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Chapter 9

Homocysteine induces reactive oxygen species-dependent apoptosis of arterial smooth muscle cells coinciding with (peri)nuclear NOX4 translocation

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Abstract

Elevated levels of homocysteine (Hcy) form a vascular risk factor. It has been shown that Hcy induces cell death in vascular smooth muscle cells (SMCs). Recent studies have shown that NADPH oxidase (NOX)-mediated reactive oxygen species (ROS) play a role in Hcy-induced apoptosis in cardiomyocytes and endothelial cells. In this study we have analyzed the role of Hcy on the different NOX isoforms in arterial SMCs.
Human arterial SMCs isolated from the human umbilical cord were incubated with 100 μM Hcy during 24 hours and were analyzed for cell viability using FACS analysis and caspase 3 activity. Diphenylene iodonium (DPI) and NOX4 small interference RNA (siRNA) were used to reduce NADPH oxidase activity and to analyze NOX4 in Hcy-reduced cell viability. The effects of Hcy on the expression of different NOX isoforms and NOX-mediated ROS production were then studied using digital-imaging microscopy.
Incubation of isolated arterial SMCs with 100 μM Hcy during 24 hours induced a significant increase in single Annexin V positive cells and caspase 3 activity. Furthermore, Hcy resulted in a significant shift of NOX4 from the cytoplasm to (peri)nuclear regions coinciding with significantly increased ROS production at these sites. No effects of Hcy on expression levels, nor location of NOX1 and NOX2 were found. Inhibition of NOX-mediated ROS production using DPI and knock-down of NOX4 using siRNA led to significant decreased apoptosis, NOX4 expression and nitrotyrosine levels. Hcy induced apoptosis of human arterial SMCs in which (peri)nuclear NOX4-mediated ROS plays a central role.

Introduction

It has been shown that elevated levels of homocysteine (Hcy) have a jeopardizing effect on the vasculature facilitating atherosclerosis.[1] High levels of Hcy namely promote oxidative stress in the vasculature causing endothelial cell injury and recruitment of leukocytes to the vessel wall, that subsequently infiltrate into the arterial wall.[2] With progression of atherosclerotic lesions, Hcy stimulates migration and proliferation of vascular SMCs, resulting in the production of extracellular matrix (ECM) components.[3] In contrast, it was also shown that Hcy decreased SMC viability *in-vitro*[4/5] and *in-vivo* in humans,[6] indicating contradicting effects of Hcy on SMCs. Reactive oxygen species (ROS) are known contributors in Hcy-induced injury, also in vascular SMCs.[3] Hcy (0.5 mM applied during 24 hours) namely decreased cell viability as measured via a tetrazolium (MTT) assay of rat aortic SMCs, coinciding with elevated levels of intracellular ROS, based on the oxidation of 2',7'-dichloroflu-

orescein (DCF).[4] In human aortic SMCs incubation with 1 mM Hcy during 48 hours also induced intracellular ROS (DCF oxidation) and apoptosis (Annexin V positivity).[5] Albeit, in both studies a relatively high concentration of Hcy was used.[4/5] Hyperhomocysteimia in humans namely is defined to be servere in case Hcy concentrations in blood reach levels of >100 μM or more.

An important source of ROS in the vasculature are the NADPH oxidase (NOX) isoforms.[7/8] We have shown previously that NOX-mediated ROS is involved in Hcy-induced apoptosis of endothelial cells (NOX2 and NOX4)[9/10] and cardiomyocytes (NOX2).[11-13] Edirimanne *et al.*[14] has found in homogenates of isolated rat aortas that increased levels of Hcy (induced by high-methionine diet) induced increased ROS levels and NOX activity, as measured via lucigenin chemiluminescence. Furthermore, they showed that transfection of cultured human vascular SMCs with $p22^{phox}$ siRNA effectively abolished Hcy-induced ROS production.[14] Since $p22^{phox}$ is known to provide membrane stabilization of NOX1, NOX2 and NOX4, a role of NOX in Hcy-induced ROS was suggested. However, to the best of our knowledge, the different NOX isoforms have not been studied directly in SMCs, under Hcy conditions.

Therefore, in the present study we have studied the effects of pathophysiological concentrations of Hcy (100 μM) on the activation of different NOX isoforms related to arterial SMC viability.

Materials and Methods

Cell cultures

Human umbilical arteries were isolated from umbilical cords and transferred to 0.1% gelatin coated wells. After 2 weeks SMCs migrated out of the artery. Arterial SMCs were subsequently cultured in Dulbecco's Modified Eagles Medium (DMEM; BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWhittaker), 10% heat-inactivated human serum (HS; Sanquin, Amsterdam, The Netherlands), 100 IU/ml penicillin (Yamanouchi Europe BV, Leiderdorp, Netherlands) and 100 μg/ml streptomycin (Radiopharma-Fisiopharma, Palomonte, Italy) (complete DMEM) at 37°C under a 5% CO_2 atmosphere.

Arterial SMCs were stained with smooth muscle α-actin (SMA) to confirm that they were smooth muscle cells.[15] 98% of the cells were positive for SMA (data not shown). Cells were subsequently exposed to 2 different conditions; control and Hcy (D,L-Hcy, 24 hours 100 μM).

Flow cytometry

After treatment arterial SMCs were collected and resuspended in 200 μl serum free culture medium (DMEM without FCS/HS) containing 10 μl Annexin V-FITC (Bender Med Systems, Burlingame, CA, USA) and incubated for 15 minutes at 37°C in the dark. The cells were then resuspended in 400 μl serum free culture medium containing 10 μl propidium iodide (PI; Bender Med Systems) and subsequently analyzed by flow cytometry using a FACSCallibur (BD Biosciences, San Jose, CA, USA).

Detection of caspase 3 activity

Caspase 3 activity in arterial SMCs was measured using a caspase 3 assay kit (Homogenous caspase 3/7 assay kit, Roche, Mannheim, Germany). After treatment an equal number of cells was lysed and incubated with DEVD-rhodamine 110 substrate (Roche, Mannheim, Germany) for one hour at 37°C. The amount of free rhodamine was determined using a microplate fluorescence reader (TECAN spectra Fluor, Switzerland) with an absorption filter of 492 λ, an emission filter of 535 λ and a gain of 93. Diphenylene iodonium (DPI; 10 μM, Sigma) was used to inhibit NOX-related ROS and rotenone (50 μM, Sigma) was used to inhibit mitochondrial ROS.

Detection of different NOX isoforms and nitrotyrosine expression

Arterial SMCs were cultured in 4-well chamber slides (Nalge Nunc International, Naperville, IL, USA). After treatment, cells were fixated for 10 minutes with 4% paraformaldehyde, permeabilized with acetone-methanol (70%-30%) for 10 minutes and incubated with the primary antibodies goat-anti NOX1 (1:25, Santa Cruz, CA, USA), mouse-anti NOX2 (1:28),[16] goat-anti NOX4 (1:40, Santa Cruz) and rabbit anti-nitrotyrosine (1:50, Invitrogen, Eugene, OR, USA) overnight at 4°C. The following day, cells were washed and subsequently incubated with the secondary antibodies donkey-anti goat Cy3 (1:40, Alexa Fluor 568, Invitrogen), donkey-anti mouse FITC (1:40, Alexa Fluor 488, Invitrogen) and donkey-anti rabbit Cy5 (1:40, Alexa Fluor 647, Invitrogen) for 30 minutes 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 mounting medium 4',6-Diamidino-2-Phenylindole (DAPI, Vector Laboratories Inc, Burlingame, CA, USA) was added, where after the slides were covered.

Live cell analysis of H_2O_2 generation

Since the presence of nitrotyrosine residues is an indirect marker for ROS production we also determined the generation of H_2O_2, using 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) 17 (Molecular Probes, Leiden, The Netherlands). CM-H_2DCFDA, being nonpolar, diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases, and is trapped within the cell. In this status it provides a substrate for oxidation by H_2O_2, resulting in the production of a highly fluorescent intracellular product emitting fluorescence with intensity proportional to the level of intracellular H_2O_2.
Arterial SMCs were grown in Delta-T dishes (0.17 mm, clear; Bioptechs Inc; Butler, PA, USA). After treatment, the cells were loaded with CM-H_2DCFDA (10 μM) 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 minutes at 37°C. Next, cells were incubated in ADS buffer for 25 minutes at 37°C, allowing the oxidized CM-H_2DCFDA to accumulate in the cells. Fluorescence microscopy was performed with a 3I Marianas™ digital-imaging microscopy workstation with a 10× air objective (see below).

siRNA transfection and RT-PCR

Arterial SMCs were transiently transfected with Lipofectamine (Invitrogen), according to the manufactures' protocol. Briefly, 50 μmol RNA was mixed with 5 μl Lipofectamine in 500 μl DMEM without serum and incubated for 20 minutes at RT, after which the transfection mix was applied to 50-60% confluent cells. Cells were transfected with NOX4-siRNA or control-siRNA for 72 hours as previously described[18] (siNOX4, sense sequence: 5'-ACUGAGGUACAGCUGGAUGUU-3', custom designed by Ambion). Subsequently, cells were incubated during 24 hours with 100 μM Hcy. Knock-down of NOX4 mRNA was validated via RT-PCR (data not shown). In short, total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturers' protocol. RNA quality was checked by analysis on the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA extracts were DNase treated (Promega) prior to cDNA synthesis. RT-PCR was performed as described previously[18] using the following primers for NOX4; forward 5'-CTGGAGGAGCTGGCTCGCCAACGAAG-3' and reverse 5'-GTGATCATGAGGAATAGCACCACCACCATGCAG-3'.

Digital-imaging microscopy

Optical sections were acquired and analyzed with a 3I Marianas™ digital-imaging microscopy workstation (Zeiss Axiovert 200M inverted microscope; Cark Zeiss, Sliedrecht, The Netherlands) equipped with a nanostepper motor (Z-axis 10 nm) and a thermo-electrically cooled EMCCD camera (QuantEM: 512C, 512×512 pixels; Photometrics, Tucson, AZ, USA). Exposures, objectives and pixel binning were automatically recorded with each image and stored in memory (Dell Dimension workstation: 3.0 GHz Xenon dual processor, 4 GB RAM). The microscope, camera and all other aspects of data acquisition as well as data processing were controlled by Slidebook™ software (version 4.2; Intelligent Imaging Innovations, Denver, CO, USA).

Statistics

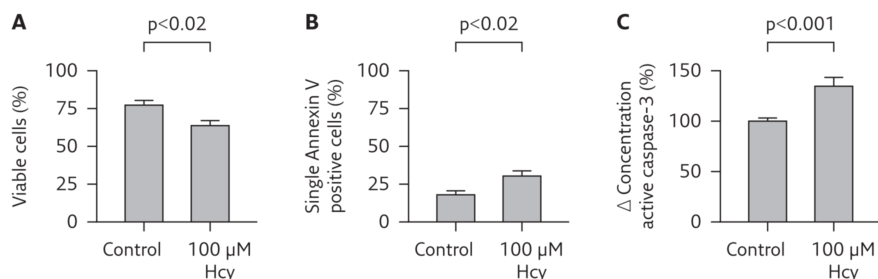
The SPSS statistics program (Windows version 9.0) was used for statistical analysis. To evaluate whether observed differences were significant, T-tests or One-way ANOVA with post hoc Bonferroni tests were used. All values are expressed as mean ± standard error of the mean (SEM). A p-value (two sided) of 0.05 or less was considered to be significant.

Results

Hcy induced flip-flop of the plasma membrane, coinciding with apoptosis

The effect of Hcy on cell viability was determined in arterial SMCs (figure 1). Flow cytometry analysis, using Annexin V/PI staining, showed that incubation of arterial SMCs with 100 μM Hcy during 24 hours significantly decreased the percentage of viable cells with 13.5±3.4% ($p<0.02$) compared to control cells (figure 1A). The percentage of single Annexin V positive cells then significantly increased with 12.5±3.4% ($p<0.02$) compared to control cells (figure 1B).

The percentage of Annexin V and PI double positive cells (late apoptotic or necrotic cells) and the percentage of single PI positive cells (necrotic cells) was limited (with a maximum of 1.8%), without significant differences between the different conditions (data not shown). As single Annexin V positive cells can represent early apoptotic cells, we subsequently analyzed the effect of Hcy on active caspase 3, as a measurement of apoptosis. Hcy induced a significant increase in active caspase 3 concentration with 34.7±8.8% ($p<0.001$) compared to control cells (figure 1C).
For this, 100 µM Hcy reduced cell viability of arterial SMCs after 24 hours, coinciding with a significant induction of apoptosis.

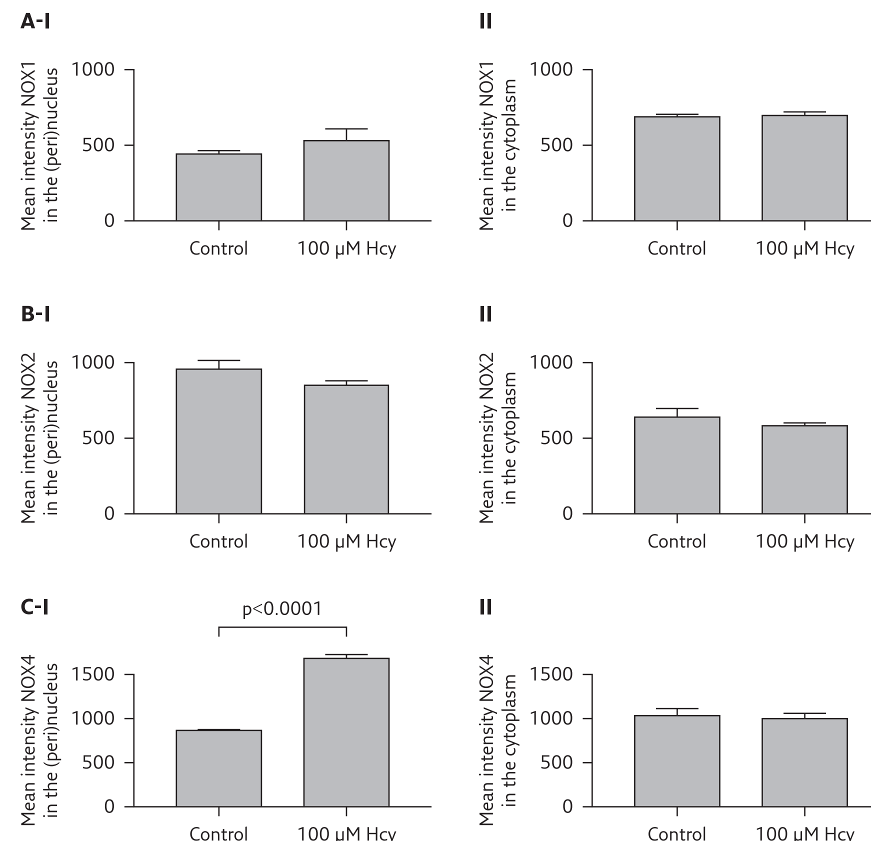


1. Hcy induced Annexin V positivity coinciding with apoptosis | Flow cytometry analysis of human arterial SMC viability, using Annexin V staining, and active caspase 3 measurements. The effects of Hcy on the percentage of (A) viable cells, (B) single Annexin V positive cells and (C) the concentration of active caspase 3. (n=6). The changes in active caspase 3 are shown as the difference (Δ) in the percentage compared to control cells set to 100%.

Hcy induced (peri)nuclear NOX4 expression in SMCs

In previous studies we have shown that Hcy-induced apoptosis of endothelial cells[9/10] and cardiomyocytes[11-13] was related to NOX-mediated ROS production. For this we subsequently analyzed the effect of Hcy on the expression and cellular localization of the NOX isoforms NOX1, NOX2 and NOX4 in SMCs using digital-imaging microscopy (figure 2).
NOX1 and NOX2 expression were both found in (peri)nuclear regions as well as the cytoplasm in control arterial SMCs. However, Hcy had no significant effect on their particular localization nor expression levels (figure 2A/B).

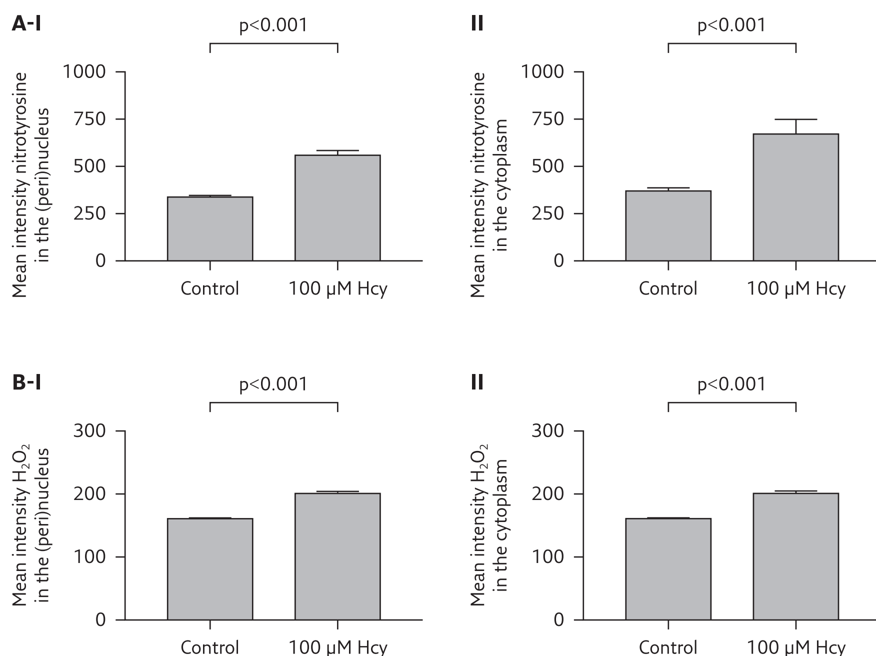
NOX4 expression was also found in (peri)nuclear regions as well as the cytoplasm in control arterial SMCs. In contrast to NOX1 and NOX2, Hcy did induce a significant shift of NOX4 to (peri)nuclear regions (with 722.5±38.9 mean intensity, $p<0.0001$) compared to control cells, albeit without a significant difference in expression level in the cytoplasm compared with NOX1 and 2 (figure 2C).
Thus, Hcy induced significant increased (peri)nuclear NOX4 expression in arterial SMCs, but had no effect on NOX1 and NOX2 expression levels nor their localization.



2. Hcy induced (peri)nuclear NOX4 expression in SMCs | Digital-imaging microscopy analysis of (A) NOX1, (B) NOX2 and (C) NOX4 in human arterial SMCs in the (peri)nuclear region (I) as well as the cytoplasm (II) (n=4). The results are depicted as the difference in mean intensity using Slide-book analysis.

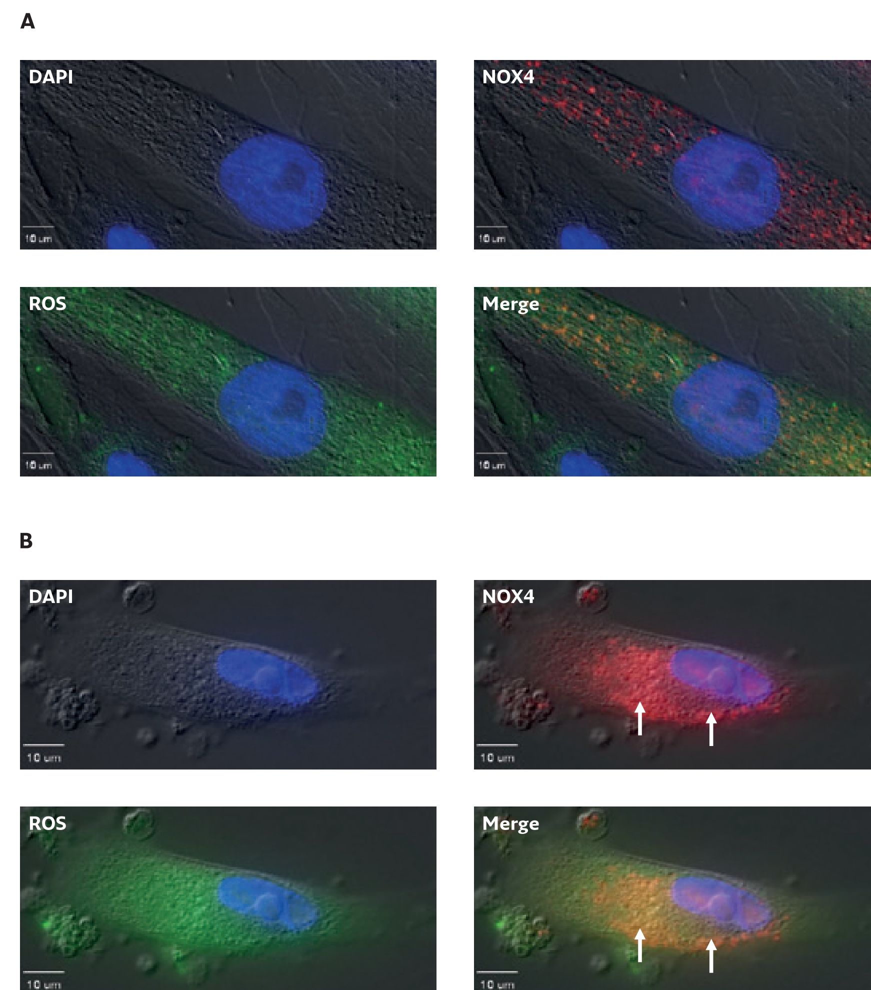
Hcy induced increased ROS production in SMCs

We subsequently analyzed whether this (peri)nuclear NOX4 expression coincided with the expression of nitrotyrosine, an indirect marker of ROS production, using digital-imaging microscopy (figure 3). We then found that incubation of arterial SMCs with 100 μM Hcy during 24 hours significantly increased nitrotyrosine expression in (peri)nuclear regions with 222.2±24.5 mean intensity ($p<0.001$) compared to control cells (figure 3A-I) and in the cytoplasm with 300.2±78.1 mean intensity ($p<0.001$) compared to control cells (figure 3A-II).
As nitrotyrosine residues form an indirect marker for the formation of ROS we also determined the generation of H_2O_2, another NOX-derived ROS.[19] Hcy indeed also induced a significant increased H_2O_2 generation in (peri)nuclear regions with 40.0±2.3 mean intensity ($p<0.001$) compared to control cells (figure 3B-I) and in the cytoplasm, albeit, less abundant with a significant increase of 9.9±2.3 mean intensity ($p<0.001$) compared to control cells (figure 3B-II).



3. Hcy induced increased ROS production in SMCs | Digital-imaging microscopy analysis of (A) nitrotyrosine expression and (B) H_2O_2 generation in human arterial SMCs in the (peri)nuclear region (I) as well as the cytoplasm (II) (n=4). The results are depicted as the difference in mean intensity using Slide-book analysis.

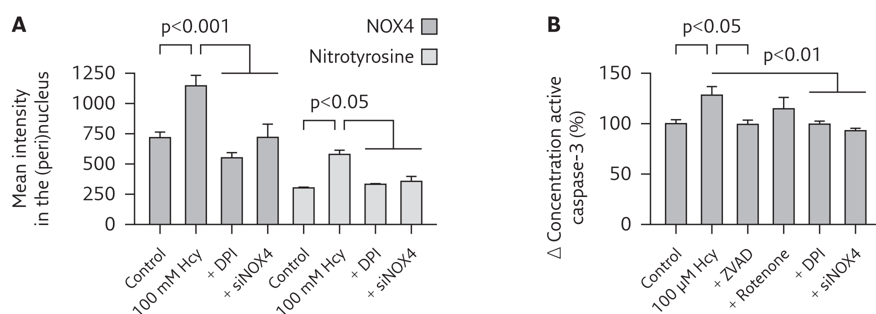
Finally we analyzed whether NOX4 expression colocalized with ROS. In control cells NOX4 expression and ROS production was found homogenously spread mainly in the cytosol (figure 4A). Hcy accumulated NOX4 staining in (peri)nuclear regions (figure 4B, red signal), colocalizing with local ROS production (figure 4B, arrows).
Thus, Hcy-induced increased (peri)nuclear NOX4 expression coincided with the generation of local ROS in that particular area in arterial SMCs.



4. (Sub)Cellular localization of NOX4 and ROS in Hcy-stimulated SMCs | Digital-imaging microscopy of NOX4 expression (red signal) and ROS production (green signal) under (A) control and (B) 100 μM Hcy. Nuclei were stained with DAPI (blue signal). Arrows indicate accumulated expression of NOX4 in (peri)nuclear regions, colocalizing with local ROS production.

Inhibition of NOX-mediated ROS production decreased Hcy-induced apoptosis

To assess whether the observed Hcy induced ROS production was indeed NOX dependent, we analyzed the effect of the general NOX inhibitor DPI hereon (figure 5). We then found that Hcy-induced (peri)nuclear NOX4 and nitrotyrosine expression was significantly reduced by DPI with 594.0±42.4 mean intensity ($p<0.001$) and 246.1±4.8 mean intensity ($p<0.05$), respectively (figure 5A). As Hcy did only cause a significant increase in NOX4 expression, we subsequently specified the role of NOX4 using NOX4 siRNA (siNOX4) herein. We then found that NOX4 knock-down, induced a significant and complete reduction of Hcy-induced (peri)nuclear NOX4 and nitrotyrosine expression with 425.4±108.6 mean intensity ($p<0.001$) and 222.9±41.2 mean intensity ($p<0.05$), respectively.



5. Inhibition of NOX-mediated ROS production decreased Hcy-induced apoptosis
(A) Digital-imaging microscopy analyse of (peri)nuclear NOX4 and nitrotyrosine expression in human arterial SMCs (n=3). The results are depicted as the difference in mean intensity using Slide-book analysis. (B) Active caspase 3 measurement in human arterial SMCs in the presence of DPI, siNOX4 and Roteneone (n=3). The changes are shown as the difference (Δ) in the percentage compared to control cells set to 100%.

In addition, both DPI and knockdown of NOX4 significantly and completely inhibited Hcy-induced caspase 3 activity with respectively 18.1±4.5% ($p<0.05$) and 24.6±2.5% ($p<0.05$), without further significant differences compared with control cells (figure 5B). We analyzed whether ROS sources other than NOX were involved also. Rotenone, an inhibitor of mitochondrial ROS, however only partly and non-significant reduced caspase 3 activity with 2.7±15.6% as compared to Hcy alone.
This therefore proves a role for NOX4-mediated (peri)nuclear ROS in Hcy-induced apoptosis of arterial SMCs.

Discussion

Elevated levels of Hcy form a vascular risk factor.[3/20] Although a jeopardizing role of Hcy on endothelial cells has been shown,[21/22] its effect on SMCs is contradicting, vary-ing from stimulation of proliferation[23/24] to reduction in cell viability.[4/5]
In the present study we found that incubation with pathophysiological concentrations of Hcy (100 µM during 24 hours) induced apoptosis of human arterial SMCs but remarkably at the same time also vasoconstricition of rat resistance arteries, coinciding with significant increased (peri)nuclear NOX4 expression and ROS production. Both apoptosis and vasoconstriction were inhibited by the NOX-dependent ROS inhibitor DPI, indicative for a role of NOX in Hcy-induced dysfunction of arteial SMCs. Furthermore, NOX4 knock-down reduced Hcy-induced SMC apoptosis and ROS production, proving that NOX4 plays a central role in ROS-mediated apoptosis of arterial SMCs induced by Hcy.
In humans hyperhomocysteinemia is usually defined as respectively moderate, intermediate or severe related to Hcy concentrations of respectively 15-30 µM, 30-100 µM or >100 µM,[25] with a maximum found of 400 µM.[26] We have found previously that concentrations of Hcy in the blood of 54±12 µM coincided with significant decreased numbers of SMCs of the media of isolated human femoral arteries, indicating that more pathophysiological concentrations of Hcy can also induce SMC death.[6] In both isolated rat[4] and human[5] aortic SMCs, Hcy was also shown to decrease cell viability as measured via MTT and Annexin V staining, although in both studies a high concentration of Hcy was used (0.5 mM during 24 hours and 1.0 mM during 48 hours, respectively). We now show that Hcy induces apoptosis of SMCs at pathophysiological concentrations of 100 µM applied during 24 hours.
In cardiovascular cells, ROS are known contributors of Hcy-induced injury.[2/14/27/28] Previously we have demonstrated in endothelial cells[9/10] and cardiomyocytes[11-13] that Hcy-induced apoptosis was related to NOX-mediated ROS. Pedruzzi *et al.*[29] did find that oxidized derivatives of cholesterol induced (peri)nuclear NOX4-derived ROS mediated apoptosis in human aortic SMC. Our results now suggest that this is also the case in Hcy-induced apoptosis of human arterial SMCs. As such, we now found that 100 µM Hcy induced a significant increase in expression and translocation of NOX4 to (peri)nuclear regions, coinciding with local ROS production in SMCs, without any effect on NOX1 and NOX2 localization or expression levels. While in endothelial cells and cardiomyocytes 2.5 mM Hcy (applied during 6 hours and 24 hours, respectively) induced apoptosis.[9/13] Compared with these concentrations, that are 25 times higher than the ones we now used in SMCs, this suggests that SMCs are more sensitive to Hcy *in-vitro* then endothelial cells and/or cardiomyocytes.

The specific intracellular NOX4 expression pattern, (peri)nuclear, we found in the present study can suggest specific functions controlled by NOX4-dependent ROS production in (peri)nuclear regions. For example, the regulation of gene expression via redox modification of transcription factors that theoretically could contribute to the induction of apoptosis in Hcy-stimulated SMCs.[30/31] In addition, a role for NOX4-related ROS in endoplasmic reticulum (ER) signaling can also be postulated, as the (peri)nuclear region is contiguous with the ER, and knock-down of NOX4 in human aortic SMCs did reduce ER stress-related proteins.[29]

It has to be noticed that ROS production was not only found in (peri)nuclear regions but also in the cytoplasm. Since the expression of the NOX isoforms NOX1, NOX2 and NOX4 were not increased in the cytosol after Hcy induction, other sources, for instance mitochondria, involved in ROS production cannot be excluded. However, Hcy-induced apoptosis was completely inhibited by the NOX inhibitor DPI, and only partially by the mitochondrial ROS inhibitor rotenone. For this a predominant role of NOX-mediated ROS can be suggested herein. Albeit, since DPI does not differentiate between the different NOX isoforms,[32] we cannot conclude from these data which NOX isoform is involved in Hcy-induced SMC apoptosis. However, knock-down of the NOX4 isoform showed significant reduction of Hcy-induced NOX4 expression and ROS production, and was found to completely abolish Hcy-induced activation of caspase 3, proving an important role for NOX4 in Hcy-induced apoptosis. However, although NOX1 and NOX2 expression did not change we cannot completely exclude these NOX isoforms from playing a role herein.

In conclusion, pathophysiological concentrations of Hcy (100 μM for 24 hours) induced ROS-dependent apoptosis of human umbilical arterial SMCs, in which (peri) nuclear NOX4 plays a central role.

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