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Radiation-induced electrolyte transport dysfunction in the rat intestine: role of nitric oxide

Alexander R. Aurora

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, within the Faculty of Medicine, Department of Physiology, of the

University of Ottawa

July, 1995

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ABSTRACT

Diarrhea is a common symptom of individuals undergoing radiation therapy for abdominopelvic malignancies suggesting that absorptive and secretory processes of the gut may be altered. Studies indicate that radiation stimulates an acute inflammatory response, releasing inflammatory mediators which may stimulate the induction of nitric oxide synthase. Nitric oxide, synthesized by constitutive nitric oxide synthase under physiological conditions, is an important modulator of intestinal electrolyte transport. However, under pathophysiological conditions, induced by radiation, inducible nitric oxide synthase may produce elevated levels of nitric oxide thereby causing an increase in the net secretion of water and electrolytes.

To determine the role of nitric oxide in the radiation-induced alterations in electrolyte transport we measured changes in nitric oxide synthase activity in the jejunum, ileum and colon of rats at various times after whole body irradiation (10 Gray) and after sham treatment. Tissues from these same animals were used for the determination of changes in the short circuit current response to neurally-evoked stimulation or to direct stimulation of the epithelium. To further determine the importance of nitric oxide in the gut, rats were treated with the competitive nitric oxide synthase inhibitor, nitro-L-arginine methylester one hour prior to irradiation or sham treatment and the above procedures repeated. To determine if an inflammatory response was caused by radiation both prostaglandin E_2 and leukotriene B_4 synthesis were measured. In addition, tissues were fixed, cut and stained for histological analysis of epithelial detachment and/or subepithelial edema.

Radiation decrease the short circuit current response to neurally-evoked stimulation in the jejunum, ileum and colon in a time-dependent fashion. The radiation-induced alterations in electrolyte transport did not coincide with changes in nitric oxide synthase activity. Radiation

transiently decreased nitric oxide synthase activity in the jejunum at 0.5h post-irradiation. In the ileum, radiation increased nitric oxide synthase activity from 2-24h post-irradiation. Radiation had no significant effect on colonic NOS activity. Nitro-L-arginine methylester pretreatment significantly attenuated nitric oxide synthase activity in the jejunum of shams and at 2h post-irradiation, in the ileum at 24h post-irradiation and in the colon of shams and at 0.5, 2, and 24h post-irradiation. Nitro-L-arginine methylester primarily targeted constitutive nitric oxide synthase with no effect on inducible nitric oxide synthase. Nitro-L-arginine methylester had no effect on jejunal electrolyte transport. However, the nitro-L-arginine methylester accentuated the hyporesponsiveness to electrical field stimulation in both the ileum and colon. Neither radiation nor nitro-L-arginine methylester significantly altered prostaglandin E_2 or leukotriene B_4 synthesis or the histological appearance of these tissues. Thus, alterations in intestinal electrolyte transport occurred in the absence of a major inflammatory response and without damage to the epithelium. Furthermore, the inducible nitric oxide synthase preferring inhibitor, aminoguanidine significantly and selectively inhibited inducible nitric oxide synthase activity in the ileum at 2h post-irradiation.

The results demonstrate that radiation can cause alterations in electrolyte transport in the rat intestine in the absence of an acute inflammatory response or significant changes in epithelial morphology. Radiation can stimulate inducible nitric oxide synthase activity in the rat ileum. The increase in nitric oxide production is likely a protective mechanism rather than damaging to epithelial function. Furthermore, we have demonstrated that the nitric oxide synthase inhibitors, nitro-L-arginine methylester and aminoguanidine are specific for the constitutive and inducible nitric oxide synthases, respectively.

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List of Abbreviations

AA	arachadonic acid
AC	adenylate cyclase
ACh	acetylcholine
AG	aminoguanidine
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BG	background
C	Centigrade
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	adenosine 3', 5'-cyclic monophosphate
CGRP	calcitonin gene-related peptide
cCOX	constitutive cyclo-oxygenase
Cl^-	chloride anion
cGMP	guanosine 3', 5'-cyclic monophosphate
cm	centimeter
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
CO_2	carbon dioxide
COX	cyclooxygenase
Cs	cesium
DAG	diacylglycerol
ddH_2O	distilled deionized water
DNA	deoxyribonycleic acid
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediamine tetraacetic acid
EFS	electrical field stimulation
EGTA	ethyleneglycol tetraacetic acid
ENS	enteric nervous system
EtOH	ethanol
FAD	flavine adenine dinucleotide
FMLP	*N*-formyl methionyl-leucyl-phenylalanine
FMN	flavine mononucleotide
GC	guanylate cyclase
G_t	conductance
Gy	Gray
h	hour
H^+	hydrogen ion
HCO_3^-	bicarbonate anion
H_2O	water
5-HT	5-hydroxytryptamine

iCOX	inducible cyclo-oxygenase
IFN-γ	interferon gamma
IgG	immunoglobulin G
I-κB	inhibitor kappa B
IL-1β	interleukin-1β
IL-4	interleukin-4
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IP_3	inositol 1, 4, 5-triphosphate
Isc	short circuit current
i.v.	intravenous
K^+	potassium ion
kDa	kiloDalton
kg	kilogram
K_m	Michaelis-Menten constant
L	litre
LT	leukotriene
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
L-NAA	nitro-L-aminoarginine
L-NAME	nitro-L-arginine methylester
L-NMA	nitro-L-methyl arginine
L-NNA	nitro-L-nitroarginine
LPS	lipopolysaccharide
min	minute
ml	millilitre
mM	millimolar
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
Na^+	sodium ion
NaCl	sodium chloride salt
NADPH	nicotinamide adenine dinucleotide
NANC	non-adrenergic non-cholinergic
NaOH	sodium hydroxide
NE	norepinephrine
nmol	nanomole
NO	nitric oxide
NOS	nitric oxide synthase
NF-κB	nuclear factor kappa B
PAF	platelet activating factor
PD	potential difference
PG	prostaglandin
PKC	protein kinase C
PLA_2	phospholipase A_2

PLC	phospholipase C
PMN	polymorphonuclear granulocytes
pmol	picomole
SNAP	s-nitroso-acetylpenicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
SP	substance P
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor alpha
TTX	tetrodotoxin
μA	microamps
μl	microlitres
μM	micromolar
μsec	microseconds
V	Volt
VIP	vasoactive intestinal peptide

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DEDICATION

This thesis is dedicated to my parents who instilled in me the ambition, courage and confidence to succeed; and to my aunt and uncle, Winder and Bachan Aurora and my cousins, Natasha, Tina and Rick Aurora who have made me welcome in their home and feel like one of the family during my stay here in Ottawa.

INTRODUCTION

A. Overview

Common symptoms of individuals suffering from radiation enteritis include abdominal pain, malabsorption and diarrhea. In fact, diarrhea presents in more than 75% of cases (Donaldson, 1989). Standard drug regimes for patients with radiation enteritis include sulphasalazine, 5-ASA, antimicrobial agents, corticosteroids and immunosuppressants. Despite these interventions, recurrences and further exacerbation may lead to the need for surgical measures (Strong and Fazio, 1993). Furthermore, a significant proportion of patients are refractory to drug treatment.

Current thinking is that inflammatory disorders of the bowel are due to a genetic predisposition which may include epithelial cell surface receptors activated by dietary allergens, diminished epithelial barrier function facilitating bacterial entry resulting in immune cell stimulation, and immunologic disorders such as a hypersensitive immune response (Strong and Fazio, 1993). However, in the case of radiation-induced intestinal dysfunction a physiological alteration is caused by radiation regardless of the health or genetic make up of the individual.

The exact etiology of radiation-induced intestinal dysfunction is unknown. Ionizing radiation can decrease expression of genes integral to the maintenance of cell structure and organization of cellular organelles (Woloschak *et al.*, 1990). In addition, radiation-induced upregulation of genes coding for inflammatory mediators and the release of chemoattractants has been identified and may be the primary mediator of a radiation-induced inflammatory response (Hallahan *et al.*, 1989; Woloschak *et al.*, 1990). Regardless of the cause, the inflammatory response to irradiation in the gut changes the net transport of ions and water in the small bowel

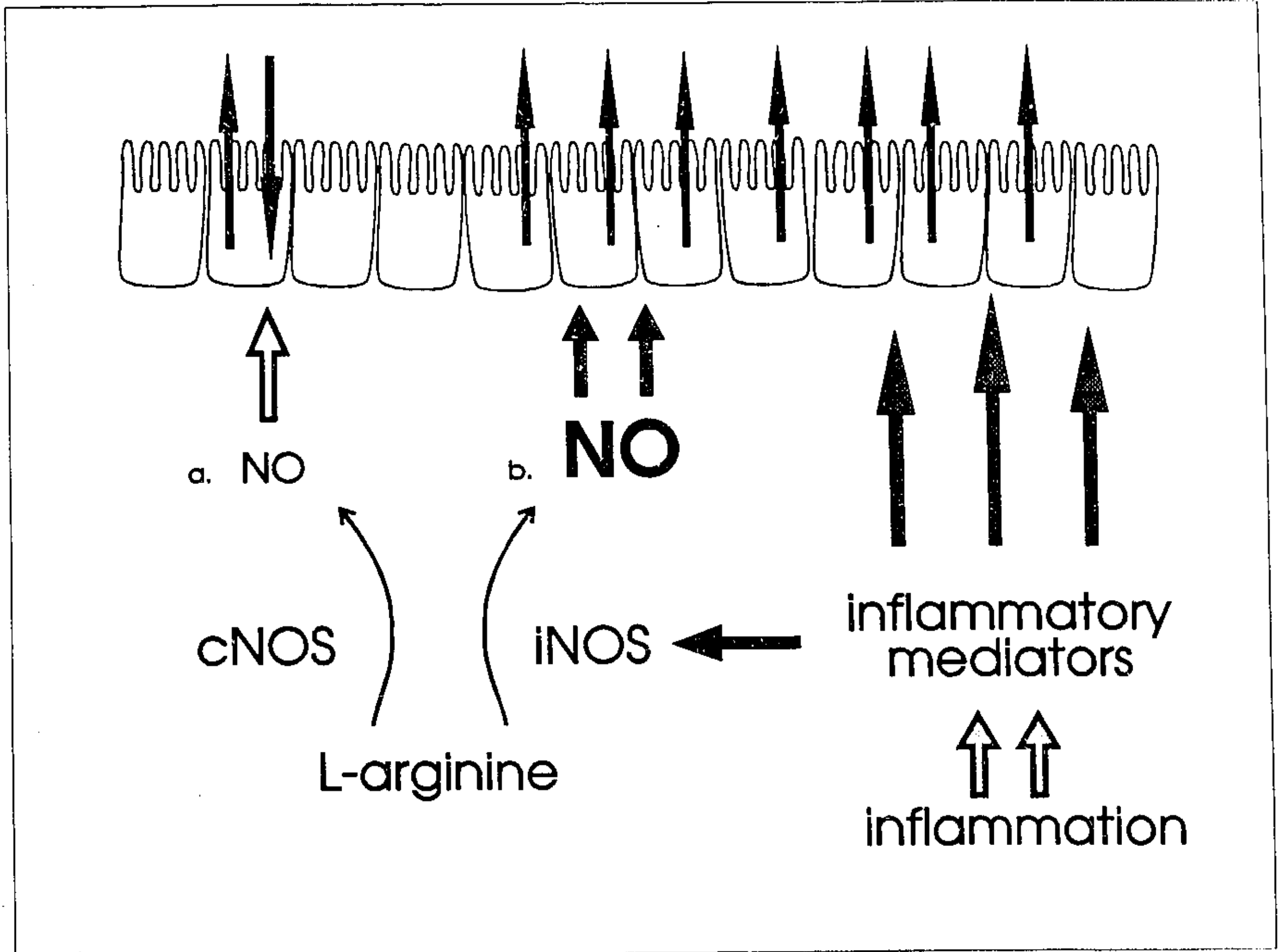
from absorptive to secretory (Gunter-Smith, 1986), resulting in diarrhea. It is hypothesized that the normal neuronal and humoral regulatory mechanisms of electrolyte transport and epithelial cell function are altered in inflammatory disorders of the bowel including those caused by radiation and are responsible for the changes in transport function (MacNaughton *et al.*, 1994; Hinterleitner and Powell, 1991). In addition, alterations in the structural integrity of the enterocyte which may loosen tight junctions, can increase mucosal permeability and therefore compromise intestinal function (Balda *et al.*, 1992; Ma *et al.*, 1993; Porvaznik, 1979). Numerous animal models of inflammatory bowel disorders are characterized by increased intestinal permeability and have identified specific immune cell types (mast cells, neutrophils) (Kanwar *et al.*, 1994; McCall *et al.*, 1989), protein kinases (protein kinase C) (Stenson *et al.*, 1993) and inflammatory mediators (nitric oxide, platelet-activating factor) (Kubes, 1992; Kanwar *et al.*, 1994; Boughton-Smith *et al.*, 1993; Filep and Földes-Filep, 1993) which can regulate intestinal permeability.

The highly diffusible gas nitric oxide (NO) has been implicated as a mediator of the inflammatory response in the gut (Gaboury *et al.*, 1993; Miller *et al.*, 1993; Caplan *et al.*, 1994; Pittner and Spitzer, 1992). NO synthases (NOS) can be placed in two main categories, the constitutive and the inducible NOS (Hirata *et al.*, 1995). The constitutive NOS produces low concentrations (pM) of NO consistent with its role as a physiological modulator. In contrast, inducible NOS produces 100-fold higher concentration of NO for prolonged periods of time compared to the constitutive enzyme. These concentrations (nM) are consistent with the role of NO as a bactericidal agent produced by macrophages (Wiegand *et al.*, 1993; Imai *et al.*, 1993; Marletta *et al.*, 1988), but may also contribute to the damage of surrounding healthy tissue

(Tepperman *et al.*, 1993; Tepperman *et al.*, 1994). These contrasting effects of NO in biological systems are exemplified by the work of Laszlo *et al.* (1994) who demonstrated that the inhibition of endogenous NO synthesis, before the induction of iNOS, increased vascular injury and plasma albumin extravasation in the gut. However, during the acute inflammatory response, once iNOS was induced, inhibition of endogenous NO production resulted in the amelioration of the acute inflammatory damage and reduced vascular albumin leakage.

Recent evidence has shown that NO is a mediator of electrolyte transport in the guinea pig (MacNaughton, 1993), mouse (Rao *et al.*, 1994) and rabbit (Barry *et al.*, 1994) ileum and the rat jejunum (Mourad *et al.*, 1993; Mourad *et al.*, 1993) and colon (Tamai and Gaginella, 1993). Since NO stimulates transport and modulates the release of other secretagogues (histamine, 5-HT and prostaglandins) (Kanwar *et al.*, 1994; MacNaughton, 1993; Tamai and Gaginella, 1993) and since NO has been found to be an important player in the inflammatory response, **we hypothesized that increased NO production under pathophysiological conditions mediates secretion responsible for diarrhea in experimental radiation enteritis** (Fig.1).

Figure 1. The effects of acute inflammation on nitric oxide (NO) synthesis and intestinal electrolyte transport. NO modulates intestinal electrolyte transport under physiological conditions (a). Inflammatory mediators released during acute inflammation may induce iNOS, increasing NO production (b) and therefore stimulate water and electrolyte transport secretion.


a. NO
b. NO
cNOS
iNOS
L-arginine
inflammatory mediators
inflammation

B. Mechanisms of intestinal electrolyte and water transport

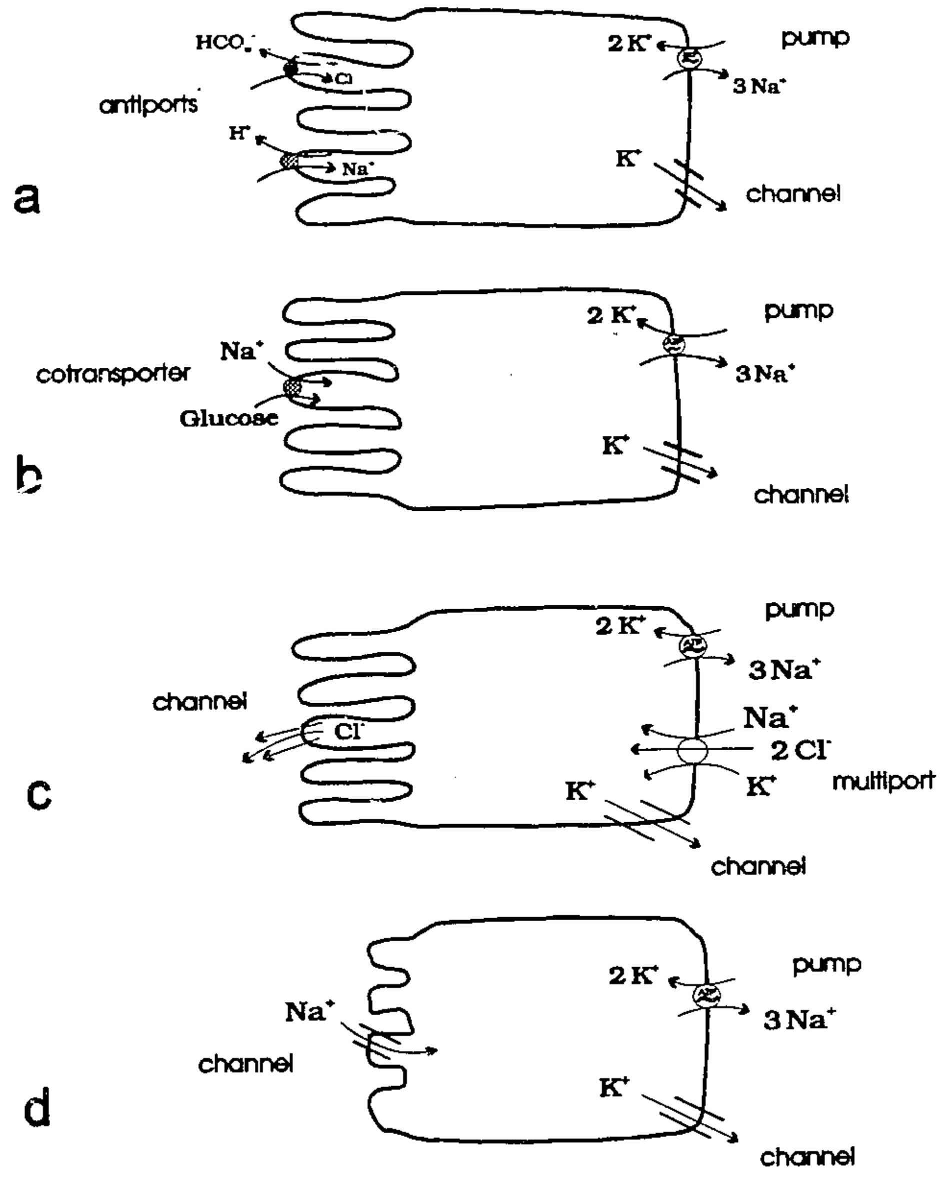
1. Ion pumps, channels, carriers

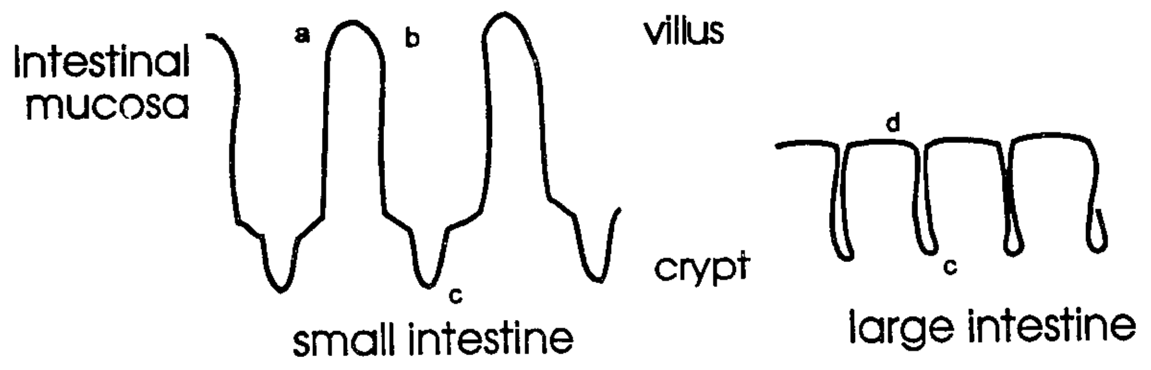
Water and electrolyte absorption and secretion in the intestine are accomplished by specific cellular transport mechanisms of the epithelial cells which line the intestinal mucosa. The major transcellular mechanisms responsible for electrolyte transport can be categorized into 3 groups: pumps, carriers and channels. These are summarized with respect to the small intestine in Figure 2.

The most common pump of the intestinal epithelial cell is the Na^+-K^+-ATPase which exchanges $3Na^+$ outward and $2K^+$ inward for every molecule of ATP hydrolyzed (Fig.2). These pumps are located on the basolateral membrane of the villus and crypt enterocytes and maintain the electrochemical gradients necessary for pathways of both simple and facilitated diffusion. Examples of facilitated diffusion in the small intestine include Na^+-glucose cotransporter on the apical membrane of villar enterocytes and the Na^+-K^+-$2Cl^-$ cotransporter on the basolateral membrane of epithelial crypt cells. In the small intestine the Na^+-H^+ and Cl^--HCO_3^- exchangers present on the apical membrane of villar enterocytes comprise the major pathway responsible for water absorption, as water follows by osmosis. The Na^+-H^+ and Cl^--HCO_3^- exchangers are also important for the effective exchange of waste products of cellular respiration as CO_2 and H_2O are converted to $H_2CO_3 \rightarrow H^+ + HCO_3^-$ and excreted in exchange for Na^+ and Cl^- (Cooke, 1989).

In the large intestine electrogenic Na^+ absorption, driven by the basolateral Na^+-K^+-ATPase, induces a serosa-positive electrical potential difference that drives Cl^- from the cytosol of the cell by a very selective anion leak pathway which allows for continuous absorption of Na^+ and Cl^- (Binder and Sandle, 1987). The amiloride-sensitive Na^+ channel of colonic enterocytes

Figure 2. Cellular transport mechanisms of intestinal epithelial cells. A. The most common pumps, exchangers, cotransporters and channels in villar and crypt enterocytes. Their relative positions on the intestinal mucosa are indicated in diagram B. *From* Binder and Sandle 1987; Dharmsathaphorn 1989.


A
a
antiports
HCO_3^-
Cl
H^+
Na^+
$2K^+$
pump
$3Na^+$
K^+
channel
b
cotransporter
Na^+
Glucose
$2K^+$
pump
$3Na^+$
K^+
channel
c
channel
Cl^-
$2K^+$
pump
$3Na^+$
Na^+
$2Cl^-$
K^+
multiport
K^+
channel
d
Na^+
channel
$2K^+$
pump
$3Na^+$
K^+
channel


B
Intestinal mucosa
a
b
villus
d
crypt
c
c
small intestine
large intestine

is present in most species except the rat which is dependent on the Na^+-H^+ and Cl^--HCO_3^- exchangers normally found in the small intestine (Binder and Sandle, 1987).

Ion channels are responsible for the passive flow of ions down their electrochemical gradients (dependent on pumps). The Cl^- channels, located on the apical membrane of intestinal epithelial crypt cells are responsible for the majority of water secretion in the gut (Cooke, 1989; Binder and Sandle, 1987).

2. Second messengers

Electrolyte transport is regulated by a small number of second messenger systems which participate in numerous complex interactions (Voet and Voet, 1990). These include adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3',5'-cyclic monophosphate (cGMP), inositol 1,4,5-triphosphate (IP_3) and calcium (Ca^{2+}) (Voet and Voet, 1990). Neurohormonal activation of G protein-coupled receptors activate adenylate cyclase (AC), phospholipase C (PLC), and guanylate cyclase (GC), which in turn result in upregulation or increased production of other intracellular messengers such as IP_3, diacylglycerol (DAG), protein kinase C (PKC), phospholipase A_2 (PLA_2), and cyclooxygenase (COX) and lipoxygenase metabolites of arachadonic acid (AA) metabolism.

The final mediator of all these changes in intracellular messengers is often intracellular calcium $[Ca^{2+}]_i$. Alterations in $[Ca^{2+}]_i$ can be regulated by both cAMP and cGMP, PLC activation of the IP_3 pathway or direct activation of Ca^{2+} channels. Mediators such as prostaglandins, leukotrienes, various neuropeptides and nitric oxide (NO) can all stimulate a rise in one or more of these second messengers and in turn stimulate a net increase in intestinal electrolyte and water secretion (Tamai and Gaginella, 1993; Cooke, 1989; Binder, 1989).

Alterations in the concentration of these mediators within enterocytes regulate the cell's permeability to Cl^- and therefore secretion. For example, an increase in $[Ca^{2+}]_i$ in villar cells results in the closure of Cl^- channels on the basolateral membrane and the inhibition of Na^+ and Cl^- absorption. Intracellular cAMP, cGMP, IP_3 as well as stimulation with muscarinic agonists, all of which stimulate an increase in $[Ca^{2+}]_i$, close the Cl^- channels on the basolateral membrane of villus cells and therefore inhibit absorption. On the other hand, a rise in $[Ca^{2+}]_i$ of crypt enterocytes will decrease the apical membrane resistance to Cl^- by opening Cl^- channels and results in Cl^- and water secretion. This event may be self limiting, due to the alteration of membrane potential, provided all other membrane permeabilities remain unchanged (Cooke, 1989). However, $[Ca^{2+}]_i$ can also activate the opening of K^+ channels on the basolateral membrane of crypt cells which results in the efflux of K^+ ions and maintenance of the membrane potential and Cl^- secretion (Cooke, 1989).

3. Paracellular transport

Another pathway of intestinal transmural ion flux is paracellular transport. Paracellular transport is a passive flow of ions, followed by water, down their electrochemical gradients via tight junctions. Tight junctions are responsible for maintaining the potential difference (PD) across the intestinal epithelium. The nature of the tight junction is different in various regions of the intestine. For example, epithelial conductance (G_t) decreases aborally with a concomitant increase in resistance and PD. The change in paracellular permeability exhibits a certain degree of selectivity with respect to the direction of ion movement (Pantzar *et al.*, 1994). Throughout the length of the intestine epithelial permeability in the serosal to mucosal direction is

consistently greater than epithelial permeability in the opposite direction (Pantzar *et al.*, 1994) (Fig.3).

C. Nitric oxide (NO)

1. Introduction

In 1980 endothelium-derived relaxing factor (EDRF) was discovered by Furchgott and Zawadzki (1980). Subsequently, Palmer *et al.* (1987) determined that EDRF was the highly diffusible, free radical gas nitric oxide (NO). NO has since been found to have a role in numerous physiological processes (Nathan, 1992; Lowenstein *et al.*, 1994; Förstermann *et al.*, 1994; Moncada *et al.*, 1991) including, embryogenesis (Lee and Juchau, 1994), vasodilation (Knowles and Moncada, 1992), neurotransmission (Gustafsson *et al.*, 1990; Boeckxstaens *et al.*, 1991), relaxation of vascular smooth muscle cells (Furchgott and Zawadzki, 1980; Bult *et al.*, 1990), regulation of vascular permeability (Boughton-Smith *et al.*, 1993), leukocyte adhesion and chemotaxis (Kubes *et al.*, 1993; Belenky *et al.*, 1993), T cell activation and activation of other inflammatory cells (Merryman *et al.*, 1993), gastric mucous secretion (Brown *et al.*, 1993) and electrolyte transport in the intestine (Tamai and Gaginella, 1993; Mascolo *et al.*, 1994; MacNaughton, 1993; Mourad *et al.*, 1993; Mascolo *et al.*, 1994; Izzo *et al.*, 1994).

NO is a co-product of the conversion of the amino acid L-arginine to L-citrulline in a reaction where the terminal guanido nitrogen of arginine reacts with molecular oxygen (Fig.4). The enzyme which catalyzes this reaction is known as NO synthase (NOS) and to date has three known isoforms which have been categorized into two groups, the constitutive (cNOS) and the inducible (iNOS).

Figure 3. Directional differences in paracellular transport along the intestinal axis. Paracellular permeability of the intestinal mucosa is greater in the serosal to mucosal direction and decreases in the oral to aboral direction as indicated by the size of the arrows. However, paracellular permeability in the mucosal to serosal direction increases aborally. *From* Pantzar *et al.* 1994.

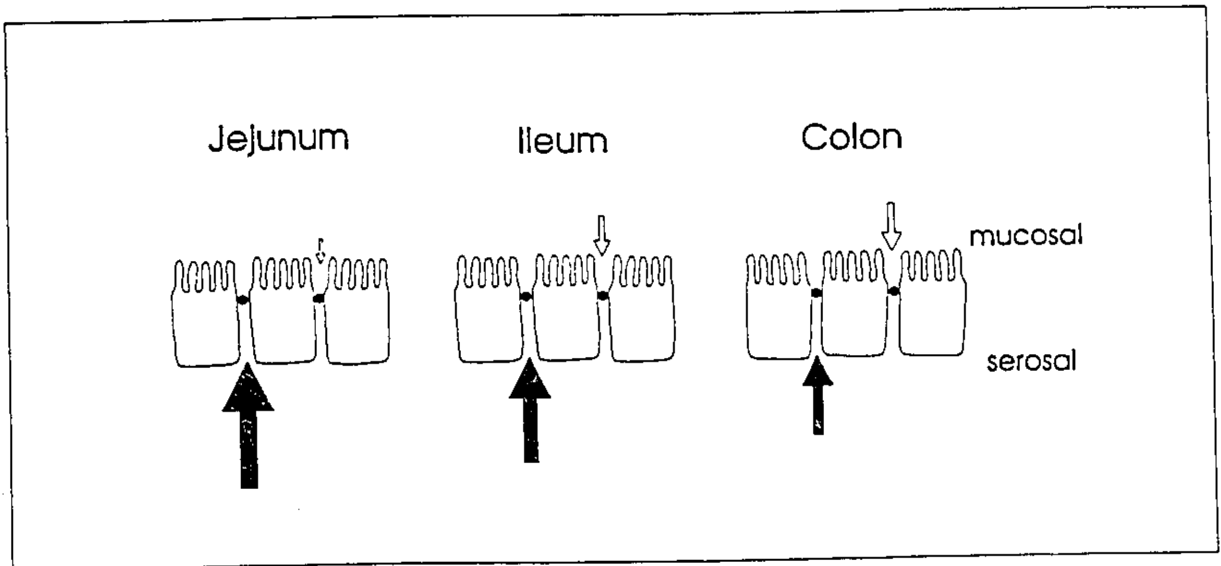
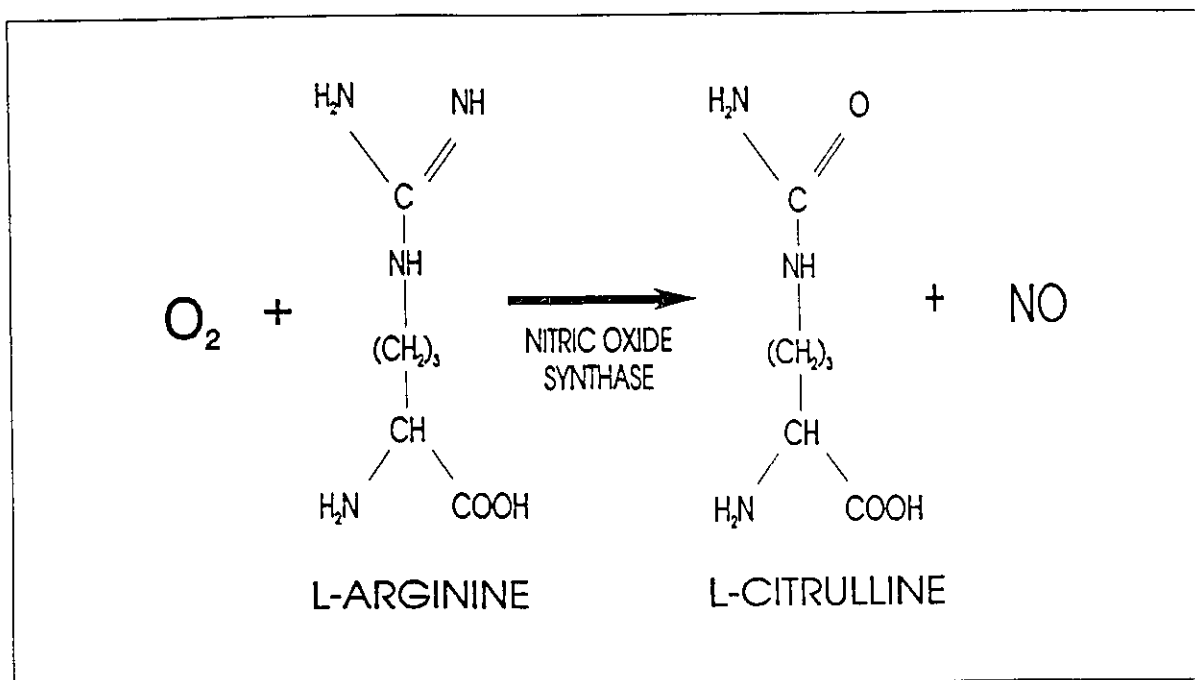

Jejunum
Ileum
Colon
mucosal
serosal

Figure 4. The nitric oxide (NO) synthase pathway. In the presence of oxygen, the amino acid, L-arginine is converted to L-citrulline and NO by the enzyme NO synthase (NOS). *From* Ignarro 1991; Nathan 1992; Knowles and Moncada 1994; Fostermann *et al.* 1994.



O2
+
H2N
NH
C
NH
(CH2)3
CH
H2N
COOH
L-ARGININE
NITRIC OXIDE
SYNTHASE
H2N
O
C
NH
(CH2)3
CH
H2N
COOH
L-CITRULLINE
+
NO

As described by Hattori *et al.* (1994), type I and type III NOS are both constitutive and Ca^{2+}/calmodulin-dependent. The constitutive form of the enzyme produces low levels (pM) of NO. Constitutive NOS is the primary producer of NO in neurons, endothelial and epithelial cells (M'Rabet-Touil *et al.*, 1993; Tepperman *et al.*, 1993). The activity of cNOS is regulated by intracellular levels of Ca^{2+} which when increased result in the ionic binding of the Ca^{2+}/calmodulin complex to the cNOS enzyme and its consequent activation.

Type I NOS, for which the gene is located on chromosome 12, has been cloned from the rat (Griscavage *et al.*, 1994) and human (Förstermann *et al.*, 1994) brain and has been identified as a 160 kDa soluble cytosolic protein found in neuronal and epithelial cells. In addition to the CNS, an extensive system of enteric non-adrenergic non-cholinergic neurons which use NO as a neurotransmitter have been identified in the rat, pig and human (Nichols *et al.*, 1993; Barbiers *et al.*, 1994; Faussone-Pellegrini *et al.*, 1994).

The gene for Ca^{2+}-dependent endothelial cNOS, identified as type III NOS, is located on chromosome 7 (Xu *et al.*, 1994). It has been cloned from bovine and human endothelial cells and has been identified in both venules and arterioles perfusing the rat and human intestine (Pollock *et al.*, 1993; Nichols *et al.*, 1994). The 135 kDa endothelial cNOS protein is membrane bound by N-myristoylation and is translocated to the cytosol upon phosphorylation, however, it is insoluble. Some reports suggest the existence of a Ca^{2+}/calmodulin-independent cNOS present in small amounts in the cytosolic fraction of bovine aortic endothelial cells (Hecker *et al.*, 1991).

Type II, inducible NOS, is the smallest of the three NOS isoforms having a molecular weight of 130 kDa, is synthesized *de novo* upon stimulation with certain cytokines and is Ca^{2+}/calmodulin-independent. However, calmodulin is non-covalently bound to iNOS, even in

the absence of Ca^{2+}.

Inducible NOS, unlike cNOS, is generally absent under normal physiologic conditions. However, once iNOS is induced it continually produces large quantities (nM) of NO. The iNOS gene, located on chromosome 17 (Chartrain *et al.*, 1994), has a number of consensus sequences for the binding of transcription factors involved in the induction by cytokines and endotoxin (Hattori *et al.*, 1994). iNOS has been cloned from a murine macrophage cell line (Gaillard *et al.*, 1992; Marletta *et al.*, 1988) as well as a number of human cell types (Nunokawa *et al.*, 1994; Tracey *et al.*, 1994; Reiling *et al.*, 1994) and is expressed in neutrophils (McCall *et al.*, 1989), platelets (Muruganandam and Mutus, 1994), fibroblasts (Wink *et al.*, 1993), vascular smooth muscle cells (Koide *et al.*, 1993; Xie *et al.*, 1993), hepatocytes (Geller *et al.*, 1993), endothelial (Knowles *et al.*, 1990) and epithelial (Tepperman *et al.*, 1993) cells after induction, with cytokines, bacterial endotoxin and interferon gamma (IFN-γ) being the most potent stimulants.

In macrophages iNOS is equally distributed between membrane bound and cytosolic (Hecker *et al.*, 1991). Interestingly, evidence suggests that the membrane bound iNOS from the macrophage J774.2 cell line is Ca^{2+}/calmodulin-dependent, in contrast to the cytosolic iNOS in these cells which is Ca^{2+}/calmodulin-independent (Hecker *et al.*, 1991).

Although the genes for type I, II and III NOS only share 50-55% homology, all three types of NOS have sequences for the binding of NADPH, FAD and FMN (Lowenstein *et al.*, 1994) and the isoform sequence is well conserved between species. However, the tertiary structure of the enzyme is still in question as conflicting reports have been published. Bredt and Snyder in 1990 isolated cNOS from rat cerebellum and determined that it was a 150 kDa monomer, whereas in 1991 Schmidt *et al.* reported the isolation of cNOS from the rat cerebellum

which was a 155 kDa homodimer.

2. NO synthesis

The production of NO is entirely dependent on the presence of the essential amino acid L-arginine. No other amino acids or guanido-containing compounds can take its place in this reaction (Iyengar *et al.*, 1987). Under physiological conditions the production of NO by iNOS may be limited by the low (80-110 μM) plasma concentration of arginine as the K_m for NO synthesis by the macrophage RAW264.7 cell line is 0.15 mM. Sites of inflammation may have lower concentrations (<0.1mM) of arginine thereby limiting NO synthesis in those regions (Tarpila, 1971).

It has been suggested that arginine may be synthesized by a number of different cell types, for example bovine aortic endothelial cells and rat peritoneal macrophages. Production of arginine by these cells is via the conversion of citrulline to arginine catalyzed by arginosuccinate synthase and lyase. Nussler *et al.* (1994) have shown that the macrophage cell line RAW264.7 express both arginosuccinate synthase and arginosuccinate lyase which actively produces L-arginine from L-citrulline used by NOS for NO production. In addition, these researchers demonstrated that arginosuccinate synthase was co-induced with NOS by both endotoxin and IFN-γ. Although arginosuccinate lyase was not upregulated by endotoxin and cytokine exposure, it appears that arginosuccinate synthase is the rate limiting step in the conversion of citrulline to arginine, as its activity is only 1/10 that of arginosuccinate lyase. Interestingly, the induction of arginosuccinate synthase was solely dependent on the cytokine activation and not on the concentration of arginine. Alternatively, arginosuccinate synthase and arginosuccinate lyase have

also recently been identified as being constitutively expressed in nitrergic neurons of the canine colon (Shuttleworth *et al.*, 1995).

Exposure to endotoxin has also been found to stimulate tetrahydrobiopterin synthesis in vascular smooth muscle cells (Gross *et al.*, 1993). Tetrahydrobiopterin is a prosthetic group tightly bound to NOS enzymes which is not essential for the activity of NOS but rather plays an integral role in maintaining the haemoprotein in the reduced state, thereby attenuating the affinity and inhibitory effect of NO on NOS (Baek *et al.*, 1993; Giovanelli *et al.*, 1991; Griscavage *et al.*, 1994).

Although L-arginine is the only physiological substrate for NO production, other forms of arginine can be used as substrate for NO production *in vitro*, such as HO-arginine, which is an intermediate in the conversion of L-arginine to L-citrulline (Knowles and Moncada, 1992; Hecker *et al.*, 1991). HO-arginine is more readily converted to citrulline and NO than is arginine. Some arginine-containing dipeptides can also act as substrate for specific isoforms of NOS. For example, arginine-phenylalanine can be used by cytosolic, Ca^{2+}/calmodulin-dependent cNOS but not by membrane bound Ca^{2+}/calmodulin-dependent iNOS (Hecker *et al.*, 1991) which suggests a difference in the active site of these isoforms.

In contrast, some analogues of L-arginine, such as *nitro*-L-methylarginine (L-NMA) or arginine methyl ester (L-NAME), can act as inhibitors of NO production by competing for the active site of NOS but not being converted to L-citrulline. L-canavanine (McCall *et al.*, 1989) and aminoguanidine (AG) (Hasan *et al.*, 1993) which are structurally similar to arginine can also act as inhibitors of NOS. Evidence suggests that AG may be a specific inhibitor of iNOS (Hasan *et al.*, 1993; Griffiths *et al.*, 1993) however more recent findings imply that AG is iNOS

preferring, not specific (Laszlo *et al.*, 1995). Thus, AG preferentially inhibits iNOS however in the absence of iNOS, AG at high doses may also inhibit cNOS activity.

A number of these compounds are commonly used to inhibit NO production in an attempt to determine the role of NO in biological systems. *Nitro*-L-aminoarginine (L-NAA) and *nitro*-L-nitroarginine (L-NNA) are 100-fold more potent inhibitors of endothelial cNOS than L-NMA whereas L-NMA and L-NAA are more potent inhibitors of iNOS than L-NNA (Joly *et al.*, 1994; Gross *et al.*, 1990). However, neither L-NMA nor L-NAA are as specific for iNOS as is AG. These findings indicate that the arginine binding site is not the same on the two isoforms of NOS.

Recently Martzen and Slemmon (1995) identified an endogenous inhibitor of neuronal cNOS. Neurogranin is a calmodulin binding peptide commonly found in neurons of the CNS including the striatum and hippocampus and is localized to the cell bodies and dendritic compartments of these neurons. Neurogranin was found to inhibit neuronal cNOS by binding calmodulin, thereby decreasing its availability for Ca^{2+} and the activation of cNOS. This effect was reversible upon phosphorylation of neurogranin. Therefore this may be a mechanism by which neurons specifically regulate NO production.

3. Induction of iNOS

The activation of the iNOS with endotoxin and cytokines has a latency of approximately 3-6 hours (Corbett *et al.*, 1993; Yang *et al.*, 1994; Salter and Knowles, 1991; Knowles *et al.*, 1990; Tepperman *et al.*, 1993; Geller *et al.*, 1993) and appears to be inactivated by 24 hours (Corbett *et al.*, 1993; Nussler *et al.*, 1994). These findings would suggest that activation of iNOS

and high concentrations of NO are primarily a factor in acute inflammation. However, it is possible that further stimulation of the system at a later time point may result in reactivation of iNOS as has been demonstrated in chronic studies of NO (Vane *et al.*, 1994).

Interestingly, ATP potentiates endotoxin-induced iNOS activity by greater than 2-fold (Tonetti *et al.*, 1994). It is suggested that ATP released from damaged cells activates P_{2y} surface receptors on murine macrophages. However the mechanism by which ATP increases nitrite production was not determined. These investigators implied that Ca^{2+} mobilization induced by purinergic receptor stimulation could not be responsible for the increase in nitrate as iNOS in macrophages is Ca^{2+}-independent. Nonetheless, they failed to consider the possible presence of the Ca^{2+}-dependent, membrane bound iNOS in macrophages (Hecker *et al.*, 1991) which could likely be the source of the increase in nitrite in their study.

Investigation into the mechanism by which endotoxin induces iNOS activity in macrophages indicates that protein kinase C (PKC) activation is partially responsible, as inhibition of PKC attenuates the induction of iNOS by endotoxin (Severn *et al.*, 1992). Furthermore, activators of PKC such as phorbol esters can induce iNOS mRNA expression and increase arginine influx in rat peritoneal macrophages in the absence of endotoxin or cytokines (Hortelano *et al.*, 1993).

Since iNOS is transcriptionally regulated, its production can be inhibited by glucocorticoids such as dexamethasone and cortisol (Cetkovic-Cvrlje *et al.*, 1993; Knowles *et al.*, 1990; Geller *et al.*, 1993) which inhibit transcription. Being a glucocorticoid, dexamethasone would inhibit transcription of iNOS and therefore inhibit mRNA production for the enzyme (Mascolo *et al.*, 1994; Knowles *et al.*, 1990; Geller *et al.*, 1993). However, both dexamethasone

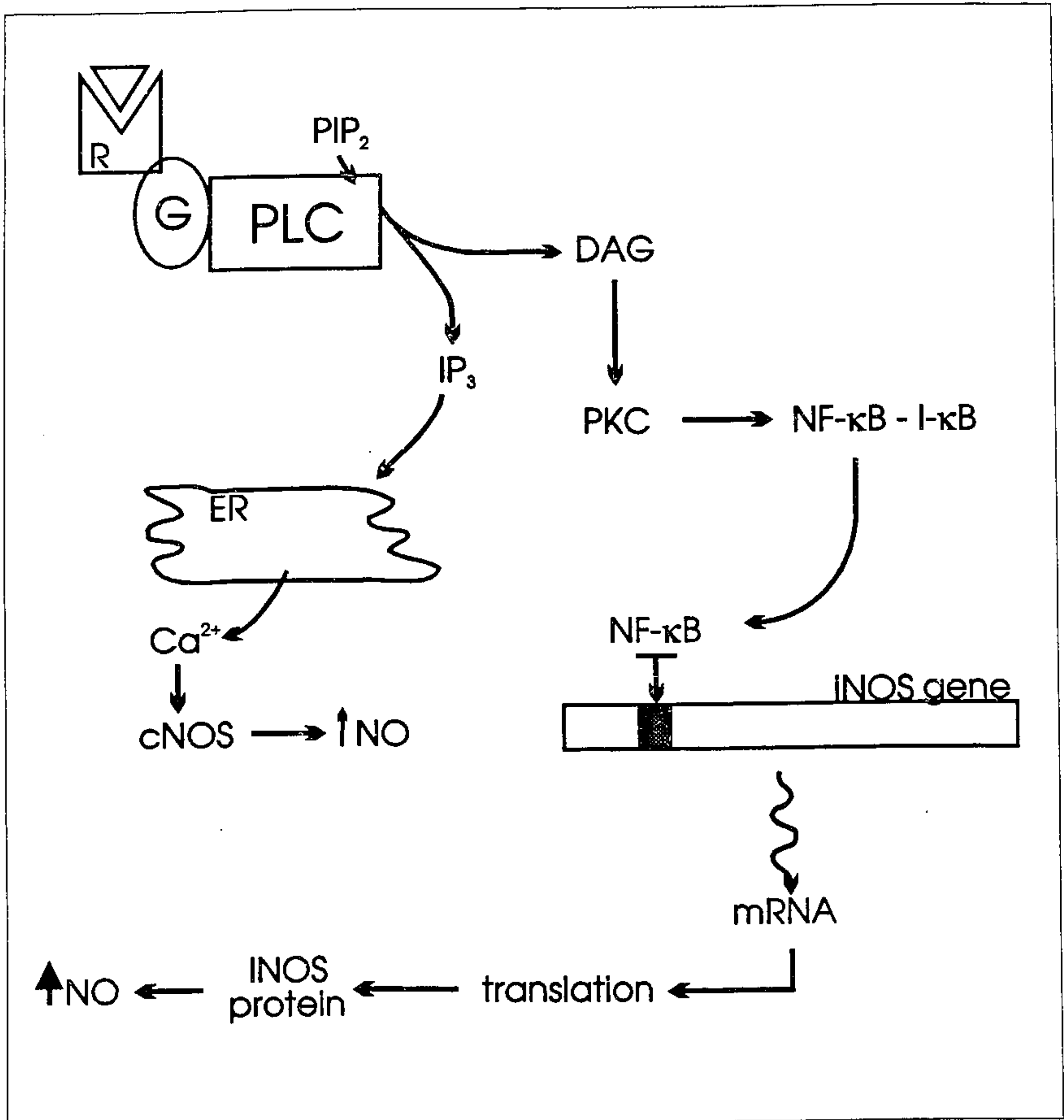
and nicotinamide have been found to inhibit NOS activity without an effect on iNOS mRNA expression induced by interleukin-1β (IL-1β) (Cetkovic-Cvrlje *et al.*, 1993). These investigators suggested that the effect of nicotinamide was due to its ability to scavenge NO. Furthermore, growth factors such as transforming growth factor-β (TGF-β) (Kanno *et al.*, 1994) have also been identified as potent inhibitors of the induction of iNOS.

The interleukins, IL-4 and IL-8 for example, are also known to inhibit the induction of iNOS in macrophages (McCall *et al.*, 1992; Bogdan *et al.*, 1994; Sands *et al.*, 1994). The studies using IL-4 have been particularly important in advancing the general working knowledge of the mechanism by which iNOS is induced.

IL-4 acts by inhibiting the translocation of PKC-ε to the plasma membrane of macrophages and hence the activation of PKC (Sands *et al.*, 1994). Protein kinases are very important in the activation of a number of proteins such as nuclear factor κB (NF-κB). In their review of NF-κB, Grimm and Baeuerle (1993) suggested that NF-κB is kept inactive by I-κB. Phosphorylation of the NF-κB-I-κB complex by PKC could release NF-κB allowing it to activate its targets (Muhl and Pfeilschifter, 1994)(Fig.5).

The gene for macrophage iNOS has two binding sites for NF-κB (Xie *et al.*, 1993), the release of NF-κB from its inhibitor, I-κB, due to phosphorylation by a protein kinase, could activate or enhance iNOS gene expression (Muhl and Pfeilschifter, 1994). Nevertheless, in the presence of stimulatory factors such as LPS or IL-1β the activation of PKC with phorbol esters decreases iNOS expression and activity (Muhl and Pfeilschifter, 1994). However, this may be due to the down regulation of receptors for IL-1β and TNF-α (stimulated by LPS) which would therefore negatively affect PKC activation of NF-κB (Muhl and Pfeilschifter, 1994). This theory

Figure 5. Potential mechanisms activating nitric oxide (NO) production. After phosphorylation by protein kinase C (PKC), nuclear factor kappa B (NF-κB) can bind to the inducible NO synthase (iNOS) gene and enhance endotoxin-induced activation of iNOS. Increases in intracellular calcium will activate constitutive NO synthase (cNOS) activity and NO production. *From* Muhl and Pfeilschifter 1994.



R
G
PLC
PIP2
DAG
IP3
PKC
NF-κB - I-κB
ER
Ca2+
cNOS
NO
NF-κB
INOS gene
mRNA
NO
INOS
protein
translation

is further supported by the findings of Hortelano *et al.* (1992) which suggest that PKC activation inhibits endotoxin-induced iNOS expression.

4. Targets for NO

The primary targets of nitric oxide in physiological systems are the iron-haem or iron-sulphur centres of the sites of activation of many proteins as well as deamination of DNA and inactivation of superoxide radicals. In these ways NO can be very damaging to cellular metabolism by inactivating enzymes of the electron transport chain such as, NADPH-ubiquinone, oxidoreductase and succinate-ubiquinone and in the Krebs cycle inactivating *cis*-aconitase. NO is thought to deenergize mitochondria by binding cytochrome *c*, the terminal heme protein in the respiratory chain (Schweizer and Richter, 1994). NO can also inhibit DNA and protein synthesis by inhibiting ribonucleotide reductase (Lepoivre *et al.*, 1991). NO can inhibit the DNA repair enzyme, Fpg, by binding at the cysteine residues within the zinc finger of the molecule (Wink and Laval, 1994). On the other hand NO may be cytoprotective in its ability to scavenge oxygen radicals (Wink *et al.*, 1993) and increase blood flow (MacNaughton *et al.*, 1989). All of these effects can contribute to the role of NO in both acute and chronic inflammation.

Since the action of NO is based on its affinity for both haem- and non-haem iron binding proteins and since NOS itself is a haem-iron containing enzyme it is only natural that NO would have an effect on its own production. Rengasamy and Johns (1993) determined that the effect of NO and NO-donating compounds on the inhibition of NO synthesis was reversible and concentration dependent. The K_m for NO synthesis by the bovine cerebellar NOS used in this study was 6.4 μM and although it was unchanged by NO inhibition of NO synthesis, the V_{max}

was significantly decreased from 80 to 45 pmol/min/mg (Rengasamy and Johns, 1993).

This evidence suggests that there is a type of feedback inhibition on NO synthesis by NO. In addition, other investigators noted that the absence of iNOS mRNA expression at 24 h post-induction could be reversed by the addition of the NOS inhibitor, L-NMA, to the induction medium (Nussler *et al.*, 1994). Thus it appears that in addition to feedback inhibition on NO synthesis, NO may inhibit the induction of iNOS mRNA.

The role of nitric oxide as a physiological messenger is primarily mediated by its activation of guanylate cyclase (GC). NO activates GC by binding the iron-haem complex of the enzyme and stimulates the production of cGMP (Schmidt *et al.*, 1991; Palmer and Higgs, 1989). The increased production of cGMP is thought to be responsible for the relaxation of vascular smooth muscle (Palmer *et al.*, 1987; Furchgott and Zawadzki, 1980) and the inhibition of platelet aggregation although the exact mechanism of activation is presently unclear (Ignarro, 1991). The activation of intracellular GC may be responsible for the prosecretory/antiabsorptive effects of NO in the small intestine (Tamai and Gaginella, 1993; MacNaughton, 1993; Field *et al.*, 1978). However, NO has also been identified as an activator of the Na^+-K^+-ATPase in vascular smooth muscle cells resulting in smooth muscle relaxation independent of GC activation and cGMP production (Gupta *et al.*, 1994). The activation of Na^+-K^+-ATPase may have been secondary to Na^+-H^+ exchange, however, the mechanism of activation was not determined.

5. NO and neurotransmission

Nitric oxide synthase (NOS) activity has been localized in neurons of the myenteric and submucous plexus and its product, NO, has been identified as a secretagogue in the gut (Furness

et al., 1994; Nichols *et al.*, 1994). Histological evidence for the neuronal role of NO in the gastrointestinal tract was provided by the identification of NADPH-diaphorase positive staining neurons in the rat and human gastrointestinal systems (Nichols *et al.*, 1993; Cuffari *et al.*, 1993). More specifically, in the rat, NO neurons have been identified in the submucosal plexus with processes that are widely spread throughout the small intestine. In the colon NO neurons are also present however fewer processes are noticeable (Nichols *et al.*, 1993).

Nitric oxide has been identified primarily as an inhibitory neurotransmitter in the gut, acting directly on the effector cell or via presynaptic inhibition (Bult *et al.*, 1990; Hryhorenko *et al.*, 1994; Soediono and Burnstock, 1994). It is likely that the nitrergic system is activated in part by cholinergic inputs (Bult *et al.*, 1990; Izzo *et al.*, 1994). Activation of nitrergic neurons is dependent on either receptor-mediated or voltage-gated activation of Ca^{2+} channels (Boeckxstaens *et al.*, 1993).

Although most functional studies of the nitrergic system in the gut identify the role of NO on smooth muscle relaxation it is likely that similar pathways regulate electrolyte transport much as they do in the cholinergics. This would suggest that the function of the nitrergic system in electrolyte transport is antisecretory or proabsorptive as has been demonstrated (Rao *et al.*, 1994). However, further study of the physiological effects of both NO donors and inhibitors indicate that NO may also be prosecretory (Tamai and Gaginella, 1993; Mascolo *et al.*, 1994a; Mascolo *et al.*, 1994b; Izzo *et al.*, 1994). Tamai and Gaginella (1993) identified the prosecretory effect of NO and the NO donor, sodium nitroprusside, on the rat distal colon. Using various models of diarrhea, Mascolo *et al.* (1994a and b) and Izzo *et al.* (1994) demonstrated the antidiarrheal effect of L-NAME, suggesting that NO contributes to the prosecretory state of the

gut in these models of diarrhea.

6. NO and prostaglandins

Since many of the prosecretory responses in the gut are mediated by prostaglandins (PG) (Miller, 1983) it is possible that NO acts via PGs (Tamai and Gaginella, 1993; MacNaughton, 1993). The interaction of NO with the heme moiety of cyclooxygenase (COX) was first discovered by Yonetani *et al.* in 1972 although its effect was not elucidated until recently. In the early 1990's it was found that NO stimulates PGE_2 production in hypothalamic fragments (Rettori *et al.*, 1992) and the murine macrophages cell line RAW264.7 (Salvemini *et al.*, 1993). In contrast, COX activity was significantly inhibited by NO in resident macrophages of the liver (Stadler *et al.*, 1993).

More recently, investigators (Corbett *et al.*, 1993) found that NOS and COX are coexpressed in Islets of Langerhans upon stimulation with IL-1β. These researchers also provide supporting evidence for the observation that NO stimulates both cCOX and iCOX activity. This may be important in the case of inflammation where infiltrating monocytes release copious amounts of IL-1β which can stimulate the induction of iNOS and iCOX and NO in turn can stimulate further production of PGs and thromboxanes which are important mediators of the inflammatory response (Corbett *et al.*, 1993; LeDuc *et al.*, 1993), mucosal blood flow (Miller, 1983) and intestinal secretion (Tamai and Gaginella, 1993; MacNaughton, 1993).

Elevated levels of intracellular cAMP have been found to enhance IFN-γ- induced iNOS mRNA expression and nitrite production in vascular smooth muscle cells (Koide *et al.*, 1993). This may be a mechanism of feed forward activation. However, prolonged elevation of cAMP

can inhibit IFN-γ and LPS-induced NOS activity in macrophages (Bulut *et al.*, 1993).

Thus, NO has many potential mechanisms by which it can alter intestinal function, in particular mucosal function. The influence of NO on epithelial and vascular permeability as well as cellular metabolism are critical to the maintenance of mucosal integrity. Furthermore, NO can stimulate the production of mediators of electrolyte transport, neural pathways involved in electrolyte transport and may act directly on intestinal epithelial cells.

D. Regulation of electrolyte and water transport

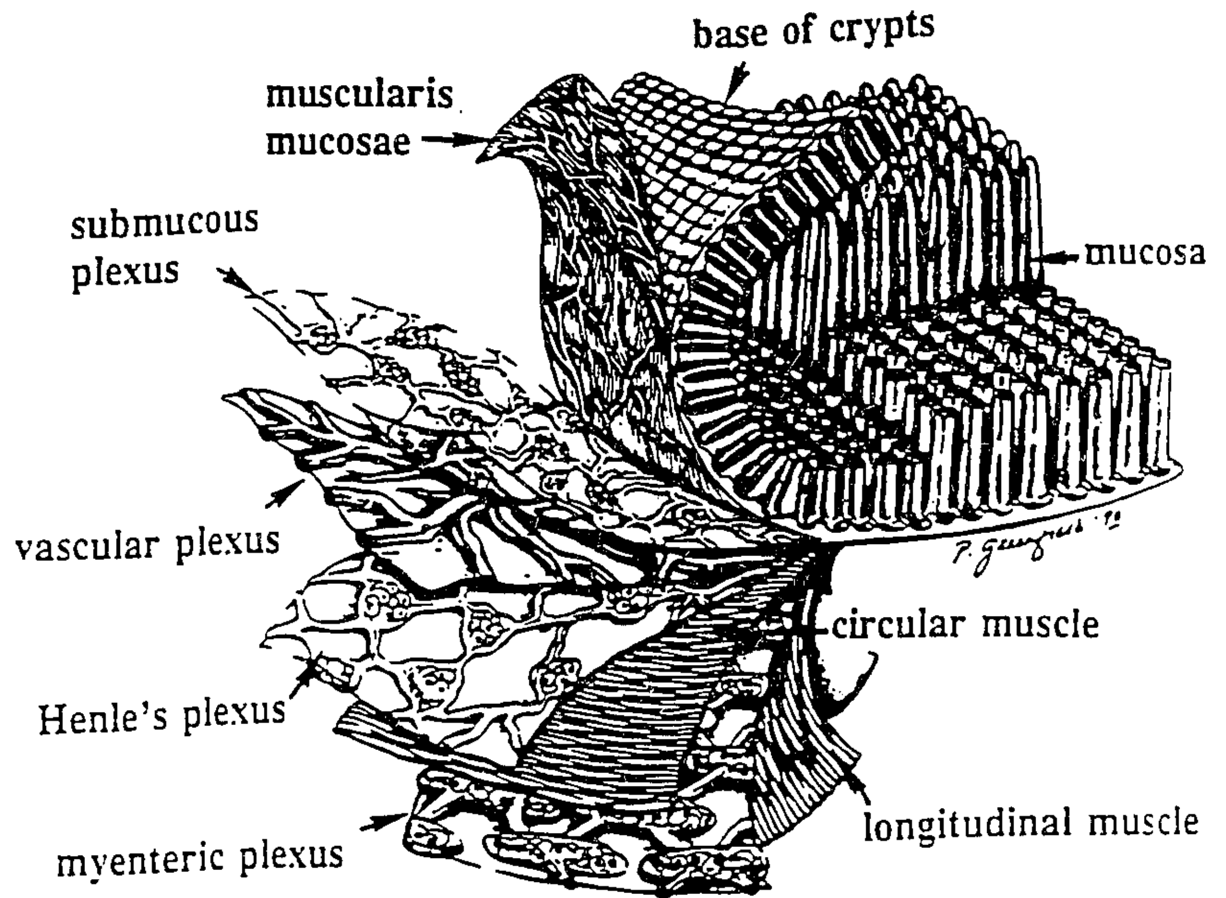
1. Neuronal mechanisms

a. Pathways

The intestine receives extrinsic innervation from the central nervous system (CNS) via both sympathetic and parasympathetic divisions of the autonomic nervous system. The sympathetic nervous system stimulates electrolyte absorption and inhibits intestinal motility via release of the neurotransmitter norepinephrine whereas the parasympathetic nervous system stimulates both intestinal secretion and motility via release of acetylcholine (ACh).

Although the gut receives inputs from the CNS, the gastrointestinal tract is a self-regulated organ and has its own "little brain" (Cooke, 1989) known as the enteric nervous system (ENS). The ENS is made up of distinct nerve layers (Fig.6). The myenteric nerve plexus which lies between the circular and longitudinal smooth muscle layers is predominately responsible for motility. Numerous nerve endings originating in the myenteric plexus directly innervate the submucosa and mucosa and therefore may influence electrolyte transport. The submucous plexus located between the submucosa and mucosa, is responsible for the majority of neuronal regulation

Figure 6. Schematic diagram of the layers which make up the gut wall. As labelled, the mucosa is made up of numerous villi which project into the lumen of the gut. The underlying section is composed of several neural and muscle layers which compose the enteric nervous system and the contractile elements of the intestinal wall, respectively. *From* Nichols *et al.* 1993.


base of crypts
muscularis
mucosae
submucous
plexus
mucosa
vascular plexus
circular muscle
Henle's plexus
longitudinal muscle
myenteric plexus

of electrolyte transport. In addition, the submucous plexus also supplies neuronal connections to the myenteric plexus but is thought to play only a minor role in enteric motility.

The autonomic and enteric nervous systems coordinate the regulation of smooth muscle contraction/relaxation, mucosal blood flow and epithelial ion and water transport. Most command signals from the CNS to the gut interface with the ENS first, although there is some direct innervation of the epithelial layer by the sympathetic nervous system. Sympathetics can inhibit secretion by presynaptic inhibition of fast (nicotinic) and postsynaptic inhibition of slow (non-cholinergic) neuronal excitation of enteric neurons (Fig.7)(Cooke, 1994).

Extrinsic reflexes within the prevertebral ganglia, spinal cord, and brain and intrinsic reflexes within the enteric nervous system play a major role in the regulation of intestinal function. Classical reflexes require a sensory afferent neuron, an interneuron and a motor neuron for transmission to effector tissues (Fig.8a)(Cooke, 1994). Axonal reflexes occur when sensory neurons are activated and cause a spread of action potential in collaterals, releasing neurotransmitters through varicosities which lie in the vicinity of mucosal effector cells (Fig.8b)(Cooke, 1994).

Neurogenic tone of active ion transport in *in vitro* preparations of the gut suggest that reflexes within the gut wall alone can control ion transport. An important way in which the ENS regulates intestinal absorption and secretion is via input from sensory afferent neurons. Sensory inputs result in a reflex cascade which is likely coordinated by both the myenteric and submucous plexuses. Substance P (SP), vasoactive intestinal peptide (VIP), and ACh are the primary neurotransmitters responsible for reflex control of the intestinal epithelium.

In response to environmental cues within the gut lumen extrinsic and intrinsic sensory

Figure 7. A common inhibitory pathway of the central nervous system on the enteric nervous system. The release of norepinephrine (NE) from sympathetic fibres act to inhibit the release of acetylcholine (ACh) at nicotinic synapses as well as inhibit post-synaptic activation of non-cholinergic effector neurons. *From* Cooke 1994.

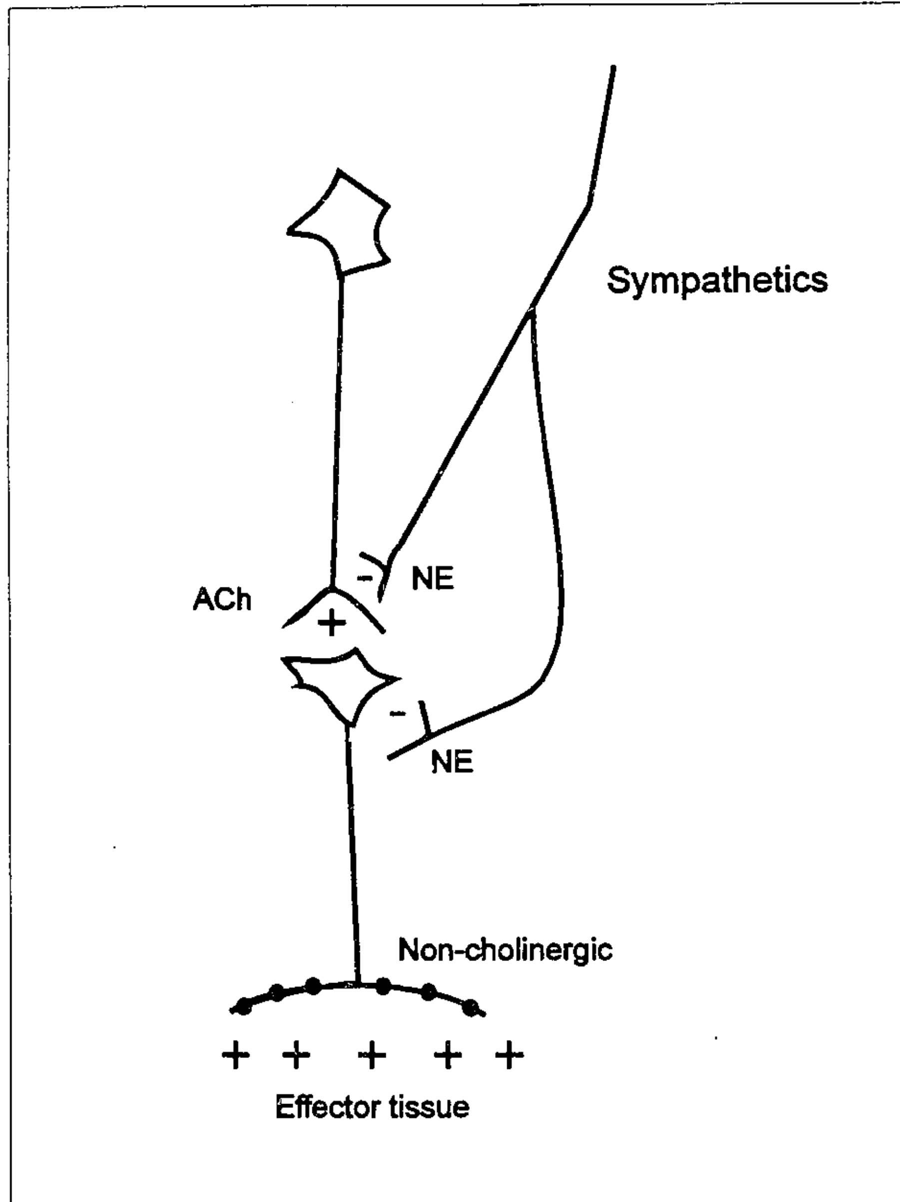
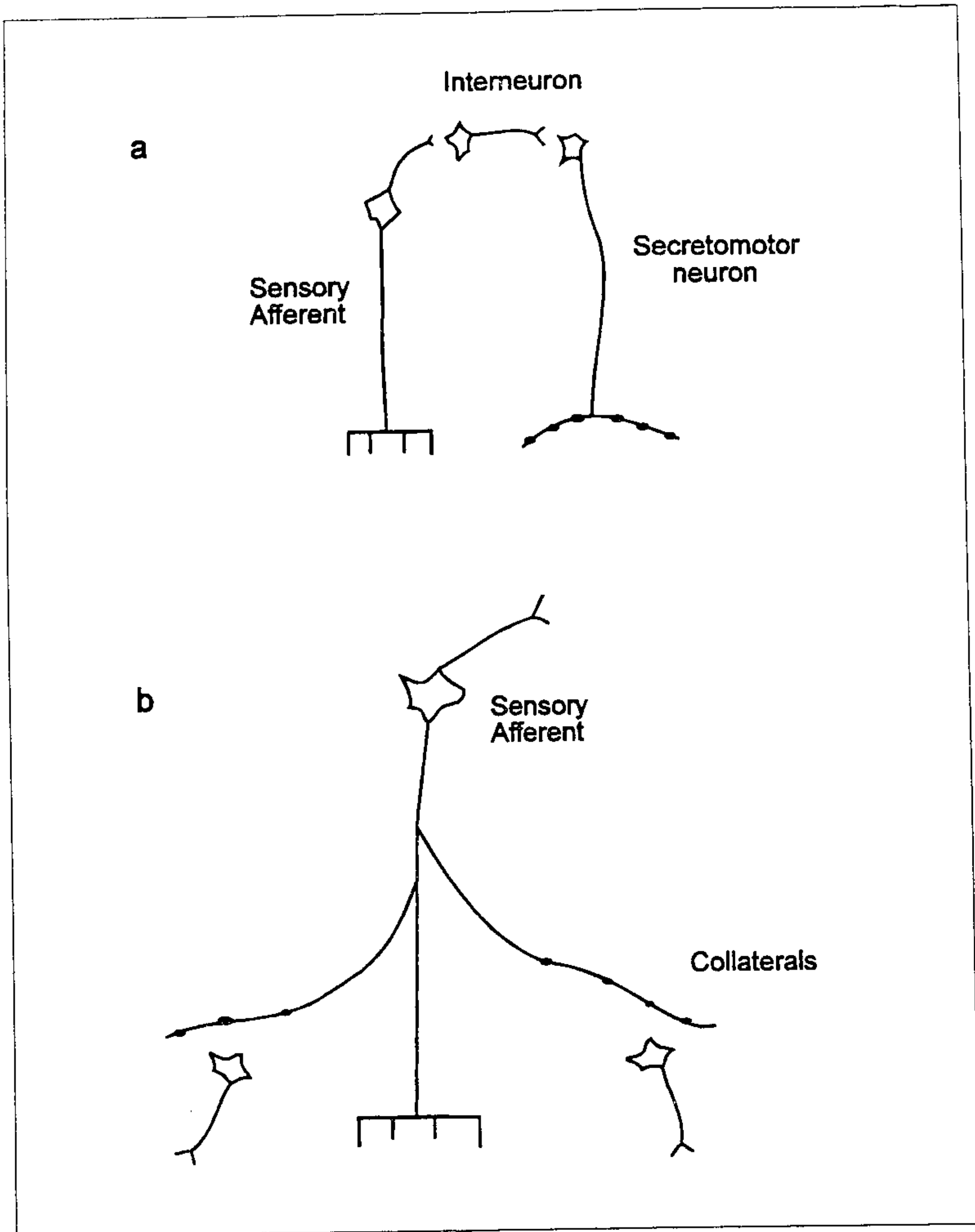

Sympathetics
ACh
+
-
NE
-
NE
Non-cholinergic
+ + + + +
Effector tissue

Figure 8. Schematic diagram illustrating classic (a) and axonal (b) neural reflexes. Sensory afferent neurons play an important role in the regulation of electrolyte transport in the gut via activation of interneurons and the subsequent activation of secretomotor neurons (a). Furthermore, the release of neurotransmitters from the collaterals of activated sensory afferent neurons can also affect electrolyte transport via stimulation of cells in the subepithelium, including neurons, and by direct stimulation of the epithelium (b). *From* Cooke 1994.


a
Interneuron
Sensory
Afferent
Secretomotor
neuron
b
Sensory
Afferent
Collaterals

afferent nerve fibres generate action potentials. Substance P containing neurons are a primary candidate in this function and act by stimulating an increase in $[Ca^{2+}]_i$ in enterocytes (Binder, 1989). Both SP and VIP neuronal activity have been identified in the villus, submucous and myenteric plexuses of the guinea pig ileum (MacNaughton *et al.*, 1994). Moreover, MacNaughton *et al.* (1994) demonstrated that stimulation of intestinal secretion by capsaicin in the guinea pig ileum is dependent on the release of SP from extrinsic sensory afferent neurons and the subsequent activation of cholinergic secretomotor neurons.

It is likely that the same afferent neuron pathways which stimulate smooth muscle contraction also stimulate epithelial secretion since both of these effects are coordinated in the response to mucosal stroking (Cooke, 1994). In addition, enteroendocrine and sensitized immune cells may also act in the detection of environmental changes and release paracrine mediators such as 5-hydroxytryptamine (5-HT) and histamine which can stimulate sensory afferent neurons. 5-HT can activate substance P neurons, via 5-HT_{1P} receptors, which impinge upon cholinergic interneurons subsequently activating VIPergic and/or cholinergic effector neurons stimulating a secretory response in crypt enterocytes. Another neurotransmitter which colocalizes with substance P in sensory afferents is calcitonin gene-related peptide (CGRP). CGRP is thought to stimulate Cl^- secretion via direct activation of epithelial cells however its effect on neural regulation of intestinal electrolyte transport is unclear (Cooke, 1992).

Tamai and Gaginella (1993) identified NO as being prosecretory in the rat colon, its effect primarily due to neuronal pathways as it was blocked by the neurotoxin, tetrodotoxin (TTX). Their studies suggested that the increase in Cl^- secretion stimulated by NO may be via VIPergic and/or cholinergic stimulation. Alternatively, NO has been identified as a proabsorptive

neurotransmitter released from non-cholinergic non-adrenergic (NANC) neurons in the mouse ileum (Rao *et al.*, 1994). Furthermore, other studies have shown that while NO can relax ileal smooth muscle stimulated by electrical transmural stimulation, NO has no effect whatsoever on neurally stimulated electrolyte transport in the same preparation (Li *et al.*, 1994).

Clearly, more work is required to fully understand the role of NO in the control of electrolyte transport. It is likely that in addition to neuronal mechanisms, NO modulates electrolyte transport at other levels i.e. epithelial and mast cells.

b. Effects of neurotransmitters on epithelial function

Neurotransmitters released in the mucosa or submucosa may modulate electrolyte transport either directly via activation of receptors on the enterocyte or indirectly via stimulation of cells in the mucosa. The release of ACh from parasympathetics, VIP, 5-HT (serotonin) and substance P from NANC neurons is known to stimulate secretion or inhibit absorption. The secretory effects of cholinergic receptor activation may in part be mediated by NO since pretreatment of rats and mice with the NOS inhibitor, L-NAME, inhibits cholinergically-mediated diarrhea in these animals (Izzo *et al.*, 1994). However the mechanism responsible for this effect was not elucidated.

NO may also mediate the effects of VIP. VIP-specific receptors have been identified on smooth muscle cells of the rabbit stomach and the G protein-coupled response of these receptors results in a Ca^{2+} influx and activation of Ca^{2+}-dependent NOS. The resulting rise NO production stimulated GC activity. It is possible that such a pathway is present in the intestinal epithelium.

Serotonin, is a known secretagogue in the gut and 5-HT neurons have been implicated as

mediators of cholinergic stimulation of smooth muscle relaxation in the guinea pig ileum via release of NO (Kanada *et al.*, 1993). A similar pathway may be present in the submucosa and responsible for electrolyte and water secretion in the gut. Siriwardena *et al.* identified 5-HT_2 receptors to be located predominantly on crypt cells of the guinea pig small bowel (Siriwardena *et al.*, 1993) whereas 5-HT_3 receptors are likely located on the glia of enteric NANC neurons (Siriwardena *et al.*, 1991). 5-HT activation of 5-HT_3 receptors appears to be responsible for part of the secretory effect of NO in the guinea pig ileum (MacNaughton, 1993). A number of studies demonstrated that 5-HT can stimulate electrolyte secretion by activating both 5-HT_3 and 5-HT_2 receptors in the gut (Beubler and Horina, 1990; Siriwardena *et al.*, 1991). The secretory effect of 5-HT appears to mediated by PGE_2 (Beubler and Horina, 1990) and possibly IP_3 (Siriwardena *et al.*, 1993) without altering cAMP (Beubler and Horina, 1990). However, both PGE_2 and IP_3 stimulate an increase in $[Ca^{2+}]_i$ leading to an inhibition of Na^+ and Cl^- absorption and increased Cl^- secretion. Increased enterocyte $[Ca^{2+}]_i$ has been identified as a stimulator of cNOS activity and NO production resulting in GC activation and Cl^- secretion (MacNaughton, 1993). Furthermore, earlier studies by Donowitz *et al.* (1980) stressed the importance of extracellular Ca^{2+} in 5-HT-induced secretory response in the rabbit ileum.

Other neurotransmitters, such as neuropeptide Y (NPY), the opiates, and norepinephrine from sympathetic nerves, are known to stimulate absorption or inhibit secretion. Norepinephrine has been identified as being able to stimulate absorption and inhibit secretion induced by electrical transmural stimulation in the porcine jejunum (Hildebrand and Brown, 1992). At low concentrations (1μM) the effect of norepinephrine was abolished by TTX and was, therefore, neuronal. However, at higher concentrations (10μM) norepinephrine proabsorptive effects were

identified at both the neuronal and the enterocyte level. Further study revealed that the proabsorptive actions of norepinephrine were mediated by the binding of norepinephrine to α_2-adrenoceptors of submucosal neurons and to the α_1-adrenoceptors on jejunal epithelial cells. These studies suggest that at higher concentrations norepinephrine may act directly on the enterocyte in addition to its neuronal mechanism. Interestingly, in the hypothalamus, norepinephrine activated NOS and NO production which subsequently activated COX (Rettori *et al.*, 1992). Whether or not such a scenario is present in the gut has yet to be determined.

2. Mucosal immune system

The lamina propria, lying between the muscularis mucosae and the epithelial cell layer of the intestine, contains a large resident population of mast cells, macrophages and polymorphonuclear granulocytes (PMN). In addition, intraepithelial leukocytes such as lymphocytes and neutrophils sit between enterocytes. These cells secrete a wide variety of secretagogues and inflammatory mediators including products of arachidonic acid metabolism, biogenic amines, cytokines, enzymes, inhibitors of various enzymes, and reactive oxygen species and NO. The majority of the effects by these secretagogues are mediated by prostaglandin E_2 and/or stimulation of second messenger pathways such as the cAMP-mediated Ca^{2+}-dependent pathway, in enterocytes. The vast majority of these primary messengers stimulate an increase in Cl^- secretion and inhibit Na^+ absorption.

A neuro-immune link has also been identified between the ENS and gut-associated lymphoid tissue, where axonal fibres containing ACh, norepinephrine, CGRP and SP all have synaptic terminals in the lymphoid fields of the lamina propria. This evidence indicates the

potential importance of enteric neurons in the regulation of the inflammatory response. The localization of neuropeptide receptors for VIP and SP on monocytes and T lymphocytes further supports the role of neuro-immunologic mechanisms which may alter electrolyte transport during the inflammatory response (Felten *et al.*, 1985). Finally, the appearance of an intimate anatomic relationship between SP/CGRP neuronal fibres and mast cells in the lamina propria establishes a definite tie between neuronal sensory inputs and mucosal protection and regulation of intestinal electrolyte transport.

MacQueen *et al.* (1989) identified the role of the CNS in regulating immune cell activation using a model of Pavlovian conditioning. After conditioning, mucosal mast cell activation was comparable in rats which had been exposed to both the audiovisual cue and the antigen and in rats which had been exposed to the audiovisual cue alone. This suggests a role for stress-induced activation of gut immune cells and further implicates the nervous system in the regulation of immune cells in the gut.

Before the upregulation of antibody production caused by antigen exposure, circulating antibodies bind to Fc receptors on mast cells and basophils. Antibodies also bind to lymphocytes, macrophages and eosinophils but with a lower affinity (Cooke, 1994). These primed immune cells can be activated and mast cells degranulated upon binding of the antigen, releasing a multitude of inflammatory mediators which stimulate a copious secretory response. For this reason mucosal immune cells, primarily mast cells, are excellent sentries and can act as sensory transducers.

Mast cells degranulate not only in response to antigen but also in response to substance P, releasing an armament of secretagogues which include histamine, 5-HT, PG, LT, PAF, NO,

and adenosine the effects of which are listed in Appendix A. Alternatively, indirect evidence suggests that NO can inhibit mast cell degranulation (Salvemini *et al.*, 1991).

A distinct population of mast cells which lie in the sympathetic ganglia degranulate in response to antigen but do not respond to SP or neural stimulation. Long lasting sympathetic potentiation stimulated in prevertebral ganglia may act as a brake on enteric neural circuits to avoid excessive loss of fluids. Therefore, this specific population of mast cells may play a vital role in the modulation of input/output characteristics of autonomic ganglia and have prolonged influences on sympathetic innervated tissues (Cooke, 1994).

Nitric oxide has been identified as an inhibitor of mast cell degranulation and an inhibitory neurotransmitter albeit the exact role of NO in the defensive mechanisms of the gut is unclear. Nevertheless, the role of NO as an irreplaceable constituent of the arsenal of host defense mechanisms was made evident by Gianotti *et al.* (1993). These researchers demonstrated that a 2% arginine supplemented diet improved survival of acutely injured mice by greater than 50%. Furthermore, arginine improved the immune competency of the mice and increased bacterial clearance of gut derived sepsis (Gianotti *et al.*, 1993).

Thus, the regulation of electrolyte and water transport in the mammalian intestine is regulated primarily by neural inputs from both the CNS and the ENS which impinge upon a number of cell types in the intestinal mucosa. These neural inputs can act directly on epithelial cells to influence transport or they may act via stimulation of immune and other cell types. Mast cells in particular play a major role in the modulation of electrolyte and water transport through direct stimulation of epithelial cells as well as neural inputs.

E. Intestinal inflammation

As NO plays a potentially important role in the modulation of intestinal electrolyte transport and since NO synthesis may be altered under pathophysiological conditions a brief discussion of intestinal inflammation is warranted. The inflammatory response is a very specific and organized response to antigenic substances. The initiation of an inflammatory response is usually due to damage of one of the body's physical barriers. The skin and intestinal epithelium are part of the primary defense mechanism with which organisms are born, and which protects them from infiltration or damage by foreign bodies. The inflammatory response itself is the most important primary defense mechanisms and its role in defending the animals systems from damage is vital.

The initial response to tissue damage is a transient vasoconstriction followed by vasodilation and hyperaemia. The efflux of cytosolic agents such as histamine and kinins from mast cells which stimulates the release of cytokines from immune cells causes secretion in the gut. The increased blood flow to the area of injury increases the capillary pressure which surpasses the colloid osmotic pressure. This results in plasma exudation into the interstitium causing tissue edema. Tissue edema in the gut can cause a breakdown of intestinal barrier function and increase the potential for bacterial infection. Cytosolic exudates released by damaged cells enhance vascular permeability in the inflamed region and further increase plasma entry to the interstitium by allowing plasma proteins to exit the vasculature to the interstitium.

Chemotactic factors released by the damaged cells initiate diapedesis of the neutrophils from the capillary bed, a process which is facilitated by the increased vascular permeability. The neutrophils which make up approximately 60% of white blood cell count, are the primary

granulocytes responsible for acute inflammation and act to phagocytize and destroy entering foreign bodies, damaged cells and debris. Neutrophils release a number of enzymes, including lysozymes and hydrolazes, in addition to being potent producers of superoxide. Superoxide stimulates mast cell degranulation and release of prostaglandins, leukotrienes, histamine, 5-HT and platelet activating factor (Salvemini *et al.*, 1991).

Exposure of the intestine to ionizing radiation for treatment of abdominopelvic malignancies can result in an acute inflammation of the gut (Cole *et al.*, 1993; Earnest and Trier, 1989). Acute radiation enteritis is characterized by hyperaemia, edema and extensive inflammatory cell infiltration of the mucosa. Abscesses involving epithelial cells, eosinophils and other acute inflammatory cells occur in the crypt region of the epithelium. Superficial ulceration may occur depending on the dose and dose rate. This type of injury is also characterized by subepithelial edema although the villi remain intact. Neutrophil influx and vacuole formation in the crypt regions of the intestinal mucosa are characteristic of an acute inflammatory response in the gut (Earnest and Trier, 1989).

The specific permeability of each region of the gut is vital to the maintenance of normal physiological function. Regulation of the permeability of tight junctions and therefore paracellular transport plays an important role in the regulation of electro-osmotic homeostasis in the gastrointestinal system. Changes in cytoskeletal structure during intestinal disease or inflammation may alter tight junctions and contribute to the increase in conductance seen in the inflamed intestine (Balda *et al.*, 1992; Ma *et al.*, 1993). Increased vascular permeability, as in the inflammatory state, can enhance epithelial permeability due to increased interstitial pressure resulting from plasma protein leakage. Several models of gut inflammation have identified

increases in both intestinal epithelial and vascular permeability after exposure to bacterial endotoxin, PAF or chemotactic peptides such as *N*-formyl methionyl-leucyl-phenylalanine (FMLP) (Boughton-Smith *et al.*, 1993; Filep and Földes-Filep, 1993; von Ritter *et al.*, 1988). von Ritter *et al.* (1988) suggested that neutrophil activation in response to FMLP is responsible for increased ileal permeability in the rat as depletion of circulating neutrophil counts to <10% with antineutrophil serum returned epithelial permeability to control levels.

NO has been implicated as an important mediator in the alteration of both epithelial and endothelial permeability (Kubes, 1992; Kanwar *et al.*, 1994; Boughton-Smith *et al.*, 1993; Filep and Földes-Filep, 1993). However, the role of NO in the regulation of vascular and epithelial integrity of the intestinal mucosa is paradoxical. In endotoxin-induced gut inflammation, NO synthase (NOS) activity was significantly increased in both jejunum and colon concomitant with plasma albumin extravasation (Boughton-Smith *et al.*, 1993). Treatment with a NOS inhibitor, L-NMA, significantly reduced NOS activity and plasma leakage suggesting that NO is detrimental to gut function. In contrast, PAF-induced plasma albumin extravasation was potentiated by NOS inhibition, using L-NAME, suggesting NO synthesis is crucial to the maintenance of vascular integrity (Filep and Földes-Filep, 1993). In addition, the role of NO as a potential mediator of epithelial integrity was confirmed by Kanwar *et al.* (1994) who demonstrated that NO secured mucosal integrity by inhibiting mast cell degranulation in the rat jejunum. In their study, treatment with mast cell stabilizers reversed the L-NAME induced increase in epithelial permeability suggesting the importance of endogenous NO as a mast cell stabilizer. The effects of the L-NAME-induced mast cell degranulation were mediated by PAF, histamine and superoxide.

In contrast to the study by von Ritter *et al.* (1988), Kanwar *et al.* (1994) determined that neutrophils had very little to do with the regulation of epithelial permeability, as treatment with antineutrophil serum did not significantly diminish the increase in epithelial permeability. Furthermore, Kubes (1992) also showed that inhibition of leukocyte adherence with the monoclonal antibody IB_4 had no effect on L-NAME-induced increases in epithelial permeability.

The disparity between the findings of Kubes group (Kubes, 1992; Kanwar *et al.*, 1994) and those of Boughton-Smith *et al.* (1993) and von Ritter *et al.* (1988) with respect to the role of NO in both vascular and epithelial permeability, respectively, is likely due to the specific isoform of NOS involved. The studies by Kubes group were under physiological conditions when constitutive NOS produces physiological levels of NO, whereas, Boughton-Smith's work used a pathologic model when the inducible form of NOS was activated, synthesizing cytotoxic amounts of NO. Similarly, von Ritter also employed a pathologic model, which may suggest that it was not the presence of neutrophils which increased epithelial permeability in their study, rather, it was the increased production of NO by neutrophils induced by FMLP which increased epithelial permeability. This hypothesis is further supported by Higa *et al.* (1993) who reported that neutrophils were involved in alterations in acute colonic epithelial injury induced by trinitrobenzene sulphonic acid. However, treatment with the prostaglandin analogue, misoprostol, attenuated the increase in colonic permeability even though neutrophil infiltration was still evident. As PGE_2 prevents the induction of iNOS (Tetsuka *et al.*, 1994), the efficacy of misoprostol in attenuating the increase in colonic epithelial permeability may be due to its inhibitory effect on the induction of iNOS in rat neutrophils. On the contrary, Gaillard *et al.* (1992) suggest that PGE_2 significantly stimulates NO production in rat liver macrophages.

The marked difference between NO production in physiological and pathological conditions were exemplified by the work of Laszlo, Whittle and Moncada (1994) who noted time-dependent differences of the effects of L-NAME treatment. When L-NAME was administered (i.v.) concomitant with LPS, therefore reducing cNOS activity, LPS-induced vascular damage was increased. However, if L-NAME was administered only 3 h after LPS, the known time point for induction of iNOS, L-NAME significantly decreased albumin extravasation (Laszlo *et al.*, 1994). Laszlo and Whittle (1994) went on further to elucidate that NO, under physiological conditions was a major contributor in opposing the vasoconstrictor effect of arginine-vasopressin. Therefore, NO was determined to be integral to the maintenance of intestinal vascular and mucosal integrity (László and Whittle, 1994). These studies stress the importance of identifying the enzymatic source of NO and being able to target a specific isoform with NOS inhibitors.

In support of a role for NO in inflammatory disorders of the bowel, elevated levels of neuronal NOS activity and luminal nitrite have been identified in patients with ulcerative colitis and Crohn's disease (Boughton-Smith *et al.*, 1993; Roediger *et al.*, 1986). Furthermore, clinical findings suggest that patients with inflammatory disorders of the bowel are characterized by increased plasma protein and IgG levels in stool samples of patients with inflammatory bowel disease (O'Mahony *et al.*, 1991). This confirms the findings of experimental models which identify increases in vascular and epithelial permeability of the inflamed intestine and provides the impetus for further study of the role of NO in this area.

F. Effects of radiation on the gut and electrolyte transport

It is well understood that radiation treatment for abdominopelvic malignancies results in

altered intestinal function, clinically characterized by diarrhea and abdominal pain. Approximately 20% of all patients undergoing abdominopelvic irradiation present with such severe diarrhea that the planned course of treatment must be interrupted and may reduce the chance of cure. Common alterations in intestinal function include, decreased bile acid and vitamin B_{12} absorption, increased fat excretion and more rapid small and whole intestinal transit time (Yeoh *et al.*, 1993). The pathology of radiation enteritis at the acute stage is characterized by decreased crypt cell proliferation resulting in decreased villus height and thinning of the mucosal layer. In addition, hyperaemia, edema, extensive infiltration of neutrophils and occasionally epithelial cell sloughing are noted shortly after radiation exposure (Earnest and Trier, 1989). Although radiation enteritis is characterized by severe diarrhea as is characteristic of chronic inflammatory bowel disorders, the transmural inflammation and complete denuding of the epithelial layer are all but absent in radiation enteritis. This suggests that exposure to radiation must alter some of the underlying regulatory mechanisms responsible for the maintenance of physiological gut function.

Although in the clinical setting patients usually receive multiple small doses with varied periods of recovery between exposures, a single large dose of ionizing radiation to the rat results in very similar pathophysiological effect. In 1989 Buell and Harding characterized the pathophysiological alterations in the rat intestine after acute exposure to 10 Gy γ-radiation. This study identified the induction of an acute inflammatory response characterized by extravasation of plasma into the gastrointestinal interstitium, vasodilation and increased neutrophil accumulation in the lamina propria, all of which occurred within the first 12 h post-irradiation. The infiltration of neutrophils consistently preceded the increase in vascular permeability suggesting that

may contribute to the increase in vascular permeability. The influx of neutrophils was apparent as early as 2 h post-irradiation in pericryptal regions and by 4 h post-irradiation in the villar regions. Submucosal edema was evident from as early as 8 h post-irradiation until 24 h post-irradiation, the end point of the study. All of these results were seen in the jejunum, ileum and colon of the rat. There was no evidence of epithelial denudation and the villar epithelium appeared normal. However, vacuole formation in the pericryptal region was apparent. These data suggest that this model of single dose exposure to ionizing radiation provides a simple and clinically relevant model to study radiation-induced intestinal pathophysiology.

Experimental evidence demonstrating the effects of radiation on basal electrolyte transport in the rabbit ileum indicated that the effect was dose-dependent (Gunter-Smith, 1986). Whole body exposure to 10 Gy γ-irradiation resulted in a significant increase in net basal secretion that was maximal at 24 h post-irradiation. The increase in net secretion was due to decreased Cl^- absorption and increased Cl^- secretion.

Measurement of key inflammatory mediators in intestinal tissues from rats after exposure to 10 Gy γ-irradiation revealed that prostaglandins, leukotrienes, PAF, histamine and 5-HT are altered from as early as 2 h post-irradiation up to 72 h post-irradiation (MacNaughton *et al.*, 1992; Borowska *et al.*, 1979; Harding *et al.*, 1990; Harding *et al.*, 1990). Thus, alterations in both the histology and physiology of intestinal tissue can be induced by a non-invasive non-chemical insult, such as ionizing radiation.

Research into the mediators of intestinal electrolyte transport which may be altered in response to radiation suggest that products of arachidonic acid metabolism are a primary target (Borowska *et al.*, 1979; MacNaughton *et al.*, 1992; Harding *et al.*, 1990; Harding *et al.*, 1990).

Levels of both PGE_2 and LTB_4 reportedly increase post-irradiation in the rat gut, however, LTC_4 synthesis decreases (MacNaughton *et al.*, 1992; Borowska *et al.*, 1979; Harding *et al.*, 1990). Eldor *et al.* (1987) identified the production of a trypsin-insensitive chemotactic factor, possibly LTB_4, post-irradiation in bovine aortic endothelial cells. Furthermore, Harding *et al.* (1990) demonstrated an increase in PAF in the rat jejunum, ileum and colon that was maximal within the first 24 h post-irradiation.

Studies into the cellular source of the radiation-induced alterations in mediators of intestinal inflammation and electrolyte transport have pinpointed the mast cell as a likely candidate. Early studies by Conte *et al.* (1956) demonstrated the effects of x-irradiation on the mesenteric mast cell population. Exposure of rats to 6 Gy (whole body) resulted in a significant decrease in mesenteric mast cell numbers within 2 days of exposure suggesting that radiation induced mast cell degranulation and release of inflammatory mediators (Conte *et al.*, 1956). This evidence is further supported by the findings of MacNaughton *et al.* (1994) who also reported that mast cell numbers in the lamina propria of the rat ileum were almost non-existent by 24 h post 10 Gy γ-irradiation. Mast cells are the major source of histamine in the rat and guinea pig intestine. Histamine levels in the rat jejunum, ileum and colon have been shown to be decreased as early as 2 and up to 24 h after whole body exposure to 10 Gy γ-irradiation (MacNaughton *et al.*, 1992; Harding *et al.*, 1990). Moreover, 5-HT, another secretagogue synthesized by both mast cells and enterochromaffin cells is also significantly decreased at 2 h post-irradiation in the jejunum (MacNaughton *et al.*, 1992) although increased in the colon at 6 and 24 h post-irradiation (Harding *et al.*, 1990). As previously mentioned, NO is thought to stabilize mast cells and therefore alterations in NO synthesis by γ-irradiation may play a

significant role in mediating the radiation-induced alteration in electrolyte transport. However, the effect of radiation on NO synthesis has not yet been determined.

Research in our lab (MacNaughton *et al.*, 1994; MacNaughton *et al.*, 1992) using the model of acute radiation enteritis developed by Buell and Harding (Buell and Harding, 1989) has demonstrated that aside from altering basal electrolyte transport, radiation alters stimulated electrolyte transport as well. Jejunal and ileal Isc response to PGE_2 and the ileal Isc response to electrical field stimulation (EFS) are decreased as early as 2 h post-irradiation. These findings suggest that radiation can induce functional changes, at the enterocyte level and possibly at the neuronal level, in the regulation of electrolyte transport.

The mechanism(s) by which γ-irradiation induces intestinal inflammation and changes in mediators of electrolyte transport is not clear. Buell and Harding (1989) put forward two theories which may explain radiation-induced inflammation: i) the degeneration of crypt cells followed by vacuole formation occurs as early as 2 h post-irradiation which renders the pericryptal regions vulnerable to microbial invasion, hence the reason for the influx of inflammatory cells; ii) radiation-induced endothelial cell stimulation of lipoxygenase pathway and the production of neutrophil chemotactic factors. In support of the former hypothesis, studies by Porvaznik (1979), using electron microscopy, described the disruption of intestinal epithelial tight junctions within 24 h and their subsequent repair by 9 days after exposure to 5 Gy γ-irradiation. The likelihood that radiation stimulates production of a chemotactic factor by cells of the intestine has also been confirmed and is presumably LTB_4 (Eldor *et al.*, 1987; Harding *et al.*, 1990).

The intestinal microvasculature is integral to gut function (Filep and Földes-Filep, 1993; Boughton-Smith *et al.*, 1993) and therefore is also a possible target for the effects of radiation

on intestinal electrolyte transport. Although the evidence is lacking for the acute effect of radiation on intestinal microvasculature, studies have identified chronic radiation-induced changes in the microvasculature of the intestinal mucosa (Carr *et al.*, 1984). Carr *et al.* (1984) showed that ionizing radiation altered the architecture of the intestinal microvasculature in the mucosal and submucosal layers predominantly. Villus blunting was also noted and may contribute to the decreased absorptive capacity commonly found in patients with radiation enteritis.

Finally, ionizing radiation has also been found to alter gene expression, with activation of the c-*jun* early response gene (Manome *et al.*, 1993), TNF-α (Hallahan *et al.*, 1989) and IL-1 (Woloschak *et al.*, 1990), while down-regulating expression of β-actin (Woloschak *et al.*, 1990). Although the significance of radiation-induced expression of c-*jun* is not understood it is hypothesized that since c-*jun* expression is temporally related to the appearance of internucleosomal DNA fragmentation, c-*jun* represents one of the signals responsible for internucleosomal DNA fragmentation and conceivably other aspects of apoptosis.

TNF-α and IL-1 are both known mediators of inducible NOS activation (Lowenstein *et al.*, 1993; Koide *et al.*, 1994; Welsh, 1994) and therefore the activation of these genes by γ-irradiation may be of vital importance to the alterations in intestinal electrolyte transport and the role of NO in gut inflammation including vascular and epithelial integrity. Furthermore, the ability of ionizing radiation to decrease expression of genes of structural importance, such as β-actin, may be the way in which γ-irradiation initiates cellular destruction (Morgan, 1994).

G. Summary of Rationale

Diarrhea is a common symptom of patients undergoing radiation therapy for abdominopelvic malignancies. Radiation causes an acute inflammatory response the effects of which may contribute to the altered absorptive state of the gut. However, the exact etiology of radiation-induced diarrhea is unknown.

Nitric oxide (NO) has been documented as a mediator of electrolyte transport (secretory and absorptive) and acute inflammation in the intestine, suggesting that NO may be of primary significance in radiation-induced alterations in electrolyte transport and acute inflammation. Cytokines released during the inflammatory response are known inducers of iNOS which is responsible for the synthesis of pathophysiological levels of NO. As NO is a major mediator of intestinal microvascular and epithelial integrity an increase in NOS activity may result in tissue damage and altered function.

Thus, identification of changes in both total NOS activity and the enzymatic source of NOS activity in the gut post-irradiation may be crucial to clarifying the role of NO in the gut. Furthermore, inhibition of radiation-induced NO synthesis *in vivo* may reduce or abolish the detrimental effects of radiation on gut function and morphology.

Therefore, it was hypothesised that **ionizing radiation stimulates an increase in NO synthesis in the gut by activation of iNOS, and that increased levels of NO, a known mediator of electrolyte transport, are responsible for radiation-induced alterations in electrolyte transport.**

H. Summary of Objectives

The pathology of radiation-induced changes in electrolyte transport is not completely understood. NO has been implicated as a mediator of both intestinal inflammation and electrolyte transport therefore it is conceivable that NO plays a role in the pathogenesis of these radiation-induced effects. Furthermore, LTB_4 and PGE_2 are known mediators of the inflammatory response which may be upregulated by radiation and interact with NO. For these reasons the following objectives were established. To determine the effects of:

1) ionizing radiation on NOS activity and the role of NO in electrolyte transport in the rat jejunum, ileum and colon

2) the NOS inhibitor, L-NAME, *in vivo* on NOS activity and electrolyte transport in the jejunum, ileum and colon of sham and irradiated rats

3) ionizing radiation and NO on LTB_4 and PGE_2 synthesis in the rat jejunum, ileum and colon

4) ionizing radiation and NO on subepithelial edema of the rat jejunum, ileum and colon

MATERIALS AND METHODS

A. Animals

Male Sprague-Dawley rats (Charles River, Montreal) weighing 240-310g were housed in wire-topped plastic cages and fed standard chow and water *ad libitum*. The rats were given at least 5 days to acclimatize to the housing conditions (room temperature: 22°C; photoperiod: 12 h light/ 12 h dark). All experimental procedures were carried out in accordance with the guidelines set by the Canadian Council on Animal Care.

B. Models of intestinal injury

1. Radiation model and drug treatment

The model of radiation injury used in this study was developed and described by Buell and Harding in 1989. They showed that exposure to 10 Gy γ-irradiation resulted in an acute inflammatory response characterized by extravasation of plasma into the gastrointestinal interstitium, vasodilation and increased neutrophil accumulation in the lamina propria, all of which occurred within the first 12 h post-irradiation. These effects were apparent in the jejunum, ileum and colon of the rat. Vacuole formation was apparent in the pericryptal region, however the villar epithelium appeared normal. This model of single dose exposure to ionizing radiation is comparable to the pathology of acute stage radiation enteritis which is characterized by decreased crypt cell proliferation resulting in decreased villus height and thinning of the mucosal layer. In addition, hyperaemia, oedema, extensive infiltration of neutrophils and occasionally epithelial cell sloughing are noted shortly after radiation exposure (Earnest and Trier, 1989).

Rats were injected subcutaneously with either nitro-L-arginine methyl ester (L-NAME;

100 or 300 mg/kg) or with an equal volume of the vehicle, 0.9% NaCl (saline 1 ml/kg), one hour prior to irradiation. The rats were irradiated (whole body) with 10 Gy gamma radiation using a ^{137}Cs source (Gammacell 40, Nordion, Kanata, Ont.) at a dose rate of 1.137 Gy/min. This dose has previously been found to induce an acute inflammatory response in rats (MacNaughton *et al.*, 1994). Shams were placed in the same chamber but were not exposed to the source. In further studies rats were irradiated as above and then immediately given aminoguanidine (AG; 100 mg/ml/kg i.v.), which preferentially inhibits iNOS (Joly *et al.*, 1994), by intravenous injection via the tail vein. For the injection of AG, rats were anaesthetized with halothane (4%) delivered in oxygen (1L/min). The rats tail was heated with warm water to facilitate visualizing the lateral veins of the tail. A 25G needle was used to inject AG into either the left or right tail vein. The injection of AG was complete within 12 minutes post-irradiation.

2. Endotoxin model and drug treatment

Intravenous (i.v.) administration of bacterial endotoxin to rats is known to induce an acute inflammatory response characterized by both intestinal vascular damage (Boughton-Smith *et al.*, 1993) and induction of iNOS (Tepperman *et al.*, 1993; Tepperman *et al.*, 1994). A dose of 3 mg/kg i.v. induced a significant increase in NOS activity in the jejunum and colon of the rat by 4 h after injection. The increase was primarily Ca^{2+}-independent in the jejunum, however in the colon both Ca^{2+}-dependent and Ca^{2+}-independent NOS activity were increased (Tepperman *et al.*, 1993; Boughton-Smith *et al.*, 1993; Tepperman *et al.*, 1994).

In the present study, rats were injected with *Salmonella typhimurium* lipopolysaccharide (LPS; 4 mg/kg i.v.) or with an equal volume of the vehicle, 0.9% NaCl (saline 1 ml/kg), via the

tail vein while under halothane anesthesia (as above). In other studies bacterial endotoxin was administered (as above), immediately followed by aminoguanidine (100 mg/ml/kg i.v.).

C. Experimental procedures

1a. Ussing chamber preparation

Previous studies have shown that the acute inflammatory response in the intestine of the rat occurs as early as 2 h and up to 24 h post-irradiation (Buell and Harding, 1989). For this reason these time points were chosen for this study. The effects of irradiation at 48 h were also investigated. Other studies in our lab have shown that there are no differences in either basal or the stimulated electrolyte transport response between shams studied at 2 or 24 h, therefore all sham studies were performed at 2 h.

After sham treatment or at 2, 24 or 48 h post-irradiation, animals were killed by cervical dislocation and segments (10 cm) of jejunum and ileum and colon were removed. Jejunal segments were taken 4 cm distal to the ligament of Treitz, ileal segments beginning 4 cm proximal to the ileocecal junction and colonic segments from 4 cm distal to the caecum. All tissues were placed in Krebs buffer (see reagents) at 4°C and gassed (5% CO_2, 95% O_2).

The segments of jejunum, ileum and colon were washed of feces with ice cold Krebs. Using a 1 ml glass pipette inserted into the lumen of the intestinal segment for structural support, a cut was made through the longitudinal and circular muscle layers along the mesenteric border and the muscle layers peeled away from the submucosa using fine forceps. The tissue was kept moist by application of cold Krebs. After the muscle layers were removed, the submucosal and mucosal layers were cut through along the mesenteric border, leaving a flat sheet of intestine.

The tissues were then cut into squares (~1 cm^2) and mounted between halves of standard Ussing flux chambers (Fig.9).

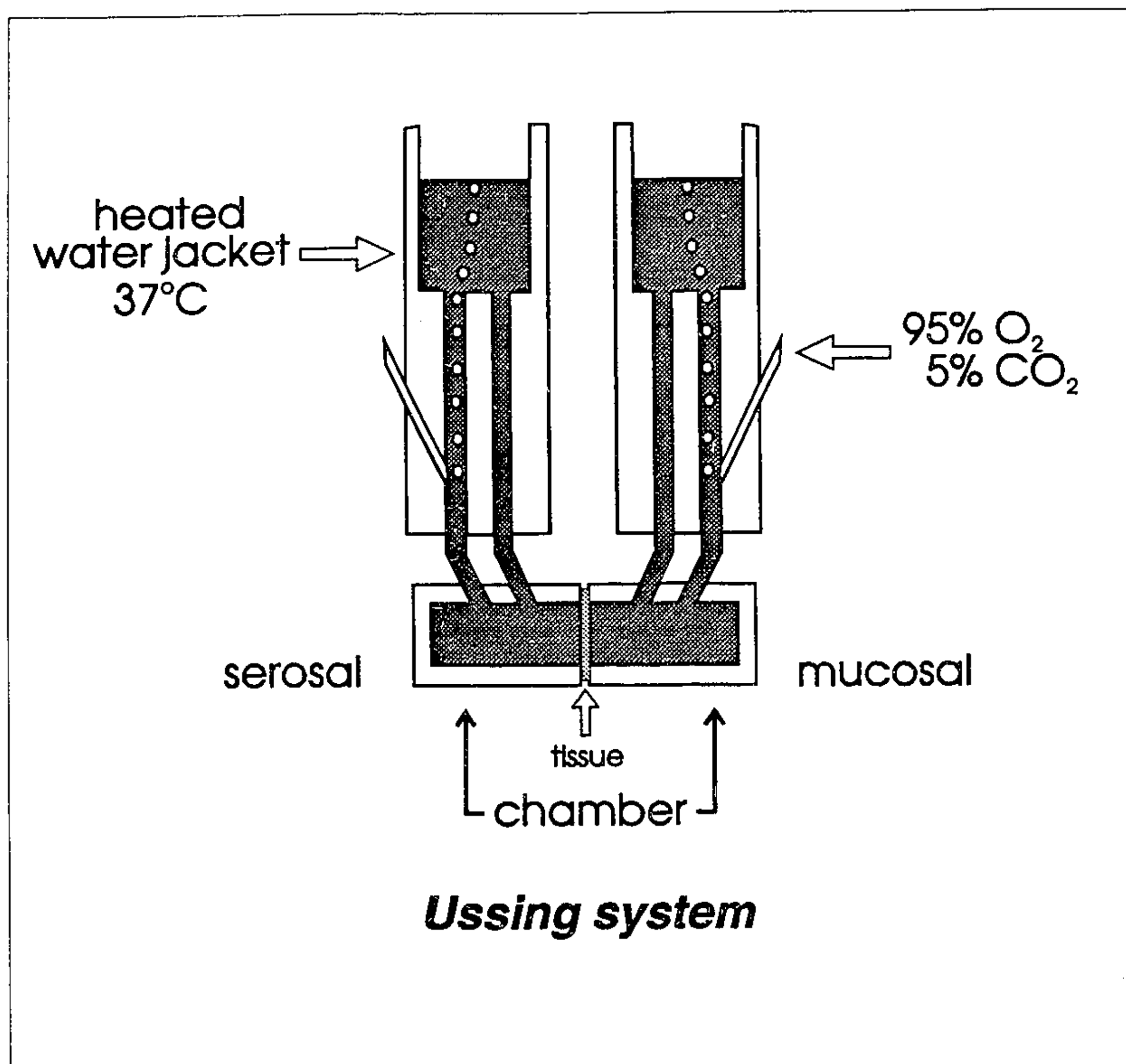
The serosal side of the tissue was bathed with Krebs buffer containing 10 mM glucose and the mucosal side of the tissue was bathed with Krebs buffer containing 10 mM mannitol (pH 7.4). The solutions were aerated with a gas lift system (5% CO_2, 95% O_2) and maintained at 37°C. The transmural potential difference was clamped to zero volts by applying a short circuit current (Isc) using a voltage clamp apparatus (DVC-1000, World Precision Instruments). Changes in Isc were monitored as the indicator of changes in net active ion transport by the tissue (Ussing, 1949; Ussing and Zerahn, 1951). Changes in Isc, in response to the serosal application of prostaglandin E_2 and electrical field stimulation at different frequencies, were recorded on a Kipp & Zonnen chart recorder (johns scientific, Canada) and expressed as $\mu A/cm^2$ tissue.

The use of these two different stimuli allowed us to determine if the alterations caused by radiation or L-NAME treatment were due to changes at the epithelial level or at the level of neural control of epithelial function.

1b. Electrical Field Stimulation

To determine whether irradiation affected neurally-evoked electrolyte transport, tissues were challenged with an electrical field stimulation (EFS) which results in the depolarization of neurons in the tissue. As the frequency of EFS is increased more neurons are recruited thus resulting in a greater, frequency-dependent response. The result of neural stimulation by this method is a composite of the release of neurotransmitters which are both stimulatory and

Figure 9. The Ussing chamber system. A heated water jacket maintains the buffer bathing the tissue at a physiological temperature. The buffers are continuously gassed with oxygen and carbon dioxide to maintain tissue oxygenation. The serosal and mucosal sides of the tissue are isolated allowing for the determination of the effects of a particular agent on the mucosal or serosal side alone. Most importantly, the separation of mucosal and serosal compartments allows us to measure changes in net active ion transport across the tissue.



Ussing system

inhibitory to absorption or secretion (Cooke, 1989). The released neurotransmitters can then activate other effector cells in the subepithelium or act directly on epithelial cells.

After mounting in the Ussing chambers, tissues were allowed an equilibration period of at least 15 minutes in order to establish a stable baseline and the conductance (G_t) was recorded. A stimulus of 100 V was applied across the tissue using a dual impedance research stimulator (Harvard). The tissue was stimulated for 3 sec with 500 μsec pulses at various frequencies (1, 2.5, 5, 10, 25 Hz) with at least 3 minutes between each frequency to re-establish baseline Isc.

1c. Response to PGE_2

Prostaglandin E_2 (PGE_2) acts directly on intestinal epithelial cells (Karayalcin *et al.*, 1990) stimulating an increase in Cl^- secretion via activation of adenylate cyclase, increasing cAMP production and/or $[Ca^{2+}]_i$ (Binder and Sandle, 1987). Therefore, PGE_2 was used to determine the direct effects of radiation or L-NAME on epithelium function.

As in the EFS experiments, after mounting in the Ussing chambers, tissues were allowed an equilibration period of at least 15 minutes in order to establish a stable baseline and the conductance (G_t) was recorded. 100 μl of PGE_2 stock solution was added to the serosal side of the tissue in the Ussing chamber in a final concentration of 0.1 μM. This concentration causes a submaximal increase in Isc (Appendix B) thus eliminating the possibility of any non-specific effects of this eicosanoid.

2. Nitric oxide synthase (NOS) assay

Nitric oxide synthase (NOS) activity was determined by measuring the conversion of ^{14}C-

L-arginine to ^{14}C-L-citrulline modified from the method of Salter and Knowles (1991) (Appendix C). The measurement of NOS activity by this method is an indicator of the total available NOS activity in the tissue but may not be a precise measurement of NO production in the tissue as conditions *in vivo* may limit NO synthesis. After sham treatment or at 0.5, 2, 6, 24 or 48 h post-irradiation animals were killed by cervical dislocation and segments (10 cm) of jejunum, ileum and colon were removed. The extra time points of 0.5 and 6 h were added to investigate any early changes in NOS activity (0.5 h) and because iNOS induction reportedly occurs between 4 and 6 h (Salter and Knowles, 1991; Boughton-Smith *et al.*, 1993). To validate the NOS assay, we employed the well established model of endotoxin-induced NOS activity. Similar to the radiation studies, animals were killed by cervical dislocation and segments (10 cm) of jejunum, ileum and colon were removed 4 hours after LPS, LPS+AG or saline i.v. injection.

As in the Ussing chamber experiments jejunal segments were taken 4 cm distal to the ligament of Treitz, ileal segments beginning 4 cm proximal to the ileocecal junction and colonic segments from 4 cm distal to the caecum. All three segments were immediately placed in Krebs buffer (Appendix C) at 4°C. The segments of jejunum, ileum and colon were washed of feces with ice cold Krebs. Segments of gut were weighed (30-90 mg), placed in Eppendorf microfuge tubes containing 250 µl of homogenization buffer (Appendix C) at 4°C and were homogenized at high speed for 20 sec using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario). The homogenates were immediately centrifuged (14000 x g) in a microfuge for 10 min at 4°C. After centrifugation, 20 µl of the supernatant was added to an Eppendorf microfuge tube containing 50 µl of prewarmed incubation buffer (Appendix C) with ^{14}C-L-arginine, vortexed and incubated at 37°C for 10 min in a metabolic shaking incubator.

To determine the profile of NOS activity (Appendix D), samples were co-incubated in incubation buffer containing the divalent cation chelator ethyleneglycol tetraacetic acid (EGTA). NOS activity in the presence of EGTA was considered to be due to the inducible form of the enzyme (iNOS) which is Ca^{2+}-independent. Constitutive NOS (cNOS) activity was determined by subtracting iNOS activity from total NOS activity (EGTA-free buffer). As an internal control an Eppendorf containing 50 µl of prewarmed incubation buffer with ^{14}C-L-arginine and 20 µl of ddH_2O was vortexed and incubated with the rest of the samples at 37°C for 10 min. The internal control was used as a means of measuring any background (BG) activity which may have been eluted.

To separate ^{14}C-L-arginine from ^{14}C-L-citrulline, 1 ml Dowex 50W-X8 ion exchange resin (Na- form, 100-200 mesh, Bio-Rad; Appendix C) columns at pH ~8.0 were prepared during the centrifugation and incubation period. After incubation, samples were loaded onto the ion exchange columns and were subsequently rinsed with 2 ml ddH_2O. Aliquots (1 ml) of the eluate were added to 14 ml liquid scintillation fluid and the samples were counted for activity (^{14}C) in a Beckman LS-9000 scintillation counter. NOS activity was calculated and expressed as nmol L-citrulline/min/g tissue (Appendix D). Determination of NOS activity per unit area was not deemed necessary as there were no major changes in tissue thickness.

3. Prostaglandin E_2 and Leukotriene B_4 Synthesis

Both PGE_2 and the chemotactic leukotriene, LTB_4, are important inflammatory mediators and previously documented studies suggest that endogenous synthesis of these mediators are altered post-irradiation (MacNaughton *et al.*, 1992; Harding *et al.*, 1990; Eldor *et al.*, 1987).

After sham treatment or at 2, 24 or 48 h post-irradiation samples (100-200 mg wet weight) of jejunum and ileum and colon were taken from rats used for the Ussing chamber and/or NOS experiments.

Samples were placed in a 1.5 ml Eppendorf microfuge tube containing 1 ml of phosphate buffer (10 mM) at pH 7.4 and room temperature. Samples were minced with fine scissors for ~30 sec and then incubated at 37°C for 20 min with moderate shaking. After incubation samples were centrifuged for ~60 sec at 4°C. The supernatants were pipetted into screw cap Eppendorf tubes, frozen in liquid nitrogen and stored at -70°C. Samples were assayed for PGE_2 and LTB_4 using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, Michigan)(Appendix C). Values for PGE_2 and LTB_4 were expressed as pg/mg tissue. Measurement of eicosanoid synthesis per unit area were deemed unnecessary as there were no major changes in tissue thickness.

4. Histology

After sham treatment or at 2, 24 or 48 h post-irradiation segments (10 cm) of jejunum, and ileum and colon were taken from rats used for the Ussing chamber and/or NOS assay. Tissues were cut into 3 cm segments and pinned out on a wax surface and fixed in Carnoy's fixative (Appendix C) for 2 h and then 60% EtOH for 10 min, cut into small squares (4 mm^2) and stored in 70% EtOH until embedding. Fixed tissues were embedded in paraffin wax, sectioned (6 μm) using a microtome (American Optical) and stained with Harris haematoxylin, phloxine B and saffran. Sections were scored (single blind) for epithelial detachment and/or subepithelial oedema. Epithelial detachment and/or subepithelial oedema was considered to be an index of damage as this alters the architecture of the villar region and may be responsible for

functional alterations of intestinal electrolyte transport. Scoring was as follows: positive villi were those which had obvious epithelial detachment and/or subepithelial oedema. There were between 30 and 70 villi per section. The percentages of positive villi per animal for each experimental group were added to obtain an average percent of positive villi per experimental group. At least one section was counted per animal and at least 4 animals per experimental group.

D. Drugs and Reagents

Glacial acetic acid, $CaCl_2$, chloroform, dithiothreitol, HEPES, KCl, KH_2PO_4, $MgCl_2$, NaCl, $NaHCO_3$, NaOH, glucose, mannitol, and sucrose were purchased from BDH, Montreal, Quebec. Ethanol was purchased from Commercial Alcohols Inc., Montreal, Quebec.

Ethylenediamine-tetraacetic acid (EDTA), ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), L-arginine, Nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), β-nicotinamide adenine dinucleotide phosphate (reduced form), *Salmonella typhimurium* lipopolysaccharide (LPS), prostaglandin E_2, soybean trypsin inhibitor, leupeptin, aprotinin and valine were purchased from Sigma, St. Louis, Missouri. ^{14}C-L-arginine was from NEN Dupont, Mississauga, Ontario. Dowex 50W-X8 resin (Na- form, 100-200 mesh) and the Poly-Prep chromatography columns were purchased from Bio-Rad Laboratories, Richmond, California.

E. Statistics

Data are expressed as the mean ± standard error. Comparison between two groups was

calculated using the Students t-test for unpaired or paired data. Multiple comparisons were conducted using a one-way analysis of variance (ANOVA) with a post hoc Neuman-Keuls multiple range test.

RESULTS

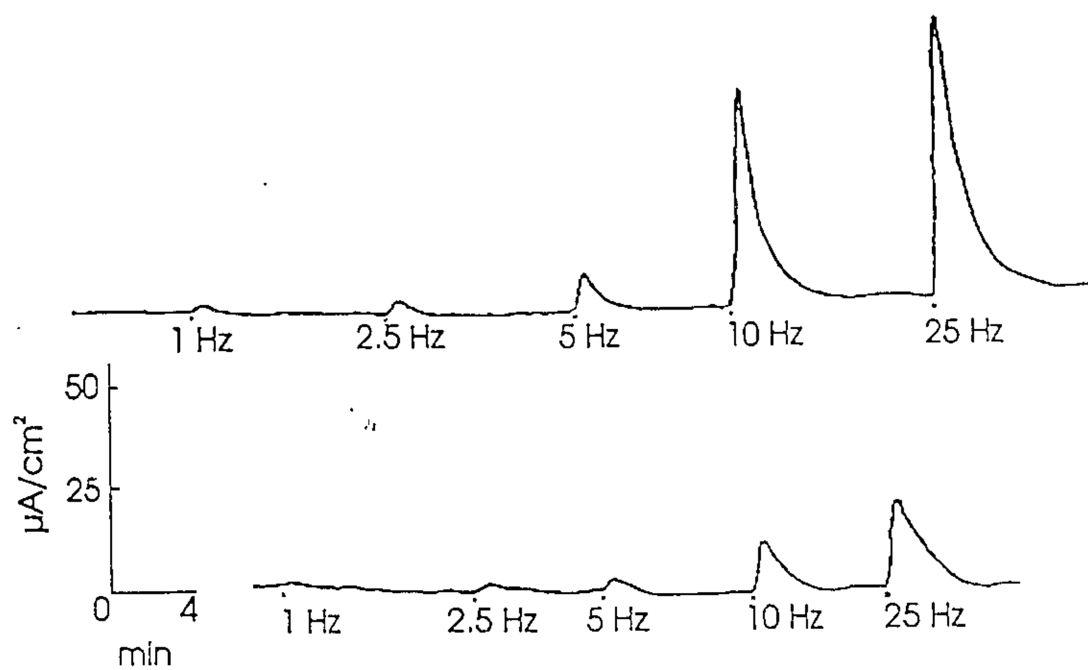
A. Intestinal electrolyte transport

Radiation is known to alter electrolyte transport and stimulate the production of inflammatory mediators in the gut which influence electrolyte transport. Radiation has also been found to have negative effects on epithelial barrier function of the gut as well as being able to induce alterations in the microvascular architecture of the intestine. For these reasons the acute effects of 10 Gy γ-irradiation on jejunal, ileal and colonic stimulated electrolyte transport were studied at 2, 24 and 48 h post-irradiation. Transmural electrical field stimulation (EFS) at various frequencies (1, 2.5, 5, 10, 25 Hz) was used to identify any radiation-induced alterations in neurally-evoked electrolyte transport, and serosal application of PGE_2 was used to study the effects of radiation at the enterocyte level.

The Isc response to EFS was frequency dependent. The response was immediate, reaching its maximal amplitude within 20 sec, returning to baseline within 2 min (Fig.10). The response to PGE_2 was slower, peaking between 1 and 2 min and lasting longer than 10 min. In the present study, radiation significantly decreased the amplitude of the responses to these stimuli at certain time points but had no effect on the curve shape of the response. Thus, all results are records of a change in the amplitude of the response only.

As NO is known to influence electrolyte transport in the gut at both the neuronal and enterocyte level, and NOS can be activated by inflammatory mediators known to be induced by irradiation, the possible role of alterations in endogenous NO production in the intestine was studied. The competitive inhibitor of NOS, L-NAME (100 mg/kg s.c.), which is presumed to act on both inducible and constitutive NOS isoforms was used to inhibit endogenous NO production.

Figure 10. A representative tracing of the Isc response to EFS in the rat intestine. The jejunal Isc response to various frequencies of EFS from a saline pretreated sham (upper) and at 48 h post-irradiation (lower). Note the obvious decrease in the responsiveness of the irradiated tissue. The red spot indicates the moment of stimulation and the scale is the same for both tracings.



1 Hz
2.5 Hz
5 Hz
10 Hz
25 Hz
50
25
µA/cm²
0
4
min
1 Hz
2.5 Hz
5 Hz
10 Hz
25 Hz

1. JEJUNUM

a. Effects of radiation

The electrolyte transport response to both EFS and PGE_2 at 2 and 24 h post-irradiation was not significantly different from shams. However, radiation significantly reduced the response to EFS of 25 Hz by 44% ($p<0.05$) and the response to PGE_2 by 81% ($p<0.05$) at 48 h post-irradiation (Fig.11 and 12).

b. Effects of L-NAME pretreatment

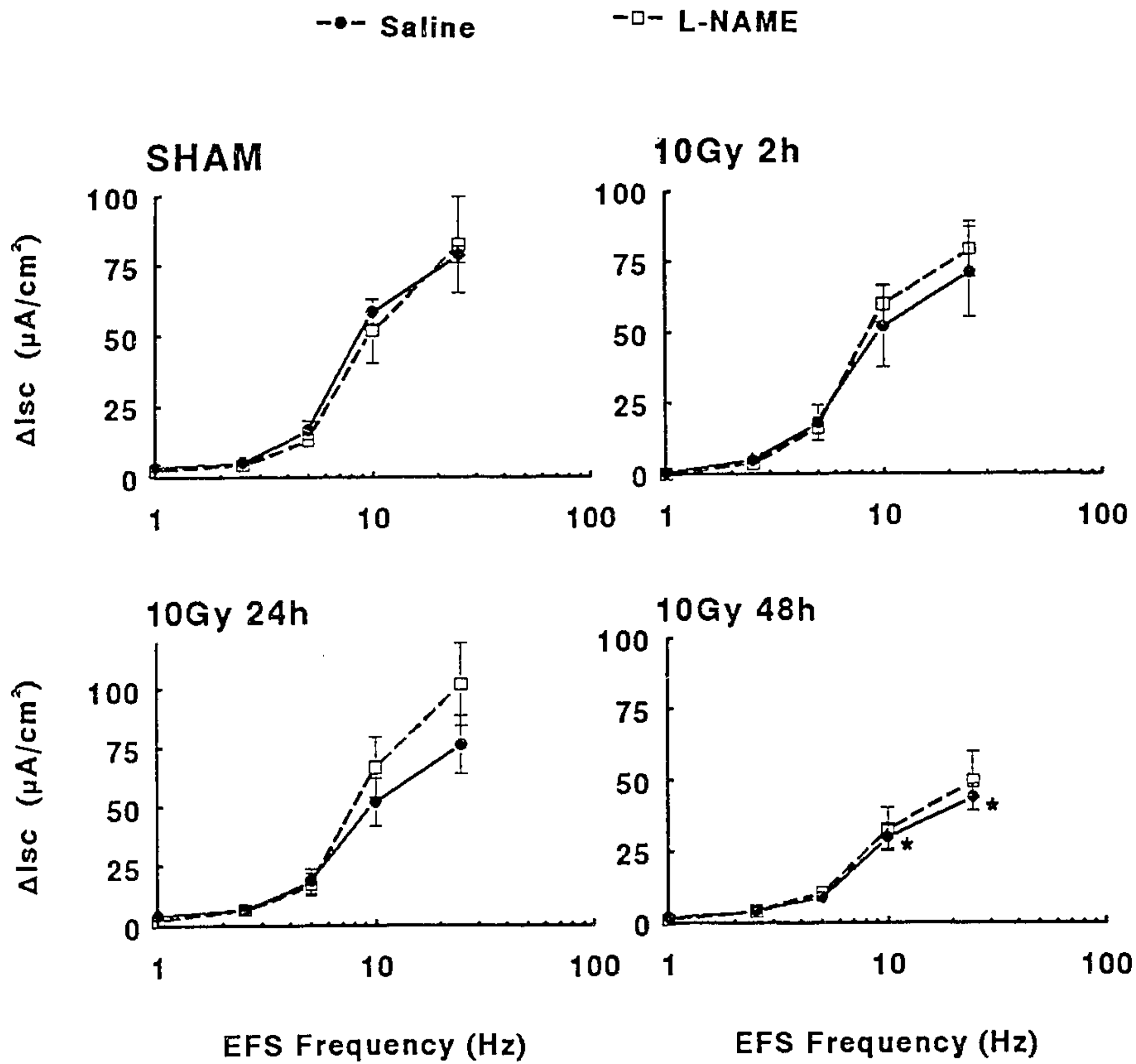
L-NAME pretreatment did not significantly alter the response to EFS or PGE_2 in tissue from shams. Furthermore, L-NAME had no significant effect on the radiation-induced alterations in response to EFS or PGE_2 (Fig.11 and 12).

2. ILEUM

a. Effects of radiation

As in the jejunum, there was no significant decrease in response to EFS at 2 or 24 h post-irradiation in the ileum. However, by 48 h post-irradiation the response to EFS of 25 Hz had decreased by 86% ($p<0.05$)(Fig.13). The change in Isc in response to PGE_2 was decreased by 54% ($p<0.05$) and 81% ($p<0.05$) at 2 and 48 h post-irradiation, respectively (Fig.14). However, at 24 h post-irradiation the response to PGE_2 was similar to that of shams (16.0 ± 3.6 $\mu A/cm^2$ in shams vs 13.1 ± 2.2 $\mu A/cm^2$ at 24 h post-irradiation)(Fig.14).

Figure 11. Isc response to EFS (1, 2.5, 5, 10, 25 Hz) in jejunal segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (circles) or L-NAME 100 mg/kg (squares). Radiation significantly depressed the response to EFS at 48 h ($^{*}p<0.05$ compared to shams). L-NAME pretreatment had no significant effect on the response EFS. Values represent the mean ±SEM for $n \geq 6$.



-•- Saline
-□- L-NAME
SHAM
10Gy 2h
10Gy 24h
10Gy 48h
ΔIsc (μA/cm²)
0
25
50
75
100
1
10
100
*
EFS Frequency (Hz)

Figure 12. Isc response to the serosal application of 0.1 μM PGE_2 in jejunal segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly depressed the response to PGE_2 at 48 h ($^*p<0.05$ compared to shams). L-NAME pretreatment had no significant effect on the response to PGE_2. Values represent the mean±SEM for $n \geq 6$.

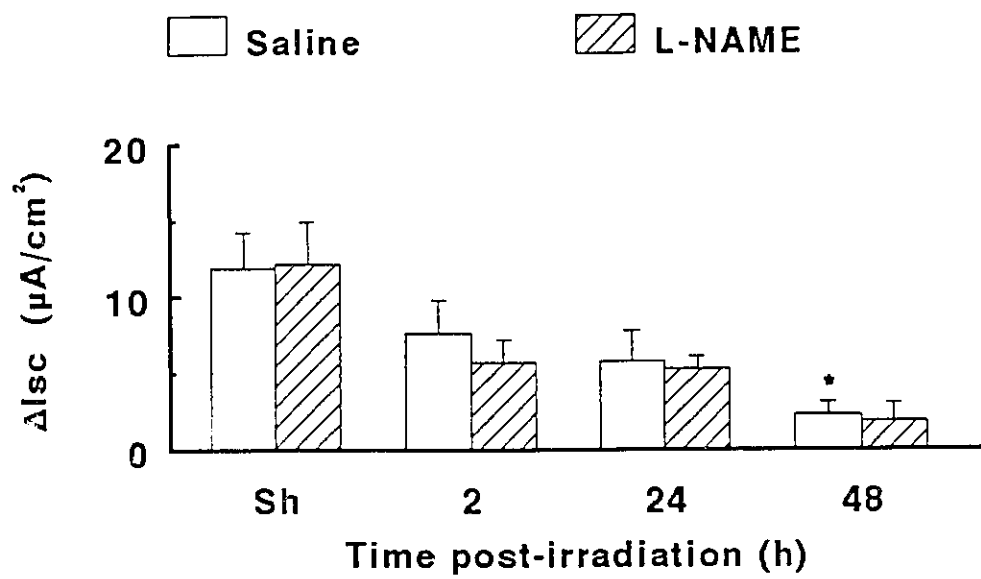
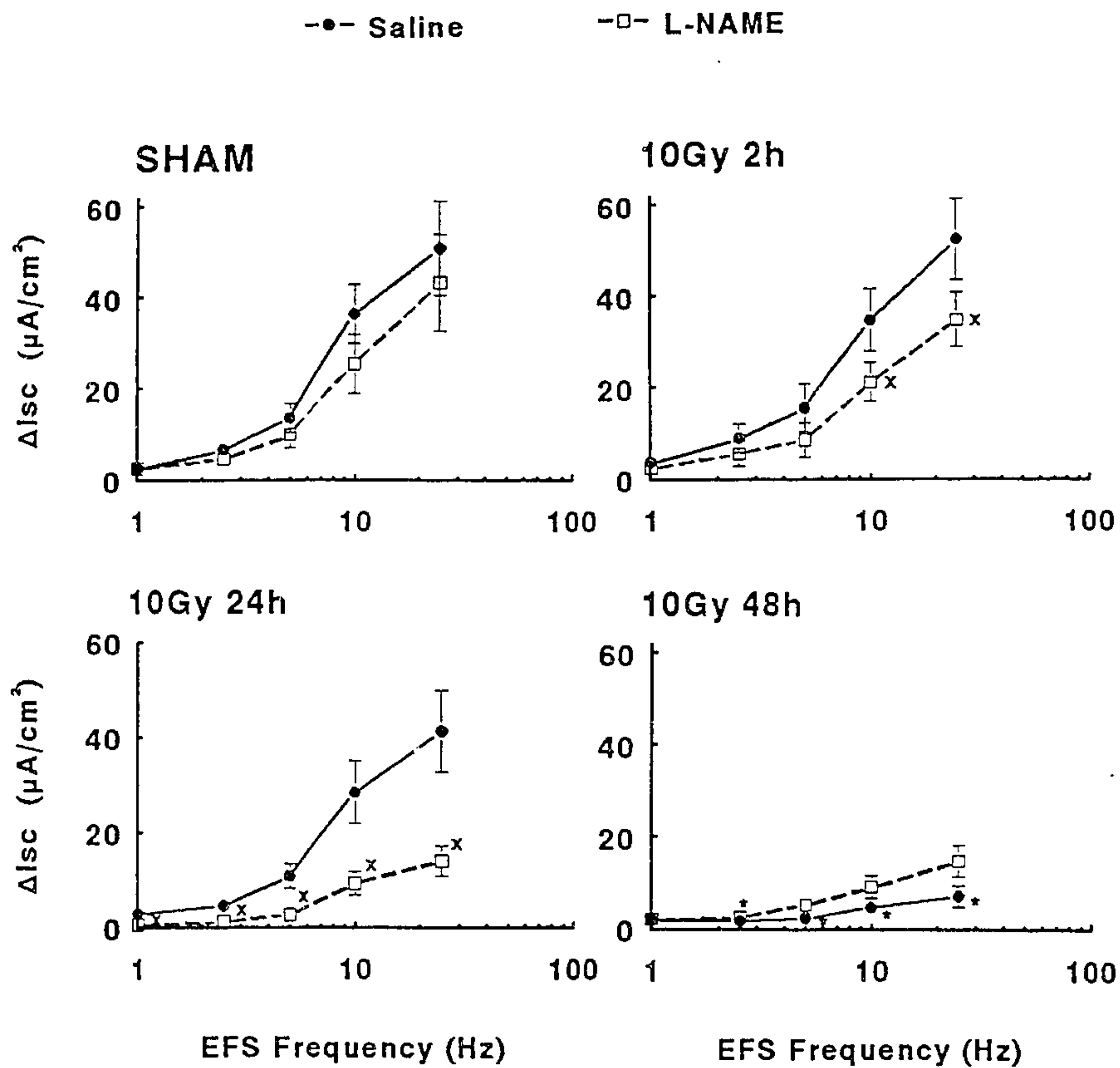
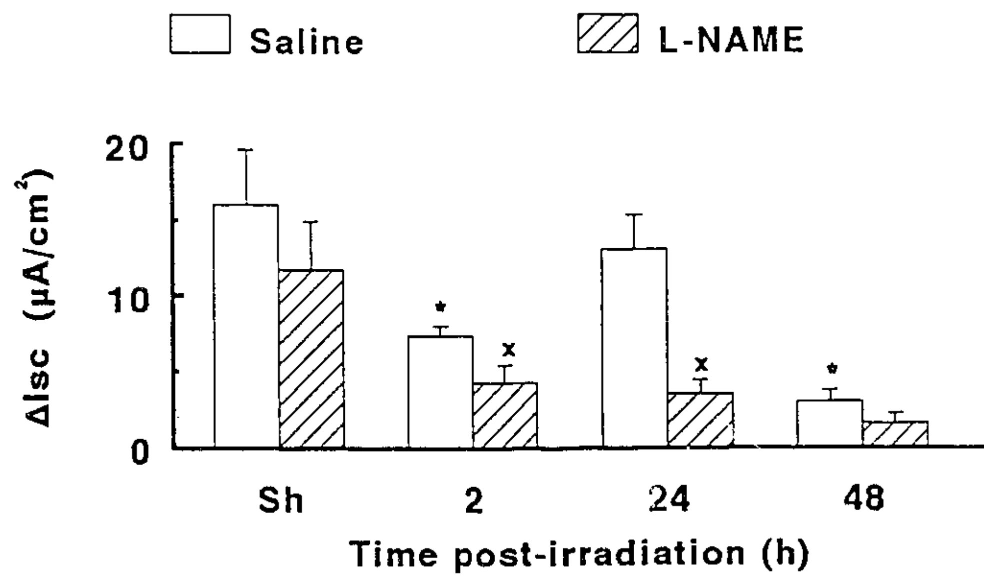

Saline
L-NAME
20
10
0
ΔIsc (μA/cm²)
*
Sh
2
24
48
Time post-irradiation (h)

Figure 13. Isc response to EFS (1, 2.5, 5, 10, 25 Hz) in ileal segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (circles) or L-NAME 100 mg/kg (squares). Radiation significantly depressed the response to EFS at 48 h ($^{*}p<0.05$ compared to shams). L-NAME pretreatment significantly decreased the response to EFS at 2 and 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated group). Values represent the mean±SEM for $n \geq 6$.



Saline
L-NAME
SHAM
10Gy 2h
10Gy 24h
10Gy 48h
ΔIsc (μA/cm²)
0
20
40
60
1
10
100
x
*
EFS Frequency (Hz)

Figure 14. Isc response to the serosal application of 0.1 μM PGE_2 in ileal segments from rats at 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly depressed the response to PGE_2 at 2 and 48 h ($^{*}p<0.05$ compared to shams). L-NAME pretreatment significantly reduced the response to PGE_2 in irradiated tissues at 2 and 24 h ($^{x}p<0.05$ compared to the saline pretreated group). Values represent the mean±SEM for $n \geq 6$.



Saline
L-NAME
20
10
0
ΔIsc (μA/cm²)
*
x
x
*
Sh
2
24
48
Time post-irradiation (h)

b. Effects of L-NAME pretreatment

L-NAME pretreatment of shams did not significantly alter the Isc response to either EFS or PGE_2 when compared to saline pretreated shams. However, in segments from irradiated rats L-NAME pretreatment significantly depressed the response. L-NAME pretreatment attenuated the response to EFS of 25 Hz by 45% ($p<0.05$) and 59% ($p<0.05$) in segments from rats at 2 and 24 h post-irradiation, respectively (Fig.13). Similarly, the response to PGE_2 was significantly decreased by L-NAME pretreatment at 2 and 24 h ($p<0.05$) post-irradiation (Fig.14). L-NAME pretreatment did not significantly change the response to EFS or PGE_2 at 48 h post-irradiation, however, these responses were already significantly depressed by greater than 80% due to the effects of irradiation alone (Fig.13 and 14).

3. COLON

a. Effects of radiation

Of the 3 tissues studied the colon appeared to be most sensitive to 10 Gy γ-irradiation as the onset of the hyporesponsiveness to EFS occurred earliest in this tissue. At 2 h after exposure to 10 Gy γ-irradiation there was no significant difference in response to EFS when compared to shams. However, by 24 h post-irradiation the response to EFS had decreased significantly. The response to EFS of 25 Hz was decreased by 42% and 57% ($p<0.05$) at 24 and 48 h post-irradiation, respectively (Fig.15). The response to the serosal application of PGE_2 was only significantly decreased at 24 h post-irradiation ($p<0.05$)(Fig.16).

Figure 15. Isc response to EFS (1, 2.5, 5, 10, 25 Hz) in colonic segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (circles) or L-NAME 100 mg/kg (squares). Radiation significantly decreased the response to EFS at 24 and 48 h ($^{*}p<0.05$ compared to shams). L-NAME pretreatment significantly decreased the response to EFS in shams and at 2 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated group) but did not further reduce the radiation-induced suppression at 24 and 48 h. Values represent the mean ±SEM for $n \geq 6$.

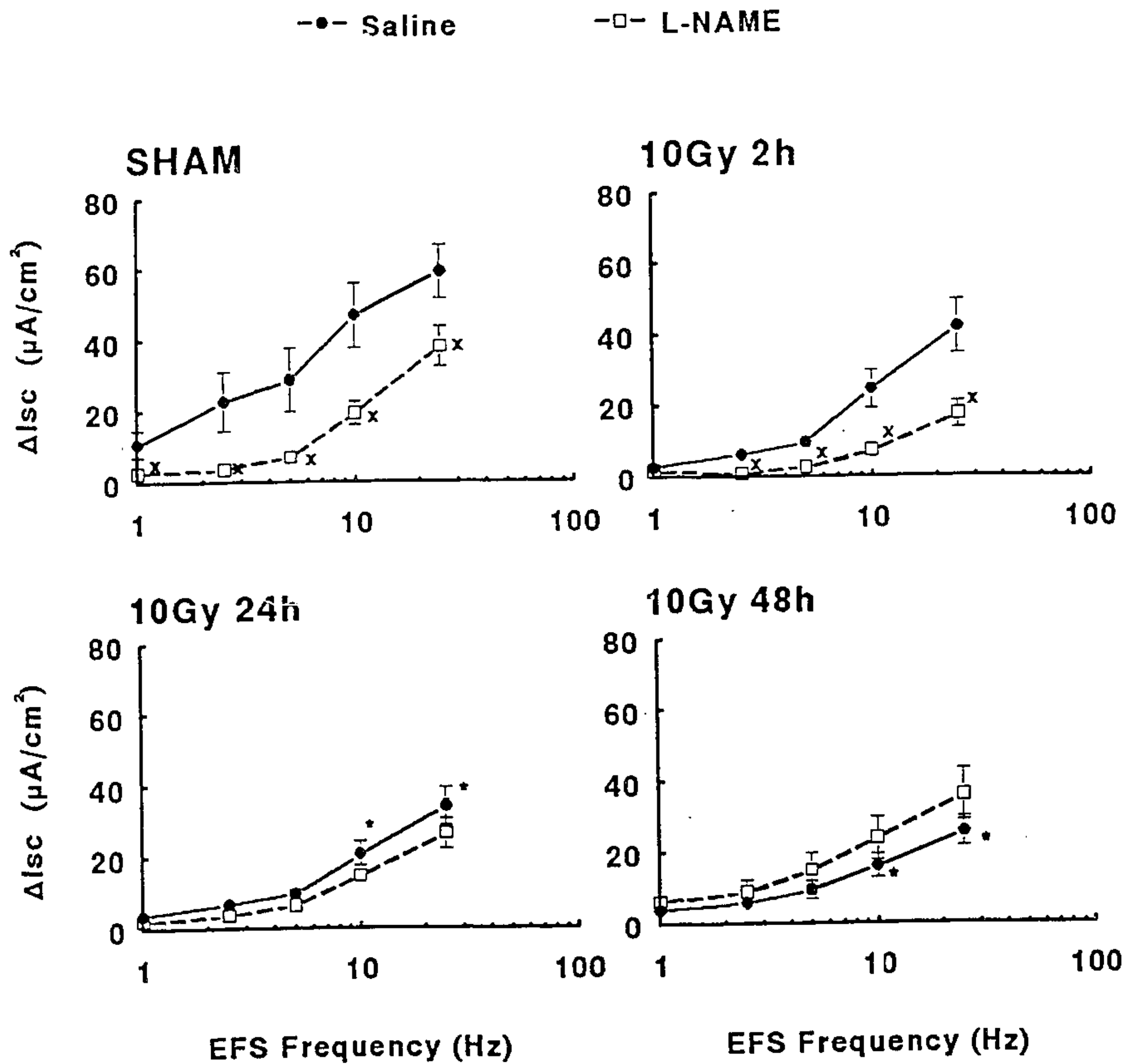
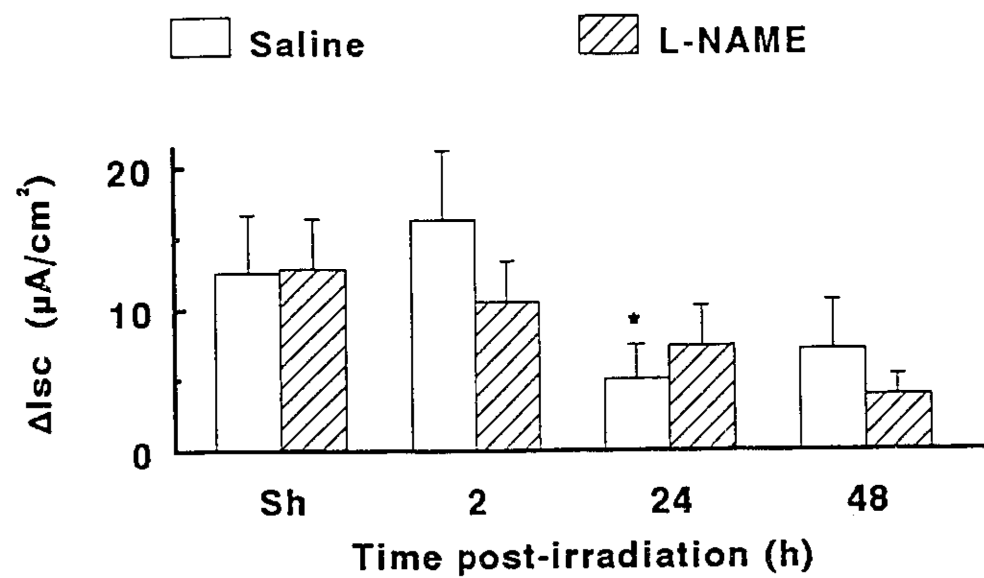

-•- Saline
-□- L-NAME
SHAM
10Gy 2h
10Gy 24h
10Gy 48h
ΔIsc (μA/cm²)
EFS Frequency (Hz)
0
20
40
60
80
1
10
100

Figure 16. Isc response to the serosal application of 0.1 µM PGE_2 in colonic segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly depressed the response to PGE_2 at 24 h (*$p<0.05$ compared to shams). L-NAME pretreatment had no significant effect on the response to PGE_2 in irradiated tissues. Values represent the mean±SEM for $n \geq 6$.


Saline
L-NAME
ΔIsc (μA/cm²)
20
10
0
*
Sh
2
24
48
Time post-irradiation (h)

b. Effects of L-NAME pretreatment

L-NAME pretreatment attenuated the response to EFS of 25 Hz in shams and at 2 h post-irradiation by 36% and 63% respectively ($p<0.05$)(Fig.15). However at 24 and 48 h post-irradiation L-NAME pretreatment had no significant effect on the response to EFS, albeit this response was already greatly attenuated by the effects of radiation at these time points (Fig.15). Furthermore, L-NAME pretreatment had no significant effect on the Isc response to PGE_2 in shams or irradiated rats (Fig.16).

B. Radiation-induced NOS activity

1. Validation of NOS assay

To validate the NOS assay in this study, the endotoxin model of NOS activation was employed. The administration of bacterial endotoxin or lipopolysaccharide (LPS) to rats is known to stimulate an acute inflammatory response which is accompanied by an increase in iNOS mRNA expression and NO production in the intestines of these animals (Tepperman *et al.*, 1993; Boughton-Smith *et al.*, 1993; Wink and Laval, 1994). *Salmonella typhimurium* endotoxin (LPS, 4 mg/kg i.v.) was administered to rats and 4 h later intestinal tissues were removed for determination of NOS activity. In addition, to determine if endotoxin-induced NOS activity could be inhibited, AG (100 mg/kg i.v.) was administered concomitantly. AG is thought to preferentially inhibit iNOS.

a. JEJUNUM

Endotoxin caused a significant increase in total NOS (tNOS) activity from 0.64±0.04 to

1.36±0.16 nmol NO/min/g tissue ($p<0.05$). This was due to an almost 4-fold increase in iNOS activity ($p<0.05$)(Fig.17). Although co-administration of AG did not significantly attenuate the increase in tNOS activity, it did significantly decrease iNOS activity by 48% ($p<0.05$)(Fig.17) and return the profile of NOS activity to control levels (Fig.18). Endotoxin administration had no significant effect on cNOS activity in these tissues (Fig.17).

b. ILEUM

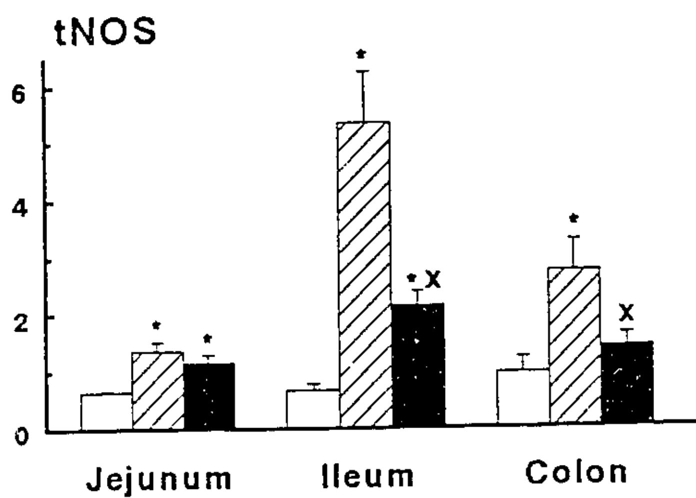
Similar to the jejunum, endotoxin induced a significant increase in tNOS activity in the ileum. However tNOS activity in this tissue increased 8-fold, from 0.66±0.11 to 5.34±0.89 nmol NO/min/g tissue ($p<0.05$)(Fig.17) as a result of a greater than 14-fold increase in iNOS activity ($p<0.05$)(Fig.18). In contrast to the jejunum, AG significantly decreased both tNOS and iNOS activity in these tissues by 60% ($p<0.05$) and 74% ($p<0.05$), respectively (Fig.17). Endotoxin alone had no significant effect on cNOS activity. However, when endotoxin was co-administered with AG, cNOS activity was significantly increased compared to the saline group ($p<0.05$)(Fig.17). AG administration reversed the endotoxin-induced alteration in the profile of NOS activity (Fig.18).

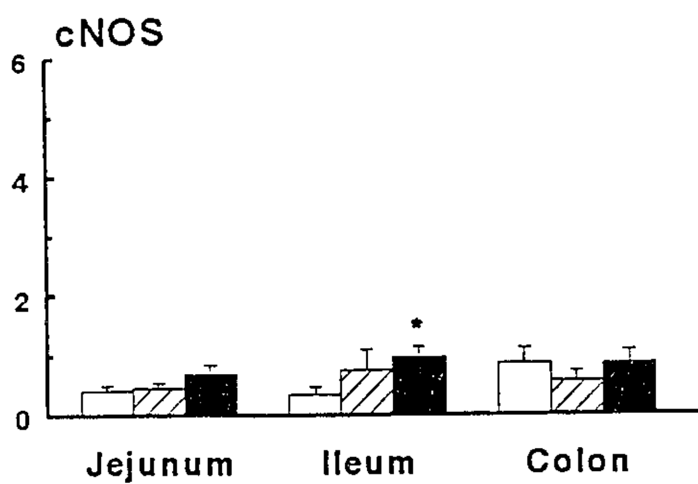
c. COLON

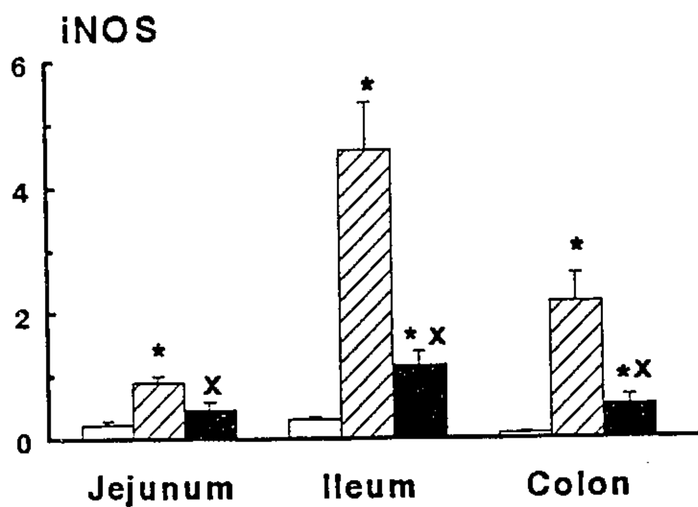
Endotoxin administration significantly increased tNOS activity by almost 2-fold from 0.95±0.26 to 2.72±0.53 nmol NO/min/g tissue ($p<0.05$)(Fig.17). This increase was due to a significant rise in iNOS activity ($p<0.05$) with no significant effect on cNOS activity (Fig.17). AG significantly depressed tNOS activity by 49% ($p<0.05$)(Fig.17) and iNOS activity by 75%

Figure 17. Effect of bacterial endotoxin on total NOS (tNOS) activity and constitutive and inducible NOS (cNOS and iNOS) activity in the rat jejunum, ileum and colon. Saline (open), *Salmonella typhimurium* lipopolysaccharide (LPS, 4 mg/kg i.v.)(hatched) or LPS+aminoguanidine (AG, 100 mg/kg i.v.)(solid) was administered 4 h prior to sampling. Endotoxin significantly increased tNOS and iNOS activity in all regions ($^*p<0.05$ compared to saline) whereas cNOS activity was not changed. AG, which preferentially inhibits iNOS, significantly attenuated endotoxin-induced tNOS activity in the ileum and colon ($^xp<0.05$ compared to LPS alone) and iNOS activity in all regions ($^xp<0.05$ compared to LPS alone). cNOS activity in the ileum was significantly increased by AG ($^xp<0.05$ compared to LPS alone). Values represent the mean±SEM for n=4 for saline and n=6 for treatment groups.

Saline LPS LPS+AG

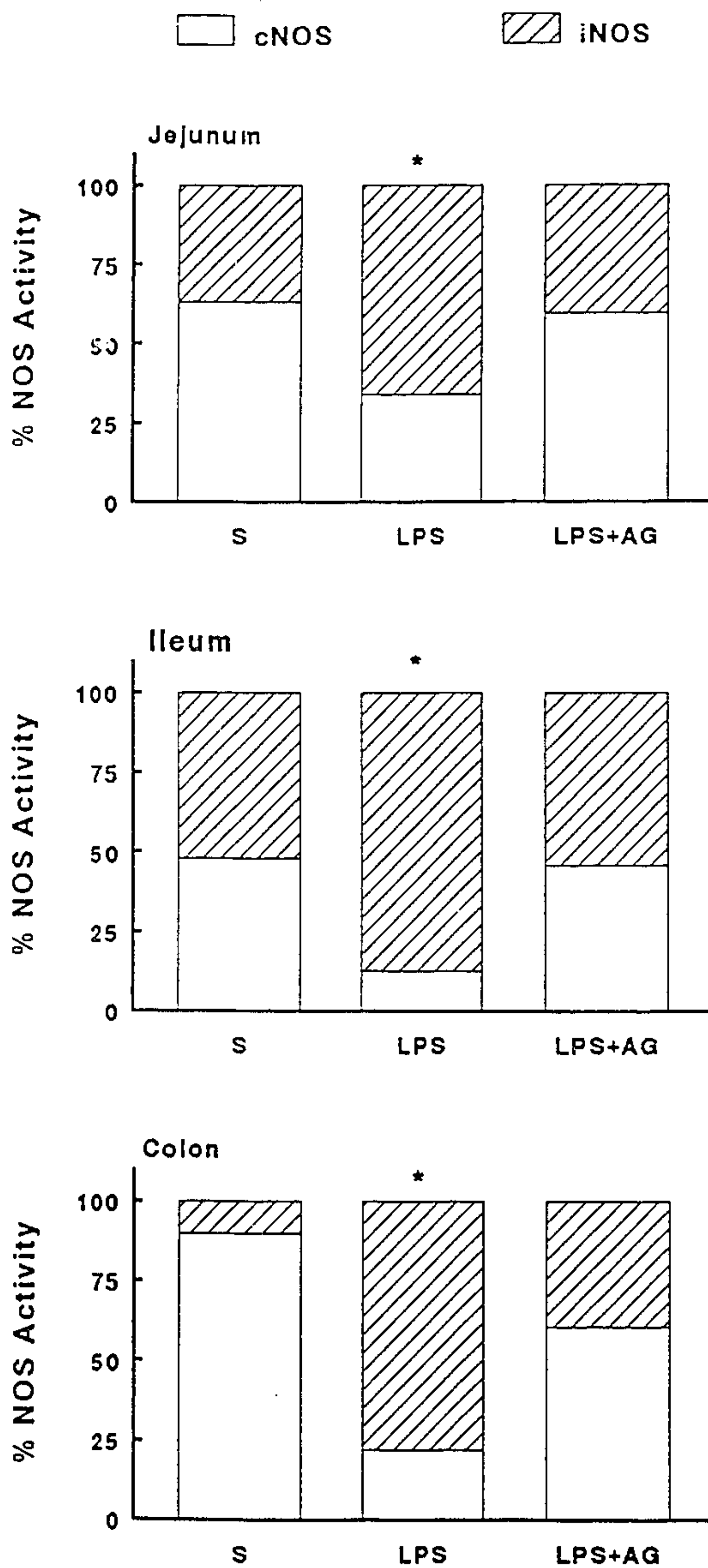






nmol ^{14}C-L-citrulline/min/g tissue

Figure 18. Profile of NOS activity in the jejunum, ileum and colon in the endotoxin model. Saline (S), *Salmonella typhimurium* lipopolysaccharide (LPS, 4 mg/kg i.v.) or LPS+aminoguanidine (AG, 100 mg/kg i.v.) was administered 4 h prior to sampling. The graph shows the percentage of total NOS activity that was due to cNOS (open) and iNOS (hatched). Endotoxin significantly altered the profile of NOS activity in all regions (*$p<0.05$ compared to saline as a percentage). AG returned the profile of NOS activity to saline treated levels in all regions. Values represent the mean for saline (n=4) and treatment groups (n=6).


cNOS
iNOS
Jejunum
% NOS Activity
100
75
50
25
0
*
S
LPS
LPS+AG
Ileum
% NOS Activity
100
75
50
25
0
*
S
LPS
LPS+AG
Colon
% NOS Activity
100
75
50
25
0
*
S
LPS
LPS+AG

($p<0.05$)(Fig.17). The inhibitory effects of AG on iNOS activity returned the profile of NOS activity to control levels (Fig.18).

2. Effects of NOS inhibition with L-NAME

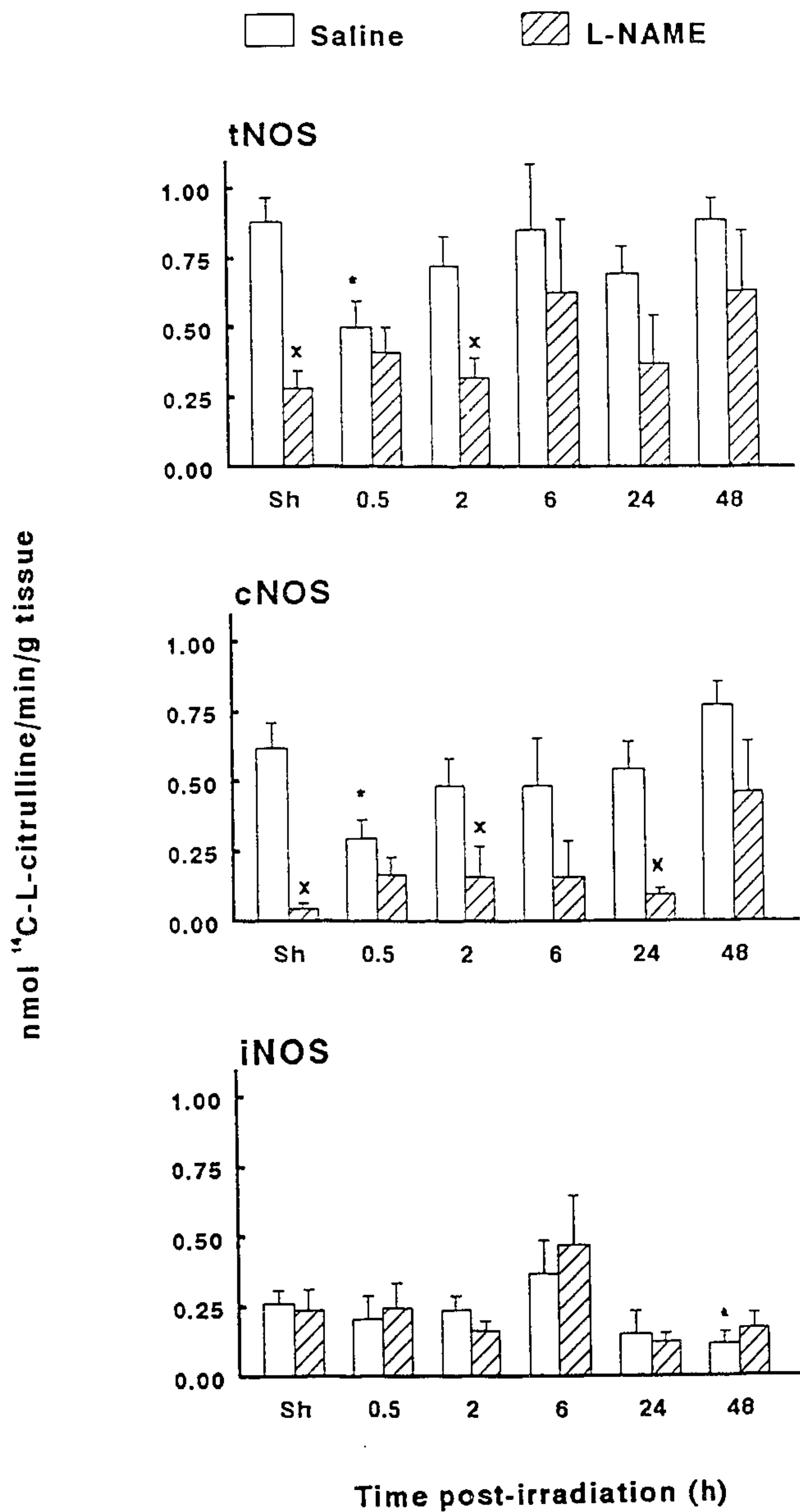
As the results of the Ussing chamber experiments clearly indicate that in some regions of the intestine endogenous NO does effect intestinal electrolyte transport it was of interest to determine what affect irradiation had on endogenous NO production. Therefore, using the same model of irradiation in the rat, segments of tissue from jejunum, ileum and colon were processed for the determination of NOS activity post-irradiation. NOS activity was determined in all tissues at 0.5, 2, 6, 24, 48 h post-irradiation in both saline and L-NAME (100 mg/kg s.c.) pretreated animals. L-NAME is thought to be a fairly sticky inhibitor of NOS but it is not considered irreversible, albeit, the exact metabolism of L-NAME has not yet been determined. To further elucidate the role of NO, NOS activity was determined both with and without the Ca^{2+} chelator, EGTA, added to the incubation buffer to determine the enzymatic profile of NOS activity at each time point.

a. JEJUNUM

i. Effects of radiation

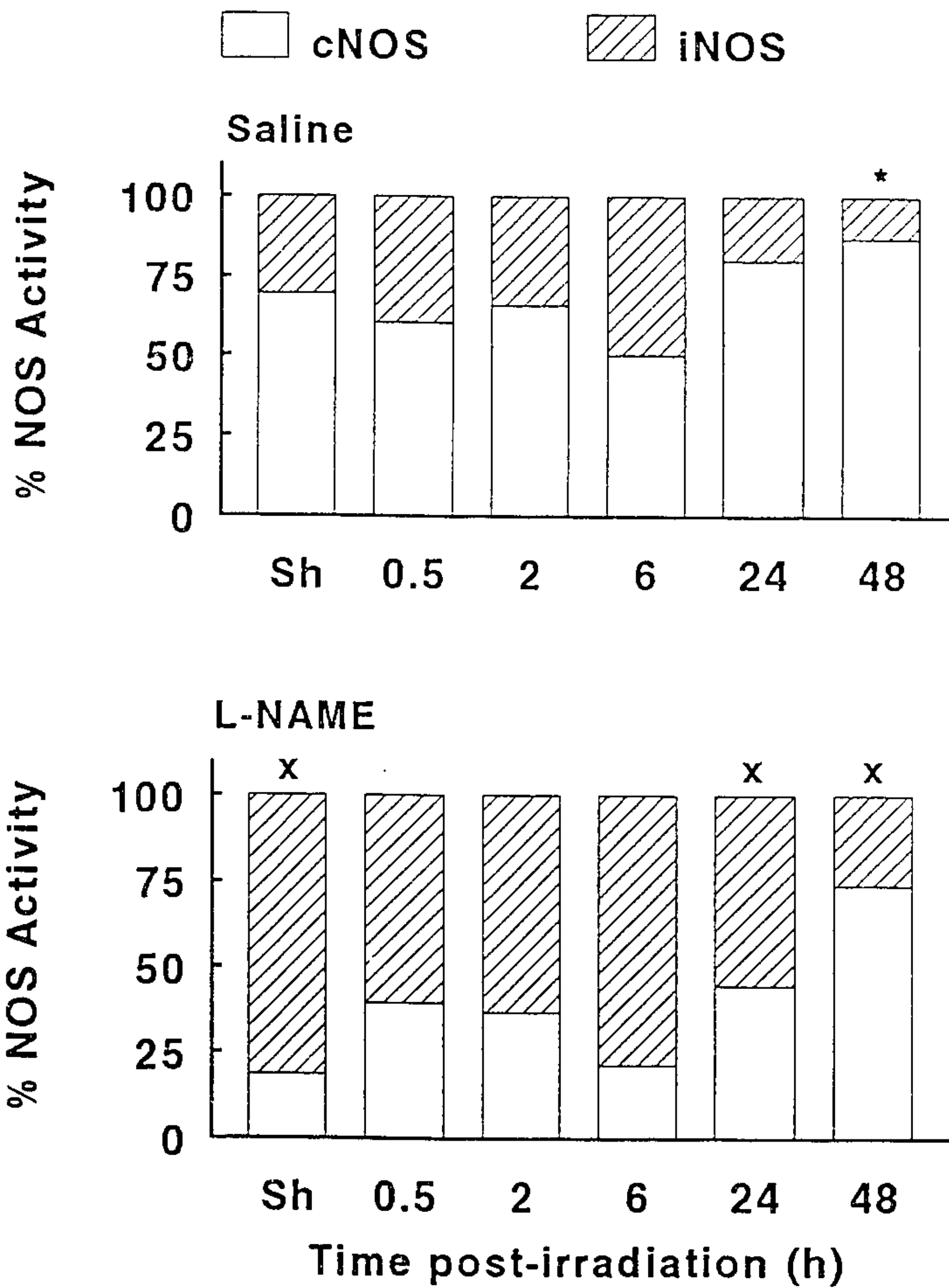
Total NOS activity was significantly decreased by 42% ($p<0.05$) immediately post-irradiation (0.5 h) (Fig.19). This decrease was due to a significant depression of cNOS activity at 0.5 h ($p<0.05$)(Fig.19) however this did not significantly alter the profile of NOS activity (Fig.20).

Figure 19. Total NOS (tNOS) activity and constitutive and inducible NOS (cNOS and iNOS) activity in jejunal segments from rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly decreased tNOS and cNOS activity at 0.5 h and iNOS activity at 48 h ($^{*}p<0.05$ compared to shams). L-NAME pretreatment decreased tNOS activity in shams and at 2 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups) and cNOS activity in shams and at 2 and 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups). L-NAME had no effect on iNOS activity. Values represent the mean±SEM for n=6.



Saline
L-NAME
tNOS
cNOS
iNOS
1.00
0.75
0.50
0.25
0.00
Sh
0.5
2
6
24
48
nmol ^{14}C-L-citrulline/min/g tissue
Time post-irradiation (h)

Figure 20. Profile of NOS activity in the jejunum of rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline or L-NAME 100 mg/kg. The graph shows the percentage of total NOS activity that was due to cNOS (open) and iNOS (hatched). Radiation significantly altered the profile of NOS activity at 48 h ($^{*}p<0.05$ compared to shams as a percentage). L-NAME pretreatment significantly altered the profile of NOS activity in shams and at 24 and 48 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups as a percentage). Values represent the mean (n=6).


cNOS
iNOS
Saline
% NOS Activity
100
75
50
25
0
*
Sh
0.5
2
6
24
48
L-NAME
x
x
x
% NOS Activity
100
75
50
25
0
Sh
0.5
2
6
24
48
Time post-irradiation (h)

Total NOS activity returned to sham levels by 2 h and did not significantly change within the 48 h of this study (Fig.19). However the enzymatic profile of NOS activity was altered at 48 h post-irradiation when %cNOS increased significantly (Fig.20) as a result of a decrease in iNOS activity ($p<0.05$) at 48 h post-irradiation compared to shams (Fig.19).

ii. Effects of L-NAME pretreatment

In sham irradiated animals L-NAME pretreatment significantly attenuated tNOS activity by 68% ($p<0.05$)(Fig.19). As a direct result of a 93% decrease in cNOS activity ($p<0.05$)(Fig.19) the profile of NOS activity was significantly altered in shams (Fig.20).

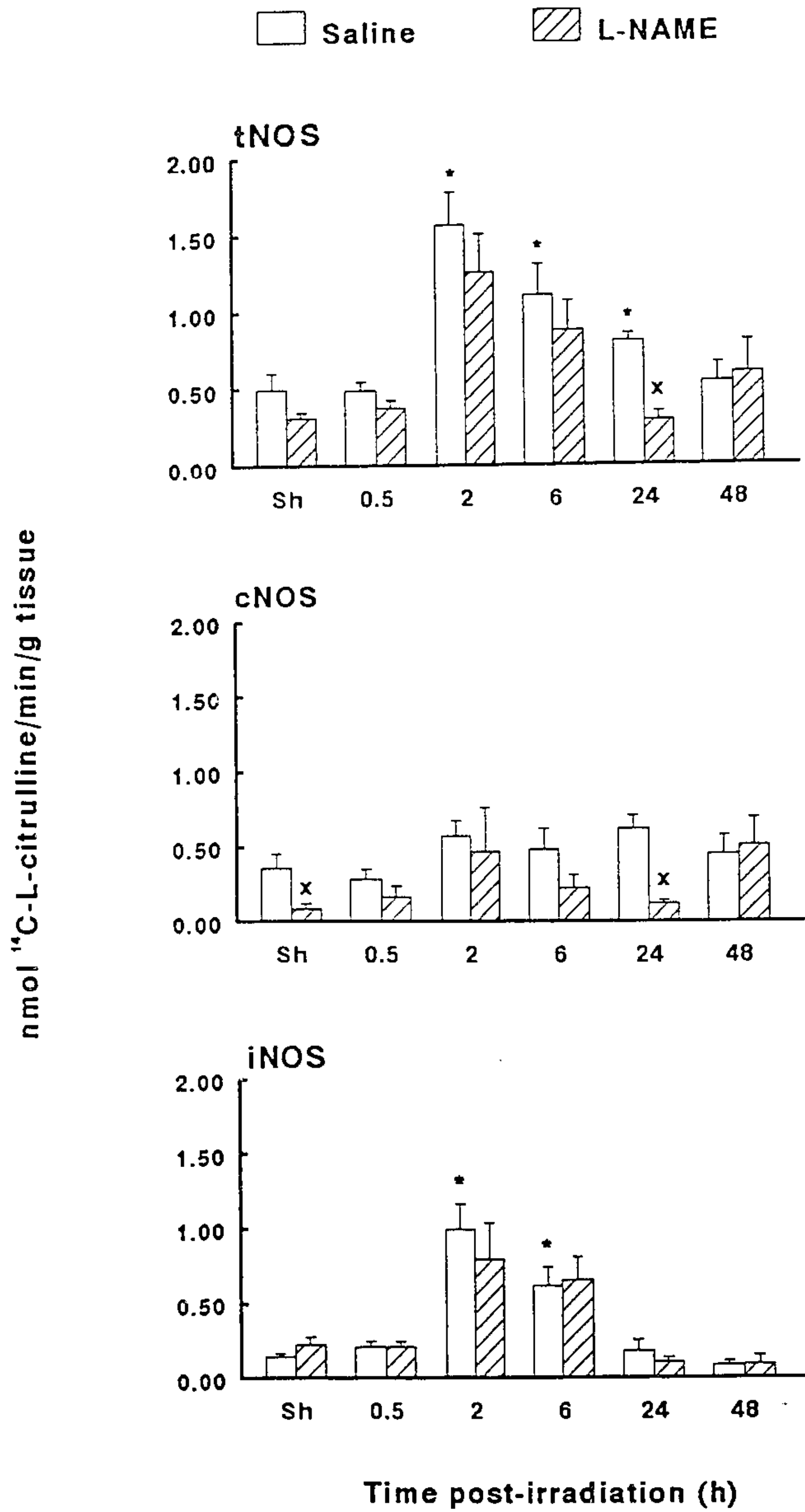
At 2 h post-irradiation tNOS activity was attenuated by 55% ($p<0.05$)(Fig.18) by L-NAME pretreatment. The decrease in tNOS activity was attributable to a decrease in cNOS activity at this time point. cNOS activity was also significantly decreased by L-NAME pretreatment at 24 h post-irradiation. The profile of NOS activity was significantly altered at 24 and 48 h with an increase in %iNOS compared to saline pretreated rats (Fig.20).

b. ILEUM

i. Effects of radiation

In contrast to the jejunum, in the ileum radiation induced a significant increase in tNOS activity at 2, 6, and 24 h post-irradiation ($p<0.05$)(Fig.21). The increase was maximal at 2 h when tNOS activity was 214% greater than shams. The radiation-induced increase in tNOS was less by 6 h (121%>sham) and even less by 24 h (61%>sham), returning to sham levels by 48 h post-irradiation (Fig.21).

Figure 21. Total NOS (tNOS) activity and constitutive and inducible NOS (cNOS and iNOS) activity in ileal segments from rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly increased tNOS activity at 2, 6, and 24 h ($^{*}p<0.05$ compared to shams) and iNOS activity at 2 and 6 h ($^{*}p<0.05$ compared to shams). Radiation had no effect on cNOS activity. L-NAME significantly attenuated tNOS activity at 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated group) and cNOS activity in shams and at 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups). L-NAME pretreatment had no effect on iNOS activity. Values represent the mean±SEM for n=6.


Saline
L-NAME
tNOS
2.00
1.50
1.00
0.50
0.00
Sh
0.5
2
6
24
48
cNOS
iNOS
nmol ^{14}C-L-citrulline/min/g tissue
Time post-irradiation (h)

The enzymatic profile of NOS activity in the ileum was significantly altered with an increase in %iNOS at 2 and 6 h post-irradiation (Fig.22). The altered profile of NOS activity was due to an increase in iNOS activity of 7-fold at 2 h and 4-fold at 6 h post-irradiation (Fig.21).

ii. Effects of L-NAME

L-NAME pretreatment only significantly attenuated tNOS activity at 24 h post-irradiation and at no other time was tNOS activity significantly altered by L-NAME pretreatment ($p<0.05$)(Fig.21). However, cNOS activity was significantly decreased by 76% and 80% ($p<0.05$) in shams and at 24 h post-irradiation, respectively (Fig.21). The decrease in tNOS activity at 24 h was attributable to the inhibition of cNOS activity at this time point whereas the inhibition of cNOS activity in shams altered the profile of NOS activity in these tissues (Fig.22). The enzymatic profile of NOS activity was not significantly affected by L-NAME pretreatment at any point post-irradiation (Fig.22).

c. COLON

i. Effects of radiation

Exposure to 10 Gy γ-irradiation did not significantly alter tNOS activity in the colon. However, it is worth noting that this region of the gut consistently produced results of high variability (Fig.23). Therefore, an accurate interpretation of the effects of radiation on NOS activity in the colon is difficult.

Similar to tNOS activity, the enzymatic profile of NOS activity in the colon was not

Figure 22. Profile of NOS activity in the ileum of rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline or L-NAME 100 mg/kg. The graph shows the percentage of total NOS activity that was due to cNOS (open) and iNOS (hatched). At 2 and 6 h post-irradiation the profile of NOS activity was significantly altered ($^{*}p<0.05$ compared to shams as a percentage). L-NAME pretreatment significantly altered the profile of NOS activity in shams ($^{x}p<0.05$ compared to the saline pretreated group as a percentage). Values represent the mean (n=6).

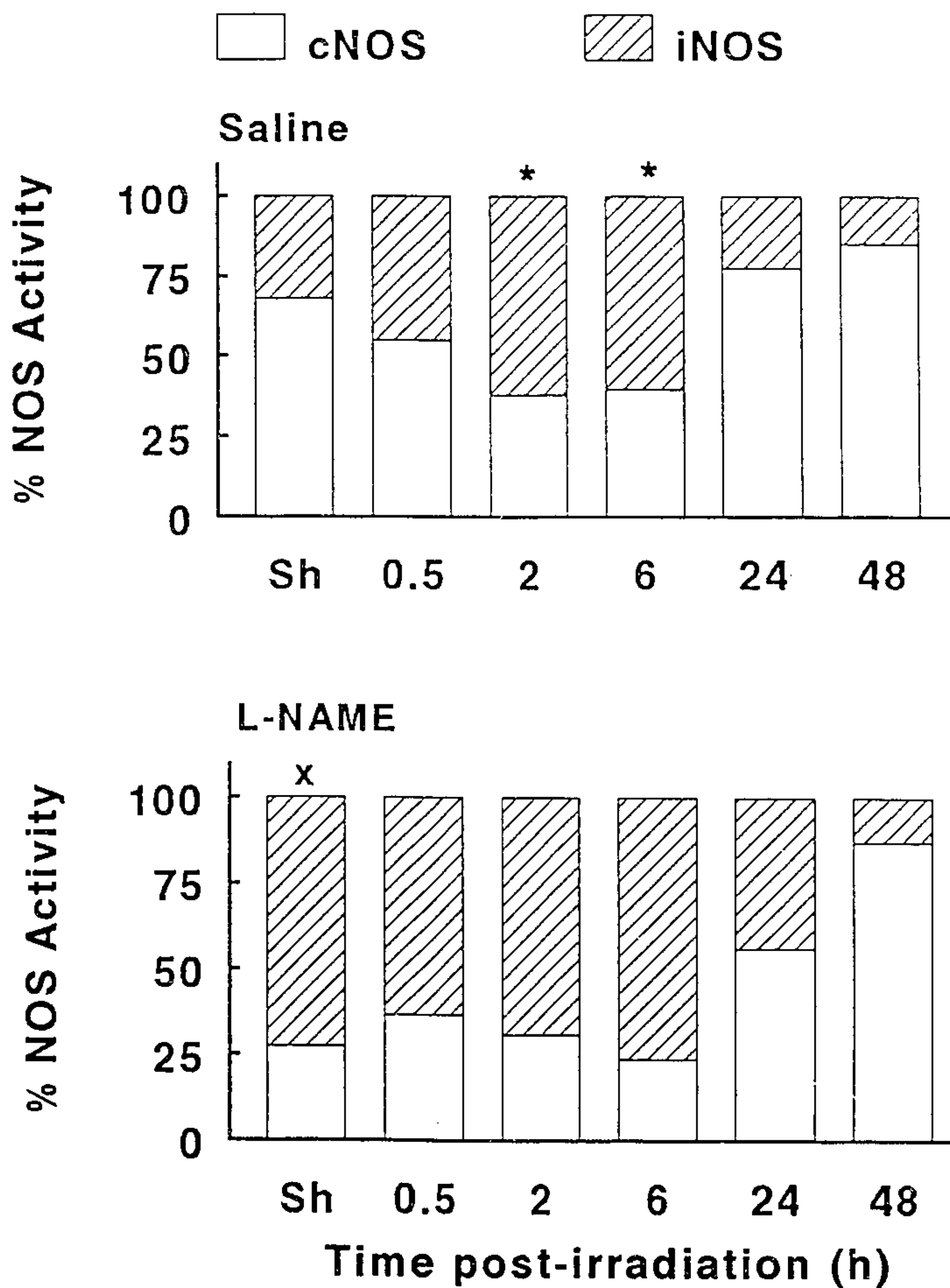
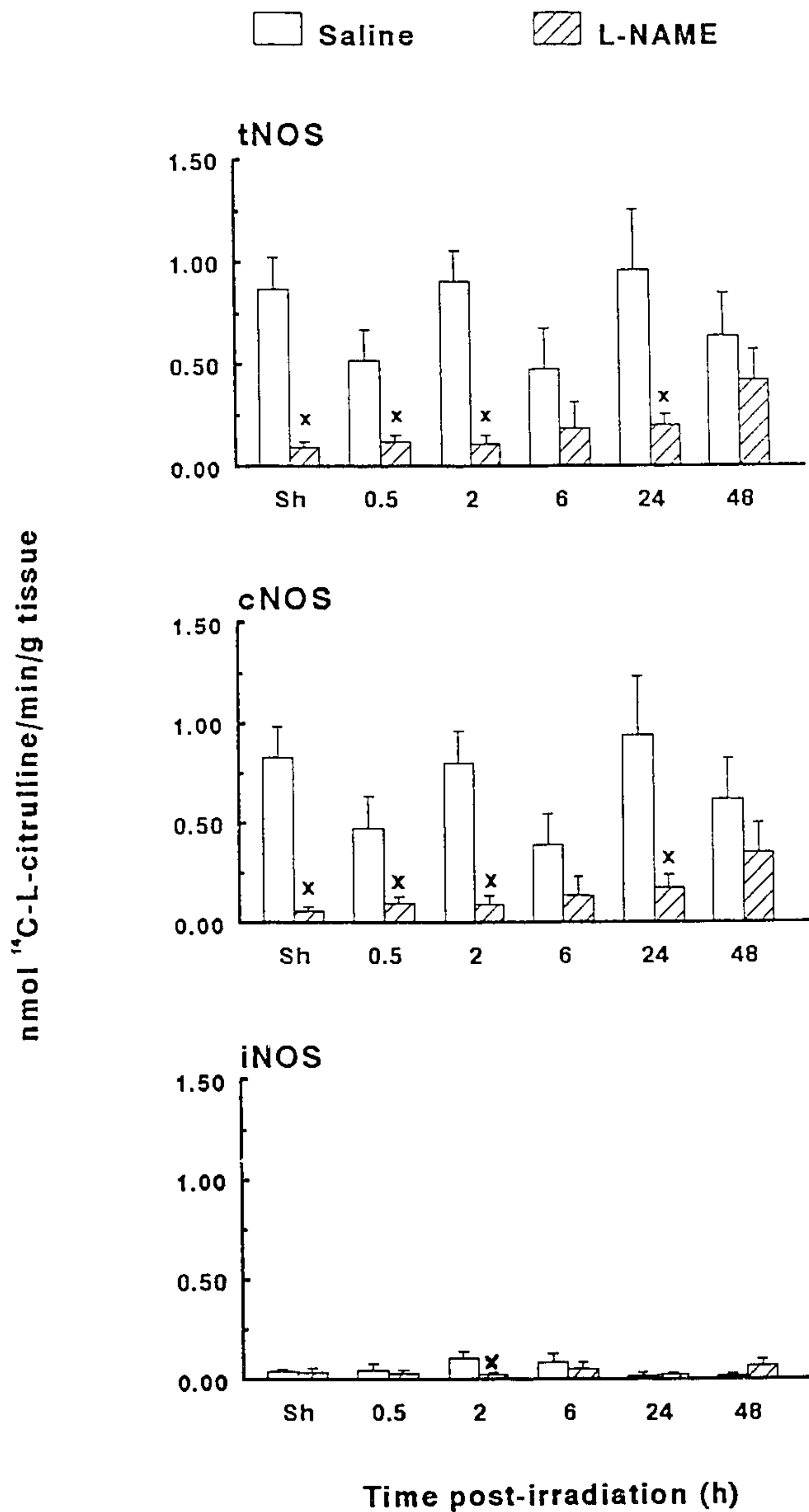

cNOS
iNOS
Saline
% NOS Activity
100
75
50
25
0
*
*
Sh
0.5
2
6
24
48
L-NAME
x
% NOS Activity
100
75
50
25
0
Sh
0.5
2
6
24
48
Time post-irradiation (h)

Figure 23. Total NOS (tNOS) activity and constitutive and inducible NOS (cNOS and iNOS) activity in colonic segments from rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation had no significant effect on tNOS, cNOS or iNOS activity when compared to shams. L-NAME significantly attenuated tNOS activity in shams and at 0.5, 2 and 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups) and cNOS activity in shams and at 0.5, 2, 6, and 24 h post-irradiation ($^{x}p<0.05$ compared to the saline pretreated groups). L-NAME pretreatment significantly decreased iNOS activity at 2 h. Values represent the mean±SEM for n=6.


Saline
L-NAME
tNOS
cNOS
iNOS
1.50
1.00
0.50
0.00
Sh
0.5
2
6
24
48
x
nmol ^{14}C-L-citrulline/min/g tissue
Time post-irradiation (h)

significantly altered by irradiation (Fig.24) however it appeared that iNOS activity was increased. This is further supported by the increase in mean iNOS activity at 2 and 6 h post-irradiation relative to shams however the standard deviation masks any detectable significant difference in these tissues (Fig.23).

ii. Effects of L-NAME

In sham irradiated animals L-NAME pretreatment significantly attenuated tNOS activity by 89% ($p<0.05$)(Fig.23). However, there was no significant change in the profile of NOS activity (Fig.24). The decrease in tNOS activity in shams was solely attributable to the inhibition of cNOS activity (Fig.23).

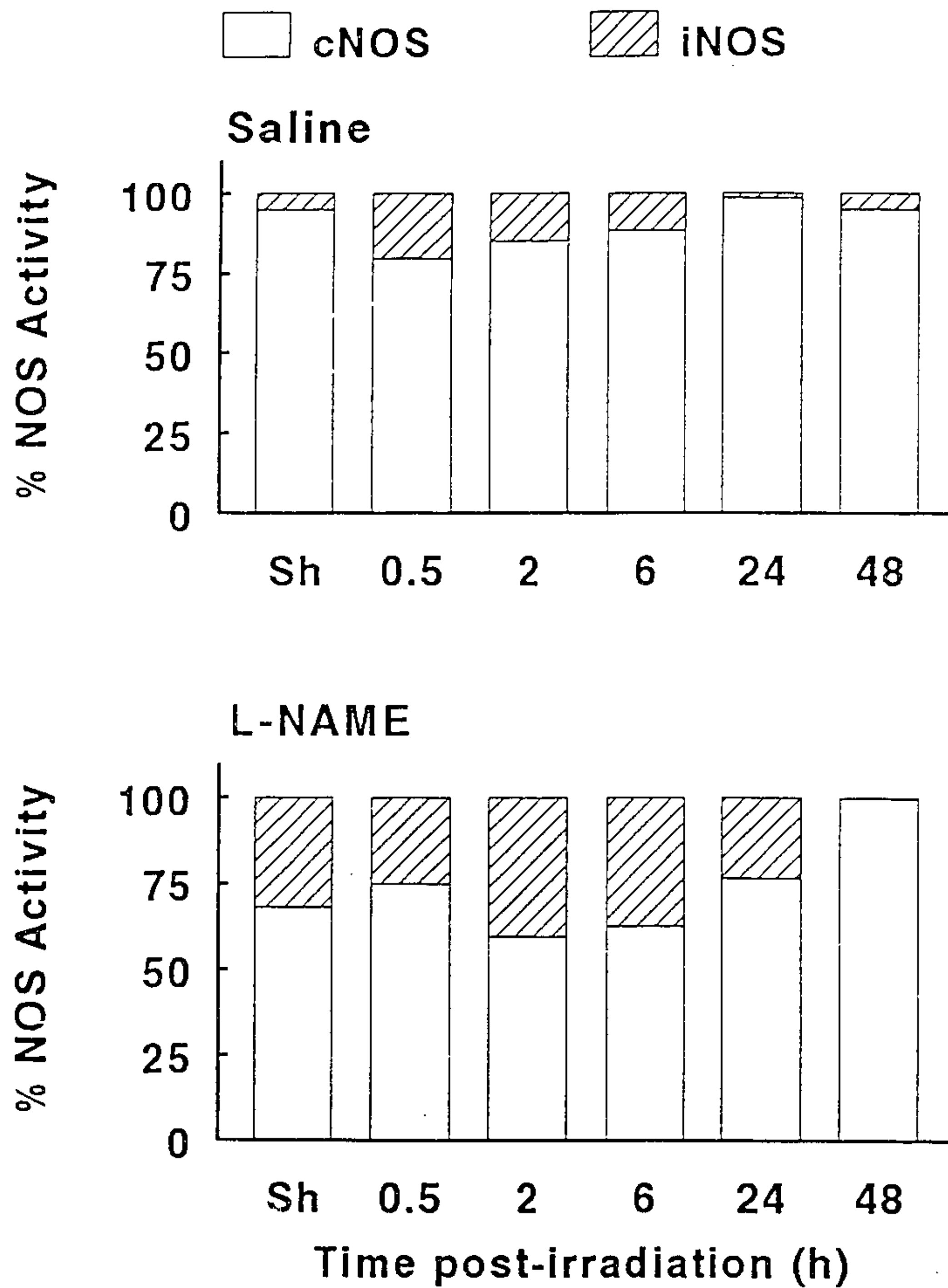
At 0.5, 2, and 24 h post-irradiation L-NAME pretreatment significantly depressed tNOS activity by 77%, 88% and 80% respectively ($p<0.05$)(Fig.23). Note that the mean NOS activity at 6 h post-irradiation was attenuated by 61% (0.476 ± 0.197 to 0.185 ± 0.127 nmol/min/g tissue) but due to a high standard deviation this decrease was not statistically significant (Fig.23).

L-NAME pretreatment appeared to increase %iNOS activity in irradiated tissues up to 24 h post-irradiation however none of these increases were statistically significant (Fig.24). As L-NAME pretreatment greatly depressed tNOS activity, the apparent increase in %iNOS activity was primarily due to a significant decrease in cNOS activity (Fig.23).

3. Maximal response to radiation: studies on selective NOS inhibition

As pretreatment with L-NAME at a dose of 100 mg/kg s.c. did not significantly attenuate the increase in NOS activity in the ileum at 2 or 6 h post-irradiation, the effect of a higher dose

Figure 24. Profile of NOS activity in the colon of rats 2 h after sham treatment and at 0.5, 2, 6, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline or L-NAME 100 mg/kg. The graph shows the percentage of total NOS activity that was due to cNOS (open) and iNOS (hatched). Radiation did not significantly alter the profile of NOS activity compared to shams. L-NAME also had no significant effect on the profile of NOS activity. Values represent the mean (n=6).


cNOS
iNOS
Saline
% NOS Activity
100
75
50
25
0
Sh
0.5
2
6
24
48
L-NAME
% NOS Activity
100
75
50
25
0
Sh
0.5
2
6
24
48
Time post-irradiation (h)

of L-NAME was studied. L-NAME at a dose of 300 mg/kg s.c. was administered 1 h prior to irradiation. NOS activity was determined in all tissues at 2 h post-irradiation as this was the time point at which NOS activity was maximally stimulated by irradiation. Furthermore, as the radiation-induced increase in tNOS activity in the ileum was primarily due to an increase in iNOS, the effect of AG, an iNOS preferring inhibitor, was also studied.

a. JEJUNUM

L-NAME significantly attenuated tNOS activity by 39% ($p<0.05$) which was no different than the effect of 100 mg/kg L-NAME. AG had no significant effect (Fig.25). L-NAME also significantly altered the profile of NOS activity, greatly decreasing %cNOS (Fig.26). This effect was due to a 90% decrease in cNOS activity in this tissue ($p<0.05$)(Fig.25). iNOS activity in this tissue was not significantly altered by L-NAME or AG (Fig.25).

b. ILEUM

Both L-NAME and AG significantly decreased tNOS activity by 47% and 37% ($p<0.05$), respectively, however they targeted different isoforms of NOS (Fig.25). L-NAME significantly decreased cNOS activity by 56% ($p<0.05$) but did not significantly alter iNOS activity (Fig.25). Whereas AG did not significantly alter cNOS activity but significantly decreased iNOS activity by 36% ($p<0.05$)(Fig.25). Neither of these inhibitors had any significant effect on the profile of NOS activity (Fig.26)

Figure 25. The effect of L-NAME and AG on total NOS (tNOS) and constitutive and inducible (cNOS and iNOS) activity at 2 h post-irradiation in the rat jejunum, ileum and colon. Rats were either pretreated (1 h prior to irradiation) with saline (open), L-NAME 100 mg/kg (course hatched) or 300 mg/kg s.c. (fine hatched) or treated immediately post-irradiation with AG 100 mg/kg i.v. (solid). L-NAME 100 mg/kg significantly decreased tNOS activity in the jejunum and tNOS, cNOS and iNOS activity in the colon ($^{x}p<0.05$ compared to the saline pretreated group) but had no effect in the ileum. At a dose of 300 mg/kg L-NAME significantly decreased tNOS and cNOS activity in all tissues ($^{x}p<0.05$ compared to the saline pretreated group) but had no effect on iNOS activity. AG significantly decreased tNOS and iNOS activity in the ileum ($^{x}p<0.05$ compared to the saline pretreated group) but had no effect on the jejunum and colon. cNOS activity was not altered by AG. Values represent the mean±SEM for $n \geq 4$.

Saline
L-NAME 100 mg/kg
L-NAME 300 mg/kg
AG 100 mg/kg

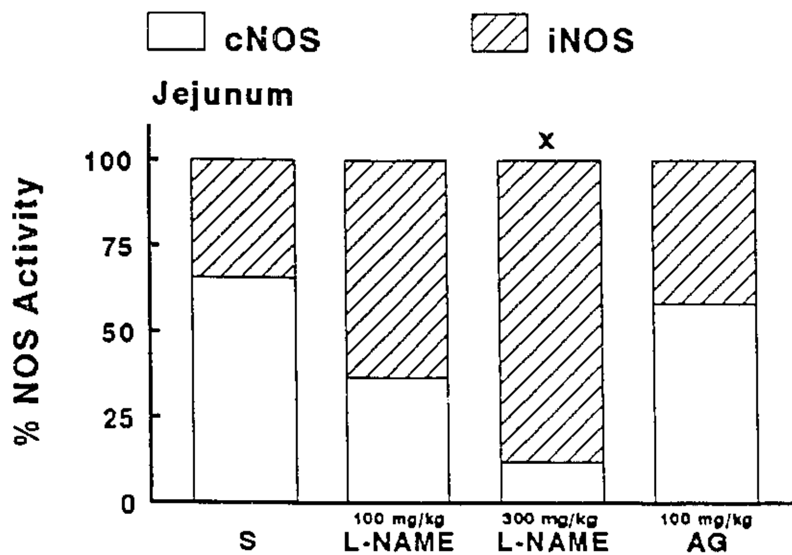
nmol ^{14}C-L-citrulline/min/g tissue

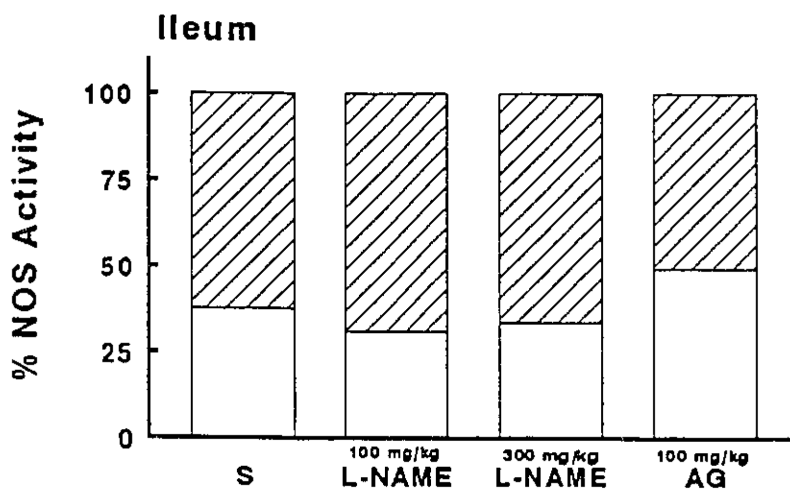
tNOS
2.00
1.50
1.00
0.50
0.00
x x x x x x
Jejunum Ileum Colon

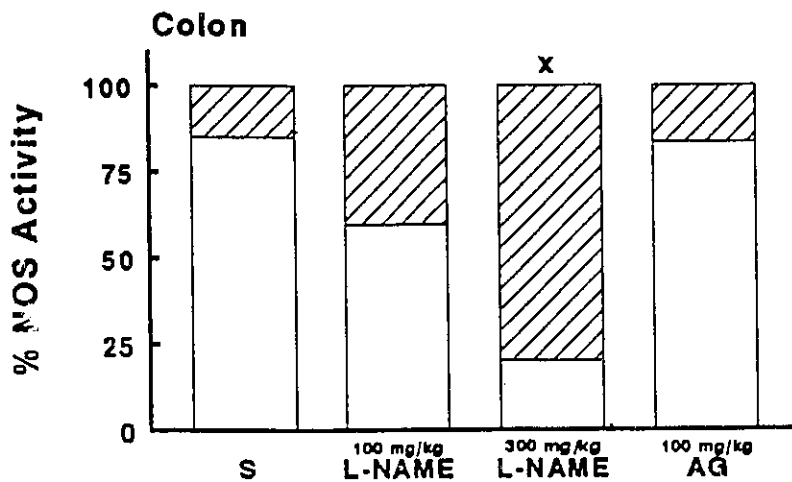
cNOS
2.00
1.50
1.00
0.50
0.00
x x x x
Jejunum Ileum Colon

iNOS
2.00
1.50
1.00
0.50
0.00
x x
Jejunum Ileum Colon

Figure 26. The effect of L-NAME and AG on the profile of NOS activity at 2 h post-irradiation in the rat jejunum, ileum and colon. Rats were either pretreated (1 h prior to irradiation) with saline (open), L-NAME 100 mg/kg (course hatched) or 300 mg/kg s.c. (fine hatched) or treated immediately post-irradiation with AG 100 mg/kg i.v. (solid). The graph shows the percentage of total NOS activity that was due to cNOS (open) and iNOS (hatched). L-NAME 300 mg/kg significantly altered the profile of NOS activity in the jejunum and colon ($^{x}p<0.05$ compared to the saline pretreated group as a percentage). AG had no significant effect on the profile of NOS activity. Values represent the mean (n=6).


cNOS
iNOS
Jejunum
% NOS Activity
100
75
50
25
0
x
S
100 mg/kg
L-NAME
300 mg/kg
L-NAME
100 mg/kg
AG


Ileum
% NOS Activity
100
75
50
25
0
S
100 mg/kg
L-NAME
300 mg/kg
L-NAME
100 mg/kg
AG


Colon
% NOS Activity
100
75
50
25
0
x
S
100 mg/kg
L-NAME
300 mg/kg
L-NAME
100 mg/kg
AG

c. COLON

L-NAME significantly attenuated tNOS activity by 82% ($p<0.05$) however, as in the jejunum, AG had no significant effect (Fig.25). The effect of L-NAME was due to a 95% decrease in cNOS activity from 0.80±0.16 to 0.04±0.03 nmol NO/min/g tissue ($p<0.05$)(Fig.25). The decrease in cNOS activity due to the inhibitory effects of L-NAME significantly altered the profile of NOS activity ($p<0.05$)(Fig.26). In contrast to the lower dose of L-NAME, 300 mg/kg L-NAME did not inhibit iNOS activity (Fig.25). AG also had no effect on iNOS activity in this tissue (Fig.25).

C. Effects of radiation on eicosanoid synthesis

Since radiation can stimulate PGE_2 and LTB_4 production and these eicosanoids are known mediators of the inflammatory process it was of interest to determine the effects of radiation on the synthesis of these inflammatory mediators in the gut. Furthermore, as NO plays an important role in the inflammatory response and has been identified as regulator of cyclooxygenase activity and vice versa, the effects of the NOS inhibitor, L-NAME (100 mg/kg), on PGE_2 synthesis were studied. In addition, as NO inhibits neutrophil adhesion and migration the effects of its inhibition on the production of the chemotactic leukotriene, LTB_4, in radiation-induced intestinal inflammation were also investigated. These mediators were studied at the same time points as in the electrolyte transport experiments, sham, 2, 24 and 48 h post-irradiation.

1. PGE_2 production

a. JEJUNUM

Although radiation did not significantly alter PGE_2 production at 2, 24 or 48 h post-irradiation when compared to shams, there appeared to be an increase in PGE_2 synthesis at 2 and 48 h and a decrease at 24 h post-irradiation. PGE_2 synthesis at 24 h when compared to PGE_2 synthesis at 2 and 48 h post-irradiation was significantly reduced ($p<0.05$) (Fig.27)

L-NAME pretreatment significantly attenuated PGE_2 synthesis by 59% from 131±28 to 54±12 pg PGE_2 /mg tissue ($p<0.05$) at 24 h post-irradiation (Fig.27). L-NAME had no significant effect on shams or at 2 or 48 h post-irradiation.

b. ILEUM

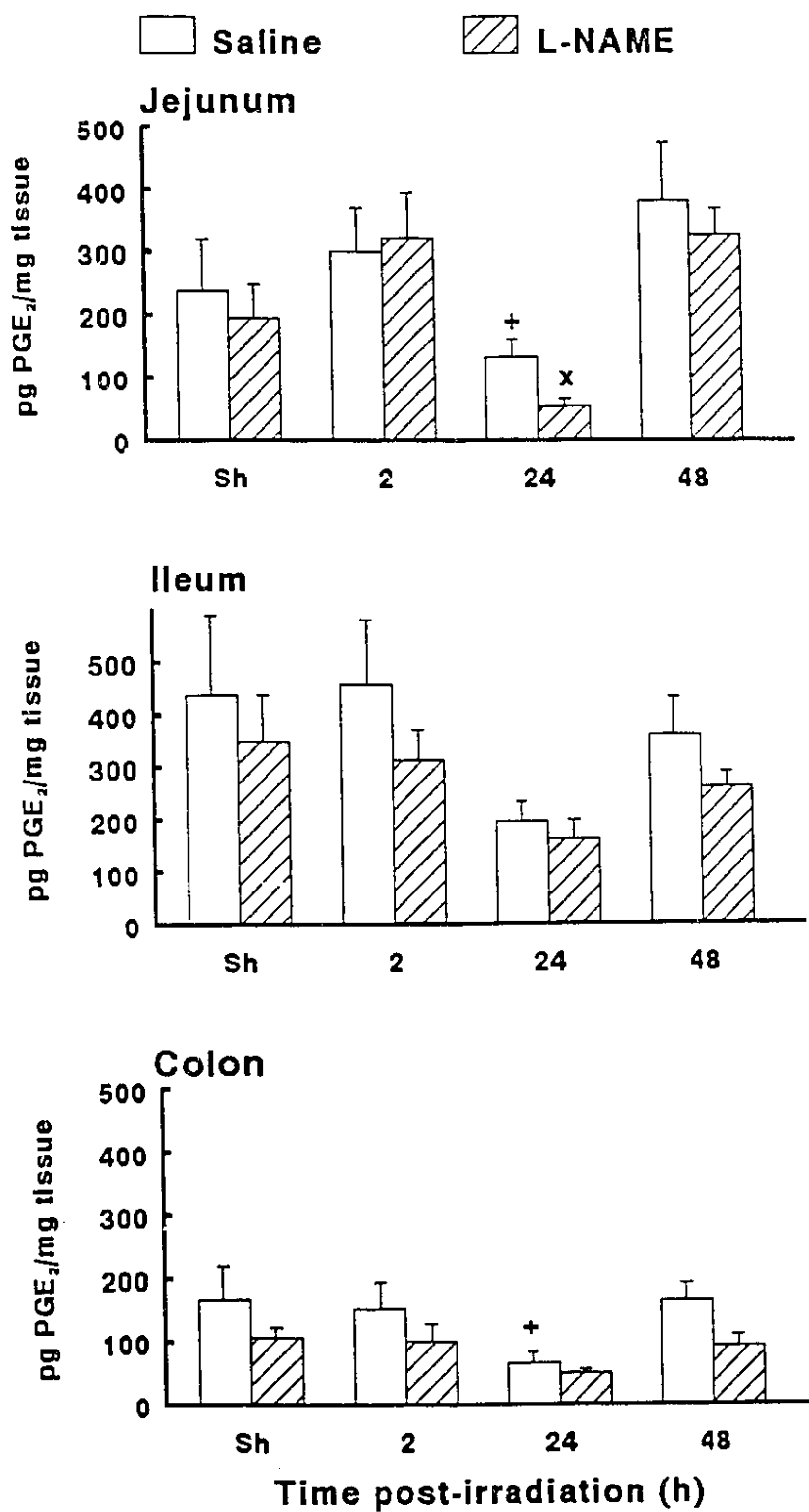
Although there was an apparent decrease in PGE_2 synthesis at 24 h post-irradiation (436±154 to 193±39 pg PGE_2/mg tissue) the effects of radiation were not significant at any point post-irradiation (Fig.27).

L-NAME had no significant effect on PGE_2 synthesis in shams or at any point post-irradiation (Fig.27).

c. COLON

Radiation did not significantly alter PGE_2 production at 2, 24 or 48 h post-irradiation when compared to shams. However, radiation appeared to induce a decrease at 24 h post-irradiation. When compared to PGE_2 synthesis at 2 and 48 h post-irradiation, PGE_2 synthesis at 24 h was significantly reduced by radiation ($p<0.05$)(Fig.27)

Figure 27. PGE_2 synthesis in jejunal, ileal, and colonic segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation significantly decreased PGE_2 synthesis in the jejunum and colon at 24 h ($^{+}p<0.05$ compared to 2 and 48 h post-irradiation). L-NAME pretreatment significantly decreased PGE_2 synthesis at 24 h post-irradiation in the jejunum ($^{x}p<0.05$ compared to the saline pretreated group). Values represent the mean±SEM for $n \geq 5$.


Saline
L-NAME
Jejunum
pg PGE2/mg tissue
500
400
300
200
100
0
+
x
Sh
2
24
48
Ileum
pg PGE2/mg tissue
500
400
300
200
100
0
Sh
2
24
48
Colon
pg PGE2/mg tissue
500
400
300
200
100
0
+
Sh
2
24
48
Time post-irradiation (h)

L-NAME had no significant effect in PGE_2 synthesis in shams or at any point post-irradiation (Fig.27).

2. LTB_4 production

a. JEJUNUM

Radiation had no significant effect on LTB_4 production at any point post-irradiation (Fig.28).

L-NAME pretreatment attenuated LTB_4 synthesis by 62% at 24 h however this decrease was not statistically significant (6.0±2.6 to 2.3±0.8 pg LTB_4/mg tissue)(Fig.28).

b. ILEUM

Radiation had no significant effect on LTB_4 production at any point post-irradiation (Fig.28).

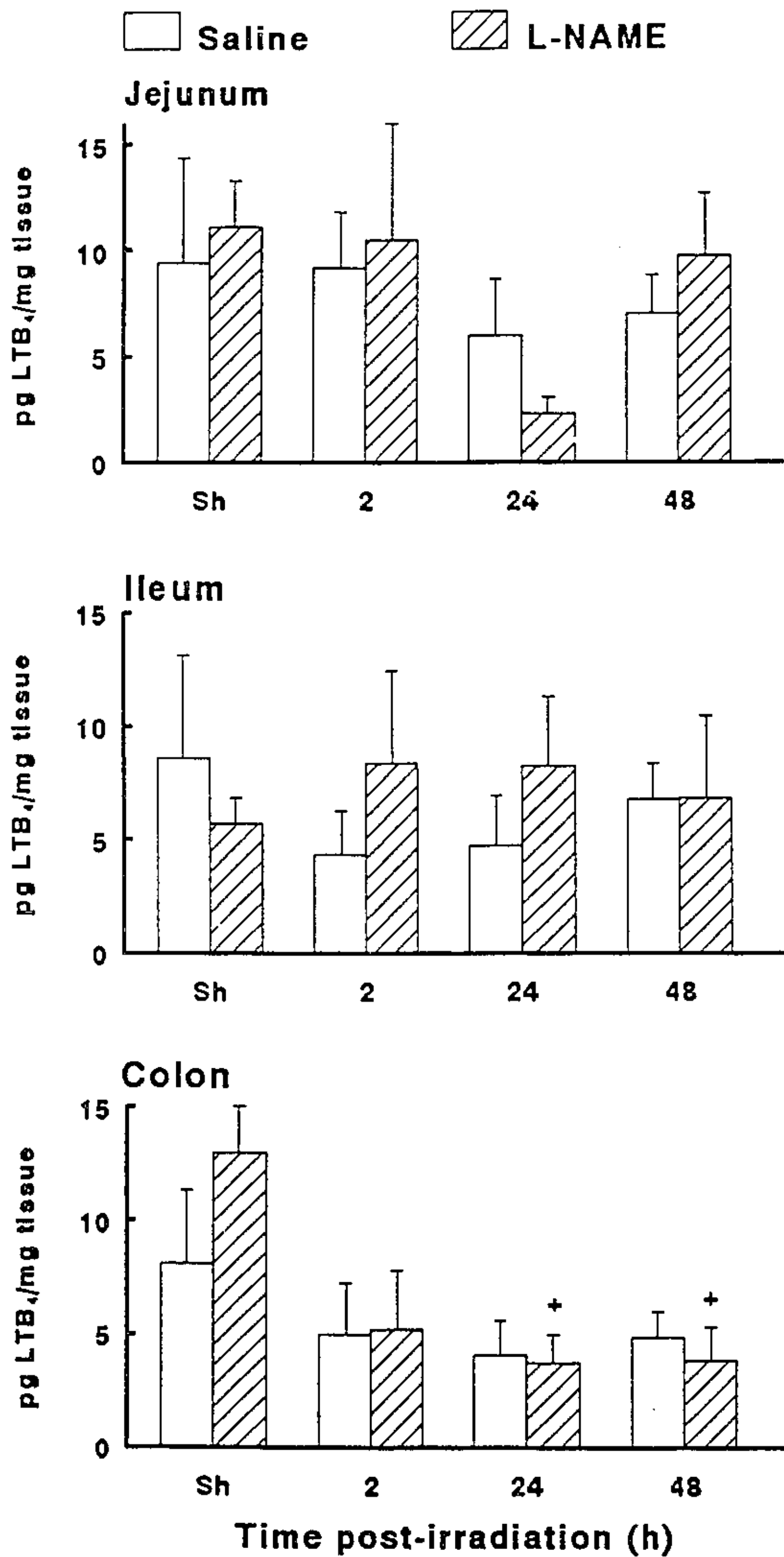
L-NAME had no significant effect on LTB_4 in shams or at any point post-irradiation (Fig.28).

c. COLON

Radiation had no significant effect on LTB_4 production at any point post-irradiation (Fig.28).

L-NAME had no significant effect on LTB_4 production in shams or at any point post-irradiation. However, it appeared that L-NAME pretreatment stimulated an increase in LTB_4 production in shams (8.1±3.2 to 13.0±2.0 pg LTB_4/mg tissue) although this effect was not

Figure 28. LTB_4 synthesis in jejunal, ileal, and colonic segments from rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Radiation had no significant effect in saline pretreated rats. Alternatively, in L-NAME pretreated rats radiation significantly decreased LTB_4 synthesis in the jejunum at 24 h and the ileum at 24 and 48 h post-irradiation ($^{*}p<0.05$ compared to L-NAME pretreated shams). Values represent the mean ±SEM for $n \geq 5$.


Saline
L-NAME
Jejunum
Ileum
Colon
pg LTB4/mg tissue
0
5
10
15
Sh
2
24
48
+
Time post-irradiation (h)

statistically significant (Fig.28). In comparison to L-NAME pretreated shams, tissue from L-NAME pretreated animals at 24 and 48 h post-irradiation produced significantly less LTB_4. LTB_4 production was decreased by 71% at both 24 and 48 h post-irradiation ($p<0.05$)(Fig.28). It should be noted that this effect was an effect of radiation on L-NAME pretreated rats rather than an effect of L-NAME.

D. Histology

The acute effects of gamma radiation on the mammalian intestine are without any major histological change. Our results confirm previous research on the acute effects of radiation on intestinal morphology. We scored histological sections on the basis of villar epithelial detachment and/or subepithelial oedema Figure 29. Representative histological sections of jejunum, ileum and colon are presented in Figures 30, 31 and 32, respectively.

There were no differences between shams and irradiated tissues nor saline and L-NAME treatment groups in any region at any of the time points (2, 24, 48 h post-irradiation) studied. In general, there was some epithelial detachment in all sections, however, the majority of the villi appeared normal. There was no epithelial denuding nor any obvious villar shortening in any region.

Figure 29. Percentage of villi with epithelial detachment or subepithelial edema. Histological sections from the jejunum and ileum of rats 2 h after sham treatment and at 2, 24 and 48 h post-irradiation. Rats were pretreated (1 h prior to sham treatment or irradiation) with either saline (open) or L-NAME 100 mg/kg (hatched). Neither radiation nor L-NAME caused any major alterations in epithelial detachment or subepithelial edema ($^{x}p<0.05$ compared to the saline pretreated group). Values represent the mean±SEM for $n \geq 4$.

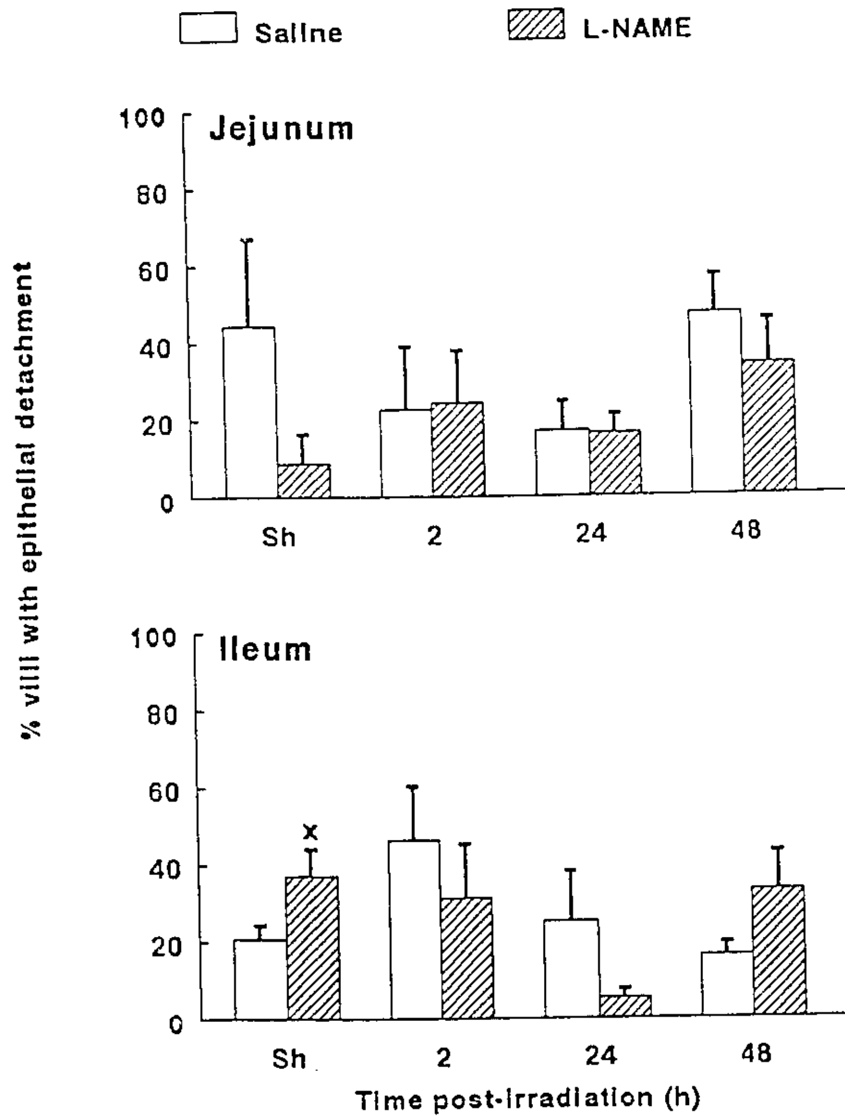
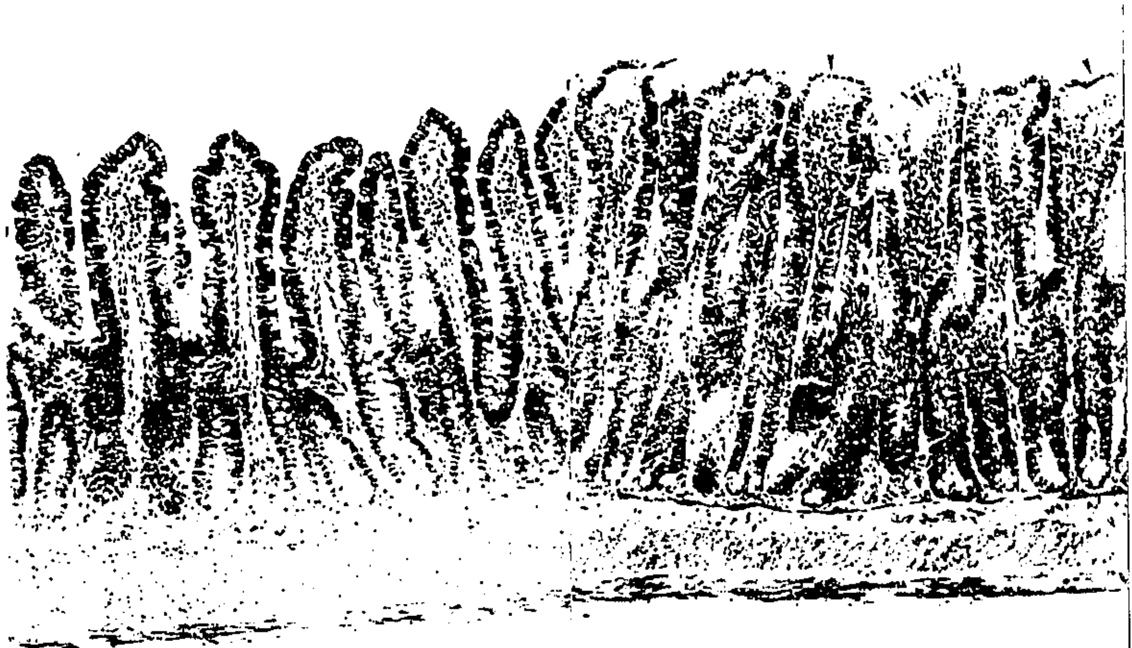

Saline
L-NAME
Jejunum
100
80
60
40
20
0
Sh
2
24
48
% villi with epithelial detachment
Ileum
100
80
60
40
20
0
x
Sh
2
24
48
Time post-irradiation (h)

Figure 30. Full thickness cross section of jejunal tissues from rats after sham treatment (a) or at 2 (b), 24 (c), or 48 h (d) post-irradiation. Representative sections from either saline or L-NAME pretreated rats are displayed as there were no major changes in villar architecture, epithelial detachment or subepithelial edema between treatment groups. Epithelial detachment (arrowheads), subepithelial edema (double arrowheads) and breaks in the epithelial barrier (small arrows). Note epithelial sloughing in (a) marked by the (*). The scale bar =100 μm.

a
b

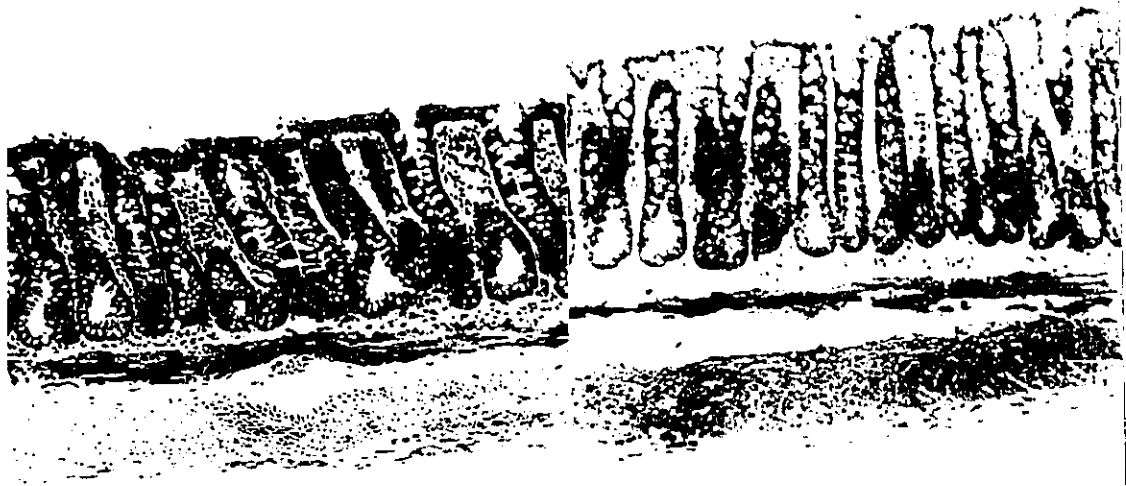
c
d

Figure 31. Full thickness cross section of ileal tissues from rats after sham treatment (a) or at 2 (b), 24 (c), or 48 h (d) post-irradiation. Representative sections from either saline or L-NAME pretreated rats are displayed as there were no major changes in villar architecture, epithelial detachment or subepithelial edema between treatment groups. At 48 h post-irradiation epithelial cell rounding is noticeable (d). Epithelial detachment (arrowheads), subepithelial edema (double arrowheads) and breaks in the epithelial barrier (small arrows). The scale bar =100 μm.


a
b

c
d

Figure 32. Full thickness cross section of colonic tissues from rats after sham treatment (a) or at 2 (b), 24 (c), or 48 h (d) post-irradiation. Representative sections from either saline or L-NAME pretreated rats are displayed as there were no major changes in surface architecture, epithelial detachment or subepithelial edema between treatment groups. There were no noticeable flaws in the colonic epithelium. The scale bar =100 μm.



a b

c d

DISCUSSION

The overall objective of this study was to determine if the radiation-induced decrease in epithelial responsiveness could be attributable to an increase in nitric oxide synthase (NOS) activity in the gut. The effects of inhibiting NOS activity were investigated in order to test the hypothesis that decreased NOS activity would ameliorate the radiation-induced decrease in stimulated electrolyte transport.

The major findings of this study were 3-fold. First, radiation altered NOS activity in the gut in a region-specific manner. Total NOS (tNOS) activity in the jejunum was significantly attenuated within 0.5 h post-irradiation but returned to control levels thereafter. In the ileum, tNOS activity was significantly increased at 2 h post-irradiation, and gradually returned to control levels between 24 and 48 h post-irradiation. In the colon, radiation had no significant effect on NOS activity. Second, systemic administration of the NOS inhibitor, L-NAME, significantly attenuated tNOS activity in the gut via inhibition of cNOS specifically. Third, contrary to the hypothesis, the inhibition of NOS activity accentuated the decreased Isc response to neurally-evoked stimulation. The inhibitory effects of L-NAME were the greatest in the colon of shams where cNOS activity was 95% of tNOS. L-NAME had less effect on the jejunum and ileum of shams where cNOS activity was only 70% tNOS.

Effects of radiation

Radiation significantly decreased the short circuit current (Isc) responses to EFS and PGE_2 in the jejunum and ileum 48 h post-exposure, confirming and extending our previous studies (MacNaughton *et al.*, 1994; MacNaughton *et al.*, 1992). In addition, radiation decreased the Isc

response to EFS in the colon by 24 h post-irradiation. Thus, the results indicate that the effects of radiation on the Isc response to EFS are consistent throughout the gut and appear to be time-dependent. Similarly, studies on the rabbit ileum by Gunter-Smith (1986) indicate that the effects of irradiation on basal Isc are time-dependent. This investigator demonstrated that radiation caused a dose-dependent increase in basal Isc between 1 and 24 h post-irradiation that was due to elevated net Cl^- secretion.

Radiation is known to inhibit DNA replication and the gut is a primary target for this effect as the intestinal epithelium is renewed approximately every 4 days. Radiation can therefore inhibit the regenerative processes of the intestine, increasingly compromising the barrier and transport function of the gut with time post-irradiation. Acutely, radiation exposure may result in villar shortening and a decreased nutrient absorptive capacity (Thomson *et al.*, 1989). However, histological evidence suggests that there is no actual denuding of the epithelium (Buell and Harding, 1989), although, radiation does loosen tight junctions (Mourad *et al.*, 1993) and cause crypt abscess formation within 24 h post-irradiation (Buell and Harding, 1989). Furthermore, radiation has been shown to decrease epithelial resistance in the rat ileum within 2 h and up to 48 h post-irradiation (MacNaughton *et al.*, 1994). Thus, epithelial barrier function may be disrupted altering epithelial transport function as a result of decreased transepithelial resistance. In addition, abscess formation may allow for greater microbial infiltration through the compromised crypt barrier region thereby stimulating an inflammatory response and intestinal secretion. The prosecretory state of the gut under these conditions may cause diarrhea.

Diarrhea, nausea and vomiting are among the chief symptoms of patients undergoing radiation therapy (Tarpila, 1971). Research suggests that the effects of radiation on gut transport

are mediated by an increased Cl^- flux in the serosal to mucosal direction (Gunter-Smith, 1986). In our study, we did not measure individual ion fluxes and therefore we cannot identify which particular ions are responsible for the secretory response. However, it is most likely that stimulation of our tissue preparation led to an inhibition of Na^+ and Cl^- absorption with a concurrent increase in Cl^- secretion as this is the common mechanism by which a secretory response in the mammalian intestine is elicited (Cooke, 1989; Binder and Sandle, 1987).

An initial hypothesis which the present study was designed to test was that radiation-induced electrolyte transport dysfunction was due to the development of an acute inflammatory response. This was based on earlier studies by Buell and Harding (1989) who demonstrated, using the same model of irradiation, that an acute inflammatory response was induced by 10Gy γ-irradiation, however, histologically little change occurred at the epithelial level. The inflammation was characterized by the infiltration of polymorphonuclear granulocytes as early as 2 h and up to 24 h post-irradiation in the jejunum, ileum and colon. Later work by Harding *et al.* (1990) identified an increase in the production of the inflammatory mediator LTB_4 in the intestine as early as 6 h after 10Gy γ-irradiation. Other research (MacNaughton *et al.*, 1992) has shown that myeloperoxidase activity (MPO), an indicator of granulocyte infiltration, is increased in the jejunum at 2 h post-irradiation and is associated with a decrease responsiveness to exogenous PGE_2.

Contrary to the inflammatory effects of radiation mentioned above, an important observation of the present study was that the hyporesponsiveness of the gut occurred in the absence of a major inflammatory response. This was concluded by the lack of any major increase in PGE_2 or LTB_4 synthesis. Furthermore, the histological data revealed remarkably

normal morphology. At each time post-irradiation, and in shams there was only mild oedema with isolated areas of epithelial detachment and little loss of mucosal integrity. This phenomenon of altered physiological mechanics in the absence of inflammation and/or tissue damage has been previously documented in our laboratory (MacNaughton *et al.*, 1994). MacNaughton *et al.* (1994) reported that, although radiation did not increase MPO activity in the rat ileum, it did cause a decrease in the Isc response to EFS and PGE_2. However, that study and others (Harding *et al.*, 1990) identified a decrease in tissue histamine levels 24 h post-irradiation suggesting that mast cell degranulation may be involved. Early studies by Conte *et al.* (1956) demonstrated that mesenteric mast cell numbers decreased with increasing dose and time post-irradiation. Thus, in the absence of increased synthesis of inflammatory mediators and/or morphological changes, radiation caused a significant change in electrolyte transport which may be in part attributable to mast cell degranulation. It is clear that in the present study the transport dysfunctions observed occur in the absence of the acute inflammatory response which likely contributes to the secretory anomalies in other inflammatory diseases of the gut such as Crohn's disease or ulcerative colitis (Shanahan, 1993; Sartor, 1994; Boughton-Smith *et al.*, 1993).

Regional differences in epithelial mucosal to serosal permeability (Pantzar *et al.*, 1994) of the intestine may contribute to the differences in radiation-induced alterations in electrolyte transport in the present study. Radiation is known to increase epithelial permeability by loosening tight junctions (Porvaznik, 1979) which may facilitate microbial penetration of the epithelial barrier. As the colon is leakier than the small intestine, the colon would be more susceptible to microbial invasion upon further increases in epithelial permeability. In addition, the lumenal environment of the colon predisposes this region to microbial invasion.

Radiation, electrolyte transport and NOS activity: Jejunum

NO has been identified as a secretagogue in the jejunum of the guinea-pig *in vitro* (MacNaughton, 1993) and the rat *in vivo* (Mourad *et al.*, 1993). In the guinea-pig the Isc response to the NO donor, sodium nitroprusside (SNP), appeared to be mediated by both prostaglandins and serotonin, which ultimately stimulated an increase in net Cl^- secretion. On the contrary, Mourad *et al.* (1993) determined that the inhibition of NO synthesis by luminal instillation of the NOS inhibitor, L-NAME, in the rat jejunum *in vivo* stimulates secretion suggesting that NO is proabsorptive. Thus, the role of NO as a modulator of electrolyte transport is not clear. The current uncertainty may be due to species or experimental differences. Thus, the concept of NO as a secretory modulator warrants further attention.

The first objective of this study was to determine if radiation-induced alterations in electrolyte transport were due to corresponding increases in NOS activity in the gut. It appears that NO is not crucial to jejunal electrolyte transport. Although total NOS (tNOS) activity was significantly decreased in the jejunum at 0.5 h post-irradiation it is unlikely that electrolyte transport was altered as a comparable decrease in tNOS activity in L-NAME pretreated shams had no effect on electrolyte transport. Thus, it appears that endogenous NO does not play a major role in the modulation of electrolyte transport in the rat jejunum. This is further substantiated by the lack of any change in electrolyte transport at 2 h post-irradiation when NOS activity was significantly attenuated by L-NAME. The inhibitory effect of L-NAME was specific to cNOS and appeared to decrease NOS activity consistently in the jejunum up to 24 h post-irradiation. However, at 0.5 h the radiation-induced decrease in cNOS precludes the effect of L-NAME. These results provide information for the future use of L-NAME as an inhibitor of NO

both with respect to its preferential target and the duration of its effects.

In the absence of a role for NO in the jejunal electrolyte transport, radiation alone decreased the Isc response to both EFS and PGE_2 by 48 h post-irradiation. At this time point there were also no noticeable changes in PGE_2 or LTB_4 synthesis in this tissue. Thus, in the absence of a major inflammatory response or an increase in NO synthesis, changes in the jejunal Isc response may be attributable to radiation-induced alterations of the jejunal microvasculature (Carr *et al.*, 1984), epithelial barrier (Porvaznik, 1979; MacNaughton *et al.*, 1994), and/or mast cell stability (Conte *et al.*, 1956; MacNaughton *et al.*, 1994).

With respect to NO, the decrease in jejunal tNOS activity at 0.5 h post-irradiation was solely attributable to a decrease in Ca^{2+}-dependent cNOS activity. Other studies have identified a decrease in NO synthesis as early as 1 h post-irradiation (Veovodskaya and Vanin, 1992). These investigators suggested that the acute increase of radical oxygen species which occurs post-irradiation may have scavenged NO, making it undetectable by electroparamagnetic resonance spectrometry. However, in our system for measuring NOS activity, based on citrulline production, the scavenging effects of radical oxygen species would not interfere significantly with the measurement of NOS activity. Therefore, if radical oxygen species were responsible for the decrease in NOS activity at 0.5 h post-irradiation their effect may have been on the enzymatic activity of cNOS. However, it is unlikely that these effects would be region specific as rats underwent whole body exposure to radiation, which would preclude differential exposures of various regions of the gut.

Radiation , NOS activity and electrolyte transport: Ileum

In the ileum, radiation stimulated iNOS activity by 2 h, resulting in a tripling of tNOS activity. iNOS activity decreased with time returning to control levels between 6 and 24 h post-irradiation, whereas tNOS activity remained elevated until after 24 h. These time points for the activation of iNOS at 2 h, and the subsequent inactivation by 24 h, correspond exactly with the findings of Tomlinson *et al.* (1994). These researchers, using a rat model of carrageenin-induced pleurisy, reported the induction of iNOS at 2 h and the inactivation by 24 h in the cell pellet of pleural exudates. These investigators also suggested that the increased synthesis of NO may be responsible for the decrease in iNOS activity at 24 h (Tomlinson *et al.*, 1994).

The negative feedback effects of NO on NOS activity have been described for both rat cerebellar cNOS (Rengasamy and Johns, 1993) and iNOS from the rat pulmonary macrophage cell line (NR8383) (Griscavage *et al.*, 1993). In these studies the addition of NO or commonly used NO donors such as S-nitroso-*N*-acetylpenicillamine (SNAP), dose-dependently inhibited NOS activity in a reversible fashion. Furthermore, Luss *et al.* (1994) demonstrated that in the liver, inhibition of NOS activity can increase iNOS mRNA and protein expression, implying that NO can also inhibit the induction of iNOS (Luss *et al.*, 1994). Indeed, we have shown that L-NAME pretreatment increases iNOS mRNA expression in the rat intestine (Aurora *et al.* unpublished observations). Thus, radiation-induced NO production may cause the decrease in iNOS activity which occurs by 24 h post-irradiation in our study.

Numerous studies have identified NO as a stimulator of water and electrolyte absorption in the mammalian ileum (Mailman, 1994; Rao *et al.*, 1994; Barry *et al.*, 1994). *In vivo* perfusion of the rat (Mailman, 1994) and the rabbit (Barry *et al.*, 1994) ileum with NOS inhibitors stimulate

secretion, suggesting that endogenous NO is proabsorptive. In these studies the addition of NO synthesis substrate, L-arginine, reversed the secretory effect of NOS inhibitors. However, L-arginine alone did not significantly alter water and electrolyte absorption suggesting that endogenous levels of L-arginine are maximal for NO synthesis. *In vitro* studies on the mouse ileum also identified NOS inhibitors as prosecretory, the effect being reversed by L-arginine but not D-arginine. The prosecretory effect of NOS inhibition was neurally mediated as the neurotoxin, tetrodotoxin (TTX), blocked L-NMA-induced Cl^- secretion (Rao *et al.*, 1994). Furthermore, the addition of the NO donor, sodium nitrite, caused a decrease in Isc which was mediated by cGMP. Thus, it appears that NO has a proabsorptive role in the ileum under physiological conditions.

In the present study, the radiation-induced increase in iNOS activity in the ileum did not have any effect on the Isc response to EFS, however, changes in iNOS activity at 2 and 24 h post-irradiation were negatively correlated with the Isc response to PGE_2. For example, tNOS activity was increased at 2 h when the Isc response to PGE_2 was decreased. In addition, when iNOS activity normalized at 24 h post-irradiation the Isc response to PGE_2 returned to control levels. This qualitative, temporal negative correlation provides circumstantial evidence that increased levels of NO may be responsible for the decreased Isc response of the ileum to exogenous PGE_2.

While the mechanism whereby NO may downregulate the response to PGE_2 is unclear, it may be related to the stimulatory effect of NO on PGE_2 synthesis which may result in a decrease in EP_2 receptor density. Increased PGE_2 synthesis has been found to downregulate eicosanoid receptors in the liver (Barry *et al.*, 1994). Furthermore, eicosanoid synthesis by both

the constitutive and inducible isoforms of cyclo-oxygenase (cCOX and iCOX) can be stimulated by NO as determined by studies in the Islets of Langerhans (Corbett *et al.*, 1993) and the macrophage cell line RAW264.7 (Salvemini *et al.*, 1993). Thus, the stimulatory effects of NO on COX activity may have resulted in an increase in PGE_2 synthesis causing a downregulation of EP_2 receptors and the subsequent decreased Isc response to PGE_2 in the ileum.

Similarly, research suggests that PG synthesis is increased in macrophages as iCOX expression is induced after treatment with the cytokine IL-1β (Salvemini *et al.*, 1993). Tomlinson *et al.* (1994) also noted the induction of iCOX activity as early as 2 h, in the pleural exudates of rats injected with carrageenin, and that this activity was lost by 24 h. This suggests that a decrease in iCOX activity and therefore PG synthesis at 24 h may return EP receptor density to normal levels. Thus, aside from the effects of NO on COX activity the pathophysiological time course of iCOX alone may be integral to the changes in the response to exogenous PGE_2 found in the present study. Further studies on iCOX expression in this model will be necessary in order to substantiate this possibility.

In spite of evidence which suggests that NO is stimulatory to COX, at 2 h post-irradiation when radiation-induced NOS activity was maximal in the ileum, PGE_2 synthesis was not significantly increased. In fact, PGE_2 synthesis was significantly decreased at 24 h post-irradiation. It is possible that 2 h post-irradiation is not sufficient time for iCOX expression. However, 24 h is sufficient for iCOX expression (Vane *et al.*, 1994). This may suggest that NO is inhibitory rather than stimulatory, with respect to COX activity in this tissue. Indeed, Stadler *et al.* (1993) demonstrated the inhibitory effects of NO on PGE_2 synthesis in rat Kupffer cells (Stadler *et al.*, 1993). Therefore, in contrast to NO stimulating PGE_2 synthesis perhaps the

inhibitory effect of NO on COX resulted in decreased PGE_2 synthesis at 24 h post-irradiation, and a resultant upregulation of EP_2 receptors caused a normal secretory response to exogenous PGE_2 masking the radiation-induced hyporesponsiveness.

By 48 h post-irradiation ileal NOS activity and PGE_2 synthesis had returned to control levels. In turn the ileal Isc response to PGE_2 was decreased again at this time point reflecting the effects of radiation on electrolyte transport in the absence of changes in NOS and/or COX. The effects of NO on electrolyte transport and PGE_2 synthesis in this tissue remain undetermined although in this study it does appear that NO is more likely an inhibitor than a stimulator of COX activity.

Effect of L-NAME pretreatment: Ileum

The inhibition of NOS activity at 24 h post-irradiation by pretreatment with L-NAME significantly decreased the Isc response to EFS and PGE_2 suggesting that the increase in NO synthesis caused by radiation is vital to the maintenance of normal intestinal transport mechanisms in the ileum. L-NAME also significantly attenuated the Isc response to EFS and PGE_2 at 2 h post-irradiation without significantly decreasing NOS activity at this time. This suggests that L-NAME pretreatment alone cannot entirely account for the decreased Isc response at 2 and 24 h post-irradiation. These results imply that the lack or decrease of NO synthesis exacerbates the detrimental effects of radiation on the Isc response to EFS and PGE_2 but may not significantly alter electrolyte transport on its own. Therefore, it appears that NO has a cytoprotective role in the irradiated rat ileum. Such a protective effect may be due to a number of physiological mechanisms which NO modulates including vascularization, epithelial

permeability, mast cell degranulation, neurotransmission, electrolyte transport and neutrophil infiltration.

In the present study, any affects of L-NAME on intestinal vasculature would only have direct effects *in vivo*, some of which may have effected the tissue function *in vitro*. L-NAME has been demonstrated to cause macroscopic and microscopic ischemia in the rat jejunum (Mourad *et al.*, 1993). The importance of NO in counteracting the injurious vascular actions of endogenous arginine-vasopressin by maintaining intestinal mucosal integrity have also been documented (László and Whittle, 1994). Furthermore, NO is integral to the maintenance of intestinal epithelial integrity as Kubes (1992) demonstrated that the inhibition of endogenous NO synthesis by treatment with L-NAME resulted in an almost six-fold increase in mucosal permeability (Kubes, 1992). This group went on to identify the mast cell as a key player in the L-NAME-induced increase in epithelial permeability (Kanwar *et al.*, 1994). In addition, MacNaughton *et al.* (1994) demonstrated that pyrilamine, an H_1 receptor antagonist, inhibited the radiation-induced decrease in neurally-evoked transport (MacNaughton *et al.*, 1994) suggesting a role for histamine. In the present study, during the time before experimentation, 2 - 48 h, L-NAME may have had affects which altered mucosal function and mast cell stability *in vivo*. These effects may have subsequently been carried over to the function of the tissue *in vitro*.

Since the L-NAME effect at 24 h post-irradiation was associated with a decrease in cNOS activity, physiological mechanisms such as neurotransmission which are solely cNOS-dependent would likely be affected. However, L-NAME pretreatment of irradiated rats not only decreased neurally-evoked transport but also PGE_2 stimulated transport. Thus, the effects of L-NAME were not solely attributable to its effect on neurotransmission as this would not alter the Isc response

to PGE_2. It is likely that L-NAME inhibition of NO synthesis affected the epithelial cells directly.

Cyclic GMP is known to be a potent stimulator of intestinal secretion in the rat small intestine (Field *et al.*, 1978) and NO stimulates guanylate cyclase (GC) activity (Schmidt *et al.*, 1991; Palmer and Higgs, 1989). As GC activity is primarily located in the microvillar plasma membrane of the absorptive villar enterocytes (Quill and Weiser, 1975), it is likely that the effects of NO on epithelial regulation of electrolyte transport are via inhibition of NaCl absorption. Thus, NO may stimulate GC activity resulting in an increase in cGMP production and the consequent inhibition of the primary absorptive mechanism in the small intestine. The net effect would be an increase in net fluid and electrolyte secretion and L-NAME would inhibit this secretory effect.

The prosecretory role of NO in the gut is supported by the findings of Capasso's group (Mascolo *et al.*, 1994; Izzo *et al.*, 1994). These investigators demonstrated that intraperitoneal administration of the NOS inhibitor, L-NAME, significantly attenuated sodium-choleate- or carbachol-induced diarrhea by approximately 80% in rats and mice, respectively. Furthermore, Donowitz *et al.* (1980) identified the importance of extracellular calcium in 5-HT-induced ileal electrolyte secretion in the rabbit ileum (Donowitz *et al.*, 1980). 5-HT, a secretagogue released from both mast cells and enterochromaffin cells, increased the serosal uptake of Ca^{2+} resulting in a decrease in NaCl absorption and an increase in Cl^- secretion. In addition, the secretory effect of serotonin was inhibited by the Ca^{2+} channel blocker verapamil or the addition of Ca^{2+}-chelator, EGTA, to the serosal bathing medium. Others have shown that the 5-HT_3 receptor antagonist, BRL43694, inhibits NO-induced Cl^- secretion in the guinea pig ileum *in vitro* (MacNaughton,

1993). In addition, these investigators concluded that the NO-induced secretory response may be mediated by the activation of GC. Thus, the role of Ca^{2+} in intestinal electrolyte transport and a potential role for NO in the Ca^{2+}-mediated pathway of net Cl^- secretion in the mammalian ileum is evident.

Neutrophil infiltration is a hallmark of acute inflammation and results in the release of inflammatory mediators which can cause a copious secretory response in the intestine. NO has been found to inhibit leukocyte adhesion in the rat mesentery and human umbilical vein endothelial cells (Kubes *et al.*, 1993). Systemic administration of the NOS inhibitor, L-NAME, induced leukocyte adhesion, an effect that was both superoxide and mast cell dependent. These investigators suggested that NO neutralizes superoxide-mediated mast cell degranulation, thereby inhibiting the release of pro-inflammatory agents. Thus, the inhibition of NOS with L-NAME administration in the present study, in addition to augmenting mast cell degranulation, may have induced leukocyte influx. The inflammatory cell infiltrate may have contributed to the altered Isc response in this tissue at 2 and 24 h post-irradiation. However, we did not observe any granulocyte influx at any time point in this study.

These results indicate that the role of NO in electrolyte transport may be more tightly linked to the regulation of endogenous levels of NO predominantly synthesized by cNOS. This is evidenced by the lack of elevated iNOS activity or PGE_2 and LTB_4 at 24 h post-irradiation suggesting that the changes in transport are not due to inflammation.

Radiation, electrolyte transport and NOS activity: Colon

Radiation significantly attenuated the colonic Isc response to EFS at 24 and 48 h and

PGE_2 at 24 h post-irradiation. NO and the NO donor, SNP, have been identified as stimulators of electrolyte transport in the rat colon *in vitro* (Tamai and Gaginella, 1993). Both NO and SNP stimulated Cl^- secretion in a concentration dependent fashion which was partially prostaglandin-dependent. Furthermore, TTX and methylene blue (an inhibitor of guanylate cyclase) blocked this response suggesting that the secretory responses to NO and SNP were neurally mediated and cGMP dependent (Tamai and Gaginella, 1993).

In the present study, however, radiation did not significantly alter NOS activity in the colon at any time post-irradiation. Changes in NOS activity cannot account for the radiation-induced decrease in the Isc response to EFS and PGE_2 at 24 h and EFS at 48 h in this tissue. Furthermore, radiation did not significantly alter PGE_2 or LTB_4 synthesis in the colon. Thus, the radiation-induced decrease in the Isc response to EFS was independent of changes in NO, PGE_2 or LTB_4 synthesis. As previously documented in the ileum (MacNaughton *et al.*, 1994), the alterations in electrolyte transport in the absence of any obvious inflammatory response may be due to radiation-induced mast cell degranulation (Conte *et al.*, 1956) and the release of histamine (MacNaughton *et al.*, 1994).

Effect of L-NAME pretreatment: Colon

In our study, the inhibition of NOS activity by pretreatment with L-NAME significantly attenuated tNOS activity in the colon of shams and irradiated rats for up to 24 h post-irradiation. This is similar to the inhibitory effect of L-NAME on NOS activity in the jejunum and confirms the duration and efficacy of a single prophylactic dose of L-NAME. In addition, L-NAME pretreatment significantly decreased the Isc response to EFS in shams and at 2 h post-irradiation

suggesting that NO plays a major role in the regulation of neurally-evoked colonic electrolyte transport. However, L-NAME pretreatment had no effect on the Isc response to exogenous PGE_2. This evidence implies the primary regulatory role of NO in colonic electrolyte transport is a component of the enteric nervous system. This is further supported by the fact that the decrease in tNOS activity was attributable to the inhibitory effects of L-NAME on cNOS, the sole source of NO in neurons. Furthermore, the results provide more evidence for the role of L-NAME as a preferential inhibitor of cNOS.

In the gut, NO acts via presynaptic inhibition of cholinergic motor neurons (Bult *et al.*, 1990; Hryhorenko *et al.*, 1994) suggesting that L-NAME would enhance neurally-evoked secretion if similar pathways are present at the mucosal level. This is in contrast to our results and there are a number of possibilities which may account for this incongruity. First, there may be prosecretory nitrergic pathways in the gut which are as yet unidentified. Second, the removal of endogenous inhibitory neuronal inputs by pretreatment with L-NAME may result in a prosecretory state of the gut, causing a decreased ability to respond to further neuronal input. Third, both epithelial and neuronal mechanisms, unique to each region and dependent on cNOS activity, may be altered. Fourth and most probable is that the decrease of endogenous NO resulted in further mast cell degranulation in addition to that already induced by radiation alone. Kanwar *et al.* (1994) demonstrated the effects of L-NAME-induced mast cell degranulation on epithelial integrity identifying the role of NO in stabilizing mast cells. Histamine released from mast cells could act as a presynaptic inhibitor at nicotinic synapses as well as inhibit the release of 5-HT from enterochromaffin cells via H_3 receptors (Cooke, 1994). Alternatively, histamine may also act at H_1 receptors on enterocytes stimulating Cl^- secretion and at nicotinic synapses

to potentiate synaptic transmission.

At the epithelial level NO may modulate colonic electrolyte transport by a number of mechanisms. NO is thought to stimulate PGE_2 synthesis or release (Salvemini *et al.*, 1995), resulting in a secretory response at crypt enterocytes. NO also binds GC stimulating cGMP production (Schmidt *et al.*, 1991; Palmer and Higgs, 1989), which in villar enterocytes could inhibit absorption. Therefore, L-NAME would inhibit both these mechanisms resulting in an overall decreased secretory response. However, exogenous PGE_2 could override the inhibitory effects of L-NAME resulting in a normal secretory response thus explaining the difference in response to EFS and PGE_2.

Selective NOS isoform inhibition

NO is an important mediator of numerous physiological functions but at higher concentrations NO can be cytotoxic (Nathan, 1992; Moncada *et al.*, 1991). Major increases in NO synthesis are usually due to the induction of Ca^{2+}-independent iNOS. Under inflammatory conditions it may be of use to target the specific isoform of NOS responsible for increased NO synthesis without altering the activity of the other.

In the present study, L-NAME did not significantly inhibit the radiation-induced increase in tNOS activity in the ileum at 2 h but did significantly inhibit tNOS activity in the jejunum and colon at this time. The increase in tNOS activity in the ileum at 2 h post-irradiation was predominantly due to iNOS suggesting that regional differences in the effects of L-NAME may be due to a lack of affinity of L-NAME for iNOS. Evidence in the literature suggests that L-NAME is not effective on iNOS (Wu *et al.*, 1995) but does affect the vasculature (László *et al.*,

1994) which is primarily cNOS-dependent under physiological conditions. L-NAME has not yet been identified as an inhibitor of a specific NOS isoform. It is possible that the concentration of L-NAME used in the present study was inadequate to inhibit the increased NOS activity in the ileum at 2 h post-irradiation. Pretreatment with 3 times the dose of L-NAME (300 mg/kg s.c.) significantly attenuated the radiation-induced increase in tNOS activity in the ileum at 2 h. This higher dose of L-NAME decreased both cNOS and iNOS activity, however, the inhibitory effect of L-NAME was only significant on the constitutive form of the enzyme. This evidence suggests that L-NAME selectively targets cNOS in the rat gut.

In our study, L-NAME primarily targeted cNOS, although high concentrations of L-NAME inhibited the radiation-induced increase in iNOS activity, however this did not reach statistical significance. By increasing the concentration of L-NAME further, it may have been possible to significantly inhibit iNOS activity at 2 h post-irradiation in the ileum. However, administration of a higher dose of L-NAME was contraindicated, as at 300 mg/kg the effects of L-NAME were stressful to the animal. It is likely that a high dose of L-NAME would increase vascular resistance to critical levels.

An important factor to consider when attempting to inhibit NOS activity is the enzymatic source(s) of NOS activity which is producing the augmented levels of NO. For example, in the present study iNOS activity was elevated in the ileum but not in the jejunum or colon and was the source of increased tNOS activity. Thus, systemic administration of an inhibitor which may inhibit tNOS activity but does not specifically target iNOS may be detrimental to tissues relying on physiological levels of NO. This appeared to be the case with the administration of the high concentration of L-NAME which did significantly inhibit tNOS activity but also caused

distress to the animals. This was likely due to the inhibitory effect of L-NAME on cNOS activity as evidenced by our results. As cNOS is the major source of NO production for both vascular homeostasis and numerous neuronal pathways among other physiological processes it is likely that the overall effect of L-NAME was more damaging than beneficial. Considering the inefficacy of L-NAME at inhibiting iNOS activity and the injurious effects it may cause at higher doses the search for a more specific iNOS inhibitor was warranted.

Aminoguanidine (AG) has been identified as a selective inhibitor of iNOS (Griffiths *et al.*, 1993), which can inhibit the lethal vasodilatory effects of endotoxic shock in rats, increasing survival by nearly 70% (Wu *et al.*, 1995). However, others (Laszlo *et al.*, 1995) have shown that in the absence of iNOS, AG increased vascular resistance suggesting that AG can inhibit the constitutive form of NOS *in vivo*. Thus AG should be considered as an iNOS preferring, not selective, inhibitor. To determine if radiation-induced iNOS activity in the ileum could be selectively inhibited, we administered AG (100 mg/kg) systemically. AG attenuated tNOS activity, in the ileum at 2 h post-irradiation, via inhibition of iNOS and without altering cNOS activity. Furthermore, AG did not alter cNOS activity in the jejunum or colon. These results clearly identify L-NAME as a selective cNOS inhibitor and AG as a selective iNOS inhibitor in the rat intestine.

As other researchers have demonstrated the inhibitory effects of AG on cNOS in the absence of elevated levels of iNOS activity caution should be taken with respect to the conditions of administration of a particular inhibitor. AG increased blood pressure and plasma leakage from both the ileum and colon (Laszlo *et al.*, 1995). In our study, the possibility that AG may have had other effects during the first few hours before the induction of iNOS activity cannot be

disputed as we did not take blood pressure measurements or determine plasma leakage at any time point in this study. However, we did observe a significant difference between rats treated with endotoxin and those treated with AG and endotoxin. The endotoxin treated rats which received AG were noticeably less stressed and appeared more comfortable than did rats which received endotoxin alone. This simple qualitative observation suggests that at the dose administered, AG effectively counteracted the endotoxin-induced discomfort the rats encountered and did not appear to have any injurious effects which may be outwardly displayed.

Regional differences in the profile of NOS activity

The regional differences in the profile of NOS activity and the differences in the effects of the induction of iNOS activity in the radiation model were supported by NOS activities determined using the endotoxin model. A number of investigators have described the induction of NOS activity in the intestine after systemic administration of bacterial endotoxin (Tepperman *et al.*, 1993; Boughton-Smith *et al.*, 1993; Boughton-Smith *et al.*, 1994). Ca^{2+}-independent NOS activity was increased by 4 h after endotoxin treatment in rat jejunum and colon (Tepperman *et al.*, 1993; Boughton-Smith *et al.*, 1993; Boughton-Smith *et al.*, 1994). The increases were comparable to those seen in our experiments with endotoxin-induced NOS activity. Interestingly, in our experiments we measured NOS activity in full thickness tissue whereas Whittle's group measured NOS activity in isolated epithelial cells in some experiments (Tepperman *et al.*, 1993). When compared, their studies (Tepperman *et al.*, 1993; Boughton-Smith *et al.*, 1993) suggest that the majority of inducible NOS activity in the gut is located in the epithelial cells. Indeed, when comparing the induction of NOS activity in the colonic muscle layers to mucosal tissue these

researchers demonstrated that Ca^{2+}-independent iNOS activity was predominantly mucosal (Boughton-Smith *et al.*, 1994). Furthermore, these investigators also noted that of the isolated epithelial cells, iNOS activity was primarily present in villar enterocytes (Tepperman *et al.*, 1993). Similarly, Zembowicz *et al.* (1995) identified strong iNOS immunoreactivity in the villar epithelial cells lining the ileum of endotoxin treated rats whereas crypt cells had a very weak signal. Other unidentified cells in the lamina propria were positive for iNOS immunoreactivity but minimal compared to that of the epithelial layer.

In the present study, basal NOS activity in the colon was ~95% cNOS dependent whereas basal NOS activity in the jejunum and ileum was only ~70% cNOS dependent in both the endotoxin and the radiation model. Similar to NOS activity in the radiation model, in the endotoxin model there were regional differences in the induction of NOS activity. However, endotoxin caused the induction of iNOS in all regions of the gut with the ileum being most responsive and the jejunum being the least. In addition to supporting the findings of the radiation study these results confirm that regional differences in the distribution of cNOS and iNOS exist in the rat intestine. These differences may provide clues to unanswered questions of earlier studies as well as providing important information for future studies of the gut. These findings imply that the ileum has a greater capacity to synthesize NO. Whether this supply of NO is important with respect to its cytotoxic effect on infiltration of microbes or its influence on the vasculature, neurotransmission, and epithelial cell transport are important questions which need to be addressed. One may hypothesize that since the jejunum does not encounter a great deal of enteric flora, it does not require a great cytotoxic resource. The colon, on the other hand, has a large resident population of microbes and may require more protection against potential

infiltration of pathogenic organisms. By and large the ileum would be most susceptible to the detrimental effects of invasive pathogens as it does not normally encounter such organisms. In the case of reflux from the colon or caecum, pathogenic organisms could very easily access the ileal lumen and possibly penetrate the epithelial barrier eventually causing sepsis. The ileum, having a high capacity for the induction of NOS, could better defend against such an invasion. In addition, the influence of NO on ileal vascularization may facilitate the clearance of toxic substances from this region and provide a greater blood supply for the impending secretory response.

In the present study, systemic administration of AG inhibited tNOS activity in the ileum and colon of endotoxin treated rats. The effect of AG was entirely due to its inhibitory action on iNOS, having no effect on cNOS in any tissue. AG also significantly attenuated iNOS in the jejunum but not to a great enough extent to inhibit tNOS activity. These results further support the findings of the radiation study which identify AG as an iNOS preferring inhibitor. In addition, the specific inhibitory effects of this inhibitor *in vivo* have not yet been documented in the literature.

Conclusion

These studies have identified a very definite role for NO in the radiation-induced alterations in electrolyte transport in the rat intestine. However, the role of NO in the modulation of electrolyte transport varies from the jejunum to the colon. Although NOS activity is present and inhibitable with L-NAME in the jejunum, NO does not appear to play a major role in the mediation of electrolyte transport in this tissue. In the ileum and colon NO mediates electrolyte

transport as inhibition of NOS activity with L-NAME significantly depressed the Isc response to the applied stimuli in irradiated tissues. Furthermore, the regulation of colonic electrolyte transport appears to be more dependent on NO than the ileum as the inhibition of NOS activity with L-NAME significantly decreased the Isc response in shams. These findings suggest that NO is an important physiological mediator of electrolyte transport in the ileum and colon but the avenues by which NO elicits its control are likely different between regions.

Similarly, these studies have identified regional differences in the profile of NOS activity in the gut which may in some way reflect the differential importance of NO in each region with respect to electrolyte transport. Basal NOS activity in the jejunum and ileum appears to be less due to cNOS than in the colon, where basal NOS activity is almost entirely due to cNOS. This is likely why L-NAME treatment had the most effect on the colon. At the same time, the fact that L-NAME had the most effect on the colon further supports the likelihood of L-NAME being a cNOS specific inhibitor.

In addition to there being regional differences in the profile of NOS activity the results also demonstrate regional differences in the distribution or possibly the sensitivity to induction of iNOS in the rat intestine. In both the endotoxin model and the radiation model it was clear that iNOS was most plentiful or most sensitive to induction in the ileum. However, the significance of iNOS activity being so much greater in this region requires further investigation. In the radiation model NOS activity was not induced in either the jejunum or colon. However, in the endotoxin model the colon was more sensitive than the jejunum, thus responding with a greater increase in iNOS activity than the jejunum. The increases in NOS activity were not inhibited by L-NAME, however AG significantly and specifically inhibited iNOS activity in all

tissues. Thus, we have shown that L-NAME and AG are specific inhibitors of cNOS and iNOS, respectively, in the rat *in vivo*.

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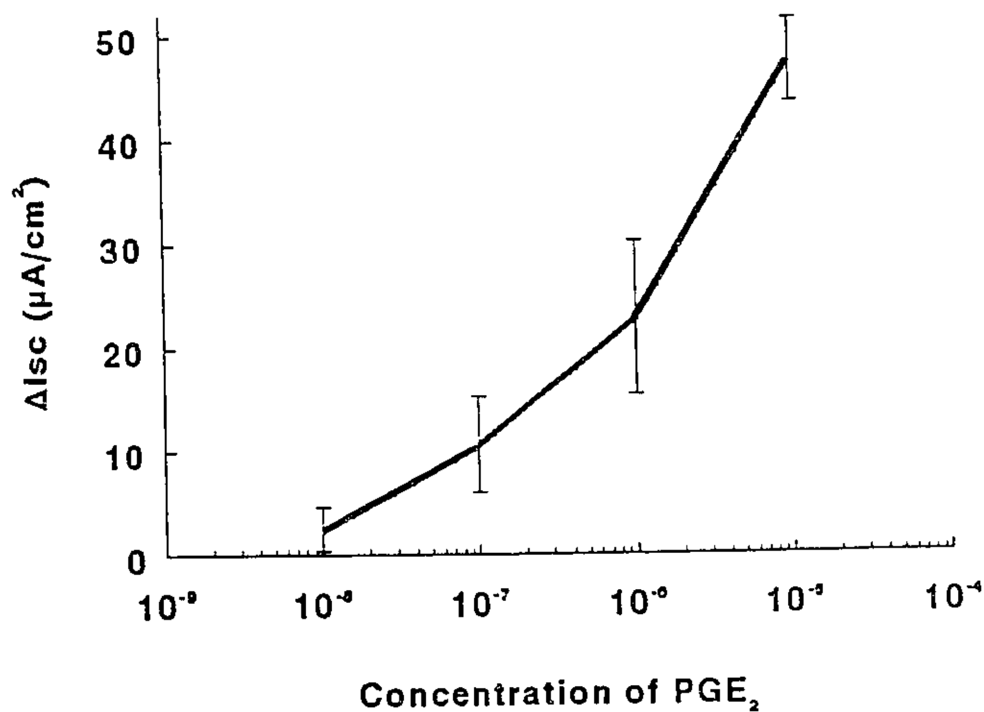
Appendix A (next page) outlines a number of the primary effects elicited by mediators released from mucosal mast cells. This table is based on a review article by Cooke 1994 which outlines the possible roles of these mediators in the gastrointestinal system.

Appendix A

Mediator	Target receptor/cell	Primary effect
Histamine	H_1 / enterocytes	increases $[Ca^{2+}]_i$ which increases basolateral K^+ conductance and apical Cl^- secretion
	H_1 / neurons	potentiates fast synaptic transmission at nicotinic synapses
	H_2 / submucosal neurons	enhances excitability and long lasting depolarization
	H_3 / cholinergic and non-cholinergic	presynaptic inhibition at nicotinic synapses
	H_3 / enterochromaffin	decreases release of 5-HT
Serotonin	neurons	presynaptic inhibition at nicotinic synapses
	HT_{1P} / intrinsic afferents	postsynaptic activation
	HT_2 and HT_4 / enterocytes	stimulate secretion
	HT_3 / submucosal neurons	stimulates fast depolarization of cholinergic and non-cholinergic pathways
Prostaglandins		
PGD_2	DP_2 / enterocytes	stimulates Cl^- secretion
PGI_2	IP_2 / enterocytes	stimulates Cl^- secretion
PGE_2	EP_2 / neurons	stimulates secretion
Leukotrienes		
LTC_4	LTC_4 / enterocytes	inhibits Na^+/ Cl^- absorption and stimulates anion secretion via release of TBX A_2 and PGE_2
LTD_4	LTD_4 / enterocytes	inhibits Na^+/ Cl^- absorption and stimulates anion secretion via release of TBX A_2 and PGE_2
LTE_4	LTE_4 / enterocytes	inhibits Na^+/ Cl^- absorption and stimulates anion secretion via release of TBX A_2 and PGE_2
Platelet Activating Factor	PAFr / enterocytes	inhibits Na^+/ Cl^- absorption and stimulates Cl^- secretion via release of PGE_2 and PGI_2
Adenosine	A_1 / neurons	presynaptic inhibition of ACh and NE release
	A_2 / neurons	excitation of postsynaptic neuron
	A_2 / enterocytes	stimulates arachadonic acid metabolism resulting in Cl^- secretion
Radical Oxygen Intermediates	enterocytes and neurons	stimulates Cl^- secretion via release of PGE_2 and PGI_2
Nitric Oxide	enterocytes and neurons	stimulates Cl^- secretion via release of PGE_2 and PGI_2; may inhibit absorption via activation GC in villar enterocytes; presynaptic inhibition
Endothelins		
E-1	ET_A / neurons	stimulates secretion via release of PGI_2
E-1, 2 and 3	ET_B / enterocytes	decreases Na^+/Cl^- absorption and increases basolateral K^+ conductance and apical Cl^- secretion

Appendix B



Dose response curve to the serosal application of prostaglandin E_2 in rat jejunum. The dose of 10^{-7} was selected for the comparison of control versus treatment groups as this dose was submaximal and would be unlikely to elicit any non-specific effects ($n \geq 4$ for each dose).

Appendix C

Drugs and Solutions

Nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) were dissolved in 0.9% NaCl to a final concentration of 100 mg/ml. *Salmonella typhimurium* lipopolysaccharide (LPS) 4 mg/ml was dissolved in 0.9% NaCl. These solutions were prepared on a weekly basis and kept refrigerated. Prostaglandin E_2 (PGE_2) stock solution was made up in ddH_2O to a final concentration of 1 mM and frozen at -70°C in aliquots of 10 µl. Before use one aliquot of the PGE_2 stock solution was thawed and diluted to 1 ml with ddH_2O.

Dowex 50W-X8 resin (Na- form,100-200 mesh, Bio-Rad) was prepared on a weekly basis. Preparation of the resin involved mixing it with concentrated NaOH (pH>11) for at least 20 min and then rinsing the resin numerous times (10-20) with filtered, deionized water (ddH_2O) in a 1:3 ratio until the pH reached ~8.0. The ionized resin was kept refrigerated and the pH was readjusted using 2.5% NaOH immediately prior to each experiment.

Krebs buffer: 115 mM NaCl, 2 mM KH_2PO_4, 2.4 mM $MgCl_2$, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 8 mM KCl. Serosal and mucosal solutions for the Ussing chamber experiments contained Krebs buffer and glucose or mannitol, respectively, in a final concentration of 10 mM.

Homogenization buffer: 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor 10 µg/ml, leupeptin 10 µg/ml, and aprotinin 2 µg/ml. The pH was adjusted to 7.4 using 1 M NaOH and 1 ml aliquots were stored at -70°C. Aliquots were thawed immediately prior to use.

Incubation buffer: 38 mM KH_2PO_4, 6 mM valine, 948 µM $MgCl_2$, 190 µM $CaCl_2$, 975 µM NADPH, 58 µM L-arginine and 0.046 µCi ^{14}C-L-arginine. To determine whether the enzyme

was of the constitutive or inducible nature, ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) was added to the incubation buffer in a final concentration of 977 μM.

PGE_2 and LTB_4 assays: X can be either PGE_2 or LTB_4. The assay employed the use of a mouse monoclonal antibody to X, bound to the assay plate, which acts as a receptor for the specific antiserum to X. The assay is based on competition between X and the acetylcholinesterase tracer linked to X. Competitive binding took place over 18 h and any free X or acetylcholinesterase tracer linked to X was washed off. Plates were developed with the addition of Ellman's reagent which reacted with acetylcholinesterase tracer to produce a bright yellow colour with a maximum absorbance of 412 nm. The absorbance was inversely proportional to the concentration of X in the sample (absorbance α [bound X tracer] α 1/[sample X]).

Carnoy's: 60% ethanol, 30% chloroform and 10% glacial acetic acid.

Appendix D

Enzymatic profile of NOS activity

When estimating NOS activity using the ^{14}C-L-arginine to ^{14}C-L-citrulline conversion assay one starts with total NOS (tNOS) activity which is the total NOS activity as assayed in the group without EGTA in the incubation buffer. By adding the calcium chelator, EGTA, the total activity can be broken down to reflect the percentage of the total which is Ca^{2+}-dependent and that which is Ca^{2+}-independent. In this study Ca^{2+}-dependent NOS activity was considered to be due to the constitutive enzyme, cNOS, and that which was Ca^{2+}-independent was considered to be due to the inducible enzyme, iNOS. It was assumed that of tNOS activity, activity which was not iNOS was due to cNOS. Therefore, percentages of iNOS and cNOS were always complimentary to each other (if iNOS=30% then cNOS=70% of tNOS). The percentages of iNOS and cNOS provided a "profile" of NOS activity with respect to tNOS activity of a particular treatment group.

Changes in the profile of NOS activity did not always reflect an equivalent change in the actual activity of the specific isotype, iNOS or cNOS. If the profile of NOS activity was unchanged after an increase in tNOS clearly the actual activity of both iNOS and cNOS was increased. Similarly, if there was an increase in %iNOS activity it could not be assumed that iNOS activity increased, since tNOS activity may have decreased and the relative increase in %iNOS may have been solely due to a decrease in cNOS activity. Thus, in these studies, both the relative proportions of cNOS and iNOS, as well as enzymatic activity, must be taken into consideration in order to gain a clear appreciation of the effects of a given experimental treatment on NOS activity.

Calculating NO production from L-^{14}C-citrulline activity

If you have followed the protocol exactly, using the same volumes, the following calculations should apply.

1. Counts per minute (CPM) of each sample multiplied by two (x2) minus (-) CPM of the background multiplied by two (x2) (to give you net activity, ie. citrulline - arginine)
2. Multiplied by the dilution factor 250/20=12.5 (20µl of 250µl of homogenate)(to give you activity of whole tissue sample).
3. Multiplied by total activity added 46.4×10^{-9} Ci * / total CPMs (to give you net activity hot arginine converted).
4. Divided by 331.2 Ci (which is the activity per mol ^{14}C-arginine; to give you moles of hot arginine converted).
5. Multiplied by moles of cold arginine added 2.92×10^{-9} * / moles of hot arginine added 0.14×10^{-9} * (to give you moles of cold arginine converted which is = citrulline = NO).
6. Divided by tissue weight multiplied by 1000 (to go from mg to grams).
7. Divided by 10 min (to get per min.)

* we have 0.05mCi/500µl stock and 9.5µl hot arginine is added to stock incubation buffer; therefore 9.5×10^{-7} Ci in stock incubation buffer (vol.=1023.5µl) therefore in 50µl (incubation vol.) there is **46.4nCi** (available to be converted total).
* arginine 210.7 g/mol; we use 31.5 mg/10ml stock (~14.95mM), we add 4µl stock to the homogenization buffer which is 59.8nmol cold arginine therefore in 50µl (incubation vol.) there is **2.92×10^{-9} moles of cold arginine** available to be converted.
* hot arginine 331.2mCi activity/mmol arginine; 46.4nCi = **0.14×10^{-9} moles hot arginine** available to be converted.

NOTE: These calculations are based on the specific activity of the isotope I am using therefore if the specific activity of your isotope is not the same you should insert the specific activity of your isotope and recalculate as required.