ATP antagonism of thrombin-induced endothelial barrier permeability

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Abstract

Objectives: Thrombin induces endothelial barrier failure by activating the contractile machinery of endothelial cells. Contractile activation is due to an increase in myosin light chain (MLC) phosphorylation. Here, it was investigated whether stimulation of endothelial cells with ATP can interrupt this thrombin-induced pathomechanism. Methods: In cultured human umbilical vein endothelial cells, cytosolic calcium [Ca2+] (Fura 2 method), phosphorylation of MLC, isometric tension and permeability for albumin were studied. Results: Thrombin (0.2 U/ml) increased [Ca2+] from a basal level of 78±6 to 570±63 nM (mean±S.D., n=5, P<0.05), MLC phosphorylation from 71±7 to 163±18%, isometric tension from 157±17 to 232±26 μN, and permeability from 2.8±0.4 to 11.6±1×10−6 cm/s. Co-presence of ATP (10 μM) and thrombin did not alter the [Ca2+] rise, but reduced MLC phosphorylation to 59.8±10%, isometric tension to 174±14 μN, and permeability to 5.4±0.6×10−6 cm/s. The thrombin-induced rise in MLC phosphorylation was sensitive to reduction of [Ca2+]. It was accompanied by an increase in Rho activation, and was inhibited by Y-27632 (10 μM), a Rho-kinase blocker. The ATP-induced decrease in MLC phosphorylation was not sensitive to [Ca2+]. It was not accompanied by changes in RhoA activation, and could not by suppressed by Y-27632. Conclusions: ATP antagonizes the Ca2+- and Rho-dependent effects of thrombin on MLC phosphorylation most likely by a Ca2- and Rho-independent activation of MLC phosphatase. It thereby functionally antagonizes the thrombin-induced increase in monolayer tension and permeability. © 2003 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Thrombin represents a central vascular mediator in inflammation and hemostasis. By stimulation of specific receptors, it activates the vascular endothelium. The endothelial lining of blood vessels forms a permeability barrier between plasma and interstitial space. In presence of thrombin, the endothelial barrier becomes permeable to water, solutes and macromolecules. The loss of endothelial barrier function, induced by presence of thrombin, can locally cause tissue edema; systemically it can contribute to a general capillary leakage syndrome. In endothelial cells, thrombin activates the contractile machinery of endothelial cells resulting in the opening of intercellular gaps which allow the passage of plasma components [1]. It has been demonstrated that thrombin mobilizes Ca2+ from intracellular stores and thus increases intracellular Ca2+ concentration [2–4]. This leads to activation of Ca2+/calmodulin-dependent MLC kinase. Phosphorylation induces a conformational change in MLC that enables actin–myosin interaction and activation of the Mg2+-ATPase activity of myosin [5]. Besides MLC kinases, MLC associated phosphatase plays also a major role in the control of the phosphorylation state of MLC in endothelial cells. Endothelial cells contain 3 phosphatase isoforms of interest, i.e. PP1, PP2A, PP2B. Work by others has shown [6,7] that PP1 but not PP2A regulate acute endothelial contractile responses. PP2B may be induced under prolonged thrombin exposure [8].

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The regulatory subunit of MLC phosphatase can be phosphorylated and thereby inactivated by Rho-kinase which is itself a downstream target of the GTPase Rho [9]. Previous work indicated that the Rho/Rho-kinase pathway contributes to cell contraction under thrombin stimulation and that this is a Ca\(^{2+}\)-independent signaling [10,11]. Among Rho isoforms RhoA is of predominant importance in endothelial cells [12]. The dual effect of thrombin on MLC kinase (activation) and MLC phosphatase (inactivation) causes a strong activation of the contractile machinery, which leads to a loss of endothelial barrier function.

Effective means to antagonize thrombin-induced endothelial hyperpermeability have not yet been identified. We showed recently that exogenously applied ATP can stabilize the endothelial barrier [13]. As causal mechanism we identified a strong net dephosphorylation of MLC [14]. This activation of MLC phosphatase dominates over another ATP signaling effect which consists in the activation of MLC kinase elicited by a transient rise in cytosolic Ca\(^{2+}\). This observation lead to the present study in which the question was addressed if ATP can antagonize the permeability increasing effect of thrombin. As our experimental model we used confluent monolayers of human umbilical vein endothelial cells.

2. Methods

2.1. Cell cultures

Human endothelial cells were isolated from umbilical cords and cultured according to van Hinsbergh et al. [15]. Confluent cultures of primary endothelial cells were trypsinized in phosphate-buffered saline (PBS, composed of (mM) 137 NaCl, 2.7 KCl, 1.5 KH\(_2\)PO\(_4\), and 8.0 Na\(_2\)HPO\(_4\), at pH 7.4, supplemented with 0.05% (w/v) trypsin, and 0.02% (w/v) EDTA) and seeded at a density of 7×10\(^4\) cells/cm\(^2\) on either 24-mm round polycarbonate filters (pore size 0.4 \(\mu\)m), 25-mm round glass coverslips, 30-mm, or 100-mm culture dishes for determination of albumin permeability, [Ca\(^{2+}\)], MLC phosphorylation, Rho activity assay, and protein phosphatase translocation assay, respectively. Experiments were performed with confluent endothelial monolayers of passage 1 or 2, 4 days after seeding.

2.2. Experimental protocols

The basal medium used in incubations was modified Tyrode’s solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.0 CaCl\(_2\), and 30.0 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.4, 37 °C) supplemented with 5% (v/v) heat-inactivated newborn calf serum (10 min, 60 °C). Basal MLC phosphorylation was determined after an initial equilibration period of 10 min. Agents were added as indicated. Stock solution of ATP was prepared immediately before use with basal medium. Stock solutions of xestospongin C were prepared with dimethyl sulfoxide (DMSO). Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations ≤0.1% (v/v). Same final concentrations of DMSO were also included in all respective control experiments. Stock solutions of all other substances were prepared in basal medium (composition as described above). Appropriate volumes of these solutions were added to the cells. Identical additions of basal medium were included in all respective control experiments.

2.3. Macromolecule permeability

The permeability of trypan blue-labeled albumin across endothelial monolayers was studied in a two-compartment system separated by a filter membrane as previously described [13,16,17] with one modification, i.e. the change of the abluminal compartment from 13 to 9.6 ml. Trypan blue-labeled albumin (60 \(\mu\)M) was added to the luminal compartment. The appearance of labeled albumin in the abluminal compartment was continuously monitored photometrically.

2.4. Free cytosolic Ca\(^{2+}\) concentration

[Ca\(^{2+}\)]\(_i\) was determined using the fluorescent Ca\(^{2+}\) indicator, Fura 2 [18], as previously described [14].

2.5. Preparation of collagen lattices and force measurement

Collagen lattices were prepared for force measurement as described [19–21] with minor modifications. Briefly, endothelial cells were seeded at 300 000 cells/cm\(^2\) on the collagen lattices and grown to confluence within 2 days. Then, the lattices were connected to a force transducer (KG 7A with bridge-amplifier DUBAM 7C; Scientific Instruments, Heidelberg, Germany) [19]. The whole setup was moved to an incubator (37 °C).

2.6. Determination of MLC phosphorylation

The MLC phosphorylation was determined by glycerol–polyacrylamide gel electrophoresis and Western blot analysis using an anti-MLC antibody as previously described [14].

2.7. RhoA activation assay

RhoA activation was determined by binding of RhoA in its active form to rhotekin as previously described [22,23]. Briefly, confluent endothelial monolayers were stimulated as indicated in the text. Cells were lysed (composition of
the lysis medium: 1% (v/v) Triton X-100, 0.5% (v/v) deoxycholate, 0.1% (v/v) SDS, 500 mM NaCl, 10 mM MgCl₂, 100 mM Tris–HCl, pH 7.4) and lysates were cleared by centrifugation (14 000 ×g, 10 min, 4 °C). A small portion of the supernatant (‘total lysate’) was taken to determine the amount of total RhoA. Then, the supernatants were incubated with the Rho binding domain of rhotekin immobilized on glutathione-coupled sepharose beads for 45 min at 4 °C. The beads were washed twice by centrifugation at 14 000 ×g at 4 °C for 30 s in wash medium (composition of the wash medium: 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 100 mM Tris–HCl pH 7.4), the pellet containing the beads with active RhoA was eluted in Laemmli sample buffer and analyzed by western blots using an anti-RhoA antibody (‘pull-down’).

2.8. Detection of protein phosphatases in the myosin-enriched and depleted cell fraction

The content of protein phosphatases 1 and 2A (PP1 and 2A) in the myosin-enriched cell fraction were determined as previously described [6]. Briefly, confluent endothelial monolayers on 10-cm dishes were stimulated as indicated in the text. Afterwards, the monolayers were rinsed twice with PBS to remove the incubation medium, 200 µl of homogenisation buffer (0.1 mM EDTA, 28 mM mercaptoethanol, 1 µg/ml pefabloc; Tris–HCl pH 7.4) was added, and dishes were cooled immediately to −80 °C. Afterwards the cells were scraped and homogenized. Homogenates were incubated with a high salt buffer (0.6 M NaCl, 0.1% (v/v) Tween 20, 1 µg/ml pefabloc) for 1 h at 4 °C and was centrifuged at 4500 ×g for 30 min at 4 °C. Supernatants were diluted 10-fold with assay buffer (0.1 mM EDTA, 28 mM mercaptoethanol, Tris–HCl pH 7.0) and centrifuged again at 8200 ×g for 40 min at 4 °C. The pellet, the myosin-enriched fraction, and the supernatant, the myosin-depleted fraction were eluted in Laemmli sample buffer and analysed by western blot using antibodies against the catalytic subunits of PP1 and PP2A. In accordance with previous reports [6] the myosin-enriched fractions only contained PP1 but not PP2A.

2.9. Materials

Falcon plastic tissue culture dishes were from Becton Dickinson (Heidelberg, Germany); ATP, from Boehringer (Mannheim, Germany); xestospongin C from Calbiochem (Bad Soden, Germany); human thrombin from Aventis Behring (Marburg, Germany), and Y-27632 from Biotrend (Köln, Germany). Transwell™ polycarbonate filter inserts (24 mm diameter, 0.4 µm pore size) from Costar (Bodenheim, Germany); newborn calf serum, medium 199, penicillin–streptomycin, and trypsin–EDTA were from Gibco Life Technologies (Eggenstein, Germany); Fura 2/AM was from Molecular Probes (Leiden, The Netherlands); pefabloc was from Merck (Darmstadt, Germany), polyvinylidene difluoride (PVDF) was from Millipore (Eschborn, Germany); dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), anti-phosphatase 1 and phosphatase 2 antibodies were from Santa Cruz (Heidelberg, Germany), and thioglycolate were from Sigma (Deisenhofen, Germany). All other chemicals were of the best available quality, usually analytical grade.

2.10. Statistical analysis

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

3. Results

3.1. Antagonism of ATP and thrombin

Under control conditions endothelial monolayers exhibited a stable permeability for albumin (Fig. 1). When ATP (10 µM) was added, permeability rapidly declined. Conversely, permeability rapidly rose when thrombin (0.2 U/ml) was applied. When both agents were applied

![Fig. 1. Effects of ATP (10 µM), thrombin (Thr, 0.2 U/ml), or simultaneous addition of both agents, on albumin permeability of human endothelial monolayers. Data are means±S.D. of n=5 separate experiments with independent cell preparations. At times between 2.5 and 30 min, permeability in presence of Thr and ATP plus Thr are significantly different P<0.05.](https://academic.oup.com/cardiovascres/article-abstract/59/2/470/291276)
simultaneously, the thrombin effect was attenuated to about one third of that seen without ATP. The dose of 0.2 U/ml was chosen for thrombin as this concentration provided a marked effect on permeability. Fig. 2 shows that the antagonistic effect of ATP is dose-dependent.

Measurement of isometric force produced by endothelial monolayers revealed a similar pattern of changes as seen for monolayer permeability. ATP caused a decline of force, thrombin a rise, and simultaneous additions of ATP and thrombin greatly attenuated the response to thrombin (Fig. 3).

Since MLC phosphorylation controls activation of the endothelial contractile machinery we analyzed changes in this biochemical parameter in the described experiments (Fig. 4). We found that ATP causes a dephosphorylation of MLC, thrombin an increase in phosphorylation, and a combination of ATP and thrombin cause a degree of phosphorylation slightly below the control level. Fig. 5 shows that the antagonistic effect of ATP is dose-dependent.

Analysis of cytosolic Ca²⁺ changes showed that both, ATP and thrombin, elicited a transient rise of cytosolic Ca²⁺ (Fig. 6). Their combined application did not significantly alter this response. This indicates that they mobilize Ca²⁺ from the same store, and that the functional antagonism on permeability and force is not due to interaction at this Ca²⁺ release mechanism. We used xestospongin C (Xe, 3 μM), a selective inhibitor of the IP₃ sensitive release channel of endoplasmic reticulum, to identify the source of the Ca²⁺ rise. Both, the responses to ATP and thrombin were greatly attenuated in the presence of Xe, indicating that both induce a rapid Ca²⁺ release from the endoplasmic reticulum.

3.2. Main routes to MLC phosphorylation

In control cultures, MLC phosphorylation amounted 72% (Fig. 7). Note that the maximum percentage is 200%, as MLC can become mono- or diphosphorylated. Then, 10 min after addition of ATP, MLC phosphorylation was reduced to half its control value, and the reduction was unaffected by the presence of Xe, demonstrating that it is not dependent on the ATP-induced rise in cytosolic Ca²⁺. This is in agreement with our previous results [14]. Presence of the Rho-kinase inhibitor, Y-27632 (10 μM), reduced the basal MLC-phosphorylation from 71 to 27%, indicating that the basal steady state phosphorylation is already controlled by a Rho-dependent pathway. The concentration of 10 μM Y-27632 was chosen since dose-finding experiments showed that this provided the maximal effect for basal MLC phosphorylation (data not shown). Addition of ATP to Y-27632-treated cultures reduced MLC phosphorylation to about one third of the last level. Identical experiments were also performed with a chemically different Rho-kinase inhibitor, HA 1077 (10 μM). The results were the same as with Y-27632 (not shown).

To analyse whether ATP interferes with RhoA activation we applied a pull-down assay based on the binding of activated RhoA (RhoA-GTP) to rhotekin. Under control conditions only small amounts of activated RhoA was found (Fig. 8). Exposure of endothelial cells to ATP (10
μM) for 5 min does not affect this amount, indicating that ATP does not alter basal RhoA activity.

Thrombin increased MLC phosphorylation within 5 min after addition to 168% (Figs. 4 and 9). The rise in MLC phosphorylation was partially attenuated in presence of Xe likely due to failure to activate the Ca^{2+}/calmodulin-dependent myosin light chain kinase. When both inhibitors Xe and Y-27632 were combined, a thrombin-induced rise in MLC phosphorylation was completely abolished. Exposure of endothelial cells to thrombin (0.2 U/ml) for 5 min increased significantly the amount of activated RhoA (Fig. 8). These experiments show that the thrombin effect on MLC phosphorylation is partially due to a Ca^{2+}- and Rho-dependent mechanism.

MLC dephosphorylation requires translocation and bind-
Fig. 7. Endothelial MLC phosphorylation under control conditions or after 10 min exposures to ATP (10 μM), xestospongin C (Xe, 3 μM), the inhibitor of Ca mobilization, Y-27632 (Y, 10 μM), the Rho kinase inhibitor, or combinations of these agents. Incubations with Xe and Y were started 20 min before other additions. Data are means±S.D. of n=4 separate experiments with independent cell preparations. * P<0.05.

Fig. 8. RhoA activation in presence of ATP (10 μM), thrombin (Thr, 0.2 U/ml), or simultaneous addition. Upper panel: (Top) Western blot analysis of active RhoA in the rhotechn pull-down fraction after 5 min and total RhoA in total lysates. Lower panel: RhoA activation, determined as densitometric ratio between active and total RhoA from Western blots and expressed as percentage of control value. Data are means±S.D. of n=5 separate experiments with independent cell preparations. * P<0.05.

4. Discussion

The present study has investigated the interaction of thrombin and ATP stimulation on the contractile response of endothelial cells. The major findings are that (i) thrombin causes activation and ATP causes inactivation of endothelial contractile elements, (ii) the contractile activation obtained by thrombin stimulation can be effectively antagonized by ATP stimulation, (iii) the thrombin/ATP antagonism is largely due to antagonistic effects on MLC phosphorylation, (iv) thrombin causes a rise in MLC phosphorylation by two mechanisms, a Ca2+-dependent and a Rho-dependent one, while ATP causes MLC dephosphorylation by a Ca2+-independent and by a Rho-independent mechanism, as well.

We showed in previous studies that the purine receptor agonist ATP causes a reduction of barrier permeability in endothelial monolayers of various origins [13]. The analysis of intracellular signaling revealed that ATP induces a dephosphorylation of MLC in endothelial cells, likely due to a Ca2+-independent activation of MLC phosphatase.

ATP activates also, but weakly, MLC kinase by a Ca2+-dependent mechanism, yet its effect on net MLC dephosphorylation prevails. MLC represent the regulatory units of the endothelial contractile apparatus. Their phosphorylation has been suggested to initiate force development in endothelial cells [2,21,24], in analogy to the contractile machinery in smooth muscle cells. In the present study, direct measurements of mechanical tension developed within endothelial monolayers were performed. We could show for the first time that ATP causes a mechanical relaxation in endothelial monolayers. Reduction of force induced by ATP was found closely related in time and magnitude to the changes in MLC phosphorylation. In the case of thrombin, the transient increase in
in MLC phosphorylation as being due to phosphatase activation, by a pharmacological approach in intact cells. In order to act on MLC, phosphatases must form a complex with myosin, through a myosin binding subunit (MBS). In the present study we analyzed, therefore, binding of protein phosphatases to myosin. We found that phosphatase 1 (PP1) but not 2A binds to myosin in presence of ATP. Thrombin alone reduces basal PP1 binding. Co-presence of ATP plus thrombin exhibited again an increased PP1 binding. These findings indicate directly PP1 activation at the site of myosin in presence of ATP. The negative result on PP2A are in accordance with other studies, which could not show a role of PP2A in regulation of the endothelial contractile machinery [6]. The inducible form of protein phosphatase (PP2B) is not involved in the early response to thrombin [8,25]. After induction PP2B exerts a delayed influence, which was not here analyzed.

The RhoA/Rho-kinase pathway represents a modulator of endothelial phosphatase activity. Thrombin is known to activate this pathway in endothelial cells, confirmed in the present study. The RhoA/Rho-kinase pathway acts on thrombin (0.2 U/ml), xestospongin C (Xe, 3 μM), Y-27632 (Y, 10 μM) MLC phosphatase in an inhibitory manner. The exact mechanism is not clear. In smooth muscle cells, it has been shown that Rho-kinase can phosphorylate MBS of the phosphatase holoenzyme complex and that this inhibits MLC phosphorylation activity. Rho-kinase may also directly phosphorylate MLC and, thereby, change the state of phosphorylation as well. MLC phosphorylation [26]. When ATP is co-present with thrombin, thrombin effects on force and MLC phosphorylation are greatly reduced. There is, however, no absolute match between both parameters, i.e. MLC phosphorylation is reduced more than force. This difference may be due to factors other than MLC activation contributing to development of mechanical tension in presence of thrombin, i.e. a reorganization of the actin cytoskeleton [10].

In a previous study [14], we characterized the reduction of isometric force goes along with an increase in MLC phosphorylation as well. When ATP is co-present with thrombin, thrombin effects on force and MLC phosphorylation are greatly reduced. There is, however, no absolute match between both parameters, i.e. MLC phosphorylation is reduced more than force. This difference may be due to factors other than MLC activation contributing to development of mechanical tension in presence of thrombin, i.e. a reorganization of the actin cytoskeleton [10].

Fig. 9. Endothelial MLC phosphorylation after 5 min exposures to thrombin (0.2 U/ml), xestospongin C (Xe, 3 μM), Y-27632 (Y, 10 μM) or combinations of these agents. Incubations with Xe and Y were started 20 min before other additions. Data are means±S.D. of n=4 separate experiments with independent cell preparations. * P<0.05 vs. control. †, ‡ P<0.05.

Fig. 10. Translocation of protein phosphatase 1 (PP1) to myosin after 5 min exposure of endothelial cells to ATP (10 μM), thrombin (Thr, 0.2 U/ml) or both. Western blots of myosin-enriched and myosin-depleted cell fraction with use of PP1 antibody (top) or MLC antibody (below). Similar blots were obtained in three other experiments with independent cell preparations.
extracellular ATP are, e.g. autonomous nerve endings [27], platelets or endothelial cells themselves [28,29]. Endothelial cells release ATP in response to shear stress, mechanical stretch, osmotic swelling or hypoxia [30]. Platelets release ATP in the process of aggregation. In the vicinity of aggregating platelets ATP concentrations may reach 50 μM [28–30], i.e. values that are higher than the ATP concentrations here applied. High levels of thrombin are also present at the site of thrombus formation. At this site therefore thrombin and ATP are present simultaneously. Results of the present study indicate that they act antagonistically at the endothelial lining of the affected vessel, since ATP inhibits the development of thrombin-induced hyperpermeability.

The results of this study are of interest not only because of the aforementioned local antagonism. Thrombin-induced endothelial barrier failure is a major contributor to vascular failure in systemic status of inflammation and disseminated coagulation. The identified antagonistic principle may therefore be exploited as an adjunct to anti-inflammatory therapy. ATP itself may not be the antidote of choice since it is rapidly degraded and has also many other effects.

As shown by others, thrombin-induced hyperpermeability can also be antagonized by manoeuvres increasing cellular cAMP-levels [31]. We and others have shown that ATP causes an increase in cAMP in porcine aortic endothelial cells, but in this study suppression of the cAMP rise did not alter the ATP-induced reduction of permeability [13]. Therefore it is most likely to assume that ATP can antagonize the thrombin effect on permeability by an cAMP-independent mechanism.

The ATP activated pathway of MLC phosphatase activation represents an important mechanism for a contractile inactivation of endothelial cells, but further studies to confirm this mechanism are necessary. In the future, other agents may be identified, which use this signaling mechanism for therapeutic approaches to the capillary leakage syndrome.

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