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Homocysteine Induces Phosphatidylserine Exposure in Cardiomyocytes through Inhibition of Rho Kinase and Flippase Activity

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Key Words

Homocysteine • PS exposure • RhoA • ROCK • Flippase • Heart failure

Abstract

Aims: Increased levels of homocysteine (Hcy) form an independent risk factor for cardiovascular disease. In a previous study we have shown that Hcy induced phosphatidylserine (PS) exposure to the outer leaflet of the plasma membrane in cardiomyocytes, inducing a pro-inflammatory phenotype. In the present study the mechanism(s) involved in Hcy-induced PS exposure were analyzed. Methods: H9c2 rat cardiomyoblasts were subjected to 2.5 mM D,L-Hcy and analyzed for RhoA translocation and activity, Rho Kinase (ROCK) activity and expression and flippase activity. In addition, the effect of ROCK inhibition with Y27632 on Hcy-induced PS exposure and flippase activity was analyzed. Furthermore, GTP and ATP levels were determined. Results: Incubation of H9c2 cells with 2.5 mM D,L-Hcy did not inhibit RhoA translocation to the plasma membrane. Neither did it inhibit activation of RhoA, even though GTP levels were significantly decreased. Hcy did significantly inhibit ROCK activation, but not its expression, and did inhibit flippase activity, in advance of a significant decrease in ATP levels. ROCK inhibition via Y27632 did not have significant added effects on this. Conclusion: Hcy induced PS exposure in the outer leaflet of the plasma membrane in cardiomyocytes via inhibition of ROCK and flippase activity. As such Hcy may induce cardiomyocytes vulnerable to inflammation *in vivo* in hyperhomocysteinaemia patients.

Introduction

Homocysteine (Hcy) is an amino acid derived from the methionine metabolism and is normally remethylated to methionine or otherwise converted to cysteine. Due to either genetic mutations in the converting enzymes or due to shortages of vitamin B6, B12 or folate which are important co-factors in the metabolism, Hcy levels can rise [1]. For instance, genetic mutations in the cysthatione

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β–synthase (CBS) gene can result in highly increased Hcy plasma levels of up to 400 μM, which is known as hyperhomocystinuria [2]. These patients usually develop severe cardiovascular complications as young adults. Most patients however suffer from moderately increased levels of Hcy, known as hyperhomocysteinaemia (HHC) which are mostly caused by co-factor shortages. Population studies have revealed that HHC, even in mild forms can be an independent risk factor for the development of atherosclerosis and heart failure [3-5]. HHC has been shown to induce heart failure including diastolic and systolic dysfunction, with myocardial fibrosis and subsequent ventricular hypertrophy and increased wall thickness [5-7]. The mechanisms underling this HHC-induced heart failure however remain for a large part unknown.

Previously we have demonstrated that Hcy induced apoptosis and necrosis, but also reversible exposure of phosphatidylserine in the outer plasma membrane leaflet in cardiomyocytes (PS exposure) [8]. In eukaryotic cells plasma membrane phospholipids are distributed asymmetrically in the bilayer. The outer leaflet predominantly contains phosphatidyl-choline (PC), whereas anionic PS and phosphatidyl-ethanolamine (PE) are kept within the inner leaflet [9]. Exposure of PS in the outer plasma membrane leaflet occurs in apoptotic cells, but can also be reversible and occur independently of apoptosis [10]. Exposed PS becomes a target for inflammation, and we have shown previously that in the presence of inflammation, reversible PS exposure can result in additional cardiomyocyte death after myocardial infarction [11, 12]. This Hcy-induced (reversible) PS exposure by cardiomyocytes, thereby facilitating local inflammation and resulting cardiomyocyte death, potentially also is a contributing factor to HHC-induced heart failure.

We have recently found that inhibition of Rho - ROCK signaling induced both apoptotic and non-apoptotic PS exposure in cardiomyocytes via inhibition of flippase [13]. Flippase is an ATP-dependent transmembrane protein that has been found to regulate transbilayer phospholipid asymmetry also in cardiomyocytes, by translocating PS from the outer to the inner membrane layer [14]. This study showed a regulatory role for a novel signaling route, Rho - ROCK - flippase signaling, in maintaining asymmetrical membrane phospholipid distribution in cardiomyocytes. In the present study we have investigated the possible involvement of this signaling route in Hcy-induced PS exposure in cardiomyocytes.

Materials and Methods

Cell culture

Rat cardiomyoblasts (H9c2 (2-1) cells; ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagles Medium (DMEM; BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWhittaker), 2 mM L-glutamine (GIBCO, Paisley, UK), 100 IU/ml penicillin (Yamanouchi Europe BV, Leiderdorp, Netherlands) and 100 μg/ml streptomycin (Radiopharma-Fisiopharma, Palomonte, Italy), and grown at 37°C in a humidified 5% CO_2/95% air atmosphere. Experiments were performed with cells grown to a confluency of 60 - 80%.

Cells were incubated with 2.5 mM D,L-homocysteine (Hcy) (Sigma, St. Louis, MO, USA) in growth medium for 14, 18 or 24 hours at 37°C in a humidified 5% CO_2/95% air atmosphere, according to our previous study [8]. Cells were incubated with ROCK inhibitor 10 μM Y27632 (Calbiochem, Nottingham, UK) for 24.5 hours at 37°C in a humidified 5% CO_2/95% air atmosphere. In case cells were co-incubated with Y27632 and Hcy, Y27632 was added to cells 30 minutes prior to Hcy.

Flow cytometry

PS exposure was assessed with FITC-labeled human recombinant annexin-V (Bender Med Systems, Vienna, Austria), while propidium iodide (PI; Bender Med Systems) was used to determine the permeability of the cellular membrane. Experiments were performed in the presence or absence of Z-Asp-Glu-Val-Asp-fluoromethylketone (Z-VAD FMK, 25 μM, Alexis Biochemicals, Lausen, Switzerland), a general caspase inhibitor, to differentiate between reversible and irreversible PS exposure.

After treatment with Hcy, the cells were trypsinized and centrifuged at 400xg for 5 minutes at room temperature. Cells were then washed with serum-free DMEM, and resuspended in serum free DMEM containing annexin V (1:40) for 30 minutes in the dark at 37°C in a humidified 5% CO_2/95% air atmosphere. Cells were subsequently washed and resuspended in serum free DMEM containing PI (1:40), and were measured with a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Results were analyzed by Cell Quest Pro software (Becton Dickinson).

To determine flippase activity, cells were harvested after incubation with or without Hcy and resuspended in serum free DMEM medium. NBD labeled phosphatydylserine (PS-NBD: (1-palmitoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine) (Avanti Polar Lipids, Alabaster, AL, USA) was added in a final concentration of 1 μmol/L at 37°C for 30 minutes. The cells were then transferred to ice and 5 ml ice-cold 1% (w/v) BSA in PBS added to wash away excess probe. After 15 minutes the cells were pelleted and resuspended in serum-free culture medium and then analyzed by flow cytometry (FACSCalibur). With each experiment, part of the harvested cells were analyzed for membrane flip flop as described before.

To determine whether the PS-NBD negative cells are in fact annexin V positive, the cells were labeled with Cy5-labeled annexin V for 15 minutes at 37°C subsequent to PS-NBD staining and measured at once as described before.

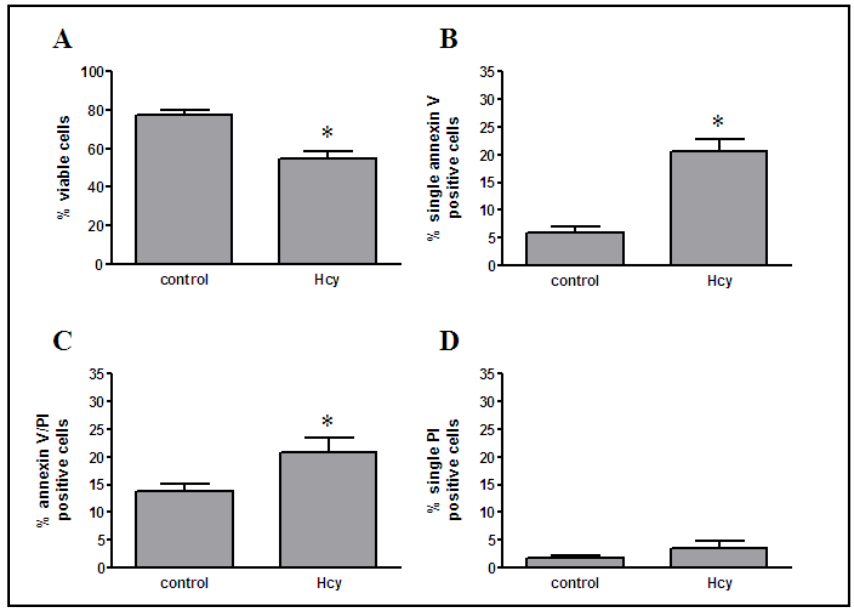

Fig. 1. Effect of Hcy on cell viability. Cell viability of H9c2 cells, incubated for 24 hours with (Hcy) or without (control) 2.5 mM D,L-Hcy. (A) percentage of viable cells, *p<0.0001; (B) percentage of single annexin V positive cells, *p<0.0001; (C) percentage of annexin V/PI positive cells, *p<0.02; (D) percentage of single PI positive cells. Data represent mean ± SEM (n=10).

Determination of ATP and GTP levels

After treatment, 5 x 10E6 cells per condition were centrifuged at 400 x g for 2 minutes. The pellet was resuspended in 150 µl of ice-cold perchloric acid (PCA) 0.4 M, kept on ice for 30 minutes and subsequently centrifuged at 200 x g for 5 minutes at 4°C. To the isolated supernatant 7.5 µl of K_2CO_3 5 M was added to neutralize pH. Samples were subsequently stored at -80°C. ATP levels were determined using a Luciferase-Luciferin assay (Biaffin GmbH & Co KG, Kassel, Germany) according to the manufacturers' protocol. Samples were analyzed in a FluoroNunc MaxiSorp plate (Nalge Nunc International, Rochester, NY). Luminescence was measured in a Tecan GENios Plus reader (Tecan Benelux, Mechelen, Belgium). GTP levels were determined using High Pressure Liquid Chromatography (HPLC) as described before [15]. The columns used were prepacked Partisphere (partisil-5) SAX cartridges (4.6 x 125 mm internal dimensions; Whatman Int. Ltd., Maidstone England). The GTP concentrations were calculated by comparison with highly purified standards (Sigma).

RhoA activity assay

RhoA activity was determined in H9c2 cell protein extracts, using the luminescence-based G-LISA™ RhoA activation assay biochemistry kit (Cytoskeleton, Inc. Denver, CO, USA) according to the manufacturer's instructions. Protein concentration was determined according to the manufacturer's protocol, and cell extracts were equalized. Samples were measured in a FluoroNunc MaxiSorp plate (Nalge Nunc International).

Cell fractioning and Western Blot analysis

For whole cell lysates, cells were harvested into modified ELB lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES pH 7.0, 5 mM EDTA, 0.5 mM DTT) with added protease inhibitor cocktail (PIC, 1:25; Sigma), mixed thoroughly and then incubated on ice for 30 minutes. To obtain membrane and cytosolic fractions, cells were harvested into lysis buffer (5 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$) and homogenized using a dounce tissue grinder (Wheaton, Millville, NJ, USA). Samples were centrifuged at 500 x g for 5 minutes at 4°C. The supernatant was then centrifuged at 40,000 rpm using a Beckman centrifuge (50 Ti rotor; BeckmanCoulter, Mijdrecht, The Netherlands) for 20 minutes at 4°C to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant). The membrane fraction was dissolved in incubation buffer (25 mM Tris, 1 mM EGTA, 20 mM $MgCl_2$). After determination of the protein concentration with the BCA protein assay kit (Pierce, Rockford, IL, USA), the samples were diluted in β-mercaptoethanol-containing sodium dodecylsulfate (SDS) sample buffer. 20 µg protein of each sample was then subjected to SDS-PAGE, blotted onto nitrocellulose membranes and analyzed for RhoA, ROCK-1 and ROCK-2 expression using mAb against RhoA (26c4; 1:100 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), mAb against ROCK-1 (G-6; 1:250 dilution; Santa Cruz) or pAb (goat) against ROCK-2 (C-20; 1:400 dilution; Santa Cruz), followed by horseradish-peroxidase-conjugated rabbit-anti-mouse immunoglobulins (RαM-HRP; 1:1000 dilution; DakoCytomation, Glostrup, Denmark) for RhoA and ROCK-1 or Swine-anti-Goat-HRP (1:500 dilution; DakoCytomation) for ROCK-2. The blots were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences AB; Uppsala; Sweden). Staining was quantified with a charge-coupled device camera (Fuji Science Imaging Systems; Düsseldorf, Germany) in combination with AIDA Image Analyzer software (Isotopenmessgeräte; Staubenhardt, Germany). For detection of total and phosphorylated ezrin, radixin and moesin (ERM) proteins, rabbit pAbs against total ERM and phospho-ERM were used (both 1:1000 dilution; Cell Signaling Technology, Inc) followed by swine-anti-rabbit-HRP (1:1000; DakoCytomation).

Digital imaging fluorescent microscopy of RhoA

Cells were cultured in Lab-Tek II 4-well chamber CC2 glass slides (Nalge Nunc International, Naperville, IL, USA). After

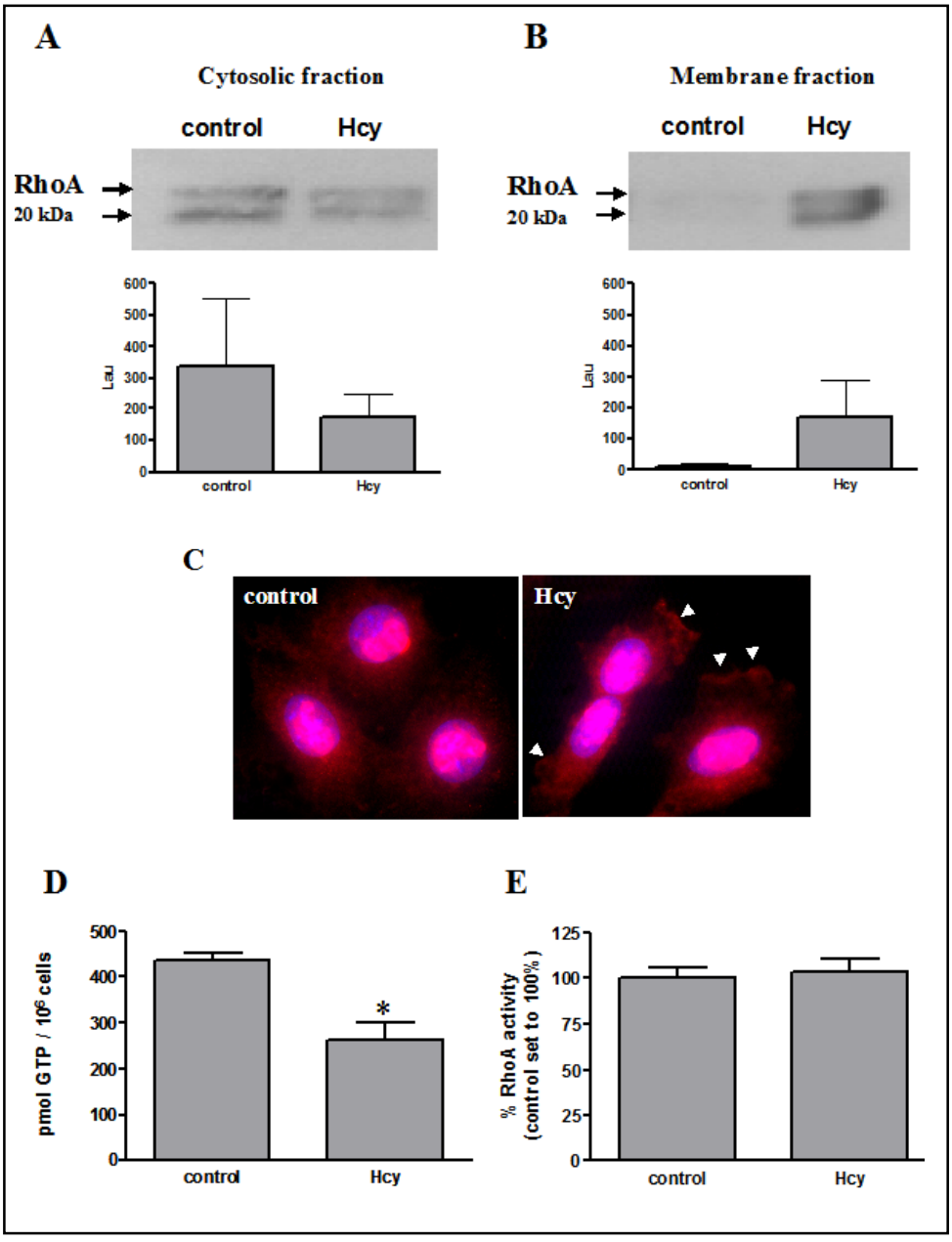

Fig. 2. Effect of Hcy on RhoA location and activity. H9c2 cells were incubated for 24 hours with (Hcy) or without (control) 2.5 mM D,L-Hcy and presence of RhoA (24 kDa) was determined and quantified (Lau= light arbitrary units) in isolated cytosol and membrane fractions via western blot analysis (n=4). (A) cytosol fractions, diluted in 2 ml, Lau recalculated to 10 µl. (B) Membrane fractions; diluted in 150 µl, Lau (Light arbitrary unit) recalculated to 10 µl. (C) Digital imaging fluorescent microscopy analysis of RhoA in H9c2 cells that were incubated for 24 hours with (Hcy) or without (control) 2.5 mM D,L-Hcy. Arrowheads indicate membranous RhoA localization. (D) GTP levels were determined in whole cell lysates of control cells and cells incubated with 2.5 mm D,L-Hcy for 24 hours (n=4). (E) RhoA activity was determined in whole cell lysates of control cells and cells incubated with 2.5 mm D,L-Hcy for 24 hours (n=3). Data represent mean ± SEM.

treatment, cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C, then permeabilized with 0.2% Triton for 10 minutes at RT and subsequently incubated with mAb against RhoA (26c4; 1:50 dilution; Santa Cruz) for 1 hour at RT followed by incubation overnight at 4°C. The cells were then incubated with Cy3-labeled Goat-anti-mouse immunoglobulins (Invitrogen Corporation, Carlsbad, CA, USA) for 30 min at RT in the dark. Negative controls with only the secondary antibody were included to assess nonspecific binding. All negative controls showed no staining (data not shown). Before visualization, HardSet mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; H-1500, Vector Laboratories Inc, Burlingame, CA, USA) was added and the cells were covered. The cells were analyzed with a 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200 M inverted microscope; Cark Zeiss, Sliedrecht, The Netherlands) equipped with a thermo-electrically cooled EMCCD camera (QuantEM: 512C, 512x512 pixels; Photometrics, Tucson, AZ, USA). Data was processed with Slidebook™ software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA).

Statistics

Statistics were performed with the SPSS statistical program (windows version 15.0). To evaluate whether observed differences were significant, independent student T-tests or One Way ANOVA with post hoc Bonferroni tests were used. In the text and relevant figures values are given as means ± SD. A *p*-value (two-sided) of 0.05 or less was considered to represent a significant difference.

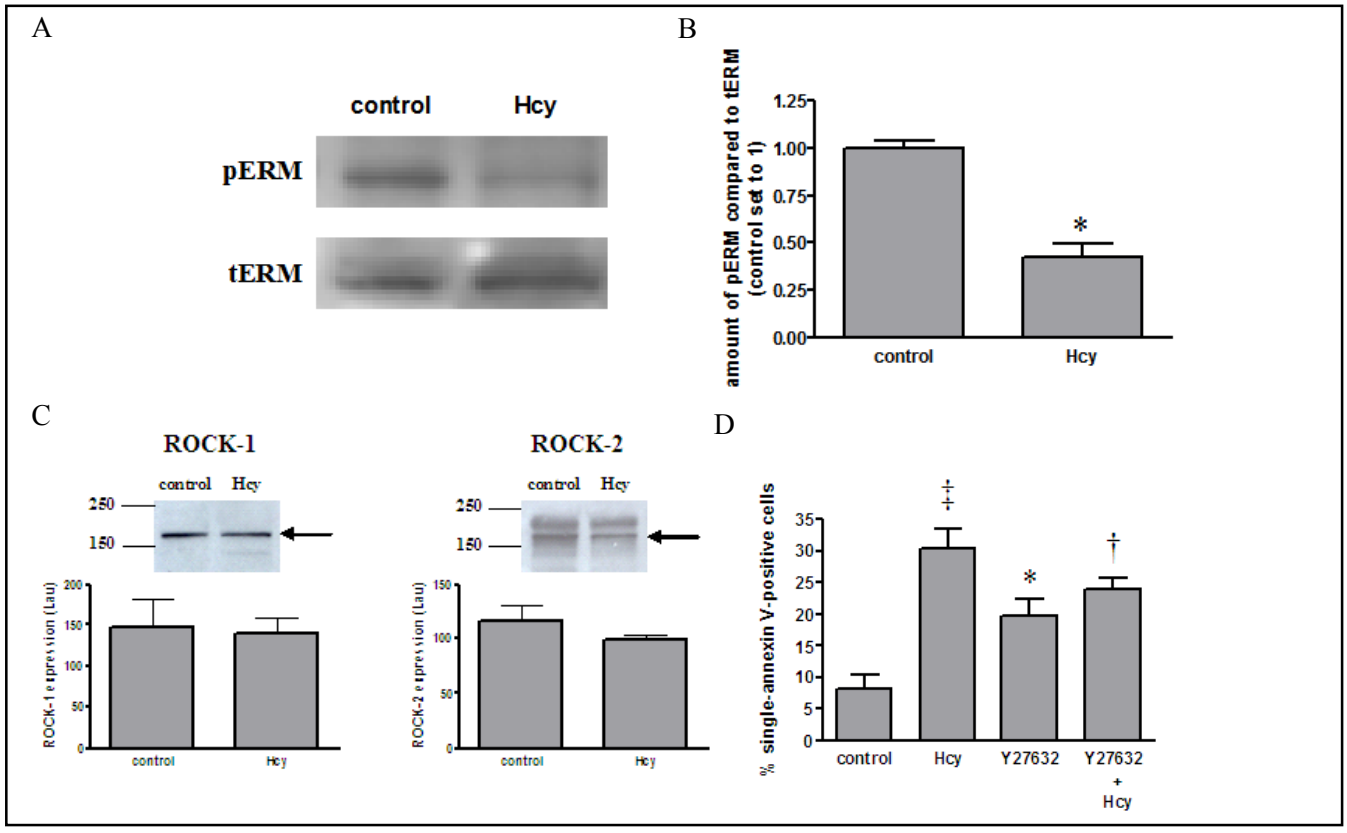

Fig. 3. Effect of Hcy on ROCK activity and expression. (A) Western blot analysis for phosphorylated ERM (pERM) and total ERM (tERM) of H9c2 cells incubated with (Hcy) or without (control) 2.5 mM D,L-Hcy for 24 hours. (B) Quantification of the intensity of the bands is shown in the graph where the amount of pERM was calculated in comparison with tERM, and the control was set to 1. *$p<0.002$. (C) Western blot analysis for ROCK-1 and ROCK-2 in H9c2 cells incubated with (Hcy) or without (control) 2.5 mM D,L-Hcy for 24 hours. Quantifications of the protein bands are shown in the graphs (Lau= light arbitrary units). (D) Percentages of single-annexin V-positive H9c2 cells, incubated with Hcy, with Y27632 or both. ‡$p<0.001$, *$p<0.05$, †$p<0.05$. Data represent mean ± SEM (n=4).

Results

Hcy induced PS- exposure

We first wanted to validate our previous finding of induced PS exposure in H9c2 cells by 2.5 mM D,L-Hcy for 24 hours [8]. We found a significant decrease of viable cells from 77.5 ± 2.4% in control cells to 54.9 ± 3.7% in Hcy treated cells (Fig. 1A; *$p<0.0001$; n=10) and a significant increase of single annexin V-positive cells from 5.8 ± 1.2% in control cells to 20.6 ± 2.2% in Hcy treated cells (Fig. 1B; *$p<0.0001$; n=10). We also found a significant increase in annexin V/PI-positive cells from 13.8 ± 1.4% in control cells to 20.9 ± 2.5% in Hcy treated cells (Fig. 1C; *$p<0.02$) and a minor, non-significant increase of single PI-positive cells from 1.7 ± 0.3% in control cells to 3.2 ± 1.0% in Hcy treated cells (Fig. 1D). The induction of single annexin V-positive cells induced by Hcy was not inhibited by Z-VAD fmk (data not shown), this indicates reversible PS exposure, as shown previously [8].

Effect of Hcy on RhoA location and activity

We have recently found that inhibition of Rho protein activity by Toxin B and exoenzyme C3 transferase resulted in PS exposure in H9c2 cells [13]. To determine whether Hcy-induced PS-exposure results from an effect on Rho - ROCK signaling, we first examined whether Hcy affects RhoA translocation -via cell fractioning - and activity - via cellular GTP measurements and a RhoA activity assay.

In control cells, RhoA was mainly present in the cytosolic fraction (Fig. 2A, B; intensity of the bands was defined as light arbitrary units (Lau); n=4). Notably, via immunoblotting two bands were detected, indicative for

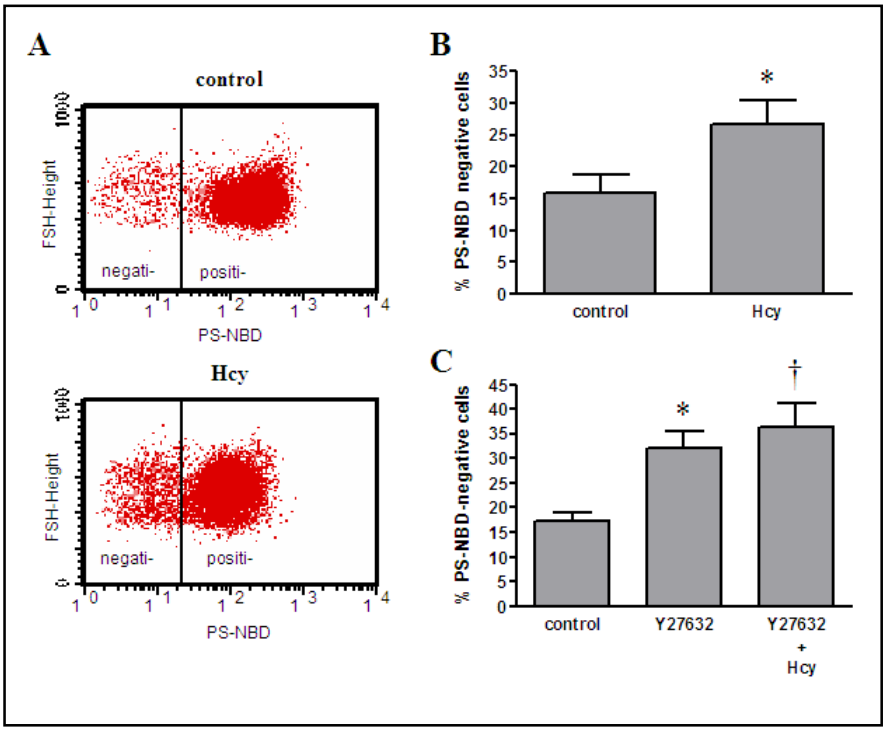

Fig. 4. Effect of Hcy on flippase activity. Flow cytometry analysis of flippase activity in H9c2 cells incubated with or without 2.5 mM D,L-Hcy for 24 hours using PS-NBD. (A) Dot plots show PS-NBD positivity against cell size (FSH-Height). (B) percentage of PS-NBD negative cells in control cells and Hcy-treated cells. *p=0.033. Data represent mean ± SEM (n=10). (C) Percentages of NBD-negative H9c2 cells, incubated with Hcy, with Y27632 or both. *p<0.05, †p<0.01. Data represent mean ± SEM (n=4).

RhoA (24 kD) and the calpain cleaved form RhoA (20 kD), which has lost its normal RhoA function as described before [16]. For this, we only quantified the 24 kD RhoA band. Hcy induced a non-significant decrease in RhoA in the cytosolic fraction and a clear but nonsignificant increase in RhoA in the membrane fraction (Fig. 2A, B), indicating that Hcy did not inhibit RhoA translocation to the plasma membrane. Although the Hcy effects were not significant, we found this Hcy-induced RhoA translocation to the membrane fraction in each of the four independent experiments. These results were confirmed via digital imaging fluorescent microscopy (Fig. 2C). In control cells, RhoA was found mainly in the cytosol, whereas Hcy induced a marked increase in RhoA located at the plasma membrane (arrowheads).

In addition to membrane-anchorage, RhoA needs GTP for its activity. We therefore also determined the effects of Hcy on cellular GTP levels. Incubation with Hcy induced cellular GTP levels to decrease significantly from 437 pmol/10^6 cells in control cells to 262 pmol/10^6 cells in Hcy treated cells (Fig. 2D; p=0.05; n=4). Notwithstanding this decrease there was still considerable residual cellular GTP. To analyze whether this residual GTP still is sufficient for RhoA activation, a RhoA activity assay was performed. RhoA activity in cells incubated with Hcy did not differ significantly from control cells (Fig. 2E; n=3). From these data we can conclude that Hcy does not inhibit RhoA.

Effect of Hcy on ROCK activity

ROCK is a downstream effector of RhoA. Previously we have shown that inhibition of Rho-kinase with the specific ROCK inhibitors Y-27632 and H1152 induced PS exposure in non-ischemic cardiomyocytes [13]. We analyzed a putative effect of Hcy on ROCK activity and expression. As ROCK phosphorylates the ezrin, radixin and moesin (ERM) proteins [17], Rho-kinase activity can be determined by measuring the level of phosphorylation of ERM (pERM).

Incubation with Hcy for 24 hours induced a significant decrease of 45% in ERM phosphorylation (Fig. 3A, B; SD ± 9.540; *p=0.002; n=4), determined as the relative amount of phosphorylyzed ERM (pERM) related to the total amount of ERM (tERM). These data thus indicate that Hcy inhibits ROCK activity. This inhibitory effect of Hcy on ROCK activity may relate to an inhibitory effect on ROCK expression. Therefore, ROCK-1 and ROCK-2 expression levels were analyzed via Western blot. There was no significant effect of Hcy on the expressions of ROCK-1 and ROCK-2, although Hcy treatment appeared to lower the expression levels of ROCK-2 (Fig. 3C).

In addition, the effect of (pre-)inhibition of ROCK on Hcy-induced PS exposure was analyzed, using the ROCK inhibitor Y27632. As reported previously [13], Y27632 induced a significant increase in single-annexin V-positive cells to 19.9 ± 2.6% compared to 8.1 ± 2.1%

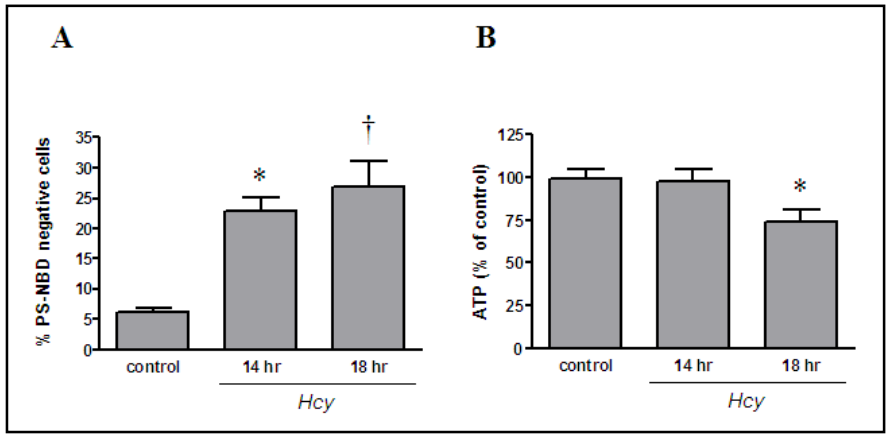

Fig. 5. Flippase activity and ATP dependency. Flow cytometry analysis of flippase activity and determination of cellular ATP levels in H9c2 cells incubated with or without 2.5 mM D,L-Hcy for 14 and 18 hours using PS-NBD. (A) percentage of PS-NBD negative cells, *p<0.01 vs control, †p<0.001 vs control; (B) ATP levels, *p<0.05 vs control. Data represent mean ± SEM (n=6).

in control cells (*p<0.05; Fig. 3D). Y27632 pre-treatment followed by Hcy incubation also induced a significant increase in single-annexin V-positive cells to 23.9 ± 1.8% (†p<0.05), but this percentage of single-annexin V-positive cells did not differ significantly from that induced by Y27632 alone. The percentage of single-annexin V-positive cells induced by Hcy alone (30.4 ± 3.1%; ‡p<0.001 vs control) was higher than those induced by Y27632 alone or Y27632 followed by Hcy, but not significantly. These results support a role for ROCK inhibition in Hcy-induced exposure of PS.

Effect of Hcy on flippase activity

We then wanted to determine whether the inhibitory effect of Hcy on ROCK activity also affected flippase activity. Flippase activity was determined by flow cytometry analysis using NBD-labeled PS. In cells with flippase activity this PS-NBD is internalized resulting in PS-NBD-positive cells, whereas cells with inactive flippase will not internalize PS-NBD and remain PS-NBD-negative [13] (Fig. 4A).

Hcy induced a significant increase in the amount of PS-NBD-negative cells, form 16 ± 3% in control cells to 27 ± 4% (Fig. 4B; *p=0.033; n=10), indicating that Hcy significantly decreased flippase activity. In addition, the effect of (pre-)inhibition of ROCK on Hcy-induced flippase activity was analyzed. Y27632 induced a significant increase in PS-NBD-negative cells to 32.1 ± 3.3% compared to 17.3 ± 1.8% in control cells (*p<0.05; Fig. 4C). Y27632 pre-treatment followed by Hcy incubation also induced a significant increase in PS-NBD-negative cells to 36.3 ± 4.8% (†p<0.01), but this percentage of PS-NBD-negative cells did not differ significantly from that induced by Y27632 alone, again supporting a role for ROCK inhibition in Hcy-induced inhibition of flippase.

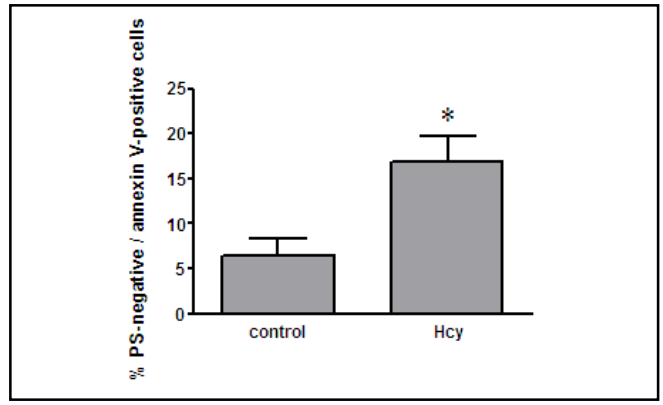

Fig. 6. Flippase activity and PS exposure. Flow cytometry analysis of H9c2 cells that were incubated without (control) or with 2.5 mM D,L-Hcy for 24 hours. Cells were then incubated with PS-NBD and subsequently with Cy5-labeled annexin V. Shown is the relative size of the PS-NBD-negative/Annexin V-positive cell population. *p=0.022. Data represent mean ± SEM (n=4).

As flippase activity is ATP dependent [18], we analyzed whether Hcy-induced flippase activity is caused by ATP. Flippase activity and cellular ATP levels were determined at several time points during the total Hcy incubation period of 24 hours. A significant decrease in flippase inactivity was measured after 14 hours of Hcy incubation (Fig. 5A; *p<0.01), at which time-point cellular ATP levels were comparable with those in control cells (Fig. 5B). A significant decrease in cellular ATP occurred only after 18 hours of Hcy incubation (Fig. 5B; *p<0.05). Therefore, Hcy-induced flippase inhibition is not due to a lack in cellular ATP.

To establish whether this flippase inactivation coincided with PS exposure, double stainings were performed with PS-NBD and Cy5-labbeled annexin V. Incubation with Hcy indeed resulted in a population of

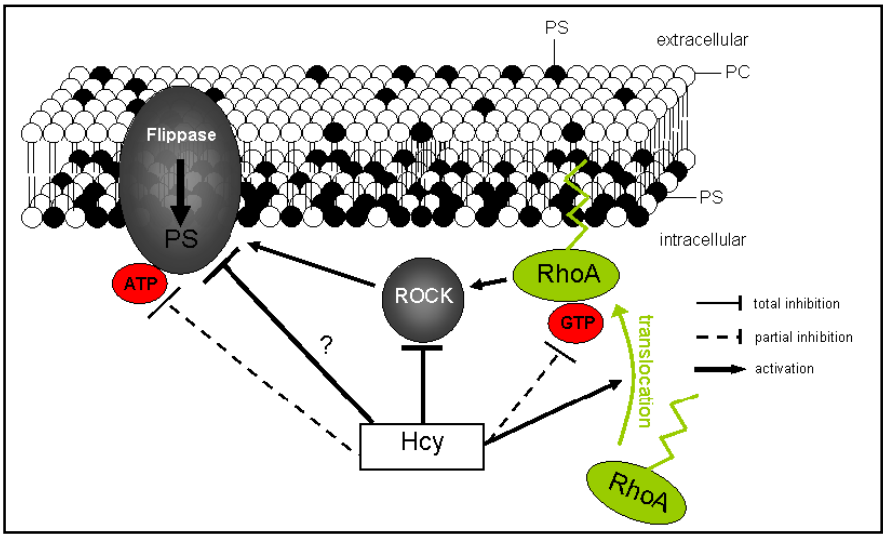

Fig. 7. Hypothetic scheme for Hcy induced PS exposure via ROCK inactivation. The scheme shows that Hcy induces RhoA translocation to the plasma membrane (green arrow); Hcy directly inhibits ROCK activity (in black, blocking line); Hcy inhibits flippase activity via ROCK, although we cannot exclude the possibility of an Hcy effect on flippase activity independent of ROCK (in black, blocking line with ?); Hcy also depletes ATP and GTP levels, but only partly (in black, broken blocking line).

cells that were PS-NBD negative but annexin V-positive (Fig. 6; *p=0.022; n=4), indicating that Hcy-induced flippase inactivity coincided with PS exposure.

Discussion

We were the first to describe that Hcy induced apoptosis, necrosis but also reversible PS exposure in cardiomyocytes [8]. In the present study, we analyzed the mechanism(s) through which Hcy induced this PS exposure in cardiomyocytes. We have found that Hcy induced inhibition of Rho-kinase activity and flippase activity (Fig. 7) which to the best of our knowledge has never been described before.

In a previous study we found that impaired Rho – ROCK signaling resulted in PS exposure in the outer plasma membrane leaflet in cardiomyocytes [13]. This concurs with the findings of Grounds et al. who showed that inhibition of Rho activity by the Rho(A,B,C) specific inhibitor exoenzym C3 transferase induced annexin V positivity [19]. In the present study however we found that although Hcy did induce RhoA translocation to the plasma membrane, it did not alter RhoA activity, indicating that the Hcy-induced PS exposure occurred independent of impaired RhoA. On the other hand, Hcy did inhibit ROCK activity resulting in decreased phosphorylation of the ERM proteins. As Hcy did not significantly decrease the expression of ROCK-1 and ROCK-2, this inhibitory effect of Hcy on ROCK activity appears to be due mainly to blocking of its activity. Since Hcy did appear to lower the expression levels of ROCK-2, we cannot rule out a partial effect of decreased ROCK-2 expression on Hcy-induced ROCK inactivation. Previously we showed that inhibition of ROCK via Y27632 or H1152 did lead to PS exposure [13]. Here we show that inhibition of ROCK via Y27632, did not significantly affect Hcy-induced PS exposure, indeed supporting a role for ROCK inhibition in Hcy-induced PS exposure.

As we have previously shown, PS exposure represents a pro-inflammatory status of the cell [12]. This suggests that Hcy induced inhibition of Rho-kinase would put cardiomyocytes at risk as targets for inflammation. In the past several rat and mice *in vivo* studies have been performed in which the effect of commercially available ROCK inhibitors was analyzed related to the prevention of vascular endothelial disease independent of Hcy [20-23]. However, in none of these studies the effect on PS exposure in cardiomyocytes and/or replacement fibrosis was studied as a measure of putative loss of cardiomyocytes. Therefore, our *in-vitro* findings suggesting a jeopardizing effect of inhibition of Rho-kinase on cell viability of cardiomyocytes still have to be extrapolated *in-vivo*.

Hcy inhibited flippase activity in cardiomyocytes, in advance of a decrease in ATP, suggesting that Hcy does so through mechanisms independent of ATP. We have shown earlier that ROCK inhibitors Y27632 and H1152 did inhibit flippase activity without altering cellular ATP levels [13]. In this study we additionally show that inhibition of ROCK via Y27632, did not significantly affect Hcy-induced inhibition of flippase activity. This suggests that Hcy inhibits flippase via its inhibitory effect on ROCK.

A limitation of the present study is the use of relatively high concentrations of Hcy. However, in our

previous study we have already shown that the added Hcy is rapidly degraded by cardiomyocytes [8]. Furthermore short-term exposure to high Hcy concentrations may reflect a prolonged exposure to moderately elevated Hcy concentrations as occur life-long in patients.

Hcy has been shown to be an independent risk factor for heart failure [5, 7, 24, 25]. The Framingham Heart Study showed a positive correlation between HHC and new-onset heart failure not due to ischemia [5]. These authors suggested that elevated Hcy levels induced left ventricular hypertrophy. This hypertrophy can be caused directly by inducing cardiac interstitial and replacement fibrosis [26], the latter of which is indicative for cardiomyocyte loss [24]. This concurs with our findings that Hcy can induce apoptosis and necrosis in cardiomyocytes, but also reversible pro-inflammatory PS exposure [8]. These latter cells are at risk in the presence of inflammatory cell clearance [12,27]. Furthermore, Hcy not only propagates inflammation in the heart via induction of this pro-inflammatory PS exposure in cardiomyocytes, but also induces the release of pro-inflammatory cytokines such as IL-6, IL-8 and TNFα, and as such promotes leukocyte recruitment to the endothelium [28-30]. Hcy also increases inflammatory markers in plasma, such as MCP-1 and VCAM-1 [29-34]. Taken together, this means that Hcy propagates inflammation in the heart by making cardiomyocytes susceptible to inflammation, but also by increasing inflammatory activity.

In conclusion, we have now shown that Hcy induces PS exposure in cardiomyocytes via inhibition of Rho-kinase and flippase activity. As such Hcy may induce cardiomyocytes vulnerable to inflammation *in vivo* in HHC patients.

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