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Chapter 4

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

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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), and (3) incubation with 2.5 mM D,L-Hcy (resulting in 68 nM intracellular SAH and 1513 μM extracellular L-Hcy). 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.

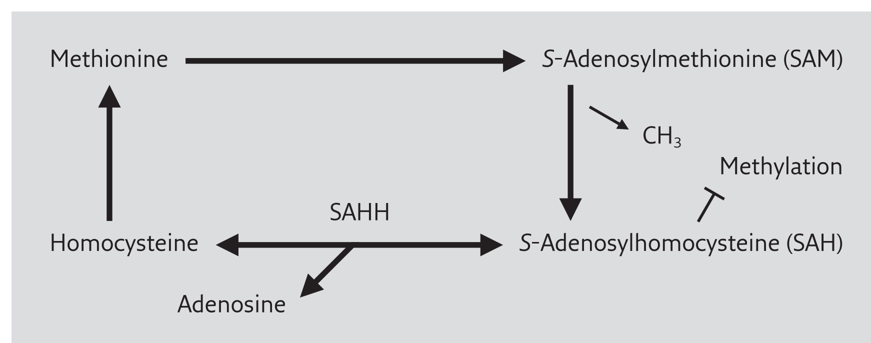
HHC-induced membrane flip-flop and apoptosis in cardiomyocytes is due to increased Hcy levels and not primarily related to increased SAH, which plays a crucial role in nuclear $p47^{phox}$ translocation.

Introduction

Hyperhomocysteinemia (HHC) as a risk factor for cardiovascular disease has been described in many *in-vitro* and *in-vivo* studies. Patient and animal studies have shown a positive correlation between increased Hcy levels with increased risk for cardiovascular complications including endothelial dysfunction, atherosclerosis and myocardial infarction.[1-6] *In-vitro* studies have proven that homocysteine (Hcy) causes proliferation of SMC,[7-10] but also oxidative stress[11] and cell death in endothelial cells.[12-16]

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.[17] Once the methylgroup is transferred to methyltransferases, *s*-adenosylhomocysteine (SAH) remains. SAH is subsequently

hydrolyzed to Hcy and adenosine (figure 1). Due to either genetic defects or deficiencies in co-factors such as vitamin B6, B12 and folate, accumulation of Hcy occurs.[18-20] 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.[21] Castro *et al.* have demonstrated in endothelial cells that increased SAH induced hypomethylation of DNA,[22] which may lead to inappropriate gene expression and ultimately promotion of endothelial dysfunction. James *et al.* suggested that SAH induced DNA hypomethylation is the main causative factor for HHC induced cardiovascular pathogenesis.[23] 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.



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 normally is responsible for the generation of reactive oxygen species (ROS) during the respiratory burst in phagocytic cells, but it has also become clear that NOX is also involved in signaling and more pathological processes in other cell types.[24-26] The NOX complex subunits $p22^{phox}$, $gp91^{phox}$ (NOX2), $p67^{phox}$ and $p47^{phox}$ have been identified in cardiomyocytes,[27-29] and were found to increase after induction of hypertrophy and heart failure in guinea pig.[30] 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.[31] In a subsequent study we also revealed that the induction of membrane flip-flop was due to inactivation of flippase (unpublished results). Taken together these findings in part could explain the development of heart failure, found in HHC patients.[32-34] 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 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.[35] 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) or with 50 μM adenosine-2,3-dialdehyde (ADA) (both from Sigma, St. Louis, MO, USA) in growth medium for 24 hours 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 hours of incubation.

Determination of intracellular SAM and SAH

We determined the intracellular concentration of SAM and SAH in H9c2 cells after 24 hours 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.[36]

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 with Hcy or ADA, the cells were trypsinized and centrifuged at 400×*g* 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. 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[37] (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 mol/L at 37°C for 30 minutes 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 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 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 minutes. Cells were then counted and equal amounts were taken per condition. After centrifugation for 2 minutes (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 minutes and then centrifuged again for 5 minutes (2000 g) at 4°C. To the isolated supernatant 7.5 μl of K_2CO_3 5M 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-wells plate (20,000 cells/well). After treatment with Hcy or ADA, cells were lysed and incubated with DEVD-rhodamine 110 substrate (Roche, Mannheim, Germany) for one hour 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 3 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 hours in the 4-well chamber slides (Nalge Nunc International, Naperville, IL, USA). Cells were washed with PBS and fixated with 4% formaldehyde for 10 minutes at 37°C. Cells were subsequently washed with PBS, permeabilized with acetone-methanol (70-30%) for 10 minutes 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 minutes 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 minutes 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.
Subsequently, 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).

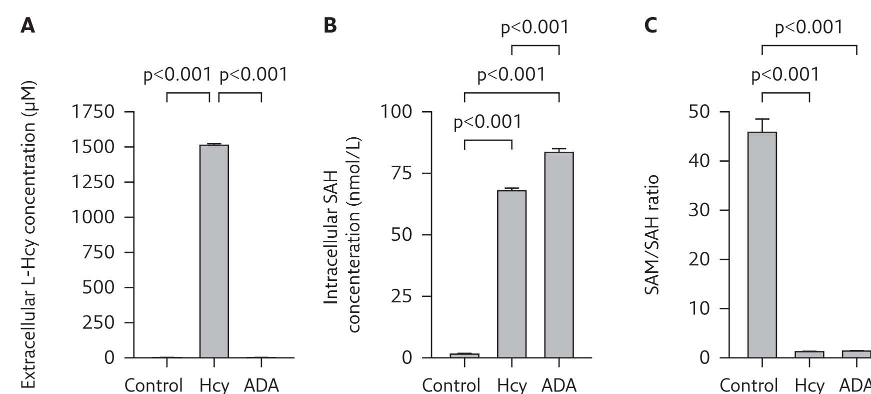
Statistics

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 were 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 (Hcy), intracellular *S*-adenosylhomocysteine (SAH) 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 hours. We found that incubation of H9C2 cells with Hcy resulted in a significant increase in SAH from 1.5 nM (SEM ±0.8) in control cells to 68 nM (SEM ±2.2) in Hcy treated cells (figure 2B, $p<0.001$; n=4), while incubation with 50 µM ADA resulted in an even higher significant increase to 83.5 nM (SEM ±3.1) SAH compared to control and 2.5 mM D,L-Hcy (both $p<0.001$; n=4).

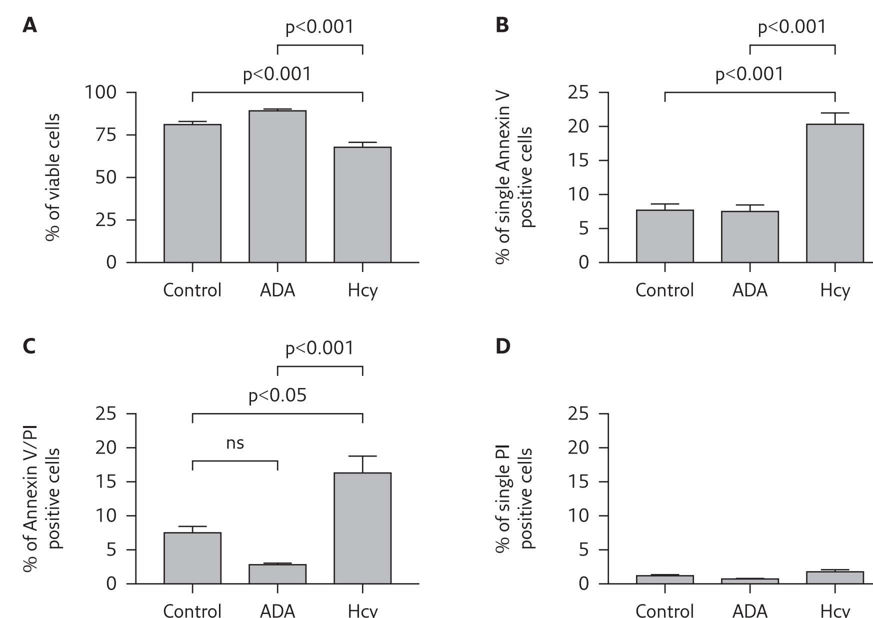


2. Extracellular L-homocysteine (Hcy) concentrations, intracellular S-adenosylhomocysteine (SAH) concentrations and SAM/SAH ratio | H9C2 cells were incubated with 2.5 mM D,L-Hcy or 50 µM ADA during 24 hours and (A) the extracellular L-Hcy was determined in cultured medium of the different conditions, (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).

We next determined the extracellular L-Hcy concentration in culture medium after incubation with Hcy or ADA for 24 hours. Incubation with 2.5 mM D,L-Hcy resulted in a significant increase of up to 1513 µM L-Hcy in medium (figure 2A, SEM ±10.2; p<0.001; n=4). Incubation with 50 µM ADA[38] however did not result in an increase of Hcy. In fact it resulted in a slight decrease to 1.6 µM L-Hcy (SEM ±0.2) compared to 2.8 µM L-Hcy (SEM ±0.3) 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.[39]
In control cells a high SAM/SAH ratio was found of 45.9 (figure 2C, SEM ±2.7) indicating the presence of more SAM than SAH. In contrast this ratio was significantly decreased to 1.3 in Hcy and ADA treated cells (both SEM ±0.1; 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.[22/40]

Effect of ADA and Hcy on cell viability

Previously we have shown that incubation of H9c2 cells with 2.5 mM Hcy during 24 hours induced plasma membrane flip-flop, apoptosis and necrosis.[31] We now found that 50 µM ADA did not result in a significant decrease of viable cells compared to the control, whereas 2.5 mM Hcy did induce a significant decrease compared to control after 24 hours (figure 3A, decrease of 13.3%, SEM ±2.9; p<0.001; n=15). The same was true for the amount of single Annexin V cells. No significant difference was found between ADA and control, whereas we did find a significant increase in the amount of single-Annexin-V-positive cells of 12.6% with Hcy compared to control (figure 3B, SEM ±1.7; p<0.001). Also the number of Annexin V/PI positive cells did not differ significantly between ADA and control. We even found a slight, but not significant reduction in Annexin V/PI positivity by ADA while a significant increase of 5.7% of Annexin V/PI positive cells was induced by Hcy compared to control (figure 3C, SEM ±2.5; p<0.05). Finally, the number of single PI positive cells did not differ between these three different groups (figure 3D).
In line with this, ADA did neither induce apoptosis as measured via caspase-3 activity, whereas Hcy did induce a significant increase of 77.7% in caspase-3 activity compared to control cells (figure 4A, p<0.001; SEM ±6.6; n=9).
In conclusion, ADA did not induce membrane flip-flop, necrosis and/or apoptosis in cardiomyocytes, while Hcy did.

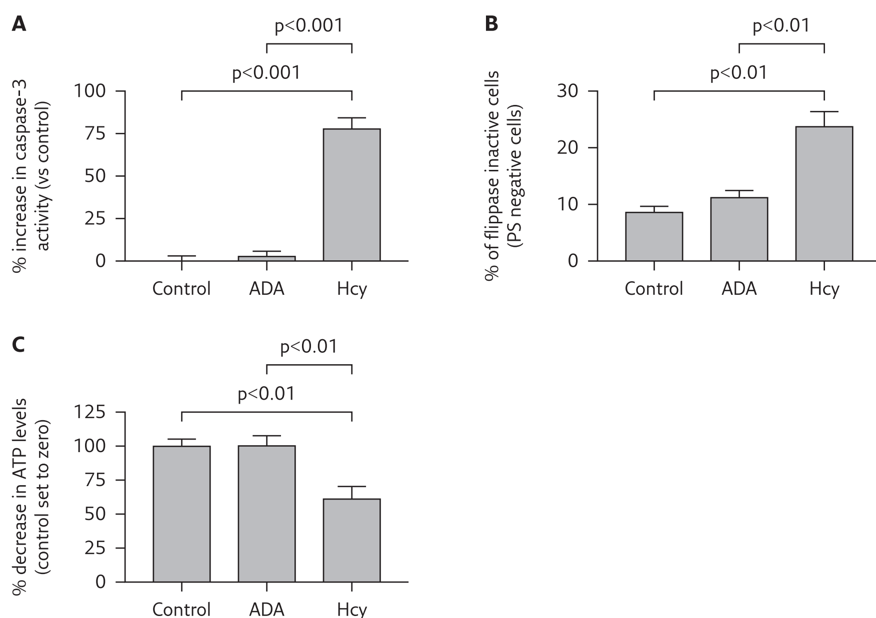


3. Effect of Hcy and ADA on cell viability | (A–D) Flow cytometry analysis of H9c2 cells incubated with 2.5 mM D,L-Hcy or 50 µM ADA during 24 hours. A total of 10,000 cells were measured per sample (n=15). (A) Percentage of viable cells, which are Annexin V and propidium iodide (PI) negative. (B) Percentage 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 ADA and Hcy on flippase activity

In a previous study, we found that Hcy induced membrane flip-flop by inhibiting flippase activity in cardiomyocytes. Flippase is one of the membrane proteins that has been found to regulate transbilayer phospholipid asymmetry in an ATP-dependent manner, by translocating phosphatidyl-serine (PS) from the outer to the inner leaflet of the plasma membrane.[41] We examined flippase activity via flow cytometry analysis using a PS-NBD probe, which is internalized when flippase is active. As such the number of PS-NBD negative cells is a measure for flippase inactivity. Treatment with ADA 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 and ADA incubated cells (figure 4B, 15.1%, SEM ±2.7; and 12.5%, SEM ±1.3 respectively; both $p<0.01$; n=4). For this, the inhibition of flippase activity by Hcy and the lack of flippase inhibition by ADA in part can explain their contradicting effects on membrane flip-flop.



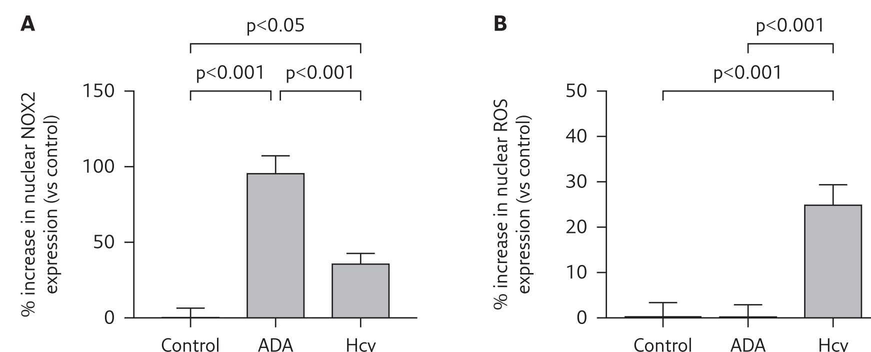
4. Effect of Hcy and ADA on caspase-3 activity, flippase activity and ATP levels (A) Caspase-3 activity in H9C2 cells that were incubated during 24 hours with 2.5 mM D,L-HCY or with 50 μM ADA (n=9). Caspase-3 activity was measured as indicated under Methods. (B) Flow cytometry analysis of flippase activity in H9C2 cells incubated with 2.5 mM D,L-HCY or 50 μM ADA for 24 hours using a PS-NBD probe. PS negative cells represent cells where flippase in inactive since the probe is no longer internalized. Percentage of PS negative cells in control cells and cells incubated with 2.5 mM D,L-HCY for 24 hours. (C) ATP levels in H9C2 cells that were incubated during 24 hours with 2.5 mM D,L-HCY or with 50 μM ADA (n=8). ATP levels were measured as indicated under Methods. All data represent mean percentages ± SEM.

Effect of ADA and Hcy on ATP levels

Since flippase is ATP dependent, we also determined ATP levels in H9C2 cells after 24 hours 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[31] we did find a significant decrease of 38.9% in ATP levels after incubation with Hcy (figure 4C, SEM ±9.3; $p<0.01$).

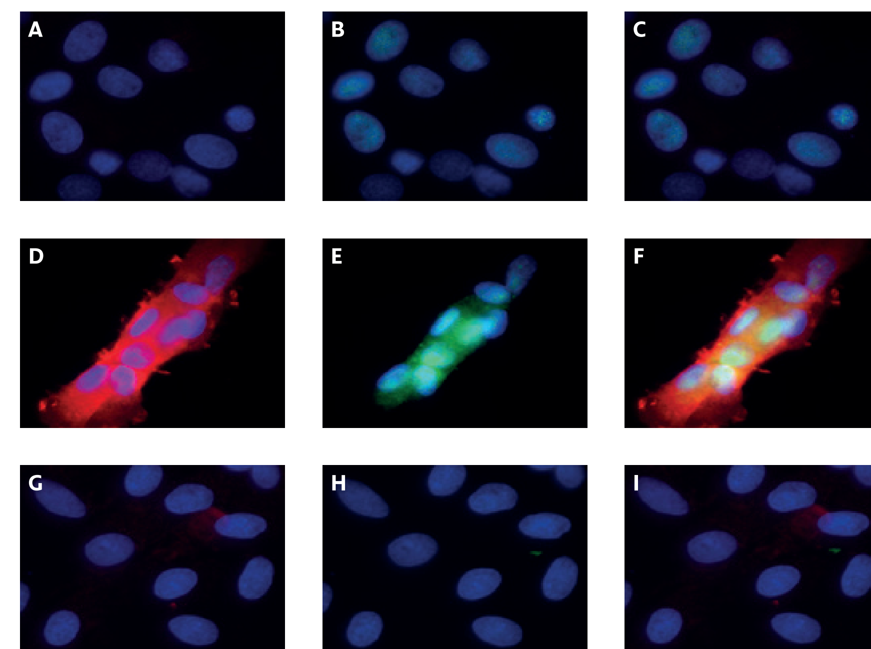
Effect of ADA and Hcy on nuclear NOX2 expression, ROS production and p47^{phox} expression

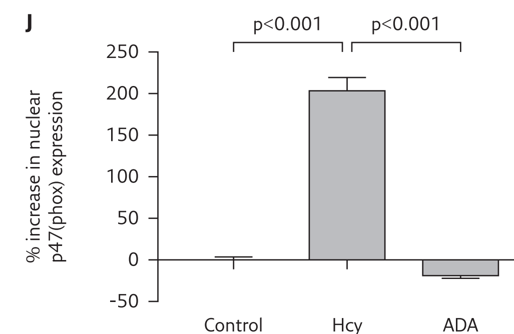
Previously, we have also found that Hcy induced nuclear NOX2 expression coinciding with nuclear ROS production and apoptosis.[31] As we did not find an effect of ADA on apoptosis induction, we analyzed whether this could be explained by a lack of nuclear NOX2 translocation. Remarkably, incubation of H9C2 cells with ADA resulted in a significant increase of 113.1% in nuclear NOX2 levels when compared to control cells (figure 5A, SEM ±14.0; $p<0.001$). This increase was even significantly more (70.9%) when compared to Hcy treated cells (figure 5A, SEM ±8.4; $p<0.001$; n=4). Hcy induced a significant increase of 42.2% in nuclear NOX2 expression compared to control (figure 5A, SEM ±7.3 for control; $p<0.05$; n=4). Notwithstanding this, ADA did not induce nuclear ROS production, which might explain the lack of apoptosis induction by ADA, while we did find a significant increase of 26.2% in nuclear ROS production in H9C2 cells incubated with Hcy when compared to controls (figure 5B, SEM ±4.7; $p<0.001$; n=4).



5. Effect of Hcy and ADA on intracellular location of NOX2 and nitrosylation | (A) Analysis of the effect of 2.5 mM D,L-HCY or 50 μM ADA during 24 hour incubation on the nuclear localization of NOX2 expression. (B) Analysis of the effect of 2.5 mM D,L-HCY or 50 μM ADA during 24 hour incubation on the nuclear localization of nitrotyrosine residues. Data represent mean ± SEM (n=4).

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.[42/43] 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 (figure 6J, 203.4%; $p<0.001$; SEM ±15.9; n=4), coinciding with ROS production, absent in control cells. In contrast, no $p47^{phox}$ expression or ROS production was found in ADA-treated cells (figure 6A-I). From this we can conclude that albeit an induction of nuclear NOX2 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.





6. Effect of Hcy and ADA on intracellular localization of $p47^{phox}$ in H9c2 cells | (A-I) Digital Imaging microscopy pictures (magnification 40x) of H9c2 cells where red signal (cy3) indicates $p47^{phox}$ expression (A/D/G), green (FITC) indicates nitrotyrosine expression as a marker for ROS production (B/E/H), and (C/F/I) shows merge picture of $p47^{phox}$ and nitrotyrosine. (A-C): Cells incubated in normal growth medium. (D-F): H9c2 cells incubated with 2.5 mM D,L-Hcy. (G-I): H9c2 cells incubated with 50 μM ADA. In all pictures blue DAPI staining for DNA (nuclei) is shown. (J) Analysis of the effect of 2.5 mM D,L-Hcy or 50 μM ADA during 24 hour incubation on the nuclear localization of $p47^{phox}$ expression measured by means of digital imaging microscopy. The arbitrary units are defined as sum intensity per nucleus and depicted as % increase vs control where control was set to 0 (n=4). Data represent mean ± SEM.

Discussion

It has been suggested that SAH, rather than Hcy is the culprit in HHC-induced cardiovascular pathogenesis.[23] In a previous study we found that increased Hcy resulted in membrane flip-flop, apoptosis and necrosis of cardiomyocytes.[31] 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 figure 2).[22] 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.

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 el.* 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,[44] however the effect on other subunits such as NOX2 was not determined. Reinehr *et al,* have shown in p47^{phox} knock-out mice that hepatocytes no longer produced ROS.[45] Furthermore, Heymes *et al.* 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,[46] 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.[47/48] 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).[49-51] It has been shown that Hcy induces activation of PKC[52] and as such activates the NOX complex in neutrophils by translocation of p47^{phox} and p67^{phox} to the plasma membrane.[53] 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.[31] 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 hour incubation.[31] Furthermore short-term exposure to high Hcy concentrations may reflect a prolonged exposure to moderately elevated Hcy concentrations as occur life-long 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.[54] 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.[22/40]

Increased levels of Hcy as a risk factor for cardiovascular disease and especially heart failure has been described by several studies.[32/34/55/56] 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[57/58] which could act as a possible disease mechanism.[23/59/60] 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 occurs 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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