S-ADENOSYLHOMOCYSTEINE INDUCES APOPTOSIS AND PHOSPHATIDYLSERINE EXPOSURE IN ENDOTHELIAL CELLS INDEPENDENT OF HOMOCYSTEINE

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S-Aenosylhomocysteine induces apoptosis and phosphatidylserine exposure in endothelial cells independent of homocysteine

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ABSTRACT

Objective: 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-Aenosylhomocysteine (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.

Method: MTT assays showed human umbilical vein endothelial cells (HUVECs) were exposed to the following conditions: (1) non-treated control (resulting in 2.8 mM intracellular SAH and 3.1 μM extracellular (-)-Hcy); (2) incubation with (2) 50 μM adenosine-2′,3′-diphosphate (ADP); resulting in 17.7 mM intracellular SAH and 3.1 μM extracellular (-)-Hcy; (3) 2.5 mM Hcy (resulting in 20.9 mM intracellular SAH and 1.8 mM extracellular (-)-Hcy); and (4) 10 and 100 μM SAH. We then determined the effect of treatment on annexin V positivity, caspase-3 activity, cytokine secretion (subcellular expression of NOX2, NOX4, p47phox and nitrotyrosine, and Hcys). Both Hcy and ADP significantly increased PS exposure (n = 5), caspase-3 activity (n = 6), and cytokine secretion (n = 3). Incubation with extracellular SAH alone did not affect cell viability. Both Hcy and ADP also induced similar increases in nuclear NOX2 and peroxynitrite NOX4, coinciding with peroxynitrite p47phox expression and local reactive oxygen species (ROS) (n = 3). Inhibition of NOX2-mediated ROS by the flavonone inhibitor diphenyleneiodonium (DPI) significantly decreased apoptosis induction (n = 3) and ROS production (n = 3).

Conclusion: 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.

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1. Introduction

Hyperhomocysteinemia 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 hyperhomo-
cysteinemia and endothelial dysfunction [1, 2], atherosclerosis [3, 4] and myocardial infarction [5–7].

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 [8–10]. Several studies have suggested a role for different NODPH oxidase (NOX) isoforms herein [9, 11, 12]. Recently, we have found that Hcy induces phosphatidylserine (PS) exposure and apoptosis in human endothelial cells, which was accompanied by nuclear NOX2/p47phox and peroxynitrite NOX4/p47phox [13].

The amino acid, Hcy, is produced as a byproduct of the methionine metabolism pathway (Fig. 1), which serves as a main source of cellular DNA, RNA and protein methylation [14, 15]. Methionine...
Methionine → S-Adenosylmethionine (SAM) → CH₃ → Methylation

Homocysteine ⇌ SAH

SAH → S-Adenosylhomocysteine (SAH)

Adenosine

Fig. 1. Homocysteine metabolism. Schematic representation of methio-
nine/homocysteine metabolism. Methionine is activated to S-Adenosylmethi-
nine (SAM), a potent methyltransferase for methylation reactions. After donating the methyl-
group S-Adenosylhomocysteine (SAH) remains, which in turn is a potent inhibitor of
S-Adenosylhomocysteine hydrolase (SAHH) which is a reversible reaction and will
favor SAH when concentration of Hcy is increased.

is first activated to S-Adenosylmethionine (SAM), and further via
methyltransferase activity to S-Adenosylhomocysteine (SAH). SAH is
then hydrolized by SAH hydrolase to Hcy and adenosine [10].
Accumulation of Hcy (which can occur for a variety of reasons, e.g.
genetic defects or deficiencies in co-factors such as vitamin B₆, vita-
mim B₁₂ and folic) results in an increase in SAH. Due to its ability to
bind to methyltransferases, SAH acts as a potent inhibitor of methylata-
tion n cells, including endothelial cells [17,18]. However, whether
cardiovascular damage is specifically due to Hcy accumulation or
increased SAH levels is currently not clear. This distinction may
be important for putative future therapy development targeting
endothelial dysfunction in patients with hyperhomocysteinemia.
In the present study we therefore have studied putative differ-
ences between Hcy- and SAH-induced cell damage of endothelial
cells in vitro, including the role of different NOX isoforms herein.

2. Methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated
from umbilical cords and cultured in Medium 199 (BioWhit-
taker, Verviers, Belgium) supplemented with 10% heat-inactivated
fetal calf serum (FCS; BioWhitaker), 10% heat-inactivated human
serum (Sanquin, Amsterdam, The Netherlands), 5 μg/ml lepirin
(Leo Pharma BV, Breda, The Netherlands), 50 μg/ml endothel-
ial cell growth factor (Sigma, St. Louis, MO, USA), 100 IU/ml
penicillin (Yamanouchi Europe BV, Leiden, The Netherlands) and
100 μg/ml streptomycin (Radiopharma-Phispharma, Palomonte,
Italy) at 37 °C in a humidified 5% CO₂/95% air atmosphere. Experi-
ments were performed at 100% confluence of the cells.

Cells were incubated for 6 h either with 2.5 mM Hcy, 50 μM adenosine, 2.5-maldehyde (ADA), or with 1 μM, 10 μM, or 100 μM S-Adenosylhomocysteine (SAH) (all from Sigma, St. Louis, MO, USA). ADA inhibits the hydrolization of SAM to Hcy and adenosine, therefore increasing the SAH concen-
trations in the cell without increasing Hcy concentrations [17,19].
Since it has been suggested that Hcy-induced endothelial dysfunc-
tion is due to adenosine depletion, adenosine (100 μM, Sigma) was
used as a control [20].

2′OD (z-Asp-Glu-Val-Asp-fluoromethylketone: 25 μM, Alexis Biocemicals) was used as a pan-caspase inhibitor and diphenylene iodonium (DPI; 10 μM, Sigma) was used to inhibit NOX-mediated ROS production [21].

2.2. 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 [22,23], we
determined the concentrations of extracellular L-Hcy. L-Hcy was
measured by the Abbott IMx fluorescence polarization immunoas-
say (IMX, Abbott Laboratories, Abbott Park, IL, USA). Intralu-
ner and interassay CVs were less than 2 and 4%, respectively. The concentra-
tion of Hcy in culture medium was measured before incubation
(r=0) and after 6 h of incubation.

2.3. Determination of intracellular SAM and SAH

We determined the intracellular concentration of
S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH)
in HUVECs after 6 h of incubation with 2.5 mM L-Hcy or 50 μM
ADA. Tandem mass spectrometry (MS/MS) was used for the determination of SAM/SAH concentrations as previously described
[24].

2.4. Hoechst staining

Plasma membrane flip-flop and thus phosphatidylserine (PS)
exposure was assessed with FITC-labeled human recombinant
annexin V (Bender Med Systems, Vienna, Austria), while propid-
um iodide (PI, Bender Med Systems) was used to determine the
permeability of the cellular membranes.

After treatment, cells were trypsinized and centrifuged at
400 × g for 5 min at room temperature. Cells were then resus-
pended in serum free Medium 199 and incubated with annexin
V (1:40) for 30 min. Shortly before measuring PI was added to the
cell suspension (1:40). Cells were measured with a FACS-Calibur
(Becton Dickinson, San Jose, CA, USA). Results were analyzed by
Cell Quest Pro software (Becton Dickinson).

2.5. Detection of cytochrome c release

Cells were grown in 4-well chamber slides (Nalge Nunc
International, Naperville, IL, USA). After treatment, cells were
fixed with 4% formaldehyde for 10 min at 37 °C and subsequently
permeabilized with acetone–methanol (70–300) for 10 min at −20 °C.

Cells were then incubated with the primary antibodies polyclonal
anti-cytochrome c (1:100, Santa Cruz Biotechnology, Inc., CA, USA)
and monoclonal mitochondrial Heat Shock Protein (HSP) (70:1:100,
Thermo scientific, Rockford, IL, USA) for 60 min at room tempera-
ture followed by incubation overnight at 4 °C. Negative controls
were performed with the only the secondary antibody were included to assess non-
specific binding. All negative controls showed no staining (data not
shown). The following day, cells were washed and incubated with the secondary antibodies Cy2-labeled anti-mouse (1:75; Invitrogen,
Leiden, Netherlands) and Cy5-labeled anti-rabbit (1:50, Jackson
Immuno Research, West Grove, PA, USA) for 30 min at room tem-
perature. After subsequent washes, cells were covered in mounting
medium containing DAPI (Vector laboratories inc., Burlingame, CA, USA) to visualize nuclei.

2.6. Detection of caspase-3 activity

Cells were grown in a 96-wells plate (20,000 cells/well). After
treatment, cells were lysed and incubated with DEVD-roydase
110 substrate (Boehr, Mannheim, Germany) for one hour at 37 °C.
Subsequently the amount of free rhodamine was determined at a
microplate fluorescence reader (TICAN spectra Fluor, Switzerland).
The developed fluorochnone was proportional to the concentration
of activated caspase-3 and could be quantified by a calibration curve
of diluted free rhodamine.
2.7. Detection of NOX2, NOX4, p47(phox) and nitrotyrosine expression

Cells were grown in 4-well chamber slides (Nalge Nunc International). After treatment, cells were fixed with 4% formaldehyde for 10 min at 37 °C and subsequently permeabilized with acetone–methanol (70–30%) for 10 min at –20 °C. Cells were then incubated with the primary antibodies monoclonal 48 (NOX2 [25]; 128, Signaling Research at CLB), polyclonal anti-nitrotyrosine (ROS: 1:50, Invitrogen), polyclonal anti-p47(phox) (1:50, Santa Cruz) and polyclonal anti-NOX4 (1:50, Santa Cruz) for 60 min at room temperature followed by incubation overnight at 4 °C. Negative controls with only the secondary antibody were included to assess non-specific binding. All negative controls showed no staining (data not shown). The following day, cells were washed and incubated with the secondary antibodies Cy3-labeled anti-mouse (1:75, Invitrogen), Cy5-labeled anti-rabbit (1:50, Jackson Immunoresearch) and Cy3-labeled anti-goat (1:40, Invitrogen) for 30 min at room temperature. After subsequent washes, cells were covered in mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA) to visualize nuclei.

2.8. Digital-imaging microscopy

All immunologically stained slides were analyzed by means of a 3i Marinas™ digital-imaging microscopy workstation (Zeiss Axiosvert 200M inverted microscope; Carl Zeiss, Sliedrecht, Netherlands), equipped with a nanostepper motor (Z-axis 10mm) and a cooled CCD camera (Cooke Sensicam, 1280 x 1024 pixels; Cooke Co, Tonawanda, NY, USA). Visualization was performed with a 40 x obj lens. The microscope, camera and data viewing process were controlled by SlideBook™ software (version 4.0.8.1; Intelligent Imaging Innovations, Denver, CO, USA).

2.9. Live cell analysis of H2O2 generation

Since the presence of nitrotyrosine residues is an indirect marker for ROS production we also determined the generation of H2O2, using 5-(5-hydroxy-1,1,3,3-tetramethylindol-2-yl) -2-(2-nitrophenyl) -2H-benzimidazol-4-ylidene) (CM-H2DCFDA) (Molecular Probes, Leiden, The Netherlands). CM-H2DCFDA, 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 H2O2, resulting in the production of a highly fluorescent intracellular product emitting fluorescence with intensity proportional to the level of intracellular H2O2.

HUVECs were grown in Delta-T dishes (0.17 mm, clear; Bioplates Inc.; Butler, PA, USA). After treatment, the cells were loaded with CM-H2DCFDA (10 μM) in ADS buffer (116 mM NaCl, 5.3 mM KCl, 1.2 mM MgSO4, 1.13 mM Na2HPO4, 0.2 mM HEPES, and 1 mM CaCl2, pH 7.4) and incubated for 15 min at 37 °C. Next, cells were incubated in ADS buffer for 25 min at 37 °C, allowing the oxidized CM-H2DCFDA to accumulate in the cells. Fluorescence microscopy was performed with a 3i Marinas™ digital-imaging microscopy workstation with a 10× air objective (see below).

2.10. 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.

3. Results

3.1. Measurement of extracellular Hcy concentrations, intracellular SAIM concentrations, and intracellular SAIM/SAH ratio

After 6 h incubation of HUVECs with either Hcy or ADA the concentrations of extracellular t-Hcy (from the conditioned medium) and intracellular SAM and SAH were determined. Only incubation with Hcy resulted in a significant increase of extracellular t-Hcy to 1.8 ± 0.4 mM (Fig. 2A, p < 0.001, n = 4) compared to control. Incubation with ADA had no effect on extracellular t-Hcy concentration (0.003 mM). In contrast, the amount of intracellular SAM was significantly increased both with Hcy: 20.9 ± 9.3 mM and with ADA to 17.7 ± 3.6 mM (Fig. 2B, p < 0.01 and p < 0.05, respectively, n = 4) compared to control. Thus, ADA treatment results in an increase in intracellular SAH but not extracellular t-Hcy, while Hcy treatment results in an increase in both intracellular SAM and extracellular t-Hcy.

Fig. 2. Extracellular Hcy and intracellular SAIM concentrations, and SAIM/SAH ratio. HUVECs were incubated with 2.5 mM L-Hcy (Hcy) or 50 μM ADA (ADA) for 6 h and subsequently (A) extracellular t-Hcy concentrations and (B) intracellular SAIM concentrations and (C) SAIM/SAH ratios were determined (n = 4).

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In line with this, the SAM/SAH ratio was reduced from 2.2 ± 0.5 in control to 1.0 ± 0.3 with Hcy (non-significant) and to 0.5 ± 0.02 with ADA (Fig. 2C, p < 0.01, n = 4).

3.2. Effect of Hcy, ADA, adenine and extracellular SAH on phosphatidylserine exposure

Previously, we have shown that incubation of HUVECs with 2.5 mM Hcy induced PS exposure and apoptosis [13]. Here we show that 6 h of incubation with Hcy resulted in a significant decrease of viable cells by 16.9 ± 3.7% (Fig. 3A, p < 0.001, n = 5), and a significant increase in single annexin V-positive cells by 15.2 ± 3.1% (Fig. 3B, p < 0.001, n = 5), compared to control. Incubation with ADA also resulted in a significant decrease in viable cells by 6.1 ± 1.7% (Fig. 3A, p > 0.05), and a significant increase in single annexin V-positive cells by 5.6 ± 0.9% (Fig. 3B, p > 0.05), compared to control. The simultaneous addition of adenine did not change the ADA-mediated decrease in cell viability nor the increased single annexin V-positivity, compared to ADA alone (n = 3), suggesting that the main causative factor is intracellular SAH level. In addition, no
differences were found between adenosine and control (n = 3). Although the effect of Hcy was more extensive than that of ADA, no significant differences in viable or single annexin V-positive cells were found between ADA and Hcy. The effect of extracellular SAH on cell viability was also analyzed (n = 3). Unlike Hcy and ADA, increasing concentrations of extracellular SAH (1 μM, 10 μM and 100 μM) did not induce a decrease in viability, nor an increase in annexin V-positivity (Fig. 3A and 8), compared to control. The percentage of annexin V and PI double positive cells, and the percentage of single PI-positive cells were low, without significant differences between the different conditions (data not shown).

Thus, both Hcy and ADA treatment decreased the viability of HUVECs and induced PS exposure.

3.3. Effect of Hcy and ADA on cell viability in HUVECs

The exposure of PS can be a hallmark for apoptosis. Since both Hcy and ADA induced PS exposure we determined the effect of ZVAD on PS exposure, the release of cytochrome c and the caspase-3 activity, as measurements of apoptosis.

As expected, ZVAD significantly inhibited the decrease in viable cells induced by Hcy and ADA by 10.3 ± 1.7% (p < 0.05) and 11.8 ± 0.6% (p < 0.01), respectively (Fig. 3C, n = 3). In line with this, ZVAD also inhibited the increase in single annexin V-positive cells by 7.5 ± 0.9% (p < 0.05) and 8.3 ± 0.9% (p < 0.05), respectively (Fig. 3D). In addition, both Hcy and ADA induced a significant increase in cytochrome c release (187.5 ± 40%, p < 0.05 and 164.2 ± 8%, p < 0.05, respectively), compared to control (Fig. 3E, n = 3). In line with this, a significant increase in caspase-3 activity compared to control was found for both Hcy (16.5 ± 4.2%, p < 0.001) and ADA (11.7 ± 1.9%, p < 0.05) (Fig. 3F, n = 6).

3.4. Effect of Hcy and ADA on intracellular NOX2, NOX4 and p47phox expression and localized ROI production in HUVECs

We previously observed that Hcy-induced apoptosis coincided with nuclear NOX2/p47phox and (per)nuclear NOX4/p47phox expression, and was accompanied by nitrotyrosine accumulation [11]. Since incubation of HUVECs with ADA also induced apoptosis, we subsequently analyzed whether ADA-induced apoptosis resulted in expression of the same NOX isoforms as Hcy-induced apoptosis. HUVECs were incubated with Hcy or ADA and the (per)nuclear expression of NOX2, NOX4, p47phox and nitrotyrosine was determined (Fig. 4, n = 3). We found that both Hcy and ADA induced nuclear NOX2 and (per)nuclear NOX4 expression coincided with (per)nuclear p47phox expression and nitrotyrosine accumulation, indicative for local ROI production.
Fig. 5. Effect of NOX-mediated ROS inhibition on Hcy and ADA induced apoptosis. (A) Quantiﬁcation of the (per)nuclear expression of nitrotyrosine (n = 3), (B) generation of H_{2}O_{2} (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 L-hcy or 50 μM ADA for 6 h, 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.

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

To analyze whether the increased (per)nuclear NOX4 and NOX2 expression indeed leads to enhanced ROS production and induction of apoptosis, we measured (per)nuclear nitrotyrosine expression, H_{2}O_{2} 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 (per)nuclear nitrotyrosine expression and H_{2}O_{2} generation compared to Hcy alone (88.9 ± 5.2% p < 0.001 and 122 ± 0.1% p < 0.001, respectively) and ADA alone (73.9 ± 11% p < 0.001 and 139 ± 0.2% p < 0.001, respectively) [Fig. 5A and 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 8.3 ± 2% (p < 0.05), respectively [Fig. 5C and 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.

4. Discussion

Since the debate is ongoing whether the main causative factor in hyperhomocysteinemia-induced cardiovascular disease is due to Hcy accumulation or to increased SAH levels, in the present study we compared the effect of Hcy and intracellular SAH on cell viability in human endothelial cells in vitro. We found that incubation of HUVECs with both Hcy and ADA induced PS exposure, caspase-3 activity, cytochrome c release, and translocation of NOX2, NOX4 and p47phox to the (per)nuclear region coinciding with local ROS production, without further signiﬁcant differences between Hcy and ADA. It has been shown that ADA inhibits SAH hydrolysis, thereby blocking Hcy formation [17, 19]. Thus, ADA treatment of cells results in increased SAH levels, but not increased in Hcy levels. Therefore we can conclude that the effects of ADA treatment of HUVECs were related to increased SAH levels only, and not to Hcy accumulation.

In contrast to ADA, adding extracellular SAH to HUVECs had no effect on cell viability. However, it has been suggested that extracellular SAH does not enter cells. For example, lymphoblasts cultured in the presence of SAH (up to 25 μM) did not lead to increased intracellular SAH levels [our unpublished observations].

Increased SAH correlates strongly with the incidence of vascular disease, as shown in several epidemiological correlation studies [26–29]. In addition, several in vitro studies have also suggested SAH to be the causative factor in hyperhomocysteinemia-induced apoptosis in BV-2 microglial cells, lymphoblasts and endothelial
cells [31,1], however none of these studies were performed under the condition of increased intracellular 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 μM SAH, 1 μM Hcy and 100 μM adenosine during 24h caused apoptosis in BV-2 microglial cells that was correlated with intracellular ROS production [31]. They however did not examine different NOX isoforms in this respect. It was also shown that adenosine induced apoptosis of pulmonary endothelial cells by elevating levels of intracellular SAH and thereby causing DNA fragmentation, although in this study 100 μM Hcy was also added [30,31]. These studies thus indicate the potential toxicity of increased SAH levels in various cell types and tissues, however, as in those studies increased Hcy was also used to define proof of an Hcy independent effect of SAH was given. Moreover, it has not been shown previously in vitro or in vivo that increased intracellular SAH levels activate different NOX isoforms. Our study is the first to show that increased levels of SAH alone, independent of Hcy, can induce apoptosis in this endothelial cell model coinciding with NOX-mediated ROS production.

Previous studies have shown the activation of different pathways through which endothelial cell apoptosis is induced by Hcy, namely via the c-Jun N-terminal kinases (JNK) pathway, mitochondrial oxidative stress or NOX-mediated ROS [1,2,3,12]. We are now the first to show that hyperhomocysteinemia-induced PS exposure and endothelial apoptosis can be induced by increased intracellular SAH alone, inducing the effects that Hcy exerts on nuclear NOX2/2/p47(phox) and (peri)nuclear NOX4/p47(phox) expression and nitric oxide consumption.

The concentration of Hcy used in the current study is higher than its physiological occurrence in humans. In our previous study, however, we already have shown that Hcy concentration in growth medium decreased significantly during incubation. Furthermore, short-term exposure to high Hcy concentrations may reflect a prolonged exposure to moderately elevated Hcy concentrations as occurs during patients’ lifetime. The SAH concentrations that we measured after incubation with ADA and Hcy, however, are comparable to the concentrations found intracellularly in erythrocytes of patients with peripheral arterial occlusive disease [29].

Therefore, our study shows, based on an in vitro cell culture model that Hcy-mediated endothelial dysfunction is due to intracellular SAH accumulation and supports the hypothesis that Hcy itself is not the predictive toxic agent but rather SAH, SAH reduction, therefore, could be a possible target in a preventive therapy to circumvent endothelial dysfunction. However, further in vivo and clinical studies to prove the functionality or relevance of these results are needed.

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