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Role of Nitric Oxide as an Antioxidant in the Defense of Gastric Cells[1]

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ABSTRACT

Gatric mucosa is exposed to toxic, reactive oxygen species generated within the lumen. Nitric oxide protected acetaminophen-induced hepatotoxicity by maintaining glutathione homeostasis. The present study examined the role of nitric oxide in mediating hydrogen peroxide - induced damage to gastric cells. Hydrogen peroxide was generated by glucose oxidase acting on β-D-glucose. L-arginine, N^G-nitro-L-arginine methyl ester, or N^G-nitro-L-arginine were treated to the cells with glucose/glucose oxidase. Lipid peroxidation and nitrite release and cellular content of glutathione were determined. As a result, dose - dependent increase in lipid peroxide production as well as dose - dependent decrease in nitrite release and cellular glutathione content were observed in glucose/glucose oxidase - treated cells. Pretreatment of L-arginine, a substrate for nitric oxide synthase, prevented the increase of lipid peroxide production and the reduction of nitrite release as well as glutathione content. Inhibitors of nitric oxide synthase such as N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine did not protect hydrogen peroxide - induced cell damage. In conclusion, nitric oxide protects gastric cells from hydrogen peroxide possibly by inhibiting lipid peroxidation and by preserving cellular glutathione stores.

Key Words: Nitric oxide, Antioxidant, Gastric cells

INTRODUCTION

The gastric mucosa is continuously exposed to oxygen metabolites that are generated within the lumen. Potential sources of luminal oxidants include ingested food, catalase - negative bacteria, desquamated mucosal cells, and cigarette smoke and tar (Nayfield *et al.*, 1976; Carlsson *et al.*, 1983; Cross *et al.*, 1984; Grisham *et al.*, 1986). Oxygen metabolites also contribute to ischemia/reperfusion - induced gastric mucosal lesions (Itoh and Guth, 1985; Perry *et al.*, 1986). Despite the exposure to these oxidants, however, the gastric mucosa appears to be unaffected. This would suggest that gastric mucosa contains effective defense mechanisms against oxidant such as glutathione redox cycle, catalase, and superoxide dismutase (Hiraishi *et al.*, 1991). It was reported that reactive oxygen metabolites, particularly hydrogen peroxide (H_2O_2), or subsequent oxidizing species such as hydroxyl radical, cause injury to rat gastric mucosal cells *in vitro* (Hiraish *et al.*, 1987) and intracellular iron plays a crucial role in mediating oxygen radical damage to gastric mucosal cells (Hiraish *et al.*, 1993).

Nitric oxide (NO) is now established as a mediator of various biological actions under physiological conditions. NO increases guanylate cyclase activity so as to elevate cGMP concentration, which has diverse functions in

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various tissues (Moncada *et al.*, 1991). Recent studies have shown that NO is a regulator of the gastric mucosal microcirculation under resting and stimulated conditions (Pigue *et al.*, 1989; Whittle *et al.*, 1990) and interacts with prostanoids and sensory neuropeptides to maintain gastirc mucosal integrity (Tepperman and Whittle, 1992). For the mucosal protective action of NO, regulation of gastric mucosal blood flow is considered to be of major importance (Pigue *et al.*, 1989; Lippe and Holzer, 1992). Other defensive mechanisms induced by NO are stimulation of mucus secretion in epithelial cells (Brown *et al.*, 1992), inhibition of the aggregation and adhesion of platelets (Radomski *et al.*, 1987) and inhibition of the adherence of neutrophils to the endothelium and their emigration to blood vessels (Kubes *et al.*, 1991), which cause mucosal damage (Kvietys *et al.*, 1990; Wallace *et al.*, 1990). However, bicarbonate secretion, one of the defensive factors in the gastroduodenal mucosa, was increased by NO synthase inhibitors (Takeuchi *et al.*, 1993, 1994, 1995), which is contradictory to the mucosal protective role of NO.

Few studies on the role of NO on oxidative injury were reported. In cultured rat hepatocytes, NO showed a protective effect on acetaminophen - induced injury by modulating glutathione homeostasis (Kuo and Slivka, 1994). On the other hand, superoxide and NO rapidly react to form peroxynitrite, which decomposes and generates strong oxidant molecules (Beckman *et al.*, 1990). Addition of nitroprusside reversed N^G-nitro-L-arginine methylester (L-NAME), a NO synthase inhibitor, protection against hypoxia/reoxygenation injury of rat proximal tubules (Yu *et al.*, 1993).

The present study aims to investigate whether NO has an antioxidant defensive role against H_2O_2 - induced gastric cell injury. H_2O_2 was generated by glucose oxidase acting on β-D-glucose. Gastric cells were isolated from the corpus of rabbit stomach. Modulators of NO synthesis such as L-arginine, N^G-nitro-L-arginine methyl ester, and N^G-nitro-L-arginine were treated to gastric cells with glucose/glucose oxidase. In addition, glucose/glucose oxidase - induced cell injury were determined by treating different doses of glucose oxidase with glucose (10 mM) to gastric cells and by observing alterations in lipid peroxide (LPO) production, nitrite (NO_2) release as well as cellular content of glutathione (GSH).

METHODS

Materials

The following compounds were purchased: 2-thiobarbituric acid and 1,1,3,1-tetramethoxypropane (Fluka, Switzerland), collagenase type I (280 U/mg), L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NNA) and all other reagents (Sigma, St Louis, MO, USA). All the drugs were dissolved in distilled water for the experiment.

Solutions

The collagenase digestion solution contained: NaCl 130.0 mM $NaHCO_3$ 12.0 mM, NaH_2PO_4 3.0 mM, Na_2HPO_4 3.0 mM, K_2HPO_4 3.0 mM, $MgSO_4$ 2.0 mM, $CaCl_2$ 1.0 mM, phenol red 10 mg/l, pH 7.4. Before use, 1 mg/ml collagenase (type I), 1 mg/ml rabbit albumin and 2 mg/ml glucose were added. The incubation medium consisted of NaCl 132.4 mM, KCl 5.4 mM, Na_2HPO_4 5.0 mM, NaH_2PO_4 1.0 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.0 mM, phenol red 10 mg/l, pH 7.4. Before use, 2 mg/ml rabbit albumin and 2 mg/ml glucose were added.

Animal preparation

Male New Zealand white rabbits weighing 1.5 ~2.5 kg were fed *ad libitum* before the experiment. Anesthesia was induced with secobarbital solution (30 mg/kg intravenously). The abdomen was opened and the aorta cannulated below the celiac artery in a retrograde direction. Five ml of heparin salution, 250 U/ml, were injected through the cannula. After 1 min, the rabbit was bled through the cannula and a ligature was placed around the superior mesenteric artery. The chest was quickly opened and the thoracic aorta was clamped. Warm (37℃), oxygenated phosphate - buffered saline (PBS) was then pumped into the aorta, whereupon the portal vein was opened to allow a free outflow of the perfusate. By this procedure, most of the

500 ml PBS was forced through the gastric blood vessels. When the stomach appeared totally exsanguinated, it was rapidly removed and cut open along the lesser curvature; the contents of the stomach were discarded.

Isolation of gastric cells

Gastric cells were separated by the method of Berglindh and Obrink (1976). Briefly, the cardiac and antral regions were discarded. The corpus was rinsed several times in PBS and finally blotted with a filter paper, thereby removing the remaining gastric contents. By blunt dessection, the mucosa was easily separated from the muscular and submucosal layers and was then minced into small pieces with a pair of scissors. The pieces were washed twice in warm oxygenated PBS and transferred to a 200 ml flask containing 50 ml collagenase digestion solution, gassed with 100% oxygen and incubated in a water bath at 37℃. The whole procedure from the removal of the stomach to the start of the incubation took less than 5 min. Incubation was terminated after 90 min when most gastric cells had been separated. The remaining procedures were continued at room temperature. The cloudy cell suspension was filtered through a 200 μm nylon mesh into 15 ml conical tubes. The gastric cells were washed with incubation medium three times over about 15 min to remove all collagenase. Morphology was examined under the light microscope and viability (>90%) was checked by trypan blue exclusion.

Experimental protocol

To investigate the role of NO on H_2O_2 - induced cell injury, a substrate for NO synthase (10^{-4}M L-arginine) or inhibitors of NO synthase (10^{-3}M L-NAME, 10^{-4}M L-NNA) was added to the gastric cells suspended in incubation buffer and incubated for 30 min at 37℃. Further incubation for 2 h at 37℃ was performed following treatment with β-D-glucose (10 mM) and glucose oxidase (10 mU/ml). For dose- response curve of glucose/glucose oxidase, gastric cells were exposed to β-D-glucose (10 mM) with different doses of glucose oxidase (0.1, 1, 10, 100 mU/ml) for 2 h. After incubation, the cell suspensions were centrifuged at 3,000 g for 2 min. Incubation medium was assessed for LPO and NO_2 and the cells were homogenized with 0.1M Tris-HCl buffer (pH 7.4) for GSH and protein assay.

Assay for LPO

LPO was measured fluorometrically by adaptation of the metod of Yagi(1976) using 1, 1,3,3-tetramethoxypropane as a standard. Briefly, 20% acetic acid, adjusted to pH 3.5, was added to incubation medium to achieve a final concentration of 15%, and centrifuged at 3,000 g for 30 min. 0.2 ml of the clear supernatant was added to 0.15 ml 8% sodium dodecyl sulfate and 0.15 ml of 0.8 % 2-thiobarbituric acid. The mixture was vortexed and then boiled for 15 min at 95℃. The colored product was extracted by adding 2 ml of n-butanol, vortexing and centrifuging for 15min. The fluorescence of upper layer was read in a Model SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL, USA) at 515 nm excitation and 553 nm emission.

Assay for NO_2

NO_2 in incubation medium was quantitated colorimetrically after reaction with the Greiss reagents (Green *et al.* 1982). Samples (0.5 ml) were mixed with 0.25 ml 1% sulfanilic acid - 5% phosphoric acid and 0.25 ml 0.1% naphthylethylenediamine dihydrochloride and stand for 10~30 min at room temperature. The absorbance was measured at 550 nm in a Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England). Concentrations were determined from a linear standard curve between 1 and 25 μM sodium nitrite.

Assay for cellular GSH

Cell homogenates were deproteinated with equal volume of 10% 5-sulfosalicylic acid and centrifuged at 12,500 g for 5 min at 4℃. An aliquot of the acid - soluble supernatant was 10 - fold diluted with phosphate buffer (125 mM, pH 7.5) containing 6.3 mM EDTA. GSSG reductase in a concentration of 5 units/ml and 6 mM 5,5'-dithiobis-2-nitrobenzoic acid were added. The reaction was started with 0.3 mM NADPH.

The change of absorbance was monitored spectrophotometrically at 412nm (Griffith, 1980). Exogenous GSH was used as the standard. Results were expressed as total GSH in nmoles per mg protein, determined by the method of Lowry *et al.* (1951).

Statistical analysis

Results were presentaed as means±SE of triplicate samples from 6 experiments. The statistical difference of experimental treatments was detemined by analysis of variance followed by the Newman-Keuls test (Zar, 1984). A P value of <0.05 was considered statistically significant.

RESULT

Dose-dependent gastric cell injury generated by glucose/glucose oxidase

To determine H_2O_2 - induced cell injury, generated by glucose oxidase acting on β-D-glucose, dose-responses of glucose oxidase on LPO production, NO_2 release and cellular GSH contents were observed. The reaction mixture of 10 mM β-D-glucose and glucose oxidase (0.1, 1, 10, 100 mU/ml) resulted in a significant increase of LPO production (Fig. 1) and significant reductions of NO_2 release (Fig. 2) as well as cellular GSH content (Fig. 3) during 2 h - incubation. LPO production of gastric cells untreated and treated with glucose oxidase 0.1, 1, 10 and 100 mU/ml were 0.18±0.03, 0.19±0.10, 0.26±0.01, 0.52±0.06 and 1.53±0.04, respectively, which were expressed as nmole/ml incubation medium (Fig. 1). This dose - response of glucose oxidase for LPO production was reciprocal to those for NO_2 release and cellular GSH content. NO_2 release (nmole/ml) of untreated gastric cells was 8.77±0.28, which decreased to 8.02±0.21, 6.84±0.28, 2.88±0.10 and 2.67±0.10 at the treatment of glucose oxidase of 0.1, 1, 10 and 100 mU/ml (Fig. 2). Cellular GSH contents (nmole/mg protein) of glucose oxidase - treated cells were 72.0 ±5.9, 58.8±1.8, 37.6±1.8 and 36.7±1.5 at 0.1, 1, 10 and 100 mU/ml, respectively, which were significantly lower than cellular GSH level of untreated control, 114.5±7.9 nmole/mg protein

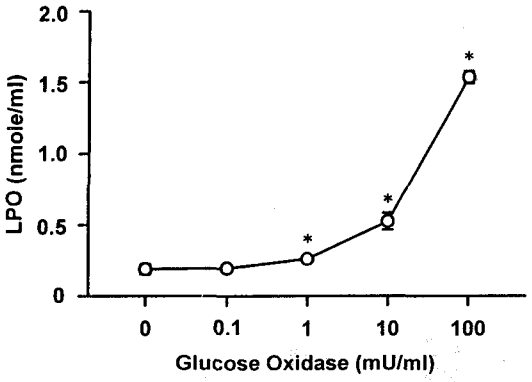

Fig. 1. Dose - response of glucose oxidase on LPO production of gastric cells.
Gastric cells were exposed to β-D-glucose (10 mM) with different doses of glucose oxidase (0.1, 1, 10, 100 mU/ml) for 2 h. After incubation, the cell suspensions were centrifuged at 3,000 g for 2 min. Incubation medium was assessed for LPO (nmole/ml). *P < 0.05 *vs* untreated control.

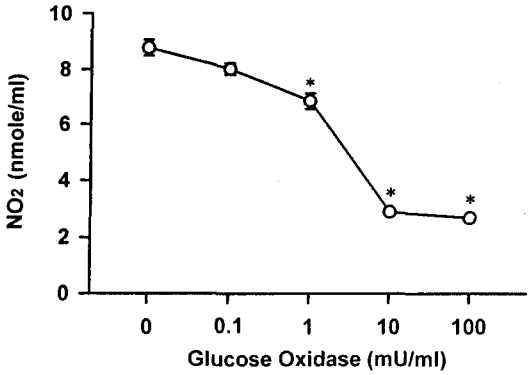

Fig. 2. Dose - response of glucose oxidase on NO_2 release from gastric cells.
Gastric cells were exposed to β-D-glucose (10 mM) with different doses of glucose oxidase (0.1, 1, 10, 100 mU/ml) for 2 h. After incubation, the cell suspensions were centrifuged at 3,000 g for 2 min. Incubation medium was assessed for NO_2 (nmole/ml). *P < 0.05 *vs* untreated control.

(Fig. 3). From 10 mU/ml of glucose oxidase, reductions both in NO_2 release and cellular GSH contents became plateur to 100 mU/ml even if

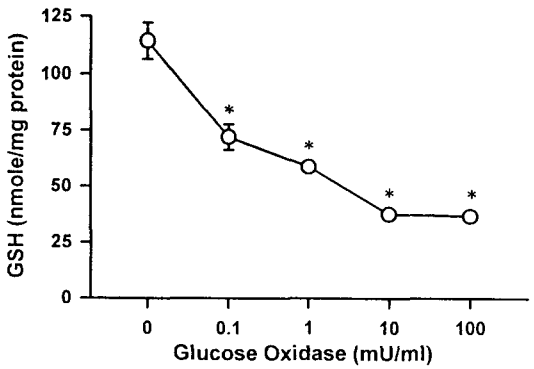

Fig. 3. Dose - response of glucose oxidase on cellular GSH content of gastric cells.
Gastric cells were exposed to β-D-glucose (10 mM) with different doses of glucose oxidase (0.1, 1, 10, 100 mU/ml) for 2 h. After incubation, the cell suspensions were centrifuged at 3,000 g for 2 min. Cell homogenate was assessed for GSH (nmole/mg protein). $^{*}P < 0.05$ *vs* untreated control.

LPO production of gastric cells increased with the dose of glucose oxidase. Therefore, 10 mU/ml glucose oxidase with 10 mM glucose was used for further experiment of NO synthase modulators on H_2O_2 - induced gastric cell injury.

Effects of NO generator and NO synthase inhibitors on LPO production, NO_2 release and cellular GSH content

Pretreatment of L-arginine (10^{-4}M) prevented glucose/glucose oxidase-induced lipid peroxidation of gastric cells but neither L-NAME (10^{-3} M) nor L-NNA (10^{-4}M) had protective effects (Fig. 4); LPO contents in incubation medium (nmole/ml) released by the cells treated with glucose/glucose oxidase alone, pretreated with L-arginine, L-NAME and L-NNA were 0.57±0.06, 0.39±0.01, 0.60±0.06 and 0.61±0.04, respectively. Decreased NO production caused by glucose/glucose oxidase, assessed as NO_2 release into medium (nmole/ml) was reversed by pretreatment of L-arginine, but inhibitors of NO synthase did not affect this alteration in NO production (Fig. 5). NO_2 release from the cells only received glucose/glucose oxidase was 2.88 ±0.10 while those pretreated with L-arginine, L-

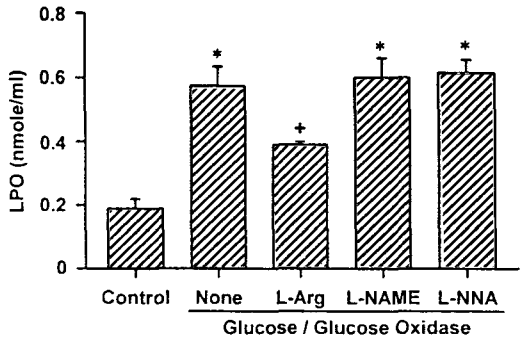

Fig. 4. Effects of NO generator and NO synthase inhibitors on LPO production of gastric cells. A substrate for NO synthase (10^{-4}M L-arginine) or inhibitors of NO synthase (10^{-3}M L-NAME, 10^{-4}M L-NNA) was added to the gastric cells and the cell suspensions were incubated for 30 min at 37℃. Further incubation for 2 h at 37℃ was performed following treatment with β-D-glucose (10 mM) and glucose oxidase (10 mU/ml). $^{*}P < 0.05$ *vs* untreated control. $^{+}P < 0.05$ *vs* glucose/glucose oxidase alone.

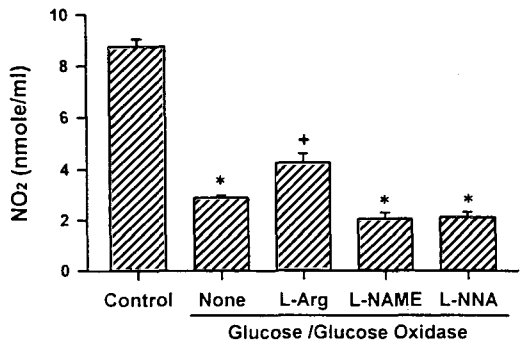

Fig. 5. Effects of NO generator and NO synthase inhibitors on NO_2 release from gastric cells. A substrate for NO synthase (10^{-4}M L-arginine) or inhibitors of NO synthase (10^{-3}M L-NAME, 10^{-4}M L-NNA) was added to the gastric cells and the cell suspensions were incubated for 30 min at 37℃. Further incubation for 2 h at 37℃ was performed following treatment with β-D-glucose (10 mM) and glucose oxidase (10 mU/ml). $^{*}P < 0.05$ *vs* untreated control. $^{+}P < 0.05$ *vs* glucose/glucose oxidase alone.

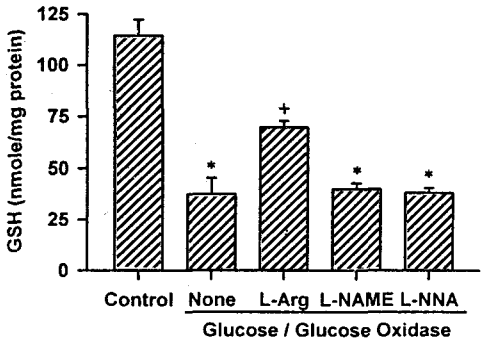

Fig. 6. Effects of NO generator and NO synthase inhibitors on GSH content of gastric cells.
A substrate for NO synthase (10^{-4}M L-arginine) or inhibitors of NO synthase (10^{-3}M L-NAME, 10^{-4}M L-NNA) was added to the gastric cells and the cell suspensions were incubated for 30 min at 37℃. Further incubation for 2 h at 37℃ was performed following treatment with β-D-glucose (10 mM) and glucose oxidase (10 mU/ml). $^{*}P < 0.05$ *vs* untreated control. $^{+}P < 0.05$ *vs* glucose/glucose oxidase alone.

NAME and L-NNA were 4.25±0.35, 2.03±0.25 and 2.10±0.20, respectively. Fig. 6 shows cellular GSH contents of gastric cells, expressed as nmole/mg protein, treated with glucose/glucose oxidase alone (37.6±7.8), pretreated with L-arginine (69.7±3.2), L-NAME (40.0±3.0) and L-NNA (38.1±2.5).

DISCUSSION

It is reported that several necrotizing agents, i.e., ethanol and aspirin, induce gastric mucosal injury by promoting the generation of reactive oxygen metabolites (Terano *et al.*, 1989; Pihan *et al.*, 1987). Furthermore, it has been indicated that compounds and enzymes that are capable of scavenging or inhibiting the generation of reactive oxygen species are able to protect the gastric mucosa from hemorrhagic lesions (Cho *et al.*, 1991; Ito *et al.*, 1992). In present study, H_2O_2, generated by glucose oxidase acting on β-D-glucose, induced gastric cell damage, which was assessed by the increment of LPO and by the reduction of NO production as well as cellular GSH level. Thus, glucose/glucose oxidase system seems to be a suitable agent for investigating reactive oxygen metabolite - induced gastric cell injury, which was also suggested by other studies using cultured rat gastric mucosal cells (Hiraish *et al.*, 1991, 1993).

GSH is present in almost all mammalian cells (Mirabelli *et al.*, 1989), existing in a combination of its reduced form and its oxidized dimer. Its particularly high concentration (about 8 mM range) in the gastric mucosa, compared with other areas of the gastrointestinal tract or in most other organs (Meister and Anderson, 1983), suggests a role for GSH in maintaining gastric mucosal integrity. GSH acts both as a nucleophilic scavenger of the superoxide anion and as a cofactor in the GSH peroxidase - mediated reduction of H_2O_2 (Meister and Anderson, 1983). Therefore, depletion to 20~30% of total GSH levels can readily impair the cell's defense against oxidative damage from H_2O_2, which may lead to cell damage and cell death (Reed and Fariss, 1984). At this point of view, NO may contribute to antioxidant defensive mechanism of gastric cells since NO showed protective effect on acetaminophen-induced hepatotoxicity by modulating GSH homeostasis (Kuo and Slivka, 1994). Present study proved this hypothesis by demonstrating that L-arginine, a substrate for NO synthase, preserved cellular GSH stores against H_2O_2 attack while inhibitors of NO synthase had no effect on GSH depletion. The possible mechanism of the inhibition of H_2O_2 - induced GSH depletion by NO in gastric cells is thought to be (1) the stimulation of GSH synthesis, (2) the result of the inhibition of gastric lesion, which may not be related to GSH, or (3) the inhibition of GSH consumption in gastric cells. In our preliminary study, treatment of a NO synthase substrate, L-arginine, did not affect gastric cellular total GSH level, suggesting that the inhibition of GSH - depletion by L-arginine might be related to its inhibitory effect on the consumption of GSH during H_2O_2 attack.

Upon exposure to excess H_2O_2, which cannot be detoxified by the cells, stored L-Fe^{3+} (ferric iron bound to ligand) may be reduced to L-Fe^{2+} (ferrous iron bound to ligand) so that H_2O_2 re-

acts with L-Fe^{2+} presumably to form a more reactive peroxo complex (which may yield hydroxyl radical or higher oxidation states of iron (Halliwell, 1982; Sutton and Winterbourn, 1989), leading to cell damage. NO may confer protection by chealating stored Fe^{3+} or reduced Fe^{2+} to form iron - nitrosyl compounds (Kanner *et al.*, 1991), without affecting endogenous antioxidant defenses. Other possibility for antioxidant role of NO is termination of radical chain propagation by direct reaction with lipid radical species ($LO^{\cdot}$, $LOO^{\cdot}$) (Rubbo *et al.*, 1994). However, the product of NO termination of lipid radicals are unstable and may mediated a different spectrum of as yet undefined target molecule and pathological reactions. Excess NO production in central nervous system during ischemia/reperfusion leads to pathological responses through combination of excess NO with superoxide to yield peroxynitrite and its secondary reactive species (Beckman and Crow, 1993). Development of better understanding of the physiological roles of NO coupled with detailed insight into NO regulation of oxygen radical - dependent reactions and the chemistry of NO, should yield a more rational basis for the present and futher therapeutic use of NO donors and inhibitors of NO syntheses.

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=국문초록=

위선세포의 항산화 방어기전으로의 Nitric Oxide의 역할

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위점막은 위강내에서 생성되는 독성이 강한 활성산소종에 노출된다. Nitric oxide는 glutathione의 항상성을 유지시킴으로써 acetaminophen 유도 간독성에 대한 보호효과를 나타내었다. 본 연구는 hydrogen peroxide로 인한 위선세포 손상에 대한 nitric oxide의 작용을 규명하고자 하였다. Hydrogen peroxide는 β-D-glucose와 glucose oxidase의 반응에 의해 생성시켰으며, 위선세포에 L-arginine, N^G-nitro-L-arginine methyl ester 및 N^G-nitro-L-arginine을 전처리 한 후, 세포외로 유리되는 지질과산화물 및 nitrite를 정량하고 세포내 glutathione 함량을 측정하였다. 결과로서, glucose/glucose oxidase를 처리한 경우 glucose oxidase 농도의존적으로 지질과산화물 생성은 증가되었으며, nitrite 유리 및 glutathione 함량은 감소되었다. NO synthase의 기질인 L-arginine 전처리시 glucose/glucose oxidase에 의한 지질과산화 및 nitrite 유리 증가와 세포내 glutathione 감소등이 방지되었다. N^G-nitro-L-arginine methyl ester 및 N^G-nitro-L-arginine등 NO synthase 억제제들은 세포손상에 보호효과를 나타내지 않았다. 결론적으로 nitric oxide는 hydrogen peroxide로 인한 세포손상에 대한 보호효과가 있으며, 이는 지질과산화 반응 및 세포내 glutathione 고갈등을 억제시킴으로써 이루어진다고 사료된다.