Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7075–7080, July 1996
Cell Biology

Hypoxia enhances stimulus-dependent induction of E-selectin on aortic endothelial cells

(ischemia/inflammation/cytokine/adhesion molecule)

GREGOR ZÜND*†, DAVID P. NELSON‡§, ELLIS J. NEUFELD¶, ANDREA L. DZUS*, JOYCE BISCHOFF||, JOHN E. MAYER†, AND SEAN P. COLGAN*,**

*Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, Brigham and Women's Hospital, Boston, MA 02115; and Departments of †Cardiac Surgery, ‡Cardiology, §Anesthesia, ¶Hematology, and ||Surgery, Children's Hospital and Harvard Medical School, Boston, MA 02115

Communicated by Judah Folkman, Harvard Medical School, Boston, MA, April 1, 1996 (received for review September 13, 1995)

ABSTRACT **In many diseases, tissue hypoxia occurs in conjunction with other inflammatory processes. Since previous studies have demonstrated a role for leukocytes in ischemia/reperfusion injury, we hypothesized that endothelial hypoxia may "superinduce" expression of an important leukocyte adhesion molecule, E-selectin (ELAM-1, CD62E). Bovine aortic endothelial monolayers were exposed to hypoxia in the presence or absence of tumor-necrosis factor α (TNF-α) or lipopolysaccharide (LPS). Cell surface E-selectin was quantitated by whole cell ELISA or by immunoprecipitation using polyclonal anti-E-selectin sera. Endothelial mRNA levels were assessed using ribonuclease protection assays. Hypoxia alone did not induce endothelial E-selectin expression. However, enhanced induction of E-selectin was observed with the combination of hypoxia and TNF-α (270% increase over normoxia and TNF-α) or hypoxia and LPS (190% increase over normoxia and LPS). These studies revealed that a mechanism for such enhancement may be hypoxia-elicited decrements in endothelial intracellular levels of cAMP (<50% compared with normoxia). Addition of forskolin and isobutyl-methylxanthine during hypoxia resulted in reversal of cAMP decreases and a loss of enhanced E-selectin surface expression with the combination of TNF-α and hypoxia. We conclude that endothelial hypoxia may provide a novel signal for superinduction of E-selectin during states of inflammation.**

Tissue hypoxia frequently accompanies a variety of vascular diseases, including thrombosis, atherosclerosis, and ischemia/reperfusion injury. Endothelial cells that line blood vessels are anatomically positioned at the interface of the blood and tissue exchange, and thus, endothelial cells are especially influenced by hypoxemia. The impact of hypoxia on endothelial cells is complex and includes changes in metabolism, gene expression, and induction of specific cell surface proteins (1). Moreover, hypoxia often occurs in conjunction with, or as a direct result of, other inflammatory processes. Under such conditions, the endothelium is bathed in a myriad of soluble factors, including cytokines, bioactive lipids, and bacterial endotoxin (2).

Significant evidence clearly defines a central role for leukocytes in tissue injury during reperfusion, termed ischemia/reperfusion injury (3). Indeed, previous studies have shown that white blood cells, especially polymorphonuclear leukocytes (neutrophils), play a prominent role in the development of acute inflammatory conditions resulting from diminished blood flow (3). During conditions of inflammation, endothelial E-selectin (ELAM-1, CD62E), mediates transient adhesion between endothelia and leukocytes (4). While it is known that endothelial E-selectin is induced by a diverse array of physiological mediators, including cytokines and bacterial endotoxin (5, 6), little is known about the role of hypoxia in the regulation of endothelial adhesion molecules such as E-selectin or whether inflammatory mediators at sites of hypoxia might differentially regulate such adhesion molecules.

Thus, we hypothesized that endothelial exposure to hypoxic stress may also serve as a physiologic signal to regulate the expression of endothelial E-selectin. We report here that hypoxia provides an additional signal for superinduction of E-selectin expression. Such enhancement occurs at the cell surface, extends to total cellular E-selectin and to mRNA levels, and is mediated, at least in part, by hypoxia-dependent decreases in endothelial intracellular cAMP.

MATERIALS AND METHODS

Cell Culture. Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) as first passage cells and were used before passage 10 for experiments. Endothelial monolayers were established, maintained, and subcultured with DMEM (GIBCO/BRL) containing 10% heat-inactivated fetal calf serum, glucose, pyruvate, glutamine, penicillin, and streptomycin (7). Confluent BAEC monolayers exhibited typical cobblestone appearance and uptake of acetylated low density lipoprotein (Biomedical Technologies, Stoughton, MA; data not shown). Where indicated, endothelial monolayers were exposed to human recombinant tumor-necrosis factor α (TNF-α; R & D Systems), lipopolysaccharide (LPS; from *Escherichia coli*; Sigma), forskolin (Sigma), or 3-isobutyl-1-methyl-xanthine (IBMX; Sigma) at indicated concentrations and durations.

Confluent BAEC monolayers were exposed to hypoxia as follows. Growth medium was replaced with fresh medium equilibrated with hypoxic gas mixture, and cells were placed in the hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI). This hypoxia chamber consisted of an airtight glove box with the atmosphere continuously monitored by an oxygen analyzer interfaced with oxygen and nitrogen flow adapters. Oxygen concentrations were as indicated with the balance made up of nitrogen, carbon dioxide [constant pCO_2 35 torr (1 torr = 133 Pa)], and water vapor from the humidified chamber.

Bovine E-Selectin Polyclonal Antibody. A polyclonal antibody against bovine E-selectin was generated by immunizing rabbits with bovine E-selectin–Ig fusion protein produced as described (8). The antiserum specifically recognized bovine and human E-selectin expressed in the COS7 cells but did not bind to P- or L-selectins. The antiserum recognizes a ≈100 kDa N-glycosylated protein in bovine capillary endothelial

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNF-α, tumor-necrosis factor α; LPS, lipopolysaccharide; BAEC, bovine aortic endothelial cell(s); IBMX, 3-isobutyl-1-methyl-xanthine; ECL, enhanced chemiluminescence; Rp-cAMPS, Rp enantiomer of cAMP; IL, interleukin.

**To whom reprint requests should be addressed at: Department of Anesthesia, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. e-mail: colgan@zeus.bwh.harvard.edu.

cells and bovine aortic endothelial cells that is up-regulated by TNF-α or LPS activation (data not shown).

Cell Surface Immunoassay. E-selectin cell surface expression was quantitated using a cell surface ELISA, as described (9). BAEC were grown and assayed for antibody binding after endothelial exposure to normoxia or hypoxia in the presence or absence of additional inflammatory stimuli, as indicated. BAEC were lightly fixed with paraformaldehyde (1% wt/vol in PBS) to preserve cell surface protein. Cells were washed with Hanks' balanced salt solution (HBSS; Sigma) and blocked with medium. Rabbit anti-bovine E-selectin antibody (1:2000 final dilution) was added to fixed cells and allowed to incubate for 2 h at 4°C. After washing with HBSS, a peroxidase-conjugated sheep anti-rabbit antibody secondary was added. Secondary antibody (1:2000 final dilution) was diluted in BSA (1% in HBSS). After washing, plates were developed by addition of peroxidase substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); 1 mM final concentration; Sigma] and read on a microtiter plate spectrophotometer at 405 nm (Molecular Devices). Controls consisted of no primary antibody (medium only, background control) and addition of equivalent dilutions of preimmune E-selectin rabbit serum (specificity control). Data are presented as mean ± SEM specific OD 405 (background subtracted).

Immunoprecipitation of Biotinylated Endothelial Membranes. BAEC were grown to confluence on six-well plates, exposed to experimental conditions, and washed with HBSS, followed by labeling of extracellular cell surface proteins with biotin (Immuno Pure Sulfo-NHS-Biotin; 1 mM; Pierce) as described (10). Unbound biotin was quenched with NH_4Cl (50 mM) in HBSS. Labeled BAEC were lysed with lysing buffer (150 mM NaCl/25 mM Tris/1 mM $MgCl_2$/1% Triton X-100/1% Nonidet P-40/5 mM EDTA/5 μg/ml chymostatin/2 μg/ml aprotinin/1.25 mM phenylmethylsulfonyl fluoride; all from Sigma). Cell debris was removed by centrifugation (10,000 × g for 5 min). BAEC lysates were precleared with 50 μl of preequilibrated protein-G Sepharose (Pharmacia) for 2 h. Immunoprecipitation of E-selectin was performed by addition of anti-E-selectin polyclonal serum (10 μl) or preimmune serum (negative control) for 2 h followed by 50 μl of preequilibrated protein-G Sepharose overnight on an end-over-end rotator. Washed immunoprecipitates were boiled in nonreducing sample buffer (2.5% SDS/0.38 M Tris, pH 6.8/20% glycerol/0.1% bromophenol blue), separated by SDS/PAGE (10% linear gel) under nonreducing conditions, and transferred to nitrocellulose using standard protocols. Biotinylated proteins were labeled with streptavidin-peroxidase and visualized by enhanced chemiluminescence (ECL; Amersham). Resulting E-selectin bands were quantitated from scanned images using IMAGE software (National Institutes of Health, Bethesda).

Total E-Selectin Western Blotting. BAEC were grown to confluence on six-well plates, exposed to experimental conditions, washed, lysed, and debris was removed as described above. BAEC lysates were separated by nonreducing SDS/PAGE, transferred to nitrocellulose, and blocked overnight in blocking buffer (250 mM NaCl/0.02% Tween 20/5% goat serum/3% BSA). Primary antibody (1:500 rabbit 61B anti-E-selectin polyclonal E-selectin serum) was added for 3 h, blots were washed, and species-matched peroxidase-conjugated secondary antibody was added. Labeled bands from washed blots were detected by ECL. Resulting E-selectin bands were quantitated from scanned images using IMAGE software (National Institutes of Health).

RNase Protection Assay. Total RNA was extracted from the confluent BAEC by the guanidinium-thiocyanate method of Chomczynski and Sacchi (11) with RNAzol B (Cinna/Biotecx Laboratories, Friendswood, TX), resuspended in 0.5% SDS, and quantitated by spectrophotometry. Bovine E-selectin and γ-actin cDNA templates were designed to be of different lengths to allow multiplex RNase protection analysis from each BAEC sample. The uncut E-selectin template (including polylinker) was 317 bp with a 186-bp-long protected sequence, corresponding to nucleotides 324–510 of bovine E-selectin (2); the template was generated by PCR. The E-selectin PCR fragment was cloned into the plasmid pCRII using the TA Cloning kit (Invitrogen). The uncut γ-actin template consisted of 162 bp with a 124-bp protected sequence, from bp 372 (*Xho*I site) to bp 496 (*Xba*I site) of bovine γ-actin cDNA (12), generously provided by David Morris (University of Washington, Seattle). The fragment was cloned into pBluescript II KS (Stratagene).

E-selectin and γ-actin templates were linearized with *Xba* and *Xho*, respectively. Riboprobes were synthesized by runoff transcription with the appropriate RNA polymerase (SP6 for antisense E-selectin riboprobe and T7 for antisense γ-actin riboprobe; Boehringer Mannheim). Sense riboprobes from each template were also synthesized and hybridized with sample RNA as controls (data not shown). Equal amounts of total RNA (7.5 μg) from each sample condition were analyzed in triplicate. A sample of yeast tRNA (10 μg) was used as a negative control. Signals were quantified using IMAGEQUANT software after 24-h exposures on PhosphorImager screens (Molecular Dynamics). The signals were controlled for background by subtraction of the tRNA signal.

Modulation and Measurement of Intracellular cAMP Levels. Confluent BAEC on 24-well plates were exposed to indicated experimental conditions and washed. BAEC cAMP was elevated during incubation in hypoxia or normoxia using forskolin (Sigma; 10 μM final concentration) and IBMX (Sigma; 5 mM final concentration). After incubation, cells were cooled to 4°C, cAMP was extracted from washed monolayers with extraction buffer (66% EtOH/33% HBSS containing 5 mM IBMX), and lysates were cleared by spinning at 10,000 × g for 5 min. cAMP was quantitated using a displacement ELISA (ELISA Technologies, Lexington, KY) according to the manufacturer's instructions. Data were converted to concentrations using a daily standard curve, and concentrations were expressed as cAMP per cm^2 confluent BAEC.

Where indicated, similar conditions were used to elevate (forskolin and IBMX as above) or reduce (Rp-cAMPS obtained from Biomol, Plymouth Meeting, PA), followed by measurement of E-selectin surface expression using the immunoassay described above.

Data Presentation. ELISA data and cAMP data were compared by two-factor ANOVA or by Student's t test. Values are expressed as the mean ± SEM of n experiments.

RESULTS

Hypoxia enhances TNF-α/LPS-induced E-selectin expression. Consistent with previous reports (7, 13, 14), BAEC monolayers tolerated exposure to hypoxia well [pO_2 = 7 mmHg (1 mmHg = 133 Pa), up to 24 h]. No changes in morphology were observed, and no evidence of cell death was apparent (based on soluble lactate dehydrogenase measurements from supernatants and trypan blue exclusion; data not shown). We first examined the induction of BAEC E-selectin surface expression by LPS (Fig. 1 *A* and *C*) or TNF-α (Fig. 1 *B* and *D*) with or without hypoxia. Using an ELISA (described in *Materials and Methods*), LPS and TNF-α brought about a dose-dependent induction of E-selectin surface expression (for both, $P < 0.01$, one-way ANOVA). No significant baseline E-selectin (i.e., unstimulated) expression was detected on confluent BAEC. The E-selectin ELISA signal was specific since addition of equivalent dilutions of preimmune E-selectin serum yielded no significant signal ($P < 0.001$ compared with immune serum; Fig. 1 *A* and *B*).

We next determined the impact of hypoxia on E-selectin surface expression using the ELISA. As shown in Fig. 1 *C* and

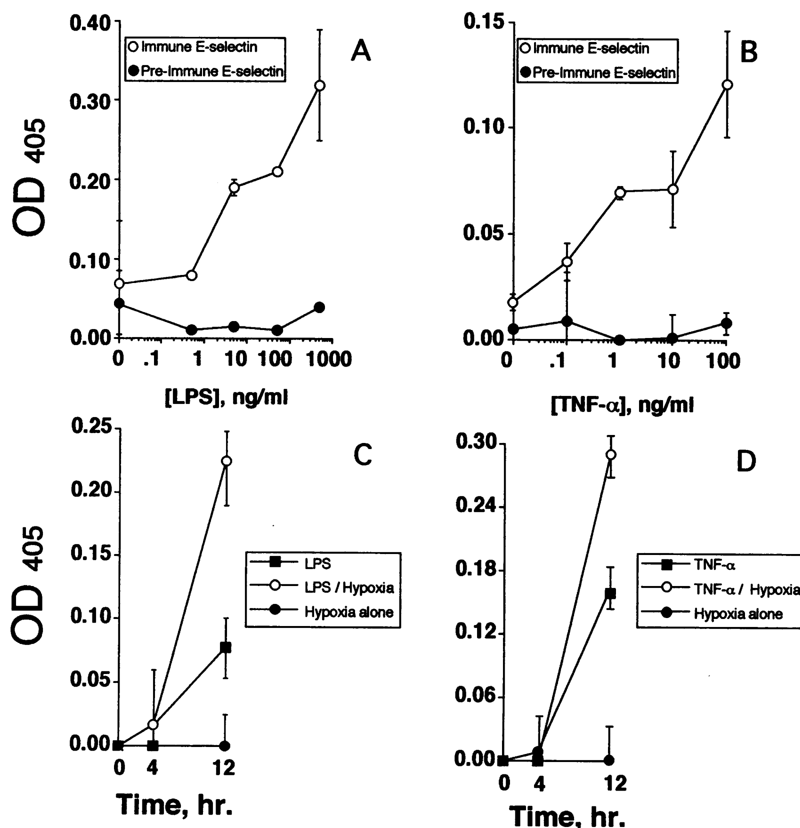

FIG. 1. Induction of BAEC E-selectin by LPS/TNF-α and hypoxia. (*A* and *B*) Confluent BAEC monolayers were exposed to LPS alone (*A*; range = 0–500 ng/ml, 12 h) or TNF-α alone (*B*; range = 0.1–100 ng/ml, 12 h) at 37°C. (*C* and *D*) Confluent BAEC monolayers were exposed to LPS (*C*; 100 ng/ml) or TNF-α (*D*; 100 ng/ml) in the presence (○) or absence (■) of hypoxia (pO2 7 mmHg) or hypoxia alone (●) for indicated periods of time. Monolayers were washed and lightly fixed (1% paraformaldehyde) followed by addition of immune serum (polyclonal E-selectin, ○) or control serum (preimmune E-selectin, ●) and assayed for BAEC expression of E-selectin by ELISA. Results are presented as the mean ± SEM OD_{405} (background subtracted) from 8–12 monolayers in each condition from at least three experiments.

D, hypoxia alone failed to induce E-selectin surface expression (P = not significant compared with normoxic controls). BAEC monolayers exposed to hypoxia in conjunction with TNF-α or LPS, however, resulted in a time-dependent, synergistic induction of endothelial E-selectin expression (85 ± 10% and 105 ± 8% over LPS or TNF-α alone, respectively; for both, $P < 0.001$ compared with either stimulus alone). These data indicate that hypoxia, in combination with other stimuli, provides an additional signal for induction of BAEC E-selectin surface expression.

Hypoxia-Elicited Increase in Surface E-Selectin. To confirm our ELISA results, we examined E-selectin surface protein using immunoprecipitation of E-selectin from surface biotinylated BAEC. As shown in Fig. 2, BAEC exposure to LPS (100 ng/ml, 12 h) under normoxic conditions revealed immunoprecipitation of a 100-kDa biotinylated protein, consistent with BAEC E-selectin. No discernible expression was apparent from control samples not exposed to LPS, consistent with our ELISA findings (Fig. 1). Confluent BAEC exposed to a combination of LPS and hypoxia followed by biotinylation and immunoprecipitation with an E-selectin-specific antibody revealed a 100-kDa band of increased density over normoxic BAEC exposed to LPS (Fig. 2). Immunoprecipitation of biotinylated BAEC exposed to hypoxia alone resulted in no distinct E-selectin band. Densitometric analysis of these bands (Fig. 2*B*) revealed a 270% increase in the hypoxia/LPS condition over LPS alone, confirming our ELISA results and indicating that the combination of hypoxia and inflammatory stimuli such as LPS act synergistically in induction of BAEC cell surface E-selectin.

Hypoxia-Elicited Synergism at Level of Total Cellular E-Selectin. We next used Western analysis to quantitate total cell-associated E-selectin. As depicted in Fig. 3*A*, no immunoprecipitable E-selectin was detected in normoxic or hypoxic cells in the absence of added stimuli. In the presence of LPS or TNF-α activation, a readily detectable 100-kDa band was observed. The combination of LPS or TNF-α and hypoxia resulted in the appearance of a band with increased density. Analysis of these bands by densitometry (Fig. 3*B*) revealed that hypoxia increased E-selectin by 280% and 190% for TNF-α- and LPS-activated BAEC, respectively. These data indicate that this hypoxia-elicited impact on E-selectin expression extends to total cell-associated protein.

Impact of Hypoxia on BAEC E-Selectin mRNA. To examine whether the hypoxia-elicited synergism was present at the mRNA level, we developed an RNase protection assay using a 186-bp segment of the bovine E-selectin gene. As shown in Fig. 4*A*, a time course of TNF-α activation revealed the appearance of quantifiable mRNA bands by 6 h of activation. Hypoxia alone did not induce BAEC E-selectin mRNA at any time point examined. The combination of hypoxia and TNF-α brought about increased levels of mRNA with similar kinetics as normoxic cells. Quantitation of E-selectin bands (Fig. 4*B*) revealed that hypoxia elicited a 190% increase over cytokine alone in E-selectin mRNA at both the 6-h and the 12-h time points. No synergism was evident at earlier time points. These data demonstrate that the observed synergism of hypoxia and inflammatory stimuli is at least partially regulated at the transcriptional level.

Mechanism of Hypoxia-Elicited E-Selectin Induction. Others have reported that hypoxia results in decreased generation of intracellular cAMP (15). In addition, endothelial cAMP levels are reported to modulate endothelial E-selectin surface expression (16). Thus, we examined whether hypoxia-elicited decreases in cAMP could account for our observed synergism with TNF-α. As shown in Fig. 5*A*, hypoxia elicited a >50% decrease in baseline cAMP compared with normoxic controls ($P < 0.025$). Incubation of hypoxic BAEC monolayers with forskolin and IBMX maintained cAMP levels to within the normoxic range (P = NS compared with baseline normoxic controls). TNF-α alone or in combination with hypoxia or forskolin/IBMX did not influence intracellular cAMP levels (P = NS). As shown in Fig. 5*B*, cell surface expression of E-selectin was inversely related to intracellular cAMP levels.

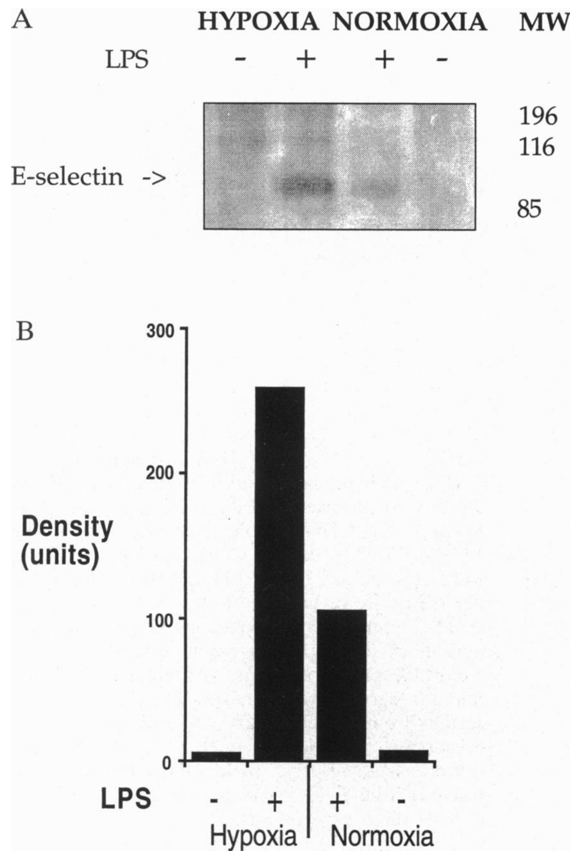

FIG. 2. Immunoprecipitation of cell surface biotinylated BAEC E-selectin induced by LPS in normoxia and hypoxia. Confluent BAEC monolayers were exposed to media or media containing LPS (500 ng/ml) under conditions of normoxia (pO_2 147 mmHg) or hypoxia (pO_2 7 mmHg) for 12 h. Cells were cooled to 4°C, and total cell surface proteins were biotinylated followed by immunoprecipitation of E-selectin. Blots were probed with streptavidin-peroxidase and developed by ECL. (*A*) The resulting blots from immunoprecipitation of normoxic and hypoxic BAEC with and without addition of LPS. (*B*) Densitometry tracings from immunoprecipitated bands shown in *A*. One of four experiments is represented.

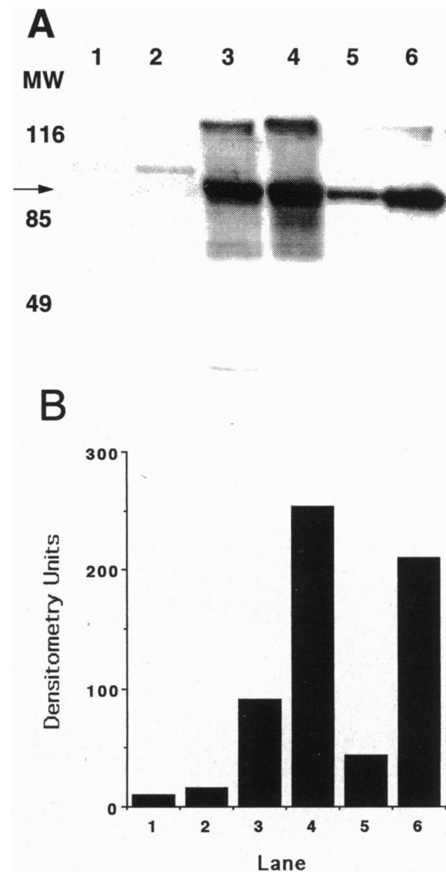

FIG. 3. Immunoblot of total cellular BAEC E-selectin induced by LPS or TNF-α in normoxia and hypoxia. Confluent BAEC monolayers were exposed to media or media containing LPS (500 ng/ml) or TNF-α (100 ng/ml) under conditions of normoxia (pO_2 147 mmHg) or hypoxia (pO_2 7 mmHg) for 12 h. Cells were cooled to 4°C, lysed, and scraped. Lysates were separated by SDS/PAGE under nonreducing conditions, and blots were probed with polyclonal bovine E-selectin serum, labeled with peroxidase-conjugated secondary antibody, and developed by ECL. (*A*) Resulting blots of control normoxic (lane 1), control hypoxic (lane 2), normoxia with LPS (lane 3), hypoxia with LPS (lane 4), normoxia with TNF-α (lane 5), and hypoxia with TNF-α (lane 6). (*B*) Densitometry tracings from resultant bands shown in *A*. One of three experiments is represented.

Indeed, decreased cAMP in hypoxic monolayers correlated with increased TNF-α-induced E-selectin. Moreover, BAEC exposure to forskolin/IBMX resulted in attenuated TNF-α-elicited expression of E-selectin in both normoxic and hypoxic monolayers and in an observable loss of synergism (P = NS between normoxic and hypoxic monolayers in the presence of TNF-α and forskolin/IBMX). Finally, experiments were performed to determine the influence of intracellular cAMP antagonists on E-selectin surface expression. As shown in Fig. 6, the addition of a combination of LPS or TNF-α with Rp-cAMPS, a potent inhibitor of kinases that elevate cAMP (17, 18), resulted in a dose-dependent enhancement of E-selectin surface expression. No significant increases were observed in the absence of LPS or TNF-α (Fig. 6), indicating that Rp-cAMPS alone does not enhance E-selectin. These results indicate that decreased intracellular cAMP during hypoxia is, at least in part, responsible for synergism between TNF-α and hypoxia in E-selectin surface expression. Moreover, we can partially recapitulate such observations using pharmacologic antagonists to cAMP.

DISCUSSION

During many disease states, tissue hypoxia occurs in conjunction with other inflammatory events. Reperfusion injury is associated with leukocyte accumulation; thus, in this study we examined regulation of endothelial E-selectin expression under conditions of hypoxia. These studies provide a number of important insights. First, our findings indicate that hypoxia alone fails to induce E-selectin at any level. At several different levels (cell surface protein, total cellular protein, and message), however, hypoxia synergizes with other inflammatory mediators to enhance induction of E-selectin. We provide evidence that implicates intracellular cAMP as a mechanism of such hypoxia-elicited enhancement.

Vascular endothelial cells exist at the crucial interface between tissue and blood, and because of this critical location, endothelia serve as a buffering monolayer during episodes of decreased oxygen tension. Thus, the ability of these cells to tolerate and respond to hypoxia is critical to their survival. Significant evidence clearly defines a central role for leukocytes in ischemia/reperfusion injury (3). Indeed, end organ damage of reperfused tissue exceeds that of ischemia alone, and blockade of leukocyte adhesion molecules appears to afford some protection (19–22). At present, the mechanisms that contribute to leukocyte accumulation under such conditions are poorly understood. We were unable to demonstrate an hypoxia-elicited induction of E-selectin in the absence of additional stimuli. These data confirm previous studies, indi-

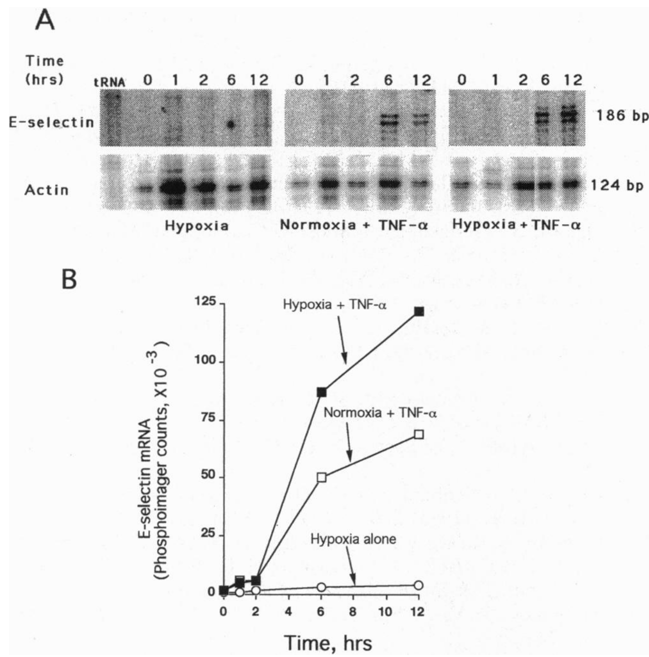

FIG. 4. Time course of BAEC E-selectin mRNA induced by TNF-α in normoxia and hypoxia. (*A*) RNase protection assay of E-selectin mRNA; conditions are as specified. The internal control for each sample was bovine γ-actin. BAEC were exposed to either hypoxia or normoxia in the presence or absence of TNF-α. Controls consisting of normoxia alone were not different than hypoxia alone. (*B*) Phosphorimager quantitation of the 186-bp E-selectin bands from RNase protection assays. Each point is the average of triplicate determinations.

cating that short-term endothelial exposure to hypoxia alone either failed to elicit increased levels of E-selectin (23–25) or increased expression only slightly. Such conditions, however, have been shown to increase E-selectin-dependent polymorphonuclear adhesion with reoxygenation (24–26).

In many disease states, hypoxia occurs in conjunction with, or as a result of, other inflammatory processes. In such settings, the endothelial cell surface is exposed to a myriad of soluble mediators (i.e., cytokines, bioactive lipids, and endotoxin) at concentrations that can influence cellular function (2). Thus, a relevant *in vitro* model of tissue hypoxia might include the combination of hypoxia and additional defined mediators. Under such conditions, we find that hypoxia promotes effects of TNF-α and endotoxin for induction of endothelial E-selectin.

The lack of induction of E-selectin by hypoxia alone provides important insight into mechanisms by which hypoxia might synergize with other inflammatory mediators. E-selectin is readily induced by a number of diverse stimuli, including cytokines and endotoxin (5, 6). With regard to hypoxia as a relevant activator, previous studies have demonstrated that hypoxia/reoxygenation stimulates endothelial cells to secrete proinflammatory cytokines such as interleukin 1α (IL-1α; refs. 23 and 24), IL-6 (27), and IL-8 (28). Such endogenous release of cytokine (especially IL-1α) and concomitant induction of E-selectin through autocrine mechanisms (24) are, however, an unlikely explanation for our observation, since hypoxia alone resulted in no detectable E-selectin induction, either at the protein or message level (Figs. 1–4). The E-selectin gene is under the control of a number of transcription factors, including AP-1, GATA-binding proteins, and NFκB (29, 30). It is unlikely, however, that hypoxia directly regulates the function of these transcription factors, since no detectable mRNA signal was demonstrated in the absence of additional mediators. We cannot, however, rule out possible mechanisms

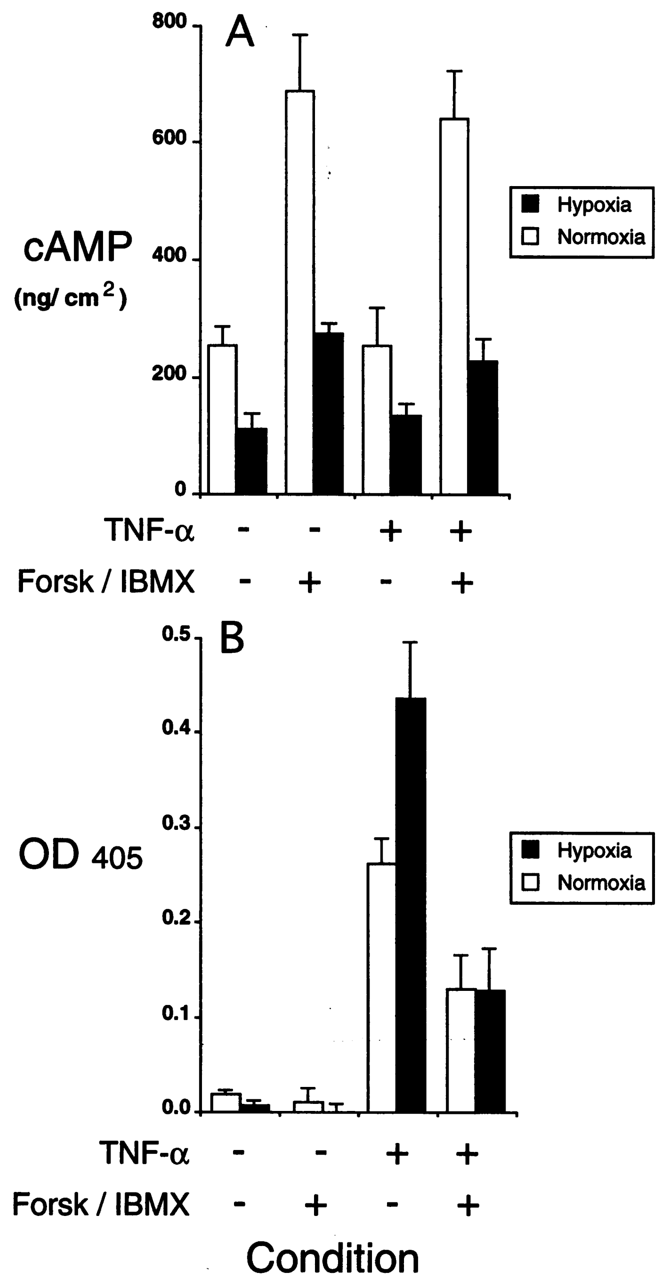

FIG. 5. Stimulation of BAEC cAMP during hypoxia results in a loss of synergism toward TNF-α. BAEC monolayers were grown to confluence on 24-well plates (*A*) or on 96-well plates (*B*) and exposed to media or TNF-α (100 ng/ml) in the presence or absence of forskolin (10 μM final concentration) and IBMX (5 mM final concentration). Monolayers were exposed to normoxia (pO_2 147 mmHg) or hypoxia (pO_2 7 mmHg) for 12 h. (*A*) BAEC were lysed in the presence of IBMX, and cAMP was determined from cell lysates. (*B*) BAEC monolayers were analyzed for specific E-selectin surface expression by ELISA. Results are presented as the mean ± SEM from 8–12 monolayers in each condition from three experiments.

by which hypoxia may decrease rates of protein turnover. While RNase protection assays revealed that hypoxia-elicited synergism with TNF-α is readily detectable at the level of mRNA, we have not determined whether this is due to increased transcription or increased mRNA stability.

These studies provide evidence that enhanced E-selectin may be mediated by hypoxia-elicited decreases in intracellular cAMP levels. Recently, it was shown that agents that elevate intracellular cAMP levels inhibit TNF-α-induced E-selectin expression (16). This effect maps to the cAMP-responsive element/activating

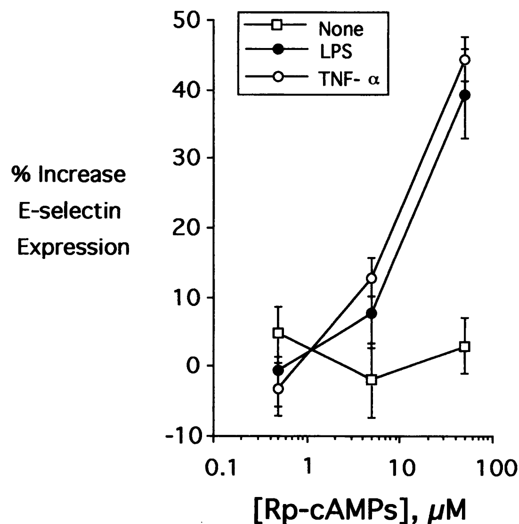

FIG. 6. Influence of cAMP antagonist Rp-cAMPS on TNF-α- or LPS-stimulated E-selectin surface expression. BAEC monolayers were grown to confluence on 96-well plates, exposed to media (□), LPS (100 ng/ml, ●), or TNF-α (100 ng/ml, ○) in the absence or presence of indicated concentrations (in μM) of the cAMP antagonist Rp-cAMPs for 12 h in normoxia (pO_2 147 mmHg). BAEC monolayers were analyzed for specific E-selectin surface expression by ELISA. Results are presented as the mean ± SEM percent increase in E-selectin expression from 8–10 monolayers in each condition from three experiments.

transcription factor within the E-selectin gene (31), and recently it was shown that this cAMP-responsive element controls the transcription rate of many genes (23). cAMP-responsive element activation increases or decreases transcription in a gene- and cell-specific manner, and thus, our results extend such findings to indicate that the hypoxia-elicited decrease in cAMP may serve as a physiologically relevant signal for stimulus-dependent E-selectin expression. Increased cAMP resulted in a loss of such synergism, suggesting a role for cAMP in this response. Some precedence exists to support our findings. For instance, others have shown that hypoxia-elicited decreases in cAMP may promote endothelial barrier function defects (15). The specific mechanism(s) for reduced adenylate cyclase activity in hypoxic endothelia remains to be elucidated, although a role for G proteins has been suggested (15). Thus, it appears that cAMP signaling may serve as a important intracellular messenger for the regulation of variety of cellular functions important in the inflammatory response.

In conclusion, our findings indicate that hypoxia may serve as a relevant pathophysiological stimulus and, thus, may provide an additional mechanism for regulation of E-selectin. At present, it is not known how such mechanisms might influence other endothelial adhesion molecules. Further investigation, both *in vivo* and *in vitro*, will shed on light on the regulatory role of hypoxia during disease states.

We thank Drs. Greg Stahl and Andrew Neish for helpful discussions. This work was supported by National Institutes of Health Grants DK50189 (to S.P.C.), GM46757 (to J.B.), and DK01977 (to E.J.N.). Dr. Zünd was supported by a grant from the Swiss National Foundation.

1. Stevens, T. & Rodman, D. M. (1995) *Endothelium* **3,** 1–11.
2. Pober, J. S. & Cotran, R. S. (1990) *Transplantation* **50,** 537–541.
3. Welbourne, C. R. B., Goldman, G., Valeri, C. R., Shepro, D. & Hechtman, H. B. (1991) *Br. J. Surg.* **78,** 651–655.
4. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A. & Seed, B. (1989) *Science* **243,** 1160–1165.
5. Pober, J. S., Gimbrone, M. A., LaPierre, L. A., Mendrick, D. L., Fiers, W., Rothlein, R. & Springer, T. A. (1986) *J. Immunol.* **137,** 1893–1896.
6. Pober, J. S., Bevilacqua, M. P., Mendrick, D. L., LaPierre, L. A., Fiers, W. & Gimbrone, M. A. (1986) *J. Immunol.* **136,** 1680–1687.
7. Ogawa, S., Gerlach, H., Esposito, C., Pasagian-Macaulay, A., Brett, J. & Stern, D. (1990) *J. Clin. Invest.* **85,** 1090–1098.
8. Aruffo, A., Kolanus, W., Walz, G., Fredman, P. & Seed, B. (1991) *Cell* **67,** 35–44.
9. Bevilacqua, M. P., Pober, J. S., Mendrick, D. L., Cotran, R. S. & Gimbrone, M. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9238–9242.
10. LeBivic, A., Francisco, X. R. & Rodriguez-Boulan, E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9313–9317.
11. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
12. Degen, J. L., Neubauer, M. G., Degen, S. J. F., Seyfried, C. E. & Morris, D. R. (1983) *J. Biol. Chem.* **258,** 12153–12162.
13. Shreeniwas, R., Ogawa, S., Cozzolino, F., Torcia, G., Braunstein, N., Butura, C., Brett, J., Leiberman, H. B., Furie, M. B., Joseph-Silverstein, J. & Stern, D. (1991) *J. Cell. Physiol.* **146,** 8–17.
14. Tretyakov, A. V. & Farber, H. W. (1995) *J. Clin. Invest.* **95,** 738–744.
15. Ogawa, S., Koga, S., Kuwabara, K., Brett, J., Morrow, B., Morris, S. A., Bilezikian, J. P., Silverstein, S. C. & Stern, D. (1992) *Am. J. Physiol.* **262,** C546–C554.
16. Pober, J. S., Slowik, M. R., DeLuca, L. G. & Ritchie, A. J. (1993) *J. Immunol.* **150,** 5114–5123.
17. Botelho, L. H. P., Rothermel, J. D., Coombs, R. V. & Jastorff, B. (1988) *Methods Enzymol.* **159,** 159–172.
18. Schaap, P., van Ments-Cohen, M., Soede, R. D., Brandt, R., Firtel, R. A., Dostmann, W., Genieser, H. G., Jastorff, B. & van Haastert, P. J. (1993) *J. Biol. Chem.* **268,** 6323–6331.
19. Welbourne, C. R. B., Goldman, G., Kobzik, L., Paterson, I., Valeri, C. R., Shepro, D. & Hechtman, H. B. (1990) *J. Immunol.* **145,** 1906–1911.
20. Horgan, M. J., Wright, S. D. & Malik, A. B. (1990) *Am. J. Physiol.* **259,** L315–L319.
21. Weyrich, A. S., Ma, X., Lefer, D. J., Albetine, K. H. & Lefer, A. M. (1993) *J. Clin. Invest.* **91,** 2620–2629.
22. Kukielka, G. L., Youker, K. A., Hawkins, H. K., Perrard, J. L., Michael, L. H., Ballantyne, C. M., Smith, C. W. & Entman, M. L. (1994) *Ann. N.Y. Acad. Sci.* **723,** 258–270.
23. Clark, E. T., Desai, T. R., Hynes, K. L. & Gewertz, B. L. (1995) *J. Surg. Res.* **58,** 675–681.
24. Shreeniwas, R., Koga, S., Karakurum, M., Pinsky, D., Kaiser, E., Brett, J., Wolitzky, B. A., Norton, C., Plocinski, J., Benjamin, W., Burns, D. K., Goldstein, A. & Stern, D. (1992) *J. Clin. Invest.* **90,** 2333–2339.
25. Yoshida, N., Granger, D. N., Anderson, D. C., Rothlein, R., Lane, C. & Kvietys, P. R. (1992) *Am. J. Physiol.* **265,** H699–H703.
26. Palluy, O., Morliere, L., Gris, J. C., Bonne, C. & Modat, G. (1992) *Free Radical Biol. Med.* **13,** 21–30.
27. Yan, S. F., Tritto, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L. & Stern, D. (1995) *J. Biol. Chem.* **270,** 11463–11471.
28. Karakurum, M., Shreeniwas, R., Chen, J., Pinsky, D., Yan, S.-D., Anderson, M., Sunouchi, K., Major, J., Hamilton, T., Kuwabara, K., Rot, A., Nowygrod, R. & Stern, D. (1994) *J. Clin. Invest.* **93,** 1564–1570.
29. Collins, T. (1993) *Lab. Invest.* **68,** 499–508.
30. Collins, T., Palmer, H. J., Whitley, M. Z., Neish, A. S. & Williams, A. J. (1993) *Trends Cardiovasc. Med.* **3,** 92–97.
31. DeLuca, L. G., Johnson, D. R., Whitley, M. Z., Collins, T. & Pober, J. S. (1994) *J. Biol. Chem.* **269,** 19193–19196.