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A SIMPLE ASSAY FOR ECTO-5′-NUCLEOTIDASE USING INTACT PULMONARY ARTERY ENDOTHELIAL CELLS

EFFECT OF ENDOTOXIN-INDUCED CELL INJURY

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Abstract—Adenosine may be protective in acute vascular injury by inhibiting platelet aggregation and neutrophil oxidant release. In contrast, adenine nucleotides, which may be released with acute vascular injury, stimulate platelet aggregation and neutrophil oxidant release. Ectonucleotidases, membrane enzymes that catabolize extracellular nucleotides, are the primary mechanism for degrading circulating nucleotides to adenosine. Ecto-5′-nucleotidase converts extracellular AMP to adenosine. We hypothesized that endothelial cell injury alters ecto-5′-nucleotidase activity. Using a novel assay first reported by Jamal *et al.* (*Biochem J* **250**: 369–373, 1988) with rat adipocytes, we studied the properties of ecto-5′-nucleotidase in intact monolayers of cultured bovine pulmonary artery endothelial cells (BPAEC) and examined the effect of endotoxin on enzyme activity. The assay uses a fluorescent analog of AMP, 1,N^6-etheno-AMP (E-AMP), as the substrate for ecto-5′-nucleotidase, and measures etheno-adenosine (E-Ado) formation. Etheno-AMP in Hepes buffer, pH 7.4, at 22°, was added to confluent monolayers of BPAEC; samples of supernatant were collected after various intervals, and E-AMP and E-Ado were quantitated by HPLC. Using these methods we found a K_m of 15 ± 6 μM, a pH optimum of 7.48, minimal effect of $MgCl_2$ or $CaCl_2$ at physiologic pH, and inhibition by α,β-methylene ADP, a known 5′-nucleotidase inhibitor. We established that the monolayer assay was indeed measuring cell surface associated 5′-nucleotidase. To determine the effect of endotoxin, we incubated confluent monolayers with endotoxin in Minimal Essential Medium plus 10% fetal bovine serum for 24 hr, washed them, and assessed the conversion of E-AMP to E-Ado by the endotoxin-injured cells. Endotoxin stimulated endothelial ecto-5′-nucleotidase activity. This increase in 5′-nucleotidase activity in response to endotoxin injury may represent an important clearance mechanism for circulating adenine nucleotides and may be protective in acute vascular injury by increasing adenosine production.

Adenine nucleotides are released into the circulation during acute vascular injury [1]. Circulating extracellular nucleotides may exacerbate vascular injury by increasing neutrophil adherence to endothelium [2], superoxide release from activated neutrophils [3, 4], and platelet aggregation [5]. In addition, adenine nucleotides alter vascular reactivity, dependent on the integrity of the endothelium [6]. Ectonucleotidases, integral membrane enzymes that degrade extracellular nucleotides, represent the primary mechanism for adenine nucleotide degradation in the extracellular space [7]. Among endothelial ectonucleotidases are ATPase, ADPase, and AMPase or 5′-nucleotidase (5′-ribonucleoside phosphohydrolase, EC 3.1.3.5) [7, 8]. Of these, ecto-5′-nucleotidase, which catalyzes the conversion of circulating AMP to adenosine, has been studied most extensively and has been well characterized [7]. The end product of ectonucleotidase activity, adenosine, may protect against vascular endothelial cell injury by inhibiting platelet aggregation [9] or by blunting neutrophil adherence to endothelium [10] and neutrophil superoxide release [11]. The action of ecto-nucleotidases may thus be beneficial during acute vascular injury by depleting extracellular adenine nucleotides and by increasing circulating adenosine levels.

Various assays have been developed to study the activity of 5′-nucleotidase, using purified enzyme and cell membrane preparations [7, 8]. Studies using intact cells and nucleotides as substrate assess cell surface 5′-nucleotidase (ectonucleotidase) activity because nucleotides are not transported across cell membranes [7]. However, assessment of ecto-5′-nucleotidase activity in intact living cells is complicated by the fact that the product of the reaction, adenosine, is transported rapidly into cells [12, 13] and metabolized by cellular enzymes. We have established an assay for ecto-5′-nucleotidase based on the method of Jamal *et al.* using 1,N^6-etheno-adenosine monophosphate (E-AMP),§ a fluorescent AMP analog, as the substrate [14, 15]. The main advantage of this substrate is that the reaction product, 1,N^6-etheno-adenosine (E-Ado), is not further metabolized by adenosine deaminase [14]. Therefore, extracellular E-Ado can be quantitated accurately. Another advantage of this assay is that it can be used with intact cell monolayers, allowing assay of ectonucleotidase. In contrast to

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§ Abbreviations: E-AMP, 1,N^6-etheno-adenosine monophosphate; E-Ado, 1,N^6-etheno-adenosine; and BPAEC, bovine pulmonary artery endothelial cells.

assays using plasma membrane preparations, this assay using intact cells avoids measurement of stored intracellular nucleotidase.

In this study, we have established a simple ecto-5′-nucleotidase assay utilizing E-AMP as substrate and using intact cultures of bovine pulmonary artery endothelial cells (BPAEC) and have employed it to define the properties of the enzyme. In addition, we have examined the effects of an endotoxin model of cell injury on ecto-5′-nucleotidase activity to test the hypothesis that cell injury alters the activity of this enzyme.

METHODS

Chemicals. E-AMP, E-Ado, α,β-methylene ADP, Levamisole, and NaF were purchased from the Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* endotoxin lipopolysaccharide (W-*E. coli* 055iB5) was purchased from Difco (Detroit, MI).

Cell culture technique. Endothelial cells were isolated from the main pulmonary artery of freshly slaughtered cows by scraping the luminal surface gently with a scalpel blade using the methods described by Ryan *et al.* [16]. Cells were grown to confluence in Minimal Essential Medium (MEM) with 10% fetal bovine serum (FBS), penicillin (10,000 U/mL), streptomycin (10,000 μg/mL), and amphotericin B (25 μg/mL) at 37° in 5% CO_2 and 95% air. Using phase contrast microscopy, we observed cobblestone morphology typical of vascular endothelium. The cells bound fluorescein-labeled acetylated low density lipoprotein, and displayed factor VIII antigen. Cells were passaged with 0.05% trypsin containing 5.3 mM EDTA. Assays were performed on cells plated in either T25 flasks or 35-mm wells (endotoxin experiments). After plating, cultures grew to confluence in 4 days. Experiments were performed on day 5 after plating. Thirteen different endothelial cell lines were used. The passage number for the cells used ranged between 6 and 28. There were no apparent differences in results based on passage number or cell line.

Ecto-5′-nucleotidase assay. The assay is based on the method of Jamal *et al.* [14]. Confluent monolayers of BPAECs in T25 flasks were washed twice with 2 mL of phosphate-buffered saline (PBS). A known concentration of E-AMP in modified Hepes buffer (pH 7.40) containing 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 μM dipyridamole was added. The cells were rocked gently at room temperature (22°) on a serological rotator and 100-μL samples of supernatant were collected at various times. The samples were centrifuged for 3 min, and the supernatants were immediately frozen at −20°.

High pressure liquid chromatography (HPLC) was used to quantitate E-AMP and E-Ado. We used a reverse phase HPLC column (μBondpack C_{18}, 3.9 × 300 mm; purchased from Waters Associates, Milford, MA) with a 0–50% methanol gradient. Time per run was approximately 30 min. Flow rate was 2 mL/min. E-AMP and E-Ado were quantitated in the supernatant using a fluorescence detector (Spectroflow Fluorometer, model 980) with an excitation monochromator set at a wavelength of 270 nm and a secondary filter with a cut off below 418 nm.

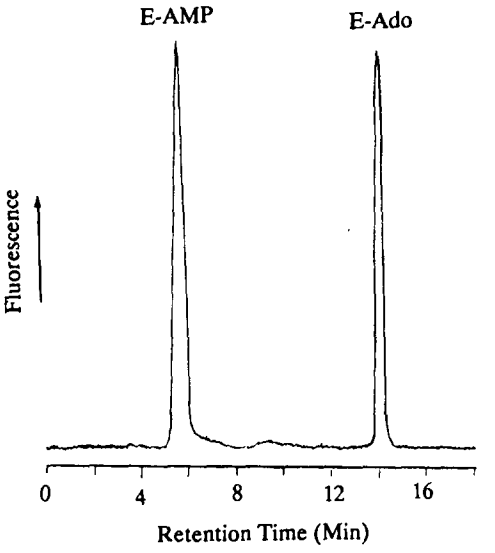

Fig. 1. Representative HPLC chromatograph of E-AMP and E-Ado. A mixture of E-AMP and E-Ado was analyzed by HPLC using a reverse-phase column, mobile phase, 0–50% methanol (30 min) with a flow rate of 2 mL/min. Fluorescence was detected using a fluorescence detector at an excitation wavelength of 270 nm and an emission wavelength of 418 nm. E-AMP and E-Ado were used as standards to quantitate the amounts in the experimental samples.

The retention time for E-AMP was about 6 min and for E-Ado was 14 min, as illustrated in Fig. 1. Peak areas for E-AMP and E-Ado from HPLC were determined for 10-μL samples. The calculated starting E-AMP concentration was 10 μM, yielding 100 pmol in 10 μL. We found partial spontaneous hydrolysis in our standard (0 time) samples. We therefore calculated the percent E-AMP and E-Ado from the peak areas for E-AMP and E-Ado on the HPLC chromatograph. This percent corresponded to the number of picomoles of E-AMP and E-Ado in the standard sample.

To calculate the concentration of E-AMP and E-Ado at a given time, we used the following ratio:

$$\frac{\text{pmol E-AMP or E-Ado at time 0}}{\text{Peak area E-AMP or E-Ado at time 0}} = \frac{Y}{\text{Peak area E-AMP or E-Ado at time x}}$$

where Y = picomoles of E-AMP or E-Ado per 10 μL at time x.

Cell counts were determined by hematocytometer after detachment of cells with trypsin/EDTA. Enzyme activity was expressed as picomoles E-Ado formed per minute per 10^4 cells.

Experiments to confirm measurement of ectonucleotidase. In other experiments we established that our assay was indeed measuring cell surface (ecto) 5′-nucleotidase activity, according to the criteria

outlined by Pearson [7]. We first assessed the possibility that the enzyme was shed from the cell surface into culture supernatants. Three endothelial cell cultures in T25 flasks were incubated with assay buffer without added E-AMP substrate for 60 min. The "conditioned" buffer was removed from the flasks and centrifuged at 2000 *g* for 5 min to remove any nonadherent cells. Substrate E-AMP was added to the conditioned buffer for a final calculated concentration of 10 μM. The conditioned buffer was then incubated at room temperature for 15 min. Aliquots of buffer were removed for HPLC analysis at times 0 and 15 min. The remaining cell monolayers were assayed for ectonucleotidase activity in the usual manner, as outlined above.

In other experiments we assayed cell lysates for E-AMP, E-Ado, and for products of E-Ado metabolism by the cells. Three endothelial cell monolayers in T-25 flasks were incubated with assay buffer containing E-AMP for 60 min at room temperature. After 0, 15, 30, and 60 min of incubation at room temperature, aliquots of the culture supernatants were removed for HPLC analysis. The monolayers were washed twice with ice-cold Hepes buffer containing 10 μM dipyridamole, and the monolayers precipitated with 1 mL of ice-cold perchloroacetic acid (4% PCA, to inactivate phosphatases). The cell extract was removed, and the flask was washed once with 1 mL of ice-cold PCA; then the cell extract plus wash was centrifuged at 2000 *g* for 5 min. The cell extract and pellet were frozen until further analysis. Prior to HPLC analysis, the acid-soluble and -insoluble cell samples were neutralized to pH 7.0 with K_2CO_3, and $KClO_4$ precipitate was removed by centrifugation. Samples were then analyzed by HPLC for E-AMP, E-Ado, or other possible etheno products of E-AMP metabolism.

Finally, we used the ^{51}Cr release technique to assess whether the enzyme assay conditions were somehow injurious to the endothelial cells, in which case intracellular nucleotidases might be released into the cell medium. Three endothelial cell cultures in T-25 flasks were preincubated overnight at 37° with MEM plus 10% FBS containing 3 μCi/mL of sodium ^{51}chromate. The monolayers were washed twice with Hepes buffer containing 10 μM dipyridamole, and control monolayers were then incubated with assay buffer without added E-AMP substrate for 60 min at room temperature. Experimental flasks were incubated with complete assay buffer containing E-AMP. The culture supernatants were removed and washed once with Hepes buffer, and supernatant plus wash was centrifuged. The remaining cell monolayer was lysed with NaOH, and the flask washed once. Cell lysate plus wash, culture supernatant plus wash, and pellet of culture supernatant were counted in a gamma counter. The percent ^{51}Cr release was determined from the ratio × 100 of cpm in supernatant/total cpm (supernatant + cell lysate + pellet).

Endotoxin-induced cell injury. Bovine pulmonary artery endothelial cells, grown to confluence in 35-mm wells, were incubated with various concentrations of *E. coli* endotoxin (0.01 to 0.1 μg/mL) in MEM with 10% FBS at 37° in 5% CO_2 and 95% air for 24 hr. The cells were then examined under phase microscopy and a visual assessment of cell injury was made. The cells were washed twice with 2 mL of PBS, and ecto-5'-nucleotidase activity was assayed as described above. A calculated E-AMP concentration of 10 μM was used as the starting concentration for all experiments. Adherent cell counts were obtained for each individual 35-mm well.

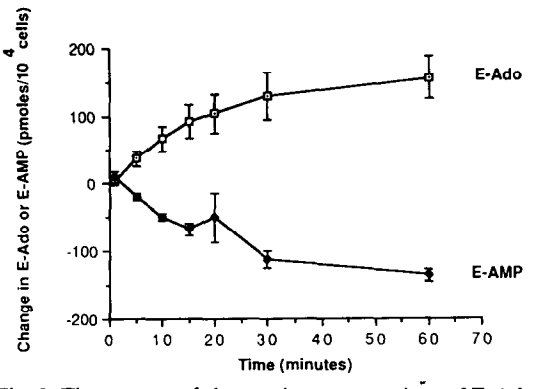

Fig. 2. Time course of changes in concentrations of E-Ado and E-AMP. Confluent monolayers of BPAECs were incubated with 5 μM E-AMP, and culture supernatant samples were obtained over 60 min and analyzed by HPLC for content of E-Ado and E-AMP. Cell number was determined at the end of the experiments. Data are means ± SEM (N = 6, each group).

Statistics. Data are expressed as means ± SEM. Data were analyzed for differences among means using analysis of variance and the Fisher least significant difference multiple comparison test. Differences were considered significant at $P < 0.05$.

RESULTS

The time course of ecto-5'-nucleotidase action, as measured by the conversion of E-AMP to E-Ado, is shown in Fig. 2. There was complete conversion of E-AMP to E-Ado over a 60-min period. The reaction was linear for the first 15 min. In addition, as many as four consecutive assays on the same endothelial monolayer yielded similar results (data not shown). Ecto-5'-nucleotidase assays were performed with and without dipyridamole to determine whether exposure to this drug, which blocks nucleoside transport into cells, would lead to higher measurable levels of extracellular E-Ado. There was only a slight increase in the amount of extracellular E-Ado measured when the cells were incubated with dipyridamole (Table 1). At physiologic pH (7.4) and room temperature (22°), we found only a small stimulatory effect of $MgCl_2$ (1–20 mM) and no stimulatory effect of $CaCl_2$ (1–10 mM) on enzyme activity (Table 1). To study the effect of pH on enzyme activity, we incubated confluent monolayers in 35-mm wells in the reaction mixture containing Hepes buffer of different pH for 15 min. The data demonstrate optimum ecto-5'-

Table 1. Effects of various agents on etheno-adenosine formation

Agent	Concentration	E-Ado formation (% Change from control)
Dipyridamole	10 μM	3.6 ± 2.9 (3)
$MgCl_2$	1 mM	0.4 ± 4.5 (3)
	10 mM	6.3 ± 1.9 (3)
	20 mM	12.5 ± 3.3 (3)
$CaCl_2$	1 mM	−0.2 ± 4.4 (3)
	5 mM	0 ± 4.2 (3)
	10 mM	0.33 ± 7.3 (3)
NaF	100 mM	−1.28 ± 1.98 (4)
Levamisole	5 mM	1.2 ± 4.4 (4)

Confluent monolayers of BPAECs in T25 flasks were incubated with E-AMP at room temperature (22°), pH 7.40, and samples of supernatant were collected at 15 min. E-AMP and E-Ado were measured by HPLC. The control T25 flask contained only assay medium and E-AMP. Control ectonucleotidase activity was 167 ± 47 pmol E-Ado formed/10^4 cells/15 min. Data are means ± SEM. Numbers in parentheses = the number of experiments.

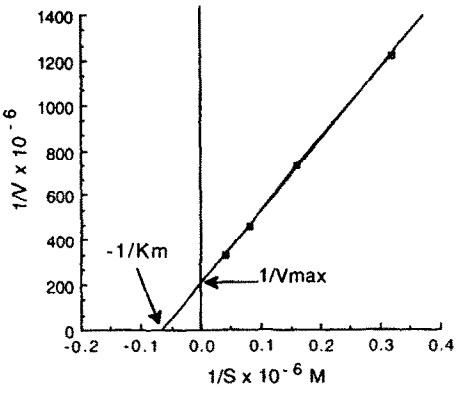

Fig. 4. Representative Lineweaver–Burk plot demonstrating K_m and V_{max} of ecto-5′-nucleotidase for E-AMP. Confluent monolayers of BPAECs were incubated with various concentrations of E-AMP (3.2, 6.3, 12.6, and 25.2 μM), and culture supernatant samples were obtained at 15 min. E-AMP and E-Ado were then quantitated by HPLC. V represents pmoles of E-Ado/min/10^4 cells and S represents μM E-AMP. The values obtained from this plot: K_m 15.6 μM; V_{max}, 4.9×10^3 pmol/min/10^4 cells.

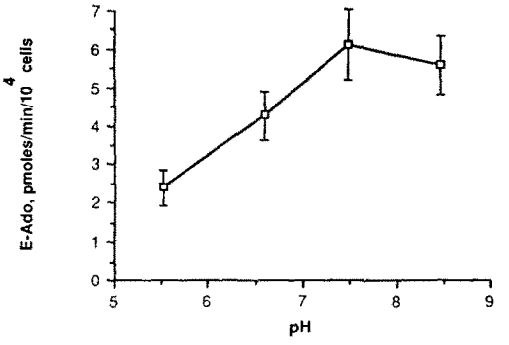

Fig. 3. Effect of pH on ecto-5′-nucleotidase activity. Confluent cultures of BPAECs in 35-mm wells were incubated at room temperature (22°) with 5 μM E-AMP in HEPES buffer at varying pH. Culture supernatant samples were collected at 15 min, and E-Ado formation was measured by HPLC. Data are means ± SEM (N = 4, each group).

nucleotidase activity to be at pH 7.48 at room temperature (Fig. 3).

To determine the kinetic parameters, confluent monolayers of BPAECs were incubated with various concentrations of E-AMP (1–40 μM) for 15 min. From Lineweaver–Burk plots of this data, we determined the K_m of the enzyme for E-AMP to be 15.0 ± 6.0 μM (N = 6). Mean V_{max} was $4.0 \pm 0.8 \times 10^3$ pmol/min/10^4 cells. A representative plot demonstrating K_m and V_{max} is shown in Fig. 4.

To confirm that our assay indeed reflected 5′-nucleotidase activity, we added 50 μM α,β-methylene ADP, a known 5′-nucleotidase inhibitor [7, 17]. We found that α,β-methylene ADP decreased E-AMP conversion to E-Ado, with 93 ± 3% inhibition at a 50 μM concentration (N = 4). To determine a possible contribution of nonspecific phosphatase activity to our ecto-5′-nucleotidase assay results, confluent monolayers were incubated with the nonspecific phosphate inhibitors NaF and Levamisole [18]. Neither agent significantly inhibited the conversion of E-AMP to E-Ado, indicating that this assay did not measure nonspecific phosphatase activity (Table 1).

The assay did indeed appear to be measuring cell surface associated ectonucleotidase activity in endothelial cell monolayers. There was no measurable hydrolysis of E-AMP in conditioned media removed from endothelial cell cultures, indicating that nucleotidase activity did not leak into the assay buffer under the conditions of these experiments. There were no measurable E-AMP, E-Ado, or etheno derivatized metabolites in PCA lysates of endothelial cell monolayers, indicating that the E-AMP substrate or E-Ado product was not taken up into cells. However, in both sets of experiments the intact monolayers metabolized E-AMP in the usual fashion (data not shown). Finally, the assay substrate did not cause cell lysis, as assessed by ^{51}Cr release from endothelial cells (control: 2.6 ± 0.4%/hr; experimental: 2.6 ± 0.9%/hr, N = 3, P = NS).

After incubation of confluent monolayers of BPAECs for 24 hr with various concentrations of endotoxin, there was marked stimulation of ecto-5′-nucleotidase activity (Fig. 5). These experiments show that endotoxin did cause cell injury, as assessed by phase contrast morphology and by adherent cell counts (Table 2). With increasing endotoxin concentration, there were increasing degrees of cell detachment, pyknosis, and fragmentation. There was a significant increase in E-Ado formation despite decreased cell number after endotoxin injury (Table 2). The increased nucleotidase activity seen after endotoxin injury was largely due to ecto-5′-

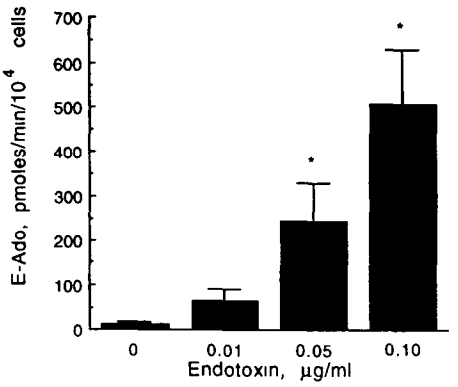

Fig. 5. Stimulation of ecto-5'-nucleotidase activity by endotoxin. Confluent monolayers of BPAECs in 35-mm wells were preincubated for 24 hr with various concentrations of endotoxin in MEM/10% FBS, and 5'-nucleotidase assay was performed. Culture supernatant samples were collected at 1 min and analyzed by HPLC. N equals 7 monolayers for 0, 0.01, and 0.10 μg/mL endotoxin, and 6 monolayers for 0.05 μg/mL endotoxin. Data are means ± SEM. Key: (*) significantly different from control (no endotoxin) at $P < 0.05$.

Table 2. Effect of endotoxin on cell count and etheno-adenosine formation

Endotoxin (μg/mL)	Cell count × 10^4 cells	E-Adenosine (pmol/min)
0.00	49 ± 5	581 ± 303
0.01	37 ± 6	1750 ± 570
0.05	24 ± 6*	3783 ± 769*
0.10	11 ± 2*	4455 ± 637*

Confluent cultures of BPAECs in 35-mm wells were preincubated for 24 hr with various concentrations of endotoxin. Cultures were washed, and 5'-nucleotidase assay was performed using E-AMP (4–6 μM) as substrate. Samples of supernatant were collected at 1 min, and conversion of E-AMP and E-Ado was measured by HPLC. Adherent cell counts were then obtained. Data are means ± SEM. N equals 7 monolayers for 0, 0.01, and 0.10 μg/mL endotoxin, and 6 for 0.05 μg/mL endotoxin.

* Significantly different from control (no endotoxin) at $P < 0.05$.

nucleotidase, since the 5'-nucleotidase inhibitor, α,β-methylene ADP (50 μM), inhibited E-AMP hydrolysis by 76 ± 6% (N = 4) after incubation of monolayers with 1 μg/mL of endotoxin for 24 hr. When endotoxin (1 μg/mL) was added to endothelial monolayers immediately prior to ecto-5'-nucleotidase assay, without preincubation, no increase in the enzyme activity was seen (data not shown). This suggests that the increased ecto-5'-nucleotidase activity was not due to a direct effect of endotoxin on the enzyme, but that cell injury was required. In addition, when monolayers were incubated with endotoxin (1 μg/mL) for various times (15 min, 1 hr, 6 hr, and 24 hr), we found that 24 hr of incubation was required in order to observe significant stimulation of ecto-5'-nucleotidase activity (data not shown).

To determine if inhibition of protein synthesis blocked the increase in ecto-5'-nucleotidase activity seen in response to endotoxin, endothelial monolayers were preincubated for 24 hr with one of the following: 1 μM cycloheximide, 0.05 μg/mL endotoxin, cycloheximide and endotoxin together, or MEM/10% FBS (medium) alone. Monolayers were washed twice, and ecto-5'-nucleotidase assay was performed. Ecto-5'-nucleotidase activity at 1 min was 187 ± 77 pmol/min/10^4 cells after incubation with endotoxin (N = 4). This represented a 44% increase in activity over control (medium alone). After incubation with endotoxin and cycloheximide, ecto-5'-nucleotidase activity was 409 ± 172 pmol/min/10^4 cells (N = 4). The difference between endotoxin alone and endotoxin plus cycloheximide was not statistically significant, indicating that cycloheximide did not prevent endotoxin-induced stimulation of ecto-5'-nucleotidase activity.

DISCUSSION

Ecto-5'-nucleotidase is a plasma membrane enzyme that is attached to the plasma membrane by a glycosyl phosphatidylinositol anchor [19]. In contrast to cytosolic 5'-nucleotidase, the K_m of ecto-5'-nucleotidase is lower, its activity is less dependent upon the presence of divalent cations, and the pH optimum is in the alkaline range [20]. Using E-AMP as a substrate for the enzyme on intact bovine pulmonary artery endothelial cells, we found a K_m for ecto-5'-nucleotidase activity (15 ± 6 μM) similar to that reported for ecto-5'-nucleotidase in intact cultured porcine aortic endothelial cells (25–28 μM) by Chesterman *et al.* [21] and Gordon *et al.* [8], who used [^{3}H]AMP as a substrate. The assay which we report, based on that of Jamal *et al.* [14] using E-AMP as a substrate for enzyme assays on intact cells has the advantage over radiolabeled AMP as a substrate in that the product, E-Ado, is not further metabolized by adenosine deaminase. This markedly simplifies kinetic analysis and allows repeated assays using the same monolayer. It is clear that our assay is indeed measuring ecto-5'-nucleotidase activity as demonstrated by inhibition of E-AMP conversion by a specific 5'-nucleotidase inhibitor and lack of inhibition by nonspecific phosphatase inhibitors. Furthermore, our assay does indeed measure cell surface ecto-5'-nucleotidase activity, since there was no measurable activity released into culture supernatants, there were no detectable intracellular products of metabolism, and the assay conditions did not cause cell injury.

We observed only mild stimulation of enzyme activity by Mg^{2+} and Ca^{2+} at physiologic pH and room temperature. Although it has been reported that 5'-nucleotidase activity is stimulated by Ca^{2+} and Mg^{2+} [7, 17], Ipata [22] showed no effect of Mg^{2+} and 16.8% inhibition by Ca^{2+} (5 mM). Variability in cation dependence may be due to differences in experimental conditions such as temperature, pH, species, or subset of enzyme measured, that is, endo- versus ecto-enzyme. In this

regard, Pearson *et al.* [23] observed no calcium dependence of porcine endothelial ecto-5′-nucleotidase.

A problem that we had not anticipated was the lack of purity of our substrate, which contained 50–90% E-AMP and the rest E-Ado in the standard samples. This appears to have resulted from spontaneous hydrolysis of E-AMP secondary to chemical instability, light sensitivity, or degradation secondary to freeze–thaw cycles. We accounted for this problem in our calculations by determining the percent E-AMP in the material at time 0.

Upon application of our assay to a model of cell injury, we found marked stimulation of ecto-5′-nucleotidase activity in endothelial cells incubated with endotoxin for 24 hr prior to assay. The stimulation was seen in association with cell injury, as assessed by adherent cell counts. This increase in activity was not likely due to release of cytosolic 5′-nucleotidase from lysed cells, since the monolayers were washed prior to assay. The cause of the endotoxin-induced increase in ecto-5′-nucleotidase activity is not clear at this time, but the lack of effect of co-incubation with cycloheximide suggests that it was not due to synthesis of new enzyme protein. Other possible mechanisms include increased display of ecto-enzyme on the cell membrane, a change in the conformation of the membrane-anchored protein, or a direct effect of endotoxin on ecto-5′-nucleotidase. The latter explanation is unlikely given our data showing no increase in ecto-5′-nucleotidase activity when the assay was performed immediately after adding endotoxin, without preincubation. Definite determination of the cause of increased ecto-5′-nucleotidase activity is the subject of ongoing research in our laboratories at this time.

Others have reported that exposure of cultured human umbilical vein endothelial cells to the superoxide generating system, hypoxanthine/xanthine oxidase, slows the rate of disappearance of radiolabeled ATP from culture medium [24]. This effect was inhibited by 250 μM α,β-methylene ADP. This study suggests that exposure of endothelial cells to non-injurious amounts of superoxide inhibits endothelial cell ecto-5′-nucleotidase activity. In contrast, our studies of endotoxin-induced injury demonstrated stimulation of ecto-5′-nucleotidase activity. The reasons for these differences are not clear, but may be due to different effects of superoxide and endotoxin on cell membrane associated enzymes. In addition, we found that 24 hr of incubation with endotoxin was necessary to see an increase in ecto-5′-nucleotidase activity. At this time cell injury is present, as manifested by decreased adherent cell counts and phase microscopic appearance of monolayers. Thus, cell injury may be necessary to see stimulation of ecto-5′-nucleotidase activity.

We chose to study the model of endotoxin injury to cultured endothelial cells because this well-described model is analogous to the syndromes of multi-system organ failure and adult respiratory distress syndrome in humans [25, 26]. However, we do not know whether endotoxin injury increases endothelial ecto-5′-nucleotidase activity *in vivo*. If so, this could have important consequences in enhancing conversion of circulating AMP to adenosine, a potentially protective substance. There are multiple possible sources of circulating nucleotides, which have been reported to be increased after vascular injury [1]. These include release from activated platelets [4], neuronal release [27], and release from injured endothelial cells [28]. Since circulating nucleotides and adenosine may modulate vascular injury [2, 3, 10, 11, 13], increased ecto-5′-nucleotidase activity could have important modulating effects on such injury by enhancing degradation of circulating nucleotides to adenosine and its metabolites.

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REFERENCES

1. Born GVR and Kratzer MAA, Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits, and man. *J Physiol (Lond)* **354**: 419–429, 1984.
2. Rounds S, McGowan-Jordan J and Bullard S, Adenine nucleotides increase granulocyte adherence to cultured endothelium. *Am Rev Respir Dis* **143**: A370, 1991.
3. Kuhns DB, Wright DG, Nath J, Kaplan SS and Basford RE, ATP induces transient elevations of $[Ca^{2+}]_i$ in human neutrophils and primes these cells for enhanced O_2^- generation. *Lab Invest* **58**: 448–453, 1988.
4. Ward PA, Cunningham TW, McCulloch KK, Phan SH, Powell J and Johnson KJ, Platelet enhancement of O_2^- response in stimulated human neutrophils. *Lab Invest* **58**: 37–47, 1988.
5. Agarwal KC, Haskel EJ and Parks RE Jr, Effects of adenosine analogs and adenine nucleotides on adenosine 5′-diphosphate-induced rat platelet aggregation. *Biochem Pharmacol* **29**: 1799–1805, 1980.
6. Gordon JL, Extracellular ATP: Effects, sources and fate. *Biochem J* **233**: 309–319, 1986.
7. Pearson JD, Ectonucleotidases. In: *Methods in Pharmacology* (Ed. Paton DM), pp. 83–107. Plenum Press, New York, 1985.
8. Gordon EL, Pearson JD and Slakey LL, The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. *J Biol Chem* **261**: 15496–15504, 1986.
9. Agarwal KC and Parks RE Jr, Adenosine analogs and human platelets. *Biochem Pharmacol* **24**: 2239–2248, 1975.
10. Cronstein BN, Levin RI, Belanoff J, Weissmann G and Hirshorn R, Adenosine: An endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J Clin Invest* **78**: 760–770, 1986.
11. Roberts PA, Newby AC, Hallett MB and Campbell AK, Inhibition by adenosine of reactive oxygen metabolite production by human polymorphonuclear leucocytes. *Biochem J* **227**: 669–674, 1985.
12. Dieterle Y, Ody C, Ehrensberger A, Stalder H and Junod AF, Metabolism and uptake of adenosine triphosphate and adenosine by porcine aortic and pulmonary endothelial cells and fibroblasts in culture. *Circ Res* **42**: 869–876, 1978.
13. Andreoli SP, Liechty EA and Mallett C, Exogenous adenine nucleotides replete endothelial cell adenosine

triphosphate after oxidant injury by adenosine uptake. *J Lab Clin Med* **115**: 301–313, 1990.
14. Jamal Z, Afkham-Ebrahimi A and Saggerson ED, 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**: 369–373, 1988.
15. Secrist JA, Barrio JR, Leonard NJ and Weber G, Fluorescent modification of adenosine-containing coenzymes. *Biochemistry* **11**: 3499–3506, 1972.
16. Ryan US, Clements E, Halliston D and Ryan JW, Isolation and culture of pulmonary artery endothelial cells. *Tissue Cell* **10**: 535–554, 1978.
17. Burger RM and Lowenstein JM, Preparation and properties of 5′-nucleotidase from smooth muscle of small intestine. *J Biol Chem* **245**: 6274–6280, 1970.
18. Richardson DJ, Smith GP, Meade TW, Langley P and Peters TJ, Assay, kinetics and properties of plasma adenosine diphosphate. The relationship to acid and alkaline phosphatase and variations in disease. *Clin Chim Acta* **121**: 87–94, 1982.
19. Low MG and Finean JB, Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C. *Biochim Biophys Acta* **508**: 565–570, 1978.
20. Piec G and Le Hir M, The soluble 'low-K_m' 5′-nucleotidase of rat kidney represents solubilized ecto-5′-nucleotidase. *Biochem J* **273**: 409–413, 1991.
21. Chesterman CN, Ager A and Gordon JL, Regulation of prostaglandin production and ectoenzyme activities in cultured aortic endothelial cells. *J Cell Physiol* **116**: 45–50, 1983.
22. Ipata PL, Sheep brain 5′-nucleotidase. Some enzymic properties and allosteric inhibition by nucleoside triphosphates. *Biochemistry* **7**: 507–515, 1968.
23. Pearson JD, Carleton JS and Gordon JL, Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial and smooth-muscle cells in culture. *Biochem J* **190**: 421–429, 1980.
24. Aalto TK and Raivio KO, Metabolism of extracellular adenine nucleotides by human endothelial cells exposed to reactive oxygen metabolites. *Am J Physiol* **264**: C282–C286, 1993.
25. Brigham KL and Meyrick B, Endotoxin and lung injury. *Am Rev Respir Dis* **133**: 913–927, 1986.
26. Meyrick BO, Ryan US and Brigham KL, Direct effects of *E coli* endotoxin on structure and permeability of pulmonary endothelial monolayers and the endothelial layer of intimal explants. *Am J Pathol* **122**: 140–151, 1986.
27. Von Kugelgen I and Starke K, Noradrenaline-ATP co-transmission in the sympathetic nervous system. *Trends Pharmacol Sci* **12**: 319–324, 1991.
28. Pearson JD and Gordon JL, Vascular endothelial and smooth muscle cells in culture selectively release adenine nucleotides. *Nature* **281**: 384–386, 1979.