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Cyclooxygenase inhibition in human monocytes increases endotoxin-induced TNFα without affecting cyclooxygenase-2 expression

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Abstract

Human endotoxin-stimulated adherent monocytes were used in order to determine whether or not NSAIDs influence cyclooxygenase-2 and/or tumor necrosis factor (TNF)α expression within the range of inhibitor concentrations that are required to suppress prostaglandin biosynthesis. Exogenous prostaglandin E_2 (IC_{50}<5 nM) inhibited endotoxin-induced TNFα mRNA and protein while, up to 1 μM, it did not significantly affect cyclooxygenase-2 mRNA expression. Similar results were obtained using the membrane-permeable cAMP analogue db-cAMP, which caused preferential inhibition of TNFα expression.

Indomethacin or lysine-acetylsalicylic acid concentration-dependently inhibited prostaglandin E_2 biosynthesis and, at concentrations causing near-complete inhibition, enhanced TNFα mRNA and protein expression without significantly influencing cyclooxygenase-2 mRNA. In addition, by facilitating endotoxin-induced TNFα expression, indomethacin or lysine-acetylsalicylic acid counteracted dexamethasone-induced inhibition of TNFα biosynthesis, thereby exhibiting an effect opposite to that of exogenous prostaglandin E_2. The results suggest that in human endotoxin-stimulated monocytes, NSAIDs can enhance TNFα expression through inhibition of cyclooxygenase and the resulting decrease in prostanoid biosynthesis.

Keywords: NSAID; Prostaglandin E_2; TNFα; Cyclooxygenase; Glucocorticoid; Monocyte, human

1. Introduction

Tumor necrosis factor (TNF)α has been suggested to be a major mediator in the pathogenesis of several inflammatory conditions including rheumatoid arthritis. In fact, drugs that block endogenous TNFα have proven effective to improve symptoms and slow progression of rheumatoid arthritis, and may provide therapeutic benefit for other chronic inflammatory diseases (Feldmann, 2002; Smolen and Steiner, 2003). In view of the central role of TNFα in disease progression it seems reasonable to ask to which extent traditional anti-inflammatory therapeutics affect TNFα biosynthesis in inflamed tissue.

There are several indications that anti-inflammatory drugs which inhibit cyclooxygenase might in fact stimulate TNFα biosynthesis. First, it is well known that exogenous prostaglandin E_2 inhibits TNFα expression (e.g., Meja et al., 1997; Kunkel et al., 1988; Seldon et al., 1995). Secondly, it has been suggested that prostaglandin E_2 can enhance the expression of the inducible cyclooxygenase isoform, cyclooxygenase-2, thus facilitating its own biosynthesis in inflamed tissue (Hinz et al., 2000a,b). This positive feed back loop could be expected to increase the concentration of prostaglandin E_2, and, in turn, underpin TNFα inhibition.

However, this hypothesis is challenged by a number of observations: On one hand, there are conflicting results with regard to the possible effects of prostaglandin E_2 on cyclooxygenase-2 expression. The results of studies showing enhanced cyclooxygenase-2 expression (Hinz et al., 2000a,b) are difficult to reconcile with those showing no effect (Panara

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et al., 1995) or even suppression (Callejas et al., 1999; Pang and Hoult, 1996, 1997) of cyclooxygenase-2 by prostaglandin E_2. On the other hand, studies aimed at determining the effects of endogenous prostaglandin E_2 on TNFα expression have also yielded diverging results. Recently, Uematsu et al. (2002) have shown in vivo and in vitro that endotoxin-induced TNFα expression is not altered in membrane-bound glutathione-dependent prostaglandin E synthase (mPGES)-deficient mice in spite of the fact that endotoxin-stimulated prostaglandin E_2 biosynthesis is abolished in these mice. Furthermore, there are studies that have employed cyclooxygenase inhibitors to block endogenous prostaglandin E_2 and find no detectable effect (Caughey et al., 1997; Jozefowski et al., 2003) or even a decrease (Lozanski et al., 1992; Osnes et al., 1996; Shackelford et al., 1997) of TNFα expression. The absence of detectable effects of cyclooxygenase inhibitors in spite of clear TNFα inhibition by exogenous prostaglandin E_2 has led to the suggestion that thromboxane A_2 counteracts the inhibitory effect of prostaglandin E_2; thus cyclooxygenase inhibition would suppress in parallel the biosynthesis of stimulatory (thromboxane A_2) and inhibitory (prostaglandin E_2) factors explaining the absence of a detectable net effect on TNFα expression (Caughey et al., 1997).

The studies mentioned above seem in contrast to a number of other studies (e.g., Endres et al., 1996; Fieren et al., 1992; Hart et al., 1989; Spatafora et al., 1991), which have employed cyclooxygenase inhibitors to block endogenous prostaglandin biosynthesis and find clear stimulation of TNFα. Thus, a nearly 6-fold increase of endotoxin-stimulated TNFα formation by 10 μM indomethacin has been reported in a macrophage cell line (Ikegami et al., 2001). Although this provides an impressive example of a possible pro-inflammatory effect of indomethacin, it remains open whether stimulation of TNFα correlated with inhibition of prostaglandin biosynthesis by indomethacin, or was due to a non-cyclooxygenase related effect of high concentrations of the drug on intracellular signaling mechanisms (Tegeder et al., 2001).

In view of the wide variation of published results on this topic, which can be explained only to some extent by cell type-dependent differences, it seemed reasonable to test the hypothesis that cyclooxygenase inhibition affects cyclooxygenase-2 and/or TNFα expression in an experimental model that allows pharmacological analysis of drug effects on cyclooxygenase-2, TNFα and prostaglandin biosynthesis in parallel.

Therefore, this study was conducted using human endotoxin-stimulated adherent monocytes in order to determine whether or not cyclooxygenase inhibitors influence cyclooxygenase-2 and/or TNFα expression within the range of inhibitor concentrations that are required to suppress prostaglandin biosynthesis. In addition, a possible interference of cyclooxygenase inhibition with dexamethasone-induced TNFα suppression was investigated.

2. Materials and methods

2.1. Preparation of human peripheral blood monocytes

Volunteer blood donors were healthy normal subjects, who were taking no systemic medication and showing normal values of C-reactive protein. Blood was prepared according to Heinemann et al. (2003): citrated blood (40 ml) was centrifuged at 300×*g* for 20 min at room temperature to remove platelet-rich plasma, after which erythrocytes were removed by addition of 6% Dextran T-500 (Sigma, Vienna, Austria) in 0.9% saline (50 ml). For separation of polymorphonuclear leukocytes (containing neutrophils and eosinophils) from peripheral blood monocytic cells (comprising monocytes and lymphocytes), the supernatant was layered on 15 ml Histopaque (1077 Density, Sigma). After centrifugation at 350×*g* for 20 min, the mononuclear cells of the interface were carefully removed, washed twice with phosphate-buffered saline (PBS) containing 5.5 mM glucose and 2.7 mM KCl, resuspended in RPMI 1640 (Roswell Park Memorial Institute Media; PAA, Linz, Austria) supplemented with penicillin (100 U/ml, Sigma), streptomycin (100 μg/ml, Sigma), glutamine (20 mM), non-essential amino acids, HEPES 0.05 M and sodium pyruvat 10 mM (all obtained from PAA) and counted using a Minos Vet cell counter (Roche, Vienna, Austria). Aliquots of 3.5×10^6 mononuclear cells/ml were placed in microwell plates (24-well format; Corning/Costar, Corning, NY) and 25 μl of human AB serum (Sigma) was added. After 120 min of incubation at 37 °C (5% CO_2), the non-adherent cells (about 85% of the original mononuclear cells) were removed by aspiration and the plate was washed three times with 500 μl PBS. The adherent monocytes were cultured in supplemented RPMI 1640 medium at 37 °C (5% CO_2). Using this method, monocytes represent >95% of total adherent cells by microscopical examination of Giemsa-stained cells. Cell viability, assessed by the 3-(4,5-dimethylthiazo-2yl)2,5-diphenyltetrazolium bromide (MTT) test, was not significantly affected by test drugs.

2.2. Experimental protocol

Cells were exposed to endotoxin (lipopolysaccharide, *Escherichia coli* serotype 055:B5; Sigma) 1 ng/ml for 4 h. Thereafter, the supernatants were collected for radioimmunological determination of prostaglandin E_2, thromboxane B_2 or TNFα, and RNA was extracted from cells. Drugs were added 30 min before addition of endotoxin.

2.3. Radioimmunoassay

Immunoreactive prostaglandin E_2 and thromboxane B_2 were determined as described previously (Amann and Schuligoi, 2000; Schuligoi et al., 1998) using [5,6,8,11,12,14,15(*N*)-3*H*] prostaglandin E_2 or [5,6,9,11,12,14,15(*N*)-3*H*] thromboxane B_2 (both from Amersham, Vienna,

Austria) as tracer and synthetic prostaglandin E_2 and thromboxane B_2 (both Cayman, Ann Arbor, MI, USA) as standards. The detection limit (defined as 10% inhibition of binding of tracer to antibody) was 4 pg for prostaglandin E_2 and 0.4 pg for thromboxane B_2; the EC_{50} values with interassay variation (S.E.) were prostaglandin E_2: 29 pg (S.E. 3%) and thromboxane B_2: 6 pg (S.E. 5%).

2.4. TNFα enzyme-linked immunosorbent assay (ELISA)

TNFα immunoreactivity in the supernatants was determined by sandwich ELISA. Ninety six well round-bottom plates (Greiner Labortechnik, Kremsmuenster, Austria) were coated with 50 μl of mouse anti-human TNFα monoclonal antibody (BD Biosciences, Schwechat, Austria) 2 μg/ml in 0.1 M sodium phosphate buffer pH=9.0 and incubated overnight at 4 °C. After removal of the antibody, 200 μl of blocking buffer (1% bovine serum albumin in PBS) was added to the wells and incubated for 2 h at room temperature. Then the wells were washed with PBS containing 0.04% Tween 20 (Sigma) TNFα standards (62.5–2000 pg/ml) and unknown samples (diluted in assay buffer: PBS containing 1% BSA and 0.05% Tween 20) were added and incubated at 4 °C overnight. Thereafter plates were washed as described above and incubated with 100 μl biotinylated monoclonal mouse anti-human TNFα antibody (BD Biosciences) 1 μg/ml, diluted in assay buffer for 1 h at room temperature, washed again and then incubated at room temperature for 30 min with 100 μl of avidin–peroxidase conjugate (Sigma) diluted in assay buffer. Plates were washed once more and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate system (Kirkegaard and Perry Laboratories [KPL], Gaithersburg, MD, USA) according to the manufacturer's instructions. The reaction was stopped by adding 100 μl of 1 M phosphoric acid. TNFα was measured colorimetrically at 450 nm and quantified by interpolation from the standard curve.

2.5. RNA extraction and reverse transcription

Total RNA was extracted using Masterpure™ Complete RNA purification kit (Epicentre, Madison, WI, USA) including DNase treatment to remove contaminating DNA. RNA was quantified with RiboGreen™ RNA quantitation kit (Molecular Probes, Eugene, OR, USA). First strand cDNA synthesis was performed with Omniscript™ reverse transcriptase kit (Qiagen, Hilden, Germany) with 0.1 μg RNA and 20 pmol oligo dT primers, according to the manufacturer's instructions.

2.6. Real-time polymerase chain reaction (PCR)

Real-time PCR was performed with the LightCycler™ Instrument (Roche, Mannheim, Germany) using fluorescence resonance energy transfer (FRET) detection. Amplification was performed in a total volume of 19 μl of LightCycler-FastStart DNA Master Hybridization Probes (Roche) mix containing 4 mM $MgCl_2$, 50 pmol of the specific primers, 20 pmol of the specific hybridization probes (Table 1) and 1 μl of an 1:10 dilution of cDNA obtained from reverse transcription. Specific fluorogenic hybridization probes were used, one labeled with fluorescein on the 3′ end and the second with LC-Red640 on the 5′ end (Table 1). Primers and hybridization probes were designed and synthesized by TibMolbiol (Berlin, Germany). For hot start the samples were kept at 95 °C for 6 min, the cycling parameters for cyclooxygenase-2 and TNFα were: 95 °C 3 s, 65 °C 10 s, 72 °C 10 s, and for glyceraldehyde 3-phoshate dehydrogenase (G3PDH): 95 °C 3 s, 67 °C 10 s, 72 °C 10 s and 50 cycles were performed.

For quantification the crossing point (CP) was used. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence indicating that the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. In this study the Second Derivate Maximum Method was performed for CP determination using LightCycler Software 3.5 (Roche, Mannheim, Germany).

For relative quantification of the target genes, standard curves were prepared from a stock of cDNA obtained from reverse transcribed RNA isolated from monocytes in 1:10 dilution steps. The same stock cDNA was used for all amplifications to determine the relative quantities across multiple runs. For each sample, the amount of target genes

Table 1
Oligonucleotide primers used in polymerase chain reaction

	Forward primer 5′–3′	
COX-2	ATG AGA TTG TGG GAA AAT TGC T	
TNFα	CCT GTA GCC CAT GTT GTA GCA A	
G3PDH	ACC ACA GTC CAT GCC ATC AC	
	Reverse primer 5′–3′	
COX-2	GGT AGA TCA TCT CTG CCT GAG TAT C	
TNFα	CCT TGG TCT GGT AGG AGA CG	
G3PDH	TCC ACC ACC CTG TTG CTG TA	
	Hybridization probes 5′–3′	
COX-2 FL	ATC CCC AGG GCT CAA ACA TGA TGT	
COX-2-LC640	CAT TCT TTG CCC AGC ACT TCA CGC	
TNFα FL	GCC CCT CCA CCC ATG TGC TCC	
TNFα LC640	CAC CCA CAC CAT CAG CCG CAT C	
G3PDH FL	TAC ACT GAG CAC CAG GTG GTC TCC TCT	
G3PDH LC640	CTT CAA CAG CGA CAC CCA CTC CTC	
	GenBank accession #	**Positions amplified**
COX-2	M90100	581–857
TNFα	M10988	358–565
G3PDH	J02642	601–1033

and endogenous reference (G3PDH) gene were determined from the appropriate standard curves in arbitrary units. Thereafter samples were normalized to G3PDH. (The target amounts were divided by the endogenous reference amount to obtain a normalized target value.) In each amplification run, reagent controls (comprising PCR-grade water instead of cDNA) were included.

2.7. Drugs

Stock solutions of 1 mM prostaglandin E_2 (Sigma) and 10 mM SQ 29,548 [1*S*-[1*a*,2*a*(*Z*),3*a*,4*a*]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazine]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptanoic acid; Sigma) were prepared in ethanol. Dexamethasone (Sigma) was dissolved in dimethyl sulfoxide (10 mM stock solution). Dibutyryl-cAMP (N_6,O_2-dibutyryl adenosine 3′,5′-cyclic monophosphate) obtained from Tocris Cookson (Bristol, UK), DL-lysine-mono-acetylsalicylic acid (Aspisol™) obtained from Bayer Leverkusen, Germany, and sodium salicylate (Sigma) were dissolved in water. Indomethacin (Sigma) was dissolved in 0.1 M phosphate buffer pH 7.4.

2.8. Data evaluation and statistical analysis

Within each experiment, samples obtained from one individual donor were assayed in duplicate for immunoreactive prostaglandin E_2, thromboxane B_2, and TNFα; cells of two wells were pooled for RNA extraction. The resulting average was used to calculate means±S.E.M. of *n* independent determinations. In order to determine the effect of drug treatment, data were calculated as percent of control (endotoxin stimulation alone) and expressed as means± S.E.M. Statistical analysis was performed using one-way analysis of variance, and multiple comparisons were done with Student–Newman–Keuls test (SigmaStat, SPSS, Chicago, IL, USA). A value of $P<0.05$ was considered significant. EC_{50} values were calculated using SigmaStat 8.0 pharmacology module.

3. Results

3.1. Effect of endotoxin on prostaglandin E_2 biosynthesis, cyclooxygenase-2 and TNFα expression

The concentration of immunoreactive prostaglandin E_2 in supernatants of non-stimulated cells (4-h incubation period) was 0.52±0.05 nM (*n*=35). In order to validate radioimmunological measurements, in one group of experiments (*n*=3) the recovery of exogenous prostaglandin E_2 was determined (3 nM prostaglandin E_2; added to the preparation immediately before supernatants were collected) and found to be 95–98% (results not shown).

After exposure to endotoxin (1 ng/ml; 4 h) the concentration of immunoreactive prostaglandin E_2 in the supernatants was 2.65±0.22 nM, (*n*=35), suggesting enhanced prostaglandin E_2 biosynthesis by endotoxin-stimulated monocytes. Exposure to endotoxin also caused a pronounced increase in TNFα mRNA (27.9±4.2-fold increase over control; *n*=34) and cyclooxygenase-2 mRNA (54.7±8.1-fold increase over control; *n*=34). Immunoreactive TNFα was not detectable in supernatants of non-stimulated cells (detection limit of the assay 190 pg/ml sample). In endotoxin-stimulated preparations it was 2590±236 pg/ml (*n*=49) indicating that increased expression of TNFα mRNA resulted in increased TNFα biosynthesis.

3.2. Effects of exogenous prostaglandin E_2 on endotoxin-induced cyclooxygenase-2 and TNFα expression

Prostaglandin E_2 caused a concentration-dependent inhibition of the expression of TNFα mRNA and immunoreactivity, the apparent IC_{50} being 4.2 nM (CI 95%: 1.1–17.5 nM) and 4.8 nM (CI 95%: 2.1–8.0 nM), respectively. A more than 80% inhibition of TNFα expression was obtained at 1 μM prostaglandin E_2 (Fig. 1A,B). However, up to a

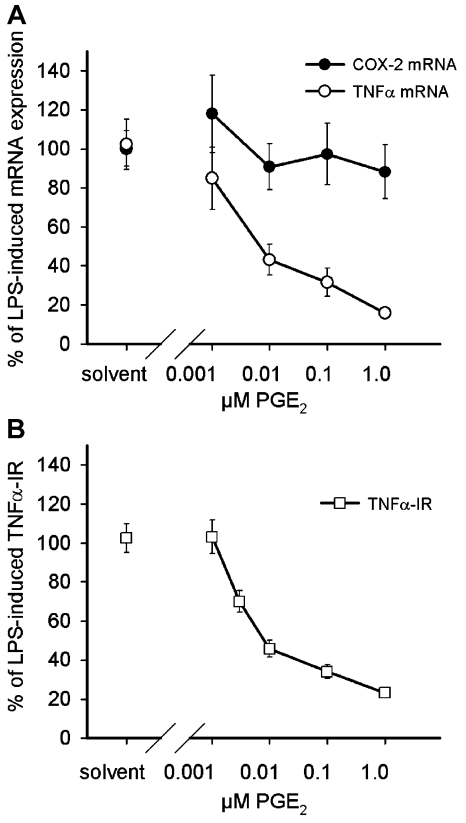

Fig. 1. Effects of prostaglandin E_2 on endotoxin-induced cyclooxygenase-2 and TNFα mRNA and TNFα protein. Adherent human monocytes were exposed to endotoxin (lipopolysaccharide=LPS; 1 ng/ml) in the presence of prostaglandin (PG)E_2 or vehicle. Following a 4-h incubation period RNA was extracted from the cells and RT-PCR was performed to determine the expression of cyclooxygenase (COX)-2 and TNFα mRNA (A). The culture supernatants were collected and assayed for immunoreactive TNFα (B). Data points represent mean±S.E.M. (*n*=4–7).

concentration of 1 μM, prostaglandin E_2 did not significantly affect cyclooxygenase-2 mRNA (Fig. 1A).

The membrane permeable cAMP analogue db-cAMP (10 μM) also decreased endotoxin-induced TNFα mRNA and immunoreactivity (Fig. 2A,B) without significantly affecting cyclooxygenase-2 mRNA (Fig. 2A). At 100 μM db-cAMP, a concentration that virtually abolished TNFα induction, there seemed to be a moderate increase of cyclooxygenase-2 mRNA, an effect that did not reach statistical significance (Fig. 2A).

In contrast to prostaglandin E_2 or db-cAMP, dexamethasone suppressed endotoxin-induced TNFα mRNA as well as cyclooxygenase-2 mRNA expression (Fig. 3).

3.3. Effects of cyclooxygenase inhibition on endotoxin-stimulated prostaglandin E_2 biosynthesis, cyclooxygenase-2 and TNFα mRNA expression

Indomethacin and lysine-acetylsalicylic acid decreased the concentration of prostaglandin E_2 in the supernatants of endotoxin-stimulated preparations. The apparent IC_{50} for indomethacin and lysine-acetylsalicylic acid was 6.8 nM (CI 95%: 3.9–11.8) and 12.1 μM (CI 95%: 7.5–19.4), respectively. Parallel determination of TNFα mRNA expression in monocytes indicated that inhibition of prostaglandin E_2 biosynthesis was accompanied by enhanced expression of TNFα mRNA, which reached statistical significance at 100 nM indomethacin and 300 μM lysine-acetylsalicylic acid (Fig. 4A,B). Within the range of concentrations tested, neither compound significantly affected the expression of cyclooxygenase-2 mRNA (Fig. 4A,B).

In order to determine a possible role of endogenous thromboxane A2, additional experiments were conducted using the TP receptor antagonist SQ 29,548. Determination of the formation of immunoreactive thromboxane B2, the stable thromboxane A2 metabolite, showed an endotoxin-induced increase of immunoreactive thromboxane B2 (control: 0.2±0.03 nM; endotoxin: 5.3±1.1 nM; n=10), which was completely inhibited by 100 nM indomethacin as well as by 300 μM lysine-acetylsalicylic acid (data not shown). In order to test the hypothesis that endogenous thromboxane A2 facilitates TNFα expression, the TP receptor antagonist SQ 29,548 was employed at a concentration (10 μM) shown previously to be effective in causing a more than 60% decrease of TNFα formation in human monocytes (Caughey et al., 1997). However, in our experimental model, SQ 29,548 on its own did not significantly alter endotoxin-induced TNFα mRNA expression. Furthermore, SQ 29,548 did not significantly influence the effect of indomethacin (100 nM) on endotoxin-induced TNFα mRNA expression (Table 2). As a positive control, SQ 29,548 was tested in human platelet-rich plasma, where it completely prevented TP agonist (U46619=9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; 5 μM)-induced platelet aggregation (results not shown).

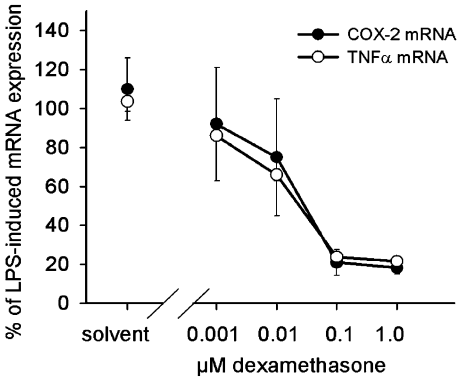

Fig. 3. Effects of dexamethasone on endotoxin-induced cyclooxygenase-2 and TNFα mRNA expression. Adherent human monocytes were exposed to endotoxin (lipopolysaccharide=LPS; 1 ng/ml) in the presence of dexamethasone or vehicle. Following a 4-h incubation period RNA was extracted from the cells and RT-PCR was performed to determine the expression of cyclooxygenase (COX)-2 and TNFα mRNA. Data points represent mean± S.E.M. (n=3–6).

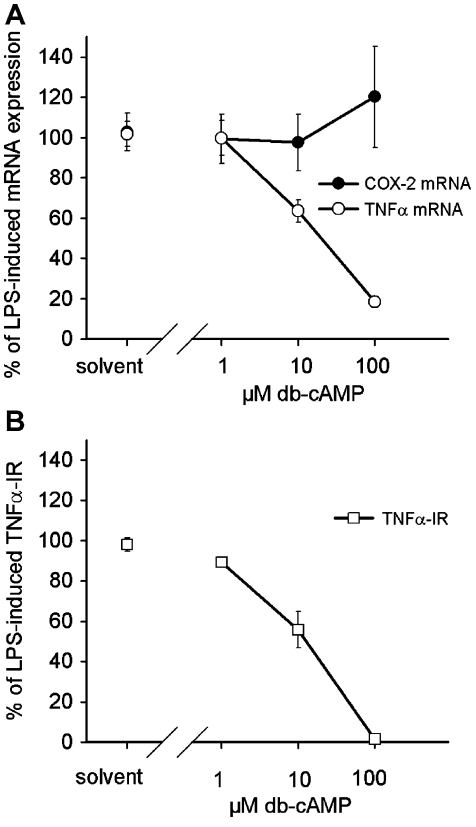

Fig. 2. Effects of db-cAMP on endotoxin-induced cyclooxygenase-2 and TNFα mRNA and TNFα protein. Adherent human monocytes were exposed to endotoxin (lipopolysaccharide=LPS; 1 ng/ml) in the presence of db-cAMP or vehicle. Following a 4-h incubation period RNA was extracted from the cells and RT-PCR was performed to determine the expression of cyclooxygenase (COX)-2 and TNFα mRNA (A). The culture supernatants were collected and assayed for immunoreactive TNFα (B). Data points represent mean±S.E.M. (n=4).

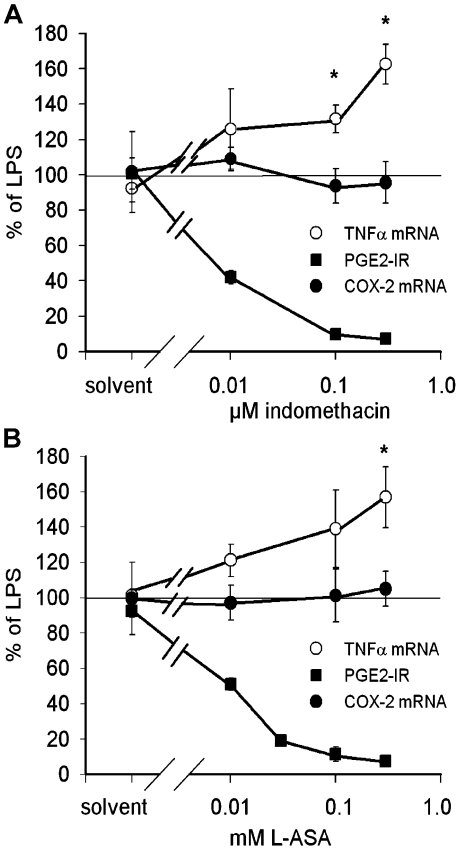

Fig. 4. Effects of NSAIDs on endotoxin-induced cyclooxygenase-2 and TNFα mRNA expression and prostaglandin E_2 immunoreactivity. Adherent human monocytes were exposed to endotoxin (lipopolysaccharide=LPS; 1 ng/ml) in the presence of indomethacin (A), lysine-acetylsalicylic acid (L-ASA) (B), or the respective vehicle. Following a 4-h incubation period, RNA was extracted from the cells and RT-PCR was performed to determine the expression of cyclooxygenase (COX)-2 and TNFα mRNA. The culture supernatants were collected and assayed for immunoreactive prostaglandin (PG)E_2. Data points represent mean±S.E.M. (n=4–29). *P<0.05 as compared to the corresponding solvent.

3.4. Effects of cyclooxygenase inhibition on dexamethasone-induced suppression of TNFα biosynthesis

Since our results so far have suggested that the concentration of endogenous prostaglandin E_2 released by stimulated monocytes is sufficient to attenuate TNFα expression without significantly affecting cyclooxygenase-2, further experiments were conducted in order to determine to which extent inhibition of endogenous prostaglandin E_2 biosynthesis interferes with dexamethasone-induced inhibition of TNFα release.

In supernatants of endotoxin-stimulated preparations, dexamethasone concentration-dependently reduced the concentration of immunoreactive TNFα with an IC_{50} of 11.4 nM (CI 95%: 4.6–28; Fig. 5A), which corresponds to results obtained at the mRNA level (see Fig. 3). In the presence of prostaglandin E_2 (3 nM), endotoxin-induced release of TNFα was attenuated (Fig. 5A), but the inhibitory potency of dexamethasone was not significantly affected (IC_{50}: 21.6; CI 95%: 8.0–57.9).

Indomethacin (300 nM), on its own, enhanced the release of immunoreactive TNFα by nearly 40% (Fig. 5A) suggesting that the observed increase of TNFα mRNA resulted in enhanced protein synthesis. In the presence of indomethacin, the inhibitory potency of dexamethasone was

Table 2
Effects of the TP receptor antagonist SQ 29,548 on endotoxin-induced TNFα mRNA expression

Treatment	TNFα mRNA (% of control)
Solvent (0.1% ethanol)	105±7 (8)
SQ 29,548 (10 μM)	107±7 (8)
Indomethacin (100 nM)	132±6 (5)[a]
Indomethacin and SQ 29,548	136±11 (5)[a]

Adherent human monocytes were exposed to endotoxin (1 ng/ml) in the presence of either solvent or drugs indicated. After a 4-h incubation period, RNA was extracted from the cells and RT-PCR was performed to determine the expression of TNFα mRNA.
Data points represent mean±S.E.M.; n in parenthesis.
[a] P<0.05 vs. solvent group.

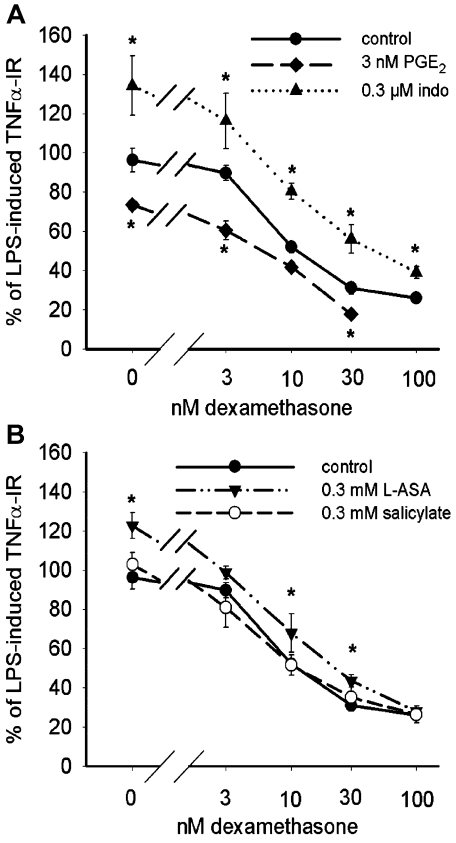

Fig. 5. Interaction of prostaglandin E_2 or NSAIDs with dexamethasone-induced inhibition of TNFα biosynthesis. Adherent human monocytes were exposed to endotoxin (lipopolysaccharide=LPS; 1 ng/ml) and incubated with dexamethasone in the absence (control) or presence of A: prostaglandin (PG)E_2 or indomethacin (indo), and B: lysine-acetylsalicylic acid (L-ASA) or sodium salicylate (salicylate). Following a 4-h incubation period, the culture supernatants were collected and assayed for immunoreactive TNFα. Data points represent mean±S.E.M. (n=6–23). *P<0.05 as compared to dexamethasone alone.

not significantly altered (IC_{50}: 12.5 nM; CI 95%: 2.7–58.0). However, due to enhanced basal TNFα release in the presence of indomethacin, about 3-fold higher concentrations of dexamethasone were required to reduce TNFα to similar levels as in preparations without indomethacin exposure (Fig. 5A). The results obtained with 300 μM lysine-acetylsalicylic acid were similar to those obtained with indomethacin (Fig. 5B). Additional experiments were conducted using the aspirin metabolite salicylate, which is a very weak inhibitor of cyclooxygenase in vitro but exhibits anti-inflammatory activity in vivo (cf. Amann and Peskar, 2002): sodium salicylate (0.01–0.3 mM) had no significant effect on endotoxin-stimulated prostaglandin E_2 biosynthesis (results not shown), and, at 0.3 mM, did not significantly affect TNFα release or interfere with dexamethasone-induced inhibition (Fig. 5B).

4. Discussion

In the present study, we have used adherent human monocytes that were exposed for 4 h to a submaximal concentration (1 ng/ml) of endotoxin. We have shown previously that using this protocol, endotoxin activates adherent monocytes selectively through a CD14 receptor-dependent pathway (Ulcar et al., 2003). The concentration of prostaglandin E_2 present in the supernatants of endotoxin-stimulated cells was quite similar to the concentration of exogenous prostaglandin E_2 required to produce half maximal inhibition of endotoxin-induced TNFα biosynthesis. This suggests that in this experimental model, endogenous prostaglandin E_2 may be effective to inhibit TNFα induction. In contrast, cyclooxygenase-2 mRNA expression was not significantly influenced by prostaglandin E_2 up to 1 μM, which is more than 200-fold the IC_{50} for TNFα inhibition.

Since an increase of intracellular cAMP has been linked to inhibition (Pang and Hoult, 1996; Pang and Hoult, 1997) as well as stimulation of cyclooxygenase-2 induction (Hinz et al., 2000a,b) additional experiments were conducted using the membrane-permeable cAMP analogue db-cAMP. Similar to the results obtained with prostaglandin E_2, db-cAMP at a concentration (10 μM) sufficient to cause half-maximal inhibition of endotoxin-induced TNFα had no significant effect on cyclooxygenase-2 induction. Only at 100 μM db-cAMP, a concentration that prevented TNFα induction, there was a tendency towards increased cyclooxygenase-2 expression (see Fig. 2A). In contrast to prostaglandin E_2 and db-cAMP, which caused preferential inhibition of TNFα induction, dexamethasone reduced endotoxin-induced TNFα and cyclooxygenase-2 with similar potency.

Since it has been shown that prostaglandin E_2 inhibits TNFα expression through a cAMP-dependent pathway (Ikegami et al., 2001; Kunkel et al., 1988; Seldon et al., 1995; Tannenbaum and Hamilton, 1989), it seems likely that the comparatively low sensitivity of cyclooxygenase-2 expression to elevated intracellular cAMP can explain the absence of detectable effects of prostaglandin E_2 within the range of concentrations employed in the present experiments.

In agreement with the results obtained with exogenous prostaglandin E_2, there was no significant effect of the two cyclooxygenase inhibitors on cyclooxygenase-2 expression. Up to concentrations of indomethacin or lysine-acetylsalicylic acid that caused maximal inhibition of prostaglandin E_2 biosynthesis, cyclooxygenase-2 mRNA expression remained stable, although, at the same time, the cyclooxygenase inhibitors caused a marked increase of TNFα mRNA and protein expression.

The absence of significant effects of cyclooxygenase inhibition on cyclooxygenase-2 mRNA in the present experiments agrees with results of Panara et al. (1995), who found no detectable effects of complete inhibition of prostaglandin E_2 biosynthesis on human monocyte cyclooxygenase-2 protein in Western blot analysis. However, the present results are at variance with observations that, in macrophages, exogenous prostaglandin E_2 suppresses and indomethacin enhances endotoxin-induced cyclooxygenase-2 (Pang and Hoult, 1996, 1997). In this context it seems noteworthy that in these studies significant effects were observed at 10–100 μM exogenous prostaglandin E_2 (Pang and Hoult, 1997) and 100 μM indomethacin (Pang and Hoult, 1996), respectively. In view of the fact that in these studies the concentration of endogenous prostaglandin E_2 was reported to be in the range of 15–50 nM, and the cyclooxygenase selective range of concentrations of indomethacin was not determined, the possibility cannot be excluded that observed effects of indomethacin were not related to cyclooxygenase inhibition.

To some extent, these points may also help to explain apparent differences between the present results and those of Hinz et al. (2000b) who observed that, in human monocytes, exogenous prostaglandin E_2 stimulates and the cyclooxygenase-2 inhibitor NS-398 attenuates endotoxin-induced cyclooxygenase-2. Stimulation of cyclooxygenase-2 mRNA was obtained between 1 and 10 μM prostaglandin E_2, inhibition of cyclooxygenase induction by 10 μM NS-398. Unfortunately, neither the concentration of endogenous prostaglandin E_2 nor the cyclooxygenase selective range of concentrations of NS-398 was determined in parallel with the expression of cyclooxygenase-2 mRNA. Therefore, also in this instance, non-cyclooxygenase related effects of the employed cyclooxygenase inhibitor cannot be ruled out. Another explanation may be provided by the fact that in the studies of Hinz et al. (2000a,b) the concentration of endotoxin used to stimulate cyclooxygenase-2 was 10,000-fold higher than in the present study, and exposure time was 24 h instead of 4 h employed in the present study. Therefore, the amount of endogenous prostaglandin E_2 released by activated monocytes may be considerably higher in the study of Hinz et al. (2000b) than under the present conditions. Supporting this assumption is the

observation of Hinz et al. (2000b) that, in the whole blood assay, endotoxin stimulation raised the concentration of prostaglandin E_2 to 140 nM, which compares to about 3 nM prostaglandin E_2 present in the supernatants of stimulated monocytes in the present experiments. It remains open whether or not these factors may have contributed to the diverging results.

Taken together, under the present experimental conditions we find no detectable effect of cyclooxygenase inhibition on endotoxin-induced cyclooxygenase-2 but significant stimulation of TNFα biosynthesis. Stimulation of TNFα biosynthesis was observed within the cyclooxygenase selective range of concentrations of indomethacin and lysine-acetylsalicylic acid. In contrast, sodium salicylate, which has similar anti-inflammatory potency as acetylsalicylic acid but is only a weak inhibitor of cyclooxygenase (cf. Amann and Peskar, 2002), did not significantly influence TNFα biosynthesis.

The magnitude of the effect of cyclooxygenase inhibition on TNFα expression, an about 50% increase, was compatible with the concentration of prostaglandin E_2 determined in supernatants and the potency of exogenous prostaglandin E_2 to suppress TNFα. The hypothesis that concomitant suppression of prostaglandin E_2 and thromboxane A_2 by cyclooxygenase inhibitors would have opposing effects on TNFα expression (Caughey et al., 1997; see Introduction) was not supported by the results obtained in the present experimental model, because there was no detectable effect of a thromboxane TP receptor antagonist on endotoxin induced TNFα or its facilitation by indomethacin. Our results are, therefore, in agreement with those of Meja et al. (1997), who found no significant effects on endotoxin-induced TNFα by a TP receptor agonist in human monocytes.

Additional experiments showed that TNFα stimulation by cyclooxygenase inhibitors could also be observed in the presence of dexamethasone. Neither indomethacin nor lysine-acetylsalicylic acid significantly altered the inhibitory potency of dexamethasone. However, in the presence of indomethacin, an about 3-fold higher concentration of dexamethasone was required in order to obtain similar TNFα values than in the absence of indomethacin. Similar results were obtained with lysine-acetylsalicylic acid, although its effect seemed less pronounced than that of indomethacin (see Fig. 5). These results not only add statistical weight to the effect of cyclooxygenase inhibitors determined in the absence of dexamethasone, but may also provide an interesting aspect on their own: Since cyclooxygenase inhibitions are frequently co-administered with glucocorticoid treatment, interference with TNFα suppression may be of pharmacological relevance.

This notion seems in contrast to the results of a recent study using membrane-bound prostaglandin E synthase (mPGES)-deficient mice (Uematsu et al., 2002). This study has convincingly demonstrated that mice lacking an essential pathway for cyclooxygenase-2 derived prostaglandin E_2 biosynthesis do not respond to endotoxin with increased prostaglandin E_2 biosynthesis, although showing normal endotoxin-induced elevation of TNFα. However, in these experiments, the response to endotoxin of genetically modified mice with permanent impairment of prostaglandin E_2 biosynthesis was determined. It seems possible that these results do not allow drawing direct conclusions as to the effects of drug-induced cyclooxygenase inhibition in human cells.

In conclusion, the present study in human endotoxin stimulated monocytes shows that inhibition of cyclooxygenase enhances TNFα biosynthesis but does not significantly affect cyclooxygenase-2 mRNA expression. Cyclooxygenase inhibitor-caused facilitation of TNFα biosynthesis is detectable also in the presence of dexamethasone, and may thereby interfere with its ability to suppress TNFα biosynthesis in inflammatory disease.

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