Heat Preconditioning Attenuates Oxygen Free Radical-Mediated Alterations in the Intestinal Brush Border Membrane Induced by Surgical Manipulation

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Background. The intestine is highly 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 and mucosal damage along with alterations in the brush border membrane (BBM). Heat preconditioning is known to offer protection against various stresses including oxidative stress and this study looked at the effect of heat preconditioning on the intestinal BBM alterations following surgical manipulation.

Methods. Control and rats heat preconditioned were subjected to surgical manipulation by opening the abdominal wall and handling the intestine. BBM were isolated from the intestine and structural and functional alterations to these membranes were assessed.

Results. Surgical manipulation resulted in oxidative stress suggested by a decrease in alkaline phosphatase activity and α-tocopherol content, accompanied by an increase in lipid peroxidation. A decrease in glucose transport by the isolated BBM vesicles suggested functional impairment. Surgical manipulation resulted in phospholipid degradation with generation of arachidonic acid along with appearance of cPLA₂ in the membrane. These changes were prevented by heat preconditioning of the animal prior to surgical manipulation.

Conclusion. These results suggest that heat preconditioning offers protection from damage to the intestinal BBM following surgical manipulation and mild whole body hyperthermia might prevent postsurgical complications.

Key Words: heat preconditioning; brush border membrane; oxidative stress; phospholipids; cytosolic phospholipase A₂.

INTRODUCTION

The microvillus (brush border) membrane (BBM) of the intestine plays an important role in the digestion and absorption of essential nutrients. It also acts as a barrier that excludes bacteria and other foreign substances from the lumen. This barrier can be adversely affected under a number of pathological conditions including burn trauma, hemorrhagic shock, and surgical stress [1]. In recent years, the intestine has assumed great importance in the development of postoperative complications such as sepsis, systemic inflammatory response syndrome (SIRS), and multiple organ failure (MOF). Our earlier work has shown that surgical manipulation of the intestine can induce widening of intercellular spaces, increased permeability, and oxidative stress in the mucosa [2]. Surgical manipulation also leads to structural and functional alterations in the intestinal BBM including altered lipid composition and sugar transport [3].

Hyperthermia plays an important role in basic cellular defense mechanisms and consequently prevents tissue damage. This protection by hyperthermia is either mediated by synthesis of highly conserved set of proteins known as heat shock proteins (HSP) [4] and augmentation of synthesis of endogenous antioxidants [5]. HSPs are also induced by unrelated environmental stress [6, 7] (ethanol or sodium arsenite). It has been shown that heat stress-induced protection against intestinal ischemia/...
reperfusion injury involves inhibition of leukotriene B4 production and subsequent prevention of neutrophil activation and chemotaxis [8].

Oxygen free radical-mediated damage has been implicated in the pathophysiology of the gastrointestinal mucosa. It has been shown that heat shock proteins play an important role in cytoprotection against oxidative stress-induced injury [6, 9]. Heat pretreatment has been shown to provide tolerance to subsequent nonthermal stress and this is illustrated by gastric cytoprotection against ethanol-induced damage [7] and reduced organ damage in sepsis-induced lung injury [10].

Our previous study has shown structural and functional alterations in the intestinal brush border membrane [3] and mitochondria [11] following surgical manipulation of the intestine, which is due to generation of oxygen free radicals. The objective of this study was to determine whether heat preconditioning offers protection to the intestinal brush border membranes against oxidative stress-induced damage following surgical manipulation.

MATERIALS AND METHODS

Tris (hydroxymethyl) aminomethane (Tris), N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (Hepes), bovine serum albumin (BSA), p-nitrophenyl phosphate, peroxidase, O-dianisidine, glucose oxidase, thiobarbituric acid (TBA), guanidine hydrochloride, phenylmethylsulfonyl fluoride, pepstatin-A, and lipid standards were obtained from Sigma Chemical Co. (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). Rabbit polyclonal antibody against cPLA2 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG and bromochloroindolyl phosphate (BCIP) was obtained from Banglore Genei (India). Polyethylene glycol (PEG) 4000 was obtained from Fluka AG, Switzerland. 14C-labeled glucose was obtained from Bhaba Atomic Research Center (Bombay, India), Millipore membranes (pore size 0.45 μm) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and piperazine-(2-ethanesulfonic acid) (BSA), pH 7.1. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM.

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: (1) sham control (laparotomy without intestinal handling), (2) surgical manipulation (laparotomy and intestinal handling), (3) heat preconditioned sham control (whole body hyperthermia prior to laparotomy alone), (4) heat preconditioning followed by surgical manipulation (whole body hyperthermia prior to laparotomy and intestinal handling). This study was approved by the Animal Experimentation Ethics Committee of the Institution.

Whole Body Hyperthermia

Rats allocated to the heat-preconditioning group were anesthetized by an injection of ketamine (50 mg/kg body weight, ip) and placed in a prewarmed humidified heating chamber for 15 min and maintained at 40°C. The body temperature was monitored by a rectal thermometer which was raised to 39°C. Following 15 min, the animals were removed from the heating chamber and placed for 30 min at room temperature [12]. Laparotomy was then performed on the animals.

Surgical Manipulation of the Small Intestine

This was carried out as described [2]. Briefly overnight fasted rats were anesthetized by ketamine injection. 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 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 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 this surgical procedure [2].

Isolation of Brush Border Membranes

BBM were isolated from the small intestine of control and surgically manipulated rats by the PEG precipitation method [13]. Briefly, luminal contents were washed thoroughly with ice-cold saline and the mucosa was scraped using a glass slide. Approximately 3% homogenate of the mucosa was prepared in 2 mM Tris-HCl containing 50 mM mannitol, pH 7.1 using a Porter-Elvehjem homogenizer for 2–3 min at full speed. This was allowed to remain at 4°C for 15 min and filtered using nylon cloth. To this, a 50% PEG solution was added to make a final concentration to 10% PEG, stirred for 15 min, and centrifuged at 7500g for 15 min. The pellet was discarded and the supernatant was spun at 27,000g for 40 min. To the pellet, 30 ml suspension buffer (10 mM Tris-HCl and 300 mM mannitol, pH 7.1) was added, centrifuged at 27,000g for 40 min. The pellet was washed twice with the same suspension buffer and finally suspended in 1 ml of the same buffer using a syringe fitted with a 26-gauge needle. Purity of the isolated BBM was checked by enrichment of the marker enzyme alkaline phosphatase (ALP), sucrase and maltase. Protein was estimated using bovine serum albumin as standard [14].

Enzyme Assays

Activity of ALP [15], sucrase [16], and maltase [16] was assayed as described. The enzyme activity is expressed as units per milligram protein (units are expressed as μmol/min/mg protein for ALP and nmol/min/mg protein for maltase and sucrase).

Oxidative Stress Parameters

Various peroxidation parameters were measured in the isolated BBM. Malonaldehyde (MDA) was measured using thiobarbituric acid [17]. The amount of MDA formed was calculated from the standard curve prepared using 1,1′,3,3′-tetramethoxypropane (values expressed as nmol/mg protein). For conjugated diene measurement, total lipids from BBM were extracted as described [18], 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 × 104 and (expressed as μmol/mg protein) [19]. Protein carbonyl content was measured using 2,4-dinitrophenylhydrazide (DNPH) [20]. The amount of protein carbonyl content formed was calculated using an extinction coefficient of 22 mM-1 cm-1. Protein carbonyl content was measured using HPLC. Membrane tocopherol was estimated as described for liver microsomes [21] and quantitated using Shimadzu 6A HPLC [22].

Lipid Analysis

BBM lipids were extracted by the Bligh and Dyer method [18]. The lower organic phase was concentrated using nitrogen, resuspended
in a small volume of chloroform:methanol (2:1) and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexanediethyl ether:acetic acid (80:20:1, v/v). Spots corresponding to the standard were identified by iodine exposure and eluted. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Individual phospholipids were separated on 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 (23).

**RESULTS**

Figure 1 shows activity of BBM enzymes, alkaline phosphatase, sucrase, and maltase and as can be seen, ALP activity decreased to half after surgical manipulation as compared to heat preconditioning of the animal prior to surgery (Fig. 1A). There was no change in the activity of other BBM enzymes, sucrase and maltase (Fig. 1B). Activity of BBM enzymes and other parameters studied between sham control and heat-preconditioned sham control were found to be identical and hence data are shown only with heat-preconditioned sham control. Figure 2 shows oxidative stress parameters in the BBM and as can be seen, heat preconditioning prevented the increase in MDA, conjugated diene, and protein carbonyl and the decrease in \( \alpha \)-tocopherol following surgical manipulation. The functional integrity of BBM was assessed by measuring the glucose transport ability of the membrane vesicles after surgical manipulations with and without heat preconditioning. This resulted in a decrease in glucose transport ability of the membrane, which was significant when compared to that of sham control (Fig. 3) and this was prevented by heat preconditioning of the animal prior to surgical manipulation. The uptake indicated the classical "uphill transport" of \( \alpha \)-glucose. Oxidative stress-induced alterations in the BBM following surgical manipulation may be associated with structural alterations, and because lipids are an important constituent of membranes, the lipid composition of BBM was analyzed in different group of animals. Figure 4 shows the level of certain phospholipids in BBM following surgical manipulation. A decrease in phosphati-
dycholine (PC) and phosphatidylethanolamine (PE) and an increase in lyso PC and lyso PE were seen following surgical manipulation. This was associated with an increase in free fatty acids, especially linoleic and arachidonic acids (Fig. 5). These lipid alterations were prevented by heat preconditioning. There was no significant change in neutral or other phospholipids in the BBM following surgical manipulation (data not shown). Since arachidonic acid is released in BBM following surgical manipulation and cPLA₂ is specific for arachidonic acid containing phospholipids, the presence of cPLA₂ in the BBM was tested by immunoblotting. As shown in Fig. 6, cPLA₂ was detected in the BBM following surgical manipulation, which was absent in BBM from control, sham control, and heat preconditioning prior to surgical manipulation.

Mild heat preconditioning used in these experiments did not result in induction of heat shock proteins as evidenced by immunoblotting using monoclonal antibody against HSP70 and HSP90 (data not shown).

**DISCUSSION**

The intestine is highly susceptible to surgical manipulation and free radical-mediated damage has been implicated in the pathophysiology of postoperative complications such as SIRS and MOF. Our earlier work has shown that during surgical manipulation of...
the intestine, oxygen free radicals are generated due to activation of xanthine oxidase (XO) [2] and mitochondrial dysfunction [11], which in turn leads to various biochemical changes in the BBM, including degradation of phospholipids and generation of arachidonic acid [3]. It was also shown that these events can be prevented by pretreating the animal with XO inhibitor, allopurinol, suggesting a role for oxidative stress in the mucosal damage following surgical manipulation [2]. Heat preconditioning is known to offer protection against different forms of stress including oxidative stress and this may be due to either induction of heat shock proteins [26–28] or increase in the activity of antioxidants [5]. Expression of stress proteins prior to any form of injury is supported by various studies which showed a protective role of HSP72 against oxidant and I/R-induced damage in the small intestine [29, 30]. The role of HSPs in the gastrointestinal mucosal defense has been studied recently [31].

In the present study heat preconditioning of the animal prior to surgical manipulation protected from surgical manipulation-induced BBM alterations, which was shown by various parameters of peroxidation as well as structural and functional alterations. Functional alteration was seen by glucose uptake in the isolated BBM. Glucose uptake was decreased following surgical manipulation, which was prevented by heat preconditioning. We have earlier shown that in vitro exposure of BBM to oxidative stress decreased glucose and amino acid transport [32] and lipid peroxidation of BBM decreases sodium-dependent glucose transport [33]. Whole body hyperthermia prior to surgical manipulation did not result in induction of heat shock proteins. It is likely either that HSPs are not induced by mild hyperthermia used in this study or that the immunoblot method used for identification was not able to detect the small amount of HSP induced. This is similar to a recent observation in which the authors were unable to detect heat shock proteins following whole body hyperthermia [5]. Heat stress is known to induce antioxidants [30] and we have observed an increase in antioxidants such as superoxide dismutase and catalase by mild heat preconditioning prior to surgical manipulation (unpublished observation).

One of the observations following surgical manipulation of the intestine was change in the BBM phospholipids leading to generation of lyso phospholipids and arachidonic acid. This suggested a possible role for PLA2 in BBM lipid alterations. It is known that oxygen free radicals can activate PLA2 [34] and increased lyso PC and lyso PE were evident following lipid peroxidation of PLA2-containing liposomes and microsomes [35]. Since arachidonic acid was the main fatty acid released by the action of PLA2 and cPLA2 specifically acts on the arachidonic acid-containing phospholipids, the presence of cPLA2 in the isolated BBM was checked by immunoblotting using antibody to cPLA2. It was...
observed that cPLA₂ was absent in the control BBM but appeared in the BBM isolated after surgical manipulation. Moreover heat preconditioning prevented the appearance of cPLA₂ in the BBM after surgical manipulation. This suggests a possible translocation of cPLA₂ on to the BBM following surgical manipulation of the intestine, resulting in phospholipid degradation and arachidonic acid release which was prevented by heat preconditioning prior to surgical manipulation. It is known that cPLA₂ preferably hydrolyzes phospholipids containing arachidonic acid and responds to physiological increments of Ca²⁺ with translocation on to the membrane [36].

It is likely that arachidonic acid released following surgical manipulation can act as a precursor of various bioactive lipids, which are important mediators of distant organ damage [37, 38]. Our earlier study has shown that following surgical manipulation of the small intestine, lung damage occurs due to oxidative stress and this lung damage can be prevented by inhibiting xanthine oxidase, PLA₂ or cyclooxygenase enzymes prior to surgical manipulation [39]. In the present study also, it was seen that heat preconditioning could prevent BBM phospholipid degradation and generation of arachidonic acid by cPLA₂, which in turn might prevent lung damage.

In conclusion, this study has shown that during surgical manipulation of the intestine, structural and functional alterations occur in the BBM including lipid peroxidation, altered transport, and lipid composition. This is associated with arachidonic acid release by the action of cPLA₂ on membrane lipids. These biochemical events in the intestinal mucosal cells can be prevented by heat preconditioning and suggest that prior mild whole body hyperthermia might offer protection from postsurgical complications.

**REFERENCES**


**FIG. 5.** Free linoleic (□) and arachidonic acid (■) in the BBM following surgical manipulation with and without heat preconditioning. (Each value represents mean ± SD of three separate animal experiments with duplicate estimations. *P < 0.05 when compared to sham control. #P < 0.05 when compared to surgical manipulation.)

**FIG. 6.** Immunoblot of cPLA2 in BBM under various experimental conditions. (Lane 1, sham control; Lane 2, surgical manipulation; Lane 3, heat-preconditioned sham control; and Lane 4, heat preconditioning and surgical manipulation.)


