Intestinal Mitochondrial Dysfunction in Surgical Stress

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Background. Surgical stress is associated with altered intestinal function. Our earlier study using a rat model indicated that oxidative stress plays an important role in this process. Since mitochondria are crucial to cellular function and survival and are both a target as well as a source of reactive oxygen species, the present study looks at the changes in enterocyte mitochondria during surgical stress.

Methods. Surgical stress was induced by opening the abdominal wall and handling the intestine as done during laparotomy. Mitochondria were prepared from the isolated enterocytes at different time periods after surgical stress. The effect of surgical stress on enterocyte mitochondrial ultrastructure, respiration, anti-oxidant enzyme activity, thiol redox status, calcium flux, permeability, and matrix enzymes was then studied.

Results. Surgical stress resulted in alterations in mitochondrial respiration and thiol redox status. It was also associated with altered mitochondrial matrix enzyme activity, decreased superoxide dismutase activity, induction of mitochondrial permeability transition, and swelling, as well as impairment of mitochondrial calcium flux. These alterations were seen at a maximum of 60 min following surgical stress and were reversed by 24 h.

Conclusions. Laparotomy and mild intestinal handling itself results in enterocyte mitochondrial damage. Since mitochondria are important cellular organelles, this damage can probably lead to compromised intestinal function.

Key Words: surgical stress; intestine; mitochondria.

INTRODUCTION

The intestine functions as an effective barrier to enteric bacteria under physiologic conditions, preventing their translocation to the systemic circulation. The gut is sensitive to surgical stress even if the surgical procedure is done at a remote location, and studies have shown that bilateral lower extremity ischemia/reperfusion results in increased intestinal permeability [1]. Surgical stress results in neuroendocrine responses [2], which can affect hepatic circulation. Hepatic mitochondrial redox status is altered due to tissue hypoxia in surgical stress, leading to shortage of energy substrates and an energy crisis [3]. The mitochondria are extensively studied organelles due to their central role in cellular energy production, and this organelle also plays an important role in calcium homeostasis [4]. Due to these important functions, any damage to mitochondria can have grave consequences for cellular function. Mitochondria are sensitive to a wide variety of insults, but among them oxidative stress is important since mitochondria consume about 90% of the oxygen utilized by the cell and are an important source of cellular oxygen free radicals [5]. Most of the studies on surgical stress deal with change occurring at the tissue level, and details of cellular damage during this process are scant. Our earlier observations have indicated that oxidative stress in enterocytes might be responsible for altered intestinal permeability seen during surgical stress induced by laparotomy with bowel manipulation [6]. It is probable that these changes at the functional level are due to damage to intestinal mitochondria, which play a number of important cellular roles. In order to understand changes occurring at the subcellular level in the intestine during surgical stress, this study has looked at various mitochondrial functions in the enterocyte after inducing surgical stress by laparotomy and bowel manipulation in the rat.
**METHODS**

Nicotinamide adenine dinucleotide (NAD), its reduced form (NADH), nicotinamide adenine dinucleotide phosphate (NADP), isocitric acid, adenosine diphosphate (ADP), adenosine triphosphate (ATP), succinate, 2,6 dichlorophenol indophenol (DCIP), iodonitrotetrazolium (INT), ethyleneglycol-bis-(z-aminoethyl ether) N,N,N',N'-tetraacetic acid, N-[2-hydroxymethyl]piperazine-N'-[2-ethanesulfonic acid] (Hepes), bovine serum albumin (BSA), 1-fluoro-2,4-dinitrobenzene, reduced glutathione (GSH), oxidized glutathione (GSSG), Arsenazo-III, and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents used were of analytical grade.

Animals. Adult Wistar rats of both sexes (200-250 g), exposed to a daily 12-h light-dark cycle and fed water and rat chow ad libitum, were used for the study. The rats were randomly divided into five groups (n = 6): control (no operation), surgical stress (intestinal handling) and sacrificed after 30 min, surgical stress and sacrificed after 60 min, surgical stress and sacrificed after 120 min, and surgical stress and sacrificed after 24 h. This study was cleared by the Institutional Animal Experimentation Ethics Committee.

Induction of surgical stress. Overnight fasted rats were anesthetized by ketamine injection (50 mg/kg body weight) and the abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was then gently moved and the ileocecal junction identified identically. 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 into the abdominal cavity. The whole process was completed within 1-2 min. Following this, the abdominal wall was repaired and the animals were allowed to recover from anesthesia. The animals were then sacrificed by decapitation at 30 min, 60 min, 120 min, and 24 h after the surgical procedure. For control, rats were injected with ketamine and killed by decapitation at different times, parallel to the tests, without opening the abdominal wall. Controls killed at different times were similar in all mitochondrial parameters assessed. The intestine from control and surgically stressed rats was removed and enterocytes isolated.

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 ethylenediaminetetraacetic acid (5 mM) and incubated at 37°C for 12 minutes. 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 in a plastic beaker on ice and gently rubbing the intestine in a modified Krebs-Henseleit buffer containing ethylenediaminetetraacetic acid (5 mM), and incubated at 37°C for 20 min. Mitochondria were then prepared from isolated enterocytes as described [7]. The final mitochondrial pellet was washed twice with a solution containing 250 mM sucrose and 5 mM Hepes pH 7.4 and suspended in the same solution.

Measurement of mitochondrial function using MTT. Mitochondrial function was assessed by MTT reduction using a microplate reader as described [8]. In a total volume of 150 μl in each well, mitochondria corresponding to 150–200 μg protein was taken, 15 μM of 10 mM succinate and 6 μl of 1.25 mM MTT were added, and the volume was made up with 25 mM phosphate-buffered saline (PBS). MTT was dissolved in PBS and filtered to remove the small amount of insoluble residue in some batches of MTT. Plates were incubated at 37°C for 20 min followed by the addition of 150 μl of dimethyl sulfoxide and mixed thoroughly to dissolve the formazan. The plates were read on a multiwell scanning spectrophotometer (enzyme-linked immunosassay reader) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The amount of MTT formazan formed was calculated from the standard curve prepared using authentic MTT formazan.

Measurement of oxygen uptake. Oxygen uptake was determined polarographically using a Clark-type electrode in 2 ml of respiratory medium (225 mM sucrose, 5 mM MgCl2, 10 mM K2HPO4, 20 mM KCl, 10 mM Tris, and 5 mM Hepes pH 7.4) containing 5 mM succinate as respiratory substrate. A mitochondrial protein of 2 mg/ml was used. Oxygen uptake was stimulated by 0.3 mM ADP. Oxygen uptake during both state 3 (in the presence of succinate and ADP) and state 4 (in the presence of succinate alone) was measured and the ratio of state 3/state 4 respiratory rate was used to calculate the respiratory control ratio.

Mitochondrial enzyme assays. The mitochondria from control and surgically stressed rats were used for assay of the following enzymes. NADH dehydrogenase is an important component of the mitochondrial electron transport chain and its activity was measured spectrophotometrically by the rate of NADH-dependent DCIP reduction at 600 nm [9]. One unit of NADH dehydrogenase activity is defined as that amount required to oxidize 1 μmol of NADH per minute at room temperature. Isocitrate dehydrogenase, both NAD and NADP specific, plays important roles in mitochondrial metabolism, and the activity of isocitrate dehydrogenase to both NAD [10] and NADP [11] was measured using thiocarbonate as substrate by following the increase in absorption at 340 nm. One unit of enzyme activity in this case is defined as that amount that causes a change of 0.01 in optical density per minute at 37°C. Succinate dehydrogenase also is an important component of the mitochondrial respiratory chain, and its activity was assayed using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride as the electron acceptor [12]. One unit of enzyme here is defined as the amount required to convert 1 μmol of INT to its formazan per minute under standard assay conditions. Adenosine triphosphatase is an important in mitochondrial ATP metabolism and the activity was measured by its ability to remove inorganic phosphate from the substrate ATP [13]. One unit of enzyme here is defined as the amount which catalyzes the release of 1 μmol of inorganic phosphate from the substrate ATP per minute under the standard assay procedure.

Measurement of superoxide dismutase and glutathione peroxidase. Mitochondrial superoxide dismutase (SOD) is an important antioxidant enzyme and its activity was assayed by measuring inhibition of superoxide-dependent reduction of the tetrazolium dye MTT as described [14]. One unit of SOD is defined as the amount required to inhibit MTT reduction by 50%. Glutathione peroxidase is also an antioxidant enzyme in mitochondria, and its activity was determined by following the oxidation of NADPH at 340 nm using H2O2 [15]. One unit of enzyme here is defined as the amount needed to oxidize 1 nmol of NADPH per minute.

Measurement of calcium transport. Calcium flux was followed by measuring the changes in the absorption spectrum of Arsenazo-III at 675–685 nm [16]. Mitochondria (1 mg protein/ml) were suspended in reaction medium containing 250 mM sucrose, 5 mM Hepes, 5 mM succinate, and 40 μM Arsenazo-III (pH 7.4). Calcium (10 μM) was added as indicated in figure 6.

Mitochondrial swelling. Swelling of control and surgically stressed mitochondria was determined by the decrease in absorbance at 540 nm up to 10 min [17]. Mitochondrial suspension was added to cuvettes containing sucroseHepes buffer (250 mM sucrose, 5 mM Hepes pH 7.4) and the decrease in absorbance at 540 nm was followed in a Shimadzu UV160 spectrophotometer.

Glutathione estimation. The mitochondrial protein was precipitated using trichloroacetic acid (5%), and glutathione in the acid supernatant was quantitated using high-performance liquid chromatography (HPLC) after derivatization as described [18]. Derivatization involves the initial formation of 5-carboxyl methyl derivatives of free thiols, followed by the conversion of free amino groups to the 2,4-dinitrophenyl derivative. Dinitrophenyl derivatives were separated by HPLC on an Ultrasil NH2 column using a gradient of methanol and sodium acetate and detected at 365 nm.

Protein was estimated using the method of Lowry et al., with BSA as standard [19].

Histological studies. Mucosal tissues from control rats and those obtained at 30, 60, and 120 min and 24 h after surgical stress were
fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and embedded in araldite (epoxy resin). One-micrometer-thick sections were cut and stained with toluidine blue. Suitable areas for ultrastructural study were chosen after examining the 1-μm sections under the light microscope.

For ultrastructural study, ultrathin sections (60–90 nm) were cut on an LKB UM4 ultramicrotome with a diamond knife (Diatome, Switzerland). The 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 electron microscope (Eindhoven, The Netherlands). Areas of the extrusion zone and a few adjacent cells were avoided to exclude any changes due to senescence.

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was performed with Student's t-test to compare changes.

RESULTS

Mitochondrial Respiration and Enzyme Activities

Figure 1A indicates MTT dye reduction by control and surgically stressed mitochondria. In the presence of succinate as substrate, there was a significant increase in dye reduction by mitochondria obtained from enterocytes, 60 min after surgical stress. This increase was transient and was not seen at 120 min and 24 h after surgical stress. Figure 1B shows the respiratory control ratio in both control and surgically stressed mitochondria. There was a decrease in the respiratory control ratio of the mitochondria isolated 60 min after stress and the ratio improved by 120 min and 24 h. The increased MTT reduction seen at 60 min after surgical stress could be due to uncoupling of the mitochondria. Mitochondrial matrix enzymes play important roles in mitochondrial function and it was seen that surgical stress induced a decrease in activity of these enzymes, 60 min after stress, and this returned to control level by 24 h (Fig. 2).

Mitochondrial Antioxidant Status

To assess the status of mitochondrial antioxidant defenses, the activities of superoxide dismutase and glutathione peroxidase were assayed. It was found that there was a significant decrease in SOD activity by 60 min after surgical stress and that this tends to return to control level by 24 h (Fig. 3). Only a small decrease was seen in the activity of glutathione peroxidase. Another important cellular antioxidant defense system is the tripeptide glutathione. It was seen that there was an increase in the level of GSH and GSSG at 30 and 60...
min following surgical stress. By 120 min, the level decreased again (Fig. 4). Interestingly, at 24 h, the level of GSSG was again found to increase.

Mitochondrial Swelling and Calcium Flux

Mitochondrial swelling is another indicator of mitochondrial damage and it was seen that there was a significant decrease in mitochondrial absorbance at 540 nm (an indicator of swelling) at 60 and 120 min after stress, and this again was restored to the control level by 24 h (Fig. 5). Figure 6 shows the calcium flux measurements in enterocyte mitochondria from control and surgically stressed rats. Control mitochondria showed normal uptake in the presence of succinate as the respiratory substrate but this uptake was not seen by 60 min, and by 120 min after surgical stress, an efflux of calcium was seen instead. By 24 h there seemed to be a slight influx of calcium, which indicates recovery of the function.

Ultrastructural Studies

To confirm these functional changes, structural studies using electron microscopy were also done, and mitochondrial swelling with distorted cristae were very clearly evident in samples obtained 60 and 120 min after surgery. In samples taken 24 h after surgery, the mitochondria were of normal size, with well-delineated cristae except in occasional cells which showed mild swelling of mitochondria (Fig. 7).

DISCUSSION

Mitochondria are considered recognition organelles of cell stress [20] and our earlier study implicated oxidative stress as a possible factor in intestinal dysfunction after surgical stress [6]. A prominent feature of cell damage caused by oxidative stress is damage to mitochondria [21]. Mitochondria are the primary sites of cellular energy generation and oxygen consumption and are an important target as well as a source of reactive oxygen species. Mitochondrial function is affected in sepsis [22, 23] and it has been shown that septic mitochondria increase the rate of H$_2$O$_2$ production, accompanied by generation of hydroxyl radicals [24]. Activity of the electron transport chain is diminished after septic shock [25] and hepatic mitochondrial $\alpha$-glycerophosphate dehydrogenase levels were altered after surgical stress [26]. Mitochondrial DNA synthesis also decreased after surgery [27].
To investigate enterocyte mitochondrial function after surgical stress, MTT reduction by isolated mitochondria was measured. MTT is a tetrazolium dye that can be reduced to a colored formazan. Since this reduction is dependent on the respiratory chain function [28], it gives an indication of the functioning of the electron transport chain. MTT reduction in the presence of respiratory substrate, succinate, was increased within an hour after surgical stress, which was not seen at 120 min and 24 h after stress. This was also reflected in the oxygen uptake studies, which indicated that by 60 min after stress, there is a decrease in the respiratory control ratio (RCR), which could be due to uncoupling of respiration from oxidative phosphorylation in the mitochondria. Mitochondrial redox potential has been found decreased after surgery [29] and activities of succinate dehydrogenase and the RCR were decreased after organ damage or multiple organ failure syndrome [30]. The state 3 oxygen uptake of muscle mitochondria was also shown to be decreased in sepsis, along with lowered activities of muscle antioxidant enzymes such as Mn-superoxide dismutase, catalase, and glutathione peroxidase [31]. Since there was evidence for damage occurring to the mitochondria by 60 min after surgical stress, the activity of some mitochondrial matrix enzymes was assayed, which showed a decrease in activity. This may explain the uncoupling of the electron transport chain, since enzymes like NADH dehydrogenase are part of the respiratory chain [32].

Uncoupling of mitochondrial respiration could contribute to generation of free radicals, and these active
species have been implicated in damage induced by surgical stress and are also capable of altering mitochondrial function [33]. All organisms that metabolize oxygen contain superoxide dismutases, which catalytically remove superoxide, and these are important components of the cellular defense against oxygen-derived free radicals [34]. Superoxide dismutase exists in two forms in mammalian cells: a copper- and zinc-containing form (Cu-Zn SOD) in the cytosol and a manganese-containing form (MnSOD) in the mitochondria [35]. MnSOD has been hypothesized to offer protection to cells against cytotoxicity due to tumor necrosis factor [36], as well as other cytokines [37]. In the present study, a decrease in MnSOD activity was seen by 60 min after surgical stress, which may compromise mitochondrial antioxidant functions and increase susceptibility of mitochondria to oxidative stress.

Glutathione is an intracellular thiol, which plays an

![FIG. 6. A representative tracing of the calcium flux in the rat enterocyte mitochondria isolated at different time periods following surgical stress. Assay was done as described under Methods, and 10 μM calcium was added as indicated in the figure.](image)
FIG. 7. Electron microscopy of mitochondria from intestinal epithelium at various time periods after surgical stress, showing normal mitochondria (arrowhead) with well-delineated cristae in controls (A), 30 min (B) and 24 h (E) after laparotomy. Swollen mitochondria, with distorted cristae (arrow) are seen at 60 min (C) and 120 min (D) after stress (original magnification ×34,000).
important antioxidant function, and mitochondrial GSH in the presence of GSH peroxidase is a major defense against toxic oxygen intermediates [38]. During oxidative stress, the ratio of GSH to GSSG decreases due to increased conversion of GSH to GSSG [39], and large doses of GSH have been shown to offer protection against endotoxin or traumatic shock in rats [40]. In the present study, an increase in GSH level was observed at 30 min after surgery, which continued to rise till 120 min and then decreased back to the control level by 24 h. The GSSG level, on the other hand, showed a considerable increase by 30 min when compared to control and then decreased by 120 min. Interestingly, the GSSG level increased again by 24 h. This could be due to the fact that the conversion of GSSG to GSH is affected due to nonavailability of reduced pyridine nucleotides and since the mitochondria cannot export GSSG [41], it accumulates in the mitochondria.

Mitochondrial swelling results from the opening of a megapore in the inner mitochondrial membrane, which causes the mitochondrial permeability transition [42], which can be induced by oxidants [42]. Surgical stress has been associated with the production of reactive oxygen species [33], which may induce mitochondrial swelling. Studies have shown enlarged mitochondria with distorted cristae after multiple organ failure, and with advanced injury, the inner membrane was found fragmented [43]. Structural evaluation of mitochondria in fixed liver slices by electron microscopy in sepsis also showed mitochondrial swelling [44]. Studies have also shown that the mitochondrial membrane permeability transition can be induced by decreased respiratory function [45]. In our study, it was observed that mitochondrial swelling occurred by 60 and 120 min after surgical stress and returned to control level by 24 h. This was confirmed by electron microscopy, which clearly shows swollen mitochondria at 60 and 120 min after stress and by 24 h, normal mitochondria reappear.

Mitochondria are involved in the maintenance of cellular calcium homeostasis [46], and uptake of calcium by mitochondria is through the electrogenic uniporter, driven by the electrochemical transmembrane potential generated during respiration [47]. In this study, it was seen that calcium flux across the mitochondria is affected by surgical stress and by 60 min, the uptake is not seen. This could be due to the fact that altered respiratory chain function may hamper maintenance of the transmembrane potential and affect functioning of the uniporter. It has also been shown that changes in mitochondrial Na\(^+\)–Ca\(^{2+}\) exchanger activity can alter oxidative phosphorylation [48].

In conclusion, these results indicate that the intestinal mitochondria are susceptible to damage during surgical stress, being maximum at 60 min and reversing by 24 h after stress. The reversibility may be due to the minimal time taken for the surgical procedure and it is likely that in major abdominal surgeries, the time taken may be much longer so that the structural and functional alterations in the intestine may be more extensive and sometimes irreversible.

REFERENCES


