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The peroxynitrite donor 3-morpholinosydnonimine activates Nrf2 and the UPR leading to a cytoprotective response in endothelial cells

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ABSTRACT

Endothelial dysfunction is associated with the formation of peroxynitrite, described to be toxic. Recent data also suggests that peroxynitrite is able to activate the protective Nrf2 pathway and/or the unfolded protein response (UPR). The aim of our work was to study the response of human endothelial cells to 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor, and to highlight the possible protective roles of Nrf2 or the UPR pathway in this response.

Immortal and primary human umbilical vein endothelial cells were exposed to SIN-1. SIN-1 incubation led to Nrf2 activation and to the overexpression of Nrf2-regulated genes, heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1. We also demonstrated that this defensive response protected cells against cell death induced by serum starvation, by reducing apoptosis (monitored by caspase-3 activity and DNA fragmentation) and favoring autophagosome formation, as evidenced by LC3-II accumulation. Interestingly, we observed an activation of the UPR, with a rapid and significant overexpression of CHOP in serum starved cells stimulated with SIN-1. While siRNA mediated knockdown of CHOP had no effect on DNA fragmentation, the invalidation of Nrf2 or HO-1 by siRNA strongly increased DNA fragmentation, but also reinforced the SIN-1-induced LC3-II accumulation.

This study shows that peroxynitrite, at least at sublethal concentrations and within a narrow concentration range, could exert protective effects on endothelial cells by modulating the balance between autophagy and apoptosis, through Nrf2-dependent pathways.

1. Introduction

Molsidomine (N-ethoxycarbonyl-3-morpholino-sydnonimine) has been clinically used as a vasodilatatory drug for more than 30 years. Molsidomine is enzymatically converted in the liver to its bioactive metabolite SIN-1 [1]. During its decomposition, SIN-1 releases superoxide anion ($O_2^{\bullet -}$) and nitric oxide (NO•), that spontaneously combine to form a highly reactive molecule peroxynitrite [2]. In physiological conditions, endothelial cells enzymatically produce very low levels of $O_2^{\bullet -}$ and NO [3]. Oxidative damage is minimized by endogenous antioxidant defenses [4]. But in pathological conditions NO• production drastically increases and the produced NO• competes with the superoxide dismutases (SODs) for $O_2^{\bullet -}$ to form peroxynitrite. Interestingly, protein nitration, considered as a "footprint" of peroxynitrite generation, has been detected in human atherosclerotic lesions using anti-nitrotyrosine antibodies [5,6]. Peroxynitrite can attack biological molecules such as lipids, DNA and proteins via direct oxidative reactions or indirect radical-mediated mechanisms [7]. Moreover peroxynitrite formation leads to endothelial nitric oxide synthase (eNOS) uncoupling, converting the enzyme into an $O_2^{\bullet -}$ producing enzyme, exacerbating the oxidative stress [4]. In addition peroxynitrite has deleterious effects on both the activity and function of prostacyclin synthase via tyrosine nitration, again worsening endothelial dysfunction [7]. Hence, peroxynitrite plays a central role in endothelial dysfunction.

Peroxynitrite-induced damage leads to cell death by apoptosis and necrosis [8]. In addition, reactive oxygen species (ROS) could also induce cell death by macroautophagy (autophagy in this paper) [9]. Autophagy does not necessarily lead to cell death: it is present at a basal rate in most cells as a cytoplasmic quality-control mechanism to eliminate protein aggregates and damaged organelles [10]. LC3 is a major constituent of the autophagosome, a double membrane structure

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that sequesters the target organelles/proteins and then fuses with a lysosome where its content is degraded. That is why the conversion of LC3-I in LC3-II is often used as an indicator of autophagic activity [11].

But before becoming toxic, ROS can be beneficial for cells [3,12]. ROS can induce expression of genes encoding proteins with antioxidant and xenobiotic detoxification activities through the activation of the transcription factor Nrf2, essential for antioxidant response element (ARE)-mediated induction of these genes [13]. Exposure of cells to xenobiotics or oxidants such as peroxynitrite [14,15] leads to the Nrf2 activation and the expression of its target genes encoding protecting enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) or heme oxygenase-1 (HO-1) [13]. HO-1 has important anti-oxidant and anti-inflammatory, but also vasodilatory properties, attenuating atheroma formation [16].

Oxidative stress can also affect the ER [17], inducing an accumulation of unfolded proteins in the ER lumen, a condition referred to as "ER stress" [18]. To be able to cope with this stress, cells have developed an adaptive response called the unfolded protein response (UPR) that reduces the overall protein synthesis, while favoring the neo-synthesis of chaperones and proteolytic enzymes to face the accumulation of unfolded proteins. During the UPR, intracellular signaling pathways are triggered by the activation of three proteins, IRE1 (inositol requiring enzyme 1), ATF6 (activating transcription factor 6) and PERK (PKR-like ER kinase), leading to the overexpression of UPR target genes such as the chaperone BiP (also named glucose-regulated protein 78 or Grp78). However, if the stress becomes too intense or too long, UPR can lead to cell death by apoptosis and overexpression of CHOP (CCAAT/enhancer-binding protein-homologous protein) [19,20]. Overexpression of CHOP has been correlated with the induction of cell death in many models of ER stress [21,22,23], but is not always sufficient for inducing apoptosis [24]. Moreover, although primarily considered as a pro-apoptotic factor, CHOP has also been shown to be able to exert anti-apoptotic functions [25] depending on the environment and cellular type involved.

As described above, peroxynitrite generation *in vivo* in the vascular wall represents a crucial pathogenic mechanism in atherosclerosis, but responses of endothelial cells to peroxynitrite are far of being completely elucidated. So, the aim of this work was to study whether peroxynitrite was able to act as a second messenger in human endothelial cells leading to a beneficial protective response, at least at sublethal concentrations. The present report reveals that peroxynitrite modulates gene expression by activating the Nrf2 and UPR pathways. Moreover, CHOP protein expression did not affect cell viability. We could demonstrate that Nrf2 pathway induced by SIN-1, protected the EAhy926 cells from cell death induced by serum starvation, by limiting apoptosis and favoring autophagy in a Nrf2 dependent way, while CHOP invalidation did neither improve nor compromise cell survival.

2. Methods

2.1. Chemical reagents

3-Morpholinosydnonimine hydrochloride (SIN-1) (Molecular Probes, USA) was solubilised in Hank's Balanced Salt Solution (0.137 M NaCl, 5.36 mM KCl, 440 nM KH_2PO_4, 5 mM glucose, 299 nM Na_2HPO_4, 4.2 mM $NaHCO_3$, 0.16 M $MgSO_4$, 0.24 M $CaCl_2$, pH 7.35). Uric acid (Sigma) was stored at 50 mM in NaOH and diluted at final concentration in culture medium, pH 7.4.

2.2. Cell culture

The EAhy926 cell line (hybridoma between human umbilical vein endothelial cells (HUVECs) and A549 cells) was kindly provided by Dr Edgell (South Carolina University) [26]. HUVECs were purchased from Clonetics (Cambrex). Cells were plated and amplified as previously described by Gustin et al. [27].

2.3. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was measured with the cytotoxicity detection kit from Roche Molecular Biochemical (Indianapolis, IN) according to the manufacturer's protocol. After incubation with SIN-1, the culture media were removed and centrifuged to pellet the cell fragments and apoptotic bodies. PBS — 2% Triton X-100 (Merck) was added to lyse the pellet and the cells remaining in the wells. The percentage of LDH released was calculated as follow: LDH activity in medium (1) + LDH activity of cell fragments (2)/(1) + (2) + LDH activity of cells remaining in the wells.

2.4. Peroxynitrite detection

Peroxynitrite was measured using the peroxynitrite—sensitive Hydroxyphenyl fluorescein (HPF) probe (Bachem, CA, USA). Before stimulation, cells were incubated with 5 μM HPF for 30 min at 37 °C in a humidified atmosphere composed of 5% CO_2 according to the manufacturer's protocol.

2.5. Immunofluorescence and confocal microscopy

Cells were fixed in 4% paraformaldehyde in PBS, permeabilized in PBS — 1% Triton X-100, stained with rabbit anti-Nrf2 antibody (H300 Santa Cruz) or mouse anti-CHOP antibody (Santa Cruz) and then incubated with Alexa Fluor-488-conjugated goat anti-rabbit IgG antibody or anti-mouse IgG antibody. The coverslips were finally mounted in Mowiol (Sigma) and observed by confocal microscopy (Leica TCS SP5) equipped with a Zeiss oil immersion 63× objective.

2.6. Reporter plasmids and small interfering RNA (siRNA) transfections

Cell transfections were performed with the pNQO1-ARE-luciferase plasmid, graciously provided by Prof Leonard [28]. Transfections were performed with the SuperFect transfection reagent (Qiagen, Netherlands) according to the manufacturer's protocol. After 3 h of transfection, cells were stimulated during 20 h. Thereafter cells were lysed with Glo lysis buffer (Promega, Madison, WI). The cell lysate was mixed with the luciferase Bright Glo substrate (Promega, Madison, WI) and the luminescence was measured with a luminometer (Luminoskan Ascent, Thermo Scientific). Protein concentration was measured by the Bradford assay and used to normalize the measured relative light unit values (RLU/μg protein).

SiRNA targeting human Nrf2 (NM_006164), HO-1 (NM_002133), CHOP (NM_004083) or negative controls (siCONTROL Non-Targeting or Risk Free) were obtained from Upstate/Dharmacon (Lafayette, USA). Briefly, cells were transfected with 10 nM siRNA using INTERFERin (250× dilution; Polyplus, Belgium). The cells were then incubated in the presence of siRNA at 37 °C in a humidified atmosphere of 5% CO_2 for 36 h for Nrf2 and HO-1 knockdown, and 24 h for CHOP knockdown. Efficiency of RNA interference on Nrf2, HO-1 and CHOP expression was determined by real-time RT-PCR with specific primers and by Western blot.

2.7. RNA analysis

Total RNA was extracted using the Total RNAgent extraction kit (Promega). mRNA contained in 5 μg total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (InVitrogen) according to the manufacturer's instructions. Gene expression was analyzed by quantitative RT-PCR. Amplification reaction samples contained 1 X SYBR Green PCR Mastermix (Applied Biosystem). A hot start at 95 °C for 5 min was followed by 40 cycles at 95 °C for 15 s and 65 °C for 1 min using an ABI PRISM 7000 SDS thermal cycler (Applied Biosystem).