Inhibition of NFκB activation with antioxidants is correlated with reduced cytokine transcription in PTC

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Rangan, Gopala K., Yiping Wang, Yuet-Ching Tay, and David C. H. Harris. Inhibition of NFκB activation with antioxidants is correlated with reduced cytokine transcription in PTC. Am. J. Physiol. 277 (Renal Physiol. 46): F779–F789, 1999.—We recently reported that inhibition of the transcription factor nuclear factor-κB (NFκB) with pyrrolidinedithiocarbamate (PDTC) reduced interstitial monocyte infiltration in rats with proteinuric tubulointerstitial disease, whereas N-acetylcysteine (NAC) was not effective. Here we investigate the effects of antioxidants (PDTC, NAC, and quercetin) on NFκB activation and cytokine transcription in primary cultured rat proximal tubular epithelial cells (PTC) stimulated with lipopolysaccharide. Antioxidant-mediated inhibition of NFκB activation (PDTC, 20–100 µM; NAC, 100 mM; and quercetin, 50 µM) diminished the induction of both pro-inflammatory cytokines IL-1β, tumor necrosis factor-α, monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1α, and MIP-2, and anti-inflammatory (IL-10, transforming growth factor-β1) cytokine transcription in PTC (RT-PCR analysis). PDTC and quercetin did not affect PTC viability, but NAC (100 mM) caused a threefold increase in lactate dehydrogenase leakage (P < 0.001). We conclude that NAC is unable to suppress NFκB activation in PTC at subtoxic and physiologically relevant concentrations. Furthermore, antioxidant-mediated inhibition of NFκB is correlated with the nonselective reduction of cytokine transcription in activated tubular cells. These data might explain the protective effects of PDTC-mediated NFκB inhibition in tubulointerstitial disease in vivo.

N-acetylcysteine; pyrrolidinedithiocarbamate; quercetin; primary culture; osteopontin; proximal tubular cells; nuclear factor-κB

THE ACCUMULATION of macrophages within the interstitial space of the renal cortex plays a pathogenic role in the development of tubular injury and interstitial fibrosis in progressive chronic renal diseases (47). Proximal tubular epithelial cells (PTC) are thought to mediate the interstitial macrophage infiltration because of their anatomic proximity and ability to produce chemotactic cytokines and other proinflammatory mediators (36).

In PTC, the ubiquitous proinflammatory transcription factor nuclear factor-κB (NFκB) has a pivotal role in the regulation of chemokines [cytokine-induced neutrophil chemoattractant (CINC), regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein (MCP)-1] (33, 49, 52) and adhesion molecules [intercellular adhesion molecule (ICAM)-1 and vascular cellular adhesion molecule-1] (32). In other cell types, NFκB is known to regulate the production of other cytokines [such as interleukin (IL)-1β and tumor necrosis factor (TNF)α], chemokines [such as macrophage inflammatory protein (MIP)-1α and MIP-2], and many early response genes (2).

The antioxidants pyrrolidinedithiocarbamate (PDTC), N-acetylcysteine (NAC), and quercetin inhibit NFκB activation in a variety of cells, possibly by suppressing the production of intracellular reactive oxygen species (2, 41, 42, 46). In murine and porcine (LLC-PK1) PTC lines, inhibition of NFκB by PDTC reduced the expression of inducible nitric oxide synthase (iNOS) and RANTES, respectively (1, 52). NAC also suppressed NFκB activation in LLC-PK1 cells after stimulation with cytokine S-conjugates (35). However, in activated rabbit alveolar macrophages, PDTC increased TNF-α despite inhibition of NFκB (6), and in an adenocarcinoma cell line, NAC paradoxically increased NFκB (11). Together, these data suggest that antioxidants are cell specific in their ability to inhibit NFκB and in the cytokines that are modulated.

Less studied are the effects of antioxidants on the expression of cytokines with macrophage deactivating properties, such as IL-10 and transforming growth factor (TGF)-β1 (3). In vitro, in human monocytes, PDTC reduced lipopolysaccharide (LPS)-induced IL-10 secretion and increased TNF-α production (23). In contrast, in vivo, in rats with LPS-induced endotoxemia, PDTC increased in vivo plasma levels of IL-10 but suppressed TNF-α, IL-12, MIP-1α, and nitric oxide production and had no effect on IL-1α, IL-6, and interferon-γ induction (31). In another study, diethylidithiocarbamate (a related dithiocarbamate) did not affect the increase in transcription of TGF-β1 in the postischemic myocardium of rats but reduced IL-1β, IL-6, TNF-α, and iNOS together with NFκB DNA-binding activity (7). The differential modulation of pro- and anti-inflammatory cytokine transcription by antioxidant-mediated NFκB inhibition may explain the cytoprotective effects of dithiocarbamates in both models (7, 31).

Recently, we reported that PDTC reduced renal cortical NFκB activation, tubular injury, and interstitial monocyte infiltration in rats with doxorubicin-induced chronic glomerular disease (39). In contrast, NAC had no significant effect on these parameters. By immunohistochemical staining, tubular cells are the predominant cellular source of activated NFκB (p50) in this model (unpublished observations). Therefore, we hypothesized that antioxidants (NAC, PDTC, and quer-
INHIBITION OF NFkB ACTIVATION WITH ANTIOXIDANTS

METHODS

Isolation and primary culture of rat PTC. PTC were isolated and cultured from normal male Wistar rats with the use of isoenzymic centrifugation, as previously described (8, 48, 49). The cells were grown on plastic tissue culture dishes (coated with rat tail collagen) in DMEM supplemented with epidermal growth factor (10 ng/ml), insulin (5 µg/ml), transferrin (5 mg/ml), and hydrocortisone (5 x 10^-8 M) in a 5% CO2 atmosphere at 37°C. The medium was supplemented with 5% FCS for the first 24 h, after which point it was replaced with serum-free media. The cells formed dome structures in culture, confirming their PTC origin, as described previously (8, 48, 49). The cells were grown on plastic tissue culture dishes (coated with rat tail collagen) in DMEM supplemented with epidermal growth factor (10 ng/ml), insulin (5 µg/ml), transferrin (5 mg/ml), and hydrocortisone (5 x 10^-8 M) in a 5% CO2 atmosphere at 37°C. The medium was supplemented with 5% FCS for the first 24 h, after which point it was replaced with serum-free media. The cells formed dome structures in culture, confirming their PTC origin, as described previously (8, 48, 49).

Experiments were commenced when cells reached confluence, ~4–5 days after isolation and initial plating.

Experimental protocol. LPS (Escherichia coli, serotype 026:B6; Sigma-Aldrich, Sydney, Australia) was used as a stimulant of NFkB activation in PTC (1, 49). Confluent cultures of PTC were incubated with LPS (5 µg/ml) or vehicle, with or without the antioxidants (NAC, PDTC, or quercetin), and harvested after 8 h for analysis of NFkB activation, cytokine gene transcription, and cell viability (48, 49). The antioxidants were added 1 h before LPS and were continued until the end of the experiment. In some experiments, catalase (1,000 units/ml; human erythrocyte source), desferrioxamine (DFO; 200–800 µM), and hydrogen peroxide (H2O2; 200 µM) were added 1 h before the addition of NAC without LPS.

The antioxidants were dissolved in media, filter sterilized, and prepared immediately before the experiment. The pH of NAC in the medium solution was 2.4, and this was corrected to pH 7.4 with 6 M sterile sodium hydroxide. The concentrations of NAC (5–100 mM), PDTC (5–100 µM), quercetin (2–50 µM), and the other agents were determined from previous reports (1, 11, 17, 30, 35, 41, 42, 46, 50, 52) and pilot studies.

Preparation of nuclear protein extracts. Nuclear proteins were extracted from PTC with the use of methods described by Dignam et al. (12) with minor modification (49). Approximately 1 x 10^6 cells were washed in PBS and then scraped from culture plates and transferred to microcentrifuge tubes. The cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl2·6H2O, and 0.1 mM EDTA) containing a cocktail of protease inhibitors (0.5 mM dithiothreitol (DTT), 0.1 mM pepstatin A, 1 µM phenylmethylsulfonyl fluoride (PMSF), 0.05 µg/mi leupeptin, and 0.01 mM aprotonin) and lysed by 10 even strokes of a glass-Teflon homogenizer. Successful release of nuclei was checked by phase-contrast microscopy. The mixture was centrifuged for 6 min at 6,000 rpm at 4°C, and the resultant pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% (vol/vol) glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.01 mM KCl, and 300 mM NaCl) and incubated on ice for 30 min. After the addition of buffer D (20 mM HEPES, 19% glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF), the mixture was centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant (containing nuclear protein extract) was removed and placed in a separate tube. The protein concentration was determined by the Bradford method (Bio-Rad) (5) and stored in diluted aliquots (3 µg/µl) at -70°C.

Electrophoretic mobility shift assay. Double-stranded oligonucleotide consensus for the NFkB binding site of the k-immunoglobulin light-chain gene (5’-AGT TGA GGG GAC TTT CCC AGG-3’; Promega, Madison, WI) was end-labeled with [γ-32P]ATP (Amersham Life Science, Sydney, Australia) with the use of T4 kinase (Promega, Sydney, Australia). Unincorporated label was removed with a G-50 Sephadex spin column. The binding reaction was performed for 30 min at room temperature and contained 5 µg of nuclear protein, 2 µl of binding buffer (5 mM MgCl2, 50 mM Tris·HCl, 250 mM NaCl, 20% glycerol, 2.5 mM EDTA, 2.5 mM DTT, and 0.25 µg/ml poly[d(l-dC)], 1 µl of 32P-labeled NFkB probe (5,000 counts/min, Cerenkov counting), and distilled water (DW) to a total volume of 10 µl. The DNA-protein complexes were resolved by electrophoresis on a 10 x 12-cm, 7% polyacrylamide gel (1 x TBE buffer; TBE is 89 mM Tris base + 89 mM boric acid + 2 mM EDTA). The gel was run at 10 V/cm for 60 min and then dried onto filter paper under vacuum with a gel dryer (80°C for 1 h). Autoradiographs were prepared by exposing the dried gel to X-ray film (Hyperfilm HP film; Amersham Life Science) with an intensifying screen for 3–6 h at -70°C (49).

RT-PCR. Total RNA was extracted from cell monolayers with the use of a one-step phenol-guanidinium isothiocyanate procedure based on the method of Chomczynski and Sacchi (9), using RNAzol B (Teltest, Friendswood, TX). The total RNA concentration was determined by absorbance at 260 nm with the use of a spectrophotometer (Beckman DU-68; Beckman Instruments, Fullerton, CA).

First-strand complementary DNA (cDNA) synthesis was performed in a 20-µl reaction containing 1 µg of total RNA, 50 U murine leukemia virus reverse transcriptase, 2.5 µM oligo(dT)12-18, 2 µl of 10× PCR buffer II (100 mM Tris·HCl and 500 mM KCl), 1 µl of 25 mM MgCl2 (Perkin-Elmer, Melbourne, Australia), 1 µM dNTP and dithioerythritol (DEPC)-treated water. The reaction was performed at 25°C for 10 min, 42°C for 90 min, and 99°C for 5 min with the use of a thermocycler (PTC-100; MJ Research, Boston, MA). Two negative controls were included with all reverse transcription reactions (total RNA replaced with DEPC-treated water, and reverse transcriptase replaced with DEPC-treated water containing RNA). The resultant cDNA was diluted to 100 µl with DW and stored at -20°C. Two or four percent of the diluted cDNA was amplified by PCR with the use of primers specific for rat GAPDH or cytokine cDNA, respectively (Table 1). The PCR reactions were performed in a final volume of 50 µl and contained cDNA, 0.2 mM dNTP, 1.5 mM MgCl2, 0.4 µM each of upstream and downstream primers, 1.5 U thermostable DNA polymerase (red hot Taq DNA polymerase; Advanced Biotechnologies, Surrey, UK), 75 U Tris·HCl, 20 mM (NH4)2SO4, 0.01% (vol/vol) Tween 20, 1.5 mM MgCl2 (Advanced Biotechnologies, Surrey, UK) and DW. Two-step cycling program was used and consisted of the following: initial template melting step at 94°C for 3 min, denaturation at 94°C for 30 s, annealing and extension at 60–68°C (depending on the primer pair; see Table 1) for 1 min and 30 s, and final extension at 72°C for 5 min.

The PCR products (20% of the amplified product) were analyzed by agarose gel electrophoresis (1.6% in 1 x TAE buffer) and visualized by ethidium bromide staining (0.5 µg/ml) under ultraviolet light. The gels were photographed with a camera, using positive/negative film (type 665; Polaroid, Cambridge, MA). The negatives were scanned by a laser densitometer (Molecular Dynamics, Sunnyvale, CA) with the use of image analysis software (ImageQuaNT, Applied Biosystems).
Table 1. Sequences of upstream and downstream rat primers for RT-PCR analysis

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<th>Reference</th>
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<td>26</td>
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<td>24</td>
<td>328–846</td>
<td>15</td>
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<tr>
<td>TNF-α</td>
<td>5'-cag aac tcc agg ggt tgt ct 3'-cga atc gtc tga cgg tgt g</td>
<td>64</td>
<td>33</td>
<td>73–485</td>
<td>14</td>
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<td>5'-agt ggt ctg ctc tgt cac gc 3'-aag tgt tga acc agg att cac a</td>
<td>64</td>
<td>32</td>
<td>39–633</td>
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<td>64</td>
<td>32</td>
<td>39–633</td>
<td>48</td>
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<td>139–484</td>
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IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TGF, transforming growth factor; T*, optimal annealing temperature; C–, no. of PCR cycles for linear amplification; position, position of amplified PCR product according to known cDNA sequences.
Effect of antioxidants N-acetylcysteine (NAC), propylthiouracil (PTU), and quercetin on LPS-induced NF-κB activation in rat proximal tubular epithelial cells (PTC) was determined by EMSA. PTC were treated with LPS (5 µg/ml) for 8 h, with or without NAC (5, 20, and 100 µM), PTU (5, 20, and 100 µM), or quercetin (50 µM). The incubation of PTC with NAC (5–20 mM) and quercetin (5–100 mM) before LPS completely prevented LPS-induced activation of NF-κB (data not shown). Activation of NF-κB in a dose-dependent manner (Fig. 1, lane 2) increased NF-κB binding (EMSA) in nuclear extracts of PTC (Fig. 1, lane 2). The specificity of NF-κB EMSA was demonstrated in competition experiments. Incubation of nuclear extracts from LPS-stimulated PTC were also included with a 100-fold excess of unlabeled consensus for activation protein-1 (AP-1, lanes 3–5), LPS (5 µg/ml, lane 1), NF-κB (lanes 2, 4). After 8 h, nuclear protein was extracted and an electrophoretic mobility shift assay (EMSA) was performed using a consensus 32P-labeled NF-κB DNA-binding oligonucleotide. Results are representative of three independent experiments. The specificity of the NF-κB EMSA was demonstrated in competition experiments by the addition of unlabeled consensus for NF-κB (Fig. 1, lane 1) or a 100-fold excess of an irrelevant labeled oligonucleotide consensus for activator protein-1 (AP-1, lane 12). A faster migrating band could represent specific binding by a non-NF-κB transcription factor. The specificity of the NF-κB EMSA was also confirmed by supershift analysis (49). The slower migrating band represents specific binding by a non-NF-κB transcription factor. The specificity of the NF-κB EMSA was also confirmed by supershift analysis (49). The slower migrating band represents specific binding by a non-NF-κB transcription factor. The specificity of the NF-κB EMSA was also confirmed by supershift analysis (49). The slower migrating band represents specific binding by a non-NF-κB transcription factor. The specificity of the NF-κB EMSA was also confirmed by supershift analysis (49). The slower migrating band represents specific binding by a non-NF-κB transcription factor.
The intra-assay coefficient of variation of RT-PCR amplification followed by agarose gel electrophoresis and densitometry was <10% (Fig. 2C). Negative PCR and RT controls that accompanied the PCR reactions produced no bands on agarose gel electrophoresis (data not shown). The housekeeping gene GAPDH was constitutively expressed in all experimental groups (Fig. 3). In resting PTC, there was weak expression of IL-1β mRNA, which increased 29-fold after exposure to LPS (5 µg/ml) for 8 h (P < 0.001; Figs. 3 and 4A). NAC, PDTC, and quercetin alone had no significant effect on IL-1β transcription in

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<th>LPS</th>
<th>NAC (mM)</th>
<th>PDTC (µM)</th>
<th>Quercetin (µM)</th>
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<td>100 20 5 100</td>
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<td>GAPDH</td>
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<td>IL-1beta</td>
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<td>Osteopontin</td>
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</tr>
<tr>
<td>IL-10</td>
<td>1 2 3 4 5 6 7 8</td>
<td>9 10 11 12 13 14 15 16</td>
<td>182-2 bp</td>
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Fig. 2. Validation of method to semiquantitate cytokine gene transcription in rat PTC by RT-PCR analysis (10, 21). Total RNA was extracted from PTC stimulated with LPS (5 µg/ml) for 8 h, and RT-PCR was performed with primers specific for rat monocyte chemoattractant protein (MCP)-1. PCR products were visualized by electrophoresis on an ethidium bromide-stained (0.5 µg/ml) 1.6% agarose gel and photographed under ultraviolet light illumination with uniform exposure times. Volume density of band was quantitated by densitometry of negative film. Positive and negative film are shown at top, and numerical values for volume density (in arbitrary units) are plotted on graphs at bottom. A: serial dilutions of total RNA (62.5–4,000 ng) were reverse transcribed and amplified by PCR for 32 cycles. B: determination of optimal cycle number for PCR amplification with the use of 1 µg of total RNA. C: total RNA (1 µg) from a sample (L1) was divided into 8 portions and underwent RT and PCR amplification (32 cycles) in separate tubes to determine intra-assay coefficient of variation (standard deviation divided by mean: L1 = 7.03/171.60 = 4.1%).

Fig. 3. Effect of NAC, PDTC, and quercetin on LPS-induced cytokine transcription in rat PTC. RT-PCR was performed, using specific primers for GAPDH, proinflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and osteopontin], chemokines [MCP-1, macrophage inflammatory protein (MIP)-1α, and MIP-2], and anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF)-β1]. PCR products were analyzed by 1.6% agarose gel electrophoresis stained with ethidium bromide. Lanes 1–2, control; lanes 3–4, LPS; lanes 5–7, NAC+LPS; lane 8, NAC+vehicle; lanes 9–11, PDTC+LPS; lane 12, PDTC+vehicle; lanes 13–15, quercetin+LPS; lane 16, quercetin+vehicle. Molecular size (in bp) of PCR products is shown at right. PCR reactions not containing cDNA or RT reactions not containing RNA or reverse transcriptase produced no bands (data not shown). Results are representative of 3 separate experiments.
unstimulated cells (P = 0.14, by 1-way ANOVA). Exposure of PTC to 100 mM NAC 1 h before LPS reduced IL-1β induction almost to the level of control cells (P = 0.006 compared with LPS alone). Lower doses of NAC (5 and 20 mM) partially increased IL-1β transcription (P = 0.01 and 0.04, respectively) in LPS-stimulated cells. Similarly, PDTC (20 and 100 µM) dramatically reduced IL-1β induction in LPS-treated PTC (P = 0.005 and 0.003, respectively). Likewise, quercetin (50 µM only) also reduced IL-1β induction (P = 0.003). Concentrations of PDTC (5 µM) and quercetin (10 µM) that did not inhibit NFκB activation had no effect on the increase in IL-1β in LPS-stimulated PTC (Figs. 3 and 4A).

TNF-α mRNA was not detectable in control cells but was strongly induced after stimulation with LPS (P = 0.003; Figs. 3 and 4B). Incubation of PTC with antioxidants alone had no effect on the basal transcription of TNF-α. The induction of TNF-α by LPS was completely abolished by 100 mM NAC (P = 0.004) and 20 and 100 µM PDTC (P = 0.004 and 0.002, respectively) and was partially reduced by 50 µM quercetin (P = 0.012). Lower doses of the antioxidants were not effective, and, in cells treated with 20 mM NAC and LPS, TNF-α was increased slightly (P = 0.035; Fig. 4B).

The cytokine osteopontin is chemotactic for macrophages and may play an important role in the pathogenesis of tubulointerstitial disease in chronic glomerular disease, but it is not known to be regulated by NFκB (2, 40). Osteopontin mRNA was constitutively expressed in resting PTC (Figs. 3 and 4C). The expression of osteopontin was not altered by LPS (P = 0.14), antioxidants alone (P = 0.18, by 1-way ANOVA), or a combination of the antioxidants and LPS (P = 0.24, by 1-way ANOVA) compared with the control group (Fig. 4C).

LPS significantly increased the transcription of the chemokines MCP-1 (9-fold), MIP-1α (not detectable in control cells), and MIP-2 (43-fold) (all P < 0.001 compared with unstimulated PTC; Figs. 3 and 5, A–C). Prophylactic treatment with any of the three antioxidants at NFκB inhibitory concentrations only prevented the increase in MCP-1, MIP-1α, and MIP-2 (all P < 0.05 compared with LPS alone; Figs. 3 and 5A).

LPS caused a twofold increase in the transcription of TGF-β1 in PTC, but this did not reach statistical significance (P = 0.08; Figs. 3 and 6A). Antioxidants alone had no significant effect on the basal expression of TGF-β1 mRNA (P = 0.09, by 1-way ANOVA). In LPS-treated cells, both 20 and 100 mM NAC reduced the transcription of TGF-β1 (P = 0.023 and 0.009, respectively). In contrast to NAC, PDTC and quercetin suppressed TGF-β1 only at concentrations that reduced NFκB activation (PDTC, 20 and 100 µM, P = 0.035 and 0.027; quercetin, 50 µM, P = 0.018) (Fig. 6A).

IL-10 mRNA was not detectable by RT-PCR in resting PTC or those exposed to the antioxidants (Figs. 3 and 6B), whereas it was strongly induced by LPS (P < 0.001). The induction of LPS was reduced by prophylactic treatment with 100 mM NAC (P = 0.005) and 20 and 100 µM PDTC (P < 0.005 for both). Quercetin reduced IL-10 induction at all concentrations, including those that did not inhibit LPS-induced NFκB activation (P = 0.003, 0.04, and 0.023 for 2, 10, and 50 µM, respectively; Fig. 6B).

Effect of LPS, NAC, PDTC, and quercetin on PTC viability. To assess whether the doses of LPS or those of the antioxidants affected cell viability, the effect of LPS, NAC, PDTC, and quercetin on LDH leakage was determined. LDH leakage was similar to that in control cells after an 8-h exposure to LPS (5 µg/ml), PDTC (100 µM), or quercetin (50 µM) alone (Fig. 7). In contrast, NAC
(100 mM) increased LDH leakage up to threefold. The increase in LDH leakage was dose dependent, starting at 20 mM ($P = 0.004$) and peaking at 100 mM ($P < 0.001$). Because NFκB inhibition can unmask TNF-α-induced cytotoxicity in LLC-PK1 cells (51), LDH leakage was also assessed in LPS-stimulated PTC treated with each of the antioxidants. LDH leakage in LPS-stimulated PTC exposed to either PDTC (100 µM) or quercetin (50 µM) for 8 h was the same as that of control cells (data not shown).

Transmission electron microscopy was undertaken to determine the clinical significance of the increased LDH leakage in NAC-treated cells. Cells exposed to vehicle alone had normal PTC morphology with numerous mitochondria and abundant microvilli (Fig. 8). Very occasionally, cells in the vehicle-treated group had evidence of cellular damage and necrosis. The ultrastructure of PTC exposed to NAC (100 mM), PDTC (100 µM), or quercetin (50 µM) for 8 h was the same as that of control cells (data not shown).

**Effect of H$_2$O$_2$, DFO, and catalase on NAC-induced LDH leakage.**

NAC can undergo metal-catalyzed autooxidation, resulting in the formation of superoxide...
anion and H$_2$O$_2$. H$_2$O$_2$ can then cause cellular damage directly or lead to the formation of ·OH via the Haber-Weiss and Fenton reactions (17, 30, 45). Therefore, we investigated the effect of catalase and DFO on NAC-induced LDH leakage. Exposure of PTC to catalase (1,000 U/ml) or DFO (200 µM) alone did not induce LDH leakage (Fig. 8). This dose of catalase has previously been reported to attenuate thiol-induced autooxidation in other cell types (30), and we have previously shown that 200 µM DFO can prevent iron-induced toxicity in PTC (8). Treatment of PTC with either DFO or catalase 1 h before NAC did not affect NAC-induced LDH leakage (Fig. 9). A higher dose of DFO (800 µM) was also not effective (14.2 ± 2.7%; P value not significant compared with NAC, 100 mM alone). A higher dose of catalase alone (5,000 U/ml) increased LDH leakage in PTC compared with control cells (4.1 ± 0.2 vs. 2.9 ± 0.2% in control cells; P < 0.05). Also, neither catalase (1,000 U/ml) nor DFO (200 µM) was able to prevent LDH leakage induced by a lower dose of NAC (20 mM) (data not shown).

Due to the fact that catalase may not penetrate cellular membranes easily because of its molecular size, we sought to determine the effect of exogenous peroxide on PTC. Exogenous H$_2$O$_2$ can accelerate thiol oxidation and potentially worsen cellular cytotoxicity induced by NAC (30). The addition of H$_2$O$_2$ (200 µM) alone at doses that increase intracellular peroxide levels to those achieved after treatment with NAC (11) did not increase LDH leakage in PTC (Fig. 9). However, paradoxically, the addition of H$_2$O$_2$ with NAC (100 mM) in PTC reduced NAC-induced LDH leakage (P = 0.046). This effect was not inhibited by the addition of catalase (1,000 U/ml) (Fig. 9).

**DISCUSSION**

This study has investigated the effects of three structurally diverse antioxidants (NAC, PDTC, and quercetin) on NFκB activation, cytokine transcription, and cell viability in rat PTC in primary culture. Similar studies (1, 33, 35, 50, 52) were performed in cell lines, and the differential effects of antioxidant-mediated NFκB inhibition on cytokine transcription were not known. The results of the present paper show that both PDTC and quercetin are potent suppressors of NFκB activation, whereas NAC is ineffective at subtoxic concentrations. Furthermore, and contrary to our original hypothesis, antioxidant-mediated inhibition of NFκB was associated with the reduction of both pro-(IL-1β, TNF-α, MCP-1, MIP-1α, and MIP-2) and anti-inflammatory (TGF-β1 and IL-10) cytokine transcription.

Quercetin is a prototypical polyphenolic plant flavonoid that has potent antioxidant and anti-inflammatory effects (20, 24, 41, 44). On the basis of the ability of quercetin to suppress NFκB activation in nonrenal cells (41), we predicted that it would have similar effects in PTC. In vitro, quercetin prevented cisplatin-induced cellular injury in LLC-PK1 cells (20). In vivo, in rats with acute renal ischemia, the prophylactic administration of quercetin prevented tubular injury and the upregulation of chemokines (MCP-1 and RANTES) in the renal cortex (44). Data from the present study suggest that the mechanism of these effects could, at least in part, involve the suppression of NFκB activation and the reduction of cytokine and chemokine transcription in PTC. Further studies are needed to determine whether the continuous administration of quercetin could attenuate the chronic upregulation of NFκB in renal tubulointerstitial disease in vivo (29, 39).

Consistent with recent data reported by Woods and colleagues (50), our results showed that at least 100 mM NAC was required to suppress NFκB activation in
tubular epithelial cells. The reasons why NAC is a less potent inhibitor of NFκB in PTC than either PDTC or quercetin are not certain. In a previous study (39), we found that NAC (150 mg/kg twice daily ip injection for 14 days) was not able to attenuate renal NFκB activation and tubulointerstitial injury in rats with doxorubicin-induced nephrosis. The treatment regimen used in the latter study may have resulted in a peak plasma concentration of NAC between 3 and 15 mM (4, 45). Higher doses were limited by systemic toxicity in nephrotic rats (39). Although it is not possible to directly extrapolate the findings of the present in vitro study, the results suggest that the failure of NAC to inhibit renal NFκB activation in vivo may have been because therapeutic concentrations were not attained in the kidney cortex.

The mechanisms by which PDTC, quercetin, and NAC suppress NFκB activation in PTC are not known. Although all three agents are antioxidants, recent evidence suggests that this property may not be responsible for their ability to inhibit NFκB in tubular epithelial cells (50). Paradoxically, the prooxidant and metal-chelating properties of PDTC could be involved in its ability to inhibit NFκB (37). In this regard, PDTC appears to act catalytically with micromolar amounts to cause the oxidation of several hundred molar equivalents of intracellular glutathione (37). The latter may explain the steep concentration gradient of PDTC-induced NFκB inhibition. Similarly, the suppression of protein tyrosine kinases and protein kinase C could play an important role in quercetin-induced NFκB inhibition (24).

Having demonstrated that NAC, PDTC, and quercetin suppressed NFκB activation in PTC, we next examined their effects on cytokine gene transcription. The promoter regions of several proinflammatory cytokine genes contain binding sites for NFκB (2). We have recently shown that the 5′-flanking end of the rat MCP-1 gene (derived from the genomic DNA of PTC) contained at least two putative binding sites for NFκB (49). Nevertheless, it cannot be assumed that NFκB plays an essential role in the transcription of these cytokines (1, 6). For example, in PTC stimulated with LPS, NFκB was necessary but not sufficient for induction of the iNOS gene (1). In the present study, activation of NFκB was associated with the upregulation of IL-1β, TNF-α, MCP-1, MIP-1α, and MIP-2 transcription. In contrast, the latter were suppressed by prophylactic treatment with the antioxidants, but only at NFκB inhibitory concentrations. These data suggest that NFκB is likely to have a positive role in the transcriptional regulation of these cytokines in PTC, as demonstrated for CINC and RANTES (35, 52).

In contrast to other proinflammatory cytokines and chemokines, osteopontin is not known to be regulated by NFκB (2, 40). Consistent with this hypothesis, the modulation of NFκB DNA binding activity in LPS-stimulated PTC treated with or without the antioxidants did not affect the basal transcription of osteopontin. Our data also provide direct confirmation of the results reported by Madsen et al. (22), who showed that LPS did not increase osteopontin expression in PTC in vivo. Because osteopontin is increased in experimental models of proteinuric renal disease (40), including that induced by doxorubicin hydrochloride (unpublished observation), its persistent expression may explain why interstitial monocyte infiltration was only partially reduced by PDTC-mediated NFκB inhibition in vivo (39).

Activation of NFκB with LPS in PTC was correlated with an increase in IL-10 and TGF-β1 transcription, cytokines with macrophage-deactivating properties (3). Whereas the posttranslational activation of the latent form of TGF-β1 is regulated by NFκB (25), neither IL-10 nor TGF-β1 transcriptions are known to be directly controlled by NFκB in rats (2). An unexpected result of this study, therefore, was that antioxidant-mediated inhibition of NFκB was associated with reduced IL-10 and TGF-β1 transcription. These data suggest that in rats, the transcriptional control of IL-10 and TGF-β1 could directly or indirectly involve NFκB.

In support of this possibility, the mouse IL-10 gene was found to have three NFκB-like binding sites (20). In addition, in monocytes, the induction of IL-10 by LPS is mediated by the autocrine effects of TGF-α (23), and, as is shown in the present study, the latter is correlated with NFκB activation.

However, the inhibition of TGF-β1 and IL-10 transcription by NAC and quercetin, respectively, also occurred at non-NFκB inhibitory concentrations (20 mM and 10 μM). Hence other signal-transducing factors specifically targeted by NAC and quercetin, such as protein tyrosine kinase (24) or activator protein-1 (6, 31, 37, 42), could also be involved in the transcriptional regulation of these cytokines. For these reasons, transfection studies and gene reporter assays are needed to further define the role played by NFκB in anti-inflammatory cytokine gene transcription in PTC.

Because the concentration of NAC required to inhibit NFκB in PTC was 10-fold higher than that needed in other cell types (35, 45, 46), we investigated its effect on PTC viability. With the use of a sensitive marker of cell toxicity, our results clearly showed that NAC caused a dose-dependent increase in LDH leakage that was not accompanied by significant ultrastructural damage. The latter is not surprising, because LDH leakage is a relatively early marker of cell damage (18). Therefore, the injury induced by NAC during the time points of the study was clinically mild and probably reversible. In contrast, neither PDTC or quercetin caused biochemical or ultrastructural damage to PTC at NFκB inhibitory concentrations.

Autooxidation and the generation of reactive oxygen intermediates (particularly peroxides and iron-generated hydroxyl radicals) are the most common explanation of thiol-mediated toxicity in cells (13, 18, 31, 45). However, our results do not support the involvement of peroxide in NAC-induced injury of PTC because 1) neither catalase nor DFO prevented NAC-induced LDH leakage, 2) concentrations of H2O2 that increase intracellular peroxide to levels similar to those induced by NAC (11) did not cause LDH leakage in PTC, and 3)
H$_2$O$_2$ did not exacerbate NAC-induced LDH leakage and paradoxically had a mild but significant protective effect. The latter has been observed in other studies and is due to the reaction of peroxides with thiols (17). Alternative hypotheses to explain NAC-induced cytotoxicity include the generation of toxic cysteine metabolites (35), formation of nitric oxide intermediates (8) or copper-catalyzed hydroxyl radicals (17), or the induction of intracellular hypoxia by high-dose antioxidants (45).

Cell- and stimulus-specific effects may explain why NAC has been shown to reduce NF-$kappaB$ activation in vivo in rats with ureteric obstruction (27) and in other nonrenal experimental models (2). For example, monocytes isolated from normal rats treated with NAC have reduced adhesion in vitro and increased NF-$kappaB$ DNA binding activity. The latter is due to an increase in nontransactivating p52 homodimers (28). Alternatively, the cytoprotective effects of NAC may involve mechanisms other than NF-$kappaB$ suppression, as demonstrated in endotoxin-induced acute lung injury (45).

In conclusion, we have demonstrated that PDTC and quercetin potently suppress NF-$kappaB$ activation in PTC. In contrast to what has been shown in other cell types, NAC was not able to suppress NF-$kappaB$ activation in PTC at subtoxic and physiologically relevant concentrations. Furthermore, antioxidant-mediated inhibition of NF-$kappaB$ was associated with the nonselective reduction of cytokine transcription in activated tubular cells. Together, these data provide a possible explanation for the protective effect of PDTC in chronic tubulointerstitial inflammation and the failure of NAC to inhibit renal cortical NF-$kappaB$ activation in vivo (39).

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