Critical Role of cAMP Response Element Binding Protein Expression in Hypoxia-elicited Induction of Epithelial Tumor Necrosis Factor-α

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Tissue hypoxia is intimately associated with a number of chronic inflammatory conditions of the intestine. In this study, we investigated the impact of hypoxia on the expression of a panel of inflammatory mediators by intestinal epithelia. Initial experiments revealed that epithelial (T84 cell) exposure to ambient hypoxia evoked a time-dependent induction of the proinflammatory markers tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and major histocompatibility complex (MHC) class II (37 ± 6.1%, 7 ± 8.8%, and 9 ± 0.9-fold increase over normoxia, respectively, each p < 0.01). Since the gene regulatory elements for each of these molecules contains an NF-κB binding domain, we investigated the influence of hypoxia on NF-κB activation. Cellular hypoxia induced a time-dependent increase in nuclear p65, suggesting a dominant role for NF-κB in hypoxia-elicited induction of proinflammatory gene products. Further work, however, revealed that hypoxia does not influence epithelial intercellular adhesion molecule 1 (ICAM-1) or MHC class I, the promoters of which also contain NF-κB binding domains, suggesting differential responses to hypoxia. Importantly, the genes for TNF-α, IL-8, and MHC class II but not ICAM-1 or MHC class I, contain cyclic AMP response element (CRE) consensus motifs. Thus, we examined the role of cAMP in the hypoxia-elicited phenotype. Hypoxia diminished CRE binding protein (CREB) expression. In parallel, T84 cell cAMP was diminished by hypoxia (83 ± 13.2% decrease, p < 0.001), and pharmacologic inhibition of protein kinase A induced TNF-α and protein release (9 ± 3.9-fold increase). Addback of cAMP resulted in reversal of hypoxia-elicited TNF-α release (80 ± 3.2% inhibition with 3 mM 8-bromo-cAMP). Furthermore, overexpression of CREB but not mutated CREB by retroviral-mediated gene transfer reversed hypoxia-elicited induction of TNF-α defining a causal relationship between hypoxia-elicited CREB reduction and TNF-α induction. Such data indicate a prominent role for CREB in the hypoxia-elicited epithelial phenotype and implicate intracellular cAMP as an important second messenger in differential induction of proinflammatory mediators.

The human intestine is lined with a single layer of protective epithelial cells that possess properties such as barrier and ion (and subsequent fluid) transport functions (1–3). Such epithelial functions are tightly controlled by an array of immune-derived factors within the intestinal microenvironment, and regulation of such conditions can vary greatly during active episodes of inflammation. A number of previous studies have revealed specificity with regard to regulation of epithelial end point function, including tight junction permeability, electrogenic chloride secretion, and neutrophil transmigration (4–8). This work utilizing intestinal epithelial cells in vitro has revealed that ligation of receptors for IFN-γ, interleukin-4, or interleukin-13 results in increased macromolecular permeability (4, 7–9). On the whole, other cytokine responses (i.e. regulation of Cl− secretion, fluid transport, MHC, and MHC-like molecule expression, polymorphonuclear leukocyte transmigration, etc.) appear to reveal a unique “functional fingerprint” with respect to individual cytokines. Others have demonstrated the presence of functional receptors for IL-2 (10, 11), transforming growth factor-β (12), and hepatocyte growth factor (13) on intestinal epithelial cells. Based on this previous work, we have proposed that epithelial cells have the unique ability to “phenotype-switch,” whereby epithelia lose classic qualities of epithelia (i.e. barrier function, ion transport properties, etc.) and assume features resembling immune-type cells (i.e. surface expression of MHC class I and II, antigen presentation properties, regulated polymorphonuclear leukocyte trafficking, etc.) (5).

Tissue ischemia/hypoxia often occurs concomitantly with other inflammatory processes, and thus, appropriate models to study mechanisms of inflammation should also account for conditions of cellular hypoxia. Surprisingly little is known about the mechanisms of hypoxic signal transduction. Several reports indicate that hypoxia down-regulates activity of cellular cAMP-generating machinery and that such diminished cAMP signaling can influence gene expression and end point cellular functions (14–18). At the transcriptional level, a primary target for cAMP-mediated signaling is cAMP response element binding proteins, a family of 43-kDa leucine zipper transcription factors that share certain structural motifs, bind DNA as dimers, and regulate transcription of target genes (19). At present, it is not known whether hypoxia-mediated gene

1 The abbreviations used are: IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; HBSS, Hanks’ balanced salt solution; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; EMSA, electrophoretic mobility shift assay; MHC, major histocompatibility complex; ICAM-1, intercellular adhesion molecule 1; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; PKI, protein kinase A inhibitor.

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expression is directly related to CREB activity or CREB expression.

Recently, we have studied the role of epithelia in intestinal ischemia using a model epithelium, T84 cells. In response to conditions of ambient hypoxia, epithelia express a more classical immune-like phenotype, including release of proinflammatory cytokines and chemokines (20, 21), regulated neutrophil transmigration (20), diminished ion transport (14), and induction of major histocompatibility complex class II expression (21). The basis for these changes in epithelial function are not well understood, and thus, in the studies defined here, we hypothesized a basic mechanism of hypoxia-induced phenotype switch. These studies revealed that hypoxia specifically induces a panel of epithelial proteins that bear cyclic AMP response elements (CRE) within the region important for regulation of gene expression. Further mechanistic insight was defined with one gene product, epithelial-derived TFN-α, and revealed a critical role for CRE and CRE-binding proteins (CREB) in induction of TFN-α. Such results define a primary role for CRE in the hypoxia-elicited epithelial phenotype and implicate CREB down-regulation as a mechanism of differential induction of proinflammatory mediators by cellular hypoxia.

**MATERIALS AND METHODS**

**Growth and Maintenance of T84 Intestinal Epithelial Cells**—The T84 cell line is a human colonic carcinoma cell line (22) which, when plated on permeable membrane supports, forms polarized monolayers of columnar intestine-like epithelial cells. T84 cells are functionally well differentiated with regard to electrogenic Cl\(^{-}\) secretion and ion transport and serve as excellent models of crypt columnar epithelial cells (22, 23). T84 cells were grown as monolayers in a 1:1 mixture of Dulbecco/Vogt modified Eagle's medium and Ham's F-12 medium, supplemented with 15 mm HepES buffer (pH 7.5), 14 mm NaHCO\(_3\), 40 mg/ml penicillin, 8 mg/ml ampicillin, 90 mg/ml streptomycin, and 5% newborn calf serum. Monolayers were subcultured from flasks every 7–14 days by brief trypsin treatment (0.1% trypsin and 0.9 mM EDTA in Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline).

**Epithelial Exposure to Hypoxia**—Epithelial exposure to hypoxia was performed as described previously (20). T84 cell growth media (1:1 Ham's F-12/Dulbecco/Vogt modified Eagle's medium and Ham's F-12 medium, supplemented with 15 mm HepES buffer (pH 7.5), 14 mm NaHCO\(_3\), 40 mg/ml penicillin, 8 mg/ml ampicillin, 90 mg/ml streptomycin, and 5% newborn calf serum) were analyzed by Western blot analysis for antibody binding following exposure to normoxia or hypoxia for the indicated periods. Cells were washed with ice-cold HBSS (Sigma), for antibody binding following exposure to normoxia or hypoxia for the indicated periods. Cells were washed with ice-cold HBSS, and separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. DNA-protein complexes were incubated with nuclear lysates for 10 min at 37 °C and separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. DNA-protein complexes were electrophoresed to nitrocellulose, and blocked overnight in 5% nonfat dry milk in TBS-T (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) prior to antibody binding. Blots were washed in TBS-T, and probed with anti-digoxigenin-peroxidase, and developed by ECL. Resulting bands were quantified from scanned images using NIH Image software (Bethesda).

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts of cells exposed to indicated experimental conditions were obtained as described above. The following synthetic oligonucleotide probes were synthesized (Genosys Biotechnologies, Inc.; The Woodlands, TX) and used as probes in EMASs: the CRE-like motif (bold) lies at −115/−93 relative to the transcription start site in the TFN-α promoter, 5′-GTTCAGACTCCAGATGACATCTAGGGTTGTC-3′. Oligonucleotide probes for EMSA were digoxigenin-labeled according to manufacturer's instructions (Gel Shift kit, Roche Molecular Biochemicals). Labeled oligonucleotides were incubated with nuclear lysates for 10 min at 37 °C and separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. DNA-protein complexes were electrophoresed to nitrocellulose, and blocked overnight in 5% nonfat dry milk in TBS-T (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) prior to antibody binding. Blots were washed in TBS-T, and probed with anti-digoxigenin-peroxidase, and developed by ECL. Resulting bands were quantified from scanned images using NIH Image software (Bethesda).

**Western Blotting**—Following experimental treatment of epithelial cells, whole cell extracts for examination of CREB and phospho-CREB (Ser133) were obtained as described previously (26). For Western blot analysis, cells were reconstituted in assay buffer, and cAMP was quantified using a daily standard curve, and concentrations were expressed as cAMP per μg of total protein.

**Pharmacological Interference with Cellular cAMP Signaling**—Epithelial cells grown on permeable supports were exposed basolaterally to Rp-cAMPs (0–50 μM Sigma), protein kinase A inhibitor amide (0–30 μM Calbiochem), or 8-bromo-cAMP (0–3 mM Sigma) for 48 h prior to harvesting of cells for CREB determination or basal/basolateral supernatants for TFN-α assay as described above.

**Analysis of Messenger RNA Levels by PCR**—Total RNA was purified according to the manufacturer's procedure from normoxic and hypoxic (6, 12 or 24 h) T84 cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). 10–50 μg of each RNA sample was treated with DNase 1 (GenHunter Corp., Nashville, TN), and protein contaminants were removed through phenol-chloroform extraction step. RNA samples were then removed through phenol-chloroform extraction step. RNA samples were then ethanol-precipitated and resuspended in diethyl pyrocarbonate-treated water. The reaction was set up according to Promega's Reverse Transcription System protocol (Promega Corp., Madison, WI). Briefly, 1 μg of RNA was added to the reaction mixture consisting of 4 μl of 25 mMol/liter MgCl\(_2\), 2 μl of 10× Reverse Transcriptase Buffer, 2 μl of 10 mMol/liter of each dNTP, 0.5 μl of RNase in ribonuclease
inhibitor (20 units total), 15 units of avian myeloblastosis virus reverse transcriptase, and 0.5 μg of oligo(dT)\textsubscript{12} primer in a total volume of 20 μl. The single-stranded cDNA was synthesized (MJ Research, Inc., Thermocycler Model PTC-200) using one cycle at 25 °C for 10 min, one cycle at 42 °C for 45 min, and one cycle at 95 °C for 5 min followed by a final cycle at 4 °C for 5 min. The Platinum\textsuperscript{TM} Taq DNA Polymerase High Fidelity PCR System from Life Technologies, Inc., was used in the amplification step. The PCR reaction for human TNF-α contained 1 μM each of the sense primer (5’-CGGGAGCTGGACGTGCGGAGGAG-3’) and the antisense primer (5’-CACCAAGCTGTATCTCTAGCTC-3’), 5 μl of Reverse Transcriptase reaction, 5 μl of 10× Platinum\textsuperscript{TM} Taq high fidelity PCR buffer, 2 μl of 50 mM MgSO\textsubscript{4}, 0.2 μM of dNTP, and 2.5 units of Platinum\textsuperscript{TM} Taq High Fidelity enzyme mix in a total volume of 50 μl. The amplification reaction included a 5-min denaturation at 94 °C and a 5-min annealing at 60 °C, followed by 30 cycles at 72 °C of 1.5 min, 94 °C for 45 s, and 60 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR reactions were then visualized on a 1% agarose gel containing 5 μg/ml ethidium bromide. A 355-base pair fragment corresponding to TNF-α was observed. In order to ensure that an equal amount of template was used in each amplification reaction, 5 μl of Reverse Transcriptase reaction was used as a template with 1 μM each of human β-actin sense primer (5’-TGCAGGGTGTCACCACACTGTCGCCATCTA-3’) and antisense primer (5’-CTAGAACGGTTGCGGATGAGGAGG-3’) in identical reactions; a 661-base pair amplified fragment with equal intensity was observed in all samples.

Generation of Retroviral CReB Overexpressing Cells—Retroviral-mediated gene transfer of T84 cells with CReB and mutant CReB (serine to alanine mutated at site 133, the PKA phosphorylation site) containing vectors was performed using a previously described technique (28). Briefly, CReB or mutant CReB cDNA (a kind gift from Dr. Marc Montminy, Harvard Medical School) was expressed under the control of the CMV-IE promoter, and the cDNA for the dominant selectable marker (neomycin resistance) was expressed under control of the viral long terminal repeat. 10\textsuperscript{6} epithelial cells were plated 24 h prior to infection. Cells were washed once, and then 3–4 ml of fresh, 0.45-μm filtered, viral supernatant supplemented with 4 μg/ml Polybrene were added to the adherent cells. After 8 h, 5 ml of fresh complete medium was added, and the cells were cultured for 48 h before drug selection.

Data Presentation—Cytokine, nucleotide, and cell-surface ELISA data were evaluated by analysis of variance and by Student’s t test with p < 0.05 considered significant. All values are given as mean ± S.E. for n experiments.

RESULTS

Hypoxia Selectively Induces Epithelial Proinflammatory Proteins—Recently, we demonstrated that hypoxia enhances epithelial responses to the cytokine IFN-γ, and such responses were subsequently attributed to induction of epithelial TNF-α release from the basolateral surface (21). These studies, however, did not reveal significant insight into mechanisms that evoke induction of TNF-α, and therefore, we sought to understand such findings at the mechanistic level. As an initial screen, a panel of epithelial proinflammatory markers were examined to determine whether such signaling by hypoxia was specific for TNF-α or is generalized to other molecules. As shown in Fig. 1, responses to hypoxia were specific. Indeed, epithelial exposure to hypoxia induced TNF-α release (maximal 37.0 ± 6.1-fold increase, p < 0.01), IL-8 release (maximal 7.0 ± 0.8-fold increase, p < 0.01), and induced surface expression of MHC class II (maximal 9.0 ± 0.9-fold increase, p < 0.01). Hypoxia did not, however, influence epithelial ICAM-1 expression (p = not significant compared with normoxia) or MHC class I (p = not significant compared with normoxia) indicating a degree of specificity for signaling by hypoxia.

Hypoxia Induces Epithelial NF-κB—In an attempt to gain insight into such specificity, we began by examining induction pathways of these pro-inflammatory genes. The regulatory regions of each of these genes contain a binding site for NF-κB, a transcription factor important in induction of a number of proinflammatory genes (29). As shown in Fig. 2, Western blot analysis of nuclear extracts derived from epithelia exposed to hypoxia (measured pO\textsubscript{2} 20 torr for 0–48 h) revealed cytoplasmic-to-nuclear localization of the p65 subunit NF-κB, a reliable readout of NF-κB activation (27). Indeed, periods of hypoxia as short as 6 h revealed a significant cytoplasmic-to-nuclear localization of the p65 subunit of NF-κB. Such responses to hypoxia were maximal by 24 h (densitometric measurement of 360.5 versus normoxic control value of 82.2 relative units) and were similar to our positive control PMA (497.9 relative units).

Hypoxia Induces the Expression of TNF-α Messenger RNA—We next concentrated on elucidating pathways of epithelial gene induction by hypoxia specifically using TNF-α to define these principles. TNF-α gene induction and protein release can be regulated in a number of ways including transcriptional and post-translational pathways. Thus we examined whether hypoxia induces transcription of the epithelial TNF-α gene. As shown in Fig. 3, reverse transcriptase-PCR analysis revealed a time-dependent induction of TNF-α. Such induction
was detectable at 12 h, maximal at 24 h, and exceeded that of our positive control (PMA, 10 ng/ml for 12 h). These data reveal that hypoxia activates transcriptional pathways.

**Role of cAMP Response Element in Induction of TNF-α**—In addition to a binding site for the transcription factor NF-κB, the TNF-α, IL-8, and MHC class II genes bear a cAMP responsive consensuses binding site, termed a cAMP response element (CRE) (26, 27). Importantly, the regulatory regions of these molecules not induced by hypoxia (MHC class I and ICAM-1, see Fig. 1) do not contain a CRE. These CRE DNA consensus motifs serve to regulate transcription of genes through protein kinase A and calcium-dependent pathways. In general, increases in intracellular cAMP are associated with decreased TNF-α release (30). Phosphorylation of nuclear proteins, termed cAMP response element binding proteins (CREB), positively or negatively regulate the activation of CRE-containing genes (e.g. cAMP can increase or decrease transcription) depending on the gene (31). Since a number of different consensus motifs serve as functional CREs, we examined the contribution of the CRE found within the TNF-α promoter. To do this, we developed an EMSA using oligonucleotides flanking the TNF-α CRE (32). As shown in Fig. 4, proteins from nuclear extracts of T84 cells bind to the CRE region of the TNF-α promoter. Furthermore, addition of antibody to the phosphorylated form of CREB demonstrate that the protein is indeed CREB. This so-called “supershift” reaction indicates that the TNF-α gene bears a CRE which binds CREB of epithelial origin.

**Hypoxia Down-regulates Epithelial CREB Expression**—We next determined whether hypoxia influenced cellular CREB levels. As shown in Fig. 5A, Western blot analysis revealed that conditions which induce epithelial TNF-α, IL-8, and MHC class II (hypoxia) resulted in attenuated levels of CREB (81.3, 90.7, and 91.1% decrease by densitometry at 24, 48 and 72 h hypoxia, respectively, compared with normoxic control). Addition of the PKA agonist forskolin (1 μM, 24 h) partially protected the abrogation of CREB expression by hypoxia, particularly at the 24-h time point (117% of normoxic controls). Similarly, as shown in Fig. 5B, total levels of nuclear phospho-CREB were also influenced by hypoxia (47.4, 24.0, and 0.0% decrease by densitometry at 24, 48 and 72 h hypoxia, respectively, compared with normoxic control), and addition of forskolin partially reversed this response.

**Role of Intracellular cAMP in Hypoxia-elicted Induction of TNF-α**—To further evaluate the role of CREB in induction of epithelial TNF-α, we determined the impact of elevating intracellular cAMP. We have recently demonstrated that conditions that liberate TNF-α production (i.e. cellular hypoxia) result in parallel diminutions in intracellular cAMP (14). Thus, we reasoned if TNF-α induction is negatively regulated by cAMP, then adback of intracellular cAMP (using the analog 8-bromo-cAMP) should diminish TNF-α release. The results shown in Fig. 6 demonstrate that decreased cAMP parallels increased epithelial TNF-α (Fig. 6A). Furthermore, the addition of 8-bromo-cAMP (0–3 mM) diminished TNF-α release in a concentration-dependent manner (Fig. 6B; analysis of variance, p < 0.001). Addition of cAMP analogs to control cells did not activate cytoplasmic-to-nuclear localization of p65 and did not influence NF-κB activation by either PMA or by cellular hypoxia (as determined by cytoplasmic-to-nuclear localization of p65, data not shown). Finally, addition of 8-bromo-cAMP to hypoxic cells (8 h) partially reversed the diminution in CREB levels (Fig. 6C). Interestingly, unlike our findings with forskolin (see Fig. 5), at time points beyond 8 h of hypoxia (e.g. Refs. 24, 48, and 72), 8-bromo-cAMP did not reverse CREB levels (data not shown), suggesting that the initial hypoxic period (i.e. within 8 h) determines the signaling event for induction of TNF-α.
**CRE and Epithelial TNF-α**

**FIG. 5. Hypoxia represses expression of CREB.** T84 cells were grown to confluence on tissue culture-treated plastic wells and exposed to 0–72 h hypoxia (±1 μM forskolin, FSK and forskol. with the balance of time in normoxia. Whole cell lysates were prepared and separated by SDS-PAGE before transfer to nitrocellulose membrane. CREB and phospho-CREB were detected with specific antibodies. Integrated densitometry was utilized to quantify differential protein display. Data shown are representative of 3 experiments. CTL, control (monolayers not exposed to forskolin).

Taken together, such data indicate the likelihood that the CREB-binding site is critical to induction of epithelial TNF-α.

**Protein Kinase A Inhibitors Induce TNF-α in Normoxic Epithelia—**To directly examine the role of PKA in induction of epithelial TNF-α release, a specific PKA inhibitor (PKI) was used to diminish cAMP signaling in normoxic cells. TNF-α release and CREB expression were used as readouts for this response. As shown in Fig. 7A, PKI induced a significant increase in epithelial TNF-α release (9.0 ± 3.9-fold increase over control) albeit to a lesser degree than epithelial exposure to hypoxia (see Fig. 1E). PKI also induced parallel decreases in total cellular CREB expression (Fig. 7B). Additionally, Rp-cAMPs, another PKA inhibitor, also induced TNF-α release (50 μM; 2.4 ± 0.6-fold increase over control). As a control for these experiments, we examined the influence of Rp-cAMPs on epithelial ICAM-1 expression. Rp-cAMPs failed to increase basal (0.6 ± 0.02-fold over basal; p = not significant compared with untreated controls) or IFN-γ-induced (0.82 ± 0.06-fold over basal; p = not significant compared with untreated controls) expression of ICAM-1. Such data indicate a direct role for PKA in induction of CREB-regulated genes and suggest that hypoxia-elicited diminutions in intracellular cAMP may explain induction of epithelial TNF-α.

**Overexpression of CREB Reverses the Hypoxia-elicited TNF-α Induction.—**The above described results define a prominent role for repression of CREB and phospho-CREB expression in induction of TNF-α by hypoxia. Thus, we reasoned that overexpression of CREB should, at least in part, diminish hypoxia-elicited induction of TNF-α. T84 cells overexpressing CREB or mutant CREB (Ser-133/Ala-133) were generated by retroviral gene transfer (demonstrated by Western blot, Fig. 8A). Compared with control epithelia (12.21 ± 2.26 pg of TNF-α/mg of protein), cells overexpressing CREB (5.0 ± 0.69 pg/mg protein; p < 0.02 compared with control cells), but not mutant CREB (10.38 ± 1.74 pg/mg protein; p = not significant compared with control cells), were significantly less responsive to hypoxia. These data provide direct evidence for CREB in hypoxia-elicited induction of TNF-α.

**DISCUSSION**

Significant evidence supports a role for ischemia and resultant tissue hypoxia in development and maintenance of both chronic and acute inflammatory processes. For this reason, a detailed understanding of the mechanistic responses to hypoxia is crucial for the development of therapeutic strategies. In the studies outlined in this investigation, we sought to define pathways involved in the induction of proinflammatory gene products by cellular hypoxia, with an emphasis on TNF-α. Given the critical role of epithelia in mucosal protection, a well defined intestinal epithelial model (T84 cells) was utilized to elucidate these pathways. Three primary observations are of note. First, these data indicate that cellular hypoxia results in transcriptional induction of genes that bear a CRE motif within the regions responsible for the regulation of gene expression. Second, down-regulation of protein kinase A activity, via cellular hypoxia, results in diminution of CREB expression, and overexpression of CREB in epithelial cells exposed to hypoxia protects the cells from transformation to the hypoxic phenotype. Third, these data reveal that maintenance of intracellular cAMP levels abrogates specific influences of epithelial hypoxia.

Recently, we undertook a series of studies aimed at identifying epithelia-derived factors that promote permeability through autocrine pathways. These studies revealed a soluble, transferable factor released from the epithelial surface in a polarized manner (similar to IL-8) (20) and bound to functional basolateral epithelial receptors (similar to IL-4, IL-13, and IFN-γ) (8). This factor was subsequently defined as soluble TNF-α (21). Herein, extensions of this work revealed induction of a number of epithelial proinflammatory markers (e.g. IL-8, MHC class II) and some degree of specificity in this regard (i.e. lack of induced MHC class I or ICAM-1). Examination of the promoter region of each of these molecules revealed a binding site for NF-κB, a transcription factor important in a number of inflammation-related pathways (29). Hypoxia induced NF-κB cytoplasmic-to-nuclear localization; however, it was likely that additional factors were necessary, since both ICAM-1 and MHC
class I contain NF-κB sites but are not induced by hypoxia. We
have not determined the mechanism of epithelial NF-κB acti-
ation by cellular hypoxia. However, we recently demonstrated
a role for hypoxia-associated metabolic acidosis in proteasome
activation and subsequent NF-κB translocation in endotoxin-
activated endothelial cells (27). Whether similar mechanisms
play a role here are not known at the present time.

Closer examination of hypoxia-elicited gene sequences (IL-8,
TNF-α, and MHC class II) revealed the presence of CRE bind-
ing motifs, and thus, we determined the specific role of CRE
and intracellular cAMP in induction of TNF-α. A number of
previous studies have suggested that proinflammatory gene
products are responsive to alterations in intracellular cAMP.
For instance, macrophage activation, and in particular TNF-α
gene expression, is specifically down-regulated by agents that
elevate cAMP (prostaglandin E2, dibutyryl cAMP, cholera
toxin, and 8-bromo-cAMP) (30, 33–36). Likewise, endothelial
E-selectin, a CRE-bearing gene (37), is inversely related to
intracellular cAMP (18). Consistent with previous reports, we

![Fig. 6. Exogenous cAMP reverses induction of epithelial
TNF-α.](image)

![Fig. 7. PKA inhibition induces TNF-α release and diminishes
CREB.](image)

![Fig. 8. Overexpression of CREB in T84 cells attenuates hy-
poxia-elicited TNF-α release.](image)
CRE and Epithelial TNF-α

CRE and Epithelial TNF-α demonstrate here that physiologic responses to cellular hypoxia are diminished intracellular cAMP and a parallel decrease in protein kinase A activity (14, 15). Although the mechanisms underlying decreased cAMP and PKA activity remain to be clarified, the association of hypoxia with induction of CRE-containing genes may serve as a basic mechanism in regulating proinflammatory gene expression under these conditions.

To further elucidate the role of CRE in induction of epithelial TNF-α, we defined the impact of elevating intracellular cAMP. Addback of intracellular cAMP (using the analog 8-bromo-cAMP) diminished hypoxia-induced TNF-α release, and downregulation of PKA activity (using PKI and Rp-cAMPS) induced TNF-α, albeit to a lesser extent than hypoxia. Importantly, epithelial exposure to 8-bromo-cAMP did not influence NF-κB activation (data not shown). As a final point, it is important to note that CRE can also be regulated indirectly or directly by elevations in intracellular Ca²⁺, as would be the case with PMA exposure (31). Taken together, such data indicate the likelihood that the CREB-binding site is critical to induction of epithelial TNF-α.

To define the causal relationship between hypoxia-elicited alterations in CREB expression and TNF-α release, we generated stable, T84 CREB-overexpressing cells. Exposure of such CREB-overexpressing cells to hypoxia resulted in significantly attenuate release of TNF-α, thus indicating a direct relationship between hypoxia-elicited CREB decreases and TNF-α release. These studies also reveal a relative importance for phosphorylation (38), little is known about substrate (tight coupling through a series of specific events (PKA activation). Although it is known that CRE-related gene induction is PKA (with forskolin) under hypoxic conditions partially salvaged the repression of CREB. Thus, the inhibition of transcription to the hypoxia-elicited phenotype by overexpression of CREB gives further evidence for a causal relationship between hypoxia-elicited decreases in CREB and the induction of TNF-α release by intestinal epithelial cells.

In summary, these results indicate that hypoxia-elicited diminution of CREB expression through diminished PKA activity may serve as a basic mechanism of activating negatively regulated, CRE-bearing genes in epithelia. Such findings have implications in the pathophysiology of a number of hypoxia-related disorders and provide a potential target for the development of therapeutic strategies.

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