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Induction of heat shock protein 72 by a nitric oxide donor in guinea-pig gastric mucosal cells

Clare R. Byrne, Peter J. Hanson *

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK

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Abstract

Gastric mucosal cells may be exposed to exogenous nitric oxide (NO) from a variety of sources. The response of primary cultures of guinea-pig gastric mucosal cells to the NO donor *S*-nitroso-*N*-acetyl-penicillamine was therefore investigated. Exposure to *S*-nitroso-*N*-acetyl-penicillamine for 8 h caused a concentration-dependent induction of heat shock protein 72 (HSP 72). Induction was inhibited by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, and by blockade of transcription with actinomycin D. Induction of HSP 72 by *S*-nitroso-*N*-acetyl-penicillamine was enhanced by diethyl maleate which decreased the intracellular reduced thiol content. By contrast, HSP 72 formation after heat shock was associated with an elevation of reduced thiol. Incubation with *S*-nitroso-*N*-acetyl-penicillamine for 18 h increased detachment of cells from the culture plate. The effect of *S*-nitroso-*N*-acetyl-penicillamine on detachment was exacerbated by the presence of actinomycin D. In conclusion, exogenous NO induces HSP 72 in guinea-pig gastric mucosal cells and this response may in part protect the cells from the deleterious effects of NO.

Keywords: Nitric oxide (NO); Gastric mucosa; Stomach; Heat shock protein

1. Introduction

Nitric oxide (NO) may be produced in small amounts within gastric mucous cells as a result of transient activation of a form of NO synthase similar to the neuronal enzyme (Price et al., 1996). Production of NO under such circumstances may increase mucus secretion by the cells (Brown et al., 1993). Mucous cells may also be continuously exposed to larger amounts of NO from exogenous sources. Thus nitrite in the gastric lumen may be converted to NO in the presence of acid (McKnight et al., 1997), and in gastritis a sustained release of NO by the inducible form of NO synthase present in neutrophils and macrophages (Mannick et al., 1996), and possibly in adjacent mucous cells (Brown et al., 1994) can be expected.

Short-term exposure to exogenous NO, derived from NO donors, damages freshly prepared suspensions of gastric mucosal cells (Tripp and Tepperman, 1996), but in hepatocytes longer exposure to exogenous NO induces expression of an heat shock protein which protects the cells from apoptosis induced by tumour necrosis factor α (Kim et al., 1997). Heat shock proteins play essential roles as molecular chaperons under stressed conditions by resolubilising protein aggregates and by facilitating refolding of denatured proteins (Welch, 1992). Thus the presence of heat shock proteins can protect guinea-pig gastric mucosal cells from damage by ethanol (Nakamura et al., 1991), and in macrophages heat shock proteins may promote resistance to damage by NO (Hirvonen et al., 1996).

Induction of the synthesis of heat shock proteins involves trimerisation of the transcription factor heat shock factor 1, and its transfer to the nucleus. This process may be induced by the depression of intracellular reduced thiol content (Huang et al., 1994), and NO has been found to reduce intracellular glutathione in suspensions of rat gastric mucosal cells (Wakulich and Tepperman, 1997). Consequently some measurements of reduced thiol content were made in this study.

Heat shock proteins in mammalian cells are separated into families according to their molecular mass. The most highly inducible protein following heat shock is heat shock protein 72 (HSP 72) (Welch, 1992), which was therefore selected for this investigation. The aims of the present

* Corresponding author. Tel.: +44-121-359-3611 Ext. 5330; Fax: +44-121-359-0733; E-mail: p.j.hanson@aston.ac.uk

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work were to examine the long-term response of primary cultures of guinea-pig gastric mucous cells to exogenous NO and to determine whether there was induction of a protective response as exemplified by the presence of a heat shock protein.

2. Materials and methods

2.1. Animals

Male Dunkin–Hartley guinea-pigs of 200–250 g body weight were obtained from Charles River, Margate, Kent, UK, and were fed on SDS Economy guinea-pig diet supplied by Lillico, Betchworth, Surrey, UK.

2.2. Materials

RPMI 1640 medium, foetal calf serum, antibiotics and amphotericin B were from Life Technologies, Paisley, UK. A monoclonal antibody, which recognises only HSP 72 from among the heat shock protein 70 family, was obtained from Amersham, Little Chalfont, UK. The bicinchoninic acid protein assay kit was from Pierce, Chester, UK. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was obtained from Alexis, Nottingham, UK. Pronase E (70 000 PU units/g) was purchased from Merck, Lutterworth, UK. Other reagents were from Sigma, Poole, UK.

2.3. Isolation and culture of gastric mucous cells

The method is based on that of Nakamura et al., 1991. The stomach was removed from non-fasted guinea-pigs which had been anaesthetised with intraperitoneal sodium pentobarbitone (60 mg/kg). The mucosa was scraped off and minced with fine scissors in 4 ml of RPMI 1640 containing 2 g/l bovine serum albumin (isolation medium). The mucosal pieces were incubated with 30 ml of isolation medium containing 0.5 mg/ml pronase for 20 min at 37°C with shaking at 140 cycles/min and gassing with 95% O_2/5% CO_2. The mixture was centrifuged at $250 \times g$ for 2 min at 15°C, the sedimented tissue resuspended in 30 ml isolation medium containing 0.4 mg/ml of collagenase, and incubated with shaking as previously for 20 min. The contents of the flask were filtered through 150 μm nylon mesh, centrifuged as previously, and resuspended under sterile conditions in RPMI 1640 containing 10% foetal calf serum, 100 u/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml of amphotericin B (culture medium). After a further centrifugation the cells were resuspended in culture medium at 10^6 cells per ml, and 2 ml of suspension added to each well of a six-well culture plate.

Unattached cells were removed and fresh culture medium added after 24 h, and the medium was changed again at 48 h. At this time the cells were 75–95% confluent, and experiments were initiated by the inclusion of agents in the 2 ml of fresh culture medium added to each well.

2.4. Immunoblotting for HSP 72

The culture medium was removed, 0.2 ml of electrophoresis sample buffer without dithiothreitol (Price et al., 1996) at 90°C was added to each well, and the cells were removed with a scraper. The extract was boiled for 5 min, the protein content determined by using the bicinchoninic acid protein assay, dithiothreitol added to give a concentration of 0.1 M and the sample reboiled. Immunoblotting was performed as described previously (Price et al., 1996) using the anti-HSP 72 antibody at a dilution of 1:1000, and enhanced chemiluminescence detection. All immunoblotting experiments were repeated at least three times with similar results.

2.5. Reduced thiol content

Cells were extracted as described by Mutoh et al. (1990) and reduced thiol assayed in the extract by reaction with 5,5′-dithiobis(-2-nitrobenzoic acid) and measurement of absorbance at 412 nm.

2.6. Cell viability and number

Detached cells were centrifuged for 10 s at $10\,000 \times g$ and the supernatant carefully removed by gentle suction. Cells were resuspended in 100 μl of a mixture of equal volumes of RPMI 1640 medium and saline (9 g NaCl/l) which contained 4 mg/ml Trypan blue. Cell counting was performed in a haemocytometer chamber and the percentage of cells excluding trypan blue was used as an indicator of viability. Adherent cells were removed for counting by incubation with trypsin (0.5 g/l) and EDTA (0.2 g/l). Typsinization was terminated by addition of culture medium and cells were centrifuged and counted as above.

3. Results

3.1. Effect of incubation with NO donors for 8 h on the presence of HSP 72, reduced thiol content and the viability of guinea-pig gastric cells

After 2 days of culture the cells preparations used for experiments contained $>90\%$ cells positive for periodic acid-Schiff reagent, and were therefore predominantly made up of mucous cells. Similar results have been obtained by others (Nakamura et al., 1991). Incubation with 1 mM *S*-nitroso-*N*-acetyl-penicillamine for 8 h produced an increase in the amount of HSP in guinea-pig gastric mucous cells (compare lanes 1 and 2 in Fig. 1, and also lanes 1 and 4 in Figs. 3 and 4). The superoxide generator, 0.1

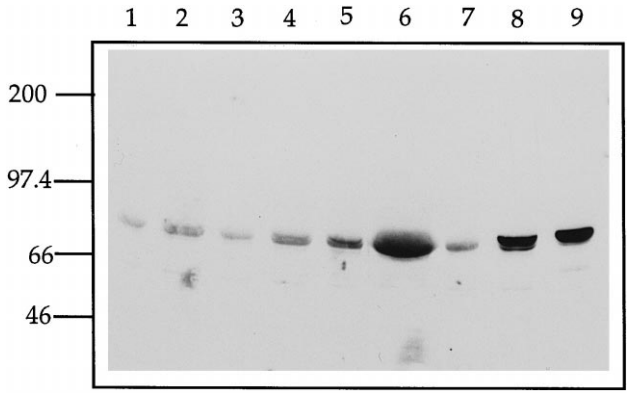

Fig. 1. Effect of incubation for 8 h with *S*-nitroso-*N*-acetyl-penicillamine (1 mM) alone, or in association with pyrogallol (0.1 mM) or diethyl maleate (1 mM), on the presence of HSP 72 in guinea-pig gastric mucosal cells. Lanes 1 and 7, control; lane 2, *S*-nitroso-*N*-acetyl-penicillamine; lane 3, pyrogallol; lane 4, *S*-nitroso-*N*-acetyl-penicillamine plus pyrogallol; lane 5, diethyl maleate; lane 6, diethyl maleate plus *S*-nitroso-*N*-acetyl-penicillamine; lane 8, incubation at 42°C for 3 h then 1 h at 37°C; lane 9, HeLa cells treated as in 8.

mM pyrogallol (Xie and Wolin, 1996), did not affect the response to *S*-nitroso-*N*-acetyl-penicillamine, but 1 mM diethyl maleate, which depletes intracellular reduced glutathione (Wakulich and Tepperman, 1997), itself produced a response and enhanced the effect of the NO donor (Fig. 1). Heat shock of guinea-pig cells for 3 h, followed by 1 h at 37°C, increased the amount of HSP 72 (Fig. 1). As expected the antibody recognised an extract of similarly heat-shocked HeLa cells, which were the source of the protein used to raise the antibody (Fig. 1).

Neither *S*-nitroso-*N*-acetyl-penicillamine, pyrogallol, or pyrogallol plus *S*-nitroso-*N*-acetyl-penicillamine affected

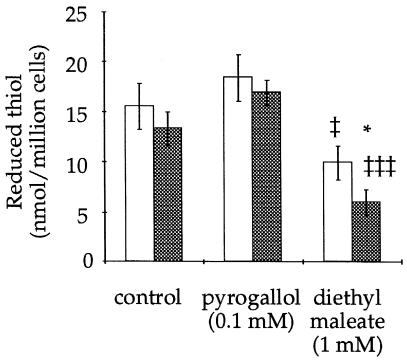

Fig. 2. Effect of incubation for 8 h with *S*-nitroso-*N*-acetyl-penicillamine (1 mM) alone, or in association with pyrogallol (0.1 mM) or diethyl maleate (1 mM), on the reduced thiol content of guinea-pig gastric mucosal cells. Clear bars: *S*-nitroso-*N*-acetyl-penicillamine absent; shaded bars: *S*-nitroso-*N*-acetyl-penicillamine present. Results are means ± S.E. of five separate experiments and were analysed by two-factor analysis of variance followed by a Newman–Keuls multiple comparison test. ‡, $P < 0.05$: ‡‡‡, $P < 0.001$ compared to the control result with no additions. *, $P < 0.05$ for effect of *S*-nitroso-*N*-acetyl-penicillamine relative to the same incubation conditions without *S*-nitroso-*N*-acetyl-penicillamine.

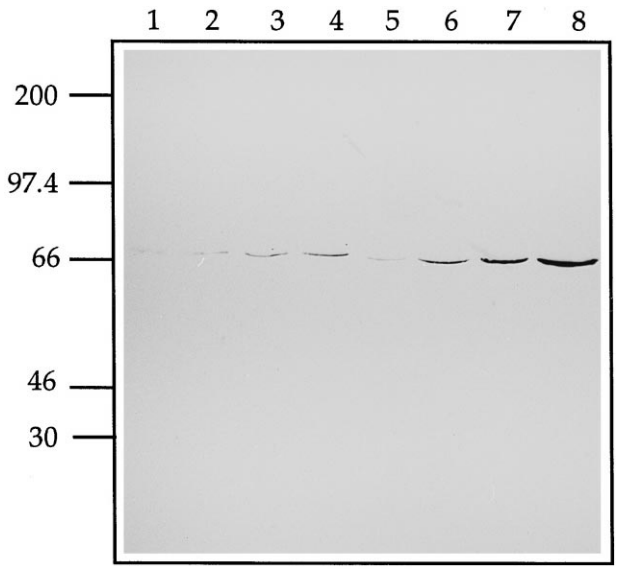

Fig. 3. Effect of the concentration of *S*-nitroso-*N*-acetyl-penicillamine, with or without diethyl maleate (0.3 mM), on the presence of HSP 72 in guinea-pig gastric mucous cells. Lanes 1 and 5, no *S*-nitroso-*N*-acetyl-penicillamine; lanes 2 and 6, 0.1 mM *S*-nitroso-*N*-acetyl-penicillamine; lanes 3 and 7, 0.3 mM *S*-nitroso-*N*-acetyl-penicillamine; lanes 4 and 8, 1.0 mM *S*-nitroso-*N*-acetyl-penicillamine. Extracts added to lanes 5–8 were from cells which had also been incubated with diethyl maleate.

the reduced thiol content of attached cells measured 8 h after addition of NO donor (Fig. 2). Reduced thiol content was diminished relative to control by diethyl maleate in both the absence ($P < 0.05$) and presence ($P < 0.001$) of

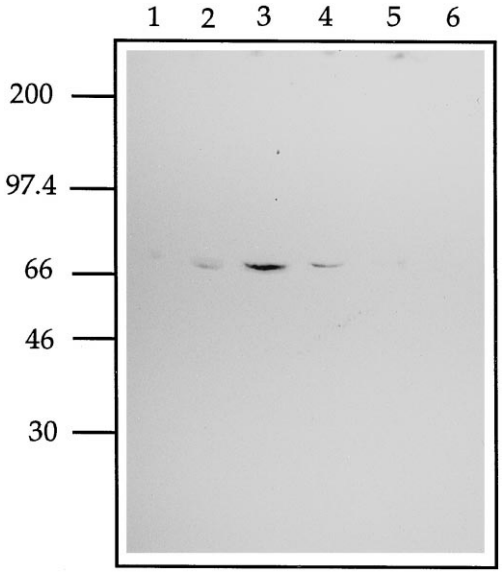

Fig. 4. Action of carboxy-PTIO (0.1 mM) and actinomycin D (5 μg/ml) on the effect of *S*-nitroso-*N*-acetyl-penicillamine (1 mM) and diethyl maleate (0.3 mM) on the amount of HSP 72 in guinea-pig gastric mucous cells. Lane 1, control; lane 2, diethyl maleate; lane 3, diethyl maleate plus *S*-nitroso-*N*-acetyl-penicillamine; lane 4, *S*-nitroso-*N*-acetyl-penicillamine; lane 5 diethyl maleate plus *S*-nitroso-*N*-acetyl-penicillamine with carboxy-PTIO: lane 6, diethyl maleate plus *S*-nitroso-*N*-acetyl-penicillamine with actinomycin D.

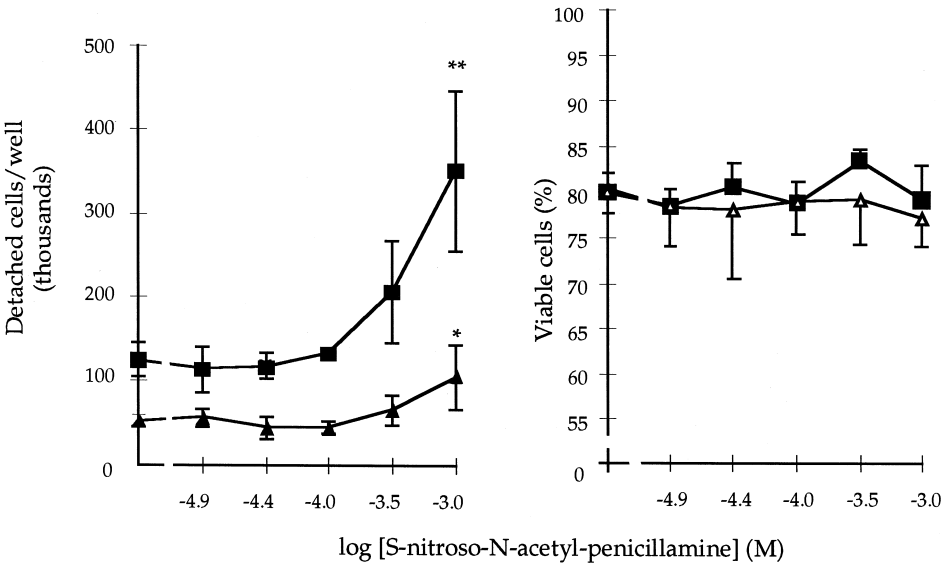

Fig. 5. Effect of incubation with *S*-nitroso-*N*-acetyl-penicillamine for 18 h on the detachment of guinea-pig gastric mucous cells from the culture plate, and on the viability of detached cells. Results are means ± S.E. of four separate preparations in the absence (triangles) and presence (squares) of actinomycin D (5 μg/ml). $*\ P < 0.05$; $**\ P < 0.01$ for a significant difference from the corresponding result without *S*-nitroso-*N*-acetyl-penicillamine by analysis of variance and Dunnett's test.

S-nitroso-*N*-acetyl-penicillamine. In the presence of diethyl maleate the addition of *S*-nitroso-*N*-acetyl-penicillamine significantly lowered reduced thiol content ($P < 0.05$) (Fig. 2). Heat shock of guinea-pig cells increased the reduced thiol content from 17.2 ± 2.1 to 29.3 ± 3.1 nmol/million cells ($P < 0.05$ by paired *t*-test, results are means ± S.E. from four separate preparations). The percentage of cells, which after detachment from the plate by treatment with trypsin/EDTA excluded trypan blue, was as follows: control, 95 ± 0.45; control plus *S*-nitroso-*N*-acetyl-penicillamine, 97 ± 0.71; pyrogallol, 97 ± 0.34; pyrogallol plus *S*-nitroso-*N*-acetyl-penicillamine, 93 ± 2.0; diethyl maleate, 95 ± 0.98; diethyl maleate plus *S*-nitroso-*N*-acetyl-penicillamine 94 ± 2.1 (means ± S.E. of five separate cultures and with no significant effect of any treatment demonstrable by analysis of variance). Further experiments, using a lower concentration of diethyl maleate (0.3 mM, Fig. 3), demonstrated that the HSP response of guinea-pig cells depended on the concentration *S*-nitroso-*N*-acetyl-penicillamine used for the 8 h incubation, in both the presence and absence of diethyl maleate.

3.2. Effect of carboxy-PTIO and actinomycin D on the HSP response to incubation with S-nitroso-N-acetyl-penicillamine for 8 h

The increased content of HSP 72 in guinea-pig gastric cells produced by incubation with 1 mM *S*-nitroso-*N*-acetyl-penicillamine and 0.3 mM diethyl maleate was inhibited by the nitric oxide scavenger carboxy-PTIO (Maeda et al., 1994) and by the inhibitor of transcription actinomycin D (Fig. 4). After the incubation period of 8 h the protein contents (mg/well) were as follows: control, 0.20 ± 0.099; 0.3 mM diethyl maleate, 0.19 ± 0.093; 1 mM *S*-nitroso-*N*-acetyl-penicillamine, 0.18 ± 0.091; 0.3 mM diethyl maleate plus 1 mM *S*-nitroso-*N*-acetyl-penicillamine, 0.16 ± 0.079; diethyl maleate, *S*-nitroso-*N*-acetyl-penicillamine and 0.1 mM carboxy-PTIO, 0.18 ± 0.091; diethyl maleate, *S*-nitroso-*N*-acetyl-penicillamine and 5 μg/ml actinomycin D, 0.20 ± 0.099 (means ± S.E. of four experiments with no effect of treatment by analysis of variance). Therefore over an 8 h period these treatments did not appear to alter the number of cells attached to the culture plate.

3.3. Effect of incubation with S-nitroso-N-acetyl-penicillamine for 18 h in the presence and absence of actinomycin D

Longer incubation with *S*-nitroso-*N*-acetyl-penicillamine demonstrated that this agent produced a dose-related detachment of cells from the culture plate in both the presence and absence of actinomycin D (Fig. 5). Furthermore a factorial analysis of variance demonstrated that the effect of *S*-nitroso-*N*-acetyl-penicillamine on detachment was greater in the presence of actinomycin D ($P < 0.05$). Neither *S*-nitroso-*N*-acetyl-penicillamine nor actinomycin D, separately or in combination, affected the proportion of detached cells which were able to exclude trypan blue (Fig. 5).

4. Discussion

The central finding of this study is that an NO donor increased HSP 72 in guinea-pig gastric mucous cells. The

inhibitory effect of the NO scavenger, carboxy-PTIO (Maeda et al., 1994), on the heat shock response is evidence that NO, or an NO-derived species, mediated the effect of *S*-nitroso-*N*-acetyl-penicillamine. The response was inhibited by actinomycin D and therefore probably involved increased transcription of the gene for HSP 72. Indeed in hepatocytes mRNA for HSP 72 was increased by treatment with 750 μM *S*-nitroso-*N*-acetyl-penicillamine (Kim et al., 1997).

General damage to the cell could have induced a heat shock response. However, incubation with the NO donor for 8 h did not affect the integrity of mucosal cell membranes as assessed by the proportion of cells capable of excluding trypan blue. Depression of intracellular reduced thiol content may activate the transcription factor heat shock factor 1 (Huang et al., 1994). Reduced thiol in the guinea-pig cells is likely to be mainly glutathione (Mutoh et al., 1990), and diethyl maleate, which conjugates reduced glutathione, both increased HSP 72 content and decreased intracellular reduced thiol. The NO donor alone did not significantly affect intracellular reduced thiol after 8 h of incubation, but it did so in the presence of diethyl maleate. Consequently the enhancement of the heat shock response to NO in the presence of diethyl maleate may have involved reduction in intracellular glutathione. Also a decrease in the cellular content of reduced thiol could have occurred soon after addition of the NO donor alone, and this change could have facilitated induction of the heat shock protein. Alternatively NO could effect the trimerisation and activation of heat shock factor 1 directly by, for example, *S*-nitrosylation (Malyshev et al., 1996). Pyrogallol generates superoxide through autoxidation, and the combination of NO with exogenous superoxide could lead to increased formation of peroxynitrite (Xie and Wolin, 1996). A role for peroxynitrite in the heat shock response to the NO donor was not supported here because the action of the NO donor was not enhanced by the addition of pyrogallol. Heat shock itself considerably increased the intracellular content of reduced thiol in the guinea-pig gastric cells, and this result suggests an apparently novel mechanism by which heat shock might protect these cells from damage by ethanol (Mutoh et al., 1990; Nakamura et al., 1991).

By 18 h of exposure to the NO donor there was a significant and dose-related increase in detachment of cells from the culture plate. However, the proportion of detached cells able to exclude trypan blue was not decreased by the NO donor. Tripp and Tepperman (1996) demonstrated a reduction in the percentage of freshly prepared rat gastric mucosal cells able to exclude trypan blue after 30 min incubation with 1 mM *S*-nitroso-*N*-acetyl-penicillamine. Both investigations therefore imply that NO can have a deleterious effect on gastric mucosal cells but this is manifested slightly differently with the two preparations. The response of the guinea-pig cells could have been affected by the period of culture before exposure to the NO donor, while the response of the rat cells could have been influenced by the trauma of the isolation process or by the presence of increased numbers of chief and parietal cells (Huber et al., 1993) compared to the cultured preparation.

Macrophages with an increased content of constitutively expressed heat shock protein 70 are more resistant to the toxic effects of NO (Hirvonen et al., 1996). The effect of inhibiting the production of heat shock protein by actinomycin D on the longer term response of the guinea-pig cells to the NO donor was investigated. The effect of NO donor on detachment was found to be greater in the presence of actinomycin D. Consequently the heat shock protein may protect against the longer term effects of NO, although increases in transcription of other potentially protective proteins such as manganese superoxide dismutase could also be involved (Sano et al., 1996). In conclusion, exogenous NO induces an increase in the HSP 72 content of guinea-pig gastric mucous cells and this response may in part protect the cells from the deleterious effects of NO.

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