Loss of ATP Diphosphohydrolase Activity with Endothelial Cell Activation

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Summary

Quiescent endothelial cells (EC) regulate blood flow and prevent intravascular thrombosis. This latter effect is mediated in a number of ways, including expression by EC of thrombomodulin and heparan sulfate, both of which are lost from the EC surface as part of the activation response to proinflammatory cytokines. Loss of these anticoagulant molecules potentiates the procoagulant properties of the injured vasculature. An additional thromboregulatory factor, ATP diphosphohydrolase (ATPDase; designated as EC 3.6.1.5) is also expressed by quiescent EC, and has the capacity to degrade the extracellular inflammatory mediators ATP and ADP to AMP, thereby inhibiting platelet activation and modulating vascular thrombosis. We describe here that the antithrombotic effects of the ATPDase, like heparan sulfate and thrombomodulin, are lost after EC activation, both in vitro and in vivo. Because platelet activation and aggregation are important components of the hemostatic changes that accompany inflammatory diseases, we suggest that the loss of vascular ATPDase may be crucial for the progression of vascular injury.

Quiescent vascular endothelial cells (EC)[1], as they normally exist in vivo, function primarily to regulate blood flow and hemostasis by the maintenance of a nonthrombogenic surface (1). These effects are mediated largely by protease inhibitors such as antithrombin, which interacts with glycosaminoglycans, including heparan sulfate (2); concurrently, there is also expression of thrombomodulin that binds thrombin and induces the protein C/protein S anticoagulant pathway (3). Activation of EC promotes vascular thrombosis by the simultaneous induction of procoagulant activity (4, 5) and the suppression of anticoagulant properties (6–8). In particular, both heparan sulfate (9) and thrombomodulin (10) are rapidly lost from the surface of the EC.

Platelet activation and aggregation are important factors in the mediation of vascular inflammation (6, 11, 12) and are specifically associated with the rejection of discordant xenografts, even in the absence of complement activation in a process termed delayed xenograft rejection (13). Progression of platelet recruitment in association with activation is enhanced by adenosine nucleotides, which are released from damaged endothelium or other vascular cells, and are secreted in high concentrations by platelets in response to exogenous ADP, collagen, thrombin, or activated complement components. This provides an important positive feedback mechanism (11, 14). A critical regulatory element in the control of platelet thrombus formation may be the expression on endothelium of an ATP diphosphohydrolase (ATPDase) (15–17). Enzymatic degradation of extracellular ATP and ADP to AMP by this ecto-enzyme would transform and reverse the proinflammatory environment brought about by interaction with purinergic receptors on platelets and vascular endothelium. The ultimate generation of adenosine results in a platelet anti-aggregatory signal and downregulation of vascular inflammation in conjunction with production of prostaglandin I_2 and nitric oxide (NO) (11, 18, 19).

Our interest in platelet activation in the setting of discordant xenograft rejection (20, 21) has led us to study the extent to which vascular ATPDase is modulated by EC activation and inflammatory mediators. Here, we show that the antithrombotic effect of the ATPDase, like heparan sul-

[1]*Abbreviations used in this paper:* ATPDase, ATP diphosphohydrolase; EC, endothelial cells; FBS, fetal bovine serum; hEC, human aortic EC; HRP, horseradish peroxidase; HUVEC, human umbilical vein EC; NO, nitric oxide; PAF, platelet activation factor; pEC, pig aortic EC; SOD, superoxide dismutase.

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fate and thrombomodulin, is lost following EC activation, both in vitro and in vivo.

We speculate that this loss, and the resultant decreased capacity to degrade ADP, could play a significant role in the extensive platelet activation and vascular inflammation seen in reperfusion processes, xenograft rejection, and other forms of vascular injury. We have recently shown common identity between CD39 and the vascular EC ATPDase (22). We propose that the now feasible expression of the CD39/ATPDase in a form that is not inhibited during EC activation, as done for thrombomodulin (23), may find therapeutic application as a valuable and novel antithrombotic agent.

Materials and Methods

Reagents

Ammonium molybdate, catalases (bovine liver), collagenases, bovine hemoglobin, dipyridamole, flutamine, malachite green, streptomycin-penicillin, superoxide dismutases (bovine erythrocyte), trypsin, Tween 20, and xanthine were bought from Sigma Chemical Co. (St. Louis, MO). ATP, ADP, and thrombin were purchased from Calbiochem Corp. (La Jolla, CA). DMEM, HBSS, RPMI, 10× PBS, fetal bovine serum (FBS; Lot#44N4044)), penicillin G–streptomycin, L-glutamine (200 mM), and EDTA-trypsin (5.3 mM/0.5%) were from GIBCO BRL (Gaithersburg, MD). Xanthine oxidase from bovine milk and ADP-β-S were from Boehringer Mannheim GmbH (Mannheim, Germany and Indianapolis, IN, respectively). Des-methyl tirilazad was a gift from The Upjohn Company (Kalamazoo, MN). Recombinant, human TNFα was a product of Sandoz Pharma, Ltd. (Basel, Switzerland). Collagen was from Chrono-Log Corp. (Havertown, PA). Agarose was from FMC Corp. (Rockland, ME). ECL enhanced luminol chemiluminescent substrate and hybond-PVDF membranes were from Amersham Corp. (Arlington Heights, IL), and X-OMAT LS film from Kodak (Rochester, NY).

Antibodies

Control mAb's IgG_1-biotin and streptavidin-PE were products of PharMingen (San Diego, CA); IgG_1-FITC, IgG_{2a}-FITC, IgG_{2a}-PE were from Becton Dickinson (San Jose, CA). Anti–human CD39 mAb (Immunotech, Westbrook, ME, and Accurate Chem. & Sci. Corp., Westbury, NY), and anti–mouse IgG conjugated with either peroxidase or fluorescein-5-isothiocyanate (Sigma Chemical Co., St. Louis, MO) were used. Peroxidase conjugated antibodies, biotinylated goat anti–mouse IgG, and streptavidine–horseradish peroxidase (HRP) were purchased and used at recommended dilution (Pierce Chem. Co., Rockford, IL). Anti–TNFα mAb used as isotype control antibody was from Endogen (Boston, MA).

A 16–amino acid peptide from the NH_2 terminus of the porcine pancreatic ATPDase (KSDTQETYGALDLGGA) with common sequence homology to human CD39 was used for the generation of rabbit polyclonal antibody (gift of Dr. A.D. Beaudoin, University of Sherbrooke, Sherbrooke, Quebec). We found that these polyclonal antibodies react with human CD39/ATPDase expressed by COS-7 transfectants (22).

Endothelial Cell Culture and Platelet Preparation

Pig aortic endothelial cells (pEC), harvested by a combined collagenase and scraping technique from adolescent male pig aortae, were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin G (50 U/ml), and streptomycin (50 μg/ml). These pEC were characterized by E-selectin expression after activation, and by morphological assessment under phase microscopy (24).

Human aortic EC (hEC), purchased from Cell Systems Corp. (Kirkland, WA), were characterized by acetylated-LDL uptake, positive staining for factor VIII/vWF, and angiotensin converting enzyme activity. Human EC culture medium (CS-3.0) containing heparin (15 μg/ml) and acidic fibroblast growth factor (50 μg/ml) with hEC passaging reagents, EDTA, trypsin, and trypsin inhibitor were also obtained from Cell Systems Corp. DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS, L-Glutamine (2 mM), penicillin G (50 U/ml) and streptomycin (50 μg/ml) was used for the culture of hEC before exposure to oxidative stress. Human umbilical vein EC (HUVEC) at the third passage were from Dr. B.M. Ewenstein (Brigham and Women's Hospital, Boston, MA). Where indicated, EC were preactivated by TNFα stimulation at 10 and 50 ng/ml for determined times before harvesting (25).

Aortic EC at ~passage 5 were prepared in suspension by EDTA-collagenase treatment (17, 26). After detachment, EC were reconstituted with an equal volume of culture medium containing FBS, and then pelleted by centrifugation at 150 *g* for 8 min at 22°C. Cells were then resuspended in 5 mM Hepes saline, pH 7.4, 5 mM KCl, and 1 mM $CaCl_2$ with 1 mM $MgCl_2$. They underwent two more wash cycles before final reconstitution at 5×10^6/ml and maintained at 22°C. EC suspensions had a viability of in excess of 90% by trypan blue exclusion.

Blood anticoagulated with 0.1 vol of 3.2% sodium citrate was collected from apparently healthy, drug free human volunteers after obtaining informed consent. Washed, gel filtered platelets, and platelet rich plasma were prepared by standard methodology (27, 28).

Platelet Aggregation Experiments with Combined Suspensions of Platelets and EC

Platelet aggregation and ATP release tests used a two sample, four channel, Whole Blood Lumi-Aggregometer (560 Ca; Chrono-Log Corp.). Platelets (2×10^8) and EC (10^6) were preincubated in siliconized cuvettes containing stirring bars at 37°C in the aggregometer. Control cuvettes contained equal numbers of human and porcine EC and the platelet–EC combinations, to correct for light absorption by the nonaggregating EC. Platelets were activated by ADP (1–5 μM), equine collagen (1–5 μg/ml) (Chrono-Log Corporation), or human thrombin (0.1 U/ml). Platelet aggregation was determined by increased light transmission (changes in OD representing aggregation), and by release of ATP (luminometry) following manufacturer's instructions (Chrono-Log Corp.).

Investigation of EC–Mediated Inhibitory Mechanisms

The influence of prostenoids and NO was determined by evaluating pEC inhibitory responses in the presence of prostaglandin synthetase inhibitors (aspirin and indomethacin) and scavengers of NO (hemoglobin), or by methylene blue that prevents effects of NO by inhibiting soluble guanylate cyclase (17). Specifically, platelet donors had blood drawn 12 h after taking 650 mg aspirin, and EC cultures were treated with 1 mM acetyl salicylic acid for 30 min under standard culture conditions. After resuspension, 10 μM indomethacin was added before study. A final concentration of 10 μM methylene blue was added to the platelets and incubated at room temperature for 30 min followed by a 15 min centrifuge at 1,450 *g* at 4°C before resuspension. Oxyhemoglobin was prepared from

bovine hemoglobin according to the method described by Martin et al. (29), and added to EC suspensions at 25 μM. The effects of an ATPDase resistant nonmetabolizable ADP analog (ADP-β-S) on platelet aggregation in the presence of EC was also studied.

Endothelial Cell ATPDase Assays

Biochemical ATPase Assays After Method of Le Bel. Intact pEC or hEC preparations were incubated with 5–200 μM ADP or ATP, and free phosphate release was determined over time (30). Copper acetate, pH 4.0, and 5% ammonium molybdate with Elon 2% in 5% sodium sulfite were added to the samples, and the color was allowed to develop for 10 min. Standard curves were constructed with an appropriate range of concentrations of KH_2PO_4 for that experiment. Absorbance was measured at 870 nm on a spectrophotometer (DU-64; Beckman Instruments, Inc., Fullerton, CA).

Biochemical ATPase Assay with Malachite Green. Reaction of enzyme was inactivated by the addition of malachite green reactive (31). Absorbance was read at 610 nm on a microplate spectrophotometer (EL 340; Bio-Tek Instruments, Inc., Winooski, VT).

TLC of Nucleotides and Nucleosides. EC cultures were incubated with [^{14}C]ADP from 1 to 50 μM (55 mCi/mmol; New England Nuclear, Boston, MA). Supernatant fluids collected at determined time points were analyzed for nucleotide degradation by TLC carried out on linear K preabsorb strip TLC plates (Whatman Laboratory Division, Clifton, NJ) using a solvent system composed of isobutyl alcohol/1-pentanol/ethylene glycol monoethyl ether/NH_4OH/water at ratios 90:60:180:90:120 (17). The separated compounds were scanned for radioactivity with a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA). Differential degradation of the [^{14}C]ADP was then determined by ImageQuant software according to manufacturer's instructions.

Western Blotting

Cell proteins obtained from lysis of EC induced by three cycles of freeze thawing, were subjected to polyacrylamide gel electrophoresis under reducing conditions, blotted onto Hybond-polyvinylidene fluoride membranes (32), and probed with polyclonal antibody directed at the NH_2-terminal peptide sequence obtained from the porcine pancreatic type I ecto-ATPDase (33). This sequence corresponds to ETNNQETFGALDLGGA within the vascular ATPDase/CD39 (sequence identity as underlined); we have shown that this antibody reacts specifically with human CD39/ATPDase expressed in COS-7 cells (22). This polyclonal antibody also cross-reacts with vascular ATPDase (34). Peroxidase conjugated antibodies were detected using the ECL enhanced luminol chemiluminescent substrate and X-OMAT LS film.

Immunocytofluorometric Analysis of CD39/ATPDase Expression

Human EC were harvested by gentle pipetting with Hanks balanced salt solution (GIBCO BRL) containing 10 mM EDTA. Cells were washed with buffer (PBS, 5% FBS, and 0.02% sodium azide), and incubated with either anti–human CD39 mAb (IgG_1) (Immunotech), or an isotype matched control mAb, anti–TNFα (Endogen) for 30 min on ice. Cells were washed twice and incubated with anti–mouse IgG conjugated with fluorescein-5-isothiocyanate (Sigma Chemical Co.) for 30 min on ice. Finally, cells were washed twice and analyzed by flow cytometry on a FACScan® bench top model using Cellquest II software (Becton Dickinson). Data were collected from viable cells only, as determined by propidium iodide uptake.

Immunocytochemical Analysis

Cells were fixed with 0.05% glutaraldehyde for 15 min, and CD39/ATPDase antigen was detected by anti–human CD39 mAb (Accurate) using biotinylated goat anti–mouse IgG and streptavidine-HRP (Pierce) as a detection system. 3-NH_2-9-ethylcarbazole (Sigma Chemical Co.) was used as the HRP substrate.

Modulation of ATPDases By Cellular Activation and Oxidative Stress

EC were exposed to TNFα, to H_2O_2 (100 μM), or xanthine oxidase (100 mU/ml) and xanthine (50–200 μM), the latter combination created to directly generate oxidative stress with all appropriate controls before determination of EC ATPDase activity (35).

The relative role of each oxidant was determined by performing parallel experiments in the presence of one of the following individual agents: superoxide radical scavenger SOD (Cu-Zn form, from bovine erythrocytes, specific activity 3,570 U/mg of protein used at 330 U/ml), the hydrogen peroxide scavenger catalase (from bovine liver, specific activity 40,000 U/mg of protein used at 1,000 U/ml), the 21-aminosteroid des-methyl tirilazad (final concentration 5 μM, an effective inhibitor of membrane peroxidation), and the iron chelator deferoxamine (100 μM). In associated experiments, the effect on activated EC ATPDase activity of the addition of β-mercaptoethanol (5 and 10 μM) was studied. The extent of lipid peroxidation was confirmed by determination of levels of malonaldehyde and 4-hydroxyalkenals in cell lysates by a colorimetric assay kit (Calbiochem Corp.).

Histology and Histochemistry

Biopsies from control rat kidney, or those rendered ischemic by renal artery occlusion under direct vision for 1 h, and then reperfused for defined time periods of up to 2 h were studied. Tissues were snap-frozen in liquid nitrogen and stored at −80°C, or fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for light microscopy. Cryostat sections were treated according to the cerium based method as previously described (36). In brief, cryostat sections were fixed with pararosaniline with $NaNO_2$ and preincubated in Tris-maleate buffer with $CeCl_3$ and $Mg(NO_3)_2$ (Sigma Chemical Co.). Sections were then incubated in the same medium with the addition of 200 μM ADP. The reaction product was converted to cerium-perhydroxide in glycine-NaOH buffer with H_2O_2. Subsequently, a diaminobenzydine amplification step was carried out to enhance the visibility of the ATPDase reaction product. Sections not incubated with ADP or ATP served as negative controls.

Statistical Analysis

Data were analyzed by graphical representation and by the Mann Whitney Rank Sum Test where indicated (Sigma Suite Version 2.0 for Windows 95; Jardel Scientific, San Rafael, CA).

Results

Quiescent Aortic Endothelial Cells (pEC and hEC) Exert an Inhibitory Effect on Human Platelet Aggregation Responses In Vitro. When control platelets were stimulated with standard agonists, aggregation was a consistent event. However, in the presence of both pEC and hEC, the aggregation of washed, gel filtered platelets in response to ADP, collagen, and low doses of thrombin was totally inhibited in a man-

ner consistent with abrogation of aggregation responses. The EC-associated factor(s) that blocked human platelet aggregation were found to be cell associated, and could not be detected in pEC or hEC culture supernatants, confirming the previously published data on HUVEC by Marcus et al. (17). This inhibitory factor completely blocked purified human platelet responses to the agonists in the presence of pEC in monolayer on the cuvettes, or on bead cultures of pEC placed into the cuvette in addition to the EC suspensions as tested above (data not shown).

Combinations of human platelet rich plasma and pEC resulted in a rapid and spontaneous aggregation response. This was observed to be a xenogeneic phenomenon, as no platelet aggregation was noted with either hEC or HUVEC when tested with human platelet rich plasma. This mode of platelet activation was found to be a result of extensive complement activation on the xenogeneic EC and thrombin generation (28).

Characteristics of the EC-associated Inhibitor. We tested four of the putative systems associated with EC that block platelet aggregation for their involvement in the inhibitory process. Incubation of pEC treated with acetylsalicylic acid and indomethacin with platelets taken from donors who had ingested aspirin, did not influence the inhibitory properties of the EC. These data suggested that the cell-associated inhibitor was "aspirin insensitive" i.e., did not represent prostenoids and were in accordance with previously published findings (17). Additionally, the inhibitory effect of EC on human platelets appeared not to be influenced by known modulators of NO; hemoglobin, which scavenges NO, and methylene blue, which inhibits soluble guanylate cyclase, had no discernible effects on the inhibitory potential of pEC and hEC.

EC-associated thrombomodulin, because of its anticoagulant potential, was considered to be a potential inhibitor of thrombin-induced platelet responses in the pEC and platelet combinations tested. Supernatants which were taken from pEC and platelets incubated with thrombin (0.1 U/ml) still contained thrombin, and induced aggregation when added to platelets alone. These observations indicated that the interaction of thrombomodulin with thrombin was not responsible for the observed inhibitory effect of pEC on platelets (data not shown).

Because of the importance of ADP as an agonist that amplifies and propagates platelet activation induced by itself or other agents (37), experiments were carried out to determine whether the inhibitory capacity of the xeno- and allogeneic aortic EC was dependent upon cellular ATPDase activity. To this end, ADP-β-S, a structural analog of ADP (38) that can activate platelet receptors but is not hydrolyzed by the ATPDase, was used to induce aggregation of platelets, both alone and in combination with pEC or hEC. The profound inhibitory effect of these EC on platelet aggregation was noted when ADP was used as the platelet agonist. However, this pattern of inhibition of platelet aggregation by EC was not observed when ADP-β-S was used as the agonist (Fig. 1). These data suggest that hydrolysis of ADP by ATPDase was responsible, at least in part, for the inhibitory potential of the EC.

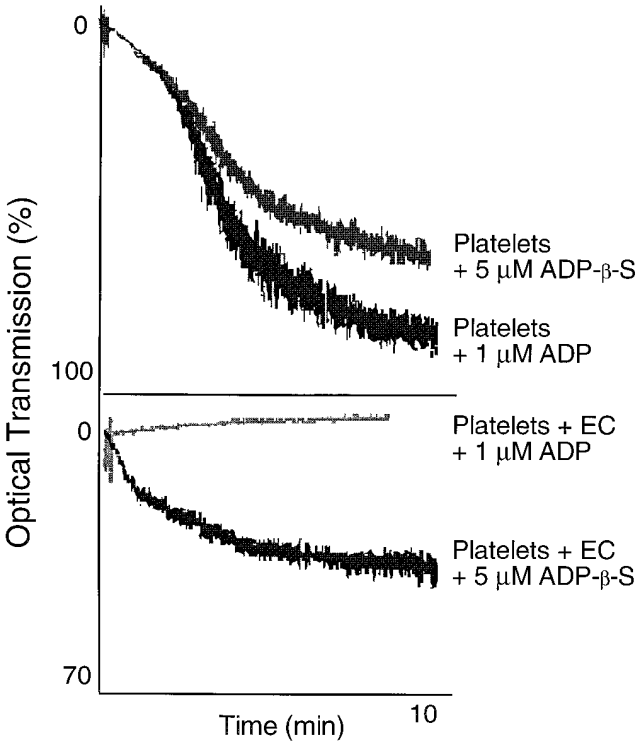


Figure 1. Effect of pEC on platelet aggregation induced by ADP and ADP-β-S (an ADP analog resistant to ATPDase activity). Platelets underwent comparable aggregation responses after stimulation with 1 μM ADP and 5 μM ADP-β-S. Addition of pEC abrogated platelet responses to ADP alone, but had minimal effects on ADP-β-S stimulated platelet aggregation. These data indicate a functional ATPDase associated with EC is responsible, at least in part, for the inhibitory effects on platelet aggregation in vitro.

Characteristics of EC-associated ATPDase. The EC associated ATPDase activity was determined by biochemical and functional parameters. The vascular ecto-enzyme was confirmed to have E-type ATPDase activity as suggested by Plesner (19) based on identical substrate specificity for ATP and ADP, the strict Ca^{2+} and Mg^{2+} dependence, and the patterns of ecto-enzymatic inactivation with various defined inhibitors (22; Table 1). The inhibitors for P-type plasma membrane ATPase (ouabain) and alkaline phosphatases (levamisole) had little effect on ATPDase enzymatic function, whereas sodium azide could block approximately half the activity. All enzymatic activities reported in Table 1 are derived by subtracting values obtained with EGTA from those with Ca^{2+}.

The hydrolysis of radiolabeled ADP to AMP and consequent catalysis to adenosine by confluent, intact pEC was measured by TLC. ADP was rapidly degraded by the ecto-enzyme associated with EC. AMP, and then adenosine, were generated during 30 min incubation with dipyridamole (10 μM; Fig. 2). Adenosine appearance was probably associated with 5′ nucleotidase activity (Fig. 2). These kinetic determinations were in concordance with those stated for

Table 1. *Effects of Selected Inhibitors on Vascular EC ATPDase Activity*

Inhibitor	Inhibitor concentration	Hydrolysis of 200 μM ADP
	mM	*%, median values*
None	-	100
NaN_3	20	48
Ouabain	3	94
Levamisole	1.5	86
AMP	5	80

Vascular EC ATPDase activity was determined by the malachite green technique as described in Materials and Methods. CA^{2+}-dependent free phosphate release from exogenous ADP was determined at the end of a 10 min incubation with pEC, with or without the specified inhibitors. All assays were performed in triplicate.

the pEC ecto-enzyme as determined by another methodology (39).

Modulation of EC ATPDase Activity By TNFα In Vitro. Activation of 10–50 ng/ml pEC or hEC by human recombinant TNFα from 1 to 8 h, resulted in rapid loss of the EC antiaggregatory phenotype and the development of a permissive environment for platelet activation in response to the standard agonists in vitro (Fig. 3). ATPDase activity, as determined by both [^{14}C]ADP hydrolysis and inorganic phosphate release from ATP and ADP, showed comparable patterns of inhibition at the time intervals examined. A trend towards inhibition was observed as early as 30 to 60 min after TNFα activation, and was maximal by 4 h; statistically significant differences between the ATPDase activities of the quiescent and TNFα stimulated EC at 2 and 4 h were confirmed (Fig. 4).

The reconstitution of EC antiplatelet aggregatory properties (Fig. 3), observed after 18 h of TNFα stimulation, was paralleled by ATPDase enzymatic activity to levels not statistically different to those in quiescent EC (data not shown).

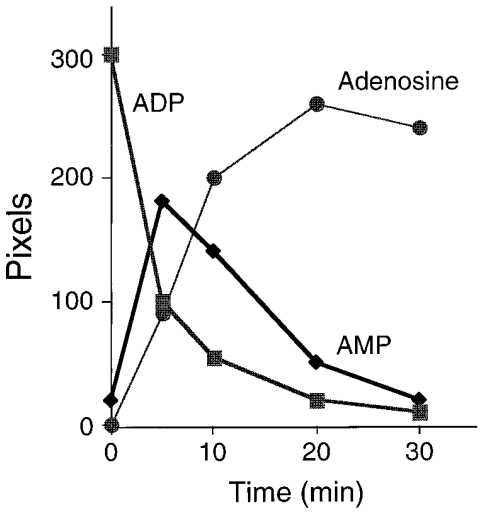


Figure 2. Hydrolysis of [^{14}C]ADP to AMP by EC-associated ATPDase. Radiolabeled ADP hydrolysis to AMP, and consequent catalysis to adenosine by pEC, was measured by TLC of supernatants from EC cultures. ADP was rapidly degraded and the radio-label appeared as AMP initially, and then adenosine over a time period of 30 min.

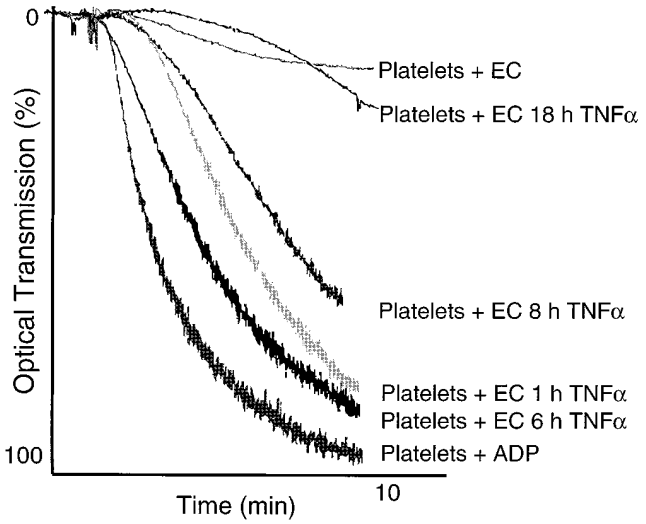


Figure 3. ATPDase antiaggregatory activity is modulated by EC responses to TNFα in vitro. Activation of quiescent porcine EC by 10 ng/ml human recombinant TNFα from 1 to 8 h in vitro resulted in rapid loss of the activated EC antiaggregatory phenotype at the time of testing. This was noted by the development of a permissive environment for platelet activation in response to 5 μM ADP in vitro. After TNFα stimulation of EC, reconstitution of functional antiplatelet aggregatory properties was observed by 18 h.

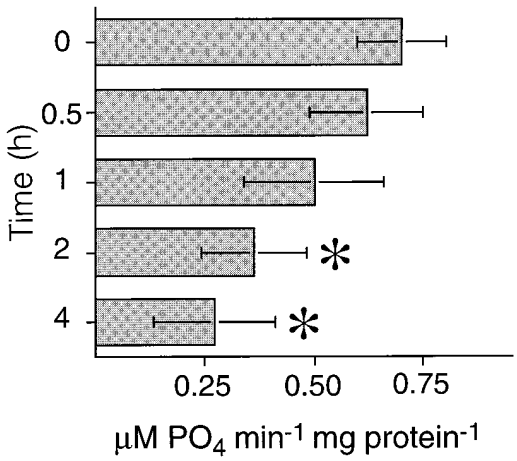


Figure 4. ATPDase enzymatic activity after EC activation by TNFα. EC ATPDase activity was determined by measuring inorganic phosphate release from ADP and ATP. An inhibitory effect was maximal by 4 h after stimulation of pEC by TNFα as depicted here for ADP (data in graph are expressed as means and SD; normality test passed). Statistical analysis confirmed significant differences to control quiescent EC values at both 2 and 4 h after EC activation; $*P < 0.005$, Mann Whitney Rank Sum Test. Experiments studying ATPDase activity by [^{14}C]ADP hydrolysis gave similar results (data not shown).

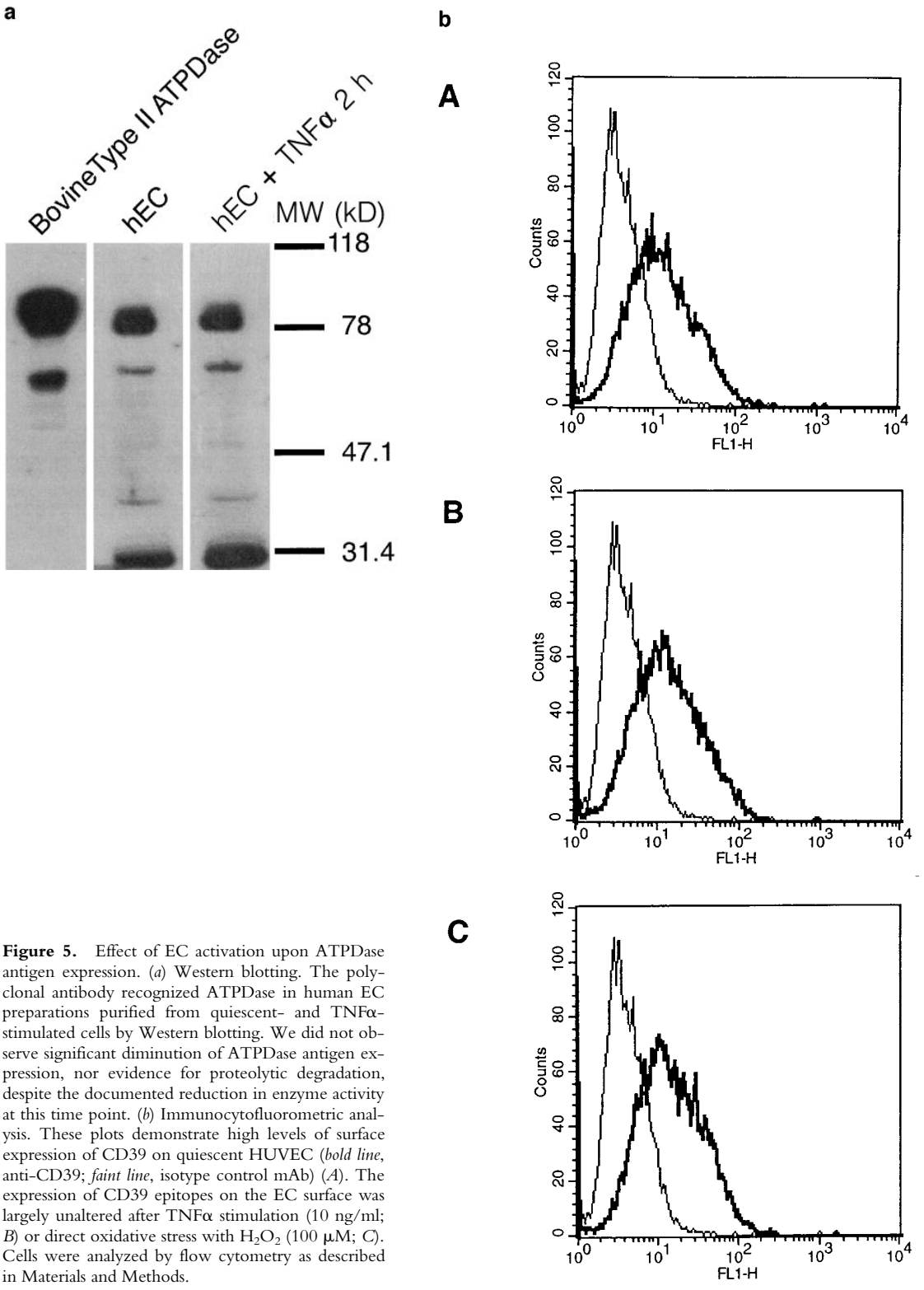
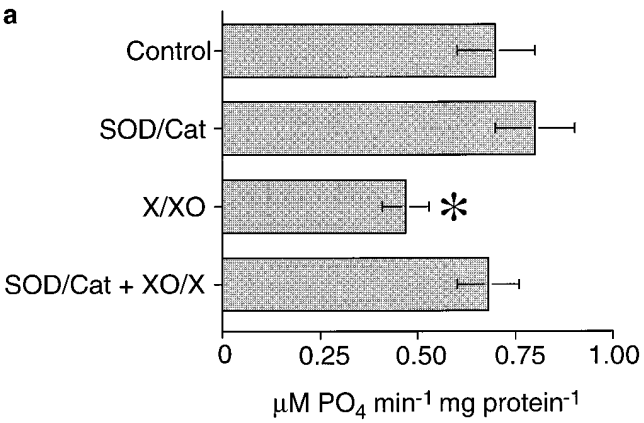


Figure 5. Effect of EC activation upon ATPDase antigen expression. (*a*) Western blotting. The polyclonal antibody recognized ATPDase in human EC preparations purified from quiescent- and TNFα-stimulated cells by Western blotting. We did not observe significant diminution of ATPDase antigen expression, nor evidence for proteolytic degradation, despite the documented reduction in enzyme activity at this time point. (*b*) Immunocytofluorometric analysis. These plots demonstrate high levels of surface expression of CD39 on quiescent HUVEC (*bold line*, anti-CD39; *faint line*, isotype control mAb) (*A*). The expression of CD39 epitopes on the EC surface was largely unaltered after TNFα stimulation (10 ng/ml; *B*) or direct oxidative stress with H_2O_2 (100 μM; *C*). Cells were analyzed by flow cytometry as described in Materials and Methods.

Expression of EC ATPDase after Cellular Activation In Vitro. The polyclonal antibody directed to peptide sequences from porcine aortic ATPDase did not react with ATPDase antigen as expressed by intact EC by immunocytochemistry (data not shown). However, this antibody did recognize denatured ATPDase from human EC lysates from quiescent and TNFα stimulated cells analyzed by Western blotting. Using this technique, we did not observe either dimi-



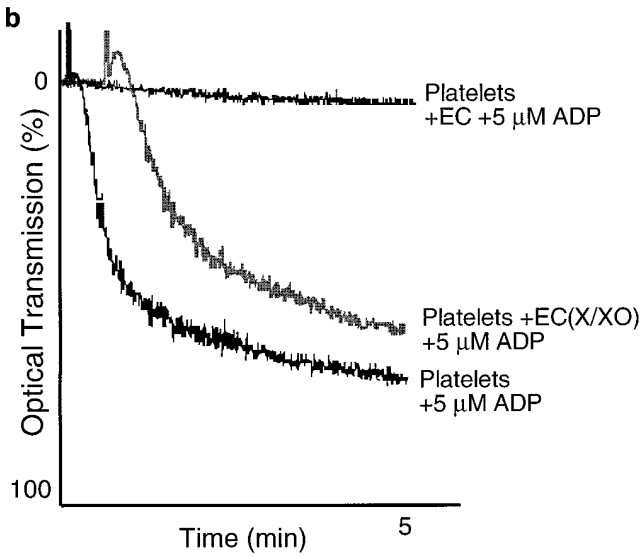


Figure 6. Effects of oxidative stress on EC ATPDase activity. (*a*) ATPDase biochemical activity assay. The decreased capacity of pEC directly perturbed by oxidative stress to express ATPDase activity was demonstrated by estimation of phosphate release from supplemental ADP by the malachite green technique. Exogenous xanthine oxidase (*XO*, 100 mU/ml) and xanthine (*X*, 100 μM) markedly inhibited pEC ATPDase activity in a statistically significant manner after 2 h oxidant exposure (*$P = 0.002$; Mann Whitney Rank Sum Test). This effect could be abrogated by the supplemental antioxidants superoxide dismutase (*SOD*; 330 U/ml) and catalase (1,000 U/ml), confirming the purely oxidant nature of the inhibition (data are expressed as means and standard deviations; normality test passed for graphical representation). (*b*) Platelet inhibitory properties of ATPDase. Further evidence for the loss of ATPDase functional activity after exposure to oxidants was derived from the demonstration that pEC exposed to such reactions for 2 h were unable to inhibit platelet aggregation responses to ADP 5 μM.

nution of ATPDase antigen expression, or evidence for protein degradation, despite the reduction in enzyme activity in EC stimulated with TNFα at 2 h. (Fig. 5 *a*).

Surface expression of CD39/ATPDase was confirmed by cytofluorometric analysis of human EC with anti–CD39 mAb (Fig. 5 *b*) in concordance with the surface biochemical activity demonstrated earlier (Fig. 4). After stimulation of EC with TNFα (10 ng/ml), the expression of CD39 epitopes, as determined by immunocytofluorometric analysis between 2 and 4 h, was largely unaltered. There were

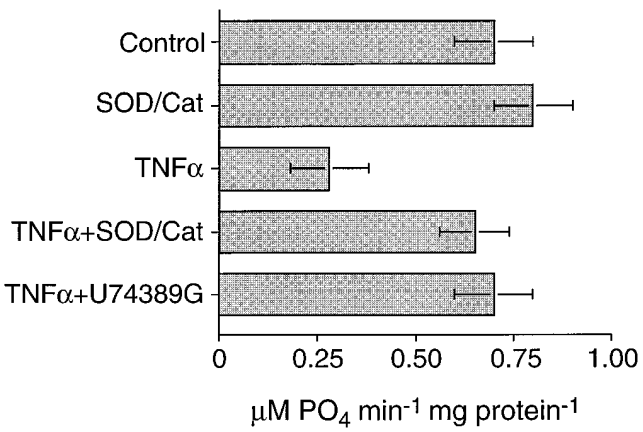


Figure 7. Maintenance of pEC-associated ATPDase activity by antioxidants after TNFα stimulation. Superoxide dismutase (*SOD*; Cu-Zn form, 330 U/ml); the hydrogen peroxide scavenger catalase (1,000 U/ml) and the 21-aminosteroid des-methyl tirilazad (U74389; final concentration 5 μM) were again used as antioxidants. Such interventions could protect against the statistically significant TNFα-mediated inhibitory changes in pEC ATPDase activity, and had minimal positive effects on quiescent pEC ATPDase levels. Inhibition of ATPDase activity following TNFα activation was consistently abrogated by these antioxidants (data are expressed as mean and standard deviations).

also no changes observed in the levels of surface CD39 staining when TNFα activated EC were examined by immunocytochemistry (data not shown).

Endogenous xanthine oxidase and other enzyme systems in pEC (e.g., NADPH oxidase) elaborate significant levels of reactive oxygen intermediates after cellular activation. For example, the generation of H_2O_2 by pEC after activation with cytokines, such as TNFα, is 0.015 nmol/min/10^6 cells (25). In light of the patterns of ATPDase functional inhibition in the setting of pEC activation and the parallels with the modulation of thrombomodulin (10), we further evaluated the effects of exogenous oxidative stress on cell-associated ATPDase expression in vitro. As demonstrated by flow cytometry, the exposure of HUVEC to H_2O_2 (100 μM) for 2 h did not alter the surface expression of CD39 (Fig. 5 *b*).

Effect of Oxidative Stress on pEC ATPDase Activity In Vitro. Although no differences in cell surface expression of CD39 were observed by flow cytometry following HUVEC activation, we tested the hypothesis that ATPDase enzymatic function could be inhibited by direct exposure to reactive oxygen intermediates. We observed that exogenous systems that generated oxidative stress and resulted in EC membrane lipid peroxidation (data not shown) could, in turn, significantly inhibit ATPDase activity in vitro. The reduced capacity of EC directly perturbed by oxidative stress to express ATPDase activity was demonstrated by both TLC analysis of radiolabeled ADP hydrolysis and by estimation of phosphate release from supplemental ADP (Fig. 6 *a*; Mann Whitney Rank Sum Test, $P = 0.002$). Further, impressive evidence for the loss of ATPDase functional activity was also derived from the demonstration that EC exposed to oxidative stress rapidly became unable to inhibit platelet responses to ADP in vitro (Fig. 6 *b*).

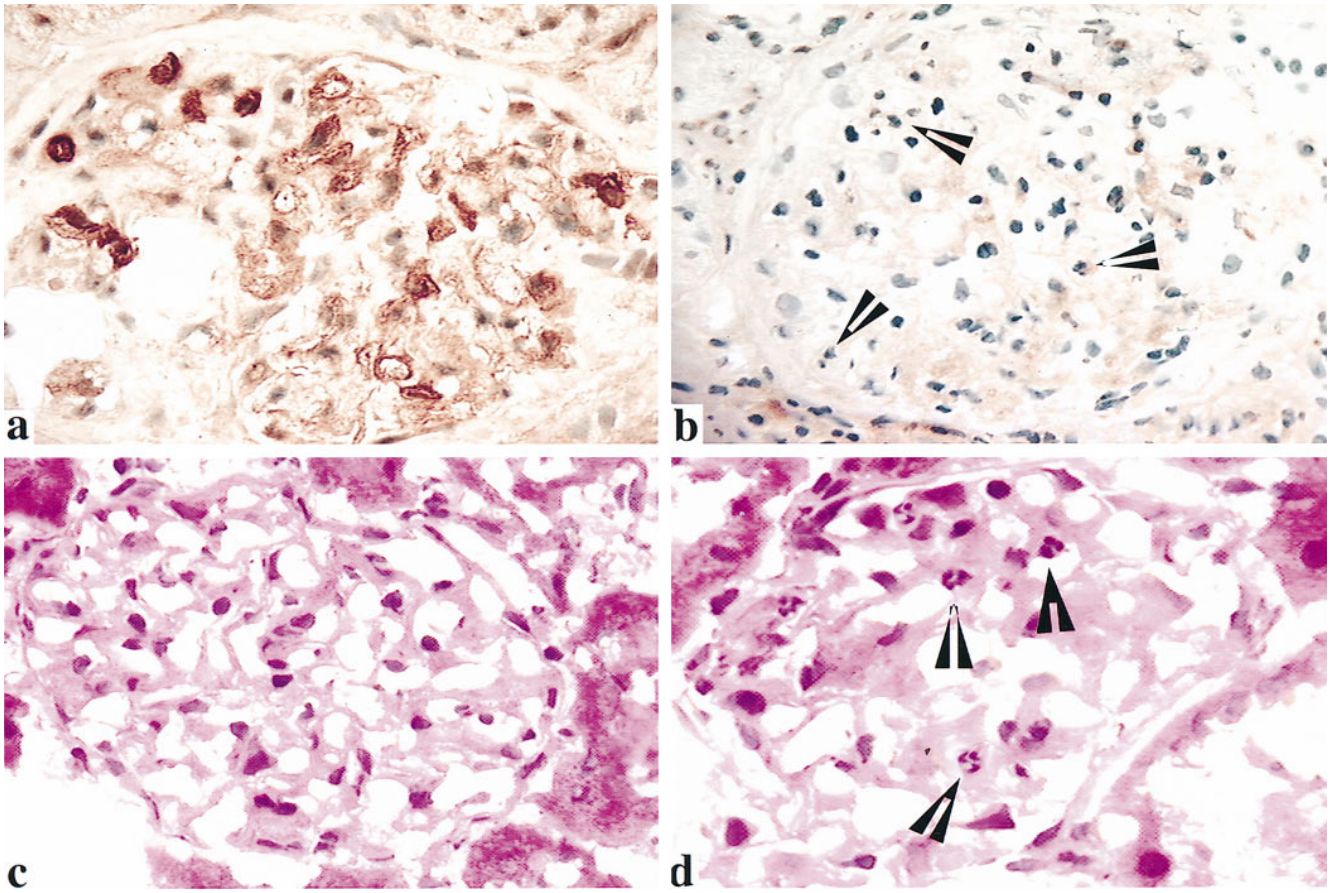


Figure 8. Loss of ATPDase activity during reperfusion injury in vivo. Upper panels show the extent of enzyme histochemical activity (cerium chloride method) within representative glomeruli of rat kidneys which were (*a*) freshly harvested, or (*b*) subjected to 1 h of ischemia and a further hour of reperfusion. Compared to the moderate to dense enzyme expression by rat vascular EC in control glomeruli (*a*), reperfusion injury and associated oxidative stress reduced ATPDase activity to negligible levels (*b*); arrows indicate neutrophils within capillary loops of kidney subjected to reperfusion injury. Lower panels are corresponding Hematoxylin- and Eosin-stained sections, showing (*c*) good preservation of glomerular structure and absence of leukocytes, and (*d*) association of reperfusion injury with focal platelet microthrombi which fill some capillary loops and the presence of neutrophils (*arrows*). All panels ×200.

Protective Effect of Antioxidants Upon ATPDase Activity. We next evaluated whether effective combinations of superoxide dismutase (SOD) and catalase, or more novel antioxidants such as 21-amino-steroids (des-methyl tirilazad) could protect EC against TNFα-mediated changes in ATPDase activity in vitro. The inhibition of ATPDase activity following TNFα activation was consistently abrogated by the selected antioxidants (Fig. 7). SOD and catalase were also able to preserve pEC ATPDase activity following the addition of the exogenous oxidant systems (Fig. 6 *a*).

In parallel experiments, the effect of a reducing agent, β-mercaptoethanol (5 and 10 μM) on ATPDase activity was studied after TNFα stimulation of pEC. Supplementation of pEC cultures with β-mercaptoethanol, before cellular activation, was as effective at maintaining enzyme activity as the antioxidant combinations.

Loss of Functional ATPDase from Vasculature. Using a cerium-based histochemical method, strongly positive staining for the ADP hydrolysis reaction product specifically generated by ATPDase activity, was observed in association with the vasculature of glomeruli from normal rat kidneys. After 1 h of ischemia and 1 h of subsequent reperfusion, a marked reduction in staining was noted (Fig. 8, *a* and *b*). In parallel, corresponding light microscopy showed that control glomeruli lacked neutrophils, whereas a considerable number of neutrophils associated with platelet deposits were noted after the episode of ischemia-reperfusion (Fig. 8, *c* and *d*).

Discussion

We have shown that the vascular ATPDase regulates both allogeneic and xenogeneic platelet aggregation in vitro, and that this activity is inhibited by EC activation and oxidant exposure. These processes are linked with ischemia-reperfusion vascular injury. The modulation of ATPDase activity is observed with cytokine-mediated EC activation and the associated elaboration of oxidants, or may occur after direct cellular perturbation with reactive oxygen intermediates. Inhibition of ATPDase was abrogated, at least in part, by pharmacologically effective levels of the antioxi-

dant agents tested. Similar beneficial effects on the maintenance of EC enzymatic activity were observed with the reducing agent β-mercaptoethanol. Comparable results have been described for nucleoside triphosphate hydrolase isozymes from the parasitic protozoan *Toxoplasma gondii* where enzyme activity has been found to be activated by dithiothreitol (40). From evaluating the experimental conditions, we have postulated that the observed inhibition of EC ATPDase functional activities could be directly linked to oxidative stress.

Our observations regarding the ATPDase are comparable to those relating to the other antithrombotic molecules, the activity of which is lost from the surface of the EC after exposure to proinflammatory mediators. Thrombomodulin activity associated with the EC surface is inhibited following similar activation responses (3, 10). Additionally, oxidative injury in xenograft rejection has been shown to exacerbate the loss of heparan sulfate, observed after the activation of pEC by xenoreactive antibody and complement (9, 41).

Acetylhydrolase, the plasma enzyme that degrades platelet activation factor (PAF), is also rapidly inactivated by oxygen radicals (35). Therefore, platelet activation, in the setting of oxidant stress, would be facilitated by the loss of EC ATPDase and would be further promoted by increased PAF. Both increased levels of PAF and the loss of the biological functions of ATPDase would tend to potentiate injury during vascular inflammatory events.

ATPDases may be considered sensitive to oxidative stress reactions because of their localization within cellular membranes (19). The documented inhibition of vascular ATPDase activity after adriamycin-mediated injury to rat glomerular endothelium in vivo (18) could represent sensitivity of the ecto-enzyme to oxidative reactions. Further, the exposure of HUVEC to reactive oxygen metabolites has been shown to result in depletion of intracellular ATP; this effect was considered to result from the interference with extracellular catabolism of adenine nucleotides and reduced adenosine entry into cells, although mechanisms were not directly examined (42).

We were not able to demonstrate any changes in the levels of surface expressed CD39/ATPDase after either cytokine activation or oxidant perturbation of human EC where loss of ATPDase function had been demonstrated in parallel (Fig. 5 *b*). Additionally, no changes were observed in the ATPDase antigen levels in TNFα-activated EC with the polyclonal antibody that was shown to recognize denatured protein of the same molecular size as the native ATPDase in Western blotting (Fig. 5 *a*). These findings are consistent with the hypothesis that the early decrease in ATPDase activity is related to oxidative damage, and thus, inactivation of the ATPDase without apparent proteolytic degradation or immediate loss from the surface membrane of the EC.

Several of our in vitro findings have been paralleled by in vivo observations. We have observed the loss of the vascular ATPDase activity following reperfusion type injury in rat kidneys where oxidative reactions are of central import. The process of vascular injury with organ reperfusion or during xenograft rejection (21) will result in the release of ATP and ADP from platelets and subendothelium (43, 44). Both ATP and ADP may activate neutrophils via purinergic type P_{2y} receptors, while ADP triggers platelets through type P_{2t} receptors (11). ATPDase activity results in hydrolysis of these nucleotides to AMP, which is further degraded to adenosine. The latter modulates both platelet and neutrophil function through adenosine type 2 receptors. ATP and ADP also trigger PGI_2 and NO release via P_{2y} receptors on the endothelium. ATP predominantly stimulates neutrophils via P_{2y} receptors, and results in the increased release of reactive oxygen intermediates, which may have profound effects on platelet reactivity as mediated through PAF and NO (45, 46). In addition, such induction of oxidative stress by the neutrophils could result in the further inhibition of the ATPDases blocking the sequential degradation of ATP and ADP, and potentiating inflammation in the setting of EC activation.

We have already confirmed that the vascular ATPDase has common identity with the previously described B cell activation marker designated as CD39 (22); others have recently shown that CD39 has apyrase activity (47). This membrane protein, CD39, has several potential targets for oxidative damage since the protein is rich in cysteine, methionine, and tyrosine residues (22, 47).

The direct relevance of CD39/ATPDase in transplantation biology and in other inflammatory vascular conditions must remain speculative at this time. It is our belief that rapid loss of ATPDase activity has an important pathogenetic role in the platelet deposition that occurs in the setting of graft preservation injury and with the repeated vascular insults associated with organ transplantation. The awareness that CD39 encodes the vascular ATPDase, and our findings above, may allow us to express CD39/ATPDase in such a manner that activity is not lost in an inflammatory environment.

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References

1. Preissner, K.T. 1988. Anticoagulant potential of endothelial cell membrane components. *Haemostasis*. 18:271–306.
2. Ihrcke, N.S., L.E. Wrenshall, B.J. Lindman, and J.L. Platt. 1993. Role of heparan sulfate in immune system-blood vessel interactions. *Immunol. Today*. 14:500–505.
3. Esmon, C.T. 1992. Protein-S and protein-C–biochemistry, physiology, and clinical manifestation of deficiencies. *Trends Cardiovasc. Med*. 2:214–219.
4. Nawroth, P.P., D.A. Handley, C.T. Esmon, and D.M. Stern. 1986. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc. Natl. Acad. Sci. USA*. 83:3460–3464.
5. Saadi, S., R.A. Holzknecht, C.P. Patte, D.M. Stern, and J.L. Platt. 1995. Complement mediated regulation of tissue factor activity in endothelium. *J. Exp. Med*. 182:1807–1814.
6. Pober, J.S., and R.S. Cotran. 1990. The role of endothelial cells in inflammation. *Transplantation (Baltimore)*. 50:537–544.
7. Cotran, R.S., and J.S. Pober. 1989. Effects of cytokines on vascular endothelium: their role in vascular and immune injury. *Kidney Int*. 35:969–975.
8. Esmon, C.T. 1993. Cell mediated events that control blood coagulation and vascular injury. *Annu. Rev. Cell Biol*. 9:1–26.
9. Platt, J.L., G.M. Vercellotti, B.J. Lindman, T.J. Oegema, F.H. Bach, and A.P. Dalmasso. 1990. Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection. *J. Exp. Med*. 171:1363–1368.
10. Moore, K.L., C.T. Esmon, and N.L. Esmon. 1989. Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. *Blood*. 73:159–165.
11. Marcus, A.J., and L.B. Safier. 1993. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. *FASEB J*. 7:516–522.
12. Marcus, A.J. 1996. Pathogenesis of atherosclerosis: special pathogenetic factors–inflammation and immunity: platelets. *In* Atherosclerosis and Coronary Artery Disease. vol. 1. V. Fuster, R. Ross, and E.J. Topol, editors. Lippincott-Raven, Philadelphia. 607–637.
13. Bach, F.H., S.C. Robson, H. Winkler, C. Ferran, K.M. Stuhlmeier, C.J. Wrighton, and W.W. Hancock. 1995. Barriers to xenotransplantation. *Nat. Med*. 1:869–873.
14. Gaarder, A., J. Jonsen, S. Laland, A. Hellem, and P.A. Owren. 1961. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature (Lond.)*. 192:531–532.
15. Born, G.V.R. 1961. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond.)*. 194:927–929.
16. Heyns, A. du P., M. Badenhorst, and F.P. Retief. 1977. ADPase activity of normal and atherosclerotic human aorta intima. *Thromb. Haemostasis*. 37:429–435.
17. Marcus, A.J., L.B. Safier, K.A. Hajjar, H.L. Ullman, N. Islam, M.J. Broekman, and A.M. Eiroa. 1991. Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. *J. Clin. Invest*. 88:1690–1696.
18. Bakker, W.W., K. Poelstra, M.A. Barradas, and D.P. Mikhailidis. 1994. Platelets and ectonucleotidases. *Platelets (Edinb.)*. 5:121–129.
19. Plesner, L. 1995. Ecto-ATPases: identities and functions. *Int. Rev. Cytol*. 158:141–214.
20. Platt, J.L., G.M. Vercellotti, A.P. Dalmasso, A.J. Matas, R.M. Bolman, J.S. Najarian, and F.H. Bach. 1990. Transplantation of discordant xenografts: a review of progress. *Immunol. Today* 11:450–456.
21. Bach, F.H., S.C. Robson, C. Ferran, H. Winkler, M.T. Millan, K.M. Stuhlmeier, B. Vanhove, M.L. Blakely, W. van der Werf, E. Hofer, and Hancock, W.W. 1994. Endothelial cell activation and thromboregulation during xenograft rejection. *Immunol. Rev*. 141:5–30.
22. Kaczmarek, E., K. Koziak, J. Sevigny, J.B. Siegel, J. Anrather, A.R. Beaudoin, F.H. Bach, and S.C. Robson. 1996. Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J. Biol. Chem*. 271:33116–33121.
23. Wrighton, C., C.W. Kopp, A. McShea, H. Vetr, and F.H. Bach. 1995. High level of functional human thrombomodulin in cultured porcine aortic endothelial cells. *Transplant. Proc*. 27:288–289.
24. Ryan, U.S., and G. Maxwell. 1986. Isolation, culture and sub-culture of endothelial cells: mechanical methods. *J. Tissue Cult. Methods*. 10:3–10.
25. Ferran, C., M.T. Millan, V. Csizmadia, J.T. Cooper, C. Brostjan, F.H. Bach, and H. Winkler. 1995. Inhibition of NF-κB by pyrrolidine dithiocarbamate blocks endothelial cell activation. *Biochem. Biophys. Res. Commun*. 214:212–223.
26. Broekman, M.J., A.M. Eiroa, and A.J. Marcus. 1991. Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: blockade of aggregation and secretion by an aspirin-insensitive mechanism. *Blood*. 78:1033–1040.
27. Timmons, S., A. Huzoor, J. Grabarek, M. Kloczewiak, and J. Hawiger. 1986. Mechanism of human platelet activation by endotoxic glycolipid-bearing mutant Re595 of *Salmonella minnesota*. *Blood*. 68:1015–1023.
28. Robson, S.C., J.B. Siegel, B.A. Lesnikoski, C. Kopp, D. Candinas, U. Ryan, and F.H. Bach. 1996. Aggregation of human platelets induced by porcine endothelial cells is dependent upon both activation of complement and thrombin generation. *Xenotransplantation*. 3:24–34.
29. Martin, W., G.M. Villani, D. Jothianandan, and R.F. Furchgott. 1985. Selective blockade of endothelium-dependent and glyceryltrinitrate-induced relaxation by hemoglobin and methylene blue in rabbit aorta. *J. Pharmacol. Exp. Ther*. 232: 708–716.
30. LeBel, D., G.G. Poirier, S. Phaneuf, P. St-Jean, J.F. Laliberte, and A.R. Beaudoin. 1980. Characterization and purification of a calcium-sensitive ATP diphosphohydrolase from pig pancreas. *J. Biol. Chem*. 255:1227–1233.
31. Geladopoulos, I.R., T.G. Sotiroudis, and A.E. Evangelous. 1991. A malachite green colorimetric assay for protein phosphatase activity. *Anal. Biochem*. 192:112–116.
32. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350–4354.
33. Sevigny, J., Y.P. Cote, and A.R. Beaudoin. 1995. Purification of pancreas type I ecto-ATP diphosphohydrolase and identification by affinity labeling with the 5′-p-fluorosulfonylbenzoyladenosine ATP analogue. *Biochem. J*. 312:351–356.
34. Sevigny, J., F.P. Levesque, G. Grondin, and A.R. Beaudoin. 1996. Purification of the blood vessel ATP diphosphohydrolase: identification and localization by immunological techniques. *Biochim. Biophys. Acta*. In press.
35. Ambrosio, G., A. Oriente, C. Napoli, G. Palumbo, P. Chiariello, G. Marone, M. Condorelli, M. Chiariello, and M. Trig-

giani. 1994. Oxygen radicals inhibit human plasma acetylhydrolase, the enzyme that catalyzes platelet-activating factor. *J. Clin. Invest.* 93:2408–2416.
36. Bakker, W.W., K. Poelstra, W. Timmerman, M.J. Hardonk, T.R. Koiter, and G.A. Schuiling. 1989. Experimental endotoxaemia in pregnancy: in situ glomerular microthrombus formation associated with impaired glomerular adenosine diphosphatase activity. *J. Lab. Clin. Med.* 114:531–537.
37. Siess, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev.* 69:58–178.
38. Goody, R.S., F. Eckstein, and R.H. Schirmer. 1972. The enzymatic synthesis of thiophosphate analogs of nucleotides. *Biochim. Biophys. Acta.* 276:155–161.
39. Gordon, E.L., J.D. Pearson, and L.L. Slakey. 1986. The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. Feed-forward inhibition of adenosine production at the cell surface. *J. Biol. Chem.* 261: 15496–15507.
40. Asai, T., S. Miura, L.D. Sibley, H. Okabayashi, and T. Takeuchi. 1995. Biochemical and molecular characterization of nucleoside triphosphate hydrolase isozymes from the parasitic protozoan *Toxoplasma gondii*. *J. Biol. Chem.* 270:11391–11397.
41. Magee, J.C., J.L. Platt, K.T. Oldham, and K.S. Guice. 1994. Oxidant stress increases susceptibility of porcine endothelial cells to injury by xenoreactive antibody and complement. *Transplant. Proc.* 26:1170.
42. Aalto, T.K., and K.O. Raivio. 1993. Metabolism of extracellular adenine nucleotides by human endothelial cells exposed to reactive oxygen metabolites. *Am. J. Physiol.* 264:C282–C286.
43. Luthje, J. 1989. Origin, metabolism and function of extracellular adenine nucleotides in the blood (erratum published 67: 558). *Klin Wochenschr.* 67:317–327.
44. Zimmermann, H. 1992. 5′-nucleotidase: molecular structure and functional aspects. *Biochem. J.* 285:345–365.
45. Pinsky, D.J., C.O. Mehmet, S. Koga, Z. Taha, M.J. Broekman, A.J. Marcus, H. Liao, Y. Naka, J. Brett, P.J. Cannon et al. 1994. Cardiac preservation is enhanced in a heterotopic rat transplant model by supplementing the nitric oxide pathway. *J. Clin. Invest.* 93:2291–2297.
46. Khimenko, P.L., T.M. Moore, L.W. Hill, P.S. Wilson, S. Coleman, A. Rizzo, and A.E. Taylor. 1995. Adenosine A2 receptors reverse ischemia-reperfusion lung injury independent of beta-receptors. *J. Appl. Physiol.* 78:990–996.
47. Wang T.F., and G. Guidotti. 1996. CD39 is an ecto-(Ca^{2+}, Mg^{2+}) apyrase. *J. Biol. Chem.* 271:9898–9901.