Thrombin-induced ATP release from human umbilical vein endothelial cells

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Gödecke S., Roderigo C., Rose CR, Rauch BH, Gödecke A., Schrader J. Thrombin-induced ATP release from human umbilical vein endothelial cells. Am J Physiol Cell Physiol 302: C915–C923, 2012. First published December 7, 2011; doi:10.1152/ajpcell.00283.2010.—ATP and its degradation products play an important role as signaling molecules in the vascular system, and endothelial cells are considered to be an important source of nucleotide release. To investigate the mechanism and physiological significance of endothelial ATP release, we compared different pharmacological stimuli for their ability to evoke ATP release from first passage cultivated human umbilical vein endothelial cells (HUVECs). Agonists known to increase intracellular Ca\(^{2+}\) levels (A23187, histamine, thrombin) induced a stable, non-lytic ATP release. Since thrombin proved to be the most robust and reproducible stimulus, the molecular mechanism of thrombin-mediated ATP release from HUVECs was further investigated. ATP rapidly increased with thrombin (1 U/ml) and reached a steady-state level after 4 min. Loading the cells with BAPTA-AM to capture intracellular calcium suppressed ATP release. The thrombin-specific, protease-activated receptor 1 (PAR-1)-specific agonist TFLLRN (10 \(\mu\)M) fully mimicked thrombin action on ATP release. To identify the nature of the ATP-permeable pathway, we tested various inhibitors of potential ATP channels for their ability to inhibit the thrombin response. Carbenoxolone, an inhibitor of connexin hemichannels and pannexin channels, as well as Gd\(^{3+}\) were highly effective in blocking the thrombin-mediated ATP release. Specifically targeting connexin43 (Cx43) and pannexin1 (Panx1) revealed that reducing Panx1 expression significantly reduced ATP release, while downregulating Cx43 was ineffective. Our study demonstrates that thrombin at physiological concentrations is a potent stimulus of endothelial ATP release involving PAR-1 receptor activation and intracellular calcium mobilization. ATP is released by a carbenoxolone- and Gd\(^{3+}\)-sensitive pathway, most likely involving Panx1 channels.

ENDOTHELIAL CELLS RELEASE ATP in response to various stimuli including alterations in mechanical strain, oxygen supply, or the action of agonists (3). In the process of regulated, non-lytic ATP release from endothelial cells, intracellular calcium ([Ca\(^{2+}\)]\(_i\)) elevation seems to play a central role (23). However, the molecular mechanism underlying the release of ATP from endothelial cells still has to be fully elucidated. Hemichannels composed of connexins or pannexin channels belong to the cellular structures intensely discussed as potential ATP channels (17). Connexons assemble in the plasma membrane into hexamers called connexons or hemichannels which by assembly with another connexon in an apposed membrane of a neighboring cell can form a functional gap junction (7). A subset of gap junction proteins has the ability to form functional hemichannels in the cytoplasmic membrane without interacting with a counterpart in the membrane of an adjacent cell (41). These hemichannels function independently of gap junctions and have been described to act as nonspecific channels for a variety of small molecules including ATP, glutamate, or NAD\(^{+}\) in several cell types (20, 21, 29, 42). Pannexins are a family of vertebrate proteins distinct from connexins. Pannexins can form ATP-permeable channels in cell membranes, whereas their role as gap junction proteins seems to be questionable (32).

The gating properties of gap junctions and hemichannels are closely connected with the concentration of calcium ions in the extra- and intracellular milieu. It has been shown for a variety of connexin hemichannels, including Cx43, Cx45, Cx30.2, and Cx32.9, that lowering extracellular calcium concentration leads to an increase in conductance (6, 47). However, the physiological relevance of this observation is still controversial. In contrast, the dependence of the gating behavior of connexin hemichannels on intracellular free calcium concentration has been clearly demonstrated (6, 14, 15). For both, Cx32 and Cx43, it could be shown that an elevation of [Ca\(^{2+}\)]\(_i\) triggers opening of hemichannels leading to a release of ATP (14, 15). The release of ATP through Cx43 hemichannels can be triggered by photoactivation of inositol 1,4,5-trisphosphate, which is known to release Ca\(^{2+}\) into the cytoplasm by mobilization of Ca\(^{2+}\) from intracellular stores (6). However, the activation of Cx43 hemichannels by [Ca\(^{2+}\)]\(_i\) is not direct but rather involves a complex intermediate signaling cascade (15). Also for pannexin1 (Panx1) channels an activation upon elevation of [Ca\(^{2+}\)]\(_i\) was shown (31). Human umbilical vein endothelial cells (HUVECs) express a repertoire of gap junction proteins capable of forming ATP-permeable pores which include connexins 37, 40, and 43 (26, 48) as well as Panx1 (45). Moreover, cellular ATP release from this cell type has been reported to be mediated by an increase in [Ca\(^{2+}\)]\(_i\) (22, 43). In this work we therefore asked which is the most robust stimulus for ATP-release in HUVECs and whether hemichannels are involved in the [Ca\(^{2+}\)]-dependent release of ATP.

MATERIALS AND METHODS

Cell culture. Human umbilical cords were obtained from normal placentas. The umbilical vein was cannulated with blunt needles and perfused to wash out all blood. The vein was then filled with 0.025 % collagenase A (Sigma), warmed at 37°C. Isolated endothelial cells were seeded in 25-cm\(^2\) flasks coated with 0.2% gelatin in endothelial cell growth medium (Promocell) and were incubated at 37°C and 5% CO\(_2\) in a humidified incubator. After reaching confluence, cells were passaged and either directly cultivated for ATP release experiments or subjected to viral infection before further experiments.

Agonist-induced ATP release assay. HUVECs were grown to confluence on six-well dishes under normal growth conditions. At the day of experiment, medium was carefully exchanged to 1.5 ml Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA) and 10% fetal calf serum (FCS), followed by incubation at 37°C for 1 h. Then, cells were subjected to viral infection before further experiments.
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balanced salt solution (HBSS) and 10 mM HEPES, pH 7.35. After an equilibration period of 2 h, 100 µl of HBSS-HEPES containing different agonists and inhibitors were carefully added and plates were gently swirled to achieve homogeneous dispersion and thereby designated end concentrations. All manipulations were carried out with caution to avoid mechanical disturbances leading to lytic ATP release.

To avoid genetic bias, each agonist experiment was performed on at least three independent primary HUVEC cultures from different individuals. For each individual experiment and agonist concentration, one well plate (six independent measurements) was used. After an incubation time of 10 min (37°C), 800 µl of the supernatant was removed, briefly centrifuged, and frozen for subsequent ATP determination. Total cellular protein amount was determined by BCA protein assay (Pierce) as a measure for cell number for each well at the end of the experiment.

**ATP measurement in cell culture supernatants.** ATP release from HUVECs was measured using a luciferin-luciferase-based assay (FLAAM, Sigma) and a Berthold Biolumat LB 9500. Cell supernatants were thawed on ice, and 100-µl aliquots were transferred to cuvettes and placed into the luminometer. One hundred microfilters of diluted ATP-assy mix (1:25) were injected into each sample, and the relative light intensity was integrated over periods of 10 s at 25°C. Calibration curves were made for each experiment using dilutions of ATP in HBSS-10 mM HEPES, pH 7.35. ATP release per microgram of total cellular protein was calculated. Statistical analyses were performed by unpaired Student’s t-test.

**Effects of agonists on luciferase activity.** A total of 0.5 pmol ATP was preincubated in 100 µl HBSS-HEPES-buffered HBSS including 100 µM ARL67156 for 10 min at 37°C to mimic experimental conditions. Like cell supernatants, these samples were briefly centrifuged, frozen, and, after thawing, used for luciferase measurements. Measurements were performed using 100 µl of diluted ATP assay mix (1:25) as described above.

**Cell viability assays.** To assess for unwanted lytic damage, cell monolayers were directly stained with Trypan blue solution. Lactate dehydrogenase (LDH) activity was determined in cell supernatants using the LDH Biotoxicity Assay Kit II (Biocat). When comparing sensitivities of LDH and ATP detection by luciferin/luciferase using serial dilutions of cell lysates, we found luciferase reaction to be 100 times more sensitive than the LDH reaction for the detection of cell lysis. Therefore, the LDH assay is not suitable to distinguish physiological from lytic ATP release.

**Thrombin protease activity assay.** Activity of thrombin was assayed using a thrombin-specific fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Human α-thrombin (American Diagnostika, Pfungstadt, Germany) was incubated in the absence and presence of gadolinium (1 – 50 µM) for 15 min in substrate solution (4 mM HEPES, 2 mM CaCl2, 12 mg/ml BSA at pH 7.35). The reactions were started by adding 20 µl of the substrate solution containing 2.5 mM Z-Gly-Gly-Arg-AMC to 80 µl of preincubated thrombin solutions in 96-well plates. Fluorescence intensities were measured in a microtiter plate fluorometer (Ascent Reader, Thermolabsystems, Helsinki, Finland) equipped with a 390/460 filter set (excitation/emission) for 60 min.

**Calcium imaging.** Wide-field fluorescent imaging was performed employing an imaging system (Till Photonics, Munich, Germany) in conjunction with an upright microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with a cooled CCD camera (Sensicam QE, PCO, Kelheim, Germany). HUVECs were seeded on gelatin-coated coverslips, cultivated for 3–5 days, and loaded with 10 µM Fura-2 AM (Molecular Probes, Invitrogen) in HBSS containing 10 mM glucose and 10 mM HEPES, pH 7.3, for 30 min at room temperature. Experiments were performed at 30°C. Cells were either directly stimulated with thrombin (1 U/ml) or preexposed to GdCl3 (50 µM) for 30 s followed by addition of thrombin (1 U/ml) under constant gadolinium concentration. Fura-2 was excited every 5 s using a monochromator (polychrome V, Till Photonics), and fluorescence emission from regions of interest (ROIs) placed around cell somata was detected by the camera. Emission intensities >440 nm were collected after alternate excitation at 357 and 380 nm, and background-corrected fluorescence ratios (F357/F380) were calculated. Background fluorescence was determined from coverslip areas devoid of cellular material. Signals from all cells located on a given coverslip were averaged. Peak calcium values and area under curve (AUC) values were determined over a period of 8 min starting with thrombin-dependent rise of intracellular calcium.

**Generation of Cx43- and Panx1-knockdown HUVECs by lentiviral transfection.** Stable knockdown of Cx43 and Panx1 was achieved by using human connexin43- or panxenin-specific mission short hairpin RNA (shRNA) plasmid collections (Sigma) and the nontarget-shRNA plasmid as a negative control containing a shRNA insert that does not target human genes. The short-hairpin sequence of the negative control shRNA contains five base pair mismatches to any known human gene (Sigma). On the basis of observations made by Ransford et al. (38), we first tested each of the five shRNA lentiviral constructs individually for their ability to target Panx1 mRNA in HUVECs. Three lentiviral constructs effectively reduced Panx1 mRNA and thus were used for further experiments. Recombinant lentiviral particles were generated by transfecting 5 µg of shRNA- and control plasmids, the helper plasmid pCD/NL-BH, and the VSV-G plasmid (kindly obtained from Jacob Reiser, New Orleans, LA, and Dirk Lindemann, Dresden, Germany, respectively) into human embryonic kidney (HEK)293T cells. Viral supernatants were harvested after 48 – 72 h and used directly to infect first passage HUVECs (250,000 cells in 25-cm² flasks) overnight. Cells underwent puromycin selection (1 µg/ml) for 5 days, starting 2 days after infection. Puromycin-resistant HUVECs were grown to confluence, passaged two to four additional times to increase cell material, and used for ATP release experiments. Protein extracts and RNA were obtained following the ATP release assay for control of knockdown efficiencies. In case of Panx1 knockdown, eight individual transfection experiments were performed.

**Western blot analysis.** Western blot analysis was performed according to standard procedures. In brief, cells were lysed in lysis buffer containing 50 mM TrisCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 mM PMSF, and protease inhibitor cocktail (Sigma), and cell lysates were centrifuged at 10,000 g for 10 min. Total protein was determined with a BCA protein assay kit (Pierce). Twenty-five micrograms of protein per sample were separated on a standard SDS-PAGE gel and transferred to nitrocellulose membranes. After blocking in TTBS (50 mM TrisCl, pH 7.4, 0.1% Tween 20, 150 mM NaCl) containing 5% nonfat milk powder, the membranes were probed in TTBS-2% milk powder with the following antibodies: rabbit-anti-human connexin43 (Sigma), goat anti-human panxenin-1 (Santa Cruz), and mouse-anti-human β-tubulin (Abcam). Quantification of Western blot signal was achieved by using a LI-COR Odyssey Infrared Imaging System and the appropriate secondary IRDye labeled antibodies (LI-COR Biosciences).

**RT-PCR.** Total RNA was isolated using the RNAeasy kit (Qiagen). Reverse transcription was performed using SuperScript II (Invitrogen) according to standard procedures. For quantitative real-time PCR, commercially available Taq-Man gene expression assays for human pannexin 1, human β-actin, and human GAPDH (Applied Biosystems) were used.

**RESULTS**

**Calcium mobilizing agonists induce ATP release from HUVECs.** We tested different agonists known to increase intracellular calcium ([Ca2+]I) for their ability to induce ATP release from HUVECs. The ecto-ATPase inhibitor ARL67156 (100 µM) was added during all measurements of extracellular ATP to avoid rapid enzymatic degradation of the released nucleotides. The calcium ionophore A23187 induced a rapid
release of ATP into the medium which peaked after 20 min (Fig. 1A). This ATP release was robust and concentration dependent (0.1–1 μM A23187 for 10 min; Fig. 1B). Lytic ATP release due to cytotoxic effects of the agonists was assessed by Trypan blue staining, supernatant LDH activity, and microscopical inspection. However, above 5 μM A23187, LDH activity was found to be increased, indicating a cytotoxic effect of this ionophore at higher concentrations (data not shown).

Histamine, a physiological stimulus acting through specific receptors on the cell membrane, is known to induce an increase in [Ca^{2+}]_i by releasing Ca^{2+} from intracellular stores (28). In our experiments, histamine was a moderate inducer of cellular ATP release (Fig. 1C) that even at very high concentrations showed no obvious signs of cytotoxicity.

Thrombin is known to rapidly release Ca^{2+} from intracellular stores in HUVECs by interaction with specific membrane receptors (34). We therefore stimulated HUVECs with thrombin (1 U/ml) and measured ATP in the supernatant every 90 s over a period of 9 min. As shown in Fig. 2A, ATP concentration increased rapidly and reached a steady-state level after 4.5 min.

ATP release induced by thrombin was concentration dependent and reached maximal values already at 1 U/ml (Fig. 2B). Trypan blue staining indicated no signs of cytotoxicity.

To further characterize the mechanism of thrombin stimulation, we used the synthetic peptide TFLLRN, which corresponds to the thrombin-specific protease-activated receptor 1 (PAR-1) tethered ligand and which can selectively mimic the actions of thrombin via this receptor (27). We found that this hexapeptide released ATP from HUVECs in a magnitude comparable to thrombin (Fig. 2C). To test for Ca^{2+} dependency of the thrombin-induced ATP release, HUVECs were preincubated with BAPTA-AM (5 μM) for 30 min for complexation of intracellular Ca^{2+}. This significantly reduced ATP release upon thrombin stimulation (1 U/ml) compared with control cells not loaded with BAPTA (Fig. 2D).

Mechanism of thrombin-mediated ATP release. To gain insight into the molecular mechanism of thrombin-induced ATP release, we used different pharmacological agonists interacting with potential transport mechanisms reported to be responsible for cellular ATP release (37). As shown in Fig. 3A, 18-β-glycyrrhetinic acid (βGA), an inhibitor of connexin function (36), did not alter thrombin-induced ATP release. However, carbadoxolone (Cbx), a derivative of αGA, at low concentrations (5 μM) fully inhibited the thrombin response.

Gadolinium, a blocker of maxi-anion channels (40), proved to be very effective in inhibiting the thrombin response on ATP release (Fig. 3B). We also tested MK571, a selective inhibitor of multidrug resistance-associated proteins (MRP) (19), and found strong inhibition. However, the observed MK571 effect was due to a direct inhibition of the luciferase activity (Table 1). From Table 1 it is also evident that neither αGA nor Cbx in the concentrations chosen in our experiments inhibited luciferase activity, while GdCl3 inhibited the enzyme activity by ~20%, which is consistent with data in the literature (4). A direct inhibitory effect of Cbx and Gd^{3+} on thrombin protease activity could be excluded (data not shown).

To study whether the effect of Gd^{3+} on thrombin-induced ATP release resulted from a suppression of intracellular calcium signaling, ratiometric calcium imaging experiments in HUVECs using Fura-2 were performed. Stimulation of HU-
VECs with thrombin is known to cause a biphasic Ca\(^{2+}\)/H\(_{11001}\) response (10). An initial elevation of \([\text{Ca}^{2+}]_{\text{I}}\) to peak within seconds is due to calcium release from intracellular stores. After a rapid decline, \([\text{Ca}^{2+}]_{\text{I}}\) reaches a long-lasting plateau, which reflects extracellular calcium influx (16). The first response can be detected by measuring peak calcium, whereas the latter is recorded by measuring AUC. We have determined both maximum peak values and AUC within the first 8 min.

![Fig. 2. Thrombin-induced ATP release from HUVECs. A: time course of thrombin-induced ATP release from HUVECs (means ± SD; n = 3). B and C: concentration-dependent effects of thrombin (B) and protease-activated receptor 1 (PAR-1)-specific peptide agonist TFLLRN (C) on ATP release. ATP release was determined after 10 min (means ± SD; n = 3–4). Open circle: ATP releases in the absence of agonist. D: effect of BAPTA on thrombin-induced ATP release. HUVECs were loaded with 5 μM BAPTA-AM 30 min before stimulation with thrombin (1 U/ml). ATP release was determined after 10 min (means ± SD; n = 6). *P < 0.05 vs. basal; **P < 0.01 vs. basal; ***P < 0.01 vs. thrombin. All experiments were carried out in the presence of ARL67156 (100 μM).](http://ajpcell.physiology.org/)

![Fig. 3. Effects of carbenoxolone (Cbx), 18-α-glycyrrhetinic acid (αGA), and gadolinium chloride (Gd\(^{3+}\)) on thrombin-induced release. A: Cbx (5 μM) but not αGA (50 μM) inhibits thrombin-induced ATP release (means ± SD; n = 4–6). ***P < 0.001 vs. basal. ***P < 0.001 vs. thrombin. B: Gd\(^{3+}\) (50 μM) efficiently blocks thrombin-induced ATP release (means ± SD; n = 6). ATP release was determined 10 min after application of the agonists. Experiments were carried out in the presence of ARL67156 (100 μM).](http://ajpcell.physiology.org/)
in the majority of the thrombin- and the thrombin
calcium response, however, was found to be variable: whereas
/H9262 M) alone did not alter baseline calcium. The pattern of the
significantly increased intracellular calcium, while gadolinium (50
cells on 6 coverslips). As expected, thrombin (1 U/ml) signif-
A 4 4
enzyme activity by different agonists was determined using 0.5 pmol ATP and
H11001
H11006 values are means ± SD; n = 6. eGA, 18-α-glycyrrhetinic acid; CBX, carbenoxolone; GdCl3, gadolinium chloride. Average inhibition of luciferase
inhibitors used above did not exclude a role of hemichannels in
channels impedes ATP release.
Since the pharmacological
preserved in the presence of gadolinium.
thrombin-induced calcium signaling in HUVECs is largely
with thrombin controls (77.1% and 89.3% of controls, respec-
tively). Data are summarized in Table 2. We conclude that
with a slightly reduced peak amplitude and AUC compared
thrombin-induced onset of [Ca2+]i increase in the
absence and presence of gadolinium (n = 123 and n = 119
cells on 6 coverslips). As expected, thrombin (1 U/ml) signif-
antly increased intracellular calcium, while gadolinium (50
µM) alone did not alter baseline calcium. The pattern of the
calcium response, however, was found to be variable: whereas
in the majority of the thrombin- and the thrombin + Gd3+
treated cells (70%), the initial peak is followed by a prolonged
plateau phase reflecting the typical biphasic response, a smaller
fraction of cells (30%) showed Ca2+ oscillations both in the
absence and presence of Gd3+. Figure 4 shows representative
registrations of both types of Ca2+ responses to thrombin (Fig.
A) and thrombin + Gd3+ (Fig. 4B). Quantitative evaluation of
the Ca2+ responses revealed that thrombin in the presence of
gadolinium still evoked calcium signals in HUVECs, albeit
with a slightly reduced peak amplitude and AUC compared
with thrombin controls (77.1% and 89.3% of controls, respec-
tively). Data are summarized in Table 2. We conclude that
thrombin-induced calcium signaling in HUVECs is largely
preserved in the presence of gadolinium.
shRNA knockdown of Panx1-channels but not Cx43 hemi-
channels impedes ATP release. Since the pharmacological
inhibitors used above did not exclude a role of hemichannels in
thrombin-mediated ATP release, we used a connexin43-spe-
cific mission shRNA vector collection and a mission shRNA
negative control vector to specifically knock down Cx43 ex-
pression. First passage HUVECs infected either with Cx43-
specific viruses or with nontarget shRNA control viruses were
subjected to a 5-day period of puromycin selection, eliminating
noninfected cells from the culture. Knockdown of Cx43 ex-
pression was then determined by quantitative Western blot
analysis on protein extracts from a part of the cells, while the
remainder of the cells was used for ATP release assays after two
additional passages. Figure 5A shows a representative Western
blot of two independent HUVEC preparations, revealing strong
downregulation of Cx43 protein to 28 ± 8% (n = 3).
Despite this pronounced reduction of connexin43 protein
levels (Fig. 5A), the amount of ATP released upon thrombin
stimulation (1 U/ml for 10 min) was not impaired. It rather
appears that lowering Cx43 protein levels increased basal as
well as stimulated ATP release (Fig. 5B). This finding was
 corroborated by repeating the experiment in triplicate using
independent HUVEC preparations to minimize the influence of
individual variations in primary cell samples.
As described above, carbenoxolone efficiently blocked ATP
release from HUVECs in low concentration (5 µM), suggest-
ing a possible participation of pannexin 1 channels in this
process (8, 38). We therefore targeted Panx1 expression in
HUVECs by shRNA using a subset of three shRNA vectors
from a Panx1-specific mission shRNA vector collection. Quan-
tifications of knockdown efficiencies were performed with
real-time Taq-Man PCR, as a commercially available anti-
human Panx1 antibody in our hands failed to detect the
appropriate band of 58 kDa in Western blots on untransfected
HUVECs.
Expression of Panx1 shRNA in eight independent experiments
led to a significant reduction of both Panx1 mRNA (50 ± 17%)
and thrombin-induced ATP release (41 ± 11%,) (Fig. 5C). The reduction of the ATP release response, however, only became evident after prolonged cell culture (4 passages under shRNA expression), whereas reduction of Panx1 mRNA did not result in a decrease in thrombin-induced ATP release after shorter culture periods (2 passages, data not shown).

DISCUSSION

In the present study we addressed the question on an adequate stimulus and the molecular mechanism underlying ATP release from HUVECs. A main challenge at the beginning of the study was to define a pharmacological stimulus leading to reproducible, non-lytic ATP release. Since a tight coupling between rise in intracellular Ca\(^{2+}\) level and ATP release has been reported for various cell types including endothelial cells (23), we tested various agonists known to increase [Ca\(^{2+}\)]\(_i\) for their ability to release ATP from HUVECs.

The [Ca\(^{2+}\)]\(_i\)-increasing ionophore A23187 reproducibly induced ATP release, but at higher concentrations the drug caused the release of LHD, a clear sign for cytotoxicity. Therefore we focused on physiological stimuli and found that both thrombin and histamine, known to rapidly increase [Ca\(^{2+}\)]\(_i\) in HUVECs via specific receptors (25), reproducibly triggered the release of ATP. Histamine induced a moderate ATP release from HUVECs which are known to express different subtypes of histamine receptors including H\(_1\) receptors (30). Stimulation of H\(_1\) receptors increases [Ca\(^{2+}\)]\(_i\) and might therefore be involved in the observed histamine-induced ATP release from HUVECs. Thrombin at physiological concentrations induced a robust ATP release. Moreover, thrombin concentrations well above physiological values were without signs of cytotoxicity. Together these findings suggest that thrombin is a very potent, most likely physiological stimulus for ATP release in HUVECs. Similar to our finding, thrombin has been reported to release ATP from human astrocytoma cells (2) and human lung epithelial cells (44). It should be noted, however, that the magnitude of basal and thrombin-stimulated ATP release showed some variability depending on the cell preparation used. This might be due to the fact that HUVECs are primary cells originating from biologically variable donors. Although we utilized only early passage cells for our experiments (passage 1–4), we also cannot exclude the influence of senescence on cell behavior.

As to the mechanism of thrombin-induced ATP release, this appears to be mediated by proteolytic cleavage of the PAR-1 receptor, the most prominent thrombin receptor expressed in HUVECs (35). We found that the tethered ligand peptide TFLLRN specific for the PAR-1 receptor effectively released ATP. Notably, maximal ATP response evoked by TFLLRN application was in the same concentration range as the thrombin-induced response, indicating that PAR-1 is the main thrombin receptor involved in this process. However, a contribution of PAR-3 and PAR-4, which are also expressed in HUVECs (13, 33), cannot completely be ruled out.

Endothelial PAR receptor activation by thrombin results in a variety of endothelial responses like changes in cell shape and permeability, expression of adhesion molecules or synthesis of cytokines (46). These cellular responses are often related to an increase in cytosolic Ca\(^{2+}\) concentration. A direct functional link between [Ca\(^{2+}\)]\(_i\) and activation of ATP release is likely, since besides thrombin, also A23187, histamine, and thapsigargin (unpublished observations) resulted in a rapid, non-lytic release of ATP, and all four substances induce—by different means—a rise in [Ca\(^{2+}\)]\(_i\). Moreover, preincubation with BAPTA, an intracellular calcium chelator, efficiently blocked the thrombin-dependent ATP release.

A possible mechanism of thrombin-induced ATP release could involve connexins which can form functional “hemichannels,” providing a transmembrane pathway for the diffusion of ATP. HUVECs express connexins 37, 40, and 43 (26). Particularly Cx43, the main gap junction protein expressed in HUVECs, has been frequently described to form ATP-permeable hemichannels (12). In addition, like most cells, HUVECs also express pannexin 1, a protein proposed to also form ATP-permeable channels (45). Both channel subtypes have been reported in the literature to open upon elevation of [Ca\(^{2+}\)]\(_i\). However, α-glycyrrhetinic acid (αGA), an established inhibitor of connexin holo-and hemichannel function, failed to reduce thrombin-induced ATP release in our experiments, whereas carbenoxolone, a less specific gap junction inhibitor, was very effective. Cbx has been reported to inhibit not only connexins, but also—especially at low concentrations—pannexin channels (8) as well as volume-regulated anion channels (1). Gadolinium ions, which are described as inhibitors of ATP-permeable maxi-anion channels (39), also very efficiently inhibited ATP release in our study.

Pharmacological inhibitors may not be sufficiently specific, and some inhibitors of channel function even interfere with the luciferase detection system, like in this work Gd\(^{3+}\) and MK571. While Gd\(^{3+}\) only weakly affected luciferase activity, the effect of MK571 could be fully attributed to luciferase inhibition. Moreover, inhibitors of gap junction proteins interfere both with hemichannel and holochannel function, thereby probably influencing cell physiology by interfering with cell-cell communication which subsequently alters the exchange of small molecules like amino acids, ions, and second messengers.

Gadolinium was reported to be a relatively unspecific inhibitor of mechanosensitive, voltage-sensitive and transient receptor potential (TRP) channels (24) that might also interfere with intracellular Ca\(^{2+}\) movements. It is therefore important to distinguish whether complete blockade of thrombin-mediated

**Table 2. Effect of gadolinium on thrombin-induced rise in intracellular [Ca\(^{2+}\)]**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>Increase (Peak)</th>
<th>%Peak Increase</th>
<th>Increase (AUC)</th>
<th>%AUC Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (n = 119 cells)</td>
<td>0.974 ± 0.028</td>
<td>0.425 ± 0.179</td>
<td>100%</td>
<td>1.606 ± 0.799</td>
<td>100%</td>
</tr>
<tr>
<td>Gd(^{3+}) + thrombin (n = 123 cells)</td>
<td>0.933 ± 0.072</td>
<td>0.327 ± 0.208</td>
<td>77.1%</td>
<td>1.435 ± 0.949</td>
<td>89.3%</td>
</tr>
</tbody>
</table>

Values are means ± SD. Cells were stimulated with 1 U/ml thrombin or 50 μM GdCl\(_3\) + 1 U/ml thrombin, respectively. Maximum peak intensities as well as integral values of 8 min (AUC, area under curve) of 357/380 nm fluorescence ratios were determined. Values are given in arbitrary units.
ATP release was due to a primary inhibition of an ATP release mechanism or was rather caused by an inhibitory effect of gadolinium on calcium mobilization. Measurements of intracellular calcium responses using Fura-2 revealed that the principal calcium response elicited by thrombin was preserved in the presence of 50 μM gadolinium, although the peak amplitude as well as the plateau of calcium was slightly reduced. Besides attenuation of calcium mobilization by Gd³⁺, we also observed a Gd³⁺-mediated moderate reduction of luciferase enzyme activity used for ATP response quantification. However, none of the observed effects can account for the nearly complete inhibition of thrombin-induced ATP release by Gd³⁺. It thus appears that Gd³⁺ in a Ca²⁺-independent manner interferes with the ATP release mechanism.

To directly probe for a role of membrane channels in mediating ATP release, we used shRNA technology to specifically downregulate the expression of Cx43 and Panx1 by stably expressing shRNAs to specifically knock down the expression of either gene. Reduction of Cx43 protein level below 30% did not alter ATP release, suggesting that hemi-channels formed by this protein are not involved in thrombin-induced ATP release. Note that a reduction of connexin43 expression will also lead to a reduction of gap junction function, which might trigger more complex cellular alterations. In contrast, suppression of Panx1 expression significantly reduced thrombin-induced ATP release. This effect, however, became apparent only after prolonged growth of targeted cells (4 passages), whereas Panx1 mRNA was already reduced after 2 passages. Similarly, targeting of Panx1 expression in airway epithelia cells by lentiviral shRNA expression lead to a marked reduction of mRNA levels while the effect on protein level and ATP release function was much less pronounced (38). It is known that the clearing of preexisting Panx1 protein from the cell membrane is slow, so that the half-life of Panx1 channels is assumed to be long (18). This might explain that the shRNA effect in our experiments became apparent only when the protein was sufficiently diluted by multiple cell divisions. However, due to the lack of protein data we cannot definitely conclude that the observed biological effect is due to a reduction in Panx1 protein.

Thrombin-mediated ATP release may be functionally important at sites of injured vessels. Here, prothrombin is cleaved to thrombin which then interacts with PAR receptors on cells in close vicinity, for example, by inducing platelet aggregation. In this process, ATP can be assumed to be released in a locally restricted manner and may either directly interact with P2 receptors or via its degradation product, adenosine, on P1 receptors on neighboring cells, i.e., endothelial cells, thromocytes, or leukocytes. Both scenarios are likely to take place, since most cell types express receptors of both subtypes. Moreover, there is a highly active ectonucleolytic pathway on endothelial cells as well as on leukocytes (CD39, CD73), by which ATP is enzymatically broken down to adenosine. In this process, platelets may become activated by ATP/ADP, whereas adenosine has antiaggregatory activity. In addition, both ATP and adenosine are potent regulators of vascular tone and may either directly interact with P2 receptors or via its degradation product, adenosine, on P1 receptors on neighboring cells, i.e., endothelial cells, thromocytes, or leukocytes. Both scenarios are likely to take place, since most cell types express receptors of both subtypes. Moreover, there is a highly active ectonucleolytic pathway on endothelial cells as well as on leukocytes (CD39, CD73), by which ATP is enzymatically broken down to adenosine. In this process, platelets may become activated by ATP/ADP, whereas adenosine has antiaggregatory activity. In addition, both ATP and adenosine are potent regulators of vascular tone and modulation cell proliferation and migration (9). Furthermore, ATP and adenosine are known to act on immune cells, regulating immunity and inflammation in a complex way (5). In this context, ATP might serve as an important messenger to recruit phagocytes to regions of vessel injury. Panx1 channels have been shown to mediate ATP release from preapoptotic Jurkat T cells (11), and it is tempting to speculate that a comparable mechanism is important for endothelial cells in regions of vessel damage.
Taken together, the present study demonstrates that stimulation of HUVECs with thrombin leads to an immediate and robust release of ATP, most likely via proteolytic activation of PAR-1 receptors. ATP is released by a carboxenoxolone- and Gd³⁺-sensitive pathway, which is probably activated by a rise in intracellular calcium. On the basis of carbenoxolone inhibitor data and shRNA experiments we conclude that Panxl channels are likely to contribute to this process.

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