Coordinated Adenine Nucleotide Phosphohydrolysis and Nucleoside Signaling in Posthypoxic Endothelium: Role of Ectonucleotidases and Adenosine A\textsubscript{2B} Receptors

Holger K. Eltzschig,\textsuperscript{1,7} Juan C. Ibla,\textsuperscript{2} Glenn T. Furuta,\textsuperscript{3} Martin O. Leonard,\textsuperscript{4} Kenneth A. Jacobson,\textsuperscript{5} Keiichi Enjyoji,\textsuperscript{6} Simon C. Robson,\textsuperscript{6} and Sean P. Colgan\textsuperscript{1}

\textsuperscript{1}Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women’s Hospital, \textsuperscript{2}Department of Anesthesiology, Perioperative and Pain Medicine, and \textsuperscript{3}Combined Program for Pediatric Gastroenterology and Nutrition, Children’s Hospital, Harvard Medical School, Boston, MA 02115
\textsuperscript{4}The Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland
\textsuperscript{5}Molecular Recognition Section, National Institutes of Health, Bethesda, MD 20892
\textsuperscript{6}Transplantation Center, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115
\textsuperscript{7}Department of Anesthesiology and Intensive Care Medicine, Eberhard-Karls-University, D-72076 Tübingen, Germany

Abstract

Limited oxygen delivery to tissues (hypoxia) is common in a variety of disease states. A number of parallels exist between hypoxia and acute inflammation, including the observation that both influence vascular permeability. As such, we compared the functional influence of activated polymorphonuclear leukocytes (PMN) on normoxic and posthypoxic endothelial cells. Initial studies indicated that activated PMN preferentially promote endothelial barrier function in posthypoxic endothelial cells (\textgreek{>}60\% increase over normoxia). Extension of these findings identified at least one soluble mediator as extracellular adenosine triphosphate (ATP). Subsequent studies revealed that ATP is coordinately hydrolyzed to adenosine at the endothelial cell surface by hypoxia-induced CD39 and CD73 (\textgreek{>}20- and \textgreek{>}12-fold increase in mRNA, respectively). Studies in vitro and in \textit{cd39}\textsuperscript{-null} mice identified these surface ecto-enzymes as critical control points for posthypoxia-associated protection of vascular permeability. Furthermore, insight gained through microarray analysis revealed that the adenosine A\textsubscript{2B} receptor (AdoRA\textsubscript{2B}) is selectively up-regulated by hypoxia (\textgreek{>}5-fold increase in mRNA), and that AdoRA\textsubscript{2B} antagonists effectively neutralize ATP-mediated changes in posthypoxic endothelial permeability. Taken together, these results demonstrate transcription coordination of adenine nucleotide and nucleoside signaling at the vascular interface during hypoxia.

Key words: adenosine • ectonucleotidase • endothelium • neutrophil • inflammation

Introduction

The study of physiologic adaptation and pathophysiologic response to hypoxia is presently an area of intense investigation. Recent reports suggest that both transcriptional and nontranscriptional hypoxia pathways may contribute to a broad range of diseases, and that a number of parallels exist between tissue responses to hypoxia and to acute inflammation (1). For example, during episodes of hypoxia, PMN are mobilized from the intravascular space to the interstitium, and such responses may contribute significantly to tissue damage during consequent reperfusion injury (2–4). Moreover, myeloid cell migration to sites of inflammation are highly dependent on hypoxia-adaptive pathways (5). As...
such, emigration of PMN through the endothelial barrier may lead to a disruption of such tissue barriers (6–8) and such a setting creates the potential for extravascular fluid leakage and subsequent edema formation (9, 10). However, with some exceptions, most episodes of hypoxia and/or ischemia–reperfusion are self-limiting, suggesting that endogenous protective mechanisms may exist to fortify the vascular barrier during such insults.

Previous studies have indicated that activated PMN release a number of soluble mediators, which regulate vascular permeability during transmigration. For example, PMN have been demonstrated to actively release both glutamate (3) and adenine nucleotides (in the form of AMP, which through a one-step metabolic conversion liberates adenosine at the vascular surface; reference 11). Glutamate and adenosine liberated by this process have been shown to promote microvascular endothelial barrier, and as such, may provide innate pathways to reestablish endothelial cell-cell contact following PMN transmigration (11–13).

In the present studies, we sought to determine whether hypoxia might differentially influence vascular permeability in response to activated PMN. Somewhat surprisingly, initial results revealed that posthypoxic endothelia demonstrated increased protective responses to activated PMN. Studies aimed at isolating potentially novel factors in this pathway revealed that PMN-derived ATP was, at least in part, responsible for the increase observed in the posthypoxic endothelium. Indeed, enhanced extracellular metabolism and signaling after hypoxia were explained by transcriptional increases in functional surface apyrase (CD39), nucleotidase (CD73), and adenosine A2B-receptor (AdoRA2B), respectively. Such studies identify a coordinated, barrier-protective response initiated by endothelial preexposure to hypoxia.

Materials and Methods

Endothelial Cell Isolation and Culture. Human microvascular endothelial cells (HMEC-1) were a gift of Francisco Candal, Centers for Disease Control, Atlanta, GA (14) and were harvested and cultured by a modification of methods previously described (3, 11). In brief, HMEC-1 were harvested with 0.1% trypsin and cultured by a modification of methods described previously (3, 14) and were harvested and endothelial cells (HMEC-1) were a gift of Francisco Candal, Centers for Disease Control, Atlanta, GA (14) and were harvested and cultured by a modification of methods previously described (3, 11).

Isolation of Human Neutrophils. PMN were freshly isolated from whole blood obtained by venipuncture from human volunteers and anticoagulated with acid citrate/dextrose (2). Platelets, plasma, and mononuclear cells were removed by aspiration from theuffy coat following centrifugation through Histopaque®-1077 at 400 g for 30 min at 25°C. Erythrocytes were removed using a 2% gelatin sedimentation technique. Residual erythrocytes were removed by lysis in cold NH4Cl buffer. Remaining cells were >97% PMN as assessed by microscopic evaluation. PMN were studied within 2 h of their isolation.

Preparation of Activated PMN Supernatants. Freshly isolated PMN (10⁶ cells/ml in HBSS with 10⁻⁶ M FMLP) were incubated end-over-end for 1 min at 37°C (based on pilot experiments; unpublished data). PMN were then immediately pelleted (1,000 g for 20 s, 4°C) and supernatants filtered (0.45 μm; Phenomenex). For initial experiments isolating active PMN fractions, 10⁶ PMN/ml were activated, cells were removed by pelleting and supernatants were filtered (0.2 μm). Resultant cell-free supernatants were resolved by high-performance liquid chromatography (model 1050; Hewlett-Packard) with an HP 1100 diode array detector by reverse-phase on an HPLC column (Luna 5-μm C18, 150 x 4.60 mm; Phenomenex) with 100% H₂O mobile phase. Ultraviolet absorption spectra were obtained throughout. 1 ml fractions were collected, evaporated to dryness by speed-vac, reconstituted in HBSS (20-fold concentrated) and bioactivity was determined by permeability assay.

In experiments measuring supernatant concentrations of ATP, 100-μl samples were taken from PMN suspensions, immediately spun (1,000 g for 20 s, 4°C), filtered (0.45 μm), and analyzed via HPLC. ATP was measured with a H₂O-CH₃CN 96:4 mobile phase (1 ml/min). E-ATP and E-AMP were measured with a 0-50% methanol/H₂O gradient (10 min) mobile phase (2 ml/min). Absorbance was measured at 260 nm. UV absorption spectra were obtained at chromatographic peaks. ATP and adenine nucleotides were identified by their chromatographic behavior (retention time, UV absorption spectra, and coelution with standards).

To measure the time course of ATP release from PMN, 10⁷ PMN/ml were activated for indicated periods of time, supernatants were collected, and ATP content was quantified using CHRONO-LUME reagent (Crono-log Corp.). Luciferase activity was assessed on a luminometer (Turner Designs Inc.) and compared with internal ATP standards.

Endothelial Macromolecule Paracellular Permeability Assay. Using a modification of methods previously described (11), HMEC-1 on polycarbonate permeable inserts (0.4-μm pore, 6.5-mm diam; Costar Corp.) were studied 7–10 d after seeding (2–5 d after confluence). Inserts were placed in HBSS-containing wells (0.9 ml), and HBSS (alone or with PMN, PMN supernatant, or ATP) was added to inserts (100 μl). At the start of the assay (t = 0), FITC-labeled dextran 70 kD (concentration 3.5 μM) was added to fluid within the insert. The size of FITC–dextran, 70 kD, approximates that of human albumin, both of which have been used in similar endothelial paracellular permeability models (15, 16). Fluid from opposing well (reservoir) was sampled (50 μl) over 60 min (t = 20, 40, and 60 min). Fluorescence intensity of each sample was measured (excitation, 485 nm; emission, 530 nm; Cyto-Fluor 2300; Millipore Corp., Waters Chromatography) and FITC–dextran concentrations were determined from standard curves generated by serial dilution of FITC–dextran. Paracellular flux was calculated by linear regression of sample fluorescence (11).

Immunoactivation. Confluent cells were labeled with biotin, lysed, and cell debris removed by centrifugation. Lysates were preclerared with 50 μl preequilibrated protein G-Sepharose (Amersham Biosciences). Immunoactivation was performed with mouse mAb to human CD39 (Research Diagnostics, Inc.; 5 μg/ml), CD73 with mAb 1E9 (5 μg/ml, a gift from Dr. Linda
Thompson, Oklahoma Medical Research Foundation, Oklahoma City, OK) or AdoRA2 with goat polyclonal (Santa Cruz Biotechnology, Inc.) followed by addition of 50 µl preequilibrated protein G-Sepharose and overnight incubation. Washed immunoprecipitates were boiled in reducing sample buffer (2.5% SDS, 0.38 M Tris, pH 6.8, 20% glycerol, and 0.1% bromophenol blue), separated by SDS-PAGE, transferred to nitrocellulose, and blocked overnight in blocking buffer. Biotinylated proteins were labeled with streptavidin–peroxidase and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Transcriptional Analysis. Semi-quantitative RT-PCR was used to verify endothelial CD39 mRNA regulation, as described previously (17). The PCR reaction contained 1 µM each of the sense primer 5′-AGG AGC TGA AAT ATG CTG GC-3′ and the antisense primer 5′-GAG ACA GTA TCT GGC GAA GTC C-3′. The primer set was amplified using increasing numbers of cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 4 min, and a final extension of 72°C for 7 min. The PCR transcriptions were visualized on a 1.5% agarose gel containing 5 µg/ml of ethidium bromide. Human β-actin (sense primer, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CT-3′) and antisense primer, 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ in identical reactions was used to control for the starting template.

In subsets of experiments, the transcriptional profile of endothelial cells subjected to normobaric hypoxia (12 h) was compared in RNA derived from control or hypoxic endothelium using quantitative genechip expression arrays (Affymetrix, Inc.) as described previously.

In Vivo Hypoxia Model. Mice deficient in cd39 on the C57BL/6/129 svj strain were generated, validated, and characterized as described previously (21). Control mice were matched according to sex, age, and weight. Total organ vascular permeability was quantified by intravascular administration of Evans blue as described previously (22). For the purpose of quantifying vascular permeability, 0.2 c.c. of Evans blue (0.5% in PBS) were injected intravenously. Animals were then exposed to normobaric hypoxia (8% O2, 92% N2) or room air for 4 h (n = 4 animals per condition). After hypoxia/normoxia exposure the animals were killed and the colon, muscle, kidney, brain, liver, and lungs were harvested. Organ Evans blue concentrations were quantified after formamide extraction (55°C for 2 h) by measuring absorbances at 610 nm with subtraction of reference absorbance at 450 nm. This protocol was in accordance with NIH guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital.

Data Analysis. CD39 and CD73 bioactivity and paracellular permeability data were compared by two-factor ANOVA, or by Student’s *t* test where appropriate. Values are expressed as the mean ± SD from at least three separate experiments.

Results

Enhanced Barrier Protection to Activated PMN in Posthypoxic Endothelial Cells. We have previously demonstrated that during the process of transmigration, activated PMNs release soluble mediators which “reseal” endothelial monolayers after transit (11, 12, 23). It was further shown that leukocyte derived AMP is at least one leukocyte-derived factor responsible for this increase in barrier function (measured as a decrease in paracellular permeability; reference 11). Given the association of hypoxia and acute inflammation (1, 24) we questioned whether posthypoxic endothelia might differentially respond to activated PMN. As such, we compared the influence of activated PMN on...
permeability of normoxic and posthypoxic (pO$_2$ 20 torr for 48 h) HMEC-1. Consistent with previous work (11), increasing numbers of activated PMN elicited a concentration-dependent increase in endothelial barrier (measured as a decrease in paracellular flux rate of 70 kD FITC-labeled dextran; Fig. 1A, P < 0.025 compared with no

**Figure 1.** Influence of PMN on permeability of normoxic and posthypoxic endothelia. (A) Activated PMN (10$^{-6}$ M FMLP) at indicated concentrations were added to the apical surface of confluent normoxic (48 h exposure to pO$_2$ 147) or posthypoxic (48 h exposure to pO$_2$ 20 torr) HMEC-1 and permeability to FITC-dextran (70 kD) was quantified. Transendothelial flux was calculated by linear regression (4 samples over 60 min) and normalized as percent of control (HBSS). Data are derived from six monolayers in each condition. Data are expressed as mean ± SD of percent control flux with HBSS only. Asterisk (*) indicates significant differences from baseline (P < 0.05) and a cross (#) indicates differences from baseline and from normoxia (P < 0.05). (B) Supernatants derived from activated PMN (10$^{-6}$ M FMLP for 10 min) were fractionated by HPLC (H$_2$O mobile phase at 1 ml/min, 1 ml samples; inset indicates representative UV spectra at 260 nm and indicated samples collected at individual arrows). Samples were concentrated 20-fold and tested for bioactivity on normoxic or posthypoxic HMEC-1 in a model of paracellular permeability. Fraction #6 promotes barrier function in normoxic and posthypoxic endothelial cells, whereas fraction #2 is relatively selective for posthypoxic endothelia (*, P < 0.01 compared with HBSS).
PMN). As shown in Fig. 1 A, parallel analysis of posthypoxic HMEC-1 revealed a significant increase in endothelial barrier protection to activated PMN, relative to normoxia ($P < 0.01$ by ANOVA). Indeed, fewer PMN were required to elicit an equivalent increase in posthypoxic endothelial barrier, with an approximate 10-fold increase in endothelial sensitivity to PMN after hypoxia. Similar results were obtained using nonimmortalized BAE as an endothelial source (unpublished data). Such results suggest that the posthypoxic endothelium is functionally distinct in this regard and that barrier protective pathways are enhanced after exposure to hypoxia.

Identification of PMN-derived ATP as a Mediator of Permeability Changes in Posthypoxic Endothelial Cells. We next sought to determine the existence of soluble mediators from activated PMN that might contribute to enhanced barrier protection after hypoxia. To do this, we used HPLC to fractionate supernatants derived from activated PMN (10$^{-6}$ M for 1 min, based on pilot experiments), and tested bioactivity of individual fractions on normoxic and posthypoxic endothelium. As shown in Fig. 1 B, of 10 fractions that were isolated, fraction 6 increased endothelial barrier function in both posthypoxic and normoxic endothelial cells. In contrast, fraction 2 was associated with an increase in barrier function.

**Figure 2.** Activated PMN rapidly release ATP. (A) Chromatographic identification of ATP in supernatants derived from activated PMN. Shown here is a representative overlay chromatogram of activated PMN supernatant (bold line) and authentic ATP (narrow line). Inset represents an overlay UV spectra derived from fraction #2 (see Fig. 1) and of authentic ATP with one dominant peak at 260 nm. (B) Time course of ATP release from activated PMN (10$^7$ PMN/ml activated with 10$^{-6}$ M FMLP). PMN were activated for indicated periods of time and supernatant ATP was quantified by luciferase assay. Data represent mean ± SD ATP/10$^7$ PMN for three separate experiments. (C) Authentic ATP selectively promotes endothelial barrier function in posthypoxic endothelia. Indicated concentrations of ATP were added to HMEC-1 preexposed to normoxia or hypoxia. As indicated, ATP influenced endothelial permeability only in posthypoxic endothelial cells ($^*$, $P < 0.025$ compared with no ATP). In contrast, the addition of adenosine (Ado, 100 µM) was associated with an increase in barrier function in both normoxic and posthypoxic endothelial cells ($^*$, $P < 0.01$ compared with buffer alone). The addition of 8-phenyl-theophylline (8-PT at 10 µM, a nonselective adenosine receptor antagonist) obviated the barrier effect of 100 µM ATP in posthypoxic endothelial cells. Data are derived from six monolayers in each condition. Data are expressed as mean ± SD of percent control flux with HBSS only.
levels 215 pernatants derived from fMLP-activated PMN (maximal says. As shown in Fig. 2 B, ATP rapidly accumulated in su-PMN, we used standard luminometric ATP detection as-
ATP standards). Indeed, UV spectra analysis demonstrated
UV absorption spectra, and coelution with internal
nucleotides identified fraction 2 as ATP (based on retention
that of fraction 6 and coinjection with authentic AMP re-
experiment. The retention time of AMP was identical to
endothelial permeability assays (11). We therefore com-
pared retention times of the HPLC fractions that were tested in this
These results indicate that activated PMN rapidly re-
and that such extracellular ATP preferentially
promotes barrier function in posthypoxic endothelial cells.
ATP Promotes Barrier Function Selectively in Posthypoxic Endothelial Cells. Having demonstrated that activated PMN
release ATP, we next examined the influence of ATP on
normoxic and posthypoxic endothelial paracellular perme-
As shown in Fig. 2 C, the addition of ATP (range
1–100 μM) did not influence normoxic endothelial per-
meability. In striking contrast, following a 48 h exposure to hyoxia, endothelial flux rates were significantly decreased in an ATP concentration-dependent fashion (P < 0.01 by ANOVA), and were similar to responses observed with 100 μM adenosine (Fig. 2 C). Similar results were obtained using posthypoxic, nonimmortalized BAE as an endothelial
source (60 ± 8% decrease in permeability with 100 μM
ATP, P < 0.01, n = 3). Such findings suggest that ATP selec-
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To gain insight into potential mechanisms of ATP regu-
lation of posthypoxic endothelial permeability, we rea-
soned that ATP may directly activate surface ATP recep-
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specific adenosine receptor antagonist 8-phenylthiophyl-
line (8-PT). As shown in Fig. 2 C, the permeability changes associated with the addition of authentic ATP (100 μM) on posthypoxic endothelial cells was neutralized by 8PT (10 μM, P = not significant compared with buffer alone), indicating that the barrier protective influence ob-
served with ATP reflects adenosine receptor activation.

Prominent Role for CD39 in ATP-modulated Permeability of Posthypoxic Endothelial Cells. Metabolism of extracellular ATP is predominantly determined by CD39, an ecto-apy-

![Figure 3](image-url)

**Figure 3.** Induction of functional CD39 is by hypoxia. (A) Confluent HMEC-1 monolayers were exposed to normoxia (pO2 147 torr, 18 h) or hypoxia (pO2 20 torr, 18 h). Total RNA was isolated, and CD39 mRNA levels were determined by RT-PCR using semiquantitative analysis (increasing cycle numbers, as indicated). As shown, β-actin transcript was determined in parallel and used as a control. (B) Real-time PCR was employed to confirm hypoxia inducibility of CD39 in cul-
tured endothelial cells (HMEC-1). Data were calculated relative to internal housekeeping gene (β-actin) and are expressed as fold increase over normoxia ± SD at each indicated time. Results are derived from three experiments in each condition. (C) Human saphenous vein was obtained from patients undergoing aorto-coronary bypass surgery and exposed ex vivo to ambient normoxia (pO2 147 torr, 24 h) or hypoxia (pO2 20 torr for 2, 8, or 24 h). After total RNA isolation, real-time PCR was per-
formed to investigate CD39 inducibility by hypoxia. Data were calculated relative to internal control (β-actin) and are expressed as fold increase over normoxia ± SD at each indicated time. Results are derived from three experiments in each condition. (D) Increase in surface CD39 surface

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eral features of this response. As shown in Fig. 3A this assessment revealed prominent induction of CD39 mRNA expression after 18 and 24 h of exposure to hypoxia (13 ± 2-fold increase of integrated band density in cells exposed to hypoxia compared with those exposed to normoxia, P < 0.01), suggesting that CD39 is induced by hypoxia. Moreover, comparison of HMEC-1 mRNA levels by real-time PCR revealed that CD39 (Fig. 3A) is prominently induced by hypoxia. Similar results of CD39 inducibility were found when human saphenous vein tissues were subjected to hypoxia, indicating that such findings are not limited to cultured endothelium. Real time PCR analysis of RNA preparations of saphenous vein revealed induction of CD39 as early as 2 h of ex vivo exposure to hypoxia (Fig. 3C, P < 0.025 by ANOVA).

We extended these findings to determine if CD39 is functionally relevant. Initially, we determined whether CD39 protein was induced by hypoxia. As shown in Fig. 3D, immunoprecipitation of CD39 from total surface biotinylated protein revealed time-dependent induction of CD39 (range 12–48 h), with maximal protein levels observed at 48 h (Fig. 3D; no additional increase at 72 h; data not shown). We then assessed whether hypoxia induced CD39 was functional. To do this, we adopted an HPLC method to compare the relative amounts of E-ATP metabolism to E-AMP (in the presence of the CD73 inhibitor 2',3'-methylene-ADP [10 μM] to inhibit further metabolism of E-AMP to E-adenosine, see method validation in Fig. 3E). As shown in Fig. 3F, exposure of HMEC-1 to hypoxia (48 h) induced an increase in functional CD39 (ANOVA, P < 0.01 relative to normoxia), and represented a 22 ± 5-fold increase in the rate of E-ATP metabolism with preexposure to hypoxia. Together, these data suggest a transcriptional induction of CD39 by hypoxia, which manifests as readily measurable increases in surface enzyme activity.

CD39 Hypoxia Induction Is Necessary for ATP Elicited Increases of Barrier Function in Posthypoxic Endothelial Cells. After having shown that functional CD39 is induced by hypoxia, we examined if CD39 induction by hypoxia is necessary for ATP-elicited increases of barrier function in posthypoxic endothelial cells. We performed CD39-specific siRNA knockdown experiments in HMEC-1 cells, and determined the relative impact of hypoxia on the functional expression of CD39.

Figure 4. Role of CD39 in ATP-elicited changes of posthypoxic endothelial permeability. (A) HMEC-1 were loaded with CD39-specific siRNA, control ribonucleotide or mock treated (control) and exposed to hypoxia or normoxia (48 h). Monolayers were washed, surface protein was biotinylated, and cells were lysed. CD39 was immunoprecipitated and resolved by SDS-PAGE, and resultant Western blots were probed with avidin-peroxidase. As a control for specificity, CD73 protein induction by hypoxia was assessed in parallel. (B) Influence of CD39 suppression by siRNA on functional surface protein. HMEC-1 were loaded with CD39-specific siRNA, control-ribonucleotide, or mock treated and CD39 activity was determined by HPLC analysis of E-ATP conversion to E-AMP (in the presence of the CD73 inhibitor 2',3'-methylene-ADP). Data are derived from five to seven monolayers in each condition, and results are expressed as E-AMP: E-ATP ratio ± SD. (C) Influence of CD39 suppression by siRNA on endothelial barrier. HMEC-1 were loaded with CD39-specific siRNA, control ribonucleotide, or mock treated (control), exposed to hypoxia or normoxia (48 h), and permeability to 70 kD FITC in the presence or absence of ATP (100 μM) was assessed (*, P < 0.01 compared with no ATP). Data are derived from six monolayers in each condition, and data are expressed as mean ± SD of percent control flux.
sary for the observed increase of barrier function in posthypoxic endothelial cells upon ATP exposure. As no specific CD39 inhibitors have come available (28), we used RNA interference to suppress CD39 induction by hypoxia and studied the functional influence on permeability. Thus, we loaded HMEC-1 with specific CD39-siRNA, and exposed the cells to hypoxia. Immunoprecipitation of biotinylated surface CD39-protein showed an induction of CD39 in untreated controls and in HMEC-1 loaded with control oligonucleotide (Fig. 4 A). In contrast, normoxic and posthypoxic CD39 levels were decreased after loading with specific CD39-siRNA. Moreover, CD73 induction by hypoxia was not affected by CD39 siRNA loading, and as such, provided a specificity control for these experiments.

As additional insight, we determined whether siRNA directed against CD39 was functional. First, we measured enzymatic conversion of E-ATP to E-AMP in siRNA-mediated CD39 deficient cells. In controls and in HMEC-1 loaded with control oligonucleotide, a 48 h exposure to hypoxia was associated with a significant increase in CD39 functional activity (ANOVA, $P < 0.01$; Fig. 4 B). In contrast, loading with specific CD39-siRNA obviated a functional induction of CD39 enzyme activity, indicating that such RNA suppression was effective at the functional protein level. To determine the relative role of CD39 in ATP-mediated changes in endothelial barrier function, we investigated the influence of ATP on barrier function in normoxic and posthypoxic endothelial cells following inhibition of CD39 with specific siRNA. In controls and in HMEC-1 loaded with a control oligonucleotide, ATP elicited an approximate 60% decrease in permeability in posthypoxic endothelial cells, but not in normoxic cells (Fig. 4 C). In contrast, no significant ATP-induced change in barrier was observed in either normoxic or posthypoxic endothelial cells after suppression of CD39 with siRNA ($P < 0.01$). These results indicate that selective targeting of CD39 by siRNA effectively neutralizes the influence of ATP on changes in endothelial barrier.

Inhibition of CD73 Blocks ATP-mediated Changes in Posthypoxic Endothelial Permeability. We next addressed the role of CD73 in ATP-mediated changes in permeability. Our previous studies indicated that CD73 is important for the maintenance of barrier function during interactions with PMN or soluble PMN mediators (e.g., AMP; reference 23). Similarly, it was previously shown that AMP enhances epithelial barrier recovery via activation of surface AdoRA2B receptors (11). As confirmation of our previous findings in epithelia (18), we observed a time-dependent induction of CD73 by hypoxia in endothelia (see Fig. 5 A). To determine the functional role of CD73 in this response, we addressed whether the specific CD73 inhibitor $\alpha,\beta$-methylene ADP might block ATP-induced changes in the posthypoxic endothelium. As shown in Fig. 5 B, the addition of increasing concentrations of $\alpha,\beta$-methylene ADP in combination with ATP (100 $\mu$M) resulted in a concentration-dependent inhibition of ATP-mediated changes in permeability selectively in posthypoxic endothelia ($P < 0.01$). These findings suggest that the increment of CD73 induced by hypoxia is relevant to endothelial functional responses (permeability).

**Hypoxia Induces AdoRA2B: Role in Coordinated Vascular Permeability Regulation.** Having demonstrated a functional role for hypoxia-regulated CD39 and CD73 in en-
dothelial permeability, we next addressed how endpoint signaling (i.e., adenosine receptor activation) is amplified in posthypoxic endothelia. First, we profiled the relative expression of adenosine receptors in normoxic and hypoxic (12 h exposure to pO$_2$ 20 torr) endothelial cells by microarray analysis. For these experiments, nonimmortalized HMVECs were used. Interestingly, these experiments demonstrated that the AdoRA$_{2B}$ was selectively induced by hypoxia (see Fig. 6 A, $P < 0.05$ by ANOVA), and that other isoforms were either not changed (AdoRA$_{3}$) or significantly down-regulated (AdoRA$_{1}$ and AdoRA$_{2A}$). As shown in Fig. 6 B, these microarray results were verified in HMEC-1 by real-time PCR in RNA derived from endothelial cells exposed to a time course of hypoxia, and consistently revealed that the AdoRA$_{1}$ isoform remained unchanged, both AdoRA$_{1}$ and AdoRA$_{2A}$ levels were decreased at later time points of hypoxia (18 and 24 h, $P < 0.025$), and that the AdoRA$_{2B}$ was increased by as much as 4 ± 05-fold ($P < 0.01$ compared with normoxia). Identical results were obtained with HMVEC as an endothelial source (unpublished data). Extensions of these findings revealed that surface protein levels of AdoRA$_{2B}$ were similarly increased by exposure of HMEC-1 to hypoxia (Fig. 6 C).

**Figure 6.** Adenosine A$_{2B}$ receptor (AdoRA$_{2B}$) induction by hypoxia enhances barrier response. (A) Microarray analysis of individual adenosine receptors in response to hypoxia (AdoRA$_{1}$, AdoRA$_{2A}$, AdoRA$_{2B}$, and AdoRA$_{3}$). Confluent HMEC-1 were exposed to normoxia or hypoxia (12 h exposure to pO$_2$ 20 torr) and the relative expression of individual adenosine receptors was quantified from total RNA by microarray analysis. Data are expressed as fold change ± SD relative to normoxia. (B) Real-time PCR analysis was employed to confirm hypoxia-regulated expression of individual adenosine receptors (AdoRA$_{1}$, AdoRA$_{2A}$, AdoRA$_{2B}$, and AdoRA$_{3}$) in HMEC-1. Data were calculated relative to internal control ($\beta$-actin) and are expressed as fold increase ± SD over normoxia at indicated time points. Results are derived from three experiments in each condition (*, $P < 0.01$ compared with normoxia). (C) Increased AdoRA$_{2B}$ surface protein with hypoxia. Confluent HMEC-1 monolayers were exposed to indicated periods of hypoxia, monolayers were washed, surface proteins were biotinylated, and cells were lysed. AdoRA$_{2B}$ was immunoprecipitated with mAb to human AdoRA$_{2B}$ and resolved by SDS-PAGE, and resultant Western blots were probed with avidin-peroxidase. A representative experiment of three is shown. (D) Hypoxia induction of AdoRA$_{2B}$ enhances barrier response of HMEC-1 to adenosine. Indicated concentrations of adenosine were added to HMEC-1 monolayers preexposed to normoxia or hypoxia (48 h). Addition of the specific AdoRA$_{2B}$-antagonist MRS 1754 (100 nM) significantly shifted the adenosine dose response in posthypoxic endothelium ($P < 0.01$ by ANOVA). Data are derived from 6 monolayers in each condition. Data are expressed as mean ± SD of percent control flux.
To determine whether such hypoxia-induced AdoRA2B expression was functional, endothelial permeability assays were employed using the selective AdoRA2B antagonist MRS1754 (29). As can be seen in Fig. 6 D, MRS 1754 (100 nM) significantly shifted the adenosine dose–response curve to the right, but had little influence on adenosine responses in normoxic endothelia. Such results suggest a relative importance for the expression level of AdoRA2B and that AdoRA2B responses are amplified in posthypoxic endothelia.

**Increased Vascular Permeability in cd39-null Animals: Influence of Hypoxia.** As proof of principle for these concepts in vivo, we compared the influence of hypoxia on vascular permeability in wild-type and cd39-null mice using a previously described model (22). After intravenous injection of Evans-blue, we exposed animals to either normoxia or normobaric hypoxia (8% O2 and 92% N2) for 4 h and measured Evans-blue tissue concentrations as a marker of endothelial permeability to albumin. Interestingly, assessment of...
permeability in nonhypoxic cd39-null animals revealed subtle, but significant increases in all tissues examined, with the exception of skeletal muscle (Fig. 7 A). Consistent with previous studies (3, 30), this model of hypoxia significantly increased permeability in all wild-type control organs examined (Fig. 7 A, P < 0.025 for all). Most importantly, and as shown in Fig. 7 A, this analysis revealed amplified increases in vascular permeability of all organs of cd39-null animals. Gross morphologic changes in overall vascular permeability (Evans blue retention) between hypoxia and normoxia as well as wild-type and cd39-null were evident in open abdominal images taken at necropsy (Fig. 7 B) and were demonstrable in dissected organs such as the colon (Fig. 7 C). These findings in vivo support our hypothesis that CD39 is a critical determinant for permeability changes associated with adenine nucleotide metabolism in the posthypoxic vasculature.

Discussion

The vascular endothelium is the predominant interface between the hypoxic insult and surrounding tissues. Simultaneously, the endothelium provides the primary determinant of vascular permeability (31). As such, PMN influx across the protective endothelium, such as occurs during ischemia-reperfusion, creates the potential for endothelial barrier dysfunction, loss of fluid and edema formation (7, 32). In this study we compared the direct influence of activated PMN on paracellular permeability in normoxic and posthypoxic endothelial cells. The results revealed the existence of PMN-derived mediator(s), which selectively promote posthypoxic endothelia. This mediator was identified as ATP based on its chemical characteristics and chromatographic behavior. Further studies demonstrated that hypoxia promotes the induction of CD39, an endothelial surface apyrase responsible for the initiation of ATP phosphohydrolysis to adenosine (33). We show here that increased activity of endothelial CD39, as seen after hypoxic exposure, is necessary for the barrier protective influence of ATP. Similarly, these studies revealed that additional metabolic and signaling molecules (CD73 and AdoRA2B, respectively) are coordinately induced by hypoxia (Fig. 8). In vivo relevance for this response revealed that cd39-null mice are more susceptible to the development of endothelial barrier dysfunction during hypoxia exposure. Taken together, these studies identify a pathway which results in both elevated tissue levels of adenosine and in amplified endothelial responses to adenosine generated at sites of hypoxia.

It is well documented that adenosine tissue and plasma levels are increased during hypoxia, however, mechanisms of this response are less clear (34, 35). For example, in human volunteers exposed to ambient hypoxia (SpO2 = 80% over 20 min), plasma adenosine concentrations increased from 21 to 51 nM in the presence of dipyridamole, an inhibitor of adenosine reuptake (36). Similarly, when measuring adenine nucleotide concentrations in the neurally and vascularly isolated, perfused skeletal muscles of anesthetized dogs, normobaric hypoxia is associated with increases of adenosine in the venous blood, but not of AMP, ADP, or ATP (37). In conjunction with these studies, we describe here a metabolic pathway, which results in increased tissue adenosine concentrations. While it is well documented that adenosine levels are increased in hypoxic tissue, the source of adenosine remains unclear. We show here, for the first time, that PMN actively release ATP to the extracellular milieu. At present, we do not know the origin of ATP release. Several mechanisms for ATP release have been proposed, including direct transport through ATP-binding cassette (ABC) proteins, transport through connexin hemichannels, as well as vesicular release (38). As part of the present experiments, some studies were done in an attempt to identify the compartmentalization of ATP (unpublished data). For example, we compared the kinetics...
of ATP release with activated release of PMN granules (myeloperoxidase, MPO). These studies indicated that while ATP levels were maximal within 1 min, activated degranulation contents such as MPO were maximal at time points >5 min, suggesting that ATP is not granule bound. Moreover, in experiments using isolated granules from unactivated PMN, >95% of MPO activity was associated with granules, <5% of ATP was measurable within this granule pool, suggesting that ATP is unlikely to be granule-bound in PMN. At present, the mechanism by which leukocytes release ATP remains unclear.

As part of these studies, we found that targeted disruption of CD39 in an animal model of ambient hypoxia was associated with barrier dysfunction at baseline and after hypoxic exposure. Such increases in permeability in the cd39-null mice may be related to decreased concentrations of adenosine and activation of endothelial adenosine receptors. In addition, activation of neutrophil A2 adenosine receptors has been shown to play a critical part for the limitation and termination of PMN-mediated systemic inflammatory responses (39, 40). Thus, it is reasonable to propose that the presence of CD39/CD73 is important in modulating inflammatory responses during hypoxia. Coordinated induction of both CD39 and CD73 by hypoxia may provide increased tissue adenosine concentrations, thus leading to increased stimulation of PMN adenosine A2-receptors and decreased leukocyte adherence and transmigration, as has been demonstrated by others (41, 42). Moreover, recent studies have indicated that adenosine A2A-receptors may contribute to attenuated vascular leak associated with leukocyte accumulation (43). As such, direct influences of adenosine via hypoxia-amplified CD39 and CD73 may provide antiinflammatory synergism with the observed protection of endothelial barrier during hypoxia.

As outlined above, leukocyte transmigration associated with hypoxic/ischemic tissues creates the potential for disturbance of the endothelial barrier. We have previously shown that adenosine activation of AdoRA2B leads to a barrier resealing response after PMN transmigration (11). Activation of the AdoRA2B is associated with increases in intracellular cAMP concentration following activation of the adenylylate cyclase (44). By inhibition of cAMP formation, the resealing of the endothelial barrier during PMN transmigration can be obviated (11). Such increases in cAMP after activation of the AdoRA2B lead to an activation of protein kinase A (PKA; reference 12). Further studies revealed a central role of PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a protein responsible for controlling the geometry of actin-filaments (45). Adenosine-receptor mediated phosphorylation of VASP is responsible for changes in the geometry and distribution of junctional proteins, thereby affecting the characteristics of the junctional complex and promoting increases in barrier function (12).

We show here that the barrier-protective response to extracellular ATP is most prevalent in posthypoxic endothelial cells. After suppression of CD39 induction with specific siRNA, this barrier-protective influence to ATP is obviated, providing insight that this molecule may efficiently coordinate permeability responses to PMN during inflammation and hypoxia. In parallel, we showed that the ectonucleotidase CD73 is similarly induced by hypoxia. These findings in endothelia coincide with recent work in epithelia identifying a predominant role for hypoxia-inducible factor-1 (HIF-1) in CD73 induction by hypoxia (18) and recently in endothelial cells (46). Currently, the mechanisms for the hypoxia-inducibility of AdoRA2B and CD39 remains unclear. Relatively little is known about the transcriptional regulation of CD39 and AdoRA2B, and while HIF-1 is currently an area of intense investigation in a variety of disorders (47), including inflammation (5, 17, 18), it remains to be seen whether the coordinated transcription of CD39, CD73, and AdoRA2B have common regulatory elements.

Some precedent exists for coordinated, metabolic responses to hypoxia. For example, in the regulation of oxygen hemostasis, HIF-1 has been shown to regulate multiple metabolic and compartmental steps in several metabolic pathways (47). As such, at least 13 different genes encoding glucose transporters and glycolytic enzymes are coordinately decreased in HIF-1 deficiency (47). Similarly in the present study, transcriptional and metabolic control-points of extracellular nucleotide metabolism and nucleoside signaling are controlled in a coordinated fashion. Interestingly, recent evidence indicates that the AdoRA2A and CD73 are coordinately regulated by mitogenic stimuli in human B cells, and that such a pathway drives increased signal transduction (48). The present results of a coordinated response to decreased oxygen availability of CD39, CD73, and the AdoRA2B suggest a similar evolutionary adaption to hypoxia.

In summary, these results define a previously unappreciated metabolic pathway prevalent at the vascular interface during inflammation/hypoxia. We show here that PMN-derived ATP selectively promotes barrier function of posthypoxic endothelia. The observed differences were attributable to a coordinated transcriptional response initiated by hypoxia, and resulting in amplified metabolism of extracellular ATP to adenosine as well as enhanced signaling through the AdoRA2B. We propose that this feed-forward mechanism may contribute an endogenous, protective pathway for highly vascular tissues during adaptation to hypoxia.

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