cAMP Targeting of p38 MAP Kinase Inhibits Thrombin-induced NF-κB Activation and ICAM-1 Expression in Endothelial Cells

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Running head: cAMP inhibits ICAM-1 expression by targeting p38 MAPK

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ABSTRACT

We investigated the mechanisms by which elevated intracellular cAMP concentration inhibits the thrombin-induced ICAM-1 expression in endothelial cells. Exposure of human umbilical vein endothelial cells (HUVEC) to forskolin or dibutyryl cAMP (dbcAMP), which increase intracellular cAMP by separate mechanisms, inhibited the thrombin-induced ICAM-1 expression. This effect of cAMP was secondary to inhibition of NF-κB activity, the key regulator of thrombin-induced ICAM-1 expression in endothelial cells. The action of cAMP occurred downstream of IκBα degradation and was independent of NF-κB binding to the ICAM-1 promoter. We observed that cAMP interfered with thrombin-induced phosphorylation of NF-κBp65 (RelA) subunit, a crucial event promoting the activation of the DNA-bound NF-κB. As p38 MAP kinase can induce transcriptional activity of RelA/p65 without altering the DNA binding function of NF-κB, we addressed the possibility that cAMP antagonizes thrombin-induced NF-κB activity and ICAM-1 expression by preventing the activation of p38 MAP kinase. We observed that treating cells with forskolin blocked the activation of p38 MAP kinase and inhibition of p38 MAP kinase interfered with phosphorylation of RelA/p65 induced by thrombin. Our data demonstrate that increased intracellular cAMP concentration in endothelial cells prevents thrombin-induced ICAM-1 expression by inhibiting p38 MAP kinase activation, which in turn prevents phosphorylation of RelA/p65 and transcriptional activity of the bound NF-κB.

Keywords: Endothelial adhesivity, polymorphonuclear leukocytes, IκBα degradation, RelA/p65 phosphorylation,
INTRODUCTION

The procoagulant serine protease thrombin, released during intravascular coagulation initiated by tissue injury or sepsis (11, 14), promotes the adhesion of polymorphonuclear leukocytes (PMN) to the endothelium by a mechanism involving the endothelial cell surface expression of intercellular adhesion molecule-1 (ICAM-1) (27, 29, 40). ICAM-1, a member of the immunoglobulin supergene family (38), serves as a counter-receptor for leukocyte β2 integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), and mediates the arrest of PMNs and other leukocytes and their migration across the vessel wall barrier (36). We showed that the transcription factor NF-κB p65 (RelA) is an essential regulator of thrombin-induced ICAM-1 gene transcription following thrombin-induced activation of the heterotrimeric G protein coupled receptor, Protease-Activated Receptor-1 (PAR-1) in endothelial cells (27, 30).

NF-κB, typically a heterodimer of 50 kDa (p50) and 65 kDa (RelA) subunits, is sequestered in the cytoplasm bound to IκB proteins that mask the nuclear localization sequence of NF-κB (3). NF-κB activity is in part regulated at the level of IκB degradation, which is accomplished through serine phosphorylation (Ser32 and Ser36) of IκBα (41) by IκBβ kinase (IKKβ) (16, 45). Phosphorylation targets IκBα for ubiquitination and proteasome-mediated degradation (31). The liberated NF-κB is translocated to the nucleus and binds to NF-κB responsive elements in genes. Studies have shown an additional regulatory pathway involving phosphorylation of RelA/p65 that stimulates the activation of NF-κB (2, 23). In contrast to IκBα phosphorylation, RelA/p65 phosphorylation can be mediated by several kinases depending upon the stimulus and cell type (12, 33, 43, 44).
The p38 mitogen-activated protein (MAP) kinases are widely expressed serine threonine kinases activated by a number of stimuli, including thrombin (21, 29, 34). Activation of p38 MAP kinase has been implicated in the mechanism of NF-κB activation (1, 29, 42). We have recently shown that inhibition of p38 MAP kinase prevented thrombin-induced transcriptional activity of NF-κB without altering its DNA binding function (29), indicating the involvement of p38 MAP kinase in promoting the transactivation of NF-κB (42).

Cyclic adenosine monophosphahte (cAMP) is an ubiquitous second messenger in cells. An important function of cAMP is to activate protein kinase A (PKA) (39), which has been implicated in the regulation of numerous genes through phosphorylation and activation of the cAMP response element binding (CREB) protein (9). Studies showed that cAMP regulates the activation of NF-κB in a cell-specific manner. In the pro-myelocytic cell line, HL 60, elevated cAMP levels induced NF-κB activation (35), whereas exposure of endothelial cells to cAMP prevented NF-κB activation (24). As cAMP modulation of NF-κB activity in endothelial cells may be important in controlling ICAM-1 expression during inflammation, in the present study we addressed the mechanism of this modulatory cAMP action. We provide evidence herein that cAMP inhibits thrombin-induced NF-κB activity and ICAM-1 expression in endothelial cells by blocking p38 MAP kinase activation, which in turns prevents the phosphorylation of RelA/p65, and activation of the DNA-bound NF-κB.
MATERIALS AND METHODS

**Materials:** Human thrombin (activity of 3170 NIH U/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). Polyclonal antibodies against p38 MAP kinase, IκBα, and NF-κB p65 and a monoclonal antibody against ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies that detect p38 MAP kinase when activated by dual phosphorylation at Thr180 and Tyr182, RelA/p65 when activated by phosphorylation at Ser536 or IκBα when phosphorylated at Ser32 and Ser36 were obtained from Cell Signaling Technology (Beverly, MA). In addition, polyvinylidene difluoride (PVDF) membrane was from Millipore Corp. (Bradford, MA); Forskolin and dibutyryl cAMP (dbcAMP) from Sigma Chemical Company (St. Louis, MO); SB203580 from Calbiochem-Novabiochem Corp. (La Jolla, CA); protein assay kit and nitrocellulose membrane were from Bio-Rad Laboratories (Hercules, CA); plasmid maxi kit from QIAGEN Inc. (Valencia, CA). All other materials were from Fisher Scientific Company (Pittsburg, PA).

**Cell Culture:** Human umbilical vein endothelial cells (HUVEC; Clonetics, La Jolla, CA) were cultured as described (29) in gelatin-coated flasks using endothelial basal medium 2 (EBM2) with bullet kit™ additives (Clonetics). Confluent cells were incubated for 2-12 h in heat inactivated 0.5-1% FBS containing EBM2 prior to thrombin challenge. All experiments, except where indicated, were made in cells under the 8th passage.

**Flow Cytometry Analysis:** Flow cytometry analysis was performed as described (27). Briefly, HUVEC monolayers in six-well tissue culture dishes were pretreated with FSK (20 µM) or dibutyryl cAMP (0.5 mM) for 30 min prior to stimulation with thrombin for 12-15 h. After completion of incubation period, cells were washed twice with cold PBS, removed by careful
trypsinization, and washed again with Ca^{2+}/Mg^{2+}-free PBS before incubating with 20% horse serum for 30 min. Following two washes, cells were incubated with a mouse mAb directed against human ICAM-1, BIRR0001 (kindly provided by Dr. Robert Rothlein, Boeringer Ingleheim, Ridgefield, CT) (32), in Ca^{2+}/Mg^{2+}-free PBS containing 3% horse serum for 30 min at 4°C. Cells were then washed twice with PBS/horse serum and incubated for 30 min at 4°C with a goat anti-mouse IgG FITC-conjugated secondary Ab. Cells were then fixed with 2% paraformaldehyde, and analyzed by flow cytometry in a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA), and the results were gated for mean fluorescence intensity above the fluorescence produced by the secondary Ab alone.

**Cell Lysis and Immunoblotting:** After treatment, the cells were lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, 1% Triton-X100, 1% sodium deoxycholate, 5 mM sodium fluoride, 1 mM PMSF, and 1 µg/ml each of leupeptin, pepstatin A, and aprotinin). Cell lysates were analyzed by SDS-PAGE and transferred onto nitrocellulose (Bio-Rad Laboratories) or polyvinylidene difluoride (PVDF; Millipore Corp., Bradford, MA) membranes, and the residual binding sites on the filters were blocked by incubating with 5% (w/v) nonfat dry milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature or overnight at 4 °C. The membranes were subsequently incubated with indicated antibodies and developed using an enhanced chemiluminescence (ECL) method as described (29).

**p38 MAP Kinase Assay:** Cells were serum-starved by overnight incubation in EBM2-1% FBS. The cells were subsequently challenged with thrombin (5 U/ml) for 5 min in the absence and presence of forskolin (20 µM) or dbcAMP (0.5 mM), which was added 30-60 min prior to
thrombin treatment. The cells were then lysed with a phosphorylation lysis buffer (50 mM HEPES, 150 mM NaCl, 200 µM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 1.5 mM magnesium chloride, 10% glycerol, 0.5-1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin). Cell lysates were immunoprecipitated with an antibody against p38 MAP kinase using protein G-Sepharose (Amersham Pharmacia Biotech) as described (29). The immunocomplexes were washed three times with phosphorylation lysis buffer and two times with kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl₂, 25 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 20 µM ATP), and resuspended in 30 µl of kinase buffer containing 5 µg of ATF-2 and 20-30 µCi of [γ³²P]ATP was added. The reaction was incubated for 15-30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of ATF-2 was detected by autoradiography.

Northern Analysis: Total RNA was isolated from HUVEC with RNeasy kit (QIAGEN Inc.) according to manufacturer’s recommendations. Quantification and purity of RNA were assessed by A₂₆₀/A₂₈₀ absorption and an aliquot of RNA (20 µg) from samples with ratio above 1.6 was fractionated using a 1% agarose formaldehyde gel. The RNA was transferred to Duralose-UV™ nitrocellulose membrane (Stratagene, La Jolla, CA) and covalently linked by ultraviolet irradiation using a Stratalinker UV crosslinker (Stratagene). Human ICAM-1 (0.96 kb SalI to PstI fragment) (38) and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1.1 kb PstI fragment) were labeled with [α³²P]dCTP using the random primer kit (Stratagene) and hybridization was carried out as described (27). Briefly, the blots were prehybridized for 30 min at 68 °C in QuikHyb™ solution (Stratagene) and hybridized for 2 h at 68 °C with random primed ³²P-labeled probes. After hybridization, the blots were washed 2x for 30 min at room
temperature in 2x SSC with 0.1% SDS followed by 2 washes for 15 min each at 60 °C in 0.1x SSC with 0.1% SDS. Autoradiography was performed with an intensifying screen at -70 °C for 12-24 h. The nitrocellulose membrane was soaked for stripping the probe with boiled water or 0.1x SSC with 0.1% SDS.

**Reporter Gene Constructs, Endothelial Cell Transfection, and Luciferase Assay:** The plasmid pNF-κB-LUC containing 5 copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene. Transfections were performed using DEAE-dextran method (22) with slight modifications (29). Briefly, 5 µg DNA was mixed with 50 µg/ml DEAE-dextran in serum-free EBM2 and the mixture was added onto cells which were 70-80% confluent. We used 0.125 µg pTKRLUC plasmid (Promega Corp., Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter to normalize the transfection efficiencies. After 1 h, cells were incubated for 4 min with 10% dimethyl sulfoxide (DMSO) in serum-free EBM2. The cells were then washed 2x with EBM2-10% FBS and grown to confluency. Cell extracts were prepared and assayed for Firefly and Renilla luciferase activities using Promega Biotech Dual Luciferase Reporter Assay System (Promega, Madison, WI). The data were expressed as a ratio of Firefly and Renilla luciferase activity.

**Cytoplasmic and Nuclear Extract Preparation:** After treatment, cells were washed 2x with ice-cold Tris-buffered saline (TBS) and resuspended in 400 µl of buffer A (10 mM HEPES, [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). After 15 min, Nonidet P-40 (NP-40) was added to a final concentration of 0.6%. Samples were centrifuged to collect the supernatants containing
cytosolic proteins for determining IκBα degradation by Western blot analysis. The pelleted nuclei were resuspended in 50 μl of buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 30 min at 4 °C, lysates were centrifuged and supernatants containing the nuclear proteins were transferred to new vials. Protein concentration of the extract was measured using a Bio-Rad protein determination kit (Bio-Rad Laboratories).

**Electrophoretic Mobility Shift Assay:** Electrophoretic mobility shift assays (EMSA) were performed as described (29). Briefly, 10 μg of nuclear extract was incubated with 1 μg of poly (dI-dC) in a binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.5 mM DTT, 10% glycerol [20 μl final volume] for 15 min at room temperature. Then end-labeled double-stranded oligonucleotides containing an NF-κB site (30,000 cpm each) were added and the reaction mixtures were incubated for 15 min at room temperature. The DNA-protein complexes were resolved in 5% native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25x Tris-borate-EDTA). The oligonucleotide used for the gel shift analysis was Ig-κB 5′-AGTTGAGGGGACTTTCCAGGC-3′. The Ig-κB oligonucleotide contains the consensus NF-κB binding site sequence. The sequence motifs within the oligonucleotides are underlined.

**PMN Adhesion Assay:** PMN adhesion assay was performed as described (4). Briefly, HUVEC grown on 12 mm circular cover slips were stimulated with thrombin, washed extensively to remove residual thrombin prior to labeling with 3 μM fluorescent (red) Cell Tracker dye for 30 min. Freshly isolated human neutrophils (PMN) were stained with 5 μM fluorescent (green) Cell Tracker dye, coincubated with endothelial cells for 20 min, washed with PBS, and visualized
using a fluorescent microscope. The adherent PMN were counted and expressed as PMN/0.8 mm² of endothelial cell.
RESULTS

cAMP inhibits thrombin-induced ICAM-1 mRNA expression in endothelial cells: We determined the effects of increasing intracellular cAMP on ICAM-1 mRNA expression in response to thrombin challenge of endothelial cells. We used forskolin, an adenylate cyclase activator, and dbcAMP, a synthetic cell-permeable cAMP analogue, to raise the intracellular cAMP by two independent means. Northern blot analysis showed that pretreatment of HUVEC monolayers with forskolin or dbcAMP, reduced thrombin-induced ICAM-1 transcript in a dose-dependent manner (Fig. 1 A&B). In another experiment, we determined if cAMP can also inhibit ICAM-1 mRNA expression in response to TNFα challenge of endothelial cells. These results showed that forskolin, in contrast to its effect on thrombin response, failed to prevent TNFα-induced ICAM-1 mRNA expression (Fig. 1C).

cAMP inhibits thrombin-induced ICAM-1 cell surface expression and endothelial adhesivity towards PMN: We next evaluated the effects of cAMP on thrombin-induced ICAM-1 cell surface expression and endothelial adhesivity towards naïve PMNs. FACS analysis showed that thrombin challenge of HUVEC resulted in increased ICAM-1 cell surface expression (Fig. 2A, panel b). Pretreatment of cells with forskolin or dbcAMP inhibited thrombin-induced ICAM-1 cell surface expression (Fig. 2A, panels c&e). In control experiments, forskolin or dbcAMP alone showed no effects on ICAM-1 cell surface expression (Fig. 2A, panels a&d). As ICAM-1 expression induces PMN adhesion to endothelial cells (27, 29), we determined whether prevention of ICAM-1 cell surface expression interfered with endothelial adhesivity. We found that increase in intracellular cAMP following pretreatment of cells with forskolin prevented the thrombin-induced endothelial adhesivity towards naïve PMN (Fig. 2B).
cAMP inhibits thrombin-induced NF-κB activity without affecting its DNA binding function: As NF-κB activation is essential for the thrombin-induced ICAM-1 gene transcription (27), we addressed the possibility that cAMP exerts its effect on ICAM-1 expression by inhibiting the NF-κB activity. HUVEC were co-transfected with pNF-κBLUC containing 5 copies of consensus NF-κB sequence linked to a minimal adenovirus E1B promoter-luciferase reporter gene. As shown in Fig. 3, thrombin-induced NF-κB activity was markedly reduced in cells pretreated with forskolin.

We also determined the effect of cAMP on thrombin-induced IκBα degradation, a requirement for NF-κB activation (6, 41). Western blot analysis showed that thrombin exposure of endothelial cells resulted in IκBα degradation (Fig. 4A). In contrast to forskolin’s effect on NF-κB activity (Fig. 3), forskolin failed to prevent the thrombin-induced IκBα degradation (Fig. 4A). As IκBα degradation results in nuclear translocation and DNA binding of NF-κB, we also evaluated the effects of cAMP on thrombin-induced nuclear uptake and DNA binding function of NF-κB. Pretreatment of cells with forskolin failed to prevent the nuclear translocation of RelA/p65 (Fig. 4B). Electrophoretic mobility shift assay showed that forskolin failed to inhibit the DNA binding activity of NF-κB (Fig. 4C) consistent with its lack of effect on nuclear uptake of RelA/p65 (Fig. 4B).

cAMP inhibits thrombin-induced phosphorylation of RelA/p65: Because activation of RelA/p65 requires phosphorylation of serine 536 in the transactivation domain (20, 44), we explored the possibility that cAMP inhibits thrombin-induced transcriptional activity of RelA/p65 by preventing its phosphorylation. We determined the effect of cAMP on the phosphorylation status of RelA/p65 using an antibody that detects RelA/p65 phosphorylated at serine 536. Co-immunoprecipitation studies demonstrated that thrombin challenge resulted in
increased phosphorylation of RelA/p65 at serine 536 and this response was inhibited in cells treated with forskolin prior to the thrombin challenge (Fig. 5A).

cAMP inhibits thrombin-induced activation of p38 MAP kinase: We have previously shown that p38 MAP kinase is a critical signaling intermediate required for thrombin-induced ICAM-1 transcription in endothelial cells (29). Our results showed that pretreatment of cells with SB203580, a p38 MAP kinase inhibitor, prevented the thrombin-induced phosphorylation of serine 536 of RelA/p65 (Fig. 5A). These findings led us to investigate if cAMP functions by interfering with the activation of p38 MAP kinase in response to thrombin challenge, and thereby inhibits phosphorylation of serine 536 of RelA/p65 and its activation. Thus, we determined the effects of cAMP on thrombin-induced p38 MAP kinase phosphorylation, and found that thrombin activated p38 MAP kinase (Thr180/Tyr182) phosphorylation in a manner dependent on cAMP (Fig. 5B). Preincubation of cells with dbcAMP also prevented the thrombin-induced phosphorylation of p38 MAP kinase (data not shown). We next determined if the effect of cAMP on p38 MAP kinase phosphorylation could be ascribed to its inhibition of p38 MAP kinase activation. In an in vitro kinase assay using ATF-2 as a substrate, we observed that the p38 MAP kinase immunoprecipitated from thrombin-challenged cells resulted in increased phosphorylation of ATF-2 (Fig. 5C) indicative of activation of p38 MAP kinase by thrombin. Pretreatment of cells with dbcAMP prevented the thrombin-induced p38 MAP kinase activation (Fig. 5C). Taken together, these data indicate that cAMP inhibits thrombin response by interfering with the activation of p38 MAP kinase.
DISCUSSION

The present study demonstrates that p38 MAP kinase is a critical target mediating the cAMP-dependent inhibition of thrombin-induced NF-κB activation and ICAM-1 expression in endothelial cells. We showed that inhibition of p38 MAP kinase by cAMP prevented the thrombin-activated phosphorylation of RelA/p65, and thus interfered with transcriptional competency of this DNA-bound NF-κB subunit.

The inhibitory effect of cAMP was only evident when thrombin was used as the agonist to elicit ICAM-1 expression. cAMP had no significant effect on the TNFα-induced ICAM-1 expression. This latter finding agrees with studies showing that increased intracellular levels of cAMP failed to inhibit ICAM-1 expression in response to TNFα challenge of endothelial cells (24, 26). However, we showed clearly that increased cAMP level following incubation of cells with forskolin significantly reduced the thrombin-induced NF-κB-dependent reporter gene activity, indicating that cAMP prevented the activation of NF-κB, and thus ICAM-1 expression. The reasons for the differential effects of cAMP in thrombin- versus TNFα-induced ICAM-1 expression are not clear; but raise important question whether raising the cAMP concentration would be protective in inflammatory diseases associated with the generation of cytokines such as TNFα. While NF-κB is essential and sufficient for thrombin-induced ICAM-1 transcription (27), the TNFα-induced ICAM-1 expression in endothelial cells required the cooperation of multiple transcription factors including NF-κB and CCAAT/enhancer binding protein (C/EBP) (19). A likely explanation for the failure of cAMP to prevent the TNFα-induced ICAM-1 expression may be that increased intracellular cAMP concentration can independently result in activation of C/EBP (5, 13). Further support for this notion comes from studies (24, 26) in which elevated cAMP levels in endothelial cells inhibited the TNFα-induced expression of genes.
encoding E-selectin and VCAM-1, which are both NF-κB-dependent but do not require the cooperation of C/EBP (8) as is the case with ICAM-1. Studies have shown that transactivation of genes by NF-κB requires DNA binding secondary to phosphorylation and degradation of IκBα and translocation of RelA/p65 to the nucleus (3, 16, 41). We determined if the increase in cAMP level could regulate NF-κB activation by influencing these events. Preincubation of cells with forskolin failed to prevent the NF-κB DNA binding activity induced by thrombin. Also increased cAMP concentration did not inhibit the thrombin-induced IκBα degradation and translocation of RelA/p65 to the nucleus. Thus, cAMP appears to exert its inhibitory effect on ICAM-1 expression without interfering with activation of NF-κB in the cytosol and its translocation to the nucleus.

Another important mechanism regulating NF-κB activity involves the phosphorylation of the DNA-bound RelA/p65 NF-κB subunit (2, 23). Studies showed that phosphorylation of RelA/p65 at serine 536 increases the transcriptional competency of NF-κB bound to the promoter (20, 44). We recently showed that inhibition of p38 MAP kinase by pharmacological and genetic approaches significantly reduced the thrombin-induced ICAM-1 expression secondary to inhibition of transactivation activity of the nuclear NF-κB, but did not interfere with its cytosolic activation (29). Thus, we addressed the possibility that elevation in cAMP levels could inhibit thrombin-induced transcriptional activity of NF-κB by preventing the phosphorylation of RelA/p65 at serine 536. Further, we determined whether this effect of cAMP could be ascribed to inhibition of p38 MAP kinase activation. We observed, consistent with our hypothesis, that increase in intracellular cAMP concentration (elicited by pretreating cells with forskolin) prevented serine 536 phosphorylation of RelA/p65 in response to thrombin. We also
found, interestingly, that cAMP prevented the activation of p38 MAP kinase, and that this was in fact responsible for inhibiting the serine 536 phosphorylation of RelA/p65 induced by thrombin. In contrast, elevated cAMP levels failed to prevent the TNFα-induced RelA/p65 phosphorylation in endothelial cells (24). Recently, Duran et al., (12) have reported that the TNFα-induced RelA/p65 phosphorylation is mediated by PKC-ζ, suggesting that other kinases are also capable of transactivating the DNA-bound RelA/p65. As PKC-ζ is required for TNFα-induced NF-κB activation in endothelial cells (2, 28), it is possible that cAMP has no effect on PKC-ζ activation, which may thus explain why cAMP had no effect on TNFα-induced RelA/p65 phosphorylation. Thus, our findings demonstrate that the mechanism of action of cAMP in preventing thrombin-induced NF-κB activation involves inhibition of p38 MAP kinase activation, which in turn prevents phosphorylation of RelA/p65 bound to the ICAM-1 promoter.

In addition to the effect of p38 MAP kinase in inducing phosphorylation of RelA/p65A as discussed above, another possible mechanism by which p38 MAP kinase can mediate NF-κB activation is through phosphorylation of TATA binding protein (TBP), a subunit of transcription factor IID (TFIID). Phosphorylation of TBP by p38 MAP kinase is necessary for TBP binding to the TATA box (7). Inhibition of phosphorylation of TBP was shown to reduce its binding to the TATA box and interaction with the NF-κB p65 subunit (7). Thus, it is possible that inhibition of p38 MAP kinase by cAMP can abrogate ICAM-1 expression by preventing the phosphorylation of TBP. This idea has support from the findings of Delgado and Ganea (10) showing that vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) prevented the lipopolysaccharide-induced NF-κB activation through the cAMP-dependent inhibition of p38 MAP kinase phosphorylation of TBP.
Yet another explanation for the inhibitory effect of cAMP on NF-κB activation is the finding of Parry and Mackman (25) showing that cAMP promotes the phosphorylation of the CRE-binding protein (CREB), and subsequent binding to the CREB-binding protein (CBP), which in turn can block NF-κB activation. CBP is a co-activator known to associate with RelA/p65 (15, 25). The presence of CBP in the nucleus may lead to a decrease in RelA-CBP complex formation, and impaired NF-κB activation (25). As the phosphorylation status of RelA/p65 is critical for its association with CBP (12, 46), it may be that cAMP-dependent inhibition of p38 MAP kinase activation and of phosphorylation of RelA/p65 can impair transcriptional competency of the bound NF-κB.

Increase in cellular cAMP levels induced by pharmacologic agents, pentoxifylline, rolipram and amrinone, suppress expression of proinflammatory genes and are beneficial in inflammatory conditions such as autoimmune encephalomyelitis and acute cardiac allograft rejection (17, 18, 37). In this study, we have shown that elevation of endothelial cell cAMP levels inhibits NF-κB activation induced by thrombin and this has important functional consequences in preventing ICAM-1 expression and endothelial adhesivity towards PMNs. We have also shown that cAMP acts by targeting p38 MAP kinase activation and preventing the phosphorylation of the RelA/p65 subunit of NF-κB bound to the ICAM-1 promoter. Thus, the present results provide a basis for the mechanism of anti-inflammatory action of cAMP, which may have implications in novel therapeutics targeting p38 MAP kinase.
ACKNOWLEDGMENTS

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8. **Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, and Maniatis T.**


ICAM-1, intercellular adhesion molecule-1; PMN, polymorphonuclear leukocytes; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor α; HUVEC, human umbilical vein endothelial cells; FSK, Forskolin; dbcAMP, dibutyryl cyclic AMP; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; LUC, luciferase; NF-κB, nuclear factor-κB; bp, base pair(s); TBP, TATA-binding protein; PAR-1, protease-activated receptor-1, CREB, cyclic AMP response element binding protein; CBP, CREB-binding protein
FIGURE LEGENDS

Figure 1. Elevated cAMP concentration in endothelial cells inhibits thrombin-induced ICAM-1 mRNA expression. Confluent HUVEC monolayers were pretreated with (A&C) forskolin (FSK) or (B) dibutyryl cyclic AMP (dbcAMP) at the indicated concentrations. After 30 min, cells were challenged with (A, B&C) thrombin (2.5 U/ml) for 3 h or (C) TNFα (100 U/ml) for 2 h. Total RNA was isolated and analyzed by Northern hybridization with a human ICAM-1 cDNA, which hybridizes to a 3.3-kb transcript. Blots were stripped and re-probed to determine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression as a measure of RNA loading. Results are representative of 3 separate experiments.

Figure 2A. Elevated cAMP concentration inhibits thrombin-induced ICAM-1 expression on endothelial cell surface. Confluent HUVEC monolayers were pretreated with (a&c) FSK (20 µM) or (d&e) dbcAMP (0.5 mM) for 30 min prior to stimulation with thrombin (2.5 U/ml) for 12-15 h. ICAM-1 expression was quantitated by flow cytometry using mAb against ICAM-1 (BIRR0001) or mAb against IgG, as described in Materials and Methods. Results are representative of two separate experiments.

Figure 2B. Elevated cAMP concentration inhibits thrombin-induced endothelial adhesivity towards PMN. Confluent HUVEC monolayers were pretreated with FSK (20 µM) prior to challenge with thrombin (5 U/ml). Expression of endothelial adhesiveness was determined by PMN adhesion assays as described in Materials and Methods. Data are mean ± S.E. (n= 3 for each condition).

Figure 3. Elevated cAMP concentration inhibits thrombin-induced NF-κB activity. HUVEC were transfected with NF-κBLUC construct using DEAE-dextran method as described (Lopata et al., 1984). Cells were pretreated with FSK (20 µM) prior to stimulation for 8 h with
thrombin (5 U/ml). Cell extracts were prepared and assayed for Firefly and *Renilla* luciferase activities using Promega Biotech Dual luciferase Assay System (Promega, Madison, WI). The data are expressed as Firefly/*Renilla* luciferase activity. Data are mean ± S.E. (n= 3 for each condition).

**Figure 4. Elevated cAMP concentration fails to prevent thrombin-induced IκBα degradation, nuclear translocation, and NF-κB DNA binding activity.** Confluent HUVEC monolayers were pretreated for 30 min with FSK (20 μM) prior to challenge with thrombin (2.5 U/ml) for indicated time periods. Cytoplasmic (*A*) and nuclear (*B&C*) extracts were prepared and assayed for IκBα degradation (*A*) and NF-κB nuclear translocation (*B*) by Western blot analysis and NF-κB DNA binding activity (*C*) by EMSA as described in Materials and Methods. Results are representative of 2-3 separate experiments.

**Figure 5A. Elevated cAMP concentration and p38 MAP kinase inhibition prevent thrombin-induced serine 536 phosphorylation of RelA/p65.** Confluent HUVEC monolayers were pretreated with FSK (20 μM) or SB203580 (10 μM) for 30 min prior to challenge with thrombin (5 U/ml) for 1 h. Cell lysates were immunoprecipitated with an antibody against p65, immunoblotted with an antibody against the phosphorylated (Ser536) form of RelA/p65. The blots were subsequently stripped and re-probed with an antibody against RelA/p65. Results are representative of 3 separate experiments.

**Figure 5B&C. Elevated cAMP concentration inhibits thrombin-induced p38 MAP kinase activation. B.** Confluent HUVEC monolayers were pretreated with FSK (20 μM) for 30 min prior to challenge with thrombin (5 U/ml) for 5 min. Total cell lysates (10 μg/lane) were separated by SDS-PAGE and immunoblotted with an antibody against phosphorylated
(Thr180/tyr182) form of p38 MAP kinase. The blots were subsequently stripped and re-probed with an antibody against p38 MAP kinase. C. Confluent HUVEC monolayers were pretreated with dbcAMP (0.5 mM) for 30 min prior to challenge with thrombin (5 U/ml) for 5 min. Cell lysates were immunoprecipitated with an antibody against p38 MAP kinase, and \textit{in vitro} kinase assays were carried out on immunoprecipitates using ATF-2 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, transferred to nitrocellulose membrane and the phosphorylated form of ATF-2 was detected by autoradiography. The blots were subsequently stripped and re-probed with an antibody against p38 Map kinase. Results are representative of 3 separate experiments.
**Figure 1**

**A**

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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>- 0.5</th>
<th>- 0.1</th>
<th>0.5</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>dbcAMP (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin (2.5 U/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

**C**

<table>
<thead>
<tr>
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<th>+</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSK (20 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin (5 U/ml)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNFα (100 U/ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 2B

PMN Adhesion
(Number of PMN/0.88 mm² of EC)

FSK (20 µM)
- - + - +
Thrombin (5 U/ml)
- - + + +

Rahman et al.
Figure 3

NF-κB Activity (Firefly/Renilla Luciferase Activity)

Thrombin

-  +  -  +

FSK

NF-κBLUC
**Figure 4**

**A**

<table>
<thead>
<tr>
<th></th>
<th>IκBα</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSK</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thrombin (h)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>p65</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSK</td>
<td>-</td>
</tr>
<tr>
<td>Thrombin (h)</td>
<td>0.5 0.5 1 1</td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th></th>
<th>NF-κB</th>
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</thead>
<tbody>
<tr>
<td>FSK</td>
<td>-</td>
</tr>
<tr>
<td>Thrombin (h)</td>
<td>1</td>
</tr>
</tbody>
</table>

**Free probe**
Rahman et al.

Figure 5A

A

**Blot: anti-p65 S536-P**

- [Image]

**Blot: anti-p65**

- [Image]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>p65</th>
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</thead>
<tbody>
<tr>
<td>IP: anti-p65</td>
<td>+</td>
</tr>
<tr>
<td>FSK (20 µM)</td>
<td>-</td>
</tr>
<tr>
<td>SB203580 (10 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Thrombin (5 U/ml)</td>
<td>-</td>
</tr>
</tbody>
</table>

+ indicates presence, - indicates absence.
**Figure 5B & C**

### B

**Blot: anti-phospho-p38**

- FSK (20 µM)
  - - + - +
- Thrombin (5 U/ml)
  - - + +

**Blot: anti-p38**

- FSK (20 µM)
  - + - +
- Thrombin (5 U/ml)
  - - + +

### C

**ATF-2**

- dbcAMP (0.5 mM)
  - - +
- Thrombin (5 U/ml)
  - + +

**p38**