Nitric Oxide Protects the Intestine from the Damage Induced by Laparotomy and Gut Manipulation

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Background. The intestine is highly susceptible to free radical-induced damage, and our earlier work has shown that surgical stress induces the generation of oxygen free radicals in enterocytes, resulting in intestinal damage along with ultrastructural changes. Since nitric oxide (NO) is an important mediator of gastrointestinal function, this study looked at the effect of NO on surgical stress-induced intestinal alterations.

Materials and methods. Control rats and rats pretreated with the NO donor L-arginine were subjected to surgical stress by opening the abdominal wall and handling the intestine as done during laparotomy. Enterocytes were isolated and homogenate prepared, and the protection offered by L-arginine against damage due to surgical stress was determined and compared with normal controls. Protection to structural as well as functional aspects of the intestine was also examined.

Results. Intestinal manipulation affected intestinal structure as assessed by electron microscopy. Functional impairment of the enterocyte was also evident, with increased xanthine oxidase activity resulting in production of superoxide anion. This impairment is more dramatic in the crypt cells. Increased protease activity was also seen following laparotomy and handling. Pretreatment with the NO synthase substrate L-arginine prevented these damaging effects. Arginine protection was abolished in the presence of the NO synthase inhibitor NG-nitro-L-arginine methyl ester, indicating the role of NO.

Conclusion. Stress in the small intestine due to any surgery can affect enterocyte structure and function. These damaging effects can be prevented by NO, an important modulator of cellular function.

Key Words: surgical stress; intestine; nitric oxide.

INTRODUCTION

The gastrointestinal (GI) tract is inhabited by a large collection of microbial species that are contained within the lumen of the gut and segregated from the internal milieu. This “gut barrier” protects the host from being invaded by its own flora or toxins. The intestine is susceptible to damage, even in response to remote organ injury, and surgery at remote locations can affect the structural and functional aspects of the intestine [1]. The gut barrier is especially vulnerable in pathological conditions such as shock, trauma, or surgical stress, where bacterial translocation and release of endotoxin into the systemic circulation plays an important role [2]. Bacterial translocation is also implicated in postoperative complications such as systemic immune response syndrome (SIRS) and multiple organ failure (MOF) [3].

GI blood flow plays an important role in absorption of nutrients, and abnormal vasoregulation can result in mismatching of oxygen delivery to consumption [4, 5]. Splanchnic ischemia and gut barrier failure play an important role in the development of both sepsis and MOF [6]. Under stress conditions such as following a major abdominal surgery, blood supply to the intestine is affected, resulting in hypoperfusion. It is known that hypoxia results in generation of oxygen free radicals and that oxidative stress has a damaging effect on cells.
and tissues. Oxygen free radicals are known to play an important role in gut epithelial damage that may facilitate bacterial translocation and release of endotoxins due to alteration in gut barrier function. Our earlier work has shown in a rat model of surgical stress that laparotomy and intestinal handling results in oxidative stress in the enterocytes and increased permeability [7].

Nitric oxide (NO), a biologically important molecule, is known to have many modulatory functions for cells and tissues, including those of the GI tract. NO has the capacity to down regulate inflammatory responses in the GI tract, to scavenge various free radical species, and to protect the mucosa from injury induced by topical irritants. NO also plays an important role in many other processes, including immune responses, neurotransmission, and adhesion of platelets and leukocytes, as well as repair of mucosal injury and regulation of intestinal mucosal barrier function [8]. The objective of this study was to explore the effect of L-arginine (substrate of NO synthase enzyme) on the enterocyte damage induced by surgical stress.

MATERIALS AND METHODS

Nicotinamide adenine dinucleotide (NAD), its reduced form, 1-chloro-2,4-dinitrobenzene, iodoacetate, reduced glutathione (GSH), oxidized glutathione (GS-SG), 1-fluoro-2,4-dinitrobenzene, NG-nitro-L-arginine methyl ester (L-NAME), xanthine, diithiothreitol (DTT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were all obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Animals. Adult Wistar rats of both sexes (200–250 g), exposed to overnight fasted rats were anesthetized by ketamine (100 μg/kg body weight, ip). The abdominal cavity was opened by a vertical incision of approximately 4 cm. The intestine was then handled along its entire length from the ileocecal junction proximally, simulating the inspection that occurs in a clinical setting. The intestine was then replaced back into the abdominal cavity and the whole process was completed within 1 to 2 min. Following this, the abdominal wall was sutured and the animals were killed by decapitation 60 min after the surgical procedure. Our earlier work has shown that maximum alteration in the intestine occurs 60 min following surgical stress [7]. For control, rats were killed by decapitation after injection of ketamine.

Enterocyte isolation. Enterocytes were isolated from the rat small intestine by the metal chelation method [10]. Briefly, the intestine was washed with Krebs-Henseleit (KH) buffer, pH 7.4, and filled with the same buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) and incubated at 37°C for 12 min in a beaker containing KH buffer alone. After incubation, the intestine was washed with cold KH buffer containing 0.25% albumin and filled with the same buffer. Enterocytes were isolated by gently rubbing the intestine along its entire length. Following this, the isolated cells were centrifuged at 900g for 5 min. The cells were washed twice and the final preparation of enterocytes was suspended in 25 mM phosphate-buffered saline (PBS), pH 7.4. Cell homogenates were prepared using Porter-Elvehjem homogenizer and used for enzyme activity measurements.

Isolation of the villus and crypt cells from the small intestine. The whole length of the small intestine was washed gently with cold physiological saline containing 1 mM EDTA. Enterocytes of various stages of maturation (villus to crypt) were isolated by the metal chelation method as described [11]. Briefly, the intestine was filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM Na3C6H5O7, 8 mM KH2PO4, 5.6 mM Na2HPO4, pH 7.3), clamped at both ends, and incubated at 37°C for 15 min. Following incubation, the luminal contents were discarded and the intestine was filled with solution B (PBS pH 7.2 containing 1.5 mM EDTA and 0.5 mM DTT) and incubated at 37°C for different time intervals of 4, 2, 3, 4, 6, 7, 10, and 15 min. At the end of each time period, the incubated solution containing cells was collected in separate tubes. All these nine fractions were pooled into three, the first three fractions being the villus, the next three fractions being the mixed population of cells, and the last three fractions being the crypt cells. These cell fractions were centrifuged at 900g for 5 min, washed with KH buffer, pH 7.4, containing 5 mM glucose and 2.5 mM calcium, and suspended in the same buffer. Separated villus, mixed, and crypt cells were identified by assaying the marker enzyme alkaline phosphatase.

Enzyme assays. Xanthine oxidase (XO) activity was measured spectrophotometrically based upon the production of uric acid, which was measured at 295 nm [12]. The reaction mixture contained 50 μM xanthine, 100 μM EDTA, 50 mM potassium phosphate buffer, pH 7.8 (all final concentration), and an aliquot of the homogenate in a total volume of 1 ml. The reaction was carried out in the presence and absence of 0.4 mM NAD to assay XO + xanthine dehydrogenase (XDH) and XO, respectively. Activity of alkaline phosphatase was assayed as described [13].

Glutathione estimation by high-performance liquid chromatography (HPLC). Trichloroacetic acid was used to precipitate cell homogenate proteins, and glutathione in the acid supernatant was quantitated using HPLC after derivatization. The derivatization method was based on the initial conversion of S-carboxyl methyl derivatives of free thiols with iodoacetate followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives. Dinitrophenyl derivatives were separated by HPLC on an Ultrasil amino column (Shimadzu, Kyoto, Japan) with a gradient of methanol and sulfuric acid and detected at 365 nm [14].

Gelatin zymography. Zymogram of protease activity was performed as described [15], with slight modification. Polyacrylamide gels (12%) containing 0.1% gelatin were cast and cystosol (25 μg protein) was applied on the gel in standard sodium dodecyl sulfate (SDS) loading buffer containing 0.1% SDS but lacking 2-mercaptoethanol. It was not boiled before loading. The gels were run at 100 V for 2 h and then soaked in 2% Triton X-100 and incubated at 20°C on a shaker for 90 min with three changes. Following this, the gels were soaked in reaction buffer (0.1 M Tris-HCl, pH 7.5) overnight at 37°C and then stained with Coomassie blue.
MTT reduction assay. MTT reduction assay was done using a microtiter plate. In a total volume of 150 µl in each well, cell suspension corresponding to 1 x 10^4 cells/ml were taken; 6 µl of 125 mM MTT was added and the volume made up with 25 mM PBS. The cells were incubated at 37°C for 20 min. The reaction was stopped by the addition of 150 µl of DMSO and mixed thoroughly to dissolve the formazan. The plates were read on a multiwell scanning spectrophotometer (enzyme-linked immunoassay reader) using a test wavelength of 570 nm and a reference wavelength of 630 nm [16]. The amount of MTT formazan formed was calculated using the molar extinction coefficient of MTT formazan E_370 of 17,000 M/cm at pH 7.4 to 8.

Protein estimation. Protein was estimated by the method of Lowry et al. using BSA as the standard [17].

Histological studies. All the tissue samples were fixed in 2.5% glutaraldehyde, postfixed with osmium tetroxide, and embedded in araldite (epoxyresin). One-micrometer sections were cut using a glass knife and stained with toluidine blue. Well-oriented areas for ultrastructural study were chosen after examining 1-µm sections under a light microscope. Ultra-thin sections (60–90 nm) were cut on an LKBUM4 ultramicrotome using a diamond knife (Diatome, Switzerland). Sections were mounted on copper grids and stained with saturated aqueous uranyl acetate and Reynolds lead citrate. The grids were examined under a Philips EM201C (Eindhoven, The Netherlands) electron microscope.

Conductance measurements. After the animals were sacrificed, four pieces of intestine were cut from the jejunum, taking care to avoid any lymph nodes in the area. Tissue samples were mounted between two halves of a Lucite Ussing chamber (World Precision Instruments, Sarasota, FL) (exposed area of 0.635 cm²). Tissues were bathed on both the mucosal and serosal sides (10 ml each) with regular Ringer’s solution (115 mmol/L sodium chloride, 2.4 mmol/L potassium monohydrate orthophosphate, 0.4 mmol/L potassium di-hydrate orthophosphate, 25 mmol/L sodium bicarbonate, 1.2 mmol/L magnesium chloride, 1.2 mmol/L calcium chloride, and 10 mmol/L glucose, pH 7.4) maintained at 37°C using a circulating water bath and were gassed with 95% O₂ and 5% CO₂. Glucose (10 mM) was added to the serosal and mucosal bathing solutions. Electrical parameters were recorded during the experiments using a four-electrode system. Transepithelial potential difference (PD) was recorded with two calomel half-cells connected to the chamber by Ringer-agar bridges placed on either side of the tissue. Current was passed across the tissue through two Ag/AgCl electrodes connected to the chamber by Ringer-agar bridges. The short-circuit current (Isc; the current required to nullify the spontaneous transepithelial PD) was continuously monitored with an automatic voltage clamp apparatus (DVC-1000, World Precision Instruments). All studies were performed under short-circuited conditions. Tissue conductance was calculated according to Ohm’s law and expressed as millisiemens per square centimeter per hour. After a 20-min initial equilibration period, measurements of transepithelial PD and Isc were made every 15 min.

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was done using Student’s t-test.

RESULTS

On ultrastructural examination, control small intestinal epithelial cells appeared normal (Fig. 1A). Following intestinal handling, epithelial cells showed diffuse widening of the paracellular spaces in the upper part of the crypts, which was more marked in the basal aspect, with unaltered apical tight junctions (Fig. 1B). Prior treatment with L-arginine, the substrate for NO synthase, showed almost complete protection of the damage and also the paracellular widening (Fig. 1C). Cells pretreated with L-arginine along with the NO synthase inhibitor L-NAME showed inhibition of the protection offered by L-arginine (Fig. 1D). Assessment of the mucosal permeability by conductance measurements in control rats and rats who had undergone intestinal manipulation in the presence and absence of L-arginine showed that the normal small intestine almost maintained a steady conductance over the period of study. The small intestine after surgical stress induced by handling showed a significant increase in conductance within 15 min after mounting in Ussing chambers. Pretreatment of L-arginine showed a slight but not significant decrease in conductance when compared to the surgically stressed rats (Fig. 2). This decrease in conductance was noted during the entire study period of 1 h.

Superoxide anion, the main oxygen free radical generated in the cell, is known to reduce MTT, a tetrazolium compound, to its colored formazan. Enterocytes isolated from rats after intestinal manipulation showed an increase in MTT reduction as compared to control enterocytes and this increase was almost completely prevented by L-arginine pretreatment. Pretreatment with L-NAME inhibited this protection by L-arginine (Fig. 3A). Superoxide generation during bowel manipulation was further confirmed by XO (source of superoxide) and XDH activity. Figure 3 shows the activity of XO and XDH in enterocytes from control and surgical stress, with and without being pretreated with L-arginine or arginine + L-NAME. Laparotomy and intestinal handling induced an approximately two-fold increase in XO activity. Pretreatment with L-arginine was able to protect against this increase, while inhibition of NO synthase activity by L-NAME abolished the protective effect of arginine (Fig. 3B). Increased XO activity was accompanied by decreased XDH activity after surgical stress, whereas the activity was normal in arginine-pretreated enterocytes. Inclusion of NAME with L-arginine prevented the protection.

The defense against oxygen free radicals is important for the normal functioning of the cells, and the intracellular nonenzymatic antioxidant tripeptide, glutathione, maintains the thiol-reduct status of the cell. The level of the reduced and oxidized form of glutathione is shown in Figs. 4A and 4B. GSH level was decreased in surgically stressed enterocytes as compared to control. Concomitant with this, an increase in the oxidized form (GSSG) was seen 60 min after bowel manipulation. These changes were not seen in arginine-pretreated rats and were similar to the control. This protection by arginine was not seen in animals pretreated with L-NAME and L-arginine. The intestine has cells at various stages of differentiation, starting from undifferentiated crypt cells to differenti-
ated villus cells, and surgical stress–induced oxidative stress in these cells was studied. It was found that free radical–mediated inactivation of alkaline phosphatase was seen in all cell fractions, although this activity was maximum in villus cells. Superoxide generation as assessed by MTT reduction was seen significantly in crypt cells after surgical stress and this was also associated with significant increase in XO activity in these cells. The increase in XO activity and MTT reduction were abolished in all fractions by pretreating the animals with L-arginine. NO synthase inhibitor L-NAME pretreatment inhibited this protection by arginine, and 88- and 22- kDa bands were prominent in zymography.

**DISCUSSION**

The intestine is highly susceptible to stress at remote locations, including burn injury, trauma, or sur-

**FIG. 1.** Electron micrographs of small intestinal epithelium of rats subjected to surgical stress in the presence and absence of L-arginine and L-NAME, showing normal appearance in the control (A). Surgically stressed cells show more widened paracellular spaces (arrow), especially in the upper part of the crypts (B). L-Arginine treatment before surgical stress results in a decrease in paracellular widening (arrowhead) (C), while L-arginine- and L-NAME-pretreated surgically stressed epithelial cells show marked widening of intercellular space (D) (original magnification \( \times12,500 \)).

Figure 6 shows the gelatin zymography of cytosolic proteases. There was an increase in the protease activity in the surgically stressed enterocyte cytosol, specifically in an 88- kDa protease, with a marginal increase in 105-, 47-, and 22- kDa proteases. This increase was completely abolished by pretreating the animals with L-arginine. NO synthase inhibitor L-NAME pretreatment inhibited this protection by arginine, and 88- and 22- kDa bands were prominent in zymography.
gical stress. The high susceptibility of the intestine to surgery plays a crucial role in the pathophysiology of postoperative complications such as SIRS and MOF [2, 18]. It is known that surgery at remote locations can lead to increased intestinal permeability, which subsequently results in luminal bacteria and endotoxin reaching the portal and systemic circulation, thereby leading to sepsis [2, 3]. Microvascular perfusion failure following surgical stress may be responsible for altered intestinal function [19]. Septic shock and surgical stress, resulting in the release of cytokines, also modulates the intestinal function [20, 21]. Our recent study has shown that laparotomy and mild intestinal handling induces generation of oxygen free radicals in the intestine, resulting in functional and biochemical alterations [7]. These changes may be the result of hypoperfusion of the following surgical stress.

NO, a free radical produced by the enzyme NO synthase [22], is now under intense investigation to decipher its varied role in organ systems. NO synthase is of two types, constitutive and inducible, and they are identified and quantified at the protein level both in the rat and human GI tract. NO has been shown to restore depressed cardiac output and regional perfusion after trauma and hemorrhage [9] and also to re-

store splenocyte function after trauma and hemorrhage, potentially by improving splenic blood flow [23]. NO also has a role in ameliorating vasoconstriction and improving the organ blood flow in the small intestine during bacteremia via an l-arginine pathway [24]. Earlier studies from our laboratory have shown a role for NO in attenuating intestinal ischemia/reperfusion injury [25]. Despite the fact that l-arginine regulates the vascular tone, blood pressure, and tissue perfusion, it is not known whether the administration of this essen-

FIG. 2. The time course transepithelial conductance measurement in small intestine of controls and rats subjected to surgical stress in the presence and absence of l-arginine. Measurement was done as described under Materials and Methods. The x axis indicates time after mounting on Ussing chamber. Data are expressed as mean ± SE from a minimum of five experiments. *P < 0.01 when compared to control.

FIG. 3. MTT reduction by enterocytes (A) and activity of XO and XDH (B and C) in the homogenate from control, control + l-arginine, surgical stress, l-arginine + surgical stress, and l-arginine + L-NAME + surgical stress. All the assays were carried out as described under Materials and Methods. *P < 0.01 when compared to control; #P < 0.01 when compared to surgical stress.
tial amino acid has any beneficial effects on the altered intestinal function during surgical stress.

We have shown earlier that 60 min after laparotomy and intestinal handling, widened intercellular spaces were seen with increased intestinal permeability, accompanied by oxidative stress in the enterocytes [7]. Since many studies support a beneficial role of NO in the intestine, the present study looked at the biochemical, functional, and histological alterations in the intestine following intestinal manipulation after pretreatment with L-arginine, the substrate for NO synthase enzyme. Administration of L-arginine prevented the histological alterations seen during surgical stress, but the protection was not complete. This was further confirmed by the electrical conductance measurements which also showed a decrease in conductance after surgical stress in rats pretreated with L-arginine, when compared to rats without pretreatment.

Studies have shown a role for NO in regulating the intestinal mucosal barrier function, and Kubes [26] has shown that NO modulates the epithelial permeability in the feline small intestine. Inhibition of NO synthesis has been shown to increase the epithelial permeability via mast cells [27], and release of NO following gut injury could contribute to functional repair of the epithelial barrier [28].

Oxidative stress seems to be a possible cause for these structural and functional alterations; this is indicated by the increased XO activity and superoxide generation after surgical stress. XO activity has been shown to be increased in blood from septic patients [29] and superoxide generated by XO decreases GSH level in the plasma and liver in pancreatitis [30]. Surgical trauma not only intensifies oxidative stress by generating reactive oxygen species (ROS), but also weakens the biological defense system against ROS attack. Damage due to generation of oxygen free radicals was further confirmed in our study by alteration in thioredoxin status that shows an increase in oxidized glutathione following surgical stress. Critical illness is associated with alterations in muscle glutathione metabolism, with a decrease in the muscle GSH concentration as well as the ratio of reduced and total

FIG. 4. Level of GSH (A) and GSSG (B) in enterocytes from control and after surgical stress in the presence and absence of L-arginine and L-NAME. Assays were carried out as described under Materials and Methods. *P < 0.01 when compared to control; #P < 0.01 when compared to surgical stress.

FIG. 5. Alkaline phosphatase activity (A), MTT reduction (B), and XO activity (C) in different cell populations of isolated enterocytes from control and after surgical stress in the presence and absence of L-arginine. All assays were carried out as described under Materials and Methods. *P < 0.01 when compared to the control; #P < 0.01 when compared to surgical stress. ND, not detectable.
The alteration in the thiol-redox status was prevented by prior treatment with arginine, but in the presence of the NO synthase inhibitor L-NAME, this protection was abolished, which suggests that the protection by L-arginine is through generation of NO. NO has the capacity to inhibit reactive oxygen metabolites, including superoxide anion, and can prevent the cellular damage attributable to hydrogen peroxide [32]. Recent studies have demonstrated that NO could play an antioxidant role in brain dopamine neurons, probably mediated by nitrosylation [33].

The intestinal mucosa contains cells at different stages of differentiation and surgical stress in the intestine has been shown to induce oxidative stress in all cell populations by activation of XO [34]. The effect was more prominent in crypt cell populations. To study the beneficial effect of NO in different cell populations, cells of different stages of differentiation were examined for oxidative stress after surgery with and without prior treatment with L-arginine. Alkaline phosphatase is a marker enzyme for differentiated cells, seen predominantly in the villus tip cells. Surgical stress induced a decrease in alkaline phosphatase and it is known that this enzyme is highly susceptible to oxidative stress [35]. Pretreatment with L-arginine protected against this decrease as well as the increase in XO activity and superoxide generation in the cell populations. However, protection by NO was more dramatic in the crypt cells.

Proteases mediate a number of important cellular functions, and oxidative stress is known to activate cellular proteases [36]. Protease activity plays an important role in modulating the surgical stress response, and plasma granulocyte elastase levels have been shown to increase after surgery [37, 38]. In the present study surgical stress was shown to activate specific cytosolic proteases and this was prevented by arginine pretreatment. Protection was abolished when the NO synthase inhibitor L-NAME was included with L-arginine. Our earlier study has shown that laparotomy and intestinal handling induce the activation of specific proteases, both in the mitochondria as well as in the cytosol of enterocytes [36]. Studies by others have also shown that local infections and surgical procedures can induce increased proteolysis [39, 40] and in trauma and sepsis, increased proteolysis is seen in skeletal muscle [41]. This study showed an increased protease activity during surgical stress, which was inhibited by NO. NO has been shown to prevent protease-induced activation of thrombin receptor-1 and increased vascular permeability in the rat [42]. The cytotoxicity of NO has been attributed to its reaction with superoxide anion to form peroxynitrite. Rubbo et al. reported recently that NO actually inhibits peroxynitrite-induced lipid peroxidation [43]. Peroxynitrite formation occurs only when the concentrations of NO and superoxide anion present are equal. When NO concentrations are increased, it inhibits the peroxynitrite formation and hence it is unlikely that peroxynitrite may have a role in this process. The protective role of NO could probably be due to its property of scavenging free radicals and possibly inhibiting XO activity. Studies have shown the inhibition of XO by NO and this inhibition may be mediated through direct binding of NO to the enzyme Fe-S moiety [44]. Another study supported this notion that NO reversibly suppresses XO activity [45].

In conclusion, this study has shown that surgical stress induced oxidative stress, accompanied by structural and functional alterations in the intestine. This can be prevented by prior treatment with L-arginine. The effect is mediated by generation of NO, since the presence of the NO synthase inhibitor L-NAME along with L-arginine prevented protease. The exact mechanism by which NO offers protection to the intestine during surgical stress may involve multiple pathways and is still under investigation.

REFERENCES


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