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N-Acetyl-Cysteine Inhibits Phospholipid Metabolism, Proinflammatory Cytokine Release, Protease Activity, and Nuclear Factor-κB Deoxyribonucleic Acid-Binding Activity in Human Fetal Membranes *in Vitro*

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The production of reactive oxygen species (ROS), prostaglandins (PGs), proinflammatory cytokines, and proteases has been implicated in the pathogenesis of term and preterm labor. The nuclear factor-κB (NF-κB) transcription pathway is activated by ROS and is a key regulator of PGs, proinflammatory cytokine release, and protease activity. *N*-Acetylcysteine (NAC) is an antioxidant that through its ability to scavenger ROS suppresses NF-κB DNA-binding activity and resultant gene expression. The aim of this study was to elucidate the effect of NAC on NF-κB DNA-binding activity, phospholipid metabolism, cytokine release, and protease activity from human fetal membranes. Human amnion and choriodecidua (n = 9 separate placentas) were treated with 0 (control), 5, 10, or 15 mM NAC in the presence of 10 μg/ml lipopolysaccharide. After 6-h incubation, the tissues were collected, NF-κB DNA binding activity was assessed by gel shift binding assays, and matrix metalloproteinase-9 and urokinase-type plasminogen activator activity were determined by zymography. The incubation medium was collected and assayed for type II phospholipase A_2 tissue content, IL-6, IL-8, TNFα, and 8-isoprostane release by ELISA. The release of $PGF_{2\alpha}$ was measured by RIA. Treatment of fetal membranes with NAC significantly suppressed lipopolysaccharide-stimulated type II phospholipase A_2 release and content; $PGF_{2\alpha}$, IL-6, IL-8, TNFα, and 8-isoprostane release; and matrix metalloproteinase-9 and urokinase-type plasminogen activator enzyme activity and suppressed NF-κB DNA-binding activity (by ANOVA, $P < 0.05$). The data presented in this study demonstrate that NAC inhibits an NF-κB-activated pathway and subsequent phospholipid metabolism, proinflammatory cytokine release, and protease activity in human fetal membranes. (*J Clin Endocrinol Metab* 88: 1723–1729, 2003)

THE PHYSIOLOGICAL FACTORS that trigger the initiation of human labor and delivery at term or preterm are not fully known; however, phospholipid-derived mediators [*e.g.* prostaglandins (PGs)], proinflammatory cytokines, and extracellular matrix (ECM) remodeling enzymes have been implicated. Collectively, they participate in the regulation of myometrial contractility, cervical ripening, and rupture of membranes (reviewed in Refs. 1 and 2). The generation of reactive oxygen species (ROS) has also been implicated in the pathogenesis of premature labor, particularly in the case of prolonged premature rupture of membranes (PPROM) (reviewed in Ref. 3). Recently, Malek *et al.* (4) demonstrated that lipopolysaccharide (LPS) stimulation of human term placenta is associated with increased production of 8-isoprostane (a marker of oxidative stress). ROS cause tissue damage by a variety of mechanisms (reviewed in Ref. 5), including DNA damage, lipid peroxidation, protein damage, and stimulation of proinflammatory cytokine release by monocytes and macrophages by depleting intracellular thiol compounds and activating nuclear factor-κB (NF-κB).

NF-κB plays a pivotal role in inducing the expression of multiple genes involved in immune and inflammatory responses (reviewed in Ref. 6). In unstimulated cells, NF-κB dimers are found in an inactive form in the cytoplasm, bound with an inhibitory protein IκB. In response to activating signals, IκB-α undergoes phosphorylation by IκB kinases, ubiquitination, and subsequent proteasome-dependent degradation. Free NF-κB dimers then rapidly translocate to the nucleus to initiate transcription by high affinity binding to regulatory κB motifs in target genes. The κB motif has been identified in the promoter region of a number of genes involved in the initiation and progression of human preterm and term labor, including components of the phospholipid-metabolizing pathway [for example, phospholipase A_2 (PLA_2) and PGE_2 production]; proinflammatory cytokines TNFα, IL-6, and IL-8; and proteases matrix metalloproteinase-9 (MMP-9) and urokinase-type plasminogen activator (uPA) (7–15).

Compounds that have antioxidant properties, such as *N*-acetyl-cysteine (NAC), have been shown to inhibit upstream events leading to NF-κB activation (16–18). NAC is a thiol-containing antioxidant that either increases intracellular glutathione concentrations (an endogenous reducing agent) and/or acts directly as a free radical scavenger (19). NAC has been used in clinical practice since the 1950s for the treatment of congestive and obstructive lung diseases such as chronic bronchitis and cystic fibrosis. Furthermore, exploratory stud-

Abbreviations: ECM, Extracellular matrix; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NAC, *N*-acetyl-cysteine; NF-κB, nuclear factor-κB; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PLA_2, phospholipase A_2; PPROM, prolonged premature rupture of membranes; ROS, reactive oxygen species; uPA, urokinase-type plasminogen activator.

ies are being performed in the treatment of a variety of immune and inflammatory disorders, such as human immunodeficiency virus infection (reviewed in Ref. 20).

LPS stimulates NF-κB DNA-binding activity, phospholipase release and activity, $PGF_{2\alpha}$ production, and proinflammatory cytokine release from human fetal membranes (21–27). The aim of this study was to investigate whether NAC suppresses LPS-stimulated NF-κB DNA-binding activity and subsequent phospholipid metabolism, proinflammatory cytokine release, and protease activity from human amnion and choriodecidua.

TABLE 1. Effect of NAC on LDH release from human amnion and choriodecidua

Treatment	% Total tissue LDH	
	Amnion	Choriodecidua
LPS control	0.7 ± 0.3	4.4 ± 1.4
5 mM NAC	0.9 ± 0.3	3.4 ± 1.4
10 mM NAC	1.0 ± 0.4	4.9 ± 2.8
15 mM NAC	0.8 ± 0.3	4.6 ± 2.6

Materials and Methods

Reagents

All chemicals were purchased from BDH Chemicals Australia (Melbourne, Australia) unless stated otherwise. RPMI 1640 (phenol red free) was obtained from Life Technologies, Inc. (Grand Island, NY). BSA (RIA grade), dithiothreitol, EDTA, gelatin, leupeptin, LPS (from *Escherichia coli* 026:B6), β-NADH (disodium salt), 3,3′,5,5′-tetramethylbenzidine, pyruvic acid (dimer free), and NAC were supplied by Sigma-Aldrich (St.

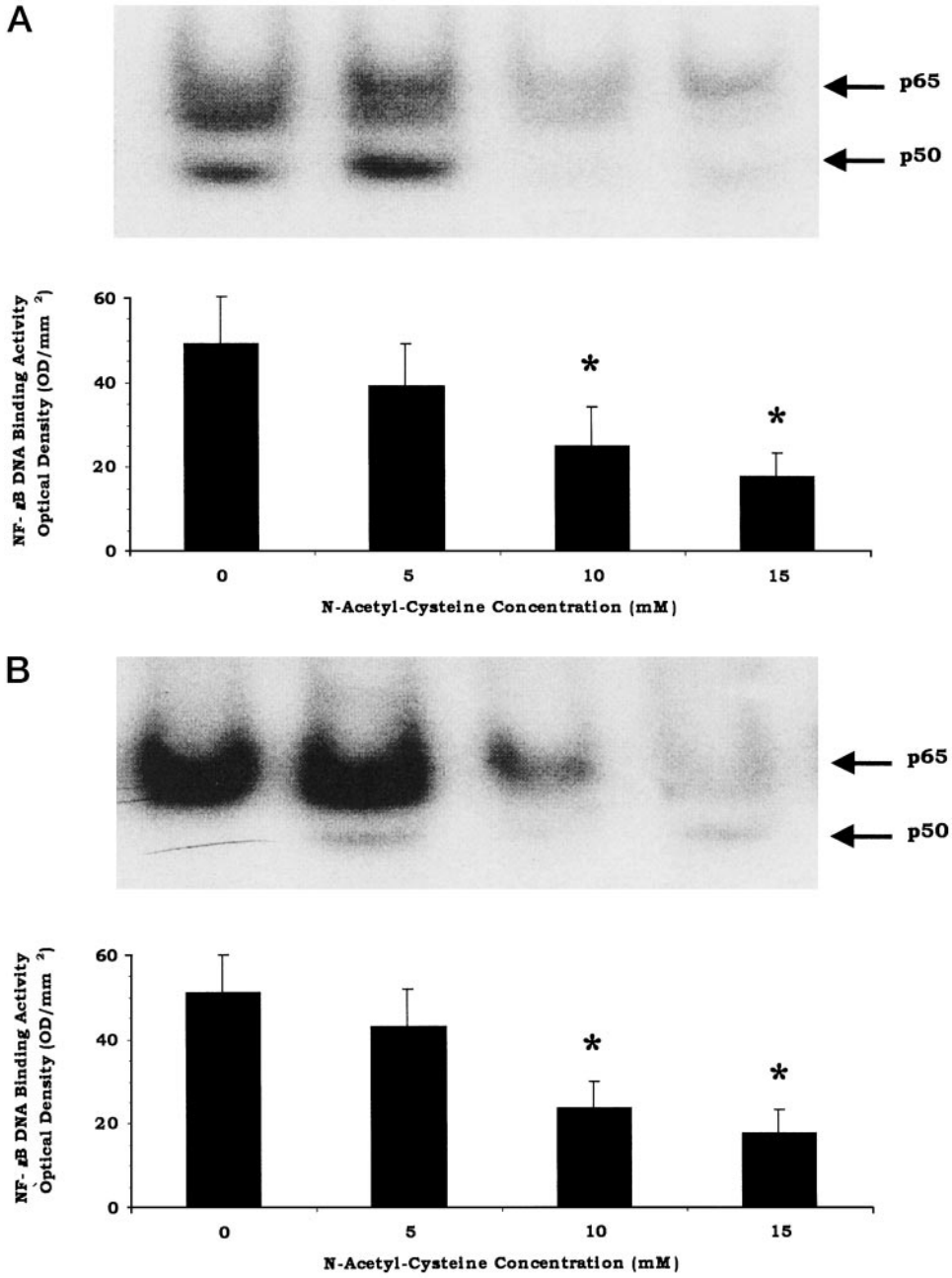

FIG. 1. Effect of NAC on LPS-stimulated NF-κB DNA-binding activity in nuclear extracts prepared from human amnion (A) and choriodecidua (B). Data are the mean ± SEM of five different EMSAs. *, Significant differences compared with control ($P < 0.05$, by ANOVA). The *top panel* in each diagram is a representative gel shift assay.

Louis, MO). Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride) was purchased from Roche (Mannheim, Germany). The transcription factor consensus oligonucleotides for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and activating protein-1 (5′-TTCCGGCTGACTCATCAAGCG-3′), HeLa scribe nuclear extract, gel shift binding buffer, and polynucleotide kinase for labeling of 5′-hydroxyl blunt-ended probes were purchased from Promega Corp. (Madison, WI). Streptavidin-horseradish peroxidase conjugate and the IL-6, IL-8, and TNFα kits were supplied by Biosource Technologies, Inc. (Camarillo, CA). The 8-isoprostane kit was purchased from Caymen Chemical Co. (Ann Arbor, MI). Human plasminogen (Glu type), MMP-9, and uPA standard were purchased from Calbiochem (San Diego, CA). Acrylamide, ammonium persulfate, bis-acrylamide, Coomassie Brilliant Blue, Rainbow protein molecular weight markers, *N*,*N*,*N*′-tetramethylethylenediamine, and [γ-^{32}P]deoxy-ATP were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Alkaline phosphatase (calf intestine grade 1) was obtained from Roche Molecular Biochemicals (Victoria, Australia). Monoclonal antibodies to human type II PLA_2 (3G3 and 2A9) were supplied by Bioquest (Sydney, Australia) and recombinantly expressed human type II PLA_2 standard was isolated from a Chinese hamster ovary cell line stably transfected with human type II PLA_2 under a metallothionine promoter. Starscint scintillation fluid was purchased from Packard (Meriden, CT), and phenol was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Human γ-globulin was provided by Commonwealth Serum Laboratories (Parkville, Australia). Goat polyclonal antiserum raised against $PGF_{2\alpha}$ was provided by Dr. Meg Ralph (Monash University, Clayton, Australia).

Tissue collection and preparation

Human placentas with the attached fetal membranes were obtained (with institutional research and ethics committee approval) from women who delivered healthy, singleton infants at term (≥37 wk gestation) undergoing elective cesarean section (indications for cesarean section were breech presentation and/or previous cesarean section). A human explant system was used to establish the effect of NAC on phospholipid metabolism, proinflammatory cytokine release, ECM remodeling enzyme activity, and NF-κB DNA-binding activity from human fetal membranes as previously described (27). Amnion and choriodecidual tissues were incubated in duplicate in 2 ml RPMI (containing 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 μg/ml LPS) in the absence (control) or presence of 5, 10, or 15 mM NAC (n = 9).

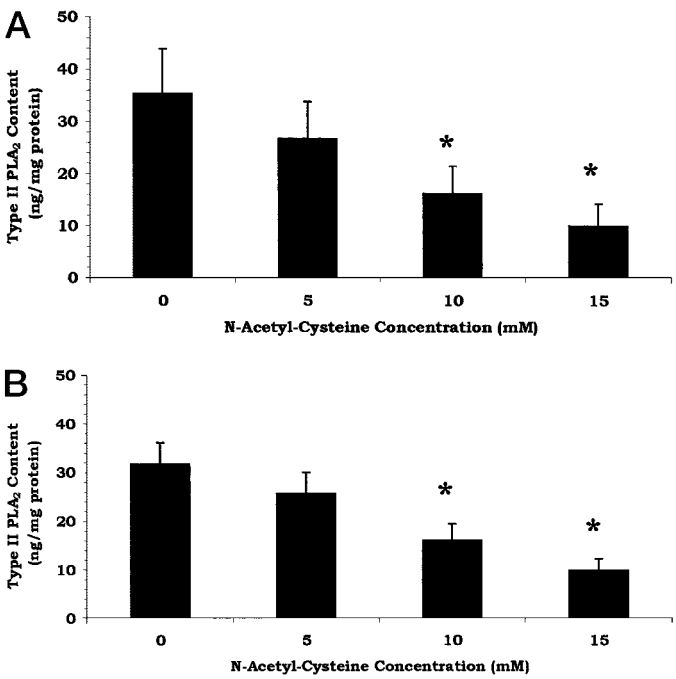

FIG. 2. Effect of NAC on LPS-stimulated type II PLA_2 tissue content from human amnion (A) and choriodecidua (B). Data are the mean ± SEM of nine independent explants. *, Significant differences compared with control ($P < 0.05$, by ANOVA).

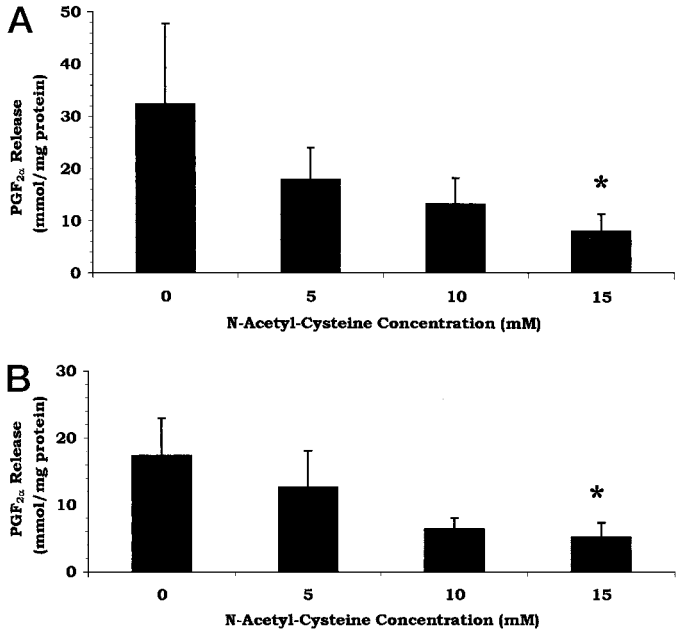

FIG. 3. Effect of NAC on LPS-stimulated $PGF_{2\alpha}$ release from human amnion (A) and choriodecidua (B). Data are the mean ± SEM of nine independent explants. *, Significant differences compared with control ($P < 0.05$, by ANOVA).

Nuclear protein extraction and EMSA

After the 6-h incubation, tissues were collected, and nuclear protein was extracted as previously described (27). Nuclear protein (12 μg) was then subjected to EMSA using a double-stranded NF-κB oligonucleotide that was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (27). After electrophoresis, the gel was dried and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) overnight at −80 C. Autoradiographs were quantified with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Inc., Richmond, CA) using the Quantity One 4.2.1 analysis program. Data were corrected for background and expressed as OD per square millimeter.

Experimental assays

Type II PLA_2 tissue content and the release of $PFG_{2\alpha}$ from fetal membranes were quantified by ELISA and RIA, respectively, as previously described (28). The release of IL-6, IL-8, and TNFα from fetal membrane tissue explants was quantified by ELISA kits according to the manufacturer's instructions (Biosource Technologies, Inc.), and the limits of detection of the IL-6, IL-8, and TNFα assays were 3, 2.8, and 7.2 pg/ml, respectively. Assessment of ECM remodeling enzymes in fetal membranes were performed by electrophoretic zymography as previously described (29), and enzyme activities were identified by comigration with authentic standards and comparison with the mobility of protein standards. Proteolytic activity was visualized as clear zones of lysis on a blue background of undigested gelatin. The release of 8-isoprostane into the incubation medium was assayed using a competitive enzyme immunoassay, according to the manufacturer's instructions (Cayman Chemical Co.), and the limit of detection of the assay was 5 pg/ml. The protein content of tissue homogenates was determined with a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), using BSA as a reference standard, as previously described (27). To determine the effect of experimental treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into the incubation medium was determined as described previously (22). Data are presented as a percentage of the total tissue LDH.

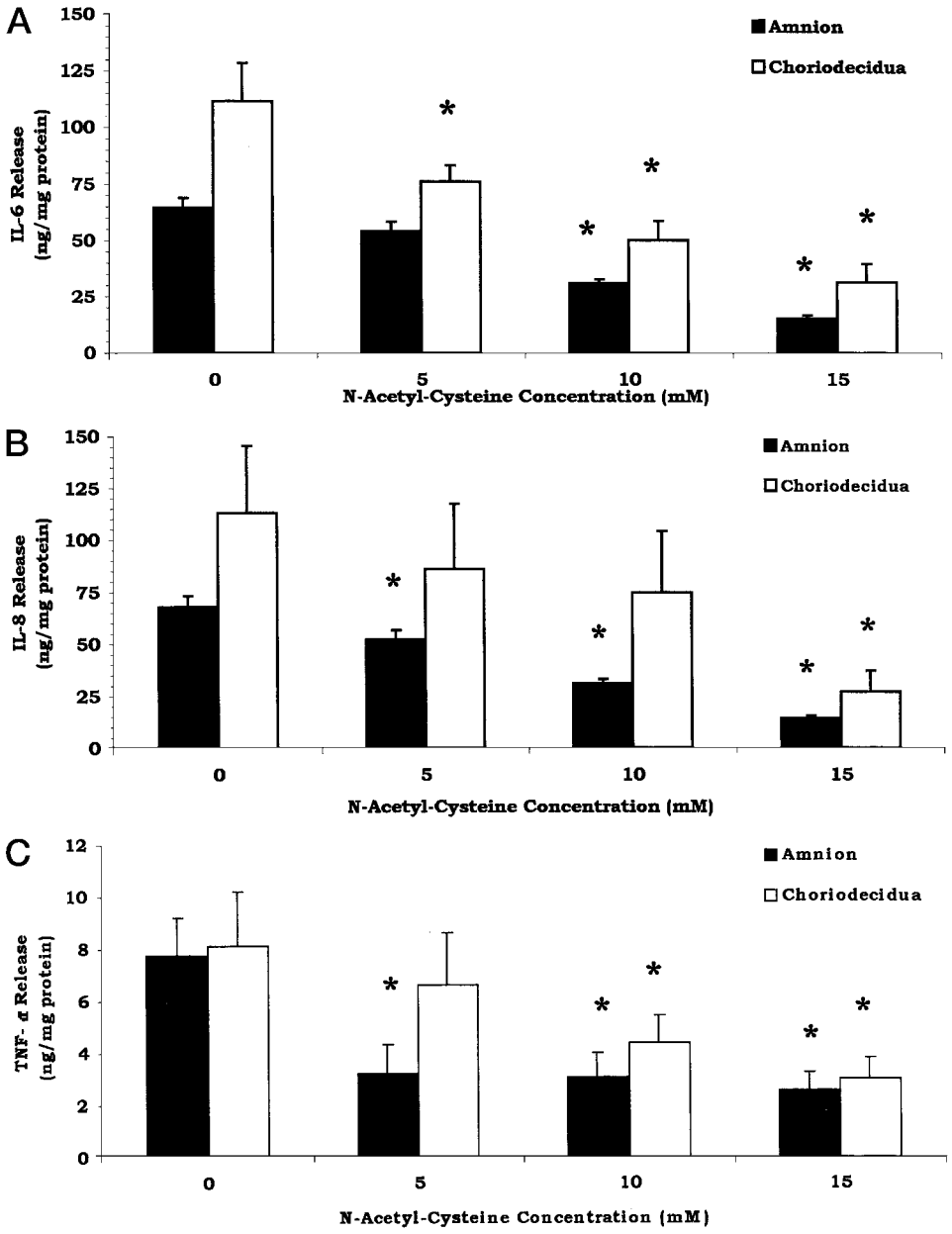

FIG. 4. Effect of NAC on LPS-stimulated IL-6 release (A), IL-8 release (B), and TNFα release (C) from human amnion (A) and choriodecidua (B). Data are the mean ± SEM of nine independent explants. *, Significant differences compared with control ($P < 0.05$, by ANOVA).

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics, STSC, Rockville, MD). The homogeneity of data was assessed by Bartlett's test (30), and when significant, data were logarithmically transformed before further analysis. Data were subjected to a one-way ANOVA. Two-sample comparisons were analyzed by *t* test. Statistical difference was indicated by $P < 0.05$. Data are expressed as the mean ± SEM of nine different placental tissues.

Results

Validation of explant cultures and viability

To validate the integrity of explants in the presence of NAC, cell viability was investigated using LDH release from explants. LDH release was investigated over the 6-h incubation period. Explants were incubated in either control medium (10 μg/ml LPS) or medium containing 5, 10, or 15 mM NAC. Compared with the LPS control, treatment with NAC, at all concentrations tested, did not significantly affect LDH release from amnion and choriodecidua, indicating that the concentration of NAC used did not affect cell viability (Table 1).

Effect of NAC on NF-κB-binding activity

Previous studies within our laboratory have demonstrated NF-κB DNA-binding activity in nuclear extracts fromhuman fetal membranes (27). Furthermore, the specificity of NF-κB DNA binding was confirmed in competition experiments. Supershift assays also established that the NF-κB p50 and p65 heterodimers are activated in gestational tissues in response to LPS. In this study treatment with 10 and 15 mM NAC significantly suppressed NF-κB DNA-binding activity in nu-

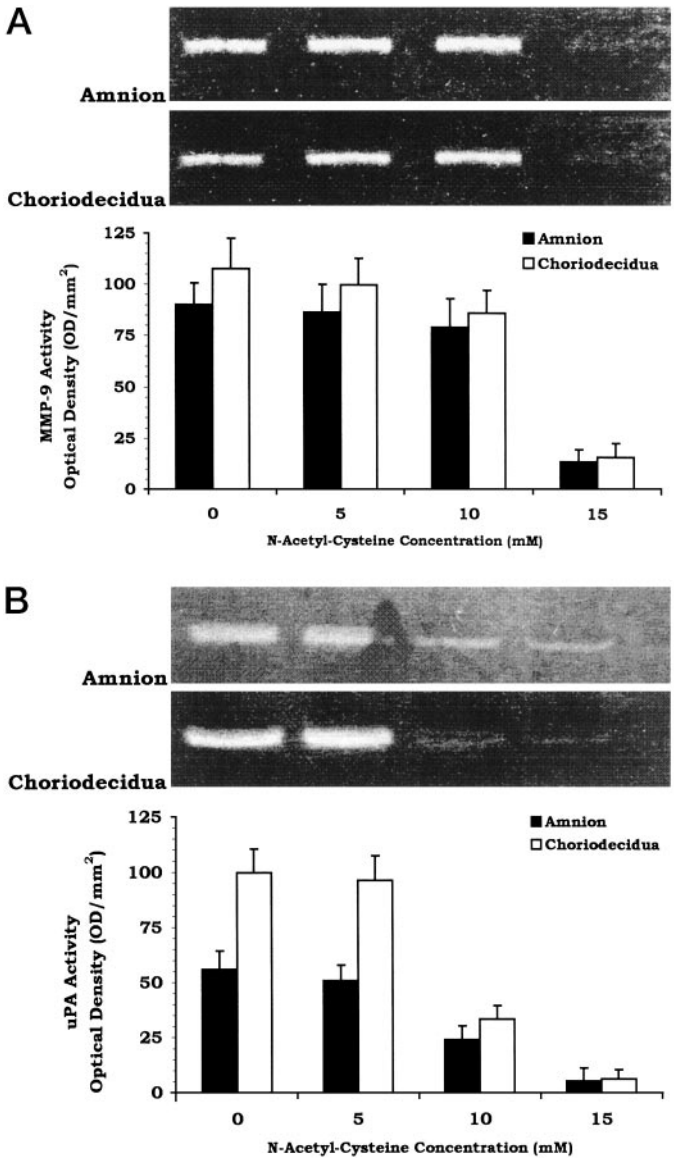

FIG. 5. Effect of NAC on LPS-stimulated MMP-9 enzyme activity (A) and uPA enzyme activity (B) in human amnion (A) and choriodecidua (B). Data are the mean ± SEM of four independent explants. *, Significant differences compared with control ($P < 0.05$, by ANOVA). The *top panel* in each diagram is a representative gelatin zymography.

clear extracts prepared from human amnion (Fig. 1A) and choriodecidua (Fig. 1B).

Effect of NAC on phospholipid metabolism

In the presence of 10 or 15 mM NAC, type II PLA_2 content was significantly inhibited in amnion (Fig. 2A) and choriodecidua (Fig. 2B). At 15 mM NAC, there were 4- and 2-fold decreases in the level of type II PLA_2 content in amnion and choriodecidua, respectively.

Compared with LPS control, treatment with 15 mM NAC significantly reduced the release of $PGF_{2\alpha}$ in amnion (Fig. 3A) and choriodecidual (Fig. 3B) tissue explants by 4- and 3-fold, respectively.

Effect of NAC on proinflammatory cytokine release

Compared with LPS control, tissues incubated in the presence of NAC greater than or equal to 10 mM caused a significant reduction of IL-6 release from amnion and choriodecidua (Fig. 4A). In the presence of 15 mM NAC, there was a 3-fold reduction in the release of IL-6 from both amnion and choriodecidua.

Treatment of samples with NAC at concentrations greater than 5 mM significantly reduced IL-8 production from amnion, whereas only 15 mM NAC was effective in choriodecidua (Fig. 4B). NAC at 15 mM caused a 5-fold decrease in IL-8 release by amnion and a 4-fold reduction in IL-8 release by choriodecidua.

In amnion, concentrations greater than 5 mM NAC significantly reduced TNFα release, with a 3-fold reduction observed at 15 mM NAC (Fig. 4C). In choriodecidua, 15 mM NAC significantly reduced TNFα release, with a 3-fold decrease (Fig. 4C).

Effect of NAC on proteolytic activity

Gelatin substrate gels copolymerized with plasminogen were used to determine the effect of NAC on protease activity in human fetal membranes. In both amnion and choriodecidua (Fig. 5A), the addition of 15 mM NAC significantly suppressed MMP-9 activity. Compared with the LPS control, concentrations of NAC equal to or greater than 10 mM significantly suppressed uPA activity in both amnion and choriodecidua (Fig. 5B).

Effect of NAC on 8-isoprostane release

The release of 8-isoprostane into the incubation medium is used as a marker of oxidative stress. Treatment with 15 mM NAC reduced LPS-induced 8-isoprostane accumulation into the incubation medium from amnion and choriodecidual tissues (Fig. 6). Compared with the LPS control, coincubation with 15 mM NAC caused a 3-fold reduction in amnion and a 5-fold decrease in choriodecidua.

Discussion

In this study treatment of fetal membranes with NAC inhibited LPS-stimulated type II PLA_2 content; $PGF_{2\alpha}$, IL-6, IL-8, TNFα, and 8-isoprostane release; and MMP-9 and uPA enzyme activities. Furthermore, this inhibition was associated with a concomitant suppression of NF-κB DNA-binding activity. Similarly, others have demonstrated that NAC inhibits the expression, release, and/or activity of proinflammatory cytokines and MMPs through suppression of NF-κB activation in a number of other systems (31–36). Similarly, the use of other antioxidants, for example, pyrrolidine dithiocarbamate, have also demonstrated the importance of the NF-κB signaling pathway in the regulation of cytokine release and protease activity (34, 35).

Although there is no common second messenger among the identifiable inducers of NF-κB, most, if not all, activating signals can be inhibited by antioxidants (reviewed in Ref. 5), suggesting that ROS act as second messengers after cellular exposure to agents that induce NF-κB activation. The interaction between ROS on the NF-κB signaling pathway has not

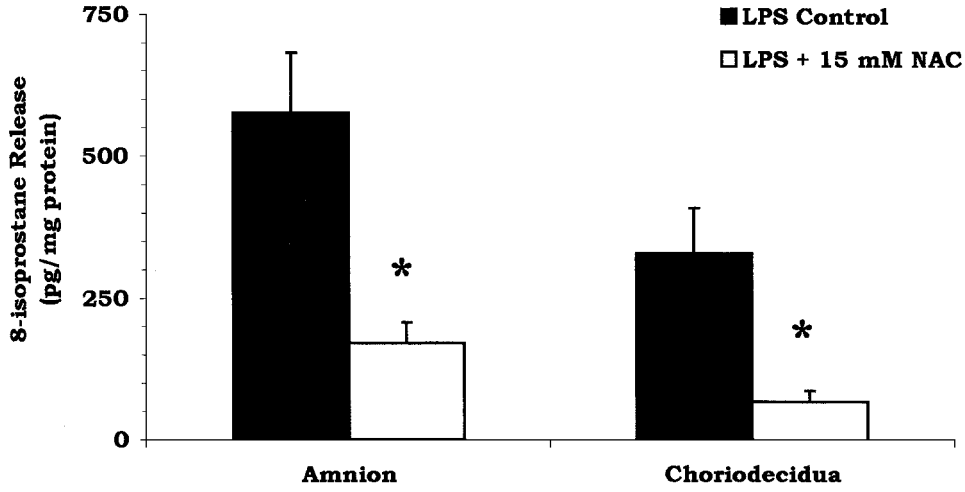

FIG. 6. Effect of NAC on LPS-stimulated 8-isoprostane release from human amnion and choriodecidua. Data are the mean ± SEM of four independent explants. *, Significant differences compared with control ($P < 0.05$, by ANOVA).

been completely defined; nevertheless, the most likely scenario is that ROS activates a critical redox-sensitive kinase, probably by the IκB kinase or NF-κB-inducing kinase signals (reviewed in Ref. 37).

As ROS have been implicated in the pathogenesis of preterm labor (reviewed in Ref. 3), and as PGs, proinflammatory cytokines, and proteases are also involved in the pathogenesis of preterm labor, interactions between ROS and NF-κB might be a component of the intracellular signaling processes that lead to activation.

The data obtained in this study are consistent with and extend studies implicating NF-κB in the regulation of human gestational tissue proinflammatory status (27). Our data establish a role for NF-κB in regulating type II PLA_2 and subsequent PG production, which are intrinsic for successful parturition. Similarly, other studies have also demonstrated the importance of NF-κB in regulating components of the phospholipid-metabolizing pathway in human gestational tissues (15, 38).

A growing body of evidence suggests that membrane rupture is partially mediated by biochemical processes, including the enzymatic breakdown of ECM components within the amnion and chorion. MMP-9 and uPA are key enzymes involved in the breakdown of these ECM components within the amnion and chorion. PPROM may be the result of ROS-induced damage to amnion epithelium or collagen in the chorioamnion (reviewed in Ref. 3). Various environmental and physical factors associated with PPROM are known to produce ROS or reduce the body's natural antioxidant defense mechanisms (*e.g.* smoking and cocaine abuse). In a recent *in vitro* study, the treatment of chorioamnion with antioxidants, including NAC, protected against ROS-induced MMP-9 activity (reviewed in Ref. 3). Similarly, in this study incubation of amnion and choriodecidua with NAC inhibited LPS-stimulated NF-κB activation of MMP-9 and uPA activities, with an associated decrease in 8-isoprostane. It is conceivable that therapies that suppress NF-κB activation may be useful in the prevention of PPROM; however, further studies are required to evaluate antioxidant therapy for the prevention of PPROM.

ROS can be generated by a number of sources, including leakage from the electron transport system in the inner membrane of a mitochondrion during cellular respiration and release by immune cells as they encircle and then kill bacteria. One of the most important sources of ROS in macrophages in response to LPS stimulation is the membrane-bound NADPH oxidase activation that occurs during phagocytosis (reviewed in Ref. 5). In response to a phagocytic stimuli, NADPH oxidase moves electrons from NADPH to reduce oxygen to superoxide. Superoxide is regarded as a weakly reactive radical, but nonetheless can attack a number of biological targets. Superoxide is removed from tissues by spontaneous dismutation to hydrogen peroxide (a process that can also be catalyzed by superoxide dismutase), then oxygen, and then sequentially to other products. However, the exact mechanism for LPS-induced ROS generation remains to be fully established.

A number of studies have identified NF-κB DNA-binding activity in human gestational tissues (14, 15, 27, 38–40). NF-κB is essential for up-regulation of IL-8 expression in human amnion-derived WISH cells and cervical epithelial cells (38, 39). Stimulation of human trophoblast cells with IL-1 leads to NF-κB activation that is functionally linked to the expression of cyclooxygenase-2 and PGs (15). It is likely that the same mechanism is operative in cytokine-induced up-regulation of PG production in gestational tissues, thereby contributing to the labor-inducing effects of proinflammatory cytokines.

These results confirm that ROS are involved in LPS-stimulated NF-κB activation and the subsequent genes that it regulates. In this study we demonstrate that in human fetal membranes, the NF-κB signaling pathway is a key regulator of phospholipid metabolism, proinflammatory cytokines, and ECM-remodeling enzymes. Consequently, the development of specific inhibitors of NF-κB will be beneficial in further dissecting the role of NF-κB in the initiation of human labor and could potentially be clinically useful in the management and/or treatment of preterm labor associated with infection.

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