Rho-kinase-dependent F-actin rearrangement is involved in the inhibition of PI3-kinase/Akt during ischemia–reperfusion-induced endothelial cell apoptosis

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Abstract Activation of cytoskeleton regulator Rho-kinase during ischemia–reperfusion (I/R) plays a major role in I/R injury and apoptosis. Since Rho-kinase is a negative regulator of the pro-survival phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway, we hypothesized that inhibition of Rho-kinase can prevent I/R-induced endothelial cell apoptosis by maintaining PI3-kinase/Akt activity and that protective effects of Rho-kinase inhibition are facilitated by prevention of F-actin rearrangement. Human umbilical vein endothelial cells were subjected to 1 h of simulated ischemia and 1 or 24 h of simulated reperfusion after treatment with Rho-kinase inhibitor Y-27632, PI3-kinase inhibitor wortmannin, F-actin depolymerizers cytochalasinD and latrunculinA and F-actin stabilizer jasplakinolide. Intracellular ATP levels decreased following I/R. Y-27632 treatment reduced I/R-induced apoptosis by 31% (P < 0.01) and maintained Akt activity. Both effects were blocked by co-treatment with wortmannin. Y-27632 treatment prevented the formation of F-actin bundles during I/R. Similar results were observed with cytochalasinD treatment. In contrast, latrunculinA and jasplakinolide treatment did not prevent the formation of F-actin bundles during I/R and had no effect on I/R-induced apoptosis. Apoptosis and Akt activity were inversely correlated (R² = 0.68, P < 0.05).

In conclusion, prevention of F-actin rearrangement by Rho-kinase inhibition or by cytochalasinD treatment attenuated I/R-induced endothelial cell apoptosis by maintaining PI3-kinase and Akt activity.

Keywords HUVEC · Cytoskeleton · Y-27632 · CytochalasinD · LatrunculinA · Jasplakinolide

Introduction

Experimental ischemia and reperfusion (I/R) induces endothelial cell apoptosis in several organs [1, 2]. We and others have previously shown that activation of RhoA and downstream effector Rho-kinase during experimental I/R plays a major role in I/R injury and associated apoptosis [3–7]. Indeed, Rho-kinase inhibition has beneficial effects on I/R injury in several organs [4, 6–12]. Endothelial cells may be essential for mediating the beneficial effects of Rho-kinase inhibition on I/R injury, because, for instance inhibition of endothelial nitric oxide synthase blocked the beneficial effects of Rho-kinase inhibition on rat myocardial infarct size [12].

Rho-kinase activation during the execution of apoptosis results in increased myosin activity, bundling of F-actin by activated myosins, actin–myosin contractile force generation, cell contraction and ultimately membrane blebbing [13–15]. Recent in vivo and in vitro studies suggest that Rho-kinase and the effects of Rho-kinase on the actin–myosin cytoskeleton may also be involved in the initiation of apoptosis [4, 12, 16–20]. Rho-kinase is a negative regulator of the pro-survival phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway and it upregulates mitochondrial death pathway protein Bax [4, 12, 16, 19]. The effects of
Rho-kinase on the cytoskeleton may therefore be important in linking Rho-kinase activation and PI3-kinase/Akt inactivation.

We hypothesized that (1) inhibition of Rho-kinase can prevent I/R-induced endothelial cell apoptosis by maintaining PI3-kinase and Akt activity and (2) prevention of F-actin rearrangement facilitates the protective effects of Rho-kinase inhibition (Fig. 1). To test this hypothesis, we assessed whether specific inhibition of Rho-kinase with Y-27632 [21] attenuates apoptosis via phosphorylation of Akt in an in vitro model of simulated I/R. The involvement of the F-actin cytoskeleton was explored by the use of F-actin depolymerizers cytochalasinD and latrunculinA and F-actin stabilizer jasplakinolide [22].

Materials and methods

Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated, cultured and characterized as previously described [23]. Briefly, HUVECs were cultured on gelatin-coated wells/glass cover slips until confluent at 37°C under 5% CO2/95% air atmosphere. Culture medium was renewed every other day (medium 199 supplemented with 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 μg/ml crude endothelial cell growth factor, 2 mM l-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin).

ATP measurement

We measured ATP after ischemia and I/R (1 h of reperfusion) in order to confirm ischemic substrate deprivation. Cells were grown in a gelatin-coated 12-well chamber and treated according to the simulated I/R protocol. ATP was measured using the ENLITEN ATP Assay System Bioluminiscence Detection Kit for ATP (Promega Corporation, Madison, Wisconsin, USA). Proteins in lysate were
measured with BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) in order to calculate nmol ATP/μg protein.

Quantification of apoptosis

The cells were grown in a 24-well chamber on gelatin-coated glass cover slips and treated according to the simulated I/R protocol (24 h of reperfusion). Cells were fixed in 4% formaldehyde and permeabilized with 0.2% triton X-100 (Sigma-Aldrich). The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield® Mounting medium (Vector Laboratories, Inc., Burlingame, California, USA). Apoptosis on each glass cover slip was quantified using a DeadEnd™ Fluorometric TdT-mediated dUTP Nick End Labeling (TUNEL) System (Promega Corporation) with fluorescence microscopy using a Marianas™ digital imaging microscope and Slidebook 4.2 software (Intelligent Imaging Innovations, Inc., Denver, Colorado, USA). The number of apoptotic nuclei (TUNEL-positive) and the total number of nuclei (DAPI-positive) were counted in three non-overlapping microscope fields/glass cover slip using a 10× air lens (Carl Zeiss B.V., Sliedrecht, The Netherlands) and averaged. The number of apoptotic nuclei was expressed as percentage of the total number of nuclei.

F-actin cytoskeleton staining

The cells were grown in a 24-well chamber on gelatin-coated glass cover slips and treated according to the simulated I/R protocol (1 h of reperfusion). Cells were fixed in 4% formaldehyde and permeabilized with 0.2% triton X-100. F-actin was stained with rhodamin-phalloidin (1:100) (Molecular Probes, Inc., Eugene, Oregon, USA) and the nucleus with DAPI in Vectashield® Mounting medium. Cells were visualized with fluorescence microscopy and Slidebook software.

Analysis of Akt phosphorylation

Cells were grown in a gelatin coated 6-well chamber, treated according to the simulated I/R protocol (1 h of reperfusion) and lysated with lysis buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 90 mM KCl, 2 mM EDTA/NaOH pH 8.0, igepal (1:200), triton X-100 (1:200), 1 mM Na3VO4, 10 mM NaF, protease inhibitors (1:100), phosphatase inhibitors (1:100)). Protein concentration was measured (BCA method) and equal amounts of protein from each sample were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California, USA). Membranes were incubated with polyclonal antibodies against pAkt (serine-473, rabbit, 1:1,000) and total Akt (tAkt, rabbit, 1:1,000) (Cell Signaling Technology, Inc., Danvers, Massachusetts, USA). tAkt was used as loading control. Goat anti-rabbit immunoglobulins horseradish peroxidase (HRP) from Dako Cytomation (Glostrup, Denmark) was used for the detection of the primary antibodies at 1:1,000. Detection of the HRP reaction was performed with ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont Buckinghamshire, UK). Imaging and analysis were performed with LAS-3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan) and AIDA Image Analyzer (Raytest GmbH, Straubenhardt, Germany). Data were expressed as pAkt/tAkt as percentage of control.

Statistical analysis

A one-way analysis of variance (ANOVA) was conducted to explore the effects of I/R, Y-27632, cytochalasinD or wortmannin treatment, and their interaction on apoptosis. The t-test was used to further analyse the differences between the groups when ANOVA indicated statistical significance. We adjusted for multiple testing with help of the Bonferroni Holm method. The paired t-test was used for paired ATP data and logarithmic transformed absolute pAkt/tAkt data. Absolute pAkt/tAkt data were logarithmic transformed to obtain a Gaussian distribution. We adjusted for multiple testing with help of the Bonferroni Holm method. The Pearson coefficient of determination (R2) was used to express the relation between the percentage of apoptotic nuclei and the logarithmic transformed absolute pAkt/tAkt data. Differences were considered significant at the P < 0.05 level. Data are presented as means ± standard error of the mean (SEM).

Results

ATP levels and apoptosis following simulated ischemia and I/R

In order to confirm ischemic substrate deprivation in simulated I/R, we measured intracellular ATP levels. Ischemia was simulated by covering the monolayer of cells with nitrogen bubbled mineral oil. Reperfusion was simulated by replacement of culture medium (Fig. 2). Intracellular ATP levels decreased following simulated ischemia compared to the levels in cells treated with medium supplemented with 10% mineral oil (control oil) (0.15 ± 0.04 nmol ATP/μg protein versus 0.22 ± 0.04 nmol ATP/μg protein, P < 0.05, n = 7). Intracellular ATP decreased even further during the
reperfusion phase (0.10 ± 0.04 nmol ATP/µg protein, \( P < 0.05 \) versus control oil, \( n = 7 \)). ATP levels of control oil cells were comparable to those of control cells (0.20 ± 0.04 nmol ATP/µg protein, \( n = 7 \)). Simulated I/R induced a significant percentage of apoptotic nuclei (ANOVA for the effect of I/R on apoptosis, \( P < 0.0001 \)), while hardly any apoptosis was observed in control and control oil cells (Fig. 3).

The effect of Rho-kinase inhibition on apoptosis

To investigate the role of Rho-kinase during I/R-induced apoptosis, HUVECs were treated with inhibitor Y-27632 during simulated I/R (Fig. 3). Y-27632 had no effect on basal apoptosis, but reduced the degree of apoptosis during I/R (ANOVA for the effects of Y-27632 and the interaction between I/R and Y-27632 on apoptosis, both \( P < 0.01 \)). To determine whether the protective effects of Rho-kinase inhibition were mediated by PI3-kinase, cells were co-treated with PI3-kinase inhibitor wortmannin. Wortmannin had no effect on basal apoptosis, but blocked the protective effect of Rho-kinase inhibition during I/R (ANOVA for the effect of wortmannin on apoptosis, \( P < 0.05 \)).

Akt activity in Y-27632-mediated protection against apoptosis

To investigate the effect of Y-27632 treatment during I/R on Akt activity, Akt phosphorylation was analyzed by western blotting (Fig. 4). Simulated I/R reduced Akt activity compared to non-treated or Y-27632-treated control cells. Y-27632 treatment maintained Akt activity in I/R cells. To determine whether this effect was mediated by PI3-kinase, cells were co-treated with wortmannin. Wortmannin blocked the Y-27632-induced maintenance of Akt activity (ANOVA for the effect of wortmannin on apoptosis, \( P < 0.05 \)).

The actin cytoskeleton in Y-27632-mediated protection against apoptosis

To investigate whether Y-27632-mediated protection against apoptosis paralleled the absence of Rho-kinase-mediated F-actin rearrangement, the F-actin cytoskeleton was visualized with rhodamin-phalloidin in non-treated or Y-27632-treated control cells, simulated ischemia and simulated I/R cells (\( n = 9 \), representative pictures are shown in Fig. 5). Y-27632-treated control cells showed a slight decrease in peripheral F-actin bundles compared non-treated control cells, indicating that in vitro inhibition of Rho-kinase was able to reduce F-actin bundle formation. After simulated ischemia in non-treated cells, some F-actin bundles appeared in the cytoplasm. Y-27632-treated ischemic cells showed only F-actin clumps. After simulated
reperfusion, even more F-actin bundles appeared in the periphery and the cytoplasm of the non-treated cells. In contrast, Y-27632-treated I/R cells, showed no F-actin bundles.

To further investigate the role of the F-actin cytoskeleton in I/R-induced apoptosis, we used F-actin depolymerizers cytochalasinD and latrunculinA and F-actin stabilizer jasplakinolide (Fig. 5). CytochalasinD-treated control cells

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**Fig. 5** Y-27632 and cytochalasinD prevent F-actin bundle formation during I/R. Shown is fluorescent imaging of rhodamin-phalloidin stained F-actin structures and DAPI stained nuclei in non-treated, Y-27632-, cytochalasinD-, latrunculinA- and jasplakinolide-treated control, simulated ischemia and simulated I/R cells. The arrow and * indicate F-actin bundles and F-actin clumps, respectively. **Control cells:** In non-treated cells, most F-actin bundles were seen in the periphery of the cell. Y-27632-treated cells showed a slight decrease in peripheral F-actin bundles. CytochalasinD-treated cells showed only F-actin clumps. Cells treated with latrunculinA or jasplakinolide showed a slight decrease and increase in F-actin bundles, respectively. **Ischemic cells:** Non-treated cells showed F-actin bundles throughout the whole cell. Cells treated with Y-27632, cytochalasinD or latrunculinA showed only F-actin clumps. Jasplakinolide-treated cells showed F-actin bundles. **I/R cells:** Non-treated cells showed F-actin bundles throughout the whole cell. In Y-27632-treated cells, no F-actin bundles were seen. CytochalasinD-treated cells showed also no F-actin bundles, only F-actin clumps were visible. LatrunculinA-treated cells showed some F-actin bundles. Jasplakinolide-treated cells showed as many F-actin bundles as non-treated I/R cells.
showed no F-actin bundles. LatrunculinA-treated control cells showed a slight decrease and jasplakinolide-treated control cells showed a slight increase in F-actin bundles compared to non-treated control cells. CytochalasinD- and latrunculinA-treated ischemic cells showed no F-actin bundles, only F-actin clumps were visible. Jasplakinolide-treated ischemic cells showed F-actin bundles throughout the whole cell. In cytochalasinD-treated I/R cells, no F-actin bundles were visible, only F-actin clumps could be seen. In contrast, latrunculinA-treated I/R cells still showed some F-actin bundles and jasplakinolide-treated I/R cells showed as many F-actin bundles as non-treated I/R cells.

Since cytochalasinD treatment prevented F-actin rearrangement similarly to Y-27632 treatment, we investigated whether cytochalasinD treatment could also prevent apoptosis during simulated I/R (Fig. 6). CytochalasinD had no effect on basal apoptosis, but reduced the degree of apoptosis during I/R (ANOVA for the effects of cytochalasinD and the interaction between I/R and cytochalasinD on apoptosis, both $P < 0.05$). Wortmannin blocked the protective effect of cytochalasinD in I/R (ANOVA for the effect of wortmannin on apoptosis, $P < 0.0001$). In contrast to the protective effect of Y-27632 and cytochalasinD on apoptosis, latrunculinA and jasplakinolide did not affect I/R-induced apoptosis, also not in co-treatment with wortmannin (data not shown). LatrunculinA and jasplakinolide had no effect on basal apoptosis (data not shown).

Furthermore, cytochalasinD maintained Akt activity during simulated I/R (Fig. 7). Co-treatment with wortmannin completely blocked the cytochalasinD-induced maintenance of Akt activity. Akt activity in cytochalasinD-treated control cells was comparable to the activity in non-treated control cells.

Interestingly, the percentage of apoptotic nuclei inversely correlated to Akt activity ($R^2 = 0.68$, $P < 0.05$). Mean percentage of apoptotic nuclei and mean logarithmic transformed absolute pAkt/Akt levels in non-treated (C), Y-27632 (C Y)- and cytochalasinD (C CytoD)-treated control cells and in non-treated (I/R), Y-27632 (I/R Y)-, cytochalasinD (I/R CytoD)- and wortmannin (I/R YW and I/R CytoDW)-treated I/R cells are shown as *.

Discussion

This study shows that (1) inhibition of Rho-kinase attenuates I/R-induced endothelial cell apoptosis by maintaining PI3-kinase and Akt activity and (2) the effects of Rho-kinase...
inhibition on PI3-kinase/Akt and apoptosis are facilitated by prevention of F-actin rearrangement.

Our data point to a novel mechanistic link between activation of Rho-kinase during I/R and negative regulation of PI3-kinase/Akt, namely the effect of Rho-kinase on F-actin rearrangement (Fig. 1). Prevention of F-actin rearrangement by Rho-kinase inhibition or by treatment with actin depolymerizer cytochalasinD, independent of Rho-kinase, both attenuated I/R-induced endothelial cell apoptosis by maintaining PI3-kinase and Akt activity. Indeed, it has been reported previously that cytochalasins inhibited in vivo and in vitro apoptosis induced by a variety of stimuli, including I/R [5, 13, 26]. This study is therefore an extension of and a link between reports on Rho-kinase as negative regulator of the PI3-kinase/Akt pathway and reports on the involvement of Rho-kinase-dependent cytoskeletal rearrangement in the initiation of apoptosis [4, 12, 16–20].

Actin depolymerizer latrunculinA did not affect I/R-induced endothelial cell apoptosis. This may be explained by the nature and degree of F-actin rearrangement during I/R, since F-actin bundles were completely absent in Y-27632- and cytochalasinD-treated I/R cells, while latrunculinA-treated cells still showed some bundles. Indeed, cytochalasinD and latrunculinA depolymerize F-actin by different mechanisms and induce different patterns in vitro [22, 27]. CytochalasinD caps the barbed end of actin filaments, severs actin filaments, sequesters actin monomers or dimers, promotes nucleation and stimulates the ATPase activity of G-actin. In vitro in hamster fibroblasts, it caused a complete collapse of the stress fibers at concentrations comparable to ours [22, 27]. LatrunculinA on the other hand binds to monomeric G-actin to form a non-polymerizable complex and caused concentration-dependent shortening and thickening of stress fibers in the fibroblasts at concentrations comparable to ours [22, 27]. Jasplakinolide, which promotes actin polymerization and stabilizes actin filaments by binding to F-actin [22], induced as many F-actin bundles in I/R as non-treated I/R cells and consequently did not affect I/R-induced endothelial apoptosis. However, in vivo it has been demonstrated that jasplakinolide prevented I/R-induced apoptosis in the rat kidney, while latrunculinB increased I/R-induced apoptosis in rat kidney [28, 29]. Furthermore, we observed hardly any apoptosis in our Y-27632-, cytochalasinD-, latrunculinA- and jasplakinolide-treated control cells, while some reports describe the induction of apoptosis in control cells by latrunculinA or jasplakinolide at much higher concentrations than ours [30, 31], by cytochalasinD in airway epithelial cells, but not in canine kidney epithelial cells [32] or by Y-27632 in airway epithelial cells [32]. Therefore, these discrepancies may be explained by the use of different concentrations, cell types or the use of an in vivo/in vitro model.

Akt activation after 1 h of reperfusion inversely correlated to the percentage of apoptosis after 24 h of reperfusion. Therefore, early reduced Akt activity may have a predictive value for the development of late I/R-induced apoptosis. The preventive effect of Y-27632 and cytochalasinD treatment probably takes place early in the process of apoptosis, because the Y-27632-induced increase in Akt activity is rapid and transient with a maximum after 30 min [12, 16]. Indeed, we could neither detect increased Akt activity in our Y-27632-treated control cells after 3 h nor in our Y-27632-treated I/R cells after 24 h of reperfusion (pilot study, data not shown), possibly because this was too late to detect the peak in Akt activity. I/R-induced apoptosis was maximal as late as after 24–48 h of reperfusion [25]. Therefore, apoptosis was quantified after 24 h of reperfusion. Indeed, we did not see any TUNEL-positive nuclei after 1 h of reperfusion (pilot study, data not shown). Our percentage of apoptotic nuclei was comparable to the percentage reported by the developers of the simulated I/R model [25]. However, less endothelial cell apoptosis was observed in a model of hypoxia/reoxygenation [33], possibly because this induces less severe metabolic deprivation. In addition, PI3-kinase inhibition did not decrease basal Akt activity nor increased basal apoptosis, while it blocked the effects of Rho-kinase inhibition on I/R-induced apoptosis and Akt activity. This suggests that the basal situation is independent of PI3-kinase, while the effects Rho-kinase inhibition during I/R are dependent on PI3-kinase.

Although it is unclear by which mechanism F-actin rearrangement facilitates the effects of Rho-kinase on PI3-kinase/Akt and apoptosis, several mechanisms can be proposed. First, RhoA and Rho-kinase may regulate pAkt levels by regulating phosphatase and tensin homologue (PTEN) activity. PTEN is in inactive state in the cytosol and translocates to the membrane upon RhoA or Rho-kinase-induced activation, where it can inhibit the phosphorylation of Akt via PI3-kinase [34]. Disruption of the F-actin bundles by Rho-kinase inhibition or cytochalasinD treatment or the direct effects of Rho-kinase inhibition on PTEN may prevent the translocation and activation of PTEN, thereby preventing its inhibitory effect on Akt phosphorylation. Furthermore, there may be some PI3-kinase independent mechanisms involved in the Y-27632-induced protection against apoptosis. It is reported that F-actin polymerization and actin–myosin contractility are necessary for membrane blebbing and chromatin condensation during execution of apoptosis induced by tumor necrosis factor-α or overexpression of Rho-kinase [13, 26]. Moreover, the actin–myosin cytoskeleton may be involved in facilitating the cellular localization of specific components of the apoptotic machinery and be required for caspase-3 activation [17, 18].
Our study carries some limitations. First, we cannot conclude on the mechanisms by which F-actin rearrangement facilitates the effects of Rho-kinase on PI3-kinase/Akt and apoptosis. Second, Rho-kinase inhibition and F-actin depolymerization could establish only a partial protection against apoptosis, in agreement with the literature [4, 5, 8, 12], possibly because other mechanisms are also involved and the I/R-induced injury is too extensive. Third, we may have underestimated the number of apoptotic cells, because endothelial cells that detached from the basement membrane during simulated I/R and did not reattach later were not counted in the quantification of apoptosis. Indeed, Prahalad et al. [35] describe this reversible cell detachment during ATP depletion of Madin-Darby canine kidney cells.

Conclusion

Prevention of F-actin rearrangement or F-actin bundle formation by Rho-kinase inhibition or by treatment with actin depolymerizator cytochalasinD, independent of Rho-kinase, attenuated I/R-induced endothelial cell apoptosis by maintaining PI3-kinase and Akt activity. The present finding suggests that the changes in the F-actin cytoskeleton play a pivotal role in the negative regulation of PI3-kinase and Akt by Rho-kinase. Further studies are required to conclude on the mechanisms by which F-actin rearrangement facilitates the effects of Rho-kinase on PI3-kinase/Akt and apoptosis.

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