TIME WAITS FOR NO ONE
Enlist the experts at Bio X Cell for Antibody Production Services
RECEIVE 10% OFF NOW with code: CONTRACT22JI
EXPLORE

The Journal of Immunology

Regulation of Endothelial CD73 by Adenosine: Paracrine Pathway for Enhanced Endothelial Barrier Function

This information is current as of February 24, 2022.

Sailaja Narravula, Paul F. Lennon, Beatrice U. Mueller and Sean P. Colgan

J Immunol 2000; 165:5262-5268; ;
doi: 10.4049/jimmunol.165.9.5262
http://www.jimmunol.org/content/165/9/5262

References This article **cites 44 articles**, 16 of which you can access for free at:
http://www.jimmunol.org/content/165/9/5262.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

Regulation of Endothelial CD73 by Adenosine: Paracrine Pathway for Enhanced Endothelial Barrier Function[1]

Sailaja Narravula,* Paul F. Lennon,* Beatrice U. Mueller,† and Sean P. Colgan[2]*

During episodes of inflammation, multiple cell types release adenine nucleotides in the form of ATP, ADP, 5′-AMP, and adenosine. In particular, following activation, polymorphonuclear leukocytes release larger quantities of 5′-AMP. Extracellular 5′-AMP is metabolized to adenosine by surface-expressed 5′-ectonucleotidase (CD73). Adenosine liberated by this process activates surface adenosine A_{2B} receptors, results in endothelial junctional reorganization, and promotes barrier function. We hypothesized that adenosine signaling to endothelia provides a paracrine loop for regulated expression of CD73 and enhanced endothelial barrier function. Using an in vitro microvascular endothelial model, we investigated the influence of 5′-AMP; adenosine; and adenosine analogues on CD73 transcription, surface expression, and function. Initial experiments revealed that adenosine and adenosine analogues induce CD73 mRNA (RT-PCR), surface expression (immunoprecipitation of surface biotinylated CD73), and function (HPLC analysis of etheno-AMP conversion to ethenoadenosine) in a time- and concentration-dependent fashion. Subsequent studies revealed that similar exposure conditions increase surface protein through transcriptional induction of CD73. Analysis of DNA-binding activity by EMSA identified a functional role for CD73 cAMP response element and, moreover, indicated that multiple cAMP agonists induce transcriptional activation of functional CD73. Induced CD73 functioned to enhance 5′-AMP-mediated promotion of endothelial barrier (measured as a paracellular flux of 70-kDa FITC-labeled tracer). These results provide an example of transcriptional induction of enzyme (CD73) by enzymatic product (adenosine) and define a paracrine pathway for the regulated expression of vascular endothelial CD73 and barrier function. ***The Journal of Immunology,*** **2000, 165: 5262–5268.**

Circulating or locally released nucleotides are rapidly metabolized on a single passage through a capillary bed, primarily through surface ectoenzymes (1). Ecto-5′-nucleotidase (CD73) is a membrane-bound glycoprotein that functions to hydrolyze extracellular nucleotides into bioactive nucleoside intermediates (2). For instance, in epithelia and endothelia, cell surface CD73 converts AMP into adenosine, which when released can activate seven-transmembrane-spanning adenosine receptors (3, 4) or can be internalized through dipyridamole-sensitive carriers (5). These pathways have been shown to activate such diverse endpoints as adenine nucleotide recycling during cellular hypoxia (6), stimulation of epithelial electrogenic chloride secretion (responsible for mucosal hydration) (7), regulation of lymphocyte-endothelial adhesion (8), and promotion of endothelial barrier function (4). Targeted overexpression of lymphocyte CD73 revealed that increased adenosine resultant from enhanced CD73 expression contributes to thymocyte apoptosis in adenosine deaminase deficiency (9).

The primary function attributed to endothelial CD73 has been catabolism of extracellular nucleotides, although CD73 may also mediate lymphocyte binding under some circumstances (8). Recent studies have revealed that when coincubated with endothelia or epithelia, neutrophils release micromolar concentrations of 5′-AMP (4, 7). 5′-AMP is rapidly converted to adenosine (via surface-expressed CD73), and adenosine liberated in this fashion directly activates surface adenosine A_{2B} receptors (4, 7, 10) and promotes endothelial barrier function through elevation of intracellular cAMP (4, 11). Rather little is known about the regulation of endothelial CD73 and whether this molecule contributes to endothelial permeability. The cloned CD73 gene promoter bears a cAMP response element (CRE)[3] (12), which is one of the consensus DNA motifs that regulate transcription through the cAMP-dependent coactivator CRE binding protein (CREB) (13). Adenosine activation of endothelia elevates intracellular cAMP (4, 14), providing the possibility that the enzymatic product of CD73 (adenosine) could transcriptionally regulate surface enzyme (CD73). To date, no direct evidence exists for this pathway. In these studies, we explored the regulation of endothelial CD73 bioactivity; surface protein expression; and transcriptional activation by adenosine, adenosine analogues, and other cAMP agonists. The results define a paracrine pathway of CD73 induction by adenosine in vascular endothelial cells.

Materials and Methods

Endothelial cell isolation and culture

Human microvascular endothelial cells (HMVEC), an endothelial primary culture isolated from adult dermis, were used throughout these studies. HMVEC were obtained from Cascade Biologics (Portland, OR) and cultured as previously described (4). Culture medium was supplemented with heat-inactivated calf serum, penicillin, streptomycin, HEPES, heparin, L-glutamine, and endothelial mitogen factor. For preparation of experimental

*Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, and †Division of Hematology and Oncology, Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, MA 02115

Received for publication February 11, 2000. Accepted for publication August 10, 2000.

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

[1] This work was supported by National Institutes of Health Grants HL03688-01 (to P.F.L.) and DE 13499 and HL60569 (to S.P.C.) and by a grant from the Crohn's and Colitis Foundation of America.

[2] Address correspondence and reprint requests to Dr. Sean P. Colgan, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Thorn Building 704, 75 Francis Street, Boston, MA 02115. E-mail address: colgan@zeus.bwh.harvard.edu

[3] Abbreviations used in this paper: CRE, cAMP response element; CREB, response element binding protein; HMVEC, human microvascular endothelial cells; E-ADO, ethenoadenosine; E-AMP, etheno-AMP; APCP, $\alpha\beta$-methylene ADP; ADAC, adenosine amine congener; CSC, 8-(3-chlorostyryl) caffeine; NECA, 5′-(*N*-ethylcarboxoamido)adenosine.

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

HMVEC monolayers, confluent endothelial cells (passages 1, 2, or 3) were seeded at $\sim 2 \times 10^5$ cells/cm^2 onto either polycarbonate permeable supports or six-well plates precoated with 0.1% gelatin. Endothelial cell purity was assessed by phase microscopic "cobblestone" appearance and uptake of fluorescent acetylated low density lipoprotein.

Measurement of 5-ectonucleotidase activity

Endothelial CD73 surface activity was assessed as before (4) by measuring the conversion of etheno-AMP (E-AMP) to ethenoadenosine (E-ADO) (15). Briefly, HBSS with or without α,β-methylene ADP (APCP) was added to HMVEC monolayers on six-well plates. After 10 min, E-AMP (final concentration 10 μM) was added for an additional 10 min, removed, acidified to pH 3.5 with HCl, spun (10,000 $\times$ *g* for 20 s, 4°C), filtered (0.45 μm), and frozen (−80°C) until analysis via HPLC. A Hewlett-Packard (Palo Alto, CA) HPLC (model 1050) with a HP 1100 diode array detector was used with a reverse-phase HPLC column (Luna 5 μm C_{18} (2), 150 $\times$ 4.60 mm, Phenomenex, Torrance, CA). E-AMP and E-ADO were measured with a 0–50% methanol/H_2O gradient (10 min) mobile phase (2 ml/min). Absorbance was measured at 260 nm and UV absorption spectra were obtained at chromatographic peaks. CD73 activity was expressed as percentage E-AMP conversion in this time frame.

CD73 immunoprecipitation

Confluent endothelial cells exposed to indicated experimental conditions (150 cm (2) confluent cells per condition) were surface labeled with biotin and lysed, and cell debris was removed by centrifugation as described previously (4). Lysates were precleared with 50 μl pre-equilibrated protein G-Sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitation of CD73 was performed with mAb 1E9 (a kind gift from Dr. Linda Thompson, Oklahoma Medical Research Foundation, Oklahoma City, OK) followed by addition of 50 μl preequilibrated protein G-Sepharose and overnight incubation. Washed immunoprecipitates were boiled in nonreducing sample buffer (2.5% SDS, 0.38 M Tris (pH 6.8), 20% glycerol, and 0.1% bromphenol blue), resolved by SDS-PAGE, electroblotted to nitrocellulose, and blocked overnight in blocking buffer. Biotinylated proteins were labeled with streptavidin-peroxidase and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Analysis of mRNA levels by PCR

RT-PCR was used to define endothelial CD73 mRNA regulation. Briefly, 1 μg of DNase I (GenHunter, Nashville, TN)-treated total RNA-derived HMVEC exposed to experimental conditions was reverse transcribed into cDNA using a reaction mixture consisting of 4 μl of 25 mM $MgCl_2$, 2 μl of 10$\times$ reverse transcriptase buffer, 2 μl of 10 mM concentrations of each dNTP, 0.5 μl of rRNase in RNase inhibitor (20 U total), 15 U of avian myeloblastosis virus reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer in a total volume of 20 μl. Thermostable *Tfl* DNA polymerase (Promega, Madison, WI) from *Thermus flavus* was used for second-strand cDNA synthesis and DNA amplification. The CD73 PCR reaction contained 1 μM concentrations each of the sense primer (5′-CAC CAA GGT TCA GCA GAT CCG C-3′) and the antisense primer (5′-GTT CAT CAA TGG GCG ACC GG-3′), 10 μl of 5$\times$ PCR buffer, 1 mM $MgSO_4$, 0.2 mM dNTP, and 5 U of *Tfl* enzyme mix in a total volume of 50 μl. Each primer set was amplified using 25 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 4 min, followed by a final extension of 72°C for 7 min. The PCR fragments were visualized on a 1.5% agarose gel containing 5 μg/ml ethidium bromide. To ensure that an equal amount of template was used in each amplification reaction, 5 μl of reverse transcriptase reaction was used as template with 1 μM concentrations each of human β-actin sense primer (5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′) and antisense primer (5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′) in identical reactions; a 661-bp amplified fragment with equal intensity was observed in all samples.

EMSA

Nuclear extracts of cells exposed to indicated experimental conditions were obtained as described before (16). Briefly, confluent monolayers of HMVEC in 100-mm petri dishes were washed in ice-cold PBS and lysed by incubation in 500 μl of buffer A (10 mM HEPES (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose, 0.5 mM PMSF, 1 μg of both leupeptin and aprotinin per ml, and 0.5% Nonidet P-40) for 5 min at 4°C. The crude nuclei released by lysis were collected by microcentrifugation (15 s). Nuclei were rinsed once in buffer A and resuspended in 100 μl of buffer C (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1.0 mM DTT, and 1 μg/ml of both leupeptin and aprotinin). Nuclei were incubated on a rocking platform at 4°C for 30 min and clarified by microcentrifugation for 5 min.

The following synthetic oligonucleotide probes were synthesized (Genosys Biotechnologies, The Woodlands, TX) and used as probes in EMSAs. The CRE motif (underlined) lies at positions −87 to −81 relative to the transcription start site in the CD73 promoter; sense probe, 5′-TCG GAT CGG GTG ACG TCG CGA ACT TGC-3′. Double-stranded oligonucleotide probes for EMSA were labeled with [^{32}P]ATP using T4 polynucleotide kinase (1 μl with specific activity of 15, 300 cpm/μl), incubated with 0.65 μg nuclear lysates for 20 min at room temperature in 26 μl annealing buffer (20 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$, 3.9 μg poly(dI-dC), and 2.5% glycerol), and separated by electrophoresis on a 4% nondenaturing polyacrylamide gel. For supershift experiments, the reaction mixtures were incubated on ice for 5 min in the presence of 3 μl specific polyclonal antisera either against CREB-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or CREB-2 (also called ATF-4, Santa Cruz Biotechnology) before addition of labeled probe. The gel was dried and autoradiographed at −80°C. As a control for specificity, a CRE site mutated probe (sense sequence 5′-TCG GAT CGG GTG AAA TCG CGA ACT TGC-3′) was used.

Endothelial macromolecule permeability assay

Endothelial paracellular permeability was assessed exactly as described previously (4) using HMVEC grown on polycarbonate permeable inserts (0.4 μm pore, 6.5 mm diameter; Costar, Cambridge, MA). Permeability of FITC-labeled dextran (70 kDa, Molecular Probes, Eugene, OR) was assessed on washed monolayers (HBSS) by sampling serosal fluid (50 μl at 5, 10, 15, 20, 30, and 60 min); sample volume was replaced with HBSS. Fluorescence intensity of each sample was measured (excitation, 485 nm; emission, 530 nm) (Cytofluor 2300, Millipore, Bedford, MA), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Paracellular flux rates were calculated by linear regression.

Other materials

Adenosine was obtained from Calbiochem (La Jolla, CA). Adenosine amine congener (ADAC), 8-(3-chlorostyryl) caffeine (CSC), alloxazine, and 5′-(*N*-cyclopropyl)-carboxamidoadenosine were obtained from Research Biochemical International (Natick, MA). E-AMP, E-Ado, APCP, 5′-(*N*-ethylcarboxiamido)adenosine (NECA), and AMP were obtained from Sigma (St. Louis, MO). FITC-dextran was obtained from Molecular Probes. Anti-CD73 Ab (mAb 1E9) was a generous gift from Dr. Linda Thompson (Oklahoma Medical Research Foundation). Transwell inserts were obtained from Corning Costar (Cambridge, MA).

Data analysis

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 and SEM of *n* monolayers from at least three separate experiments.

Results

Adenosine receptor activation induces endothelial CD73

We recently demonstrated that activated polymorphonuclear leukocyte supernatants promote endothelial barrier function (4). Characterization of this bioactivity revealed that polymorphonuclear leukocyte-derived 5′-AMP contributed to this action and required 5′-AMP conversion to adenosine via endothelial surface ecto-5′-nucleotidase (CD73). Here, we determined whether endothelial CD73 expression might be regulated by activation of adenosine A_2 receptors, thus defining a paracrine pathway of adenosine metabolism. As shown in Fig. 1*A*, HMVEC pretreatment with the adenosine A_2 receptor agonist NECA at concentrations that approximate measured adenosine plasma levels (1 μM) (17) induced a time-dependent increase in functional CD73 (ANOVA, $p < 0.01$) as determined by HPLC analysis of E-AMP conversion to E-adenosine (15), with a 5.4- $\pm$ 1.1-fold increase at 48 h following NECA exposure ($p < 0.001$). Shorter periods of incubation with NECA (i.e., <8 h) revealed no significant change in CD73 activity (data not shown). These results of increased CD73 activity by NECA were specific, since addition of the CD73 inhibitor APCP (3 μM) significantly diminished CD73 activity in both control and

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

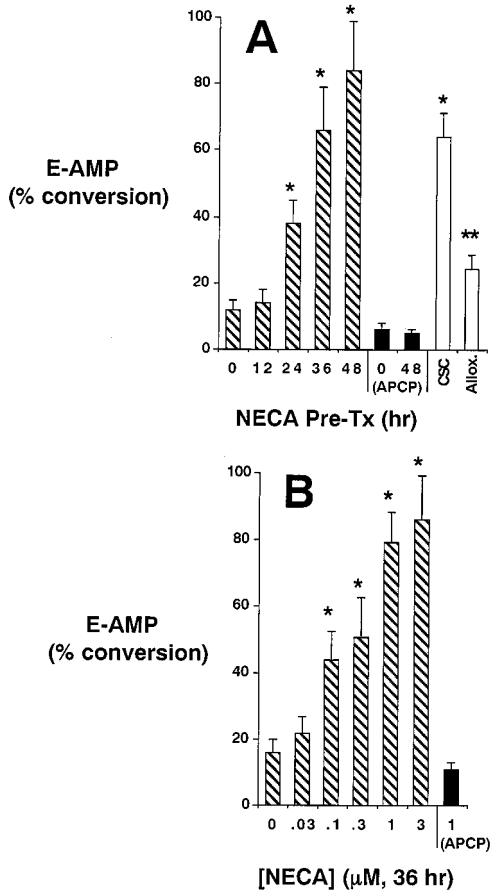

FIGURE 1. Induction of endothelial surface CD73 bioactivity by the adenosine analogue NECA. Confluent HMVEC monolayers were exposed to NECA (1 μM) for indicated periods of time (*A*) or to indicated concentrations of NECA for 36 h (*B*). Monolayers were washed, and surface CD73 activity was determined by HPLC analysis of E-AMP conversion to E-ADO (▧). To determine specificity, a similar analysis was performed in the presence of the CD73 inhibitor APCP (■) or in the presence of adenosine receptor antagonists (□) to adenosine A_{2A} receptor (CSC, 10 μM) or A_{2B} receptor (alloxazine, 10 μM). Data are derived from six to eight monolayers in each condition, and results are expressed as mean ± SEM percentage E-AMP conversion. *, Statistically different from untreated controls ($p < 0.025$). **, Statistically different from 48-h NECA treatment ($p < 0.025$).

48-h NECA-treated monolayers (Fig. 1*A*, filled bars). Similarly, as shown in Fig. 1*A* (open bars), the addition of antagonists (18) to adenosine A_{2A} receptor (CSC, 10 μM) or A_{2B} receptor (alloxazine, 10 μM) in coincubation with NECA revealed a predominant role for the A_{2B} receptor ($p < 0.025$ compared with NECA alone), since A_{2A} receptor antagonists did not influence functional CD73 induction (p = not significant compared with NECA alone). Fig. 1*B* depicts a concentration-dependent increase in CD73 activity (ANOVA, $p < 0.01$), with a maximal increase of 4.7- ± 0.6-fold at 3 μM and an apparent EC_{50} of ~0.8 μM. Addition of APCP revealed these differences to be specific for CD73 ($p < 0.001$ in the presence and absence of APCP). Similar results of increased CD73 activity were observed with endothelial exposure to 5′-AMP (2.8- ± 0.3-fold increase at 100 μM for 36 h, $p < 0.05$) as well as the adenosine A_2 receptor agonist 5′-(*N*-cyclopropyl)carboxamidoadenosine (3.6- ± 0.6-fold increase at 10 μM for 36 h, $p < 0.01$). Pulsing endothelial cells with NECA (i.e., 1-h exposure followed by removal) was found to be as effective as continuous exposure to NECA (data not shown), suggesting that continuous adenosine receptor activation is not required for this response. Previous studies have indicated that endothelial cells express adenosine A_1, A_2, and A_3 receptors. However, a time-course and concentration-response curve using the adenosine A_1 receptor agonist ADAC did not increase surface CD73 functional activity (maximal 5.5 ± 2.1% increase in CD73 activity with 3 μM ADAC at 36 h, p = not significant compared with no ADAC), suggesting that the increase in CD73 activity is specific for activation of the A_2 receptor.

To determine whether adenosine A_2 receptor-mediated CD73 surface activity represents increased surface protein, intact HMVEC monolayers were biotinylated, CD73 was immunoprecipitated, and blots were probed with avidin-peroxidase. As shown in Fig. 2, a time course of NECA pretreatment (5 μM NECA) revealed increased CD73 with increasing time of exposure (maximal induction of 17.2- ± 3.8-fold at 36 h by densitometry, $p < 0.001$). Increases in both surface activity (85.0 ± 6.2% inhibition) and surface protein levels (72.2 ± 10.5% inhibition) were inhibited by coincubation of HMVEC with NECA (3 μM) in the presence of cycloheximide (2 μg/ml, data not shown), suggesting the requirement for new protein synthesis.

Activation of HMVEC CD73 mRNA

We next extended these studies to examine CD73 mRNA. Adenosine receptor coupling is well characterized and indicates that A_1 and A_3 receptor activation decreases intracellular cAMP while A_2 receptor ligation increases cAMP (18). Moreover, we have specifically determined that adenosine A_{2B} receptor activation is coupled to increased intracellular cAMP (4). Since the CD73 promoter bears a consensus CRE (12), RT-PCR analysis was used to determine whether adenosine A_2 receptor activation induces CD73 mRNA. As shown in Fig. 3, 25 cycles of PCR revealed no detectable CD73 message in control HMVEC, but exposure to the adenosine A_2 receptor agonist NECA (5 μM) induced a time-dependent increase in CD73 mRNA (comparison of CD73-to-β-actin ratios

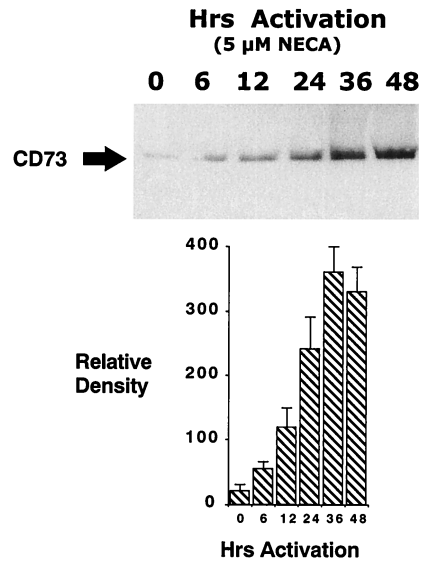

FIGURE 2. Endothelial induction of CD73 surface protein by the adenosine analogue NECA. Confluent HMVEC were exposed to NECA (5 μM) for indicated periods of time. Monolayers were washed, surface proteins were biotinylated, and cells were lysed. CD73 was immunoprecipitated (mAb 1E9) and resolved by SDS-PAGE, and resultant Western blots (*top*) were probed with avidin peroxidase. *Bottom*, Densitometry from resultant blots (pooled from three experiments); data are expressed as mean ± SEM relative densitometry units.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

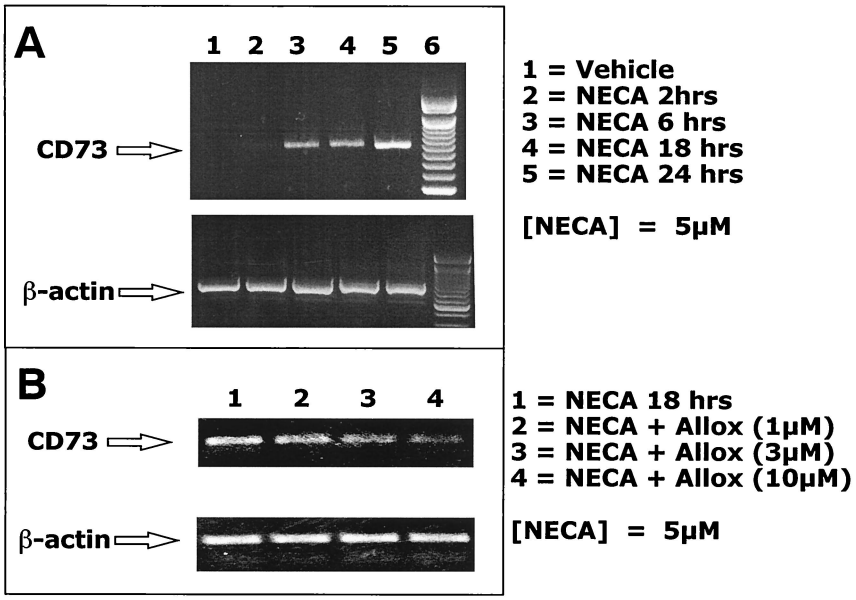

FIGURE 3. Adenosine induction of endothelial CD73 message. RT-PCR was used to determine CD73 message levels in response to the adenosine analogue NECA (*A*) or the combination of NECA and adenosine A_{2B} receptor antagonist alloxazine (Allox; *B*). Following exposure to NECA (5 μM) for indicated periods of time, total RNA was isolated, treated with DNase I, and amplified by RT-PCR using CD73-specific primers. *A,* Lanes represent (*left* to *right*) vehicle control followed by 2, 6, 18, and 24 h of exposure to NECA, respectively. *Lane 6,* 100-bp ladder. As indicated, corresponding β-actin mRNA expression was used as a housekeeping gene. *B,* Lanes represent (*left* to *right*) 18 h of exposure to NECA alone or in combination with 1, 3, or 10 μM alloxazine, respectively. Data are representative of three experiments in each case.

by ANOVA, $p < 0.01$). Additionally, this NECA-induced response was inhibited by the relatively selective adenosine A_{2B} receptor antagonist aloxazine (Fig. 3*B*). These data suggest that adenosine A_{2B} receptor activation induces CD73 mRNA.

Role of elevated cAMP in CD73 induction

To define the relationship of intracellular cAMP to CD73 induction, other cAMP agonists were used to examine HMVEC CD73 induction. Fig. 4 demonstrates the influence of the cAMP agonists forskolin and PGE_2 on CD73 bioactivity (examined by HPLC) and revealed that both forskolin and PGE_2 induced a concentration-dependent increase in surface CD73 activity (ANOVA, $p < 0.01$ for both forskolin and PGE_2). As shown in Fig. 5, forskolin (10 μM) and PGE_2 (1 μM) readily induced CD73 mRNA. As a control for specificity, endothelial exposure to the selective adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarboxoamidoadenosine, which decreases intracellular cAMP (19), did not influence CD73 mRNA levels (data not shown). These data indicate that elevation of intracellular cAMP induces CD73 mRNA and suggest that adenosine-mediated induction of CD73 is coupled to intracellular cAMP levels.

The cloned CD73 promoter bears a classical CRE (TGACGTC at positions −81 to −87 relative to the transcription start site) (12). This DNA motif is the functional binding site for the transcriptional coactivator CREB in many genes (13). Therefore, to determine whether adenosine agonists induce DNA-protein interactions at this CRE site, oligonucleotides spanning the CD73 CRE were designed and examined by EMSA using HMVEC nuclear lysates. As shown in Fig. 6, induction of CD73 DNA-binding activity was evident in monolayers pre-exposed to NECA (10 μM) for 4 or 16 h at 37°C. A similar DNA-binding activity was evident in controls not exposed to NECA, but to a far lesser extent. Forskolin (1 μM, 4 h) served as a positive control for this DNA-binding activity. Inclusion of anti-CREB-1 but not anti-CREB-2 Ab during these incubations resulted in a clear supershift, indicating the likelihood that specific DNA-binding activity represents CREB-1.

Functional impact of adenosine-induced CD73

Our previous studies revealed that adenosine A_{2B} receptor activation (via 5′-AMP) promotes endothelial barrier function, measured as a decrease in paracellular flux of labeled tracer (4). This activity required endothelial surface CD73 conversion of 5′-AMP to adenosine. It is not known whether increased CD73 (via induction by cAMP agonists) contributes to increased 5′-AMP responses. Thus, we defined whether forskolin-induced CD73 influenced endpoint functional responses in intact HMVEC monolayers. As shown in Fig. 7, endothelial pre-exposure to forskolin (5 μM for 36 h, conditions that induce CD73; see Figs. 4 and 5) resulted in a nearly 10-fold enhanced response to exogenous administration of 5′-AMP (apparent EC_{50} ~2 μM) compared with HMVEC not pre-exposed to forskolin (apparent EC_{50} ~22 μM). This response was specific for CD73, since addition of APCP blocked this response by 77.3 ± 5.6% at 30 μM 5′-AMP ($p < 0.001$ compared with no APCP). These data indicate that CD73 induced by elevations in intracellular cAMP manifests as an endpoint functional response (increased endothelial barrier function).

Discussion

Adenosine exerts paracrine and autocrine actions on most cell types. Functional sequelae of adenosine receptor activation are diverse, and for this reason, a detailed understanding of the enzymatic pathways that liberate adenosine is important to defining potential therapies. In these studies, we explored the regulation of endothelial CD73, a primary determinant for localized production of adenosine at vascular interfaces (2). These studies revealed that exogenous 5′-AMP or adenosine transcriptionally regulates CD73 expression through elevation of intracellular cAMP. Moreover, cAMP-induced CD73 is functional and promotes endothelial barrier function in response to 5′-AMP.

CD73 has been studied widely at the functional and genomic level (2, 20). Cloning of the promoter region of human CD73 identified a CRE upstream from the CD73 start codon (21). Since genes containing such elements can be regulated by changes in intracellular cAMP (22), and we have previously demonstrated that adenosine elevates endothelial intracellular cAMP (4), we examined the impact of adenosine on CD73 transcriptional responses and cell surface expression. Our results of CRE-dependent induction of CD73 confirm previous work alluding to differences in CD73 regulation between endothelial and myeloid cell types (23). For instance, original studies suggested that CD73 promoter constructs lacking the consensus CRE were still functional and that CD73 production in myeloid cell lines was not influenced by

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

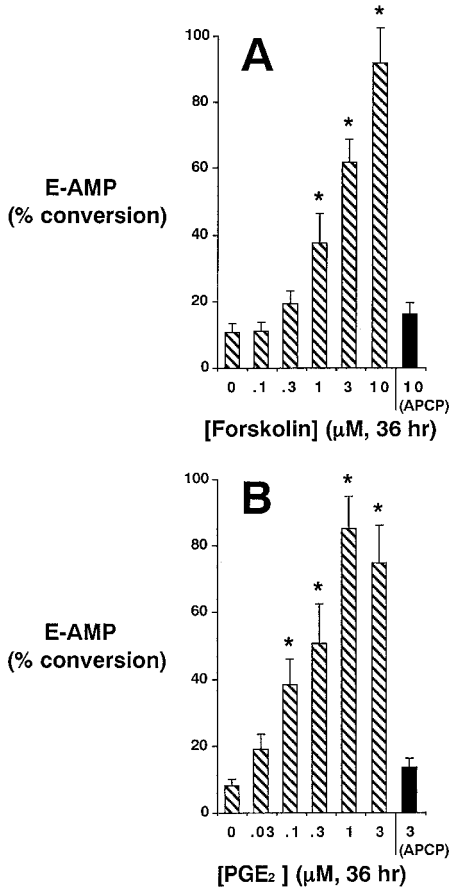

FIGURE 4. Induction of endothelial CD73 bioactivity by the cAMP agonists. Confluent HMVEC monolayers were exposed to adenylate cyclase activator forskolin (*A*) or PGE_2 (*B*) at indicated concentrations for 36 h. Monolayers were washed, and surface CD73 activity was determined by HPLC analysis of E-AMP conversion to E-ADO (▧). To determine specificity, a similar analysis was performed in the presence of the CD73 inhibitor APCP (■). Data are derived from five to nine monolayers in each condition, and results are expressed as mean ± SEM percentage E-AMP conversion. *Statistically significant vs untreated controls ($p < 0.025$).

agents that elevate intracellular cAMP (12). It has been reported, however, that agents that activate protein kinase C (i.e., PMA) strongly induce CD73 mRNA and activity in myeloid leukocytes (HL-60 cells) (24). Similarly, in Jurkat T cells, PMA induces CD73 gel shift using oligonucleotides corresponding to the CRE site (25). These latter results may be explained by the fact that induction of CRE-bearing genes can occur through many pathways, including activation both of protein kinase A and C (13). Similarly, our EMSA results demonstrating CREB-1 binding emphasize differences between endothelial cells and myeloid cells. For instance, in Jurkat T cell extracts, neither CREB-1 nor CREB-2 was found to bind to the CD73 promoter CRE site (25), suggesting some degree of cell specificity for this response. Taken together, these observations suggest that while myeloid and endothelial CD73 lack structural variability (23), significantly different regulatory properties exist.

Following activation, neutrophils release a number of biologically active mediators. Two such mediators are 5′-AMP and its metabolite, adenosine (4, 7), which can exist in human plasma at micromolar concentrations (17). Measured concentrations of 5′-AMP and adenosine are in a range sufficient to influence functions such as epithelial electrogenic Cl^- secretion and endothelial permeability (4, 7). Metabolism of 5′-AMP to adenosine requires 5′-nucleotidase; both intracellular (cytosolic 5′-nucleotidase) and cell surface (5′-ectonucleotidase/CD73) forms are expressed in most cell types (2). In the present studies, we have not addressed whether both forms are similarly regulated by adenosine. Together with other ectonucleotidases, 5′-ectonucleotidase completely hydrolyzes ATP to adenosine (1, 26). Through inhibition of endothelial 5′-ectonucleotidase with APCP, our data demonstrate that the 5′-AMP-mediated decrease in endothelial permeability requires conversion of 5′-AMP to adenosine and that CD73 may be rate limiting (i.e., increased CD73 results in parallel increases in 5′-AMP-mediated bioactivity). The competitive inhibitor APCP abolishes the influence of 5′-AMP, whereas the less potent, noncompetitive inhibitor mAb 1E9 substantially diminishes the influence of 5′-AMP (4), indicating that CD73 may influence endothelial cell permeability in addition to its previously characterized role as a cell adhesion molecule (8). These results may have broad implications. For instance, it is not presently known how CD73 expression is regulated in the healthy vasculature. A number of physiologically relevant mediators flow across the healthy endothelium at any point in time (e.g., nucleotides, hormones, chemokines, and cytokines). Many of these mediators bind and activate signal transduction pathways in the endothelium (i.e., elevation in cAMP, Ca^{2+}, etc.). Thus, it is possible that basal, low level activation of the healthy endothelium regulates CD73 expression as a bystander process and, in turn, regulates basal vascular permeability. In this regard, adenosine may serve as a feedback mechanism to directly regulate CD73.

Once liberated in the extracellular space, adenosine either is taken up into the cell (through dipyridamole-sensitive carriers) or interacts with cell surface adenosine receptors (5). Presently, four subtypes of G protein-coupled adenosine receptors exist, designated A_1, A_{2A}, A_{2B}, and A_3. These receptors are classified according to use of pertussis toxin-sensitive pathways (A_1 and A_3) or adenylate cyclase activation pathways (A_{2A} and A_{2B}) (5). Endothelial cells of many origins express constitutive adenosine receptors (5), primarily of the A_{2A} and A_{2B} subtypes (27–30). The present studies define a transcriptional pathway mediated by adenosine receptor activation, including but not limited to CD73. In fact, many gene promoters bear response elements for cAMP (13), and, therefore, it is quite likely that adenosine also activates a number of other genes. Intriguingly, it is possible that cellular hypoxia might induce endothelial CD73. While we have not studied this aspect directly, recent work has defined a role for CRE and CRE-binding protein (CREB) in hypoxia-elicited induction of a number of proteins (31, 32), and this pathway may be particularly relevant to a number of cardiovascular diseases in vivo. For instance, adenosine production in the ischemic myocardium is attributable to activity of CD73 (33), and both CD73 activity and adenosine metabolism have been implicated in cardiac preconditioning by brief periods of ischemia (34, 35). Importantly in this regard, it was recently shown that adenosine (via activation of A_1 receptors) can directly regulate extracellular adenosine levels in rat cardiac fibroblasts (36), providing a potential feed-forward loop in this metabolic process. Thus, whether adenosine-mediated CD73 induction occurs at the level of the ischemic myocardium is not known at the present time.

We show in these studies that cAMP agonist-induced CD73 enhances 5′AMP-mediated promotion of endothelial barrier function. Our recent studies revealed that 5′-AMP promotes endothelial barrier in at least two different endothelial sources (umbilical vein and microvascular cells) (4), primarily through use of the A_{2B} receptor. At present, the mechanism(s) of cAMP-mediated regulation of endothelial barrier are not clear. Endothelial cells, like other eukaryotic nonmuscle cells, contain myosin, actin, and a

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

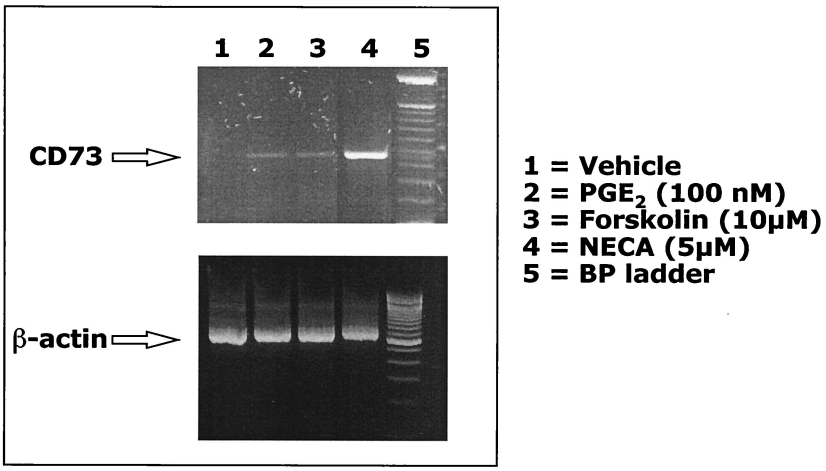

FIGURE 5. Induction of endothelial CD73 message by cAMP agonists. RT-PCR was used to determine CD73 message levels in response to indicated cAMP agonists at indicated concentrations for 18 h. Following exposure to each agonist, total RNA was isolated, treated with DNase I, and amplified by RT-PCR using CD73-specific primers. Lanes represent (*left* to *right*) vehicle control, PGE_2, forskolin, and NECA, respectively. *Lane 6*, 100-bp ladder. *Bottom*, Corresponding β-actin mRNA expression is shown to demonstrate equivalent loading. Data are representative of three experiments.

number of associated proteins that function in cell motility (37–39). It is now generally accepted that phosphorylation of the 20-kDa light chain of myosin is required to activate the myosin ATPase essential for endothelial shape changes (40). This process is catalyzed by myosin light chain kinase, a Ca^{2+}-calmodulin-dependent enzyme system that produces a transfer of the γ-phosphate of ATP onto myosin light chain. Moreover, it has been demonstrated that intracellular levels of cAMP are tightly coupled to myosin light chain activity (41). Pharmacologic maneuvers that increase intracellular levels of cAMP (42) or cGMP (43) result in a dephosphorylation of myosin, a redistribution of actin and myosin II, and a decrease in endothelial isometric tension, indicating that endothelial permeability is likely coupled to myosin light chain kinase activity. Finally, recent in vivo work has suggested that endotoxin-induced increases in vascular permeability are associated with accumulation of neutrophils and that dysfunction may be coupled to elevations in cGMP (44).

An important point of consideration is whether this adenosine-mediated CD73 induction pathway is relevant to basal and/or acute responses in vivo, particularly since the components may take up to 36–48 h for development. First, the kinetics of this response in an in vivo system is not known. Based on previous studies of other regulated endothelial surface molecules (e.g., leukocyte adhesion molecules) (45), the in vivo kinetics tend to be more rapid than those in vitro, and thus, one would predict that these responses with CD73 would occur more rapidly than in our model (24–36 h). Nonetheless, as a second point, it is important to note that endothelial cells have long been known to express functional CD73 under "basal conditions" (1) and that our work sheds light onto pathways to regulate CD73 expression. Under conditions of both

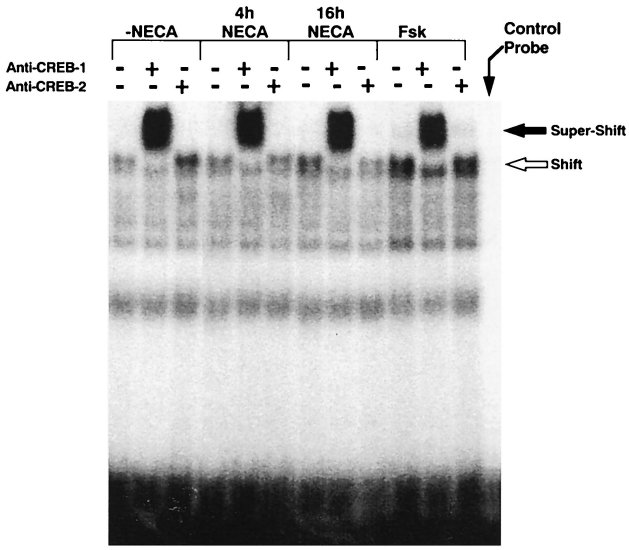

FIGURE 6. EMSA for CD73 CRE. Confluent HMVEC monolayers were exposed to (*left* to *right*) vehicle control, NECA (10 μM) for 4 or 16 h, or forskolin (Fsk, 1 μM) for 4 h. Nuclear lysates were generated and mixed with ^{32}P-labeled double-stranded oligonucleotide probes spanning positions –71 to –97 of the CD73 promoter containing CRE consensus sequence in the presence or absence of anti-CREB-1 or anti-CREB-2 Ab (as indicated) or CRE-mutated oligonucleotide (control probe). DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel followed by autoradiography. Data are representative of three experiments.

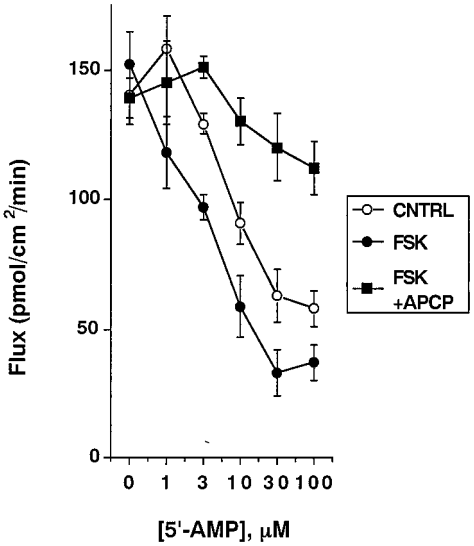

FIGURE 7. CD73 induced by elevation of cAMP enhances endothelial permeability responses to exogenous 5′-AMP. Confluent HMVEC monolayers grown on semipermeable supports were exposed to medium (CNTRL, ○) or medium containing forskolin (FSK, 5 μM, ●) for 36 h. Monolayers were washed, and the influence of indicated concentrations of 5′-AMP on monolayer permeability to 70-kDa FITC-dextran was determined. To define specificity, a similar analysis was performed in the presence of the CD73 inhibitor APCP (■). Data are derived from five to six monolayers in each condition, and results are expressed as mean ± SEM flux rate.

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022

acute and chronic inflammation, adenosine is a demonstrated mediator (46), and thus, it is reasonable to assume that adenosine may be an endogenous regulator of CD73 expression. Thirdly, these adenosine-regulated CD73 responses may well function in a chronic inflammatory setting. For example, CD73 has been shown to function as an adhesion molecule for lymphocytes (8, 23), and thus, cells that characteristically traffic later in the inflammatory response (i.e., lymphocytes and monocytes) may be orchestrated by endothelial expressed CD73. Taken together, it is not difficult to hypothesize a role for adenosine regulation of basal as well as acute induction of endothelial CD73.

These results confirm previous studies defining the function of CD73 on vascular endothelial cells and reveal a previously unappreciated mechanism of cAMP-mediated regulation of CD73 on vascular endothelia. This regulatory pathway extends to the level of transcriptional induction and unveils a potential role for CD73 regulation of vascular permeability.

Acknowledgments

We thank Dr. Linda Thompson for supplying Abs and Dr. Walter Bruyninckx for critical review of the manuscript.

References

1. Pearson, J. D., and J. L. Gordon. 1985. Nucleotide metabolism by endothelium. *Annu. Rev. Physiol. 47:617.*
2. Zimmerman, H. 1992. 5′-Nucleotidase: molecular structure and functional aspects. *Biochem. J. 285:345.*
3. Strohmeier, G. R., W. I. Lencer, T. W. Patapoff, L. F. Thompson, S. L. Carlson, S. J. Moe, D. K. Carnes, R. J. Mrsny, and J. L. Madara. 1997. Surface expression, polarization, and functional significance of CD73 in human intestinal epithelia. *J. Clin. Invest. 99:2588.*
4. Lennon, P. F., C. T. Taylor, G. L. Stahl, and S. P. Colgan. 1998. Neutrophil-derived 5′-adenosine monophosphate promotes endothelial barrier function via CD73-mediated conversion to adenosine and endothelial A_{2B} receptor activation. *J. Exp. Med. 188:1433.*
5. Stiles, G. L. 1992. Adenosine receptors. *J. Biol. Chem. 267:6451.*
6. Mertens, S., T. Noll, R. Spahr, A. Krutzfeldt, and H. M. Piper. 1990. Energetic response of coronary endothelial cells to hypoxia. *Am. J. Physiol. 258:H689.*
7. Madara, J. L., T. W. Patapoff, B. Gillece-Castro, S. P. Colgan, C. A. Parkos, C. Delp, and R. J. Mrsny. 1993. 5′-Adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J. Clin. Invest. 91:2320.*
8. Airas, L., J. Hellman, M. Salmi, P. Bono, T. Puurunen, D. J. Smith, and S. Jalkaanen. 1995. CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. *J. Exp. Med. 182:1603.*
9. Resta, R., S. W. Hooker, A. B. Laurent, S. M. Jamshedur Rahman, M. Franklin, T. B. Knudsen, N. L. Nadon, and L. F. Thompson. 1997. Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5′-nucleotidase (CD73). *J. Clin. Invest. 99:676.*
10. Strohmeier, G. R., S. M. Reppert, W. I. Lencer, and J. L. Madara. 1995. The A2b adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J. Biol. Chem. 270:2387.*
11. Stelzner, T. J., J. V. Weil, and R. F. O'Brien. 1989. Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *J. Cell. Physiol. 139:157.*
12. Hansen, K. R., R. Resta, C. F. Webb, and L. F. Thompson. 1995. Isolation and characterization of the promoter of the human 5′-nucleotidase (CD73)-encoding gene. *Gene. 167:307.*
13. Montminy, M. 1997. Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem. 66:807.*
14. Riley, M. V., B. S. Winkler, C. A. Starnes, and M. I. Peters. 1996. Adenosine promotes regulation of corneal hydration through cyclic adenosine monophosphate. *Invest. Ophthalmol. Vis. Sci. 37:1.*
15. Jamal, Z., A. Afkham-Ebrahimi, and E. D. Saggerson. 1988. A novel assay for 5′nucleotidase using 1,N^6-etheno-AMP as substrate, and comments on the properties of the reaction product, ethenoadenosine. *Biochem. J. 250:369.*
16. Zünd, G., S. Uezono, G. L. Stahl, A. L. Dzus, F. X. McGowan, P. R. Hickey, and S. P. Colgan. 1997. Hypoxia enhances endotoxin-stimulated induction of functional intercellular adhesion molecule-1 (ICAM-1). *Am. J. Physiol. 273:C1571.*
17. Guieu, R., B. Dussol, C. Devaux, J. Sampol, P. Brunet, H. Rochat, G. Bechis, and Y. F. Berland. 1998. Interactions between cyclosporine A and adenosine in kidney transplant recipients. *Kidney Int. 53:200.*
18. Fredholm, B. B., M. P. Abbracchio, G. Burnstock, J. W. Daly, T. K. Harden, K. A. Jacobson, P. Leff, and M. Williams. 1994. Nomenclature and classification of purinoceptors. *Pharmacol. Rev. 46:143.*
19. Baraldi, P. G., B. Cacciari, G. Spalluto, X. D. Ji, M. E. Olah, G. Stiles, S. Dionisotti, C. Zocchi, E. Ongini, and K. A. Jacobson. 1996. Novel N^6-(substituted-phenylcarbamoyl)adenosine-5′-uronamides as potent agonists for A3 adenosine receptors. *J. Med. Chem. 39:802.*
20. Resta, R., Y. Yamashita, and L. F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol. Rev. 161:95.*
21. Hansen, K. R., R. Resta, C. F. Webb, and L. F. Thompson. 1995. Ecto-5′-nucleotidase (CD73): genomic cloning and characterization of regions upstream of the translation start site. In *Purine and Pyrimidine Metabolism in Man.* A. Sahota and M. Taylor, eds. Plenum Press, New York, p. 689.
22. Borrelli, E., J.-P. Montmayeur, N. S. Foulkes, and P. Sassone-Corsi. 1992. Signal transduction and gene control: the cAMP pathway. *Crit. Rev. Oncog. 3:321.*
23. Airas, L., J. Niemela, M. Salmi, T. Puurunen, D. J. Smith, and S. Jalkanen. 1997. Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. *J. Cell Biol. 136:421.*
24. Clifford, E. E., K. A. Martin, P. Dalal, R. Thomas, and G. R. Dubyak. 1997. Stage-specific expression of P2Y receptors, ecto-apyrase, and ecto-5′- nucleotidase in myeloid leukocytes. *Am. J. Physiol. 273:C973.*
25. Spychala, J., A. G. Zimmermann, and B. S. Mitchell. 1999. Tissue-specific regulation of the ecto-5′-nucleotidase promoter: role of the camp response element site in mediating repression by the upstream regulatory region. *J. Biol. Chem. 274:22705.*
26. Plesner, L. 1995. Ecto-ATPases: identities and functions. *Int. Rev. Cytol. 158:141.*
27. Schiele, J. O., and U. Schwabe. 1994. Characterization of the adenosine receptor in microvascular coronary endothelial cells. *Eur. J. Pharmacol. 269:51.*
28. Sexl, V., G. Mancus, C. Holler, E. Gloria-Maercker, W. Schutz, and M. Freissmuth. 1997. Stimulation of the mitogen-activated protein-kinase via the A_{2A}-adenosine receptor on primary human endothelial cells. *J. Biol. Chem. 272:5792.*
29. Iwamoto, T., S. Umemura, Y. Toya, T. Uchibori, K. Kogi, N. Takagi, and M. Ishii. 1994. Identification of the adenosine A_2 receptor-cAMP system in human aortic endothelial cells. *Biochem. Biophys. Res. Commun. 199:905.*
30. Watanabe, H., W. Kuhne, P. Schwartz, and H. M. Piper. 1992. A_2-Adenosine receptor stimulation increases macromolecular permeability of coronary endothelial cells. *Am. J. Physiol. 262:H1174.*
31. Beitner-Johnson, D., and D. E. Millhorn. 1998. Hypoxia induces phosphorylation of the cyclic AMP response element-binding protein by a novel signaling mechanism. *J. Biol. Chem. 273:19834.*
32. Taylor, C. T., N. Fueki, A. Agah, R. M. Hershberg, and S. P. Colgan. 1999. Critical role of cAMP response element binding protein expression in hypoxia-elicited induction of epithelial TNFα. *J. Biol. Chem. 274:19447.*
33. Imai, S., M. Nakazawa, H. Imai, and H. Jin. 1987. 5′-Nucleotidase inhibitors and the myocardial reactive hyperemia and adenosine content. In *Topics and Perspectives in Adenosine Research.* E. Gerlach and B. F. Becker, eds. Springer-Verlag, Berlin, p. 416.
34. Minamino, T., M. Kitakaze, T. Morioka, K. Node, K. Komamura, H. Takeda, M. Inoue, M. Hori, and T. Kamada. 1996. Cardioprotection due to preconditioning correlates with increased ecto-5′-nucleotidase activity. *Am. J. Physiol. 270:H238.*
35. de Jonge, R., S. Bradamante, and J. W. de Jong. 1998. Cardioprotection by ischemic preconditioning: role of adenosine and glycogen. *Adv. Exp. Med. Biol. 431:279.*
36. Andresen, B. T., D. G. Gillespie, Z. Mi, R. K. Dubey, and E. K. Jackson. 1999. Role of adenosine A(1) receptors in modulating extracellular adenosine levels. *J. Pharmacol. Exp. Ther. 291:76.*
37. Dejana, E., M. Corada, and M. G. Lampugnani. 1995. Endothelial cell-to-cell junctions. *FASEB J. 9:910.*
38. Rubin, L. L. 1992. Endothelial cells: adhesion and tight junctions. *Curr. Opin. Cell Biol. 4:830.*
39. Lum, H., and A. B. Malik. 1994. Regulation of vascular endothelial barrier function. *Am. J. Physiol. 267:L223.*
40. Wysolmerski, R. B., and D. Lagunoff. 1990. Involvement of myosin light-chain kinase in endothelial cell retraction. *Proc. Natl. Acad. Sci. USA 87:16.*
41. Stevens, T., G. Brough, S. Shasby, I. McMurtry, D. Rodman, and D. Shasby. 1996. Inhibition of cAMP by intracellular Ca^{2+} corresponds with myosin phosphorylation in pulmonary artery endothelial cells. *FASEB J. 10:A110.*
42. Moy, A. B., J. V. Engelenhoven, J. Bodmer, J. Kamath, C. Keese, I. Ciaever, S. Shasby, and D. M. Shasby. 1996. Histamine and thrombin modulate focal adhesion through centripetal and centrifugal forces. *J. Clin. Invest. 97:1020.*
43. Yamada, Y., T. Furumichi, H. Furui, T. Yokoi, T. Ito, K. Yamauchi, M. Yokota, H. Hayashi, and H. Saito. 1990. Roles of calcium, cyclic nucleotides, and protein kinase C in regulation of endothelial permeability. *Arteriosclerosis 10:410.*
44. Fullerton, D. A., R. C. J. McIntyre, A. R. Hahn, J. Agrafojo, K. Koike, X. Meng, A. Banerjee, and A. H. Harken. 1995. Dysfunction of cGMP-mediated pulmonary vasorelaxation in endotoxin-induced acute lung injury. *Am. J. Physiol. 268:L1029.*
45. Henninger, D. D., J. Panes, M. Eppihimer, J. Russell, M. Gerritsen, D. C. Anderson, and D. N. Granger. 1997. Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *J. Immunol. 158:1825.*
46. Cronstein, B. N. 1994. Adenosine, an endogenous anti-inflammatory agent. *J. Appl. Physiol. 76:5.*

Downloaded from http://www.jimmunol.org/ by guest on February 24, 2022