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Research paper

Hydrogen sulphide induces HIF-1α and Nrf2 in THP-1 macrophages

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ABSTRACT

The transcription factor HIF-1α regulates the adaptive response of cells to hypoxia and oxidative stress. In addition, an important regulatory role for HIF-1α in immune reactions and inflammation is suggested. The present study attempts to investigate the effect of the gaseous signalling molecule hydrogen sulphide (H_2S) on HIF-1α in THP-1 macrophages using the slow H_2S releasing donor GYY4137.

We found that H_2S induced HIF-1α protein accumulation in THP-1 macrophages in a concentration-dependent manner. Western blot analysis of cell fractions showed that HIF-1α protein translocates into the nucleus and leads to an increase of its target protein glucose transporter-1 (GLUT-1). Activation of nuclear factor-κB (NF-κB), as well as secretion of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), were reduced in the presence of H_2S. These findings indicate that HIF-1α accumulation due to H_2S was not triggered by the NF-κB pathway. The antioxidant pathway Nrf2/HO-1 (nuclear factor erythroid 2-related factor 2/heme oxygenase-1) was activated by H_2S. Inhibition of the p38 mitogen-activated protein kinase (MAPK) reversed H_2S mediated effects, suggesting that the p38 MAPK pathway may be involved in H_2S induced HIF-1α/Nrf2 signalling pathways.

1. Introduction

The hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of α and β subunits. While HIF-1β is constitutively expressed in most cell types, HIF-1α is undetectable or present at very low levels under normal oxygen supply due to hydroxylation of specific prolyl hydroxylases and degradation *via* the proteasome. Under hypoxic conditions, the inactivation of the prolyl-hydroxylases allows HIF-1α protein stabilisation and dimerisation with the HIF-1β subunit, regulating more than 100 HIF-1 responsive target genes, which are responsible for angiogenesis, survival and metabolism and include vascular endothelial growth factor (VEGF), virtually all enzymes in the glycolytic pathway, as well as the glucose transporter (GLUT) family [1–3].

Despite its stabilisation during hypoxia, HIF-1α protein has also been shown to be upregulated under normoxia in response to mechanical stress, cytokines, growth factors and reactive oxygen and reactive nitrogen species (ROS and RNS) [4–7].

Hydrogen sulphide (H_2S) has been proposed as a third endogenous gasotransmitter, besides carbon monoxide (CO) and nitric oxide (NO) [8]. H_2S is produced mainly from cysteine by the enzymes cystathionine β-synthase, cystathionine γ-lyase and the tandem enzymes cysteine aminotransferase/3-mercatopyruvate sulphurtransferase. Production occurs in many cells but preferentially in vascular smooth muscle cells where it diffuses into the surrounding tissues. A growing number of publications report on the cytoprotective effects of H_2S under various pathophysiologic conditions and its therapeutic potential [9]. The effects of H_2S are mainly based on its anti-inflammatory, antioxidant, anti-hypertensive, regulatory and signalling functions [10,11]. H_2S has been shown to attenuate lipopolysaccharide induced formation of inflammatory mediators in murine RAW264.7 macrophages [12], to reduce the generation of pro-inflammatory mediators from human joint cells [13] and to inhibit IL-6 secretion of fibroblasts isolated from the synovial membrane of rheumatoid arthritis patients [14].

In general, two redox-sensitive signalling pathways have been reported to work together to modulate inflammatory responses,

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namely, nuclear factor-κB (NF-κB) and nuclear factor-erythroid 2-related factor 2 (Nrf2) [15]. Activation of the NF-κB pathway leads to expression of pro-inflammatory cytokines, including tumor necrosis factor−α (TNF-α) or interleukin-6 (IL-6). H_2S has been shown to inhibit or activate NF-κB nuclear translocation [16,17] and may affect the activity of numerous kinases including p38 mitogen-activated protein kinase (p38 MAPK) [18]. On the other hand, Nrf2 plays a key role in cellular antioxidant defences. Under basal conditions, Nrf2 is sequestered by Keap1 (Kelch-like ECH-associated protein) in the cytosol [19]. When challenged by oxidants, the complex disrupts and Nrf2 translocates to the nucleus where it leads to the transcription of different antioxidant defence enzymes, like glutathione S-transferase (GST) [20] and heme oxygenase-1 (HO-1) [21]. Recently, Nrf2 has been suggested as a potential mediator of the cardioprotective effect of H_2S [22].

NF-κB as well as Nrf2 signalling pathways have been reported to be involved in the activation of HIF-1α [23–25]. Whether H_2S is involved in HIF-1α regulation is not clarified so far. Exposure of *C. elegans* to H_2S resulted in accumulation of HIF-1α protein and transcription of HIF-1 targets [26]. Additionally, pro-angiogenic effects of H_2S have been reported to be mediated *via* HIF-1α stabilisation during chemically induced hypoxia in rat brain cells [27]. On the other hand, Wu et al. reported that accumulation of HIF-1α protein under hypoxia was reversed by H_2S [28].

In the present study using the slow H_2S releasing donor GYY4137, we report that H_2S induced HIF-1α and Nrf2 protein accumulation. Our results suggest that the underlying mechanism may involve activation of p38 MAPK.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) if not stated otherwise. Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (San Diego, CA). 2′,7′-dichloro-dihydrofluorescein diacetate (H_2DCF-DA) was purchased from Molecular Probes (Eugene, OR).

2.2. Production of H_2S

H_2S was generated by addition of GYY4137 [(p-methoxyphenyl) morpholino-phosphinodithioic acid] to the incubation media.

2.2.1. Synthesis of GYY4137

To a solution of morpholine (10 mmol) in dichloromethane (3 mL) Lawsson reagent (2 mmol; dissolved in 3 mL dichloromethane) was added dropwise. After 3 h the formed precipitate was filtered and washed several times with dichloromethane and dried. The reaction gave a white solid (85% yield). Melting point: 130 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.11–8.00 (m, 2H), 6.93–6.86 (m, 2H), 5.30 (s, 1H), 3.84 (s, 3H), 3.78–3.71 (m, 4H), 3.63–3.60 (m, 4H), 3.23–3.17 (m, 4H), 3.00–2.94 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 161.3, 161.2, 132.8, 132.5, 132.1, 130.1, 113.0, 112.7, 67.3, 67.0, 63.7, 55.3, 44.9, 44.9, 43.4. Anal. Calculated for $C_{15}H_{25}N_2O_3PS_2$: C, 47.85; H, 6.69; N, 7.44; S, 17.03. Found: C, 47.48; H, 6.44; N, 7.10; S, 16.81.

2.2.2. Measurement of H_2S release from GYY4137

H_2S release from GYY4137 was assessed with the use of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). GYY4137 (0.25–1 mmol/L) was incubated in HBSS in capped glass vials at 37 °C. At appropriate times, aliquots (100 μL) were mixed with DTNB (1 mmol/L, 250 μL) and HEPES buffer (50 mmol/L, 250 μL, pH 8.0), and absorbance was measured at 412 nm. The concentration of H_2S (defined as a combination of H_2S, HS^- and S^{2-}) released from GYY4137 was calculated from a standard curve of NaHS (12.5–200 μmol/L).

2.3. Cell culture

Human monocytic THP-1 cells were cultured in RPMI 1640 media containing 10% FCS (Gibco, NY), L-glutamine (2 mmol/L), streptomycin (50 U/mL) and penicillin (0.1 mg/mL; PAA, Linz, Austria). Cell viability was determined by an electronic cell counter (CASY Model TTC; Schaerfe System, Reutlingen, Germany). Cells with viability higher than 95% were used to start differentiation towards macrophages.

2.4. Cell treatment

THP-1 cells (0.5×10^6 cells/mL) were differentiated towards macrophages by addition of PMA (160 nmol/L) for 3 days [29], washed twice with Hank's Balanced Salt Solution (HBSS) and incubated with up to 1 mmol/L GYY4137 for 6 or 24 h. Hypoxic conditions were generated in a hypoxia incubator chamber (Billups Rothenberg, Del Mar, CA) which was flushed with 1% O_2, 5% CO_2 for 10 min and sealed.

For making whole cell lysates, the cells were lysed in 125 mmol/L Tris–HCl, pH 6.5 with 2% SDS, 10% glycerol, 100 mmol/L DTT, supplemented with protease inhibitor mixture. Cytosolic and nuclear cell lysates were separated by using the Active Motif nuclear extract kit (Carlsbad, CA) according to the manufacturer's protocol. Protein concentration was determined by the Bradford assay (Bio-Rad, Vienna, Austria).

2.5. Immunoblotting

Cell lysates were separated under reducing conditions on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane with 5% non-fat dry milk in TBS washing buffer (20 mmol/L Tris–HCl pH 7.4 with 145 mmol/L NaCl, 0.1% Tween-20) for 1 h and membranes were incubated with primary antibodies overnight at 4 °C.

Anti-HIF-1α antibody was obtained from BD BioSciences (San Jose, CA), anti-Nrf2, anti-GLUT-1, anti-Calnexin and anti-Lamin B1 antibodies were purchased from Abcam (Cambridge, UK). Anti-NF-κB subunits p65 and phosho-p65 (Ser536) antibodies were from Cell Signaling (Danvers, MA). Anti-β-Actin antibodies were obtained from Sigma. Next day, the membranes were incubated with anti-mouse or anti-rabbit HRP-linked secondary antibodies for 1 h at room temperature. Bands were detected by enhanced chemiluminescence (GE Healthcare, UK) and a chemiluminescence imager system (Fusion Fx7, Peqlab).

2.6. HO-1 protein detection

Heme oxygenase-1 was quantified using the HO-1 sandwich enzyme linked immunosorbent assay development set from Abcam.

2.7. Measurement of cytotoxicity

To monitor whether GYY4137 shows an impact on cell viability, we used the LDH (lactate dehydrogenase) cytotoxicity assay kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's instruction.