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Effects of Forskolin on Kupffer Cell Production of Interleukin-10 and Tumor Necrosis Factor Alpha Differ from Those of Endogenous Adenylyl Cyclase Activators: Possible Role for Adenylyl Cyclase 9

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Proinflammatory cytokines like tumor necrosis factor alpha (TNF-α) that are released from Kupffer cells may trigger liver inflammation and damage. Hence, endogenous mechanisms for limiting TNF-α expression are crucial for avoiding the development of sepsis. Such mechanisms include the anti-inflammatory actions of interleukin-10 (IL-10) as well as signaling induced by the intracellular second messenger cyclic AMP (cAMP). Kupffer cells express several receptors that activate cAMP synthesis, including E-prostanoid receptors and β-adrenergic receptors. The expression and role of specific adenylyl cyclases in the inhibition of Kupffer cell activation have so far not been subject to study. Pretreatment of rat Kupffer cell cultures with cAMP analogues [8-(4-chlorophenyl)-thio-cAMP], adenylyl cyclase activator (forskolin), or ligands for G-coupled receptors (isoproterenol or prostaglandin E_2) 30 min before the addition of lipopolysaccharide (LPS) (1 μg/ml) caused attenuated TNF-α levels in culture medium (forskolin/isoproterenol, $P \leq 0.05$; prostaglandin E_2, $P \leq 0.01$). Forskolin also reduced IL-10 mRNA and protein ($P \leq 0.05$), which was not observed with the other cAMP-inducing agents. Furthermore, we found that rat Kupffer cells express high levels of the forskolin-insensitive adenylyl cyclase 9 compared to whole liver and that this expression is down-regulated by LPS ($P \leq 0.05$). We conclude that regulation of TNF-α and IL-10 in Kupffer cells depends on the mechanism by which cAMP is elevated. Forskolin and prostaglandin E_2 differ in their effects, which suggests a possible role of forskolin-insensitive adenylyl cyclases like adenylyl cyclase 9.

Through many studies on inflammatory mechanisms, it has become increasingly clear that the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and the anti-inflammatory cytokine interleukin-10 (IL-10) are important regulatory cytokines in the initial course of inflammation following a bacterial infection (8, 37, 38, 39). IL-10 is directly involved in the repression of TNF-α release (14) and was recently shown to control the onset of irreversible septic shock after cecal ligation and puncture (19, 42). IL-10 deficiency has been observed in a vast amount of inflammatory conditions including endotoxemia (9), inflammatory bowel disease (35), peritonitis (6), preeclampsia (25), and psoriasis (22), pointing to a role for IL-10 as a general control factor against inflammatory outburst.

Kupffer cells are not only the abundant macrophage population of the liver but are also continuously in communication with circulating leukocytes. Kupffer cells may in this way contribute to a fatal systemic inflammation (18, 40) but may also set off protective mechanisms, particularly by releasing anti-inflammatory cytokines like IL-10 (16, 17). Interruption of Kupffer cell function in animal models has been associated with reduced plasma values of IL-10, indicating that Kupffer cells are primary producers of this important anti-inflammatory cytokine in the circulation (6, 27).

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The cyclic AMP (cAMP) signaling pathway has long been known as an important pathway in the repression of TNF-α production, leading to subsequent inhibition of inflammatory responses (15, 26). In addition, cAMP signaling has been reported to enhance lipopolysaccharide (LPS)-mediated IL-10 production in monocytes and macrophages through promoter elements that bind cAMP-responsive element binding protein (CREB) and CCAAT enhancer binding protein (C/EBP) (2, 24, 33). Abrogation of cAMP signaling has been demonstrated in blood from septic patients, which may contribute to the dysregulated inflammatory responses (1).

The second messenger cAMP is produced by adenylyl cyclases, which in general are regulated by activating and inhibitory G proteins (reviewed in reference 20). Several membrane receptors (G-coupled receptors) activate stimulating G proteins leading to cAMP production. Examples of two such well-known receptor families present on Kupffer cells are the adrenergic receptor family and the prostanoid receptor family (7, 11). Recently, mice deficient in the cAMP-inducing adenosine 2A receptors and pituitary adenylyl cyclase-activating polypeptide receptors were shown to be highly susceptible to developing sepsis, and the liver was identified as the organ mainly affected (5, 34).

Most adenylyl cyclases may also be directly activated by forskolin independent of G-coupled receptors (28), but at least one adenylyl cyclase isoform is insensitive to forskolin (10). Following stimulation, intracellular cAMP levels are rapidly down-regulated by phosphodiesterases that transform cAMP into inactive AMP (29). A range of downstream effects of the

TABLE 1. Primers used in the real-time PCR assays

mRNA	Sequence[a]
Rat TNFα	Forward, 5′-AGACCCTCACACTCAGATCATCTTCT-3′ Reverse, 5′-CACGCTGGCTCAGCCACT-3′ Probe, 5′-FAM-AGCCCACGTCGTAGCAAACCACC-dark quencher-3′
Rat IL-10	Forward, 5′-CCTTACTGCAGGACTTTAAGGGTTA-3′ Reverse, 5′-TTTCTGGGCCATGGTTCTCT-3′ Probe, 5′-FAM-CCTGGGGCATCACTTCTACCAGGTAAAACT-dark quencher-3′
Rat AC6	Forward, 5′-TGCTGCTGGTCACCGTGCTCAT-3′ Reverse, 5′-GGACGCTAAGCAGTAGATCATAGTTGTCAA-3′
Rat AC9	Forward, 5′-ACCTACCTTTACCCAAAGTGCACGGACAAT-3′ Reverse, 5′-CTCGGCGCTGCCTCACACACTCTTTGAGAC-3′

[a] FAM, 6-carboxyfluorescein.

cAMP signal have been described in different immune cells, regulated by strict localization of the signal (reviewed in reference 36). In macrophages, however, the pathway of the cAMP-mediated inhibition of TNF-α downstream of the different receptors is still unclear.

We aimed to assess the effects of cAMP-mediated signaling on cytokine production in Kupffer cells, focusing on proinflammatory TNF-α and anti-inflammatory IL-10, and to start examining the expression and regulation of specific adenylyl cyclases in Kupffer cells.

MATERIALS AND METHODS

Reagents. *Escherichia coli* LPS was from Sigma, and prostaglandin E_2 (PGE_2), 8-(4-chlorophenyl)-thio (CPT)-cAMP, isoproterenol, and forskolin were all from Calbiochem (San Diego, CA).

Isolation of Kupffer cells. Kupffer cells were isolated from rat liver (adult Sprague-Dawley rats, 300 to 500 g; Institute of National Health, Oslo, Norway) as previously described (23). In brief, Kupffer cell isolation was based on digestion of the liver in the presence of collagenase P (0.24 mg/ml; Roche, Mannheim, Germany), gradient centrifugation in Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and selective plating (31). Kupffer cells were plated in 96-well plates (0.2×10^6 cells/well) for cytokine assays or 6-well plates (3×10^6 cells/plate) for RNA isolation. Kupffer cells were then cultured for 48 h in RPMI 1640 medium (BioWhittaker Europe, Verviers, Belgium) in the presence of 10% fetal calf serum (GIBCO, Grand Island, NY), L-glutamine (2 mM; GIBCO), and antibiotics (penicillin and streptomycin; GIBCO) before experiments were performed. The study was approved by the Institutional Review Board for the care of animal subjects, and animals were handled and cared for in accordance with National Institutes of Health guidelines.

Kupffer cell experiments. Kupffer cells were exposed to *E. coli* LPS (1 μg/ml) for various periods of time, as indicated in the figure legends. In most experiments, 8-CPT-cAMP or cAMP-elevating drugs were added 30 min prior to the addition of LPS. All unstimulated and control samples were added an equal amount of drug solvent (dimethyl sulfoxide [1‰, vol/vol]). Cell viability was assessed by trypan blue staining at the time of harvest. Medium samples were harvested for cytokine analyses after 6 h or for RNA analyses after 2 h.

Cytokine and cAMP assays. Rat TNF-α (Biosource Ultra Sensitive kit [detection limit, 0.7 pg/ml]; Biosource, Nivelles, Belgium) and rat IL-10 (Biosource regular kit [detection limit, 20 pg/ml]) levels were measured by enzyme-linked immunosorbent assay (ELISA), and cAMP levels were measured by a competitive enzyme immunoassay (EIA; Cayman Chemical Company, Ann Arbor, MI). All analyses were done in accordance with the manufacturers' instructions.

Real-time reverse transcriptase PCR (RT-PCR). For Kupffer cell RNA preparation, cultures in 6-well plates (3×10^6 cells/plate) were scraped into RNeasy lysis buffer (QIAGEN, Hilding, Germany). Total RNA from whole liver was obtained from untreated Sprague-Dawley rats. Liver tissue was snap-frozen in liquid nitrogen and ground in a precooled mortar, and 30 mg of frozen material was homogenized by Ultra Turrax (20,000/min) in RNeasy lysis buffer (QIAGEN). All total RNA was further isolated using an RNeasy Mini kit (QIAGEN, Hilding, Germany) according to the manufacturer's protocol. TNF-α and IL-10 mRNA was analyzed using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) with sequence-specific PCR primers and probes (Table 1). In brief, for cytokine mRNA analyses, 25 ng of total RNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems), and real-time PCR was performed with 2× qPCR Master Mix (Eurogentec, Liege, Belgium), 300 nM sense and antisense primers, 100 nM TaqMan probe, cDNA, and water up to 25 μl. For adenylyl cyclase mRNA analyses, 100 ng of total RNA was reverse transcribed, and real-time PCR was performed with 2× qPCR Master Mix for SYBR green (Eurogentec, Liege, Belgium), 300 nM sense and antisense primers, cDNA, and water up to 25 μl. The specificities of all SYBR green assays were confirmed by melting-point analysis. The concentration of 18S mRNA was used for normalization of target gene expression (18S Predeveloped Assay reagents; Applied Biosystems). All samples were run in triplicate, standard curves were run on the same plate, and the standard-curve method was used to calculate the relative gene expression.

Statistical analyses. Data were analyzed with GraphPad Prism 3.0 software by one-way analysis with Tukey's multiple-comparison test. Differences with a P value of <0.05 were considered significant.

RESULTS

8-CPT-cAMP specifically down-regulates TNF-α levels without affecting the levels of IL-10 in Kupffer cell cultures. In order to characterize the effects of elevated cAMP levels on cytokine production, Kupffer cell cultures derived from male Sprague-Dawley rats were preincubated with the cell-permeable cAMP analogue 8-CPT-cAMP (1 to 100 μM) before they were subjected to LPS stimulation (1 μg/ml). As shown in Fig. 1A, a significant reduction of TNF-α levels was obtained with 1 μM of 8-CPT-cAMP, whereas no reduction of IL-10 was obtained with as much as 100 μM of the cAMP analogue (Fig. 2B). We chose to perform further experiments using 10 μM of cAMP to avoid any side effects of the analogue on cell viability. A time course study of Kupffer cells incubated with 10 μM of 8-CPT-cAMP show significantly reduced TNF-α levels ($P \leq 0.05$) at all time points after 4 h (Fig. 2A). IL-10 levels were not significantly affected but were slightly elevated at 24 and 48 h (Fig. 2B).

8-CPT-cAMP reduces TNF-α mRNA expression without influencing IL-10 mRNA levels. To find out whether LPS-mediated TNF-α gene expression was altered by cAMP pretreatment, we isolated total RNA from Kupffer cells treated with cAMP and/or LPS and analyzed TNF-α and IL-10 mRNA expression after 2 h by real-time RT-PCR. Both TNF-α (Fig. 3A) and IL-10 (Fig. 3B) gene expression levels were strongly induced by LPS. A significant (>50%) reduction in TNF-α

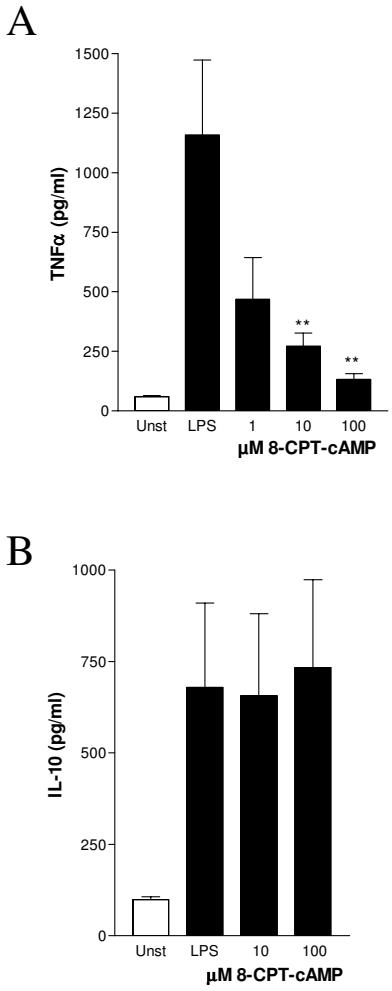

FIG. 1. Dose-dependent effects of 8-CPT-cAMP on Kupffer cell production of TNF-α and IL-10. Kupffer cells were incubated for 30 min with 8-CPT-cAMP (0, 1, 10, or 100 μM) before the addition of LPS (1 μg/ml) or were left unstimulated (Unst). Supernatants were collected after 6 h, and TNF-α (A) and IL-10 (B) levels were assessed by ELISA. Mean values ± standard errors of the mean (SEM) of five separate experiments performed in triplicate are shown.

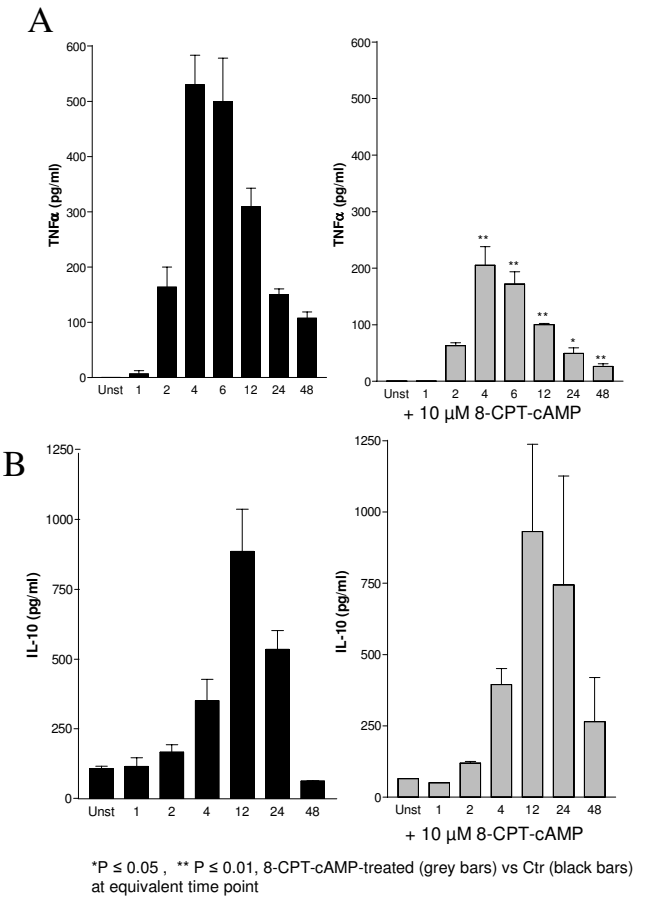

FIG. 2. Effects of 8-CPT-cAMP on culture medium levels of TNF-α and IL-10 over time. Cultured rat Kupffer cells were incubated for 30 min with 8-CPT-cAMP (10 μM, grey bars) or were left untreated (black bars) before addition of LPS (1 μg/ml). Supernatants were collected at the time points indicated (hours), and TNF-α (A) and IL-10 levels (B) were assessed by ELISA. Mean values ± SEM of triplicate samples from one representative experiment performed three times are shown. Unst, unstimulated; Ctr, control.

mRNA expression was observed following 8-CPT-cAMP pretreatment, but no effect was found on IL-10 mRNA levels.

Forskolin, isoproterenol, and PGE_2 induce cAMP production in Kupffer cells within 20 min. Our next step was to study whether other, more physiological mediators of intracellular cAMP elevation had the same effect as 8-CPT-cAMP on Kupffer cell cytokine production. For all agents, cell viability was good (>80%) at the time of harvest, demonstrating no toxic effects of the cAMP-inducing agents. To make sure our agents were effective and that they indeed caused elevated intracellular cAMP levels in Kupffer cells, we performed cAMP EIAs. As shown in Fig. 4, all agents significantly elevated cAMP levels after 20 min or less, with forskolin and PGE_2 being the most effective cAMP inducers at early time points.

Pretreatment of Kupffer cells with isoproterenol and PGE_2 reduces TNF-α levels, whereas forskolin treatment also reduces IL-10. In order to study the potential of the different cAMP-elevating agents in the regulation of TNF-α compared to that of IL-10, Kupffer cells were pretreated for 30 min with forskolin, isoproterenol, or PGE_2 in a 1 or 10 μM concentration before the addition of LPS (1 μg/ml), and medium was harvested after 6 h for TNF-α and IL-10 assays. As shown in Fig. 5A, the effectiveness of the cAMP-inducing agents in reducing TNF-α levels did not correlate directly with the cAMP-inducing potential. The strong cAMP inducer forskolin was no more potent than isoproterenol in reducing TNF-α levels, even at a concentration of 10 μM, and significantly reduced IL-10 levels. In contrast, PGE_2 (1 and 10 μM) significantly reduced TNF-α levels by more than 75% and tended to elevate levels of IL-10 (10 μM). We then aimed to assess the inhibiting effect of PGE_2 on TNF-α as posttreatment relative to LPS addition. PGE_2 (1 μM) was added at different time points relative to the addition of LPS, and the levels of TNF-α (Fig. 5C) were assayed 6 h after LPS stimulation. More than 50% reduction of TNF-α levels was obtained when PGE_2 was added 5 or 30 min

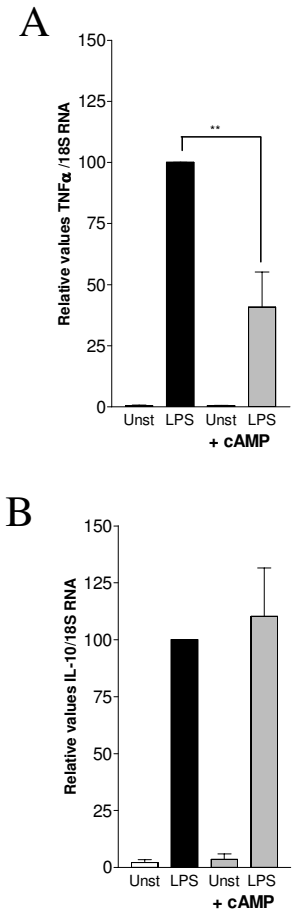

FIG. 3. Effects of 8-CPT-cAMP on TNF-α and IL-10 mRNA induction. Cultured rat Kupffer cells were either incubated for 30 min with 8-CPT-cAMP (10 μM) or left untreated followed by the addition of LPS (1 μg/ml). Total RNA was isolated after 2 h, and TNF-α (A) and IL-10 (B) mRNAs were assessed by real-time RT-PCR and normalized for 18S RNA. Cytokine mRNA expression levels in LPS-treated cells are set to 100 in each individual experiment (black bars), and means ± SEM of the relative levels found in cAMP-treated cells (gray bars) are shown. Four individual experiments are included. Unst, unstimulated.

after LPS, but the effect was less apparent when PGE_2 was added at later time points.

Both forskolin and PGE_2 reduce TNF-α mRNA in Kupffer cells cultures, whereas forskolin also reduces IL-10 mRNA. We then studied the expression of TNF-α and IL-10 mRNA by quantitative RT-PCR in Kupffer cells treated for 30 min with 1 μM of either forskolin or PGE_2 prior to LPS stimulation for 2 h. The concentration of 1 μM was chosen for both agents because 10 μM concentrations gave no additional reduction in TNF-α or IL-10 protein levels. For TNF-α mRNA expression (Fig. 6A), significant reduction was observed when cells were treated with forskolin (1 μM) or PGE_2 (1 μM) for 2 h. IL-10 mRNA expression (Fig. 6B) was inhibited only by forskolin.

Forskolin-insensitive adenylyl cyclase 9 mRNA is expressed in Kupffer cells, and the expression is inhibited by LPS treatment. The relative expression levels of two adenylyl cyclase isoforms previously found in liver, adenylyl cyclases 6 and 9,

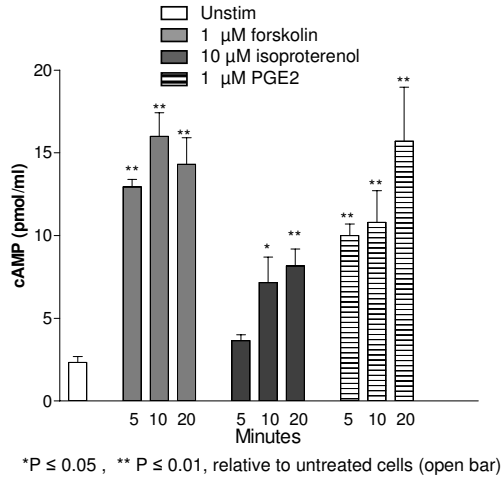

FIG. 4. Effects of forskolin, isoproterenol, and PGE_2 on cAMP production. Cultured rat Kupffer cells were incubated with forskolin (1 μM), isoproterenol (10 μM), or PGE_2 (1 μM) and harvested after 5, 10, or 20 min in 0.1% HCl. Intracellular cAMP levels were analyzed by cAMP EIA (Cayman). Representative data from one out of three experiments are shown. Unstim, unstimulated.

were studied by quantitative RT-PCR on total RNA derived from cultured primary Kupffer cells and whole rat liver and normalized for 18S rRNA. As shown in Fig. 7A, adenylyl cyclase 9 appeared to be expressed at higher levels in Kupffer cells than in whole liver, whereas adenylyl cyclase 6 appeared to be expressed at lower levels in Kupffer cells. The expression of adenylyl cyclase 9 mRNA in Kupffer cells was clearly reduced 2 h after the addition of LPS (1 μg/ml), as demonstrated in Fig. 7B.

DISCUSSION

We demonstrate here that elevating intracellular cAMP levels in primary rat Kupffer cells by cAMP analogue, PGE_2, or isoproterenol strongly reduces LPS-mediated induction of TNF-α without notably interfering with the ability of Kupffer cells to produce IL-10. In this way, cAMP-elevating agents in the liver bloodstream may offer protection against proinflammatory cytokine production in liver and may favor anti-inflammation. A different effect was obtained by the strong cAMP inducer forskolin. We demonstrate for the first time that forskolin-insensitive adenylyl cyclase 9 mRNA is highly expressed in Kupffer cells and propose that adenylyl cyclase 9 may be involved in cAMP-mediated attenuation of TNF-α release from Kupffer cells.

According to our observations and the observations of others, a primary effect of cAMP elevation in Kupffer cells appears to be the reduction of TNF-α mRNA and protein and thereby the inhibition of proinflammatory responses (15, 26, 32). Our results also tell us that although forskolin elicits higher cAMP levels than does isoproterenol 5 to 20 min after addition to Kupffer cells, forskolin is not more effective in attenuating TNF-α protein within the first 6 h of LPS treatment. However, we do observe that TNF-α mRNA levels are significantly reduced by forskolin. One possible explanation for this may be that TNF-α release or stability is potentiated by the corresponding loss of IL-10, but this remains to be confirmed.

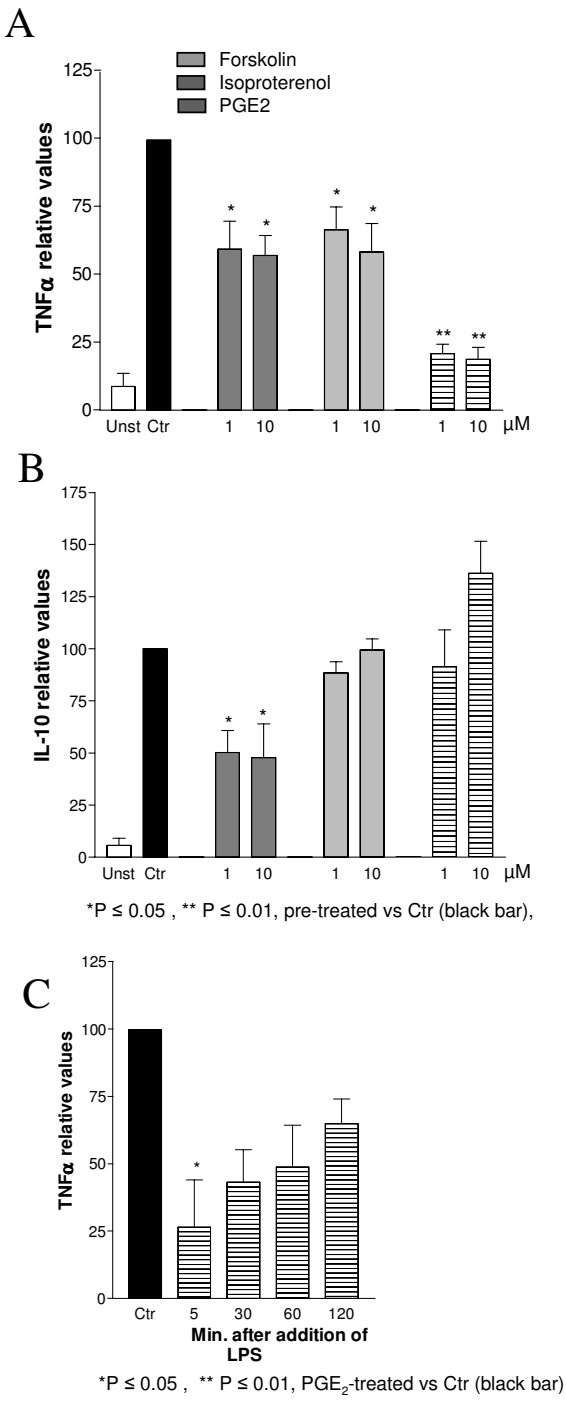

FIG. 5. Effects of forskolin, isoproterenol, and PGE_2 on TNF-α and IL-10 protein levels. Cultured rat Kupffer cells were incubated for 30 min with forskolin, isoproterenol, or PGE_2 in concentrations of 1 or 10 μM as indicated, before the addition of LPS (1 μg/ml). Supernatants were collected after 6 h, and TNF-α (A) and IL-10 (B) levels were assessed by ELISA. Mean cytokine levels following stimulation with 1 μg/ml of LPS (control [Ctr], black bars) are set to 100 for each individual experiment, and means ± SEM of relative levels found in samples preincubated with cAMP-elevating agents are shown. Data derived from triplicate samples from six independent experiments are included. (C) PGE_2 (1 μM) was added to Kupffer cell cultures at 5 to 120 min following the addition of LPS (1 μg/ml). Supernatants were collected after 6 h of LPS stimulation, and TNF-α levels were assessed by ELISA. Mean cytokine levels following stimulation with 1 μg/ml of LPS (Ctr, black bars) are set to 100 for each individual experiment, and means ± SEM of relative levels found in samples preincubated with PGE_2 are shown. Data derived from triplicate samples from three independent experiments are included. Unst, unstimulated; Ctr, control.

Augmentation of LPS-mediated production of IL-10 by cAMP analogues and PGE_2 has been demonstrated in monocytes and peritoneal macrophages (24, 33), and mechanisms for cAMP-mediated induction of IL-10 transcription through CREB and C/EBP has been shown previously (2). We could not find that cAMP-elevating agents were effective in inducing IL-10 transcription in Kupffer cells, and we observed only a nonsignificant tendency of enhanced IL-10 levels by PGE_2. Several isoforms of CREB and C/EBP with different abilities to confer cAMP-mediated gene transcription are expressed in macrophages, and even inhibitory isoforms like inducible cAMP early repressor and C/EBP liver-enriched inhibitory protein exist (2, 13, 21). The relative expression of inhibitory and activating factors has not been studied in Kupffer cells but may explain that the potential of cAMP to induce IL-10 differs from that of other macrophage populations.

In contrast to PGE_2, forskolin strongly reduced IL-10 mRNA and protein. It is still unknown whether the forskolin-specific inhibition of IL-10 production is mediated by cAMP. If so, it is surprising that the addition of an excess of cAMP analogues to the cell does not lead to any inhibitory effects on IL-10 production. It should be noted that cAMP analogues like 8-CPT-cAMP may not enter all compartments of the cell equally and may not be correctly localized or specific enough to mimic the results of adenylyl cyclase activation by forskolin. However, other, and so-far-unidentified, additional effects of forskolin on cAMP-independent processes cannot be completely ruled out. Recently, a report showed that forskolin and a cAMP analogue had distinct effects on cytokine gene expression in microglia (41). Forskolin and dibutyryl-cAMP elevated IL-10 levels and decreased TNF-α levels equally in these cells but had different effects on the regulation of nitric oxide, IL-6, and IL-1β. These studies tell us that cytokine production by different macrophage populations may be regulated by cAMP signaling in a variety of ways and that the effect of forskolin diverges from that of other cAMP-elevating agents when it comes to controlling cytokine production in different systems.

Specificity of cAMP signaling is obtained by strict compartmentalization of complexes containing adenylyl cyclase, phosphodiesterases, and downstream substrates that specify the cAMP signal (reviewed in reference 36). When synthesis, breakdown, and target of cAMP are colocalized, a spread of intracellular cAMP is prevented and specificity is obtained. Adenylyl cyclase exists in nine membrane-associated isoforms, five of which are reported to be expressed in liver (reviewed in reference 4). Of these, adenylyl cyclase 6 was reported to be expressed at high levels, whereas adenylyl cyclases 4, 5, 7, and 9 were moderately expressed. Forskolin is a general and strong activator of all but one of the membrane-associated adenylyl cyclase isoforms, namely, adenylyl cyclase 9. In fact, adenylyl cyclase 9 is reported to be inhibited by forskolin in vitro (10). It has been shown that mutations in adenylyl cyclase 9 clearly reduce β-adrenergic receptor stimulation in transfected HEK293

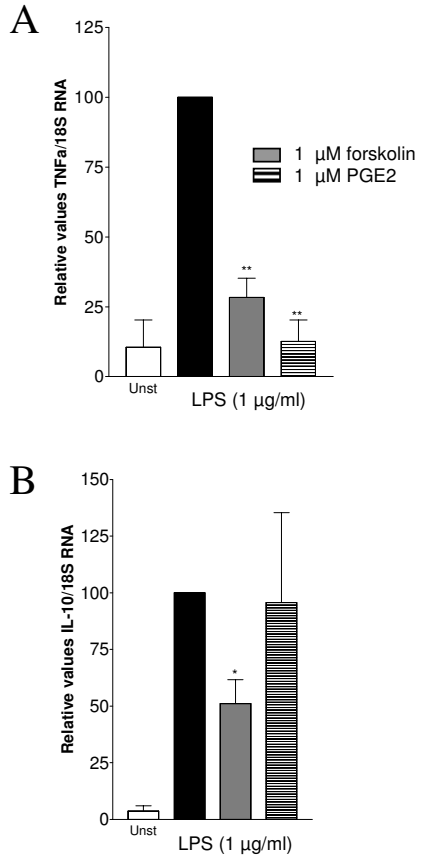

FIG. 6. Effects of forskolin and PGE_2 on TNF-α and IL-10 mRNA levels. Cultured rat Kupffer cells were incubated for 30 min with forskolin (1 μM) or PGE_2 (1 μM) before the addition of LPS (1 μg/ml). Total RNA was isolated after 2 h, and TNF-α (A) and IL-10 (B) mRNAs were assessed by real-time RT-PCR and normalized for 18S RNA. Cytokine mRNA expression levels in LPS-treated cells are set to 100 in each individual experiment (black bars), and means ± SEM of the relative levels found in pretreated cells are shown. Three individual experiments are included. Unst, unstimulated; Ctr, control.

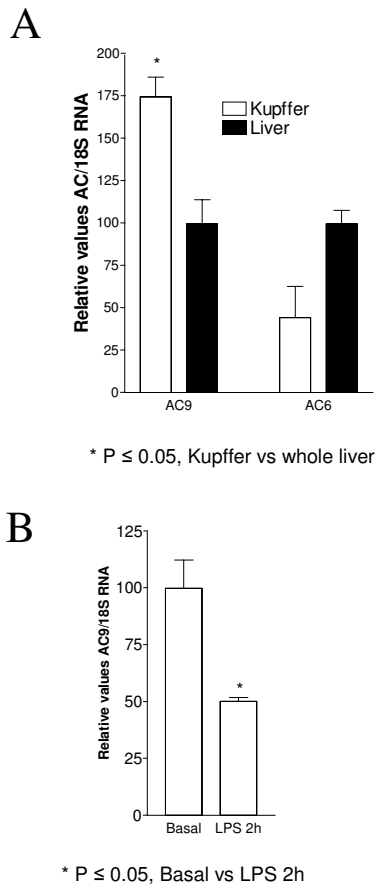

FIG. 7. Expression of adenylyl cyclase 9 mRNA in Kupffer cells. (A) Total RNA from cultured Kupffer cells or whole liver was assessed by real-time RT-PCR using primers for adenylyl cyclase 9 (AC9) and adenylyl cyclase 6 (AC6) mRNA and normalized for 18S rRNA. The mean mRNA expression of each adenylyl cyclase mRNA in whole liver is set to 100, and relative expression levels in untreated rat Kupffer cells are shown. Liver samples from four individuals and Kupffer cells from three individuals are included. (B) Cultured rat Kupffer cells were incubated with LPS (1 μg/ml) or left untreated. Total RNA was isolated after 2 h, and adenylyl cyclase 9 (AC9) mRNA was assessed by real-time RT-PCR and normalized for 18S rRNA. Means ± SEM of samples from three individuals are shown. Mean adenylyl cyclase 9 mRNA expression in untreated cells is set to 100.

cells (30), indicating that forskolin-insensitive adenylyl cyclase 9 is important downstream of isoproterenol signaling and that forskolin treatment may not induce the same cAMP-mediated mechanisms as isoproterenol. In support of this notion, inhibitory effects of forskolin on isoproterenol-mediated cAMP production have been reported in macrophages (3).

This paper shows for the very first time that forskolin differs from isoproterenol and PGE_2 in the regulation of IL-10 and TNF-α in Kupffer cells, in which adenylyl cyclase 9 mRNA is highly expressed. Adenylyl cyclase 9 mRNA expression is inhibited by LPS, and LPS treatment also reduces the inhibitory effect of PGE_2 on TNF-α production. This indicates that adenylyl cyclase 9 could be involved in cAMP-mediated attenuation of inflammatory processes in the liver.

The water-soluble forskolin derivative colforsin daropate has been tested for therapeutic purposes in patients with chronic congestive heart failure and has been shown to increase intracellular cAMP in patients insensitive to β-adrenergic receptor stimulants or phosphodiesterase inhibitors and to inhibit proinflammatory cytokine production (12). Interestingly, colforsin daropate actions were notably divergent from those of isoproterenol and induced strong coronary vasodilation (43). Our observations that forskolin may not have the same anti-inflammatory effects as isoproterenol or PGE_2 in liver according to inhibitory effects on IL-10 production in Kupffer cells indicate that treatment involving circulating forskolin derivatives should be subject to care and further study.

In conclusion, our data, along with those of others, point to the cAMP signaling pathway as a key protective mechanism against LPS-induced TNF-α production in Kupffer cells. In contrast, forskolin-mediated mechanisms may inhibit Kupffer cell production of an important control factor of inflammation, IL-10, an effect that differs markedly from that of PGE_2. Hence, highly specific cAMP signaling mechanisms, possibly generated in part by adenylyl cyclase 9, appear to control the balance between pro- and anti-inflammatory cytokine production in Kupffer cells.

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