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Homocysteine-induced cardiomyocyte apoptosis and plasma membrane flip-flop are independent of *S*-adenosylhomocysteine: a crucial role for nuclear p47^{phox}

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Abstract We previously found that homocysteine (Hcy) induced plasma membrane flip-flop, apoptosis, and necrosis in cardiomyocytes. Inactivation of flippase by Hcy induced membrane flip-flop, while apoptosis was induced via a NOX2-dependent mechanism. It has been suggested that *S*-adenosylhomocysteine (SAH) is the main causative factor in hyperhomocysteinemia (HHC)-induced pathogenesis of cardiovascular disease. Therefore, we evaluated whether the observed cytotoxic effect of Hcy in cardiomyocytes is SAH dependent. Rat cardiomyoblasts (H9c2 cells) were treated under different conditions: (1) non-treated control (1.5 nM intracellular SAH with 2.8 μM extracellular L-Hcy), (2) incubation with 50 μM adenosine-2,3-dialdehyde (ADA resulting in 83.5 nM intracellular SAH, and 1.6 μM extracellular L-Hcy), (3) incubation with 2.5 mM D,L-Hcy (resulting in 68 nM intracellular SAH and 1513 μM extracellular L-Hcy) with or without 10 μM reactive oxygen species (ROS)-inhibitor apocynin, and (4) incubation with 100 nM, 10 μM, and 100 μM SAH. We then determined the effect on annexin V/propodium iodide positivity, flippase activity, caspase-3 activity, intracellular NOX2 and p47^{phox} expression and localization, and nuclear ROS production. In contrast to Hcy, ADA did not induce apoptosis, necrosis, or membrane flip-flop. Remarkably, both ADA and Hcy induced a significant increase in nuclear NOX2 expression. However, in contrast to ADA, Hcy additionally induced nuclear p47^{phox} expression, increased nuclear ROS production, and inactivated flippase. Incubation with SAH did not have an effect on cell viability, nor on flippase activity, nor on nuclear NOX2-, p47phox expression or nuclear ROS production. HHC-induced membrane flip-flop and apoptosis in cardiomyocytes is due to increased Hcy levels and not primarily related to increased intracellular SAH, which plays a

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crucial role in nuclear p47^{phox} translocation and subsequent ROS production.

Keywords Homocysteine · *S*-adenosylhomocysteine · Cardiomyocyte apoptosis · Membrane flip-flop

Introduction

Hyperhomocysteinemia (HHC) as a risk factor for cardiovascular disease has been described in many in vivo and in vitro studies. Patient and animal studies have shown a positive correlation between increased homocysteine (Hcy) levels with increased risk for cardiovascular complications including endothelial dysfunction, atherosclerosis, and myocardial infarction [1–3]. In vitro studies have proven that Hcy causes proliferation of SMC [4, 5], but also oxidative stress [6] and cell death in endothelial cells [7, 8].

Hcy is an amino acid of the methionine metabolism. Methionine (Met) is converted into *S*-adenosylmethionine (SAM), which is the main source of methyl for methylation of DNA, RNA, and proteins [9]. Once the methylgroup is transferred to methyltransferases, *S*-adenosylhomocysteine (SAH) remains. SAH is subsequently hydrolyzed to Hcy and adenosine (Fig. 1). Due to either genetic defects or deficiencies in co-factors such as vitamin B6, B12, and folate, accumulation of Hcy occurs [10]. An increase in Hcy also results in an increase in SAH. SAH is known as a potent inhibitor of methylation of DNA, RNA, and proteins since it can bind to methyltransferases but cannot donate a methylgroup necessary for methylation to occur [11]. Castro et al. [12] have demonstrated in endothelial cells that increased SAH induced hypomethylation of DNA, which may lead to inappropriate gene expression and ultimately promotion of endothelial dysfunction. James et al. [13] suggested that SAH-induced DNA hypomethylation is the main causative factor for HHC-induced cardiovascular pathogenesis. However, none of the previous studies have given conclusive evidence that increased SAH alone is the causative factor in Hcy-induced cellular damage since these studies were performed in conditions in which increased SAH was accompanied by increased Hcy levels.

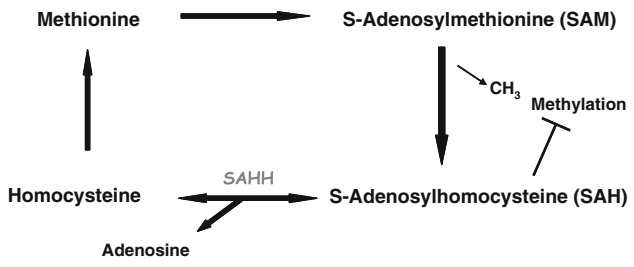

Fig. 1 Homocysteine metabolism. Schematic representation of methionine/homocysteine metabolism. Methionine (Met) is phosphorylized to *S*-Adenosylmethonine (*SAM*), a potent methyldonor for methyltransferases. After donating the methylgroup, *S*-Adenosylhomocysteine (*SAH*) remains, which in turn is a potent inhibitor of methylation. SAH is then hydrolyzed to homocysteine and adenosine by S-Adenosylhomocysteine hydrolase (*SAHH*), which is a reversible reaction and will favor SAH when concentration of Hcy is increased

The NADPH-oxidase (NOX) complex was originally found to be responsible for the generation of reactive oxygen species (ROS) during the respiratory burst in phagocytic cells, but it has become clear that NOX-derived ROS are also involved in cellular signaling (so called redox-signaling) in other cell types [14]. The NOX complex subunits p22^{phox}, gp91^{phox} (NOX2), p67^{phox}, and p47^{phox} have been identified in cardiomyocytes [15–17]. Previously, we have shown that Hcy induced apoptosis in cardiomyocytes through a nuclear NOX2-mediated ROS production mechanism and also induced membrane flip-flop and necrosis [18]. In a subsequent study, we also revealed that the induction of membrane flip-flop was due to inactivation of flippase [19]. Taken together these findings in part could explain the development of heart failure, found in HHC patients [20]. However, whether Hcy is the causative factor of the cardiomyocyte damage or the increased SAH remains to be determined. This information is important for future therapy development in heart failure in patients with HHC, since it would provide a more specified target for putative therapeutics. Therefore, in the present study, we have analyzed the role of SAH in the induction of membrane flip-flop, apoptosis, and necrosis in cardiomyocytes.

Materials and methods

Cell cultures

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%.

Antibodies and chemicals

Monoclonal antibody 48 against NOX2 IgG 1 (1:28) was obtained from Sanquin Research at CLB, Amsterdam, The

Netherlands [21]. Cy3-labeled goat-anti-rabbit-Ig (1:75) (Alexa Fluor Leiden, Netherlands) was used as a secondary antibody. Polyclonal α-Nitrotyrosine IgG (1:50) (Invitrogen, Eugene, OR, USA) was used as an antibody to measure nitrotyrosin residues, which is an indicator for ROS production. In this case, Cy5-labeled goat-anti-rabbit-Ig (1:50) (Jackson Immuno Research, West Grove, PA, USA) was used as a secondary antibody. The same procedure was followed for the immunofluorescent staining with goat anti-p47^{phox} antibody (1:50) (c-20 Santa Cruz Biotechnology Inc, CA, USA) where we used Cy3-labeled donkey-anti-goat-Ig (1:40) (Alexa Fluor 568, Invitrogen) as a secondary antibody. Again, we co-stained for nitrotyrosine; however, now we used FITC-labeled swine-anti-rabbit (1:50) (DakoCytomation, Glostrup, Denmark) as a secondary antibody. Isotype controls and PBS were used to determine aspecific binding.

Cells were incubated with either 2.5 mM D,L-Homocysteine (Hcy), with 50 μM adenosine-2,3-dialdehyde (ADA), with 100 nM, 10 μM, or 100 μM SAH or with 10 μM apocynin (all from Sigma, St. Louis, MO, USA) in growth medium for 24 h at 37°C in a humidified 5% CO_2/95% air atmosphere.

Measuring Hcy concentration in growth medium

Since we wanted to establish the effect of the inhibition of SAH hydrolase by ADA on the effect of Hcy concentrations, we determined the concentration of L-Hcy in culture medium of the incubated H9c2 cells. To this day, it is not possible to determine intracellular Hcy concentrations. Extracellular L-Hcy was measured by the Abbott IMx fluorescence polarization immunoassay (IMx Abbott Laboratories, Abbott Park, IL, USA). Intra- and interassay CVs were less than 2 and 4%, respectively. The concentration of Hcy in culture medium was measured before incubation ($t = 0$) and after 24 h of incubation.

Determination of intracellular *S*-adenosylmethionine and *S*-adenosylhomocysteine

We determined the intracellular concentration of SAM and SAH in H9c2 cells after 24 h of incubation with 2.5 mM D,L-Hcy or 50 μM ADA. Liquid chromatography tandem mass spectrometry (LC–MS/MS) was used for the determination of SAM/SAH concentrations as previously described.

Flow cytometry

Phosphatidylserine 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.

After treatment, the cells were trypsinized and centrifuged at 400×*g* for 5 min at room temperature. Cells were then washed with serum-free DMEM and resuspended in serum-free DMEM containing annexin V (1:40) for 30 min in the dark at 37°C in a humidified 5% CO_2/95% air atmosphere. Shortly before measuring, PI was added to the cell suspension (1:40). Cells 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 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 μM at 37°C for 30 min to allow internalization of the probe by flippase. Following, the cells were transferred to ice and 5 ml ice-cold PBS with BSA was added to wash off excess probe attached to the outside of the cell membrane. After 15 min, 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 was analyzed for Annexin V/PI positivity as described before.

Determination of ATP levels

After treatment, cells were collected by trypsinization and centrifugation at 400×*g* for 5 min. Cells were then counted and equal amounts were taken per condition. After centrifugation for 2 min (400×*g*), the supernatant was discarded. The pellet was resuspended in 150 μl of ice-cold perchloric acid (PCA) 0.4 M. Cells were left on ice for 30 min and then centrifuged again for 5 min (2,000*g*) 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 until measuring. The amount of ATP in the samples was determined with a luciferase–luciferin assay (Biaffin GmbH & Co KG, Kassel, Germany) according to the manufacturers' protocol. Samples were measured in a FluoroNunc MaxiSorp plate (Nalge Nunc International, Rochester, NY). Luminescence was measured by using a Tecan GENios Plus reader (Tecan Benelux, Mechelen, Belgium).

Detection of caspase-3 activity

Cells were grown in a 96-well plate (20,000 cells/well). After treatment, cells were lysed and incubated with DEVD-rhodamine 110 substrate (Roche, Mannheim, Germany) for 1 h at 37°C. Subsequently, the amount of free

rhodamine was determined at a microplate fluorescence reader (TECAN spectrafluor, 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. Each condition was measured in triplo per measurement (total of three measurements).

Immunofluoresence microscopy

To measure the expression of NOX2, $p47^{phox}$ and the putative formation of nitrotyrosine as an indirect measurement of reactive oxygen species (ROS), cells were incubated with or without 2.5 mM D,L-Hcy or 50 μM ADA for 24 h in the 4-well chamber slides (Nalge Nunc International). Cells were washed with PBS and fixated with 4% formaldehyde for 10 min at 37°C. Cells were subsequently washed with PBS, permeabilized with acetone-methanol (70–30%) for 10 min at −20°C, and then washed again with PBS/Tween-20 (0.05% (v/v) Tween-20 in PBS). Subsequently cells were incubated with primary antibodies for 60 min at room temperature followed by incubation overnight at 4°C. PBS and isotype controls were also tested to determine nonspecific binding of the antibodies and background signal. The following day, the cells were washed with PBS/Tween and incubated with the secondary antibodies for 30 min at room temperature. After subsequent washes in PBS/Tween and PBS, the slides were sealed in mounting medium containing DAPI (Vector Laboratories Inc, Burlingame, CA, USA) to visualize nuclei. Thereafter, the slides were covered with coverslips.

The generation of H_2O_2 was analyzed in live cells, grown in Delta-T dishes (0.17 mm, clear; Bioptechs Inc.; Butler, PA, USA). After treatment, the cells were loaded with 10 μM 5-(*6*)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Leiden) in ADS buffer (116 mM NaCl, 5.3 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.13 mM $NaH_2PO_4 \cdot H_2O$, 20 mM HEPES, and 1 mM $CaCl_2$, pH 7.4) and incubated for 15 min at 37°C. The cells were then incubated in ADS buffer for 25 min at 37°C, allowing the oxidized CM-H_2DCFDA to accumulate in the cells.

All cells were analyzed by means of a 3I Marianas™ digital imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope Carl Zeiss, Sliedrecht, Netherlands), equipped with a nanostepper motor (Z-axis increaments 10 nm) and a cooled CCD camera (Cooke Sensicam, 1280 × 1024 pixels Cooke Co, Tonawanda, NY, USA). Visualization of NOX2 and nitrotyrosine was performed with a 40× air lens. The microscope, camera, and data viewing process were controlled by SlideBook™ software (version 4.0.8.1 Intelligent Imaging Innovations, Denver, CO, USA).

Statistics

Statistics were performed with the SPSS statistics program (windows version 9.0). To evaluate whether observed differences were significant, one-way ANOVA with Post-Hoc Bonferroni tests was used. All values are expressed as mean ± standard error of the mean (SEM). A *P* value (two sided) of 0.05 or less was considered to be significant.

Results

Measurement of extracellular homocysteine, intracellular *S*-adenosylhomocysteine concentration, and intracellular SAM/SAH ratio

In the present study, we evaluated the role of SAH in the homocysteine-induced decrease in cardiomyocyte viability. First, we analyzed the intracellular concentrations of SAH after incubation with either un-supplemented growth medium, 2.5 mM D,L-Hcy or 50 μM ADA for 24 h. We found that incubation of H9c2 cells with Hcy resulted in a significant increase in SAH from 1.5 ± 0.8 nM in control cells to 68 ± 2.2 nM in Hcy treated cells (Fig. 2b; $P < 0.001$, $n = 4$), while incubation with 50 μM ADA resulted in an even higher significant increase to 83.5 ± 3.1 nM SAH compared to control and 2.5 mM D,L-Hcy (both $P < 0.001$, $n = 4$).

We next determined the extracellular L-Hcy concentration in culture medium after incubation with Hcy or ADA for 24 h. Incubation with 2.5 mM D,L-Hcy resulted in a significant increase of up to 1513 ± 10.2 μM L-Hcy in medium (Fig. 2a; $P < 0.001$, $n = 4$). Incubation with 50 μM ADA [22], however, did not result in an increase of Hcy. In fact it resulted in a slight decrease to 1.6 ± 0.2 μM L-Hcy compared to 2.8 ± 0.3 μM L-Hcy in control cells. Information about the intracellular Hcy concentration would have been a valuable addition to our current data. However, we were unable to assess these levels since very few laboratories are able to measure intracellular Hcy concentrations [23].

In control cells, a high SAM/SAH ratio was found of 45.9 ± 2.7 (Fig. 2c) indicating the presence of more SAM than SAH. In contrast, this ratio was significantly decreased to 1.3 ± 0.1 in Hcy and ADA-treated cells (both $P < 0.001$). There was, however, no significant difference in the SAM/SAH ratio between Hcy- and ADA-incubated cells. This indicates that both incubations with Hcy or ADA result in increased SAH levels and a decreased SAM/SAH ratio. ADA, however, does not increase extracellular Hcy concentrations, as expected, since it inhibits SAH hydrolase thereby blocking Hcy formation [12].

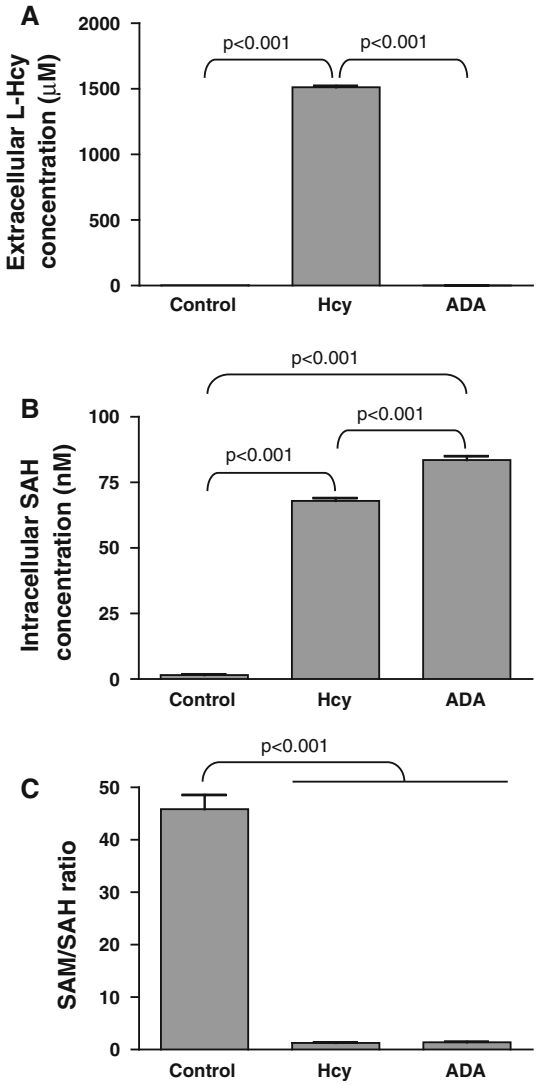

Fig. 2 Extracellular L-Homocysteine (Hcy) concentrations, intracellular *S*-Adenosylhomocysteine (*SAH*) concentrations, and SAM/SAH ratio. H9c2 cells were incubated with 2.5 mM D,L-Hcy or 50 μM ADA during 24 h and **a** the extracellular L-Hcy was determined in cultured medium of the different conditions, **b** the intracellular SAH concentrations were determined, and **c** the SAM/SAH ratio as a measure for methylation potential was determined. Data represent mean ± SEM ($n = 4$)

Effect of Hcy, ADA, and extracellular SAH on cell viability

Previously, we have shown that incubation of H9c2 cells with 2.5 mM Hcy during 24 h induced plasma membrane flip-flop, apoptosis, and necrosis [18]. We now found that whereas 2.5 mM Hcy did result in a significant decrease of viable cells compared to the control cells, 50 μM ADA did not result in a significant decrease, (Fig. 3a; $P < 0.001$, $n = 20$). Also, ADA did not induce an increases in single annexin V-positive cells, nor in annexin V/PI-positive cells, compared to control cells, whereas Hcy did (Fig. 3b and c; $P < 0.001$ and $P < 0.01$, respectively). Finally, the number of single PI-positive cells did not differ between these three different groups (Fig. 3d). The effects of extracellular SAH on H9c2 cell viability were also analyzed. Unlike Hcy, increasing concentrations of extracellular SAH (100 nM, 10 μM and 100 μM) did not induce a decrease in viability, nor an increase in annexin V and/or PI positivity (Fig. 3a–d).

In line with this, ADA did not induce apoptosis as measured via caspase-3 activity, whereas Hcy did induce a significant increase of 77.7 ± 6.6% in caspase-3 activity compared to control cells (Fig. 4a; $P < 0.001$, $n = 9$).

In conclusion, unlike Hcy, ADA, as well as extracellular SAH, did not induce membrane flip-flop, necrosis, and/or apoptosis in cardiomyocytes.

Effect of Hcy, ADA, and extracellular SAH on flippase activity

In a previous study, we found that Hcy induced membrane flip-flop by inhibiting flippase activity in cardiomyocytes [18]. Flippase is one of the membrane proteins, which has been found to regulate transbilayer phospholipid asymmetry in an ATP-dependent manner, by translocating phosphatidylserine (PS) from the outer to the inner leaflet of the plasma membrane [24]. We examined flippase activity via flow cytometry analysis using a PS-NBD probe that is internalized when flippase is active. As such the number of PS-NBD negative cells is a measure for flippase inactivity. Treatment with ADA or with extracellular SAH did not induce a significant increase in PS-NBD negative cells compared to control. Treatment with Hcy, however, did induce a significant increase in PS-NBD negative cells compared to control, ADA-incubated cells (Fig. 4c; 23.6 ± 3.6% (vs. control), 21.8 ± 3.0% (vs. ADA), 14.5 ± 2.1% (vs. SAH 100 nM), 23.6 ± 3.2% (vs. SAH 10 μM) and 18.5 ± 2.5% (vs. SAH 100 μM; $P < 0.001$ (vs control and ADA), $P < 0.05$ (vs. SAH), $n = 4$). Therefore, in line with the cell viability results, neither ADA nor extracellular SAH affected flippase activity, whereas Hcy did.

Effect of ADA and Hcy on ATP levels

Since flippase is ATP dependent, we also determined ATP levels in H9c2 cells after 24 h of incubation with either 50 μM ADA or 2.5 mM D,L-Hcy. We found that incubation with ADA did not result in a decrease of ATP compared to control. However, in concordance with our previous study, we did find a significant decrease of 38.9 ± 9.3% in ATP levels after incubation with Hcy (Fig. 4d; $P < 0.01$).

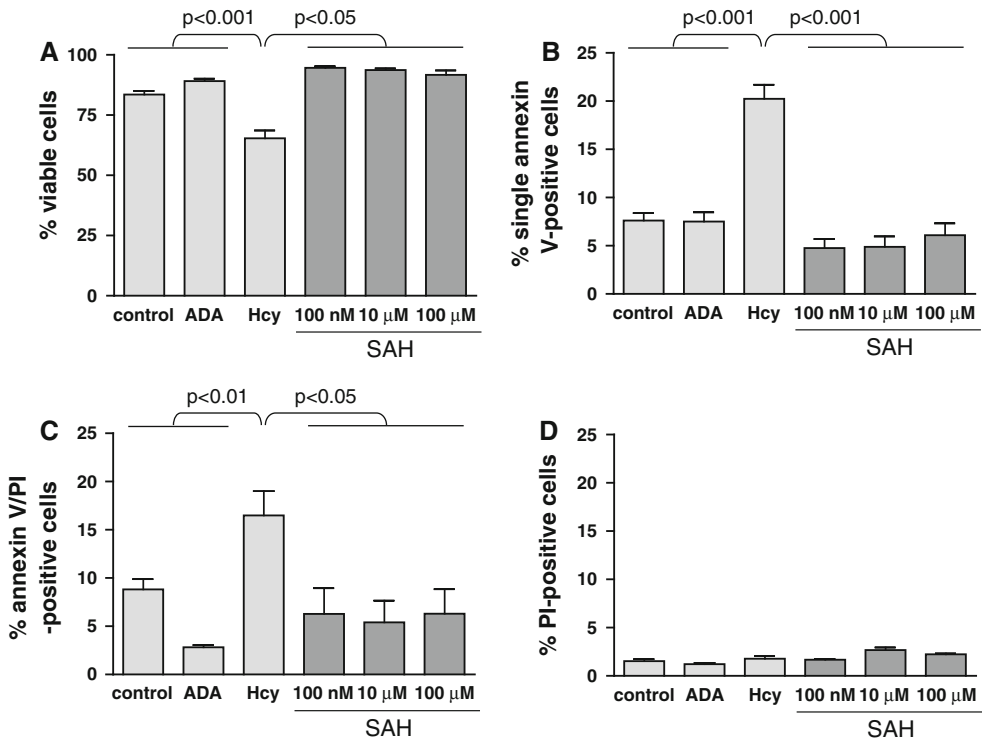

Fig. 3 Effects of Hcy, ADA, and extracellular SAH on cell viability. **a–d** Flow cytometry analysis of H9c2 cells incubated with 2.5 mM D,L-Hcy, 50 μM ADA or SAH (100 nM, 10 μM and 100 μM) for 24 h. A total of 10,000 cells were measured per sample ($n = 20$). **a** Percentage of viable cells, which are annexin V and propidium iodide (PI) negative. **b** Percentage of single annexin V-positive, PI-negative labeled cells, which is a marker of flip-flop of the plasma membrane phospholipids (reversible flip-flopped or apoptotic). **c** Percentage of double-annexin V/PI-positive cells depicting late apoptotic and/or necrotic cells. **d** Percentage of single PI-positive cells, as a measure of necrotic cells. All data represent mean percentages ± standard error of the mean (SEM)

Effect of Hcy, ADA, and extracellular SAH on nuclear NOX2 expression, ROS production, and p47^{phox} expression

Previously, we have also found that Hcy induced nuclear NOX2 expression coinciding with nuclear ROS production and apoptosis [18]. As we did not find an effect of ADA on apoptosis induction, we analyzed whether this could be explained by a lack of nuclear NOX2 translocation.

Hcy induced a significant increase of 42.2 ± 7.3% in nuclear NOX2 expression compared to control cells (Fig. 5a; $P < 0.05$; $n = 4$; Fig. 6I, II). Remarkably, also ADA resulted in a significant increase of nuclear NOX2 levels when compared to control cells (Fig. 5a; 113.1 ± 14.0%; $P < 0.001$). This increase was even significantly higher (70.9 ± 8.4%) than in Hcy treated cells (Fig. 5a; $P < 0.001$, $n = 4$; Fig. 6III). Notwithstanding this, ADA did not induce nuclear ROS production, as measured with nitrotyrosin and the generation of H_2O_2. This might explain the lack of apoptosis induction by ADA, while we did find a significant increase of nuclear ROS in H9c2 cells incubated with Hcy compared to control cells (Fig. 5b, g; $P < 0.001$, 26.2 ± 4.7% for nuclear tyrosine and 421.9 ± 17.4% for H_2O_2; $n = 4$; Fig. 6IV–VI). Extracellular SAH did not induce increased nuclear NOX2 expression (Fig. 5d), nor nuclear ROS (Fig. 5e, g).

Inhibition of ROS with apocynin did induce a significant decrease (76.0 ± 5.2%) of Hcy-induced caspase-3 activity (Fig. 4b; $P < 0.01$, $n = 6$), although the Hcy-induced active caspase-3 levels in the presence of apocynin were still significantly higher (69.0 ± 12.7%) than those in control cells ($P < 0.001$). These results indicate that ROS are necessary, at least in part, for Hcy-induced apoptosis.

Theoretically, the lack of ROS production, notwithstanding increased nuclear NOX2 expression in ADA-treated cells, could be related to a lack of nuclear p47^{phox} expression, which is a crucial non-catalytic subunit, in induction of apoptosis, also in cardiomyocytes [25]. For this, we performed 3D confocal stackings to acquire optical sections of the cells to determine the exact intracellular location of p47^{phox}. We then found that Hcy induced cytosolic and more importantly, nuclear p47^{phox} expression (Fig. 5c; 203.4 ± 15.9%, $P < 0.001$, $n = 4$ and Fig. 6-VIII) that was virtually absent in control cells (Fig. 6VII). In contrast, no increased nuclear p47^{phox} expression was found in ADA-treated cells (Figs. 5c, 6VIIII) nor in extracellular SAH-treated cells (Fig. 5f). From this, we can conclude that albeit an induction of nuclear NOX2

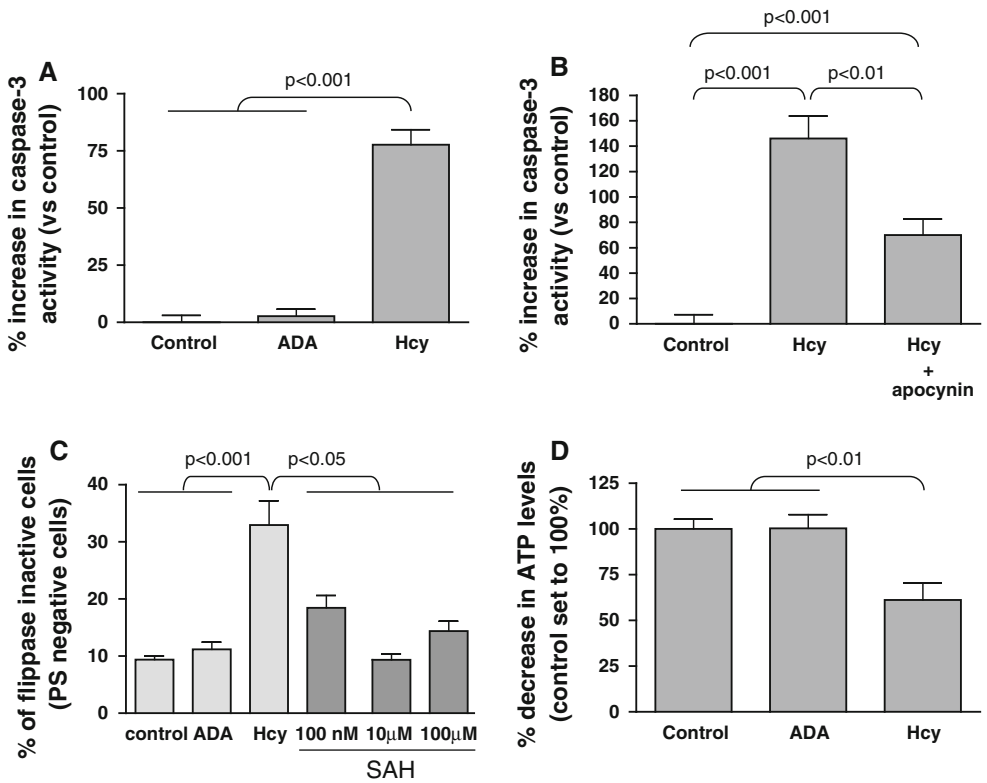

Fig. 4 Effects of Hcy, ADA, and extracellular SAH on caspase-3 activity, flippase activity, and ATP levels. **a**, **b** Caspase-3 activity in H9c2 cells that were incubated with 2.5 mM D,L-Hcy with or without 10 μM apocynin or with 50 μM ADA ($n = 9$). Caspase-3 activity was measured as indicated under "Materials and methods" section. **c** Flow cytometry analysis of flippase activity in H9c2 cells incubated with 2.5 mM D,L-Hcy, with 50 μM ADA, or with SAH (100 nM, 10 μM, and 100 μM) for 24 h using NBD labeled PS. PS-NBD negative cells represent cells where flippase is inactive since PS-NBD is no longer internalized. Percentage of PS negative cells is shown. **d** ATP levels in H9c2 cells that were incubated during 24 h with 2.5 mM D,L-Hcy or with 50 μM ADA ($n = 8$). ATP levels were measured as indicated under "Materials and methods" section. All data represent mean percentages ± SEM

expression was found, activation of the active NOX2 complex in cardiomyocytes is prohibited during ADA incubation, probably related to a lack of nuclear p47^{phox} expression, as such preventing nuclear ROS production and eventually apoptosis.

Discussion

It has been suggested that SAH rather than Hcy is the culprit in HHC-induced cardiovascular pathogenesis [13]. In a previous study, we found that increased Hcy resulted in membrane flip-flop, apoptosis, and necrosis of cardiomyocytes [18]. Incubation of cardiomyocytes with Hcy will, in addition to an increase in Hcy levels, also result in an increase of intracellular SAH levels. In the present study, we therefore analyzed the effect of ADA, a known SAH hydrolase inhibitor, which solely increases intracellular SAH since Hcy formation is blocked (see Fig. 2) [12] and the effect of extracellular SAH. In accordance with our previous study, we found that Hcy incubation resulted in membrane flip-flop (due to inhibition of flippase inactivity), induction of necrosis, a decrease in ATP levels, and induction of apoptosis due to nuclear NOX2/p47^{phox}-mediated ROS production. Remarkably, none of these Hcy effects were induced by ADA, except for induction of nuclear NOX2 expression. However, in cells incubated with ADA, the lack of nuclear p47^{phox} expression apparently prohibited nuclear ROS production and also no inhibition of flippase activity was found. Remarkably, the increase in nuclear NOX2 expression due to incubation with ADA was approximately twice as much as after incubation with Hcy. This could suggest that in response to the lack of p47^{phox} translocation to the nucleus by ADA, the cells react by increasing nuclear NOX2 expression. Like ADA, incubation with SAH did not have an effect on cell viability, nor on flippase activity, nor on nuclear p47^{phox} expression or nuclear ROS production. However, unlike ADA, SAH had no effect on nuclear NOX2 expression. This absence of effects may suggest that extracellular SAH does not enter the cells. Indeed, recently culturing of lymphoblasts in the presence of SAH (up to 25 μM) did not lead to increased intracellular SAH levels (unpublished results of our laboratory).

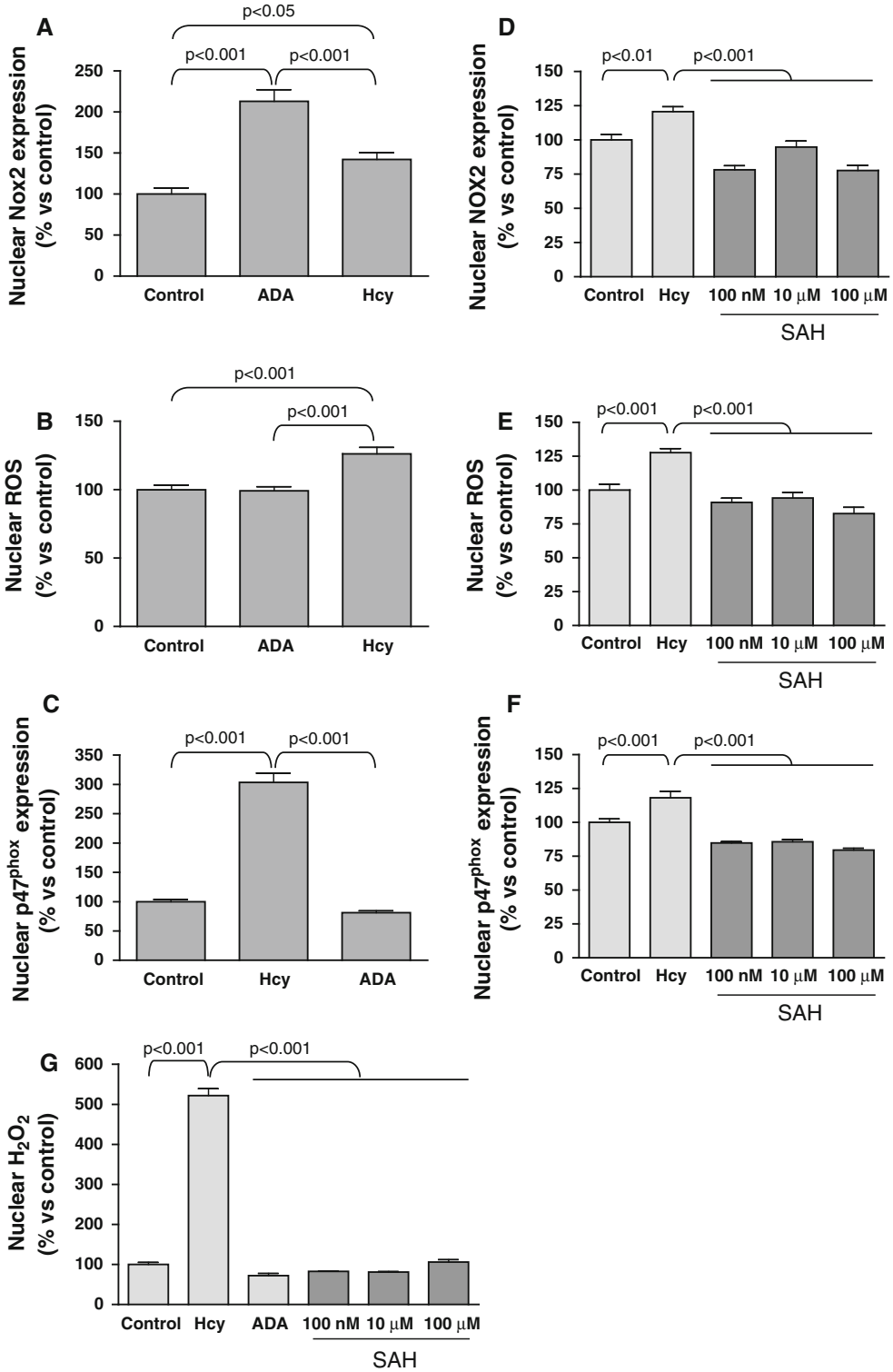

Fig. 5 Quantification of the nuclear expression of NOX2, $p47^{phox}$, and nitrotyrosine under Hcy, ADA, and extracellular SAH. H9c2 cells were treated with either 2.5 mM D,L-Hcy, 50 μM ADA or SAH (100 nM, 10 μM, and 100 μM) for 24 h, and the subcellular expression of NOX2, $p47^{phox}$, and nitrotyrosine was quantified using digital imaging microscopy. **a** and **d** Nuclear localization of NOX2 expression. **b** and **e** Nuclear localization of nitrotyrosine. **c** and **f** Nuclear localization of $p47^{phox}$. **g** Nuclear generation of H_2O_2. Data represent mean ± SEM ($n = 4$)

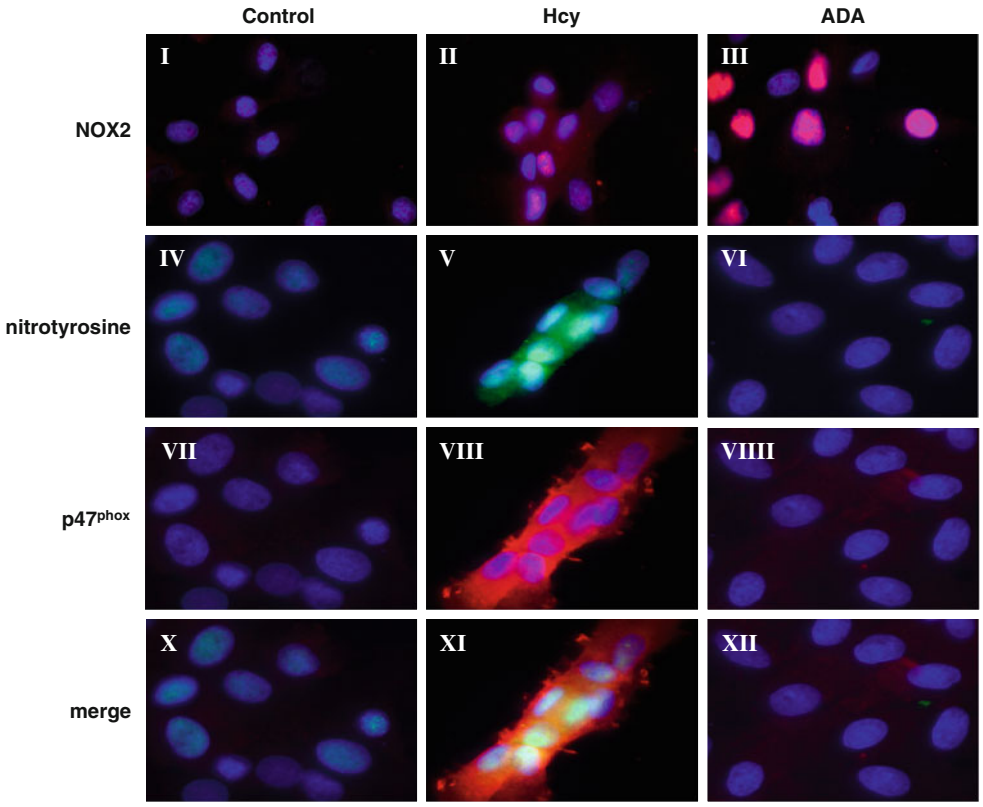

Fig. 6 Nuclear expression of NOX2, p47^{phox}, and nitrotyrosine under Hcy, and ADA. H9c2 cells were treated with either 2.5 mM D,L-Hcy or 50 μM ADA for 24 h and analyzed for the subcellular expression of NOX2, p47^{phox}, and nitrotyrosine using digital imaging microscopy. Images *I*, *II*, *III* NOX2 expression (*red*); Images *IV*, *V*, *VI* nitrotyrosine expression (*green*) as a marker for ROS production; Images *VII*, *VIII*, *VIIII* p47^{phox} expression (*red*); Images *X*, *XI*, *XII* merged images of p47^{phox} (*red*) and nitrotyrosine (*green*). In all images DAPI staining (*blue*) for DNA (nuclei) is shown. Magnification 40×. (Color figure online)

Previous studies, unrelated to Hcy, have shown in cardiomyocytes and hepatocytes that p47^{phox} is a crucial subunit in the active ROS-producing NOX complex. Qin et al. [26] have shown that Angiotensin II induced apoptosis in H9c2 cells via an increase in cytoplasmic and apparent nuclear p47^{phox} expression as determined by western blot and immunocytochemistry, which resulted in intracellular ROS production; however, the effect on other subunits such as NOX2 was not determined. Reinehr et al. [27] have shown in p47^{phox} knock-out mice that hepatocytes no longer produced ROS. Furthermore, Heymes et al. [28] found an increase in translocation of p47^{phox} to the sarcolemmal membrane of cardiomyocytes in failing human hearts compared to non-failing hearts, which coincided with increased membrane and intracellular gp91^{phox} expression, analyzed by immunohistochemistry, which could suggest that both p47^{phox} and gp91^{phox} are necessary for cardiomyocyte damage. Heymes et al. did not, however, examine nuclear expression patterns as we did in the present study, in which we show that Hcy induced an increase in nuclear expression of both NOX2 and p47^{phox} which coincided with apoptosis of the cardiomyocyte.

The function and location of the several subunits of the NOX complex have been studied extensively in polymorphonuclear neutrophils (PMNs). In PMNs, gp91^{phox} (NOX2) is located in the plasma membrane and during activation p47^{phox} translocates from the cytosol to the membrane to form a complex with NOX2 [29]. For an active NOX complex to occur, both NOX2 and p47^{phox} have to be phosphorylated, and in neutrophils, it has already been established that NOX2 is phosphorylated by protein kinase C (PKC) [30]. It has been shown that Hcy induces activation of PKC and as such activates the NOX complex in neutrophils by translocation of p47^{phox} and p67^{phox} to the plasma membrane [31, 32]. However, until now, no studies have shown PKC activation through SAH. Albeit ADA, in contrast to Hcy, did not decrease ATP levels, a lack of phosphorylation potential is unlikely to be the explanation for the lack of NOX activation by ADA. The actual effect of ADA on the phosphorylation level of p47^{phox} is now subject of further study.

We have previously found in cardiomyocytes that Hcy induced membrane flip-flop was in part due to inactivation of flippase [18]. In the present study, we have shown that

this membrane flip-flop is not related to an increase in SAH alone, since incubation with ADA did not result in a decrease in flippase activity.

The concentration of Hcy used in the current study is higher than physiologically occur in humans. However, in our previous study, we have already shown that Hcy concentration in growth medium decreased significantly during 24 h incubation. Furthermore, short-term exposure to high Hcy concentrations may reflect a prolonged exposure to moderately elevated Hcy concentrations as occur lifelong in patients. The SAH concentrations that we measured after incubation with ADA and Hcy, however, are comparable to the concentrations found intracellular in erythrocytes of cardiovascular patients [33]. Another limitation of the present study is that it was not possible to determine the intracellular concentration of Hcy since very few laboratories are able to measure this. Therefore, we cannot state that incubation with ADA does not result in increased intracellular Hcy levels. However, it is not expected that ADA increases Hcy since it inhibits SAH hydrolase thereby blocking Hcy formation [12].

Increased levels of Hcy as a risk factor for cardiovascular disease and especially heart failure have been described by several studies [20, 34]. There is an ongoing debate, however, on whether increased Hcy or its related increased SAH is the causative factor in cardiovascular disease. It has been postulated that increased SAH is the main causative factor since it induces hypomethylation of DNA, RNA, and proteins, which could act as a possible disease mechanism [35]. However, these previous studies do not provide conclusive evidence that increased SAH alone is the causative factor in Hcy-induced cellular damage, since conclusions were drawn when increased Hcy levels in patients were also present. In the present in vitro study in cardiomyocytes, we have elucidated that membrane flip-flop, apoptosis, and necrosis only occur after incubation with Hcy, with coinciding increased levels of Hcy and SAH, but not by increased SAH levels alone. This is probably due to a lack of $p47^{phox}$ translocation by SAH, preventing an active ROS-producing NOX complex. Furthermore, increased SAH alone did not inhibit flippase activity, prohibiting membrane flip-flop, while Hcy did.

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