR. Each experiment was repeated three times. (b) Densitometry was performed and each value normalized against that of GAPDH at each time point. Statistical analysis was performed with a t-test. The trend in results of three experiments showed RNA destabilization of VEGF by TA. At 3.0 hours, the effect was statically significant (P = 0.04). TA induced RNA destabilization in the presence of actinomycin D, suggesting that TA provokes this response without de novo transcription and hence through a nongenomic pathway.
conventional pathway of initiating gene expression. The classic action of steroid hormones predicts that TA binds a soluble intracellular receptor that on activation translocates to the nucleus to function as ligand-dependent transcription factor. At least in the case of estrogen, it is reported that in addition to the classic estrogen receptor, membrane-bound G-protein-coupled receptor 30 (GPR30) binds estrogen and initiates intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-triphosphate in the nucleus. The finding that TA induces VEGF mRNA instability in the presence of actinomycin D suggests that further efforts at defining the molecular mechanism of IVTA should center on the nongenomic action of steroids.

References


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