Depolarization of Endothelial Cells Enhances Platelet Aggregation Through Oxidative Inactivation of Endothelial NTPDase

Florian Krötz,* Hae Young Sohn,* Matthias Keller, Torsten Gloe, Steffen Sebastian Bolz, Bernhard F. Becker, Ulrich Pohl

Objective—The objective of this study was to investigate whether depolarization of cultured endothelial cells (human umbilical vein endothelial cells, HUVECs) affects their ectonucleotidase activity through superoxide (O$_2^-$) production.

Methods and Results—Depolarization by the cation channel gramicidin (100 nmol/L) or tetrabutylammonium chloride (1 mmol/L) induced O$_2^-$ release from HUVECs (n=4), which was decreased by superoxide dismutase (SOD, 500 U/mL). The activity of endothelial ectonucleotidases was assessed by the production of inorganic phosphate from ADP, which was decreased by chronic depolarization by 25% (n=6, P<0.05) and the amount of AMP derived from ADP in the presence of the 5'-nucleotidase inhibitor α,β-methylene-5'-diphosphate (100 μmol/L). AMP was decreased by chronic depolarization from 0.54±0.16 to 0.39±0.11 μmol/min/mg protein (n=6, P<0.05). This was abolished in the continuous presence of SOD (n=6). NTPDase protein was predominantly expressed in HUVECs (n=4). Protein abundance, viability of cells, and apoptosis rates were not altered by depolarization (n=10). Only in the presence of depolarized HUVECs, but not with control cells or with HUVECs depolarized in the presence of SOD, did 5 μmol/L of ADP cause irreversible platelet aggregation. Increases in transmural pressure induced endothelial depolarization in intact hamster small arterioles.


Key Words: endothelium ■ chronic depolarization ■ superoxide ■ ectonucleotidases ■ platelet aggregation

Platelet adhesion initiates a series of events leading to firm thrombus formation,1,2 for instance, release of platelet granule contents.3 Among the substances released, ADP is a major effector for recruitment of further platelets.4,5 Accordingly, the metabolism of ADP, which co-controls local ADP concentrations, is of substantial importance for primary hemostasis and the center of recent interest in attempts to therapeutically target thrombosis.6,8 In vitro, platelet aggregation induced by various stimuli is effectively inhibited by potato apyrase, an ectonucleotidase (ecto-NTPDase) that catalyzes ADP hydrolysis into free phosphate and AMP.9 Endothelial cells are known to express various families of ecto-NTPDases, among which the E-NTPDases (ectonucleoside triphosphate diphosphohydrolases or ecto-apyrases) have gained attention because of their substantial contribution to the inhibition of platelet aggregation.6,7,10 They can be classified according to their preferential substrates.9 CD39 (NTPDase 1) has been recognized as the major NTPDase present in endothelial cells.11 It exhibits the highest ADP:ATP substrate ratio in favor of ADP and is therefore a focus of antiplatelet research.12,13 Indeed, soluble CD39 effectively abolishes platelet aggregation in response to various agonists6 and reduces the cerebral infarct volume that is increased in CD39−/− mice.8

Endothelial NTPDases have been suggested to be inactivated by exposure to oxidative stress in vitro14 and during ischemia/reperfusion injury in rat glomeruli in vivo.15 Recently, we have reported that depolarization of human umbilical vein endothelial cells (HUVECs) leads to the activation of endothelial NAD(P)H-oxidase, resulting in enhanced O$_2^-$ release.16 This might be of importance in cardiovascular disease because hypertensive,17 diabetic,18 and injured19 vessels are thought to be chronically depolarized. In isolated vessels, increases in transmural pressure induce depolarization of smooth muscle cells30; however, whether pressure-induced depolarization also occurs in the endothelium is not
yet clear. In this study, we investigated whether O$_2^-$ release induced by endothelial cell membrane depolarization is sufficient to inactivate endothelial NTPDase activity, thereby leading to enhanced ADP-dependent platelet aggregation. In isolated resistance arteries, we further studied whether an increase in transmural pressure leads to endothelial depolarization.

**Methods**

For a detailed methods section, please see the online version of this article, which can be accessed at http://atvb.ahajournals.org.

**Endothelial Cell Culture and Platelet Preparation**

Human umbilical vein endothelial cells (HUVECs) were cultured as previously described. Platelet-rich plasma (PRP) was obtained from citrate-anticoagulated blood as previously described. Platelet counts were obtained with a resistance particle counter (Coulter Z2).

**Measurements of Superoxide (O$_2^-$) and of HUVEC Membrane Potential**

O$_2^-$ was measured using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate. Cells were preincubated with the NO-synthase inhibitor N-nitro-L-arginine (L-NA; 50 μmol/L), washed three times, and incubated with 10 μmol/L 2',7'-dichlorodihydrofluorescein diacetate for 15 minutes. In separate experiments, membrane potential was measured using the fluorescent dye bis-[1,3-dibutylbarbituric acid] trimethineoxonol (DiBac$_4$) as described previously. Fluorescence intensities were recorded on a confocal microscope (Zeiss LSM 410).

**Aggregation Studies**

Platelet aggregation was measured turbidimetrically in PRP adjusted to 200,000 platelets/μL as previously described. In addition, experiments, platelets (2×10$^9$/mL) were stimulated with ADP or control substances in the presence of endothelial cells (10$^5$/mL) and aggregation measured turbidimetrically as described by Marcus et al.

**Preparation of Supernatants for Ecto-NTPDase Activity Measurements**

HUVECs were washed 3 times in phosphate-free modified tyrode buffer and incubated with 100 μmol/L ADP for 15 minutes at 37°C. Experiments were performed in the presence of APCP (100 μmol/L) to avoid additional cleavage of inorganic phosphate (P$_i$) from the AMP formed by degradation of ADP. The supernatants were then collected and stored at −20°C.

**Measurement of Free Phosphate and of Nucleotides**

P$_i$ in supernatants was measured by the malachite green assay described by Baykov et al. AMP and ADP were measured by high-pressure liquid chromatography as described with an EC 250/4 nucleosil carbohydrate column. Retention time for AMP was 4.5 minutes and for ADP 10.5 minutes.

**Assessment of Cell Viability and Apoptosis**

A trypan blue exclusion assay was performed to assess cell viability. Cells able to exclude the dye were assumed viable. In additional experiments LDH release was assessed using a CytoTox 96® assay (Promega). Apoptosis was determined flow cytometrically using an annexin-V apoptosis detection kit (BD Pharmingen).

**Immunoblotting Studies**

HUVECs were washed once in PBS and lysed in %1 buffer Triton-X 100. Protein content was assessed, and equal amounts were separated by SDS-PAGE by using standard techniques of blotting and chemiluminescent visualization as previously described.

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**Figure 1.** Depolarization induces endothelial cell superoxide (O$_2^-$) production. A, Cultured HUVECs were depolarized by addition of 100 nmol/L of gramicidin. This resulted in significant O$_2^-$ production as assessed by DCF fluorescence (dihydrodichlorofluorescein in the presence of L-NA). Inhibition of NAD(P)H-oxidase using diphenyleneiodoniumchloride and scavenging of O$_2^-$ using SOD or SOD and catalase resulted in decreased signals. Boiled SOD had no effect. Values are percent of controls; *P<0.05, **P<0.01 vs control, ##P<0.01, #P<0.05 vs gramicidin (n=10). B, Depolarization-induced change in fluorescence of endothelial cells loaded with the membrane-potential sensitive dye DiBac$_4$. Resting membrane potential was −47±2 mV.

**Measurement of Endothelial Membrane Potential Changes in Small-Resistance Arteries**

Animal care and the conduct of the experiments were in strict accordance with the German animal protection laws. Preparation of vessels from the gracilis muscle of Golden Syrian hamsters was previously described. Cannulated small-resistance arteries were analyzed on a confocal microscope. The endothelium was loaded from the luminal side with the voltage-sensitive dye di-8-ANEPPS as described elsewhere. After subtraction of the background signal, the ratio of the two signals (>570 nm/525 to 565 nm) was calculated.

**Materials**

DiBac$_4$ and di-8-ANEPPS were from Molecular Probes (Netherlands). Polyclonal rabbit anti-NTPDase antibody (anti-CD39) was a kind gift of Prof. Adrien Beaudoin (Canada). All other substances were obtained from Sigma Chemicals Co., Germany.

**Statistical Analysis**

All data are expressed as means±SEM. Data were analyzed using one-way ANOVA or Student’s t test for paired or unpaired data. Differences were considered significant when the error probability level was P<0.05.

**Results**

**Membrane Potential in Cultured HUVECs**

In a previous work we measured the resting membrane potential of HUVEC by DiBac$_4$, and it amounted to −47±2 mV. Depolarization using gramicidin (100 nmol/L) increased basal DiBac$_4$ fluorescence by 39±21% (n=4, P<0.05, Figure 1B), corresponding to membrane potential values of about −34±2 mV. The potassium channel blocker tetrabutylammonium chloride (TBA) (1 mmol/L) increased fluorescence by 28±5% (n=4, P<0.01).

**Superoxide Production**

Chronic depolarization of HUVECs for 24 hours (gramicidin, 100 nmol/L) increased O$_2^-$ production (in the presence of
L-NA) by 54±17% over control cells (P>0.01). This increase was prevented by superoxide dismutase (SOD, U/mL) whereas boiled SOD had no effect (Figure 1A and online Figure I, which can be accessed at http://atvb.ahajournals.org, n=10). SOD (500 U/mL) plus catalase (1000 U/mL) had no additional effect. 

Ectonucleotidase Activity

The P_i concentration [P_i] in supernatants of HUVECs incubated with 100 μmol/L ADP in the presence of 5'-nucleotidase inhibitor α,β-methylene-5'-diphosphate (APCP, 100 μmol/L) for 15 minutes was 77.8±22.8 μmol/L (n=6). In the supernatants of HUVECs that had been depolarized for long periods by gramicidin (100 nmol/L), [P_i] was decreased by 25.1±6.8% (P<0.05), similar as after depolarization using TBA (1 mmol/L), which decreased it by 29.7±11.4% (P<0.05, n=5). [P_i] in supernatants immediately after depolarization was not altered. [P_i] in supernatants of gramicidin-treated cells in the presence of SOD was increased by 33.6±23.2% compared with gramicidin alone (P<0.05 versus gramicidin) and by 38.8±20.4% with gramicidin in the presence of SOD and catalase (P<0.01 versus gramicidin, n=6, all data Figure 2A), which was not significantly different from depolarization in the presence of SOD only. When depolarization was induced in the presence of catalase only, [P_i] was not different from gramicidin alone (not shown).

Endothelial ectonucleotidase activity was further assessed by the transformation of ADP to AMP in the presence of the 5'-nucleotidase inhibitor APCP (100 μmol/L). The AMP concentration in supernatants was reduced by chronic depolarization was not significantly release P_i (not shown). The amount of P_i, formed from ADP was decreased when HUVECs had been chronically depolarized using gramicidin (100 nmol/L) or TBA (1 mmol/L) but was not decreased when depolarization was performed in the presence of SOD or SOD and catalase. Without HUVECs, there was no significant hydrolysis of ADP (no cells). *P<0.05 vs control, #P<0.05 vs gramicidin (n=6).

Platelet Aggregation

When 4 μL of supernatants of depolarized cells (100 μL/100 μmol/L ADP) were used as a stimulus, aggregation reached 68.4±6% of the aggregation achieved by 4 μmol/L of ADP (set as maximal aggregation). This was significantly more than the response caused by supernatants of nondepolarized cells (52.9±4%, P<0.05, n=6). Depolarization-induced loss of ADP degradation was prevented when SOD was present. These supernatants caused 53.5±5% of maximum aggregation (P<0.05, n=6), similar as control supernatants (Figure III, which can be accessed at http://atvb.ahajournals.org).

Aggregation experiments were also performed in mixed suspensions containing HUVECs and washed platelets. In the presence of HUVECs, ADP in concentrations that otherwise induced irreversible aggregation (5 μmol/L) caused transient aggregation only. This effect was lost when HUVECs had been chronically depolarized but was again observed when depolarization was performed with SOD (n=3). Such differences were not found when other platelet stimuli, such as...
collagen, thrombin receptor-activating peptide (TRAP), or epinephrine, were used as stimuli (n=2, Figure 4).

Cell Viability
Depolarization did not result in any detectable changes in cell viability assessed by a trypan blue exclusion assay. After 24 hours, 99.1±0.8% of control cells were viable, similar as after treatment with gramicidin (99.4±0.4%) or TBA (99.1±0.5%, n=9, NS). Likewise, there were no differences in LDH release between depolarized and control cells (see online Figure IIA, which can be accessed at http://atvb.ahajournals.org). Furthermore, there was no difference in the relative amount of apoptotic cells because 9.8±1.1% of nonnecrotic cells chronically treated with gramicidin showed apoptosis as compared with 9.3±1.1% of control cells (n=16, NS, Figure IIB).

NTPDase Protein Expression
Western blots performed using a polyclonal NTPDase antibody (raised against porcine pancreatic NTPDase and provided by Prof. A. Beaudoin) did not reveal an effect of depolarization on the expression of this protein by HUVECs (Figure 5A). Densitometric analysis of 3 experiments showed equal expression of the protein under the conditions tested, that is control, gramicidin (100 nmol/L), gramicidin with SOD (500 U/mL), and gramicidin with SOD and catalase (1000 U/mL, n=3 each, see online Figure IV, which can be accessed at http://atvb.ahajournals.org).

Comparison of protein expression levels in HUVECs, PRP, and platelet-poor plasma (PPP) showed that HUVECs contained the highest levels of NTPDase protein, followed by platelets. There were no signals when PPP was used as substrate (Figure 5B).

Increases in Transmural Pressure Depolarize the Endothelium of Isolated Arteries
Perfusion of isolated arterioles with di-8-ANEPPS selectively loaded the endothelium as demonstrated by confocal micro-

Discussion
ADP is the major mediator of platelet recruitment after platelet activation. We and others have shown that after collagen stimulation of platelets, ADP is released in large amounts.22 Because ecto-NTPDases rapidly hydrolyze ADP, they are considered to be of major significance in platelet recruitment.8,26 We have recently observed that the activity of platelet ecto-NTPDase(s) might be oxidatively influenced.22 However, in quantitative terms, the ecto-NTPDase activity of endothelial cells seems to be the dominant contributor to the rate of ADP degradation in the vasculature.26 In comparison with platelets, endothelial cells express higher amounts of one specific ecto-NTPDase, CD39,27 which is thought to be the ecto-NTPDase predominantly responsible for vascular nucleotide metabolism and, therefore, inhibition of platelet activation.9–11 It was recently demonstrated in mouse microvessels that the endothelium mainly contains NTPDase 1 (CD39),12 whereas NTPDase 2 (CD39L1), a preferential NTPDase that should rather increase ADP concentrations and, conse-
observe a change in NTPDase protein expression caused by depolarization-induced endothelial reactive oxygen species (ROS) production or by depolarization itself in HUVECs.

Although we cannot ascribe our findings to endothelial CD39, an oxidative inactivation of which is discussed controversially, they support the concept of NTPDases as targets of redox regulation, a regulatory mechanism, which can also be found in NTPDases of other species. Therefore, we cannot exclude that ectonucleotidases other than CD39, for example, N-Pases (nucleotidedepyrophosphatases) were also affected. However, because the reaction catalyzed by these enzymes seems to predominantly result in the hydrolysis of 2 moles of P"9,35 their involvement seems improbable because of the complementary behavior of AMP and P" (parallel increases) in our study. A significant influence of alkaline phosphatase is unlikely as a result of the physiological pH conditions prevailing in our experiments, and 5'-nucleotidase was blocked throughout. Hence, the enzymatic activity investigated in this study is most likely that of CD39.

Inactivation of endothelial NTPDase activity was assessed by two independent methods: AMP formation and P" production from ADP. Because endothelial 5'-nucleotidase normally further degrades AMP to adenosine, thereby releasing another P",9,36 it was necessary to perform the experiments in the presence of APCP, which, at the concentration used (100 μmol/L), completely inhibits endothelial 5'-nucleotidase activity.36 Under more physiological conditions, when 5'-nucleotidase is active, the generation of adenosine would even supply another antiaggregatory agent.36 This should yet increase the proaggregatory effects of an inhibition of NTP-Dase. Pertinently, there was no significant hydrolysis of ADP in the absence of endothelial cells, which themselves did not release relevant amounts of ADP.

The depolarization-induced effects were reversed completely by addition of SOD, and this was not enhanced by catalase, which alone did also not influence the rate of formation of P". This indicates that O_2^- is the predominant ROS involved in inactivation of endothelial ecto-NTPDases. Cellular viability or apoptosis rates, which are both known to increase cellular O_2^- release,30,37 were not altered by depolarization. Furthermore, platelet reactivity to other stimuli, such as collagen, epinephrine, or TRAP, was not affected by depolarization. In two different bioassays, we demonstrated that endothelial NTPDase activity is lost on chronic depolarization and indeed results in enhanced platelet aggregation. In addition, comparison of expression of NTPDase in HUVECs, PPP, or PRP showed the highest protein expression in HUVEC, little in PRP, and none in PPP. This confirmed previous observations27 and made confounding contributions of NTPDases from other sources unlikely.

By directly measuring membrane potential changes in intact resistance vessels selectively loaded with a membrane-potential sensitive, ratiometric dye from their luminal side, we, for the first time, demonstrate that increases in transmural pressure depolarize the endothelium. Because chronic depolarization of vascular cells or attenuated endothelial hyperpolarization have been observed in spontaneously hypertensive rats,17 in endothelial injury after balloon angioplasty,18 and in diabetic patients,18 chronic or repeated depolarization on
elevated pressure is likely a mechanism of endothelial cell activation in vivo. The mechanisms underlying such endothelial depolarization remain unknown. Although there is a possibility of direct ion channel activation,38 a mechanism more likely to occur in vivo is electrical coupling through myoendothelial gap junctions that conduct electrical changes from vascular smooth muscle to endothelial cells.39,40 Evidence for a role of ROS in depolarization-induced deterioration of endothelial function comes from the observation that delivery of SOD decreases blood pressure in spontaneously hypertensive rats (SHR),41 and by increased O2− production in internal mammary arteries and saphenous veins from diabetic patients.42 We have previously observed that depolarization of HUVECs was associated with enhanced NAD(P)H-oxidase–dependent O2− production.16 As shown here, this production is sustained for 24 hours and is sufficient to inactivate endothelial ecto-NTPDases. Two substances causing depolarization by independent mechanisms induced endothelial O2− production and inactivated NTPDases. Thus, it is unlikely that the effects of gramicidin, which was used in most experiments, are caused by effects on cellular ion concentration and pH that have been reported.43 We conclude that oxidative inactivation of endothelial ecto-NTPDases as a result of chronic depolarization can result in altered ADP-dependent platelet recruitment. Such depolarization might occur in hypertensive vessels because increases in transmural pressure induce endothelial depolarization in isolated hamster arterioles. As platelet aggregation initiated by contact with ruptured endothelium depends on the release of autoactivating substances like ADP, the membrane potential and consequently the NTPDase-activity of the surrounding endothelial cells might pivotally influence further growth of the local thrombus. Moreover, in light of the observation that certain fatty acids might increase endothelial NTPDase activity,44 a potential novel role for an endothelium-derived hyperpolarizing factor, which is widely assumed to be an epoxyeicosatrienoic acid,45,46 could be postulated. Chronic vessel depolarization and the release of substances, such as endothelium-derived hyperpolarizing factor influencing endothelial membrane potential, might therefore be involved in the regulation of thrombus formation in vivo.

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References


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FIGURE LEGENDS ONLINE FIGURES

Fig. I Depolarization induces endothelial cell superoxide (O$_2^-$) production.
Representative images of DCF-loaded HUVEC after depolarization with gramicidin.

Fig. II Depolarization by gramicidin does not affect cellular viability or increase apoptosis rates. A. Gramicidin treatment did not affect cellular viability as assessed by LDH release or increase apoptosis rates (B) of cultured HUVEC as assessed by annexin-V staining (n=16 each).

Fig. III Effect of HUVEC ectonucleotidase activity on platelet aggregation. Platelets stimulated with ADP-solutions that had been coincubated with HUVEC for 15 minutes showed different aggregation responses depending on the pretreatment of HUVEC. When taken from control HUVEC (EC) a volume of the supernatants that would have yielded 4µM ADP in the aggregation cuvette only induced half as much aggregation as a solution of 4µM ADP (scaled to 100% aggregation). When the cells had been depolarized, however, the aggregation response was strong again, a change which could be prevented when depolarization was performed in the presence of SOD or SOD and catalase (n=6, * P<0.05 vs. control, # P<0.05 vs. gramicidin, n.s.).

Figure IV. NTPDase protein expression is not influenced by depolarization of HUVEC.
Average densitometric values of three independent western blotting experiments expressed as expression relative to control cells (100%) reveal no differences in NTPDase expression between depolarized cells and control cells.
IV

ATPase expression, % of control

Control, Gramicidin, + SOD, + SOD/Catalase
METHODS

Endothelial Cell Culture:

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by digestion with dispase and grown to confluence as previously described in medium 199 supplemented with 10% fetal calf serum and 20% endothelial growth medium (Promocell, Germany). Only cells of the 1st or 2nd subpassage were used for experiments.

Platelet Preparation:

Samples of venous blood were drawn from healthy volunteers who had not taken any medication for at least 10 days. Informed consent was obtained from all subjects. The blood, anticoagulated by 3.13 % sodium citrate, was centrifuged at 150g for 15 minutes. The supernatants were used as platelet-rich plasma (PRP) within two hours. Platelet counts were obtained with a resistance particle counter (Coulter Z2).

Measurement of Membrane Potential of Cultured Cells:

Membrane potential was measured using the fluorescent dye bis-[1,3-dibutylbarbituric acid] trimethineoxonol (DiBac4(3)) as described previously. HUVEC cultured on coverslips were pre-incubated with DiBac4(3) (100 nmol/L) in modified Tyrode’s buffer (135 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl2, 0.49 mmol/L MgCl2, 0.28 mmol/L NaH2PO4, 5.5 mmol/L glucose, 20 mmol/L HEPES) for 30 minutes and transferred into a perfusion chamber. The cells were continuously superfused with modified Tyrode’s buffer containing DiBac4(3). Fluorescence intensity (excitation 488 nm, emission >515 nm) was recorded using a confocal microscope (Zeiss LSM 410) and membrane potential changes were expressed as per cent change of light emission (arbitrary units) compared to control.

Superoxide (O2−) Measurements:

O2− was measured using the fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (DCF). Confluent cells were incubated with depolarizing agents for 24 hours. After 23 hours the depolarizing medium was removed and stimuli or inhibitors (see below) were added with new
medium. 15 minutes before measurements, cells were incubated with the NO-synthase inhibitor N-nitro-L-arginine (L-NA; 50 µM) to prevent DCF-oxidation through peroxynitrite formation. To apply DCF, the cells were washed once in phosphate buffered saline and then incubated in modified Tyrode’s buffer containing 10 µmol/L DCF for 15 minutes at 37 °C. ROS production was then assessed by the fluorescence intensity in several preselected regions of interest (ROI) using a confocal microscope (LSM 410 Invert, Zeiss, Germany). Fluorescence intensities are expressed as per cent increase vs. control conditions.

**Aggregation Studies:**

Platelet aggregation was measured using a turbidimetric method as previously described. PRP was adjusted to a platelet concentration of 200,000 /µL using autologous platelet-poor plasma. Aggregation was measured photometrically with a two-chamber-aggregometer (elvi 840, Logos, Milan, Italy) under continuous stirring at 1,000 rpm at 37 °C. In additional experiments, aggregation of washed platelets (2x10⁸/mL), in the presence of endothelial cells was assessed as described by Marcus et al. (JCI 1997). Endothelial cells were depolarized for 24 hours followed by novel addition of the depolarizing substances immediately before detachment. They were detached by trysin digestion for 2 minutes, which was stopped by adding serum-containing medium, they were then pelleted by centrifugation and resuspended in tyrode’s buffer (1x10⁵/mL). Then they were stimulated with ADP or control substances, and aggregation measured turbidimetrically.

**Preparation of Supernatants for Ecto-NTPDase-Activity Measurements:**

HUVEC were washed 3 times in phosphate-free modified tyrode buffer (155 mmol/L NaCl, 5.7 mmol/L KCl, 1.9 mmol/L CaCl₂, 15 mmol/L HEPES, pH 8.0). Next, they were incubated with 100 µmol/L ADP dissolved in the above mentioned buffer for 15 minutes at 37 °C. Experiments were performed in the presence of the 5’-nucleotidase inhibitor α,β-methylene-5’-diphosphate (APCP, 100 µmol/L) to avoid additional inorganic phosphate (P₃) release from
the AMP formed by degradation of ADP. The supernatants were then collected and stored at –20 °C.

**Measurement of free Phosphate:**

Measurement of free inorganic phosphate (Pᵢ) in the supernatants was performed using the malachite green assay described by Baykov et al.²² Briefly, one part of the reaction mixture, containing malachite green (1.47 g/L), ammonium molybdate (7.5 %), and tween-20 (0.17 %), was added to 4 parts of the sample volume, mixed and incubated at 37 °C in darkness for 10 minutes. Then, absorption was measured at 610 nm and the amounts of Pᵢ were determined using a standard calibration curve for Pᵢ.

**Measurement of Nucleotides:**

Adenosine monophosphate (AMP) and ADP were measured using high pressure liquid chromatography (HPLC) as previously described.²¹ After addition of perchloric acid, samples were centrifuged at 10,000 g for 5 minutes at 4 °C to remove precipitates. The supernatants were then applied to an EC 250/4 nucleosil carbohydrate column and eluted with 10 mmol/L NH₄H₂PO₄ (A; at pH 3.5) or 0.5 mmol/L NH₄H₂PO₄ (B; at pH 3.0) using a gradient of 100 % of A for 15 minutes, then 30 % of B for 1 minute, 40 % of B for 4 minutes and 100 % of A at minute 20. Retention time for AMP was 4.5 minutes, for ADP it was 10.5 minutes.

**Assessment of Cell Viability:**

A trypan blue exclusion assay was performed to assess cell viability. Cells were incubated with various depolarizing agonists for a total of 24 hours. After incubation (23h) and re-addition of the depolarizing substances (1h), cells were washed once and 0.2 % trypan blue was added for 10 minutes. Cells able to exclude the dye after this time were assumed viable and the non-viable cells were counted. Cell viability was expressed as viable cells in percent of all cells.

LDH-release was assessed using a CytoTox 96® assay (Promega, USA) according to the manufacturer’s instructions. Cells were treated as indicated and 50µl of the supernatants
mixed with 50µl of the assay’s substrate mix and incubated at RT for 30 minutes. For positive controls, cells were treated with 1% Triton-X 100 in tyrode’s buffer for 2 minutes. Then the reaction was stopped by adding 50µl of acetic acid (1M) and absorption measured at 486nM. LDH-release is expressed as OD after substraction of the background signal.

To assess HUVEC apoptosis rates a annexin V-apoptosis detection kit (BD Pharmingen, USA) was used according to the manufacturer’s instructions. Cells were detached by trypsin treatment for 2 minutes, which was stopped by adding serum-containing medium. Then they were pelleted by centrifugation, resuspended in 300µl binding buffer and incubated with 3µl of annexin V-FITC conjugate and 3µl of propidium iodide (PI, 50µg/ml) for 15 minutes at darkness and RT. They were then analyzed by flow cytometry. Cells staining positive for PI were excluded from the analysis, as they were considered necrotic. Cells negative for PI and positive for annexin V were considered apoptotic.

**Immunoblotting Studies**

HUVEC were washed once in PBS and lysed in ice-cold buffer containing Tris 50 mmol/L, EDTA 1 mmol/L, sodium orthovanadate 1 mmol/L, sodium fluoride 10 mmol/L, tetra sodium diphosphate deca-hydrate 1.5 mmol/L, disodium hydrogen phosphate 4 mmol/L, leupeptin, aprotinin, pepstatin 10 µmol/L each, phenylmethylsulfonyl fluoride 1 mmol/L, Triton-X 100 1%. Protein content was assessed and equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis using standard techniques of blotting and chemiluminescent visualisation as previously described 21.

**Measurement of Endothelial Membrane Potential Changes in Small Resistance Arteries:**

Animal care and the experimental procedures performed in this study were in strict accordance with the standards and guidelines provided by German animal protection laws. The preparation of the vessels were previously described in detail 23. Golden Syrian hamsters were anesthetized by intraperitoneal and then killed by intracardial injection of pentobarbital sodium (50 mg/kg). Segments of small resistance arteries were excised from the gracilis
muscle and cannulated with glass micropipettes. The vessel and cannulation apparatus was mounted on the stage of a confocal microscope. Hydrostatic transmural pressure of the arteriole was set to 45 mmHg.

Membrane potential changes of endothelial cells were recorded using the fluorescence ratio technique based on the voltage – sensitive dye di-8-ANEPPS described by Beach et. al. 24. A depolarization results in a shift of emission spectra to shorter wavelength. Endothelial dye loading of the arterioles was performed by intraluminar perfusion of di-8-ANEPPS (10 µM for 30 min) at darkness. Excitation was done at 488nm using an argon laser. Every 10 seconds dual-wavelength recordings were performed by using a 525–565nm bandpass filter in combination with a 570nm long pass filter. Images were analysed by using LSM – software (Zeiss, version 3.95). After subtraction of the background signal the ratio of the two signals (> 570 nm/ 525 – 565 nm) was calculated.

Materials
Superoxide dismutase was supplied by Roche Molecular Biochemicals, Germany, malachite green-oxalate and ammonium molybdate tetrahydrate were from Applichem, Germany. DiBac4(3) was from Molecular Probes (Netherlands). Polyclonal rabbit anti-NTPDase antibody (anti-CD39) was a kind gift of Prof. Adrien Beaudoin (Canada). EC 250/4 nucleosil carbohydrate columns were purchased from Macherey-Nagel (Germany). All other substances were obtained from Sigma Chemicals Co, Germany.

Statistical Analysis
All data are expressed as means +/- S.E.M. Data were analysed using one-way ANOVA or student’s t-test for paired or unpaired data. Differences were considered significant when the error probability level was P<.05.