Elevated Cyclic AMP Inhibits NF-κB-mediated Transcription in Human Monocytic Cells and Endothelial Cells*

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The NF-κB/Rel family of transcription factors regulates the inducible expression of many genes in activated human monocytes and endothelial cells. In this study, we examined the molecular mechanism by which agents that elevate intracellular cAMP inhibit the expression of the tumor necrosis factor α (TNFα), tissue factor, endothelial leukocyte adhesion molecule-1, and vascular cell adhesion molecule-1 genes. Both forskolin and dibutyryl cAMP, which elevate intracellular cAMP by independent mechanisms, inhibited TNFα and tissue factor expression at the level of transcription. Induction of NF-κB-dependent gene expression in transiently transfected human monocytic THP-1 cells and human umbilical vein endothelial cells was inhibited by elevated cAMP and by overexpression of the catalytic subunit of protein kinase A (PKA). Elevated cAMP did not prevent nuclear translocation of p50/p65 and c-Rel/p65 heterodimers, decrease nuclear translocation of p65, or significantly modify TNFα-induced phosphorylation of p65. Functional studies demonstrated that transcriptional activation of a plasmid containing multimerized κB sites by p65 was inhibited by agents that elevate cAMP and by overexpression of the catalytic subunit of PKA. This study indicates that activation of PKA reduces the induction of a distinct set of genes in monocytes and endothelial cells by inhibiting NF-κB-mediated transcription.

Human monocytes and endothelial cells can be induced to express a variety of genes involved in immune and inflammatory responses, cell adhesion, blood coagulation, and fibrinolysis (1, 2). For example, bacterial lipopolysaccharide (LPS)† stimulates monocytes to rapidly and transiently express a defined set of gene products including tumor necrosis factor α (TNFα) (3) and the transmembrane receptor tissue factor (TF) (4, 5). In endothelial cells, LPS and cytokines induce the expression of various adhesion molecules, including endothelial leukocyte adhesion molecule-1 (E-selectin), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which permit binding and transmigration of leukocytes into sites of inflammation (6–8). Activation of endothelial cells also induces TF expression, which converts the normal anticoagulant surface to a procoagulant state (9–11).

The NF-κB/Rel family of transcription factors has been implicated in the inducible expression of many genes in monocytes and endothelial cells, including TNFα, TF, E-selectin, and VCAM-1 (12–15). The NF-κB/Rel family of transcription factors includes NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB, and c-Rel (16). These factors can homo- and heterodimerize to generate distinct transcription factors that regulate expression of many different genes (16). The TNFα, E-selectin, and VCAM-1 genes are regulated by p50/p65 heterodimers (17), whereas the TF gene is regulated by c-Rel/p65 heterodimers (18). In monocytes and endothelial cells, LPS and cytokines induce the dissociation of the inhibitory protein IκBα from pre-existing cytoplasmic NF-κB-Rel complexes, allowing the transcription factors to translocate to the nucleus and initiate expression of target genes (19).

cAMP induces the expression of numerous genes through the protein kinase A (PKA)-mediated phosphorylation of CRE-binding factors, including CRE-binding protein (CREB) (20). Elevation of intracellular cAMP levels in monocytes and endothelial cells also inhibits the induction of a distinct set of genes, including TNFα, TF, E-selectin, and VCAM-1 (21–25). We (27) and others (26, 28) have shown that pharmacologic agents that elevate intracellular levels of cAMP, such as pentoxifylline and a prostacyclin analog, iloprost, inhibit LPS induction of TF expression in human monocytes. In addition, agents such as dibutylryl cAMP (Bt2cAMP), forskolin, and isobutylmethylxanthine, which increase cAMP levels by independent mechanisms, all inhibit induction of TF expression (29, 30). In endothelial cells, elevation of cAMP and activation of PKA inhibit cytokine induction of E-selectin, VCAM-1, and TF expression (21, 22, 31). Studies using both monocytes and endothelial cells indicate that cAMP inhibits transcription of this distinct set of genes (21, 23, 25, 30).

This study examined inhibition of the transcriptional activation of the TNFα, TF, E-selectin, and VCAM-1 genes in human
monocytic and endothelial cells by agents that elevate intracellular levels of cAMP.

**EXPERIMENTAL PROCEDURES**

Chemicals—LPS (Escherichia coli serotype O111:B4) and forskolin were purchased from Calbiochem. Bt²CAMP was purchased from Sigma. Human recombinant TNFα was obtained from Collaborative Biomedical Products (Bedford, MA).

Cell Culture—Human monocytic THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described (5). Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained from collagenase-digested umbilical veins or from Clonetics Corp. (San Diego, CA) and were cultured as described (11). All experiments used HUVECs between passages 3 and 5.

RNA Isolation and Northern Analysis—Total RNA was isolated from THP-1 cells using TRIzol reagent (Life Technologies, Inc.) and from HUVECs by phenol extraction as described (32). 10 μg of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and transferred to a GeneScreen membrane (DuPont NEN). Membranes were hybridized with various cDNA fragments labeled with [α-32P]dCTP (>3000 Ci/mmol; ICN, Costa Mesa, CA) using the Primet-I kit (1) (Strategene, San Diego, CA). TNFα and TF cDNA fragments have been described previously (5). An E-selectin cDNA fragment was obtained from R&D Systems (Minneapolis, MN), and a 1.3-kilobase VCAM-1 cDNA fragment was obtained by polymerase chain reaction. A c-fos cDNA fragment was kindly provided by Dr. I. Verma (Salk Institute, La Jolla, CA). Variances, cellswere transfected with the control plasmid pCMV-phosphate dehydrogenase cDNA fragment (Clontech); and a vector containing a tetracycline repressor/VP16 fusion protein and the cytochrome b oxidase promoter (34) was made by inserting the human TF promoter (121 base pairs) into the pTF-tet/VP16 vector (Clontech), kindly provided by Dr. R. Hooft van Huijsduijnen. pTF-tet/VP16 was used as a model for the TF gene. Levels of TF, E-selectin, and VCAM-1 mRNAs at 1 h (Fig. 1A) and the murine IgG1κ antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Similarly, nuclear translocation of p65 was monitored by Western blotting in THP-1 cells and HUVECs using a 1:1000 dilution of an anti-p65 antibody (Santa Cruz Biotechnology).

In Vivo Labeling of Cells and Immunoprecipitation—Confluent monolayers of HUVECs in 10-cm tissue culture dishes were washed twice with phosphate-free RPMI 1640 medium and incubated in phosphate-free medium containing 200 μCi of [32P]orthophosphate (400–800 mCi/ml; ICN) for 1 h. Cells were stimulated with TNFα (10 ng/ml) for 1 h with or without a 20-min pretreatment with forskolin (20 μM). After incubation, the cells were washed, and p65 was recovered by immunoprecipitation with an anti-p65 antibody using an immunoprecipitation kit containing protein A beads (Boehringer Mannheim). The precipitated proteins were washed several times, eluted in sample buffer containing 25 mM dithiothreitol, separated on 8–16% SDS-polyacrylamide gels (Novex), and visualized by autoradiography.

**RESULTS**

**Elevated cAMP Inhibits LPS and TNFα Induction of a Distinct Set of Genes in Human Monocytic and Endothelial Cells**—The effect of elevated intracellular levels of cAMP on LPS induction of the TF and TNFα genes in human monocytic THP-1 cells was investigated using Bt²CAMP, a membrane-permeable analog of cAMP (30), or forskolin, an activator of adenyl cyclase (21). The reagent concentration used in this study has been previously shown to increase cAMP levels in monocytes/macrophages and HUVECs (21, 25). LPS stimulation of THP-1 cells increased the steady-state levels of TNFα and TNFα mRNA at 2 h (Fig. 1A). The addition of Bt²CAMP immediately before LPS stimulation abolished induction of TF and TNFα mRNA expression. In addition, Bt²CAMP reduced TF and TNFα mRNA levels in unstimulated cells. In contrast, Bt²CAMP induced expression of c-fos mRNA in THP-1 cells, indicating that Bt²CAMP activates PKA (Fig. 1A).

TNFα stimulation of HUVECs increased the steady-state levels of TF, E-selectin, and VCAM-1 mRNAs at 1 h (Fig. 1B). Pretreatment of HUVECs with forskolin before the addition of TNFα inhibited the induction of TF, E-selectin, and VCAM-1 mRNAs by 60 ± 9, 78 ± 8, and 73 ± 14%, respectively (mean ± S.D., n = 3). In contrast, forskolin did not inhibit TNFα induction of ICAM-1 mRNA (data not shown), consistent with previous studies (21, 22). These data indicate that agents that increase intracellular levels of cAMP by two independent mechanisms inhibit the induction of a distinct set of genes in human monocytic and endothelial cells.

**Elevated cAMP Inhibits LPS-induced TNFα Gene Transcription in Monocytic Cells**—We examined the effect of Bt²CAMP on LPS-induced TNFα gene transcription in monocytic THP-1 cells using nuclear run-on assays. LPS stimulation of THP-1 cells increased the rate of transcription of the TNFα gene by 11.7-fold at 1 h (Fig. 2). Treatment of the cells with Bt²CAMP before the addition of LPS abolished the increase in the rate of transcription of the TNFα gene (Fig. 2). These results indicate that cAMP inhibition of transcription may account for most, if not all, of the observed inhibition of LPS-induced TNFα mRNA expression.

**Elevated cAMP Inhibits TNFα Induction of TF Promoter Activity in Endothelial Cells**—The human TF promoter donor plasmid upstream of the luciferase reporter gene was used as a model system to examine inhibition of promoter activity in THP-1 cells and HUVECs by Bt²CAMP and forskolin. These studies...
indicated that Bt2cAMP alone induced TF promoter activity (data not shown), suggesting that these plasmids contain cryptic CRE sites. In addition, cAMP and cAMP derivatives have been shown to interfere with the luciferase assay (38). Therefore, we employed a two-plasmid reporter system in which the first plasmid contained the human TF promoter expressing a transcription factor (Fig. 1). Elevated cAMP may inhibit all of these genes by a common mechanism involving NF-κB/Rel proteins. To determine if elevation of cAMP inhibited NF-κB-mediated induction of gene transcription, THP-1 cells and HUVECs were transfected with pSV40-tet/VP16 or pTF-tet/VP16, which contains four κB sites cloned upstream of the minimal SV40 promoter. LPS stimulation of THP-1 cells transfected with pSV40-tet/VP16 resulted in a 5.0 ± 0.7-fold (mean ± S.E., n = 5) induction of CAT activity (Fig. 4A). Treatment of the THP-1 cells with Bt2cAMP before LPS stimulation resulted in a 61 ± 5% (mean ± S.E., n = 5) inhibition of LPS-induced CAT activity. Similar results were observed using a plasmid containing four copies of the murine Igκ-B site (data not shown). No induction of CAT activity was observed in THP-1 cells transfected with pSV40-tet/VP16, which contains only the minimal SV40 promoter. In HUVECs, TNFα induced a 11.4 ± 2.3-fold (mean ± S.E., n = 5) induction of CAT activity in cells transfected with p(kB6)2-tet/VP16, whereas no induction was observed with cells transfected with pSV40-tet/VP16 (Fig. 4B). Pretreatment of HUVECs with forskolin resulted in a 61 ± 10% (mean ± S.E., n = 5) inhibition of TNFα-induced CAT activity (Fig. 4B).

Fig. 1. Elevated cAMP in THP-1 and HUVECs inhibits the induction of TF, TNFα, E-selectin, and VCAM-1 mRNA expression. A, total RNA was extracted from THP-1 cells exposed to LPS (10 μg/ml) for 2 h with or without Bt2cAMP (dbcAMP; 1 mM); B, total RNA was extracted from HUVECs exposed to TNFα (20 ng/ml) for 1 h with or without a 20-min forskolin pretreatment (Fsk; 100 μM). TF, TNFα, E-selectin, and VCAM-1 mRNA levels were determined by Northern blot analysis using the appropriate radiolabeled human cDNA probes. Blots were reprobed to determine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels as a measure of RNA loading. Similar results were observed in two independent experiments.

Fig. 2. Elevated cAMP inhibits LPS-induced TF mRNA expression. A, total RNA was extracted from THP-1 cells stimulated with LPS (10 μg/ml) for 1 h, and cells treated with Bt2cAMP (dbcAMP; 1 mM) before LPS stimulation. Labeled nuclear RNA levels were determined by hybridization to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and TNFα DNAs and a vector control (pSP73). The autoradiogram was exposed for 12 days at −80 °C with an intensifier screen. Similar results were observed in two independent experiments.

Fig. 3. Elevated cAMP inhibits the induction of the TF promoter in HUVECs. HUVECs were cotransfected with pBasic-tet/VP16 (pBasic; 10 ng) and pHG10.3CAT (10 μg) or with pTF-tet/VP16 (pTF; 10 ng) and pHG10.3CAT (10 μg) using DEAE-dextran. After 24 h, cells were stimulated with TNFα (20 ng/ml) with or without a 20-min pretreatment with forskolin (Fsk; 50 μM) and incubated for a further 24 h before determining CAT activity. Results from three independent experiments are shown. Transfection efficiencies were assessed using pCMVβ and exhibited <15% variation between samples.
Cyclic AMP Inhibition of Transcription

These data demonstrate that agents that elevate cAMP strongly inhibit NF-κB-mediated gene transcription in human monocytic and endothelial cells.

Nuclear Translocation of NF-κB/Rel Proteins Is Not Affected by Elevated cAMP—Inhibition of NF-κB-mediated transcription may be due to a block in the nuclear translocation of NF-κB/Rel proteins and/or a reduction in the transcriptional activity of these proteins in the nucleus. To examine the effect of an increase in intracellular levels of cAMP on the nuclear translocation of these NF-κB/Rel complexes, electrophoretic mobility shift assays were performed using a κB site from the murine Igκ enhancer, which binds p50/p65 heterodimers, and a κB site from the human TF gene, which binds c-Rel/p65 heterodimers (34). Previous competition and antibody supershift experiments have established the protein composition of these complexes (11, 34). Binding of nuclear proteins to these κB sites in THP-1 cells was examined 1 h after stimulation because the maximal rate of transcription of the TF and TNFα genes was previously shown to occur 1 h after LPS stimulation (3, 5). LPS stimulation of THP-1 cells for 1 h induced the nuclear translocation of p50/p65 and c-Rel/p65 heterodimers that bound the Igκ-κB and TF-κB sites, respectively (Fig. 5A). No statistically significant differences were observed between the levels of p50/p65 (p < 0.49) and c-Rel/p65 (p < 0.20) heterodimers in nuclear extracts from cells treated with LPS in the presence and absence of Bt2cAMP. Nuclear extracts from unstimulated THP-1 cells and Bt2cAMP-treated cells showed no binding of NF-κB-Rel complexes (Fig. 5A).

The TF, E-selectin, and VCAM-1 genes are rapidly and transiently expressed in endothelial cells (6, 11, 17). Binding of nuclear proteins from HUVECs was examined 1 h after stimulation because transcription of these genes is increased at this time (11, 17). TNFα stimulation of HUVECs induced the nuclear translocation of p50/p65 and c-Rel/p65 heterodimers (Fig. 5B). The addition of forskolin to HUVECs before TNFα stimulation had no effect on the nuclear translocation of p50/p65 and c-Rel/p65 heterodimers (Fig. 5B). No statistically significant differences were observed between the levels of p50/p65 (p < 0.44) and c-Rel/p65 (p < 0.14) in nuclear extracts from cells treated with TNFα in the presence and absence of forskolin. Forskolin alone did not induce the nuclear translocation of NF-κB-Rel complexes in HUVECs (Fig. 5B). Similarly, forskolin did not inhibit the nuclear translocation of p50/p65 induced by LPS stimulation of HUVECs (data not shown). Antibody supershift experiments indicated that elevated cAMP did not change the composition of the p50/p65 and c-Rel/p65 heterodimeric complexes (data not shown). In addition, antibody supershift experiments using an anti-CREB antibody excluded the possibility that elevation of cAMP induced binding of CREB to the NF-κB-Rel complexes (data not shown). These results indicate that Bt2cAMP and forskolin do not affect either the nuclear translocation or DNA binding of NF-κB-Rel complexes in monocytic and endothelial cells.
To confirm that elevated cAMP did not selectively inhibit the nuclear translocation of p65, cytoplasmic and nuclear extracts from THP-1 cells were examined by Western blotting using a p65-specific antibody. LPS stimulation induced the nuclear translocation from THP-1 cells were examined by Western blotting using a 1:1000 dilution of a specific anti-p65 antibody. LPS stimulation induced the nuclear translocation of p65, cytoplasmic and nuclear extracts were determined by Western blotting using a 1:1000 dilution of a specific anti-p65 antibody. LPS stimulation induced the nuclear translocation of p65, cytoplasmic and nuclear extracts were determined by Western blotting using a 1:1000 dilution of an anti-p65 antibody.

To determine if elevated cAMP affected IxBα proteolysis, IxBα levels in cytoplasmic extracts from HUVECs were analyzed by Western blotting. TNFα induced the proteolytic degradation of IxBα (Fig. 6B). Pretreatment of the cells with forskolin (20 μM) did not affect the proteolytic degradation of IxBα (Fig. 6B). Similarly, forskolin (20 μM) did not affect the proteolytic degradation of IxBα in THP-1 cells stimulated with LPS for 1 h (data not shown).

TNFα-induced Phosphorylation of p65 Is Not Affected by Elevated cAMP—A recent study showed that p65 is strongly phosphorylated during the activation of NF-kB in vivo (39). More detailed studies indicated that p65 contains two transactivation (TA) domains in the carboxyl-terminal end (40). TA1 is constitutively phosphorylated, whereas stimulation of cells induces phosphorylation of TA2 and is correlated with increased transcriptional activity (40). To determine if elevated cAMP inhibited the functional activity of nuclear NF-kB-Rel complexes by modifying the phosphorylation of p65, we examined the phosphorylation of p65 in TNFα-stimulated HUVECs with or without forskolin. p65 was immunoprecipitated from unlabelled HUVECs and detected by Western blotting using an anti-p65 antibody to confirm selective recovery of p65 (data not shown). In addition, immunoprecipitation of p65 was prevented by the addition of the peptide (Santa Cruz Biotechnology) that was used to raise the antibody (data not shown). TNFα induced a strong phosphorylation of p65 in HUVECs, which was not affected by pretreatment of the cells with forskolin (Fig. 7). A low constitutive phosphorylation of p65 was observed in unstimulated HUVECs, which was not increased by treatment of the cells with forskolin alone (Fig. 7). Additional phosphorylated proteins observed in these studies may represent immunoprecipitation of other NF-kB/Rel proteins associated with p65 as shown previously (39). These data indicate that activation of PKA does not modify the TNFα-induced phosphorylation of p65, although we cannot exclude the possibility that forskolin changes the sites of phosphorylation of p65.

NF-kB-mediated Transcription Is Inhibited by Elevated cAMP and by Activation of PKA—The effect of increased levels of intracellular cAMP and expression of the catalytic subunit of PKA on NF-kB-mediated transcription was measured in HUVECs. Cells were cotransfected with p(κB)4-tet/VP16, pUHG10.3CAT, and pCMVp65. Expression of p65 increased the transcriptional activity of p(κB)4-tet/VP16 by 22.1 ± 1.3-fold (mean ± S.E., n = 3) (Fig. 8A), but did not affect the transcriptional activity of the control plasmid, pSV40-tet/VP16 (data not shown). Forskolin reduced the level of p65 transcription by 74 ± 10% (mean ± S.E., n = 3) (Fig. 8A). To exclude the possibility that forskolin nonspecifically reduced transcription of the cytomegalovirus promoter, which was used to express p65, we determined CAT activity in the presence and absence of forskolin in HUVECs transfected with pCMV-tet/VP16, which expresses the tet/VP16 transcription factor from the cytomegalovirus promoter. Forskolin did not affect cytomegalovirus promoter activity as measured by the level of CAT activity (Fig. 8A).

To determine if activation of PKA was required to inhibit NF-kB-mediated transcription, we examined the TNFα induction of CAT activity in HUVECs overexpressing the catalytic subunit of PKA on NF-kB-mediated transcription was measured in HUVECs. Cells were cotransfected with p(κB)4-tet/VP16, pUHG10.3CAT, and pCMVp65. Expression of p65 increased the transcriptional activity of p(κB)4-tet/VP16 by 22.1 ± 1.3-fold (mean ± S.E., n = 3) (Fig. 8A). To determine if activation of PKA inhibited p65 transcription, HUVECs were cotransfected with p(κB)4-tet/VP16, pUHG10.3CAT, and pCMVp65 in the presence and absence of PKA. p65 transcription was inhibited in a dose-dependent manner by coexpression of the catalytic subunit of PKA (Fig. 8B). Expression of maximal levels of PKA inhibited p65 transcription by 75 ± 10% (mean ± S.E., n = 3). These data indicate that activation of PKA either by an increase in intracellular levels of cAMP or by expression of the catalytic subunit of PKA inhibits transcription mediated by endogenous NF-kB/Rel complexes or by overexpressed p65.
In this study, we demonstrated that activation of PKA by agents that elevate cAMP in human monocyctic and endothelial cells inhibited the expression of a distinct set of NF-κB/Rel-regulated genes, including TNFα, TF, E-selectin, and VCAM-1. A common inhibitory mechanism was identified that involved reduction in NF-κB-mediated transcription. Our studies demonstrated that nuclear translocation of NF-κB/Rel complexes was not affected by elevated cAMP. In addition, forskolin did not modify the TNFα-induced phosphorylation of p65. However, transactivation by p65 was inhibited by activation of endogenous PKA and by overexpression of the catalytic subunit of PKA. These data indicate that activation of PKA reduced NF-κB-mediated transcription in human monocyctic and endothelial cells.

Our studies show that elevation of cAMP by both Bt2cAMP and forskolin inhibited the functional activity of endogenous NF-κB/Rel complexes in human monocyctic and endothelial cells. A recent study also showed that forskolin inhibits NF-κB-mediated transcription in HUVECs (21). Inhibition of NF-κB-mediated transcription may be due to a block in the nuclear translocation of NF-κB/Rel complexes and/or a reduction in the transcriptional activity of these proteins in the nucleus. Here, we showed that agents that elevate cAMP did not affect the nuclear translocation of NF-κB/Rel proteins or proteolytic degradation of IκBα. Similarly, two recent reports indicated that forskolin does not reduce the nuclear translocation of NF-κB in human promyelocytic HL-60 cells and HUVECs (21, 41). Forskolin and Bt2cAMP did not alter the composition of the NF-κB/Rel complexes or induce the binding of CRE-binding protein to the NF-κB/Rel complexes (data not shown). Instead, we demonstrated that elevation of cAMP or expression of the catalytic subunit of PKA strongly inhibited transcription mediated by endogenous NF-κB/Rel complexes and overexpressed p65.

A recent study using Jurkat T-cells suggested that cAMP inhibition of interleukin-2 gene expression is due to a small change in the amount of IκBα, which selectively decreases the nuclear translocation of p65 (42). It should be noted that nuclear translocation of NF-κB and p65 in Jurkat T-cells did not appear to be affected 40 min after stimulation, which is similar to our studies using monocytic and endothelial cells (Figs. 5 and 6), and only a small change was noted 2 h after stimulation (42). Functional studies using a chimeric Gal4-p65 protein, which contains the transactivation domain of p65, indicated that forskolin did not inhibit transactivation by Gal4-p65 in human E14 T lymphoma cells (42). However, the use of Gal4-binding sites and a chimeric protein in place of κB-binding sites and wild-type p65 may not recapitulate cAMP-mediated inhibition. The use of different cell types may account for these different inhibitory mechanisms. Alternatively, costimulation of Jurkat cells and E14 T-cells with phorbol ester and ionomycin rather than LPS or cytokines may explain these differences.

Our study and those of others (21, 22, 25) have shown that elevated cAMP in macrophages and endothelial cells selectively inhibits the induction of a distinct set of genes. In contrast, elevated cAMP does not inhibit other inducible genes such as ICAM-1 and interleukin-1 (21, 22, 25). Interestingly, induction of the ICAM-1 gene in endothelial cells is regulated, at least in part, by NF-κB/Rel proteins (17). However, inhibition of TNFα-induced nuclear translocation of NF-κB in HUVECs by aspirin reduces VCAM-1 and E-selectin expression.
expression without affecting ICAM-1 expression (43). In addition, unlike VCAM-1, E-selectin, and TF, ICAM-1 is constitutively expressed by HUVECs, and induction of ICAM-1 is delayed compared with these other genes (17), suggesting that the ICAM-1 gene is regulated by a different combination of transcription factors.

Elevated cAMP may also regulate the activity of other transcription factors that are required for expression of the TNFα, TF, E-selectin, and VCAM-1 genes. In monocytic THP-1 cells, Bt2cAMP abolished LPS induction of TNFα and TF mRNA expression and TNFα gene transcription, but only partially inhibited NF-κB-mediated transcription in transfected cells, suggesting the possibility that cAMP may inhibit other transcription factors. Changes in the composition of proteins binding to the CRE/ATF site in the E-selectin promoter have been reported to contribute to the cAMP inhibition of the E-selectin gene in bovine aortic endothelial cells (44). The TNFα and TF promoters contain AP-1 sites that contribute to the induction of these genes (13, 45, 46). We cannot exclude the possibility that elevated cAMP may, in part, reduce TNFα and TF gene transcription by modulating protein binding to these AP-1 sites and/or by inhibiting AP-1 activity. Our preliminary studies indicate that cAMP increases protein binding to both AP-1 sites in the TF promoter, but so far, no changes in protein composition have been detected. Further studies are required to determine if cAMP inhibits expression of this distinct set of genes via multiple mechanisms.

PKA can modulate the activity of a number of intracellular signaling pathways (47). In yeast, the PKA-dependent phosphorylation of the transcription factor ADR1 inactivates its transcriptional activity without altering its binding to DNA (48, 49). In our studies, PKA may directly phosphorylate p65 or initiate a signaling cascade involving other kinases that reduce the functional activity of NF-κB-Rel complexes. Indeed, c-Rel, p65, and p50 all contain a conserved serine in a consensus PKA recognition site (X-Arg-Arg-X-Ser-X) near the carboxyl-terminal end of the rel homology domain (50). However, the role of this PKA site is unclear because it is not present in all NF-κB/Rel proteins. Mutation of serine 266 to alanine in c-Rel reduces DNA binding and transactivation, suggesting that serine 266 contributes to protein dimerization (50). Similarly, mutation of serine 276 to alanine in p65 abolishes its transactivation activity in HUVECs, making it impossible to determine if elevated cAMP inhibited NF-κB activity by direct phosphorylation of the consensus PKA site of p65.

Phorbol-ester-dependent phosphorylation of the TNFα transactivation domain of p65 enhances its transactivating activity, suggesting that phorbol-ester-activated protein kinase C directly phosphorylates TA2 on serine residues (40). We showed that forskolin did not modify the TNFα-induced phosphorylation of nuclear p65 in HUVECs. However, changes in the phosphorylation pattern of p65 would not be detected in this experiment. PKA may modulate NF-κB activity by phosphorylating cofactors that are required for transactivation. Alternatively, cAMP may induce the synthesis or activation of transcriptional inhibitors of NF-κB-mediated transcription such as Bcl3 (51, 52) and p202, which has recently been shown to inhibit NF-κB enhancers (53). Finally, activation of the PKA signaling pathway may inhibit NF-κB-mediated transcription by squelching coactivators.

Pharmacologic agents that elevate cellular levels of cAMP, such as pentoxifylline and rolipram, have been observed to inhibit LPS-induced TNFα and TF gene transcription in mono-}

2O. V. Ollivier and N. Mackman, unpublished data.
3G. C. N. Parry and N. Mackman, unpublished data.
