GENERATION OF NITRIC OXIDE AND ITS PROTECTIVE AND TOXIC ACTIONS IN THE GASTROINTESTINAL TRACT

CLARE ROSEMARY BYRNE

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF ASTON IN BIRMINGHAM
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Nitric oxide is a free-radical gas which can exert both protective and damaging effects. The objectives of the thesis were: (i) to investigate arginine metabolism in isolated rat gastric mucosal cells, (ii) to investigate the role of NO in the induction of ornithine decarboxylase in the rat gastric mucosa damaged by hypertonic saline *in vivo*, (iii) to expose primary cultures of guinea-pig gastric mucosal cells to oxidative challenge and an NO donor, and to investigate the response in terms of heat shock protein 72 (HSP 72) induction, and (iv) to investigate the induction of iNOS and the role of potential modulators of activity in gastric cell lines.

Isolated rat gastric mucosal cells converted exogenous arginine to ornithine and citrulline. This metabolism of arginine was not affected by a range of NO synthase inhibitors, but was reduced by the arginase inhibitors N^G-hydroxy-L-arginine and L-ornithine. Thus, the predominant pathway of arginine metabolism involves arginase and ornithine transcarbamoylase, not NO synthase.

Pretreatment of rats with N^G-nitro-L-arginine promoted activation of ornithine decarboxylase after intragastric hypertonic saline, but did not increase acid phosphatase release (damage). NO may therefore restrict activation of ornithine decarboxylase in response to damage.

Exposure of primary cultures of guinea-pig gastric mucosal cells to S-nitroso-N-acetyl-penicillamine (SNAP) caused a concentration dependent induction of HSP 72, which was inhibited by an NO scavenger and blockade of transcription. The effect of SNAP was enhanced by decreasing the intracellular reduced thiol content with diethyl maleate, which itself also induced HSP 72 formation. Substantial amounts of NO may induce defensive responses in cells.

Induction of iNOS was not detected in HGT-1 or AGS cells exposed to cytokines.

Conclusions

An arginase pathway may restrict availability of arginine for NO synthase in gastric mucosa or may be present to supply ornithine for polyamine synthesis. NO may modulate the response to damage of the stomach epithelium *in vivo*. Exogenous NO may induce a defensive response in gastric mucosal cells.

Key words: Gastric mucosa, arginase, hypertonic saline, ornithine decarboxylase, heat-shock protein, nitric oxide synthase.

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CHAPTER 1

INTRODUCTION

1.0. INTRODUCTION

This chapter is intended as a general introduction to nitric oxide, damage and cytotoxicity, with particular reference to the gastrointestinal tract. The results chapters 3-6 describe studies carried out to investigate various actions of nitric oxide in the gastrointestinal tract, and contain short introductory sections which relate more specifically to the work carried out in each study.

1.1. Identification of NO as a novel biological messenger.

Where specific references are not given, information for sections 1.1 to 1.3 can be found in the following reviews: Moncada et al. (1991), Feldman et al. (1993), Marletta (1993), Bredt and Snyder (1994), Nathan and Xie (1994ab), Stamler (1994), Fostermann et al. (1995), Aoki et al. (1995), Knowles (1997).

Nitric oxide (NO) is a small, inorganic, unstable, gaseous diatomic free radical, which performs an unprecedented number of functions as a biological messenger. It is considered a novel biological messenger in that it is not stored in synaptic vesicles and does not bind to receptors on membranes, but dissolves in cellular fluids, permeates lipid membranes, and freely diffuses in all directions from its site of origin. NO is highly reactive and labile with a biological half-life of only a few seconds, and consequently may have a very transient mode of action. Its action is terminated by oxidation to nitrate or nitrite by oxygen. Oxyhaemoglobin also has an extremely high affinity for NO and will prevent any further activity upon binding. Due to the rapid termination of NO activity, uptake mechanisms are not required to control removal of NO. The key to regulating activity of NO therefore lies with control of its synthesis, which is achieved by a group of enzymes known as NO synthases.

Two major lines of research resulted in the conclusion that NO fulfilled physiological roles. Furchgott and Zawadski, (1980) first demonstrated the importance of the endothelium in blood vessel relaxation in response to acetylcholine, and suggested that the effect was mediated by a labile factor named endothelium-derived relaxing factor. This endothelium-derived relaxant factor released from vascular endothelial cells was later measured by bioassay studies, and identified as NO by chemiluminescence (Palmer et al., 1987). The second approach involved work with germ-free rats, in which the urinary excretion of nitrates was maintained, and could not be accounted for by bacterial metabolism, and the observation that nitrates increased after exposure of rats to endotoxin

(Wagner et al., 1993). Metabolism of arginine by macrophages was identified as the source of nitrate which was formed by breakdown of NO (Snyder and Bredt, 1991).

1.2. NO Synthases

1.2.1. Isoforms of NO Synthases.

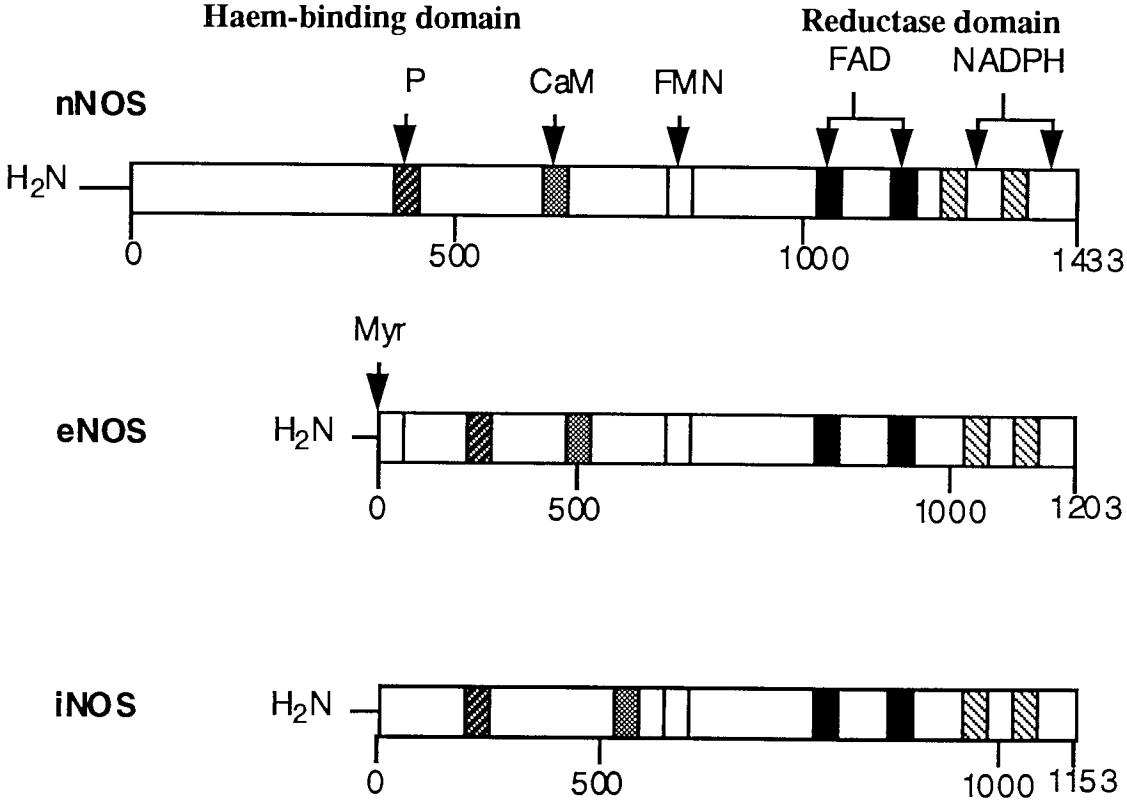
There are three distinct isoforms of NO synthase, which have now been purified, cloned and expressed, and are distinct in antigenicity (Figure 1.2.1). In general terms, the synthases may be divided into two broad categories: two constitutive types that are Ca^{2+} and calmodulin-dependent, and an inducible type that typically does not display a dependence on Ca^{2+} in the physiological range of concentration (Knowles and Moncada, 1994). Considering the range of tissues where NO synthases are localised, and that all three isoenzymes may be "induced" by different stimuli under some circumstances, a range of nomenclature has arisen using either a numeric or tissue-specific mode of classification. nNOS (also known as Type I) is a "constitutive" isoform which may be induced, and is so named due to its original identification in neuronal tissue. eNOS (or Type III) also is "constitutive" but may be induced, and is so named after its original localisation in vascular endothelial cells. iNOS (also known as Type II), was first identified in macrophages (Hevel et al., 1991), and may be constitutively expressed, but is more commonly induced in response to cytokines and endotoxin in a wide range of cell-types.

All NO synthase isoforms consist of two domains, a reductase domain which is similar to cytochrome P-450 reductase, and a haem-binding domain or oxygenase domain. There are two consensus binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), two for flavin adenine dinucleotide (FAD), one for flavin mononucleotide (FMN) and one for calmodulin (Figure 1.2.1). nNOS, the first isoenzyme to be purified and cloned (Bredt and Snyder, 1990), is widespread in brain, particularly cerebellum, but is also distributed throughout peripheral neural systems such as myenteric plexi in the gut. nNOS may be regulated by phosphorylation, and possesses a consensus sequence for cyclic AMP-dependent protein kinase (PKA), although regulation by protein kinase C (PKC) and calmodulin-dependent kinase (CaMK) may also occur (Bredt et al., 1992).

Although eNOS is "constitutive", both Ca^{2+} and calmodulin-dependent and appears to be functionally similar to nNOS, it shares only 57% amino acid identity

with nNOS (Marsden et al., 1992). The significant difference between the enzymes lies within the N-terminus, which for eNOS, is considerably shorter, and possesses a consensus N-myristoylation site. eNOS exists predominantly as a particulate enzyme (Hecker et al., 1991) due to N-terminal myristoylation and nearby palmitoylation (Robinson and Michel, 1995) which anchors the enzyme to membranes.

Figure 1.2.1. Schematic representation of cofactor recognition sites on NO synthase isoforms



Legend to Figure 1.2.1

Cofactor recognition sites on the three isoforms of NO synthase.

CaM = Calmodulin binding site. FMN, FAD, NADPH = binding sites for these cofactors. P = Protein kinase A phophorylation site. Myr = Myristoylation site.

iNOS activity may be induced in a wide range of cell-types in response to cytokines or microbial products, sometimes in combination, such as interferon-γ, tumour necrosis factor-α, interleukin-1ß, and bacterial lipopolysaccharide. Induction of activity may take several hours of exposure to occur, but once initiated may sustain the release of nanomolar amounts of NO over long periods.

This high-output path of NO by iNOS contributes to host-defence and inflammation. NO may be cytotoxic to bacteria and tumour cells. iNOS activity has been extensively studied in rodent macrophages and differs from the constitutive isoenzymes in that it demonstrates Ca^{2+} independence due to tightly-bound calmodulin (Xie et al., 1992), and shares only 51% and 54% sequence identity with eNOS and nNOS respectively.

1.2.2. Biosynthesis of NO.

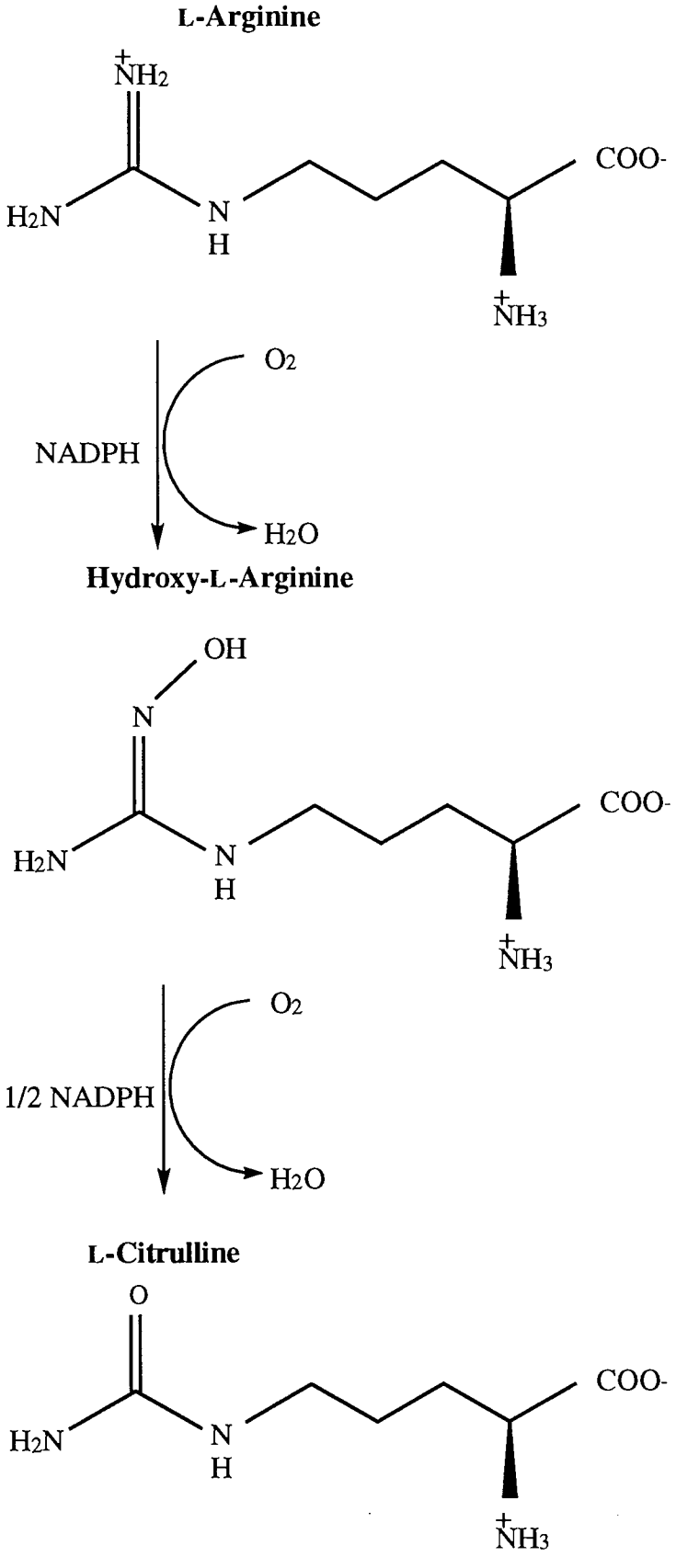
The NO synthases catalyse a complex reaction using substrates L-arginine and molecular oxygen to form NO, with stoichiometric formation of L-citrulline (Figure 1.2.2). NO synthases also require NADPH, FAD, FMN, tetrahydrobiopterin (BH_4), and haem as cofactors for activity. Biosynthesis of NO occurs via a two-step reaction involving a five-electron oxidation of the terminal guanido-nitrogen atom of L-arginine (Marletta, 1993). The first step, a two-electron oxidation, is a hydroxylation that forms the enzyme-bound intermediate N^G-hydroxy-L-arginine (Stuehr et al., 1991). The second step involves an overall three-electron oxidation to form L-citrulline and NO. NADPH is required as an electron donor for both steps, and two molecules of oxygen are utilised in one catalytic turnover to generate NO.

The synthesis of NO by eNOS or nNOS is stimulated by a transient increase in intracellular Ca^{2+}, with binding of intracellular Ca^{2+} to calmodulin allowing it to bind to NO synthase dimers at a site between the haem-containing and reductase domains. Binding of calmodulin brings about a conformational change in NO synthase, which allows electron flow between the domains and promotes NO biosynthesis (Su et al., 1995). As calmodulin is bound very tightly to the iNOS isoform, even at the trace levels of Ca^{2+} found in resting cells, the main switch for its activation is the level of mRNA, which may be induced by cytokines or lipopolysaccharide.

1.2.3. Inhibition of NO Synthases.

The activity of all isoforms of NO synthase can be reduced by certain L-arginine analogues (Figure 1.2.3) which cause competitive inhibition of the enzyme by binding to the active site. Consequently, inhibition can be overcome by simultaneous application of excess exogenous L-arginine. The most widely used NO synthase inhibitors are: N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA) and the methyl ester of L-NA (L-NAME).

Figure 1.2.2. Synthesis of NO



Some inhibitors exhibit selectivity, for example N^G-iminoethyl-L-ornithine (L-NIO) and N-(3-(aminomethyl)-benzyl)acetamidine (1400W) (Garvey et al., 1997) inhibit iNOS more potently than eNOS or nNOS. In addition, NO synthase may be inhibited in biological systems by limitation of substrate and cofactors, and induction of iNOS can be prevented by the glucocorticoid dexamethasone.

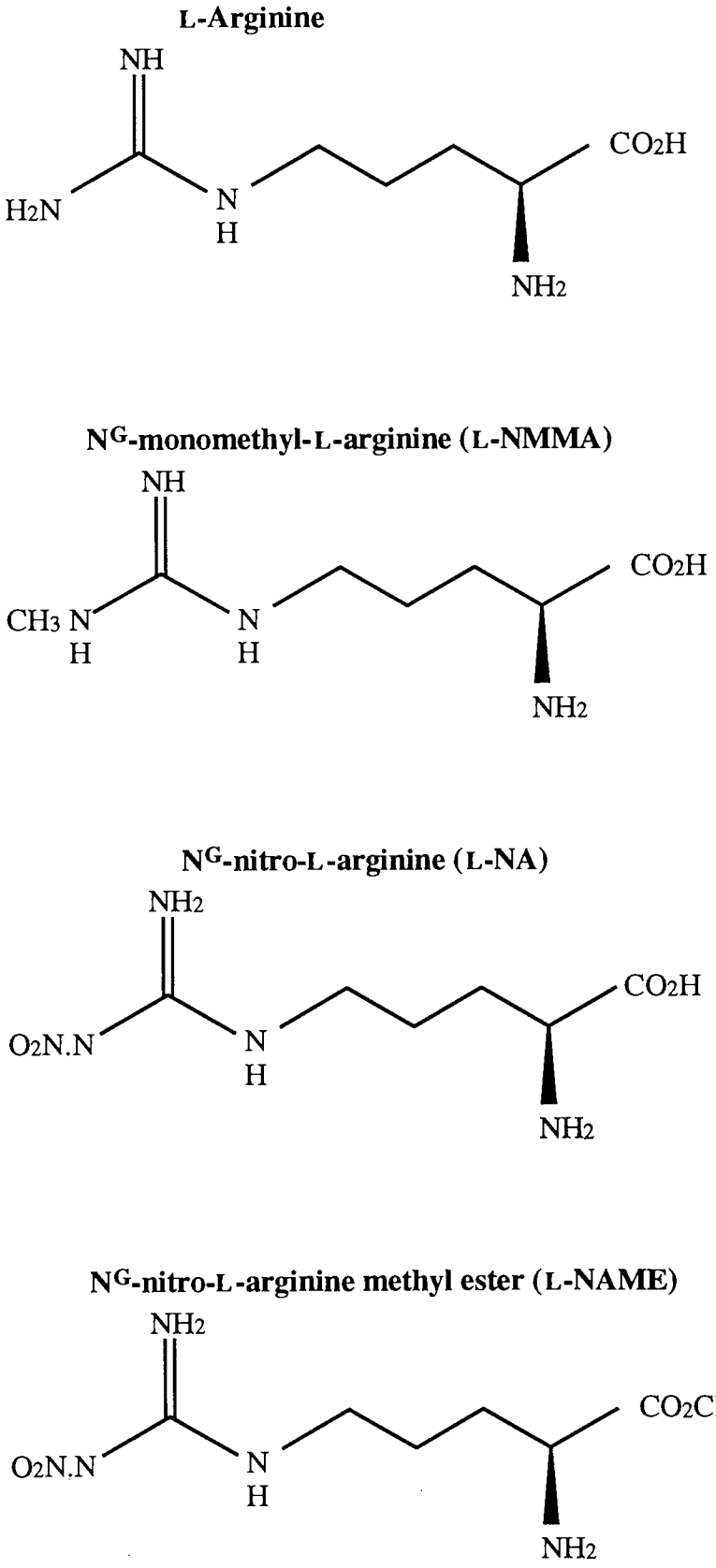
1.2.4. Regulation of production of NO Synthase.

Evidence suggests that regulation of NO biosynthesis may occur at the levels of transcriptional, post-transcriptional, translational and post-translational control (Nathan and Xie, 1994a). A complex promoter region has been identified in murine iNOS, allowing regulation of transcription by various agents, such as lipopolysaccharide, which increases expression of iNOS by activation of the transcription factor NF-κB. Postranscriptional control has been demonstrated by the ability of TGF-ß to destabilise mouse macrophage iNOS mRNA, and TNF-α to destabilise eNOS mRNA in rat vascular smooth muscle. Control is also exerted in mouse and human brain nNOS at the level of alternative splicing. All isoforms of NO synthase may be regulated by phosphorylation, leading to a rapid loss of enzymatic activity. This may occur by action of either cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), or calmodulin-dependent kinase (CaMK), each stoichiometrically phosphorylating NO synthase on a distinct serine (Bredt et al., 1992). Additionally, as all NO synthase isoforms have been associated with both soluble and particulate cell fractions, regulation may occur at the level of subcellular localisation. Regulation such as this is demonstrated by the phosphorylation of eNOS in response to bradykinin which follows its translocation from particulate to cytosolic subcellular fractions (Michel et al., 1993).

1.3. Sites of NO action.

NO exerts a wide variety of effects in biological systems by interacting with signalling proteins, enzymes, ion channels, receptors and transcription factors. These effects are achieved by binding, by oxidation events and by covalent modification of proteins, mediated either directly by reaction with NO, or with products of NO metabolism such as peroxynitrite.

Figure 1.2.3. Structure of L-Arginine and NO Synthase Inhibitors.



1.3.1. Physiological activation of guanylate cyclase

The majority of the effects of NO in signal transduction are mediated through activation of soluble guanylate cyclase. NO activates guanylate cyclase by binding to the iron in the haem moiety at the enzyme's active site, causing a conformational change and a subsequent elevation in 3',5'-cyclic guanosine monophosphate (cGMP). NO induces vasodilation by increased cGMP initiating a signalling pathway which results in the dephosphorylation of myosin light chain kinase, and therefore vascular relaxation. This action may be blocked by soluble guanylate cyclase inhibitors or haemoglobin, mimicked by cGMP, and potentiated by selective inhibitors of cGMP phosphodiesterase.

1.3.2. Cytotoxicity involving a direct action of NO.

Under normal conditions, NO has a number of important physiological roles, however under pathological conditions the production of NO, superoxide, and peroxynitrite can increase, contributing to the disease state. Disease states associated with excessive production of NO include: septic shock, asthma, inflammatory bowel disease, arthritis, and cerebral ischemia (Knowles, 1997). Cytotoxic actions generated by NO directly include: disruption of mitochondrial function (Xie and Wolin, 1996), inhibition of NADH respiration, and prevention of DNA synthesis through inhibition of deoxyribonucleotide synthesis. It was originally thought that NO attacked key iron-sulphur centres on aconitase of the tricarboxylic acid, thus causing its inhibition. However, other evidence suggests that peroxynitrite and superoxide may cause aconitase inactivation, but NO does not (Hausladen and Fridovich, 1994; Castro et al., 1994). NO is linked to inhibition of DNA synthesis, an important cytotoxic effect of macrophage-derived NO on tumour cells. This is achieved through the ability of NO to oxidise the non-haem iron of ribonucleotide reductase (Kwon et al., 1991), thus preventing formation of precursors for DNA synthesis. NO can cause the S-nitrosylation of numerous proteins, thus regulating their function. NO may also be metabolised to form a variety of high and low molecular weight thiols. Species such as S-nitrocysteine and S-nitroglutathione may be formed by oxidation of NO to an NO^+ donor, which is followed by its reaction with thiol. Due to its instability, S-nitrocysteine breaks down to produce NO and a disulphide. NO is capable of DNA damage through base deamination. NO induced DNA damage leads to the activation of the nuclear enzyme poly (ADP ribose) synthase, which results in energy depletion and cell death. Another site of NO reaction is with superoxide which leads to the formation of peroxynitrite, an oxidant with cytotoxic potential, and which forms nitrotyrosine in proteins.

1.3.3. Cytotoxicity mediated via peroxynitrite formation.

Superoxide is produced by the reduction of oxygen by one electron, which may occur by flavins and other redox centres in enzymes in biological systems. Superoxide is also produced by the plasma membrane enzyme NADPH oxidase in phagocytes where it functions in the process of pathogen killing. Under normal physiological conditions superoxide is kept at low levels by the action of superoxide dismutase which scavenges superoxide. However, a more rapid reaction occurs between superoxide and NO, so that NO may out-compete superoxide dismutase for superoxide. In spite of this, peroxynitrite formation is limited in normal tissue. This is due to a higher tissue concentration of superoxide dismutase compared to the NO concentration, and due to the NO-scavenging ability of haemoglobin in red blood cells.

Under pathological conditions NO production greatly increases due to the induction of iNOS activity in macrophages and other cells, and superoxide production increases due to activation of NADPH oxidases in inflammatory cells. Under such conditions where there is an increased availability of NO and superoxide, peroxynitrite formation will occur at a faster rate. Although peroxynitrite has a short half life of about one second, it rapidly oxidises many important biological molecules such as lipids, sulfhydryls, iron sulphur centres, and zinc fingers, thus modulating their function. Peroxynitrite can also act as an oxidant in the presence of metal catalysts such as Fe^{3+} to form a reactive intermediate. The resulting nitronium-ion like intermediate nitrates protein tyrosine residues, which prevents their phosphorylation, leading to a block of signal transduction.

Cytochrome oxidase is the terminal complex of the mitochondrial respiratory chain, and passes electrons from cytochrome c to oxygen. As NO competes with oxygen for binding to the same site on cytochrome c oxidase, it may therefore cause competitive inhibition of the enzyme. A possible mechanism of cell death and damage involves the action of NO, superoxide and peroxynitrite. NO affects respiration at complex IV where it causes inhibition of mitochondrial respiration through inhibition of cytochrome c oxidase. In the presence of mitochondrial inhibitors, such as NO, rotenone or cyanide, the rate of superoxide formation increases. If this occurs under circumstances of sustained NO production, an increased formation of peroxynitrite will result, causing irreversible inhibition of respiratory complexes I and II, and finally cell death (Knowles, 1997).

1.3.4. Cellular defences against cytotoxicity of NO.

Under most circumstances, cells are able to survive oxidant stresses due to the action of efficient defence systems. When subjected to oxidative threat, eukaryotic cells can respond with the upregulation of specialised genes such as manganese superoxide dismutase, glucose-6-phosphate-dehydrogenase and glutathione-S-transferase (Hiraishi et al., 1994). In addition, transcriptional control of glutathione-S-transferase is mediated by the transcription factor AP-1, which is under the control of NO. Reduced glutathione is important in antioxidant defence where it scavenges peroxynitrite and binds other free radicals, and acts in combination with glutathione peroxidases to break down hydrogen peroxide and lipid hydroperoxides (Munday and Winterbourn, 1989; Starke and Farber, 1985). Removal of NO is controlled through the scavenging ability of oxyhaemoglobin, forming methaemoglobin and nitrate, and by deoxyhaemoglobin forming nitrosohaemoglobin. The existence of an alternative NO-oxidation mechanism, which requires a high molecular-weight species and converts NO to nitrate and nitrite has recently been suggested (Knowles, 1997), however this mechanism necessitates further identification. One aim of this thesis was to determine whether NO itself could provoke defensive responses in gastric mucous cells, for example by causing the synthesis of a heat shock protein (see chapter 5 for detailed review and results).

1.4. Structure of the gastrointestinal tract.

1.4.1. Basic structure

The structure and function of the gastrointestinal tract is explained in depth in many textbooks and reviews, and the following references were used to write this section: Ito (1987), Wheater et al. (1987), Vander et al. (1990).

The gastrointestinal tract conforms to the same basic structure throughout the entire length. Although the tract shows marked regional variations, it is essentially composed of a muscular tube lined by a mucous membrane. The tract can be divided into four functional layers: mucosa, submucosa, muscularis and adventitia. The mucosa consists of an epithelial lining, below which lies the supporting connective tissue, the lamina propria, which sits on a smooth muscle layer known as the muscularis mucosa. The submucosa is a layer of loose, connective tissue which supports the mucosa and contains the nerve cell network the submucous plexus, as well as blood and lymphatic vessels. The muscularis propria consists of an inner circular layer, and an outer longitudinal layer of smooth muscle opposed at right angles to one another. Between the two muscle layers is the network of

nerve cells known as the myenteric plexus. The outer layer of connective tissue of the gastrointestinal tract is the adventitia, which is referred to as the serosa when exposed to the abdominal cavity.

1.4.2. The stomach

The stomach is responsible for reducing ingested food to chyme by mechanical and chemical processes. Three distinct regions comprise the rat stomach: the fundus, corpus and antrum. The fundus or forestomach is lined by an epithelium which is of the stratified squamous type. This stomach region is non-glandular, and does not secrete acid or pepsinogen. The corpus consists of gastric glands responsible for acid and pepsinogen secretion. The antrum is also glandular, and contains G cells which secrete the hormone gastrin.

1.4.3. Cell types of the gastric glandular mucosa.

The mucosa of the glandular stomach is composed of tubular glands, containing a mixed cell population, which synthesise and secrete gastric juice. The luminal surface of the stomach and the gastric pits are lined by mucous cells, which are involved in the secretion of mucus and bicarbonate and are of two types, surface mucous cells and mucous-neck cells. Parietal or oxyntic cells, which are distributed in the neck and base regions of gastric glands, secrete hydrochloric acid. Parietal cells are large and rounded, with a luminal plasma membrane which forms deep, branching caniculi which extend throughout the cytoplasm. Chief cells, which secrete pepsin by the process of exocytosis, are clustered at the base of gastric glands. The numerous ribosomes synthesise the inactive pepsin precursor pepsinogen, which is stored in zymogen granules. Additionally, a number of secretory cells such as G, enterochromaffin, and enterochromaffin-like cells, are located in the gastric mucosa, which release gastrin, 5-HT and histamine respectively.

1.5. Mechanisms of gastric mucosal defence.

The mechanisms of mucosal protection employed in the gastrointestinal tract prevent autodigestion by endogenous aggressors such as hydrochloric acid and pepsin, and act to limit damage from exogenous aggressors such as drugs and alcohol. Where specific references are not mentioned, information for sections 1.5. and 1.6. was taken from the following reviews: Stark and Szurszewski (1992), Whittle (1993 and 1994), Allen et al. (1993), Wallace and Bell (1995).

1.5.1. Mucus and bicarbonate

The continuous mucus gel layer which lies over the gastric epithelial surface, provides a visco-elastic, unstirred layer which functions as a physical barrier against the luminal contents and secreted pepsin. The adherent mucus gel layer, consists of: mucin, a glycoprotein which accounts for the viscous and gel-forming properties, and protein, lipid, and nucleic acid, which are mostly derived from exfoliated cells. Gastric mucins are large molecular weight glycoproteins, which link with each other via disulphide bridges, to form complex polymers possessing physical strength. Gastric mucin subunits are composed of a central protein core with glycosylated and non-glycosylated regions, and an overall strong net negative charge. The glycosylated region is resistant to proteolysis due to the attachment of many carbohydrate side chains, and the non-glycosylated region participates in the interchain disulphide bridge linkages. The gastric mucosa secretes bicarbonate, which neutralises acid diffusing into the mucus gel unstirred layer, such that with an acidic pH in the lumen, a near neutral pH can exist at the mucus-epithelium interface. Thus, the mucus-bicarbonate layer provides a physical barrier to pepsin and enables neutralisation of acid at the surface of the epithelium.

1.5.2. Proliferation and restitution.

The epithelial layer itself contributes to mucosal integrity by providing a barrier to H^+ which is maintained through the processes of proliferation and restitution. Epithelial turnover is rapid in the stomach, due to the constant exfoliation of cells and their replacement by cell division. Restitution is the rapid protective process that occurs in response to superficial epithelial damage. This involves the migration of any remaining viable epithelial cells from areas adjacent to the lesioned region, to re-establish epithelial continuity. Mucus plays a role in this process, by forming a thick protective mucoid cap over the damaged area.

1.5.3. The gastric microcirculation

An extensive microcirculation functions to provide oxygen and nutrients to the epithelium, and to remove toxins or hydrogen ions derived from acid-back diffusion. Acid-back diffusion occurs when the gastric protection afforded by the mucus-bicarbonate barrier by the epithelial layer, and by the alkaline tide have been insufficient to prevent hydrogen ions from penetrating the tissue. A situation such as this might result from gastric damage due to excessive acid load, ethanol, or non-steroidal anti-inflammatory drugs. In response, blood flow is increased and the extra hydrogen ions are removed in the blood. A number of vasoactive substances are reported to regulate the gastric microcirculation. NO, calcitonin-gene related peptide (CGRP), and prostacyclin (PGI_2) function as vasodilators and

are protective, whereas vasoconstrictors such as noradrenaline, neuropeptide Y and endothelin enhance damage.

1.5.4. Epidermal growth factor and prostanoids.

Epidermal-growth factor (EGF) is a potent mitogen which is synthesised and secreted by salivary glands. A role for EGF in mucosal protection is suggested due to its ability to stimulate proliferation and inhibit acid secretion. Prostanoids are also important for mucosal protection in that PGE_2 is involved in the mucosal alkaline response following acid exposure, and can increase mucus secretion, and PGI_2 exerts a vasodilator action on the microcirculation.

1.6. The role of NO in the gastric mucosa.

1.6.1. Detection of NO synthase in gastric mucosal tissue.

Ca^{2+}-dependent NO synthase activity has been detected in rat whole stomach, in addition, gastric mucosal cells isolated by a method of pronase digestion and intermittent Ca^{2+} chelation possess Ca^{2+}-dependent NO synthase activity (Brown et al., 1992). Surface cells of the corpus mucosa express a form of NO synthase that resembles nNOS (Price et al., 1996). eNOS is present in blood vessels in the mucosa and submucosa (Price et al., 1996). Although NO synthase has been detected in gastric mucosa by direct assay and by immunohistochemistry very little is known about how the enzyme might be regulated in intact gastric mucosal cells. One aim of this thesis was to investigate the metabolism of radioactively-labelled arginine by such cells with a view to using citrulline formation as an index of NO synthase activity in intact tissue (for more detail and results see chapter 3). iNOS can be induced in gastric mucosal cells by pretreatment of rats with lipopolysaccharide *in vivo* (Brown et al., 1994). Another aim of this thesis was to try to identify mechanisms regulating induction of iNOS in cultured gastric mucosal cells (for more detail and results see chapter 6). Initial experiments were carried out on pulmonary cells because procedures for inducing iNOS in this system are established and because the emphasis of research in NO at the industrial collaborator (Glaxo-Wellcome) had changed from gastrointestinal to pulmonary after this thesis was initiated.

1.6.2. The role of NO in the maintenance of the gastric mucosal microcirculation.

Intravenous administration of the NO synthase inhibitor L-NMMA into rats dose-dependently reduced resting gastric mucosal blood flow, as determined by hydrogen gas clearance (Pique et al., 1989). This result suggested an involvement of endogenous NO in modulation of the resting gastric microcirculation. Indeed, gastric mucosal damage can result from treatment of rats with indomethacin followed by L-NMMA (Whittle et al., 1990), although neither agent alone had an effect. The mucosal hyperaemia which occurs in response to intravenous infusion of pentagastrin may be attenuated by the concurrent administration of L-NMMA or L-NAME. This result implicates NO as a mediator of the vasodilatation associated with the stimulation of acid secretion (Pique et al., 1992). In addition, L-NMMA pretreatment increased gastric mucosal damage, and decreased the hyperaemic response to damage by NaCl, in the corpus and fundus, but not the antrum in cats (Gislason et al., 1996).

The direct effect of intraluminal ethanol administration in rats is a reduction in Ca^{2+}-dependent NO synthase, and mucosal damage characterised by vasocongestion and hemorrhagic necrosis (Tepperman and Soper, 1994). Injury by ethanol was exacerbated by the use of NO synthase inhibitor L-NA (Masuda et al., 1995). Water immersion stress, which induces gastric lesions, led to a significant reduction in NO synthase activity and injury was greater in the presence of a NO synthase inhibitor (Tachi et al., 1996). Damage inflicted on feline small intestine by ischemia-reperfusion was exacerbated in the presence of L-NAME, supporting the concept that endogenous NO limits injury (Kubes, 1993). One major mechanism by which protection against effects of ethanol and water-immersion stress occurs is probably by the maintenance of mucosal microcirculation: thus the release of NO from nitrovasodilator agents or NO donors, has been shown to relax vascular smooth muscle and protect against mucosal injury. Application of these NO-releasing agents such as glyceryl trinitrate or nitroprusside protected against hydrochloric acid-induced mucosal injury (Kitagawa et al., 1990) and reduced the severity of gastric mucosal damage caused by ethanol (MacNaughton et al., 1989). Infusion of endothelin-1 into rats induces gastric mucosal vasoconstriction and haemorrhagic injury, which can be prevented by administration of NO donors (Lopez-Belmonte et al., 1993). Such protective effects are attributed to the vasorelaxant effect of NO.

The gastroprotection afforded with the use of anti-ulcer compounds such as sucralfate, carbenoxolone, and aluminium containing antacids appears to be

mediated by NO (Konturek et al., 1992; 1993; 1994), because L-NA is capable of attenuating this protection. The anti-ulcer effects exhibited by the immunosuppressive drug FK506 are related to its ability to preserve gastric NO synthase when the mucosa is subjected to damage (Hisanaga et al, 1996). It is unclear whether these agents exert their effect solely via modulation of the microcirculation.

NO and CGRP may interact in regulating the gastric microcirculation. Thus chronic capsaicin treatment which destroys sensory neurons, augments the reduction in mucosal blood flow induced by NO synthase inhibitors (Tepperman and Whittle, 1992). Furthermore, sensory nerve activation and the subsequent vasodilation which occurs in response to acid-back diffusion, involves both NO and CGRP (Lippe and Holzer, 1992). A synergistic interaction leading to relaxation of vascular smooth muscle may be occurring between NO and CGRP, in addition, NO may be involved in the modulation and release of CGRP (Whittle, 1993).

1.6.3. Role of NO in bicarbonate and mucus secretion.

NO has been implicated in the control of bicarbonate secretion, although the full role it plays in this process requires further study. The ability of cGMP to stimulate gastric bicarbonate secretion (Flemstrom, 1994) may suggest a stimulatory role for NO, however other evidence suggests an inhibitory action of NO. Application of NO synthase inhibitors resulted in an increase in rat duodenal bicarbonate secretion, which was reversed by L-arginine (Takeuchi et al., 1993). This result however may be attributed to an increased plasma bicarbonate leakage due to epithelial disruption. A physiological depressant role for NO on neuronal activity that regulates bicarbonate secretion has also been suggested. A role for NO in the modulation of mucus secretion, which involves a cGMP mechanism, has been proposed. The release of mucin from suspensions of isolated gastric mucosal cells was shown to be enhanced in the presence of NO donors, dibutryl cGMP, and a cGMP phosphodiesterase inhibitor (Brown et al., 1993). In addition, the intragastric administration of NO donors can increase the thickness of the mucus layer (Brown et al., 1992a), and NO synthase inhibition reduces the thickening of the gastric mucus layer which occurs in response to luminal instillation of carbachol (Price et al., 1994). Formation of the mucoid cap during the process of restitution after hypertonic salt injury has also been attributed to the action of endogenous NO (Yanaka et al., 1995).

1.6.4. Role of NO in acid secretion.

NO has been implicated in control of acid secretion, although reports have been conflicting, and the role NO plays, if any, is far from clear. Administration of a high dose of L-NMMA results in a decrease in acid secretion (Pique et al., 1992), although this is thought to be an indirect effect relating to the decreased gastric mucosal blood flow. Histamine-stimulated acid secretory activity in isolated rat parietal cells, was inhibited by NO released from a NO donor through a cGMP-dependent mechanism (Brown et al., 1993). Administration of endotoxin to anaesthetised rats abolishes the acid secretory response induced by pentagastrin. However, pretreatment with a NO synthase inhibitor restored the secretory response, suggesting a role for NO in the endotoxin-induced inhibition of acid secretion (Martinez-Cuesta et al., 1992). Exposure of the rat stomach to the damaging agent taurocholate results in an adaptive cytoprotective response involving a reduction in potential difference, acid secretion, and an increase in luminal bicarbonate. It is suggested that a constitutive type of NO is involved in this mechanism of acid inhibition following damage (Takeuchi et al., 1995). An aim of this work was to establish whether damage by hypertonic NaCl *in vivo*, which is known to induce ornithine decarboxylase activity, causes release of NO to act as an effector of gene induction.

1.7. Summary of aims of this thesis.

1. To investigate arginine metabolism by isolated rat gastric mucosal cells, with a view to using citrulline formation as an index of NO synthase activity in intact tissue.
2. To investigate the role of NO in the induction of ornithine decarboxylase in rat gastric mucosa damaged by hypertonic saline *in vivo* .
3. To expose primary cultures of guinea-pig gastric mucosal cells to oxidative challenge and an NO donor, and investigate the cellular response in terms of reduced glutathione content, viability and heat shock protein 72 induction.
4. To establish the role of potential modulators on iNOS activity, induced by cytokines, in gastric and pulmonary cells in culture.

CHAPTER 2

GENERAL METHODOLOGY

2.1 Bicinchoninic acid protein assay

The bicinchoninic acid (BCA) protein assay was used under circumstances where it was necessary to measure dilute protein samples in the presence of detergents. The detection range of the assay is from 20 µg/ml to 1200 µg/ml. Protein concentrations were determined by this method in tissue homogenates, cell pellets and samples prepared for gel electrophoresis. The BCA protein assay was carried out by using the Pierce BCA protein assay reagent kit.

The method involves the use of bicinchoninic acid, a selective and sensitive detection reagent for the cuprous ion (Cu^{+}). The detection principle is based on the protein present in the sample reducing Cu^{2+} to Cu^{+} at an alkaline pH. The BCA then chelates with the Cu^{+} ion, where one Cu^{+} interacts with two molecules of BCA. The reaction product is a water soluble, purple coloured complex, which exhibits a strong absorbance at 562 nm. The amount of coloured complex formed is directly proportional to the protein present in the sample and so quantitation is possible by spectrophotometric measurement.

The standard Pierce assay protocol was used. For this, a set of 6 protein standards in the range of 0 µg/ml to 100 µg/ml were prepared in duplicate in borosilicate glass tubes. This was achieved by appropriate dilution of a 2 mg/ml stock solution of BSA with distilled water to give a final volume of 95 µl. 5 µl of the solvent for the protein samples was then added to each tube of protein standard. Each unknown was prepared in duplicate by adding 5 µl of sample to 95 µl distilled water. 2 ml working reagent was then added to each tube of standard and unknown and mixed well before incubation at 37°C in a water bath for 30 min. Working reagent consists of reagent A and B combined to give a 50 : 1 ratio. Reagent A contains sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate (unknown concentrations) in 0.2 M NaOH. Reagent B is 4% (w/v) copper sulphate solution. On completion of the 30 min incubation period, 200 µl was removed from each tube and transferred to a corresponding well in a flat-bottomed 96 well plate. Absorbance was read at 570 nm in a plate reader.

For each sample the mean absorbance was calculated from the corresponding set of duplicates, after which, the reagent blank was subtracted from each of the standards and unknowns. To construct the calibration curve, the concentration of BSA (µg/ml) in each standard was plotted against absorbance to give a straight line. From this, protein concentration in unknowns was determined by reading concentration from the curve for the absorbance value obtained.

2.2 Coomassie blue protein assay

The Coomassie blue protein assay was used under circumstances where the solution requiring protein determination contained reducing agents. Tissue samples used in the ornithine decarboxylase assay were prepared in homogenisation buffer containing DTT, and as the Coomassie blue protein assay is compatible with reducing agents, this was the protocol of choice.

The detection principle is based on the direct binding of the Coomassie dye Brilliant Blue G250 to protein in an acidic solution which involves a colour change from brown to blue and a shift of the absorbance maximum from 465 nm to 595 nm. The detection range is in the region of 1 μg/ml to 1500 μg/ml.

A set of 9 protein standards in the concentration range of 0 μg/ml to 80 μg/ml were prepared in duplicate. This was achieved by diluting a 2 mg/ml BSA stock solution into a total volume of 50 μl. Care was taken to ensure that protein standards and unknowns were present in the same buffer. Blank tubes contained this buffer alone. The Coomassie blue dye reagent concentrate (Sigma) was diluted in a 1:4 ratio with distilled water. The assay involved adding 2.5 ml of the diluted reagent to 50 μl of either sample or standard, mixing well, and leaving for 5 min at room temperature. Absorbance was measured at 595 nm, after which, a calibration curve was constructed so that the protein concentrations of the unknowns could be determined.

2.3. Estimation of cell viability using trypan blue

Live, viable cells possess the ability to exclude the trypan blue stain, whereas dead cells or those with a damaged cell membrane will appear stained blue when viewed under a light microscope. This technique has been shown to provide a reliable index of gastric cell injury (Tepperman et al., 1991). A solution of trypan blue (4 mg/ml) was made up in 0.9 % w/v NaCl. In order to estimate viability, a 20 μl aliquot of cell suspension was added to 20 μl of trypan blue solution and the cells resuspended. A 20 μl volume was removed, and total and non-viable cells were counted in a standard haemocytometer chamber. To obtain a reliable estimation of cell number and viability, cells were suspended in sufficient volume so that a minimum of 200 cells per large central square were counted. The large central square (1 mm^2) in a standard haemocytometer is divided by double lines into 5 x 5 squares, each of which is composed of 5 x 5 smaller squares. Regulation

of cell counting was achieved by counting only the cells which touched the top and left middle lines of each subdivided square. The volume of the large central square is known to be 1 x 10^{-4} ml, so that once the cell number in this small volume is obtained, the estimated cell number in the cell suspension sample can be calculated.

Figure 2.3.1 Calculation used in estimation of cell viability

$$\text{Number of cells/ml} = \frac{\text{Total cells}}{\text{No. of large squares}} \times 1 \times 10^4 \times 2$$

where 1 x 10^4 is the volume of a large square
x 2 accounts for the dilution by trypan blue.

2.4 Nitric Oxide Synthase Assay

The formation of NO from L-arginine and molecular oxygen is catalysed by NO synthase. This is a complex, two-stage reaction via a hydroxyarginine intermediate to yield L-citrulline and NO as final products. The assay works on the principle that with the formation of each NO molecule there is concomitant release of L-citrulline. In this way, NO synthase activity can be measured indirectly, by determining the conversion of radiolabelled L-arginine to L-citrulline. To confirm that the L-citrulline produced is due to NO synthase activity, and has not occurred independently of this process, a specific NO synthase inhibitor is used to block the activity. This technique was first used to monitor purification of the enzyme in the cerebellum (Bredt and Snyder, 1989), and the assay used here is a modification on that first described by those authors.

In outline, the assay involves preparation of either tissue or cell homogenate, an aliquot of which is added to a reaction mixture containing L-[^{14}C]arginine and cofactors in assay buffer. The reaction is then allowed to proceed for a set time, after which, there is addition of distilled water and newly formed L-[^{14}C]citrulline is separated from L-[^{14}C]arginine by the adsorption of unreacted L-[^{14}C]arginine on to DOWEX resin, (AG 50-W8, Na^+-activated). L-[^{14}C]citrulline is left in the supernatant, the amount present can be determined by scintillation counting, and the activity of NO synthase in the reaction mixture can be calculated.

2.4.1 Preparation and activation of DOWEX resin.

To prepare a suspension of DOWEX capable of binding L-[^{14}C]arginine, the resin must first be activated. To do this, 200 ml of 1 M NaOH was added to 50 g DOWEX AG 50-W8 and the mixture was gently swirled to form a suspension. The resin was allowed to settle for 1 hour, after which, the aqueous layer was carefully decanted off, and a further 200 ml of 1 M NaOH was added. The combination was gently mixed as before, and left to stand for 1 hour. After the second equilibration period, the aqueous layer was decanted off and 300 ml of distilled water was added. The DOWEX was gently mixed and allowed to settle. The resin was repeatedly washed with water in this way until the pH of the DOWEX suspension was approximately 6.8. The water was then poured off and a 1:1 (v/v) suspension of DOWEX with distilled water was prepared. The suspension was stored in a covered container at 4°C until required.

2.4.2 Preparation of homogenates

2.4.2.1 Preparation of tissue homogenates

A male Wistar rat was anaesthetised with sodium pentobarbitone (60 mg/kg i.p.). A midline incision was made, gentle pressure was applied to the left side of the rat until the stomach came into view, and it was then carefully cut free. The non-glandular region was removed and the remaining stomach section was cut open along the greater curvature, it was then immediately washed in ice cold saline (NaCl, 9 g/l). The tissue was kept cold by performing the following procedure on a glass plate over ice. The luminal surface of the tissue was first blotted with filter paper to remove excess debris and saline. It was then scraped with a scalpel blade to remove the corpus mucosa. The tissue was weighed, and a volume (ml) of NO synthase homogenisation buffer (10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 6 µg/ml aprotinin, 0.1 mg/ml PMSF in 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.5) was added which was equal to 5 x the weight (g) of the tissue (i.e. 5 ml buffer per 1 g tissue). The tissue was homogenised in the above buffer, on ice, for 30 s by using an Ultraturrax homogeniser running at full speed. The homogenate was centrifuged at 10,000 x g_{av} for 20 min at 4°C. The resulting supernatant was removed and kept on ice (a period no longer than 30 min) until required as the source of NO synthase for the assay. Samples of other tissues for NO synthase assay were homogenised in a similar way.

2.4.2.2 Preparation of homogenates for enhanced assay protocol.

Two extracts were prepared; a 100,000 x g_{av} supernatant and a pellet fraction. Rat tissue was obtained and homogenised for 30 s in buffer with an Ultraturrax as

described in section 2.4.2.1. The homogenate was then centrifuged at 100,000 x g_{av} for 30 min at 4°C in an ultracentrifuge. The supernatant obtained was removed, put in a fresh tube and kept on ice until needed. Homogenisation buffer, as described in section 2.4.2.1, but with the addition of 10% (v/v) glycerol was added to the remaining pellet. The volume of buffer added equalled the initial volume of homogenate that had been centrifuged. The pellet was resuspended in this fresh homogenisation buffer by the action of an Ultraturrax microhomogeniser running at full speed for 30 s. The preparation was kept cool during homogenisation by placing the microfuge tube in a beaker of ice.

2.4.2.3 Preparation of cell pellet homogenates

Rat gastric mucosal cells obtained from the cell isolation procedure (see section 2.6) were also assayed for NO synthase. A volume of cell suspension was added to a microfuge tube and centrifuged at 10,000 x g_{av} for 2 s to loosely pellet the cells. The supernatant was discarded and a volume of homogenisation buffer (constituents as described in section 2.4.2.1) was added to give 1 x 10^8 cells/ml. Cells were split open into the buffer by the process of repeating a cycle of rapid freeze/thawing, (by the use of liquid nitrogen) 3 times, or by the action of an Ultraturrax microhomogeniser running at full speed for 30 s. As described above, the resulting homogenate was centrifuged at 10,000 x g_{av} for 20 min at 4°C. The supernatant obtained was removed and kept on ice (a period no longer than 30 min), after which the usual procedure for the NO synthase assay was followed.

2.4.3 Standard assay protocol

In the standard protocol, microfuge tubes were set up under 3 different assay conditions: NO synthase uninhibited, L-NMMA (NO synthase inhibitor) inhibited and EGTA (calcium chelator) inhibited. Each assay condition was set up in triplicate. To perform the assay, 50 µl of either uninhibited or the inhibited assay buffers were added to the relevant 1.5 ml microfuge tubes and pre-equilibrated to 37°C in a waterbath. The basic assay buffer contained 40 mM KH_2PO_4, 0.20 mM $CaCl_2$, 1mM $MgCl_2$ (pH 7.4). The reaction was started by addition of 20 µl of supernatant homogenate (preparation described in sections 2.4.2.1 and 2.4.2.2) and halted after an incubation period of 10 min at 37°C by addition of 500 µl DOWEX suspension (section 2.4.1) and 1.0 ml distilled water. The time course was staggered so that each subsequent addition of homogenate was made at 30 s intervals, to ensure the same length of reaction time in each individual microfuge tube. The microfuge tubes were left to stand for a few minutes whilst the resin settled, after which 975 µl of the supernatant was transferred to scintillation vials. An addition of 5 ml Optiphase HiSafe II scintillant was made to each vial, which

was capped and shaken, and the content of radio-labelled citrulline was determined by liquid scintillation counting. A "blank" was included with 5 ml scintillant alone in order to establish the background radioactivity count. This value was then subtracted from the total counts obtained with each vial.

Table 2.4.3 Constituents of standard NO Synthase assay buffer

Reagent	Reagent stock conc.	Final conc. in assay buffer	Final conc. after addition of sample
ß-NADPH	50 mM	1 mM	0.714 mM
L-[^{14}C]arginine	297 mCi/mmol 50 µCi/ml	1.515 µM	1.082 µM
L-Arginine	5 mM	20 µM	14.28 µM
L-Valine	500 mM	10 mM	7.14 mM
L-NMMA	50 mM	1 mM when added	0.714 mM when added
EGTA	50 mM	1 mM when added	0.714 mM when added

2.4.4 Enhanced assay protocol

The enhanced assay protocol is based on that described by Hecker et al. (1994), the major difference being the presence of exogenous FAD, calmodulin and 6-R-tetrahydrobiopterin (6R-BH_4)(see Table 2.4.4). The basic assay buffer was composed of: 50 mM HEPES, 1mM dithiothreitol, 1 mM EDTA, 1.25 mM $CaCl_2$). Microfuge tubes containing 100 µl of uninhibited, L-NMMA-inhibited or EGTA-inhibited assay buffer, in triplicate were set up and allowed to equilibrate to 37°C. To initiate the reaction, 25 µl of enzyme was added sequentially at 30 s intervals to each tube of assay buffer and left to incubate for 10 min at 37°C. The reaction was halted with the addition of 0.5 ml of a 1:1 suspension of DOWEX and 0.5 ml of distilled water. This was followed by centrifugation of assay tubes at 10,000 x g_{av} for 5 min at 4°C. This ensured that the DOWEX with associated labelled arginine did not contaminate the supernatant and was instituted because of potential interference between particulate matter in the homogenate and sedimentation of DOWEX. 0.5 ml of the resulting supernatant was removed from each tube for scintillation counting as described previously (section 2.4.3).

Table 2.4.4 Constituents of enhanced protocol NO Synthase assay buffer

Reagent	Reagent stock conc.	Final conc. in assay buffer	Final conc. after addition of sample
ß-NADPH	50 mM	1 mM	0.8 mM
L-[^{14}C]arginine	297 mCi/mmol 50 µCi/ml	1.515 µM	1.212 µM
L-Arginine	5 mM	20 µM	16 µM
L-Valine	500 mM	10 mM	8 mM
L-NMMA	50 mM	1 mM when added	0.8 mM when added
EGTA	50 mM	1 mM when added	0.8 mM when added
FAD	1 mM	1 µM	0.8 µM
6R-BH_4	15 mM	15 µM	12 µM
Calmodulin	100 µM	1 µM	0.8 µM

2.4.5 Calculations used in NO Synthase assay

A mean dpm value, above background radiation, was first calculated from each set of triplicate results obtained from each assay condition investigated (uninhibited, L-NMMA-inhibited and EGTA-inhibited). The citrulline formation in nmol/min/g wet weight was then calculated for each mean value obtained. Citrulline formation in the presence of inhibitor was then subtracted from the uninhibited value to give a value for NO synthase activity in sample.

2.4.5.1 Calculation of Specific Activity

The specific radioactivity of L-[^{14}C]arginine in the assay buffer was calculated in the following way;

$$\text{Radioactivity in assay (µCi/ml)} = \frac{\text{radioactivity of stock}}{\text{Total vol.assay buffer}} \times \text{vol.stock in buffer}$$

Radioactivity of stock L-[^{14}C]arginine = 50 µCi/ml
Total volume of assay buffer = 1029.09 µl
Volume of L-[^{14}C]arginine stock in assay buffer = 9.09 µl

By converting the value obtained above for radioactivity in assay (μCi/ml) into dpm/ml (conversion factor = 2.22 x 10^6), specific activity can be calculated as follows;

$$\text{Specific activity (dpm/nmol)} = \frac{\text{Radioactivity in assay}}{\text{Conc. of total arginine}}$$

Radioactivity in assay = 980468 μCi/ml (from previous equation)
Concentration of hot and cold arginine in assay buffer = 20.515 nmol/ml

2.4.5.2 Calculation of NO Synthase activity

Using the value obtained for specific activity of L-[^{14}C]arginine in section 2.4.5.1, NO Synthase activity was calculated in the following way;

$$\text{NO Synthase Activity (nmol/min/ml homogenate)} = \frac{\text{Mean dpm counts}}{\text{Specific activity}} \times \frac{\text{FV}}{\text{IT}} \times \frac{\text{VL}}{\text{VS}}$$

Mean dpm counts = mean counts obtained from each set of triplicates
Specific activity (dpm/nmol) = activity of radiolabelled arginine per nmol
FV = factor homogenate volume is multiplied by to give 1 ml.
IT = incubation time of assay (min)
VL = volume (ml) liquid in assay tube (DOWEX is added in a 1:1 suspension), 1 part solid to 1 part liquid.
VS = volume (ml) liquid in sample taken for scintillation counting.

Therefore to calculate NO synthase activity in the standard assay, the following values were put into the above equation;

$$\text{NO Synthase Activity (nmol/min/ml homogenate)} = \frac{\text{Mean dpm counts}}{47088} \times \frac{50}{10} \times \frac{1.320}{0.975}$$

To express NO synthase activity per g wet weight, the value obtained from the above equation was multiplied by a factor of 5.8. It is assumed that 1 g wet weight of tissue is composed of approximately 80% water. In the assay, 5 ml of homogenisation buffer was used per 1 g of tissue. So, from this it is estimated that there is 1 g wet weight in 5.8 ml. To express NO synthase activity as nmol/min/mg protein, the activity per ml homogenate was divided by the protein concentration of homogenates (in mg/ml), as measured by the BCA protein assay.

2.5 Polyacrylamide Gel Electrophoresis and Immunoblotting

The techniques of polyacrylamide gel electrophoresis (PAGE) and immunoblotting were used to separate proteins obtained from primary culture and freshly isolated cell samples, and then using antibody to probe proteins immobilised on a filter to determine presence or quantity of a particular protein of interest. There are a number of stages in this process which involves: solubilising the sample with detergents and reducing agents so that the proteins can be separated by SDS-PAGE, transfer of proteins from the gel to a nitrocellulose filter, exposing filter to antibody specific for the target protein and then developing the blot so that targeted proteins can be visualised.

2.5.1 Preparation of samples for PAGE

Cell samples were prepared under conditions that ensured dissociation of the proteins present into their individual polypeptide subunits with minimal aggregation. This was achieved by using the strongly anionic detergent SDS in combination with a reducing agent and heat to dissociate the proteins. In this way, the denatured polypeptides bound a quantity of SDS proportional to their mass and could therefore be separated by SDS-PAGE according to size.

Rat gastric mucosal cells obtained from the cell isolation procedure (section 2.6), guinea-pig primary cultured gastric mucosal cells (section 5.2.2), and HeLa cells were used to prepare samples for SDS-PAGE. To prepare rat gastric mucosal cells for electrophoresis, a volume of cell suspension was placed in a microfuge tube and centrifuged at 10,000 x g_{av} for 2 s to loosely pellet the cells. The required volume of hot 1 x electrophoresis sample buffer without dithiothreitol, (62.5 mM Tris.HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS, 0.0125 mg/ml bromophenol blue, made up in glass vial and brought to boil for 5 s on a heating block) (2 x 10^7 cells/ml sample buffer) was then added to the cell pellet. Guinea-pig primary cultured gastric mucosal cells, and HeLa cells were grown in 6-well culture plates. These cells were harvested by the addition of 100 µl hot 1 x electrophoresis sample buffer, without dithiothreitol, to each individual well. A cell scraper was used to ensure removal of all cells from the plate and the cell contents of each well was then transferred to the appropriate microfuge tube. All the above samples, were placed on a heating block, to give a temperature of 100 °C inside microfuge tubes, for 5 min. A small sample was then removed from each tube to determine protein concentration by the BCA protein assay (section 2.1). A volume of 1 M dithiothreitol was then added to each tube to give a final dithiothreitol concentration in tube of 0.1 M. Sample tubes were then placed back

on the heating block to reboil all constituents for 2 min. After cooling to room temperature samples could be loaded on to a gel directly or stored frozen at -20°C.

On each electrophoresis gel, one lane was used to run a sample of markers of known molecular mass so that it was possible to later estimate the molecular mass of the protein of interest. Rainbow coloured protein molecular weight markers (Amersham) were used. This product consists of a mixture of individually coloured and purified proteins, combined to give bands of approximately equal intensity when separated by SDS-PAGE. The coloured bands can be visualised on the gel and after transfer to nitrocellulose filter. In this way, the satisfactory running of the gel and transfer of proteins can be checked. By measuring the distance travelled by each coloured band, a calibration curve of electrophoretic mobility versus molecular mass can be drawn allowing accurate measurement of the molecular mass of bands of interest. To prepare samples, 10 µl of rainbow marker solution was boiled for 2 min on a heating block with 10 µl of complete 2 x electrophoresis sample buffer (125 mM Tris.HCl pH 6.8, 20% v/v SDS, 0.2 M dithiothreitol, 0.025 mg/ml bromophenol blue).

2.5.2 Casting and running gel

The polyacrylamide gel is composed of chains of polymerised acrylamide that are cross-linked with N,N'-methylenebisacrylamide. This forms pores in the gel which allow the SDS-polypeptide complexes to pass through, and creates a gel with tensile strength.

Two glass plates (16 cm x 16 cm), cleaned with acetone, separated by two 0.75 mm spacers were sandwiched together and clamped vertically in the gel stand. A separating gel solution was prepared, gently stirred, and carefully poured into the gap between the glass plates to give a gel height of 11.5 cm. The gel solution was then carefully overlaid with a 1 cm layer of methanol : water (1:1), and allowed to set for 40 min. When polymerisation was completed, a piece of filter paper was inserted into the gap between the plates to remove the overlay. A stacking gel was prepared, carefully mixed and poured directly onto the surface of the polymerised resolving gel. A clean gel comb was immediately inserted into the stacking gel solution, without the introduction of air bubbles, to create the lanes in which to load the protein. Polymerisation was complete after 30 min after which the comb was removed, and the wells carefully washed and filled with running buffer (0.025 M Tris, 0.2 M glycine, 0.1% (w/v) SDS, pH 8.3).

Table 2.5.2.1 Resolving gel components

Reagent	Volume	Final Concentration
1.5 M Tris.HCl (pH 8.8)	5 ml	375 mM
10 % (w/v) SDS	0.2 ml	0.1 %
Acrylamide/N,N'-bisacrylamide (30 %/ 0.8 %)	5.34 ml	8 % acrylamide
Fresh ammonium persulphate (100 mg/ml)	0.1 ml	0.5 mg/ml
Distilled water	9.36 ml	
N,N,N',N'-tetramethylenediamine	7.5 µl	

Table 2.5.2.2 Stacking gel components

Reagent	Volume	Final Concentration
0.5 M Tris.HCl (pH 6.8)	2.5 ml	125 mM
10 % (w/v) SDS	0.1 ml	0.1 %
Acrylamide/N,N'-bisacrylamide (30 %/ 0.8 %)	1.0 ml	3 % acrylamide
Fresh ammonium persulphate (100 mg/ml)	0.05 ml	0.5 mg/ml
Distilled water	6.35 ml	
N,N,N',N'-tetramethylenediamine	5 µl	

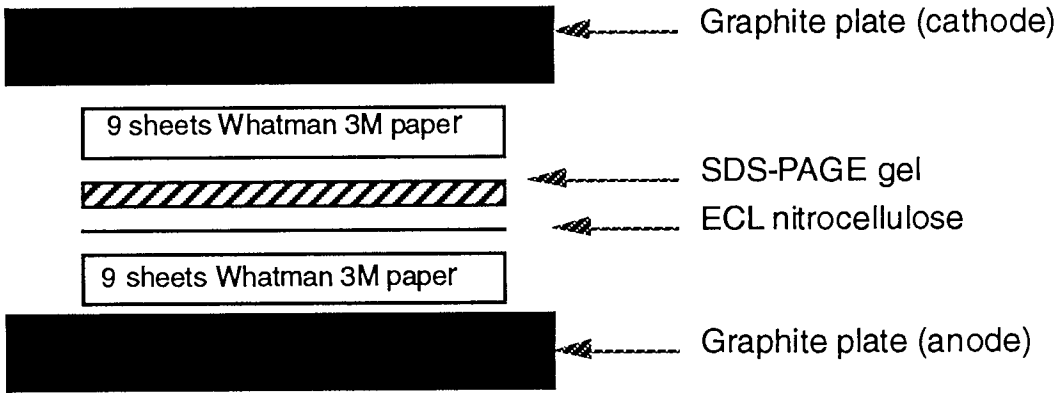
Protein samples (section 2.5.1) were added to the appropriate wells in volumes to ensure equal protein loading in each well. Wells were topped up with complete 2 x electrophoresis buffer so that each well held an equal volume. An upper reservoir, containing running buffer was clamped to the top of the gel and the unit immersed in a tank of running buffer (0.025 M TRIS, 0.2 M glycine, 0.1% (w/v) SDS, pH 8.3). The electrophoresis unit was connected to a power supply set at 25 mA constant current with maximum voltage set at 500 V, and left to run until the dye front reached the bottom of the resolving gel, usually 2 h. A cooling system was employed to ensure temperature of the unit was kept at 10 °C.

2.5.3 Transfer of proteins to nitrocellulose

After completion of electrophoresis, the top glass plate was carefully prised away, and the stacking gel was removed. The resolving gel was overlaid with a piece of nitrocellulose filter (soaked for a few min prior to use in transfer buffer; (39 mM glycine, 0.0375 % w/v SDS, 48 mM Tris in methanol:distilled water (1:4)) cut exactly to the same size. The resolved proteins in the gel were transferred to the nitrocellulose filter by direct, semi-dry electrophoretic transfer performed using a

LKB electrophoresis transfer unit (Multiphor II). Eighteen pieces of Whatman 3MM paper were cut to the exact size of the resolving gel. A gel sandwich was constructed, described here running from top to bottom, which consisted of: a stack of nine pieces of Whatman 3MM paper soaked in transfer buffer, the resolving gel, the nitrocellulose filter, and another stack of nine pieces of Whatman 3MM paper soaked in transfer buffer (Figure 2.5.3). Care was taken to exactly align each component of the sandwich during assembly and to ensure that no air bubbles were trapped, in order to optimise transfer. The sandwich was constructed between graphite plate electrodes on the transfer unit, with the nitrocellulose filter on the anodic side. A power pack supplied 130 mA (0.8 mA per cm^2) constant current for 1 h, which was sufficient for complete protein transfer. After transfer, the nitrocellulose filter was dried, and the position of each of the molecular weight markers was noted before storing the filter in an air-tight container at 4 °C.

Figure 2.5.3 Composition of gel sandwich used for protein transfer.



2.5.4 Immunoblotting with antibody

Western blotting involves incubating the proteins immobilised on the nitrocellulose filter with an antibody specific to the protein of interest, after first blocking non-specific sites. The nitrocellulose is then incubated with an anti-species IgG secondary, horseradish peroxidase-labelled antibody. A detection system can then be employed to visualise the targeted protein-antibody complexes.

The nitrocellulose sheet was transferred to a polystyrene specimen container (250 ml), with the protein surface facing inside of the drum, and placed on a roller, operating at a slow speed, for each of the incubation steps. This allowed uniform coverage of the sheet with the incubation solution. The following protocol was

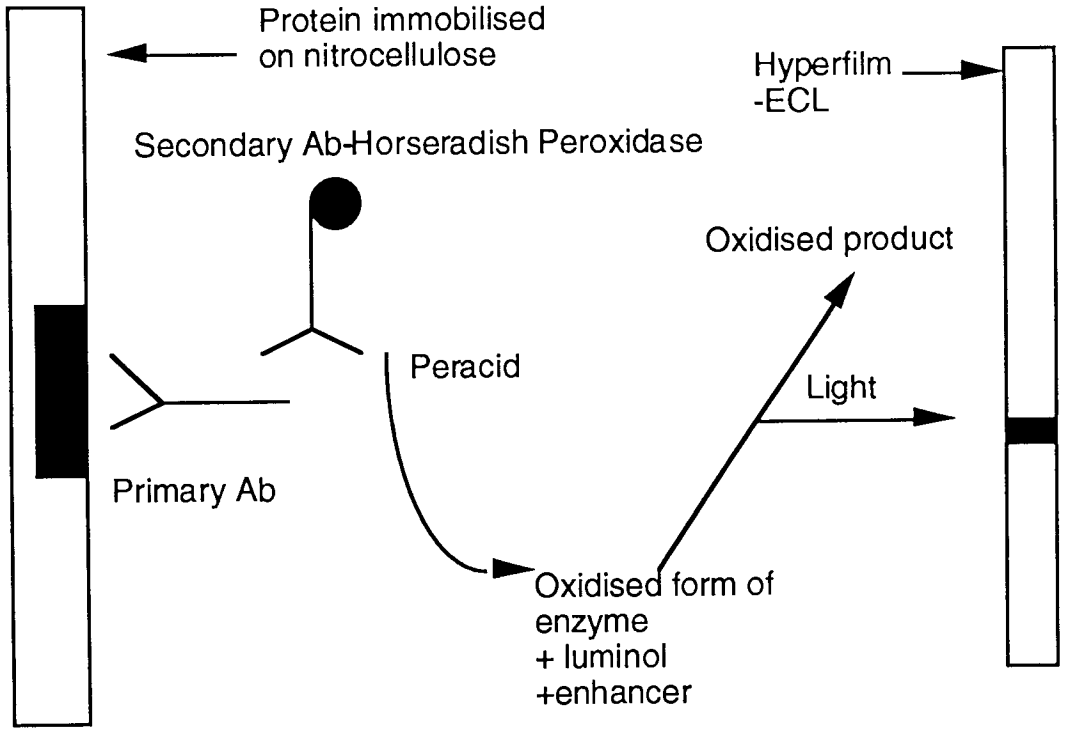
used to develop the blot: 10 ml blocking agent (5 % w/v defatted milk in Tris buffered saline/Tween (TBS/Tween: 0.1 % v/v Tween 20 in 10 mM Tris, 100 mM NaCl, pH 7.5)) was added to the drum and left to incubate for 1 hour. This was followed by removal of blocking agent, and addition of 2.5 ml primary antibody, diluted in TBS/Tween milk to an appropriate concentration, again for an incubation period of 1 hour. This was followed by several wash stages with 20 ml TBS/Tween to remove all excess primary antibody: 1 x 1 min wash and 3 x 10 min washes. The secondary antibody (dilution of 1:1000 made up in 5 ml TBS/Tween milk) was then employed in a 1 hour incubation period. This was followed by a repetition of the washing procedure except that there were 4 x 10 min washes.

2.5.5 Enhanced chemiluminescence detection system

The protein-antibody complexes immobilised on the nitrocellulose sheet were detected by the system of enhanced chemiluminescence (ECL) using a commercial kit (Amersham). ECL involves horseradish peroxidase (HRP)/hydrogen peroxide catalysed oxidation of luminol in alkaline conditions. Immediately after its oxidation, the luminol is in an excited state which then decays to the ground state via a light emitting pathway (Figure 2.5.5). This chemiluminescence is enhanced 1000 fold in the presence of phenols, resulting in a peak of light emission after the enhanced chemiluminescence reaction, followed by a slow decay with a half-life of approximately 60 min. This light emission can be detected by a short exposure to blue-light sensitive autoradiography film.

The steps in the following protocol were undertaken in a darkroom using only red light. The nitrocellulose membrane was placed, protein side up, on a sheet of SaranWrap (Dow Chemical Co.), and covered with a solution containing an equal volume of detection solution 1 mixed with detection solution 2 (containing luminol, hydrogen peroxide and a chemical enhancer, unknown concentrations, as provided by ECL kit, Amersham), to provide a covering of 0.125 ml solution mix/cm^2. This was incubated for exactly 1 min after which excess detection reagent was drained off and the membrane was wrapped in SaranWrap, taking care to prevent air pockets forming. The nitrocellulose in SaranWrap envelope, was placed in the film cassette, again, protein side up and exposed to Hyperfilm-ECL (Amersham) for 1 min. The film was then removed from the cassette and developed and fixed (developing and fixing agents, Kodak). On the basis of the strength of the signal, fresh sheets of film were put in the cassette with the membrane and the exposure time was varied accordingly until a good clear image with low background signal was obtained.

Figure 2.5.5 Reaction sequence of Enhanced Chemiluminescence.



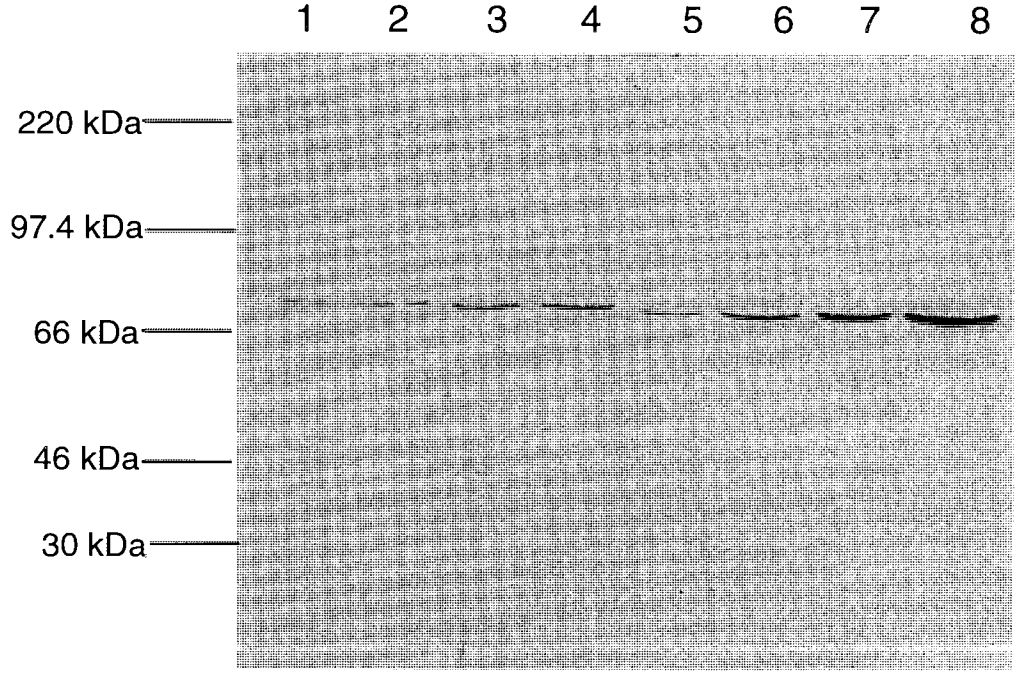
2.5.6. Presentation of immunoblots

Immunoblots were scanned using an Agfa Colour Scanner linked to an Apple Macintosh Computor running the programme M.C. colouview. A suitable scale of resolution was chosen in order to obtain a good reproduction of the blot without using excessive memory. This was usually at a level of between 100 and 200 dots per inch. A TIFF uncompressed file was obtained which could later be opened in MacDrawPro so that the legend could be added. Immunoblots scanned in this way were deemed to provide sufficient resolution for presentation of data. Plate 2.5.6. provides a comparison of an immunoblot presented both as a photograph and a scan. This immunoblot is presented with accompanying legend later in this thesis (section 5.3).

2.6. Isolation and preparation of a crude suspension of rat gastric mucosal cells

The method used for the isolation is a modification of that described by Trotman and Greenwell (1970) and has also been described in detail by Atwell (1991) and Brown (1994). Cells were isolated by subjecting everted stomach sacs to pronase digestion with intermittent chelation of Ca^{2+} with EDTA and cell collection.

Figure 2.5.6.
Immunoblot presented as both a scan and a photograph for comparative purposes



2.6.1 Media preparation

The cell culture medium, Eagle Minimum Essential Medium (EMEM, Sigma) with various additions for particular stages, was used to prepare a suspension of rat gastric mucosal cells. EMEM was prepared fresh the day before use and was stored in clean glass, air tight flasks at 4°C. EMEM is supplied as powdered media, and was reconstituted in double distilled water as described by the manufacturer. The medium was constantly gassed with 95 % oxygen and 5 % carbon dioxide throughout reconstitution. EMEM was used as the basic medium to prepare media A, B and B' required for the isolation procedure; and also used for medium C, 10 x tissue culture medium (TCM), and iso-osmotic Percoll which were required for the cell enrichment procedure in addition to media A, B and B'. The pH of all media was adjusted to pH 7.4 prior to storage.

Table 2.6.1.1. Composition of media used for gastric mucosal cell isolation

Medium	Concentration of additions in EMEM
A	HEPES 20 mM, $NaHCO_3$ 20 mM, EDTA 2 mM, Soybean Trypsin Inhibitor (SBTI) 0.1 % w/v, Dextran 3 % w/v
B	HEPES 20mM, $NaHCO_3$ 20mM, BSA 3 % w/v
B'	HEPES 20mM, $NaHCO_3$ 20mM, BSA 0.1 % w/v

Table 2.6.1.2. Composition of supplementary media used for cell enrichment procedure

Medium	Concentration of constituents
C	BSA 0.1 % w/v, EGTA 3 mM, DTT 0.5 mM in EMEM
10 x tissue culture medium (TCM)	Contents of 1 bottle EMEM reconstituted in 100 ml double distilled water
Iso-osmotic Percoll	$NaHCO_3$ 26 mM in 2 ml TCM + 18 ml Percoll

2.6.2 Preparation of a crude suspension of rat gastric mucosal cells.

Anaesthesia in male Wistar rats was achieved by sodium pentobarbitone (60 mg/kg i.p.). A midline incision was made and gentle pressure was applied to the left side of the rat until the stomach came into view, when it was carefully cut free. The forestomach and antrum were removed, and the remaining corpus section was washed in ice cold saline (NaCl, 9 g/l), and everted onto a fixed glass rod. A

length of silk thread was used to tie the stomach section around the bottom of the glass rod, after which it was gently eased off to produce an enclosed stomach sac. The sac was inflated with a pronase solution (1000 PUK units/ml medium A) by introduction of the solution via a 26 gauge needle through the stomach wall. For each cell isolation three stomach sacs were prepared in this way. The sacs were placed in a beaker containing 40 ml medium A, previously equilibrated to 37°C , and left to incubate in a water bath for 30 min. During this incubation period the incubation medium was constantly gassed with 95 % oxygen and 5 % carbon dioxide and the water bath was set to shake at 80 cycles per min while the temperature was maintained at 37°C. After 30 min sacs were removed, blotted on filter paper and transferred to 30 ml medium B in a covered plastic beaker for a further 30 min. During this time the air space above the sacs was gassed constantly and the incubation medium was subjected to stirring with a small magnetic bar (80 oscillations per min) in order to release cells. After this harvesting period the sacs were removed, blotted, and transferred back to 40 ml fresh medium A warmed to 37°C.

The cycle of the 30 min digestion period followed by the 30 min harvesting period was repeated twice more. Meanwhile, the cells isolated in medium B were filtered through 150 μm mesh gauze into polystyrene centrifuge tubes, which were then subjected to centrifugation at 100 x g_{av} for 7 min at 15°C. The resulting cell pellets were resuspended in 10 ml medium B and transferred to a Nalgene conical flask (polycarbonate). This was left to incubate, gassed with 95 % oxygen and 5 % carbon dioxide, in a 37°C waterbath set to shake at 120 cycles per min to avoid clumping of the cells. Subsequent batches of cells were added to this pool and after 3 h, when all harvesting stages were completed, the cells were centrifuged at 100 x g_{av} for 7 min at 15°C. The resulting pellet was resuspended in the incubation buffer required for the experiment, usually medium B' or Krebs bicarbonate buffer (KRB) (KRB pH 7.4, consisting of; NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.25 μM, glucose 11 mM, BSA 0.1% w/v). Cell viability was established by trypan blue exclusion and numbers of cells were estimated using a standard haemocytometer chamber (section 2.3). Cells were centrifuged again, as before, and were finally resuspended in incubation medium to give a cell concentration of 5 x 10^6 cells/ml.

2.6.3 Percoll density cell separation

Cells in the crude cell suspension can be separated further by use of a self-forming Percoll gradient. Fractionation involves resuspension in Percoll containing

medium and centrifugation to produce a high density fraction and a low density fraction rich in parietal cells (Brown et al., 1992).

The entire crude cell suspension was resuspended in 28 ml medium C after the final harvesting stage. 4.5 ml of this cell suspension was then added to each of six high-speed centrifuge tubes containing 3 ml of iso-osmotic Percoll. Tubes were weighed and balanced before centrifugation at 30,000 x g_{av} for 13 min at 4°C using a 20° angle rotor in an MSE superspeed 50 centrifuge. After centrifugation the top 1.5 ml cell layer was removed from each tube and evenly distributed amongst 4 clean sterilin tubes to give the low density fraction. The remaining cells, which banded close to the bottom of the original high-speed tubes, were distributed amongst another set of fresh sterilin tubes and labelled the high density fraction. Both sets of cells were then resuspended in a quantity of medium B' which was added to give a final volume of 10 ml in each tube. Both sets of cell suspensions were centrifuged at 100 x g_{av} for 7 min at 15°C. The cell pellets resulting from each set were combined to give one cell pellet per fraction, and each was resuspended in 10 ml medium B'. A 20 µl volume was removed from each tube for assessment of cell viability by trypan blue exclusion and estimation of numbers of cells (section 2.3). A final centrifugation spin was performed, again at 100 x g_{av} for 7 min at 15°C, and each cell pellet was resuspended in the appropriate volume of B' to give the cell concentration required.

2.7 Cell culture and maintenance of cell lines

A variety of cell lines and primary culture were used in this project and the source of these cells and the type of media used in their maintenance is described in the relevant chapters. Sterile cell culture technique was observed in the maintenance of all cell lines and primary cultures by use of a Gelair laminar flow hood.

Cells were maintained in the media of choice, supplemented with 10 % foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine and 2.5 µg/ml amphotericin B. Cell lines were grown in 75 cm^2 cell culture flasks with media change every 2-3 days, whereas primary cultures were grown in either 6 or 12 well culture plates with a daily media change. Both flasks and plates were kept in a LEEC CO_2 humidified incubator at 37°C in 95 % air :5 % CO_2.

Primary cells were freshly cultured for each experiment, but cell lines were continuously sustained and subcultured on a weekly basis. To do this, the cellular

monolayer was exposed to a solution of pre-warmed 0.05 % trypsin (w/v) and 0.2 % EDTA (w/v) for 2-5 min at 37°C. Cell detachment was assessed by use of a phase contrast microscope. To halt the trypsinisation reaction an equal volume of fresh medium containing 10 % FCS was added to the flask, after which the whole suspension was removed, transferred to a sterile centrifuge tube and subjected to centrifugation at 100 x g_{av} for 5 min at 15°C. The supernatant was discarded and the pellet was resuspended in 10 ml fresh medium, 1 ml of which, with the addition of a further 35 ml fresh medium, was used to seed a new 75 cm^2 cell culture flask. Cell lines were grown to confluence, as assessed by microscopy, with regular medium changes, at which point they were ready for experimentation.

For storage purposes, cells from cell lines were detached as above and slowly frozen at -140°C in culture medium containing 10 % FCS and 10 % v/v dimethylsulphoxide (DMSO) at a cell concentration of 1 x 10^7 cells /ml. Cells were reseeded by rapidly thawing at 37°C in a water bath and diluting 1 ml of suspension into 20 ml culture medium containing 10 % FCS. Cells were centrifuged at 100 x g_{av} for 5 min at 15°C and the supernatant containing the DMSO was discarded. The cell pellet was then resuspended in fresh medium and used to seed a 25 cm^2 flask.

CHAPTER 3

NO SYNTHASE AND ARGINASE IN RAT GASTRIC MUCOSAL CELLS

3.1. INTRODUCTION

3.1.1. Metabolism of arginine in the rat

The metabolic fate of L-arginine follows two major and distinct pathways: oxidation by NO synthase to NO and L-citrulline, and hydrolysis by arginase to L-ornithine and urea. L-Arginine is also required by all tissues for protein biosynthesis, and by some tissues as an amidine group donor in transamidination reactions (Windmueller and Spaeth, 1981). Finally, metabolism to argamine by arginine decarboxylase is an alternative L-arginine pathway which has been identified in rat brain (Wu and Meininger, 1995). Gastric mucosal cells isolated by a method of pronase digestion and intermittent Ca^{2+} chelation (Hatt and Hanson, 1989) possess Ca^{2+}-dependent NO synthase activity (Brown et al., 1992). In cases where the source of isolated cells was from a rat previously treated for a 4 h period with LPS, Ca^{2+}-independent iNOS activity, which also metabolises arginine to NO, was exhibited (Brown et al., 1994).

Although mammalian arginase is present at high activity in the liver, some activity is also present in most peripheral tissues, and it has been identified in the small intestine (Konarska and Tomaszewski, 1975), red blood cells, lactating mammary gland and kidney (Reczkowski and Ash, 1994). Hepatic arginase is involved in the fifth and final reaction in the urea cycle of ureotelic animals (Grody et al., 1989). Isolated rat liver arginase contains tightly bound Mn^{2+}, and the reversible binding of additional Mn (II) cations causes further activation of the enzyme, which is proportional to the bound Mn^{2+} content (Reczkowski and Ash, 1994). X-ray crystallographic studies have indicated a trimeric structure of identical or nearly identical subunits. A fully activated arginase enzyme has been shown to contain 6 Mn^{2+} per trimer. The purpose of these ions is to form a binuclear metal centre, which is essential for its catalytic activity (Daghigh et al., 1994).

Two arginase isozymes, AI and AII, have been identified that possess similar physiochemical properties, but differ electrophoretically and antigenically (Grody et al., 1989). It is thought that a family of arginase genes exists, resulting in differential expressions of various forms of the enzyme (Shinomya et al., 1990). AI, a cytoplasmic protein that is principally located in liver and red blood cells, plays an important role in ureagenesis. The second isozyme AII, has a mitochondrial matrix location, and is present in smaller amounts in extrahepatic tissue. Extrahepatic tissues do not possess urea cycle activity and the function of arginase in such tissues is unclear. L-ornithine is required by ornithine

decarboxylase, the first and rate-limiting enzyme of the polyamine biosynthetic pathway (Tabor and Tabor, 1984). Thus, the function of arginase in extrahepatic tissue may be to provide the initial substrate in the production of proline, spermine and spermidine, which are necessary for cellular growth and repair (section 4.1).

3.1.2. Interaction between NO synthase and arginase pathways.

Arginase and NO synthase have been found together in a variety of cell types including: macrophages (Wand et al., 1995), neurons (Wu and Meininger, 1995), hepatocytes (Wu and Meininger, 1995), and small intestinal mucosal cells (Blachier et al., 1991). The active centres of arginase and NO synthase are similar in the structural fitting of substrate to enzyme, and stereochemical configuration. The free α-amino group of L-arginine is essential in the fitting of the substrate to the binding sites in both enzymes, and if N-α-substituted groups are applied the sterical fitting is not possible (Hbrak et al., 1994). However, despite these similarities hepatic arginase and NO synthase differ where substrate affinities are concerned. Thus NO synthases have Km values of 0.01-0.1 mM for arginine, whereas the Km values of hepatic arginases are above 1 mM (Hbrak et al., 1994). Furthermore the intermediate in the biosynthetic pathway of NO from L-arginine, N^G-hydroxy-L-arginine, is a potent competitive inhibitor of arginase (Daghigh et al., 1994). The presence of a positively charged δ-guanidoxy group on N^G-hydroxy-L-arginine is an important factor involved in the recognition of the L-arginine binding site on arginase (Boucher et al., 1994).

Considering that both NO synthase and arginase share a common substrate, that they are located together in many cell types, and that the intermediate of the NO synthase pathway inhibits arginase activity, there exists the possibility of competition between these pathways in tissues containing both NO synthase and arginase. Exposure to lipopolysaccharide (LPS) resulted in the co-induction of arginase and NO synthase in macrophages (Wang et al., 1995). However, the increased arginase activity could potentially reduce NO production by the activated macrophages. Conversely, increased iNOS activity, could result in the inhibition of arginase activity, by the action of N^G-hydroxy-L-arginine. The current view is that both arginase and NO synthase play a role in the process of inflammation and repair that follows tissue injury (Albina et al., 1990; Cook et al., 1994). Thus, a temporal expression of the pathways is exhibited during wound healing, where an early phase of high NO synthase activity is followed by a later increase in arginase activity. In this situation, using an inhibitor of NO synthase

resulted in an increased activity of arginase, demonstrating competition for available substrate.

This study was undertaken for the following reasons. Firstly, to determine the form of NO synthase present in isolated gastric mucosal cells. Secondly, to investigate the metabolic fate of L-arginine in isolated gastric mucosal cells with a view to determining whether citrulline production could be used as an index of NO synthase activity. Thirdly, to determine whether any interaction between arginase and NO synthase pathways might take place in gastric mucosal cells.

3.2 METHODS

3.2.1 General experimental procedure with isolated cells

Freshly isolated rat gastric mucosal cells (section 2.6) were resuspended in Krebs bicarbonate buffer (KRB) (KRB pH 7.4, consisting of: NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.25 mM, glucose 11 mM, BSA 0.1% w/v) after the final spin to give a concentration of $5x10^6$ cells/ml. The isolation procedure involved subjecting the cells to a variety of stresses including several centrifugation spins and vigorous stirring. For this reason, cells were allowed a recovery period during which they were equilibrated in a 37°C waterbath with gassing of the air space (95 % O_2 / 5 % CO_2) and shaking at 120 cycles per min for a period of 15 min before commencing the experiment. If a preincubation period was required for the experiment the cells were split at this stage into control flasks with medium alone and test flasks with the agents under investigation added to the medium.

After the recovery and preincubation phase, 1.5 ml of cell suspension was transferred into individual 20 ml plastic vials containing L-[2,3,4,5-^{3}H]-arginine (3 µCi/ml, 43 nM) and the agents under investigation or appropriate vehicle controls. All agents tested were dissolved in KRB, with the exception of: ionomycin, BAPTA-AM (1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid) tetrakis (acetoxymethyl) ester), digitonin and 7-nitroindazole which were all made up in DMSO (final concentration in incubation medium: DMSO 0.13% w/v). All treatments were carried out in triplicate. Vials were incubated in a 37°C waterbath, with shaking at 120 cycles per min, for 15 min. Cells in the vials were then gently resuspended, and 1 ml samples of cells were removed from each vial and transferred to prelabelled microfuge tubes on ice. Microfuge tubes were centrifuged at 10,000 x g_{av} for 2 s at room temperature in order to loosely pellet the cells. The supernatant was decanted and discarded, after which the cell pellet was washed without resuspension with 1.0 ml ice cold KRB to remove as much of the extracellular L-[^{3}H]-arginine as possible.

Amino acids were extracted from the cell pellets by the addition of 200 µl of 65% ethanol v/v, in 35 % distilled water, containing 5 mg/ml of each of the following unlabelled amino-acids: arginine, ornithine and citrulline, to each microfuge tube. Samples were left to extract overnight at 4°C. Prior to use, extracts were centrifuged at 10,000 x g_{av} for 2 min and the following volumes of supernatant were removed: 25 µl for analysis by thin layer chromatography (TLC) (section

3.2.2), 100 µl for ion-exchange analysis (section 3.2.3) and 25µl for determination of total radioactivity by transferring the aliquot directly to vials for scintillation counting containing 2 ml Optiphase HiSafe III. 25µl was kept in reserve in the event of re-analysis.

3.2.1.1 Use of volume marker

In experiments where the intracellular content of L-[^{3}H]-arginine was a factor which could influence results (e.g. those involving preloading with ornithine and N^G-hydroxy-L-arginine) a volume marker was required. Polyethylene glycol 4000 (PEG) is a large molecule which is unable to enter the isolated cells, and can therefore be used as a volume marker in order to determine the volume of extracellular fluid associated with the cell pellet. This was achieved by addition of radiolabelled [^{14}C]-PEG 4000 (0.4 µCi/ml) to each vial along with L-[^{3}H]-arginine. In this way, if the ^{14}C concentration of the incubation medium, and the ^{14}C content of the pellet is known, the volume of extracellular fluid can be calculated. Supernatants obtained after the centrifugation of cells were retained, and 100 µl volumes of these supernatants were transferred to vials with 2 ml Optiphase HiSafe III for liquid scintillation counting. A dual label counting protocol was set up on the scintillation counter so that samples could be counted for both ^{3}H and ^{14}C content simultaneously.

The first step was to calculate the extracellular volume of the pellet:

$$\text{Extracellular fluid in pellet (µl)} = \frac{\text{Amount } ^{14}\text{C-PEG in pellet extract (dpm)}}{^{14}\text{C-PEG conc. in supernatant (dpm/µl)}}$$

Then the amount of [^{3}H]-arginine in the extracellular fluid was calculated:

$$\text{Extracellular } ^3\text{H (dpm)} = \text{} ^3\text{H conc in supernatant (dpm/µl)} \times \text{extracellular fluid volume (µl)}$$

Finally the total ^{3}H in the pellet was corrected for extracellular [^{3}H] and the result was taken as intracellular [^{3}H]:

$$\text{Corrected intracellular counts (dpm)} = \text{Amount } ^3\text{H in pellet (dpm)} - \text{extracellular } ^3\text{H (dpm)}$$

3.2.2 Thin layer chromatography

A 25µl aliquot of cell extract (section 3.2.1) from each sample was analysed by the process of thin layer chromatography (TLC). A closed TLC tank containing a solvent mix consisting of: chloroform, methanol, and ammonium hydroxide in a 2:2:1 ratio by volume was set up to allow the air in the tank to become saturated. 25 µl of each sample was loaded in a concentrated spot on an aluminium backed Silica Gel 60 TLC plate. Standards containing known amounts of either [^{3}H]-arginine or [^{3}H]-citrulline in 65% ethanol v/v in distilled water, were also loaded. The TLC plate was then placed in the tank and left for a period of approximately 2 hours to allow the solvent front to reach the top of the plate. At this point the plate was removed, sprayed evenly with ninhydrin solution in a fume cupboard and heated to about 70°C until the plate had dried. Spots for arginine, ornithine and citrulline from each sample were cut from the plate. Each cut section was placed into a scintillation vial with 2.0 ml distilled water and left for a period of 1 hour to allow any radioactive material present to pass into solution. After which, 2.0 ml of Optiphase HiSafe III scintillant was added, the vials were capped and shaken and the amount of radioactivity determined by liquid scintillation counting.

To verify the TLC procedure, control samples in the absence of cells, were run on the TLC plates. 25µl of the control solution: that is, a known amount of L-[^{3}H]-arginine in 65% (v/v) ethanol, containing 5 mg/ml each of arginine, ornithine and citrulline, was loaded onto the plate. Once run, the amount of radioactivity present in each amino-acid spot was analysed. A check could then be made that the amount of radiolabel known to be added to the plate, tallied with the value obtained by analysis. It also served to establish what quantity of added label ended up associated with either the ornithine or citrulline spot.

3.2.3 Analysis by ion-exchange chromatography

Using the process of ion-exchange chromatography, the newly formed radiolabelled citrulline was separated from the original radiolabelled arginine substrate. To achieve this, 10 ml plastic Biorad columns were loaded with 2 ml of the activated strongly-acidic cation-exchange resin, DOWEX AG 50-W8 (see section 2.4.1). The cell extracts were then added to the columns which were able to bind the arginine, but not the citrulline allowing it to pass through and be collected and counted for radioactivity content.

0.8 ml distilled water and 1.0 ml HEPES buffer pH 6.0 were added to each 100 µl sample of pellet extract retained previously for ion-exchange chromatography analysis. 1.8 ml of this solution was then added to the column and the elutant collected in a scintillation vial underneath. The column was then washed with a further 6.0 ml distilled water to ensure the removal of all ^{3}H-citrulline. 8.0 ml of Optiphase HiSafe 3 scintillant was added to the vials which were capped and shaken and the amount of radioactivity determined by liquid scintillation counting. For each new batch of resin, control columns were employed to determine the efficiency with which ^{3}H-arginine remained bound to the column and the efficiency with which ^{3}H-citrulline was washed off. The results for extracts were then corrected accordingly. As a routine control procedure, a 65% (v/v) ethanol solution containing amino-acids and a known amount of either ^{3}H-arginine or ^{3}H-citrulline was prepared. 100 µl of the control solution was added to 0.8 ml distilled water and 1.0 ml HEPES buffer and added to the column, this gave a result for the column in the absence of cells.

3.2.4 Expression and analysis of data

Results are expressed as means ± S.E. either for triplicate determinations on a single batch of cells, or for n separate batches of cells with triplicate determinations for each batch. Absence of error bars from figures is due to the S.E. being too small to plot. Arginine metabolism, as analysed by ion-exchange chromatography and TLC is expressed as % distribution of label between arginine, citrulline and ornithine. For TLC experiments, this involved dividing the amount of radioactivity obtained in each spot by the total amount of radioactivity loaded onto the plate, for that particular sample. With ion-exchange experiments, % label in citrulline is calculated by dividing the amount of citrulline eluted from column, after correction, by the total amount of radioactivity loaded onto the column. Arginine uptake into the cells (expressed as dpm) varied between cell batches and for this reason results are expressed as % distribution of label in an attempt to normalise data. Statistical analysis was used to determine any significant difference from controls caused by the agents tested. This analysis was achieved by use of a paired t-test or by ANOVAR followed by Dunnett's test.

3.2.5 Assay for arginase activity

Arginase, an enzyme of the urea cycle, catalyses the conversion of L-arginine to L-ornithine and urea. Since arginase catalyses the hydrolysis of the guanidino group of arginine, the activity of this enzyme can be determined by following the conversion of radiolabelled [Guanido^{14}C]-arginine to [^{14}C]-urea. It is important that uniformly radiolabelled L-arginine is not used, otherwise the radiolabel would become equally incorporated into both products, which would lead to detection complications. Enzyme activity was assayed essentially according to Cook *et al.* (1994), with radiolabelled urea being separated by means of ion-exchange chromatography from the substrate.

3.2.5.1 Preparation of homogenates

The organ under investigation (either duodenum or stomach) was removed from an anaesthetised male Wistar rat and washed in ice-cold saline and then scraped with a scalpel blade to remove the tissue (section 2.4.2.1). For some experiments, differential scraping of the rat gastric mucosa was performed. This first involved a light scrape to remove the surface mucosal cells, followed by a second heavier scrape to remove the remaining tissue. For other experiments freshly isolated rat gastric mucosal cells (section 2.6) were used to make homogenates (section 2.4.2.3) which were assayed for arginase activity. The tissue was weighed, and a volume (ml) of arginase homogenisation buffer (10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 6 µg/ml aprotinin, 0.1 mg/ml PMSF in 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM $MnCl_2$ pH 7.5) was added which was equal to 10 x the weight (g) of the tissue (i.e. 10 ml buffer per 1 g tissue). The tissue was homogenised in the above buffer, on ice, for 30 s by an Ultraturrax homogeniser, or by an Ultraturrax microhomogeniser for surface scraped tissue, both running at full speed. The resulting homogenate was centrifuged at 10,000 x g_{av} for 20 min at 4°C, to obtain a supernatant for use in the assay.

3.2.5.2 Arginase assay protocol

The DOWEX AG 50-W8 (H^+ form) resin, necessary to separate out radiolabelled product, was first prepared. This was achieved by repeated washing of 50 g of the H^+ form of the resin (as supplied by Sigma) with distilled water until pH 5.5 was obtained. A 1:1 v/v suspension of resin and distilled water was prepared. The assay protocol used was as follows: microfuge tubes containing 150 µl assay buffer (100 mM glycine, pH 9.7) and 50 µl homogenate were warmed for 3 min in a 37°C waterbath. To commence the reaction, 50 µl of 250 mM arginine containing [Guanido^{14}C]-arginine (111,000 dpm, 1 µCi/ml) was added to each

tube. An incubation period of 15 min was allowed before halting the reaction by the addition of 0.8 ml stop buffer (250 mM acetic acid, 100 mM urea, 10 mM L-arginine, pH 4.5) and 400 µl H^+ DOWEX AG 50-W8 suspension. Sequential additions were made to each tube at 30 s intervals, this was to ensure that the reaction progressed for the same time interval in each tube. Assay tubes were set up in triplicate, so that a mean arginase activity value could be obtained for each homogenate. Triplicate control tubes were also set up in which 50 µl of homogenisation buffer was used in the assay to obtain a "blank" value. This basal value was then subtracted from the triplicate mean obtained in the presence of enzyme. Once the resin had settled in the tubes, 1.0 ml of supernatant was removed and transferred to scintillation vials to which 1.0 ml Optiphase HiSafe III scintillant was added. The vials were capped and shaken and the amount of [^{14}C]-urea, used as an index of arginase activity, was determined by scintillation counting.

3.2.5.3 Calculation of arginase activity

Specific activity of [Guanido^{14}C]-arginine and arginase activity was calculated in a similar way as for the NO synthase assay (section 2.4.5):
Concentration of stock [Guanido^{14}C]-arginine = 48 mCi/mmol

$$\text{Specific activity (dpm/nmol)} = \frac{\text{Radioactivity in assay}}{\text{Conc. of total arginine}}$$

Radioactivity in assay = 111,000 dpm/ml
Concentration of hot and cold arginine in assay buffer = 12376.05 nmol/ml

Using the value obtained for specific activity of [Guanido^{14}C]-arginine, arginase activity was calculated in the following way;

$$\text{Arginase Activity (nmol/min/ml homogenate)} = \frac{\text{Mean dpm counts}}{\text{Specific activity}} \times \frac{FV}{IT} \times \frac{VL}{VS}$$

Mean dpm counts = mean counts obtained from each set of triplicates
Specific activity (dpm/nmol) = activity of radiolabelled arginine per nmol
FV = factor homogenate volume is multiplied by to give 1 ml.
IT = incubation time of assay (min)
VL = volume (ml) liquid in assay tube (DOWEX is added in a 1:1 suspension), 1 part solid to 1 part liquid.
VS = volume (ml) liquid in sample taken for scintillation counting.

Therefore to calculate arginase activity in the assay, the following values were put into the above equation;

$$\text{Arginase Activity (nmol/min/ml homogenate)} = \frac{\text{Mean dpm counts}}{8.968} \times \frac{20}{15} \times \frac{1.250}{1.0}$$

To express arginase activity per g wet weight, the value obtained from the above equation was multiplied by a factor of 10.8. It is assumed that 1 g wet weight of tissue is composed of approximately 80% water. In the assay, 10 ml of homogenisation buffer was used per 1 g of tissue. So, from this it is estimated that there is 1 g wet weight in 10.8 ml. To express arginase activity as nmol/min/mg protein, the activity per ml homogenate was divided by the protein concentration of homogenates (in mg/ml), as measured by the coomassie blue protein assay (section 2.2).

3.2.6 Assay for ornithine transcarbamoylase activity

Ornithine carbamoyltransferase catalyses the nucleophilic addition of ornithine to the carbonyl group of carbamyl phosphate to produce citrulline and the release of a phosphate group. This enzyme is part of a multistep pathway in the biosynthesis of arginine from glutamate, and also constitutes part of the urea cycle. Ornithine carbamoyltransferase activity was assayed as the stimulation of citrulline formation from radiolabelled ornithine by carbamoyl phosphate as described previously (Wu and Meininger, 1995). The liver is known to have a high ornithine carbamoyltransferase activity and a homogenate from this tissue was used to validate this assay.

3.2.6.1 Ornithine transcarbamoylase assay protocol

10 % tissue homogenates (section 2.4.2.1) or freshly isolated rat gastric mucosal cell homogenates (section 2.4.2.3) were prepared using homogenisation buffer (10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 6 µg/ml aprotinin, 0.1 mg/ml PMSF in 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.5). For each homogenate used, nine assay tubes were required, resulting in: assay tubes, assay "blanks" and control tubes, each in triplicate (Table 3.2.6.1). The ornithine carbamoyl-transferase reaction progressed in the assay tube, and commenced on addition of 100 µl homogenate to microfuge tubes containing 100 µl assay reagent (100 mM potassium phosphate pH 7.5 containing L-[U-

^{14}C]ornithine (0.22 μCi/ml), 40 mM ornithine, 20 mM carbamoyl phosphate) prewarmed to 37 °C in a waterbath. Assay "blanks" used addition of 100 μl homogenate to assay reagent in the absence of carbamoyl phosphate, and control tubes used the addition of 100 μl homogenisation buffer to the full assay reagent. Tubes were left to incubate for 30 min in a 37°C waterbath, after which 371 μl ethanol was added to each tube to halt the reaction. Extraction took place over 30 min at 4°C. After extraction, 25 μl of a solution containing 5 mg/ml of each of the unlabelled amino-acids; arginine, ornithine and citrulline dissolved in 65 % ethanol (v/v), was added to each microfuge tube. Tube contents were mixed, then subjected to centrifugation at 10,000 x gav for 2 min at room temperature. 25 μl aliquots were removed from each tube and analysed by thin layer chromatography (TLC, section 3.2.2).

Table 3.2.6.1 Ornithine carbamoyltransferase assay protocol

Additions to assay	**Assay Conditions**		
	Assay Tube	Assay "blank"	Control
Tissue/Cell homogenate	100 μl	100 μl	
Homogenisation buffer			100 μl
Assay Reagent + Carbamoyl Phosphate	100 μl		100 μl
Assay Reagent without Carbamoyl Phosphate		100 μl	
	Incubate at 37 °C for 30 min		
Ethanol	371 μl	371 μl	371 μl

3.2.6.2 Calculation used for ornithine carbamoyltransferase activity

Triplicate values obtained for assay "blanks" and controls were similar, so a mean value of both blanks and control was calculated, which was subtracted from the mean dpm count obtained from assay tubes for each homogenate.

Specific activity of L-[U-^{14}C]ornithine and ornithine carbamoyltransferase activity was calculated in a similar way as for the NO synthase assay (section 2.4.5)

Concentration of stock L-[U-^{14}C]ornithine = 56 mCi/mmol

Radioactivity in assay = 0.225 μCi/ml

Specific activity (dpm/μmol) = 24970

Using the calculated value for specific activity of L-[U-^{14}C]ornithine, ornithine carbamoyltransferase activity was calculated in the following way;

$$\text{Enzyme Activity } (\mu\text{mol/min/ml homogenate}) = \frac{\text{Mean dpm counts}}{\text{Specific activity}} \times \frac{FV}{IT} \times \frac{VL}{VS}$$

Mean dpm counts = mean counts obtained from each set of triplicates
Specific activity (dpm/μmol) = activity of radiolabelled ornithine per μmol
FV = factor homogenate volume is multiplied by to give 1 ml.
IT = incubation time of assay (min)
VL = volume (ml) liquid in assay tube
VS = volume (ml) liquid in sample taken for scintillation counting.

Therefore to calculate ornithine carbamoyltransferase activity in the assay, the following values were put into the above equation;

$$\text{Enzyme Activity } (\mu\text{mol/min/ml homogenate}) = \frac{\text{Mean dpm counts}}{24970} \times \frac{10}{30} \times \frac{0.596}{0.025}$$

3.2.7 Nitric Oxide Synthase Assay

The standard assay was performed as described previously (section 2.4.3) using tissue homogenates (section 2.4.2.1) and cell pellet homogenates (section 2.4.2.3).

3.2.8 Protein Blotting

Polyacrylamide gel electrophoresis (section 2.5) was performed on tissue samples and on a preparation of freshly isolated gastric mucosal cells (section 2.6). Samples were used to probe for the presence of eNOS and nNOS.

3.3. RESULTS

3.3.1. Detection of NO synthase, arginase and ornithine transcarbamoylase in isolated mucosal cells.

Preliminary work was undertaken to ensure that the crude gastric mucosal cell suspension contained NO synthase, and to investigate whether arginase was also present. NO synthase and arginase activity (Figure 3.3.1.1) together with ornithine transcarbamoylase activity (93 ± 32 pmol/min/million cells, n = 5) were subsequently detected in unenriched suspensions of gastric mucosal cells. Arginase activity was found to be substantially higher ($P < 0.01$) than NO synthase activity, with activity at 2080 pmol/min/million cells for arginase, and at 0.25 pmol/min/million cells for NO synthase. Both arginase and NO synthase activities demonstrated differential distribution between cell-types. Thus, the high-density fraction produced by centrifugation on a Percoll gradient, exhibited a significantly lower arginase activity ($P < 0.01$) than the unenriched fraction, whereas activity in the low-density fraction was comparable to the unenriched fraction. By contrast, NO synthase activity was greater in the high-density fraction ($P < 0.01$) as compared to the unenriched fraction.

To obtain a crude estimate of the distribution of NO synthase and arginase activity in the gastric mucosa, homogenates were prepared from a first scrape obtained from the surface mucosa and a second scrape from the remaining tissue. It was assumed that the scraping technique was performed in the same way each time as the protein content of the scrapes were repeatable, with 18.3 ± 4.0 % of protein in the surface scrape (n=5). No significant difference was seen between the first and second scrapes assayed for NO synthase activity. However, a significant difference ($P < 0.05$) was observed for arginase activity between the scrapes, the surface scrape showing a lower activity (0.0218 ± 0.004 nmol/min/μg protein) compared to that of the second scrape (0.037 ± 0.008 nmol/min/μg protein). Arginase activity was substantially higher than that of NO synthase; with arginase in the nmol range and with NO synthase in the pmol range (Figure 3.3.1.2).

Immunoblotting was used to verify the presence of the NO synthase protein in isolated gastric mucosal cells. Monoclonal antibodies to both nNOS and eNOS were used to investigate a range of cell fractions (Figure 3.3.1.3). A form of NO synthase recognised by a monoclonal antibody directed to neuronal NO synthase was detected in whole gastric mucosal homogenate, unenriched gastric mucosal cells and cells from the high-density fraction. Endothelial-type NO synthase was

detected in the gastric mucosal homogenate but not in any of the isolated cell fractions.

3.3.2. Radiolabelled arginine uptake and its rate of conversion to citrulline in isolated gastric mucosal cells

To determine the time course of [^{3}H]-arginine uptake into cells, and [^{3}H]-arginine conversion to citrulline, a pilot experiment was undertaken (Figure 3.3.2). There was an approximately linear increase in total cell-associated label over a 30 min time period (graph A). The isolated gastric mucosal cells converted arginine to citrulline, as demonstrated by the appearance of material which behaved chromatographically as citrulline (graph B). The proportion of label in citrulline increased rapidly for the first 5 min, after which it slowed down. An incubation period with [^{3}H]-arginine of 15 min was chosen for future studies.

3.3.3. Verification that the thin-layer chromatography and ion-exchange procedures demonstrate arginine metabolism by isolated cells.

In the absence of cells the vast majority of radioactive material was localised to the arginine spot on the TLC plate (Figure 3.3.3). Radioactivity (% of total loaded) in the presence of cells was 8.5 ± 1.1% (ornithine) and 15 ± 2.1% (citrulline) and in the absence of cells 0.18 ± 0.013% (ornithine) and 0.12 ± 0.04% (citrulline). There was a significant effect of cells ($P < 0.05$, by t-test). Citrulline content by ion-exchange chromatography was 13 ± 1% of total counts loaded in the presence of cells and 0.33 ± 0.1% in the absence of cells ($P < 0.01$). Results with control samples made up in 65% ethanol and those made up in distilled water were similar when analysed by ion-exchange chromatography.

3.3.4. Lack of effect of N^G-nitro-L-arginine on metabolism of arginine by isolated gastric mucosal cells.

After a 15 min period of incubation, ornithine production by isolated cells was 6.2 ± 1.7% (% of total radioactivity loaded by TLC) and citrulline production was 11.2 ± 2.7% (% of total radioactivity loaded by TLC) or 12.4 ± 2.2% (by ion-exchange chromatography). Treatment with the NO synthase inhibitor N^G-nitro-L-arginine (L-NA, 300 μM) did not significantly affect either the ornithine or citrulline

formation. In the presence of L-NA, the label in citrulline was 11.8 ± 2.8% by TLC and 14.0 ± 2.2% as analysed by ion-exchange. These data are means ± S.E.M. from 5 separate cell preparations (Figure 3.3.4).

3.3.5. Lack of effect of antibiotic gentamicin on metabolism of arginine by gastric mucosal cells

The effect of the non-selective antibiotic gentamicin was investigated in order to determine whether bacterial activity might be responsible for metabolism of arginine (Figure 3.3.5). Isolated cells were split into 2 flasks after each hourly harvesting step: either into a control flask with no antibiotic, or a test flask containing 50 μg/ml gentamicin. Cells from the test flask were resuspended in incubation media, again with 50 μg/ml gentamicin, at the start of the experiment. The experimental procedure was conducted as normal in the presence and absence of L-NA (300μM). Use of gentamicin had no effect on arginine metabolism, that is, no difference from controls was seen in either ornithine or citrulline production as analysed by TLC, or in citrulline production as analysed by ion-exchange. The NO synthase inhibitor L-NA may have had a slight effect of reducing the total label associated with the cell pellet, although the difference from controls was not significant (paired t-test).

3.3.6. Lack of effect of a variety of potential modulators of NO synthase on arginine metabolism.

3.3.6.1. Pilot experiment 1

Preincubation for 15 min with either the NO synthase inhibitors L-NA, L-nitroarginine-methyl-ester (L-NAME), 7-nitroindazole, or addition of digitonin after the preincubation stage to cells in both the presence and absence of L-NA, did not affect ornithine or citrulline formation from arginine (Figure 3.3.6.1). No significant differences in total label associated with cell pellet after the different treatments was observed.

3.3.6.2. Pilot experiment 2

Extracellular Ca^{2+} was removed from cells by using Ca^{2+}-free incubation media with EGTA (2 mM). Intracellular as well as extracellular Ca^{2+} was removed from cells by using the cell permeable Ca^{2+} chelator BAPTA-AM and Ca^{2+} free incubation media in the presence of EGTA. Control cells were maintained in the presence of Ca^{2+} (1.25 mM). The effect of N^G-monomethyl-L-arginine (L-

NMMA) and L-valine were also investigated. No significant differences on arginine metabolism from controls were observed with any treatment (Figure 3.3.6.2). L-NMMA did not reduce the % of label in citrulline, although total label associated with cells was significantly reduced ($P < 0.01$).

3.3.6.3. Pilot experiment 3

The calcium ionophore, ionomycin (2 μM), or the protein kinase C inhibitor, bisindolylmaleimide (5μM) did not affect arginine metabolism (Figure 3.3.6.3). No differences due to ionomycin or L-NA were apparent. Use of bisindolylmaleimide did not modify the effect of ionomycin. Use of these agents, singly or in combination, resulted in no significant effects on either arginine metabolism or uptake.

3.3.7. Inhibition of arginine metabolism by N^G-hydroxy-L-arginine and L-ornithine

A preliminary experiment was undertaken to test the effects of a 15 min incubation with N^G-hydroxy-L-arginine (4 mM), L-valine (50 mM) and L-ornithine (1 mM) on label associated with the cells. The agents probably competed with [^{3}H]-arginine for uptake as cell-associated label was very low (dpm/10^6 cells < 700). For this reason, cells were preloaded with the agent, washed and then exposed to [^{3}H]-arginine. In this way cells should contain the agent in their intracellular environment, and competition for [^{3}H]-arginine uptake should be minimised.

When a pilot experiment (Figure 3.3.7.1) was performed in which cells were preloaded for 15 min with ornithine (1 mM) after which they were washed, and then further incubated for either 5 or 15 min with [^{3}H]-arginine, the volume marker ^{14}C-PEG 400 was used to enable precise estimation of the amount of label in the intracellular environment. Arginine metabolism to ornithine and citrulline (as assayed by TLC), and citrulline production (as assayed by ion-exchange) "appeared" reduced compared with cells not exposed to ornithine. Intracellular label associated with the cells, was apparently reduced by preloading with ornithine. Results are means ± S.E.M. of triplicate determinations on a single batch of cells.

Repetition of this experimental protocol with 5 min exposure to radiolabel, the use of volume marker and the arginase inhibitors, L-ornithine and N^G-hydroxy-L-

arginine reduced arginine metabolism to ornithine and citrulline, and caused a reduction in total label uptake (Figure 3.3.7.2).

3.3.8. Effect of preincubation with calcium ionophore and NO Synthase inhibitor on arginine metabolism.

In order to make a direct comparison between the effect of inhibitors of arginase and NO synthase, again, using the revised protocol (section 3.3.7) the effect of preincubation with either the calcium ionophore A23187 (2 μM) or L-NA (300 μM) or a combination of them both for 15 min, on arginine metabolism and uptake were investigated (Figure 3.3.8). The proportion of label as ornithine was slightly, but significantly increased above control ($P < 0.05$, by analysis of variance and Dunnett's test) by preincubation with both agents, alone and in combination, but there was no effect on the proportion of label as citrulline. These treatments did not have a significant effect on the total cell associated label.

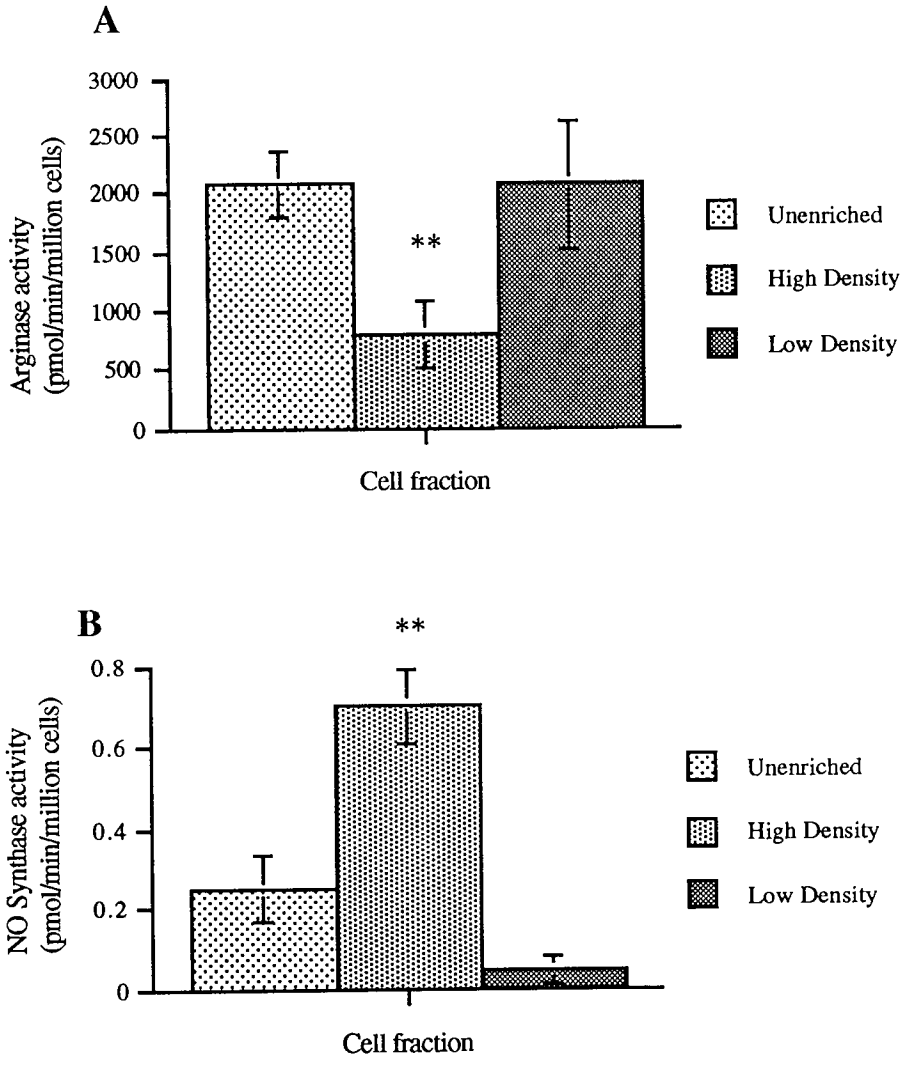
3.3.9. The effect of the NO synthase inhibitor L-NMMA on arginase activity.

The NO synthase assay depends on measurement of citrulline formation which is inhibitable by the NO synthase inhibitor L-NMMA (100 μM). To ensure that the assay was not being compromised by the inhibition of the potential arginase pathway leading via ornithine to citrulline, the effect of L-NMMA (100 μM) on arginase activity was investigated (Figure 3.3.9). Arginase activity, in gastric mucosal homogenates, in the presence of L-NMMA, was not significantly different from that obtained in its absence (determined by t-test, $n = 4$, graph A). A similar result was obtained with homogenates of isolated gastric mucosal cells (graph B).

3.3.10. Conversion of labelled citrulline to arginine.

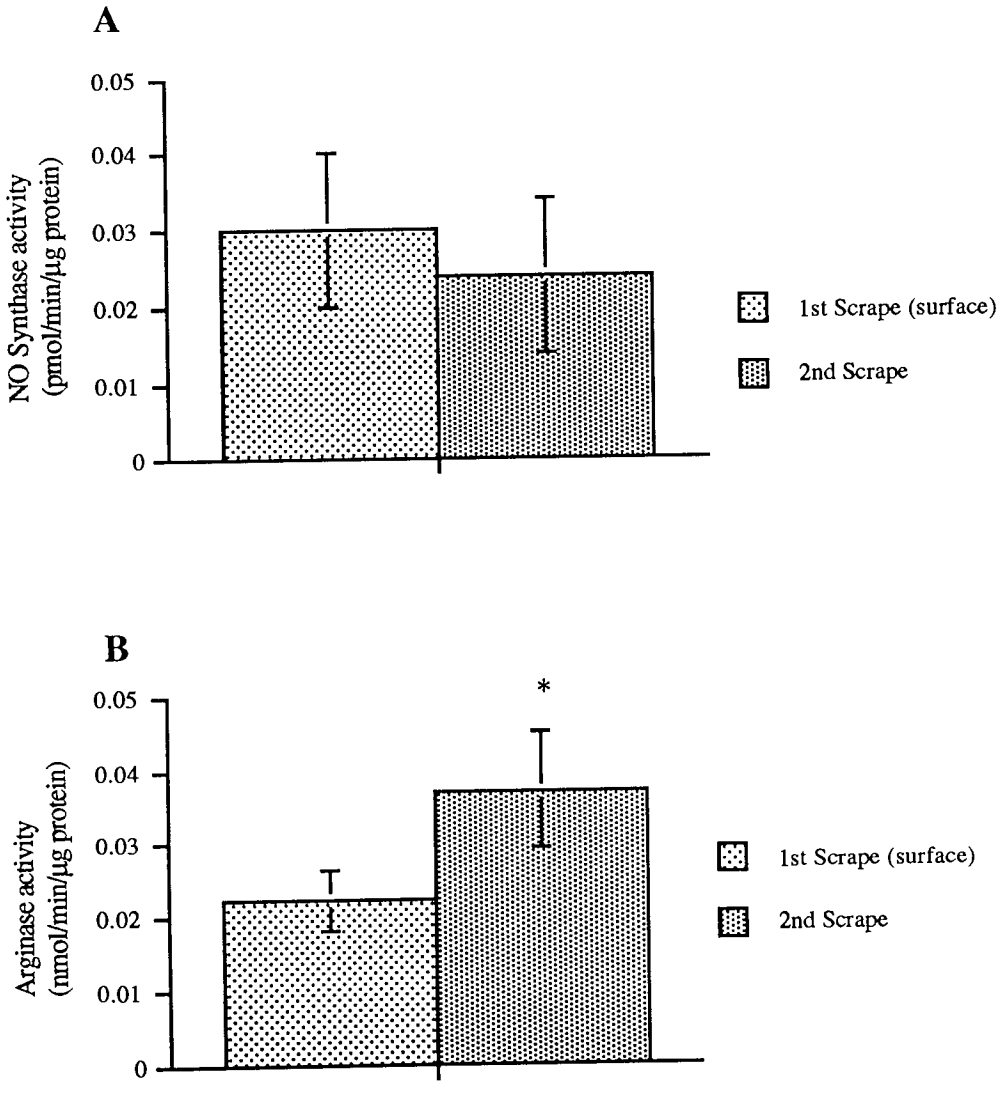
Taking into account the rate of metabolism of arginine to ornithine and citrulline, the possibility of the presence of a pathway converting citrulline back to arginine was investigated (Figure 3.3.10). There was a small, but significant ($P < 0.05$ by t-test, $n = 4$), conversion of [^{3}H]-citrulline to [^{3}H]-arginine after 15 min of incubation: 0.85 ± 0.29% of label as arginine in the presence of cells, 0.31 ± 0.07% with the cells absent (analysis by TLC).

Figure 3.3.1.1.
Arginase (A) and NO Synthase (B) activities in cell fractions isolated from the rat gastric mucosa.



Arginase (A) and NO synthase (B) assays were performed on rat gastric mucosal cell fractions. Results are means ± S.E.M. of 5 separate preparations of cells for measurement of arginase and of 10 preparations for NO synthase. ** $P < 0.01$ for comparison of activity in the high density fraction with that in the unenriched fraction by analysis of variance and Dunnett's test.

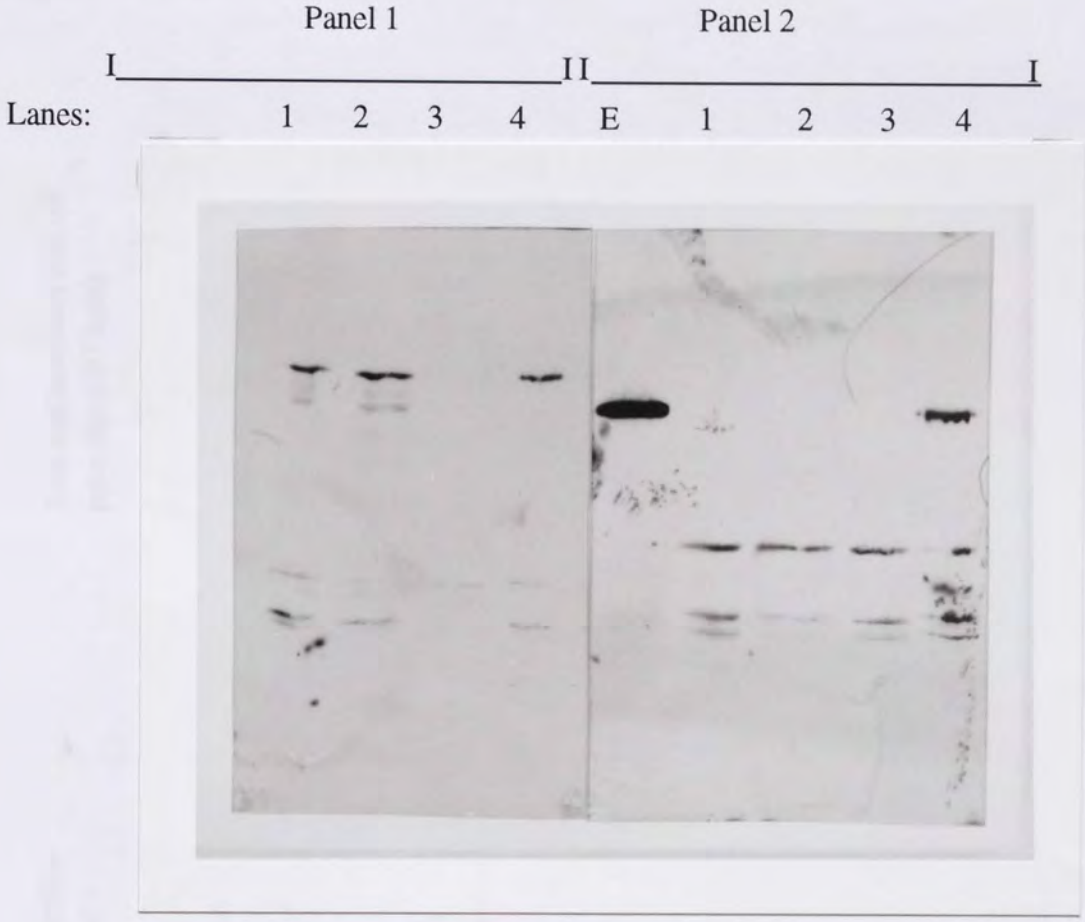
Figure 3.3.1.2.
Comparison of NO synthase activity (A) and arginase activity (B) in rat gastric mucosal homogenates prepared by differential scraping.



Two homogenates were prepared from rat gastric mucosa: a surface scrape and a scrape taken from the remaining tissue. Homogenates were assayed for both NO synthase and arginase activity. Results are means ± S.E. from 5 separate animals. Data are analysed by paired t-test, in B * $P < 0.05$.

Figure 3.3.1

Immunoblotting of NO synthase extracted from unenriched isolated gastric mucosal cells and from fractions separated on a Percoll density gradient.



A crude preparation of cells were separated using a Percoll density gradient into high-density and low-density cells. Monoclonal antibodies to nNOS and eNOS were used to blot the following samples: an unenriched fraction, high-density cells, low-density cells and whole gastric mucosa.

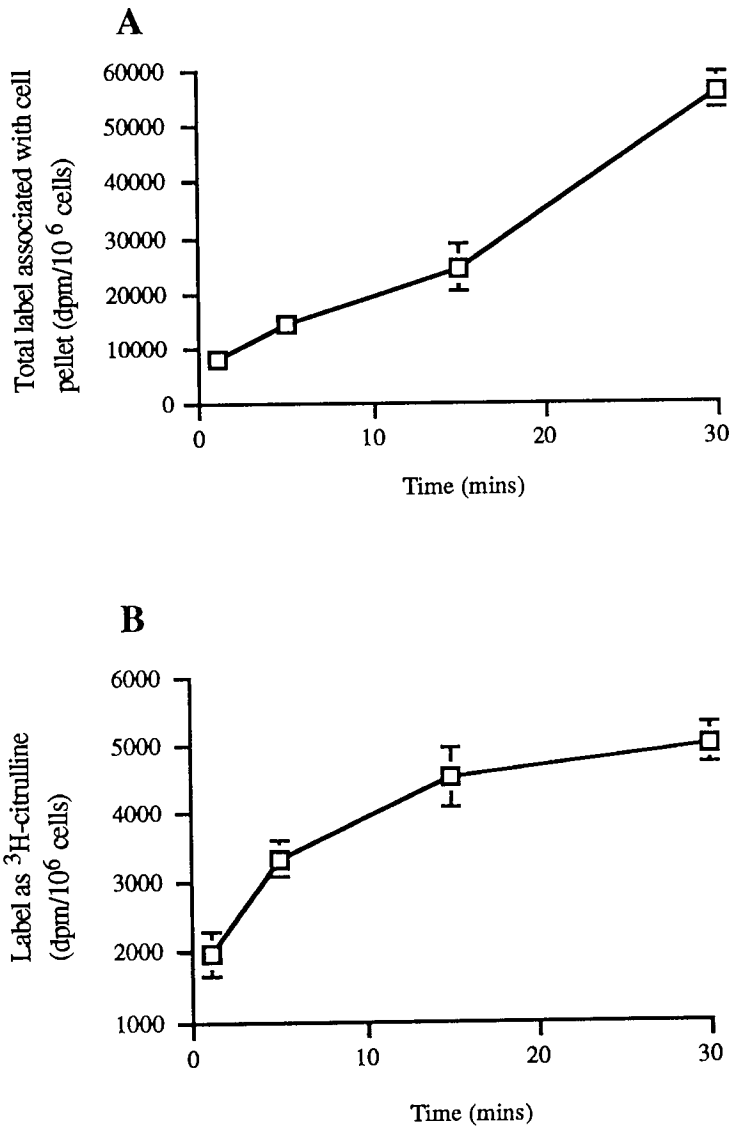
Panel 1: nNOS antibody

Panel 2: eNOS antibody

Lanes: 1. Unenriched fraction
2. High-density Percoll fraction
3. Low-density Percoll fraction
4. Whole gastric mucosal homogenate
E. Cultured endothelial cells.

Figure 3.3.2

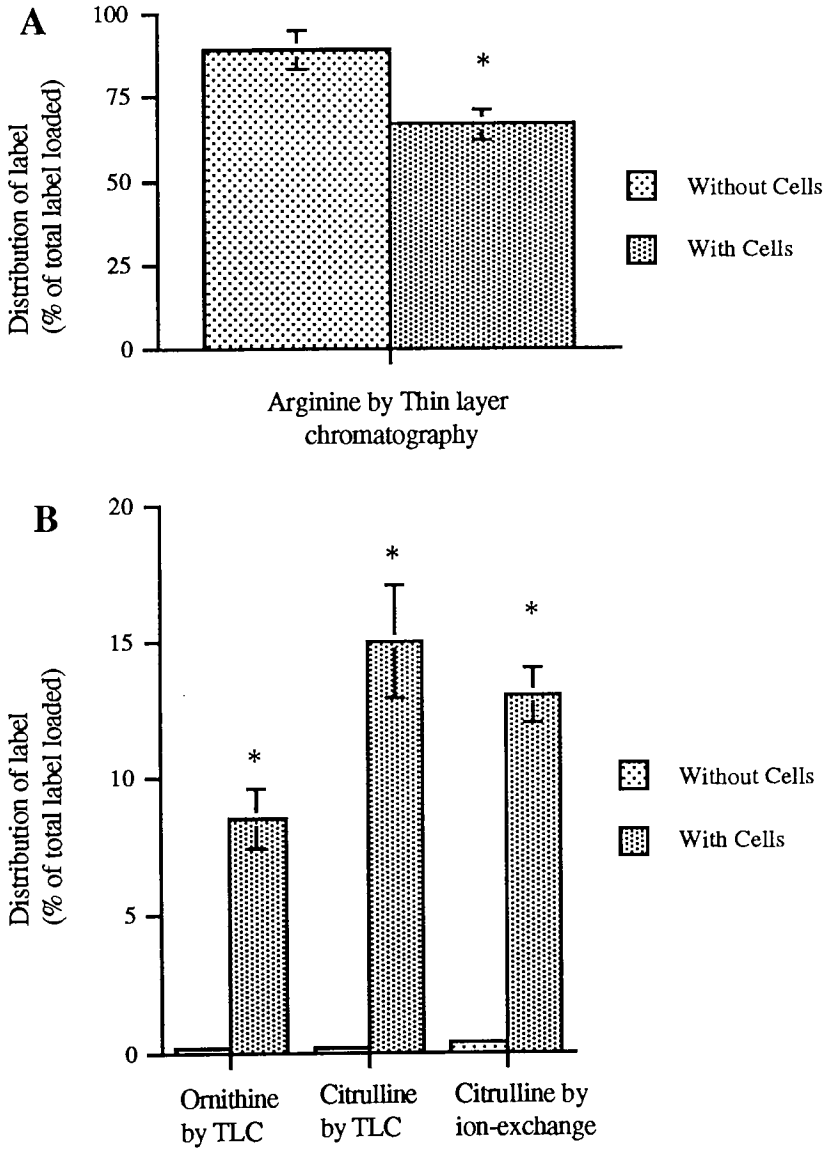
Effect of incubation time on ^{3}H-arginine uptake (A) and ^{3}H-arginine conversion to citrulline as analysed by ion exchange chromatography (B) by a suspension of gastric mucosal cells.



A suspension of gastric mucosal cells was incubated with [^{3}H]-arginine for varying time periods. Results are means ± S.E. of triplicate determinations on a single batch of cells.

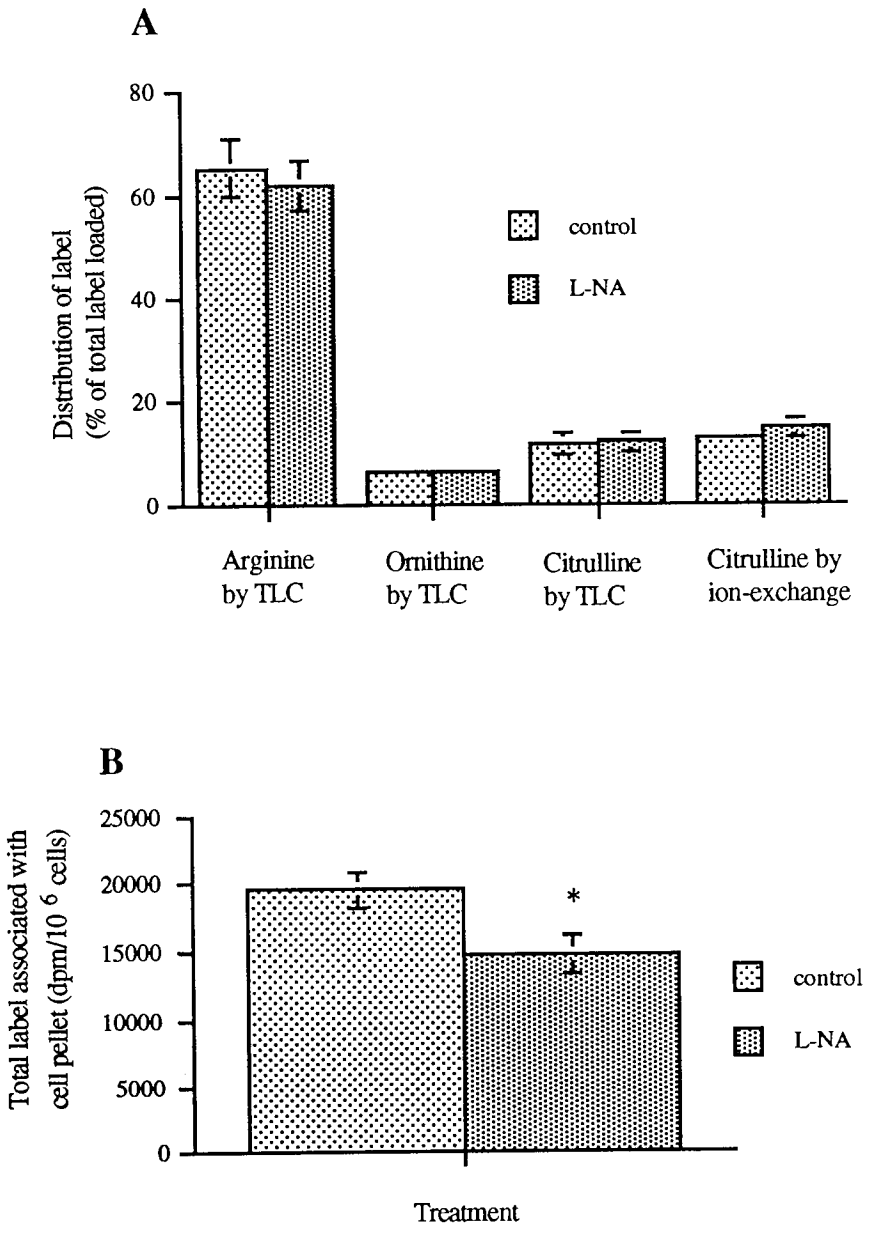
Figure 3.3.3

Distribution of radioactive label after a 15 min incubation with [^{3}H]-arginine in the presence and absence of cells as analysed by thin layer or ion-exchange chromatography. (A) shows distribution of label in arginine, and (B) shows distribution of label in ornithine and citrulline.



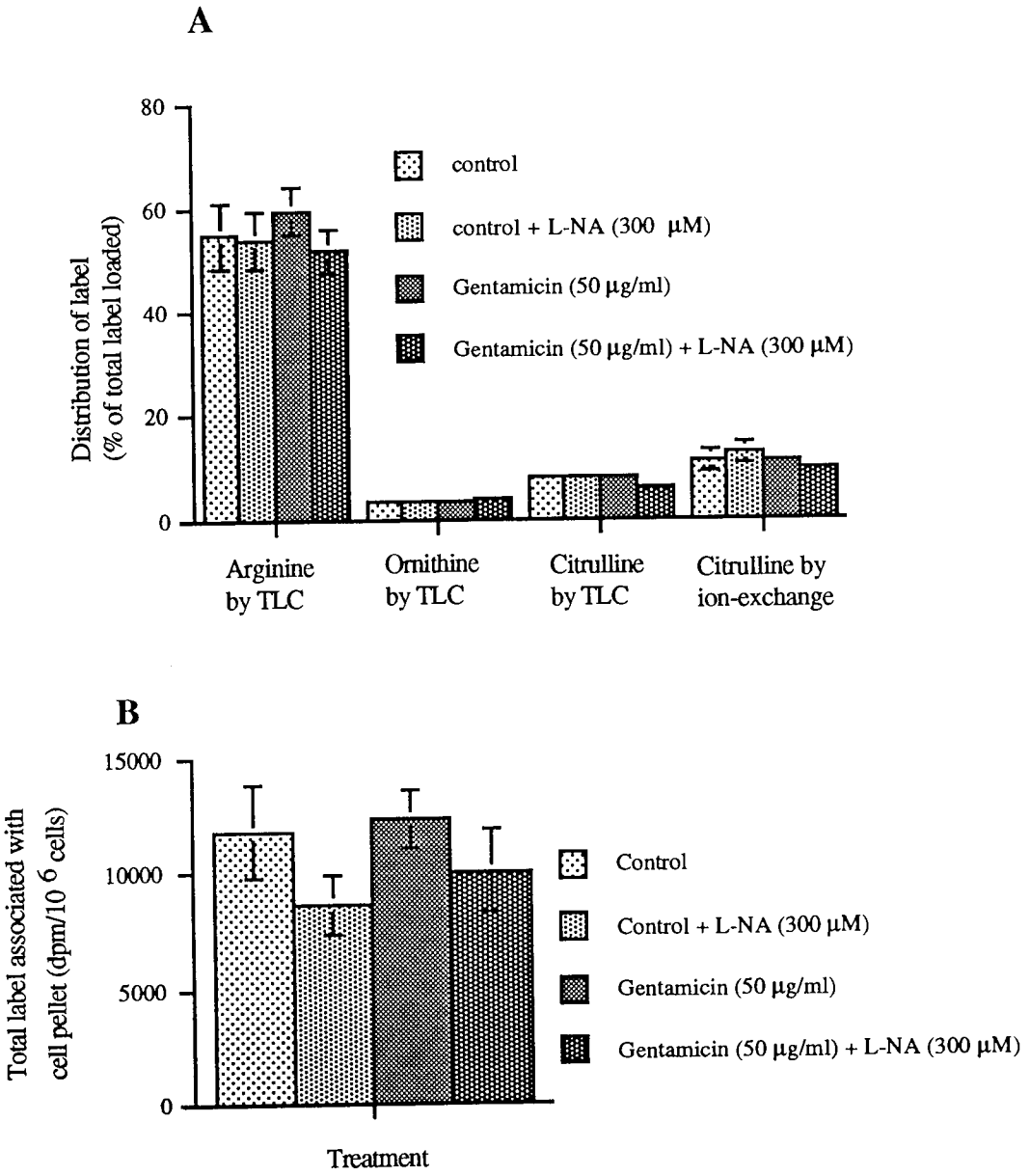
Suspensions of gastric mucosal cells or media alone were incubated with [^{3}H]-arginine for 15 min after which extracts of pellets, or label present in incubation medium alone were analysed by thin-layer chromatography or ion-exchange chromatography to determine the distribution. Results are means ± S.E. for 4 separate batches of cells. * $P < 0.05$ by t-test, for difference in label distribution in the presence of cells. Total label added to TLC plate was 150,000 dpm.

Figure 3.3.4.
Effect of NO synthase inhibitor L-NA on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine associated with the cell pellet (B).



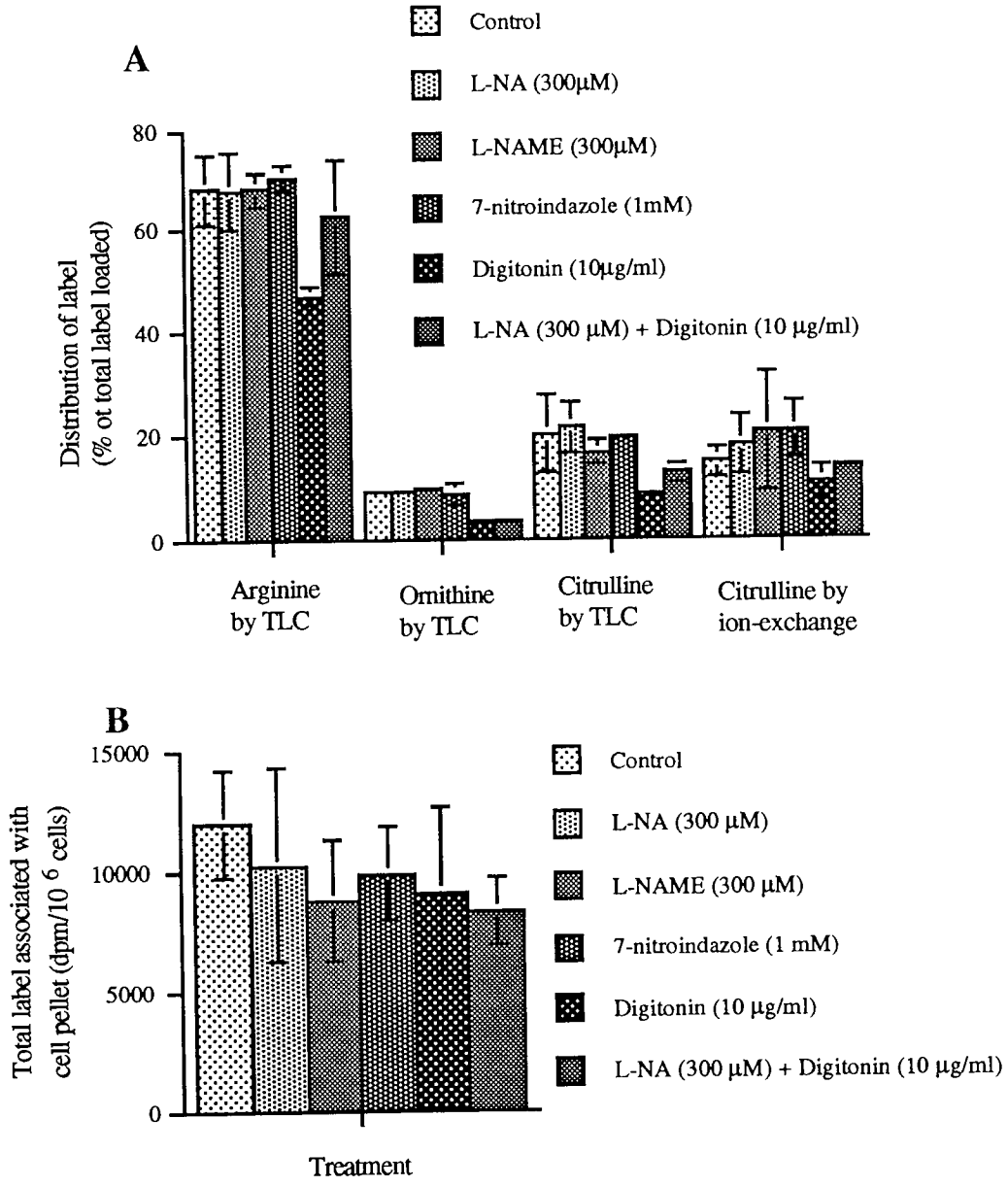
A suspension of gastric mucosal cells were preincubated in the presence and absence of L-NA (300 μM) for 15 min at 37°C and then with [^{3}H]-arginine for a further 15 min. Results are means ± S.E. for 5 separate batches of cells with triplicate determinations for each batch. Data were analysed by paired t-test, in graph B * $P < 0.05$.

Figure 3.3.5.
Effect of the antibiotic gentamicin on [^{3}H]-arginine metabolism (A) and [^{3}H]-arginine uptake (B).



Gastric mucosal cells were split after each harvesting stage in the isolation procedure to give 2 batches: a control cell flask and a test flask containing 50 μg/ml of the antibiotic gentamicin. The experimental procedure was conducted under normal conditions in the presence and absence of L-NA (300 μM). Results are means ± S.E. for 3 separate batches of cells with triplicate determinations for each batch. No effect of gentamicin was evident by paired t-test.

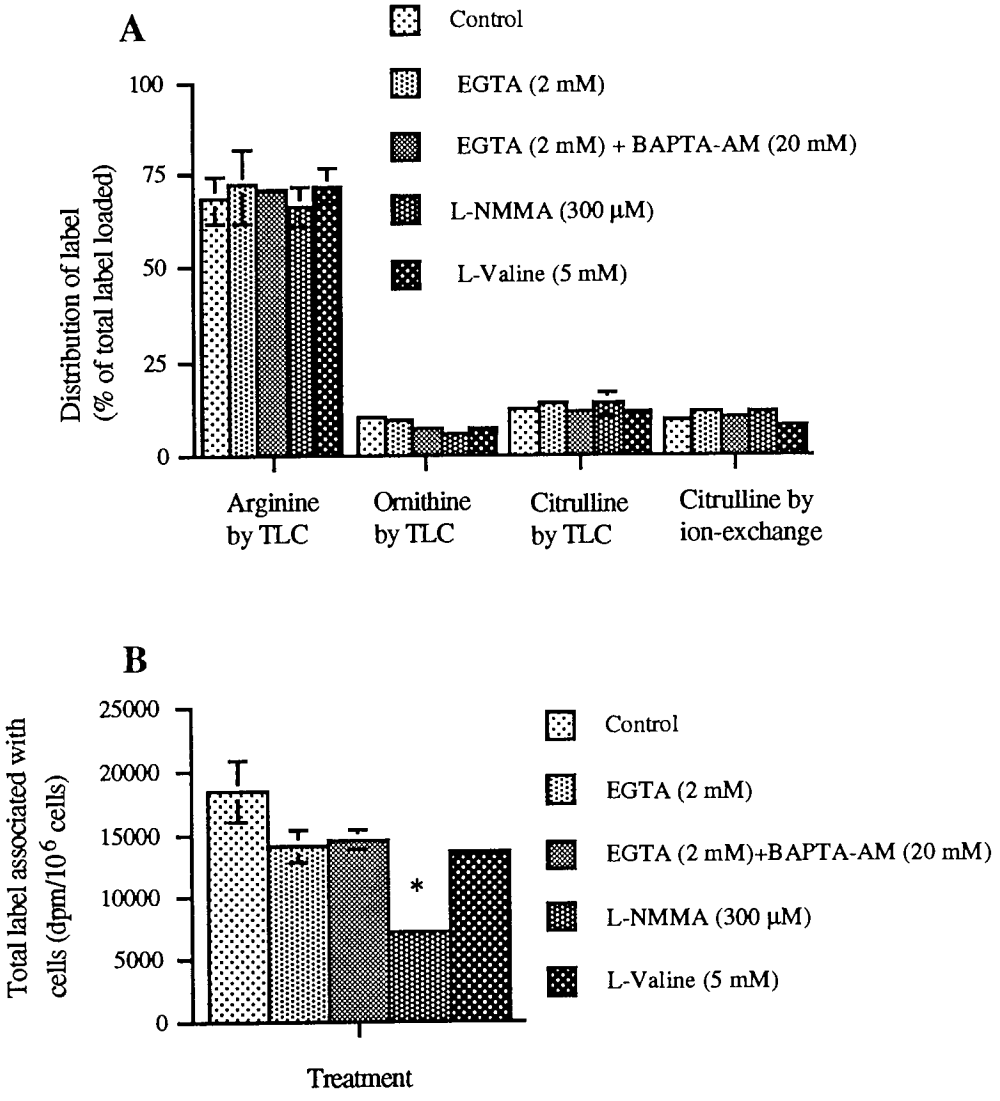
Figure 3.3.6.1.
Lack of effect of NO synthase inhibitors on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



A suspension of gastric mucosal cells were incubated at 37°C with [^{3}H]-arginine for 15 min after 15 min preincubation with: control cells, L-NA (300μM), L-NAME (300μM), 7-nitroindazole (1mM), digitonin (10μg/ml), and L-NA (300 μM) with digitonin (10 μg/ml). Results are means ± S.E. of triplicate determinations on a single batch of cells. No significant effect of treatments was detectable by analysis of variance.

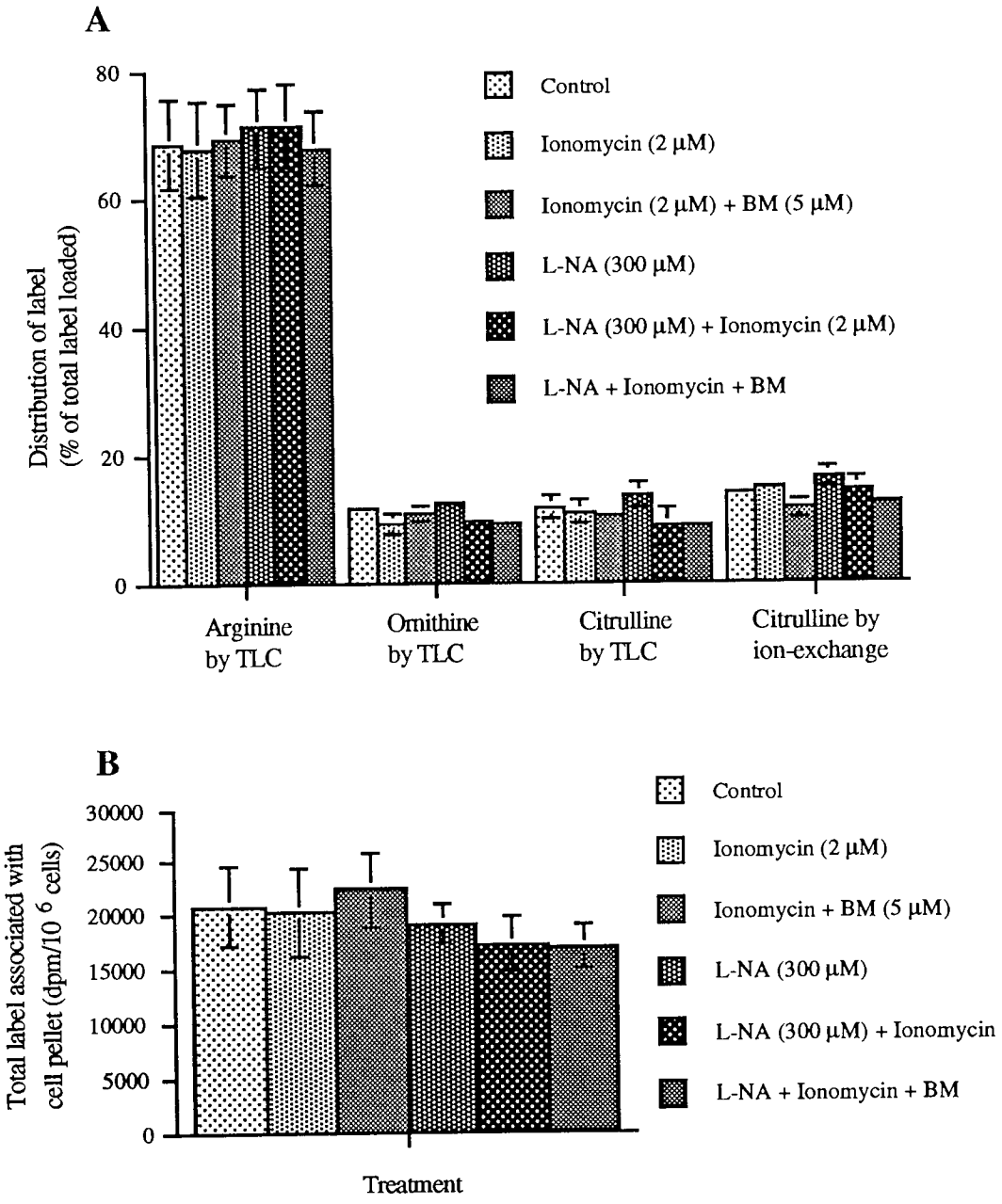
Figure 3.3.6.2.

Effect of Ca^{2+} removal and NO synthase inhibition on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



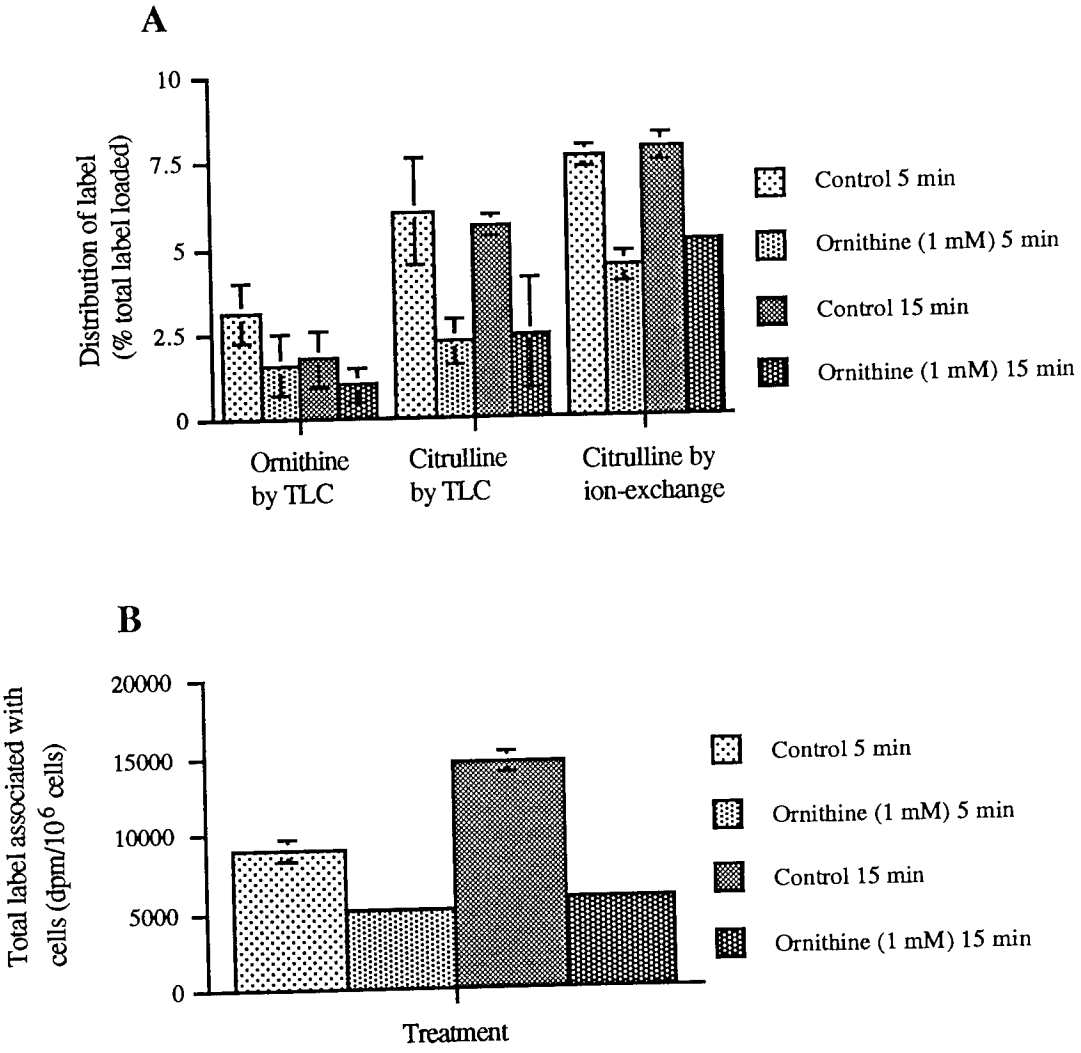
A suspension of gastric mucosal cells were incubated at 37°C with[^{3}H]-arginine for 15 min under the following conditions: control cells, EGTA (2 mM) in Ca^{2+} free media, EGTA (2 mM) and the cell permeable Ca^{2+} chelator BAPTA-AM (20 mM) in Ca^{2+} free media, L-NMMA (300 µM) and L-valine (5 mM). Results are means ± S.E. of triplicate determinations on a single batch of cells. Data was compared with control by analysis of variance and Dunnett's test. * $P < 0.01$.

Figure 3.3.6.3.
Effect of ionomycin and bisindolyl maleimide on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



A suspension of gastric mucosal cells were incubated at 37°C with [^{3}H]-arginine for 15 min under the following conditions: control cells, ionomycin (2 μM), ionomycin (2 μM) and the protein kinase C inhibitor bisindolyl maleimide (BM) (5 μM), L-NA (300 μM), L-NA and ionomycin, and also L-NA with ionomycin and bisindolyl maleimide. Results are means ± S.E. of triplicate determinations on a single batch of cells. No effect of treatments were evident by analysis of variance.

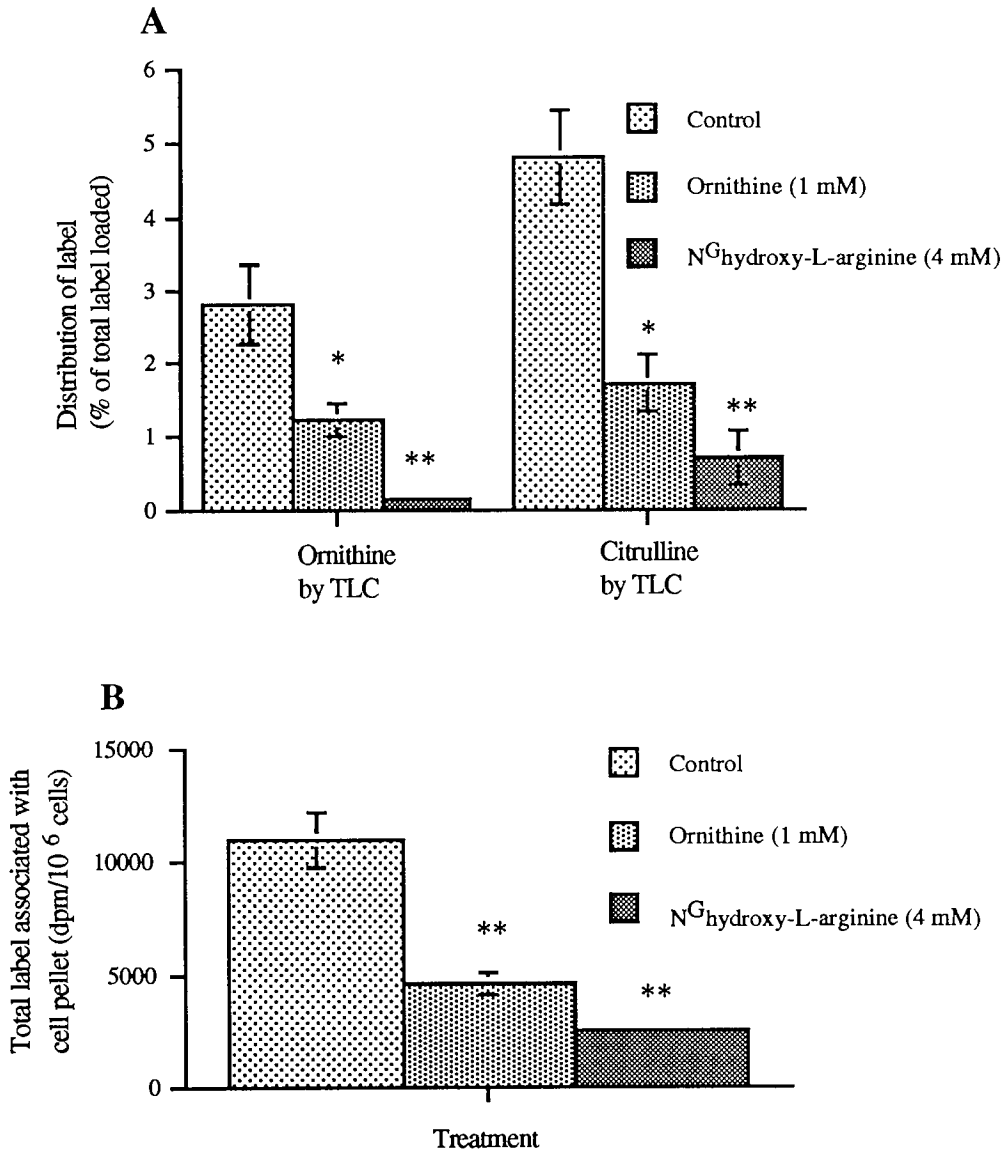
Figure 3.3.7.1.
The effect of ornithine preloading on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



Gastric mucosal cells were split after the final harvesting step to give a control flask and a test flask containing 1 mM ornithine. Cells were incubated for 15 min, after which cells were washed and then exposed to [^{3}H]-arginine for either 5 or 15 min. Results are means ± S.E. of triplicate determinations on a single batch of cells. Data was analysed by analysis of variance and Dunnett's test, and although an effect of ornithine is suggested, this did not reach significance.

Figure 3.3.7.2.

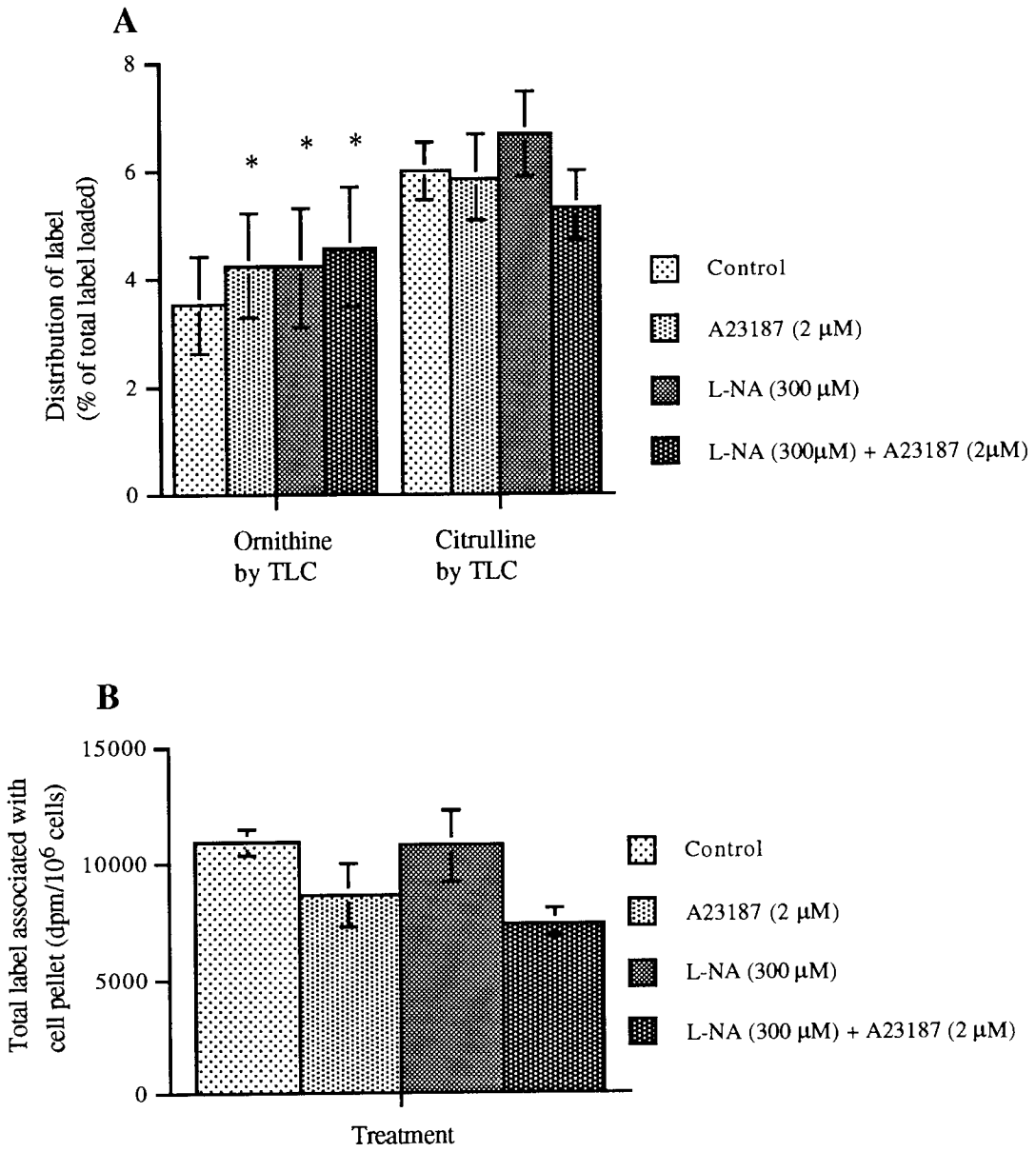
The effect of preincubation with ornithine and N^G-hydroxy-L-arginine on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



Gastric mucosal cells were split after the final harvesting step to give a test flask containing 1 mM ornithine, a test flask containing 4 mM of the arginase inhibitor N^G-hydroxy-L-arginine and a control flask. Cells were preincubated with treatment for 15 min after which they were washed and then exposed to [^{3}H]-arginine for a further 5 min. Results are means ± S.E. for 4 separate batches of cells with triplicate determinations for each batch. * $P < 0.05$, ** $P < 0.01$ for difference from control by analysis of variance and Dunnett's test.

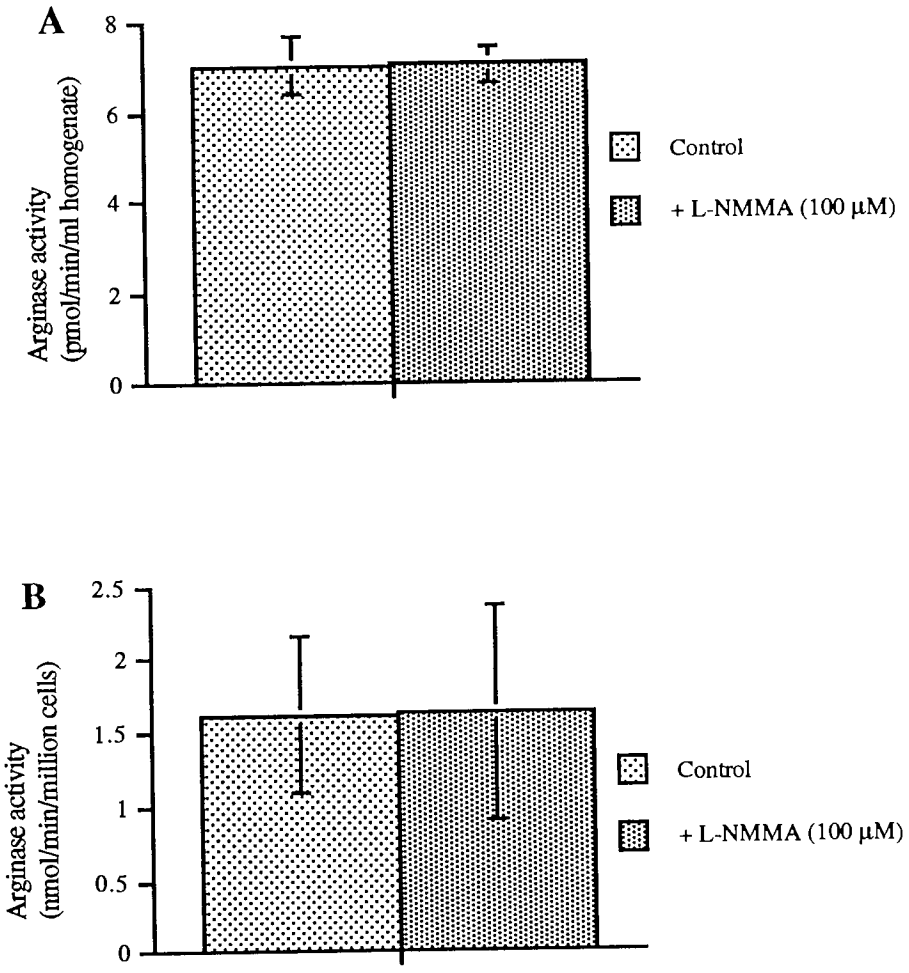
Figure 3.3.8.

The effect of preincubation with Ca^{2+}-ionophore A23187 and NO synthase inhibitor N^G-nitro-L-arginine on [^{3}H]-arginine metabolism (A) and on [^{3}H]-arginine uptake (B).



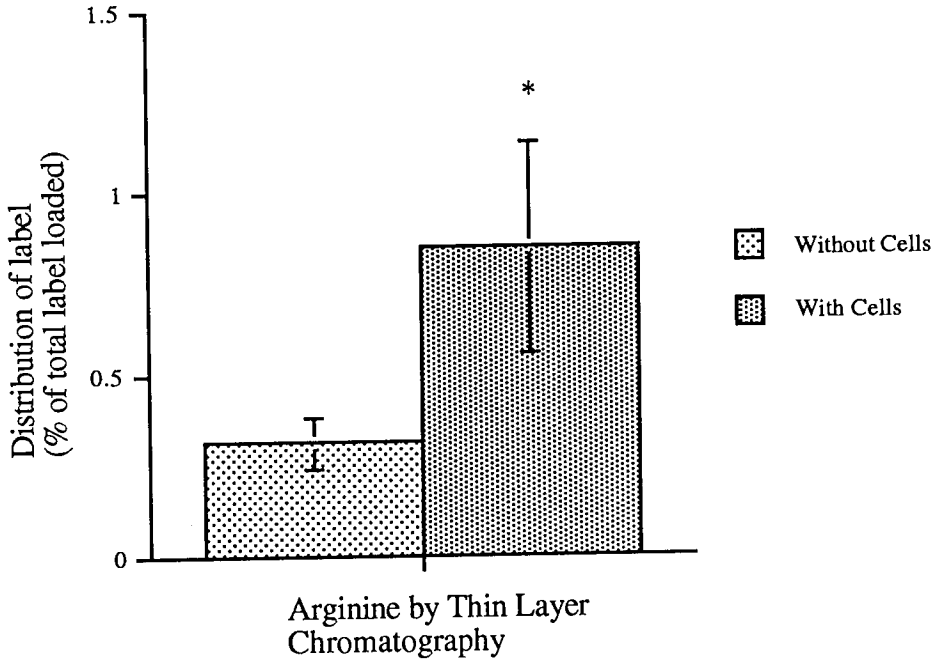
Gastric mucosal cells were split after the final harvesting step to give a control flask and test flasks containing either Ca^{2+}-ionophore A23187 (2 μM), or L-NA (300 μM), or a combination of both A23187 and L-NA. Cells were preincubated with treatment for 15 min after which they were exposed to [^{3}H]-arginine for a further 5 min. Results are means ± S.E. for 4 separate batches of cells with triplicate determinations for each batch. * $P < 0.05$ for difference from control by analysis of variance and Dunnett's test.

Figure 3.3.9.
Lack of effect of L-NMMA on arginase activity in gastric mucosal homogenates (A) and suspensions of isolated gastric mucosal cells (B).



The NO synthase inhibitor L-NMMA (100 μM) was included in arginase assays on rat gastric mucosal homogenates (A) and in homogenates of unenriched isolated gastric mucosal cells (B). L-NMMA had no significant effect on arginase activity in either preparation as analysed by t-test. n = 4.

Figure 3.3.10.
Conversion of [^{3}H]-citrulline to [^{3}H]-arginine after a 15 min incubation period in the presence and absence of cells as analysed by thin layer chromatography.



Incubation of radiolabelled [^{3}H]-citrulline for 15 min in the presence of cells resulted in a small but significant conversion to arginine, when compared with conversion in the absence of cells. * $P < 0.05$ by t-test. $n = 4$.

3.4. DISCUSSION

3.4.1. Presence of arginine metabolising enzymes in the gastric mucosa.

In order to ascertain whether isolated gastric mucosal cells provided a suitable system for the investigation of arginine metabolism, it was necessary to establish the presence of the metabolic enzymes involved. The two potential pathways of L-arginine metabolism of interest in this study were its conversion to either NO and L-citrulline by the action of NO synthase, or the production of L-ornithine and urea by the action of arginase (Wu and Meininger, 1995). The presence of NO synthase and arginase enzyme activities were demonstrated both in isolated cell suspensions and in gastric mucosal homogenates. A significant ornithine transcarbamoylase activity, which acts to convert L-ornithine to L-citrulline was also detected. Immunoblotting experiments confirmed the presence of NO synthase, and demonstrated that it was of the neuronal type.

Unenriched gastric mucosal cells resolved by a Percoll density gradient form a low-density fraction rich in parietal cells, and a high-density fraction containing mucous cells (Brown et al., 1992). The distribution of arginase and NO synthase activities in the unenriched cell suspension, and the Percoll density gradient separated fractions, were compared and were found to be different. Arginase was present at a much higher total activity, and found to be concentrated in the low density fraction, whereas NO synthase activity was more pronounced in the high density fraction. This distribution of arginase activity was reflected in the result obtained with homogenates prepared from gastric mucosal scrapes. The surface scrape, probably containing surface mucous cells was found to contain low arginase activity, but the second scrape, which should contain parietal cells enriched in the low-density Percoll fraction, had higher arginase activity. So, although both arginine metabolising enzymes are present in gastric mucosal tissue, it is possible that they may not colocalise in the same cell type. Indeed arginase and NO synthase activities appear to be concentrated in different cell types, with perhaps the majority of arginase activity residing in parietal cells and gastric glands, and NO synthase activity residing in surface mucus cells.

The presence of NO synthase was confirmed by results obtained from immunoblotting for the NO synthase protein. Immunoblots of extracts of isolated cells, when exposed to a monoclonal antibody directed against nNOS, gave bands at a 160 kDa position, which corresponded to the presence of NO synthase. The 160 kDa material was enriched in the high-density Percoll fraction but depleted

from the low-density fraction. This result of a concentration of nNOS protein in the high-density fraction corresponds with the data obtained with the enzyme assay. Use of the eNOS antibody gave a band at the appropriate position when exposed to extracts of gastric mucosa, but not with extracts of isolated cells. An explanation is that endothelial cells are the source of this eNOS, which are present in the gastric mucosa but are absent from isolated cell preparations. In conclusion, the form of NO synthase present in gastric cells isolated from the glandular corpus mucosa belongs to the nNOS type.

After detection of nNOS protein and activity, and arginase and ornithine transcarbamoylase activity in isolated gastric mucosal cells, experiments were conducted to investigate the fate of labelled arginine in these cells. It was of interest to determine which was the dominant pathway of arginine metabolism and whether there was any interaction between the arginine metabolising enzymes.

3.4.2. Pilot experiments.

Pilot experiments confirmed the uptake of sufficient radiolabelled arginine into gastric mucosal cells and its conversion into radiolabelled citrulline to establish a suitable system within which to study arginine metabolism. The association of label with the cells increased approximately linearly up to 30 min, but the proportion of label in citrulline which was used as an index of metabolism, reached a plateau with time, one explanation of this would be efflux of citrulline, although none was detectable in incubation medium run on TLC plates. Radiolabelled arginine was only converted into radiolabelled ornithine or citrulline in the presence of cells, and the lack of effect of gentamicin suggested that contamination of the cell suspensions with bacteria was not responsible.

3.4.3. Arginine metabolism by NO synthase in gastric mucosal cells.

Isolated cells displayed a basal metabolism of arginine to ornithine and citrulline when incubated with [^{3}H]-arginine. Citrulline is a product of NO synthase but its formation could not be inhibited by a variety of NO synthase inhibitors. A significant effect observed in studies with NO synthase inhibitors was with L-NMMA, which resulted in a reduction in the total label associated with cells ($P < 0.01$) but not the proportion of label in citrulline. A cationic L-amino acid cellular uptake pathway termed y+, has been identified in a number of cell-types including:

fibroblasts, kidney, erythrocytes and heptoma cell lines (Kim et al., 1991). Other transport systems are responsible for mediating the uptake of neutral and acid amino acids. L-arginine gains access to endothelial cells by the y+ saturable transport system. Use of L-NMMA, which is a cationic L-arginine analogue, in porcine aortic endothelial cells, resulted in a reduced arginine uptake due to the NO synthase inhibitor sharing the same uptake system as arginine (Bogle et al., 1992). The consequences of this interaction were apparent in gastric mucosal cells, and consequently L-NA which does not enter cells by y+ (Bogle et al., 1992) was used as the predominant NO synthase inhibitor.

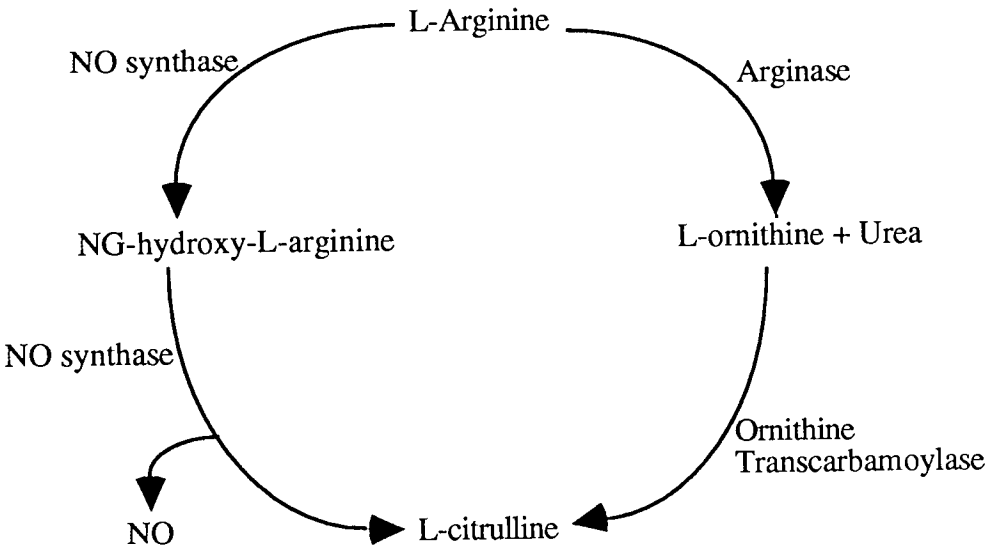
There is an obligatory requirement for calcium in order for nNOS, the presence of which was detected in gastric mucosal cells (section 3.4.1), to function (Knowles and Moncada, 1994. section 1.2). However, the reduction of intracellular and extracellular calcium, by use of calcium chelators, did not inhibit the metabolism of arginine to citrulline. An attempt to activate NO synthase by causing a large and rapid increase in intracellular Ca^{2+} was made with the use of the calcium ionophores ionomycin and A23187, or by permeabilising cells with digitonin. However, no corresponding increase in citrulline production was observed. This result is in marked contrast with data obtained using ionomycin on bovine aortic endothelial cells (Shimizu et al., 1993), and mouse striatal neurons (Marin et al., 1992), or with A23187 on rat cerebellar primary cultures (Paltrons et al., 1995), canine arterial endothelial cells (Cosentino et al., 1994), or pig aortic endothelial cells (Weisbrod et al., 1993), where use of ionophores stimulated an increase in cGMP and citrulline production. Protein kinase C inhibition was examined in case its activation had been preventing the activation of NO synthase. Such an inhibitory effect of protein kinase C on Ca^{2+}-dependent NO synthase activity has previously been demonstrated in rabbit gastric muscle cells (Murthy et al., 1994). In addition, activated protein kinase C is capable of NO synthase inhibition in cultured bovine pulmonary artery endothelial cells (Davda et al., 1994). An inhibitor was therefore used to prevent any protein kinase C related inhibition of NO synthase, which could possibly uncover an underlying NO synthase activity. However, with the use of a bisindolylmaleimide, a potent and selective inhibitor of protein kinase C (Toullec et al., 1991), no increase in citrulline production was apparent.

Even when experiments investigating the effects of L-NA and the ionophore A23187 were conducted using the preloading protocol, that was used successfully to demonstrate effects of arginase inhibitors (see below), no effect on citrulline production was found. A slight, but significant increase in ornithine production

was observed with L-NA, A23187, or the two in combination, over control. Inhibition of any NO synthase activity with L-NA might be sufficient to increase availability of arginine substrate, with the consequence of an increased conversion by arginase to ornithine. However the similar effect of A23187 alone, and the absence of an accompanying change in citrulline production do not make this explanation convincing.

It was concluded that the citrulline produced by the crude cell suspension was not due to NO synthase activity. The fact that NO synthase was not responsible for the observed citrulline formation raises the possibility of an alternative pathway of arginine metabolism in gastric mucosal cells. The arginase inhibitors L-ornithine and N^G-hydroxy-L-arginine, both evoked an inhibitory response. L-ornithine is capable of evoking a strong competitive inhibition of isolated arginase (with a K_i of 1 mM for the liver enzyme) (Reczkowski and Ash, 1994), but has no such effect on NO synthase (Hbrak et al., 1994). N^G-hydroxy-L-arginine, the only known intermediate of the NO synthase enzyme reaction, is reported as a uniquely potent competitive inhibitor of rat liver arginase (Daghigh et al., 1994) with a 25-fold higher binding affinity for arginase than L-ornithine, and with a K_i for competitive inhibition of liver arginase equal to 150 μM (Boucher et al., 1994). Both arginase inhibitors were capable of reducing ornithine and citrulline production and the uptake of arginine by the cells. These results suggest that arginase activity initiates the pathway responsible for arginine metabolism in gastric mucosal cells. The observed reduction in total label uptake with ornithine and N^G-hydroxy-L-arginine, is accounted for by arginine and the agents tested sharing the same y+ carrier for uptake into cells. However, preloading cells with test agents prior to exposure to radiolabel, ensured that the agents gained intracellular access, and that sufficient [^{3}H]-arginine was taken up to enable measurements to be made. Since ornithine and citrulline are expressed as a proportion of total cellular label it is likely that changes in metabolism rather than uptake are responsible for the effects of the arginase inhibitors. The presence of significant ornithine transcarbamoylase activity in isolated cells, and the inhibitory effects of the arginase inhibitors on citrulline formation, suggest that arginase acts on L-arginine with the production of urea and L-ornithine, and the L-ornithine is then converted to L-citrulline by the enzyme ornithine transcarbamoylase (Figure 3.4.3). In this way a basal citrulline formation is seen which is not altered by NO synthase inhibition.

Figure 3.4.3.
Metabolic transformation of L-arginine to L-citrulline involving NO synthase and arginase.



3.4.4. Possible interaction between NO synthase and arginase

The evidence suggests that arginase is present in isolated gastric mucosal cells, and that it is the dominant pathway responsible for arginine metabolism. The existence of nNOS protein and arginase activity in isolated gastric cells raises the potential for interaction between the two pathways. This could take place in a number of ways depending on location of the two enzymes, and their relative activities *in vivo*. One possibility is that both NO synthase and arginase are active simultaneously in different cells. Taking into account the differential distribution of arginine metabolising enzymes, a situation such as this could occur in cells in the gastric glands where metabolism of arginine might restrict the supply of arginine to NO synthase in surface mucous cells. If the arginase containing cells were so localised as to remove arginine from the blood supply *in vivo* before it reached cells containing NO synthase then arginase could reduce NO synthase activity. Such an interaction would not be visible in an isolated cell system where plentiful arginine was available in the medium. Evidence in support of this theory comes from a study investigating the effect of L-arginine administration on gastric mucosal integrity (Ferraz et al., 1994). The *in vivo* supplementation of L-arginine resulted in the production of large amounts of NO, unrelated to iNOS activation, and a dose-dependent increase in the susceptibility of the rat stomach to damage.

This suggests that availability of substrate for NO synthase may be limiting, which when supplied exogenously, resulted in the release of cytotoxic amounts of NO. A complex relationship exists between NO and mucosal integrity (sections 1.4 and 1.7), where small amounts are necessary for the maintenance of a healthy mucosal perfusion, and where large amounts exacerbate mucosal injury. It is possible that the purpose of arginase activity in the gastric mucosa is to regulate the production of NO, by metabolising arginine and thereby limiting the availability of substrate for NO synthase. This provides another mechanism of NO regulation, whereby its excessive production and subsequent potential cytotoxic effects are prevented.

Alternatively, if both enzymes are present in the same cell, it raises the possibility that there could be interaction between the two pathways, resulting in competition for substrate with a regulated balance of products. Arginase has a high Km for arginine (Reczkowski and Ash, 1994) which is in the mmolar range, whereas the Km value for NO synthase is much lower, in the μmolar range. In intact gastric mucosal tissue, this difference in Km values could mean that arginase may not be as active at such a high rate as the results on homogenates may suggest. Interactions between the NO synthase and arginase metabolic pathways have been suggested in rat alveolar macrophages (Hey et al., 1995), and in an experimental model of glomerulonephritis (Cook et al., 1994). In cases such as this, an inversely proportional relationship exists between the enzyme pathways. Thus, induction of NO synthase activity by LPS in macrophages resulted in a substantial reduction in arginase activity. Whereas, use of L-NMMA in nephritic glomeruli resulted in an increased arginase activity, thus revealing a competition for substrate between the pathways.

It is not clear why the present results showed a complete absence of functioning NO synthase in gastric mucosal cells even with elevated Ca^{2+}. However, in rat coronary endothelial cells, which had been cultured in 0.4 mM L-arginine, the predominant L-arginine metabolic pathway was found to be arginase, with NO synthase active to a far lesser extent (Wu and Meininger, 1995), so other tissues do show some parallels with the gastric mucosal preparation. Another situation similar to that found in gastric mucosal cells, is the arginine metabolism exhibited by freshly isolated enterocytes (Blachier et al., 1991). There appears to be an intensive intestinal degradation of L-arginine resulting in the release of L-ornithine, urea and L-citrulline into extracellular fluid. The dominant metabolic fate of L-arginine in enterocytes is its conversion to L-ornithine by the action of arginase. Metabolism in these cells differs from that observed in gastric mucosal cells, in that there was a limited synthesis of L-citrulline, and the presence of calcium-

independent NO synthase was demonstrated. Isolated enterocytes were found to contain sizeable amounts of the polyamines putrescine, spermidine and spermine. Considering the high arginase activity demonstrated by gastric mucosal cells, the presence of polyamines is a possibility, although this was not a parameter measured in this study. L-ornithine is the initial substrate in the polyamine biosynthetic pathway (Pegg, 1986) which is converted to putrescine by the first, rate limiting enzyme, ornithine decarboxylase (section 4.1). Polyamines play an essential role in cell growth and repair (Tabor and Tabor, 1984) and may contribute to the process of restitution (Wang and Johnson, 1990c) in the gastric mucosa. The high arginase activity may thus maintain a basal production of polyamines.

3.4.5. Citrulline formation and metabolism in the gastric mucosa.

A continuous release of citrulline from the small intestine into the circulation has been demonstrated (Windmueller and Spaeth, 1981). Arginine is required by all tissues, and it is thought that the fate of circulating citrulline, is for uptake into tissues such as brain and kidneys, in order to be converted into arginine. The enzyme ornithine transcarbamoylase, shown to be active in gastric cells in this study, is present in only trace amounts in tissues other than liver and small intestine. Therefore, arginase in gastric cells may serve a purpose, together with ornithine transcarbamoylase, to release citrulline, which would enable resynthesis of arginine to occur in the kidney. In an attempt to determine the fate of citrulline in gastric cells, the potential presence of a pathway converting citrulline back to arginine was investigated. The presence of an intracellular pathway converting citrulline to arginine has been demonstrated in cerebral artery perivascular nerves (Chen and Lee, 1995), in cultured bovine endothelial cells (Hecker et al., 1990) and in a number of endothelial cell lines (Wu and Meininger, 1993). The pathway by which this occurs is probably by the action of by arginosuccinate synthase on L-citrulline to produce L-arginosuccinate, followed by the conversion of L-arginosuccinate to L-arginine by arginosuccinate lyase. The purpose of the citrulline to arginine conversion in these cells is to help maintain sufficient levels of L-arginine during prolonged periods of NO release. A conversion of citrulline to arginine was established in gastric mucosal cells, smaller than that obtained with other preparations mentioned above. It is possible, that similar to citrulline released from the small intestine, citrulline from gastric cells is released into the circulation to provide a L-arginine source for other tissues.

3.4.6. Summary

1. The arginine metabolising enzymes identified in gastric mucosal tissue and isolated gastric mucosal cell preparations were found to be: the neuronal form of NO synthase, arginase, and ornithine transcarbamoylase.

2. The data presented here suggests that the predominant pathway of arginine metabolism in isolated gastric mucosal cells is one involving the action of arginase and ornithine transcarbamoylase.

3. Possible reasons for gastric cells to produce large amounts of ornithine, citrulline and urea, whilst causing the inhibition of NO synthase, could be: regulation of production of cytotoxic quantities of NO, to provide a substrate for polyamine biosynthesis, or to provide a source of arginine, from citrulline, for other tissues.

CHAPTER 4

INVOLVEMENT OF NO IN THE RESPONSE OF THE GASTRIC MUCOSA TO DAMAGE BY HYPERTONIC SALINE

4.1. INTRODUCTION

4.1.1. Protection of the gastric mucosa

The gastric mucosa is constantly exposed to many noxious stimuli, such as luminal acid, hot food, alcohol, and drugs, and therefore has developed cytoprotective mechanisms and adaptive responses which come into effect under adverse conditions (Wallace and Bell, 1995). Protective factors such as mucus, bicarbonate, prostaglandins and NO have all been implicated in the process of cytoprotection (section 1.6). In addition to its cytoprotective functions in the gastric mucosa, endogenous NO is also suggested to play a role in the response of the gastric mucosa to damage. A decrease in NO synthase activity is associated with an increased susceptibility to mucosal damage and a delay in ulcer healing (Konturek et al., 1993). The greater reduction in NO synthase activity in siaoloadenectomized, as compared to sham operated rats, in response to ethanol, is associated with a corresponding increase in mucosal damage (Tripp and Tepperman, 1995). Whereas, the antigastric ulcer effects exhibited by the immunosuppressive drug, FK506, are related to its ability to preserve gastric NO synthase when the mucosa is subjected to damage (Hisanaga et al., 1996). Formation of the mucoid cap during the process of restitution after hypertonic salt injury in rats has been attributed to the action of endogenous NO (Yanaka et al., 1995). The involvement of NO in the mechanism of the gastric alkaline response after damage by 1 M NaCl has also been observed (Takeuchi and Okabe, 1995). It is possible that NO could fulfil these functions as the presence of nNOS has been demonstrated in the gastric surface cells and eNOS has been detected in blood vessels in the lower region of the gastric glands and in the sub-mucosa (Price et al., 1996).

A balance is maintained in the gastric mucosa between aggressive and protective factors, which if destroyed will lead to the formation of lesions or ulcers. The gastric and duodenal mucosa are among the most rapidly proliferating tissues in the body, where surface cells exfoliated into the gastric lumen are quickly replaced. Exposure of the gastric mucosa to a damaging luminal agent will result in the upregulation of adaptive responses to prevent further damage, followed by its rapid repair. Full repair of damaged mucosa occurs by at least two separate mechanisms, the rapid process of mucosal restitution and the much slower replacement of lost cells by the process of cell division (Wang and Johnson, 1990c).

4.1.2. Restitution

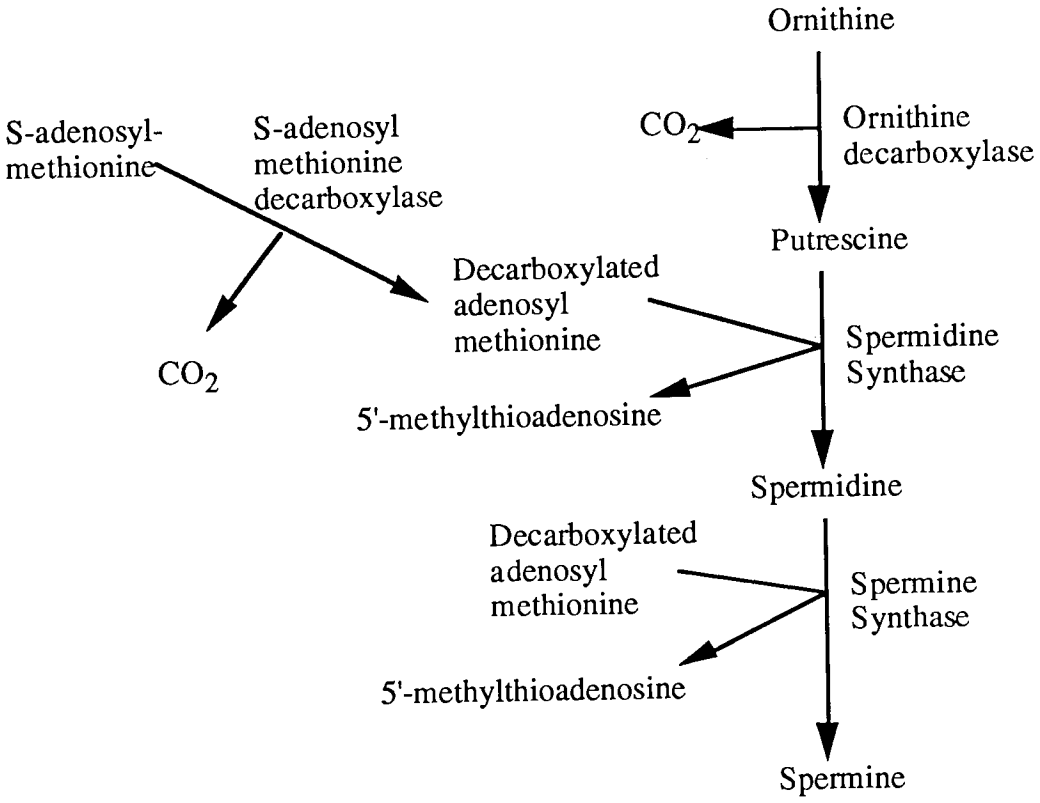
After the occurrence of superficial gastric mucosal damage, a process of re-epithelialization, named restitution, takes place to re-establish epithelial continuity and integrity (Silen and Ito, 1985). Restitution involves the migration of any remaining, viable epithelial cells from areas adjacent to and beneath gastric lesions to cover the denuded area. Restitution is reliant on the inherent properties of epithelial cells and requires that damage is limited to the surface and part of the gastric pit, whilst leaving the basal lamina intact. Restitution is an extremely rapid process, occurring within minutes in mammalian stomach *in vivo*, and therefore cannot be accounted for by cell replication. Several agents have been implicated in the process of restitution. Calcitonin-gene-related peptide, laminin and epidermal growth factor, have been shown to contribute to the restitution that occurs in response to damage with hypertonic saline in the rat gastric mucosa (Miller et al., 1994; Miller et al., 1995; Miller and Debas, 1995). Whereas restitution in the guinea-pig gastric mucosa in response to hypertonic saline involves TGF-ß (Yanaka et al., 1996) and NO (Yanaka et al., 1995).

4.1.3. Polyamines and the control of their synthesis.

The polyamines: putrescine, spermidine and spermine, play an essential role in cell growth and repair (Tabor and Tabor, 1984), and may also contribute to the process of restitution (Wang and Johnson, 1990c). Polyamines have been found to be distributed throughout all mammalian cells, where they exist in high concentrations which increase rapidly in growing tissues. Polyamines are polycationic metabolites, that possess, at physiological pH, protonated amino groups which are distributed at fixed lengths along a conformationally flexible carbon chain. Electrostatic interactions are set up between the positively charged polyamines and negatively charged cellular macromolecules such as nucleic acids which accounts for their membrane binding ability (Schuber, 1989). The activity of polyamine biosynthetic enzymes, and the subsequent synthesis of polyamines, are increased in response to a number of stimuli, such as growth factors, trophic hormones and tumour promoters (Ding et al., 1996). The first and rate limiting enzyme in the highly regulated polyamine biosynthetic pathway is ornithine decarboxylase (Pegg, 1986), which catalyses the conversion of ornithine to putrescine with the release of carbon dioxide. The initial substrate, ornithine, results from the action of arginase on the amino acid, arginine. Putrescine is converted first to spermidine and then to spermine by the respective actions of spermidine synthase and spermine synthase. The source of the propylamine groups

required for these conversions comes from the decarboxylation of S-adenosyl-methionine produced by the enzyme S-adenosylmethionine decarboxylase (Figure 4.1.3).

Figure 4.1.3. Pathway for polyamine biosynthesis



An increase in ornithine decarboxylase activity is one of the earliest biochemical indications of the onset of cell proliferation. The ornithine decarboxylase enzyme has a rapid turnover rate and changes in activity in response to various stimuli. Gastrin, an important hormonal regulator of growth in the gastrointestinal tract stimulated an increase in ornithine decarboxylase activity in rat duodenal mucosa (Wang et al., 1994). Epidermal growth factor, which provides mitogenic signals necessary for replication, healing and regeneration of damaged tissues, is capable of inducing ornithine decarboxylase in isolated rat gastric glands (Wojciechowski et al., 1995). Luminal amino acids, such as asparagine, increase ornithine decarboxylase activity and stimulate mucosal growth in intestinal cell lines (Wang et al., 1996). Parenteral administration of prostaglandin I_2 to fasted rats also results in ornithine decarboxylase induction (Kuwayama et al., 1993). Finally, ornithine decarboxylase activity exhibits a circadian rhythm, and food is one of the strongest

stimulants for ornithine decarboxylase induction and mucosal growth (Tanaka et al., 1993) in the rat small intestine.

The control of ornithine decarboxylase gene activation is highly complex and evidence has suggested transcriptional, post-transcriptional, translational and post-translational modes of regulation (Wang et al., 1996). The highly conserved, GC rich, promoter sequence for the ornithine decarboxylase gene contains multiple potential transcription factor binding sites of varying affinity where binding leads to the activation of the gene and the promotion of cell growth. The transcription factors Sp1, c-myc and AP-1 have been implicated in the regulation of the ornithine decarboxylase gene (Li et al., 1994; Kumar et al.; 1995, Mar et al., 1995). Mammalian ornithine decarboxylase has a short half life and is rapidly degraded and gene activity down regulation is associated with methylation in the promoter region (Wang et al., 1996). As it is possible that a cellular accumulation of polyamines might result in cytotoxicity or cellular transformation, ornithine decarboxylase activity is tightly regulated by negative-feedback control by its polyamine products. It is likely that polyamines regulate activity by causing the induction of an ornithine decarboxylase inhibitory protein by the mechanism of translational frameshifting (Hayashi et al., 1996). Antizyme is a 26.5 kDa protein that can reversibly bind to ornithine decarboxylase, thus causing its inhibition and playing a key role in the regulation of its synthesis Antizyme not only inactivates polyamine uptake, but stimulates ATP-dependent degradation of ornithine decarboxylase, a process catalysed by the 26S proteasome.

4.1.4. Gastric mucosal damage and ornithine decarboxylase activity.

If an increase in ornithine decarboxylase activity and polyamine synthesis is associated with increased mucosal growth, a similar increase might be expected to be associated with the cell division which accompanies mucosal repair following damage. Indeed, gastric mucosal damage inflicted by hypertonic saline (Furihata et al., 1984; Thirumalai et al., 1987; Ishibashi, 1992), corticosterone (Wang and Johnson, 1990b), and water-immersion stress (Wang and Johnson, 1990a) all lead to a marked elevation in ornithine decarboxylase activity. Intragastric administration of hypertonic saline results in irritation of the gastric mucosa, which leads to the release of endogenous NO which inhibits acid secretion (Takeuchi et al., 1994). A luminal alkalisation caused by increased bicarbonate movement into the lumen coupled with inhibition of acid secretion results in a beneficial microclimate for restitution of the damaged epithelium to occur. Taking

into account the ability of hypertonic saline to cause gastric mucosal damage resulting in an increased ornithine decarboxylase activity, as well as the release of NO leading to the reparative gastric alkaline response, it seemed reasonable to investigate a possible link between NO and ornithine decarboxylase induction. An involvement of NO in mediating the induction of ornithine decarboxylase in response to excitotoxin lesion has been shown in the cerebral cortex (Gardiner et al., 1994). Although there is no evidence of NO having a direct effect on transcription, it amplifies the effect of calcium signals on induction of c-fos in neuronal cells (Peunova and Enikolopov, 1993).

4.1.5. Experimental strategy.

The intention was to treat fasted rats with hypertonic saline *in vivo* in the presence and absence of inhibitors of NO synthase, and to measure both the change in ornithine decarboxylase activity and also mucosal damage. If these experiments were to satisfactorily investigate whether there was an involvement of NO in the change in ornithine decarboxylase activity, it was important first to establish whether NO synthase was present in the gastric mucosa of fasted rats, and secondly whether its activity was reduced by hypertonic saline. Gastric mucosal damage inflicted by intraluminal ethanol administration in rats is reported to reduce Ca^{2+}-dependent NO synthase (Tepperman and Soper, 1994), as does damage due to water immersion stress (Tachi et al., 1996). Clearly if hypertonic saline removed most of the NO synthase activity it would be difficult to propose a role for NO in changing ornithine decarboxylase activity. Finally, to ensure that the NO synthase inhibitor was active *in vivo* the effect of L-NA on stimulation of cyclic GMP content by carbachol was investigated.

4.2 METHODS

4.2.1 In vivo experimental procedures

This chapter investigates aspects of the response of the rat gastric mucosa to damage inflicted by hypertonic saline *in vivo*. The general pattern of the experimental protocol consisting of: subjecting an anaesthetised animal to the damaging agent or control solution for a set period of time, removing the tissue of interest and then assaying for chosen enzyme activity (either ornithine decarboxylase, acid phosphatase or NO synthase) or cyclic GMP content.

4.2.1.1 Anaesthetising and preparation of animal

Male Wistar rats (Bantin and Kingman, Hull, UK), of between 150 - 200 g body weight were used. Animals were fasted but allowed free access to drinking water, for 18 h prior to experimentation, so that the gastric mucosa could be fully exposed to the damaging agent. A maximum of 4 animals were used for a single experiment per day. Rats were weighed so that exactly the correct volume of anaesthetic (sodium pentobarbitone (Sagatal) 60 mg/kg i.p.) could be administered to maintain a level of anaesthesia in which the animal showed an absence of the usual reflexes, but was still breathing easily and regularly. This level of anaesthesia was maintained for a period of up to 4 h with a top up dose of anaesthetic after approximately 3 h. During the anaesthesia, rats were kept warm on a heated metal plate. Their temperatures were monitored throughout the experiment by means of a rectal probe.

4.2.1.2 Experimental protocol

Animals were weighed, given an identifying mark on the tail, and in most experiments dosed with a pharmaceutical agent or the vehicle 1 h prior to administration of the damaging agent. To achieve this, sterile solutions of either saline (0.9 g/l NaCl) or N^G-nitro-L-arginine (L-NA, 20 mg/kg made up in 0.9 g/l NaCl) were injected at 5 ml solution /kg rat body weight, into the intraperitoneal cavity. Rats were left for 1 h, fully conscious, for the treatment to take effect. At the end of this period animals were anaesthetised (section 2.4.2.1), and a small midline incision was made and gentle pressure was applied to the left side of the rat until the stomach came into view. The treatment (a sterile solution of either control 0.154 M (0.9 g/l) NaCl, or damaging agent 2.5 M NaCl, at 5 ml/kg) was then introduced into the stomach lumen by the means of a 26 gauge needle. Four different treatment regimes were used (Table 4.2.1.2). For cyclic GMP determinations a different treatment regime was used, consisting of intragastric

administration of either 0.154 M NaCl or carbachol (150 μg/kg in saline 5 ml/kg) to a rat for 5 min, before removal of tissue. For those experiments where it was necessary to retain stomach contents (for acid phosphatase assay, section 4.2.3), a ligature was used to tie off the pylorus before introduction of agent into stomach lumen. The body cavity of the rat was then stitched up using sterile sutures and left for either a 10 min or 4 h period (for acid phosphatase measurements, section 4.2.3, only a 10 min incubation period was observed) before removal of tissue or luminal contents for assay.

Table 4.2.1.2. Generalised treatment regime for in *vivo* protocol

	Treatment Number			
Type of treatment	1	2	3	4
Preliminary i.p. dose (5ml/kg)	0.154 M NaCl	0.154 M NaCl	L-NA (20 mg/kg)	L-NA (20 mg/kg)
	Rats left for 1 h for preliminary dose to take effect.			
Intra-gastric dose (5 ml/kg)	0.154 M NaCl	2.5 M NaCl	0.154 M NaCl	2.5 M NaCl
	Intra-gastric treatment left in anaesthetised rat for either a 10 min or 4 hour period.			
	Luminal contents collected for acid phosphatase assay. Homogenates prepared for ODC and NO synthase assays.			

N.B. No preliminary dosing used prior to NO synthase assay.

4.2.1.3. Collection of tissue and luminal contents prior to assay

At the end of the required time period, the rat was cut open again by removal of the stitches along the midline incision made previously for assay of ornithine decarboxylase and NO synthase. The stomach was carefully cut free, the non-glandular region was removed and the remaining stomach section was cut open along the greater curvature. The tissue was immediately washed in ice cold saline (NaCl, 9 g/l). A 10 % tissue homogenate (section 2.4.2.1) was prepared using the necessary homogenisation buffer (section 4.2.2 and 4.2.4). For determination of acid phosphatase (section 4.2.3), luminal contents were collected. Once the midline incision was reopened, the oesophagus was ligated and the stomach sac was removed intact by cutting proximal to the oesophageal and distal to the pylorus ligatures. The luminal contents were filtered through 150 μm mesh gauze, directly into a small, clean glass measuring cylinder, after which the contents were

transferred into the appropriately labelled centrifuge tube kept on ice. A record was made of the volume of luminal contents collected, after which the tubes were subjected to centrifugation at 10,000 x g_{av} for 5 min at 4°C and the supernatant used for assay.

4.2.2 Assay for ornithine decarboxylase activity

The ornithine decarboxylase (ODC) assay protocol followed is similar to that described by Thirumalai et al. (1987). The activity of the enzyme was estimated by a radiometric technique involving the trapping of $^{14}CO_2$ liberated from radiolabelled ornithine in the assay buffer, on a piece of filter paper impregnated with KOH and suspended above the reaction mixture. The assay procedure was verified using duodenal tissue taken from rats which were starved overnight and the following morning either refed for 8 h or were remained fasted. Alternatively, duodenal tissue was removed 3 h before the beginning of the dark period (i.e. 17.00 h) for analysis of ODC activity. The assay was also verified using a commercially obtained ODC enzyme.

4.2.2.1 Preparation of homogenates

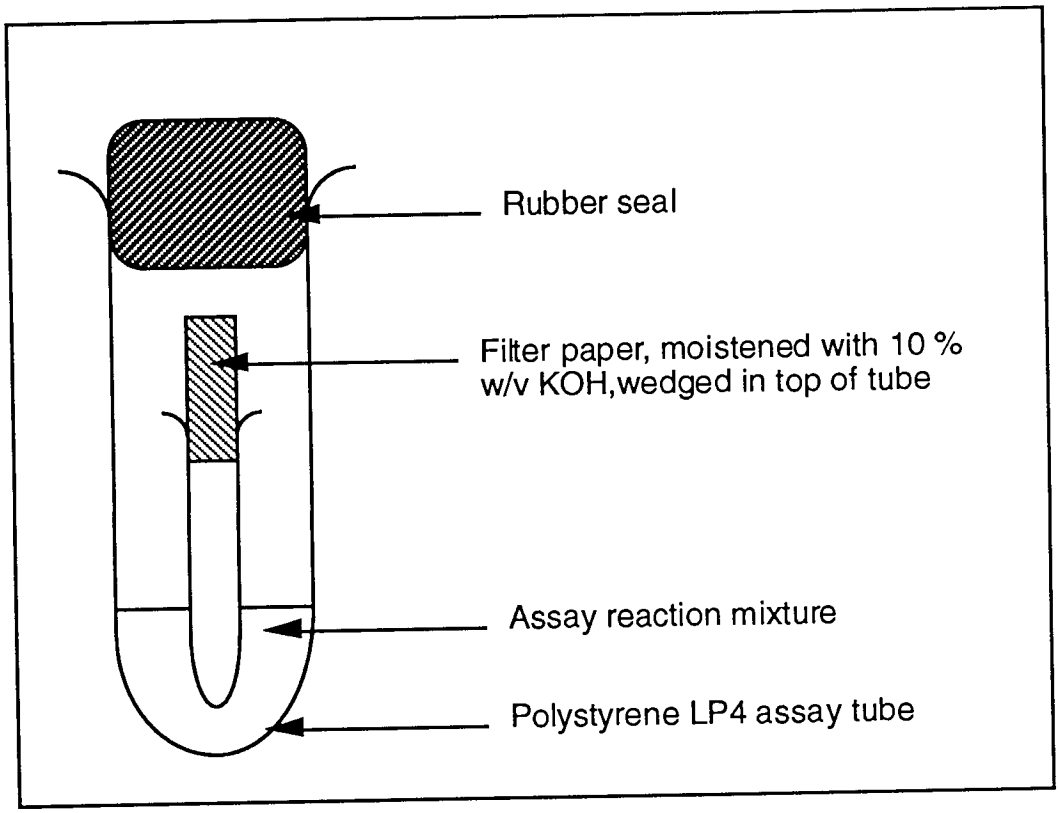
Tissue obtained (either duodenal or gastric mucosal from fed and fasted animals, or gastric mucosal from *in vivo* (4.2.1.2) protocol) was weighed, and a volume (ml) of ODC homogenisation buffer (25 mM TrisHCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.01 % v/v Tween 80, pH 7.4) was added which was equal to 10 x the weight (g) of the tissue (i.e. 10 ml buffer per 1 g tissue). The tissue was homogenised in the above buffer, on ice, for 30 s by an Ultraturrax homogeniser running at full speed. The resulting homogenate was centrifuged at 30,000 x g_{av} for 30 min at 4°C.

4.2.2.2 Ornithine decarboxylase assay protocol

Assay tubes were set up in triplicate and consisted of a piece of filter paper (4 mm x 20 mm), moistened with 10 µl of 10 % w/v KOH wedged in the top of a tall, thin test tube, placed inside a polystyrene LP4 tube (fig. 4.2.2.2). The assay was started with the addition of 200 µl supernatant homogenate to 50 µl assay solution (4 mM L-ornithine containing 4 µCi/ml L-^{14}C-ornithine) at the bottom of the LP4 tube. Assay tubes were capped with a rubber seal and shaken gently to mix the contents. Assay "blanks" were set up by the addition of homogenisation buffer with no enzyme to the full reaction mixture. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 200 µl 5 M HCl. This was introduced directly into

the reaction mixture by means of a long syringe needle piercing the rubber seal. Assay tubes, still sealed, were shaken and left to incubate for a further 20 min at 37°C. The filter paper from each tube was transferred to a labelled vial, 5 ml of Optiphase HiSafe II was added and the content of radiolabelled $^{14}CO_2$ determined by scintillation counting. The protein content of homogenates was determined using the Coomassie blue protocol (section 2.2).

Figure 4.2.2.2. Diagram of assay tube used for ornithine decarboxylase assay.



4.2.2.3 Calculation of ornithine decarboxylase activity

A mean dpm value was calculated for each set of triplicates. The control value obtained with homogenisation buffer alone, was subtracted from each mean dpm value.

Specific activity of L-[U-^{14}C]ornithine and ornithine decarboxylase activity was calculated in a similar way to the NO synthase assay (section 2.4.5)

Concentration of stock L-[U-^{14}C]ornithine = 56 mCi/mmol

Total ornithine in assay = 4 μmol/ml + 0.0714 = 4.071 μmol

Specific activity (dpm/μmol) = 2.181×10^6

Using the calculated value for specific activity of L-[U-^{14}C]ornithine, ornithine decarboxylase activity was calculated in the following way;

$$\text{Enzyme Activity (pmol/h/ml homogenate)} = \frac{\text{Mean dpm counts}}{\text{Specific activity}} \times \frac{\text{FV}}{\text{IT}} \times \text{FM}$$

Mean dpm counts = mean counts obtained from each set of triplicates
Specific activity (dpm/µmol) = activity of radiolabelled ornithine per µmol
FV = factor homogenate volume is multiplied by to give 1 ml.
IT = incubation time of assay (h)
FM = factor multiplied by to give value in pmol/h/ml
Therefore to calculate ornithine decarboxylase activity in the assay, the following values were put into the above equation;

$$\text{Enzyme Activity (pmol/h/ml homogenate)} = \frac{\text{Mean dpm counts}}{2.181 \times 10^6} \times \frac{5.0}{1.0} \times 1 \times 10^6$$

Activity was also expressed as pmol/min/mg protein. This was done by dividing enzyme activity by the protein concentration in homogenate (mg), obtained by performing the Coomassie blue protein assay.

4.2.3 Assay for acid phosphatase activity

Acid phosphatase is one of the many acid hydrolases which reside in lysosomes. When a cell is dead or damaged, its lysosomes rupture, and consequently the activity of the lysosomal enzyme acid phosphatase can be used as an index of gastric mucosal damage (Tepperman and Soper, 1994). The assay protocol used here is similar to that described by Brown (1994). The detection principle employed is based on an absorbance change due to the conversion of p-nitrophenylphosphate by acid phosphatase to p-nitrophenol, under acid conditions.

4.2.3.1. Acid phosphatase assay protocol

A volume of centrifuged luminal contents (section 4.2.1.3) obtained after a 10 min intra-gastric treatment (section 4.2.1.2) was used to assay for acid phosphatase activity. For each treatment six assay tubes were set up: two tubes for each of three time points. For assay "blanks" a 0.1 ml volume of 0.9 % NaCl (9g/l) was used instead of sample. To perform the assay a 0.1 ml volume of sample was added to 0.4 ml p-nitrophenylphosphate (1.33 mg/ml) in citrate buffer (0.1 M citric acid pH

4.8). Contents of assay tubes were vortexed and left to incubate for either 10, 30 or 50 min in a 37°C waterbath. The enzyme reaction was brought to a halt by the addition of 0.5 ml 1 M NaOH to each assay tube, and the tube was removed from the waterbath. After 50 min, the absorbance of each sample from each time point, was measured at 400 nm using a spectrophotometer. The extinction coefficient, ε (l/mole/cm), was calculated at three different concentrations of p-nitrophenylphosphate, and the mean from these calculations was used as the value of ε in subsequent experiments.

4.2.3.2. Validation of acid phosphatase assay for this investigation.

A pilot assay was performed to ensure that the intra-gastric treatments used in the *in vivo* protocol (section 4.2.1.2) did not affect the activity of acid phophatase. To do this a 10 % homogenate (homogenisation buffer: 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 6 μg/ml aprotinin, 0.1 mg/ml PMSF in 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.5) using a gastric mucosal scrape was prepared with tissue from an untreated rat (section 2.4.2.1). After centrifugation at 10,000 x g_{av} for 20 min at 4°C, the resulting homogenate supernatant was used to prepare two solutions, A and B. Solution A comprised a 1:1 ratio v/v, of supernatant with 0.9 % NaCl (9g/l), whereas solution B comprised a 1:1 ratio v/v, of supernatant with 2.5 M NaCl. The pH of these solutions was checked to ensure citrate assay buffer was maintaining the pH at 4.8. A 0.1 ml aliquot of each of these solutions was used instead of sample in the assay.

4.2.3.3 Calculation of acid phosphatase activity

A mean value for each set of absorbance readings was calculated and from this the mean value obtained with the "blanks" was subtracted. Using this corrected absorbance reading and the value ε, obtained earlier (section 4.2.3.1), the concentration of p-nitrophenol present in assay reaction mixture was calculated.

Using the formula: $A = \varepsilon \times C \times l$

where A = mean absorbance at 400 nm

ε = Extinction coefficient (l/mole/cm) = 15151

C = concentration (moles/l)

l = length of light path (cm)

the concentration of product formed can be calculated.

This is expressed as μg product in total volume using the following expression;

μg product in total volume = C x Mw x DF x VR x MF

where Mw = Mw of p-nitrophenol = 139.1

DF = dilution factor used in assay = 10

VR = volume luminal contents recovered (ml)

MF = factor multiplied by to give μg = 1000.

The amount of product formed (μg) due to acid phosphatase activity was plotted against each time point (min) for each treatment, to obtain a straight line. A value for the gradient of the line (μg p-nitrophenol formed in total volume per min) was obtained, and used as an index of acid phosphatase activity.

4.2.4 NO Synthase assay

The NO synthase assay was used to investigate the differences between fed and fasted rats. For each comparison two homogenates were prepared: one using a gastric mucosal scrape from a fed rat, the other using tissue from a rat fasted for a period of 18 h, but having free access to drinking water during that time period. In some experiments both the standard (section 2.4.3) and the enhanced (section 2.4.4) NO synthase assay protocols were used to establish enzyme activity. Homogenates were prepared as described previously (section 2.4.2) for the relevant assay.

The NO synthase assay was also used to establish enzyme activity in tissue taken from animals used in the *in vivo* protocol (section 4.2.1.2). For this set of experiments a control treatment of 0.154 M (0.9 g/l) NaCl at 5 ml/kg i.g. for 10 min was compared with the damaging agent, 2.5 M NaCl at 5 ml/kg i.g. for 10 min and for 4 h. The enhanced assay protocol (section 2.4.4) was used to establish enzyme activity. Homogenates used in the assay were prepared as described in section 2.4.2.2.

4.2.5. Cyclic GMP assay.

4.2.5.1. Principle of assay.

The cyclic GMP content of tissue pieces was determined using a commercially available enzymeimmunoassay kit (Amersham). The principle of the assay is

based on the competition between unlabelled cGMP and a fixed quantity of peroxidase-labelled cGMP for a limited number of binding sites on a cGMP specific antibody. As the amount of antibody and peroxidase-labelled cGMP is fixed, the amount of peroxidase-labelled ligand bound by the antibody is inversely proportional to the concentration of added unlabelled ligand. The kit consists of a 96-well microtitre plate with donkey anti-rabbit antibody immobilised at the base of each well. Either standard cGMP-peroxidase conjugate or unknown cGMP from sample will bind to rabbit anti-cGMP antibody in the assay reagent, which recognises the anti-rabbit antibody at the base of the well. The amount of peroxidase labelled cGMP bound to the antibody is determined by addition of the substrate, tetramethylbenzidine. Further addition of an acid solution results in a colour change, the absorbance of which can be read at 450 nm in a microtitre plate reader.

4.2.5.2. Sample preparation.

As described previously (section 4.2.1) rats were dosed with preliminary agent (0.154 M NaCl or L-NA at 20 mg/kg) 1 h prior to intragastric treatment with 0.154 M NaCl or carbachol (150 μg/kg in 0.9 % saline 5 ml/kg). After exposure to agent for 5 min, a section of whole wall was removed, weighed (between 120-200 mg) and frozen immediately in liquid nitrogen. Tissue sections must be rapidly frozen after collection so as to prevent alteration to the cGMP content before analysis. Extraction was achieved by adding a volume of 6% (w/v) trichloroacetic acid to the frozen tissue to give a 10% (w/v) homogenate. The homogenate was subjected to centrifugation at 2,000 x g_{av} for 15 min at 4°C, after which a 500 μl aliquot of supernatant was removed and added to 200 μl Freon/octylamine (1:1 ratio), and mixed well. 2 x 200 μl samples were removed from the upper aqueous layer, transferred to polypropylene LP3 tubes and dried under a stream of nitrogen at 60°C.

4.2.5.3. Assay protocol.

Reagent preparation and assay protocol were carried out according to the instruction booklet accompanying the enzymeimmunoassay kit. Briefly, the dried extracts were reconstituted into 1 ml assay buffer (0.05 M acetate buffer pH 5.8, 0.02% (w/v) bovine serum albumin, 0.005% thimerosal), and cyclic GMP standards ranging from 0-512 fmol/50 μl were prepared. A 100 μl volume of acetylation reagent (acetic anhydride:triethylamine in 1:2 ratio) was added to 1 ml of each standard and sample, and vortexed immediately. The assay was started by duplicate additions of 50 μl of standards and samples to the appropriate wells of the microtitre plate, followed by the addition of 100 μl antiserum (anti-cGMP

serum in assay buffer). Non-specific binding wells contained 200 µl assay buffer. The plate was covered and incubated at 4°C for 2 h, after which 100 µl peroxidase conjugate (cGMP-horseradish peroxidase in assay buffer) was added to each well. The plate was covered and a further incubation period of 60 min at 4°C was observed, followed by aspiration and washing of wells (4 x 400 µl washes with wash buffer: 0.01 M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween 20). After a further addition of 200 µl enzyme substrate (3,3'5,5'-tetramethylbenzidine /hydrogen peroxide in 20% (v/v) N,N-dimethylformamide), the plate was covered and incubated at room temperature with shaking for 30 min. The reaction was halted by addition of 100 µl sulphuric acid (1 M) to each well, after which the absorbance at 450 nm was determined.

4.2.5.4. Calculation of results

The average optical density (OD) for each set of replicate wells was calculated, and the percent bound for each standard and sample was calculated using the following formula:

$$\frac{\%B}{B_0} = \frac{\text{(standard or sample OD - NSB OD)}}{(B_0 \text{ OD - NSB OD})} \times 100$$

Where NSB = Non-specific binding.

A standard curve was constructed by plotting % B/B_0 against fmol cGMP standard per well. The cGMP content of samples (fmol/well) was then read directly from the graph.

4.3. RESULTS

4.3.1. Effect of fasting on nitric oxide synthase activity in rat gastric mucosal homogenates.

Basic or enhanced assay protocols (see 2.4.3 and 2.4.4) were used to compare NO synthase activity in gastric mucosal scrapes obtained from either fed or 18 h-fasted rats (Figure 4.3.1). Results were compared by an unpaired t-test. No significant differences between fed and fasted results were evident with the basic assay protocol or with either supernatant or pellet data obtained with the enhanced assay procedure. However, comparison of values obtained with whole homogenates in the basic assay and 100,000xg supernatants in the enhanced assay resulted in a significant, ($P < 0.05$) effect of assay (paired t-test) for both fed and fasted rats.

4.3.2. Effect of NO synthase inhibitor N^G-nitro-L-arginine (L-NA) on the intragastrically administered carbachol stimulated increase in cyclic GMP content.

Intragastric treatment with 0.154 M NaCl for 5 min in anaesthetised rats treated 1 hour previously with isotonic saline i.p. resulted in a basal cyclic GMP content of 1.61 ± 0.12 fmol/mg wet weight (Figure 4.3.2). Intraluminal administration of carbachol (150 µg/kg in 5ml/kg in isotonic saline) in rats resulted in a significant increase in cyclic GMP content above control levels as analysed by one-way analysis of variance followed by Dunnett's test. Cyclic GMP content was reduced back to control levels when carbachol was administered to rats pretreated one hour previously with L-NA (20 mg/kg i.p.).

4.3.3. Effect of intragastric treatment with 2.5 M NaCl on NO synthase activity of rat gastric mucosa.

Intragastric treatment with 2.5 M NaCl for 4 h resulted in some haemorrhagic damage, as determined by macroscopic examination by eye. Such effects which were absent from the stomach of rats treated for either 10 min or 4 h periods with intragastric 0.154 M NaCl. No differences in either total or Ca^{2+}-independent NO synthase activity were evident between rats treated with 2.5 M NaCl and 0.154 M NaCl (Single factor analysis of variance)(Figs. 4.3.3.1, 4.3.3.2). Ca^{2+}-independent enzyme activity was defined as that remaining in the presence of 1 mM EGTA.

4.3.4. Effect of intragastric 2.5 M NaCl on ornithine decarboxylase activity in gastric mucosa of rats pretreated with or without an inhibitor of NO synthase.

Ornithine decarboxylase activity was 14.49 ± 6.31 pmol/h/mg protein in rat gastric mucosa after pretreatment with 0.154 M NaCl i.p. followed by intragastric treatment with 0.154 M NaCl for 4 h (treatment 1 (T1); for treatment regime refer to table 4.2.1.2). There was no significant difference in activity from control when the intragastric treatment was exposure to 2.5 M NaCl for 4 h (T2) or when pretreatment was 20 mg/kg L-NA i.p. followed by intragastric 0.154 M NaCl (T3). However, the combination of pretreatment with 20 mg/kg L-NA and intragastric exposure to 2.5 M NaCl (T4) resulted in a substantial increase in ornithine decarboxylase activity above basal level (Fig. 4.3.4.1). Pretreatment with the inactive enantiomer D-NA at 20 mg/kg in combination with intragastric 2.5 M NaCl gave an ornithine decarboxylase activity similar to basal levels. The concentration dependence of L-NA in eliciting the increase in ornithine decarboxylase activity in response to 2.5 M NaCl was demonstrated with L-NA at 2 mg/kg and 6.3 mg/kg. Both these concentrations were capable of increasing ornithine decarboxylase activity above basal levels (Fig. 4.3.4.2), but not in the region of that obtained with the inhibitor at 20 mg/kg.

Samples were produced for analysis of ornithine decarboxylase mRNA by Northern blotting by Dr. J. De Belleroche (London), but no definitive signal was obtained.

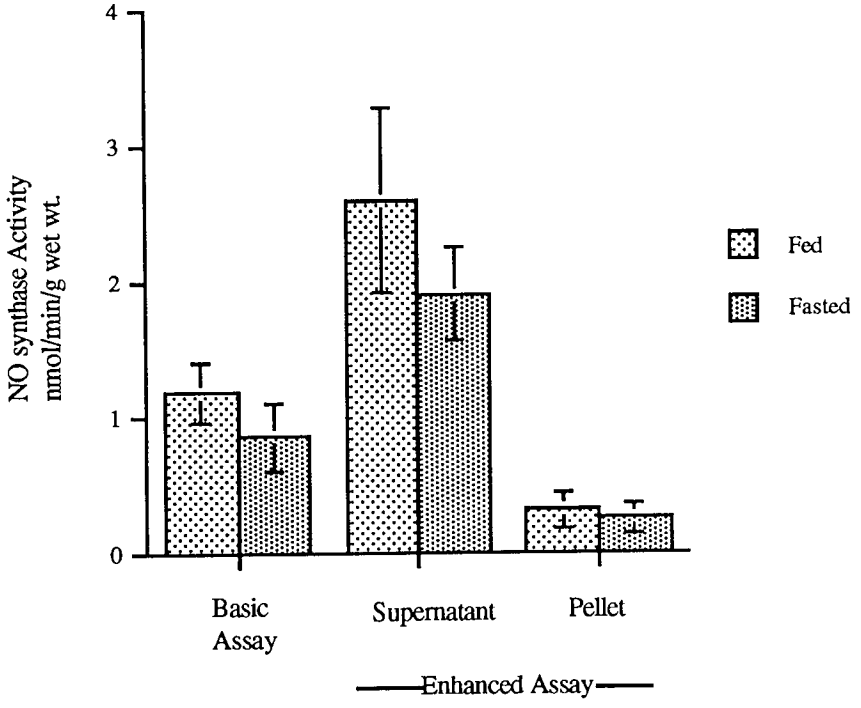
4.3.5. Effect of intragastric treatment for 10 min with 2.5 M NaCl on acid phosphatase activity and the volume of the luminal contents.

A control value for acid phosphatase activity was established by pretreating with 0.154 M NaCl i.p. followed by intragastric administration of 0.154 M NaCl (T1). An increase in acid phosphatase activity above control levels was found if 2.5 M NaCl was administered whether or not animals had been pretreated with L-NA (fig. 4.3.5, graph A). The acid phosphatase activity in tissue from rats subjected to intra-gastric treatment with 0.154 M NaCl was very similar in both the presence and absence of L-NA. This result indicates that the hypertonic saline, and not the NO synthase inhibitor, is responsible for the increase in luminal activity.

However, the NO synthase inhibitor L-NA did have a significant effect on the volume of luminal contents recorded compared to appropriate controls both when 2.5 M NaCl or 0.154 M NaCl were present in the gastric lumen. A significant effect of 2.5 M NaCl versus 0.154 M NaCl in the gastric lumen was only seen when rats had been treated with L-NA (Fig. 4.3.5.B).

Figure 4.3.1

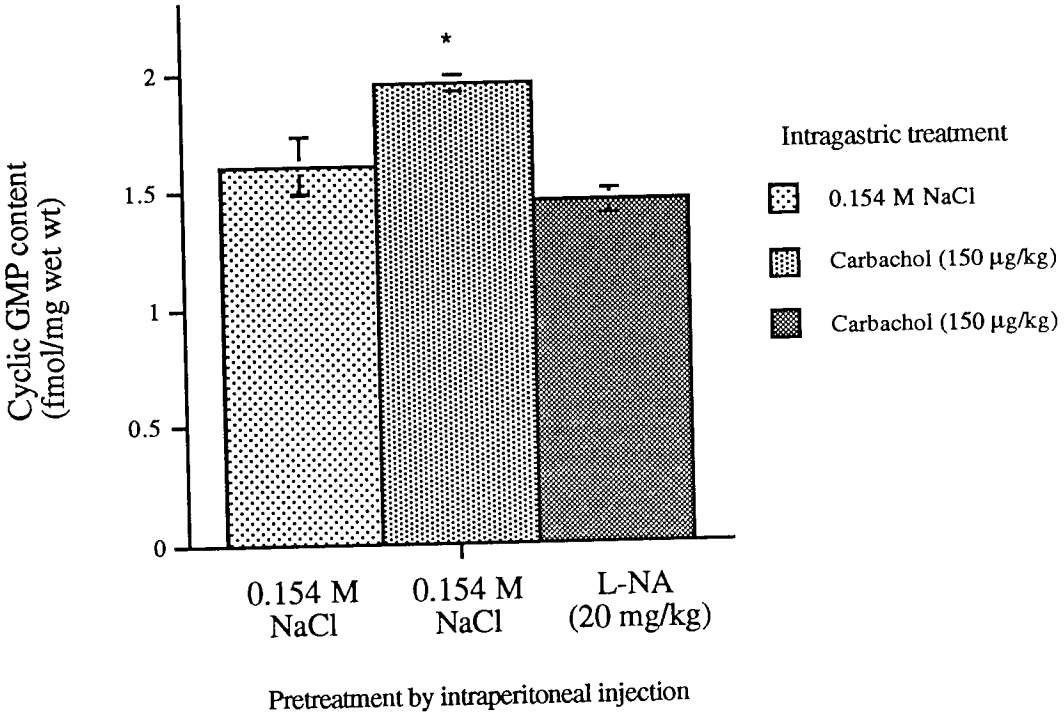
Effect of fasting for 18 h on nitric oxide synthase activity in rat gastric mucosal homogenates as determined by both basic and enhanced assay protocols.



Basic or enhanced assay protocols were used to compare NO synthase activity in gastric mucosa obtained from either fed or 18 h-fasted rats. Results are means ± S.E.M. for 7 animals for each basic assay determination using whole homogenates and 3 animals for each enhanced assay determination using 100,000 xg supernatant and pellet. Results compared by an unpaired t-test showed no significant effect of fasting. However, comparison of values obtained with the basic assay and the supernatant from the enhanced assay by a paired t-test resulted in a significant effect of assay ($P < 0.05$).

Figure 4.3.2

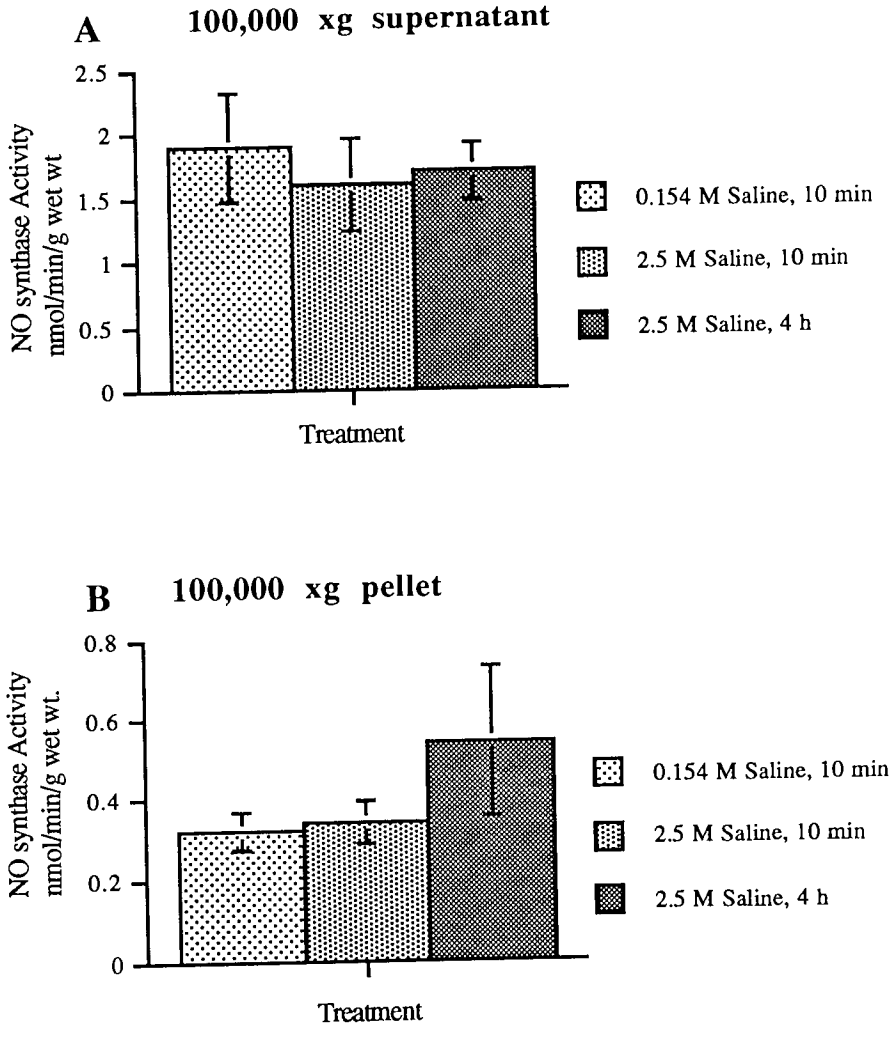
Effect of NO synthase inhibition with L-NA pretreatment on the intragastric carbachol stimulated increase in cyclic GMP.



The cyclic GMP content was determined in sections of rat gastric whole wall removed from rats pretreated (i.p. injection) with either 0.154 M NaCl or L-NA (20 mg/kg) 1 hour before intragastric treatment for 5 min with either 0.154 M NaCl or carbachol (150 µg/kg in saline 5 mg/kg). Data are means ± S.E.M from 4-5 rats for each condition. *P<0.05 for difference from control by Dunnett's test after one way analysis of variance.

Figure 4.3.3.1

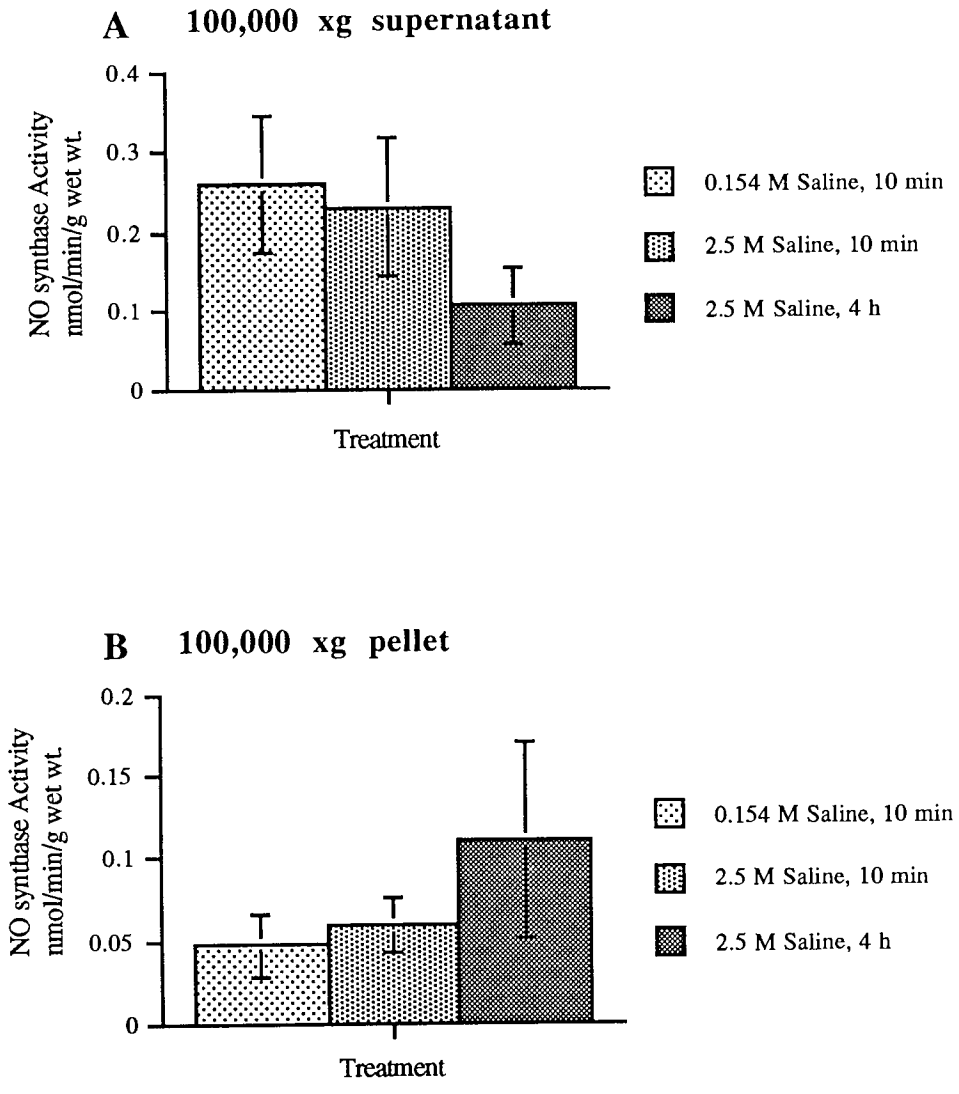
Effect of intragastric treatment with 0.154 M or 2.5 M NaCl in anaesthetised rats on total NO synthase activity in gastric mucosa.



The enhanced NO synthase assay protocol was used to establish supernatant (A) and pellet (B) enzyme activity in rat gastric mucosal homogenates after intragastric treatment with 0.154 M NaCl for 10 min or 2.5 M NaCl for 10 min and 4 h. Single factor analysis of variance was performed to compare each treatment but no significant effect of treatments was found. Results are means ± S.E. from 4 rats.

Figure 4.3.3.2

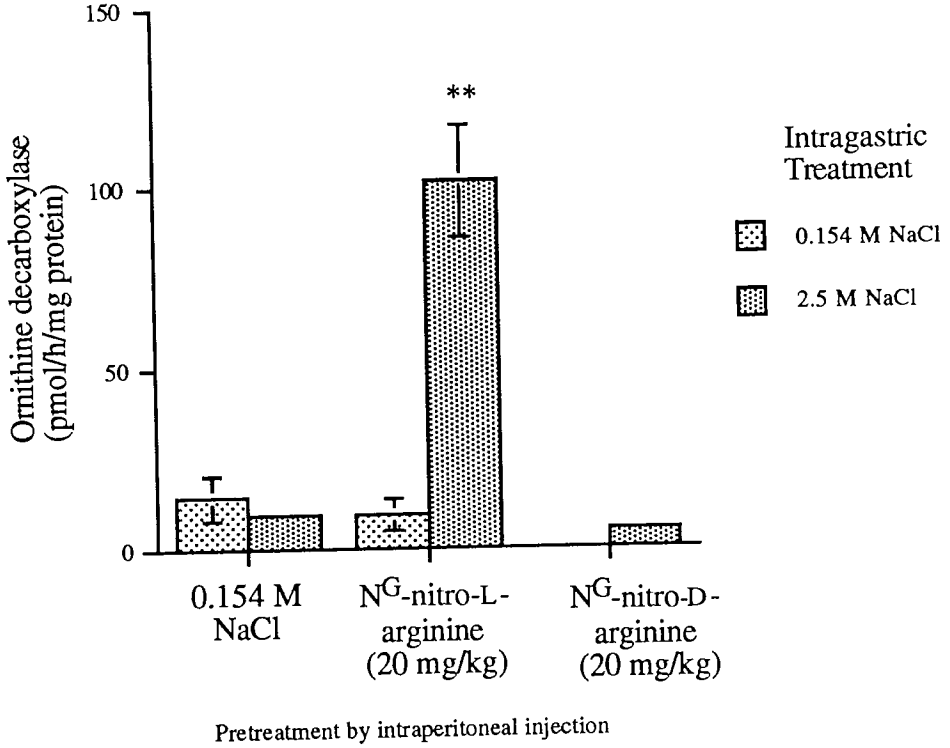
Effect of intragastric treatment with 0.154 M or 2.5 M NaCl in anaesthetised rats on Ca^{2+}-independent nitric oxide synthase activity in gastric mucosa.



The enhanced NO synthase assay protocol was used to establish supernatant (A) and pellet (B) Ca^{2+}-independent enzyme activity in rat gastric mucosal homogenates after intragastric treatment with 0.154 M NaCl for 10 min or 2.5 M NaCl for 10 min and 4 h. Ca^{2+}-independent enzyme activity is that remaining in the presence of 1 mM EGTA. Single factor analysis of variance was performed to investigate any effect of treatment but no significant effects were found. Results are means ± S.E. for 4 rats.

Figure 4.3.4.1.

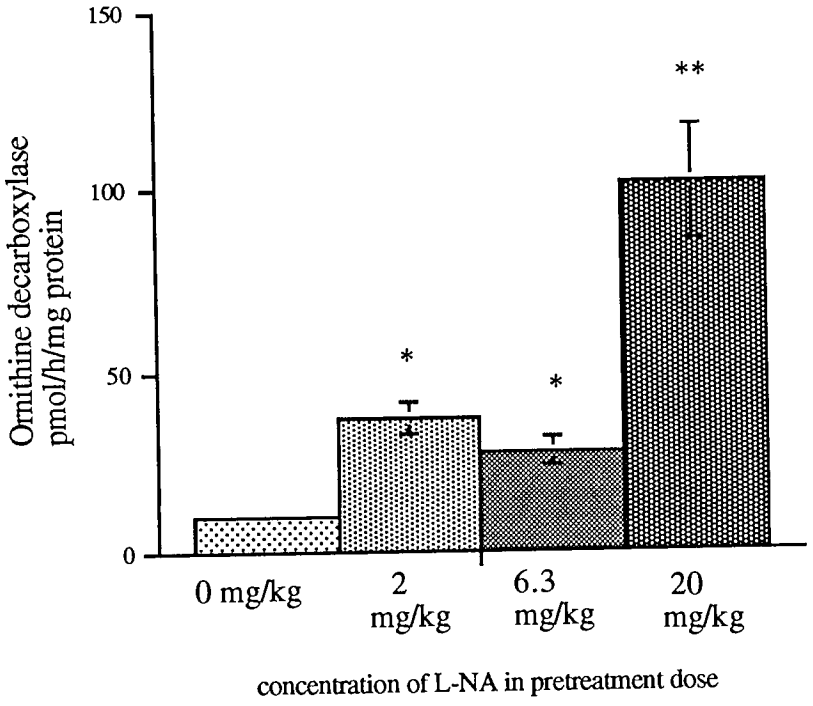
Effect of intragastric treatment for 4 h with either 0.154 M or 2.5 M NaCl in anaesthetised rats on ornithine decarboxylase activity in gastric mucosa.



Rats were pretreated with intraperitoneal 0.154 M NaCl, or L-NA or D-NA, at 20 mg/kg. 1 hour later intragastric administration of 0.154 M NaCl or 2.5 M NaCl took place in an anaesthetised animal. Each result is mean ± S.E.M. for six separate animals. **P<0.01 by a Kruskal-Wallis test followed by a non-parametric equivalent of Dunnett's test for comparison with the control result obtained with intragastric and i.p. 0.154 M saline. Where error bars are not visible they were too small to show up.

Figure 4.3.4.2.

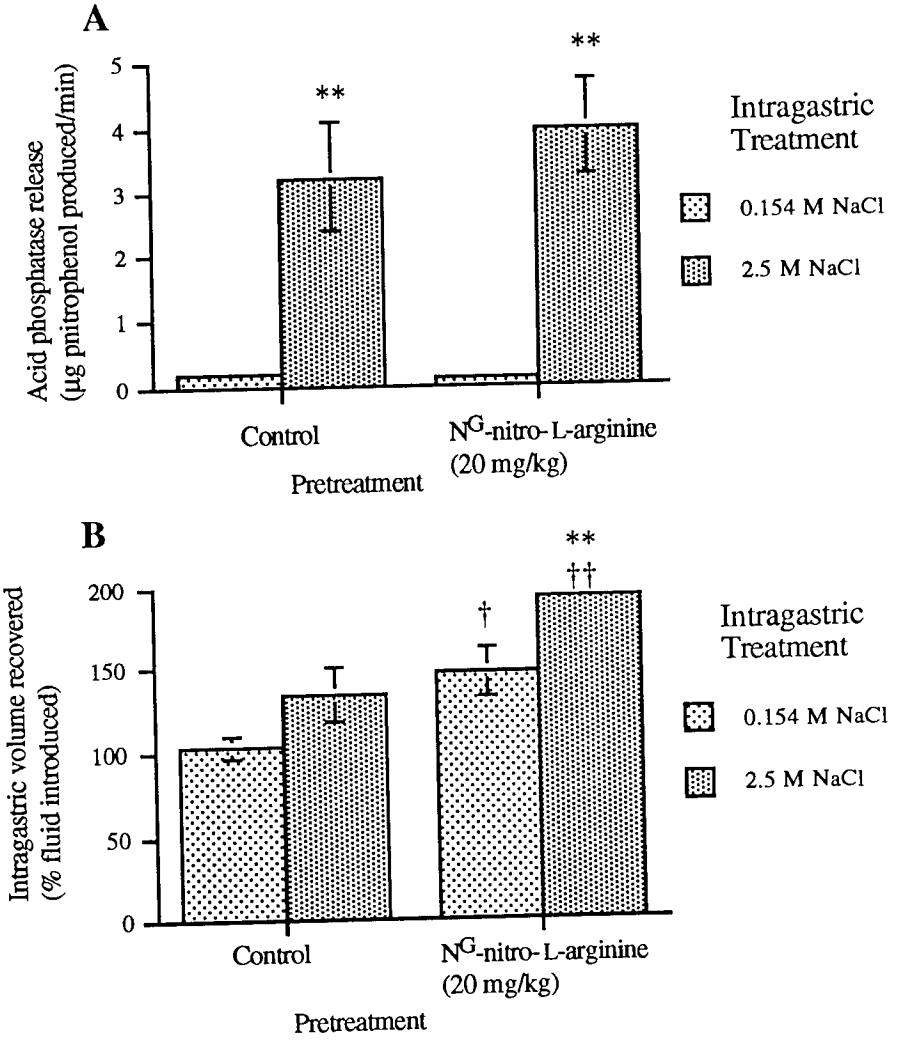
Effect of the dose of L-NA used for pretreatment of rats on ornithine decarboxylase activity in gastric mucosa taken from anaesthetised rats subjected to intragastric treatment with 2.5 M NaCl for 4 h.



Rats were pretreated with either 0.154 M NaCl, or L-NA at 2 mg/kg, 6.3 mg/kg or 20 mg/kg. 1 hour later intragastric administration of 2.5 M NaCl took place in an anaesthetised animal. Each result is mean ± S.E.M. for six separate animals. Data was analysed by a Kruskal-Wallis test followed by a non-parametric equivalent of Dunnett's test. * $P < 0.05$, ** $P < 0.01$ for effect of dose. Where error bars are not visible they were too small to show up.

Figure 4.3.5.

Effect of intragastric treatment for 10 min with either 0.154 M or 2.5 M NaCl in anaesthetised rats on acid phosphatase activity (A) and volume of luminal contents (B).



Rats were pretreated with agent or vehicle 1 hour prior to anaesthetic and intragastric administration of either 0.154 M or 2.5 M NaCl for 10 min. Luminal contents were collected and expressed as a % of the initial volume administered. Graph A shows acid phosphatase activity. ** $P < 0.01$ for effect of 2.5 M NaCl vs 0.154 M NaCl with the same pretreatment. No effect of L-NA was found in either the presence or absence of 2.5 M NaCl. Graph B shows % volume recovered where ** $P < 0.01$ for effect of 2.5 M NaCl vs 0.154 M NaCl in the presence of L-NA and †† $P < 0.01$, † $P < 0.05$ for effect of pretreatment with L-NA on the result obtained with the same intragastric treatment. Each result is mean ± S.E.M. for 4-6 separate animals which was analysed by ANOVA followed by Student-Newman-Keuls multiple comparisons test. Where error bars are not visible they were too small to show up.

4.4. DISCUSSION

4.4.1. Pilot experiments.

The pilot experiments were conducted to determine the most suitable conditions under which to investigate damage by hypertonic saline of rat gastric mucosa. The presence of luminal contents provides a barrier to the potentially damaging treatment gaining access to the mucosal surface, a problem which can be avoided by the use of fasting. Consequently, rats were fasted for 18 h. Ornithine decarboxylase activity exhibits a circadian rhythm in the rat small intestine (Tanaka et al., 1993), and the onset of feeding is associated with a dramatic rise in activity (Kuwayama and Naito, 1993). NO synthase activity exhibited no similar variation, and there was clearly sufficient activity present to use fasted rats to investigate the involvement of NO synthase activity in mucosal responses to hypertonic saline.

Muscarinic acetylcholine receptor agonists are capable of raising intracellular Ca^{2+} in gastric mucosal cells (Seidler and Pfeiffer, 1991) and subsequently causing NO synthase activation. Instillation of carbachol into the rat gastric lumen *in vivo* stimulated an increase in mucus secretion, an effect which was inhibitable with NO synthase inhibitor L-NAME (Price et al., 1994). Evidence that carbachol stimulated NO synthase under these circumstances was provided here by the significant increase in the cyclic GMP content of gastric mucosa above basal, due presumably to activation of guanylate cyclase by NO (section 1.3). The ability of L-NA to inhibit the carbachol-stimulated increase in cyclic GMP supported the contention that L-NA had inhibited gastric NO synthase. L-NA was used at the same dose and for the same time period to inhibit gastric NO synthase in further experiments involving damage.

4.4.2. The effect of hypertonic saline on gastric mucosal NO synthase activity.

A number of adaptive responses occur in gastric mucosa exposed to hypertonic saline. Intragastric treatment of feline stomach with 2 M NaCl for 10 min resulted in extensive superficial damage, back-diffusion of acid, release of histamine and gastric hyperemia (Gislason et al., 1996). With anaesthetised rats, an increased gastric hyperemia and an increased gastric residual volume as well as exfoliation of surface cells was observed after 30 min treatment with 2 M NaCl (Endoh et al., 1993). These particular parameters were not quantified in this study, but areas of

mucosal haemmorhagic lesion were noticed after 4 h treatment with 2.5 M NaCl. The failure of intragastric 2.5 M NaCl after 10 min or 4 h to elicit any significant change in NO synthase activity in this study is somewhat surprising given the supposed location of nNOS in surface epithelial cells (Price et al., 1996). The exposure of rat stomach to 1 M NaCl for 10 min in an ex vivo chamber is suggested to increase endogenous NO production (Takeuchi et al., 1994). Another possibility is that after 4 h exposure to the damaging agent, no further loss of NO synthase activity was evident because an efficient process of restitution had begun to take effect. Mucosal restitution is a rapid event and therefore 4 h is a reasonable time span in which to expect the protective effects of restitution to be underway, as demonstrated by restoration of epithelial surface continuity between 2 and 4 h after a 10 min exposure to 1 M NaCl (Thirumalai et al., 1987). The rapid gastric mucosal healing ability was also shown by evidence of repair in rats after inducing stress by water immersion for 4 h (Wang and Johnson, 1990c). Considering a number of reports implicating a role for NO in restitution and the hyperemic response associated with damage (sections 1.7 and 4.1.1), after 4 h exposure to hypertonic NaCl compensatory changes in NO synthase activity might be expected. Finally, localised changes in NO synthase activity close to lesions may have taken place, however the entire surface mucosa was assayed for NO synthase in this study and results are indicative only of global activity. One possible explanation is that although the total amount of enzyme is reduced, that which remains is somehow permanently activated and remains so in the homogenate.

Intragastric administration of 40% w/v ethanol to rats for 1 h, resulted in a reduction in NO synthase activity (Tepperman et al., 1993). Similarly, a reduction in gastric NO synthase activity was observed in rats subjected to water immersion stress (Tachi et al., 1996), although this decrease did not reach significance until the 6 h time point. In a separate study rat gastric mucosal damage by water immersion stress resulted in both early and late changes in NO synthase activity (Hisanaga et al., 1996). After 30 min of damage a significant increase in activity was demonstrated, activity then returned to control levels after 1 and 3 h, which was followed by a decrease in activity after 6 h. A possible reason for the variable effect of damage on NO synthase activity in the literature may be the difference in damaging insult received. Water immersion is a highly stressful event and will involve a whole host of hormonal changes not necessarily related to events in the stomach, and while hypertonic saline is presumed to damage the mucosal surface (Ishibashi, 1992), ethanol has effects on the vasculature. Another potentially complicating factor is that damage might cause recruitment of neutrophils from the

vasculature and the expression of Ca^{2+}-independent iNOS activity although no evidence was obtained for that response to 2.5 M NaCl in this work.

4.4.3. Involvement of NO in the response of ornithine decarboxylase to damage with hypertonic saline.

The aim of this section was to determine the involvement of NO in the induction of ornithine decarboxylase in response to hypertonic saline, and to determine whether any effects could be dissociated from the degree of damage as assessed by release of acid phosphatase activity. If this dissociation was found then a specific effect of NO on the induction process might be indicated. Hypertonic saline alone did not induce ornithine decarboxylase activity in this study which is in direct conflict with results of other workers (Furihata et al., 1984, Thirumalai et al., 1987, Ishibashi, 1992, Ding et al., 1996). The methodology used here differs from previous reports in that the damaging agent was not introduced by gavage to a conscious rat but by a needle into the gastric lumen of an anaesthetised rat. The anaesthetic used here may have exerted some inhibitory effects on the induction of ornithine decarboxylase in response to hypertonic saline.

Despite these differences, the major novel finding of this study was that inhibition of NO synthase allowed the induction of ornithine decarboxylase in rat gastric mucosa in response to hypertonic saline. Repair of damaged gastric mucosa occurs too rapidly to be accounted for by cell division alone, which replaces lost cells slowly. Instead, noxious luminal agents are prevented from causing deeper necrotic damage by the mechanism of early mucosal restitution. A proportion of the newly synthesised polyamines function in restitution to stabilise cell membranes and provide a matrix for cell migration into damaged areas. The protonated amines of the polyamines are the functional groups involved in the cross linking and the promotion of healing (Wang and Johnson, 1990c). It might therefore be expected that the dramatic rise in ornithine decarboxylase activity observed with L-NA and hypertonic saline is a consequence of the damage inflicted by these agents, however this does not account for the lack of induction observed with hypertonic saline alone, which produced equivalent alkaline phosphatase release. The ornithine decarboxylase induction effect was likely to be a direct result of NO synthase inhibition, firstly because L-NA at the concentration used had been shown in a pilot experiment to be an effective inhibitor of gastric NO synthase, and secondly because the inactive D-enantiomer, D-NA, failed to reproduce such an effect. Finally the effect of L-NA was concentration dependent.

A link between NO and ornithine decarboxylase induction has previously been suggested (Gardiner et al., 1994) in the cerebral cortex. However, in this tissue NO was shown to have a positive effect on induction, as L-NA administration at time of neuronal excitotoxin lesion attenuated induction of ornithine decarboxylase.

Acid phosphatase activities and epithelial permeability measurements were taken as an index of gastric mucosal damage. The presence or absence of L-NA had no significant effect on the release of acid phosphatase activity in response to 2.5 M NaCl. This suggested that the severity of damage inflicted by 2.5 M NaCl was not increased after the removal of NO. However, the combination of L-NA and 2.5 M NaCl resulted in a significantly increased epithelial permeability, evidenced by the increase in luminal content volume, compared with 2.5 M NaCl alone. However interpretation of this result is complicated by an effect of L-NA alone on permeability. Changes in epithelial permeability had been previously observed in a number of cases, both in relation to gastric damage and NO synthase inhibitors. Gastric damage elicited by HCl in rat proximal duodenum led to a disturbance of mucosal integrity and an increase in mucosal permeability (Nylander et al., 1994). Administration of NO synthase inhibitor L-NAME into rat small bowel results in a rapid increase in epithelial permeability (Kanwar et al., 1994). Injury of feline small intestine by means of ethanol or endotoxic shock was enhanced by NO synthase inhibitors, and accompanied by a significant increase in mucosal permeability (Kubes, 1992).

The increased epithelial permeability attributed to NO synthase inhibition is probably due to mucosal mast cell activation. NO has an inhibitory effect on mast cells, and therefore removal of NO by use of L-NA will result in mast cell degranulation. This leads to the release of inflammatory agents such as platelet-activating factor and histamine which are known to increase epithelial permeability (Kanwar et al., 1994). Another mechanism by which NO synthase inhibitors may increase permeability is by the removal of NO reducing cGMP-dependent dephosphorylation of myosin light chain, thus causing endothelial cell contraction. The result is increased interendothelial cell junctions and microvascular permeability (Kubes, 1993). Superoxide production is a feature of various inflammatory conditions and it may be higher with L-NA, because NO is no longer available to react with superoxide. Superoxide is another factor capable of causing mast cell degranulation and the subsequent increase in permeability (Kanwar et al., 1994). Increased permeability may also occur in an effort to dilute the noxious luminal agent and thus minimise damage.

There are a number of potential mechanisms by which inhibition of NO synthase might have enabled the induction of ornithine decarboxylase. One explanation for the ornithine decarboxylase activity observed with L-NA and hypertonic saline is that the removal of the cytoprotective effects of NO (section 1.7) results in enhanced damage, consequently leading to increased enzyme induction. The degree of gastric damage inflicted by hypertonic saline has previously been shown to be directly in proportion to the subsequent increase in ornithine decarboxylase activity and polyamine synthesis (Thirumalai et al., 1987). Alternatively, NO synthase inhibition could either have caused the release of a local mediator which promoted induction of ornithine decarboxylase, or caused inhibition of release of a negative modulator of ornithine decarboxylase. Finally, NO synthase inhibition may have directly affected transcription or translation of the message for ornithine decarboxylase.

Evidence against an enhancement in surface damage by hypertonic NaCl, after inhibition of NO synthase, from this work was that acid phosphatase release into the gastric lumen was not increased by the administration of L-NA prior to hypertonic saline. Furthermore the presence of L-NAME did not affect the decline and recovery of potential difference in response to 1 M NaCl (Takeuchi et al., 1994) nor did a NO synthase inhibitor prevent the gastric hyperaemia induced by 2 M NaCl (Endoh et al., 1993). It is possible that NO may be having an inhibitory effect on ornithine decarboxylase gene activation, which is uncovered by the removal of NO and the subsequent increase in ornithine decarboxylase activity. The possibility of an inhibitory interrelationship between NO and polyamine metabolic pathways has been suggested previously where use of the NO donor sodium nitroprusside resulted in inhibition of ornithine decarboxylase activity in human colon carcinoma HT-29 cells (Blachier et al., 1996). Further work should be performed to investigate the action of local mediators involved in the induction of ornithine decarboxylase. One potential candidate is histamine which is implicated in mediating the induction or ornithine decarboxylase in the rat small intestine in response to ischemia and reperfusion (Fujimoto et al., 1995). In support of this, the release of histamine is enhanced by inhibition of NO synthase in gastric mucosa damaged by 20 mM taurocholate (Takeuchi et al., 1997). In conclusion, the possible physiological significance of this work is that NO negatively modulates a component of the proliferative response of the gastric mucosa to surface damage.

4.4.4. Summary

1. Gastric mucosal damage in fasted rats caused by exposure to 2.5 M NaCl for either 10 min or 4 h did not affect NO synthase activity measured subsequently in homogenates.

2. Inhibition of NO synthase by prior administration of L-NA, but not D-NA, allowed the induction of ornithine decarboxylase by intragastric 2.5 M NaCl.

3. The release of acid phosphatase into the gastric lumen in response to 2.5 M NaCl was not enhanced by prior inhibition of NO synthase, suggesting that NO might act to promote ornithine decarboxylase induction by a mechanism more specific than one involving gross damage to the mucosa.

CHAPTER 5

INDUCTION OF HEAT SHOCK PROTEIN 72 BY NO IN GASTRIC MUCOSAL CELLS

5.1 INTRODUCTION

5.1.1. Cellular protective systems.

Biological systems are not always subjected to ideal environmental conditions, and consequently have developed different strategies to deal with any adverse changes in their surroundings. An example of this is the heat-shock response which is universally conserved across all animal species (Ang et al., 1991). Cells respond to potentially lethal changes in the external environment, such as a sustained rise in temperature, by increasing expression of the heat shock genes. This is referred to as the heat shock or stress response and the resulting proteins as heat shock or stress proteins. The gastric epithelium is frequently exposed to physiological and chemical stressors derived from ingested food (Cross et al., 1984), and gastric mucosal cells provide a mucous coat in the stomach which is the first line of defence against these irritants. The mechanism by which these cells defend themselves against irritant induced injury is of interest. It is thought that heat shock proteins may play a role in the pathophysiology of gastric ulceration (Teramae et al., 1991), as tissue taken from gastric ulcers shows higher levels of the inducible heat shock protein transcript (HSP 72) as compared to normal human mucosa.

Another source of tissue or cell injury is the production of intracellular free radicals by noxious agents. Mammalian cells possess an array of antioxidant enzymes which act to detoxify such reactive species and prevent their harmful effects. The ubiquitous thiol glutathione, is involved in many cellular functions, including protection from oxidative stress. This chapter is concerned with an investigation of the possible protection of guinea-pig gastric mucosal cells by stress proteins and glutathione when exposed to exogenous NO.

5.1.2. The Heat Shock Response.

The existence of the heat shock response was first noted in *Drosophila melanogaster* (Ritossa, 1962), when a new pattern of polytene chromosomal puffing resulted from exposure of the fly's isolated salivary glands to a temperature above its physiologic norm. This puffing was due to the enhanced expression of genes encoding the group of proteins known as the heat shock proteins (HSP's). Subsequent studies have shown that there is an increased synthesis of this set of proteins when cells are subjected to a wide variety of insults including:

temperature shift, exposure to protein damaging agents, various ionophores, toxic metals, alcohol, metabolic poisons, agents that modify protein sulphydryls and oxidative stress (Minowada and Welch, 1995).

5.1.2.1. Induction of the heat shock response.

Hightower (1980), suggested that the heat shock response was initiated by the intracellular accumulation of abnormally folded proteins, and indeed injection of denatured proteins into frog oocytes activated this stress response (Anathan et al., 1986). The subsequent increase in stress protein levels results in the identification and degradation, or in less severe cases the correct refolding, of proteins adversely affected by the stress inducing agent (Ang et al., 1991). The heat shock proteins are often described as "molecular chaperones", and under normal conditions several operate to form complexes with proteins as they are synthesised and thus prevent misfolding or aggregation before translation is complete.

In higher eukaryotes the induction of the stress response is initiated by activation of a heat shock transcription factor (HSF). Two HSF's have been identified in humans: the monomeric HSF-1 and the dimeric HSF-2 (Schuetz et al., 1991). HSF-1 mediates the response to heat shock, whereas HSF-2 is involved in the control of heat shock gene expression during differentiation and development (Huang et al., 1994). HSF-1 is composed of two highly conserved regions: an amino terminal DNA-binding domain, and an adjacent trimerization domain with an array of three leucine zippers. When the cell is subject to stress there is a transition from the monomeric form to the activated homotrimeric state. NO may play a role in this conversion by catalysing the trimerisation of HSF, by accelerating the formation of disulphide bonds between HSF molecules (Malyshev et al., 1996). The stress response is mediated by the activated HSF translocating to the nucleus and binding to the heat shock promoter element (HSE) which is comprised of multiple nGAAn binding sites and located about 80-150 base pairs upstream from the RNA transcription promoter start site (Craig et al., 1994). This binding of transcription factor to the promoter leads to the rapid synthesis of stress proteins. The exact mechanism by which the stressed cell senses the accumulation of denatured or misfolded polypeptides which leads to the synthesis of stress proteins is unknown. A possible model is that under normal homeostatic conditions, HSF remains bound to one of the stress proteins which locks the factor in an inactive state. Under stressful circumstances, the available stress proteins are recruited, including those loosely associated with HSF, to bind to the accumulating denatured proteins. The released HSF is no longer maintained in an inactive form

and is able to trimerise, bind HSE and activate transcription (Georgopoulos and Welch, 1993).

5.1.2.2. The HSP 70 family and HSP 72.

Heat shock proteins are divided up into families on the basis of their molecular mass on SDS-PAGE (Schlesinger, 1990). The HSP 70 family includes the constitutively synthesised HSP 73 also known as cognate HSP 70, and HSP 72 which is sometimes referred to as the inducible form of HSP 70. The rapid synthesis of HSP 72 only occurs in a cell experiencing stress and was therefore the heat shock protein chosen for this study. Proteolysis and X-ray crystallography has revealed structural information on HSP 72 (Craig et al., 1994). The protein consists of two major domains: a highly conserved and compact amino terminal end and a more variable carboxyl portion. The amino terminal region comprises an ATP-binding site, which has weak ATPase activity, and a calmodulin binding site which is connected by a protease sensitive portion to the carboxyl-terminal region which is required for binding of peptides. The newly synthesised HSP 72 locates to the nucleolus in the stressed cell, and it is suggested that HSP 72 in the ADP-bound state recognises and binds short hydrophobic regions of amino acids in extended conformation. In the stressed cell protein remains stably bound to the HSP 72 chaperone, until a time when conditions are favourable for release, which occurs by ADP-ATP exchange. This is followed by ATP hydrolysis which returns the component to a high affinity state (Palleros et al., 1993).

5.1.2.3. Involvement of NO with induction of the heat shock response.

Several studies have also reported the ability of NO donors to induce a heat shock response. Injection of the NO donor dinitrosyl iron complex into rats was accompanied by HSP 70 accumulation in the heart, and addition of this donor to the Hep G2 human hepatoblastoma cell line also led to an increase in HSP 70 (Malyshev et al., 1996). Similarly, exposure of a primary culture of rat hepatocytes to the NO donor SNAP resulted in induction of HSP 70 mRNA and protein (Kim et al., 1997) and protection against apoptosis induced by tumour necrosis factor α. Endogenous NO synthesised after induction of iNOS has also been shown to lead to an increase in HSP 70 in isolated rat islet cells (Hao et al., 1996). 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). The aim of the present work was therefore to examine the response of gastric mucous cells to exogenous NO, and in particular to determine whether there was induction of a protective response as exemplified by the presence of heat shock protein.

Exogenous NO may be derived from a sustained release of NO by the inducible form of NO synthase present in neutrophils and macrophages, as associated with gastritis (Mannick et al., 1996), and possibly in adjacent mucous cells (Brown et al., 1994). In addition, nitrite in the gastric lumen may be converted to NO in the presence of acid (McKnight et al., 1997).

5.1.3. Prevention of the damaging effects of free radicals by antioxidant enzymes.

Naturally occurring free radicals are usually oxygen or nitrogen based reactive species with an unpaired electron (Lander, 1997). Free radicals such as these maybe generated as a result of normal cell metabolism or as a consequence of pathological conditions. If allowed to accumulate unchecked they are capable of causing cell damage. Thus treatment of a variety of cell types including gastric, hepatocytes, cardiac and muscle cells, with hydrogen peroxide resulted in DNA damage, loss of intracellular NAD^+, a reduction in intracellular ATP, activation of poly (ADP-ribose) polymerase, and a depression of the glycolytic pathway (Abe et al., 1995). Making use of these cell damaging properties, free radicals produced by macrophages and neutrophils perform a vital function in immunological host defence.

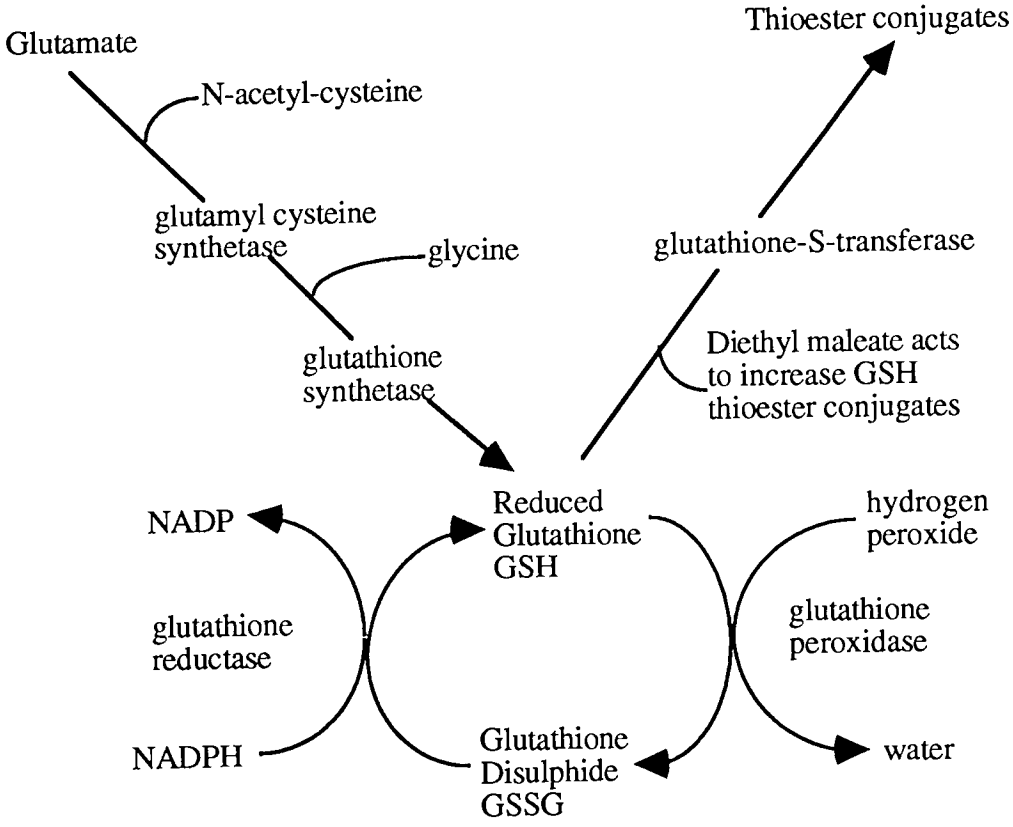
In times of oxidative stress, the potentially damaging effects of free radicals in mammalian cells are minimised by the action of antioxidant enzymes which are: enzymes of the glutathione redox cycle, catalase and superoxide dismutase (SOD). Catalase converts hydrogen peroxide to water and oxygen, and superoxide dismutase converts superoxide radicals to hydrogen peroxide (Munday and Winterbourn, 1989). The glutathione redox cycle is pertinent to the actions of NO and is discussed in more detail below.

5.1.3.1. The synthesis and function of glutathione

Glutathione is a sulphydryl containing tripeptide, accounting for the majority of intracellular non-protein thiols (Szabo, 1981). Important roles include involvement in: synthesis of proteins and DNA, transport, enzyme activity, metabolism and protection of cells (Meister and Anderson, 1983). Glutathione is formed from glutamate, N-acetyl-cysteine and glycine by the action of two enzymes. The enzyme γ-glutamylcysteine synthetase, in the presence of ATP, catalyses the formation of a peptide linkage between the γ-carboxyl group of glutamate and the amino group of N-acetyl-cysteine. This results in an acyl phosphate intermediate,

which by the action of ATP and glutathione synthetase, condenses with the amino group of glycine to yield glutathione. Glutathione can exist in either its reduced form (GSH) or its oxidised form, glutathione disulphide (GSSG). Glutathione is converted from its reduced form to the oxidised dimer by the action of glutathione peroxidase, and regeneration of GSH is catalysed by glutathione reductase (Figure 5.1.3.1). Diethyl maleate is an electrophilic agent which forms thioester conjugates with GSH, thus depleting the GSH cellular concentration, a process catalysed by glutathione-S-transferase (Mutoh et al., 1990).

Figure 5.1.3.1. Synthesis of glutathione and the glutathione redox cycle.



Glutathione peroxidase is an antioxidant enzyme with a seleno-cysteine active site, which has the ability to scavenge various peroxides. Glutathione peroxidase exists as three isoenzymes, of which the cellular isozyme is the best characterised. Glutathione peroxidase functions to reduce hydrogen peroxide or lipid peroxides to water or lipid alcohols with glutathione. By causing these conversions with the simultaneous conversion of GSH to GSSG, the enzyme plays a key role in the maintenance of cellular redox homeostasis and free radical detoxification.

5.1.3.2. The role of glutathione in the gastric mucosa.

In the gastric glandular mucosa the concentration of glutathione is in the region of 7-8 mM (Mutoh et al., 1991). This comparatively high concentration relative to other tissues suggests a possible role for glutathione in gastric mucosal resistance to damage. A protective role of glutathione in preventing ethanol-induced injury both *in vitro*, with cultured rat gastric mucosal cells (Mutoh et al., 1990), and *in vivo* (Miller et al., 1985) has been reported. Glutathione has also been shown to aid the defence of cultured rat gastric mucosal cells against acid-induced injury (Mutoh et al., 1991).

In primary cultures of guinea-pig gastric mucosal cells, specific proteins undergo S-thiolation when exposed to oxidative stress, an effect which is prevented by glutathione depletion (Rokutan et al., 1994). S-thiolation of proteins such as actin prevent irreversible reorganisation of microfilaments and thereby serve to protect normal function of cells when faced with oxidative threat. Glutathione may influence the physical properties of protective gastric mucus as it is composed of large molecular weight glycoproteins whose subunits are joined by disulphide bridges.

5.1.3.3. Possible role for reduced glutathione in the protection of gastric mucosal cells from the toxicity of NO.

Toxic effects of NO in rat and rabbit gastric mucosal cells have been shown to occur when iNOS is induced or when the cells are exposed to the NO donor SNAP, with the result of a decreased gastric cellular viability (Tripp and Tepperman, 1996; Wakulich and Tepperman, 1997). Damage by NO was exacerbated by depletion of intracellular glutathione (Wakulich and Tepperman, 1997). NO also has an inhibitory effect on glutathione peroxidase, the enzyme responsible for hydrogen peroxide removal, due to its ability to modify the selenium catalytic centre of the enzyme (Asahi et al., 1995). Removal of the detoxifying function of glutathione peroxidase could have potentially cell damaging consequences. An aspect of NO metabolism concerns its interaction with thiol-containing proteins, whereby a variety of high and low molecular weight nitrosothiols are formed, and the functions of the proteins are modified (Stamler, 1994; section 1.3). NO has the ability to react with GSH with the consequent formation of GSNO, and in this way NO may reduce the GSH/GSSG ratio, which may be a determinant of HSP 72 induction (Liu et al., 1996). For these reasons the role of reduced glutathione has been incorporated into the investigation of the induction of heat shock protein in gastric cells by NO.

5.1.4. Summary of aims.

1. To investigate whether exogenous NO could induce the formation of HSP 72 in gastric mucosal cells.
2. To investigate the involvement of glutathione in the above response of cells to exogenous NO.

5.2 METHODS

5.2.1 HSP 72 induction in a crude suspension of rat gastric mucosal cells

Rat gastric mucosal cells were resuspended in a volume of medium B' (Table 2.6.1.1) with the addition of the antibiotic gentamicin (50 μg/ml), to give a final concentration of 5 x 10^6 cells/ml (section 2.6). Aliquots of stock solutions of the NO donors, SNAP, GSNO and SIN-1, in the incubating medium B', were added to the appropriate 20 ml vials. 1.5 ml cell suspension was added and incubated for either 2 h or 4 h in a 37°C waterbath, with shaking at 120 cycles per min. Cell samples were resuspended and gassed with 95 % O_2, 5 % CO_2 after 2 h and 4 h of incubation. One vial of cells was subjected to heat shock by incubation at 43°C for a 3 h period, followed by a 1 h recovery period in a 37°C water bath, after which the cells were collected for analysis by immunoblotting. 1.0 ml of cell suspension was removed from each vial and placed in a microfuge tube. Cells were centrifuged for 10 sec at 10,000 xg_{av}, the supernatant decanted and the cell pellet resuspended in the required volume of hot 1 x electrophoresis sample buffer without DTT (62.5 mM Tris.HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS, 0.0125 mg/ml bromophenol blue) to give a concentration of 2 x 10^7 cells/ml sample buffer (section 2.5.1).

5.2.2 Preparation of a primary culture of guinea-pig gastric mucosal cells

Guinea-pig gastric mucosal cells were isolated using a modification of procedures described in the literature (Nakamura et al., 1991; Hirakawa et al., 1996). The procedure involved a preliminary, non-sterile, digestion phase using RPMI 1640 medium with additions, followed by a sterile culturing phase using complete RPMI 1640 containing 10 % foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2.5 μg/ml amphotericin B. To prepare media for the digestion phase, 100 ml RPMI 1640 was first gassed with 95 % O_2, 5 % CO_2 for 20 min, after which 0.2 % (w/v) bovine serum albumin (BSA) was added. The digestion media, one containing 0.5 mg/ml pronase E, the other containing 0.4 mg/ml collagenase Type I, were then prepared using the gassed RPMI 1640 containing 0.2 % (w/v) BSA.

Anaesthesia in a male Dunkin-Hartley guinea-pig (200-400g) was achieved with sodium pentobarbitone (60 mg/kg i.p.). The stomach was removed and opened by cutting along the greater curvature, and all of the stomach contents were removed.

The tissue was gently washed in ice-cold isotonic saline (9g/l NaCl) to remove all excess debris. A glass slide, having first been sterilised in 70 % v/v ethanol, was used to scrape all mucosal tissue away from the underlying muscle layer. The preparation was kept cold by performing the procedure on a glass plate over ice.

Once removed, the tissue was then minced finely using sharp, alcohol sterilised scissors in 5 ml RPMI 1640 medium containing 0.2 % bovine serum albumin in a small sterile petri dish. The resulting tissue and media were transferred to 50 ml Nalgene flask, which had been rinsed with ethanol and dried, using a plastic transfer pippete. The tissue was suspended in a further 30 ml RPMI 1640 containing 0.5 mg/ml pronase E. The flask was incubated in a water bath at 37°C with continual gassing of the air space with 95 % O_2 and 5 % CO_2 and shaking at 140 cycles per minute for 20 min. At the end of the incubation period the contents were centrifuged at 100 x g_{av} for 1 min at 15°C in sterile 30 ml universal tubes, the supernatant was then discarded and tissue pieces were resuspended in 30 ml RPMI 1640 containing 0.4 mg/ml collagenase. A further incubation period of 20 min followed, again with shaking and gassing of the air space as described previously. Then the flask contents were filtered through 150 μm nylon mesh (sprayed with alcohol and dried prior to use) into sterile centrifuge tubes. This procedure was carried out under sterile conditions in a cell culture hood. Filtered cells were centrifuged at 250 x g_{av} for 3 min at 15°C. Resulting cell pellets were resuspended in 20 ml fresh RPMI 1640 containing 10 % foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2.5 μg/ml amphotericin B. A small volume of the suspension was removed for cell counting before cells were centrifuged again at 250 x g_{av} for 3 min at 15°C. Cell numbers and viability was established by counting in a haemocytometer chamber and by trypan blue exclusion (section 2.3).

The cells were then resuspended under sterile conditions, in a volume of complete RPMI 1640 to give a concentration of 1.5 x 10^6 cells /ml. 2 ml of cell suspension was added to each well of a 6 well cell culture plate and cells were left to adhere and grow in a cell culture incubator at 37°C under 95% air 5% CO_2. Non-adherent cells and media were removed and replaced with fresh complete RPMI 1640 medium 24 h after the initial set up of culture. Cells were left to grow for a further 24 h before commencing experimental work.

5.2.3 General experimental protocol used with guinea-pig gastric mucosal primary cultured cells.

Primary cells were used for experimental purposes after 48 h of growth. At this point they had reached between 75-95 % confluence. At the beginning of each experiment non adherent cells and media were removed and replaced with 2.0 ml fresh complete RPMI 1640 medium per well with or without agents. In experiments with NO donors, stock solutions of freshly prepared NO donors were made using the relevant medium and cells were dosed with either control media or NO donor by addition of a further 0.5 ml/well. In each six well plate, two treatments were investigated using three wells per treatment. Cells were exposed to treatments for a period of 8 h before analysis. After incubation cells were harvested (section 2.5.1) by pooling from wells subjected to the same treatment, resulting in one sample per treatment.

5.2.4. Heat shock of HeLa cells and guinea-pig cells.

The monoclonal antibody used to probe for HSP 72 was raised against heat-shocked HeLa cells and exhibited a strong signal against protein from this source. HeLa cells were grown in six well culture plates and maintained in Dulbecco's MEM supplemented with 10 % foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. To heat shock either HeLa cells or guinea-pig primary cells, the six-well plate was placed in a separate cell culture incubator which had the temperature maintained at 43°C for a 3 h period, after which the cell plate was returned to the 37°C incubator for 1 h prior to cell collection. After a plate of cells had been subject to the relevant treatment, cells were collected for either electrophoresis (section 2.5.2) or reduced glutathione (GSH) (section 5.2.5) determinations. Cell samples were prepared for SDS-PAGE as described in section 2.5.1.

5.2.5. Analysis of guinea-pig primary culture samples

Guinea-pig primary culture samples were analysed as described in section 2.5., namely: electrophoresis by SDS-PAGE and transfer of proteins to nitrocellulose, followed by immunoblotting with HSP 72 antibody and ECL detection. Prior to electrophoresis, it was ensured that there was equal protein loading in each well on the gel, although there was variance in protein loading between gels (between 10

µg/well - 100 µg/well) according to the protein content in the particular set of samples. The monoclonal anti-72 kDa heat shock protein antibody (Amersham) was used to develop blots. This is a IgG_1 antibody raised in mice against 72 kDa heat shock proteins from HeLa cells. This antibody was incubated with nitrocellulose filters at a dilution of 1:1000. A secondary anti-mouse IgG peroxidase conjugated antibody (Sigma) was used at a 1:1000 dilution.

5.2.6. Assay for reduced glutathione

The majority of soluble-reduced sulphydryls in cells are composed of glutathione (Wong and Tepperman, 1994). Consequently, reduced glutathione levels were estimated by measuring total soluble-reduced thiol content without identifying the component sulphydryl species. The assay protocol followed is similar to one described by Mutoh et al. (1991). Medium was removed from culture plates and cells were washed with ice-cold phosphate buffered saline (PBS: 10 mM NaH_2PO_4, 9g/l NaCl, pH 7.5). The PBS wash was removed and cells were harvested by addition of 0.7 ml extraction buffer (PBS pH 7.5 containing 0.2 % Triton-X-100 and 2.5 % (w/v) sulphosalicylic acid) to each well. A cell scraper was used to scrape all cells into the extraction buffer, after which the plate was checked by microscopy to ensure cell removal. Cells from wells subjected to the same treatment were pooled to give one sample for analysis.

Cell extracts were transferred into microfuge tubes and sonicated at 50 W for 30 sec with tubes on ice using a Soniprep. Microfuge tubes were then subject to centrifugation at 10,000 x g_{av} for 5 min at 4°C. Assay tubes were set up in duplicates, containing 1.0 ml 0.3 M Na_2HPO_4 and 0.125 ml detection reagent (0.04 % w/v 5,5'dithiobis(2-nitrobenzoic acid), 1 % (w/v) sodium citrate). 0.5 ml of cell extract was added to the relevant assay tubes, the contents were mixed and 200 µl of each assay mix was transferred to a well in a 96 well plate. The absorbance was read immediately at 412 nm. Each sample was assayed in duplicate and the absorbance of each assay tube was read in duplicate, resulting in four absorbance readings for each sample.

Assay "blanks" were set up by the addition of 0.5 ml extraction buffer to 1.0 ml Na_2HPO_4 and 0.125 ml detection reagent. For each experiment a set of 6 GSH standards in the range of 0 nmol/ml to 50 nmol/ml were prepared in duplicate in assay tubes. This was achieved by making the relevant dilutions from a 0.25 mM standard stock of GSH in extraction buffer. The standards were assayed, as above,

by adding 0.5 ml of each standard to tubes containing assay mix. From the readings obtained with the GSH standards, a calibration curve of concentration plotted against absorbance at 412 nm was constructed. In some experiments cells from only two wells from the same treatment were pooled and cells from the third well were detached by trypsinisation (section 2.7) and were used to estimate cell numbers and viability by the trypan blue exclusion technique (section 2.3).

5.2.7. Periodic Acid-Schiff staining

To determine whether the guinea-pig primary cells cultured were of the mucus type, the Periodic Acid Schiff system of staining was employed. This involved removing and fixing cells before staining using periodic acid solution and Schiff reagent.

To prepare Schiff's reagent 100 ml distilled water was brought to the boil, and removed from the heat for a few sec before 0.5 g basic Fuchsin was added. The solution was allowed to cool to 50°C, at which point 1.5 g sodium metabisulphite and 15 ml 1 M hydrochloric acid were introduced. The solution was mixed, cooled to room temperature and left overnight in the dark. The following day 0.5 g decolourising charcol was added, the solution was mixed and left for a few min before filtration. The final product was stored in the dark at 4°C.

Cells were removed from the plate by trypsinisation as described previously (section 2.7). The cells were transferred to microscope slides by subjecting 200 µl of primary cells at a concentration of 1 x 10^6 cells/ml to centrifugation at 300 x g_{av} for 5 min. Cytospins were fixed using 3 % paraformaldehyde in PBS for 10 min followed by several washes in PBS. Slides were then immersed in periodic acid solution (1 g/100 ml) for 20 min after which they were rinsed for 5 min in running tap water. Finally cytospins were placed in Schiff's reagent for 20 min and rinsed again in tap water for a further 10 min. Slides could then viewed using a light microscope.

5.3 RESULTS

5.3.1. Effect of incubation with NO donors on amount of HSP 72 in a suspension of rat gastric mucosal cells.

The presence of HSP 72 in rat gastric mucosal cells after a 4 h incubation period with the following NO donors: 1 mM SNAP, 1mM GSNO and 100 μM SIN-1 was compared with that after 2 and 4 h control incubations of cells (Fig. 5.3.1). The monoclonal anti-72 kDa HSP antibody reacted with a 72 kDa protein in each lane, with the strongest signal exhibited with the HeLa control (lane 13) indicating that the immunoblotting procedure had been performed satisfactorily.

Despite some variability between the results of duplicate incubations, the NO donors at the concentrations and time periods used did not result in an obviously increased HSP 72 content as compared to appropriate controls (compare lanes 5 and 6 with 7 and 8 or 9 for example). When the result obtained with lane 12, where a sample was taken immediately after isolation without any incubation, was compared with the 2 h controls (lanes 1 and 2) and with the 4 h controls (lanes 5 and 6), it was seen that the HSP 72 signal increased with incubation time under control conditions. The results are from a single experiment.

5.3.2. Immunoblotting experiments with primary cultures of guinea-pig gastric cells.

5.3.2.1 Effect of incubation with NO donor in serum free medium on amount of HSP 72.

A pilot experiment was conducted to investigate whether to challenge cells with agents in either the presence or absence of serum. Primary cultures were exposed to NO donor (0.1 and 1 mM GSNO) in serum free medium for 4 or 8 h. The blot (Fig. 5.3.2.1) clearly shows that guinea-pig cells express HSP 72 in response to serum deprivation (compare lane 4 with lane 7). The bands seen in lanes 1-6 appear to be of similar intensity and no clear-cut effect of concentration of NO donor or incubation time was apparent in the absence of serum.

5.3.2.2. Effect of incubation with SNAP on the presence of HSP 72 under conditions capable of promoting superoxide generation or decreasing cellular glutathione.

These experiments were repeated three times, and the result obtained with one immunoblot is shown in Figure 5.3.2.2. Pyrogallol, at 0.1 mM, was used to give conditions under which superoxide generation was likely to be increased (Xie and Wolin, 1996), and diethyl maleate, at 1 mM, was used to decrease cellular glutathione (Mutoh et al., 1991).

Incubation with 1 mM SNAP for 8 h using culture medium containing 10 % foetal calf serum, resulted in an increased amount of HSP 72 as compared to controls. Addition of 0.1 mM pyrogallol did not alter the effect of SNAP on induction of HSP 72. Furthermore 0.1 mM pyrogallol alone did not appear to have an effect on the amount of HSP 72. With medium containing 1.0 mM diethyl maleate, there was an increase in the amount of HSP 72 compared to the control cells. Furthermore the effect of 1.0 mM SNAP was considerably enhanced (Fig. 5.3.2.2) by the addition of 1.0 mM diethyl maleate. When guinea-pig cells were subjected to heat shock (3 h at 43°C, followed by 1 h incubation at 37°C) there was induction of HSP 72 (Fig. 5.3.2.2, lane 8) the molecular weight of which was similar to the protein present in heat-shocked HeLa cells.

5.3.2.3. Effect of incubation with S-nitrosoglutathione on the amount of HSP 72 under conditions likely to increase superoxide generation or to decrease intracellular glutathione concentration.

Another set of three experiments were conducted to investigate whether the effect of a different NO donor, S-nitrosoglutathione (GSNO), on the amounts of HSP 72 were affected in the same way by pyrogallol and diethyl maleate as those obtained above with SNAP. The most obvious result was, as before (Fig. 5.3.2.3.), that the presence of diethyl maleate enhanced the effect of the NO donor (compare lanes 1 and 2 with 5 and 6).

5.3.2.4. Effect of concentration of SNAP on the amount of HSP 72 in cells incubated in the presence and absence of diethyl maleate.

In some experiments the combination of SNAP and 1.0 mM diethyl maleate caused substantial detachment of cells from the plate. Therefore diethyl maleate was reduced to 0.3 mM in further experiments. This experiment was repeated three times, and the immunoblot shown in figure 5.3.2.4 is a typical example of the results obtained. Lane 1 shows the weak basal signal exhibited under control conditions. Lanes 2, 3 and 4 show an increasing HSP 72 content with SNAP at 0.1,

0.3 and 1 mM. Lanes 6, 7 and 8 show that in the presence of 0.3 mM diethyl maleate the HSP 72 response increases with SNAP at concentrations of 0.1, 0.3 and 1 mM and is enhanced relative to the results obtained in the absence of diethyl maleate.

5.3.2.5. Effect of the NO scavenger carboxy PTIO and of actinomycin D on induction of HSP 72 by SNAP and diethyl maleate.

The transcriptional inhibitor actinomycin D (5 μg/ml) and the NO scavenger [2-(4-carboxyphenyl) -4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide potassium salt] (carboxy PTIO) (0.1 mM) inhibited the stimulatory effect of 1 mM SNAP and 0.3 mM diethyl maleate (Fig. 5.3.2.5). This experiment was repeated three times with similar results.

5.3.3. Effect of diethyl maleate, pyrogallol and NO donors on the protein content per well of the tissue culture plate.

In one series of experiments with GSNO at 1 mM (Fig. 5.3.3.1, graph A), there were no differences in protein content per well with treatment, but in another series of experiments use of SNAP at 1 mM in combination with 1 mM diethyl maleate (Fig. 5.3.3.1, graph B), resulted in an obvious reduction in the protein content per well when compared to control. If diethyl maleate was used at a reduced concentration of 0.3 mM, then SNAP had no effect on protein content per well (Fig. 5.3.3.2).

5.3.4. Effect of heat shock on reduced thiol content, viability and number of guinea-pig gastric cells attached to the culture plate.

Subjecting cells to heat shock resulted in a significant increase ($P < 0.05$) in reduced thiol content, but had no significant effect on either the viability or the number of cells attached to the plate (Fig. 5.3.4).

5.3.5. Effect of SNAP on reduced thiol concentration, viability and number of attached cells when incubated in the presence and absence of pyrogallol or diethyl maleate.

Since virtually all cellular reduced thiol exists as GSH (Mutoh et al., 1991) this will be referred to as the species assayed. Diethyl maleate alone, and in combination with SNAP, resulted in a significant reduction ($P < 0.05$ and $P < 0.001$ respectively) in GSH content from the control (Fig. 5.3.5.1). Only when diethyl maleate was present did SNAP exert a significant reduction of GSH content ($P < 0.05$, Fig. 5.3.5.1) there being no such effect of SNAP in the absence of other additions or in the presence of 0.1 mM pyrogallol. SNAP, neither in the presence nor absence of pyrogallol or diethyl maleate, did not affect the viability or number of cells attached to the culture plate (Fig. 5.3.5.2). Comparison of the control cell number result of 1.6 ± 0.31 millions with the result for diethyl maleate and SNAP of 1.1 ± 0.4 millions, suggests that some effect of diethyl maleate and SNAP may have been occurring, but this did not reach significance because of one experiment where no such detachment occurred.

5.3.6. Periodic-Acid Schiff staining of a primary culture of guinea-pig gastric mucosal cells.

Three days after plating the majority of cells harvested from the culture plate stained pink with the Periodic-Acid Schiff reagent (Plate 5.3.6).

Plate 5.3.6. Cytospins of guinea-pig gastric mucosal cells stained with Periodic-Acid Sciff reagent.

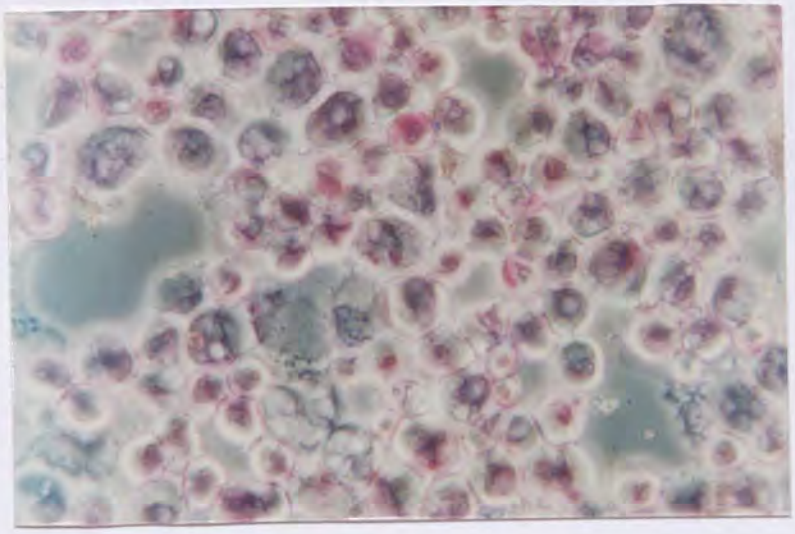
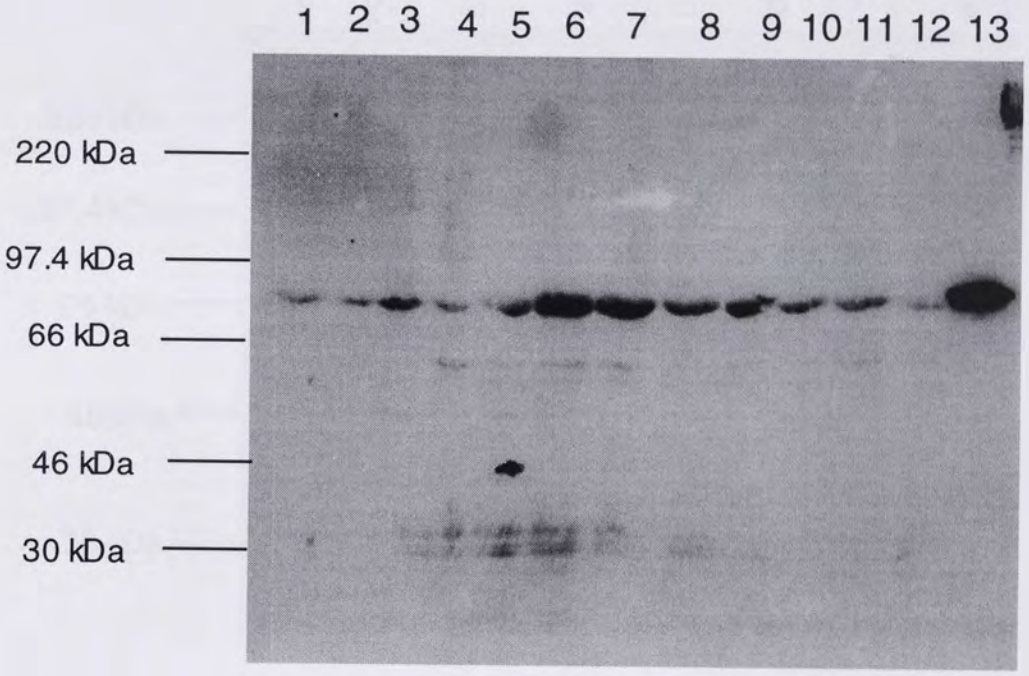
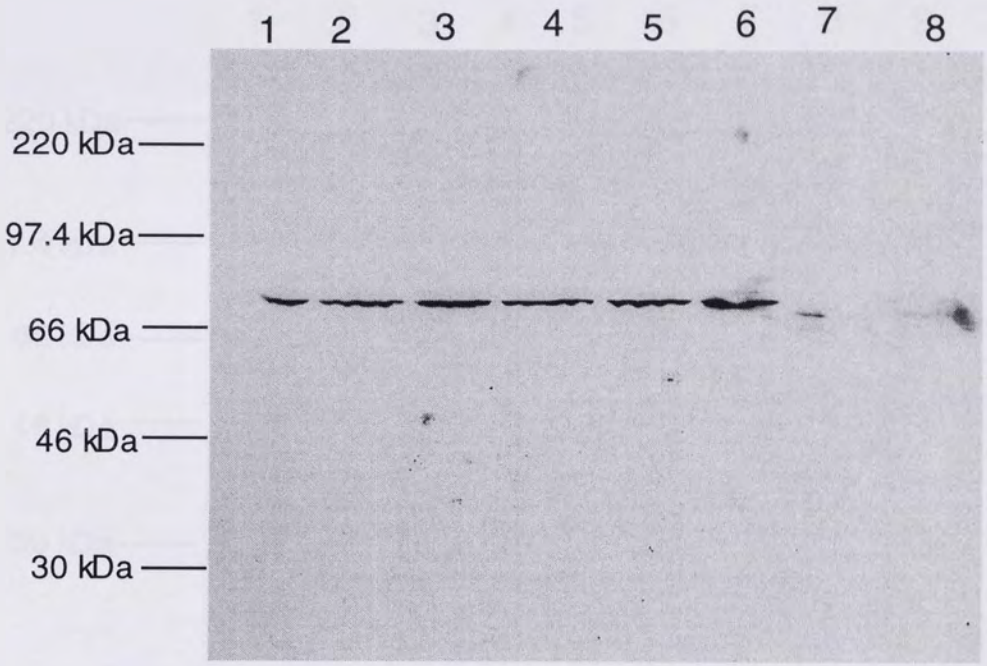


Figure 5.3.1.
Effect of incubation with NO donors on HSP 72 induction by a suspension of rat gastric mucosal cells.



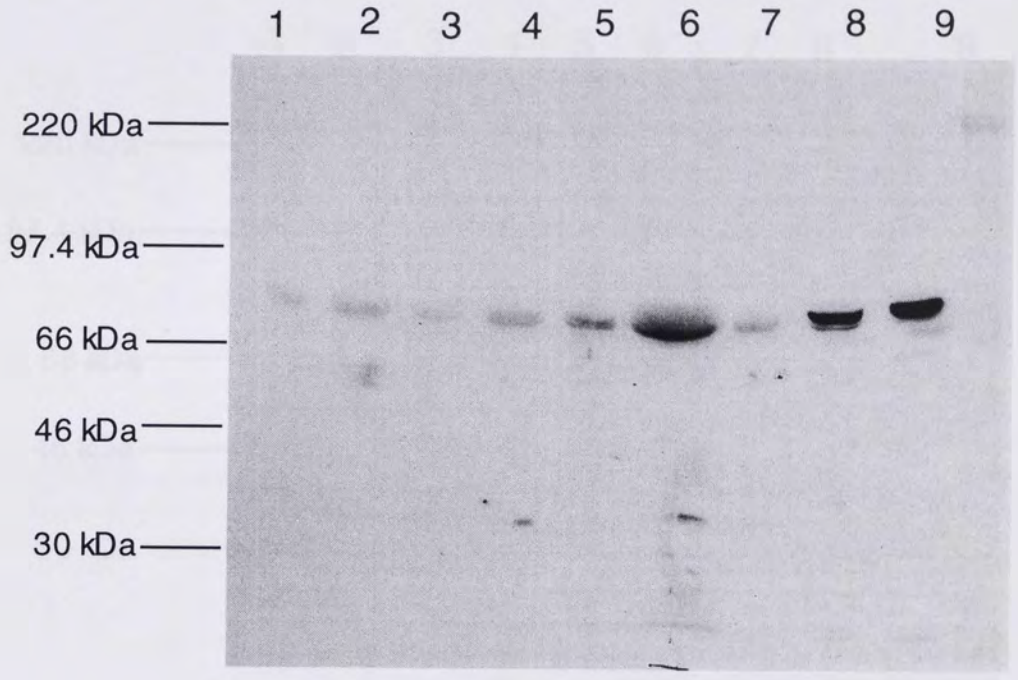
Individual vials containing 1.5 ml rat gastric mucosal cell suspension were exposed to NO donors for either 2 h or 4 h before measurement of HSP 72 by immunoblotting.
100 µg of protein was loaded to each lane.
Lanes 1 + 2, Control, 2 h; **Lanes 3 + 4**, SNAP (1 mM), 2 h; **Lanes 5 + 6**, Control 4 h; **Lanes 7 + 8**, SNAP (1 mM), 4 h; **Lane 9**, GSNO (1 mM), 4 h; **Lanes 10 + 11**, SIN-1 (100 µM), 4 h; **Lane 12**, Cell sample placed in sample buffer after isolation (equivalent to t = 0); **Lane 13**, Heat shock standard (HeLa cells incubated at 43°C for 3 h followed by 1 h at 37°C)

Figure 5.3.2.1.
Effect of incubation with NO donors in the presence and absence of serum on the amount of HSP 72 in a primary culture of guinea-pig gastric mucosal cells.



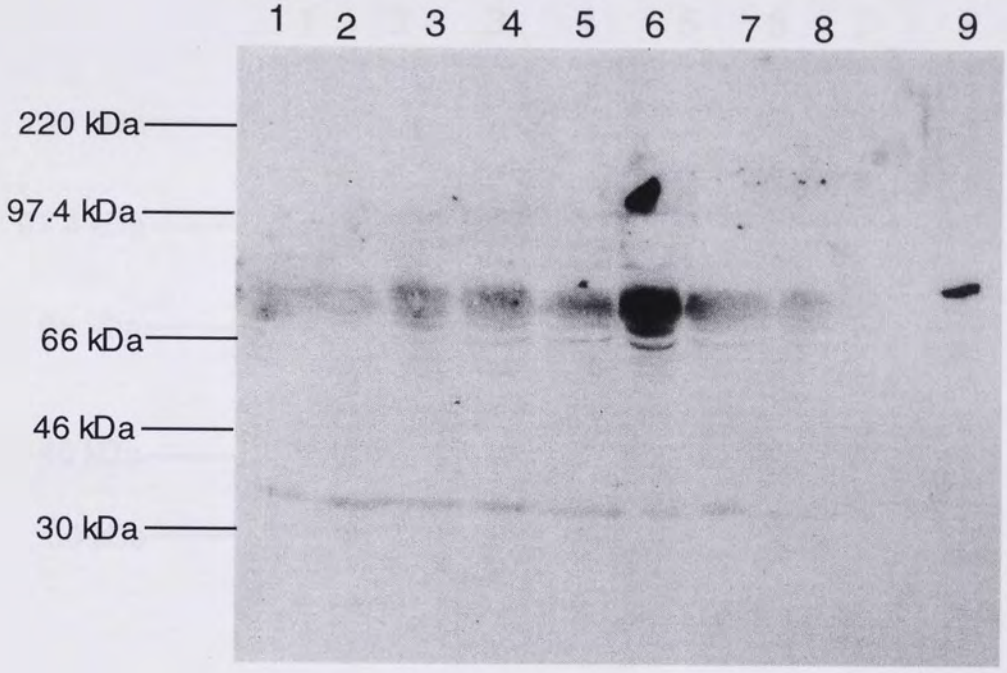
80 µg of protein was loaded to each lane.
Lanes 1-6: Samples from incubation with serum-free medium; Lanes 7 + 8: Samples from incubation in medium with 10% foetal calf serum.
Lane 1, Control, 4 h; **Lane 2**, GSNO (0.1 mM), 4 h; **Lane 3**, GSNO (1 mM), 4 h; **Lane 4**, Control, 8 h; **Lane 5**, GSNO (0.1 mM), 8 h; **Lane 6**, GSNO (1 mM), 8 h; **Lane 7**, Control, 8 h; **Lane 8**, Control, 8 h.
Samples 1-7 were from cells attached to the plate, sample 8 was from cells which were in suspension in the well.

Figure 5.3.2.2.
Effect of incubation with SNAP on the amount of HSP 72 in primary cultures of guinea-pig gastric mucosal cells incubated under conditions likely to increase superoxide generation or to decrease intracellular glutathione concentration.



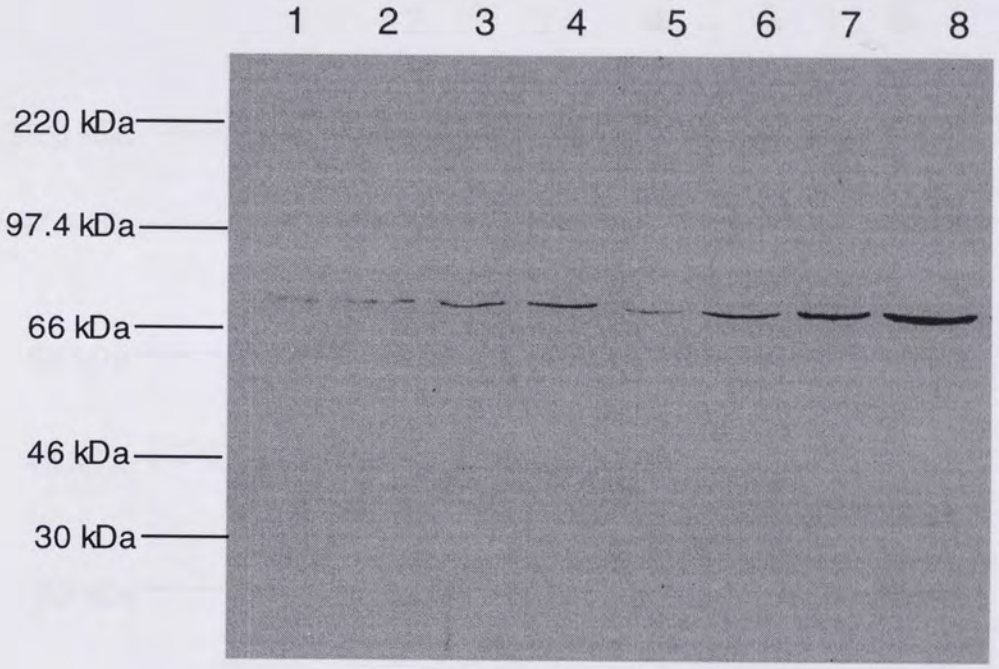
13 µg of protein was loaded in each lane.
Lane 1, Control; **Lane 2**, SNAP (1 mM); **Lane 3**, pyrogallol (0.1 mM); **Lane 4**, pyrogallol (0.1 mM) and SNAP (1 mM); **Lane 5**, diethyl maleate (1 mM); **Lane 6**, diethyl maleate (1 mM) and SNAP (1 mM); **Lane 7**, Control; **Lane 8**, Heat-shocked guinea-pig cells; **Lane 9**, Heat-shocked HeLa cells. Incubation with agents was for 8 h and attached cells were used for assay.

Figure 5.3.2.3.
Effect of incubation with S-nitrosoglutathione on the amount of HSP 72 in primary cultures of guinea-pig gastric mucosal cells incubated under conditions likely to increase superoxide generation or to decrease intracellular glutathione concentration.



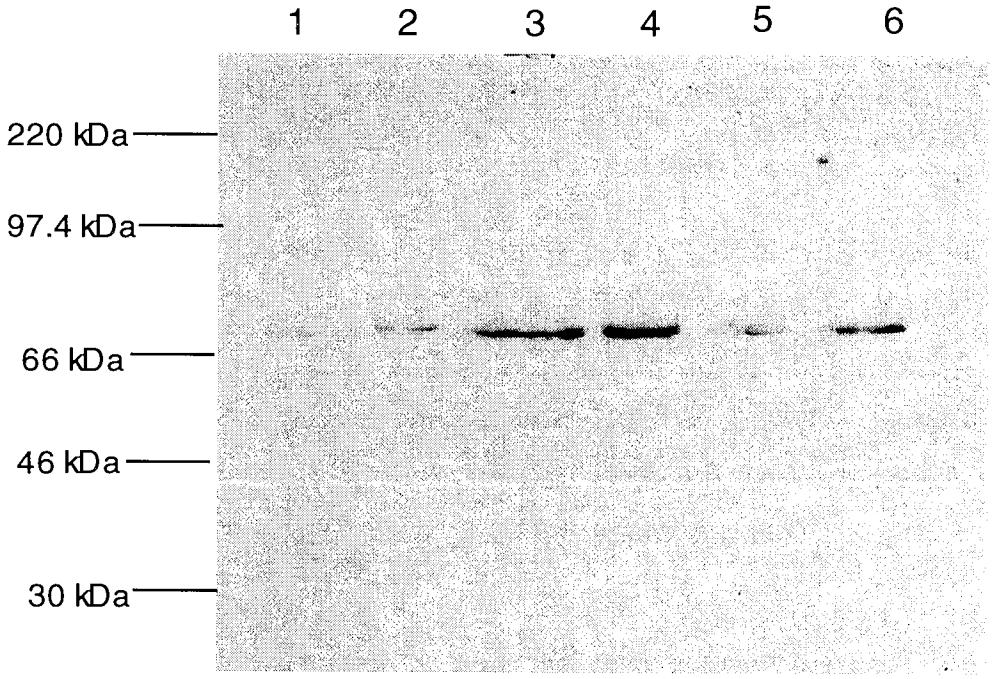
78 µg of protein was loaded in each lane.
Lane 1, Control; **Lane 2**, GSNO (1 mM); **Lane 3**, pyrogallol (0.1 mM); **Lane 4**, pyrogallol (0.1 mM) and GSNO (1 mM); **Lane 5**, diethyl maleate (1 mM); **Lane 6**, diethyl maleate (1 mM) and GSNO (1 mM); **Lane 7**, pyrogallol (0.1 mM) and diethyl maleate (1 mM); **Lane 8**, pyrogallol (0.1 mM), diethyl maleate (1 mM) and GSNO (1 mM); **Lane 9**, Heat-shocked HeLa cells.
Incubation with agents was for 8 h and attached cells were used for assay.

Figure 5.3.2.4.
Effect of concentration of SNAP on the amount of HSP 72 in the presence and absence of diethyl maleate in primary cultures of guinea-pig gastric mucosal cells.



70 µg of protein was loaded in each lane.
Lane 1, Control; **Lane 2**, SNAP (0.1 mM); **Lane 3**, SNAP (0.3 mM); **Lane 4**, SNAP (1 mM); **Lane 5**, diethyl maleate (0.3 mM); **Lane 6**, diethyl maleate (0.3 mM) and SNAP (0.1 mM); **Lane 7**, diethyl maleate (0.3 mM) and SNAP (0.3 mM); **Lane 8**, diethyl maleate (0.3 mM) and SNAP (1 mM).
Incubation with agents was for 8 h and attached cells were used for assay.

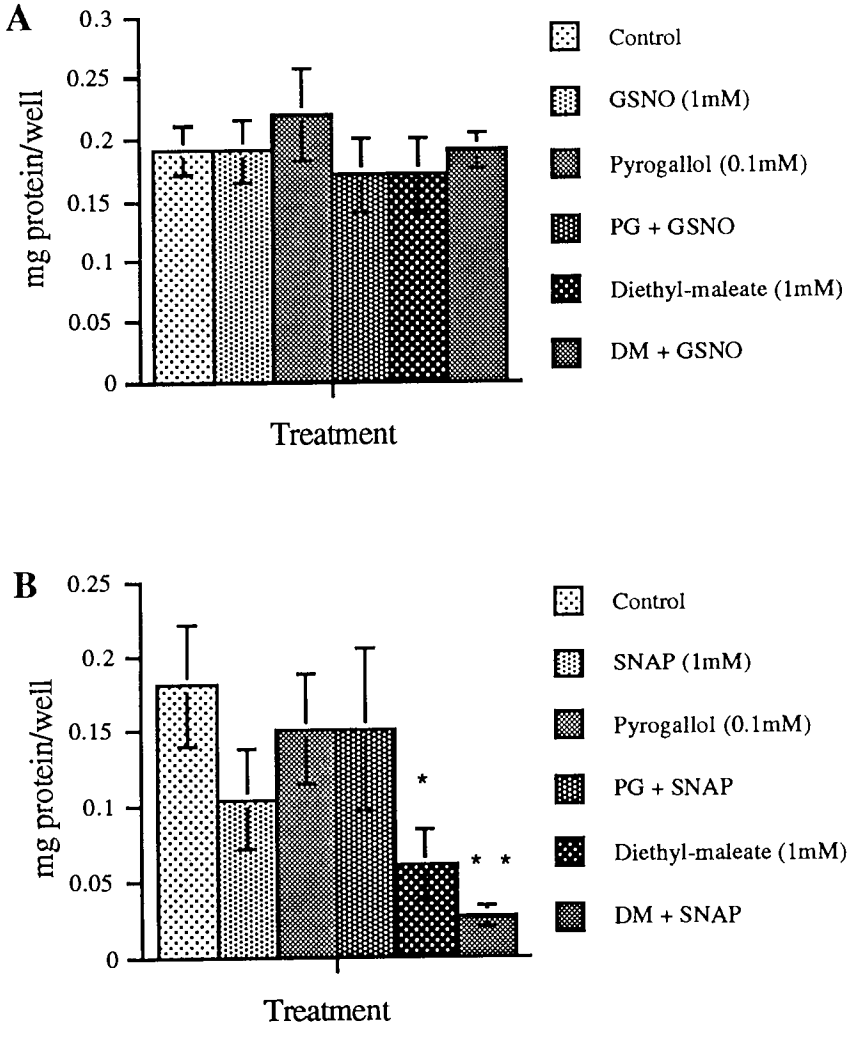
Figure 5.3.2.5.
Effect of the NO scavenger carboxy-PTIO and actinomycin D on the induction of HSP 72 by SNAP and diethyl maleate in primary cultures of guinea-pig gastric mucosal cells.



22 µg of protein was loaded in each lane.
Lane 1, Control; **Lane 2**, diethyl maleate (0.3 mM); **Lane 3**, SNAP (1 mM); **Lane 4**, diethyl maleate (0.3 mM) and SNAP (1 mM); **Lane 5**, diethyl maleate (0.3 mM), SNAP (1 mM), actinomycin D (5 µg/ml); **Lane 6**, diethyl maleate (0.3 mM), SNAP (1 mM) and carboxy-PTIO (0.1 mM).
Incubation with agents was for 8 h and attached cells were used for assay.

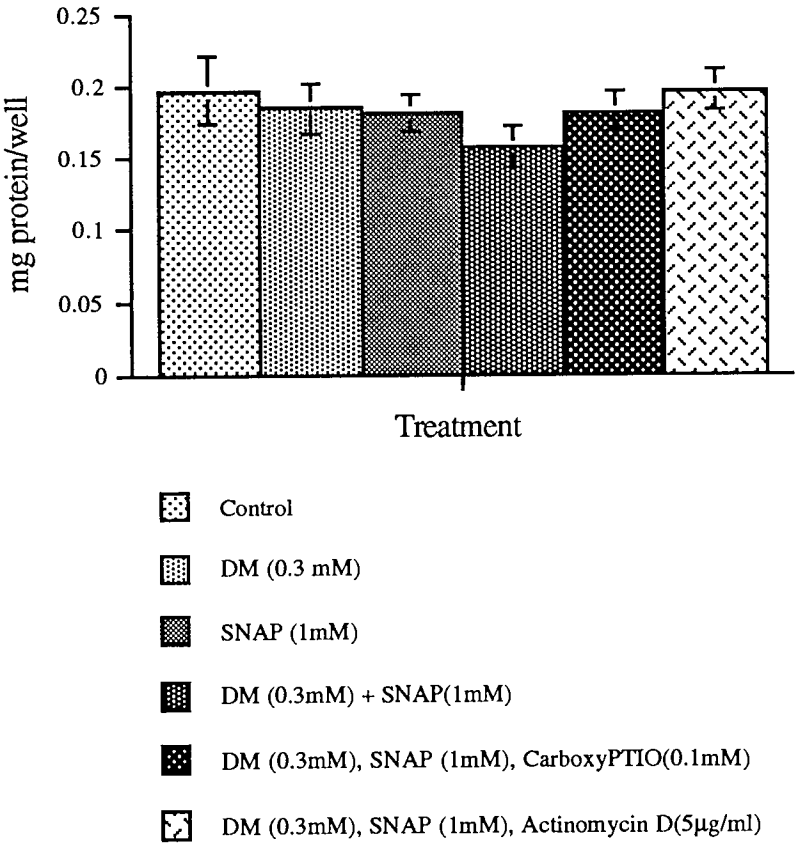
Figure 5.3.3.1.

The effect of the NO donors GSNO (A) and SNAP (B) in combination with 1.0 mM diethyl maleate or pyrogallol on the protein content per well.



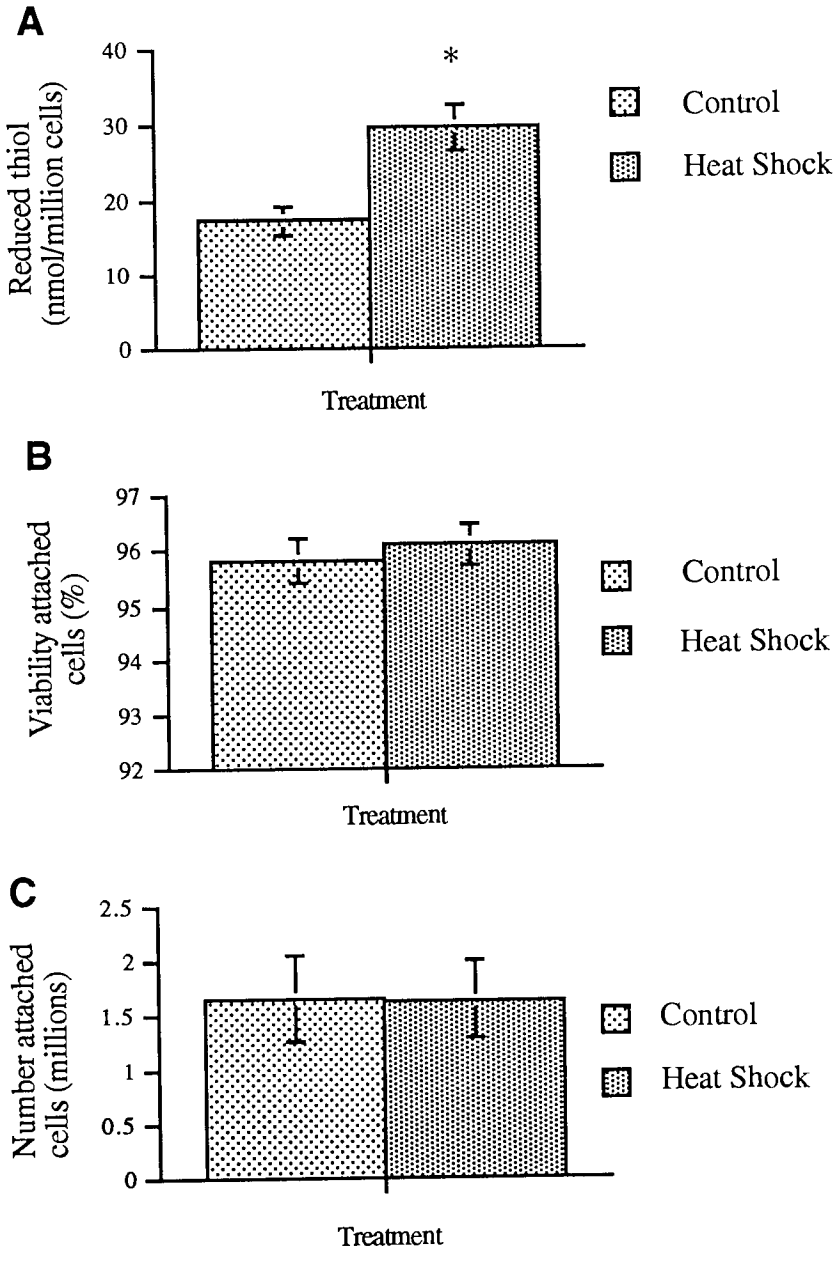
Agents were added 8 h before harvesting attached cells by trypsinisation from 6-well culture plates. In figure legend: DM= diethyl maleate and PG= pyrogallol. Results are means ± S.E. from 3 experiments. Data were analysed by 2 factor analysis of variance followed by Dunnett's test. * $P < 0.05$ and ** $P < 0.01$ for difference from control.

Figure 5.3.3.2.
The effect of 0.3 mM diethyl maleate and SNAP, carboxy PTIO and actinomycin D on the protein content per well of the culture plate.



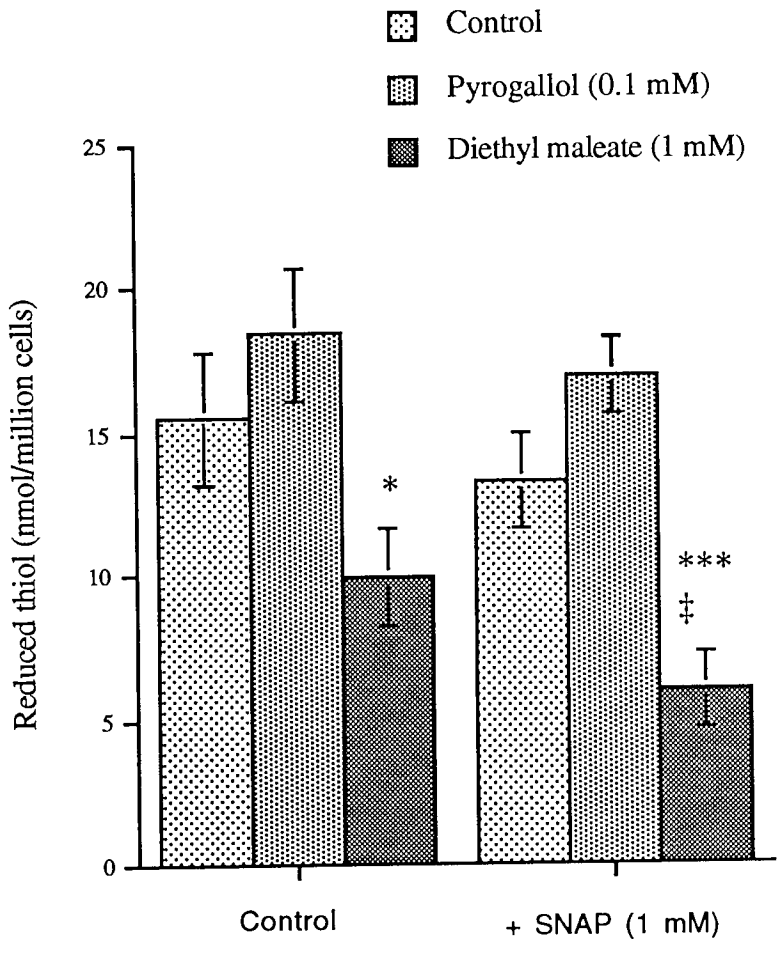
Agents were added for 8 h before harvesting of attached cells by trypsinisation. Results are means ± S.E. from 4 experiments. In figure legend: DM = diethyl maleate. Analysis of variance demonstrated no significant effect or treatment.

Figure 5.3.4. Effect of heat shock on reduced thiol content, viability and number of guinea-pig gastric epithelial cells attached to the culture plate.



Guinea-pig gastric mucosal cells were incubated for 3 h at 43°C, followed by a recovery period of 1 h at 37°C. Control cells were incubated at 37°C for 4 h. Measurements were then taken for reduced thiol content (A), ability of attached cells to exclude trypan blue after detachment from the plate by trypsinisation (B) and number of attached cells per three wells (C). Results are means ± S.E.M from four separate cell cultures. * $P < 0.05$ for comparison with control by a paired t-test.

Figure 5.3.5.1.
Effect of SNAP on reduced thiol concentration when incubated with guinea-pig gastric epithelial cells.in the presence and absence of pyrogallol or diethyl maleate

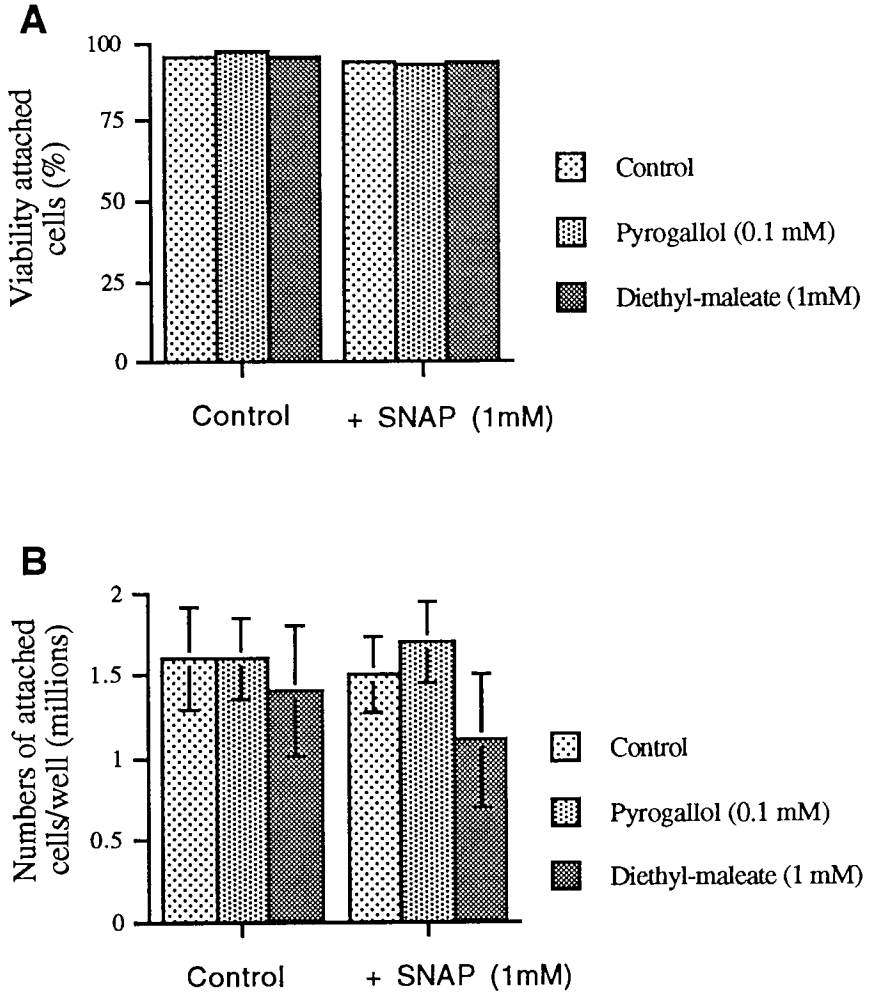


Cells were subjected to treatments for 8 h after which attached cells were harvested. Results are means ± S.E.M of five separate cell cultures and were analysed by two-factor analysis of variance followed by a Newman-Keuls multiple comparison test.

*, $P < 0.05$; ***, $P < 0.001$ compared to cells incubated wihtout additions.

‡ $P < 0.05$ for effect of SNAP relative to same condition without SNAP.

Figure 5.3.5.2.
Effect of SNAP on viability and numbers of guinea-pig gastric epithelial cells attached to the plate.



Cells were subjected to treatments for 8 h after which they were harvested and the ability of attached cells to exclude trypan blue was determined (A), and their number (B). Results are means ± S.E.M of five separate cell cultures. Two-factor analysis of variance with percentages transformed using the Arcsin transformation before analysis, did not show any effect of treatments on cell viability or number. Errors were too small in Graph A to show up.

5.4 DISCUSSION

5.4.1. Rat gastric mucosal cells and HSP 72.

Rat gastric mucosal cells expressed HSP 72 under basal conditions and this expression was not obviously increased in response to a variety of NO donors. The stress of the cell isolation process itself, independent of any treatment the cells were subsequently subjected to, probably caused the induction of heat shock protein. Thus at the end of the isolation period of 2 h there was little HSP 72, but a gradual darkening in intensity of the heat shock band took place with time as the cells were incubated. The cell isolation process involved exposure to proteases and Ca^{2+}-chelators plus shaking and centrifugation of cells. Exposure of cells to these unfavourable conditions therefore probably caused them to undergo stress and consequently express heat shock proteins. This heat shock effect in response to cell isolation has been noted previously (Wolffe et al., 1984) with a preparation of *Xenopus* hepatocytes. However a less vigorous cell isolation method using *in situ* collagenase perfusion of rat liver, did not result in detectable HSP 70 expression in hepatocytes (Takahashi et al., 1994).

A degree of variability in the presence of HSP 72 was observed between different vials of cells exposed to the same treatment. The cell population held in each vial may not have been exactly the same, or the degree of dispersion may have varied between vials and consequently a variable amount of HSP 72 induction may have been obtained. The high background levels of HSP 72 obtained with controls may have prevented effects of NO donors from being observed. Consequently, rat gastric mucosal cells were considered an unsuitable system in which to study induction of heat shock protein by NO donors. Guinea-pig gastric cells in primary culture were used in subsequent experiments because the period of culture after initial isolation of the cells should allow any HSP 72 induced during isolation to be degraded.

5.4.2. Heat shock and HSP 72 in guinea-pig gastric mucous cells.

With guinea-pig gastric mucosal cells in primary culture for two days before challenge, the amount of HSP 72 expressed under control conditions was very low, if visible at all. Therefore the system provided an appropriate basal level against which any increase due to treatments should be detectable. However, agents had to be added in the presence of 10 % foetal calf serum as its withdrawal promoted an

induction of HSP 72 in the absence of agents. Hepatocytes also induce HSP 72 when placed in culture without serum (Van Remmen, 1996). The results obtained with Periodic Acid Schiff staining (section 5.3.6) show that after culture for 2 days the vast majority of attached cells were of the mucus-secreting variety. The system therefore appears suitable to investigate possible protective responses induced by exogenous NO on mucous cells.

Guinea pig cells in this study, as shown previously (Rokutan et al., 1996), showed a HSP 72 response to increased temperature. A sample of these heat shocked cells were used in each experiment to provide validation of preparation and immunoblotting procedure. The mechanism by which heat shock induces HSP 72 is thought to involve activation of the transcription factor HSF-1 and its transfer to the nucleus. One suggestion is that heat shock induces a sharp, transient, increase in NO production prior to the accumulation of heat shock proteins (Malyshev et al., 1995). This increase in NO is proposed as a trigger signal, and therefore NO may be involved in the heat shock activation of HSP 72. The ability of the NO scavenger carboxy-PTIO to inhibit oxidative-stress linked induction of HSP 72 supports this. It may be that removal of the NO trigger signal by the scavenger prevents HSP 72 activation. Whether guinea-pig gastric mucous cells produce NO in response to heat shock is unknown.

5.4.3. HSP 72 and NO

Both heat shock proteins and glutathione, as explained earlier (section 5.1) are proposed to have protective roles in the gastric mucosa. NO has also been linked with the gastric mucosa, and modulatory roles in stress protein activation and the glutahione redox cycle. As previous work has established that guinea-pig gastric mucosal primary cultures are capable of HSP 72 induction under conditions other than sustained heat shock (Hirakawa et al., 1996), the effect of NO donors and other treatments on the heat shock response was investigated. The primary cells used in this study were exposed to chemical agents in an attempt to create conditions of oxidative stress and investigate any interaction with exogenous NO. Diethyl maleate is an electrophilic agent which has the ability to deplete reduced glutathione (GSH) without the formation of glutathione disulphide (GSSG). Thus diethyl maleate has previously been shown to deplete GSH levels in cultured gastric mucosal cells (Mutoh et al., 1991) by the conversion of GSH to thioester conjugates by a reaction catalysed by glutathione-S-transferase. Depletion of intracellular GSH stores by use of diethyl maleate therefore results in the removal

of the protective effects stemming from removal of hydrogen peroxide by glutathione peroxidase, with the subsequent accumulation of reactive oxygen species. Another chemical agent used in this study, pyrogallol, releases superoxide anion, which in the presence of NO can result in formation of peroxynitrite which is often cytotoxic (Xie and Wolin, 1996).

There was a consistent and dose-related induction of HSP 72 by NO donors in guinea-pig gastric mucous cells. Heat shock protein expression has previously been demonstrated in primary cultures of guinea-pig gastric mucous cells which had been subjected to oxidative stress as a result of hydrogen peroxide and diamide exposure (Rokutan et al., 1995). Oxidative injury imposed on human amniotic cultured cells by treatment with hydrogen peroxide resulted in HSP 72 induction (Abe et al., 1995), an effect which was enhanced during the depletion of GSH by diethyl maleate. Use of the alkylating toxicant iodoacetamide in a porcine renal epithelial cell line (Liu et al., 1996) was linked to depletion of GSH, induction of oxidative stress, lipid peroxidation and HSP 72 transcriptional activation. The mechanism by which NO donors induced the heat shock protein in guinea-pig cells is discussed below. Firstly, it is important to establish that it was NO donation, and not some other property of the NO donor, which was causing the heat shock response.

Carboxy-PTIO is a NO scavenger, which has been shown to be inhibitory against a series of the biological actions of NO (Maeda et al., 1994) such as NO-mediated cytotoxicity in murine macrophage J774 cells (Zamora et al., 1997). Carboxy-PTIO inhibited the SNAP elicited HSP 72 response obtained in the presence of diethyl maleate. The ability of the NO scavenger to inhibit the response demonstrates that there is a direct involvement of NO in the induction of HSP 72.

Induction of HSP 72 was shown to be a transcriptional effect, because the transcriptional inhibitor actinomycin D reduced the intensity of the heat shock band. Prevention of heat shock protein induction by transcriptional inhibition such as this, has been demonstrated in cultured rat hepatocytes (Kim et al., 1997). The transcription factors, HSF-1 and HSF-2, tightly regulate activation of the heat shock genes. Control is regulated in several ways (Baler et al., 1993) such as at the level of HSF activation, their state of oligomerisation and their subsequent acquisition of DNA binding activity. As HSF-1 must translocate into the nucleus before it can exert an effect, this provides another point of regulation. HSF-1 also experiences regulation by modification, both by heat and post translationally in the absence of stress.

NO may be involved in the induction of the heat shock response by catalysing the trimerisation of the heat shock transcription factor HSF (Malyshev et al., 1996). Once HSF has been activated in this way it is able to enter the nucleus and initiate transcription of HSP 70 genes. The mechanism by which NO causes activation of HSF-1 in gastric mucous cells is not entirely clear. An involvement of a reduction in GSH content in activating HSF-1 has been proposed (Liu et al., 1996). Although the present data were suggestive, the NO donor SNAP at a concentration of 1 mM, did not significantly reduce intracellular GSH content at 8 h. The possibility that NO may interact with the SH-groups of HSF-1, with this nitrosylation effect causing the eventual activation of HSF-1, should be considered.

5.4.4. The involvement of diethyl maleate, pyrogallol and NO with the HSP 72 response.

Use of pyrogallol did not result in a consistent effect on the heat shock response of guinea-pig gastric mucous cells. Pyrogallol is an $O_2^{\cdot -}$ releasing agent, which can produce $ONOO^-$ in the presence of NO (Xie and Wolin, 1996). Unless this potent oxidising agent is removed from the system lipid peroxidation and cellular membrane damage may result (Wakulich and Tepperman, 1997). However, as cells remained attached to the culture plate throughout the experiment and HSP 72 was not routinely induced, it must be assumed that the primary cells were able to remove the oxidative threat, by use of superoxide dismutase and subsequent removal of hydrogen peroxide by catalase or glutathione peroxidase. Certainly no reduction in cellular GSH was detected with pyrogallol whether or not NO donors were present, despite exogenous NO having been reported to cause the inactivation of purified bovine cytosolic glutathione peroxidase (Asahi et al., 1995).

Depletion of intracellular GSH by the agent diethyl maleate was found as expected (Wakulich and Tepperman, 1997) and was associated with a substantial induction of HSP 72 above control levels. Initial experiments investigated HSP 72 induction with the NO donors SNAP and GSNO in combination with 1 mM diethyl maleate. Use of diethyl maleate at this high concentration was shown in most cultures to cause detachment of cells in the presence of NO donor, and the HSP response to NO donor under these circumstances was not always reproducable. That is, the NO donor did not always succeed in eliciting a further increase in HSP 72 levels above that with diethyl maleate alone. However, with 0.3 mM diethyl maleate the variability was very much decreased, there was no significant cellular detachment

and a consistent difference in HSP 72 induction between diethyl maleate alone and diethyl maleate in combination with SNAP was demonstrated.

The increase in HSP 72 effected by NO donor was potentiated in the presence of diethyl maleate.This potentiation was consistently produced with diethyl maleate at a concentration of 0.3 mM, but only intermittantly produced with 1 mM diethyl maleate. The combined treatment of 1 mM SNAP and 1 mM diethyl maleate reduced cellular GSH further than the reduction produced by diethyl maleate alone. Enhancement of the effect of SNAP by diethyl maleate may have been related to this further reduction in GSH, which as discussed above may regulate HSP 72 induction. Variability in the presence of 1 mM diethyl maleate may have been a consequence either of non-specific toxic effects associated with promotion of cell detachment, or of excessive depletion of GSH which may actually inhibit HSP 72 induction in gastric mucous cells (Rokutan et al., 1996).

The precise way in which oxidative stress increases the transcriptional activity of heat shock genes is unknown. It has been reported (Teshima and Rokutan, 1995) that the redox sensitive transcription factor NF-κB may participate in the stress response in primary cultures of guinea-pig gastric mucosal cells. This is confirmed by another study (Malyshev et al., 1995) which suggests that NF-κB generation in response to heat shock leads to heat shock protein induction. It has also been proposed (Rokutan et al., 1995) that c-fos and c-jun mRNA expression in response to increased GSH oxidation inflicted by diamide is involved in HSP 70 induction in guinea-pig gastric mucosal primary cultured cells. Direct effects on HSF-1 as mentioned previously are also possible.

5.4.5. Physiological significance of results.

In this final section of the discussion the effects of the various treatments on cell viability and the physiological significance of the results will be discussed. Subjecting primary cultured cells to heat shock resulted in an almost doubling of GSH content measured after 4 h, when compared to control. Heat shocked cells did not experience any reduction in cell number or loss in viability as detected from their ability to exclude trypan blue. This is probably due to the efficient induction of the heat shock response and the subsequent protective mechanisms that ensue. It would appear that a sustained increase in temperature not only resulted in an increase in HSP 72, but also possible upregulation of another protective system, the GSH redox cycle, as evident by the increase in the GSH

content. Heat shock has been shown to protect gastrointestinal mucosa and mucosal cells from damage (Nakamura et al., 1991, Stojadinovic et al., 1995, Hirakawa et al., 1996), apparently the possibility that the mechanism may involve elevation of GSH has not been mentioned previously.

Previous studies using isolated rat gastric mucosal cells have reported a reduction in viability in response to NO donors (Tripp and Tepperman, 1996). However, in this study no such loss of viability was evident with attached guinea-pig mucous cells. Although variable cell detachment was observed under conditions of reduced GSH produced by diethyl maleate, this did not result in a loss of viability. Induction of HSP 72 by NO may have a protective role, a possibility supported by the trypan blue variability data, or it may simply be a response to damage. Future work should address the effects of preventing the heat shock response to NO, by use of actinomycin D, on the viability of cells at 8 and 24 h after challenge.

5.4.6. Summary

1. Exposure of primary cultures of guinea-pig gastric mucosal cells to the NO donor SNAP for 8 h caused a concentration-dependent induction of HSP 72.

2. Induction was inhibited by the NO scavenger carboxy-PTIO, and by blockade of transcription with actinomycin D.

3. The effect of SNAP was enhanced by decreasing the intracellular reduced thiol content with diethyl maleate, which by itself also induced HSP 72 formation.

4. HSP 72 formation by heat shock was associated with a elevation of reduced thiol.

CHAPTER 6

INDUCTION OF Ca^{2+}-INDEPENDENT NO SYNTHASE IN GASTRIC AND PULMONARY CELL LINES AND IN TRACHEAL EPITHELIUM

6.1. INTRODUCTION

6.1.1. Role of NO in the lungs (for gastric mucosa see section 1.7).

The extensive localisation of both the constitutive and inducible isoforms of NO synthase in multiple cell types in human and rat lungs suggests an important role for NO synthase activity in these tissues. Immunocytochemical and histochemical techniques have demonstrated the uniform distribution of iNOS in epithelium of normal, large, cartilaginous airways of human and rat samples and in alveolar macrophages, whereas constitutive NO synthase has been identified in rat lung nerves, endothelium and airway epithelium (Kobzik et al., 1993). The importance of constitutive NO synthase activity in pulmonary tissues has been demonstrated by the presence of both eNOS and nNOS gene expression in bronchiolar epithelium (North et al., 1994), which is subject to developmental regulation. NO has a number of beneficial actions in the lungs. Thus, NO relaxes airway smooth muscle, causes pulmonary arterial vasodilatation, increases ciliary beat frequency (Jain et al., 1993), and regulates neurotransmission (Nagaki et al., 1995). Endogenous NO stimulates mucus glycoconjugate secretion from airway submucosal glands (Nagaki et al., 1995).

Although NO is an apparently important regulator of normal physiological function in the lung it may also be associated with pathological events occurring in these tissues. Bronchial biopsy specimens obtained from asthmatic patients showed increased iNOS expression in epithelial cells as compared to normal controls. In addition, evidence suggests that the level of NO exhaled in humans increases with asthma (Nagaki et al., 1995).

6.1.2. iNOS in lungs and gastrointestinal tract.

The uniform distribution of iNOS in the epithelium of large, cartilaginous airways of humans and rats has been demonstrated (Kobzik et al., 1993). The induction of iNOS has also been demonstrated in response to administration of LPS *in vivo* in rat gastric mucosal cells (Brown et al., 1994), and rat intestinal epithelial cells (Tepperman et al., 1993). Cytokines are capable of iNOS induction in a number of pulmonary cell lines, including the A549 human lung epithelial cell line (Berkman et al., 1996), and the LA-4 murine lung epithelial cell line (Robbins et al., 1994b), and in rat pulmonary artery smooth muscle (Thomae et al., 1996).

The combination of cytokines best capable of stimulating the induction of iNOS in animals has been reported to be: interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1ß), and lipopolysaccharide (LPS) (Bredt and Snyder, 1994; Asano et al., 1994; Warner et al., 1995). Therefore, with reference to these publications a "cytomix" of these agents was chosen to attempt induction of iNOS. IFN-γ is a multifunctional protein produced by T lymphocytes and natural killer cells which regulates numerous immunological functions, such as the upregulation of MHC complex antigens (Pellegrini and Schindler, 1993). TNF-α is produced by a range of cells including: neutrophils, activated lymphocytes, macrophages, endothelial cells and smooth muscle cells. It has the ability to kill certain tumour cells directly, and plays a central role, along with IL-1, in initiating the cascade of cytokines that comprise the immune response to infection (Fiers, 1991). The cytokine interleukin-1 beta (IL-1ß) is produced mainly by activated macrophages, but lymphoid, vascular and epithelial tissues can synthesise it. Similarly to TNF-α, it has a key role in mediating immune and inflammatory responses in response to microbial infection and tissue injury, such as facilitating the responses of B and T cells to antigen, and inducing other cytokines (Dinarello, 1988). LPS is a component of the bacterial cell wall, sections of which may be shed during microbial infection, leading to local immune reactions, and induction of iNOS in some cell types (Bredt and Snyder, 1994).

The exact mechanisms by which cells produce high levels of NO as a result of inducing NO synthase are not yet fully elucidated. Transcriptional induction of iNOS has previously been demonstrated in rodent cells (Nathan and Xie, 1994), and it is thought that the enzyme possesses a complex promoter region due to the ability of a range of diverse stimuli to cause induction. Potentially responsive sites to NF-κB, IFN-γ, NF-IL6, and AP-1, among others, exist on the promoter region of the inducible isoform of NO synthase (Simmons et al., 1996). In cultured rat astrocytes it has also been shown that induced iNOS activity is dependent on the concomitant induction of the CAT-2 gene which encodes the high affinity System y^+, L-arginine transporter (Stevens et al., 1996). Exposure of cardiac microvascular endothelial cells to cytokines resulted in an increased cellular availability of L-arginine (Simmons et al., 1996). This cytokine-induced L-arginine availability was due to a 2-fold increase in arginine uptake, which was associated with an increased expression of the mRNA for the L-arginine transporters CAT-1 and CAT-2B, as well as induction of arginosuccinate synthetase mRNA, the rate limiting enzyme in the de novo synthesis of arginine.

6.1.3. Aims of this section.

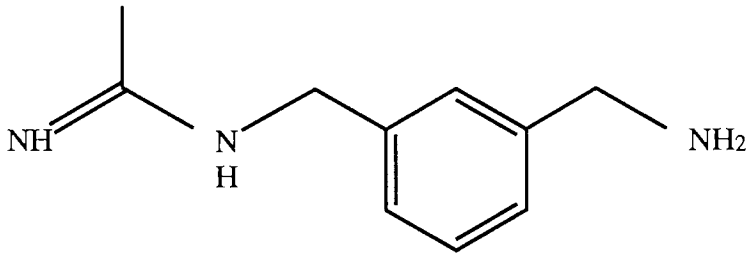
The experiments undertaken in this chapter aimed to stimulate induction of iNOS in pulmonary and gastric epithelial cells by the use of cytokines, and to investigate the effects of several potential modulatory agents, and a specific iNOS inhibitor, on this induction. The modulatory agents were: interleukin-8 (IL-8), prostaglandin E_2 (PGE_2), indomethacin and epidermal growth factor (EGF). The reasons for selecting these four agents are outlined below.

Infection of the stomach with *Helicobacter pylori* , particularly strains expressing cagA, induced levels of IL-8 in the mucosa. IL-8 could contribute to the recruitment of inflammatory neutrophils into the tissue (Yamaoka et al., 1997). IL-8 has also been suggested to inhibit the *ex vivo* induction of iNOS in rat peritoneal neutrophils (McCall et al., 1992). Therefore its action on induction of iNOS in pulmonary and gastric cells was studied here. Prostanoids are known to have cytoprotective effects in the gastric mucosa (section 1.6), and therefore determining the effect of the prostanoid PGE_2, and the effect of inhibiting any endogenous synthesis with indomethacin, on the induction of iNOS was of interest. Indomethacin has also been reported to enhance the cytokine stimulated induction of NO synthase in vascular endothelial cells (McCall et al., 1992). Epidermal growth factor (EGF) is a potent growth stimulatory peptide, which is secreted by salivary and Brunner's glands, and has a role as a mediator of mucosal protection and repair in the stomach and duodenum (Allen et al., 1993). Due to its stimulatory effects on proliferation and PGE_2 release in guinea-pig gastric mucous cells (Sakamoto et al., 1994), and its secretion by a novel cell lineage associated with mucosal ulceration (Wright et al., 1990), any potential effects on iNOS induction were thought worthy of investigation.

Induction of iNOS was to be monitored by the production of the stable degradation products of NO, namely nitrate and nitrite, from the cells. The proportion of these products present in culture media is variable depending on the pathway of NO inactivation. A reaction of NO with superoxide anions will lead to the accumulation of nitrate, whereas reaction with molecular oxygen will predominantly yield nitrite (Schmidt, 1995). To ensure that nitrate and nitrite were produced by iNOS, N-(3-(aminomethyl)benzyl) acetamidine (1400W) (Figure 6.1.3) which is a novel and highly selective inhibitor for iNOS (Garvey et al., 1997) was employed. It exhibits slow, tight binding of iNOS, which is 5000-fold and 200-fold selective for iNOS, over eNOS and nNOS respectively. Inhibition of

iNOS using 1400W has been demonstrated both *in vitro* and *in vivo*, where its use inhibited the growth of solid tumours expressing iNOS (Thomsen et al., 1997).

Figure 6.1.3. Structure of iNOS inhibitor 1400W.



This work was performed at Glaxo-Wellcome Medicines Research Centre, Stevenage. The emphasis on pulmonary tissue reflected changes in the direction of research within the company since the studentship was taken up with Wellcome plc. The A549 cell line, in which induction of iNOS had previously been demonstrated (Asano et al., 1994; Robbins et al., 1994a) was used in this study to provide a positive control, whereby the effects of the modulators mentioned above on iNOS induction by a combination of cytokines could be examined. A549 cells are an alveolar type II epithelium-like cell line derived from a human lung adenocarcinoma. After the validation of the procedure with A549 cells, next a comparison could be made with gastric cell lines, and finally the work would be extended into primary cultures of guinea-pig tracheal and gastric mucosal cells. The AGS and HGT-1 cell lines were both originally established from gastric adenocarcinomas and the cells exhibit some epithelial cell characteristics. Induction of iNOS in these gastric cell lines has not been reported in the literature, and therefore the investigation of the potential modulators on cytokine-stimulated iNOS represented novel work. Primary cultures of guinea-pig tracheal epithelial cells respond to inflammatory mediators with the production of mucin, via a mechanism involving the intracellular production of NO (Adler et al., 1995). This, taken together with the identification of iNOS in large, cartilaginous, airways in both human bronchi and rat trachea (Kobzik et al., 1993), and the identification of constitutive NO in the BEAS-2B human tracheal epithelial cell line (Chee et al., 1993), made a primary culture of guinea-pig tracheal cells a suitable candidate in which to investigate iNOS induction in a primary culture.

6.2. METHODS

6.2.1. Use and maintenance of cell lines

Both A549 (American Type Culture Collection) and AGS (European Culture Collection) cell lines were cultured and maintained in Ham's F12 media with additions of: foetal calf serum (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The HGT-1 cell line (Gift from Christian Gespach) was maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with foetal calf serum (5%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell lines were cultured and maintained as described previously (section 2.7). Cell lines were grown to confluence within 75 cm^2 cell culture flasks, after which they were transferred to 24 well culture plates (1 ml medium/well) and left to adhere and grow for a further 1-2 days. Cell lines were then serum-starved for 24 h after which they were used immediately for experimental purposes. In experiments where inserts were used, sterile 12-well culture plates complete with 1 ml inserts were obtained direct from the supplier (Costar). When collagen coating was required, a 1:10 dilution of Vitrogen (synthetic collagen) with sterile distilled water was made, and a 200 μl volume was added to each insert of a 12-well plate. The plates were left to dry overnight in a sterile environment in a laminar flow hood.

6.2.2. Primary culture of guinea-pig tracheal epithelium.

Anaesthesia in a male Dunkin-Hartley guinea-pig (200-400 g) was achieved with sodium pentobarbitone (60 mg/kg i.p.). The trachea was rapidly removed and placed immediately into sterile Hank's Balanced Salt Solution (HBSS) (No Ca^{2+} or Mg^{2+}). Using a laminar flow hood to maintain sterility, the trachea was cleaned to remove excess membranes and blood clots from the exterior surface. The trachea was then placed in a sterile 30 ml Universal tube containing 10 ml HBSS (no Ca^{2+} or Mg^{2+}) supplemented with Pronase (1 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml). The trachea was totally immersed in the solution and left at 4°C for 16-24 h. On completion of the incubation period two Universal tubes each containing 10 ml Ham's F12 with foetal calf serum (10%) were prepared. Again under sterile conditions, the trachea was first rinsed using the medium in one of the tubes. Using a sterile petri dish and cell scraper, cells were then harvested from the epithelial surface of the trachea and collected in the second Universal tube. Both tubes were subjected to centrifugation at 12,000 rpm for 5 min at 4°C. The cell pellets were combined and resuspended in

sufficient Ham's F12 (serum-free) containing penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), amphotericin B (2.5 μg/ml), insulin (10 μg/ml), hydrocortisone (10^{-6} M), epidermal growth factor (25 ng/ml), triiodothyronine (3 x 10^{-8} M), transferrin (5 μg/ml) and cholera toxin (10 ng/ml), to seed a 12 well culture plate. Primary cultured cells were fed with fresh media every 24 h, and left to grow to confluence (usually 4 days) before commencing experimental work. Primary cells were not serum starved before use, however a variety of different treatment regimes were investigated to establish the optimal conditions for response to cytokines.

6.2.3. General experimental procedure used to challenge cells.

6.2.3.1. Cell lines

For experiments where cell lines were used, medium was replaced by exactly 1 ml serum-free medium per well 24 h before the start of experiment. A general treatment regime of 6 treatments per plate, and 4 wells per treatment was observed.

Table 6.2.3.1. Generalised treatment regime used for cell lines

Treatment	No. of wells	Content of wells
1	4	Control: Vehicle only
2	4	Modulatory agent under investigation
3	4	"Cytomix" (see below)
4	4	"Cytomix" + agent under investigation
5	4	"Cytomix" + 1400W
6	4	"Cytomix" + agent under investigation + 1400W

All agents were made up in sterile phosphate-buffered saline (PBS: 10 mM NaH_2PO_4, 9g/l NaCl, pH 7.5), and were added to the wells in a total volume of no more than 60 μl. Cytokines and other agents were diluted into sterile PBS immediately upon acquisition, aliquoted at suitable concentrations into sterile tubes and stored at -20°C, or as suggested by the supplier, until used. The cytomix used was a combination of 4 different cytokines, final concentration in parenthesis: human IFN-γ, (10 ng/ml in well), human IL-1β, (10 ng/ml in well), human TNF-α, (10 ng/ml in well) and E.Coli LPS, (10 μg/ml in well). The modulatory agents were: human (IL-8, 30 ng/ml in well), PGE_2, (1 μM in well), indomethacin (5 μM in well), and human EGF, (30 ng/ml). All cytokines and other agents were added to give a total equal volume in wells, using sterile pipettes, so that the pipette tip was just under the surface of the medium in the well. On completion of challenge

with agents, the sides of the plates were gently tapped to evenly mix the contents, and the plates were placed back in a humidified incubator at 37°C in 5% CO_2. At the required time intervals, usually 8, 24 and 48 h, 100 µl of incubation medium was removed from each well and transferred to a corresponding well on a 96-well culture plate, after which the experimental plates were returned to the incubator. The 96-well plates containing the 100 µl samples were stored at 4°C, to prevent any change in contents, until the end of experiment when they were assayed for nitrite and nitrate.

6.2.3.2. Primary Cultures

Table 6.2.3.2. Description of media used with primary cultured cells.

Name of Media	Contents of Media
Growth media	Ham's F12 + standard additions for primary culture (6.2.2)
Hydrocortisone-free growth media	Ham's F12 as above, except with hydrocortisone omitted
Basal media	Ham's F12 + foetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), amphotericin B (2.5 µg/ml)
Serum-free basal media	Ham's F12 as above, except with foetal calf serum omitted

The generalised treatment regime used during experiments is shown in table 6.2.3.3. The media used in particular experiments are given in the results section.

Table 6.2.3.3. Generalised treatment regime used for primary cells.

Treatment	No. of wells	Content of wells
1	3	Control: PBS additions only
2	3	1400W
3	3	Cytomix
4	3	Cytomix + 1400W

Additions were made to wells as described previously. 100 µl aliquots of incubation media were removed from each well and transferred to corresponding wells on a 96-well plate, at the required time intervals, usually after 8, 24 and 48 h, as described previously.

6.2.4. Determinations of nitrite and nitrate.

Nitrate was reduced to nitrite using nitrate reductase, and nitrite was determined spectrophotometrically using the Griess reaction (see below). The total nitrate and nitrite content is referred to as NOx. The method detailed here is similar to one described previously (Robbins et al., 1994).

At the start of the assay three solutions were prepared: assay buffer, the "assay mix" and the Griess reagent. Assay buffer (14 mM sodium phosphate pH 7.4) was used to prepare 10 ml assay mix (2.5 mM glucose-6-phosphate, 400 U/L glucose-6-phosphate dehydrogenase, 200 U/L NADPH-dependent nitrate reductase). To prepare the Griess reagent equal volumes of 0.1% w/v naphthylethylene-diamine dihydrochloride in double distilled water was mixed with 1% w/v sulphanilamide in 5% concentrated H_3PO_4.

Table 6.2.4.1. Concentration of reagents in nitrite assay.

Assay constituent	Stock solution conc.	Concentration in well
Nitrate reductase	200 units/litre	80 units/litre
Glucose-6-phosphate-dehydrogenase	400 units/litre	160 units/litre
Glucose-6-phosphate	2.5 mM	1 mM
NADPH	10 μM	1 μM

Two sets of eight standards were also prepared, one for nitrate and one for nitrite, both in the presence and absence of NADPH dependent nitrate reductase, in the range of 0-200 μM. With the use of a 96-well plate this required 32 wells for standards leaving 64 wells for samples. Where more than one 96 well plate was necessary for all samples, only one set of standards was used per experiment, and this determined nitrite concentration in all plates. To perform the assay, 50 μl of sample culture media was transferred to a fresh 96-well plate. The nitrite and nitrate standards were prepared in 50 μl volumes in the 96-well plate. To both samples and half the standards, 40 μl assay mix and 10 μl of 10μM NADPH (made fresh, final concentration in well = 1 μM) was added. In cases where the content of nitrite alone was being measured, such as half the remaining nitrite and nitrate standards, NADPH dependent nitrate reductase was omitted, and 40 μl assay buffer was substituted for assay mix.

Table 6.2.4.2. Summary of nitrite assay

Assay constituent	Nitrite determinations	Total NOx determinations
Sample/ standard	50 µl	50 µl
Assay mix	omitted	40 µl
Assay buffer	40 µl	omitted
NADPH	10 µl	10 µl
	45 min incubation at room temperature	
Griess Reagent	100 µl	100 µl
	15 min incubation at room temperature	
	Read plate at 550 nm	

The plates were left to incubate, with gentle shaking, at room temperature for 45 min, after which 100 µl Griess reagent was added to each well and plates were incubated for a further 15 min. On completion of the incubation period the optical density of each well was read at 550 nm. Using the standard curves the total NOx concentration (µM) per sample was calculated.

6.2.5. Determination of protein content per well.

On completion of each experiment, the culture plates were retained, and the protein content per well was determined (section 2.2). All remaining culture medium was removed from the plate, and cells were washed several times with 0.5 ml PBS. An addition of 250 µl NaOH (1 M) was made to each well, and the plate was left to incubate at room temperature with gentle shaking, to allow the protein present to dissolve. A duplicate set of six protein standards using BSA in the range of 0-0.8 mg/ml were prepared using 1 M NaOH. To perform the assay 10 µl of standard or sample was added to the appropriate well of a 96-well plate, to which 200 µl of dilute Bradford reagent (1:4 ratio with distilled water) was added, and the contents were mixed well. Absorbance was measured at 595 nm, after which a calibration curve was constructed so that protein concentrations of the unknowns could be determined.

6.3. RESULTS

6.3.1. Pilot experiment with A549 cells.

A pilot experiment was conducted to determine the most suitable time points to assay for nitrite, and whether to measure nitrite only, or the total NOx (nitrite and nitrate) concentration (Figure 6.3.1.1.A and B). The nitrite concentration alone remained low, with no significant effect of treatment or time for the duration of the experiment. However, when total NOx concentration (B) was assayed, a significant increase ($P < 0.01$) in concentration above control levels, was observed in response to cytomix challenge. No such increase was observed with cytomix in the presence of 1400W. The NOx concentration was found to be significantly higher than the analogous measurements for nitrite alone at all time points ($P < 0.01$) by analysis of variance followed by Dunnett's test. Cytomix, both in the presence and absence of 1400W resulted in a significant decrease ($P < 0.05$) in protein content from the control value (Figure 6.3.1.2.A). When the nitrite and total NOx at 24 h was re-expressed as a function of the protein concentration (Figure 6.3.1.2.B), no significant effect of treatment was observed with nitrite measurements alone, but with total NOx measurements, cytomix and cytomix in the presence of 1400W, resulted in a significant increase in concentration above the control value ($P < 0.01$).

6.3.2. Effect of cytokine challenge and potential modulators on total NOx concentration and protein concentration in A549 cells.

Cytomix in both the presence and absence of IL-8, resulted in a significant increase in NOx concentration ($P < 0.01$) above the control value (Figure 6.3.2.1). Cytomix in combination with the iNOS inhibitor 1400W, in both the presence and absence of IL-8, resulted in NOx concentrations which were not significantly different from control values. Cytomix alone, or in combination with any other treatment, resulted in a signifcant reduction ($P < 0.05$) in the mean protein (mg/well) concentration, as measured after 48 h (Figure 6.3.2.1.B). When the total NOx concentration at 48 h was re-expressed to account for this difference in protein concentration (Figure 6.3.2.1.C), cytomix in the presence and absence of IL-8, resulted in a signifcant increase ($P < 0.05$) in total NOx above the control value. A similar pattern of results was seen in experiments involving cytomix and PGE_2 (Figure 6.3.2.2), cytomix and indomethacin (Figure 6.3.2.3), and cytomix

and EGF (Figure 6.3.2.4). No evidence for any modulatory effects of IL-8, PGE_2, indomethacin or EGF was found.

6.3.3. Lack of effect of cytokine challenge on total NOx concentration in the HGT-1 human gastric adenocarcinoma cell line.

The effect of cytomix: IFN-γ (4.75×10^4 units/μg), TNF-α (1.43×10^5 units/μg), IL-1ß (all at 10ng/ml) and LPS (10 μg/ml), and the iNOS inhibitor 1400W (10^{-4} M) on the total NOx concentration, was determined after 8, 24 and 48 h. This experiment was repeated twice with HGT-1 cells with similar results, and the data presented in Figure 6.3.3. shows the results from one of these experiments. Figure 6.3.3.A. demonstrates the lack of effect of cytokine challenge in the presence and absence of 1400W, on the total NOx concentration. Similarly, these agents had no effect on the protein content per well (Figure 6.3.3.B). Two factor analysis of variance did demonstrate a significant effect of time ($P < 0.01$), although this was not consistently either a progressive decrease of increase with time. After 48 h the mean protein concentration (mg/well) was determined and found to range from 3.43 - 3.75 mg/well, with an overall mean protein concentration of 3.62 ± 0.054 mg/well.

6.3.4. Lack of effect of cytokine challenge on total NOx concentration and on protein content of wells after 48 h, in the human AGS adenocarcinoma cell line.

Cytokine challenge did not affect total NOx concentration in the medium used to culture the AGS adenocarcinoma cell line. Two factor analysis of varinace did however demonstrate a significant effect of incubation time ($P < 0.01$), where a reduction in NOx concentration is demonstrated after the 8 h time point. Figure 6.3.4.B shows the lack of effect of cytomix alone and in combination with 1400W, on the protein content of wells after 48 h. Due to the lack of effect of cytomix on NOx concentration, the effect of further modulating agents was not investigated.

6.3.5. Lack of effect of cytokine challenge on total NOx concentration in a primary culture of guinea-pig tracheal epithelial cells.

Guinea-pig tracheal cells adhered to the culture plate overnight and grew to confluence usually within 4 days. The appearance of these cells as seen growing in a well of a 12 well culture plate is shown in Plate 6.3.5. When cells were grown on collagen coated (Figure 6.3.5.1.A) and uncoated (Figure 6.3.5.1.B) inserts there was no significant effect of cytomix in either cells grown on collagen-coated or uncoated inserts. However, a significant effect of time was demonstrated with both collagen-coated ($P < 0.05$) and uncoated ($P < 0.01$) data. Single factor analysis of variance showed no significant effect of treatment on the mean protein concentration determined for each treatment after 48 h. The protein concentration for each treatment was very similar ranging from 1.53 - 2.12 mg/well, with a mean concentration of 1.73 ± 0.13 mg/well.

When cells were preincubated in one of four different types of media and grown on plastic culture plates (as outlined in table 6.2.3.2) for 24 h prior to cytokine challenge, no significant effect of cytomix was found (Figure 6.3.5.2). Single factor analysis of variance demonstrated no significant difference in the mean protein concentration (mg/well) determined after 48 h, between treatment groups. The protein concentration for each treatment was very similar ranging from 2.80 - 3.03 mg/well, with a mean concentration of 2.93 ± 0.07 mg/well. Since results with tracheal cells were negative, there was no progression onto experiments with primary cultures of gastric mucous cells.

Plate 6.3.5. Primary culture of guinea-pig tracheal cells.

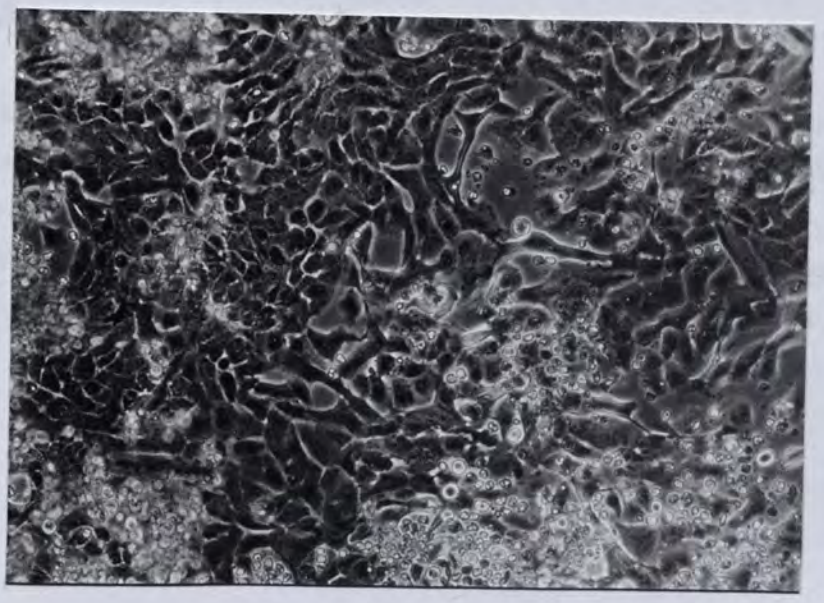
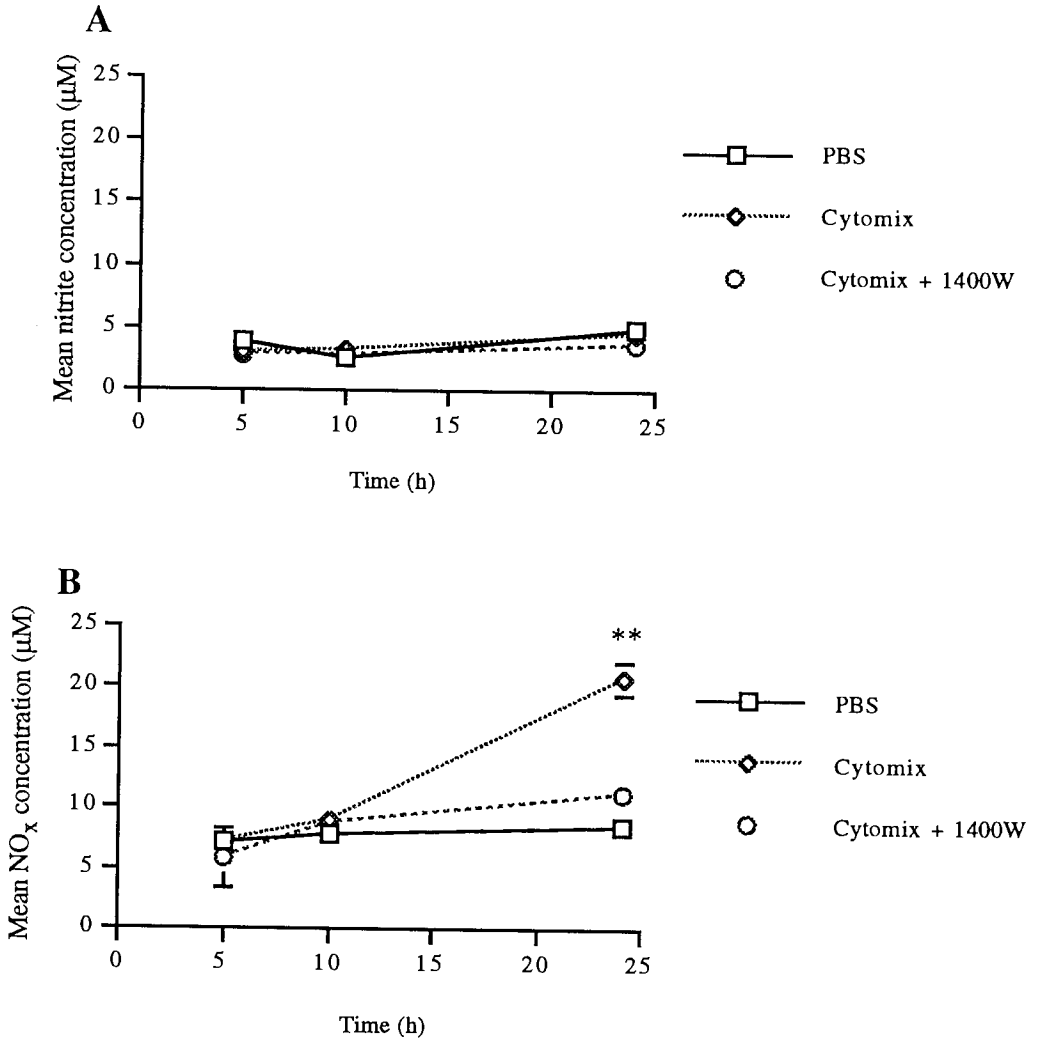


Figure 6.3.1.1.

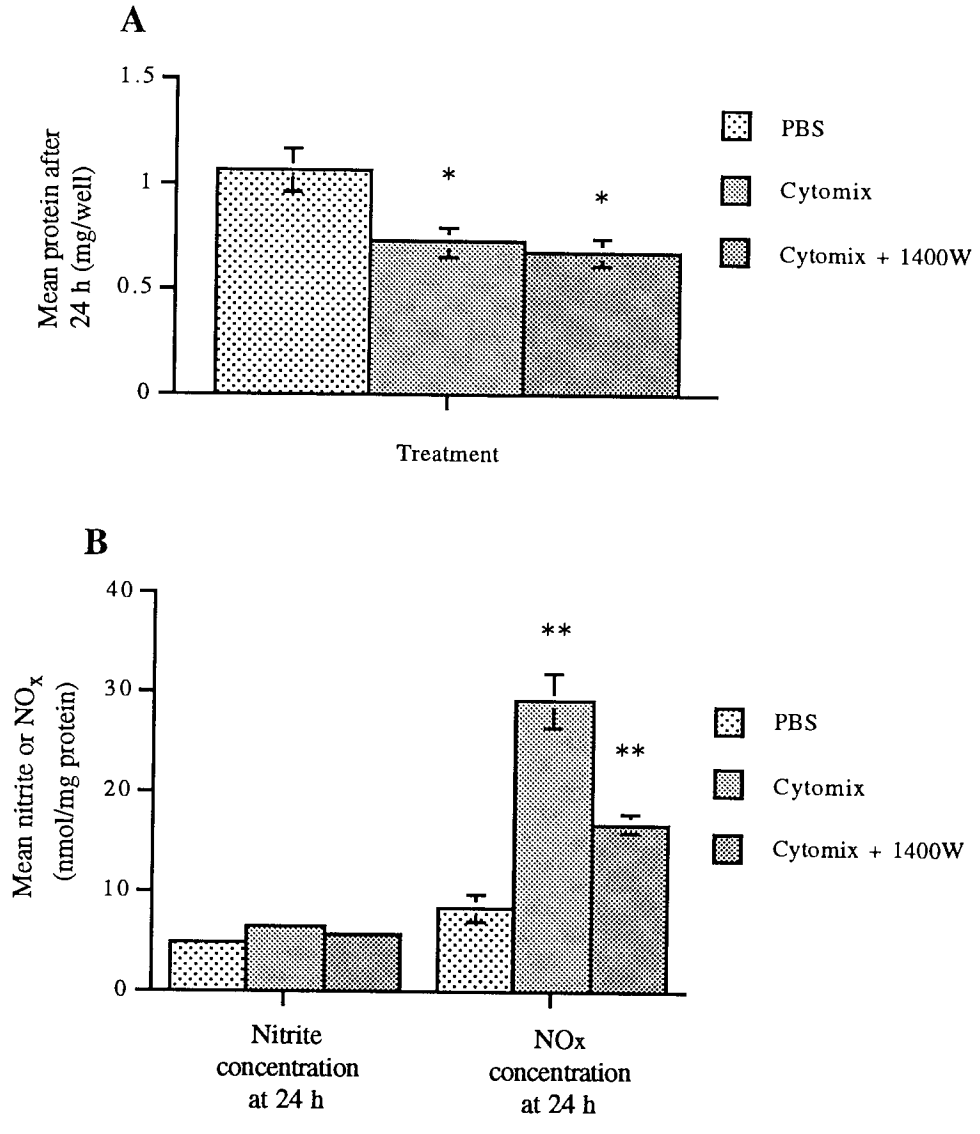
Effect of cytokine challenge and iNOS inhibitor on the change in nitrite (NO_2) (A) and total NOx (nitrate and nitrite) (B) with time in the A549 human lung adenocarcinoma cell line.



Data show means ± S.E. from one experiment with 4 wells per treatment. Medium was removed from individual wells after 5, 10, and 24 hours. The iNOS inhibitor used was 1400W (10^{-4}M in well). Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml), and LPS (10 µg/ml). The data were analysed by 2 way analysis of variance followed by Dunnett's test. ** $P < 0.01$ for difference from result with PBS.

Figure 6.3.1.2.

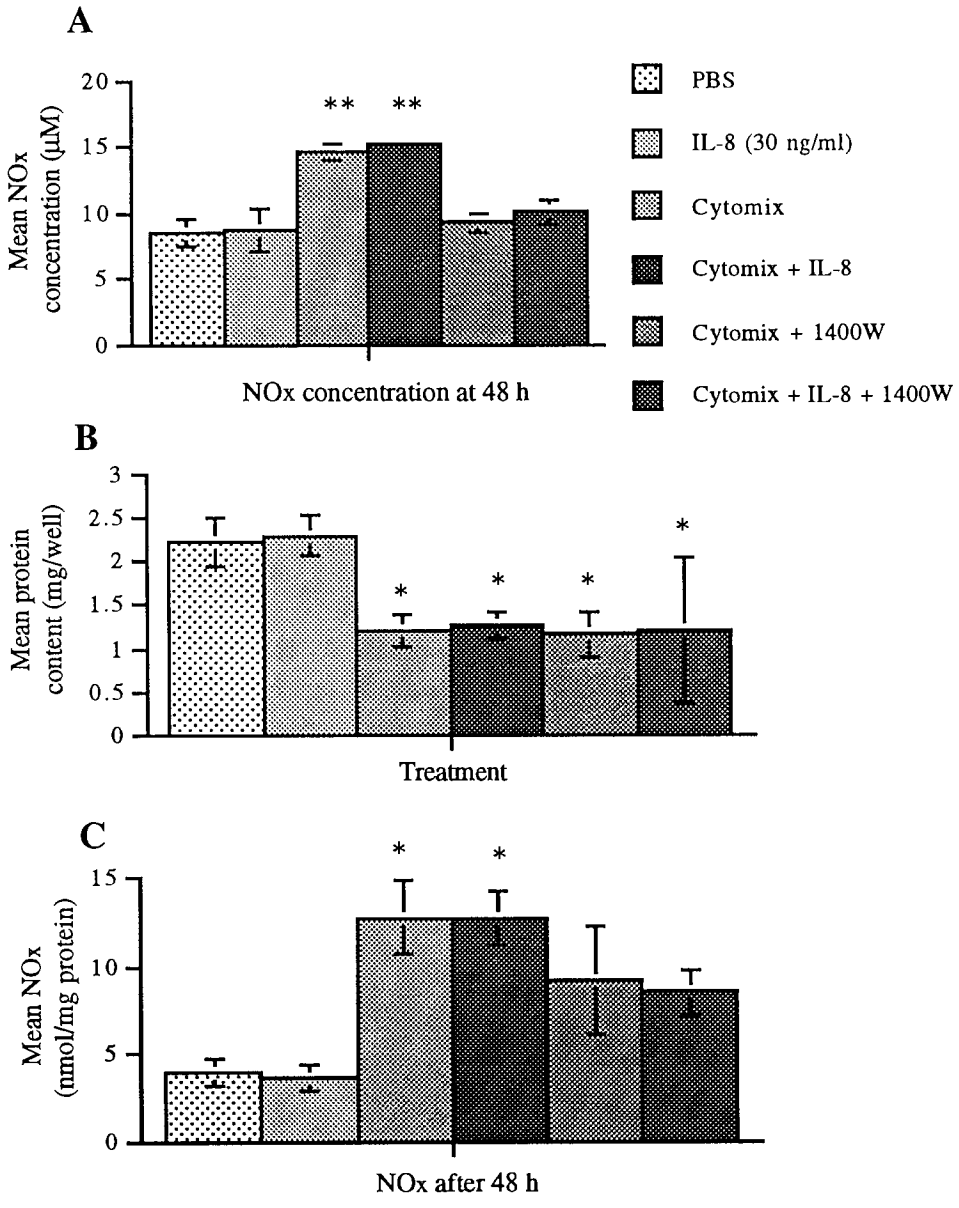
Effect of cytokine challenge and iNOS inhibitor on protein content (A), and NOx expressed as nmol/mg protein (B) after 24 h in the A549 human lung adenocarcinoma cell line.



Data show means ± S.E. from one experiment with 4 wells per treatment. Protein per well was determined after 24 h (A). The nitrite and total NOx at 24 h was recalculated to express data as nmol/mg protein (B). The iNOS inhibitor used was 1400W (10^{-4}M in well). Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml) and LPS (10 μg/ml). The data were analysed by 2 way analysis of variance followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$ for difference from result with PBS.

Figure 6.3.2.1.

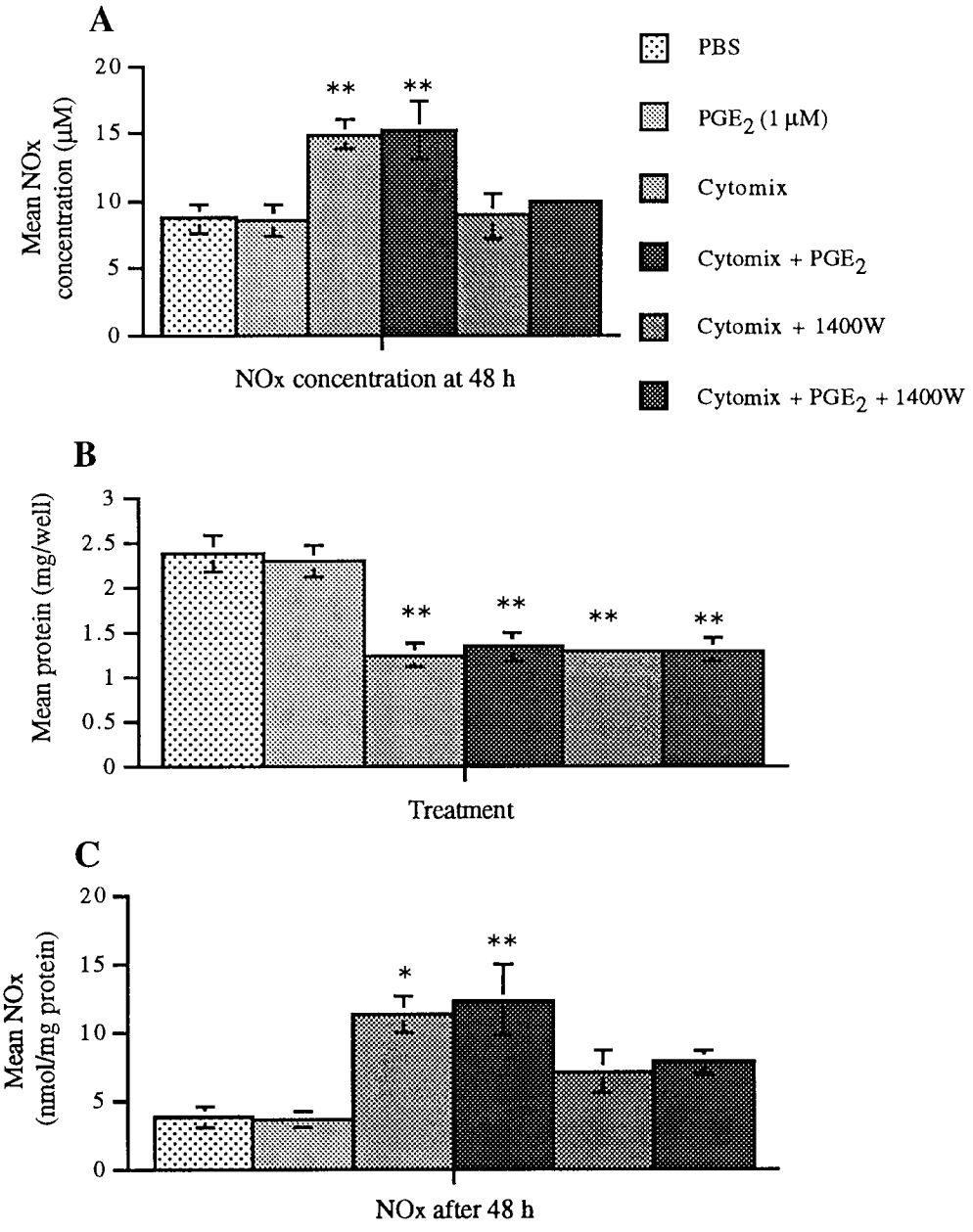
Effect of cytokine challenge, and lack of effect of IL-8 on NOx concentration (A), protein content (B), and NOx expressed as nmol/mg protein (C) after 48 h in A549 cells.



Data show means ± S.E. of 3 separate experiments with 4 wells per treatment in each experiment. Protein concentration per well was determined after 48 h (B), and total NOx (A) was recalculated to express data as nmol/mg protein (C). Cytomix = IFN-γ, TNF-α, IL-1β (at 10 ng/ml) and LPS (10 μg/ml). Concentration of agents: IL-8 (30 ng/ml), 1400W (10^{-4} M in well). The data were analysed by 2 way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, for difference from control.

Figure 6.3.2.2.

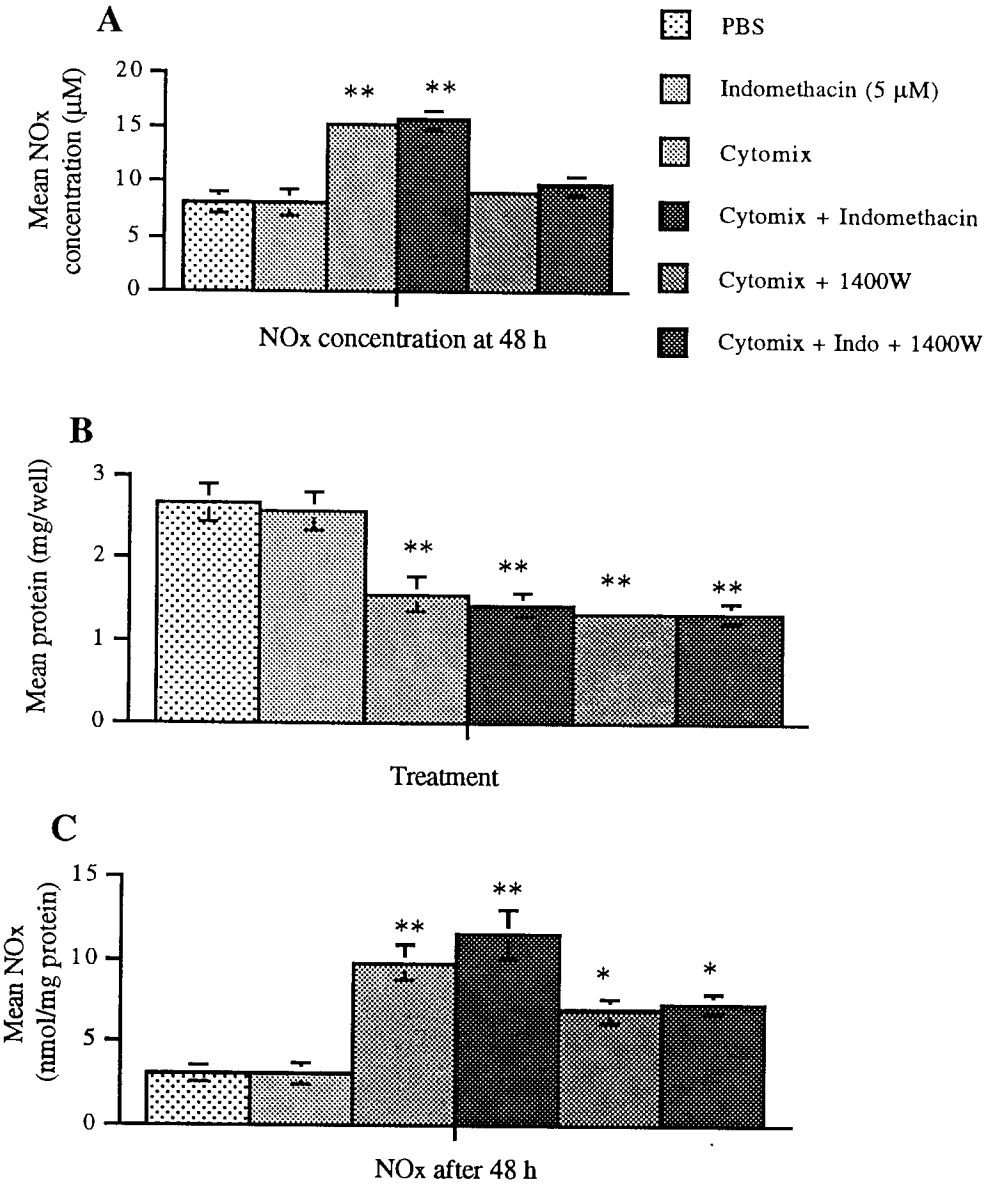
Effect of cytokine challenge, and lack of effect of PGE_2 on NOx concentration (A), protein content (B), and NOx expressed as nmol/mg protein (C) after 48 h in A549 cells.



Data show means ± S.E. of 3 separate experiments with 4 wells per treatment in each experiment. Protein concentration per well was determined after 48 h (B), and total NOx (A) was recalculated to express data as nmol/mg protein (C). Cytomix = IFN-γ, TNF-α, IL-1β (at 10 ng/ml) and LPS (10 μg/ml). Concentration of agents: PGE_2 (1 μM), 1400W (10^{-4} M in well). The data were analysed by 2 way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, for difference from control.

Figure 6.3.2.3.

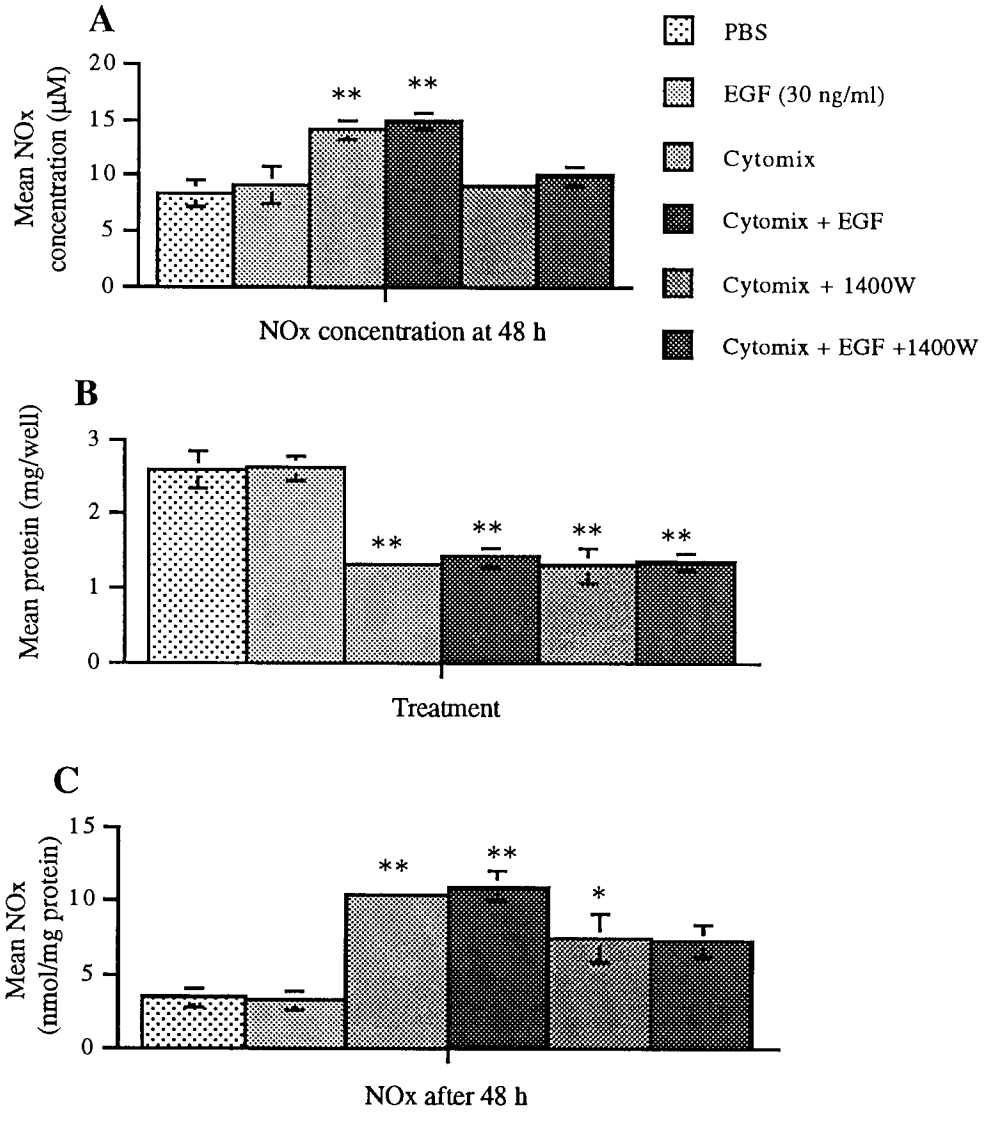
Effect of cytokine challenge, and lack of effect of Indomethacin on NOx concentration (A), protein content (B), and NOx expressed as nmol/mg protein (C) after 48 h in A549 cells.



Data show means ± S.E. of 3 separate experiments with 4 wells per treatment in each experiment. Protein concentration per well was determined after 48 h (B), and total NOx (A) was recalculated to express data as nmol/mg protein (C). Cytomix = IFN-γ, TNF-α, IL-1β (at 10 ng/ml) and LPS (10 μg/ml). Concentration of agents: Indomethacin (5 μM), 1400W (10^{-4} M in well). The data were analysed by 2 way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, for difference from control.

Figure 6.3.2.4.

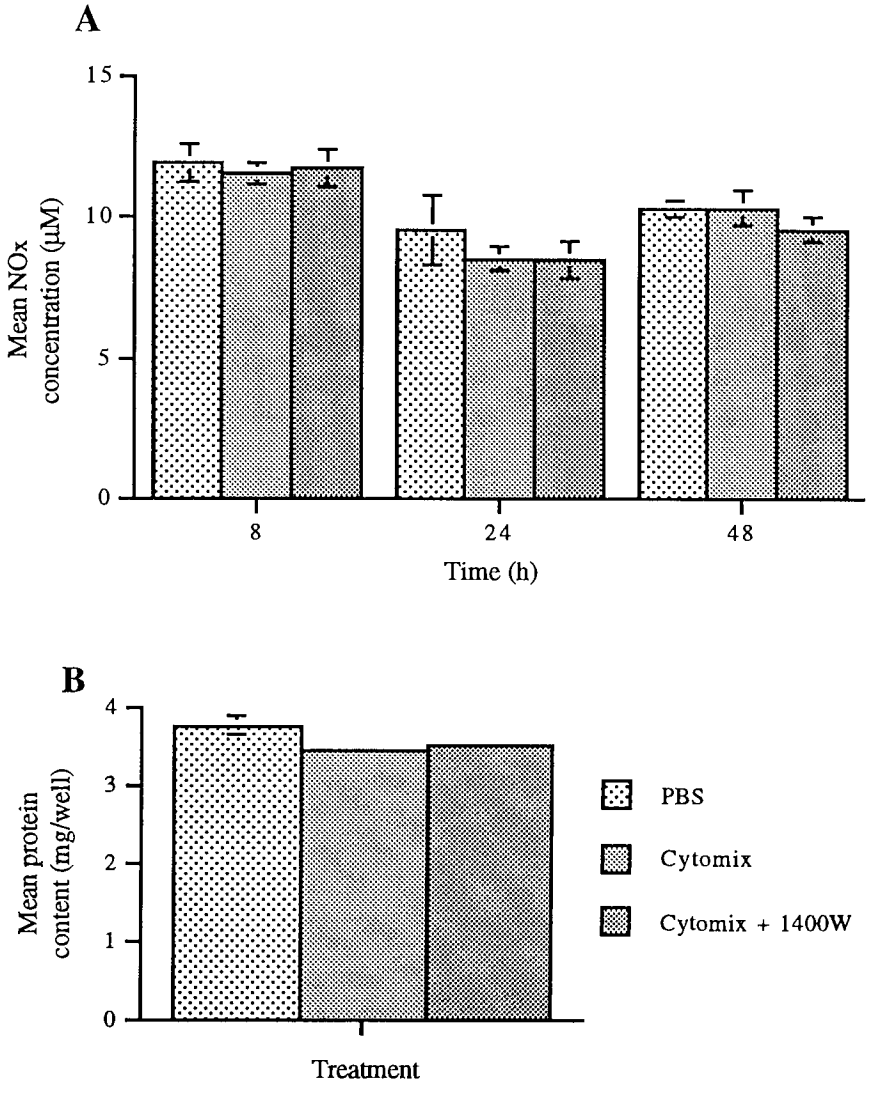
Effect of cytokine challenge, and lack of effect of EGF on NOx concentration (A), protein content (B), and NOx expressed as nmol/mg protein (C) after 48 h in A549 cells.



Data show means ± S.E. of 3 separate experiments with 4 wells per treatment in each experiment. Protein concentration per well was determined after 48 h (B), and total NO x (A) was recalculated to express data as nmol/mg protein (C). Cytomix = IFN-γ, TNF-α, IL-1β (at 10 ng/ml) and LPS (10 μg/ml). Concentration of agents: EGF (30 ng/ml), 1400W (10^{-4} M in well). The data were analysed by 2 way ANOVA followed by Dunnett's test. * $P < 0.05$, ** $P < 0.01$, for difference from control.

Figure 6.3.3.

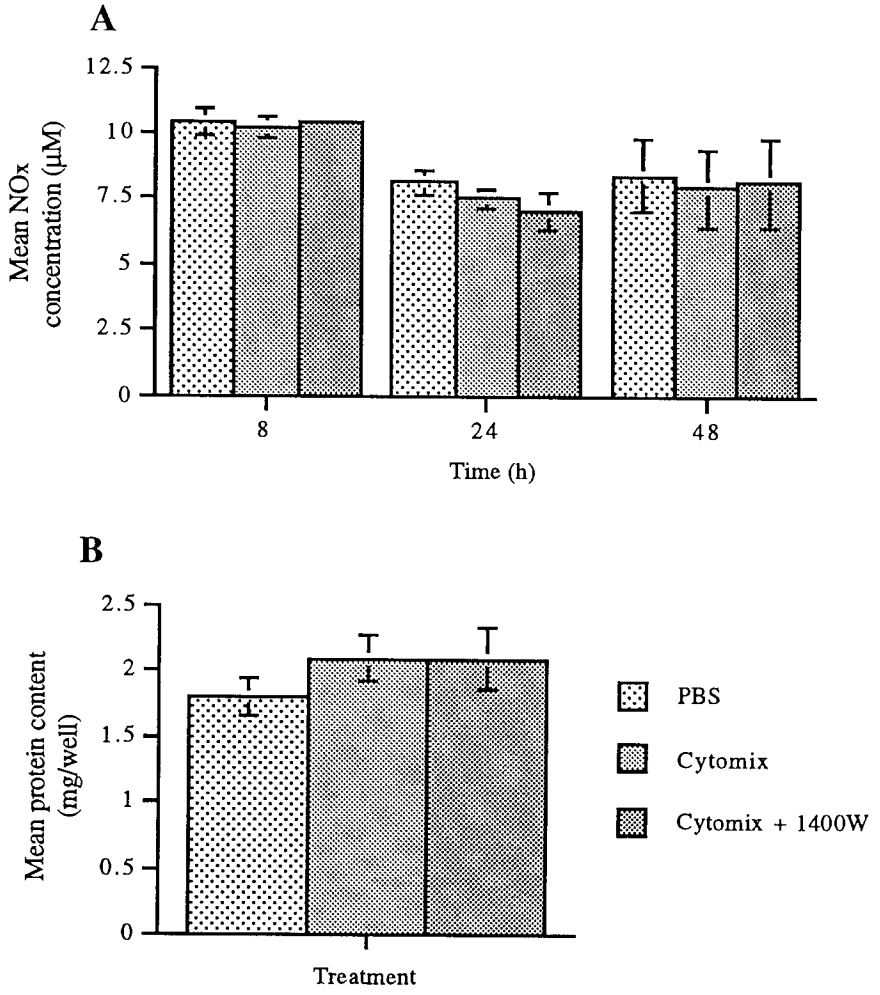
Lack of effect of cytokine challenge on total nitrite and nitrate (NOx) concentration in the medium used to culture the HGT-1 human gastric adenocarcinoma cell line (A), and on protein content of wells after 48 h (B).



Data show means ± S.E. from one experiment with 12 wells per treatment. Medium was removed from individual wells after 8, 24, and 48 h to ascertain change in NOx production with time. Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml) and LPS (10 µg/ml). 1400W was used at 10^{-4}M in well. The data were analysed by 2 way analysis of variance which showed a significant effect of time, but not of treatment. Single factor analysis of variance showed no effect of treatment on protein content after 48 h.

Figure 6.3.4.

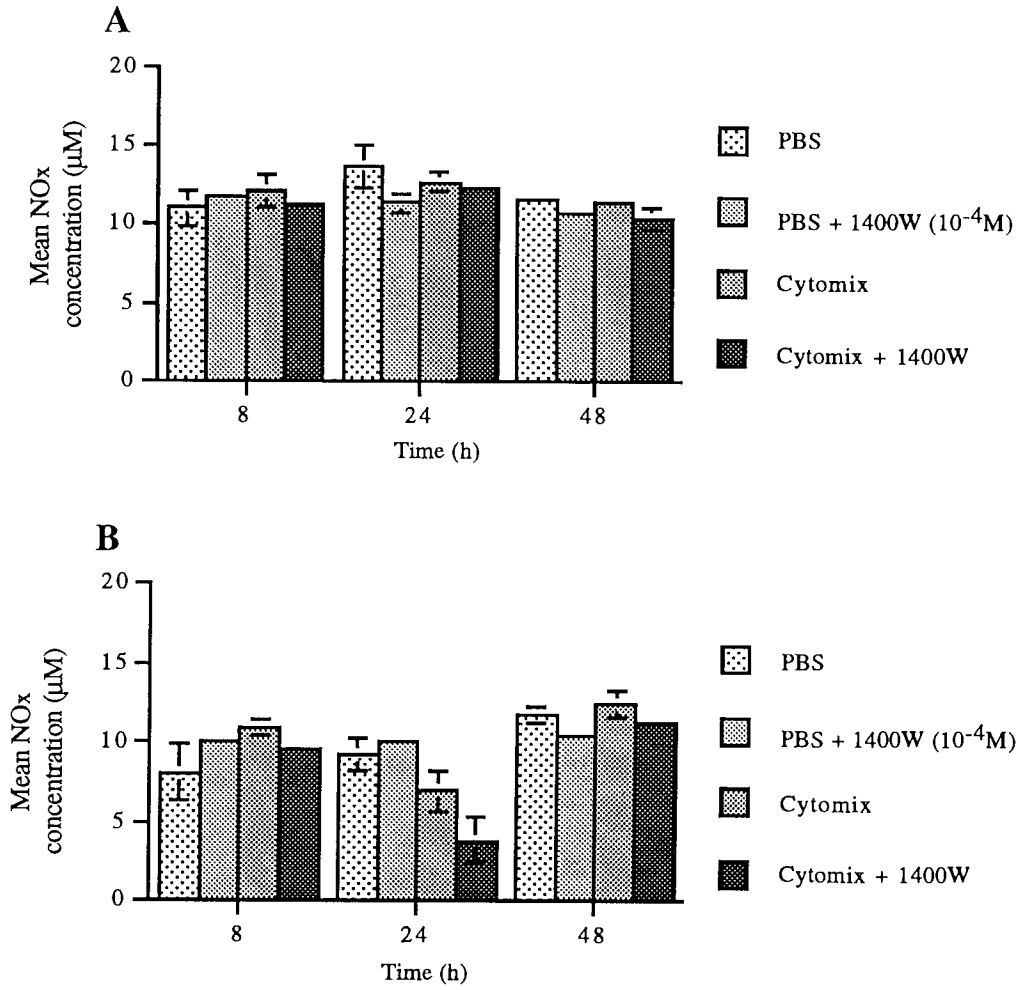
Lack of effect of cytokine challenge on total nitrite and nitrate (NOx) concentration in the medium used to culture the human AGS adenocarcinoma cell line (A), and on protein content of wells after 48 h (B).



Results are presented as means ± S.E.M. of values from 4 separate multiwell plates in one experiment, and with the value for each plate being the average of 4 separate wells. Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml) and LPS (10 μg/ml). 1400W was used at 10^{-4}M in well. Two factor analysis of variance showed a significant effect of incubation time ($P < 0.01$), but not of treatment (A). Single factor analysis of variance showed no effect of treatment on protein in the wells after 48 h (B).

Figure 6.3.5.1.

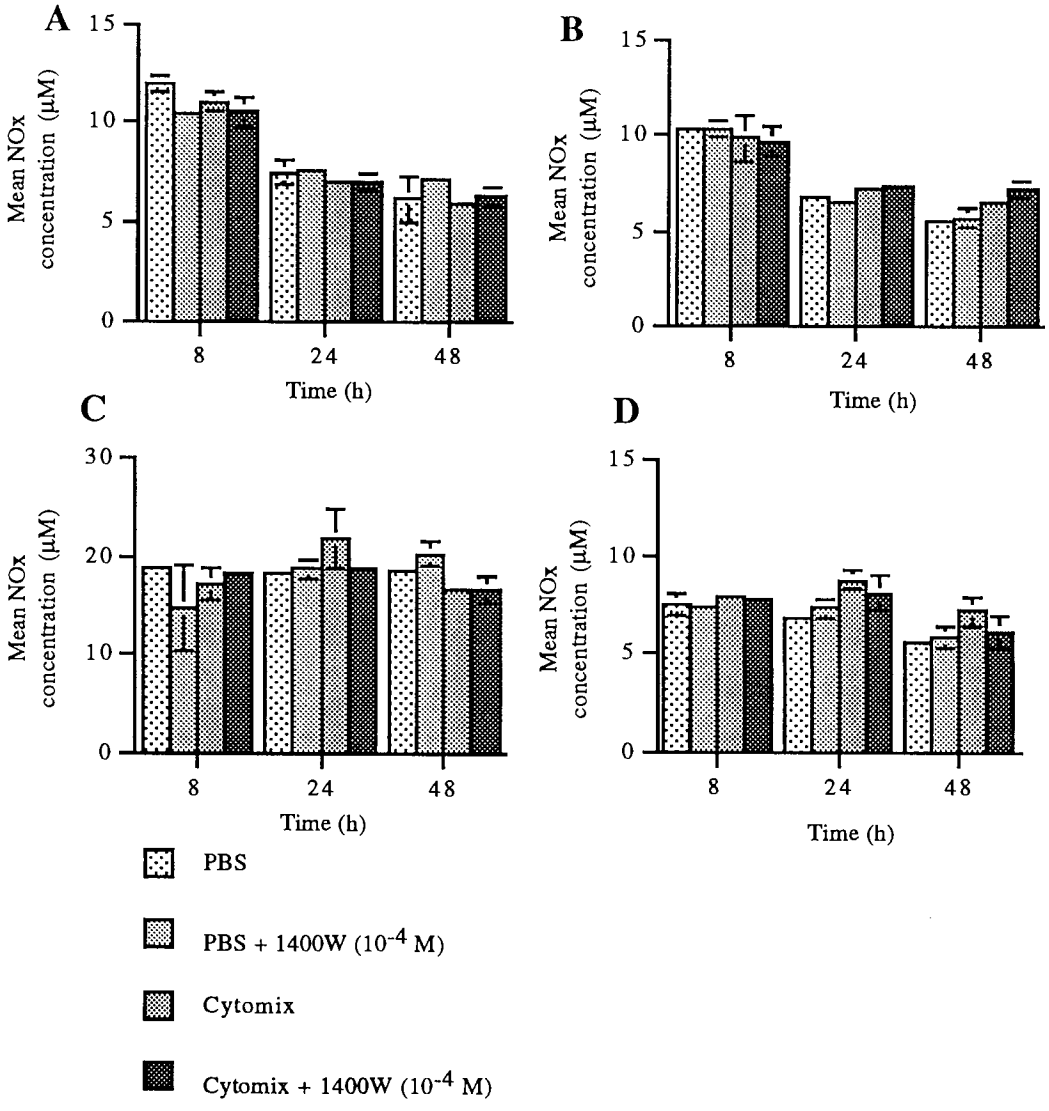
Lack of effect of cytokine challenge on total nitrite and nitrate (NOx) concentration in a primary culture of guinea-pig tracheal epithelial cells grown on collagen-coated (A) and uncoated (B) inserts in a 24 well plate.



Data shows means ± S.E. from one experiment with 3 wells per treatment. Medium was removed from individual wells after 8, 24 and 48 h to ascertain change in NOx concentration with time. The iNOS inhibitor used was 1400W (10^{-4}M in well). Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml) and LPS (10 µg/ml). Two factor analysis of variance showed a significant effect of incubation time ($P < 0.05$), but no effect of treatment with either collagen coated (A) or uncoated (B) data.

Figure 6.3.5.2.

Effect of incubation conditions and lack of effect of cytokine challenge on total nitrite and nitrate (NOx) concentration in a primary culture of guinea-pig tracheal epithelial cells.



Data shows means ± S.E. from one experiment with 3 wells per treatment. The iNOS inhibitor used was 1400W (10^{-4}M in well). Cytomix = IFN-γ, TNF-α, IL-1ß (all at 10 ng/ml) and LPS (10 μg/ml). Within one experiment cells were preincubated in 4 different types of media for 24 h prior to cytokine challenge: growth media (A), growth media without hydrocortisone (B), basal media (C), basal media without serum (D) (see section 6.2.3.2). Two factor analysis of variance showed a significant effect of incubation time ($P < 0.05$), but no effect of cytomix or 1400W with any of the treatment groups.

6.4. DISCUSSION

6.4.1. Response to cytokines and modulating agents in A549 cells.

The induction of iNOS in the A549 cell line in response to cytokines has previously been reported (Kobzik et al., 1993; Robbins et al., 1994a; Asano et al., 1994; Berkman et al., 1996). In the present work the ability of cytomix to signifcantly increase the total NOx concentration above the control level, is suggestive of an induction of iNOS activity. This induction of iNOS was confirmed by the use of the selective iNOS inhibitor 1400W (Garvey et al., 1997), which was able to reduce the cytokine elicited increase in NOx concentration to a level in most cases, not significantly different from the control. This suggests that the increase in total NOx concentration observed with cytomix is due to the action of inducible NO synthase, and that this response is inhibitable with 1400W. The A549 pilot experiments (section 6.3.1) demonstrate that little nitrite was produced, and that cytomix appeared to have no significant effect until nitrate was also converted to nitrite, and the total NOx production was measured. Although some previous studies concerned with iNOS induction in cell culture have not found it necessary to measure nitrate production in addition to nitrite (Robbins et al., 1994a and 1994b; Cunha et al., 1992), in a separate study using A549 cells (Asano et al., 1994) a similar pattern of nitrate and nitrite production in response to cytomix was observed, to that presented here. In cell cultures producing NO, both nitrite and nitrate are produced, with the proportion of each species varying between cell types and under different conditions, thus reflecting the pathway of NO oxidation (Schmidt,1995). If NO is oxidised by molecular oxygen, nitrite is the sole product. *In vivo*, NO targets a variety of biological species, resulting in a series of reactions, with nitrate as the final product (Feldman et al., 1993). The high proportion of nitrate production in comparison to nitrite in A549 cells suggests predominantly such an indirect pathway of NO oxidation. In further experiments with A549 cells no effect of the agents IL-8, PGE_2, indomethacin and EGF, to modulate the response to cytomix was found.

This result with IL-8 in A549 cells is in contrast to that obtained with rat peritoneal neutrophils (McCall et al., 1992), where IL-8 caused a concentration-dependent inhibition of the induction of iNOS. However, addition of indomethacin to these cells, gave a result similar to that obtained with A549 cells, where the agent had no effect on the induction of NO synthase. In a separate study using A549 cells, interleukins have been shown to modulate the activity of iNOS (Berkman et al., 1996). The ability of the products of T-helper (Th2) cells: IL-4, IL-10 and IL-13,

to inhibit the response of A549 cells to cytomix was investigated. IL-4 and IL-13, but not IL-10, were shown to inhibit both iNOS mRNA expression and nitrite release in these cells. By contrast, in murine macrophage J774 cells IL-10 inhibited the IFN-γ induction of iNOS activity, an effect also seen with IL-4 (Cunha et al., 1992). A similar reduction in nitrite generation by cytomix-stimulated iNOS activity, was observed with IL-4 and IL-10 in rat alveolar macrophages (Warner et al., 1995). Thus A549 cell iNOS expression can be modulated by some cytokines. The lack of effect of IL-8 seen here may reflect the lack of a receptor for this cytokine or the generation of inappropriate or insufficient intracellular signals.

Use of cytomix in all of the A549 experiments resulted in a significant reduction in the protein content per well, when measured either after 24 h (pilot experiment) or after 48 h. As a similar level of reduction in protein content was observed with cytomix in the presence of 1400W, it is unlikely that loss of protein is due to cytotoxic effects of induced NO. Although the cytotoxic effects of NO are well documented (section 1.4), it appears that the cytokines themselves are cytotoxic to A549 cells after prolonged exposure. For this reason total NOx concentrations were re-expressed to account for the change in protein content after 48 h. Despite the cytotoxicity of the cytokines causing a reduction in protein content, the remaining viable cells demonstrated an increased NOx production, which was inhibitable with 1400W.

6.4.2. Response to cytokines and modulating agents in gastric cell lines.

Gastric cell lines were exposed to the same combination of cytokines as for A549 cells, for the same duration, in an attempt to induce iNOS, and to compare the effect of modulating agents. In contrast to the results obtained with A549 cells, treatment of HGT-1 and AGS cell lines with cytomix did not cause the induction of iNOS, as evidenced by the lack of increase in total NOx concentration, and the lack of effect of iNOS inhibitor 1400W. iNOS can be induced in monolayers of the human intestinal epithelial cell line DLD-1, derived from a human intestinal adenocarcinoma, in response to a combination of IFN-γ and IL-1ß (Salzman et al., 1996), but the gastric cell lines used here were negative, although human derived. Possible explanations are that the cells were transformed and had lost the components necessary for cytokines to induce iNOS, that the cytokines used were not the optimal combination of agents to induce activity, or that one of the agents used acted to down-regulate induction in these cells. In A549 cells the cytotoxic effect of cytokines was not linked to production of NO. The absence of

cytotoxicity with HGT-1 cells and AGS cells suggests a general inability of these cells to sense cytokines.

6.4.3. Response to cytokines and modulating agents in primary cultures of guinea-pig tracheal cells.

In order to establish the most favourable conditions for investigating the effects of cytokines and modulating agents in primary cultures of guinea-pig tracheal epithelial cells, a number of pilot experiments were conducted. Many primary cultures are grown in culture dishes first coated by a collagen support which enables adherence, or grown on plastic inserts which fit inside the wells on the culture plate, which enables polarisation and differentiation (Steele et al., 1986). Cell lines are routinely serum-starved for 24 h prior to cytokine challenge (Robbins et al., 1994a, 1994b), in order to remove any endogenous factors which may effect the response under investigation. Glucocorticoids, particularly dexamethasone (Simmons et al., 1996), as well as hydrocortisone (O'Connor and Moncada, 1991) are known to suppress the cytokine induction of iNOS in many cell types. Therefore, in some experiments primary cells were incubated in hydrocortisone-free growth media for 24 h prior to challenge, to determine whether this glucocorticoid was responsible for the lack of iNOS induction. The pilot experiments in this study investigated the effects of collagen coating, inserts, and a variety of media, on the response of the primary cells to cytomix. Although the response of the cells were tested under a variety of conditions, no change in total NOx concentration was observed in response to cytomix in the presence or absence of 1400W. Cytokines have not previously been shown to induce iNOS in human or guinea-pig trachea. However, exhaled human breath contains NO derived from iNOS, some of which originates from the trachea, although this was found to be 8-fold lower than that in the nose, and not cytokine induced (Dillon et al., 1996). In addition, evidence suggests that the i.v. administration of LPS to rats led to iNOS expression in the trachea (Bernareggi et al., 1997), which contrasts with the lack of iNOS induction in response to LPS in combination with cytokines, in guinea-pig tracheal cells, presented here. As with the gastric cell lines, but again in contrast to A549 data, no change in the protein content per well was observed with any of the treatments tested.

The lack of cytotoxicity of cytokine action alone observed with primary cells, raises the question as to whether the primary cells were able to recognise and respond to the cytokines used in any way at all. As cytomix alone was toxic to

A549 cells, it might be expected that a similar response would be observed with primary cells. It is possible that the human derived cytokines used in this study are sufficiently different from those in guinea-pig so as to be unable to effect a response in guinea-pig cells. However, evidence from the literature suggests that rodent cells are capable of responding to human cytokines (Pfeilschifter et al., 1992; Robbins et al., 1994b). In a study using rat renal mesangial cells, a type of vascular smooth muscle cell, use of the human recombinant cytokines IL-1ß and TNF-α, caused the induction of iNOS (Pfeilschifter et al., 1992). Whereas, the LA-4 murine lung epithelial cell line was stimulated to produce iNOS in response to recombinant human IL-1ß and TNF-α, and murine recombinant IFN-γ (Robbins et al., 1994b). If the lack of iNOS induction in guinea-pig tracheal primary cells is not due to an inability of the cells to respond to human cytokines, the lack of response may be due to the dedifferentiation of cells in the primary culturing process with loss of receptors, or may signify a species difference between guinea-pig and human.

6.4.4. Summary

1. The human adenocarcinoma A549 cell line produces predominantly nitrate in response to "cytomix", an effect attributed to iNOS induction due to the ability of the iNOS inhibitor 1400W to reduce NOx production to basal levels.

2. The agents: IL-8, PGE_2, indomethacin and EGF, failed to modulate this iNOS induction in A549 cells.

3. "Cytomix" failed to induce iNOS activity in the HGT-1 and AGS gastric cell lines, and also in a primary culture of guinea-pig tracheal cells.

CHAPTER 7

CONCLUSIONS

7.0. CONCLUSION

The potential for gastrointestinal damage from factors in the local environment, and the existence of effective mucosal defence mechanisms which preserve integrity, have been constant themes throughout this thesis. The action of a variety of agents, such as reactive oxygen intermediates (nitric oxide, peroxynitrite and superoxide), alcohol, hypertonic or acidic solutions, pathogens and cytokines involved with inflammatory disease, all threaten the integrity of the gastric mucosa. However, the concerted action of a number of defence mechanisms and mediators including: the mucous-bicarbonate layer, restitution and polyamine synthesis and subsequent cell division, the glutathione cycle, inducible heat shock proteins, prostanoids and EGF, are able to prevent excessive damage, in all but extreme cases. The cytoprotection or cytotoxicity afforded by NO, associated with some of these mechanisms has been discussed at the end of each individual chapter.

A novel finding of this thesis is that the dominant pathway of arginine metabolism in isolated rat gastric mucosal cells is one involving the action of arginase and ornithine transcarbamoylase, and not NO synthase (Chapter 3). Determination of arginase activity in homogenates prepared from gastric mucosal scrapes, confirmed that arginase was present in the intact tissue and had not been induced as a result of damage inflicted during the isolation process. Evidence that the isolation process is causing trauma to these cells, is the increased expression of inducible HSP 72 with time after isolation (Chapter 5). Arginase acts to convert arginine to ornithine, which is the substrate required by ornithine decarboxylase, the first and rate limiting enzyme in polyamine synthesis. A substantial induction of ornithine decarboxylase activity in the gastric mucosa was observed in response to *in vivo* exposure to hypertonic saline, in rats which had been pretreated with the NO synthase inhibitor L-NA prior to challenge (Chapter 4). The synthesis of polyamines, which are required for growth and repair, has been shown to increase in response to superficial damage in some rodent models. It is possible that a role of the basal arginase activity in the gastric mucosa, a tissue which is constantly replacing sloughed and damaged cells, is to provide a supply of ornithine substrate, for use by ornithine decarboxylase, to provide the polyamines necessary for regeneration. Indeed, exposure to hypertonic saline did not result in a significant loss of NO synthase activity, measured 4 h later, although evidence suggests that this enzyme resides in surface mucosal cells, presumably due to the action of efficient restitution and defence systems (Chapter 4). The induction of ornithine decarboxylase activity observed in this study however is not solely as a result of

damage inflicted, as hypertonic saline alone, in animals where there was no prior inhibition of NO synthase, did not exhibit ornithine decarboxylase induction. The dose dependence of the ornithine decarboxylase response on the L-NA concentration, and the inability of the inactive isomer D-NA to induce activity, demonstrates that NO has a modulatory role in control of this enzyme. It is possible that inhibition of NO synthesis results in release of a local mediator, perhaps histamine, which may induce ornithine decarboxylase activity directly or inhibit the release of a negative modulator. NO is known to have an inhibitory effect on mast cells, so its removal by use of L-NA, which was responsible for the increased epithelial permeability and luminal volume, was probably due to mast cell degranulation and the subsequent release of inflammatory agents such as histamine and platelet-activating factor. In conclusion, the response of the rat gastric mucosa to hypertonic saline exposure did not result in global NO synthase changes, and an involvement of NO in the response was demonstrated by the ability of L-NA to promote induction of ornithine decarboxylase activity in response to damage (Chapter 4). Apart from providing ornithine for polyamine biosynthesis, arginase activity could potentially limit the supply of arginine to NO synthase. This interaction has been established in macrophages but we were unable to establish this effect in gastric mucosal cells due to the activity of the NO synthase pathway being below the level of detection.

A role for constitutive NO has been implicated in the preservation of gastric mucosal integrity, however, higher levels of NO resulting from NO donation or iNOS induction have been associated with cytotoxicity (Chapter 1). Indeed, high NO output accounts for the cytotoxic abilities of macrophages (Chapter 1), and induction of iNOS activity comprises part of the host innate immune reaction and can occur in response to bacterial infection and cytokines (Chapter 6). Experiments conducted in this thesis using primary cultures of guinea-pig gastric mucosal cells demonstrated the involvement of NO and oxidative challenge in the induction of HSP 72. Oxidative threat was imposed on these cells with the use of the agent diethyl maleate, which reduced intracellular levels of the protective antioxidant, reduced glutathione, in combination with the NO donor SNAP (Chapter 5). An induction of HSP 72 resulted, which was dependent on the dose of SNAP administered and on the presence of diethyl maleate. Induction was inhibitable by the NO scavenger carboxy-PTIO, suggesting a direct involvement of NO in the induction. This signifies the ability of gastric mucosal cells to upregulate a defence mechanism, the heat shock response, when under threat to NO or other agents capable of causing oxidant damage.

In an effort to further elucidate the protective mechanisms employed in the gastrointestinal tract, and the role played by NO, an attempt was made to induce iNOS activity using cytokines, and to investigate the effects of potential modulatory agents on this induction (Chapter 6). Although cytokines stimulated iNOS induction in the A549 pulmonary cell line, a system similar to the gastrointestinal tract in its extensive employment of NO and defence requirements, no effects were observed with the agents tested, and no induction was observed with gastric cell lines.

In conclusion, an arginase pathway may restrict availability of arginine for NO synthase in gastric mucosa, or may be present to supply ornithine for polyamine synthesis. NO may modulate the response of the gastric epithelium to damage *in vivo*. Exogenous NO may induce a defensive response in gastric mucosal cells. NO has many varied roles in the gastrointestinal tract, and the regulation and control of these mediatory roles is complex. The work presented in this thesis contributes to previous evidence suggesting that NO is involved in protective mechanisms in the gastrointestinal tract, although these roles are far from straight forward and require further evaluation.

REFERENCES

8.0. REFERENCES

Abe T, Konishi T, Hirano T, Kasai H, Shimizu K, Kashimura M, Higashi K. (1995) Possible correlation between DNA damage induced by hydrogen peroxide and translocation of heat shock 70 protein into the nucleus. *Biochem. Biophys. Res. Commun.*;**206**: 548-555.

Adler KB, Fischer BM, Li H, Choe NH, Wright DT. (1995) Hypersecretion of mucin in response to inflammatory mediators by guinea-pig tracheal epithelial cells *in vitro* is blocked by inhibition of nitric oxide synthase. *Am. J. Respir. Cell Mol. Biol.*; **13**: 526-530.

Albina GE, Mills CD, Henry WL, Caldwell MD. (1990) Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.*; **144**: 3877-3880.

Allen A, Flemstrom G, Garner A, Kivilaakso E. (1993) Gastroduodenal mucosal protection. *Phys. Revs.*; **73**: 823-857.

Anathan J., Goldberg A.L., Voellmy R. (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science Wash. DC* **232**: 252-254.

Ang D, Liberek K, Skowyra D, Zyliez M, Georgopoulos C. (1991) Biological role and regulation of the universally conserved heat shock proteins. *J.Biol. Chem.*; **266**: 24233 - 24236.

Aoki E, Takeuchi IK, Shoji R. (1995) Nitric oxide: an attractive signalling molecule. *Acta. Histochem. Cytochem.*; **28**: 97-106.

Asahi M, Fujii J, Suzuki K, Seo HG, Kuzuya T, Hori M, Tada M, Fujii S, Taniguchi N. (1995) Inactivation of glutathione peroxidase by nitric oxide. *J.Biol. Chem.*; **270**: 21035 - 21039.

Asano K, Chee CBE, Gaston B, Lilly CM, Gerard C, Drazen JM, Stamler JS. (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci.*; **91**: 10089-10093.

Atwell M.M. (1991) PhD Thesis, Aston University.

Baler R, Dahl G, Voellmy R. (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Mol. Cell Biol.*; **13**: 2486 - 2496.

Berkman N, Robichaud A, Robbins RA, Roesems G, Haddad EB, Barnes PJ, Chung KF. (1996) Inhibition of inducible nitric oxide synthase expression by interleukin-4 and interleukin-13 in human lung epithelial cells. *Immunology*; **89**: 363-367.

Bernareggi M, Mitchell JA, Barnes PJ, Belvisi MG. (1997) Dual action of nitric oxide on airway plasma leakage. *Am. J. Resp. Critical Care Med.*; **155**: 869-874.

Blachier F, Darcy-Vrillon B, Sener A, Duee P-H, Malaisse WJ. (1991) Arginine metabolism in rat enterocytes. *Biochim. Biophys. Acta.*; **1092**: 304-310.

Blachier F, Robert V, Selamnia M, Mayeur C, Duee PH. (1996) Sodium nitroprusside inhibits proliferation and putrescine synthesis in human coclon-carcinoma cells. *FEBS Letts.*; **396**: 315-318.

Bogle RG, Moncada S, Pearson JD, Mann GE. (1992) Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.*; **105**: 768-770.

Boucher J-L, Custot J, Vadon S, Delaforge M, Lepoivre M, Tenu J-P, Yapo A, Mansuy D. (1994) N^{ω}-hydroxy-L-arginine, an intermediate in the L-arginine to nitric-oxide pathway, is a strong inhibitor of liver and macrophage arginase. *Biochem. Biophys. Res. Commun.*; **203**: 1614-1621.

Bredt DS, Ferris CD, Snyder SH. (1992) Nitric oxide synthase regulatory sites. *J. Biol. Chem.*; **267**: 10976-10981.

Bredt DS, Snyder SH. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA.*; **87**: 682-685.

Bredt DS, Snyder SH. (1994) Nitric oxide: a physiologic mesenger molecule. *Annu. Rev. Biochem.*; **63**: 175-195.

Bredt, D.S., Snyder S.H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA.* **86**: 9030-9033.

Brown J.F. (1994) PhD Thesis, Aston University.

Brown JF, Hanson PJ, Whittle BJR. (1992a) Nitric oxide donors increase mucus gel thickness in rat stomach. *Eur. J. Pharmacol.*; **223**: 103-104.

Brown JF, Hanson PJ, Whittle BJR. (1993) The nitric oxide donor, s-nitroso-n-acetyl-penicillamine, inhibits secretory activity in rat isolated parietal cells. *Biochem. Biophys. Res. Commun.*; **195**: 1354-1359.

Brown JF, Keates AC, Hanson PJ, Whittle BJR. (1993) Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *Am. J. Physiol.*; **265**: G418-G422.

Brown JF, Tepperman BL, Hanson PJ, Whittle BJR, Moncada S. (1992) Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. *Biochem. Biophys. Res. Commun.*; **184**: 680-685.

Brown JF, Tepperman BL, Hanson PJ, Whittle BJR. (1994) Lipopolysaccharide induces Ca^{2+}-independent nitric oxide synthase activity in rat gastric mucosal cells. *Eur. J. Pharmacol.*; **292**: 111-114.

Castro L, Rodriguez M, Radi R. (1994) Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem.*; **269**: 29409-29415.

Chee C, Gaston B, Gerard C, Loscalzo J, Kobzik L, Drazen J, Stamler J. (1993) Nitric oxide is produced by a human epithelial cell line. *Am. Rev. Respir. Dis.*; **147**: A433.

Chen F-Y, Lee TJ-F. (1995) Arginine synthesis from citrulline in perivascular nerves of cerebral artery. *J. Pharmacol. Exp. Ther.*; **272**: 895-901.

Cook H.T, Jansen A, Lewis S, Largen P, O'Donnell M, Reaveley D, Cattell V. (1994) Arginine metabolism in experimental glomerulonephritis: interaction between nitric oxide synthase and arginase. *Am.J.Physiol.* **267**: F646-F653.

Cosentino F, Sill JC, Katusic ZS. (1994) Role of superoxide anions in the mediation of endothelium-dependent contractions. *Hypertension*; **23**: 229-235.

Craig EA, Weissman JS, Horwich AL. (1994) Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. *Cell* ; **78**: 365 - 372.

Cross C.E.B, Halliwell B, Allen A. (1984) Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. *Lancet* ; **1**: 1328-1330.

Cunha FQ, Moncada S, Liew FY. (1992) Interleukin-10 inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochem. Biophys. Res. Commun.*; **182**: 1155-1159.

Daghigh F, Fukuto JM, Ash DE. (1994) Inhibition of rat liver arginase by an intermediate in NO biosynthesis, N^G-hydroxy-L-arginine: implications for the regulation of nitric oxide biosynthesis by arginine. *Biochem. Biophys. Res. Commun.*; **202**: 174-180.

Davda RK, Chandler LJ, Guzman NJ. (1994) Protein kinase C modulates receptor-independent activation of endothelial nitric oxide synthase. *Eur. J. Pharmacol.*; **266**: 237-244.

Dillon WC, Hampl V, Shultz PJ, Rubins JB Archer SL. (1996) Origins of breath nitric oxide in humans. *Chest.*; **110**: 930-938.

Dinarello CA. (1988) Biology of interleukin 1. *FASEB J.*; **2**: 108-115.

Ding X-Q, Chen C, Rosengren E, Persson L, Håkanson R. (1996) Comparison between activation of ornithine decarboxylase and histidine decarboxylase in rat stomach. *Am. J. Physiol.*; **270**: G476-G486.

Endoh K, Kao J, Domek M.J, Leung F.W. (1993) Mechanism of gastric hyperemia induced by intragastric hypertonic saline in rats. *Gastroenterol.* ; **104**: 114-121.

Esplugues JV, Whittle BJR, Moncada S. (1992) Modulation by opiods and by afferent sensory neurones of prostanoid protection of the rat gastric mucosa. *Br. J. Pharmacol.*; **106**: 846-852.

Feldman PL, Griffith OW, Stuehr DJ. (1993) The surprising life of nitric oxide. *Chem. Eng. News.*; **71**: 51, 26-38.

Ferraz J-GP, Tigley A, Wallace JL. (1994) Paradoxical effects of L-arginine on gastric mucosal integrity. *Eur. J. Pharmacol.*; **260**: 107-111.

Fiers W. (1991) Tumour necrosis factor. Characterisation at the molecular, cellular and in vivo level. *FEBS Letts.*; **285**:199-212.

Flemstrom G. (1994) Gastric and duodenal mucosal secretion of bicarbonate. In: Johnson LR. (ed), The Physiology of the gastrointestinal tract. Raven, New York, 267-294.

Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H. (1995) Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control. *Biochem. Pharmacol.*; **50**: 1321-1332.

Fujimoto K, Sakata Y, Tsunada S, Koyama T, Morita H, Ogata SI, Matsunaga C, Gotoh Y, Iwakiri R. (1995) Newly synthesised histamine accelerates ornithine decarboxylase activity in rat intestinal-mucosa after ischemia-reperfusion. *Dig. Dis. Sci.*; **40**: 717-721.

Furchgott RF, Zawadski JV. (1980) The obligatory role of endothelial cells in therelaxation of arterial smooth muscle by acetylcholine. *Nature*; **288**: 373-376.

Furihata C, Sato Y, Hosaka M, Matsushima T, Furukawa F, Takahashi M. (1984) NaCl induced ornithine decarboxylase and DNA synthesis in rat stomach mucosa. *Biochem. Biophys. Res. Commun.*; **121**: 1027-1032.

Gardiner IM, Adams J, Castell F, De Belleroche JS. (1994) Nitric oxide synthase inhibitors attenuate the neuronal injury response in cerebral cortex. *Biochem. Soc. Trans.*; **22**:150S

Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJR, Knowles RG. (1997) 1400 is a slow, tight binding, and highly selective inhibitor of inducible nitric oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.*; **272**: 4959-4963.

Georgopoulos C, Welch W.J. (1993) Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* **9**: 601-34.

Gislason H, Varhaug P, Sørbye H, Waldum L, Svanes K. (1996) Role of adenosine and nitric oxide in the hyperemic response to superficial and deep gastric mucosal injury and H^+ back-diffusion in cats. *Gastroenterol.*; **31**: 14-23.

Grody WW, Argyle C, Kern RM, Dizikes GJ, Spector EB, Strickland AD, Klein D, Cederbaum SD. (1989) Differential expression of the two human arginase genes in hyperargininemia. Enzymatic, pathologic and molecular analysis. *J. Clin. Invest.*; **83**: 602-609.

Hao W, Myhre A.P, Palmer J.P, (1996) Nitric oxide meediates IL-1ß inhibition of glutamic acid decarboxylase. *Diabetologue* ; **45**: 870.

Hatt JF, Hanson PJ. (1989) Sites of action of protein kinase c on secretory activity in rat parietal cells. *Am. J. Physiol.*; **256**: G129-G138.

Hatt JF. (1989) PhD Thesis Aston University, Birmingham.

Hausladen A, Fridovich I. (1994) Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J. Biol. Chem.*; **269**: 29405-29408.

Hayashi S, Murakami Y, Matsufuji S. (1996) Ornithine decarboxylase antizyme: a novel type of regulatory protein. *TIBS.*; **21**: 27-30.

Hbrak A, Bajor T, Temesi A. (1994) Comparison of substrate and inhibitor specificity of arginase and nitric oxide synthase for arginine analogs and related compounds in murine and rat macrophages. *Biochem. Biophys. Res. Commun.*; **198**: 206-212.

Hecker M, Mulsch A, Busse R. (1994) Subcellular localisation ans characterisation of neuronal nitric oxide synthase. *J. Neurochem.*; **62**: 1524-1529.

Hecker M, Sessa WC, Harris HJ, Anggard EE, Vane JR. (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci.*; **87**: 8612-8616.

Hecker M, Walsh DT, Vane JR. (1991) Substrate specificity of nitric oxide synthase. *FEBS Letts.*; **294**: 221-224.

Hevel JM, White KA, Marletta MA. (1991) Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.*; **266**: 789-791.

Hey C, Wessler I, Racké K. (1995) Nitric oxide synthase activity is inducible in rat, but not rabbit alveolar macrophages, with a concomitant reduction in arginase activity. *Naunyn-Schmiedeberg's Arch. Pharmacol.*; **351**: 651-659.

Hightower, L.E. (1980) Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesise several polypeptides. *J. Cell Physiol.* **102**: 407-424.

Hiraishi H, Terano A, Sugimoto T, Harada T, Razandi M, Ivey KJ. (1994) Protective role of intracellular superoxide dismutase against extracellular oxidants in cultured rat gastric cells. *J. Clin. Invest.*; **93**: 331-338.

Hirakawa T, Rokutan K, Nikawa T, Kishi K. (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology*; **111**: 345-357.

Hisanaga Y, Goto H, Tachi K, Hayakawa T, Sugiyama S. (1996) Implication of nitric oxide synthase activity in the genesis of water immersion stress-induced gastric lesions in rats: the protective effects of FK506. *Aliment. Pharmacol. Ther.*; **10**: 933-940.

Hu JG, Mahmoud MI, Elfakahany EE. (1994) Polyamines inhibit nitric oxide synthase in rat cerebellum. *Neurosci. Letts.*; **175**: 41-45.

Huang L.E, Zhang H, Bae S.W, Liu A.Y-C. (1994) Thiol reducing agents inhibit the heat shock response. *J. Biol. Chem.*; **269**: 30718-30725.

Ishibashi Y. (1992) Accumulation of ornithine decarboxylase mRNA in rat stomach mucosa treated with hypertonic NaCl. *Biomed. Res.*; **13**(3):185-190

Ito S. (1987) Functional gastric morphology in *Physiology of the Gastrointestinal Tract.* 2nd Edn. (ed., Johnson LR)

Jain B, Rubinstein I, Robbins RA, Leise KL, Sisson JH. (1993) Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem. Biophys. Res. Commun.*; **191**: 83-88.

Johnson JA. (1995) Pathogenesis of bacterial infections of the respiratory tract. *Br. J. Biomedical Science.*; **52**: 157-161.

Kanwar S, Wallace JL, Befus D, Kubes P. (1994) Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am. J. Physiol.*; **266**: G222-G229.

Kim JW, Closs EI, Albritton LM, Cunningham JM. (1991) Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature*; **352**: 725-728.

Kim Y-M, de Vera ME, Watkins SC, Billiar TR. (1997) Nitric oxide protects cultured rat hepatocytes from tumour necrosis factor-α-induced apoptosis by inducing heat shock protein 70 expression. *J.Biol. Chem.*; **272**: 1402-1411.

Kitagawa H, Takeda F, Kohei H. (1990) Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. *J. Pharmacol. Exp. Ther.*; **253**: 1133-1137.

Knowles RG, Moncada S. (1994) Nitric oxide synthases in mammals. *Biochem. J.*; **298**: 249-258.

Knowles RG. (1997) Nitric oxide, mitochondria and metabolism. *Biochem. Soc. Trans.*; **25**: 895-901.

Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS. (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localisation. *Am. J. Respir. Cell Mol. Biol.*; **9**: 371-377.

Konarska L, Tomaszewski I. (1975) Studies on L-Arginase of the small intestine. *Biochemical Medicine* **14**: 250-262.

Konturek SJ, Brzozwski T, Majka J, Szlachcic A, Czarnobilski K. (1994) Nitric oxide in gastroprotection by sucralfate, mild irritant, and nocloprost. Role of mucosal blood flow. *Dig. Dis. Sci.*; **39**: 593-600.

Konturek SJ, Brzozwski T, Majka J, Szlachcic A, Pytko-Polonczyk J, Stachura J. (1993) Inhibition of nitric oxide synthase delays healing of chronic gastric ulcers. *Eur. J. Pharmacol.*; **239**: 215-217.

Konturek SJ, Brzozwski T, Majka J, Szlachcic A, Pytko-Polonczyk J. (1993) Implications of nitric oxide in the action of cytoprotective drugs on gastric mucosa. *J. Clin. Gastroenterol.*; **17**: S140-S145.

Konturek SJ, Brzozwski T, Majka J, Szlachcic A. (1992) Nitric oxide in protection by aluminium-containing antacids. *Eur. J. Pharmacol.*; **229**: 155-162.

Kubes P (1993) Nitric oxide-induced microvascular permeability alterations: a regulatory role for cGMP. *Am. J. Physiol.*; **265**: H1909-H1915.

Kubes P. (1992) Nitric oxide modulates epithelial permeability in the feline small intestine. *Am. J. Physiol.*; **262**: G1138-G1142.

Kubes P. (1993) Ischemia/reperfusion in the feline small intestine: a role for nitric oxide. *Am. J. Physiol.*; **264**: G143-G149.

Kumar AP, Mar PK, Zhao BW, Montgomery RL, Kang DC, Butler AP. (1995) Regulation of rat ornithine decarboxylase promoter activity by binding of transcription factor SP1. *J. Biol. Chem.*; **270**: 4341-4348.

Kuwayama H, Naito T. (1993) Effects of prostaglandins on ornithine decarboxylase activity in rat small intestine. *Dig. Dis. Sci.*; **38**: 1087-1090.

Kwo P, Patel T, Bronk S.F, Gores G.J. (1995) Nuclear serine protease activity contributes to bile-acid induced apoptosis in hepatocytes. *Am. J. Physiol.*; **268**: G613-G621.

Kwon NS, Stuehr DJ, Nathan CF. (1991) Inhibition of tumour cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.*; **174**: 761-767.

Lander HM. (1997) An essential role for free radicals and deprived species in signal transduction. *Faseb J.*; **11**: 118 - 124.

Li RS, Abrahamsen MS, Johnson RR, Morris DR. (1994) Complex interactions at a GC-rich domain regulate cell type dependent activity of the ornithine decarboxylase promoter. *J. Biol. Chem.*; **269**: 7941-7949.

Lippe I.Th, Holzer P. (1992) Participation of endothelium-derived nitric oxide but not prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion. *Br. J. Pharmacol.*; **105**: 708-714.

Liu H, Lightfoot R, Stevens JL. (1996) Activation of heat shock factor by alkylating agents is triggered by glutathione depletion and oxidation of protein thiols. *J.Biol. Chem.*; **271**: 4805 - 4812.

Lopez-Belmonte J, Whittle BJR, Moncada S. (1993) The actions of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa. *Br. J. Pharmacol.*; **108**: 73-78.

MacNaughton WK, Cirino G, Wallace JL. (1989) Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. *Life Sci.*; **45**: 1869-1876.

Maeda H, Akaike T, Yoshida M, Suga M. (1994) Microbiology - Analysis by a new nitric oxide scavenger. *J. Leukocyte Biology*; **56**: 588-592.

Malyshev IY, Malugin AV, Golubeva LY, Zenina TA, Manukhina EB, Mikoyan VD, Vanin AF. (1996) Nitric oxide donor induces HSP70 accumulation in the heart and in cultured cells. *FEBS Letts.*; **391**: 21-23.

Malyshev IY, Manukhina EB, Mikoyan VD, Kubrina LN, Vanin AF. (1995) Nitric oxide is involved in heat-induced HSP70 accumulation. *FEBS Letts.*; **370**: 159-162.

Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang X-J, Ruiz B, Fontham ETH, Mera R, Miller MJS, Corea P. (1996) Inducible nitric oxide synthase, nitrotyrosine and apoptosis in Helicobacter pylori gastritis: effect of antiobiotics and antioxidants. *Cancer Res.*; **56**: 3238-3241.

Mar PK, Kumar AP, Kang DC, Zhao BW, Martinez LA, Montgomery RL, Anderson L, Butler AP. (1995) Characterization of novel phorbol ester- responsive and serum-responsive sequences of the rat ornithine decarboxylaase gene promoter. *Molecular carcinogenesis*; **14**: 240-250.

Marin P, Lafoncazal M, Bockaert J. (1992) A nitric oxide synthase activity selectively stimulated by NMDA receptors depends on protein kinase C activation in mouse striatal neurons. *Eur. J. Neurosci.*; **4**: 425-432.

Marletta MA. (1993) Nitric oxide synthase structure and mechanism. *J. Biol. Chem.*; **268**: 12231-12234.

Marsden PA, Scappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T. (1992) Molecular cloning and characterisation of human endothelial nitric oxide synthase. *FEBS Letts.*; **307**: 287-293.

Mårtensson J, Jain A, Meister A. (1990) Glutathione is required for intestinal function. *Proc. Natl. Acad. Sci. USA.*; **87**:1715.

Martinez-Cuesta MA, Barrachina MD, Pique JM, Whittle BJR, Esplugues JV. (1992) The role of nitric oxide and platelet-activating factor in the inhibition by endotoxin of pentagastrin-stimulated gastric acid secretion. *Eur. J. Pharmacol.*; **218**: 351-354.

Masuda E, Kawano S, Nagano K, Tsuji S, Takei Y, Tsuji M, Oshita M, Michida T, Kobayashi I, Nakama A, Fusamoto H, Kamada T. (1995) Endogenous nitric oxide modulates ethanol induced gastric mucosal injury in rats. *Gastroenterology*; **108**: 58-64.

Matsumoto J, Takeuchi K, Ueshima K, Okabe S. (1992) Role of capsaicin-sensitive afferent neurons in mucosal blood flow response of rat stomach induced by mild irritants. *Dig. Dis. Sci.*; **37**: 1336-1344.

McCall TB, Palmer RMJ, Moncada S. (1992) Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophils. *Biochem. Biophys. Res. Commun.*; **186**: 680-685.

McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. (1997) Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut.*; **40**: 211-216.

Meister A, Anderson ME. (1983) Glutathione. *Ann. Rev. Biochem.*; **52**: 711 - 760.

Michel T, Li GK, Busconi L. (1993) Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. Proc. Natl. Acad. Sci.; 90: 6252-6256.

Miller CA, Bunnett NW, Debas HT. (1994) Laminin mediates the restitution of rat gastric-mucosa in vitro. *Experimental Physiology*; **79**: 647-659.

Miller CA, Debas HT. (1995) Epidermal growth factor stimulates the restitution of rat gastric mucosa in vitro. *Experimental Physiology*; **80**: 1009-1018.

Miller CA, Nakashima M, Gittes GK, Debas HT. (1995) Calcitonin-gene-related peptide (CGRP) up regulates the restitution ofrat gastric mucosa. *Journal of Surgical Research*; **58**: 421-424.

Miller MJS, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL, Clark DA. (1993) Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J. Pharmacol. Exp. Ther.*; **264**: 11-16.

Miller MJS, Thompson JH, Zhang X-J, Sadowska-Krowicka H, Kakkis JL, Munshi UK, Sandoval M, Rossi JL, Eloby-Childress S, Beckman JS, Ye YZ, Rodi CP, Manning PT, Currie MG, Clark DA. (1995) Role of inducible nitric oxide synthase expression and peroxynitrite formation in guinea-pig ileitis. *Gastroenterology*; **109**: 1475-1483.

Miller T.A, Li D, Kuo Y-J, Schmidt K.L, Shanbour L.L. (1985) Nonprotein sulfhydryl compounds in canine gastric mucosa: effects of PGE2 and ethanol. *Am. J. Physiol.*; **249**: G137-G144.

Minowada G, Welch W.J. (1995) Clinical implications of the stress response. *J. Clin. Invest.* ; **95**: 3-12.

Moncada S, Palmer RMJ, Higgs EA. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Revs.*; **43**: 109-142.

Munday R, Winterbourn CC. (1989) Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. *Biochem. Pharmacol.*; **38**: 4349-4352.

Murthy KS, Jin J-G, Makhlouf GM. (1994) Inhibition of nitric oxide synthase activity in dispersed gastric muscle cells by protein kinase C. *Am. J. Physiol.*; **266**: G161-G165.

Mutoh H, Hiraishi H, Ota S, Ivey KJ, Terano A, Sugimoto T. (1990) Role of oxygen radicals in ethanol-induced damage to cultured gastric mucosal cells. *Am. J. Physiol.*; **258**: G603-G609.

Mutoh H, Ota S, Hiraishi H, Ivey KJ, Terano A, Sugimoto T. (1991) Reduced glutathione protects cultured gastric mucosal cells from suckling rats against acid. *Am. J. Physiol.*; **261**:G65-G70.

Nagaki M, Shimura S, Irokawa T, Sasaki T, Shirato K. (1995) Nitric oxide regulation of glycoconjugate secretion from feline and human airways in vitro. *Resp. Physiol.*; **102**: 89-95.

Nakamura K, Rokutan K, Marui N, Aoike A, Kawai K. (1991) Induction of heat shock proteins and their implication in protection against ethanol-induced damage in cultured guinea pig gastric mucosal cells. *Gastroenterology*; **101**: 161-166.

Nathan C, Xie Q-W. (1994a) Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.*; **269**: 13725-13728.

Nathan C, Xie Q-W. (1994b) Nitric oxide synthases: roles, tolls, and controls. *Cell*; **78**: 915-918.

North AJ, Star RA, Brannon TS, Ujiie K, Wells LB, Lowenstein CJ, Snyder SH, Shaul PW. (1994) Nitric oxide synthase type I and type III gene expression are developmentally regulated in rat lung. *Am. J. Physiol.*; **266**: L635-L641.

Nylander O, Holm L, Wilander E, Hallgren A. (1994) Exposure of the duodenum to high concentrations of hydrochloric acid. Effects of permeability, alkaline secretion, and blood flow. *Scand. J. Gastroenterol.*; **29**: 437-444.

O'Connor KJ, Moncada S. (1991) Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells. *Biochim. Biophys. Acta.*; **1097**: 227-231.

Palleros D.R., Reid K.L., Shi L., Welch W.J., Fink, A.L. (1993) ATP-induced protein-Hsp70 complex dissociation requires K^+ but not ATP hydrolysis. *Nature*; **365**: 664-666.

Palmer RMJ, Ferrige AG, Moncada S. (1987) NO release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*; **327**: 524-526.

Paltrons MA, Agullo L, Garcia A. (1995) Dexamethasone up-regulates a constitutive nitric oxide synthase in cerebellar astrocytes but not in granule cells in culture. *J. Neurochem.*; **64**: 447-450.

Pegg A.E, McCann P.P. (1982) Polyamine metabolism and function. *Am. J. Physiol.* **243**: C212-C221.

Pegg AE. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.*; **234**: 249-262.

Pellegrini S, Schindler C. (1993) Early events in signalling by interferons. *TIBS*; **18**: 338-342.

Peunova N, Enikolopov G. (1993) Amplification of calcium-induced gene transcription in neuronal cells. *Nature*; **364**:450-453

Pfeilschifter J, Rob P, Mulsch A, Fandrey J. Vosbeck K, Busse R. (1992) Interleukin 1ß and tumour necrosis factor α induce a macrophage-type of nitric oxide synthase in rat renal mesangial cells. *Eur. J. Biochem.*; **203**: 251-255.

Pique JM, Esplugues JV, Whittle BJR. (1992) Endogenous nitric oxide as a mediator of gastric mucosal vasodilatation during acid secretion. *Gastroenterology*; **102**: 168-174.

Pique JM, Whittle BJR, Esplugues JV. (1989) The vasodilator role of endogenous nitric oxide in the rat gastric microcirculation. *Eur. J. Pharmacol.*; **174**: 293-296.

Pison U, Max M, Neuendank A, Weißbach S, Pietschmann S. (1994) Host defence capacities of pulmonary surfactant: evidence for "non-surfactant" functions of the surfactant system. *Eur. J. Clin. Invest.*; **24**: 586-599.

Price K.J, Hanson P.J, Whittle BJR. (1996) Localisation of constitutive isoforms of nitric oxide synthase in the gastric glandular mucosa of the rat. *Cell Tisue Res.*; **285**: 157-163.

Price KJ, Hanson PJ, Whittle BJR. (1994) Stimulation by carbachol of mucus gel thickness in rat stomach involves nitric oxide. *Eur. J. Pharmacol.*; **263**: 199-202.

Price KJ, Hanson PJ, Whittle BJR. (1996) Localization of constitutive isoforms of nitric oxide synthase in the gastric glandular mucosa of the rat. *Cell Tissue Res.*; **285**: 157-163.

Reczkowski RS, Ash DE. (1994) Rat liver arginase: kinetic mechanism, alternate substrates, and inhibitors. *Arch. Biochem. Biophys.*; **312**: 31-37.

Reed D.J, Fariss M.W. (1984) Glutathione depletion and susceptibility. *Pharmacol. Rev.*; **36**: 25S-33S.

Ritossa, F.M. (1962) A new puffing pattern induced by a temperature shock and DNP in *Drosophila*. *Experimentia*, **18**: 571-73.

Robbins RA, Barnes PJ, Springall DR, Warren JB, Kwon OJ, Buttery LDK, Wilson AJ, Geller DA, Polak JM. (1994a) Expression of inducible nitric oxide in human lung epithelial cells. *Biochem. Biophys. Res. Commun.*;**203**: 209-218.

Robbins RA, Springall DR, Warren JB, Kwon OJ, Buttery LDK, Wilson AJ, Adcock IM, Riveros-Moreno V, Moncada S, Polak J, Barnes PJ, (1994b) Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. *Biochem. Biophys. Res. Commun.*; **198**: 835-843.

Robertson CA, Green BG, Niedzwiecki L, Harrison RK, Grant SK. (1993) Effect of nitric oxide synthase substrate analog inhibitors on rat liver arginase. *Biochem. Biophys. Res. Commun.*; **197**: 523-528.

Robinson LJ, Michel T. (1995) Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targetting. *Proc. Natl. Acad. Sci. USA.*; **92**: 11776-11780.

Rokutan K, Hirakawa T, Teshima S, Honda S, Kishi K. (1996) Glutathione depletion impairs transcriptional activation of heat shock genes in primary cultures of guinea pig gastric mucosal cells. *J. Clin. Invest.*; **97**: 2242-2250.

Rokutan K, Johnston RB, Kawai K. (1994) Oxidative stress induces S-thiolation of specific proteins in cultured gastric mucosal cells. *Am. J. Physiol.*; **266**:G247-G254.

Rokutan K, Teshima S, Hirakawa T, Nikawa T, Kishi K, Kawai K. (1995) Possible involvement of glutathione in redox control of transcription of stress-related genes in cultured gastric epithelial cells. *Gastoenterology*; **108**: A203, 2982.

Rutten MJ, Dempsey PJ, Solomon TE, Coffey JR. RJ. (1993) TGF- is a potent mitogen for primary cultures of guinea-pig gastric mucous epithelial cells. *Am. J. Physiol.*; **265**: G361-G369.

Sakamoto C, Matsuda K, Nakano O, Konda Y, Matozaki T, Nishisaki H, Kasuga M. (1994) EGF stimulates both cyclooxygenase activity and cell proliferation of cultured guinea pig gastric mucous cells. *J. Gastroenterol.*; **29**: 73-76.

Salzman AL, Denenberg AG, Ueta I, O'Connor M, Linn SC, Szabo C. (1996) Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am. J. Physiol.*; **270**: G565-G573.

Schlesinger MJ. (1990) Heat shock proteins. *J.Biol. Chem.*; **265**: 12111 - 12114.

Schmidt HHHW. (1995) Determination of nitric oxide via measurement of nitrite and nitrate in culture media. *Biochemica*; **2**: 30.

Schuber F. (1989) Influence of polyamines on membrane functions. *Biochem. J.*; **260**: 1-10.

Schuetz, T.J., Gallo, G.J., Sheldon, L, Tempst, P., and Kingston, R.E. (1991) Isolation of a cDNA for HSF2: Evidence for two heat shock factor genes in humans. *PNAS.USA*. **88**: 6911-6915.

Seidler U, Pfeiffer A. (1992) Inositol phosphate formation and $[Ca^{2+}]i$ in secretagogue - stimulated rabbit gastric mucous cells. *Am. J. Physiol.*; **260**:G133-G141.

Shimizu S, Yamamoto T, Momose K. (1993) Inhibition by methylene-blue of the L-arginine metabolism to L-citrulline coupled with nitric oxide synthesis in cultured endothelial cells. *Res. Commun. Chem. Path. Pharm.*; **82**: 35-48.

Shinomiya T, Ohara T, Wada N, Omori A, Kamada N. (1990) Rat liver arginase suppresses mixed lymphocyte reaction. *J. Biochem.*; **107**:435-439.

Silen W, Ito S. (1985) Mechanisms for rapid re-epithelialization of the gastric mucosal surface. *Ann. Rev. Physiol.*; **47**: 217-229.

Simmons WW, Ungureanu-Longrois D, Smith GK, Smith TW, Kelly RA. (1996) Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport. *J. Biol. Chem.*; **271**: 23928-23937.

Snyder SH, Bredt DS. (1991) Nitric oxide as a neuronal messenger. *TIPS.*; **12**: 125-128.

Stamler JS. (1994) Redox signalling: nitrosylation and related target interactions of nitric oxide. *Cell*; **78**: 931-936.

Stark ME, Szurszewski JH. (1992) Role of nitric oxide in gastrointestinal and hepatic function and disease. *Gastroenterol.*; **103**: 1928-1949.

Starke PE, Farber JL. (1985) Endogenous defenses against the cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. *J. Biol. Chem.*; **260**: 86-92.

Steele RE, Preston AS, Johnson JP, Handler JS. (1986) Porous-dishes for culture of polarized cells. *Am. J. Physiol.*; **251**: C136-C139.

Stevens BR, Kakuda DK, Yu K, Waters M, Vo CB, Raizada MK. (1996) Induced nitric oxide synthesis is dependent on induced alternatively spliced *CAT-2* encoding L-arginine transport in brain astrocytes. *J. Biol. Chem.*; **271**: 24017-24022.

Stojadinovic A, Kiang J, Smallridge R, Galloway R, Shea-Donohue T. (1995) Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology*; **109**: 505-515.

Stuehr DJ, Kwon NS, Nathan CF, Griffith OW, Feldman PL, Wiseman J. (1991) N^{w}-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.*; **266**: 6259-6263.

Su Z, Blazing MA, Fan D, George SE. (1995) The calmodulin-nitric oxide synthase interaction. Critical role of the calmodulin latch domain in enzyme activation. *J. Biol. Chem.*; **270**: 29117-29122.

Szabo S, Trier JS, Frankel PW. (1981) Sulfhydryl compounds may mediate gastric cytoprotection. *Science*; **214**: 200 -202.

Tabor CW, Tabor H. (1984) Polyamines. *Annu. Rev. Biochem.*; **53**: 749-790.

Tachi K, Goto H, Hayakawa T, Sugiyama S. (1996) Prevention of water immersion stress-induced gastric lesions through the enhancement of nitric oxide synthase activity in rats. *Aliment. Pharmacol. Ther.*; **10**: 97-103.

Takahashi R, Heydari A.R, Gutsmann A, Sabia M, Richardson A. (1994) The heat shock transcription factor in liver existsin a form that has DNA binding activity but no transcriptional activity. *Biochem. Biophys. Res. Commun.*; **201**: 552-558.

Takeuchi K, Kato S, Yasuhiro T, Yagi K. (1997) Mechansim of acid secretory changes in rat stomcah after damage by taurocholate. Role of nitric oxide, histamine, and sensory neurons. *Dig. Dis. Sci.*; **42**: 645-653.

Takeuchi K, Ohuchi T, Miyake H, Okabe S. (1993) Stimulation by nitric oxide synthase inhibitors of gastric and duodenal HCO_3^- secretion in rats. *J. Pharmacol. Exp. Ther.*; **263**: 1512-1519.

Takeuchi K, Ohuchi T, Okabe S. (1994) Endogenous nitric oxide in gastric alkaline response in the rat stomach after damage. *Gastroenterology*; **106:** 367-374.

Takeuchi K, Okabe S. (1995) Mechanism of gastric alkaline response in the stomach after damage. Roles of nitric oxide and prostaglandins. *Dig. Dis. Sci.*; **40**: 865-871.

Takeuchi K, Takehara K, Kaneko T, Okabe S. (1995) Nitric oxide and prostaglandins in regulation of acid secretory response in rat stomach following injury. J. *Pharmacol. Exp. Ther.*; **272**: 357-363.

Tanaka J-I, Fujimoto K, Sakata T, Fujimoto T, Tsunada S, Iwakiri R, Yamaguchi M, Okabe Y, Yoshimatsu H. (1993) Effect of vagotomy on ornithine decarboxylase activity in rat duodenal mucosa. *Am. J. Physiol.*; **265**:G1016-G1020.

Tepperman BL, Brown JF, Whittle BJR. (1993) Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am. J. Physiol.*; **265**: G214-G218.

Tepperman BL, Soper BD. (1994) Nitric oxide synthase induction and cytoprotection of rat gastric mucosa from injury by ethanol. *Can. J. Phys. Pharm.*; **72**: 1308-1312.

Tepperman BL, Vozzolo BL, Soper BD. (1993) Effect of neutropenia on gastric mucosal integrity and mucosal nitric oxide synthesis in the rat. *Dig. Dis. Sci.*; **38:** 2056-2061.

Tepperman BL, Whittle BJR. (1992) Endogenous nitric oxide and sensory neuropeptides interact in the modulation of the rat gastric microcirculation. *Br. J. Pharmacol.*; **105**: 171-175.

Tepperman, B.L., Tan, S.Y., Whittle B.J.R. (1991) Effects of Ca^{2+}-modifying agents on integrity of rabbit isolated gastric mucosal cells. *Am. J. Physiol.* **261**: G119.

Teramae N, Azuma T, Habu Y, Kodama T, Kashima K, Aoike A, Kawai K. Expression of heat shock protein in human gastric mucosa. (1991) *Gastroenterol Jpn*; **26**: 683.

Teshima S, Rokutan K, Nikawa T, Kishi K. (1995) Evidence for participation of redox sensitive transcription factor in stress response of gastric epithelial cells. *Gastoenterology*; **110**: A602.

Thirumalai C.H.R, Tseng C-C, Tabata K, Fitzpatrick L.R, Johnson L.R. (1987) Relationship between ornithine decarboxylase activity and gastric damage. *Am. J. Physiol.* **253**: G1-G6.

Thomae KR, Joshi PC, Davies P, Pitt BR, Billiar TR, Simmons RL, Nakayama DK. (1996) Nitric oxide produced by cytokine-activated pulmonary artery smooth muscle cells is cytotoxic to cocultured endothelium. *Surgery*; **119**:61-6.

Thomsen LL, Scott JMJ, Topley P, Knowles RG, Keerie A-J, Frend AJ. (1997) Selective inhibition of inducible nitric oxide synthase inhibits tumour growth in vivo: studies with 1400W, a novel inhibitor. *Cancer Res.*; **57**: 3300-3304.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. (1991) *J. Biol. Chem.*; **266**: 15771-15781.

Tripp MA, Tepperman BL. (1995) Effect of nitric oxide on integrity, blood flow and cyclic GMP levels in the rat gastric mucosa: modulation by sialoadenectomy. *Br. J. Pharmacol.*; **115**: 344-348.

Tripp MA, Tepperman BL. (1996) Role of calcium in nitric oxide-mediated injury to rat gastric mucosal cells. *Gastroenterology*; **111**: 65-72.

Trotman, C.N.A, Greenwell, J.R. (1979) *Biochem. J.* **178**: 159 -164.

Trujillo-Ferrara J, Koizumi G, Muñoz O, Joseph-Nathan P, Yañez R. (1992) Antitumor effect and toxicity of two new active-site-directed irreversible ornithine decarboxylase and extrahepatic arginase inhibitors. *Cancer Letts.*; **67**: 193-197.

Van Remmen H, Williams MD, Heydari AR, Takahashi R, Chung HY, Yu BP, Richardson A. (1996) Expression of genes coding for antioxidant enzymes and heat shock proteins is altered in primary cultures of rat hepatocytes. *J. Cell Physiol.*; **166**: 453 - 460.

Vander AJ, Sherman JH, Luciano DS. (1990) Human Physiology: The mechanisms of body function, 5th edition, McGraw Hill, New York.

Wagner DA, Young UR, Tannenbaum SR. (1993) Mammalian nitrate biosynthesis: incorporation of $^{15}NH_3$ into nitrate is enhanced by endotoxin treatment. *Proc. Natl. Acad. Sci. USA.*; **80**: 4518-4521.

Wakulich CA, Tepperman BL. (1997) Role of glutathione in nitric oxide - mediated injury to rat gastric mucosal cells. *Eur. J. Pharmacol.*; **319**: 333 - 341.

Wallace JL, Bell CJ. (1995) Gastroduodenal mucosal defence. *Current opinion in Gastoenterology*.; **11**: 479-485.

Wang J-Y, Johnson L.R. (1990.c) Luminal polyamines stimulate repair of gastric mucosal stress ulcers. *Am. J. Physiol.*; **259**:G584-G592.

Wang J-Y, Johnson LR. (1990 a) Role of ornithine decarboxylase in repair of gastric mucosal stress ulcers. *Am. J. Physiol.*; **258**:G78-G85.

Wang J-Y, Johnson LR. (1990 b) Gastric and duodenal mucosal ornithine decarboxylase and damage after corticosterone. *Am. J. Physiol.*; **258**:G942-G950.

Wang J-Y, McCormack SA, Viar MJ, Johnson LR. (1994) Secretin inhibits induction of ornithine decarboxylase activity by gastrin in duodenal mucosa and IEC-6 cells. *Am. J. Physiol.*; **267**: G276-G284.

Wang J-Y, Viar MJ, Blanner PM, Johnson LR. (1996) Expression of the ornithine decarboxylase gene in response to asparagine in intestinal epithelial cells. *Am. J. Physiol.*; **271**: G164-G171.

Wang WW, Jenkinson CP, Griscavage JM, Kern RM, Arabolos NS, Byrns RE, Cederbaum SD, Ignarro LJ. (1995) Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem. Biophys. Res. Commun.*; **210**: 1009-1016.

Warner RL, Paine III R, Christensen PJ, Marletta MA, Richards MK, Wilcoxen SE, Ward PA. (1995) Lung sources and cytokine requirements for *in vivo* expression of inducible nitric oxide synthase. *Am. J. Respir. Cell Mol. Biol.*; **12**: 649-661.

Weisbrod RM, Brown ML, Cohen RA. (1993) Effect of elevated glucose on cyclic-GMP and eicosanoids produced by porcine aortic endothelium. *Arteriosclerosis and Thrombosis*; **13**: 915-923.

Wheater PR, Burkitt HG, Daniels VG. (1987) Functional Histology. Churchill Livingstone, 2nd Edn.

Whittle BJR, Lopez-Belmonte J, Moncada S. (1990) Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br. J. Pharmacol.*; **99**: 607-611.

Whittle BJR. (1993) Neuronal and endothelium-derived mediators in the modulation of the gastric microcirculation: integrity in the balance. *Br. J. Pharmacol.*; **110**: 3-17.

Whittle BJR. (1994) in The Physiology of the Gastrointestinal Tract. (Johnson, LR. ed.) 3rd edn, Raven Press, New York.

Whittle BJR. (1994) Nitric oxide in the gastrointestinal physiology and pathology. In: Johnson LR (ed), The physiology of the gastrointestinal tract, Raven, New York, 267-294.

Windmueller HG, Spaeth AE. (1981) Source and fate of circulating citrulline. *Am. J. Physiol.* **241**: E473-E480.

Wojciechowski K, Trzeciak L, Konturek SJ, Ostrowski J. (1995) Inhibition of acid secretory response and induction of ornithine decarboxylase and its mRNA by TGF and EGF in isolated rat gastric glands. *Regulatory peptides*; **56**: 1-8.

Wolffe A.P, Glover J.F, Tata J.R, (1984) Culture shock: synthesis of heat-shock-like proteins in fresh primary cell cultures. *Exp. Cell Res..*; **154**: 581-590.

Wong HM, Tepperman BL. (1994) Reduced glutathione modulates Ca^{2+}-mediated damage to rabbit isolated gastric mucosal cells. *Am. J. Physiol.*; **267**: G1-G9.

Wright NA, Pike C, Elia G. (1990) Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature*; **343**: 82-85.

Wu G, Meininger C.J. (1993) Regulation of L-arginine synthesis from L-citrulline by L-glutamine in endothelial cells. *Am. J. Physiol.* **265**: H1965-H1971.

Wu G, Meininger C.J. (1995) Impaired arginine metabolism and NO synthesis in coronary endothelial cells of the spontaneously diabetic BB rat. *Am.J.Physiol.* **269**: H1312-H1318.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T Nathan C. (1992) Cloning and characterisation of inducible nitric oxide synthase from mouse macrophages. *Science*; **256**: 225-228.

Xie Y-W, Wolin MS. (1996) Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/ reoxygenation. *Circulation*; **94**: 2580 - 2586.

Yamaoka Y, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. (1997) Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. *Gut*; **41**: 442-451.

Yanaka A, Muto H, Fukutomi H, Ito S, Silen W. (1995) Role of nitric oxide in restitution of injured guinea pig gastric mucosa in vitro. *Am. J. Physiol.*; **268**:G933-G942.

Yanaka A, Muto H, Fukutomi H, Ito S, Silen W. (1996) Role of transforming growth factor beta in the restitution of injured guinea-pig gastric mucosa in vitro. *Am. J. Physiol. -Gastrointestinal and Liver Physiology*; **34**: G75-G85.

Yanaka A, Muto H, Fukutomi H, Silen W. (1995) Role of epidermal growth factor (EGF) in restituiton of injured guinea-pig gastric mucosa. *Gastroenterology*; **108**: 262.

Zamora R, Matthys KE, Herman AG. (1997) The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells. *Eur. J. Pharmacol.*; **321**: 87 - 96.

APPENDICES

A1: ABBREVIATIONS

Abbreviation	Meaning
BAPTA-AM	(1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic -acid) tetrakis (acetoxymethyl) ester)
BCA	bicinchoninic acid
BH_4	tetrahydrobiopterin
CaMK	calmodulin-dependent kinase
DM	diethyl maleate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
cGMP	cyclic guanosine monophosphate
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	glutathione disulphide
HRP	horse radish peroxidase
HSF	heat shock transcription factor
HSP	heat shock protein
h	hour(s)
IL	interleukin
IFN-γ	interferon-γ
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NA	N^G-nitro-L-arginine
L-NIO	N^G-iminoethyl-L-ornithine
LPS	lipopolysaccharide
min	minute(s)
NADPH	nicotinamide adenine dinucleotide
NO	nitric oxide

Abbreviation	Meaning
Carboxy-PTIO	[2-(4-carboxyphenyl)-4,4,5,5 tetramethylimidazoline-1-oxyl-3-oxide potassium salt]
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PGE_2	prostaglandins E_2
PKA	cyclicAMP-dependent protein kinase
PKC	protein kinase C
PMSF	phenylmethyl sulphonyl fluoride
s	second(s)
SDS	sodium dodecyl sulphate
SNAP	S-nitroso-N-acetyl-penicillamine
TBS	tris buffered saline
TEMED	N,N, N',N'-tetramethylenediamine
TLC	thin layer chromatography
TNF-α	tumour necrosis factor–α
TGF-ß	transforming growth factor-ß
Tris	tris(hydroxymethyl)methylamine
1400W	N-(3-(aminomethyl)benzyl)acetamidine

A2: SOURCE OF REAGENTS

Reagent	Supplier
Acrylamide	BDH
Amphotericin B	GibcoBRL
D-Arginine	Sigma
L-Arginine	Sigma
2',5'ADP-Agarose	Sigma
Ammonium persulphate	BDH
Aprotinin	Sigma
BAPTA-AM	Sigma
Bis-acrylamide	BDH
BCA protein assay reagent kit	Pierce
Bromophenol Blue	Biorad

Reagent	Supplier
BSA-fraction V	BDH
$CaCl_2$	Sigma
Calmodulin	Sigma
Carbachol	Sigma
cGMP enzymeimmunoassay kit	Amersham
Collagenase	Sigma
Coomassie Blue assay kit	Sigma
L-Cysteine	Sigma
Developer (Kodak)	Sigma
DOWEX AG W-8	Sigma
DTT	Sigma
Dulbecco's MEM	GibcoBRL
ECL Detection Reagents	Amersham
EDTA	Sigma
EGF	Sigma
EGTA	Sigma
FAD	Sigma
Fixer (Kodak)	Sigma
Foetal calf serum	GibcoBRL
Gentamicin	Sigma
Glucose-6-phosphate	Sigma
Glucose-6-phosphate dehydrogense	Sigma
Glycerol	Sigma
Glycine	BDH
GSH	Sigma
GSNO	Sigma
Ham's F12 Media	GibcoBRL
HEPES	BDH
Hisafe scintillation fluid	Wallac
Recombinant Human IFN-γ	Genzyme
Recombinant Human IL-1ß	R&D Systems
Recombinant Human IL-8	R&D Systems
Indomethacin	Sigma
Leupeptin	Sigma
$MgCl_2$	Sigma
NaCl	BDH
NADPH	Sigma

Reagent	Supplier
L-NAME	Sigma
L-NMMA	Wellcome
1400W	GlaxoWellcome
L-Ornithine	Sigma
Penicillin	GibcoBRL
Percoll	Sigma
PGE_2	Sigma
PMSF	Sigma
p-nitrophenylphosphate	Sigma
Rainbow Markers	Amersham
Radiolabelled amino acids	Amershan
RPMI1640	GibcoBRL
SDS	BDH
SIN-1	Sigma
SNAP	Sigma
Sodium citrate	BDH
Sodium pentobarbitone	May and Baker
Soybean trypsin inhibitor	Sigma
Streptomycin	GibcoBRL
Sucrose	BDH
TEMED	BDH
Tetrahydrobiopterin	Sigma
Recombinant Human TNF-α	R&D Systems
TRIS	BDH
Trypsin	GibcoBRL
L-Valine	Sigma

A3: STATISTICAL TESTS USED TO ANALYSE DATA

Several statistical tests were used in this thesis to determine the significance of results. The more advanced tests used are outlined below.

When measurements of a variable as three or more samples, from three or more populations are collected, multisame analyses are required to determine any differences between samples. It is invalid to attempt the testing of multisame hypotheses by applying two sample tests to all possible pairs of samples. In situations such as this an analysis of variance test is employed to determine whether there is a significant difference between samples. If the outcome is positive, the test is followed by a multiple comparisons test, such as Newman-Keuls or Dunnett's test, to determine which set or sets of data is/are significantly different.

ANALYSIS OF VARIANCE

1. If an experiment has more than two treatments there are statistical objections to comparing all of the means by paired or unpaired `t' tests (see above). Instead a technique called analysis of variance is used.

2. Analysis of variance relies on the assumption that the total variation in a set of data can be calculated, that the variation can be partitioned into separate components and that these components can be compared.

3. The following table shows a `one-way analysis of variance' because there is only one source of variation apart from random error.

   To perform **single factor analysis of variance**:-

Variation	Sum of squares	Degrees of Freedom	Mean Square Variation
Total	$\sum x^2 - C$	n-1	
Treatments	$\frac{\sum(\text{treatment totals})^2}{\text{results per treatment}} - C$	t-1 (t=number of treatments)	$\frac{\text{sum squares treatments}}{\text{t-1}}$
Residual (error)	S.S. total-S.S. treatments	(n-1)-(t-1)	$\frac{\text{sum squares error}}{\text{d.f. error}}$

C = the correction factor $= \frac{(\Sigma X)^2}{n}$

4. To calculate **two factor analysis of variance,** the following procedure is used:-

Source of variation	Sum of Squares	Degrees of freedom	Mean square
Total	$\Sigma x^2 - C$	n-1	
Treatments	$\frac{\Sigma(\text{treatment totals})^2}{\text{number of blocks}} - C$	t-1 (t=treatments)	$\frac{\text{sum squares treatment}}{\text{t-1}}$
Blocks	$\frac{\Sigma(\text{block sums})^2}{\text{number of treatments}} - C$	b-1 (b=blocks)	$\frac{\text{sum of squares block}}{\text{b-1}}$
Residual (error)	S.S. total-S.S. blocks- S.S. treatments	total-treatments-blocks	$\frac{\text{sum squares error}}{\text{d.f.error}}$

5. In both cases the variation due to treatments is compared with that due to random error. This is done by calculating a Variance Ratio (F).

   (F) = TREATMENTS MEAN SQUARE/ERROR MEAN SQUARE

   The result is compared with tabulated values to determine the probability that the value of F could have arisen by chance.

Analysis of variance is a single test which determines whether the treatment means differ from each other (the null hypothesis is that all column means are equal). The test does not tell us which pairs of means differ. There are multiple comparison tests or post tests to enable you to perform this second step. To compare a control mean with all others means use **Dunnett's test** and to compare all means with each other use the **Student-Newman-Kuels test**. The basis of these tests involves determining the difference between 2 means and dividing by the SE to give the value q. The possibility that this value may arise by chance is assessed by reference to a statistical table.

The Student-Newman-Kuels test.

The sample means are ranked, pairwise differences between means are determined, and a standard error is computed as:

$$\sqrt{\frac{Error.mean.square.from.ANOVAR}{number.of.results.for.each.treatment}}$$

Then the statistic, q, is calculated (see above). The determination of the critical value $q_{a, n, r}$ where r is the number of means in the range of means being tested (eg. For X_1 - X_5, a range of 5 means is considered), n is the degrees of freedom associated with the error mean square and a is the probability at which the null hypothesis is rejected.

Dunnett's test

Where the objective of experiments with k samples, or groups is to determine whether the mean of one group, designated as a "control" differs significantly from each of the means of k-1 other groups, Dunnett's test may be used. In this test, all possible comparisons of pairs of group means are not made, but only k-1 comparisons are made involving the control group. The statistic q' is calculated by dividing the difference between the pairs of means by the standard error which is given by:

$$\sqrt{\frac{2x.error.mean.square.from.ANOVAR}{number.of.results.for.each.treatment}}$$

Knowing k, the total number of groups in the experiment, and n, the error degrees of freedom from the analysis of variance the critical value of q' can be obtained from a table.

A4: REACTIONS OF NITRIC OXIDE INVOLVED IN CYTOTOXICITY

The cytotoxicity exerted both by the action of NO directly, and by peroxynitrite are discussed in detail in sections 1.3.2 and 1.3.3. The reactions of NO involved in the formation of peroxynitrite and the hydroxyl radical are outlined below.

$$NO + O_2^- \longrightarrow ONOO^- \quad (1)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (2)$$

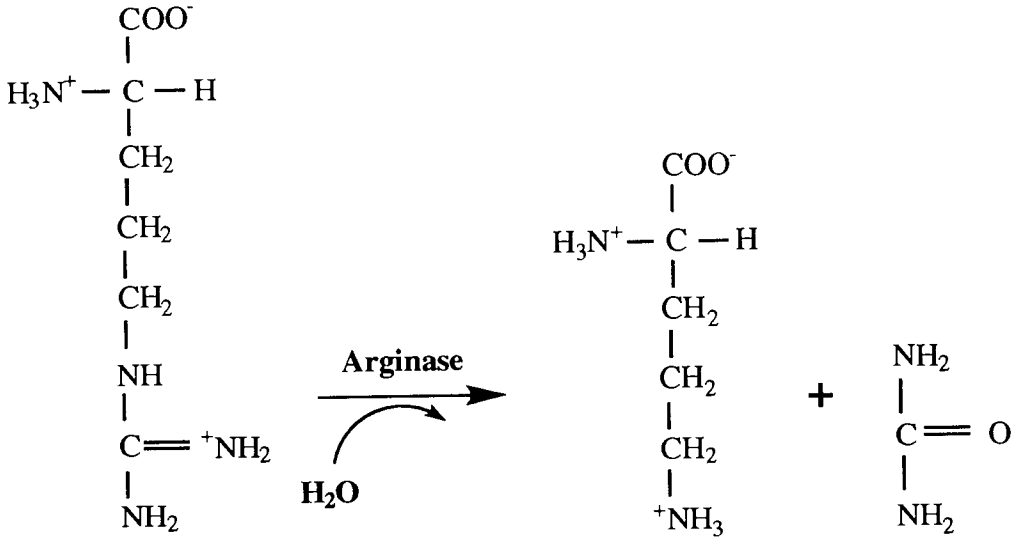
$$ONOOH \longrightarrow NO_2 + HO^\cdot \quad (3)$$

(1) NO reacts with superoxide (O_2^-) to give peroxynitrite.

(2+3) Peroxynitrite is a powerful oxidant that further decomposes to hydroxyl radicals (OH) and NO_2.

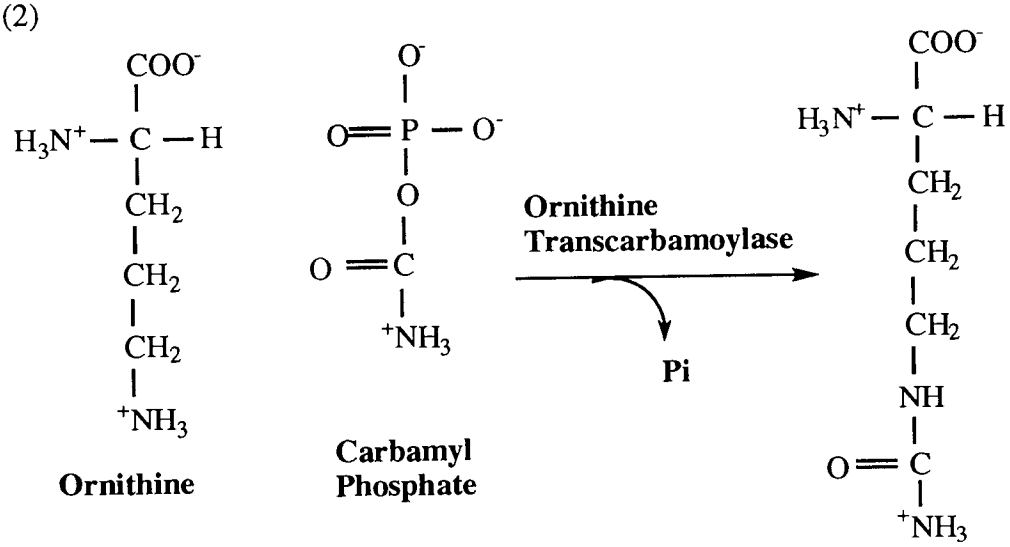
A5: STRUCTURAL FORMULAE OF SPECIES INVOLVED IN THE METABOLISM OF ARGININE TO CITRULLINE.

(1)



Arginine **Ornithine** **Urea**

(2)



Ornithine **Carbamyl Phosphate** **Citrulline**

The metabolism of arginine to ornithine and citrulline by gastric mucosal cells is discussed in detail in chapter 3.

(1) Arginase converts arginine to ornithine and urea.

(2) The resulting ornithine is converted to citrulline by the action of ornithine transcarbamoylase.