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Extracellular ATP attenuates ischemia-induced caspase-3 cleavage in human endothelial cells

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ABSTRACT

Background: Apoptotic death of endothelial cells (EC) plays a crucial role for the development of ischemic injury. In the present study we investigated the impact of extracellular Adenosine-5′-triphosphate (ATP), either released from cells or exogenously added, on ischemia-induced apoptosis of human EC.
Methods and results: To simulate ischemic conditions, cultured human umbilical vein endothelial cells (HUVEC) were exposed to 2 h of hypoxia ($Po_2 < 4$ mm Hg) in serum-free medium. Ischemia led to a 1.7-fold (+/−0.4; $P < 0.05$) increase in EC apoptosis compared to normoxic controls as assessed by immunoblotting and immunocytochemistry of cleaved caspase-3. Ischemia-induced apoptosis was accompanied by a 2.3-fold (+/−0.5; $P < 0.05$) increase of extracellular ATP detected by using a luciferin/luciferase assay. Addition of the soluble ecto-ATPase apyrase, enhancing ATP degradation, increased ischemia-induced caspase-3 cleavage. Correspondingly, inhibition of ATP breakdown by addition of the selective ecto-ATPase inhibitor ARL67156 significantly reduced ischemia-induced apoptosis. Extracellular ATP acts on membrane-bound P2Y- and P2X-receptors to induce intracellular signaling. Both, ATP and the P2Y-receptor agonist UTP significantly reduced ischemia-induced apoptosis in an equipotent manner, whereas the P2X-receptor agonist αβ-me-ATP did not alter caspase-3 cleavage. The anti-apoptotic effects of ARL67156 and UTP were abrogated when P2-receptors were blocked by Suramin or PPADS. Furthermore, extracellular ATP led to an activation of MEK/ERK- and PI3K/Akt-signaling pathways. Accordingly, inhibition of MEK/ERK-signaling by UO126 or inhibition of PI3K/Akt-signaling by LY294002 abolished the anti-apoptotic effects of ATP.
Conclusion: The data of the present study indicate that extracellular ATP counteracts ischemia-induced apoptosis of human EC by activating a P2Y-receptor-mediated signaling reducing caspase-3 cleavage.

1. Introduction

Endothelial cells (EC) form a selective barrier between the vessel lumen and the surrounding tissue. Disturbance of endothelial integrity by apoptotic cell death plays a crucial role in the development of ischemic injury of various organs including brain, heart and kidney [1–3]. It was shown that hypoxic/ischemic conditions induce the release of adenosine-5′-triphosphate (ATP) from diverse cell types including EC and cardiomyocytes [4,5]. Once released, extracellular ATP exerts its auto- or paracrine effects by acting on membrane-bound P2-receptors [6]. Based on homologies in the receptor-sequence and on the downstream signaling cascades, two types of P2-receptors can be distinguished: the P2X-receptors ($P2X_{1-7}$), acting as ligand-gated ion channels and the G-protein coupled P2Y-receptors ($P2Y_{1, 2, 4, 6, 11-14}$) [7]. Interestingly, ATP may act as pro- or anti-apoptotic signaling molecule depending on the receptor-subtype that is activated. While activation of P2Y-receptors by ATP was shown to prevent apoptosis, activation of P2X-receptors seems to enhance apoptotic cell death [8,9]. Thus, the effect of extracellular ATP depends on the P2-receptor expression pattern of a certain cell type. RNA and protein analysis revealed that EC mainly express $P2Y_1$-, $P2Y_2$-, $P2Y_{11}$- and $P2X_4$-receptors [10]. However, the net effect of extracellular ATP on ischemia-induced apoptosis of human EC is not clear. To investigate this issue, human EC of the umbilical vein (HUVEC) were exposed to simulated ischemia for 2 h. Endothelial cell apoptosis was assessed by analyzing the active cleavage products (17 and 19 kDa) of caspase-3. Its activation leads to cleavage of apoptotic key elements such as the DNA fragmentation factor or the caspase-activated DNase I resulting in the fragmentation of the chromosomal DNA [11,12]. Cleavage of

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caspase-3 is the common final pathway of both the extrinsic and the intrinsic pathway of apoptosis and its analysis represent an appropriate method to investigate apoptotic cell death [13,14].

2. Materials and methods

2.1. Cell culture

The investigation confirms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997; 35:2-3). Macrovascular endothelial cells from human umbilical veins (HUVEC) were isolated and cultured according to Peters et al. [15]. Briefly, harvested cells were cultured in PromoCell endothelial cell basal medium supplemented with 10% (vol/vol) FCS, 0.4% (vol/vol) endothelial growth supplement with heparin, 0.1 ng/ml human EGF, 1.0 μg/ml hydrocortisone, 1 ng/ml human bFGF and 2% (vol/vol) penicillin/streptomycin in a fully humidified atmosphere at 37 °C and 5% CO_2. Confluent cultures were trypsinized in phosphate-buffered saline and seeded at a density of 7×10^4 cells/cm^2. 16 h before the experiment, FCS was reduced to 1%. All experiments were performed with subconfluent endothelial monolayers of first passage.

2.2. Experimental protocols

Confluent monolayers of endothelial cells were exposed to simulated ischemia. Therefore, culture medium was replaced with serum-free Hank's buffered salt solution (HBSS; PAA) and cells were placed into a custom-made hypoxic chamber. After 30 min of rest, cells were exposed to 100% of N_2 for 2 h at 37 °C. Polarographic measurements showed that the Po_2 in the hypoxic chamber was reduced to less than 4 mm Hg within 5 min. Control cells were simultaneously cultured under normoxic conditions. After 2 h of simulated ischemia the cleavage of caspase-3 was analyzed by immunoblotting and immunocytochemistry to determine apoptotic cell death.

The MEK-inhibitor UO126, the p38-inhibitor SB203580, the JNK/c-Jun-inhibitor SP600125, the PI3K-inhibitor LY294002 (all Calbiochem) and the P2-receptor antagonists Suramin and PPADS (both Sigma) were prepared with dimethyl sulfoxyde (DMSO) and added 30 min prior to simulated ischemia. The same final concentrations of DMSO were included in all respective controls. The ectonucleotidase-inhibitor ARL67156 as well as the P2-receptor agonists ATP, UTP and αβ-me-ATP (all Tocris) were dissolved in water and added with the onset of simulated ischemia using indicated concentrations.

2.3. ATP luciferin/luciferase assay

At indicated time points of simulated ischemia samples from the supernatant were collected and stored on ice. 80 μl of the ATP luciferin/luciferase assay reagent (Calbiochem) was prepared according to manufactures' instructions, and mixed with 80 μl of the supernatant in a microcentrifuge tube. Subsequently, light emission of the luciferin/luciferase reaction was measured by using a TD-20/20 Luminometer (Turner Design). All assays were performed at room temperature.

2.4. Lactate dehydrogenase (LDH) activity

The activity of LDH was analyzed by using the CytoTox-ONE Homogeneous Membrane Integrity Assay according to the manufacturer's instructions (Promega). Samples of the supernatant were collected and equilibrated to room temperature. Subsequently 100 μl of the CytoTox-ONE® reagent was mixed with 100 ml of the respective sample and incubated for 10 min. 50 μl of a ready-made stopping solution was then added to the samples and LDH activity was analyzed by using microplate reader (Tecan).

2.5. Immunoblotting

Samples for SDS–PAGE were collected as previously described [16]. Briefly, equal amounts of the lysed cellular protein were loaded to SDS–PAGE and subsequently transferred to nitrocellulose membranes (Schleicher & Schuell). After blocking, membranes were incubated with anti-cleaved caspase-3 (Asp^{175}), anti-phospho-ERK 1/2 (Thr^{202}/Tyr^{204}), anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-phospho-c-Jun (Ser^{73}) and anti-phospho-Akt (Ser^{473}) (all Cell Signal) at 4 °C, overnight. After washing, membranes were incubated with appropriate secondary horseradish peroxidase (HRP) conjugated antibodies. Immunoreactivity was detected with enhanced chemiluminescence and quantified by densitometric analysis by using Quantity One software of ChemiDoc XRS (BioRad).

2.6. Immunocytochemistry

For immunocytochemical analysis, HUVEC were grown on glass cover slips. After the experiments, cells were fixed with 4% (wt/vol) para-formaldehyde in PBS for 20 min at room temperature and subsequently permeabilized with 0.1% (vol/vol) Triton X-100 for 8 min. After blocking with PBS containing 3% (wt/vol) BSA, slides were incubated with anti-cleaved caspase-3 monoclonal IgG antibody (R&D Systems) over night at 4 °C. After washing, slides were incubated for 1 h with AlexaFluor 488 anti-mouse IgG second antibody (Invitrogen) at room temperature. Subsequently slides were washed and mounted in PBS/glycerol (1:1, vol/vol) and analyzed by using confocal laser microscopy (LSM 510 Meta, Zeiss). TO-PRO 3 iodide (Invitrogen) was added together with the secondary antibody to stain nuclei.

2.7. Statistics

Data are given as mean ± standard error (SE) of n separate experiments using independent cell preparations. The comparison of means between groups was performed by one way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Changes of parameters within the same group were analyzed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant.

3. Results

3.1. Ischemia-induced apoptosis is accompanied by enhanced ATP release from HUVEC

First, we examined whether simulated ischemia induces apoptotic cell death of HUVEC. Therefore, cells were exposed to 2 h of hypoxia ($Po_2 < 4$ mm Hg) in serum-free medium. Apoptosis was analyzed by immunoblotting of active cleavage products of caspase-3. Simulated ischemia led to a 1.7-fold (+/− 0.4; $P < 0.05$) increase in cleaved caspase-3 (Asp^{175}) compared to control cells cultured under normoxic conditions (Fig. 1A). In addition, immunocytochemistry was performed demonstrating a clear increase in cleaved caspase-3-positive HUVEC following simulated ischemia (Fig. 1B). Subsequently, we tested whether HUVEC undergoing ischemia-induced apoptosis, release ATP. Analysis of cell culture supernatants using a luciferin/luciferase assay revealed a significant increase of extracellular ATP after 10 min of simulated ischemia compared to control cells cultured under normoxic conditions (2.3-fold +/− 0.5; $P < 0.05$; Fig. 1C). Compared to ATP standard mea-

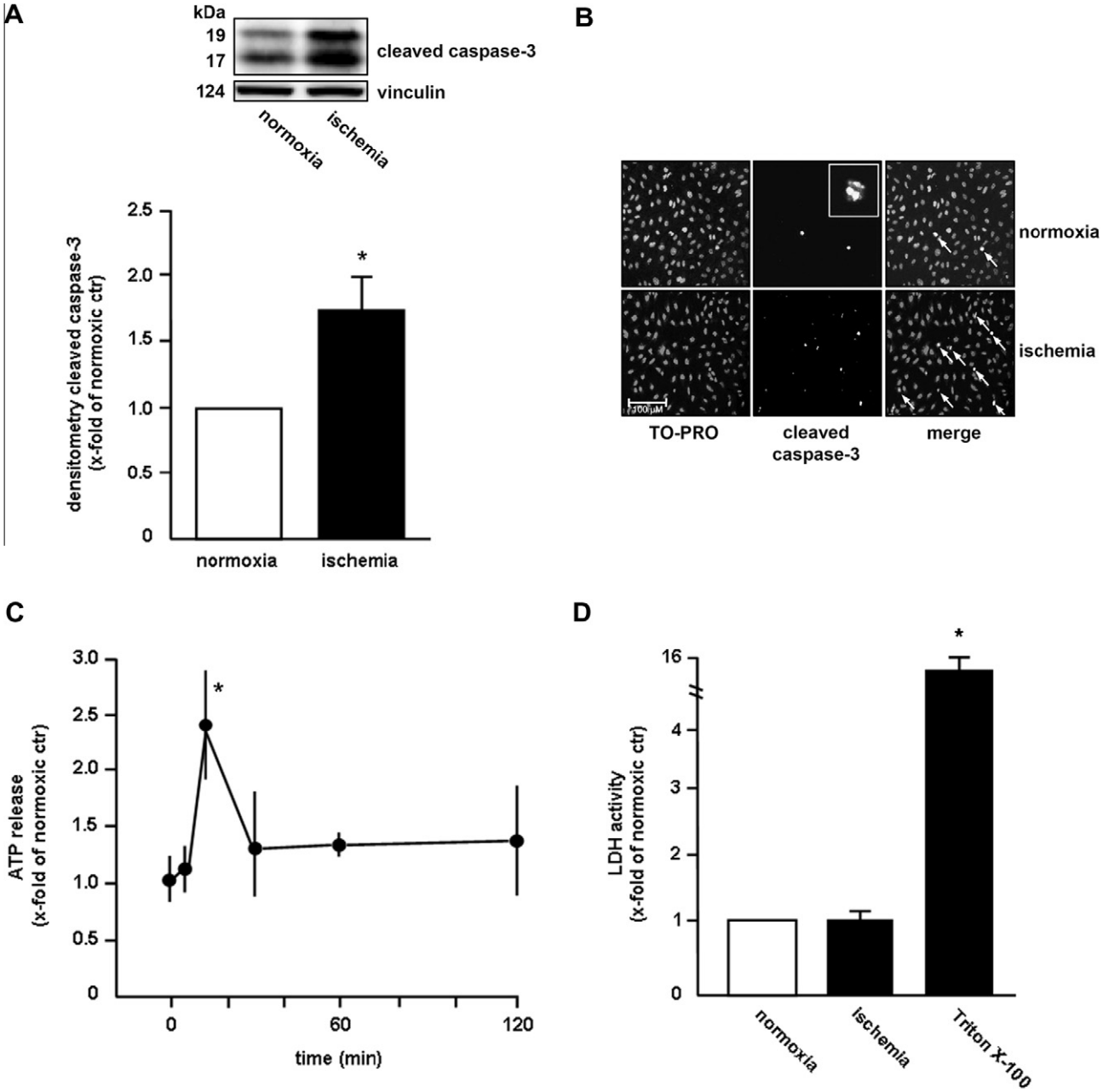

Fig. 1. Ischemia-induced apoptosis is accompanied by enhanced ATP release from HUVEC. HUVEC were exposed to simulated ischemia for 2 h. Cells simultaneously cultured under normoxic conditions served as controls. (A) Upper panel: representative immunoblot demonstrating enhanced apoptosis following simulated ischemia as indicated by an increase of caspase-3 cleavage products (17 and 19 kDa). Vinculin was used as a control to demonstrate equal loading. Lower panel: corresponding densitometric analysis of caspase-3 cleavage products relative to vinculin. The ratio of control was set to 1. (B) Representative immunocytochemistry showing an increase of cleaved caspase-3-positive cells (arrows) in response to simulated ischemia. TO-PRO-3-iodide (TO-PRO) was used for nuclear staining. In the insert a cleaved caspase-3-positive cell is depicted in high magnification. (C) Ischemia-induced apoptosis was accompanied by increased levels of extracellular ATP as detected by using a luciferin/luciferase ATP detection assay. Data represent extracellular ATP at indicated time points relative to the corresponding normoxic control. Values of ATP at the starting point were set to 1. (D) Analysis of LDH activity showed that simulated ischemia (10 min) did not induce cytolysis. For positive control, cells were treated with Triton X-100. Data are means ± SE of at least three separate experiments with independent cell preparations. *$P < 0.05$ vs. onset of simulated ischemia.

surements this equates a concentration of approximately 4 ng/ml (data not shown). Analysis of LDH activity excluded that the increase of extracellular ATP was due to ischemia-induced cytolysis (Fig. 1D).

3.2. Inhibition of ATP breakdown reduces ischemia-induced apoptosis of HUVEC

Extracellular ATP may act as a signaling molecule by activating membrane-bound P2-receptors. Signaling is controlled by a complex network of soluble and membrane-bound ectonucleotidases (ecto-ATPases) leading to a well-directed degradation of ATP [17]. To elucidate the impact of extracellular ATP on ischemia-induced apoptosis, HUVEC were treated with the selective ecto-ATPase-inhibitor ARL67156. Fig. 2 illustrates that prevention of ATP breakdown by ARL67156 reduced caspase-3 cleavage by 22% (+/− 6%; $P < 0.05$). Correspondingly, enhanced ATP degradation by application of the soluble ecto-ATPase apyrase during simulated ischemia increased caspase-3 cleavage by 42% (+/− 14%; $P < 0.05$; Fig. 2). Simultaneous addition of adenosine deaminase ruled out that the observed increase in caspase-3 cleavage following

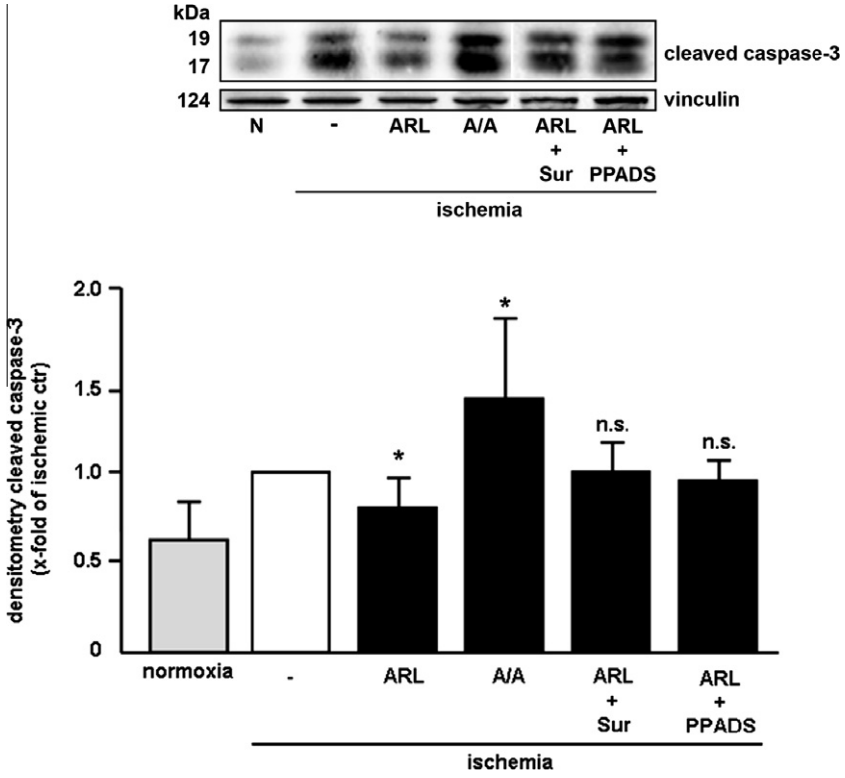

Fig. 2. Inhibition of ATP breakdown reduces ischemia-induced apoptosis of HUVEC. HUVEC were exposed to simulated ischemia for 2 h. Cells simultaneously cultured under normoxic conditions (N) served as controls. Upper panel: representative immunoblot showing that incubation of HUVEC with the 5′-ectonucleotidase-inhibitor ARL67156 (ARL, 100 μM) reduced ischemia-induced cleavage of caspase-3. In contrast, addition of the soluble ecto-ATPase apyrase plus adenosine deaminase (A/A, each 1 U/ml) enhanced caspase-3 cleavage. The reduction of caspase-3 cleavage by the 5′-ectonucleotidase-inhibitor ARL67156 (ARL, 100 μM) was abolished when cells were pre-incubated with the P2-receptor antagonist Suramin (ARL + Sur, 100 μM) or PPADS (ARL + PPADS, 5 mM). Vinculin served as a control to demonstrate equal loading. Lower panel: densitometric analysis of cleaved caspase-3 relative to vinculin. The ratio of ischemic control was set to 1. Data are means + SE of at least three separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. ischemic control; n.s. not significantly different from ischemic control.

enhanced ATP breakdown was due to an accumulation of adenosine (Fig. 2). To confirm that the observed reduction in caspase-3 cleavage by ARL67156 is based on ATP-induced activation of P2-receptors, HUVEC were pre-incubated with the pan-specific P2-receptor antagonists Suramin and PPADS, respectively. Fig. 2 shows that the blockade of P2-receptors abrogated the anti-apoptotic effect of ARL67156.

3.3. Protective effects of extracellular ATP seem to be mediated by P2Y-receptors

Extracellular ATP can act on two types of P2-receptors: G-protein coupled P2Y-receptors and P2X-receptors [7]. To identify the P2-receptor subtype mediating the anti-apoptotic effect of extracellular ATP, we used a pharmacological approach. Therefore, different P2-receptor-agonists were added to the medium with the onset of simulated ischemia. After 2 h, cells were harvested and the effect of the particular P2-receptor-agonist on ischemia-induced apoptosis was analyzed. Our results demonstrate that both, the pan-specific P2-receptor agonist ATP and the P2Y-receptor agonist UTP significantly reduced ischemia-induced caspase-3 cleavage in a concentration-dependent manner (Fig. 3A and B). In contrast, addition of the P2X-receptor agonist αβ-me-ATP did not affect ischemia-induced apoptosis (Fig. 3C). According to our previous experiments, the anti-apoptotic effect of the P2Y-receptor agonist UTP was abrogated when cells were pre-incubated with the P2-receptor antagonists Suramin or PPADS (Fig. 3D). These findings suggest that the anti-apoptotic effect of extracellular ATP in HUVEC is mediated by activation of P2Y-receptors.

3.4. Reduction of ischemia-induced apoptosis via P2Y-receptors is mediated by MEK/ERK- and PI3K/Akt-signaling pathways

As G-Protein-coupled receptors, P2Y-receptors activate different intracellular signaling pathways, including mitogen-activated protein kinase (MAPK)- and phosphoinositid-3-kinase (PI3K)/Akt-signaling [18,19]. We therefore analyzed the effect of exogenously applied ATP on these signaling pathways by using phospho-specific antibodies. Addition of ATP during simulated ischemia led to an increase in phosphorylation of ERK 1/2 (Thr^{202}/Tyr^{204}), p38 (Thr^{180}/Tyr^{182}) and Akt (Ser^{473}) in a time-dependent manner (Fig. 4A). However, ATP did not alter phosphorylation of c-Jun (Ser^{73}). Corresponding analysis of control cells exposed to simulated ischemia without addition of ATP showed no comparable phosphorylation of ERK 1/2, p38 or Akt (data not shown). To further elucidate the role of these signaling pathways for the reduction of ischemia-induced apoptosis, cells were pre-incubated with specific inhibitors. These experiments revealed that the anti-apoptotic effect of ATP was abrogated when the MEK/ERK-inhibitor UO126 or the PI3 K/Akt-inhibitor LY294002 was present during ischemia (Fig. 4B). In contrast, neither addition of the p38-inhibitor SB203580 nor addition of the JNK/c-Jun-inhibitor SP600125 did alter the anti-apoptotic effect of ATP (Fig. 4B). To elucidate whether the activation of ERK 1/2 and Akt in response to ATP are associated with each other, we analyzed the effect of UO126 and LY294002 on ERK 1/2- and Akt phosphorylation. While UO126 clearly abrogated phosphorylation of ERK 1/2 in response to ATP, it did not affect Akt phosphorylation (Fig. 4C). Correspondingly, addition of LY294002 prevented Akt-phosphorylation in response to ATP but did not alter phosphorylation of ERK 1/2,

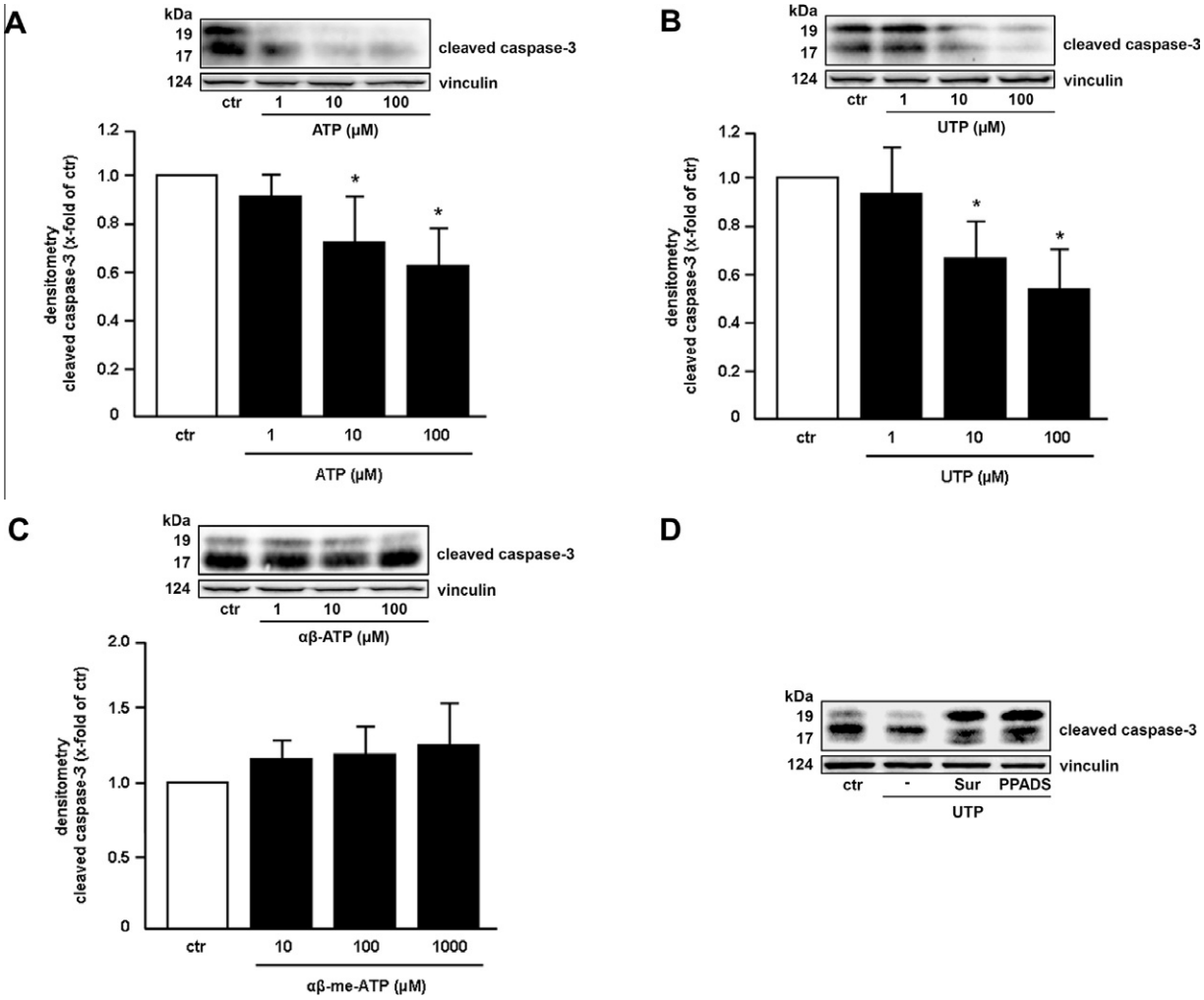

Fig. 3. Protective effects of extracellular ATP seem to be mediated by P2Y-receptors. HUVEC were exposed to 2 h of simulated ischemia alone (ctr) and in the presence of (A) ATP, (B) the P2Y agonist UTP or (C) the P2X agonist αβ-me-ATP. Upper panel: apoptotic cell death was analyzed by immunoblotting of cleaved caspase-3. Vinculin served as a control to demonstrate equal loading. Lower panel: densitometric analysis of cleaved caspase-3 relative to vinculin. The ratio of ischemic control was set to 1. Data are means + SE of at least three separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. ischemic control. (D) HUVEC were exposed to 2 h of simulated ischemia alone (ctr) and in the presence of UTP (100 μM). In part cells were pre-incubated with Suramin (Sur, 100 μM) or PPADS (PPADS, 5 mM). Apoptosis was analyzed by immunoblotting of cleaved caspase-3. Vinculin was used as a control to demonstrate equal loading.

suggesting that both signaling pathways are independently activated (Fig. 4C). In line with our results from the pharmacological approach we found that also addition of UTP reduced caspase-3 cleavage by activation of MEK/ERK- and PI3 K/Akt-signaling, emphasizing the role of P2Y-receptors in the protection of human EC from ischemia-induced apoptosis (data not shown).

4. Discussion

Endothelial cell apoptosis plays a crucial role for the induction and progression of ischemic injury occurring during brain injury, myocardial infarction or acute kidney injury [1–3] Therefore, protecting the endothelium from ischemia-induced apoptotic cell death represents a potential target for the treatment of ischemic injury. The present study demonstrates a novel anti-apoptotic mechanism in human EC counteracting ischemia-induced caspase-3 cleavage by activating a P2Y-receptor-mediated signaling.

Our data show that ischemia-induced apoptosis of human EC is accompanied by a significant increase of extracellular ATP. Regarding the time-course, a rapid increase of extracellular ATP during ischemia was observed, which was also found in pulmonary EC and neuronal cells [4,20]. Enhancement of ATP breakdown by addition of the soluble ecto-ATPase apyrase resulted in an increase in HUVEC apoptosis as shown by enhanced ischemia-induced caspase-3 cleavage. These results indicate that ATP acts as a survival signal for human EC mediating ischemic tolerance, which was previously described in the central nervous system [20]. However, this mechanism cannot completely prevent apoptosis and seems to be overrun by ischemia. This fact might be due to the rapid degradation of extracellular ATP via soluble and membrane-bound ecto-ATPases [21]. Accordingly, we found that selective inhibition of ATP breakdown by addition of the ecto-ATPase-inhibitor ARL67156 reduced ischemia-induced apoptosis.

After being released, ATP acts as a signaling molecule binding to P2-receptors, which are expressed by EC. Blockage of P2-receptors with Suramin or PPADS abrogated the anti-apoptotic effect observed under ATP-ase inhibition. These data suggest that extracellular ATP exerts its anti-apoptotic effects via a P2-receptor mediated signaling.

HUVEC express both isoforms of P2-receptors, P2Y- and P2X-receptors [10]. As these two isoforms are known to exert differential effects on EC apoptosis with P2X being pro-apoptotic and P2Y anti-apoptotic, a pharmacological approach was used to further

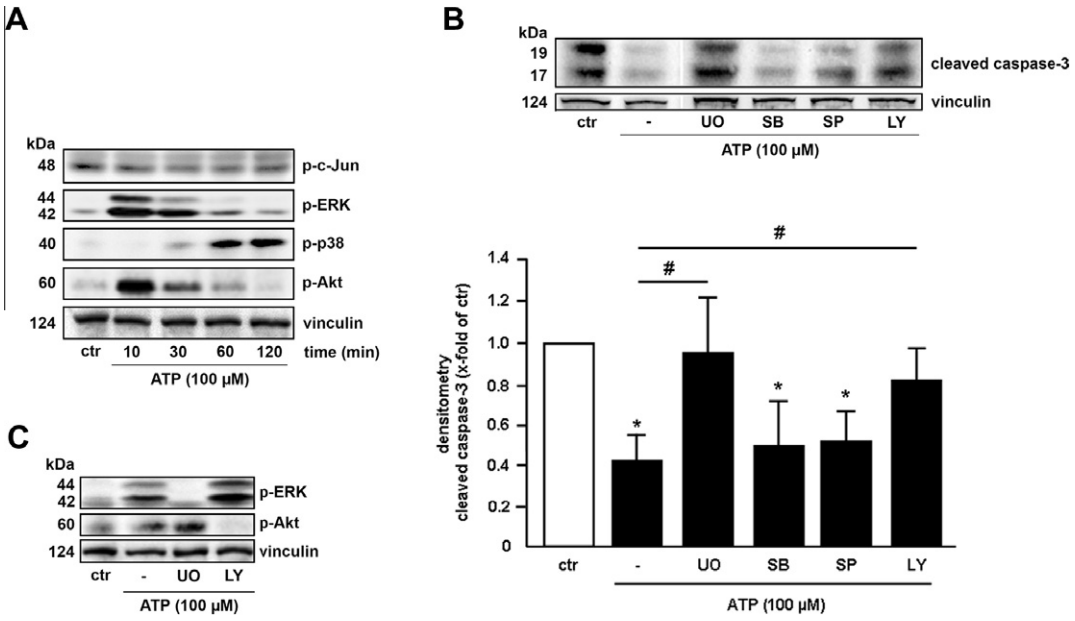

Fig. 4. Reduction of ischemia-induced apoptosis via P2Y-receptors is mediated by MEK/ERK- and PI3K/Akt-signaling pathways. HUVEC were exposed to 2 h of simulated ischemia in the presence of ATP (100 µM). (A) Representative immunoblots indicating that ATP induced a time-dependent phosphorylation of ERK 1/2 (p-ERK), p38 (p-p38) and Akt (p-Akt). In contrast, phosphorylation of c-Jun (p-c-Jun) was not increased. Vinculin served as a control to demonstrate equal loading. (B) In addition, HUVEC were partly pre-incubated with the MEK/ERK-inhibitor UO126 (UO, 10 µM), the p38-inhibitor SB203580 (SB, 10 µM), the JNK/c-Jun-inhibitor SP600125 (SP, 20 µM) or the PI3K/Akt-inhibitor LY294002 (LY, 10 µM). Upper panel: representative immunoblottings of cleaved caspase-3. Vinculin was used as a control to demonstrate equal loading. Lower panel: corresponding densitometric analysis of caspase-3 cleavage products relative to vinculin. The ratio of control was set to 1. Data are means + SE of at least three separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. ctr; $\#P < 0.05$. (C) HUVEC was exposed to 10 min of simulated ischemia alone or in the presence of ATP (100 µM). In part cells were pre-incubated with the MEK/ERK-inhibitor UO126 (UO, 10 µM) or the PI3K/Akt-inhibitor LY294002 (LY, 10 µM). Representative immunoblots demonstrating that ATP-induced phosphorylation of ERK 1/2 was abrogated by UO126 (UO). However, U0126 did not affect ATP-induced phosphorylation of Akt. In contrast, LY294002 (LY) inhibited ATP-induced phosphorylation of Akt, but had no effect on ERK 1/2 phosphorylation. Vinculin was used as a control to demonstrate equal loading.

elucidate the underlying molecular pathway of the ATP-mediated protective effects. Interestingly, treatment of HUVEC with the P2Y-receptor agonist UTP led to a comparable reduction in apoptosis as previously observed with ATP. In contrast, selective stimulation of P2X-receptors with αβ-me-ATP had no effect on ischemia-induced apoptosis. Although these results suggest that the protective effect of extracellular ATP in human EC is mediated via P2Y-receptors, further studies are required to identify the specific P2Y-receptor subtype responsible for the here described anti-apoptotic mechanism. Results from neuronal cells indicate that in this context the $P2Y_2$-receptor, which is highly expressed in HUVEC, plays a key role [9].

Stimulation of G-protein-coupled P2Y-receptors by ATP or UTP during simulated ischemia led to a significant increase in phosphorylation of the downstream-targets ERK 1/2, p38 and Akt. Interestingly, inhibition of MEK/ERK and PI3K/Akt abolished the anti-apoptotic effect of ATP and UTP, whereas inhibition of p38 did have no effect. These data are consistent with the results from Hausenloy et al. [22], demonstrating the pivotal role of ERK 1/2 and Akt for protecting the heart from ischemic injury. Neither affected inhibition of MEK/ERK-signaling Akt phosphorylation nor did inhibition of PI3K/Akt-signaling have an effect on ERK 1/2 phosphorylation. These data indicate that stimulation of EC with ATP resulted in an independent activation of the two pathways. However, blockade of one pathway was sufficient to completely abolish the anti-apoptotic effect of extracellular ATP or UTP. Consequently, an interaction of both pathways seems to be essential for the effective reduction of ischemia-induced apoptosis by extracellular nucleotides. In line with these findings Shelton et al. [23] found that a synergism of MEK/ERK- and PI3K/Akt-signaling is a requirement for effective protection of hematopoietic stem cells from apoptotic cell death. However, it is not clear how P2Y-receptor-mediated activation of MEK/ERK- and PI3K/Akt-signaling reduces ischemia-induced caspase-3 cleavage in human EC. Results from Mehrhof and colleagues suggest that simultaneous activation of both signaling pathways leads to an activation of the transcription factor cAMP response element-binding protein transcription (CREB) enhancing expression of anti-apoptotic factors such as bcl-2 [24,25]. In line with these findings Chen and colleagues demonstrated that activation of CREB is essential for ATP-dependent upregulation of survival factors in neurons, underlining the impact of CREB for ATP-mediated cell survival [26].

Taken together, our results identify a novel anti-apoptotic mechanism in human EC suggesting that selective activation of P2Y-receptors might represent a promising therapeutic strategy to protect the endothelium from ischemic injury.

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