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The induction of GSH synthesis by nanomolar concentrations of NO in endothelial cells: a role for γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase

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Abstract Nitric oxide protects cells from oxidative stress through a number of direct scavenging reactions with free radicals but the effects of nitric oxide on the regulation of antioxidant enzymes are only now emerging. Using bovine aortic endothelial cells as a model, we show that nitric oxide, at physiological rates of production (1–3 nM/s), is capable of inducing the synthesis of glutathione through a mechanism involving γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase. This novel nitric oxide signalling pathway is cGMP-independent and we hypothesize that it makes an important contribution to the anti-atherosclerotic and antioxidant properties of nitric oxide.

Key words: Glutathione; Endothelial cell; Nitric oxide; γ-Glutamylcysteine synthetase; Atherosclerosis; γ-Glutamyl transpeptidase

1. Introduction

Nitric oxide (NO) has been shown in a number of studies to protect cells from the cytotoxicity of oxidants [1–3]. This may be particularly important in chronic diseases of the vasculature in which oxidants, or their metabolic by-products, are thought to contribute to the pathophysiology of the disease [4–8]. An important example of this is atherosclerosis, initiated by hypercholesterolemia, which is associated with an early loss of responses to endothelial-derived NO [4,8–11]. NO has been shown to protect the cells of the vasculature by inhibiting the cell-dependent oxidation of low density lipoprotein (LDL) [3,12–14]. However, oxidized LDL (oxLDL) has been shown to inhibit the NO production from the endothelium as is known to occur in human atherosclerosis [15,16].

The intracellular antioxidant glutathione (GSH) protects cells against oxidative stress and its synthesis and related metabolism are now understood in some detail [17,18]. It is less clear how exposure to reactive nitrogen species affects GSH metabolism. In the vasculature, GSH protects against oxLDL by detoxifying the lipid intermediates formed during oxidation and also protects against the loss of NO production from the endothelium. Thus, there appears to be a mechanistic link between intracellular levels of GSH, the loss of NO formation in hypercholesterolemia and modified lipoproteins [19]. Since oxLDL is likely to be formed in atherosclerotic vessels, through multiple mechanisms, it is reasonable to postulate that maintenance of essential antioxidants, such as GSH, is important to retard atherosclerosis [6,15].

Under some circumstances, NO, or its metabolites, can react with oxidants produced at sites of inflammation such as superoxide or hypochlorite. It may then exert effects on the cell that have been associated with cytotoxicity as in the case of peroxynitrite [8]. These effects can also occur with the very high concentrations of NO formed on activation of the inducible or type 1 NO synthase (NOS). Little is known, however, of the effects of NO on GSH metabolism at the concentrations of NO formed in the normal vasculature.

In a recent study [20], we have shown that rates of production of NO in the order of 8–12 nM/s induce GSH synthesis in vascular smooth muscle cells (VSMCs) through the increased activity and expression of the rate limiting enzyme for its synthesis γ-glutamylcysteine synthetase (GCS). These rates of NO formation are at the upper limit of the eNOS activity and are probably not encountered under physiological conditions in the vasculature but could be readily achieved if iNOS is induced [8,21,22]. In vivo, the vasculature is responsive to 10–50 nM NO and under physiological conditions, this NO production is stimulated by the flow of blood over the endothelium. The rate of NO production in the vasculature due to blood flow has been measured directly and found to be in the order of 1–3 nM/s [23]. These rates of NO formation elicited little or no effect on the GSH levels in smooth muscle cells, therefore we decided to test their effect on endothelial cells [20].

The rate-limiting enzyme in the GSH synthesis, GCS, consists of two subunits, the catalytic (heavy) subunit and the regulatory (light) subunit. The promoter regions for the heavy and light subunits contain a number of transcription factor binding sites including AP-1, Sp-1, NFκ-B, ARE and EpRE sites, all of which are potential targets for NO-dependent reg-

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Abbreviations: NO, nitric oxide; SNAP, S-nitrosopenicillamine; BAEC, bovine aortic endothelial cell; GCS, γ-glutamylcysteine synthetase; γ-GT, γ-glutamyl transpeptidase; GSH, reduced glutathione; GSSG, oxidized glutathione; tGSH, total glutathione; DTPA, diethylene triamine penta acetic acid; DEPC, diethyl pyrocarbonate; NOS, nitric oxide synthase; ODQ, 1H-(1,2,4) oxadiazolo(4,3,-α) quinoxalin-1-one

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ulation [24–26]. Recent data indicate that exogenous NO increases the production of GSH in vascular VSMCs by increasing the levels of mRNA for both the heavy and light subunit of GCS [20].

In this study, we have investigated the potential of NO to regulate the synthesis of the antioxidant GSH in bovine aortic endothelial cells (BAECs) by treatment of the cells with chemically diverse NO donors. We find that NO in the low nM concentration range induces GSH synthesis in endothelial cells through a process that is not dependent on the cGMP signalling pathway but does require the activity of GCS and γ-glutamyl transpeptidase (γ-GT). We hypothesize that this NO-dependent induction of GSH synthesis contributes to its anti-atherogenic effects.

2. Materials and methods

2.1. Cell culture

BAECs harvested from descending thoracic aortas were maintained (37°C, 5% CO_2) in a growth medium (DMEM (1 g/l glucose, Gibco) containing 20% fetal calf serum (Atlanta Biologicals) without antibiotics [27]. Cells used in this study were between passages 5–13.

2.2. Measurement of the rate of decomposition of NO donors

S-nitrosopenicillamine (SNAP) or DetaNONOate (Alexis Biochemicals, San Diego, CA, USA) was added to cells in culture. The rate of NO release from DetaNONOate was calculated from the published half life of 20 h [28]. To measure the rate of NO release from SNAP, the NO-dependent oxidation of oxymyoglobin was measured. Oxymyoglobin (30 μM) was added along with SNAP to cells and the oxidation was followed by measuring the absorbance decrease at 580 nm over a period of 15 min incubation with cells. Concentration changes were calculated using the $\varepsilon_{580nm} = 14.4$/mM/cm. Decomposed SNAP was prepared by incubating the stock concentration of SNAP (10 mM) under light for 48 h at 22°C after which time no absorbance at 340 nm could be detected indicating the complete decomposition of the compound.

2.3. GSH and GCS activity measurements

Total GSH (tGSH) (GSH+GSSG) was measured using a spectrophotometric assay coupling the thionitrobenzoate formation from dithionitrobenzoate with the GSH reductase activity [29]. The rates were converted to tGSH concentrations using a standard curve generated with authentic GSH. For determination of the GCS activity, cultured cells were grown to confluency in six well plates and after experimental manipulation, the medium was removed and the cells were washed twice with ice-cold PBS. The cells were then scraped from the plates into a 100 μl volume of 100 mM Tris-Cl (pH 8.2), 50 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 0.1% Triton X-100, centrifuged (4°C, 20 min, $15000 \times g$) and the supernatants removed. A 300 μl aliquot of the supernatant was then added to 700 μl of a buffer containing 100 mM Tris (pH 8.2), 50 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 10 mM ATP, 5 mM glutamic acid and 5 mM α-aminobutyric acid. The solution was incubated at 37°C for 2 h before the reaction was terminated by the addition of 200 μl of 1 M perchloric acid. The activity of GCS was then determined by measuring the formation of γ-glutamyl-α-aminobutyric acid using HPLC analysis [30].

3. Results and discussion

3.1. NO induces GSH synthesis in endothelial cells

To determine whether NO induces GSH synthesis, BAECs were exposed to two chemically distinct NO donors, SNAP and DetaNONOate. Exposure of cells to SNAP for 12 h increased the tGSH in a concentration-dependent manner (Fig. 1A). Concentrations as low as 50 μM SNAP significantly increased the tGSH while 200 μM SNAP resulted in a maximal effect on tGSH levels (Fig. 1A). When the products of decomposed SNAP were used to treat BAECs, there was no increase in the tGSH indicating that NO, or NO-derived species, were responsible for these effects rather than the decomposition products of the NO donor. In a second series of experiments, the time course for induction of tGSH by SNAP was determined. The basal level of total tGSH in the cells for these experiments was approximately 30% higher than those used in the experiment shown in Fig. 1A. These differences in tGSH levels have been observed by other laboratories and are due to routine changes in the media and a consequent variation in growth rates in the cell cultures [18]. Again, an increase in the tGSH levels was observed in response to SNAP treatment detectable at 8 h and reaching a maximum at 12 h of exposure (Fig. 1B).

To determine the exposure of the cells to NO under these conditions, the rate of oxidation of oxymyoglobin was determined as a function of the SNAP concentration. Similar to other NO donors, release of NO from SNAP occurred in a cell-dependent manner and was saturable (with an apparent K_m of approximately 15 μM for SNAP). The maximal rates of NO release were 1 nM/s, with 50–100 μM SNAP, which would be a rate of NO production readily achieved during the physiological activation of eNOS [21,23].

The S-nitrosothiols have other biochemical mechanisms through which they can exert their effects including transfer of the nitrosonium group to another thiol via a transnitrosation reaction [31]. To rule out this mechanism, the compound DetaNONOate was used as an NO donor which has a different mechanism of NO release, chemistry and decomposition products than SNAP [28]. The results are shown in Fig. 2 in comparison with SNAP and indicate that DetaNONOate was also able to induce tGSH synthesis. The decomposition products of either SNAP or DetaNONOate had no significant effect on tGSH levels in the cells, nor was cytotoxicity detected in the presence of NO donors at this concentration (result not shown). The rate of NO release from 100 μM DetaNONOate was calculated from its published half life (20 h). Taking into account that two NO molecules are re-

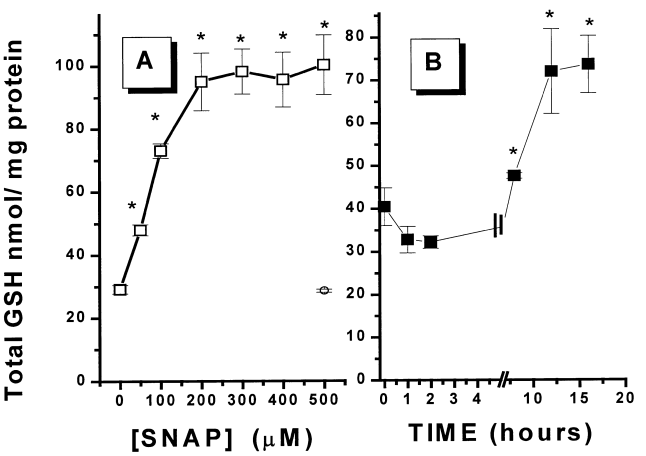

Fig. 1. The induction of total GSH in endothelial cells by NO. Endothelial cells were exposed to increasing concentrations of SNAP (□) or its decomposition products (○) (A) for a period of 12 h before the measurement of tGSH. Cells were lysed with Triton X-100 (0.1%) in PBS and the tGSH content was determined and normalized to protein. In B, the result of exposing cells to 500 μM SNAP for the periods shown on tGSH was determined. Data are represented as the mean ± S.E.M., $n = 3$–4. *Significantly different to control, ($P < 0.01$).

Table 1
Acivicin blocks the NO-dependent increase in tGSH

Sample	Cellular GSH (nmol/mg protein)	Medium GSH (nmol/mg cell protein)
Control	36.8 ± 1.3	3.8 ± 0.2
100 μM DetaNONOate	61.1 ± 2.8	10.0 ± 1.2
100 μM DetaNONOate+5μM acivicin	37.4 ± 1.5	14.3 ± 2.3
5 μM Acivicin	37.6 ± 3.4	6.0 ± 0.8

BAECs were treated in vitro with either 100 μM detaNONOate, 5 μM acivicin or both, for 16 h. Media were collected and cells were rinsed twice with ice-cold PBS plus 0.1% Triton and 10 μM DTPA and the concentration of GSH was measured. The tGSH measured in the media was predominantly (more than 90%) in the form of GSH as determined by the direct measurement of reduced thiol with DTNB. The medium GSH is normalized to the protein concentration in the cell lysate to correct for variability in the cell growth or seeding density.

leased for each molecule of the NONOate, the rate of NO release was approximately 1–1.6 nM/s, similar to that found with SNAP [28]. This rate of NO formation is approximately 250 times lower than the rates reported to be cytotoxic to cells in culture and leading to an initial depletion of tGSH [32–34].

The NO-dependent increase in tGSH may occur through affecting the activity of enzymes, such as GSH peroxidase (GPx), which use GSH as a substrate. Treatment of either VSMCs or BAECs with SNAP for 4 h did not affect the activity of either GPx or catalase (data not shown) indicating that the changes in the tGSH levels with NO treatment were not due to changes in the activity of these enzymes. Although an increase in tGSH clearly occurs in BAECs on exposure to NO, this would not result in an increased antioxidant capacity of the cell if the antioxidant is predominantly in its oxidized form (GSSG). To test for this possibility, cells were exposed to 200 μM SNAP for a period of 12 h before the measurement of GSH and GSSG by HPLC. The tGSH increased by 2.5-fold while the ratio of GSH:GSSG was 19.7 ± 0.7 for decomposed SNAP, 17.6 ± 1.2 for the control (untreated cells) and increased to 57 ± 0.7 for cells exposed to SNAP (all data = mean ± S.E.M, $n = 3$). These data indicate that GSH-dependent antioxidant defenses have been enhanced by exposure to NO.

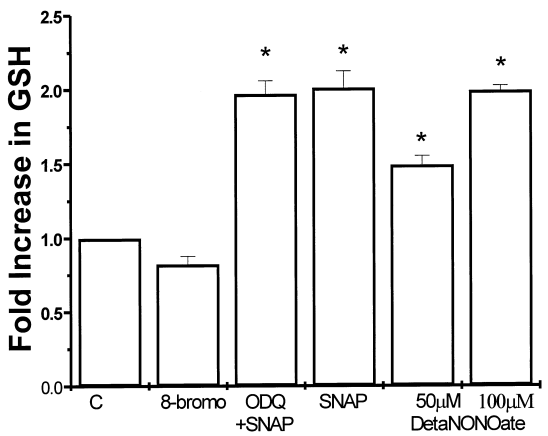

Fig. 2. The effect of cGMP analogs and inhibition of guanylate cyclase on the induction of GSH in endothelial cells. Endothelial cells were exposed to the cell permeable cGMP analog 8-bromo-cGMP (500 μM), 200 μM SNAP in the presence and absence of 3 μM ODQ, or 50 or 100 μM DetaNONOate. The vehicle for ODQ was DMSO, present at a final concentration of 0.1%. The fold increases in tGSH levels are shown following exposure to various treatments for 12 h. The control values for tGSH in untreated cells were between 18 and 25 nmol tGSH/mg protein for the different experiments. Data are represented as the mean ± S.E.M., $n = 3$–4, *significantly different to control ($P < 0.01$).

3.2. Induction of GSH synthesis by NO is cGMP- and guanylate cyclase-independent

To test the potential role of the NO soluble guanylate cyclase signalling pathway in the induction of tGSH synthesis, two approaches were taken. Firstly, endothelial cells were incubated with a cell permeable cGMP analog, 8-bromo cGMP (500 μM), followed by measurement of tGSH content, 12 h later (Fig. 2). The cGMP analog had no significant effect on tGSH levels at this concentration, which was selected to be equivalent to the maximal activation of guanylate cyclase [35]. In the second approach, the inhibitor of guanylate cyclase, 1H-(1,2,4) oxadiazolo(4,3,-α) quinoxalin-1-one (ODQ), was used in the presence or absence of SNAP. After exposure of the cells to ODQ (3 μM), in the presence or absence of the NO donor, tGSH was measured in the cells. This concentration of ODQ has been shown to inhibit guanylate cyclase in a number of biological preparations [20]. As shown in Fig. 2, ODQ did not prevent the increase in the tGSH induced by SNAP. It is clear that the tGSH levels were not affected by either treatment, indicating that the cGMP pathway was not involved in the NO-dependent induction of GSH synthesis.

3.3. NO induces GSH synthesis via an increased GCS activity

The rate-limiting enzyme for GSH synthesis is GCS and it is therefore a potential candidate for activation by the NO donors. To test whether GCS activity is increased by NO, the activity was measured in cell lysates after treatment with SNAP (100 and 200 μM) for 12 h and is shown plotted

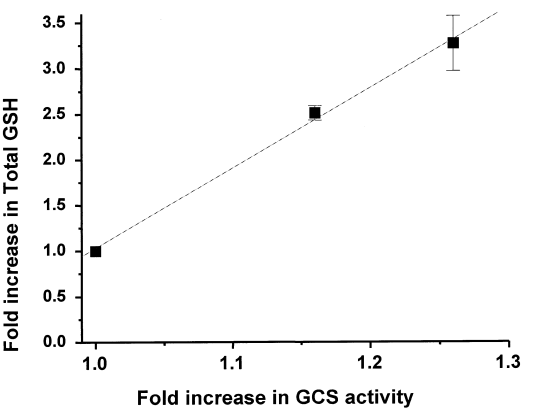

Fig. 3. Increase in the GCS activity in endothelial cells. A: GCS activity was measured as described in Section 2 and was 7.21 ± 0.3 nmol/mg/h (mean ± S.E.M., $n = 6$) for the control untreated samples. It is shown plotted as a function of the fold increase in tGSH for groups of cells treated with 100 or 200 μM SNAP (Figure 1, □). The line shows the linear fit to the data and the correlation is significant ($P < 0.03$).

against the fold increase in tGSH (Fig. 3). A small change in the GCS activity of 1–1.3-fold was associated with increases of 2–3-fold in the tGSH. These data are consistent with a number of reports using different stimuli and indicate that an increase in the activity of the enzyme of this magnitude can result in the 2–4-fold increase in tGSH that is observed [24–26]. However, this result does not rule out a contribution from other elements in the GSH biosynthetic pathway, including activation of the plasma membrane enzyme γ-GT or the cystine transporter [36–39]. To test for an effect of γ-GT, cells were treated with the inhibitor of the enzyme, acivicin, in the absence or presence of DetaNONOate and the results are reported in Table 1. Treatment with NO alone increased tGSH in both the cells and that exported to the medium. Treatment with acivicin alone had no significant effect on tGSH and caused a modest increase in the tGSH present in the medium. In combination with the NO donor, acivicin prevented the increase in the cell tGSH and increased further the concentration of tGSH in the medium. The GSH (5 ± 1.25 μM, mean ± S.E.M., $n = 3$) in the medium without cells was over 90% in the reduced form and presumably derived from the fetal calf serum. The media also contains 200 μM cystine that can be transported into cells, reduced and then serves as a further source of substrate for the GSH synthesis. These data are consistent with a requirement for the metabolism of GSH by γ-GT, although the activity of the enzyme will be needed to confirm this result, for the NO-dependent synthesis of GSH in the cell.

3.4. Summary

Both NO and GSH are thought to play an important role in the protection of the cells in the vasculature from oxidative stress. For example, the ability of macrophages and endothelial cells to resist the cytotoxicity of oxLDL is critically dependent on intracellular GSH levels [40,41]. While NO or its metabolites can react with other oxidants to promote nitration or oxidation reactions, NO per se appears to protect cells against oxidants including oxLDL [1–3]. The reactions of NO are then dependent on both its concentration and on the presence of other molecular species with which it can react. Herein, we extend recent observations with VSMCs to show that an indirect mechanism for the antioxidant action of NO is the regulation of the intracellular antioxidant GSH [20]. In contrast to the results with smooth muscle cells, the induction of GSH synthesis in endothelial cells occurred in response to a substantially lower rate of NO formation. These rates of NO formation (1–3 nM/s) are within the range required for endothelial (type III) NOS to elicit vasorelaxation [23]. Our data indicate that this NO signalling pathway functions independently of the activation of soluble guanylate cyclase.

The requirement shown here for γ-GT activity in the NO-dependent increase in GSH is similar to that found in response to redox cycling xenobiotics. For example, the γ-GT-specific activity and transcription is elevated by quinones that redox cycle or deplete GSH through conjugation such as 2,3-dimethoxy-1,4-naphthoquinone [42]. Pretreatment with acivicin, an inhibitor of γ-GT, also prevented the TBHQ-induced increase in GSH and markedly diminished the resistance to the cytotoxicity of 200 μM TBHQ. We have not determined whether the γ-GT activity is increased in response to NO but this is a likely outcome since the GSH metabolic cycle, including the cystine transporter, appears to be coordinately regulated [18].

The mediators involved in the NO-dependent signalling for GCS are not clear. If the production of secondary oxidants, such as peroxynitrite, are involved, they likely serve a signalling function since the NO exposures are two orders of magnitude lower than those concentrations that are cytotoxic. It is postulated, therefore, that a potentially cooperative interaction between NO and GSH may be important in enhancing the antioxidant properties of endothelium and to protect against the cytotoxic effects of nitrosative or oxidative stress.

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