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## **NADPH oxidases in the cardiovascular system**

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## Chapter 8

### **S-adenosylhomocysteine induces apoptosis and phosphatidylserine exposure in endothelial cells independent of homocysteine**

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## Abstract

We have previously shown that homocysteine (Hcy) induces phosphatidylserine (PS) exposure, apoptosis and necrosis in human endothelial cells. Since it has been suggested that *s*-adenosylhomocysteine (SAH) is the main causative factor in Hcy-induced pathogenesis of cardiovascular disease, we evaluate here whether the cytotoxic Hcy effect in endothelial cells is also SAH dependent.

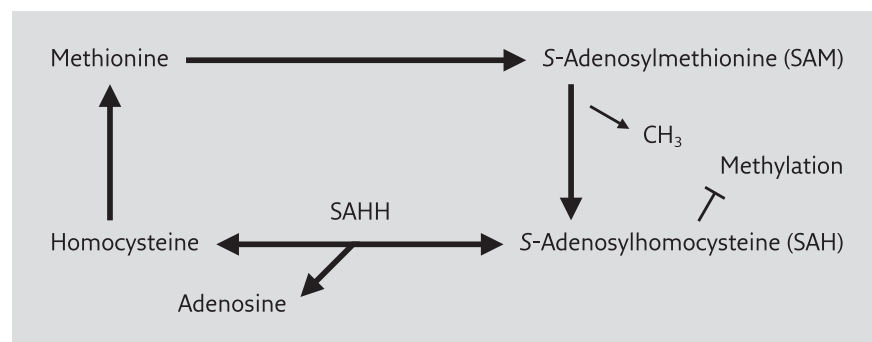
Human umbilical vein endothelial cells (HUVECs) were exposed to the following conditions: (1) non-treated control (resulting in 2.8 nM intracellular SAH and 3.1  $\mu$ M extracellular L-Hcy); and incubation with (2) 50  $\mu$ M adenosine-2,3-dialdehyde (ADA; resulting in 17.7 nM intracellular SAH and 3.1  $\mu$ M extracellular L-Hcy), (3) 2.5 mM Hcy (resulting in 20.9 nM intracellular SAH and 1.8 mM extracellular L-Hcy), and (4) 1, 10 and 100  $\mu$ M SAH. We then determined the effect of treatment on Annexin V-positivity, caspase-3 activity, cytochrome c release, (sub)cellular expression of NOX2, NOX4, p47<sup>phox</sup> and nitrotyrosine, and H<sub>2</sub>O<sub>2</sub>. Both Hcy and ADA significantly increased PS exposure (n=5), caspase-3 activity (n=6) and cytochrome c release (n=3). Incubation with extracellular SAH alone did not affect cell viability. Both Hcy and ADA also induced similar increases in nuclear NOX2 and (peri)nuclear NOX4, coinciding with (peri)nuclear p47<sup>phox</sup> expression and local reactive oxygen species (ROS) (n=3). Inhibition of NOX-mediated ROS by the flavoenzyme inhibitor diphenylene iodonium (DPI) significantly decreased apoptosis induction (n=3) and ROS production (n=3). SAH induces PS exposure and apoptosis in endothelial cells independently of Hcy. Our study therefore shows that Hcy-mediated endothelial dysfunction, as determined in the cell model used, is mainly due to SAH accumulation.

## 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 correlation between HHC and endothelial dysfunction, atherosclerosis and myocardial infarction.<sup>1-6</sup> *In-vitro* studies in endothelial cells have shown that homocysteine (Hcy) has a pathogenic effect in these cells via induction of oxidative stress resulting in cell death.<sup>7-10</sup> Several studies have suggested that the NADPH oxidase isoforms NOX2 and NOX4, and the cytosolic NADPH oxidase activating subunit p47<sup>phox</sup> are involved in Hcy-induced endothelial cell death.<sup>11-14</sup> In a previous study we have found that Hcy induces phosphatidylserine (PS) exposure (i.e. membrane flip-flop) and apoptosis in human endothelial cells, which was accompanied by NOX2/p47<sup>phox</sup> on the nucleus and (peri)nuclear NOX4/p47<sup>phox</sup>.<sup>15</sup>

Hcy is an amino acid of the methionine metabolism pathway (figure 1). In a first step methionine is activated to *s*-adenosylmethionine (SAM), which is the main methyl donor for methylation of DNA, RNA and proteins.<sup>16</sup> Once the methyl group is transferred via methyltransferases, *s*-adenosylhomocysteine (SAH) remains. SAH is then hydrolyzed by SAH hydrolase (SAHH) to Hcy and adenosine. Due to for example genetic defects or deficiencies in co-factors such as vitamin B6, vitamin B12 and folate, accumulation of Hcy can occur.<sup>17-19</sup> An increase in Hcy also results in an increase in SAH. SAH is known as a potent inhibitor of methylation since it can bind to methyltransferases, also in endothelial cells.<sup>20/21</sup> However, whether Hcy is the causative factor of the cardiovascular damage or the increased SAH is still not clear. This can be important for putative future therapy development to prevent endothelial dysfunction in HHC patients.

In the present study we therefore have studied putative differences between Hcy- and SAH-induced cell damage of endothelial cells, including the role of different NADPH oxidase isoforms herein.



**1. Homocysteine metabolism** | Schematic representation of methionine/homocysteine metabolism. Methionine (Met) is phosphorylated to *s*-adenosylmethionine (SAM), a potent methyl donor for methyltransferases. After donating the methyl group, *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.

## Materials and Methods

### Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured in Medium 199 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWhittaker), 10% heat-inactivated human serum (Sanquin, Amsterdam, The Netherlands), 5 µg/ml heparin (Leo Pharma BV, Breda, The Netherlands), 50 µg/ml endothelial cell growth factor (Sigma, St. Louis, MO, USA), 100 IU/ml penicillin (Yamanouchi Europe BV, Leiderdorp, Netherlands) and 100 µg/ml streptomycin (Radiopharma-Fisiopharma, Palomonte, Italy) at 37°C in a humidified 5% CO<sub>2</sub> / 95% air atmosphere. Experiments were performed at 100% confluence of the cells.

### Antibodies and Chemicals

Monoclonal 48 against NOX2 (1:28) was obtained from Sanquin Research at CLB, Amsterdam, The Netherlands.<sup>22</sup> Cy3-labeled goat anti-mouse (1:75; Invitrogen, Leiden, Netherlands) was used as secondary antibody. Polyclonal anti-nitrotyrosine (1:50) (Invitrogen, Eugene, OR, USA) was used as an antibody to measure nitrotyrosine residues, which is an indicator for ROS production. In this case, Cy5-labeled goat anti-rabbit (1:50; Jackson Immuno Research, West Grove, PA, USA) was used as secondary antibody. The same procedure was followed for the immunofluorescent staining with polyclonal anti-p47<sup>phox</sup> (1:50; Santa Cruz Biotechnology Inc, CA, USA) and polyclonal anti-NOX4 (1:50; Santa Cruz), where we used Cy3-labeled donkey anti-goat (1:40; Invitrogen) as secondary antibody. We co-stained for nitrotyrosine, but with FITC-labeled swine anti-rabbit (1:50; DakoCytomation, Glostrup, Denmark) as 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 6 hours at 37°C in a humidified 5% CO<sub>2</sub> / 95% air atmosphere. ADA inhibits the hydrolysis of SAH to Hcy and adenosine therefore increasing the SAH concentrations in the cell without increasing Hcy concentrations.<sup>21/23</sup>

## Measuring Hcy concentration in growth medium

Because several studies have suggested that only the L-form of Hcy is bioactive and the D-form has no cellular effect,<sup>24/25</sup> we determined the concentrations of extracellular L-Hcy. 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 6 hours of incubation.

## Determination of intracellular SAM and SAH

We determined the intracellular concentration of *s*-adenosylmethionine (SAM) and *s*-adenosylhomocysteine (SAH) in HUVECs after 6 hours of incubation with D,L-Hcy or ADA. Tandem mass spectrometry (MS/MS) was used for the determination of SAM/SAH concentrations as previously described.<sup>26</sup>

## Flow cytometry

Plasma membrane flip-flop and thus 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<sub>2</sub> / 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).

## 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 spec-

trafluor, 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 triple per measurement (total of 3 measurements).

## Immunofluorescence Microscopy

To measure the expression of NOX2, NOX4, p47<sup>phox</sup> and the putative formation of nitrotyrosine, cells were incubated with or without Hcy and ADA for 6 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. 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 covered 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<sup>TM</sup> digital imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Carl Zeiss, Sliedrecht, Netherlands), equipped with a nanostepper motor (z-axis increments; 10 nm) and a cooled CCD camera (Cooke Sensicam, 1280×1024 pixels; Cooke Co, Tonawanda, NY, USA). Visualization of NOX2, NOX4, p47<sup>phox</sup> and nitrotyrosine was performed with a 40× air lens. The microscope, camera and data viewing process were controlled by SlideBook<sup>TM</sup> software (version 4.0.8.1; Intelligent Imaging Innovations, Denver, CO, USA).

## Statistics

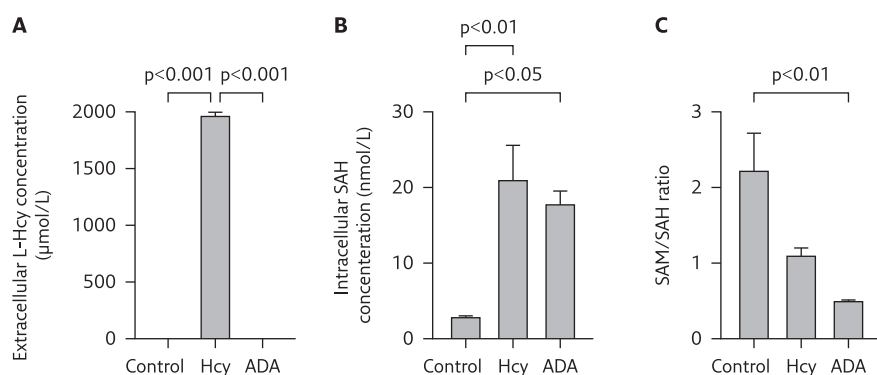
Statistics were performed with the SPSS statistics program (Windows version 9.0). To evaluate whether observed differences were significant, One Way ANOVA with Post Hoc Bonferroni tests or Student T-tests were used where appropriate. All values are expressed as mean ± standard error of the mean (SEM). A p-value (two sided) of less than 0.05 was considered to be significant.

## Results

### Measurement of extracellular Hcy and intracellular SAH concentrations and intracellular SAM/SAH ratios

After 6 hours incubation of endothelial cells with either 2.5 mM D,L-Hcy<sup>27</sup> or 50  $\mu$ M ADA<sup>28</sup> we determined the concentration of L-Hcy in the conditioned medium and the intracellular concentrations of SAM and SAH. Only incubation with D,L-Hcy resulted in a significant increase to  $1.8 \pm 0.4$  mM extracellular L-Hcy (figure 2A,  $p < 0.001$ ) compared to control. Incubation with ADA had no effect on extracellular L-Hcy concentration (0.003 mM). In contrast, the amount of intracellular SAH was significantly increased both with Hcy to  $20.9 \pm 9.3$  nM and with ADA to  $17.7 \pm 3.6$  nM (figure 2B,  $p < 0.01$  and  $p < 0.05$ , respectively) compared with control. For this, ADA incubation only results in an increase in intracellular SAH but not extracellular L-Hcy, while during incubation with 2.5 mM Hcy both intracellular SAH and extracellular L-Hcy are increased.

In line with this, the SAM/SAH ratio was reduced from  $2.2 \pm 0.5$  in control to  $1.0 \pm 0.1$  with Hcy (non significant) and to  $0.5 \pm 0.02$  with ADA (figure 2C,  $p < 0.01$ ).



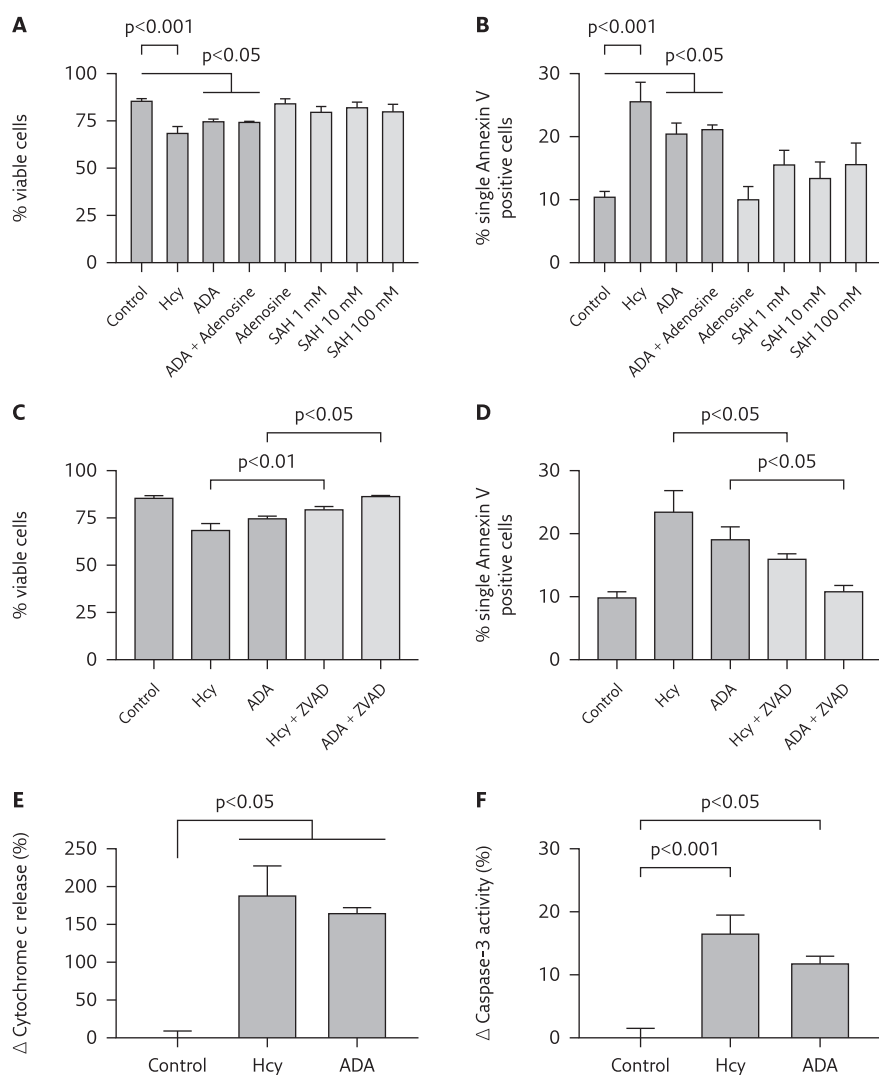
**2. Extracellular Hcy and intracellular SAH concentrations, and SAM/SAH ratio | HUVECs** were incubated with 2.5 mM D,L-Hcy (Hcy) or 50  $\mu$ M ADA (ADA) for 6 hours and subsequently (A) extracellular L-Hcy concentrations, and (B) intracellular SAH concentrations and (C) SAM/SAH ratios were determined. (n=4).

### Effect of Hcy and ADA on phosphatidylserine exposure in HUVECs

We next examined the effect of Hcy and ADA on cell viability in HUVECs. As depicted in figure 3, 6 hours of incubation with 2.5 mM D,L-Hcy resulted in a significant decrease of viable cells of  $12.3 \pm 3.3\%$  (figure 3A,  $p < 0.006$ ), and a significant increase in single Annexin v positive cells of  $9.1 \pm 2.7\%$ , compared to control (figure 3B,  $p < 0.01$ ). Incubation with ADA also resulted in a significant decrease in viable cells of  $5.35 \pm 2.30\%$  (figure 3A,  $p < 0.05$ ), and a significant increase in single Annexin v positive cells of  $4.64 \pm 1.68\%$ , compared to control (figure 3B,  $p < 0.002$ ). Although the effect of Hcy was more extensive compared to ADA, no significant differences in viable or single Annexin v positive cells were found between ADA and Hcy. The number of Annexin v/PI positive cells and single PI positive cells did not differ between the three groups (data not shown). Thus, both Hcy and ADA decreased the viability of HUVECs and induced PS exposure.

### Effect of Hcy and ADA on caspase-3 activity in HUVECs

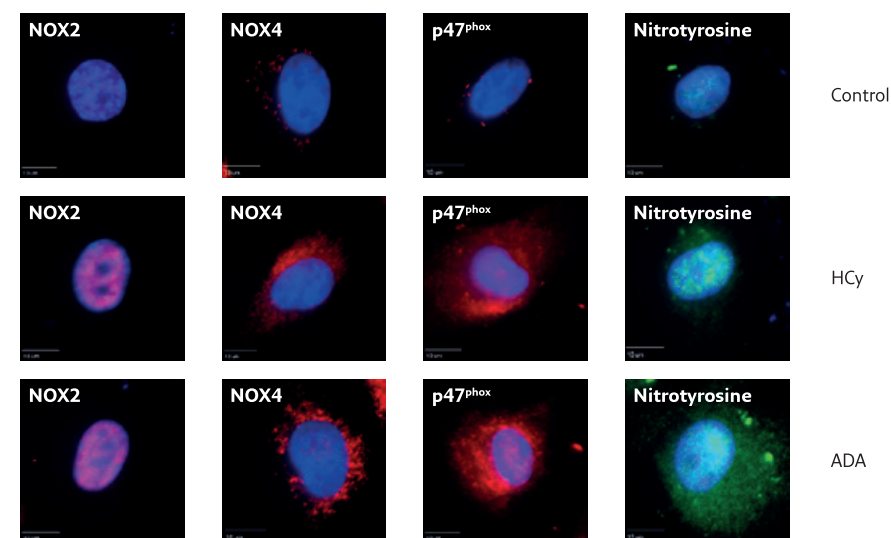
The exposure of PS can be a hallmark for apoptosis. Since both ADA and Hcy induced PS exposure we also determined the caspase-3 activity as a measurement of apoptosis. A significant increase in caspase-3 activity compared to control was found for both Hcy (with  $16.5 \pm 4.2\%$ ,  $p < 0.001$ ) and ADA (with  $11.7 \pm 1.9\%$ ,  $p < 0.05$ ) (figure 3C). Although the increase in caspase-3 activity was higher during Hcy compared to ADA, this was again not significantly different.



**3. Effect of Hcy, ADA, adenosine and extracellular SAH on cell viability** | (A-D) Flow cytometry analysis of HUVECs incubated with 2.5 mM D,L-Hcy (n=5), 50  $\mu$ M ADA (n=5), adenosine (100  $\mu$ M) or SAH (1  $\mu$ M, 10  $\mu$ M and 100  $\mu$ M) (n=3) for 6 hours. A total of 10,000 cells were measured per sample. (A) Percentage of viable cells. (B) Percentage of single Annexin v-positive cells, which is a marker for phosphatidylserine (PS) exposure. (C) Percentage of viable cells and (D) single Annexin v-positive cells in the presence of the pan-caspase inhibitor ZVAD (n=3). (E) Percentage of cytochrome c release (n=3) and (F) active caspase-3 (n=6) in HUVECs that were incubated with 2.5 mM D,L-Hcy or 50  $\mu$ M ADA. The changes are shown as the difference ( $\Delta$ ) compared to control cells set to 0%.

## Effect of Hcy and ADA on intracellular NOX2, NOX4 and p47<sup>phox</sup> expression and localized ROS production in HUVECs

We previously observed that Hcy-induced apoptosis coincided with nuclear NOX2/p47<sup>phox</sup> and (peri)nuclear NOX4/p47<sup>phox</sup> expressions, which were accompanied by nitrotyrosine accumulation.<sup>15</sup> Since incubation of HUVECs with ADA also induced apoptosis, we subsequently analyzed whether this was regulated by the same NOX proteins as did Hcy. For this, we incubated HUVECs with 2.5 mM D,L-Hcy or 50  $\mu$ M ADA and determined the (sub)cellular NOX2, NOX4 and p47<sup>phox</sup> expression and coinciding ROS production, using 3D confocal stack (figure 4). We found that both Hcy and ADA induced nuclear NOX2 and (peri)nuclear NOX4 expression coinciding with nuclear and (peri)nuclear p47<sup>phox</sup> expression and nitrotyrosine accumulation, indicative for local ROS production. Taken together, these results indicate that ADA does activate NOX proteins in endothelial cells, independent of Hcy, resulting in PS exposure and apoptosis.

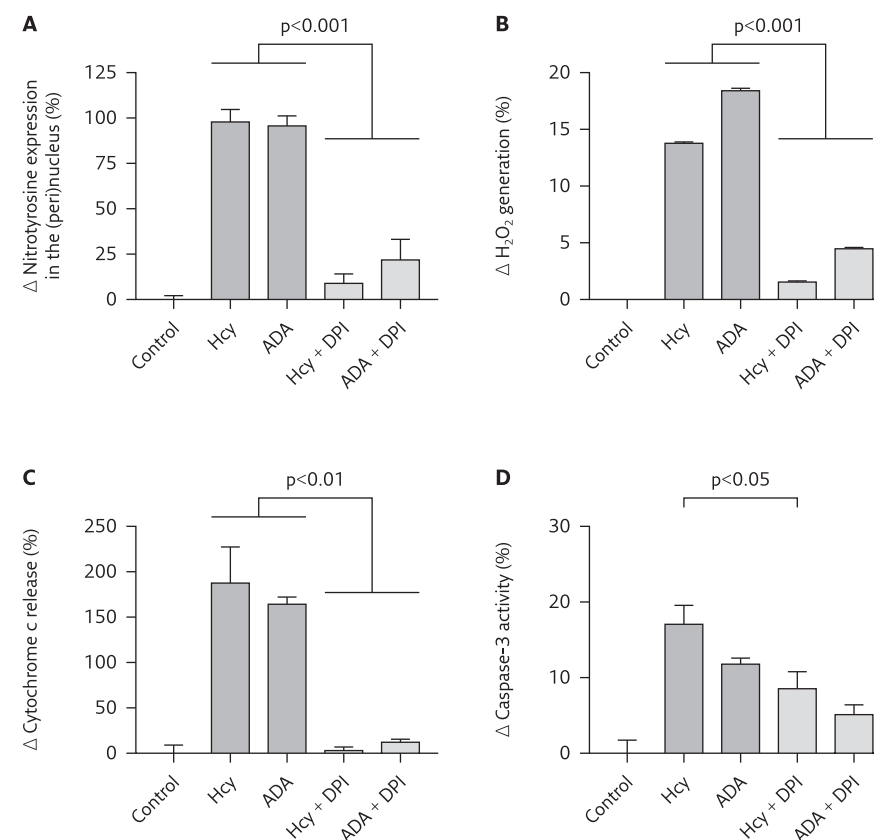


**4. Effect of Hcy or ADA on localization of NOX2, NOX4, p47<sup>phox</sup> and nitrotyrosine** | Digital-imaging microscopy pictures showing optical section of a 3D stack after deconvolution from a single nucleus (stained with DAPI, blue signal) and adjacent cellular area (stained for NOX2, NOX4 and p47<sup>phox</sup>, red signal, and nitrotyrosine, green signal) of HUVECs, which were incubated for 6 hours with 2.5 mM D,L-Hcy or 50  $\mu$ M ADA (n=3). Magnification 40x.

## Effect of NOX-mediated ROS inhibition on Hcy and ADA induced apoptosis

To analyze whether the increased (peri)nuclear NOX4 and NOX2 expression indeed leads to enhanced ROS production and induction of apoptosis, we measured (peri)nuclear nitrotyrosine expression, H<sub>2</sub>O<sub>2</sub> generation, cytochrome c release and caspase-3 activity after incubation with Hcy and ADA in the presence or absence of the antioxidant/NOX inhibitor DPI.

As expected, DPI significantly and completely inhibited both (peri)nuclear nitrotyrosine expression and H<sub>2</sub>O<sub>2</sub> generation compared to Hcy alone (88.9±5%,  $p < 0.001$  and 12.2±0.1,  $p < 0.001$ , respectively) and ADA alone (73.9±11%,  $p < 0.001$  and 13.9±0.2,  $p < 0.001$ , respectively) (figure 5A/B n=3). Compared to Hcy alone, DPI significantly inhibited Hcy-induced cytochrome c release and caspase-3 activity by 184.5±4% ( $p < 0.01$ ) and by 8.5±2% ( $p < 0.05$ ), respectively (figure 5C/D n=3). Furthermore, DPI also significantly inhibited ADA-induced cytochrome c release (151.9±3%,  $p < 0.01$ ), and resulted in a reduction of caspase-3 activity (6.7±2%), although this was not significant. This data strongly suggests, as we have previously shown for Hcy-induced apoptosis, a role for NOX-mediated ROS production in ADA-induced apoptosis.



**5. Effect of NOX-mediated ROS inhibition on Hcy and ADA induced apoptosis** | (A) Quantification of the (peri)nuclear expression of nitrotyrosine (n=3), (B) generation of H<sub>2</sub>O<sub>2</sub> (n=3), (C) percentage of cytochrome c release (n=3) and (D) caspase-3 activity (n=3) in HUVECs that were incubated with 2.5 mM D,L-Hcy or 50 μM ADA for 6 hours, in the absence or presence of the NOX-mediated ROS inhibitor diphenylene iodonium (DPI). The changes are shown as the difference (Δ) compared to control cells set to 0%.

## Discussion

Since there is an ongoing debate whether Hcy or increased SAH is the main causative factor in HHC induced cardiovascular disease,<sup>20/21/29</sup> we compared in the present study the effect of Hcy and SAH on apoptosis induction and PS exposure in human endothelial cells. In the present study we found that incubation with both Hcy and ADA induced PS exposure, caspase-3 activity and translocation of NOX2 and p47<sup>phox</sup>



to the nucleus and NOX4 and p47<sup>phox</sup> to the (peri)nuclear region coinciding with local nitrotyrosine accumulation, without further significant differences between Hcy and ADA. As ADA did not result in increased Hcy levels, we can conclude that these particular effects of ADA were related to increased SAH levels, independent of Hcy, because it was shown that ADA inhibits SAH hydrolyse, thereby blocking Hcy formation.<sup>21/3</sup>

Indeed, increased SAH correlates strongly with the incidence of vascular disease, as shown in several epidemiological correlation studies.<sup>29-32</sup> In addition, several *in-vitro* studies have also suggested SAH to be the causative factor in HHC induced apoptosis in BV-2 microglial cells, lymphoblasts and endothelial cells,<sup>33-35</sup> however none of these studies were performed under the condition of increased SAH alone. All these studies namely used a combination of SAH, adenosine and Hcy. Lin *et al.* showed that incubation of BV-2 microglial cells with combined 1  $\mu$ M SAH, 1 mM Hcy and 100  $\mu$ M adenosine during 24 hours, caused apoptosis in BV-2 microglial cells that was correlated with intracellular ROS production.<sup>33</sup> They however did not examine different NADPH oxidase isoforms in this respect. It was also shown that adenosine induced apoptosis of pulmonary endothelial cells by elevating levels of SAH and thereby causing DNA fragmentation, although in this study 100  $\mu$ M Hcy was also added.<sup>34/35</sup> These studies thus indicate the potential toxicity of increased SAH levels in various cell types and tissues, but, as in those studies increased Hcy was also present, no definite proof of an Hcy independent effect of SAH was given. Moreover, it has not been shown previously *in-vitro* or *in-vivo* that increased SAH levels activate different NADPH oxidase isoforms. Our study now is the first to show that increased levels of SAH alone, independent of Hcy, can induce apoptosis in endothelial cells coinciding with NADPH oxidase-mediated ROS.

Previous studies have shown the activation of different pathways through which endothelial cell apoptosis is induced by Hcy, namely the JNK pathway,<sup>10/11/36/37</sup> mitochondrial oxidative stress<sup>13/14/38</sup> or NADPH oxidase-mediated ROS.<sup>11-15</sup> We are now the first to show that HHC induced PS exposure and endothelial apoptosis can be induced by increased SAH alone, inducing the effects that Hcy exerts on nuclear NOX2/p47<sup>phox</sup> and (peri)nuclear NOX4/p47<sup>phox</sup> expression and nitrotyrosine accumulation. Therefore, our study shows that Hcy mediated endothelial dysfunction is due to SAH accumulation and supports the hypothesis that Hcy itself is not the preliminary toxic agent but rather SAH, and that SAH reduction could be a possible target in a preventive therapy to circumvent endothelial dysfunction.

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