Coordinate Activation of HIF-1 and NF-κB DNA Binding and COX-2 and VEGF Expression in Retinal Cells by Hypoxia

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PURPOSE. Proinflammatory signaling mechanisms are implicated in the induction of retinal neovascularization (NV) during ischemic retinopathies. This study examined transcription factor (TF) AP-1, HIF-1, and NF-κB DNA-binding in relation to cyclooxygenase (COX)-2 and VEGF RNA and protein levels in hypoxia-triggered monkey choroidal retinal (RF/6A) endothelial cells. Effects of the carboxamide CGP43182 were tested on COX2 and VEGF activation and prostaglandin (PG)E2 release.

METHODS. RF/6A cells were subjected to hypoxia for 1 and 5 hours, at which times RNA and proteins were isolated. Potential AP-1, hypoxia-inducible factor (HIF)-1 and NF-κB DNA-binding sites were identified using DNA sequence search algorithms and were analyzed using gel-shift assay. COX-2 and VEGF RNA, protein, and PGE2 levels were quantified by RT-PCR. Western analysis, and enzyme immunoassay, respectively. Tubular morphogenesis was analyzed with phase-contrast imaging microscopy.

RESULTS. Nuclear AP-1, HIF-1 and NF-κB promoter DNA binding increased 1.5-, 4-, and 3-fold, respectively, after 1 hour of hypoxia. COX-2 RNA was elevated five- and fourfold after 1 and 3 hours of hypoxia, respectively. VEGF RNA and protein abundance lagged behind COX-2 induction but were each increased two- to threefold 3 hours after hypoxia. CGP43182 was found to inhibit NF-κB DNA binding, COX-2 and VEGF gene expression, PGE2 release, and hypoxia-induced tubular morphogenesis.

CONCLUSIONS. Maximum HIF-1 and NF-κB DNA binding immediately before COX-2 expression suggests that these TFs are important regulators of COX-2 induction in hypoxic RF/6A cells. IL-1β emulated AP-1, HIF-1, and NF-κB DNA binding during hypoxia and may be a novel cytokine trigger for NV. CGP43182 appears to be an effective inhibitor of NV. VEGF expression appears to be regulated through dual interdependent mechanisms involving HIF-1 directly and indirectly through NF-kB-mediated COX-2 expression and PGE2 production.

Neovascularization (NV) is orchestrated by the coordinate induction of a family of growth factor genes and most prominently by vascular endothelial growth factor (VEGF). VEGF, one of the most potent angiogenic factors known, also possesses endothelial cell-specific mitogenic effects that closely correlate with NV during embryonic development and normal systemic physiology. However, VEGF also contributes to pathophysiologic conditions in diverse tissue types. For example, VEGF gene transcription spatially and quantitatively correlates with NV in chronic fetal anemia, in malignant tumors, in retinal NV, and in models of hypoxic ischemia, thus suggesting VEGF’s key role in growth, health, and disease.1–3 Hypoxia is thought to be a crucial physiologic stimulus for VEGF upregulation that precedes NV, and low cellular oxygen tension rapidly induces a number of transient genetic signals through which this is accomplished. Primarily, hypoxia induces the activation of hypoxia-inducible factor (HIF)-1, a transcription factor (TF) that provides the means for changes in gene expression in response to alterations in cellular oxygenation.4–7 HIF-1 recognizes the minimal cis-acting hypoxia-responsive element (HRE) core DNA sequence, 5′-RCGTG-3′ (R is any purine) that is enriched in the inducible prostaglandin synthase cyclooxygenase (COX)-2, in VEGF, and other oxygen-sensitive gene promoters.4–7 HIF-1 plays a role in the upregulation of VEGF in the ischemic retina, where increased levels of HIF-1 DNA binding show temporal and spatial correlation with enhanced VEGF gene transcription.4–7–9 NF-κB, another oxygen-sensitive TF that recognizes multiple DNA sequences of 5′-GGACTT-3′ in the COX-2 but not in the VEGF gene promoter, plays an important role in autoimmune, chronic inflammatory, and vascular diseases. NF-κB also contributes to altered hypoxia-induced signal-transduction events through the expression of target genes that encode cytokines and chemokines, such as interleukin (IL)-1, -2, -6, -8, TNFα, inflammatory enzymes such as COX-2, inducible nitric oxide synthase (iNOS), cPLA2, and cell-adhesion molecules.9–11 Hypoxia also potently triggers COX-2 transcription in the rat lung,12 in human vascular endothelium,13 and in human neural cells in primary culture.14 An important COX-2 product, prostaglandin (PG)E2, is also a strong inducer of VEGF in osteoblastic RCT-3 cells and rheumatoid synovial fibroblasts,15 in human monocytic THP-1 cells,16 in the rat lung,17 and in cultured Müller cells.18 Compounds that induce COX-2 expression and enzymatic activity also induce VEGF, and those that inhibit COX-2, such as indomethacin or methanesulfonylamide, also inhibit the induction of VEGF.19 These data suggest a key

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regulatory and potential priming role for COX-2 and PGE₂ in the VEGF-expression pathways, vascular remodeling, and NV. In the experiments presented herein, in hypoxia- or IL-1β-treated monkey choroidal-retinal endothelial (RF/6A) cells, the oxygen-sensing Tfs HIF-1 and NF-κB (but not activator protein [AP]-1) were involved in driving COX-2 gene expression and subsequent VEGF-gene induction. CGP43182, an enolized 1,3-dioxane-4,6-dione-5-carboxamide and potent secretory phospholipase (sPL)A2 antagonist also inhibited NF-
  subsequence VEGF gene induction. CGP43182, an enolized 1,3-

MATERIALS AND METHODS

RF/6A Endothelial Cells and the Hypoxia Protocol

RF/6A (monkey choroid-retinal) cells (RF/6A cell line CRL-1790; American Type Culture Collection [ATCC], Rockville, MD) were plated in a normobaric chamber (model 116885 CO₂ Incubator; Fisher Scientific, Pittsburgh, PA) containing humidified atmosphere and normal filtered air (controls) or in a hypoxic chamber (model MCO-175M Multigas Incubator; Sanyo Scientific, Itasca, IL) with humidified atmosphere. In the latter case, cells were perfused with 90% N₂, 5% CO₂, and 5% O₂ during the period specified (0-3 hours). The hypoxia-treated and age-matched control cells were kept under normoxic conditions (75% N₂, 5% CO₂, and 20% O₂). There were no apparent changes in the gross morphology of the RF/6A cells during or after hypoxia treatment.

Electrophoretic Mobility-Shift Assay, RT-PCR, and Western Immunoblot Analysis

Cellular and nuclear protein extracts (CPXTs, NPXTs) were rapidly isolated, and concentrations were quantified with a protein microassay (dot-Metric; sensitivity 0.3 ng/ml protein; Chemicon, Temecula, CA), as previously described. Oligonucleotides containing DNA-bound and mutant consensus sequences of AP-1 (5′-TGACTCA-3′) were end labeled by T4 polynucleotide kinase and [γ-32P]adenosine triphosphate (ATP; 3000 Ci/mmol; Redivue; Amersham Pharmacia Biotech, Piscataway, NJ). Electrophoretic mobility-shift assay (EMSA) reactions, using 5 μg of input CPXT or NPXT, were run out on 3.5% or 5% TBE-acrylamide gels, quantified by a fluorometric image analyzer (FLA-2000; Fuji). Tables and figures were generated on computer (Designver. 6.0 and Excel ver. 5.0; Microsoft). Statistical significance of

Analysis of Tube Formation in RF/6A Cultures

RF/6A cells were grown at 37°C in a humidified atmosphere of normal air (controls) or under conditions of hypoxia (90% N₂, 5% CO₂, and 5% O₂) in F12-K medium supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were seeded onto 24-well plates (6 x 10⁵ cells/well) coated with collagen type I. When cells were 90% to 95% confluent, the medium was replaced with fresh F12-K medium containing 1% FCS or 1% FCS containing 0.1 to 1.0 μM CGP43182 and incubated overnight. Cells were then exposed to 10 ng/ml human recombinant IL-1β or to hypoxia for 1 or 3 hours and were compared to cells maintained under normoxic conditions. At these time points CPXTs, NPXTs, total RNA and total cellular protein were prepared as previously described. To test the effects of CGP43182 on tube formation, plates were then transferred back into a standard incubator, and cells were incubated for an additional 72 hours, at which time five fields per sampling were photographed at 100 x magnification by phase-contrast microscopy (Optonphor-2; Nikon Laboratories, Melville, NY). Image-analysis software (AnalySYS 5.0 software; AnalySYS, Munster, Germany) was used to measure the length of tubes in micrometers and the area of tight cellular aggregates in square micrometers on each image obtained by phase-contrast microscopy. In the later analysis, tube lengths were measured (in micrometers) using the “polygon length setting” of the image-analysis software, and the perimeter (in micrometers) and area (in square micrometers) of each aggregate was measured using the “perimeter/area setting.” All data were saved on spreadsheets (Excel, Microsoft Corp., Redmond, WA). The average of each parameter per image was then calculated, and the average results obtained from five images characterized one well. The mean of eight wells characterized one group of cultured cells. Data on tube lengths and areas of cellular aggregates were statistically analyzed by one-way analysis of variance (ANOVA).

DNA Sequence Analysis, Data, and Statistical Analysis

DNA sequence and structure analyses identifying putative cis-acting DNA regulatory elements located between ~1100 bp and +100 bp of the COX-1, COX-2, and VEGF gene promoters (GenBank accession nos. D64068 [M31812], AF276953, and AF905785, respectively; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) were performed using sequence analysis software (DNASIS ver. 2.6; Hitachi Genetic Systems, Alameda, CA; and Transfac, ver. 5.2; Biobase, Wöhnfinnt, Germany). AP-1, HIF-1- and NF-κB-DNA consensus sequences within the COX-2 and VEGF gene promoters are described in GenBank AF276953, AF905785 and in this study (see Fig. 9). EMSA, RT-PCR, Western immunoblot, and ELISA data were quantified by phosphorescence imaging analysis and data-acquisition/statistical-analysis software provided with the molecular imager (model GS250; Bio-Rad, Hercules, CA) or a fluorescence image analyzer (model FAL-2000; Fuji). Tables and figures were generated on computer (Designver. 6.0 and Excel ver. 5.0; Microsoft). Statistical significance of
FIGURE 1. Levels of transcription factors AP-1, HIF-1, and NF-κB DNA binding were quantified in nuclear protein extracts prepared from normoxic RF/6A cells and in cells incubated under hypoxic conditions for 1 and 3 hours. Nuclear binding was analyzed on 3.5% TBE-acrylamide gels. The results are the mean plus 1 SD of results in three replicate cell experiments. Results from the normoxic and hypoxic cells at the different times were compared with a two-way factorial analysis of variance, with multiple comparisons conducted using a simulation-based method for α level correction. *P < 0.05; **P < 0.01.

EMSA, RT-PCR, or EIA data were analyzed by using a completely randomized design in a two-way factorial analysis of variance. After the finding of a significant overall F-test and a significant interaction F-test, individual interaction means (each represented by a histogram bar in the figures) were compared with protected t-tests. The α levels of these tests were corrected for the number of comparisons made, using a simulation-based multiple-comparison method. All statistical procedures were performed with the programs and procedures in a commercial statistical-analysis program (Statistical Analysis System software; SAS Institute, Cary, NC).

RESULTS

Effect of Hypoxia on Rapid Gene Signaling in RF/6A Cells

Data in Figures 1 through 6 indicate that the time points of 1 and 3 hours after hypoxia were the most useful in preliminary analyses of the earliest genetic events that involve TF translocation, HIF-1 and NF-κB DNA binding, and signal coupling of these oxygen-sensitive TFs to gene targets in monkey choroid-retinal (RF/6A) nuclei. At these time points, TF DNA binding was determined by EMSA, using AP-1-, HIF-1-, and NF-κB-specific mutant and consensus TF DNA target-containing oligonucleotides (see Figs. 1, 2, 4). Gene expression was quantified for COX-1, COX-2, and VEGF RNA message levels by RT-PCR, using β-actin as an internal control RNA message marker in the same retinal endothelial cell sample (see Figs. 3, 5). In these sample sets, COX-1, COX-2, and VEGF protein (see Fig. 6) and PGE_2 levels (see Fig. 7) were quantified by Western immunoblot analysis and enzyme-immunoassay (EIA), respectively.

EMSA of AP-1, HIF-1, and NF-κB DNA Binding and COX-2 and VEGF Gene Expression

The kinetics of the HIF-1 and NF-κB DNA-binding activities correlated both positively and temporally with COX-2 (but not COX-1) RNA message levels, and this was followed by increasing levels of VEGF RNA message in the same sample. Figure 1 shows the levels of AP-1, HIF-1, and NF-κB DNA binding in RF/6A NPXTs. These increased to 1.5-, 4-, and 3-fold, respectively, over time 0 controls, after only 1 hour of hypoxia, suggesting that nuclear HIF-1 and NF-κB, but not AP-1, activations are rapid TF-signaling events. Figure 2 shows the cellular and nuclear protein extracts (CPXT, NPXT, respectively) of HIF-1 in hypoxic RF/6A cells, as determined by EMSA. CPXT levels of AP-1, HIF-1, and NF-κB were only slightly elevated after 3 hours of hypoxia. In contrast, NPXT levels of HIF-1 were sharply elevated after 1 and 3 hours of hypoxia, implying that the major changes in HIF-1 DNA-binding levels are confined to the nuclear compartment during this time course (as are NF-κB, but not AP-1 DNA-binding profiles; data not shown). Figure 3 shows RT-PCR results for β-actin, COX-1, and COX-2 RNA message levels in RF/6A cells after 1 and 3 hours of hypoxia. The basal order of RNA message abundance was β-actin > COX-1 > COX-2 > VEGF > COX-1 RNA message at time 0, changing to β-actin > COX-2 > VEGF > COX-1 RNA message at 3 hours. COX-2 (but not β-actin or COX-1) RNA message levels rose to five- and fourfold over the control values after 1 and 3 hours of hypoxia, respectively. During this same 1- and 3-hour time course and at 3 hours after hypoxia, the latter being the longest time point reported in the study, the level of VEGF RNA was still increasing.

Hypoxia Triggering Effect of IL-1β

Figure 4 shows that the levels of AP-1, HIF-1, and NF-κB DNA binding in NPXTs isolated from hypoxic RF/6A cells were emulated by 1- and 3-hour treatment of normoxic cells with 10 ng/mL of human recombinant IL-1β. As discussed more fully later, application of the proinflammatory cytokine IL-1β has been shown to simulate hypoxia treatment, through induction
of both HIF-1 and NF-κB DNA binding in human adult neural cells and in human gingival and synovial fibroblasts, suggesting that the TFs NF-κB and HIF-1 both have roles in inflammatory signaling, possibly in cellular pathways that mediate oxidative homeostasis.

Patterns of COX-2 and VEGF gene activation, at the RNA and protein levels in RF/6A cells, were observed (Figs. 5, 6) after treatment with normobaric hypoxia (90% N₂, 5% CO₂, and 5% O₂) for 1 and 3 hours. Further experiments were performed in parallel with IL-1β-triggered RF/6A cells (data not shown). Comparison of the effects between hypoxia- or IL-1β-treated groups on AP-1, HIF-1, and NF-κB DNA binding and on COX-2 and VEGF gene expression patterns over 1 and 3 hours showed statistically nonsignificant differences (P > 0.05).

Effects of CGP43182 on TF DNA Binding and COX-1, COX-2, and VEGF Expression

Figure 4 shows that NF-κB (but not AP-1 or HIF-1) DNA binding in hypoxia-triggered RF/6A cells was significantly reduced in the presence of the enolized 1,3-dioxane-4,6-dione-5-carboxamide, CGP43182, a known sPLA2 antagonist. In the same cell homogenates, the levels of COX-2 and VEGF (but not that of COX-1 or β-actin) RNA message was reduced by approximately 50% over untreated controls in the presence of CGP43182 (Fig. 5). In control cells, increases in COX-2 and VEGF RNA message species translated into marked upregulation of COX-2 and VEGF (but not of COX-1) protein abundance in the same sample; however, the levels of COX-2 and VEGF proteins were again markedly attenuated when treated with CGP43182 (Fig. 6). Increased levels of COX-2 RNA and protein correlated with...
Effects of CGP43182 on Cellular Aggregate and Tube Formation

In the presence of CGP43182 during hypoxia, the area occupied by cellular aggregates was reduced from 79,788 ± 8,759 μm² to 21,016 ± 3,415 μm², representing a decrease in cellular aggregate area of 73% (P < 0.01; Fig. 8). Tubular morphogenesis, an indicator of NV, was also significantly inhibited at 72 hours after hypoxia. The mean plus 1 SD for tube lengths was 575 ± 92 μm for hypoxia-treated and 212 ± 32 μm for CGP43182+hypoxia-treated cells, when compared with control cultures. This represented a decrease of 63% in tube length in the presence of CGP43182 (P < 0.01; Fig. 8).

DISCUSSION

The present studies focused on the activation and binding kinetics to DNA of AP-1, a composite dimeric TF implicated in growth control and neurodegeneration\(^{6–9,14–16}\); NF-κB, a known proinflammatory TF and positive regulator of the COX-2 gene in many cells and tissue types\(^{6,9,10,11}\); and HIF-1, a master sensor for intracellular oxygen homeostasis and a key transcriptional regulator of hypoxia-sensitive genes\(^{4,5,7,14,22,27,28}\). These activation events were analyzed by EMSA, a highly sensitive radiolabel-based electrophoretic technique that can monitor real-time TF-DNA interactions in promoter DNA sequences that immediately precede transcription from that gene.\(^{5,8,9,14}\) Proximal promoter DNA sequence analysis (~1100 to +100 bp) showed that HIF-1 and NF-κB occupy three sites centered at −442, −190, and −160 bp in the human COX-2 (but not in the human COX-1) gene promoter.\(^{6,9,12–14}\) The human VEGF promoter (GenBank accession no. AF095785) contains no obvious NF-κB DNA-binding consensus sequence in this 1200-bp region, but it has at least 5 HIF-1 DNA-binding sites centered at positions −920, −440, −360, −205, and −80 bp, relative to the start of transcription at +1\(^{32}\) (Fig. 9). HIF-1- and NF-κB- (but not AP-1) DNA binding increased three- to fourfold after only 1 hour of hypoxia (or IL-1β) treatment (Fig. 1). This suggests that part of the induction of VEGF by NF-κB DNA binding may be indirect and perhaps is mediated by NF-κB DNA activation of the COX-2 promoter, the latter being a known trigger for COX-2 gene transcription.\(^{5–11,29–31}\) This would result in subsequent COX-2 gene induction, followed by the activation of VEGF gene expression through upregulation of COX-2–generated PGs, such as PGE\(_2\), a known potent activator of VEGF activity\(^{14–19,29}\) (Figs. 7, 9). In parallel, COX-2, but not β-actin or COX-1, RNA message was increased by several-fold over controls after only 1 hour of hypoxia (or IL-1β) stimulation. These phenomena were accompanied by increasing levels of VEGF RNA message and protein abundance in the same retinal cell samples, even after 3 hours of hypoxia (Figs. 5, 6). We have reported that tube formation and NV are significantly inhibited (26%–44%) by CGP43182 at 0.1 to 1.0 μM (Ottlecz A, et al. IOVS 2001;43:ARVO Abstract 515). In the present experiments, CGP43182 also inhibited NF-κB DNA binding, COX-2 and VEGF RNA and protein abundance, PGE\(_2\) levels, and tube formation in hypoxia-treated RF/6A cells (Figs. 4–8). These data collectively support the hypothesis that NF-κB is one of the key regulators of cellular aggregation and tube formation leading to pathoangiogenesis and NV in hypoxia (or IL-1β)-stimulated retinal-choroid cells.

Another emerging hypothesis substantiated by the present study is that, like NF-κB, HIF-1 DNA binding is involved in proinflammatory TF signaling and is an important transcriptional activator element in the induction of both the COX-2 and VEGF genes.\(^{6,9,14,24–28,52,53}\) HIF-1 DNA binding increased at 1 and 3 hours after hypoxia in the NPXTs of RF/6A cells, whereas changes in HIF-1 DNA-binding activities were significantly...
lower in the CPXT fractions (Fig. 2). These findings suggest nuclear- and not cytoplasmic-mediated genetic events in HIF-1–regulated gene expression. During related experiments in mixed cultures of human neural progenitor cells in primary culture and over a time course of 1 to 3 hours, the inflammatory cytokine peptide IL-1β was also noted to induce significant NF-κB and HIF-1 DNA binding and COX-2 gene expression. In doing so, it simulated the 1- and 3-hour periods of hypoxia treatment in RF/6A cells (Fig. 4). In further support of this finding, several independent laboratories have recently demonstrated this aspect of HIF-1 activation by hypoxia or IL-1β in human neural primary cells, in human proximal tubular cells, in the human hepatoma cell line HepG2, in human fetal astrocytes, and in cultured rat neonatal cardiac myocytes. In fact, in RF/6A cells, treatment with 10 ng/mL of human recombinant IL-1β simulated the hypoxic effect on HIF-1 and NF-κB DNA binding during the time course of 1 to 3 hours after its application (Fig. 4), and this was also reflected in the upregulation of both COX-2 and VEGF RNA message and protein abundance over the same time course (data not shown).

**Figure 8.** Typical cord and cellular aggregate formation in RF/6A cells. Cells were exposed to 3 hours of hypoxia and then to 72 hours of normoxia. (A) Normoxic control with no exposure to hypoxia, where no tube formation and only a few small cellular aggregates were apparent. Arrowhead: cellular aggregate. The hypoxic condition (B) reflects the typical situation of cellular aggregate and tube formation. Arrow: formed tube. The CGP43182-treated hypoxic condition shown in (C) demonstrates the inhibitory effect of CGP43182 (1 μM) on tube formation. Arrow: small formed tube. Phase-contrast photomicrographs. Magnification, ×100; scale bar, 400 μm.

**Figure 9.** Dual, interdependent mechanisms for COX-2 and VEGF gene activation in hypoxia- or IL-1β–triggered retinal endothelial cells: proposed scheme for the temporal activation of COX-2 followed by VEGF gene activation. The -1100- to +100-bp 5′ flanking region from the immediate COX-2 and VEGF gene promoters were analyzed with GenBank AF276953 and AF095785, respectively. As these and previous studies indicate, there is a large hypoxia- or IL-1β–induced increase in COX-2 gene activation that immediately precedes VEGF gene expression. This fact suggests at least a partial mediation of VEGF expression through prostaglandin (PG), the rapidly synthesized primary catalytic product of COX-2, in addition to VEGF activation by the hypoxia-generated TF HIF-1. Transcription factor IID (TFIID) binding at the TATA box is recognized by the RNA polymerase II basal transcription complex. Actual positions of AP-1, HIF, and NF-κB DNA binding sites may be slightly skewed due to space constraints. A positively acting regulatory element; dashed lines: putative pathways and sites of interaction for CGP43182.
shown). It is therefore becoming increasingly clear that because conditions of hypoxia potently induce IL-1β production and release in a wide variety of cell types, direct addition of IL-1β to the test systems studied would circumvent this first hypoxia- and cytokine-mediated gene activation step.14,34–36,38

Hypoxia-triggered AP-1, HIF-1, and NF-κB DNA binding was also assayed by EMSA in RF/6A cells pretreated overnight with CGP43182. No significant effects were noted in AP-1 or HIF-1 DNA binding; however, NF-κB DNA binding decreased to approximately 50% of that of hypoxia- or IL-1β-treated controls (Fig. 4). These latter results suggest that CGP43182 not only prevents the formation of certain AA metabolites produced by COX-2 and certain lipoxigenases by reducing the release of AA from membrane phospholipid stores, but also suppresses the genetic mechanism involving NF-κB activation. The physiologic roles of COX-2 may not be as simple as previously thought, because COX-2 may possess both pro- and anti-inflammatory properties through the generation of different forms of PGs.8,9,25 As has been widely reported, NF-κB is a major regulator of COX-2 gene transcription,8,10–20,31 whereas cyclopentenone PGs can inhibit NF-κB activation and its nuclear accumulation through the inhibition of IκB kinase.11,20,30. The mode of action of CGP43182 is that it prevents the phosphorylation and proteolytic degradation of the endogenous NF-κB inhibitor (IκB), a process necessary for NF-κB activation.11,20 This results in the cellular persistence of the IκB-NF-κB complex and suppression of NF-κB-sensitive gene activation, as we have demonstrated in these experiments (Figs. 4–6). Antioxidants such as pyrrolidine dithiocarbamate have also been shown to prevent NF-κB activation and in doing so suppress tube and capillary formation both in vitro and in vivo in microvascular endothelial cells and in the mouse retina.29–31 The present data also suggest that CGP43182 antagonizes NF-κB DNA binding without exerting any inhibitory effect on that of HIF-1 under the same treatment conditions. Further, overnight pretreatment of RF/6A cells with CGP43182 produced no significant effects on β-actin or COX-1 RNA message induction in comparison to COX-2 and VEGF RNA message levels after 1 and 3 hours of hypoxia (Fig. 5). Taken together, these data suggest that the anti-tube-forming and antiangiogenic effects of CGP43182 may not be exerted through the direct inhibition of the VEGF gene, but rather through the suppression of NF-κB-driven target gene activation, such as through the COX-2 gene and PGE2 signaling pathways (Figs. 5–7). These in turn appear to modulate VEGF expression negatively at both the RNA and protein levels of VEGF and also restrict the cellular aggregation and tube formation associated with angiogenesis and NV (Figs. 8, 9).

In summary, HIF-1 and NF-κB should both be considered novel targets for antiangiogenic and anti-NV drugs, because disruption of their binding to proangiogenic promoters may selectively diminish the activation of NF-κB- and HIF-1-driven target genes. These data also indicate that the administration of IL-1β is an effective and a more easily controllable trigger, compared with the application of hypoxia, for studies of HIF-1 and NF-κB DNA binding, COX-2 and VEGF gene expression, and PGE2 generation in RF/6A cells. IL-1β may therefore be useful for the simulation of hypoxia-triggered gene signaling alterations in models of retinal NV and thus for testing the efficacy of pharmacologic compounds for the potential management of angiogenesis and NV. Finally, these studies support the idea that VEGF expression may be regulated in part through at least two interdependent mechanisms: one involving HIF-1–VEGF promoter activation directly and the other indirectly through NF-κB–mediated COX-2 gene expression and PGE2 production.

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