Regulation of ecto-apyrase CD39 (ENTPD1) expression by phosphodiesterase III (PDE3)

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ABSTRACT **The ectoenzyme CD39 suppresses thrombosis and inflammation by suppressing ATP and ADP to AMP. However, mechanisms of CD39 transcriptional and post-translational regulation are not well known. Here we show that CD39 levels are modulated by inhibition of phosphodiesterase 3 (PDE3). RAW macrophages and human umbilical vein endothelial cells (HUVECs) were treated with the PDE3 inhibitors cilostazol and milrinone, then analyzed using qRT-PCR, immunoprecipitation/Western blot, immunofluorescent staining, radio-thin-layer chromatography, a malachite green assay, and ELISA. HUVECs expressed elevated CD39 protein (2-fold [$P<0.05$] for cilostazol and 2.5-fold [$P<0.01$] for milrinone), while macrophage CD39 mRNA and protein were both elevated after PDE3 inhibition. HUVEC ATPase activity increased by 25% with cilostazol and milrinone treatment ($P<0.05$ and $P<0.01$, respectively), as did ADPase activity (47% and 61%, $P<0.001$). There was also a dose-dependent elevation of soluble CD39 after treatment with 8-Br-cAMP, with maximal elevation of 60% more CD39 present compared to controls (1 mM, $P<0.001$). Protein harvested after 8-Br-cAMP treatment showed that ubiquitination of CD39 was decreased by 43% compared to controls. A DMSO or PBS vehicle control was included for each experiment based on solubility of cilostazol, milrinone, and 8-Br-cAMP. These results indicate that PDE3 inhibition regulates endothelial CD39 at a post-translational level.—Baek, A. E., Kanthi, Y., Sutton, N. R., Liao, H., Pinsky, D. J. Regulation of ecto-apyrase CD39 (ENTPD1) expression by phosphodiesterase III (PDE3). *FASEB J.* 27, 4419–4428 (2013). www.fasebj.org**

Key Words: vascular homeostasis · endothelium · cAMP

The processes of thrombosis and inflammation contribute significantly to the severity and progression of numerous disease states, including stroke and atherosclerosis (1, 2). Extracellular purinergic signaling through ATP and ADP has been shown to be a potent trigger for inflammatory cell recruitment and thrombosis. Modulating this pathway is the plasmalemmal ectonucleotidase, CD39/NTPDase1 (ectonucleoside triphosphate diphosphohydrolase). CD39 is expressed by a number of cell types, including endothelial cells and leukocytes, and hydrolyzes the terminal phosphate of ATP and ADP in an enzymatic cascade that generates AMP (3–5). By depleting the potent prothrombotic and proinflammatory signals of ADP and ATP, CD39 preserves homeostasis in the vascular environment.

A growing body of literature describes the involvement of CD39 and its apyrase activity in limiting thrombosis and inflammation (6), yet relatively little is known about the regulation of CD39, at either a transcriptional or translational level. We recently demonstrated that CD39 RNA and functional protein are up-regulated potently by cAMP in murine macrophages (7). To more fully understand how CD39 is regulated in cells of the vascular wall, we explored the effects of cAMP on CD39 in human endothelium. Our objective was to reveal additional facets underlying control of leukocyte-endothelial interaction in thrombosis and inflammation.

The role of cAMP in modulating vascular tone has been well established. In cardiac myocytes, increased cAMP leads to increased contractility and tachycardia. In vascular smooth muscle, cAMP acts as a vasodilator, by activating cAMP-dependent PKA, which then phosphorylates myosin light chain kinase and reduces its phosphorylation of myosin light chain, thereby inhibiting contractile function of the actin/myosin complex (8–10). cAMP also has a known important role in the endothelium, where it preserves endothelial monolayer barrier function (11).

To investigate the effect of cAMP in human endothelial cells on CD39 expression, we employed a pharmacologic approach to modulate intracellular levels of cAMP. Cilostazol has been administered as a safe and

Abbreviations: 8-Br-cAMP, 8-bromo-3′-5′-cyclic adenosine monophosphate; EBM-2/EGM-2, endothelial basal/growth medium; HUVEC, human umbilical vein endothelial cell; PDE3, phosphodiesterase 3; qRT-PCR, quantitative reverse transcription-PCR; RanBPM, Ran binding protein M; RAW cell, murine macrophage-like RAW 264.7 cell

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effective drug for the treatment of claudication in peripheral arterial disease; it inhibits phosphodiesterase 3 (PDE3), an enzyme that depletes intracellular cAMP by hydrolyzing its 3′-cyclic phosphate bond, by competitively binding to the cAMP-specific binding site of PDE3 (12, 13). Milrinone is used clinically in patients with heart failure to improve contractility. Milrinone was chosen as an independent tool to confirm the effect of PDE3 inhibition on the expression of CD39 (14). The cell membrane-permeable cAMP analog, 8-Bromo-cAMP (8-bromo-3′-5′-cyclic adenosine monophosphate), was also used to increase intracellular cAMP.

In this work, we show that increased intracellular cAMP (through the specific inhibition of PDE3) leads to the up-regulation of functional CD39 protein in human endothelial cells as well as in murine macrophages. Most significantly, we found that macrophages and endothelial cells regulate CD39 through separate pathways, with the former utilizing transcriptional up-regulation and the latter utilizing post-translational regulation.

MATERIALS AND METHODS

Cell culture

All chemical reagents were obtained from Invitrogen (Grand Island, NY, USA) unless specified. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords based on previously described methods (15). Fresh cords were washed. The umbilical vein was attached to a 10 ml syringe and flushed with isotonic saline buffer. The cord was incubated with 2% collagenase 3 (Worthington Biochemical, Lakewood, NJ, USA) in HBSS buffered with HEPES at 37°C. Veins were then flushed with isotonic buffer, and the flow through was centrifuged at 4°C, 190 *g*, 7 min. Pellets were plated onto 35 mm plates coated with 0.2% gelatin. Cells were not used beyond passage 4. Endothelial basal medium (EBM-2) and endothelial growth medium (EGM-2) supplement bullet kits (Lonza, Allendale, NJ, USA) were reconstituted according to the manufacturer's instructions. RAW cells (murine macrophage-like RAW 264.7 cells) were obtained from ATCC (Manassas, VA, USA), and cultured in RPMI 1640 medium supplemented with penicillin (50 U/ml), streptomycin (5 μg/ml), and 10% fetal bovine serum (Invitrogen). Cells were treated with 8-Br-cAMP (Sigma, St. Louis, MO, USA), PDE3 inhibitors (cilostazol or milrinone; Sigma), or DMSO (Fisher, Pittsburgh, PA, USA) control for indicated times in serum- and supplement-free EBM-2 medium or serum-free RPMI 1640 medium.

Quantitative reverse transcription-PCR (qRT-PCR)

qRT-PCR was used to quantify RNA levels. HUVECs were plated onto 0.2% gelatin-coated 6-well plates (Falcon). When cells were confluent, they were serum-starved in EBM-2 and then treated with a range of doses of cilostazol (30–100 μM) or DMSO (1 μl/ml) (16, 17). After treatment, cells were washed twice in PBS, and then total RNA was isolated using RNeasy kits (Qiagen, Valencia, CA, USA). cDNA was made using cDNA synthesis kits (Applied Biosystems, Grand Island, NY, USA). Real-time qPCR was carried out using the 7000 detection system (Applied Biosystems) with 2× universal mastermix and primers for human CD39 and human β_2 microglobulin (Applied Biosystems). All data were normalized to β_2 microglobulin.

Whole-cell protein isolation

Following treatment with PDE3 inhibitors or DMSO as a negative control, cells were washed twice with PBS (Invitrogen), scraped with a rubber policeman, and suspended in ice-cold RIPA buffer supplemented with a tablet of a complete mini protease inhibitor tablet (Roche, Branchburg, NJ, USA). Cells were homogenized by 10 strokes through a sterile 30 gauge 1 ml insulin syringe. Lysed cells were centrifuged for 10 min at 13,000 *g* at 4°C, and the resulting supernatants were transferred to new tubes. The samples were flash frozen in aliquots and stored at −80°C. Concentrations were determined *via* a colorimetric protein assay (Bio-Rad, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

HUVECs were plated on 24-well plates (Corning, Tewksbury, MA, USA) coated with 0.2% gelatin and grown until confluent. Cells were serum starved for 6 h before serum-free treatment with 100, 250, 500, and 1000 μM 8-Br-cAMP for 8 h. Medium was transferred into microcentrifuge tubes (Eppendorf, Hauppauge, NJ, USA), and samples were centrifuged (500 *g*, 5 min, 4°C). Then 100 μl of cell-free supernatants were loaded onto 96-well plates (Nunc-Nalgene, Lafayette, CO, USA) that had been coated overnight at 4°C with a rat anti-CD39 antibodies (5 μg/ml, MAB4398, R&D, Minneapolis, MN, USA), and then blocked for 1 h at room temperature with 4% BSA (Sigma) in PBS. Standard lanes were included in each assay plate, with recombinant human CD39 (R&D) in PBS. Wells were then washed 3 times with 0.05% Tween-20 in PBS, and then probed with a sheep anti-CD39 antibody (5 μg/ml, 2 h, room temperature, R&D AF4398). Wells were washed 3 times again before probing with anti-sheep biotinylated antibody for 1 h at room temperature. This was followed by 3 more washes, and then avidin conjugated to alkaline phosphatase (1:800, 1 h, room temperature, Sigma) was added. Wells were then washed 3 times, and 5 mg of p-nitrophenyl phosphate, disodium salt in 5 ml of diethanolamine buffer (Thermo Scientific, Pierce, Rockford, IL, USA). Color was allowed to develop for up to 30 min, stopped with 2 N NaOH, and then absorption measured at 405 nm by a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

LDH assay

Using a colorimetric LDH assay (Pierce Biotechnology, Rockford, IL, USA), 15,000 cells were plated in triplicate in a 96-well format, and allowed to adhere overnight before treatment with either vehicle control (Medium 199 (Gibco, Grand Island, NY, USA)), or 8-Bromo-cAMP for 8 h. LDH was then measured according to manufacturer instructions. Briefly, cells were incubated in a reaction mixture containing lactate, tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium) and diaphorase, and a red formazan product was allowed to develop. Absorption was then measured at 490 and 680 nm using a SpectraMax M5 microplate reader.

Immunoprecipitation

HUVECs were plated on 24-well plates (Corning) coated with 0.2% gelatin and grown until confluent. Cells were serum-

starved for 6 h before serum-free treatment with 250 μM 8-Br-cAMP for 18 h.

Cell supernatants were collected on ice and incubated with gentle rocking overnight with protein-G agarose beads (Roche), and then incubated with anti-human CD39 antibody (Abcam, Cambridge, MA, USA) coated beads at 4°C. Beads were washed, and purified CD39 protein was eluted and quantified *via* standard Western blotting techniques.

cAMP measurement: competitive enzyme immunoassay

HUVECs were treated with cilostazol (100 μM) or an equivalent volume of DMSO, washed twice in PBS to wash away traces of DMSO, and then intracellular cAMP levels were measured according to manufacturer instructions of the competitive enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA).

Immunofluorescence

HUVECs were plated on 4-well plastic chamber slides (LabTek), with 0.2% gelatin coating. After cells had adhered and grown to 40% confluence, cells were treated in serum-free conditions with the following: Lonza EBM-2 basal medium with DMSO as vehicle control, cilostazol (50, 100, 400 μM), or milrinone (100, 200, 400 μM). Cells were then fixed for 15 min with ice-cold methanol, and then incubated overnight with a mouse anti-CD39 antibody (1:100) at 4°C followed by biotinylated horse anti-mouse IgG (1:100; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. An avidin-biotin-peroxidase complex was formed for 30 min at room temperature and then added to samples. Finally, a tetramethylrhodamine-labeled tyramide was added (PerkinElmer, Waltham, MA, USA). Nuclei were stained with DAPI. Slides were mounted with Prolong Gold (Invitrogen) and cured for 24 h. Slides were then imaged using a Nikon Eclipse TE2000-E microscope (Nikon, Tokyo, Japan).

Radio-thin layer chromatographic (TLC) analysis of CD39 activities

TLC was used to assess CD39 enzymatic function. Total protein of HUVECs was mixed with 1.0 mM [8-^{14}C]ATP (MP Biomedicals, Santa Ana, CA, USA) and 286 μM AOPCP (Sigma) in Medium 199 (Life Technologies) and incubated at 37°C for 30 min. Reactions were stopped using 8M formic acid, and the reaction mixture was then spotted onto silica gel TLC plates (Fluka, Invitrogen). A ladder of [8-^{14}C]ATP, [8-^{14}C]ADP, and [8-^{14}C]AMP (MP Biomedicals) was used. Nucleotides were separated by thin layer chromatography with isobutyl alcohol, isoamyl alcohol, 2-ethoxyethanol, ammonia, and water (9:6:18:9:15) (5). Separation was allowed to occur for 5 h before plates were dried, exposed to a phosphorimaging screen (Eastman Kodak Co., Rochester, NY, USA), and then analyzed using a Typhoon Trio^{+} Variable Mode Imager (GE Healthcare, Livonia, MI, USA).

Western blotting assay

Total protein was quantified and added to 4× sample buffer (Invitrogen), boiled for 3 min at 100°C, separated by 10% SDS-PAGE, and electrophoretically transferred onto PVDF membranes (Invitrogen). Membranes were probed with mouse monocolonal IgG$_1$ anti-CD39 antibodies (Abcam) and HRP-conjugated anti-mouse antibodies (Sigma), and then autoradiographed using enhanced chemiluminescence (ECL detection system; Amersham Biosciences, Piscataway, NJ, USA).

Statistics

Statistical analysis was performed using analysis of variance to detect differences between groups. Groups were analyzed with a one-way ANOVA, and further *post hoc* analysis using the student Newman-Keuls method was applied (Prism). Differences were considered significant if $P < 0.05$.

RESULTS

cAMP increases CD39 in the extracellular milieu

To analyze the potential role of cAMP in regulating human endothelial CD39, we began by measuring CD39 in HUVEC supernatants as a function of cAMP levels (**Fig. 1*A***). The cell-permeant cAMP analog 8-Bromo-cAMP was used to achieve a gradation in cAMP levels. ELISA data showed that CD39 protein increased with the addition of cAMP, in what appeared to be a cAMP dose-dependent manner. These supernatant samples were measuring CD39 as released into the surrounding

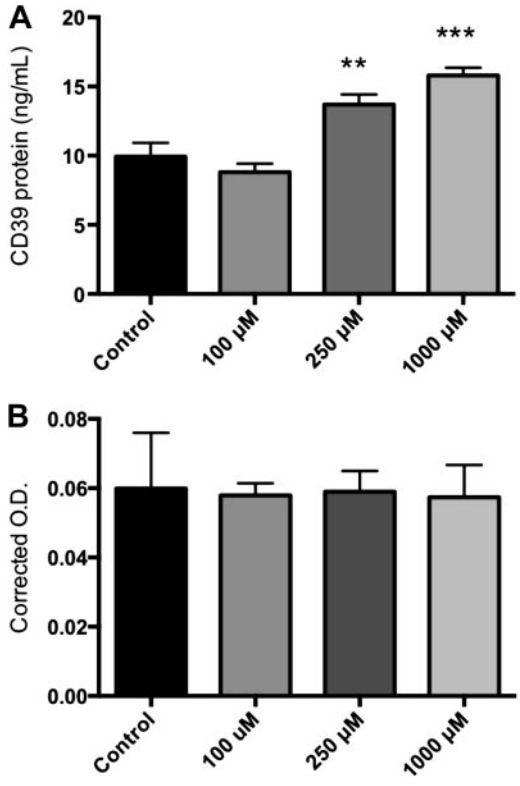

Figure 1. (*A*) CD39 protein levels measured by ELISA: Supernatants of HUVECs treated with either PBS (control) or 8-Br-cAMP were measured for CD39 *via* ELISA. Treatment with this cell permeable analogue of cAMP increases extracellular CD39. $P<0.001$ between 1 mM *vs.* control and $P<0.05$ between 250 μM and control. (*A*) LDH levels were measured in HUVECs treated with either vehicle control or 8-Br-cAMP. cAMP treatment did not change levels of LDH release.

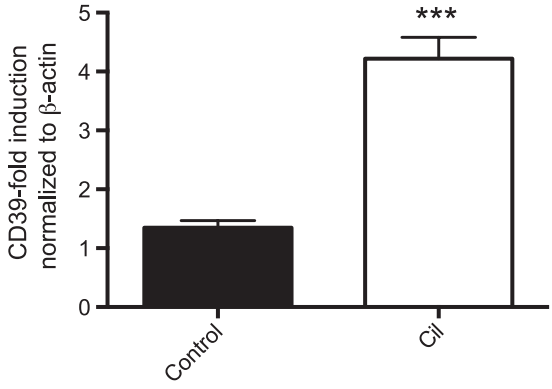

Figure 2. Effect of PDE3 inhibition on CD39 mRNA: RAW macrophages were treated with 30 μM cilostazol for 18 h and then harvested for measurement of total mRNA by qRT-PCR. $P<0.001$ compared to control, $n = 4$.

medium, thus indicating that intracellular cAMP levels regulate CD39 levels. This suggested that either more CD39 was being made or released, or less was being eliminated. LDH levels were also measured (Fig. 1*B*), in order to confirm that CD39 elevation after cAMP treatment was not a result of cytotoxicity. Results showed LDH levels in cAMP-treated samples were the same across all conditions as LDH levels in control samples.

PDE3 inhibition increases CD39 transcription

Having determined that extracellular CD39 elevated in response to cAMP, we subsequently sought to establish whether modulating intracellular cAMP would alter membrane CD39 expression. Cilostazol was a primary candidate to modulate cAMP because of its specific PDE3 inhibitory activity (13) and for its safety profile in humans. Treatment of RAW murine macrophages with cilostazol resulted in increased CD39 mRNA as well as protein as measured by qRT-PCR and Western blot, respectively (**Figs. 2** and **3**). To confirm this finding as a PDE3 class effect, RAW cells were also treated with milrinone, and CD39 protein was measured. These results were consistent with previous work, which showed that murine peritoneal macrophage CD39 is strongly up-regulated by treatment with 8-Bromo-cAMP (7) and that a defined transcriptional pathway exists for such regulation.

PDE3 inhibition increases CD39 protein expression

In human endothelial cells, there is no prior work establishing a link between cAMP and CD39. Given the significant role of endothelial cells in the maintenance of vascular homeostasis, HUVECs were used to investigate whether endothelium stimulated by cAMP can result in the up-regulation of CD39. To confirm that intracellular cAMP levels were indeed elevated after the indicated treatment times using PDE3 inhibitors in HUVECs, endogenous cAMP levels were measured with or without cilostazol (**Fig. 4**). Results demonstrated that

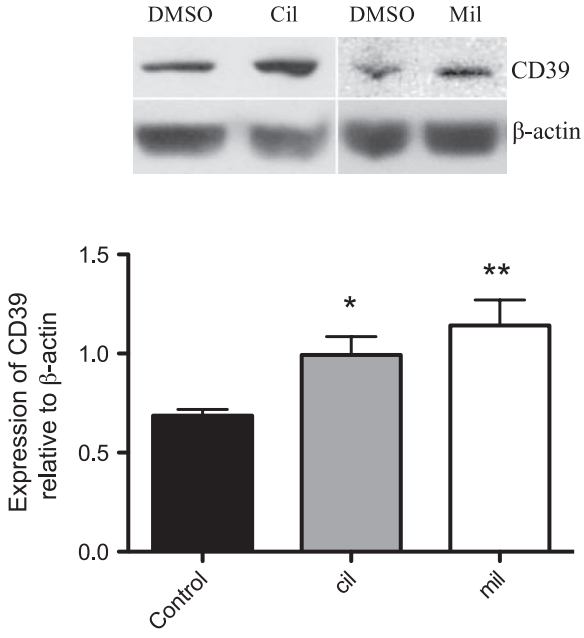

Figure 3. Effect of PDE3 inhibition on CD39 protein: RAW macrophages were treated with DMSO (control), 30 μM cilostazol, or 50 μM milrinone for 21 h, harvested for total protein, and analyzed by Western blot. RAW macrophages treated with cilostazol expressed 44% more CD39 compared to DMSO-treated controls ($P<0.05$, $n=3$). Macrophages treated with milrinone had a 74% increase in CD39 compared to DMSO ($P<0.05$, $n=3$).

cAMP levels were significantly elevated with cilostazol treatments. Surprisingly, endothelial cells exhibited markedly different responses to cAMP elevation, compared with macrophages subjected to PDE3 inhibition. There was no change in mRNA levels for CD39 (**Fig. 5**), whereas CD39 protein levels measured by Western blot elevated after treatment (**Fig. 6**), indicating that the increase in CD39 protein was not at the transcriptional level. Further evidence for the effect of PDE3 inhibition on CD39 protein is shown in **Fig. 7**. HUVECs were treated with a range of doses of either cilostazol or milrinone and compared to control wells treated with

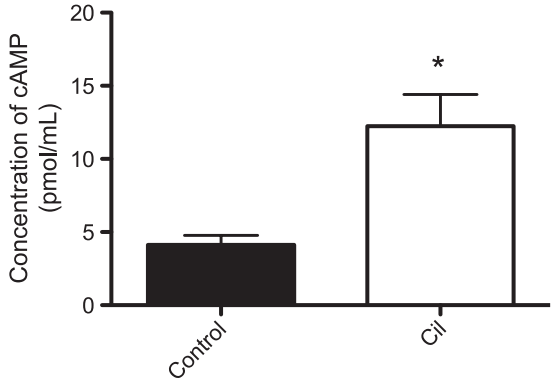

Figure 4. Quantification of intracellular cAMP level: HUVECs treated with 100 μM cilostazol show 2.97-fold increase in cAMP levels ($P<0.05$, $n=7$). Analysis of intracellular cAMP performed *via* competitive binding assay.

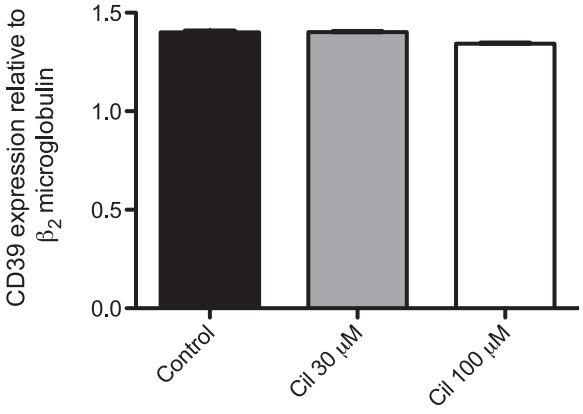

Figure 5. CD39 mRNA levels by qRT-PCR: HUVECs treated with 30 or 100 μM cilostazol showed no difference in CD39 mRNA expression compared to control (n=3).

DMSO as a vehicle control. Results from Western blots of whole cell lysates indicate a marked rise in CD39 protein levels as a function of cilostazol or milrinone dose. CD39 elevation peaks between 200 and 400 μM. Higher doses of milrinone were used in order to account for the relatively short half-life of milrinone compared to cilostazol, which has been cited as ~2.3 and 11 h, respectively (18, 19).

Immunofluorescent imaging of HUVECs treated with milrinone or cilostazol and then surface stained for CD39 with tetramethylrhodamine (**Figs. 8** and **9**) further confirmed that CD39 protein is elevated after treatment. This also demonstrated that CD39 expression is increased after PDE3 inhibition on the plasma membrane, where it has been shown to be active (20).

Since CD39 mRNA was not affected by treatment, we considered the possibility that regulation of CD39 in HUVECs may occur *via* post-translational modifications, possibly involving changes in the intracellular trafficking or release of CD39. Post-translational control was supported by an immunoprecipitation assay probing for ubiquitinated CD39 (**Fig. 10**). Here HUVECs were treated with either control DMSO or 8-Br-cAMP, followed by precipitation with beads coated with anti-human CD39 antibody. Using Western blot, samples were sequentially evaluated for ubiquitin and CD39 expression. The ratio of ubiquitin expression to CD39 expression was diminished after treatment with 8-Br-cAMP (by 43%, n=3, P<0.05). The data indicated that cAMP is likely raising CD39 levels by preventing CD39 ubiquitination in HUVECs.

Effect of PDE3 inhibition on CD39 activity

To assess whether increases in CD39 protein, as measured by Western blots of cell lysates, resulted in changes in ATPase and ADPase activity, an activity assay measuring the breakdown of radioactively labeled ATP was performed. Nucleotides were separated by TLC (**Fig. 11**). Signal density indicates the relative levels of nucleotides present in samples at the time of measurement and thus serves as a measure of CD39 activity. Peaks 1, 2, and 3 correspond to ADP measured in HUVEC samples treated with DMSO (control), cilostazol, or milrinone, respectively. Both cilostazol and milrinone increased CD39 activity significantly compared with control DMSO-treated HUVECs. As a confirmatory test of relative differences in CD39 activity after PDE3 inhibitor treatment, a malachite green assay was performed on HUVECs treated with varying doses of cilostazol or milrinone (**Fig. 12*A*, *B***). Here the presence of inorganic phosphates generated by the cleavage of ATP and ADP was measured, where higher concentrations of inorganic phosphate indicate greater apyrase activity. These measurements indicate that both ATP- and ADPase activity are elevated in cilostazol and milrinone-treated samples, thus confirming the results of the TLC assay.

DISCUSSION

CD39, a widely expressed plasmalemmal nucleotidase, occupies a unique niche in the vascular milieu, with a dichotomous role depending on physiological or diseased conditions. Under normal conditions in the absence of stressors such as hypoxia/ischemia, high shear stress, and triggers for inflammation (cytokines/chemokines), CD39 helps to maintain a homeostatic environment (21–23), maintaining blood fluidity and inhibiting inflammation. In the presence of these injurious conditions, however, one of the most potent signals for platelet aggregation, ADP, as well as proinflammatory signals such as ATP, are released into the

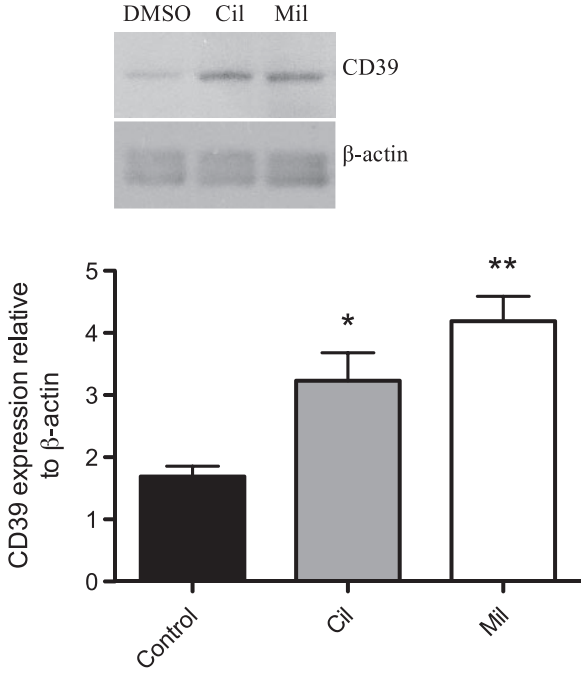

Figure 6. CD39 protein expression by Western blot: CD39 expression in HUVECs increased after 18 h of treatment with 50 μM of cilostazol or 200 μM of milrinone (2- and 2.5-fold, respectively) compared to DMSO control (P<0.05 and P<0.01, n=3).

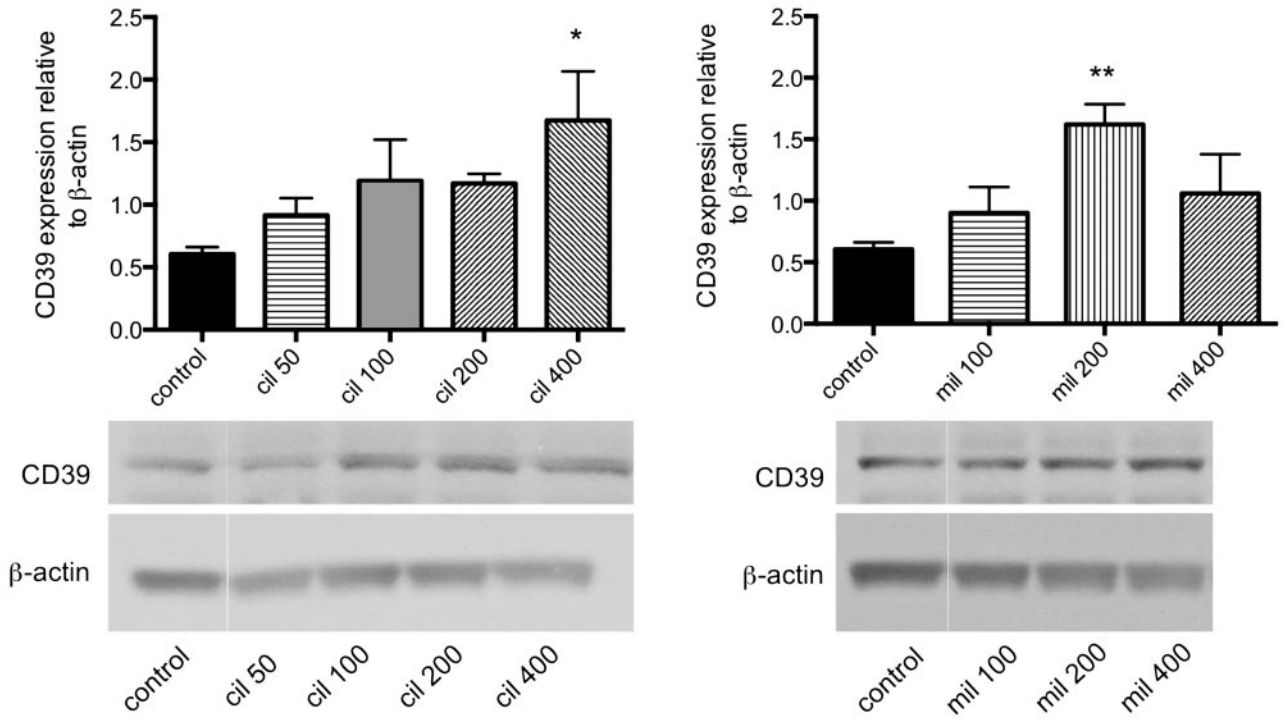

Figure 7. Effect of PDE3 inhibition on CD39 is dose dependent: HUVECs were treated with varying concentrations of cilostazol (50–400 μM) or milrinone (100–400 μM). After 18 h of treatment, cells were washed and collected for analysis *via* Western blot. The results show that CD39 increases in a dose-dependent manner (n=3, P<0.05 for cilostazol for control *vs.* 400 μM and P<0.01 for milrinone control *vs.* 200 μM).

extracellular environment. When this occurs, the extracellular concentration of ADP and ATP increases markedly. Low levels of ATP act as communication between cells that no injuries are present (24), and most receptors that bind ATP belonging to the class of P2X receptors are maximally activated at these low concentrations. However, the P2X7 receptor is activated only at the high levels of ATP (>100 μM) (25, 26) that signal danger and therefore is the main ATP-driven signaling pathway of injury. The activation of this pathway leads to increased $\alpha_m\beta_2$ integrins and increased leukocyte recruitment. In short, the presence of high local concentrations of ATP serves as an acute sign of danger and triggers greater inflammation. CD39 acts to dissipate both ADP and ATP, limiting thrombosis, coagulation, and inflammation (23). Though this role of CD39 has largely been determined, many questions surrounding the regulation of CD39 on both transcriptional and post-translational levels remain unanswered.

Pleiotropy of PDE inhibition

Endothelial cells have been described to express several of the 11 phosphodiesterase family members, including PDE2, PDE4, PDE3, and PDE5 (27), and the last 3 of these have been targeted for a diverse range of clinical uses. For example, roflumilast and cilomilast are PDE4 inhibitors developed for the treatment of chronic obstructive pulmonary disease, and other drugs targeting PDE4 inhibition have been researched as antidepressants and have been shown to improve memory in a rodent model (28, 29). PDE5 inhibitors are best known for their role in facilitating vascular smooth muscle

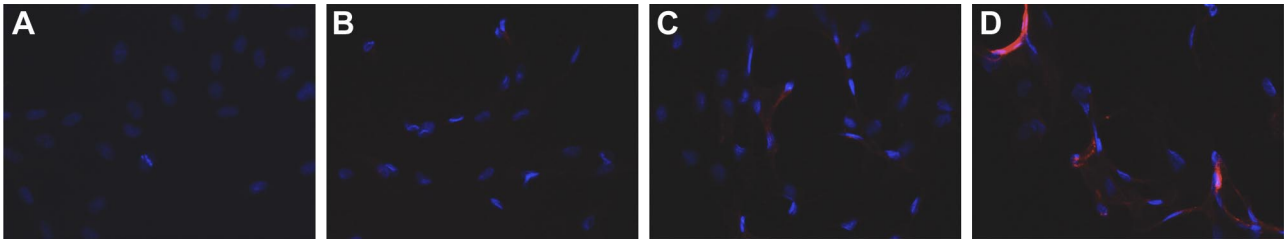

Figure 8. Effect of PDE3 inhibition on CD39 protein visualized by immunofluorescence microscopy: HUVECs were treated for 18 h with control DMSO or milrinone (100–400 μM). Cells were then fixed in methanol and labeled CD39 with tetramethylrhodamine and nuclei with DAPI before imaging using a ×20 objective. *A*) DMSO (control); (*B*) 100 μM milrinone; (*C*) 200 μM milrinone; (*D*) 400 μM milrinone.

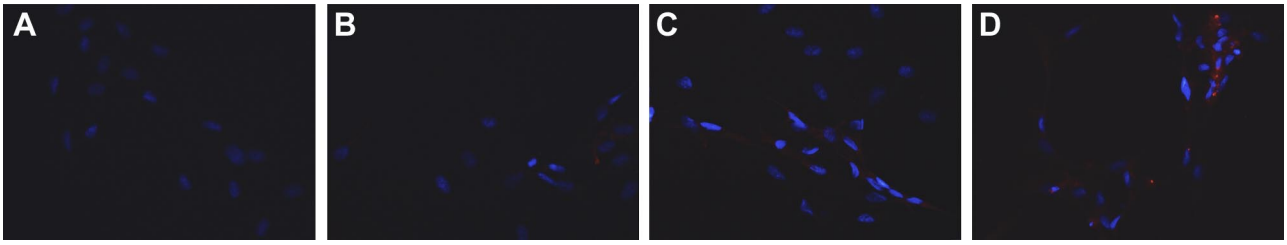

Figure 9. Effect of PDE3 inhibition on CD39 protein visualized by immunofluorescence microscopy: HUVECs were treated for 18 h with control DMSO or cilostazol (50–400 μM). Cells were then fixed in methanol and labeled CD39 with tetramethylrhodamine and nuclei with DAPI before imaging using a ×20 objective. *A*) DMSO (control); (*B*) 50 μM cilostazol; (*C*) 100 μM cilostazol; (*D*) 400 μM cilostazol.

relaxation, and drugs such as sildenafil and tadalafil have been developed for the treatment of erectile dysfunction and for treatment of pulmonary arterial hypertension (28, 30). PDE3 inhibitors have also been a target of interest, particularly useful as vasodilators in the treatment of heart failure, and two of these were specifically used herein as a tool to modulate intracellular cAMP levels. PDE3 is particularly interesting in relation to its potential effects on CD39, as it is important for regulating platelet aggregation by affecting intracellular cAMP levels. It is to this class that cilostazol and milrinone belong, the former developed as a vasodilator and platelet inhibitor and the latter as a regulator of cardiac contractility and vasodilator in the treatment of heart failure.

Effects of PDE3 inhibition

Cilostazol has been used in the treatment of intermittent claudication in the context of peripheral arterial disease (31). It is an antithrombotic that acts as an arterial vasodilator (32) and has been shown to prevent platelet aggregation stimulated by collagen, ADP, epinephrine, thrombin, arachidonic acid, and shear stress (31, 33). While the mechanism of action of cilostazol has yet to be fully elucidated, it is known that cilostazol acts as a specific inhibitor of PDE3. This inhibition of

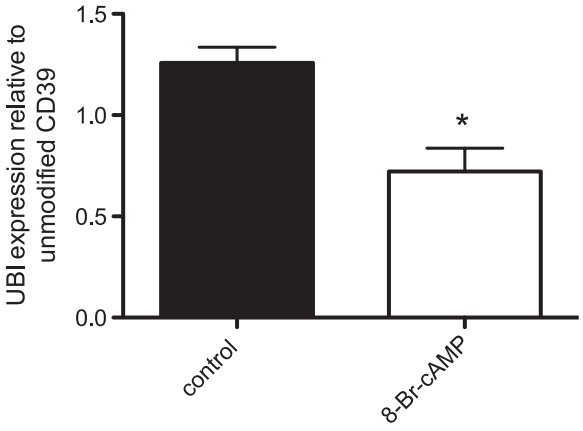

Figure 10. Effect of PDE3 inhibition on ubiquitination of CD39: HUVECs were treated for 18 h with 8-Br-cAMP or control PBS. Cells were then washed in PBS, harvested, and incubated with beads coated with anti-human CD39 antibody. Beads were then washed and the protein eluted and analyzed *via* Western blot. Membranes were first probed with anti-ubiquitin (UBI) antibody, then stripped and reprobed with anti-human CD39 antibody (n=3, P<0.05). Treatment with 8-Br-cAMP resulted in 43% less ubiquitin bound to CD39.

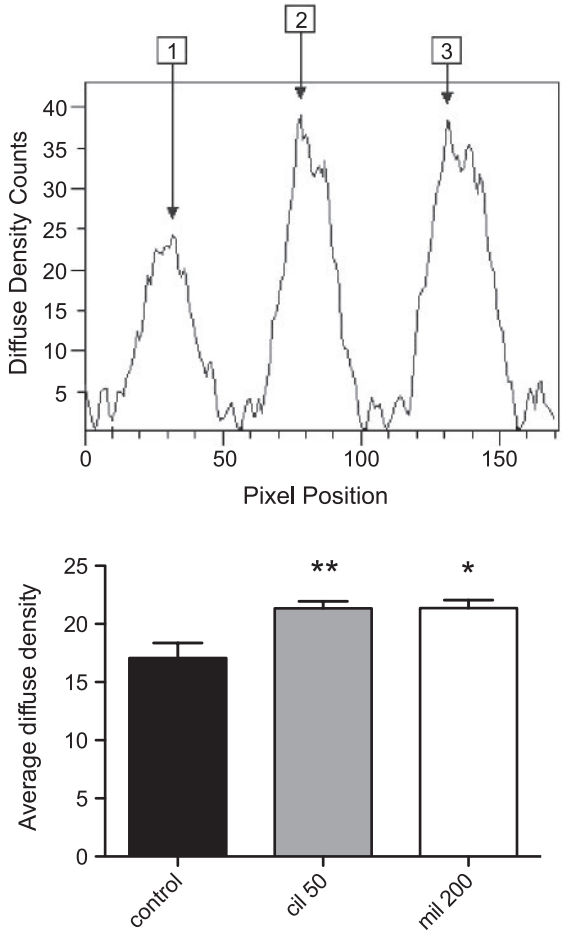

Figure 11 Functional assessment of CD39 activity following PDE3 using radio-thin-layer chromatography (TLC): Total cell lysates of HUVECs treated with either DMSO, cilostazol (50 μM), or milrinone (200 μM) were incubated with ^{14}C-labeled ATP. Total ADP was measured and normalized to total protein and total radiation per lane (n=5, *P<0.05, **P<0.01). Peak 1 indicates ADP measured in DMSO (control) treated samples, peak 2 indicates ADP measured in cilostazol-treated samples, and peak 3 indicates ADP measured in milrinone-treated samples.

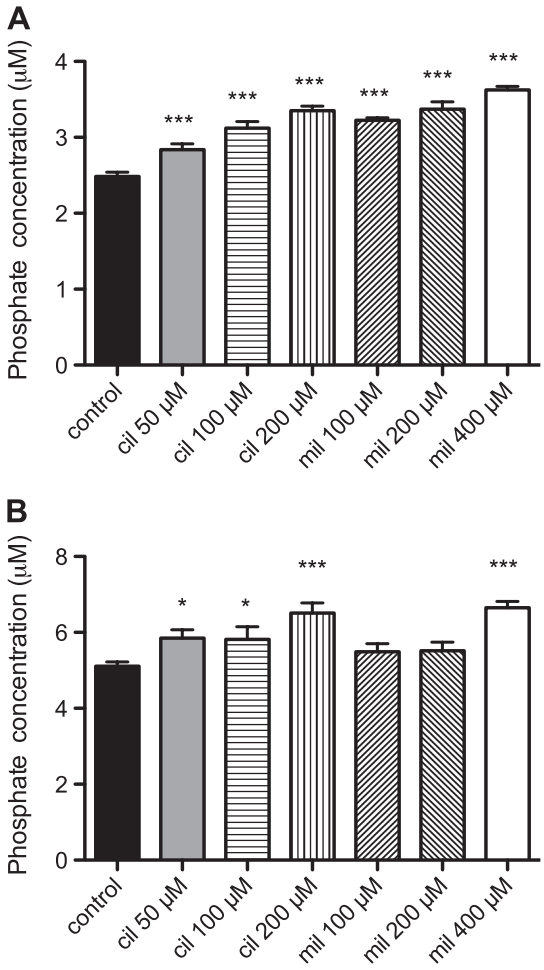

Figure 12. Measurement of inorganic phosphate generation following PDE3 inhibition using a malachite green assay: HUVECs were treated with either DMSO, along with varying doses of either cilostazol (50–400 μM) or milrinone (100–400 μM). *A*) inorganic phosphate levels after the addition of ADP, thus indicating relative ADPase activity. *B*) inorganic phosphate levels after the addition of ATP, thus indicating relative levels of combined ATPase and ADPase activity ($n=6$, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

PDE3 leads to an increase in intracellular cAMP levels, which then potentiates other signaling pathways driven by cAMP, and finally results in vasodilation and suppression of platelet aggregation (34). It is different from other drugs of its class such as milrinone, in that cilostazol may also exert beneficial effects on the endothelium by reducing adenosine reuptake into endothelial cells, platelets, erythrocytes, and myocytes, thereby increasing extracellular adenosine signaling through A_1 and A_2 receptors (35, 36). Milrinone, in contrast to cilostazol, has been used in the treatment of patients with heart failure (37). Though its half-life is considerably shorter than that of cilostazol, it has been shown to have a greater stimulatory effect on cAMP in ventricular myocytes compared to cilostazol, and to have a greater effect on contractility in hearts (17). Both drugs have been demonstrated to act specifically on PDE3 and increase intracellular cAMP and are used herein to demonstrate that specific PDE3 inhibition, as opposed to drug-specific effects, is responsible for changes to CD39 expression.

Transcriptional regulation of human CD39

Very little is currently known about the transcriptional mechanisms responsible for the expression of CD39. However, it has recently been established that CD39 mRNA and protein can be increased in murine RAW macrophages and murine peritoneal macrophages after exposing cells to 8-Br-cAMP (7). There are several cAMP response elements (38) in the murine CD39 promoter region, of which one is proximal to the transcriptional start site to regulate CD39 directly. The human CD39 promoter region, in contrast, has a handful of AP-1/cre-like sequences encoded distal to the start site, indicating that cAMP-driven effects in endothelial cells may indeed be different from the direct transcriptional pathway in murine macrophages. This is particularly evident here, where inhibition of PDE3 led to significant increases in protein expression and activity of endothelial CD39, but led to no differences in mRNA levels (Figs. 5–9, 11, and 12). This is in contrast to RAW macrophages, which showed increases in both CD39 protein and mRNA (Figs. 2 and 3).

Differential regulation of CD39 in endothelial cells: ubiquitination

The fact that CD39 can be detected in higher levels after an increase in intracellular cAMP despite relatively unaltered mRNA levels indicates that changes may be occurring on a post-translational level. However, extremely little is known about the degradation and turnover of CD39. Here we raise the possibility that CD39 may be ubiquitinated. Several lines of evidence led us to propose this as a possibility. First, the literature suggests that PDE inhibition and the subsequent modulation of intracellular cAMP can inhibit ubiquitination (39). In this case, PDE4 inhibition led to the inhibition of ubiquitin-driven protein degradation in rat skeletal muscles. This activity seems to be dependent in part on an increase in intracellular cAMP, in that cAMP levels induced by a β_2-agonist led to the suppression of two ubiquitin ligases (40). Taken together with data presented here showing that cAMP treatment leads to significantly less ubiquitinated CD39, it may be indicative of an endothelial mechanism by which CD39 expression is modulated posttranslationally. A second indication that CD39 may be regulated by ubiquitination is that CD39 protein contains a Ran binding protein M (RanBPM or RanBP9) binding site on its N terminus (41). RanBPM is a widely expressed scaffolding protein that mediates interactions between the cytoplasmic domains of proteins and other signaling moieties, and its various associated activities range from promoting apoptosis (42) to microtubule reorganization (43). Relevant to this study, others have shown that protein binding to RanBPM can inhibit ubiquitination (44, 45). These data suggest that CD39 association with

RanBPM may play a role in how CD39 expression is regulated.

In conclusion, we have shown that while the inhibition of PDE3 results in an increase in CD39 expression in both macrophages and endothelial cells, the mechanisms governing this up-regulation differs between the two. In macrophages, there is a strong basis for the direct involvement of cAMP in the transcriptional regulation of CD39. In contrast, endothelial cells are only affected at the protein level, and we have shown here that PDE3 inhibition results in differential ubiquitination, with elevation of CD39 protein levels. While further study into this mechanism is ongoing, the demonstration of CD39 modulation by PDE3 inhibition is unique. That this was achieved through the utilization of available therapeutics, cilostazol and milrinone, presents the potential of repurposing currently used PDE3 inhibitors for the targeted control of CD39 and extracellular nucleotide signaling. FJ

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