Heat Preconditioning Prevents Enterocyte Mitochondrial Damage Induced by Surgical Manipulation

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Background. The small intestine is susceptible to free radical-induced damage and our earlier work has shown that surgical manipulation of the intestine results in generation of oxygen free radicals, leading to mucosal damage. Heat preconditioning has been shown to offer protection against various stresses including oxidative stress and this study looked at the effect of heat preconditioning on surgical manipulation-induced intestinal mitochondrial alterations.

Methods. Control and rats pretreated with heat were subjected to surgical manipulation by opening the abdominal wall and handling the intestine as done during laparotomy. Mitochondria were prepared from isolated enterocytes and structural and functional alterations were assessed.

Results. Surgical manipulation of the intestine resulted in mitochondrial alterations as seen by ultrastructural changes and altered lipid composition. Mitochondria were functionally impaired as evidenced by altered calcium flux, decreased respiratory control ratio, and increased tetrazolium dye reduction and swelling. Along with this, biochemical alterations such as increased lipid and protein oxidation were seen following surgical manipulation. Mild heat preconditioning of the animal prevented these damaging effects.

Conclusion. These studies suggest that stress in the small intestine due to surgery can affect enterocyte mitochondrial structure and function and these effects can be prevented by mild whole body hyperthermia prior to surgery. © 2002 Elsevier Science (USA)

Key Words: surgical manipulation; intestine; mitochondria; heat preconditioning; oxidative stress.

INTRODUCTION

Sepsis, systemic inflammatory response syndrome (SIRS), and multiple organ failure syndrome (MOFS) are major causes of mortality in the intensive care unit. In recent years, the gastrointestinal tract has assumed great importance in the development of these complications and this organ may play a role in the initiation or amplification of these conditions [1, 2]. Splanchnic ischemia and gut barrier failure lead to the development of these complications [3] and it has been suggested that microorganisms from the intestinal lumen may be the source of bacteria and endotoxin involved in the pathophysiology of these syndromes. Reactive oxygen species generated in the gastrointestinal tract have been implicated as one of the initiating factors in the pathophysiology of these postoperative complications [4]. Gastrointestinal epithelium has been shown to be sensitive to oxidant insult arising from both the mucosa and the lumen [5, 6]. Mucosal sources include activated neutrophils, high activity of xanthine oxidase, and mitochondrial respiration. Lumen contains prooxidants derived from dietary materials such as transition metals, ascorbic acid, peroxidized lipids, and ingested drugs, along with bacterial metabolites. Any abdominal surgery involves handling of the intestine in order to reach the organs below. Our earlier work has shown that laparotomy and mild intestinal handling which can occur during any abdominal surgery are capable of causing transient damage to enterocytes by activation of the superoxide-generating enzyme, xan-
thine oxidase (XO), resulting in oxidative stress and considerable damage to intestinal mucosal structure and function [7, 8]. Surgical manipulation also affects the structure and function of subcellular organelles, mitochondria [9]. It has been shown recently that mild intestinal handling could affect distant organ injury such as lung [10].

Both eukaryotes and prokaryotes have evolved essential mechanisms to preserve cellular structure and function under various stresses. Recently, heat preconditioning has been proposed as a new strategy to prevent oxidative injury in cells and tissues [11, 12]. The heat shock response is a highly conserved molecular response that involves a profound alteration in the pattern of gene expression and increased production of heat shock proteins (HSPs) [13]. Various studies have shown the involvement of heat shock proteins in conferring protection against damage [14, 15]. Heat shock proteins are a group of highly conserved proteins, induced in prokaryotes and eukaryotes in response to elevated temperatures and a variety of other stresses, including oxidative stress [16]. Cross-tolerance, the ability of the prior sublethal heat stress to confer protection against a subsequent insult, is well documented under in vitro and in vivo conditions [17, 18]. Recent study from our laboratory has shown that mild whole body hyperthermia offers protection to the intestine and lung during surgical manipulation (unpublished observation). Mitochondria, being an important organelle involved in cellular function, has been shown to be one of the main targets for heat shock-related protection in oxidatively stressed cells [19, 20]. This study looked at the effect of whole body mild hyperthermia on enterocyte mitochondria following surgical manipulation.

MATERIALS AND METHODS

Adenosine diphosphate (ADP), bovine serum albumin (BSA), ethyleneglycol-bis(β-aminomethyl ether)-N₂N₂N₂N’-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), 1,1’-tetramethoxy propane, thiobarbituric acid (TBA), 2,4-dinitrophenyl hydrazine (DNPH), Arsenazo III, succinic acid, phenylmethylsulfonyl chloride (PMSF), pepstatin A, and lipid standards were obtained from Sigma Chemical (St. Louis, MO). Mouse monoclonal antibody against HSP70 and HSP90 and affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG were obtained from Stressgen Biotechnologies Corp. (Victoria, Canada). Bromochloroindolyl phosphate (BCIP) was obtained from Bangalore (Genevi, India). All other chemicals 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 this study. The rats were randomly divided into four groups, sham control (laparotomy without intestinal handling), surgical manipulation (intestinal handling), heated sham control (whole body hyperthermia prior to laparotomy alone), and heated surgical manipulation (whole body hyperthermia prior to intestinal handling).

This study was approved by the Animal Experimentation Ethics Committee of the institution.

Whole Body Hyperthermia

Rats allocated to the heated group were anesthetized by injections of ketamine (50 mg/kg body weight, ip) and placed in a prewarmed humidified heating chamber for 15 min and maintained at 42°C. The body temperature was monitored by a rectal thermometer which was raised to 40°C. Following 15 min, the animals were removed from the heating chamber and allowed to recover for 30 min at room temperature. Laparotomy was then performed on the animals.

Surgical Manipulation of the Small Intestine

Surgical manipulation was carried out as described [8]. Briefly overnight fasted rats were anesthetized and the abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileocecal junction identified. The intestine was then replaced in 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 manipulation [7].

Enterocyte Isolation and Mitochondrial Preparation

Enterocytes were isolated from the small intestine by the metal chelation method. Briefly, the intestine was washed with Krebs Henselet (KH) buffer, pH 7.4, and filled with the same buffer containing 5 mM 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 isolated enterocytes were homogenized and mitochondria were prepared as described [21]. The final mitochondrial preparation was suspended in a solution containing 250 mM sucrose, 5 mm Heps, pH 7.4.

Protein Estimation

Protein was estimated as described using bovine serum albumin as standard [22].

Histological Studies

Intestinal tissue samples were fixed in 2.5% glutaraldehyde, post-fixed with osmium tetroxide, and embedded in araldite (epoxy resin). One micrometer sections were cut using a glass knife and stained with toluidine blue. Well-oriented areas for ultrastructural study were chosen after examination of 1-μm sections under a light microscope. Ultrathin sections (60–90 nm) were cut on an LKBUM4 ultramicrotome using a diamond knife (Diatom, 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, Netherlands) electron microscope.

Western Blot Analysis

Aliquots of intestinal homogenates corresponding to 75 μg protein were resolved on SDS-polyacrylamide gels (7.5%) using electrophoresis over 1 h at a constant voltage of 100 V. The samples were then electrophoretically transferred to a nitrocellulose membrane (type NC, 0.45-μm pore size) using a blotting apparatus. Nonspecific bind-
ing sites were blocked overnight with wash buffer–Tween 20 (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM sodium azide, and 20% Tween 20) containing 5% w/v fat-free dry milk powder at 4°C. The membranes were incubated for 2 h at room temperature with 1:1000 diluted monoclonal mouse antibody directed against inducible HSP70 and HSP90. After a 10- to 15-min wash in wash buffer–Tween 20, the

FIG. 1. Electron micrographs of small intestinal mitochondria of rats subjected to surgical manipulation in the presence and absence of heat preconditioning, showing normal mitochondria in the control and preheated control (A and C). Surgically manipulated intestine show altered mitochondria with increased numbers of dense bodies (arrow) (B). Heat treatment before surgical manipulation results in unaltered mitochondria with well-preserved cristae (D). Original magnification ×39,500).
membranes were incubated for 2 h at room temperature with 1:1000 diluted goat anti-mouse IgG conjugated to alkaline phosphatase. The membranes underwent 10- to 15-min washings in wash buffer–Tween 20 before detection of the alkaline phosphatase activity using the substrate, bromochloroindolyl phosphate [23].

**MTT Reduction Assay**

MTT reduction assay was done using a microtiter plate. In a total volume of 150 μl in each well, mitochondria corresponding to 10–20 μg protein were taken. Six microliters of 125 mM MTT was added and the volume was made up with 25 mM PBS. Mitochondria were incubated at 37°C for 20 min. The reaction was stopped 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 (ELISA reader) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The amount of MTT formazan formed was calculated using the molar extinction coefficient of MTT formazan $E_{370}$ of 17,000 M$^{-1}$ cm$^{-1}$ at pH 7.4 to 8 [24].

**Oxygen Uptake and Swelling Studies**

Polarographic determination of oxygen uptake by isolated mitochondria was done using a Clark-type electrode in 2 ml of respiration medium (225 mM sucrose, 5 mM KH$_2$PO$_4$, 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 with 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) respiration was measured and the ratio of state 3/state 4 respiratory rate was used to calculate the respiratory control ratio [10]. Mitochondrial swelling was determined by absorbance at 540 nm [25].

**Measurement of Calcium Transport**

Calcium flux measurements were done by quantitating the changes in the absorption spectrum of Arsenazo III at 675/685 nm. Mitochondria were suspended in reaction medium containing 250 mM sucrose, 5 mM Hepes, 5 mM succinate, and 40 μM Arsenazo III (pH 7.4). A concentration of 10 μM of calcium was added to the reaction medium to initiate the flux studies [10].

**Peroxidation Parameters**

Mitochondria were used for assessment of lipid peroxidation. Malonaldehyde (MDA) was measured using the TBA method. The amount of MDA formed was calculated from the standard curve prepared using 1,1,3,3-tetramethoxypropane and values were expressed as nanomoles per milligram of protein [26]. For conjugated diene measurement, total lipids were extracted as described, dissolved in 1 ml heptane, and read at 233 nm using a Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated using a molar absorption coefficient of 2.52 × 10$^4$ and expressed as micromoles per milligram [27]. Protein carbonyl content was measured using 2,4-dinitrophenylhydrazine. The amount of protein carbonyl content formed was calculated using a molar absorption coefficient of 2.52 × 10$^4$ and expressed as micromoles per milligram [27]. Protein carbonyl content formed was calculated using an extinction coefficient of 22 mM$^{-1}$ cm$^{-1}$ [28]. α-Tocopherol content was measured using HPLC as described for liver microsomes [29] and quantitated using a Shimadzu 6A HPLC [30].

**Lipid Analysis**

Mitochondrial lipids were extracted by the method of Bligh and Dyer [31]. Lipids were separated by thin-layer chromatography. Neutral lipids were separated on silica gel G plates using the solvent system hexane:diethyl ether:acetic acid (80:20:1, v/v). Spots corresponding to the standard were identified by iodine exposure and

**FIG. 2.** A representative trace of mitochondrial swelling in rat enterocytes isolated from control and after surgical manipulation in the presence and absence of heat preconditioning. Each value represents mean ± SD of three separate experiments with duplicate estimations. All assays were carried out as described under Materials and Methods. *P < 0.05 when compared to control, #P < 0.05 when compared to surgical manipulation. a, control; b, surgical manipulation; c, heat pretreated control; d, heat pretreated surgical manipulation.
eluted. Cholesterol [32] diacylglycerol and triacylglycerol [33] were quantitated as described. Individual phospholipids were separated on a silica gel H plate using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis [34, 35].

Statistical Analysis

Data are expressed as means ± SD. The Mann-Whitney nonparametric test was used to test the significance of differences between groups. A probability of less than 0.05 was accepted as significant. Statistical calculation were performed using SPSS (version 9) software.

RESULTS

On ultrastructural examination, control small intestinal mitochondria appeared normal (Fig. 1A). Mucosa after surgical manipulation (Fig. 1B) showed increased numbers of dense bodies in the mitochondria in the upper part of the villi. Heat-pretreated control mitochondria (Fig. 1C) also appeared normal with well-preserved cristae. There was a significant decrease in the number of dense bodies in the mitochondria of the heat-pretreated surgical manipulation group (Fig. 1D). A characteristic feature of mitochondrial damage is the opening of the permeability transition pore which allows the free passage of small solutes in and out of the mitochondria, resulting in mitochondrial swelling. Spectrophotometric measurement of mitochondrial swelling showed an increased swelling after surgical manipulation and this increase in swelling was prevented by heat pretreatment (Fig. 2). Along with structural changes, the functional aspects of the mitochondria were examined after surgical manipulation. Studies on mitochondrial respiration showed that heat pretreatment protected against the decrease in mitochondrial respiratory control ratio, seen after surgical manipulation (Fig. 3A). Superoxide anion, the main oxygen free radical generated in the cell, is known to reduce MTT, a tetrazolium compound, to its colored formazan. Enterocyte mitochondria isolated from rats after intestinal manipulation showed an increase in MTT reduction as compared to control enterocyte mitochondria and this increase was almost completely prevented by heat pretreatment (Fig. 3B). Mitochondria play an important role in cellular calcium homeostasis and calcium flux studies on mitochondria showed an uptake of calcium in the control mitochondria, which was abolished in mitochondria prepared from intestine after surgical manipulation. Pretreatment with heat restored the calcium influx (Fig. 4). Uncoupling of mitochondrial respiration together
with increase in MTT reduction indicates possible superoxide generation during surgical manipulation. Since free radical generation can occur within the organelle, mitochondria were further examined for oxidative stress parameters. An increase in lipid peroxidation and protein oxidation products such as malonaldehyde, conjugated diene, and protein carbonyl content and a decrease in \( \alpha \)-tocopherol content were observed following surgical manipulation and this was prevented by heat treatment prior to surgical manipulation (Fig. 5).

Lipids are important constituents of the membrane and their integrity is crucial for normal functioning of organelles. Lipid analysis of the mitochondria showed alteration in the phospholipid composition, especially a decrease in phosphatidylcholine and phosphatidylethanolamine accompanied by an increase in lysophosphatidylcholine, lysophosphatidylethanolamine, and phosphatidic acid after surgical manipulation and heat conditioning prior to surgery prevented these lipid alterations (Fig. 6).

The heat shock response provides an ideal model to address the mechanism of cellular and subcellular protection in the face of acute pathophysiological stresses. This response is a highly conserved molecular response resulting in increased production of a family of heat shock proteins. In the present study pretreatment with mild whole body hyperthermia to a core body temperature of 40°C for 15 min did not induce the synthesis of HSP70 or 90 in the rat small intestine as assessed by immunoblot (data not shown).

**DISCUSSION**

Reactive oxygen species have been implicated in the pathophysiology of postsurgical complications such as SIRS and MOFS [4]. Mitochondria are important subcellular organelles involved in energy production and are susceptible to a variety of insults, but among them, oxidative stress is important since mitochondria consume more than 90% of the oxygen utilized by the cell. Normally oxygen free radicals are produced endogenously as a by-product of electron transport, and mitochondria have their own radical-scavenging system to neutralize these radicals [36]. Alteration in the balance between free radical production and scavenging ability is thought to lead to mitochondrial injury. Earlier studies have implicated oxidative stress as a possible factor in intestinal mitochondrial dysfunction after surgical manipulation of the bowel [8].

Recent studies have shown the importance of several endogenous cellular factors that protect the intestinal epithelial cells from the effects of stress and injury. Of these, inducible heat shock proteins have been shown to increase survival of intestinal epithelial cells against oxidant and heat-induced stress [17, 18]. Mitochondria is suggested to be one of the targets for heat-related protection in cells undergoing oxidative stress [37] and in the present study the effect of heat preconditioning on surgical manipulation-induced alterations to the intestinal mitochondria was examined. The results show that heat preconditioning is able to offer protec-
Mitochondrial function has been shown to be affected in sepsis and a decrease in respiratory control ratio was seen after organ damage and MOFS [38, 39]. It was observed that surgical manipulation affects intestinal mitochondrial function with a decrease in RCR which was prevented by heat pretreatment. This supports the earlier studies, which have shown a protective role of heat shock on rat mitochondrial respiration [19]. Mitochondria play an important role in maintaining cellular calcium [40] and uptake of calcium by mitochondria is through the electrogenic uniporter, driven by the electrochemical transmembrane potential generated during respiration [41]. Efflux of calcium from mitochondria occurs through sodium-dependent and -independent channels. Mitochondrial calcium flux is affected after surgical manipulation. Ultrastructural studies show the presence of dense bodies in the mitochondria, which indicates alteration in ion transport to enterocyte mitochondria during surgical manipulation.

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during surgical manipulation and pretreatment with heat restored the uptake of calcium as well as decreased the dense bodies in the mitochondria, indicating a protective role of heat in this process. Mitochondrial uncoupling of respiration together with increase in MTT indicates possible superoxide generation during surgical manipulation. Lipid peroxidation, the undesired oxidative modification of polyunsaturated fatty acyl chains, is a major contributor to membrane damage in cells and has been implicated as a cause and effect of many pathological processes associated with oxygen toxicity. Lipid peroxidation is considered as one of the basic mechanisms involved in reversible and irreversible cell and tissue damage. It was seen that surgical manipulation of the intestine resulted in an increase in lipid and protein peroxidation products and a decrease in \( \alpha \)-tocopherol in enterocyte mitochondria. It is likely that peroxidation of lipids can lead to degradation of membrane lipids, and studies have shown that interaction of these degradation products with intra and extracellular targets can produce new reactive species during the course of chain reactions resulting in damage to cells and tissues [42]. Functional alterations in the mitochondria may be induced by structural changes and this was checked by analyzing the lipid compositions which are essential components of the membrane. Surgical manipulation resulted in mitochondrial phospholipid degradation and generation of lysophospholipids. These degradation products are membrane lytic agents and are probably the result of phospholipase \( \Lambda_2 \) activation. In addition to these products, the level of phosphatidic acid was also increased accompanied by decreased phosphatidylethanolamine and phosphatidylcholine, suggesting activation of phospholipase D (PLD). Our earlier work has shown the presence of PLD in intestinal mitochondria,
which can be activated by oxygen free radicals and divalent metal ions [43]. These lipid alterations were prevented by heat preconditioning.

Several studies support the hypothesis that hyperthermia, followed by a period of temperature normalization, protects tissues and cells from the effects of subsequent potentially lethal conditions. This cytoprotective effect is associated with increased HSP production. HSP induction has been shown to reduce damage and enhance survival in a rat model of intraabdominal sepsis and sepsis-induced lung injury [44–46]. To look for possible factors involved in protection by heat preconditioning during surgical manipulation, HSP expression was studied using immunoblot. It was seen that both HSP70 and HSP90 were not detectable in the intestine by 40°C heat preconditioning used in this experiment. This result agrees with a recent study on whole body hyperthermia where HSP70 induction was not seen at 40°C [47]. It is likely that either the HSPs are not induced by the mild hyperthermia used in this study or the immunoblot method used for the identification was not able to detect the minute amount of HSPs present. It is also possible that during mild hyperthermia with surgical manipulation, some other mechanism may be the end effector of protection.

It has been suggested that the protection offered by heat preconditioning may be a response to the elevation of antioxidants [48, 49]. In an earlier study, heat preconditioning increased endogenous antioxidant levels in the intestine and prevented the oxidative stress damage in both the intestine and the lung following surgical manipulation (unpublished observation). Protection by heat stress against oxidative damage has been studied in various animal models [50, 51] and there is evidence showing decreased tissue damage in an animal model of acute lung injury by heat pretreatment [52, 53]. Hence it is probable that heat pretreatment can offer protection to enterocyte mitochondria by increasing the cellular antioxidant levels.

In conclusion this study has shown structural and functional alterations to enterocyte mitochondria during surgical manipulation, which can be prevented by prior mild whole body hyperthermia. Since oxidative stress occurs in the intestine following surgical manipulation and heat preconditioning increases the antioxidant enzyme activities, it is likely that upregulation of cellular antioxidants may be one of the mechanisms by which heat preconditioning offers protection.

REFERENCES


