Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia

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Abstract The disrupted metabolism of homocysteine (Hcy) causes hyperhomocysteinemia, a condition associated with the impairment of nitric oxide (NO) bio-availability, tissue hypoxia and increased risk of vascular disease. Here, we examined how Hcy modulates the induction of the stress protein haem oxygenase-1 (HO-1) evoked by NO releasing agents and hypoxia in vascular endothelial cells. We found that Hcy (0.5 mM) markedly reduced the increase in haem oxygenase activity and HO-1 protein expression induced by sodium nitroprusside (SNP, 0.5 mM) but did not affect HO-1 activation mediated by *S*-nitroso-*N*-acetyl-penicillamine. Cells pre-treated with Hcy followed by addition of fresh medium containing SNP still exhibited an augmented haem oxygenase activity. Interestingly, high levels of Hcy were also able to abolish hypoxia-mediated HO-1 expression in a concentration-dependent manner. These novel findings indicate that hyperhomocysteinemia interferes with crucial signaling pathways required by cells to respond and adapt to stressful conditions.

Key words: Heme oxygenase-1; Nitric oxide; Endothelial cell dysfunction; Stress response; *S*-Nitrosothiol; Signaling pathway

1. Introduction

Increased plasma homocysteine (Hcy) levels have become widely accepted as an independent risk factor for vascular disease [1,2]. Hyperhomocysteinemia, a condition that arises from disrupted Hcy metabolism, is caused by both genetic and dietary disorders and has been implicated in the pathophysiology of atherosclerosis [3]. Moderate hyperhomocysteinemia has been found in 20–30% of patients with coronary and peripheral vascular disease and levels of Hcy may be as high as 200–300 μM in homocysteinuria [4,5]. Hcy is a sulphur-containing amino acid whose metabolism stands at the intersection of two pathways: remethylation to methionine, which requires folate and vitamin B12, and trans-sulphuration to cystathionine, which requires pyridoxal-5′-phosphate [6]. The mechanism(s) whereby impaired Hcy metabolism leads to the development of vascular dysfunction is poorly understood. Previous studies have shown that Hcy evokes adverse vascular effects by promoting oxidative damage to endothelial cells [1,7]. These cytotoxic effects appear to be caused by an increased production of hydrogen peroxide during the auto-oxidation of the sulfhydryl group and may account for the observed endothelial dysfunction mediated by elevated Hcy levels [4–6]. Alteration of the vascular responses of normal endothelial cells by Hcy has also been attributed to the high reactivity of this metabolite with nitric oxide (NO) [1,8]. Indeed, thiols are critical sites of reaction for NO and NO-related species in biological systems, leading to the formation of *S*-nitrosothiols [9,10]. *S*-Nitrosohomocysteine is formed under physiological conditions [8] and the presence of thiols in the surrounding intracellular milieu determine its stability, reactivity and, consequently, its cytoprotective or cytotoxic action [11].

In view of its capacity to interact with NO and promote oxidative stress to the vascular wall, there exists the possibility that increased Hcy levels may affect the expression of intracellular defensive genes that are induced in response to stressful conditions. We have demonstrated that haem oxygenase-1 (HO-1), a redox sensitive inducible protein which is crucial for the resolution of several disorders, is potently induced by NO and NO-related species in aortic endothelial cells [12–15]; this effect results in increased haem oxygenase activity which protects vascular cells against oxidative stress and cell death [13,16,17]. The induction of HO-1 by exogenous and endogenously produced NO is prevented by increased glutathione production and is finely modulated by redox reactions involving the formation of *S*-nitrosothiols [12,15]. It is, therefore, plausible to suggest that other compounds containing sulphydryl residues could influence the expression of HO-1 in response to stressful stimuli. In the present study, we investigated the effect of high levels of Hcy on HO-1 expression and haem oxygenase activation mediated by NO and low oxygen tension (hypoxia) in cultured vascular endothelial cells.

2. Materials and methods

2.1. Reagents

S-Nitroso-*N*-acetylpenicillamine (SNAP) was obtained from Alexis Corporation (Bingham, Nottingham, UK). Sodium nitroprusside (SNP), Hcy and all other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise specified.

2.2. Cell culture and hypoxia

Bovine aortic endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA), cultured in 75 cm^2 flasks and grown in Iscove's modified Dulbecco's medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). For the experiments conducted under hypoxic conditions, endothelial cells at confluence were

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transferred to an air-tight chamber (Billups-Rothenberg, Del Mar, CA, USA) and flushed with a mixture of 95% N_2–5% CO_2. The gas was infused continuously into the air-tight chamber at a flow rate of 5 l/min for the first 2 h and at 2 l/min for the following hours of incubation. In previous experiments conducted under these conditions [15], it was found that the pO_2 measured in the media by an oxygen electrode was 2 mm Hg after 2 h exposure to hypoxia and did not fluctuate from this value throughout the remaining incubation period. Within the hypoxia chamber, cells were maintained in a humidified atmosphere at 37°C.

2.3. Experimental protocol

The NO releasing agents, SNP and SNAP, were used in all experiments. Cells were incubated for 6 h in complete medium (control), medium supplemented with 0.5 mM SNP or 0.5 mM SNP plus 0.5 mM Hcy. In another set of experiments, cells were pre-treated with 0.5 mM Hcy for 18 h prior to exposure to SNP (0.5 mM) for 6 h. In these experiments, SNP was added either to the original medium of cells pre-treated with Hcy or the existing medium was replaced with fresh medium containing SNP. Similar experiments were carried out with SNAP. Cells were also treated with various concentrations of Hcy for 6 or 18 h to exclude any potential effect of this thiol compound on HO-1 induction. For the experiments conducted under hypoxic conditions, cells were exposed to low oxygen tension ($pO_2 \approx 2$ mm Hg) for 18 h with or without Hcy (0.5–1 mM). At the end of each treatment, cells were collected and analysed for haem oxygenase activity and HO-1 protein expression.

2.4. Assay for endothelial haem oxygenase activity

A haem oxygenase activity assay was performed as previously described [12,15,18]. Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase and the substrate hemin. The reaction was conducted at 37°C in the dark for 1 h, terminated by the addition of 1 ml of chloroform and the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40$ mM^{-1} cm^{-1}).

2.5. Western blot analysis for HO-1

Samples of endothelial cells treated for the haem oxygenase activity assay were also analysed by Western immunoblot technique as previously described [12,15]. Briefly, 30 μg of protein from each sample were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred overnight to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in phosphate-buffered saline (PBS). Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with PBS containing 0.05% (v/v) Tween-20, blots were visualised using an amplified alkaline phosphatase kit from Sigma (Extra-3A) and the relative density of bands analysed by an imaging densitometer (Model GS-700, Bio-Rad, Herts, UK).

2.6. Statistical analysis

Differences in data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at $P < 0.05$.

3. Results

3.1. Effect of Hcy on NO-mediated HO-1 induction in endothelial cells

The effect of SNP and Hcy on haem oxygenase activity and HO-1 protein expression is shown in Fig. 1. Treatment of cells with 0.5 mM SNP for 6 h resulted in a significant increase in haem oxygenase activity from 171 ± 18 (control) to 1466 ± 165 pmol bilirubin/mg protein/h (Fig. 1A) confirming our previous studies [12]. This effect was attenuated by the presence of 0.5 mM Hcy (558 ± 47 pmol bilirubin/mg protein/h) ($P < 0.05$ versus control). Interestingly, pre-treatment of cells with Hcy for 18 h followed by addition of SNP to the original culture medium also resulted in a significant decrease in haem oxygenase activity ($P < 0.05$). Addition of fresh medium containing SNP to cells pre-treated with Hcy still showed a reduced activation of haem oxygenase; however, in this case the activity was higher compared to cells treated with SNP added to the original culture medium. Changes in haem oxygenase activity were reflected by changes in HO-1 levels as increased HO-1 protein expression by SNP was significantly reduced by the presence of Hcy in the culture media (Fig. 1B). Endothelial cells exposed to SNAP for 6 h also resulted in augmented haem oxygenase activity; however, this effect was unaltered by the presence of Hcy (Fig. 2). It is important to note that treatment of cells with Hcy (0.5–1 mM) alone for 6 or 18 h did not cause any change in haem oxygenase activity compared to control (data not shown).

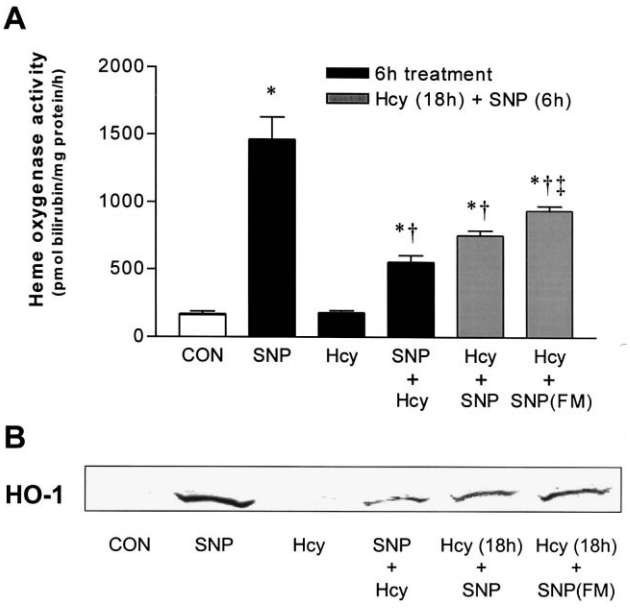

Fig. 1. Modulatory effect of Hcy on endothelial HO-1 induction mediated by SNP. Endothelial haem oxygenase activity (A) and HO-1 protein expression (B) were determined after 6 h incubation (black bars) with 0.5 mM SNP, 0.5 mM Hcy or 0.5 mM SNP+0.5 mM Hcy. In additional experiments, cells were pre-treated for 18 h with 0.5 mM Hcy followed by exposure to 0.5 mM SNP (grey bars). In these experiments, SNP was added either to the original medium of cells pre-treated with Hcy or the existing medium was replaced with fresh medium (FM) containing SNP. *$P < 0.05$ versus control (CON); †$P < 0.05$ versus SNP; ‡$P < 0.05$ versus SNP+Hcy (6 h).

3.2. Effect of Hcy on hypoxia-mediated HO-1 induction in endothelial cells

Exposure of endothelial cells to reduced oxygen tension resulted in augmented HO-1 protein expression, which was associated with a marked increase in haem oxygenase activity ($P < 0.01$) (Fig. 3A,B). After 18 h hypoxia, haem oxygenase activity increased from 171 ± 18 to 2917 ± 272 pmol bilirubin/mg protein/h ($P < 0.01$). Interestingly, the presence of Hcy in the culture media of cells exposed to hypoxia markedly reduced the increase in haem oxygenase activity in a concentration-dependent manner. Specifically, as shown in Fig. 3A, 0.5 and 1 mM Hcy suppressed hypoxia-mediated haem oxygenase activation by 79.7 and 93.7%, respectively ($P < 0.01$); this

effect was paralleled by a marked reduction in HO-1 protein expression as revealed by Western blot analysis (Fig. 3B).

4. Discussion

Recent investigations from our group have demonstrated a modulatory role of thiols and *S*-nitrosothiols in NO-mediated induction of HO-1 in vascular endothelial cells [12,13,15,19]. Here, we extend our previous findings by showing that Hcy interacts with NO to prevent the stimulation of endothelial HO-1 expression and haem oxygenase activity by SNP, an iron nitrosyl complex in which the NO is formally bound to the metal centre as nitrosonium cation (NO^+). Hcy is known to react with the NO group and stabilise it by formation of *S*-nitrosohomocysteine [8,11]; this is consistent with the notion that NO^+ can be transferred reversibly to cysteine residues (trans-nitrosation) which are preferential targets of NO and NO-related species [20]. Accordingly, compared to cells where SNP and Hcy were added simultaneously to the starting medium, cells pre-treated with Hcy for 18 h followed by addition of fresh medium containing SNP still displayed a significant elevation in haem oxygenase activity. These data suggest that attenuation of HO-1 expression caused by Hcy is the consequence of a direct interaction between the sulphur-containing amino acid and the NO donor. Our data exclude the possibility that oxidative stress mediated by hydrogen peroxide originating from Hcy [1,7] is responsible for HO-1 induction as haem oxygenase activity was unchanged after treatment of cells with high levels of Hcy alone. We also found that activation of endothelial haem oxygenase by SNAP, a nitrosating agent that release NO but to a certain extent also NO^+ and nitroxyl anion (NO^-) [21,22], was not affected by the presence of Hcy in the starting medium. This could be related to the different trans-nitrosation activities of SNP and SNAP towards sulphydryl groups [22] and is in agreement with our previous findings showing that much higher amounts of exogenous thiols are required to effectively reduce SNAP-mediated HO-1 induction in endothelial cells [12,23]. In view of the multifactorial mechanism by which hyperhomocysteinemia damages the endothelium leading to an impairment of vascular function, the data presented in this study highlight the possibility that the decrease in antioxidant potential promoted by Hcy in human endothelial cells could be related to its negative effect on the expression of important genes implicated in cytoprotection [24].

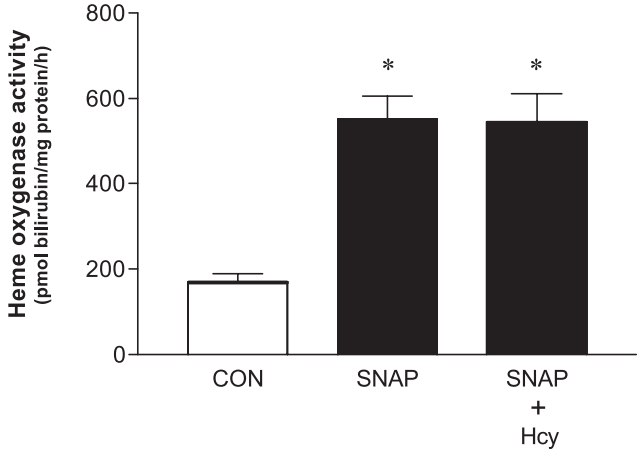

Fig. 2. Effect of Hcy on SNAP-mediated haem oxygenase activation in endothelial cells. Heme oxygenase activity was determined 6 h after incubation of endothelial cells with 0.5 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) or 0.5 mM SNAP+0.5 mM Hcy. $^*P<0.05$ versus control.

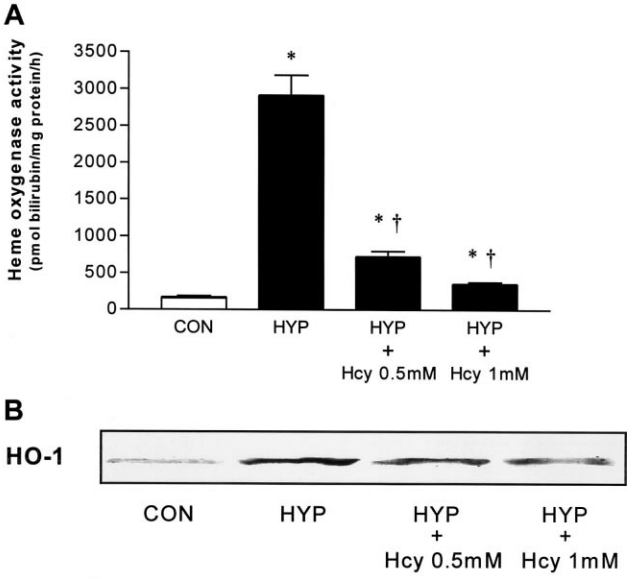

Fig. 3. Effect of Hcy on hypoxia-mediated HO-1 induction in endothelial cells. Heme oxygenase activity (A) and HO-1 protein expression (B) were determined 18 h after exposure of cells to hypoxic conditions ($pO_2 \approx 2$ mm Hg) in the presence or absence of Hcy (0.5–1 mM). Control experiments are represented by cells incubated under normoxic conditions for 18 h. $^*P<0.01$ versus control (CON); $^\dagger P<0.01$ versus hypoxia (HYP).

As several cardiovascular-related diseases are characterised by a reduction in oxygen availability, which renders the challenged tissue hypoxic, we designed experiments to examine the possible modulatory effect of Hcy on hypoxia-mediated HO-1 induction. Hypoxia is known to promote an increased generation of reactive oxygen species resulting in the induction of distinctive genes whose expression is finely regulated by redox modification of transcription factors such as hypoxia inducible factor-1 [25,26]. A previous report from our group demonstrated that over-expression of HO-1 and the consequent elevation in haem oxygenase activity of endothelial cells exposed to hypoxia are associated with a transient formation of endogenous *S*-nitrosothiols due to an early induction of the iNOS gene [15]. We have also shown that iNOS inhibitors significantly attenuate hypoxia-mediated HO-1 activation suggesting that, in conditions of low oxygen tension, both oxidative and nitrosative reactions actively contribute to the observed stimulation of the HO-1 pathway [15]. In the present study, we found that Hcy suppresses HO-1 protein expression and prevents the increase in haem oxygenase activity caused by hypoxia in a concentration-dependent manner. These data suggest that high levels of Hcy may render the cardiovascular system more vulnerable to oxidant-mediated injury since a crucial role for the HO-1/bilirubin pathway in protection against hypoxia-reoxygenation of cardiac tissue has been recently demonstrated [27,28].

In conclusion, based on the data of the present study, we propose that Hcy may contribute to vascular dysfunction by preventing the inherent ability of endothelial cells to respond

and adapt to stressful conditions. As the products of haem degradation by augmented HO-1 act as important effector molecules in the restoration of vascular activities following pathophysiological insults [14,27,29,30], chronic exposure to hyperhomocysteinemia could significantly interfere with important transduction signals responsible for the activation of HO-1 and other protective systems required to counteract the diverse forms of stress [19]. In this context, the direct interaction of Hcy with NO groups in normal or hypoxic conditions and the consequent impairment of NO's biological activity would exacerbate oxidative injury ultimately contributing to endothelial cell dysfunction.

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References

[1] Loscalzo, J. (1996) J. Clin. Invest. 98, 5–7.
[2] Clarke, R., Daly, L., Robinson, K., Naughten, E., Cahalane, S., Fowler, B. and Graham, I. (1991) N. Engl. J. Med. 324, 1149–1155.
[3] Guthikonda, S. and Haynes, W.G. (1999) Curr. Opin. Cardiol. 14, 283–291.
[4] Malinow, M.R., Sexton, G., Averbuch, M., Grossman, M., Wilson, D. and Upson, B. (1990) Coron. Artery Dis. 1, 215–220.
[5] Malinow, M.R. (1994) J. Intern. Med. 236, 603–617.
[6] Selhub, J. (1999) Annu. Rev. Nutr. 19, 217–246.
[7] Starkebaum, G. and Harlan, J.M. (1986) J. Clin. Invest. 77, 1370–1376.
[8] Stamler, J.S., Osborne, J.A., Jaraki, O., Rabbani, L.E., Mullins, M., Singel, D. and Loscalzo, J. (1993) J. Clin. Invest. 91, 308–318.
[9] Stamler, J.S., Simon, D.I., Osborne, J.A., Mullins, M.E., Jaraki, O., Michel, T., Singel, D.J. and Loscalzo, J. (1992) Proc. Natl. Acad. Sci. USA 89, 444–448.
[10] Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992) Science 258, 1898–1902.
[11] D'Emilia, D.M. and Lipton, S.A. (1999) Neurosci. Lett. 265, 103–106.
[12] Foresti, R., Clark, J.E., Green, C.J. and Motterlini, R. (1997) J. Biol. Chem. 272, 18411–18417.
[13] Foresti, R., Sarathchandra, P., Clark, J.E., Green, C.J. and Motterlini, R. (1999) Biochem. J. 339, 729–736.
[14] Sammut, I.A., Foresti, R., Clark, J.E., Exon, D.J., Vesely, M.J.J., Sarathchandra, P., Green, C.J. and Motterlini, R. (1998) Br. J. Pharmacol. 125, 1437–1444.
[15] Motterlini, R., Foresti, R., Bassi, R., Calabrese, V., Clark, J.E. and Green, C.J. (2000) J. Biol. Chem. 275, 13613–13620.
[16] Motterlini, R., Foresti, R., Intaglietta, M. and Winslow, R.M. (1996) Am. J. Physiol. 270, H107–H114.
[17] Clark, J.E., Foresti, R., Green, C.J. and Motterlini, R. (2000) Biochem. J. 348, 615–619.
[18] Motterlini, R., Foresti, R., Intaglietta, M., Vandegriff, K. and Winslow, R.M. (1995) Am. J. Physiol. 269, H648–H655.
[19] Foresti, R. and Motterlini, R. (1999) Free Rad. Res. 31, 459–475.
[20] Stamler, J.S. (1994) Cell 78, 931–936.
[21] Arnelle, D.R. and Stamler, J.S. (1995) Arch. Biochem. Biophys. 318, 279–285.
[22] Feelisch, M. (1998) Naunyn-Schmiedeberg's Arch. Pharmacol. 358, 113–122.
[23] Motterlini, R., Green, C.J. and Foresti, R. (2002) Antiox. Redox Signal., in press.
[24] Outinen, P.A., Sood, S.K., Pfeifer, S.I., Pamidi, S., Podor, T.J., Li, J., Weitz, J.I. and Austin, R.C. (1999) Blood 94, 959–967.
[25] Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C. and Schumacker, P.T. (1998) Proc. Natl. Acad. Sci. USA 95, 11715–11720.
[26] Bunn, H.F. and Poyton, R.O. (1996) Physiol. Rev. 76, 839–885.
[27] Clark, J.E., Foresti, R., Sarathchandra, P., Kaur, H., Green, C.J. and Motterlini, R. (2000) Am. J. Physiol. Heart Circ. Physiol. 278, H643–H651.
[28] Foresti, R., Goatly, H., Green, C.J. and Motterlini, R. (2001) Am. J. Physiol. Heart Circ. Physiol., in press.
[29] Motterlini, R., Gonzales, A., Foresti, R., Clark, J.E., Green, C.J. and Winslow, R.M. (1998) Circ. Res. 83, 568–577.
[30] Taille, C., Foresti, R., Lanone, S., Zedda, C., Green, C.J., Aubier, M., Motterlini, R. and Boczkowski, J. (2001) Am. J. Respir. Crit. Care Med. 163, 753–761.