Antioxidants Inhibit Interleukin-1-induced Cyclooxygenase and Nitric-oxide Synthase Expression in Rat Mesangial Cells

EVIDENCE FOR POST-TRANSCRIPTIONAL REGULATION*

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Glomerular mesangial cells produce reactive oxygen intermediates when stimulated by interleukin-1 (IL-1) or tumor necrosis factor. Recent observations suggest that reactive oxygen intermediates may play a role in IL-1 and tumor necrosis factor signaling and may up-regulate gene expression. We therefore evaluated the effects of antioxidants on IL-1β-induced cyclooxygenase-2 (Cox-2) and inducible nitric-oxide synthase (iNOS) expression in rat mesangial cells. The oxidant scavenger, pyrrolidine dithiocarbamate (PDTC), inhibited iNOS expression at the transcriptional level, since PDTC abolished iNOS mRNA accumulation. In contrast, PDTC inhibited Cox-2 expression at the post-transcriptional level, since PDTC did not affect IL-1β-induced Cox-2 mRNA levels but inhibited Cox-2 protein expression and prostaglandin E2 production. Another antioxidant, rotenone, which inhibits reactive oxygen intermediate production by inhibiting the mitochondrial electron transport system, did not inhibit IL-1β-induced iNOS and Cox-2 mRNA expression but inhibited iNOS and Cox-2 protein expression, suggesting a post-transcriptional target for the inhibition of iNOS and Cox-2 expression induced by IL-1β. These results suggest that not only transcriptional regulation but also post-transcriptional mechanisms are involved in redox-sensitive inhibition of cytokine induced Cox-2 and iNOS expression. These results suggest a novel approach for intervention in cytokine-mediated inflammatory processes.

Interleukin-1 (IL-1β) is a cytokine which mediates a variety of processes in host defense, such as inflammation and the cellular response to injury (1). During glomerular inflammation, cytokines from infiltrating macrophages and activated mesangial cells may act to sustain and promote glomerular damage. We have previously demonstrated that IL-1β induces cyclooxygenase-2 (Cox-2) and the inducible nitric-oxide synthase (iNOS) with increases in proinflammatory mediators, PGE2 (2) and NO (3), in rat mesangial cells. The molecular signaling mechanisms by which IL-1β induces Cox-2 and iNOS includes transcriptional activation of these genes to produce increased levels of mRNA species which are "unstable." This mRNA is then translated into protein and degraded. These intracellular events are therefore potentially subject to regulation at the transcriptional or post-transcriptional level. Furthermore, the factors which control message stability and translational efficiency are not well understood.

Mesangial cells produce reactive oxygen intermediates (ROI) with stimulation by endotoxin and cytokines, including IL-1 and tumor necrosis factor (4). ROI are produced during various electron-transfer reactions. When generated in excess, ROI can damage cells by peroxidizing lipids and degrading proteins and nucleic acids. However, ROI may exert signaling functions and regulate gene expression at moderate concentrations (5–10). During glomerular inflammation, ROI from activated mesangial cells may act as signaling molecules. We have therefore evaluated the mechanisms by which some antioxidants can influence the expression of the proinflammatory genes, Cox-2 and iNOS when they are up-regulated by the cytokine IL-1β.

EXPERIMENTAL PROCEDURES

Materials—Humam IL-1β (100 half-maximal units/ml) and restriction enzymes were purchased from Boehringer Mannheim. Murine cDNA probes ligated in BlueScript SK- for Cox-1 (pBS-Cox-1) and Cox-2 (pBS-Cox-2) were generous gifts of Dr. Karen Seibert, Monsanto Co. (St. Louis, MO). Pyrrolidine dithiocarbamate (PDTC) and rotenone were from Sigma. Polyclonal antibody against murine Cox-2 was from Cayman Chemical, Ann Arbor, MI. Polyclonal antibody against murine iNOS was a generous gift of Dr. Thomas Misko, Monsanto Co. Double-stranded oligonucleotides specific for the κB sequence and the affinity purified rabbit polyclonal antibody against p50 NF-κB protein were from Santa Cruz Biotechnology, Santa Cruz, CA.

Cell Culture and Treatment—Primary mesangial cell cultures were prepared from male Sprague-Dawley rats as described previously (2). Cells were grown in RPMI 1640 medium supplemented with 15% (v/v) heat-inactivated fetal calf serum, 0.3 IU/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 15 mM HEPES. Cells were used at passages between 2 and 6.

Northern Blot Analysis—The full-length Cox-1 and Cox-2 cDNA was excised from the plasmid pBS-Cox-1 and pBS-Cox-2 as a BamHI-HindIII and KpnI-BamHI digest, respectively. Excised cDNAs were purified from 1% agarose gels by GeneClean (BIO101). Mouse iNOS and rat glyceraldehyde-3-phosphate dehydrogenase cDNAs were prepared using the polymerase chain reaction as described previously (11). Cells grown in RPMI 1640 medium supplemented with 15% (v/v) fetal calf serum for the indicated periods and harvested. Total RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform method (RNA-STAT 60), Tel-Test “B,” Friendswood, TX). 20 μg of total RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred onto nylon membranes (GeneScreen, DuPont), and immobilized with UV cross-linking. cDNAs for Cox-1, Cox-2, and iNOS were radiolabeled with [32P]-dCTP by the random primed labeling method.

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1 The abbreviations used are: IL-1, interleukin-1; Cox, cyclooxygenase; Cox-1, constitutive form of Cox; Cox-2, inducible form of Cox; NO, nitric oxide; NOS, nitric-oxide synthase; PG, prostaglandin; ROI, reactive oxygen intermediates; PDTC, pyrrolidine dithiocarbamate.

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The membrane was hybridized with radiolabeled cDNA probes at 42°C overnight. To control for variability in the loaded quantity of RNA, all membranes were probed with glyceraldehyde-3-phosphate dehydrogenase cDNA to determine the steady state levels of glyceraldehyde-3-phosphate dehydrogenase gene-related sequences and used to normalize the mRNA for Cox-1, Cox-2, and iNOS.

Western Blot Analysis—Confluent cells grown in 25-cm² flasks were preincubated with antioxidants for 1 h in RPMI 1640 containing 5% (v/v) fetal calf serum then stimulated with IL-1β for indicated periods. Cells were washed twice with ice-cold phosphate-buffered saline, and lysed in 1 ml of ice-cold extraction buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 0.6% Nonidet P-40). The membranes were saturated with 5% fat-free dry milk in Tris-buffered saline (50 mM Tris, pH 8.0, 150 mM NaCl) with 0.1% (v/v) Tween 20 (TBS-T) overnight at 4°C. The membranes were then incubated with purified polyclonal rabbit IgG antibody against murine Cox-2 or iNOS at 1:1,000 dilution in the above solution for 1 h at room temperature. Blots were washed four times (15 min each) in TBS-T. Blots were further incubated for 1 h at room temperature with the goat anti-rabbit IgG antibody coupled to horseradish peroxidase (Amersham) at 1:2,500 dilution in TBS-T, followed by four washes (15 min each) in TBS-T before visualization. The enhanced chemiluminescence (ECL) kit (Amersham) was used for detection and exposed to Hyperfilm MP (Amersham).

Measurement of Nitrite and PGE2—The stable metabolite of nitric oxide, nitrite, in the medium was measured by the Griess reaction. PGE2 in the medium was determined by gas chromatography-mass spectrometry. O, basal; A, IL-1β.

Results

PDTC Inhibits IL-1β-induced iNOS Expression at the Transcriptional Level—The effect of an oxidant scavenger, PDTC, on IL-1β-induced NO production and iNOS expression was determined. PDTC inhibited nitrite production and iNOS protein expression in a dose-dependent manner (Fig. 1A and Fig. 2A). Since iNOS mRNA expression peaked at 12 h after IL-1β stimulation, the effect of PDTC on iNOS mRNA was determined at 12 h after IL-1β stimulation. 100 μM PDTC abolished IL-1β-induced iNOS mRNA expression (Fig. 2B). This data suggest that PDTC inhibits iNOS expression at the transcriptional level. Since PDTC has been reported to inhibit NF-κB activation in some cell types (8) and the promoter for the iNOS gene contains the NF-κB binding consensus (12), the effect of PDTC on IL-1β-induced NF-κB activation was determined. Somewhat surprisingly PDTC failed to inhibit IL-1β-induced NF-κB activation in rat mesangial cells at concentrations that inhibit NO and iNOS mRNA expression (Fig. 3), suggesting that PDTC may block the activation of iNOS gene by some other...
PDTC Inhibits IL-1β-induced Cox-2 Expression at the Post-transcriptional Level. The effect of PDTC on IL-1β-induced PGE2 production and Cox expression was determined. PDTC inhibited IL-1β-induced PGE2 production in a dose-dependent manner (Fig. 1B). PDTC did not affect the catalytic activity of Cox from microsomal fractions of rabbit medullary collecting duct (data not shown), indicating that the effect of PDTC is not because of the inhibition of catalytic activity of the Cox enzyme. Since Cox-2 mRNA expression peaked at 3 h after IL-1β stimulation, the effect of PDTC on Cox-2 mRNA was determined at 3 h after IL-1β stimulation. PDTC did not affect IL-1β-induced Cox-2 mRNA expression (Fig. 4A). In contrast, PDTC inhibited IL-1β-induced Cox-2 protein expression in a dose-dependent manner (Fig. 4B). These data suggest that post-transcriptional events are involved in the inhibition of IL-1β induced Cox-2 expression by PDTC.

Effect of Rotenone on Cox-2 and iNOS Expression. Cells were pretreated with rotenone for 1 h and then stimulated with IL-1β (50 units/ml) for 3 h. Nuclear protein was extracted as described under “Experimental Procedures” and used for electrophoretic mobility shift assay. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIG. 3. Effect of PDTC on NF-κB activation. Cells were pre-treated with PDTC for 1 h and then stimulated with IL-1β (50 units/ml) for 30 min. Nuclear protein was extracted as described under “Experimental Procedures” and used for electrophoretic mobility shift assay. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effect of Rotenone on nitrite (A) and PGE2 production (B). Cells were pretreated with rotenone for 1 h and then stimulated with IL-1β (50 units/ml) for 24 h. The stable metabolite of NO, nitrite, in the medium was measured by the Griess reaction. PGE2 in the medium was determined by gas chromatography-mass spectroscopy. ○, basal; ▲, IL-1β.

FIG. 4. Effect of PDTC on Cox-2 mRNA (A) and Cox-2 protein (B) expression. Cells were pretreated with PDTC for 1 h and then stimulated with IL-1β (50 units/ml). Cells were harvested at 3 h for Northern blot analysis (A) and at 24 h for Western blot analysis (B), respectively.

FIG. 5. Effect of rotenone on nitrite (A) and PGE2 production (B). Cells were pretreated with rotenone for 1 h and then stimulated with IL-1β (50 units/ml) for 24 h. The stable metabolite of NO, nitrite, in the medium was measured by the Griess reaction. PGE2 in the medium was determined by gas chromatography-mass spectroscopy. ○, basal; ▲, IL-1β.

FIG. 6. Effect of rotenone on Cox-2 mRNA (A) and Cox-2 protein (B) expression. Cells were pretreated with rotenone for 1 h and then stimulated with IL-1β (50 units/ml). Cells were harvested at 3 h for Northern blot analysis (A) and at 24 h for Western blot analysis (B), respectively. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Do PDTC and Rotenone Affect Protein Stability?—To demonstrate that PDTC and rotenone did not affect protein stability we carried out incubations of mesangial cells with IL-1β at 0, 2, 4, 8, and 24 h in the presence and absence of PDTC, 100 μM, and rotenone, 40 μM. Total cell lysates were harvested in buffer containing Nonidet P-40 (1%) as described previously. Western
blots were then performed for Cox-2 and iNOS. Fig. 9 shows the quantitative densitometry of a mean of two such experiments on the time course of protein expression for Cox-2, Fig. 9A, and iNOS, Fig. 9B. It clearly shows that these drugs simply reduce the magnitude of the increase with no effect on the time to peak expression.

**DISCUSSION**

In this study, we have demonstrated that two mechanistically distinct antioxidants, PDTC and rotenone, inhibit Cox-2 protein expression and PGE₂ production. Inhibition of Cox-2 is likely to be unrelated to alterations in its gene transcription, because neither compounds significantly reduced the level of the Cox-2 gene transcripts. Neither of these compounds directly inhibited the catalytic activity of Cox enzyme in crude microsomal preparations in vitro. These data suggest that these two antioxidants inhibit the Cox-2 expression at a post-transcriptional level.

Recent observations have demonstrated that proinflammatory cytokines, IL-1 and tumor necrosis factor, increase the production of ROI in mesangial cells (4). The mitochondrial electron transport system is one of the major sources for cellular ROI generation. Schultz-Oschoff et al. (10) showed that tumor necrosis factor α-induced cytotoxicity and NF-κB activation were abolished by the mitochondrial electron transport system inhibitor, rotenone. Changes in redox status have been reported to modulate the activation of transcription factors, such as NF-κB (7–10) and AP-1 (5, 6). Thus it is proposed that these cytokines may mediate their effects in part via ROI. However, several lines of evidence suggest that the redox status of the cell can affect post-transcriptional events in the cell (13–15).

Redox status has been shown to regulate mRNA stability and modulate translation in a cell free system (13–15). Our data suggests that similar post-transcriptional events might be involved in the regulation of Cox-2 expression in vivo. Cox-2 and iNOS mRNA have “AUUUUA” motifs in their 3’-untranslated regions (16, 17). This AU-rich element has been considered to be a mRNA instability determinant. We have shown that IL-1β stabilizes the Cox-2 message by phosphorylation of cytosolic factors which bind to the AUUUUA-rich 3’-untranslated region in rat mesangial cells (18). Furthermore, some of these AU-rich motif binding factors are known to be redox-sensitive (15). However, changes in stability of Cox-2 mRNA may not account for the effect of the antioxidants PDTC and rotenone, since the steady state levels of Cox-2 transcripts were not different in the control cells (IL-1β treated) and the antioxidant-treated (IL-1β plus antioxidant) cells. Inhibition of translation is more consistent with our observations, since the antioxidants did not inhibit Cox-2 mRNA expression but inhibited Cox-2 protein expression. Eukaryotic translation is regulated by many eukaryotic initiation factors and RNA binding proteins. One of the eukaryotic initiation factors, eukaryotic initiation factor-2, changes its function with redox status as well as phosphorylation (14). Redox status is also known to regulate the RNA-protein interaction of the iron-responsive element binding protein, which binds to the 5’-untranslated region of ferritin mRNA and 3’-untranslated region of the transferrin receptor mRNA. These cellular events control the translation of ferritin mRNA and stability of the transferrin receptor mRNA (13). Thus the change in redox status might regulate directly or indirectly some eukaryotic initiation factors and/or RNA binding proteins which regulate the translation and/or stability of Cox-2 mRNA.

**FIG. 7.** Effect of rotenone on iNOS mRNA (A) and iNOS protein (B) expression. Cells were pretreated with rotenone for 1 h and then stimulated with IL-1β (50 units/ml). Cells were harvested at 12 h for Northern blot analysis (A) and at 24 h for Western blot analysis (B), respectively.

**FIG. 8.** Effects of PDTC and rotenone as general inhibitors of translation. In vitro translation was carried out with rabbit reticulocyte lysate. Lane 1 shows no RNA added; lane 2 standard RNA from kit; lanes 3–5, PDTC at concentrations indicated under “Experimental Procedures”; and lanes 6 and 7, rotenone.

**FIG. 9.** Effect of PDTC and rotenone on the temporal expression of Cox-2 and iNOS mesangial cells were incubated with IL-1β with and without PDTC and rotenone for the times indicated. Immunoblots were performed on lysates and probed for Cox-2 (A) and iNOS (B). This figure shows the results of quantitative densitometry performed on the blots.
In the case of iNOS, PDTC and rotenone appeared to inhibit IL-1 induced iNOS expression by the different mechanisms. PDTC inhibited iNOS expression at the transcriptional level, since PDTC inhibited iNOS mRNA accumulation. However, we cannot exclude the possibility that PDTC also affects translational efficiency of the iNOS gene since the drug produced a reduction in iNOS mRNA. It has been demonstrated that PDTC inhibits NF-κB activation in many cell types and that the promoter for the iNOS gene has a κB binding consensus. However, consistent with the recent observation by Rovin (19), our data demonstrates that PDTC does not inhibit IL-1β-induced NF-κB activation in mesangial cells at concentrations which inhibit PGE2 and NO formation. Thus PDTC appeared to inhibit the iNOS mRNA expression by some other unknown mechanisms. In contrast to PDTC, rotenone inhibited iNOS expression at a post-transcriptional level, since it did not inhibit iNOS mRNA expression but suppressed iNOS protein expression.

In summary, we have shown that two mechanistically different antioxidants, PDTC and rotenone, inhibit IL-1β-induced Cox-2 and iNOS expression in rat mesangial cells. Our data suggests that a change in cellular redox status may influence gene expression at multiple levels which include transcription and post-transcriptional events. Neither PDTC nor rotenone appeared to be general inhibitors of translation (Fig. 8) but their effects appeared to be restricted to a subset of messages which include Cox-2 and iNOS. Furthermore, the effects of these agents was simply to reduce the magnitude of the increase of protein expression with an effect on the time to peak expression. These results may suggest a potentially novel mechanistic approach to therapeutically intervene and downregulate the biologic effects of the proinflammatory genes, iNOS and Cox-2, in glomerular inflammation. The observation that the 3'-untranslated region of many "unstable" messages carry motifs that may regulate translational efficiency by reversible binding of cytosolic or nuclear factors raise the possibility that some antioxidants may influence, either directly or indirectly, these RNA-protein interactions.

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