Nitric oxide (NO)¹ has been identified as an important signaling molecule that is involved in regulating a wide array of biological activities in neural, vascular, and immune cellular systems (1). NO is generated from l-arginine and molecular oxygen in the presence of the enzyme NO synthase (NOS) (2). To date three distinct NOS isoforms have been identified from molecular cloning and sequencing analyses (3). The endothelial (eNOS) and neuronal (nNOS) isoforms are expressed constitutively in endothelial and neuronal cells, respectively. The amount of NO generated by these cell types is dependent upon the cellular content of NOS (4). NO generated by endothelial cells plays an important role in the control of vascular tone, whereas in neuronal tissue NO acts to regulate cGMP-mediated neurotransmission (1, 2). The third isoform of NOS, termed inducible NOS (iNOS), was first identified in macrophages stimulated with interferon-γ and bacterial lipopolysaccharide (LPS) (3). iNOS has been identified in a wide variety of cell types including macrophages, mesangial cells, vascular smooth muscle cells, keratinocytes, chondrocytes, osteoclasts, and hepatocytes (1, 5). NO generated within these cells mediates macrophage cytotoxicity during host defense reactions, alterations in the contractile responses of mesangial cells, and in instances where NO exceeds normal physiological levels, instigates the inhibition of vascular smooth muscle tone, hepatocyte metabolism, and protein synthesis (6–8). Recent evidence indicates that elevated levels of NO play a major role in the pathogenesis of several chronic disorders and inflammatory processes. In particular, studies have indicated that an overproduction of NO in response to LPS and cytokines contributes to the development and prolongation of severe hypotension and peripheral vasodilation observed during endotoxic shock (9). The activities of nNOS and eNOS are regulated by rapid, transient elevations of intracellular free calcium which enhance the binding of calmodulin to the NOS enzyme resulting in NO release over a time frame of seconds and minutes (10). In contrast, the expression of iNOS is thought to be regulated primarily at the transcriptional level of the iNOS gene. Once induced, iNOS produces NO for periods of several hours or days. Thus, given the magnitude of the wide variety of inhibitory actions of NO, it is of considerable interest and even may be of some therapeutic utility to delineate the mechanism(s) by which the production and resultant activity of NO can be controlled or attenuated.

In the presence of LPS, Kupffer cells, the resident macrophage found in the sinusoids of the liver, produce large amounts of nitrite and nitrate, the stable end products of the NO pathway (11). It has become apparent recently that overproduction of NO by hepatic cells plays a major role in hepatic injury/necrosis associated with endotoxic shock. Kupffer cells also synthesize and release several cytokines in response to LPS which in turn stimulate neighboring hepatocytes to generate NO (8). The consequent overproduction of NO in the liver results in profound degenerative changes observed in hepatocytes (12). These changes include a decrease in total protein synthesis, cellular proliferation, and an increase in cGMP for-
mation (15). The induction of iNOS by LPS in Kupffer cells requires the initiation of gene expression and de novo protein synthesis over a period of several hours. It is unclear whether classical second messengers such as cAMP are involved in iNOS gene expression and NO formation. Recent studies have indicated that agents that elevate levels of cAMP improve circulatory function in animal models of endotoxic shock; in particular, isoproterenol was found to inhibit the development of vascular hyporeactivity in the endotoxic rat (14, 15). In contrast, certain in vitro studies have shown that elevation of cAMP caused an induction of iNOS, whereas in other studies increased levels of cAMP caused a reduction in iNOS (6, 7, 16). The present study was designed to investigate whether iNOS gene expression and/or enzymatic activity is regulated by elevated levels of cAMP in cultured rat Kupffer cells.

EXPERIMENTAL PROCEDURES

Isolation and Primary Culture of Rat Kupffer Cells—Following enzymatic digestion of the rat liver, Kupffer cells were isolated by centrifugal elutriation as described previously (17). The viability of the Kupffer cell preparation was greater than 95% as determined by trypan blue exclusion. Freshly isolated Kupffer cells were maintained at 37 °C in RPMI 1640 culture medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 112 units/ml penicillin, and 112 units/ml streptomycin in 24-well plates or 60-mm tissue culture dishes. All cells were incubated in an atmosphere of 90% air and 10% CO2. On the 2nd day of culture the RPMI medium was changed. For experimental purposes, Kupffer cells were used within 3–4 days of their establishment in culture.

Measurement of Nitrite Formation—Production of NO by iNOS was quantified by measuring the accumulation of nitrite in the culture medium using the Griess reaction (18). Kupffer cells were cultured in 24-well plastic tissue culture plates at a density of 1 × 106 cells/ml (1 ml/well). After 4 days in culture the cells were washed and complete medium without phenol red was added to each well. The cells were then exposed to several cAMP-elevating agents. After a specified incubation interval the medium from each well was removed. The cells were then washed twice in 2% dextran, 50 m M Tris-HCl, pH 7.5, 10% non-fat dried milk, and 0.1% sodium pyrophosphate, and then washed at 55 °C for 15 min at room temperature. 0.1 × SSC for 15 min. Reactivity was visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Control hybridizations were performed using a 32P end-labeled oligonucleotide complementary to rat 18 S rRNA.

Nuclear Run-on Analysis—Nuclei were isolated from treated Kupffer cells (5–6 × 106) according to standard protocols (22). Briefly, cells were rinsed twice with ice-cold phosphate-buffered saline, scraped gently in 6 ml of phosphate-buffered saline, and centrifuged at 800 × g at 4 °C for 5 min. Lysis buffer (10 mM Tris-Cl, 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40, 20 ml) was added followed by vortexing for 10 s to disrupt the cell pellets. The cells were incubated on ice for 5 min and nuclei pelleted by centrifugation at 800 × g at 4 °C for 5 min. The pellet was resuspended in storage buffer (50 ml Tris-HCl, 5 mM MgCl2, 0.1 mM EDTA, 40% (v/v) glycerol), frozen, and then stored in liquid nitrogen until needed. For in vitro transcription, freshly thawed nuclei were incubated in reaction buffer (10 ml Tris-HCl; 5 mM MgCl2; 0.3 mM KCl; 100 mM ATP, CTP, GTP; 1 mM dithiothreitol; 10 mM[3H]UTP (3,000 Ci/mmol, Amersham Corp.), shaking for 30 min at 30 °C. The reaction was quenched by the addition of RNase-free DNase I (10 mg/ml, Worthington) and proteinase K (20 mg/ml, Ambion, Austin, TX) and incubated for 30 min at 42 °C. Nascent labeled RNAs were purified by extraction with phenol/chloroform and two sequential precipitations with ammonium acetate. Equal amounts of 32P-labeled RNA were resuspended in 1 ml of TES/NaCl solution (10 mM TES, pH 7.4, 10 mM NaCl, 0.2% SDS, 0.3 mM NaCl) and hybridized for 60 h at 65 °C to denatured DNA probes immobilized on nitrocellulose filters. Following hybridization, the filters were washed twice in 0.1 M NaCl, 0.1% SDS containing 1 × SSC for 5 min, at room temperature. 0.1 × SSC for 15 min. Reactivity was visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Control hybridizations were performed using a 32P end-labeled oligonucleotide complementary to rat 18 S rRNA.

Materials—Anti-mac iNOS was obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies raised against p65 and IκBα—Cytoplasmic and nuclear samples were resolved using SDS-PAGE (11% gel). The separated proteins were electrotransfered to polyvinylidene difluoride membranes. The membranes were then incubated in blocking buffer (Tris-buffered saline, pH 7.4) containing 10% non-fat dried milk powder for 1 h and then exposed to diluted primary antibodies against the p65 subunit of NFκB. The membranes were incubated for 1 h at room temperature with 5,000-fold diluted horseradish peroxidase-conjugated anti-mouse IgG antibody. Protein bands were visualized using an enhanced chemiluminescence (ECL) assay kit.

Northern Blot Analysis—Kupffer cells were plated at a density of 7 × 106 cells/60-mm dish. After 3 days in culture and the appropriate treatment, total RNA from cultured rat Kupffer cells was isolated using TRIzol reagent (Life Technologies, Inc.). RNA (3–4 µg) was separated by electrophoresis on a 0.8% agarose, 2.2 M formaldehyde gel and transferred using a Piblottost (Stratagene, La Jolla, CA) onto a Magna nylon membrane (Micron Separations Inc. Westborough, MA). A full-length murine iNOS cDNA probe kindly provided by Dr. S. H. Snyder (The Johns Hopkins University School of Medicine, Baltimore) or IκBα cDNA kindly provided by Dr. A. Baldwin Jr. (University of North Carolina, Chapel Hill) was labeled with a multiprime DNA labeling system using [α-32P]dCTP (specific activity, 3,000 Ci/mmol). Northern blot hybridizations were performed in 50% formamide, 1 mM NaCl, 10 mM dextran sulfate, 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, and 0.2% Denhardt’s solution at 42 °C for 16 h. The membranes were washed twice in 2 × SSC, 1% SDS at 65 °C for 20 min, twice in 0.1 × SSC, 1% SDS, and at 55 °C for 30 min. The membranes were exposed to X-ray film at −70 °C with an intensifying screen. The membranes were then washed twice in 2 × SSC, 5 × SSC containing RNase A (10 mg/ml, Ambion) for 30 min, and once in 2 × SSC at 37 °C for 1 h. Reactivity was visualized using a PhosphorImager.
era toxin by ADP-ribosylation of Gs. We have used these agents 011:B4), dibutyryl cAMP, cholera toxin, forskolin, isoproterenol, IBMX, obtained from Bio-Rad. Bacterial LPS (from Escherichia coli, serotype 011:B4), dibutyryl cAMP, cholera toxin, forskolin, isoproterenol, IBMX, (Santa Cruz, CA). Goat anti-mouse and goat anti-rabbit IgG horseradish peroxidase conjugate and prestained SDS-PAGE standards were obtained from Bio-Rad. Bacterial LPS (from Escherichia coli, serotype 011:B4), dibutyryl cAMP, cholera toxin, forskolin, isoproterenol, IBMX, and actinomycin D were purchased from Sigma.

RESULTS

cAMP-elevating Agents Inhibit LPS-induced Nitrite and iNOS Protein Formation by Kupffer Cells—Cultured unstimulated Kupffer cells exhibited a low basal level of nitrite production, whereas LPS caused an 8-fold increase in nitrite formation during the 24-h observation period (Table I). The addition of various cAMP-elevating agents attenuated LPS-induced nitrite formation by Kupffer cells, each in a dose-dependent manner (data not shown). The effect of the highest concentration of each agent used in this study is noted in Table I. The diterpene forskolin activates adenylyl cyclase directly, resulting in an increase of intracellular cAMP levels; forskolin inhibited LPS-induced nitrite formation strongly. The membrane-permeable cAMP analog dibutyryl cAMP reduced LPS-induced nitrite formation by 34%. Both isoproterenol and cholera toxin stimulate adenylyl cyclase via the stimulatory G-protein Gs, isoproterenol by binding to cell surface β-adrenergic receptors and cholera toxin by ADP-ribosylation of Gs. We have used these agents previously to demonstrate cAMP-dependent changes in platelet-activating factor binding in Kupffer cells, and we have confirmed that isoproterenol causes an increase in intracellular cAMP in these cells (23). It is important to note that none of the cAMP-elevating agents (at the indicated concentrations) caused 1) any significant change in the rate of formation of nitrite above the control value when added to Kupffer cells in the absence of LPS (Table I) and 2) any morphological alterations or detachment of Kupffer cells after an incubation period of 24 h. In addition, LPS-induced nitrite formation by Kupffer cells in the combined presence of the aforementioned cAMP-elevating agents and the phosphodiesterase inhibitor IBMX was inhibited to a greater degree than in the absence of IBMX (Table I). Thus cAMP-elevating agents clearly are capable of decreasing LPS-induced iNOS activity in Kupffer cells.

TABLE I

| Treatment                              | Nitrite production (nmol/10^9 Kupffer cells/24 h) |
|----------------------------------------|---------------------------------------------------|
| No addition                            | 5.1 ± 0.9                                          |
| LPS (10 ng/ml)                         | 39.7 ± 1.1                                         |
| Forskolin (50 μM)                      | 5.4 ± 0.9                                          |
| LPS + forskolin                        | 16.3 ± 2.0                                         |
| LPS + forskolin + IBMX                 | 11.3 ± 1.5                                         |
| Bt2cAMP (10 μM)                        | 6.1 ± 1.1                                          |
| LPS + Bt2cAMP                          | 26.1 ± 1.4                                         |
| LPS + Bt2cAMP + IBMX                   | 17.3 ± 0.9                                         |
| Cholera toxin (1 ng/ml)                 | 5.9 ± 0.5                                          |
| LPS + cholera toxin                    | 24 ± 0.4                                           |
| LPS + cholera toxin + IBMX             | 15.7 ± 0.9                                         |
| Isoproterenol (10 μM)                   | 4.2 ± 1.3                                          |
| LPS + isoproterenol                    | 26 ± 1.8                                           |
| LPS + isoproterenol + IBMX             | 17.5 ± 2.0                                         |

Elevated cAMP levels might exert direct metabolic control on preexisting iNOS or might decrease the synthesis of iNOS protein. To distinguish between these alternatives, the nitrite inhibition experiments were repeated, and the levels of iNOS protein in Kupffer cells were analyzed by immunoblotting with anti-iNOS antibody. As depicted in Fig. 1, in untreated Kupffer cells immunoreactive iNOS was undetectable, whereas LPS-treated cells produced a heavily stained band. The iNOS protein bands from Kupffer cells that had been treated with LPS, cAMP-elevating agents, and IBMX appeared to be lightly stained (lanes 3–6), indicating a substantial decrease in the amount of iNOS protein formed after 24 h. As forskolin was the most effective inhibitor of LPS-induced nitrite and iNOS protein formation by cultured Kupffer cells (Table I and Fig. 1) it was used for all subsequent experiments.

Forskolin Inhibited LPS-induced Nitrite Formation and iNOS mRNA Levels in a Time-dependent Manner—Kupffer cells were treated with either LPS or LPS and forskolin; nitrite formation was measured at the time intervals indicated in Fig. 2A. In agreement with other research groups, LPS stimulated nitrite formation by Kupffer cells in a time-dependent fashion. The inhibitory effects of forskolin on LPS-induced nitrite formation by Kupffer cells became apparent after 6–8 h of treatment and continued up to 24 h. The time dependence of LPS-induced iNOS mRNA accumulation in Kupffer cells is shown in Fig. 2B, C, and D; the iNOS mRNA level increased rapidly between 3 and 6 h and then declined by 24 h. The addition of forskolin caused a considerable decrease in the accumulation of iNOS mRNA, and the levels of message were barely detectable at 24 h (Fig. 2B, C, and D).

Kinetics of Inhibition of LPS-induced Nitrite and iNOS mRNA Formation by Forskolin—Forskolin was added to Kupffer cells before, at the same time as, or at different times after the addition of LPS to determine the optimum time for the inhibition of LPS-induced nitrite production and iNOS mRNA levels. Nitrite accumulation in the culture medium was measured 24 h after the addition of LPS. Fig. 3A shows that maximal suppression of LPS-induced nitrite formation by Kupffer cells occurred when forskolin was present 1 h before the addition of LPS. When forskolin was added after LPS, its inhibitory effect decreased gradually with time. Fig. 3B depicts a similar experiment except iNOS mRNA was isolated and analyzed by Northern blotting. When Kupffer cells were pretreated with forskolin for 1 h before the addition of LPS, the level of iNOS mRNA formed after 6 h was barely detectable. However, the intensities of the iNOS mRNA bands increased when forskolin was added at the same time as LPS and subsequently 2 and 4 h after the addition of LPS.

Effects of LPS and Forskolin on the Half-life of iNOS mRNA—To examine whether forskolin attenuated LPS-induced steady-state levels of iNOS mRNA by decreasing its stability, we assessed the effects of forskolin on the half-life of LPS-induced iNOS mRNA by coinubation of Kupffer cells with the transcriptional inhibitor actinomycin D. Kupffer cells were treated with LPS in the presence and absence of forskolin for
6 h to induce maximal iNOS mRNA accumulation. Actinomycin D (10 ng/ml) was added to the cells at this point to inhibit further transcription. At different times after the addition of actinomycin D, total RNA was isolated and examined by Northern analysis. To allow for differences in loading, the signal density of each RNA sample hybridized to the iNOS probe was corrected by that hybridized to the 18 S probe. Fig. 4 shows the decay of iNOS mRNA as ln (relative intensity) against time. Under these conditions, the half-life of iNOS mRNA can be calculated as £ $\frac{\ln \left(\frac{2}{I(t)}\right)}{\text{gradient of regression line}}$ £. The calculated half-lives of iNOS mRNA in LPS-stimulated Kupffer cells in the absence and presence of forskolin were 2.4 and 2.3 h, respectively; therefore, the reduction of LPS-induced iNOS mRNA levels by forskolin in Kupffer cells was not caused by a decrease in message stability.

Effect of Forskolin on Transcription of the iNOS Gene—iNOS gene transcription in Kupffer cells was measured directly using a nuclear run-on assay to confirm that forskolin caused inhibition of this process. Kupffer cells were incubated either alone, or with LPS or LPS and forskolin for 3 h and 5 h; cells were lysed and nuclei isolated. The transcription of iNOS and $\beta$-actin by isolated nuclei was determined by hybridizing the elongated, labeled RNA transcripts to iNOS- and $\beta$-actin-specific cDNA fragments that had been slot blotted onto a nitrocellulose membrane. Fig. 5A shows that iNOS gene transcription was barely detectable in control cells (lane 1), was increased greatly by 3-h exposure to LPS (lane 2), and that pretreatment with forskolin for 1 h attenuated the LPS effect (lane 3). In a similar experiment using a 5-h stimulation with LPS, a 1-h pretreatment with forskolin caused no apparent attenuation of iNOS gene transcription (compare lanes 2 and 3 in Fig. 5B). These results are in agreement with time-dependent changes in iNOS mRNA. Fig. 2D shows that after a 3-h stimulation with LPS a pulse of iNOS gene transcription has just commenced, and the effect of added forskolin will be maximal, i.e. the gradients of the curves with and without forskolin differ considerably. After 5 h of LPS stimulation the pulse of iNOS gene transcription is ending; mRNA levels fall after 6 h. At this time
the effect of added forskolin will be minimal; the gradients of the curves with and without forskolin will differ very little as both approach their highest values. Taken together, the above findings indicated that forskolin attenuates LPS-induced iNOS mRNA formation, mainly at the transcriptional level.

**Forskolin Decreased the Translocation of the p65 Subunit of NF-κB from the Cytoplasm into the Nucleus**—Recent studies by Xie et al. (24) have shown that activation of the transcription factor NF-κB and its binding to the promoter region of the iNOS gene are critical steps in the induction of iNOS synthesis by LPS in macrophages. In addition, Tran-Thi et al. (25) reported NF-κB-binding activity in Kupffer cells treated previously with LPS. In view of these findings, we decided to investigate the effect of forskolin on NF-κB activation in the presence and absence of LPS. Kupffer cells were either untreated, treated with LPS for 30 min, pretreated with forskolin for 1 h and then stimulated with LPS for 30 min, or pretreated with forskolin for 1 h and then stimulated with vehicle for 30 min; cytoplasmic and nuclear proteins were then isolated. A representative experiment is depicted in Fig. 6. Panel A represents cytoplasmic extracts, and panel B represents nuclear extracts. In untreated cells (lane 1) the p65 subunit of NF-κB was located mainly in the cytoplasm. A residual amount of p65 was detected in the nuclear extract. In contrast, LPS (lane 2) increased greatly the amount of p65 detected in the nucleus of Kupffer cells. Interestingly, in forskolin-treated Kupffer cells, both in the presence (lane 3) and absence (lane 4) of LPS, the amount of p65 detected in the nuclear extract was greatly diminished compared with LPS-treated cells (lane 2). Practically all of the p65 was retained in the cytoplasm (lanes 3 and 4), suggesting that forskolin interfered with the translocation of p65 from the cytoplasm into the nucleus.
levels of IκB and nuclear protein (3 μg/ml) for 30 min (both in the presence (in untreated cells. However, in forskolin-treated Kupffer cells

The above results showed that in the presence of forskolin, the IκB protein in the cytoplasm of Kupffer cells remains intact and remains complexed to the NF-κB proteins.

Effect of Forskolin and LPS on IκB mRNA Levels in Kupffer Cells—The above results showed that in the presence of forskolin, the integrity of IκB protein in the cytoplasm of Kupffer cells was maintained. We then investigated whether forskolin actually initiated IκB gene expression in Kupffer cells. Kupffer cells were stimulated with either forskolin or LPS or LPS and forskolin and at the times indicated in Fig. 8. RNA was isolated and employed in Northern blot analyses. The mRNA for IκBα in Kupffer cells appeared as a single band at approximately 1.6 kilobases. It is important to note here that in untreated Kupffer cells the level of IκBα mRNA was undetectable (data not shown). In the presence of forskolin (Fig. 8, panels A and D), IκBα mRNA levels peaked after 2 h of stimulation and then declined to negligible levels by 6 h. In contrast, in the presence of LPS (panels B and D), IκBα mRNA levels increased just after 30 min of stimulation, reached a peak by 1 h, and then remained elevated for up to 6 h. When Kupffer cells were pretreated for 1 h with forskolin and then stimulated with LPS over a period of 6 h (panels C and D), it was observed that forskolin up-regulated LPS-induced IκBα mRNA levels for up to 4 h after the addition of LPS (Fig. 8D). These findings suggest that forskolin has the capacity to induce IκBα mRNA synthesis and in turn increase the levels of protein in the cytoplasm of Kupffer cells. In the event of pretreating Kupffer cells with forskolin and subsequent stimulation by LPS the cytoplasmic levels of IκBα are maintained and thereby attenuate the translocation of NF-κB into the nucleus.

DISCUSSION

The release of NO by Kupffer cells under conditions of endotoxic shock represents an important contribution to the patho-

physiology of the liver (27). Thus it is of considerable interest to investigate the intracellular signaling pathways that regulate the induction/suppression of iNOS gene expression. In Kupffer cells, agents that elevate intracellular cAMP levels attenuated both LPS-induced nitrite formation (Table I) and LPS-induced iNOS protein (Fig. 1). Furthermore, in the presence of forskolin LPS-induced iNOS mRNA formation was suppressed greatly
(Fig. 2, C and D). This suggested that elevated levels of cAMP may have 1) directly suppressed the onset of iNOS gene transcription, 2) decreased transcription of the iNOS gene, or 3) destabilized iNOS mRNA following transcription. The half-life of iNOS mRNA was essentially the same in the presence or absence of forskolin, eliminating the possibility of altered mRNA stability (Fig. 4). Experiments in which forskolin was added to cultured Kupffer cells before, with, or at different times after LPS indicated that maximal inhibition of iNOS mRNA required the presence of forskolin before the addition of LPS (Fig. 3B). This favors alternative 1) above, i.e. suppression of the initiation of transcription. Direct measurement of iNOS gene transcription showed that at 3 h after LPS stimulation forskolin decreased (Fig. 5A) the transcriptional process substantially, whereas by 5 h after LPS stimulation forskolin had little effect.

The promoter region of the recently cloned rat gene encoding iNOS has been found to contain consensus sequences for the binding of numerous transcription factors (28). Activation of these factors is critical in the induction of iNOS by LPS or cytokines. One transcription factor of paramount importance required during the induction of iNOS by LPS in macrophages is NF-κB. The promoter region of the rat gene encoding iNOS contains two copies of the NF-κB binding site consensus sequence (24). NF-κB is an inducible, ubiquitous transcription factor present in the cytoplasm of cells. It is composed of a dimer of p50 and p65 (Rel A) subunits. In resting cells the NF-κB complexes are sequestered in the cytoplasm by association with a family of inhibitory proteins which includes mainly IκBα and IκBβ (26). Activation of NF-κB can be initiated by a variety of agents including mitogens such as phorbol 12-myristate 13-acetate and inflammatory cytokines such as LPS and tumor necrosis factor α. After cellular activation the IκB proteins undergo phosphorylation and subsequent proteolytic degradation via the ubiquitin pathway (29). Free NF-κB protein rapidly translocates into the nucleus and binds to its consensus DNA sequence(s) (26) regulating a variety of genes responsible for immunological and inflammatory reactions (30).

As noted above, pretreatment of Kupffer cells by forskolin resulted in maximal attenuation of LPS-induced iNOS mRNA in Kupffer cells. We surmised that forskolin inhibited the LPS-stimulated nuclear translocation of NF-κB in Kupffer cells, resulting in decreased transcription of the iNOS gene and consequently decreased steady-state levels of iNOS mRNA. Western blot analysis of cytoplasmic and nuclear extracts (Fig. 6) confirmed that in Kupffer cells forskolin pretreatment greatly reduced the amount of NF-κB (p65 subunit) which was translocated to the nucleus by LPS stimulation. A similar result was obtained using cytoplasmic and nuclear extracts from Kupffer cells that had been treated only with forskolin. These findings confirmed our conjecture that forskolin inhibits LPS-induced iNOS mRNA formation in Kupffer cells by functionally inactivating NF-κB.

Attenuation of NF-κB activity by forskolin could occur by two alternative pathways: either forskolin could directly prevent the degradation of IκBα proteins and thus the translocation of NF-κB into the nucleus, or forskolin could cause cytoplasmic retention of NF-κB by some other mechanism without preventing degradation of the IκBα protein. LPS treatment of Kupffer cells decreased levels of cytoplasmic IκBα, and this decrease coincided with the appearance of the p65 subunit of NF-κB in the nucleus. In cytoplasmic extracts of Kupffer cells that had been pretreated with forskolin there appeared to be no decrease in IκBα upon stimulation with LPS (compare lanes 2 and 3, Fig. 7). These results indicate that forskolin protects IκBα presumably by preventing its phosphorylation and degradation.

Nuclear NF-κB itself can induce the expression of the IκBα gene, thus rapidly replenishing the depleted IκBα protein pool in the cytoplasm of cells (31). The newly synthesized IκBα proteins then complex with NF-κB in the cytoplasm, limiting its translocation into the nucleus. This IκB homeostasis operates in LPS-treated stimulated Kupffer cells, where IκBα mRNA levels remained elevated for longer than 6 h (Fig. 8, panel B); it provides the most likely explanation for why LPS-induced iNOS mRNA levels in Kupffer cells are not expressed indefinitely but were observed to decrease substantially by 24 h (Fig. 2, B and D). Forskolin alone induced transient IκBα mRNA formation in Kupffer cells, with the mRNA levels peaking after 2 h of stimulation and decreasing to control values by 6 h (Fig. 8, panels A and D). Interestingly, pretreatment of Kupffer cells for 1 h with forskolin up-regulated LPS-induced IκBα mRNA levels (Fig. 8, panels C and D). It is important to note that maximal up-regulation of IκBα mRNA levels in Kupffer cells pretreated with forskolin for 1 h and then stimulated with LPS occurred after 1 h (Fig. 8, panel D), but in fact the Kupffer cells had been contact with forskolin for 2 h, at which time forskolin induces maximum amounts of IκBα mRNA in Kupffer cells (Fig. 8D). It may be of importance to consider that the time at which maximal inhibition of LPS-induced iNOS mRNA levels in Kupffer cells occurs is when the cells have been pretreated with forskolin for 1 h compared with the situation where forskolin was added after LPS (Fig. 3B), which coincides with maximal IκBα mRNA levels in the cells. In contrast, when forskolin was added to Kupffer cells several hours after LPS, the transcriptional and translational pathways of iNOS formation are already well established, and thus there is no opportunity for forskolin to exert its inhibitory mode of action on iNOS synthesis.

Recent reports have shown that LPS stimulation of Kupffer cells activates NF-κB within 1 h, both in vitro (32, 33) and in the intact rat (34). Moreover, CD18/ICAM-1-dependent NF-κB activation leads to nitric oxide production in Kupffer cells (35). To our knowledge, the present report is the first detailed analysis of the NF-κB control system during in iNOS response to endotoxin in the Kupffer cell. Also, this is the first report to characterize the effects of cAMP on this regulatory system; a previous study using Kupffer cells reported no effect of dibutyryl cAMP on LPS-stimulated iNOS induction (36).

The effects of cAMP on iNOS production are of increasing interest since the first report (37) that cAMP-elevating agents induced iNOS in cultured vascular smooth muscle cells and that this induction was synergistic with that elicited by inflammatory cytokines. cAMP elevation has been shown to have similar effects in renal mesangial cells (6) and in rat brown adipocytes (38). Although cAMP alone does not induce iNOS in unstimulated cardiac myocytes, it augments iNOS induction in interleukin 1β-stimulated cells (39). NF-κB/Rel is regulated positively by the cAMP cascade to help initiate iNOS gene expression in response to LPS stimulation of the macrophage line RAW 264.7, and inhibition of adenylate cyclase attenuates the activation of iNOS in these cells (40). In 3T3 fibroblasts different signaling pathways including elevation of cAMP lead to the induction of iNOS by NF-κB mediation (41). In contrast, elevation of cellular cAMP has been shown to down-regulate iNOS in endotoxin-activated cultures of rat microglia (quiescent brain macrophages (42)), rat primary astrocytes (43), and J774 cells (murine macrophage line (44)).

Our present findings suggest that pretreatment of Kupffer cells with forskolin both prevents the degradation of IκBα and induces IκBα mRNA formation, thereby increasing the pool of IκBα proteins in the cytoplasm. When these same cells are...
In Kupffer cells, the ability of cAMP to attenuate LPS-induced NO formation provides a model in which to characterize secondary shock-related effects on NF-κB. cAMP elevation abrogates the ability of cAMP to attenuate LPS-induced NO formation by Kupffer cells and for typing this manuscript. We are grateful to Dr. Stephen A. K. Harvey for thoughtful criticism of the manuscript.
