Protease Activation during Surgical Stress in the Rat Small Intestine

Anup Ramachandran, M.Sc., and K. A. Balasubramanian, Ph.D.

The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Vellore-632 004, India

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Background. Surgical stress affects intestinal permeability and our earlier study using a rat model indicated that oxidative stress plays an important role in this process. Proteases are important mediators of cellular damage and are known to be activated in oxidative stress. This study looked at protease activity in enterocytes after surgical stress.

Methods. Surgical stress was induced by opening the abdominal wall and handling the intestine as done during laparotomy, in normal and xanthine oxidase-deficient rats. Enterocytes at various stages of differentiation were isolated and protease activity and protection offered by xanthine oxidase inhibitors were determined. Mitochondria and cytosol were prepared from total isolated enterocytes at different periods after surgical stress and protease activation was studied.

Results. Surgical stress induced activation of proteases in both the villus and crypt cells. Protease activation is seen in both mitochondria and cytosol, and similar to the other alterations in mucosal cells, protease activation was maximum 60 min after stress, returning to normal by 24 h. Thiol compounds modulate protease activity in both mitochondria and cytosol and the activation is not seen in xanthine oxidase-deficient animals.

Conclusions. Surgical stress induces activation of proteases in villus and crypt cells of the small intestine. Both mitochondrial and cytosolic proteases are activated and free radicals generated by xanthine oxidase may mediate protease activation after surgical stress in the intestine.

Key Words: surgical stress; intestine; protease.

INTRODUCTION

Under physiological conditions, the gastrointestinal tract functions to absorb nutrients and act as a barrier against the invasion of luminal bacteria into the systemic circulation. The gut is known to be susceptible to stress during surgery and studies have shown that surgical trauma induces oxidative stress and damage through enhanced production of reactive oxygen species [1]. Surgical stress has been shown to increase intestinal permeability and induce bacterial translocation into the systemic circulation [2], which has been implicated in the multiorgan failure syndrome (MOFS) [3]. Surgical stress results in increased PML elastase activity [4] and, also, increased production of H₂O₂ by 7 days after trauma [5]. Urinary trypsin inhibitor ameliorated the increase in protease activity in skeletal muscle caused by laparotomy in the mouse [6] and also inhibited the elevation of interleukin (IL)-8 and PMN elastase seen after surgery [7]. The protease inhibitor gabexate mesilate was also observed to reduce the systemic inflammatory response syndrome [8]. Proteases are involved in various cell functions as diverse as processing of viral polypeptide precursors, polypeptide processing, and regulation of enzyme turnover. Proteases have been implicated in damage caused by oxidative stress and mitochondria are known to contain a proteolytic system that recognizes and degrades oxidatively denatured proteins [9]. Proteases have also been implicated in the mitochondrial permeability transition induced by Ca²⁺ and tertiary butyl hydroperoxide [10].

Although a number of studies have shown damage to the intestine during surgical stress, the biochemical mechanism involved in this damage has not been investigated. Our earlier study showed oxidative stress and increased permeability in the intestine after surgical stress [11]. Since protease activation may occur after oxidative stress, this study has looked at the
proteolytic activity in enterocytes from rats subjected to surgical stress.

**MATERIALS AND METHODS**

Ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (Hepes), bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione (GS(G), tris(hydroxy methyl)aminomethane (Tris), synthetic protease substrates, dithiothreitol (DTT), allopurinol, xanthine, xanthine oxidase, and gelatin were obtained from Sigma Chemical Company. All other chemicals and solvents used were of analytical grade.

**Inhibition of Xanthine Oxidase Activity in Animals**

Xanthine oxidase-inhibited animals were produced by treatment with either allopurinol or sodium tungstate. For allopurinol treatment, rats were injected with 100 mg/kg body wt allopurinol intraperitoneally, 1 h before induction of surgical stress. For tungstate treatment, animals were fed sodium tungstate (0.7 g/kg body wt) in drinking water for 5 days before surgical stress.

**Induction of Surgical Stress**

For laparotomy, overnight fasted rats were anesthetized by ketamine injection (50 mg/kg body wt) and the abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently handled over its entire length while being raised from the abdominal cavity. The intestine was then placed into the abdominal cavity. The entire process was completed within 1–2 min. Following this, the abdominal wall was sutured and the animals were allowed to recover from anesthesia. In this model, surgical stress is induced by opening only the abdominal wall, without inflicting injury to the intestine per se, and this simulates intestinal handling during abdominal surgery. The animals were then sacrificed by decapitation 30 min, 60 min, 120 min, and 24 h after the surgical procedure.

**Isolation of Villus and Crypt Cells from the Small Intestine**

The entire length of the small intestine from surgically stressed rats as well as control were washed gently with cold physiological saline containing 1 mM DTT. Enterocytes at various stages of maturation (villus to crypt cells) were isolated by the metal chelation method as described [12]. Briefly, the intestine was filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH2PO4, and 5.6 mM Na2HPO4, pH 7.3), clamped at both ends, and incubated at 37°C for 15 min. Following incubation, the luminal contents of the intestine were discarded and the intestine was filled with solution B (phosphate-buffered saline, pH 7.2, containing 1.5 mM EDTA and 0.5 mM DTT) and incubated at 37°C for different intervals of 4, 2, 3, 4, 6, 7, 10, and 15 min. At the end of each period, the incubated solution containing cells was collected in separate tubes. These cell fractions were centrifuged at 900g for 5 min, washed with PBS, and suspended in the same buffer. These fractions were numbered 1 to 9, fraction 1 being villus tip cells, fraction 9 being crypt stem cells, and the middle fraction containing cells at various stages of differentiation. Separated villus and crypt cells were identified by assaying the marker enzyme alkaline phosphatase.

**Total Enterocyte Isolation and Mitochondrial Preparation**

Total enterocytes were isolated from the small intestine of the control and surgically stressed rats by the metal chelation method. Briefly, the intestine was filled with modified Krebs–Henseleit buffer containing EDTA (5 mM) and incubated at 37°C for 12 min. After incubation, enterocytes were isolated by placing the intestine in a plastic beaker on ice and gently rubbing the intestine along the sides. Mitochondria were prepared from isolated enterocytes as described [13]. The final mitochondrial pellet was washed twice with a solution containing 250 mM sucrose, 5 mM Hepes, pH 7.4, and suspended in the same solution. The postmitochondrial supernatant was spun at 100,000g for 1 h to obtain the cytosolic fraction.

**Assay of Protease Activity**

Enzyme activity was measured using various synthetic substrates as described [14]. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.5), 50 μM substrate, and mitochondrial or cytosolic protein, in a final volume of 1 ml. Production of p-nitrophenol was measured at 400 nm in a Shimadzu spectrophotometer. Enzyme activity was expressed as nanomoles of p-nitrophenol released per minute per milligram of protein.

**Gelatin Zymography**

Zymography of protease activity was performed as described [15], with slight modification. Polyacrylamide gels (12%) were cast containing 0.2% gelatin. Mitochondria or cytosol (100–150 μg protein) was applied on the gel in standard 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 on a shaker for 1 h with three changes at 20°C. 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 brilliant blue. For pretreatment with sulphydryl reagents, equal aliquots of mitochondria from control and surgically stressed rats were incubated with 1 mM (final concentration) modifier for 15 min at 37°C and then run on the gel. For the xanthine (X)–xanthine oxidase (XO) system, control mitochondria were incubated with 1 mM xanthine and 100 μM xanthine oxidase (both final concentrations) for 15 min at 37°C.

**Statistical Analysis**

Data are expressed as means ± SD. Statistical analysis was performed with Student’s t test to compare changes.

**RESULTS**

Initial studies using various substrates indicated that intestinal mitochondrial protease activity is specific for substrates with glutamate or aspartate at the P1 position (Table 1). Intestinal epithelial cell mitochondria isolated at various periods after surgical stress were assayed for protease activity using the two substrates with glutamate and aspartate at the P1 position. Increased protease activity, especially against the aspartate-containing substrate, was evident in mitochondria by 60 min after surgical stress (Fig. 1). This increase was transient, and a return to control levels occurred by 24 h. To further characterize this protease...
activity, gelatin zymograms were run using mitochondria from control and surgically stressed rats. It was observed that normal enterocyte mitochondria showed a prominent band of protease activity of approximately 105 kDa. By 60 min after surgical stress three additional bands of 88, 47, and 22 kDa appeared (Fig. 2). These bands persist 120 min after stress, and disappear by 24 h, confirming the reversibility seen in the colorimetric assay. Our earlier study showed that surgical stress leads to increased activity of the free radical-generating enzyme xanthine oxidase (XO), accompanied by changes in thiol status [11]. To determine whether XO or thiol status can modulate protease activity, zymograms were run after pretreatment of mitochondria with the xanthine–xanthine oxidase system as well as thiol compounds. It was seen that the X–XO system activated the 105- and 88-kDa proteases. Dithiothreitol also activated both these enzymes in control mitochondria, while reduced glutathione activated the 105-kDa protease. The 88-kDa protease in mitochondria, 60 min after stress, was also further activated by GSH and DTT and to a slight extent also by GSSG (Fig. 3). The 47- and 22-kDa proteases were unaffected by these treatments.

A number of proteases are known to be present in the cytosolic compartment, and to investigate whether protease activation was confined only to the mitochondria, enterocyte cytosol from control and surgically stressed rats was also analyzed. Gelatin zymograms were run as described in the text. Equal amounts of protein were loaded in the lanes.

**FIG. 1.** Mitochondrial protease activity in rat enterocytes at various periods after surgical stress. The assay was performed as described in the text, using the two substrates, N-t-Boc-Benz-Glu-pNPE (A) and N-t-Boc-Benz-Asp-pNPE (B). Values represent means ± SD of three separate estimations. *P < 0.05 when compared with control.

**FIG. 2.** Zymogram of mitochondrial protease activity in rat enterocytes at various periods after surgical stress. Gelatin zymography was performed as described in the text. Equal amounts of protein were loaded in the lanes.

**FIG. 3.** Zymogram of mitochondrial protease activity from control and 60 min after surgical stress in the presence of the superoxide-generating system and sulfhydryl reagents. Equal aliquots of mitochondria from control and surgically stressed rat intestine were incubated with 1 mM (final concentration) modifier for 15 min at 37°C and then run on the gel as described under Materials and Methods. For the X–XO system, control mitochondria were incubated with 1 mM xanthine and 100 mu xanthine oxidase for 15 min at 37°C.

**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
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<tr>
<td>Benz-Tyr-pNA</td>
<td>–</td>
</tr>
<tr>
<td>Benz-Arg-pNA</td>
<td>–</td>
</tr>
<tr>
<td>N-t-Boc-Benz-Glu-pNPE</td>
<td>+</td>
</tr>
<tr>
<td>N-t-Boc-Benz-Asp-pNPE</td>
<td>+</td>
</tr>
<tr>
<td>t-Boc-Ala-Ala-pNA</td>
<td>–</td>
</tr>
<tr>
<td>t-Boc-Phe-Ala-Ala-pNA</td>
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rats was assayed for protease activity. As seen in Fig. 4, a pattern similar to that of mitochondria was obtained, with increased proteolytic activity at 60 min after stress and a return to the control level by 24 h. Gelatin zymography of enterocyte cytosol identified three prominent bands of 105, 88, and 22 kDa, of which the 88- and 22-kDa bands showed increased activity by 60 and 120 min after surgical stress and returned to control levels by 24 h (Fig. 5). The 22-kDa band was further found to be inhibited by dithiothreitol and activated by oxidized glutathione (Fig. 6). We had earlier observed that activation of xanthine oxidase occurs throughout the villus–crypt axis of intestine after surgical stress and this was inhibited by pretreatment of animals with the xanthine oxidase inhibitors (unpublished observation), and the present study indicated that the X–XO system can induce an increase in activity of high-molecular-weight proteases in enterocyte mitochondria. On assaying protease activity in cells along the villus–crypt axis after surgical stress, it was seen that protease activity was increased after surgical stress, especially in the villus cells, and returned to control levels by 24 h. Pretreatment of animals with allopurinol or sodium tungstate was able to prevent the increased protease activity induced by surgical stress, especially in cells of the villus region (Fig. 7). Allopurinol or sodium tungstate pretreatment also inhibited the 88-kDa mitochondrial protease activated by surgical stress, and sodium tungstate treatment also seems to inhibit the 22-kDa protease in cytosol (Fig. 8).

**DISCUSSION**

Gastrointestinal surgery results in profound metabolic changes, organ dysfunction, and immunosuppression, which form part of the surgical stress response to trauma [16]. The intestine has been suggested to be the “motor” for surgical stress-induced trauma and the multiple-organ failure syndrome [17]. Surgical stress is also associated with microvascular hypoperfusion, which leads to altered intestinal function [18]. Protease activity plays an important role in modulating the surgical stress response, and plasma granulocyte elastase levels have been shown to increase after surgery [19, 20], the levels increasing with the severity of the operative intervention, accompanied by decreases in α1-antitrypsin levels [21]. Salivary kallikrein levels were also significantly elevated after surgical stress and this increase is probably associated with the stress...
response to surgery [22] along with release of granulocyte elastase [23].

The intestine is highly susceptible even to surgery at remote locations and our earlier study indicated that mild handling of the intestine following laparotomy caused oxidative stress in the enterocyte, accompanied by functional alterations in the intestine [11]. Free radical-induced damage has been indicated in surgical stress [24] and increased serum lipid peroxide levels have been seen after surgery [25]. Radical scavengers like EPC-K1 were able to reverse liver metastasis induced by surgical stress [26] and antioxidants were also able to prevent surgical stress in lung cancer patients [27]. A prominent feature of cell damage caused by oxidative stress is damage to mitochondria [28] and our earlier work had indicated enterocyte mitochondrial dysfunction after surgical stress (unpublished observation).

A number of proteases have been localized in mitochondria [29–31] and to determine the role played by proteases in surgical stress-induced damage, protease activity was assayed in intestinal mitochondria isolated from rats at various periods after surgical stress. While activity against a substrate with glutamate at the P1 position showed a mild increase by 30 min and then a decrease, there was a considerable increase in activity at 60 min against an aspartate-containing substrate, which also decreased 24 h after stress. To further characterize the proteolytic activity gelatin zymography was done with the enterocyte mitochondria, which showed a prominent proteolytic activity of around 105 kDa. In addition to this, proteolytic activities of 88, 47, and 22 kDa were also seen 60 min after stress, which persisted at 120 min and disappeared by 24 h after surgery. Exposure of cells to various forms of oxidative stress like \( \text{H}_2\text{O}_2 \) and superoxide was found to significantly increase the intracellular degradation of both short- and long-lived cellular proteins [32] and reactive oxygen species were seen to trigger activation of metalloproteases in the vascular interstitium [33].

Our earlier study showed an increased activity of the free radical-generating enzyme xanthine oxidase after surgical stress accompanied by changes in thiol status [11]. Glutathione is an intracellular thiol and normally reduced glutathione is maintained at a much higher level than oxidized glutathione for proper cellular function [34]. Earlier, we observed that surgical stress results in an increase in oxidized glutathione levels 60 min after stress and since thiol redox status can affect enzyme activity, the modulation of proteolytic activity by these factors was also investigated. The X–XO system and dithiothreitol were found to activate the mitochondrial high-molecular-weight proteases (105 and 88 kDa), while GSH activated the 105-kDa enzyme. A number of proteolytic activities have been described in cytoplasm, and surgical stress was seen to activate cytosolic proteases also. Similar to mitochondria, an increase in activity against the aspartate-containing substrate was seen and zymography showed the prominent activation of a 22-kDa protease, along with activation of an 88-kDa protease. As in mitochondria, these cytosolic activities returned to control levels by 24 h. Interestingly, the 22-kDa protease was inhibited by DTT and activated by GSSG, indicating that it is different from the mitochondrial protease observed earlier. Mixed disulfide formation has been shown to

FIG. 7. Protease activity in various cell populations along the villus-crypt axis isolated from control rats and 60 min after surgical stress, using XO inhibited animals. Assay was done as described in the text. Each value represents the mean ± SD from three separate experiments.quare, Control; □, 60 min after stress; △, 60 min after stress in allopurinol-pretreated animals; □, 60 min after stress in sodium tungstate-pretreated animals. *P < 0.05 when compared with control; #P < 0.05 when compared with 60 min after stress.
activate enzymes like trypsin and collagenase and also conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) [35]. Reversible S-thiolation has also been detected on proteins in cells exposed to free radicals [36]. Hence it is possible that oxidation of SH groups in the cytosolic protease might have increased its activity.

The intestinal epithelium consists of cells at various stages of differentiation, from the undifferentiated crypt stem cells to the differentiated villus tip cells. We had earlier observed increased xanthine oxidase activity throughout the villus–crypt axis (unpublished observation). To elucidate the relationship between xanthine oxidase activation and protease activity during surgical stress, xanthine oxidase deficient animals were developed and subjected to surgical stress. Xanthine oxidase activity can be completely inhibited by allopurinol [37] and studies using allopurinol have found to provide protection against bacterial translocation following challenge with a lethal dose of zymosan [38] as well as decreased intestinal mucosal lipid peroxidation and polymorphonuclear-derived myeloperoxidase activity due to surgical stress [39]. Allopurinol is also an antioxidant, in addition to its inhibitory effect on XO [40], and hence experiments were also done with another XO inhibitor, namely, sodium tungstate, which completely inhibits xanthine oxidase activity by depleting molybdenum, which is a cofactor for the enzyme [41]. It was observed that xanthine oxidase activity was absent in the intestine of both control animals and animals subjected to surgical stress after pretreatment with allopurinol or sodium tungstate (data not shown).

Protease activity was increased 60 min after stress throughout the villus–crypt axis, though it was more prominent in the villus cells, indicating that this cell population probably bears the brunt of the injury during surgical stress. Pretreatment with allopurinol or sodium tungstate inhibited this increase in the villus cells. Allopurinol or sodium tungstate treatment also inhibited the 88-kDa mitochondrial protease activity, which was activated by 60 min after surgical stress. This indicates that protease activation is probably mediated by oxygen free radicals generated by activation of xanthine oxidase. Once the protease is activated, the conversion of XDH to XO may increase, since this conversion can be mediated by proteolysis [42], thus amplifying the damage. The present study indicates increased proteolytic activity after surgical stress in mitochondria and these organelles are sensitive targets of oxidative stress, which leads to induction of the mitochondrial permeability transition (MPT) [43]. Studies have shown that proteases can activate MPT [10] and our earlier study showed an increased MPT 60 min after surgical stress in enterocyte mitochondria from rats after surgery (unpublished data). Thus, the activated protease may play a role in inducing the MPT after surgical stress.

**CONCLUSION**

This study has shown that proteases in enterocyte mitochondria are activated by oxygen free radicals generated by increased activity of xanthine oxidase observed in surgical stress. This was further corroborated using xanthine oxidase deficient animals in which protease activation was not observed. Increased mitochondrial protease activity may be responsible for induction of mitochondrial permeability transition and mitochondrial dysfunction, which is seen during surgical stress. Surgical stress also activates cytosolic proteases, which, like the mitochondrial enzymes, are modulated by thiol redox status and are maximally activated by 60 min after surgical stress, returning to normal by 24 h.
REFERENCES


