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Nuclear Factor Kappa B Regulation of Proinflammatory Cytokines in Human Gestational Tissues In Vitro[1]

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ABSTRACT

Proinflammatory cytokines are implicated in the initiation and progression of human labor and delivery, particularly in relation to infection-induced preterm labor. In nongestational tissues, the nuclear factor kappa B (NF-κB) transcription pathway is a key regulator of proinflammatory cytokine release. In these tissues, sulfasalazine (SASP), through its ability to inhibit NF-κB activation, inhibits release of interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF)-α. Therefore, the aim of this study was to investigate whether or not NF-κB activation regulates the formation of proinflammatory cytokines in human gestational tissues. Human placenta, amnion, and choriodecidua (n = 9 separate placentas) were incubated with 10 μg/ml of lipopolysaccharide (LPS) in the absence (control) or presence of SASP (0.1, 1, 5, or 10 mM). After 6 h of incubation, the tissues were collected, and NF-κB DNA binding activity in nuclear extracts was assessed by electromobility shift binding assay. The incubation medium was collected and the release of IL-6, IL-8, and TNF-α was quantified by ELISA. Treatment of placenta, amnion, and choriodecidua with SASP at concentrations 5 mM or greater significantly inhibited the release of IL-6, IL-8, and TNF-α, and NF-κB activation (ANOVA, $P < 0.05$). The data presented in this study demonstrate that the NF-κB transcription pathway is a key regulator of LPS-stimulated IL-6, IL-8, and TNF-α release from human gestational tissues. The control of NF-κB activation may therefore provide an alternative therapeutic strategy for reducing the release of proinflammatory mediators in infection associated preterm labor.

cytokines, decidua, gene regulation, parturition, placenta

INTRODUCTION

Maternal cervical and intrauterine infection and inflammation may have a primary causative role in many cases of preterm labor (reviewed in [1]). Briefly, in the presence of infection, bacteria or bacterial products may activate resident macrophages (ubiquitous cells present in the maternal, fetal, and placental compartments) and lymphocytes. The resultant secretion of proinflammatory cytokines (e.g., interleukin [IL]-1β, IL-6, and tumor necrosis factor [TNF]-α), and chemotactic factors (e.g., IL-8) establishes a chemokine gradient that promotes further recruitment of polymorphonuclear (PMN) cells, macrophages, neutrophils, and eosinophils, and local cytokine release. These proinflammatory cytokines act locally on intrauterine cells to 1) induce phospholipid metabolizing enzymes and stimulate the ongoing release of prostaglandins; and 2) activate extracellular matrix (ECM) remodeling enzymes involved in the processes of human labor (such as urokinase type plasminogen activator [uPA] and matrix metalloproteinases [MMPs]). Proinflammatory cytokines have been identified throughout human pregnancy and labor at the fetal-maternal interface. In nongestational tissues, the nuclear factor-kappa B (NF-κB) transcription signaling pathway is a common central pathway involved in promoting the formation of inflammatory cytokines [2]. The aim of this study was to elucidate whether or not NF-κB activation regulates the release of proinflammatory cytokines in human gestational tissues.

At least five genes belong to the NF-κB family, with the most common NF-κB dimer composed of the RelA (p65) and p50 subunits [3]. NF-κB dimers are usually sequestered in the cytoplasm bound to an inhibitory subunit, IκB-α [4]. Upon stimulation with bacterial endotoxin lipopolysaccharide (LPS), mitogens, or viral proteins [5], IκB-α is phosphorylated [6], ubiquitinated, and rapidly degraded [7], resulting in the rapid translocation of NF-κB to the nucleus where it binds specific DNA elements (κB motifs) in the promoter/enhancer region of target genes to initiate, enhance, or suppress the transcriptional process [3].

NF-κB activation initiates both extracellular and intracellular regulatory events that result in autoregulation of the inflammatory cascade. In the case of infection, microbial products can cause NF-κB activation that enhances TNF-α and IL-1 production and release, both of which are capable of activating NF-κB (reviewed in [8]). This positive regulatory loop may amplify and perpetuate local inflammatory reactions.

The in vitro activation of NF-κB can be regulated by anti-inflammatory agents such as salicylates (reviewed in [9, 10]). Consistent with the essential role of NF-κB in inflammation, it is a target of many anti-inflammatory compounds, including sulfasalazine (SASP). SASP was synthesized in 1942 as a combined antibiotic, sulfapyridine, and an anti-inflammatory agent, 5-aminosalicylic acid. SASP has been used successfully in the clinical treatment of ulcerative colitis, inflammatory bowel disease, and rheumatoid arthritis for nearly 50 years (reviewed in [11]). SASP has been shown to inhibit granulocyte activation; lymphocyte proliferation; and synthesis of IL-1, IL-2, and TNF-α [12–14]. Recent studies have demonstrated that the anti-inflammatory effects of SASP could be attributed to its ability to inhibit NF-κB DNA binding activity [12, 13].

The hypothesis to be tested is that inhibition of NF-κB DNA binding activity suppresses the release of proinflammatory cytokines IL-6, IL-8, and TNF-α in human gesta-

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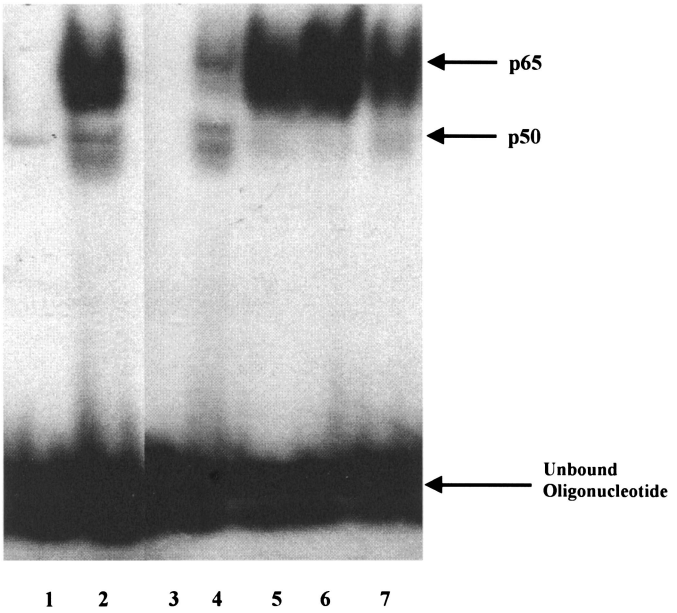

FIG. 1. NF-κB binding activity in placenta, amnion, and choriodecidua nuclear protein extracts. Both the p50 and p65 (RelA) subunits of NF-κB were detected in all nuclear protein extracts prepared from placenta (lane 5), amnion (lane 6), and choriodecidua (lane 7). Lane 1: negative control; lane 2: positive control (^{32}P-labeled NF-κB oligonucleotide); lane 3: ^{32}P-labeled NF-κB oligonucleotide plus unlabeled NF-κB oligonucleotide (specific competitor); lane 4: ^{32}P-labeled NF-κB oligonucleotide plus unlabeled AP-1 oligonucleotide (nonspecific competitor).

tional tissues. Human placenta, amnion, and choriodecidua tissues were incubated in the presence of increasing SASP concentrations (0.1, 1, 5, or 10 mM). NF-κB binding activity in nuclear extracts was analyzed by a gel shift assay and the release of IL-6, IL-8, and TNF-α into the incubation medium was quantified by ELISA.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from BDH Chemicals Australia (Melbourne, Victoria, Australia) unless stated otherwise. RPMI 1640 (phenol red-free) was obtained from Gibco Laboratories (Grand Island, NY). BSA (radioimmunoassay grade), dithiotheitol (DTT), ethylenediamine tetraacetic acid (EDTA), leupeptin, LPS (from *Escherichia coli* 026:B6), β-NADH (disodium salt), 3,3′,5,5′-tetramethylbenzidine (TMB), pyruvic acid (dimer free), and SASP were supplied by Sigma Chemical Company (St. Louis, MO). Pefabloc SC (AEBSF) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). The transcription factor consensus oligonucleotides for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-TTCCGGCTGACTCATCAAGCG-3′), HeLa scribe nuclear extract, gel shift binding buffer, and polynucleotide kinase for labeling of 5′OH blunt-ended probes were purchased from Promega (Madison, WI). Streptavidin-horseradish peroxidase conjugate and the IL-6, IL-8, and TNF-α kits were supplied by Biosource International (Camarillo, CA). Acrylamide, ammonium persulphate, bis-acrylamide, TEMED, and [γ-^{32}P]dATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

Tissue Collection and Preparation

Human placentas and attached fetal membranes were obtained (with institutional Research and Ethics Committee approval) from women who delivered healthy, singleton infants at term (≥37 wk gestation) via elective cesarean delivery (indications for cesarean delivery were breech presentation, previous cesarean delivery, or both). Tissues were obtained within 10 min of delivery and dissected fragments were placed in ice-cold RPMI. Placental tissue was blunt-dissected to remove visible connective tissue and calcium deposits. Choriodecidua was separated from amnion by blunt

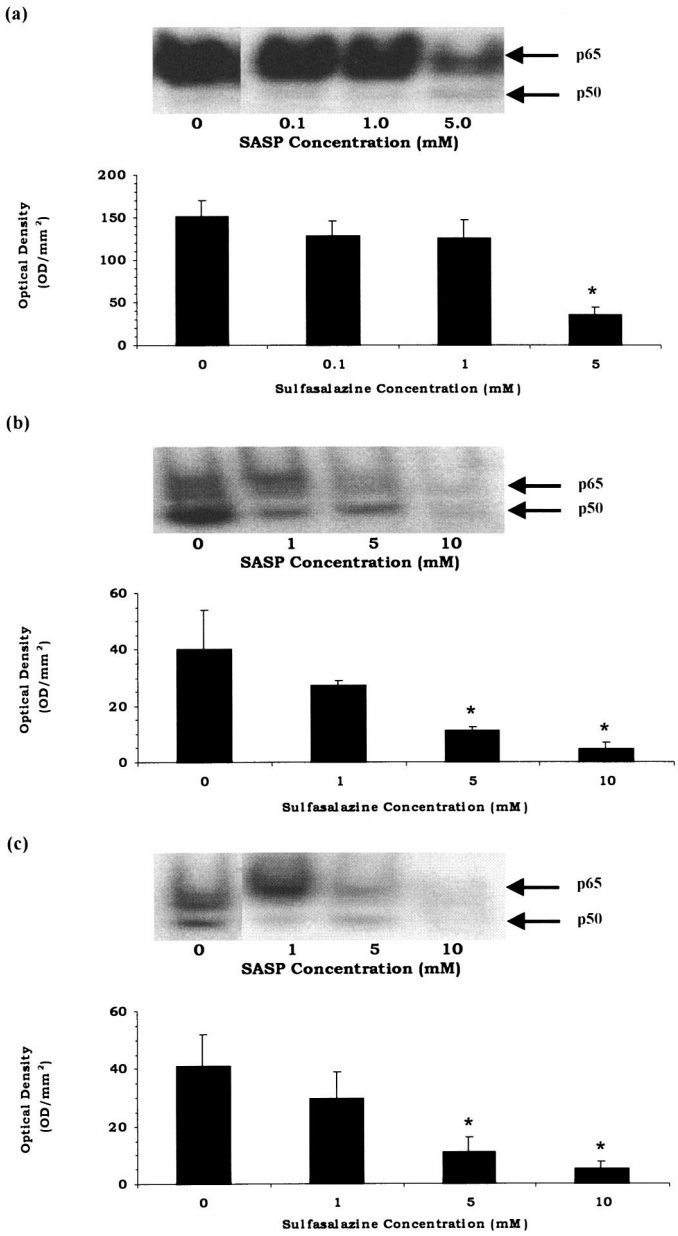

FIG. 2. Effect SASP on NF-κB activation from nuclear extracts prepared from human placenta (**a**), amnion (**b**), and choriodecidua (**c**). NF-κB DNA binding activity was significantly inhibited by SASP concentrations of 5 mM or greater in all three tissues. Significant differences, as compared to control, are represented by * ($P < 0.05$, ANOVA). The top panel in each diagram is a representative gel shift assay.

dissection, and explants were prepared by sharp dissection of 2.5 cm^2 squares. Tissue fragments were placed in RPMI at 37°C in a humidified atmosphere of carbogen gas (95% O_2 and 5% CO_2) for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (200–250 mg wet weight/well). The explants were incubated in duplicate in 2 ml of RPMI containing penicillin G (100 U/ml) and streptomycin (100 μg/ml). Explant incubation was performed in the presence of 10 μg/ml of LPS to achieve maximal stimulation of NF-κB.

Nuclear Protein Extraction

After the 6-h incubation, placental tissues were homogenized in 1:5 w/v TBS (150 mM NaCl, 50 mM Tris) by three 20-sec bursts with a metal blade homogenizer (T25 Ultra-Turrax and S25N 8G dispersing tool; Jenke and Kontel GmBH, Staufen, Germany). All subsequent steps were performed at 4°C. The homogenate was centrifuged at 1200 × *g* for 10 min, and the supernatant was collected and stored at −20°C until assayed for

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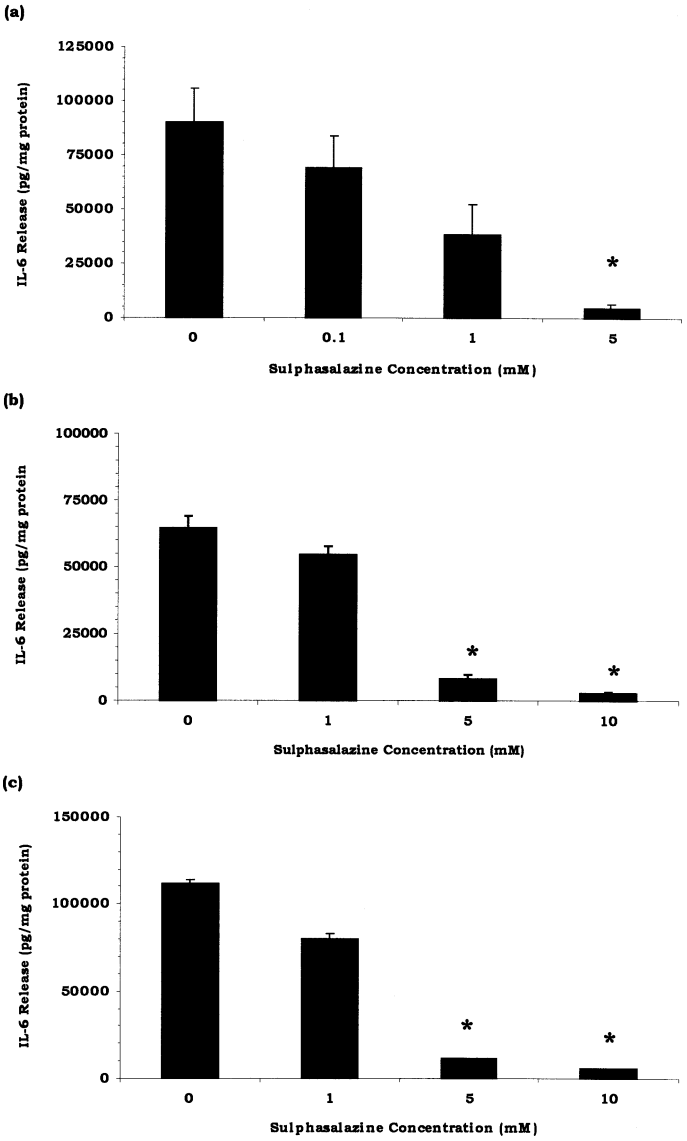

FIG. 3. Effect of SASP on LPS-induced IL-6 secretion from human placenta (**a**), amnion (**b**), and choriodecidua (**c**). Compared to control, concentrations of SASP greater than or equal to 5 mM caused a significant reduction in IL-6 secretion from all three tissues. Significant differences compared to control are represented by * ($P < 0.05$, ANOVA).

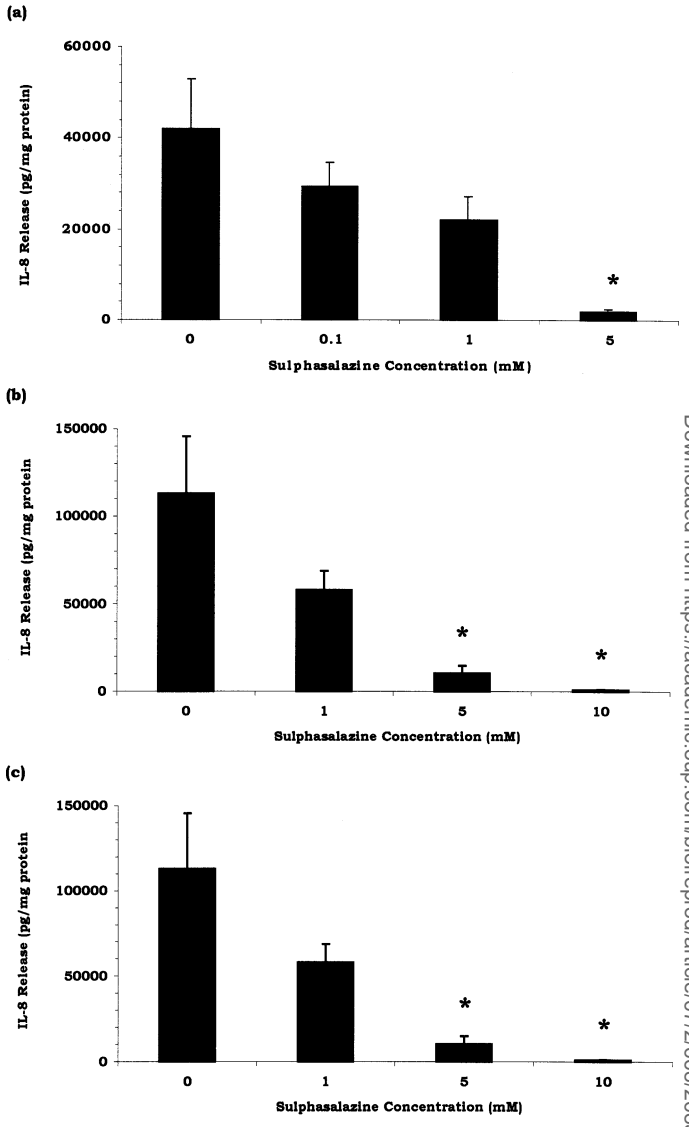

FIG. 4. Effect of SASP on LPS-induced IL-8 released from human placenta (**a**), amnion (**b**), and choriodecidua (**c**). Incubation of placenta, amnion, and choriodecidua tissues with SASP concentrations of 5 mM or greater resulted in significantly less IL-8 being released into the incubation medium. Significant differences compared to control are represented by * ($P < 0.05$, ANOVA).

protein content. The pellet was washed with 1 ml of TBS and centrifuged at 14 000 × *g* for 15 sec. The pellet was resuspended in 800 μl of Buffer A (10 mM Hepes pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM AESBF, and 4 μg/ml leupeptin), and incubated for 15 min. Fifty microliters of 10% Nonidet P-40 was added, samples were vortexed for 30 sec, and then centrifuged at 14 000 × *g* for 15 sec. The pellet was resuspended in 50 μl of Buffer B (50 mM Hepes pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM AESBF, 4 μg/ml leupeptin, and 1% glycerol), mixed gently for 20 min, and then centrifuged at 14 000 × *g* for 5 min. The supernatant was collected and stored at −80°C. Protein concentration was determined using the BCA protein assay system (Pierce, Rockford, IL), using BSA as a reference standard, as described below.

Electrophoretic Mobility Shift Assay

The double-stranded NF-κB oligonucleotide was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Electrophoretic mobility shift assay (EMSA) was performed using approximately 2 ng of labeled NF-κB oligonucleotide (20 000 dpm), 12 μg of nuclear extract, and 2 μl of gel shift binding buffer. The reactions were incubated at room temperature for 20 min, and 2.5 μl of gel loading buffer (250 mM Tris-HCl pH 7.5, 0.2% bromophenol blue, and 40% glycerol) was added to each reaction. Complexes were resolved on a 4% polyacrylamide gel using 0.5× TBE running buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) at 150 V for 3 h. After electrophoresis, the gel was dried and exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) overnight at −80°C. Autoradiographs were quantified using a GS-800 calibrated densitometer (Bio-Rad, Richmond, CA) using the Quantity One 4.2.1 analysis program (Bio-Rad). Data were corrected for background, and expressed as optical density (OD/mm^2).

Using supershift assays, previous studies in our laboratory had confirmed that the NF-κB p50 and p65 heterodimers are activated in gestational tissues in response to LPS (unpublished results). Antibodies to NF-κB heterodimers p50 and p65 were added to nuclear protein extracts. Antibody binding resulted in a higher shift, or supershift, on EMSAs with a reciprocal decrease in the intensity of the NF-κB band. Furthermore, the addition of both p50 and p65 antibodies eliminated the NF-κB band. Antibodies to other Rel-related proteins, specifically C-Rel and p52, did not result in supershifts.

IL-6, IL-8, and TNF-α ELISA

The concentration of IL-6, IL-8, and TNF-α in the explant incubation medium was performed by sandwich ELISA (Biosource) using monoclo-

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nal mouse immunoglobulin G antibodies at a concentration of 0.5 μg/ml for capturing and detecting antibodies. The procedure was followed according to the manufacturer's instructions. A Bio-Rad Benchmark Microplate Reader was used to read the sample absorbance at 450 nm. The limit of detection of the IL-6, IL-8, and TNF-α assays (defined as 2 SD from the zero standard) was 3, 2.8, and 7.2 pg/ml, respectively.

Tissue Homogenate Protein Assay

The protein content of placental tissue supernatant was determined using the BCA protein assay (Pierce) with BSA as a reference standard. Tissue supernatants were solubilized in 2 M sodium hydroxide (1:1 v/v) and boiled for 10 min. Hydrochloric acid (1M, 1:1 v/v) was added to neutralize the sample. Samples were diluted in distilled water (1:2 v/v), and 10 μl of this sample was assayed for protein content.

Lactate Dehydrogenase

To determine the effect of experimental treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously [15].

Statistical Analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics; STSC, Rockville, MD). Homogeneity of data was assessed by the Bartlett test [16], and when significant, data were logarithmically transformed before further analysis. Data were subjected to a one-way ANOVA. Statistical difference was indicated by a P value of less than 0.05. Data are expressed as means ± SEM of nine different placental tissues.

RESULTS

To determine whether SASP suppresses the release of proinflammatory cytokines from human placenta, amnion, and choriodecidua by inhibiting NF-κB activation (n = 9 independent placentas), tissues were incubated in the absence or presence of increasing concentrations of SASP (0.1, 1, 5, or 10 mM). The concentrations of SASP used in this study were chosen according to previous studies [12]. Following a 6-h incubation, tissues were collected and nuclear protein was immediately extracted to determine NF-κB DNA binding activity by EMSA while the incubation medium was collected and assayed for IL-6, IL-8, and TNF-α release. Data represent means ± SEM. Significant differences, as compared to control, are represented by $P < 0.05$ (ANOVA).

Effect of SASP on NF-κB Binding Activity

Previous studies in our laboratory have demonstrated higher NF-κB DNA binding activity in nuclear extracts of human gestational tissues incubated with 10 μg/ml of LPS (unpublished results). Thus, in this study, to achieve maximal NF-κB DNA binding activity, all experiments were performed in the presence of 10 μg/ml of LPS.

To establish the role of NF-κB in the regulation of cytokine production in human gestational tissues, it was first necessary to determine NF-κB DNA binding activity in nuclear extracts from these tissues. Figure 1 is a representative gel illustrating the detection of NF-κB activation in nuclear extracts prepared from placenta (lane 5), amnion (lane 6), and choriodecidua (lane 7).

The specificity of NF-κB DNA binding was confirmed in competition experiments. Incubation with an excess of an unrelated oligonucleotide spanning AP1 binding site did not antagonize NF-κB binding (Fig. 1, lane 4), whereas competition with excess unlabeled NF-κB oligonucleotide inhibited binding activity (Fig. 1, lane 3). Negative and positive (Fig. 1, lanes 1 and 2, respectively) controls were run in parallel.

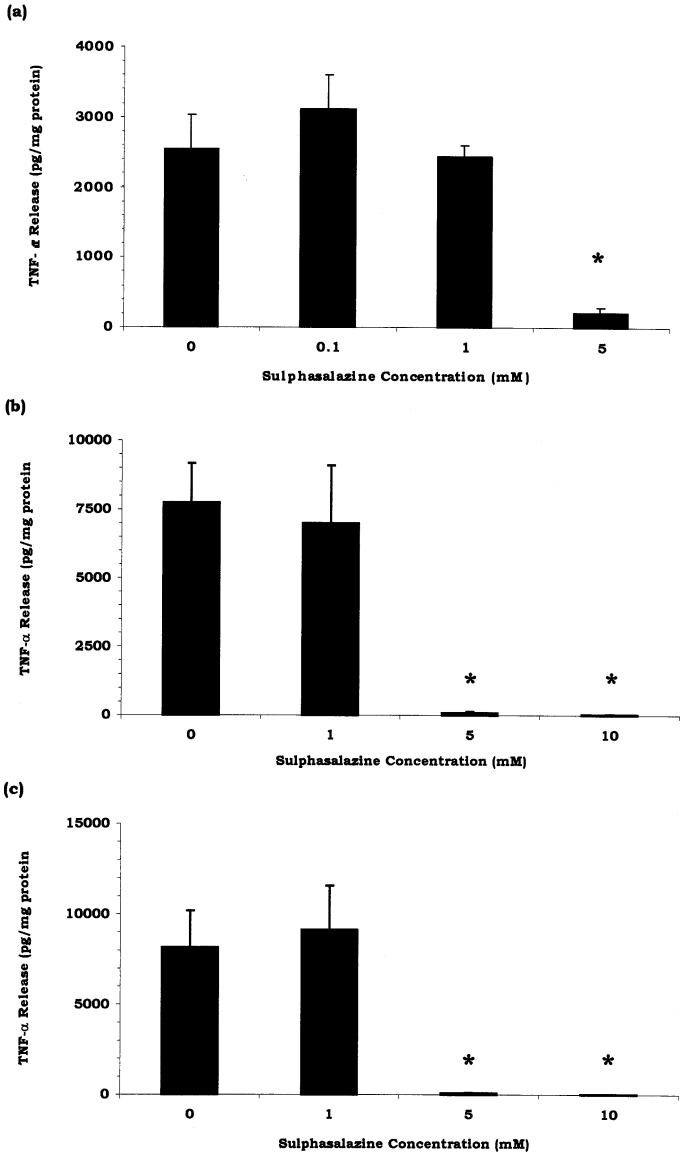

FIG. 5. Effect of SASP on LPS-induced TNF-α secretion by human placenta (**a**), amnion (**b**), and choriodecidua (**c**). Compared to control, SASP concentrations of 5 mM or greater caused a significant reduction in TNF-α secretion in all three tissues tested. Significant differences compared to control are represented by * ($P < 0.05$, ANOVA).

Treatment of tissue explants with SASP concentrations equal to or greater than 5 mM caused a significant suppression of NF-κB DNA binding activity in nuclear extracts prepared from human placenta (Fig. 2a), amnion (Fig. 2b), and choriodecidua (Fig. 2c).

TABLE 1. Effect of SASP on LDH release from human gestational tissues.

	Total tissue LDH (%)		
Treatment	Placenta	Amnion	Choriodecidua
LPS control	6.9 ± 1.7	0.67 ± 0.28	4.4 ± 1.4
0.1/1 mM SASP	5.0 ± 1.1	1.13 ± 0.37	3.3 ± 1.8
1/5 mM SASP	4.3 ± 1.4	1.70 ± 0.62	3.6 ± 1.0
5/10 mM SASP	5.7 ± 1.7	2.10 ± 0.94	5.9 ± 1.7

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Effect of SASP on Proinflammatory Cytokine Release

Compared to control, IL-6 (Fig. 3), IL-8 (Fig. 4), and TNF-α (Fig. 5) released into the conditioned media from placental, amnionic, and choriodecidual tissue explants was significantly reduced by the addition of SASP at concentrations of 5 mM or greater.

Validation of Explant Cultures and Viability

To validate the integrity of explants in the presence of SASP, cell viability was investigated using LDH release from explants. LDH release was investigated over the 6-h time course of placental, amnion, and choriodecidual explants. Explants were incubated either in control media (10 μg/ml LPS) or media containing SASP (Table 1). Data are presented as a percentage of total tissue LDH, which was calculated for tissues collected at time zero and homogenized to release cytosolic LDH.

Compared to the LPS control, treatment with SASP at all concentrations tested did not significantly affect LDH release from placenta, amnion, and choriodecidua, indicating that the concentrations used were not toxic to the tissue explants.

DISCUSSION

NF-κB subunits p50 and RelA have been demonstrated in amnion-derived WISH cells [17, 18], human myometrial cells [19], and human cytotrophoblasts [20, 21]. King et al. [22] have demonstrated the differential expression of mRNA transcripts encoding for NF-κB pathway intermediates throughout the peripartum period. The identification of the NF-κB signaling pathway in human intrauterine tissues suggests that it plays a functional role in these tissues; however, very little evidence is available to support this. The available data have, however, demonstrated the importance of NF-κB in the expression of COX-2 in human myometrial cells and amnion WISH cells [17, 19, 21] and IL-8 gene expression in amnion cells obtained before and after labor [18]. The κB motif has been identified in the promoter regions of a number of proinflammatory cytokines, including TNF-α [23], IL-6 [2], and IL-8 [18], and all have been implicated in the initiation or progression of human preterm and term labor. Thus, the aim of this study was to determine whether NF-κB regulates proinflammatory cytokine release from human intrauterine tissues.

In this study, SASP at concentrations of at least 5 mM suppressed NF-κB DNA binding activity in human placenta, amnion, and choriodecidua. Furthermore, this inhibition was associated with a significant and concomitant inhibition of IL-6, IL-8, and TNF-α release. This is in agreement with previous studies that have demonstrated that inhibition of IL-2, IL-12, and TNF-α using SASP was associated with concomitant decreases in NF-κB activation [12–14]. Furthermore, other anti-inflammatory salicylates such as aspirin and sodium salicylate have also been found to inhibit both basal and LPS-stimulated NF-κB-dependent cytokine transcription [24, 25]. The precise mechanism by which SASP and other salicylates inhibit NF-κB activation has not yet been clearly established, although it is postulated that inhibition is the result of preventing nuclear translocation of RelA due to the inhibition of IκB-α phosphorylation and subsequent degradation. It is proposed that SASP exerts a direct effect on an IκB-α kinase or on another upstream signal [12].

IL-6, IL-8, and TNF-α protein and mRNA transcripts have been identified in human gestational tissues (reviewed in [26]). In this study, NF-κB has been identified as a transcription factor that is important for the formation of proinflammatory cytokines in human placenta, amnion, and choriodecidua. This has important implications, because the available evidence is consistent with the involvement of cytokines in the processes of labor and delivery, both at term and preterm. Preterm labor is associated with cervicovaginal infection [27, 28]. It is proposed that bacteria, bacterial products, or both may elicit inflammatory reactions in gestational tissues, resulting in a greater release of chemokines such as IL-8 [29], which form a chemotactic gradient to promote the recruitment of PMN cells and monocytes, and the release of proinflammatory cytokines IL-1β, IL-6, and TNF-α [15, 30], which can then further induce 1) cytokine release through a positive feed-forward mechanism; 2) the expression of adhesion molecules; 3) phospholipid metabolizing enzymes (phospholipase A_2 and cyclooxygenase isozymes) and their products [31–33]; and 4) ECM matrix remodeling enzymes uPA and MMP-9 [34]. This provides a network that facilitates remodeling of gestational tissues to facilitate delivery.

In this study we have established that in human placenta, amnion, and choriodecidua, NF-κB activation is a key regulator of the proinflammatory cytokines IL-6, IL-8, and TNF-α. Consequently, the development of specific inhibitors of NF-κB will be both 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 treatment of preterm labor associated with infection, and should, as a result, reduce the side effects associated with drugs such as nonsteroidal anti-inflammatory drugs and glucocorticoids.

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