INHIBITION OF RHO-ROCK SIGNALING INDUCES APOPTOTIC AND NON-APOPTOTIC PS EXPOSURE IN CARDIOMYOCYTES VIA INHIBITION OF FLIPPASE


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Inhibition of Rho–ROCK signaling induces apoptotic and non-apoptotic PS exposure in cardiomyocytes via inhibition of flippanse

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Subsequent to myocardiud infarction, cardiomyocytes within the infarcted areas and border zones expose phosphatidylserine (PS) in the outer plasma membrane leaflet (flip-PS). We showed earlier that in addition to apoptosis, this flip-PS can be reversible in cardiomyocytes. We now investigated a possible role for Rho and downstream effector Rho-associated kinase (ROCK) in the process of (reversible) PS exposure and apoptosis in cardiomyocytes. In rat cardiomyocytes (HT152 cells) and isolated adult ventricular rat cardiomyocytes Cardiomyocytes Ccl5-stimulated differentiated Toad cells, a Rho GTPase family inhibitor, C3 transferase (C3), a Rho (AC), Rho kinase inhibitor, and the ROCK-activating Y262 and H1152 were used to inhibit Rho-ROCK signaling. PS exposure was assessed via flow cytometry and fluorescent digital imaging microscopy using annexin V. Akt expression and phosphorylation were analyzed by Western blot, and Akt activity was inhibited by wortmannin. Expression was determined as a measure of apoptosis, and flippanse activity was assessed via flow cytometry using NBD-labeled PS. Toad, C3, Y262, and H1152 all significantly increased PS exposure. Toad, Y262, and H1152 all significantly inhibited phosphorylation of the anti-apoptotic protein Akt and Akt inhibition by wortmannin led to increased PS exposure. However, only Toad and C3, but not ROCK- or Akt inhibition led to caspase 3 activation and thus apoptosis. Notably, pro-caspase inhibitor zVAD-fmk only partially inhibited Toad-induced PS exposure indicating the existence of apoptotic and non-apoptotic PS exposure. The induced PS exposure coincided with decreased flippanse activity as measured with NBD-labeled PS flip-PS. In this study, we show a regulatory role for a novel signaling route, Rho-ROCK-flippanse signaling, in maintaining asymmetrical membrane phospholipid distribution in cardiomyocytes.

1. Introduction

It is well known that in normal cells, the phospholipids are normally asymmetrically distributed across the inner and outer leaflet [2, 3]. The outer leaflet predominantly contains hydrophobic phospholipids such as phosphatidylycerine (PC), whereas anionic phospholipids such as phosphatidylserine (PS) and, to a lesser extent, phosphatidylethanolamine (PE) are kept within the inner leaflet. Loss of this phospholipid asymmetry occurs in some activated cells [4] and under pathological conditions. This process, also called membrane flip-flop, involves the exposure of PS and PE in the outer plasma membrane leaflet. PS exposure at the cell surface was reported to occur in both apoptotic cells [5, 6] as well as necrotic cells [7], presumably to potentiate recognition by phagocytes [8]. However, we and others have observed that plasma membrane PS exposure in cardiomyocytes is not always a determinant of death but can in fact be reversible [9, 15].

After acute myocardial infarction (AMI) cardiomyocytes within the infarcted areas, as well as morphologically normal cardiomyocytes in the border zones of infarction, lose their membrane phospholipid asymmetry [16]. We have observed in vivo as well as in vitro that the...
inflammatory mediator type IIA secretory phospholipase A2 (sPLA2-IA) binds to and induces death in these flip-flopped cardiac myocytes, but not in cardiomyocytes that maintain their plasma membrane phospholipid asymmetry [17,18]. Therefore, PS exposure is pro-inflammatory and targets the inflammatory response to cells that do so, including cells that reversibly expose PS. Through PS exposure, these reversibly damaged cardiomyocytes fall victim to the inflammatory response leading to additional damage in the reperfused heart after AMI. Thus, insight into systems that regulate transblayer phospholipid asymmetry may offer clues for preventing propagation of inflammation in the heart after AMI.

Three classes of membrane proteins, i.e. two ATP-dependent (flipases and floppases) and an ATP-independent (scramblases), have been found to regulate transblayer phospholipid asymmetry [19]. The flipase prefers PS over other lipids [20]. In addition, there is evidence supporting a role for phospholipid binding proteins such as membrane-associated cytoskeleton elements [21] or annexin V [22,23] in maintaining phospholipid asymmetry.

However, the majority of studies maintaining asymmetrical phospholipid distribution in plasma membranes have been done in erythrocytes and platelets and little is known about the regulation of transblayer phospholipid asymmetry in cardiomyocytes. Recently Rho member RhoA was shown to participate in PS exposure in megakaryocytes [24], RhoA, Rhb and RhoC (RhoA/BC) are three closely related Rho proteins that together with Rac and CDC42 are part of the family of Rho-GTPases [25]. Here we have investigated a putative regulatory role for Rho, its downstream effector Rho-associated kinase (ROCK), the anti-apoptotic kinase Akt and flipase in regulating transblayer phospholipid asymmetry in the rat neonatal cardiomyoblast line H9c2 and in isolated adult rat ventricular cardiomyocytes.

2. Methods

2.1. Cell culture

Neonatal rat cardiomyoblasts (H9c2; 2-3 cells; ATCC, Manassas, Virginia, USA) were cultured in culture medium, that is Dulbecco’s Modified Eagles Medium (DMEM; BioWhittaker, Verviers, Belgium) containing 10% (v/v) heat-inactivated Fetal Calf Serum (FCS; BioWhittaker, Verviers, Belgium), 100 IU/ml penicillin (Yamanouchi Europe BV, Leiden, The Netherlands), 100 g/ml streptomycin (Radiopharmaceutica-Hio-pharma, Falomonte, Italy) and 2 mmol/L L-glutamine (Gibco, Paisley, England), under a 5% CO2 atmosphere at 37°C. For experiments, cells were used at 60 to 80% confluence.

Tox-2 from Claudiunum difficile (Tox-2; List Biological Laboratories Inc, Campbell, CA, USA), cell permeable exoenzyme C3 transfinance from Clostridium botulinum (CS; Cytokeleton Inc., Denver, CO, USA), Y27632 (Calbiochem, Nottingham, UK), wortmannin (LC Laboratories, Woburn, MA, USA) and zVAD-fmk (zVAD; Alexis Biochemicals, San Diego, CA, USA) were all incubated for 24 h under a 5% CO2 atmosphere at 37°C. H1152 (Calbiochem) was incubated for 4 h under a 5% CO2 atmosphere at 37°C. Throughout the study final concentrations of 1 mg/ml for Tox-2, 1 mg/ml for CS, 10 μmol/L for Y27632, 1 μmol/L for H1152, 100 μmol/L for wortmannin and 25 μmol/L for zVAD were used.

2.2. PS exposure analysis by flow cytometry

FITC-labeled human recombinant (hr) annexin V and RPE-labeled hr-annexin V (Bender Med Systems, Vienna, Austria) were used to assess PS exposure of the cell membrane. Propidium iodide (PI; Bender Med Systems) was used to assess membrane permeability, and thus cell death. A CaspaseGlo caspase 3/7 in situ kit (Chemicon Int. Inc. Temecula, CA, USA), a green fluorescent probe that binds active caspase 3 (FAM-DEVD-fluor), was used to stain for active caspase 3.

H9c2 cells were incubated in culture medium. All cells (attached- as well as detached cells) were then collected and stained with either annexin V-PTC (1:40) or annexin V-PE (1:40) for 30 min at 37°C. Cells were stained for active caspase 3 according to the manufacturer’s protocol. Briefly, cells were stained with FAM-DEVD-fluk for 1 h at 37°C in serum-free culture medium and subsequently washed twice with serum-free culture medium (hr supplied wash buffer was not used since it interfered with the annexin V-PE staining). Fix (1:40) was added only few minutes prior to measurement. The cells were then analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA).

2.3. Total Akt/phosphorylated Akt protein expression

The expression of total Akt and phosphorylated Akt was quantified using Western blot. H9c2 cells were lysed in a buffer containing 250 mmol/L NaCl, 0.1% Nonidet P-40, 50 mmol/L HEPES pH 7.6, 5 mmol/L EDTA and 0.5 mmol/L DTT in H2O, supplemented with protease inhibitor cocktail (1:40; Sigma, St. Louis, MO, USA). Protein concentrations in the samples were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). After adding SDS sample loading buffer containing β-mercaptoethanol as a reducing agent, the samples were stirred, and heated for 10 min at 95°C. 36 μg protein of each sample was subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted using either a rabbit polyclonal Ab against Akt (1:1000, overnight 4°C; Cell Signaling, Danvers, MA, USA) or a rabbit polyclonal Ab against phospho-Akt (ser473) (1:1000, overnight 4°C; Cell Signaling) followed by horseradish peroxidase-conjugated swine-anti-rabbit immunoglobulins (1:1000, 60 min; DakoCytomation, Glostrup, Denmark). The blots were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences AB; Uppsal, Sweden). Staining was quantified with a charge-coupled device camera (Fuji Image Imaging Systems; Duoscan Micron, Germany) in combination with AIDA image analysis software (virotyping software; Stäuberhardt, Germany).

2.4. Caspase 3 measurements

Caspase 3 activity was determined using a fluorometric homogeneous caspase assay (Roche, Mannheim, Germany), according to the manufacturer’s instructions. Cells were lysed and incubated with DEVD-royhaemine 110 substrate for 1 h at 37°C. Subsequently, the amount of free rhodamine was determined at a microplate luminometer reader (TECAN spectraflor, Hombretshoven, Switzerland). The developed fluorochrome was proportional to the concentration of activated caspase 3 and could be quantified by a calibration curve of diluted free rhodamine [formula: caspase concentration (nmol/L) = (absorption–2273.4) × 1000]. Active caspase 3 concentration levels were indicated as the difference (Δ) in caspase 3 concentration levels relative to control cells.

2.5. ATP measurements

Sample ATP measurements were performed as follows. The described steps were all performed at 4°C. After treatment, the cells (n=6–10) were collected and counted and the cell concentrations were equalized accordingly. After centrifugation (400-g) the cells were incubated in 500 μL 0.4 mol/L Perchloric Acid (Sigma) for 30 min and subsequently centrifuged (1600-g). The supernatants were collected and the pH was restored to 7.4 by adding 25 μL 5 mol/L K2CO3 (Sigma, St. Louis, MO, USA). The samples were frozen (-80°C) until measurement. ATP levels were measured either in a bioluminescence assay, using a sensitive ATP determination kit following the manufacturer’s protocol (Blaufin GmbH & Co KG, Eassel, Germany), or using high pressure liquid chromatography (HPLC), which was also
used to measure GTP levels. For the AIP assay, luminescence was measured using a Tecxan GENios Plus reader (Tecan Benelux, Mecelen, Belgium). For HPLC, the columns were used prepacked Partisphere (partial-5) SAX cartridges (4.6 x 125 mm internal dimension, Whatman International Ltd., Maidstone England). The concentration of the different substances was calculated by comparison with highly purified standards (Sigma).

2.6.5 Exposure analysis in isolated adult ventricular rat cardiomyocytes

PI exposure was determined using annexin V-FITC and PI in adult rat cardiomyocytes. Animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the VU University of Amsterdam. The adult rat cardiomyocytes were isolated as described previously [9] and were seeded onto laminin-coated Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY, USA) and allowed to adhere to the substratum overnight. After treatment, the cells were incubated with annexin V-FITC (1:40) in serum-free DMEM for 30 min under a 5% CO₂ atmosphere at 37 °C. Afterwards, the cells were washed twice with PBS (37 °C) and then incubated with serum-free DMEM, PI was added immediately prior to analysis. The cells were analyzed by use of a 3i Marinus™ digital imaging microscopy workstation.

2.7. Analysis of flipase activity by flow cytometry

For the PS-NBD staining, HK2 cells were harvested and resuspended in serum-free culture medium. For measurement of flipase activity, cells were incubated with NBD-labeled phosphatidylethérine [26]. PS-NBD (1-palmitoyl-2-sn-[7-nitro-2,1,3-benzoxadiazole-4-y])- amino(caproyl)-sn-glycero-3-phosphoehérine) (Avanti Polar Lipids, Alabaster, AL, USA)] in a final concentration of 1 μmol/L at 37 °C for 30 min. The cells were then transferred to ice and 5 mL ice-cold 1% (w/v) BSA/PBS solution was added to extract label present in the outer leaflet of the membrane. To measure total fluorescence, 5 mL ice-cold PBS without BSA was added. After 15 min the cells were pelleted and resuspended in serum-free culture medium and then analyzed by flow cytometry (FACS Calibur). With each experiment, part of the harvested cells were analyzed for PS exposure as described before.

2.8. Statistics

Statistics were performed with the SPSS statistical program (windows version 5.0). To evaluate whether observed differences were significant, one-way ANOVA analysis combined with Bonferroni’s multiple comparison test, Mann-Whitney analysis or independent T-test was used when appropriate. In the text and relevant figures, values are given as means ± SE. A p-value (two-sided) of less than 0.05 was considered to represent a significant difference.

3. Results

3.1. Inhibition of Rho proteins by TocdB and by C3 induces PS exposure in HK2 cells

The effects of Rho GTase family inhibitor TocdB and Rho(A,B,C) inhibitor C3 transface (C3) on PS exposure in HK2 cells were analyzed using flow cytometry. FITC-labeled human recombinant annexin V (annexin V; a marker for membrane flip-flop) and propidium iodide (PI; a marker for cell death) to determine whether the cells were viable (negative for both annexin V and PI), damaged (single-annexin V-positive) or dead (annexin V/PI-positive or single-PI-positive).

TocdB as well as C3 induced a significant increase in the percentage of single-annexin V-positive cells to 33 ± 5% (TocdB) and 31 ± 2% (C3) compared to control cells (5 ± 1% (TocdB) and 9 ± 1% (C3); p<0.0001; Figs. 1(b) and (c). On the flow cytometry dot plot, the PS exposure induced by TocdB and C3 manifested itself mainly as a population of single-annexin V-positive cells (Fig. 1(a)). TocdB and C3 both induced a significant decrease in the percentage of viable cells to 44 ± 6% (for TocdB: p<0.0001) and 41 ± 2% (for C3: p<0.0001) compared to control cells (77 ± 3% and 84 ± 3% respectively), but had no effect on the percentage of PI-positive cells (not shown). Thus, inhibition of the Rho proteins by TocdB or C3 induced PS exposure in HK2 cells. We subsequently investigated whether inhibition of ROCK, a downstream effector of Rho, also has an effect on PS exposure in HK2 cells.
3.2. Inhibition of ROCK by Y27632 and H1152 induces PS exposure in H9c2 cells

The effect of inhibition of ROCK by the selective ROCK inhibitors Y27632 and H1152 on PS exposure in H9c2 cells was analyzed via flow cytometry using annexin V and PI. Similar to TcT8, both Y27632 and H1152 induced a significant increase in the percentage of single-annexin V-positive cells compared to control cells (p < 0.001). Y27632 increased the percentage of single-annexin V-positive cells to 17 ± 3% (Fig. 1(d)) and H1152 increased the percentage of single-annexin V-positive cells to 21 ± 1% (Fig. 1(c)). Both Y27632 and H1152 did not have a significant effect on the percentages of viable or PI-positive cells (not shown). These data suggest that interference with Rho-ROCK activation leads to loss of transmembrane phospholipid asymmetry.

3.3. Inhibition of Rho-ROCK signaling leads to reduced Akt phosphorylation in H9c2 cells

The PS exposure due to inhibition of Rho-ROCK signaling is indicative of cell stress. In endothelial cells, PS exposure and apoptosis were found to be inhibited through Akt activity [27]. The effect of Rho-ROCK inhibition on the expression of Akt and on Akt activation via the expression of phosphorylated Akt (pAkt) was therefore quantified in H9c2 cells using Western blot analysis. Total Akt protein migrated to approximately 60 kDa (Fig. 2(a)). Neither Rho protein inhibition by TcT8 nor ROCK inhibition by either Y27632 or H1152 altered Akt expression significantly compared to control cells (Fig. 2(b)). Maximal activation of Akt is achieved through phosphorylation on serine 473 (ser473) [28]. pAkt(ser473) protein migrated to approximately 60 kDa (Fig. 2(a)). Quantification of pAkt levels revealed that Rho protein inhibition by TcT8 significantly reduced the amount of pAkt to 1 ± 1% (p < 0.001; Fig. 2(c)). Similarly, ROCK inhibition by both Y27632 and H1152 significantly reduced the amount of pAkt to respectively 9 ± 7% (p < 0.001) or 41 ± 20% (p < 0.05) of control cells. No significant difference in the amount of pAkt was found between TcT8, Y27632 or H1152 treated cells. Thus inhibition of Rho-ROCK signaling does not affect Akt expression, but leads to inhibition of Akt activity. To investigate whether this effect on Akt activation is related to the observed PS exposure, H9c2 cells were treated with wortmannin, a PI3-Kinase/Akt pathway inhibitor, and analyzed via flow cytometry using annexin V and PI. Wortmannin...
induced a significant increase in single-annexin V-positive cells from 9 ± 1% (control) to 17 ± 1% (Fig. 2d; p ≤ 0.001). Wortmannin induced a significant decrease in the percentage of viable cells (p ≤ 0.01) but had no effect on the percentage of PI-positive cells (not shown). These results suggest that the PS exposure induced through Rho-ROCK inhibition may involve inactivation of Akt.

3.4. TcdB and C3 but not Y27632 or H1152 induce caspase 3 activation

PS exposure is a hallmark of apoptosis and inhibition of Akt has been shown to lead to apoptosis [27, 29]. To assess whether the observed inhibition of Rho-ROCK signaling-induced PS exposure and Akt inhibition is related to apoptosis we quantified putative caspase 3 activation in H9c2 cells.

TcdB significantly increased the active caspase 3 concentration to 386.8 ± 92 nmol/L above that in control cells (p = 0.01; Fig. 3a)). In cells treated with TcdB in combination with pan-caspase inhibitor zVAD, the active caspase 3 concentration was completely reduced to control level (p = 0.05 compared to TcdB alone). Similarly, C3 significantly increased the active caspase 3 concentration to 1720 ± 87 nmol/L above that in control cells (p = 0.001; Fig. 3b)). However, the active caspase 3 concentration in C3-treated cells was significantly lower than the 3318 ± 164 nmol/L found in TcdB-treated cells (p = 0.001; Fig. 3b)). Wortmannin did not induce caspase 3 activation. In contrast to TcdB and C3, Y27632 nor H1152 did significantly increase the active caspase 3 concentration (Figs. 3b) and c).

Thus, Rho protein inhibition by TcdB or C3, but not ROCK inhibition by Y27632 or H1152, induced apoptosis in H9c2 cells. The PS exposure induced by ROCK inhibition therefore is not related to caspase 3 activity.

3.5. The effect of caspase 3 inhibition on TcdB-induced PS exposure

To assess whether the observed membrane flip-flop, as induced by impaired Rho protein signaling, is solely the result of concurrently induced apoptosis, we quantified the effects of zVAD on TcdB-induced membrane flip-flop in H9c2 cells, using flow cytometry analysis with double staining of FITC-labeled human recombinant annexin V and PI. zVAD significantly reduced the percentage of single-annexin V-positive cells induced by TcdB alone to 36 ± 3% compared with 50 ± 3% of cells treated with TcdB alone (p = 0.05; Fig. 4). Interestingly however, the percentages of single-annexin V-positive cells after treatment with TcdB + zVAD were still significantly higher than 5 ± 1% in control cells (p = 0.001).

In addition, we analyzed H9c2 cells treated with TcdB by flow cytometry, using a double labeling with RPE-labeled human recombinant annexin V (annexin V-PE) and a green fluorescent probe that binds active caspase 3 (FAM-DEVQ-flmk). In line with the caspase 3 activity assay results, TcdB induced a population of cells positive for annexin V-PE and active caspase 3. In addition, TcdB induced a population of cells positive for annexin V-PE but negative for active caspase 3 (not shown).

Thus, a significant part of the TcdB-induced plasma membrane flip-flop is not dependent upon activation of caspase 3.

3.6. The effect of inhibition of Rho-ROCK signaling on cellular ATP content

ATP-dependent systems are involved in maintaining plasma membrane asymmetry and loss of cellular ATP content may result in PS exposure, while ATP is necessary for completing the process of apoptosis [30]. We therefore measured the effects of Rho-ROCK signaling inhibition on cellular ATP content in H9c2 cells.

TcdB almost completely depleted cellular ATP to 4 ± 3% of control cells (p < 0.001; Fig. 5a)). In contrast to TcdB, both ROCK inhibitors Y27632 as well as H1152 did not significantly reduce the cellular ATP content in H9c2 cells. The ATP-depleting effect of TcdB was confirmed via nucleotide analysis using HPLC where TcdB induced a significant decrease in cellular ATP to 191 ± 53 pmol ATP/106 cells compared to the 1790 ± 71 pmol ATP/106 cells in control cells (p = 0.005; Fig. 5b).

The effects of C3 and TcdB on the cellular ATP levels were also analyzed over time in H9c2 cells (Fig. 5c)). ATP levels in TcdB treated

![Fig. 3](image-url) Effects of TcdB, C3, Y27632, H1152 and wortmannin on active caspase 3 concentration in H9c2 cells. (a-d): Concentrations (nmol/L) of active-caspase 3 are depicted as the difference (Δ) in caspase 3 concentrations relative to control cells. (a) TcdB 1 ng/ml, 24 h, zVAD 25 μmol/L, 24 h (4 experiments); p < 0.01; p < 0.05; C3 1 ng/ml, 24 h, TcdB 1 ng/ml, 24 h, wortmannin (wort) 100 μmol/L, 24 h (8 experiments); p < 0.001; p < 0.001; Y27632 10 μmol/L, 24 h (4 experiments); p < 0.001; p < 0.001; H1152 1 μmol/L, 24 h (4 experiments).
cells declined with increasing incubation time (12 h: p<0.02; 24 h: p<0.01, compared to control), whereas in C3-treated cells ATP levels did not significantly differ compared to control cells. After 12 h and 24 h of incubation, ATP levels in TcDB treated cells were also significantly lower than in C3-treated cells (12 h: p<0.02; 24 h: p<0.005).

3.7. The effect of inhibition of Rho-ROCK signaling on PS exposure in isolated adult ventricular rat cardiomyocytes

To extrapolate our findings in H9K2 cells to adult cardiomyocytes, the effect of Rho-ROCK signaling by C3, Y27632 and H1152 was analyzed in isolated adult ventricular rat cardiomyocytes, using fluorescent digital imaging microscopy. To visualize PS exposure FITC-labeled annexin V was used combined with PI for identification of cell death.

Similar as in H9K2 cells, Rho(ABC) inhibition by C3 and ROCK inhibition by Y27632 and H1152 induced a significant increase in PS exposure in adult cardiomyocytes, mainly identified as single-annexin V-positive cells (p<0.001; Fig. 6(a)). The percentage of single-annexin V-positive cells was increased by 39±5% by C3, with 21±4% by Y27632 and with 47±8% by H1152, compared to control cells (Fig. 6(b)). There was no significant difference in PI-positive cells between C3, Y27632 and H1152 and controls.

3.8. The effect of inhibition of Rho-ROCK signaling on flipase activity

Flipase selectively transports PS from the outer to the inner plasma membrane layer and thereby maintains transbilayer phospholipid asymmetry [10,20]. As flipase activity is ATP-dependent and TcDB depletes cellular ATP, whereas the Rho(ABC) inhibitor C3 and...
ROCK inhibitors had no effect on cellular ATP, the effect of ROCK inhibition on flip-flop activity was subsequently analyzed in H9c2 cells, treated with either C3, Y27632 or H1152. These cells were incubated with NBD-labeled PS and then analyzed by flow cytometry. Internalization of PS-NBD results in PS-NBD-positive cells and lipid flip-flop activity. PS-NBD-negative cells therefore are cells in which flip-flop activity is inhibited.

4. Discussion

There is accumulating evidence that plasma membrane alterations such as the exposure of anionic phospholipids, for instance PS, in the outer leaflet (flip-flop) of jeopardized cardiomyocytes target the inflammatory response to these cells in the reperfused myocardium [17,36,31]. We and others however, have shown that this flip-flop can be a reversible phenomenon and may precede commitment to death [9-11,14]. Thus, (reversible) plasma membrane flip-flop may serve as a handle by which inflammatory mediators bind and induce additional damage in the reperfused heart after AMI. We now show evidence that ROCK-Rock signaling is involved in the regulation of transbilayer phospholipid asymmetry both in H9c2 cells and in isolated adult ventricular rat cardiomyocytes, which may involve regulation of flip-flop activity.

In the present study, we namely show that Rho GTPase family inhibitor TcdB, Rho(A,B,C) inhibitor exoenzyme C3 transerase and ROCK inhibitors Y27632 and H1152 induced increased apoptotic and non-apoptotic plasma membrane PS exposure in cardiomyocytes. In addition, TcdB, Y27632 and H1152 also induced a significant decrease in the activity of the anti-apoptotic protein Akt, as shown by decreased phosphorylation on serine 473. Studies have shown before that in various cells, i.e. rat mast cells, human vascular smooth muscle cells, HL-60 cells and rat fibroblasts, Akt phosphorylation and translocation are inhibited by TcdB, perhaps through inhibition of Rac [32-35]. In our study, TcdB also led to ATP depletion, which may contribute to decreased phosphorylation of Akt, whereas in the mentioned studies the effect of TcdB on ATP levels was not investigated. The inhibition of ROCK appears to have different effects on Akt activity in different cells. For instance, ROCK inhibitor fasudil inhibited Akt phosphorylation in bovine retinal microvascular endothelial cells whereas in human endothelial cells fasudil activated Akt [35,36]. In neonatal rat cardiomyocytes transfected with a constitutively active ROCK1 vector Akt phosphorylation was inhibited [37]. Loss of Akt signaling has been shown to lead to apoptosis, also in cardiomyocytes [29]. However, whereas TcdB and C3 induced caspase 3 activation, indicative for increased apoptosis, Y27632 and H1152 did not. Similarly, inhibition of P38 MAPK/Akt signaling by wortmannin induced significant PS exposure, but not caspase 3 activation. This implies that decreased Akt activity through impaired ROCK signaling leads to PS exposure, but need not necessarily coincide with apoptosis. Indeed, pan-caspase inhibitor zVAD could only partially inhibit TcdB-induced PS exposure, again indicating that PS exposure does not necessarily coincide with apoptosis. Recently we showed that ischemia induced PS exposure can be a non-apoptotic and reversible phenomenon in cardiomyocytes [9]. Therefore, in H9c2 cells Rho GTPase family inhibition by TcdB induced both apoptotic and non-apoptotic PS exposure.

The apoptosis inducing properties of TcdB and C3 have been shown in several different cell types [38-42], indicating that inhibition of the Rho can induce apoptosis. Although Y27632 has been reported to induce apoptosis in human airway epithelial cells and H4IECs [43,44], in our experiments neither Y27632 nor H1152 induced a significant increase in caspase 3 activation in cardiomyocytes. These data may suggest a mechanism wherein impaired Rho signaling (by TcdB or C3) leads to non-apoptotic PS exposure, probably through impaired ROCK signaling, as assessed by Y27632 and H1152, and impaired flip-flop activity and in addition leads to apoptosis and apoptotic PS exposure (Fig. 6).

TcdB, but not C3, Y27632 or H1152, significantly lowered cellular ATP levels. It does therefore appear that the effect of TcdB on cellular ATP involves signaling pathways other than Rho-ROCK. Earlier studies have shown cellular ATP depletion induced by C. difficile Toxin A [45,46]. How these compounds interfere with cellular ATP levels we do as yet not know, however they both may interfere with mitochondrial respiratory function [40,45]. This ATP depletion effect of TcdB possibly also down-regulates known ATP-dependent regulators of transbilayer phospholipid asymmetry such as flippases and floppases. This may therefore contribute to TcdB-induced membrane flip-flop. In contrast, the Rho(A/B/C) inhibitors C3 and ROCK inhibitors Y27632 and H1152, which induced PS exposure, did not lower cellular ATP. This indicates involvement of Rho-ROCK signaling independent of putative ATP lowering effects in maintaining membrane asymmetry.
Fig. 7. The effect of C3, Y27632, H1152, and resveratrol on flipase activity in H9c2 cells. The effect of (a) C3 (1 μg/mL, 24 hour incubation), (b) Y27632 (10 μmol/L, 24 hour incubation), (c) H1152 (1 μmol/L, 4 hour incubation) and (d) resveratrol (wort; 100 μmol/L, 24 hour incubation) as the percentage of cells negative for NBD-labeled PS. (a) 5 experiments; *p < 0.02; (b) 6 experiments; *p < 0.004; (c) 4 experiments; *p < 0.004; (d) 5 experiments; *p < 0.05. (e) Example of Y27632 (10 μmol/L, 24 hour incubation) induced PS exposure (panel B) versus control cells (panel A), as determined via flow cytometry using annexin V-FTC and propidium iodide (PI). The PI exposure manifests itself as increased population of single-annexin V-positive cells (arrow, panel B). This PS exposure coincides with Y27632 (10 μmol/L, 24 hour incubation)-induced increase in the percentage of PS-NBD-negative cells (arrow, panel B) versus that in control cells (panel A).

Flipase-catalyzed transport translocates preferably PS from the outer to the inner plasma membrane layer and this transport requires ATP [20]. C3, Y27632 and H1152, which as mentioned earlier did not deplete ATP, significantly increased the percentage of PS-NBD-negative cells, i.e., cells that were incapable of internalizing PS-NBD, which is a measure for flipase inhibition [47]. Interestingly,
Fig. 6. Rho-ROCK–Rip progress signaling is involved in maintenance of membrane phospholipid asymmetry. Interference leads to PS exposure and apoptosis. Our data point to a role for Rho-ROCK signaling via Rip progress activity in maintenance of phospholipid transbilayer asymmetry (a). Impaired Rip progress signaling through depletion or C3 leads to PS exposure, in part through induction of apoptosis (apoptosis 3 activation) and in part through a non-apoptotic route probably via impaired ROCK signaling and impaired Rip progress activity (b). Impaired ROCK signaling through direct inhibition by Y27632 or H1122 leads to indirect loss of PS exposure (c).

Pi3K/Akt inhibitor wortmannin also significantly inhibited flippase, suggesting that the effects of Rho–ROCK signaling on flippase activity involve Akt.

In conclusion, in recent years we have provided evidence for an importance of (reversible) plasma membrane PS exposure in jeopardized cardiomyocytes in the border zones of AMI for the propagation of inflammation and the additional damage this brings about [57,178]. In this study we now provide evidence for a role for Rho-ROCK signaling in maintaining the asymmetrical distribution of myocardial phospholipids in cardiomyocytes, which directly involves flippase activity.

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