NITRIC OXIDE PREVENTS OXIDATIVE STRESS-INDUCED DAMAGE TO ENTEROCYTE MITOCHONDRIA DURING SURGICAL STRESS

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Key Words: α-Tocopherol, enterocyte, free radical damage, gastrointestinal function, L-arginine, mitochondria, N⁶-nitro-L-arginine methyl ester (L-NAME), nitric oxide, oxygen free radicals, prevention of oxidative stress, surgical stress.

Abstract

The intestine is highly susceptible to free radical-induced damage. Earlier work has shown that surgical stress induces generation of oxygen free radicals in enterocytes, resulting in intestinal damage along with changes in mitochondrial structure and function. Nitric oxide is an important mediator of gastrointestinal function. This study looks at the effect of nitric oxide on oxidative stress-induced intestinal mitochondrial alterations during surgical stress. Mild handling of the intestine resulted in oxidative stress within the mitochondria and this was confirmed by uncoupling of respiration, increased tetrazolium dye (MTT) reduction, increased lipid and protein oxidation products, altered lipid composition and decreased α-tocopherol content in the mitochondria. Activation of mitochondrial proteases was also observed following surgical stress. Pretreatment with nitric oxide synthase substrate, L-arginine prevented these effects of surgical stress. Protection with L-arginine was abolished by the nitric oxide synthase inhibitor N⁶-nitro-L-arginine methyl ester. These results indicate a protective role of nitric oxide against surgical stress-induced enterocyte mitochondrial dysfunction.

Introduction

Gastrointestinal epithelium has been shown to be sensitive to oxidant insult arising from both mucosa Parke (1989) and the lumen (Koningsberger, Mark et al., 1988).

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Mucosal sources include activated neutrophils, high activity of xanthine oxidase and mitochondrial respiration. Lumen contain pro-oxidants derived from dietary materials such as transition metals, ascorbic acid, peroxidized lipids and ingested drugs, along with bacterial metabolites. Reactive oxygen species generated in the gastrointestinal tract have been implicated as one of the factors in the pathophysiology of post-operative complications such as multiple organ failure (MOF) and systemic inflammatory response syndrome (SIRS) (Zhi-Youngl, Dong et al., 1992). Under stress conditions, such as following a major abdominal surgery, or surgery even at remote locations, hypoperfusion of the intestine results in impairment of mucosal barrier function, which permits translocation of bacterial pathogens into the systemic circulation. It is well known that hypoxia results in generation of oxygen free radicals, and oxidative stress has a damaging effect on cells and tissues. At the cellular level, mitochondria are important source as well as target of free radicals, and mitochondrial dysfunction could thus play a role in initiation of cellular damage. Our earlier work using a rat model has shown that laparotomy with mild intestinal handling can result in increased intestinal permeability and oxidative stress in the enterocytes (Anup, Aparna et al., 1999). Surgical stress also affects the sub-cellular organelle, mitochondria (Anup, Susama et al., 2001) and this may be the instigating factor for the later development of evident clinical complications such as SIRS and MOFS.

Nitric oxide (NO), a nitrogen free radical, has emerged as an important signal and effector molecule in mammalian physiology. NO is known to have many modulatory functions on cells and tissues, including those of the gastrointestinal (GI) tract (Wallace, Reuter et al., 1994; Andrews, Malcomplex-Wilson et al., 1994). It has the capacity to down regulate inflammatory responses in the intestinal tract, and also to protect cells against the detrimental effects of reactive oxygen species (Kubes, Suzuki et al., 1991; Gaboury, Woodman et al., 1993). Its beneficial and damaging effect on cells and tissues has been extensively studied, and this dual effect of nitric oxide is also seen on the mitochondria (Podesser, Linder et al., 1999; Schweizer and Richter 1994). L-arginine administration reverses the deleterious effect of nitric oxide synthase inhibition on mucosal barrier function (Vromen, Szabo et al., 1996). Our earlier work has shown that oxidative stress-induced damage to the intestinal mitochondria following ischemia-reperfusion injury can be ameliorated by the luminal presence of nitric oxide donor (Madesh, Ramachandran et al., 2000). The objective of the present study was to look at the effect of surgical stress-induced oxidative stress on intestinal mitochondrial structure and function and the protective role of nitric oxide in this process.

**Materials and Methods**

Adenosine diphosphate (ADP), bovine serum albumin (BSA), ethyleneglycol-bis-(B-aminoethyl ether) N, N, N, N'-tetra acetie acid (EGTA), dimethyl sulfoxide (DMSO), 
MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), 1', 1', 3, 3' 
tetramethoxy propane, gelatin, thiobarbituric acid (TBA), N^6-nitro-L-arginine methyl ester (L-NAME), succinic acid and lipid standards were obtained from Sigma Chemical (St Louis, Mo). All other chemicals used were of analytical grade.

**Animals**

Adult Wistar rats of both sexes (200-250 g), exposed to a daily 12-hr light-dark cycle and fed water and rat chow ad libitum were used for the study. The study has been divided in to two parts. The first part consist of five groups of rats: control (laparotomy
alone) and various time periods such as 30, 60, 120 min and 24hr after surgical stress (laparotomy with intestinal handling). The time periods indicates the time of sacrificing the animal following surgical stress. In the second part, the rats were randomly divided into four groups: control (arginine pretreated and laparotomy alone performed without intestinal handling), surgical stress (laparotomy and intestinal handling), arginine (surgical stress after arginine pretreatment) and L-NAME (surgical stress after arginine and L-NAME pretreatment). The animals were sacrificed 60 minutes following surgical stress since our earlier work indicated maximum alterations at this time period. This study has been cleared by the Animal Experimentation Ethics Committee of the institution.

**L-arginine and L-NAME pretreatment**

Rats were given an intra-peritoneal injection of 0.3g/kg body weight of L-arginine (Angeli et al., 1998) 30 minutes prior to surgery or administered with N\(^{\circ}\)-nitro-L-arginine methyl ester (L-NAME) (0.1 mg/ml) for 9 days in drinking water prior to experimentation.

**Induction of surgical stress**

Surgical stress was induced as described (Anup, Susama et al., 2000). Briefly, overnight fasted rats were anesthetized by ketamine injection (50mg/kg body weight, IP). The abdominal wall was opened by a vertical incision of approximately 4cm. The intestine was gently moved and the ileocaecal junction identified. The intestine was then handled along its entire length from the ileocaecal junction proximally, simulating the "inspection" that occurs in a clinical setting. The intestine was then replaced back into the abdominal cavity and the whole process was completed within one to two minutes. Following this, the abdominal wall was sutured and the animals were killed by decapitation at various time periods such as 30, 60, 120 min and 24 hr after the surgical procedure.

**Enterocyte Isolation and mitochondrial preparation**

Enterocytes were isolated from the small intestine by metal chelation method. Briefly, the intestine was washed with Krebs Henseleit (KH) buffer pH 7.4 and filled with the same buffer containing 5mM EDTA and incubated at 37\(^\circ\) C for 12 minutes 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 900 x g for 5 minutes. Mitochondria were prepared from isolated enterocytes (Masola and Evered 1984). Protein was estimated by Lowry’s method using bovine serum albumin as standard (Lowry, Rosebrough et al., 1951).

**Lipid Analysis**

Mitochondrial lipids were extracted by the method of (Bligh and Dyer 1959). 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 eluted. Cholesterol (Zlatkis, Zak et al., 1953) diacylglycerol and triacylglycerol (Synder and Stephen 1959) were quantitated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. 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 (Shipski Peterson et al., 1964; Barlett (1959); Cohen, Derksen et al., 1969).

**MTT Reduction Assay**

MTT reduction assay was done using a microtitre plate. In a total volume of 150 μl in each well, mitochondria corresponding to 10-20μg protein were taken. 6 μl of 125 mM MTT added and the volume made up with 25mM PBS. Mitochondria were incubated at 37°C for 20 min. The reaction was stopped by the addition of 150μl of dimethyl sulfoxide (DMSO) and mixed thoroughly to dissolve the formazan. The plates were read on a multiwell scanning spectrophotometer (ELISA reader) using a test wavelength of 570nm and a reference wavelength of 630nm (Madesh, Lakshmi et al., 1997). 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.

**Oxygen uptake and swelling studies**

Polarographic determination of oxygen uptake by isolated mitochondria was done using a Clark type electrode in 2ml of respiration medium (225mM sucrose, 5mM KH_{2}PO_{4}, 20mM KCl, 10mM Tris and 5mM Hepes pH 7.4) containing 5mM succinate as respiratory substrate. A mitochondrial protein of 2mg/ml was used. Oxygen uptake was stimulated with 0.3mM 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 (Anup, Susama et al., 2001). Mitochondrial swelling was determined by absorbance at 540 nm (Takeyama, Matsno et al., 1993).

**Peroxidation parameters**

Mitochondria were used for assessment of lipid peroxidation. Malonaldehyde (MDA) was measured using TBA method (Ohkawn, Ohishi et al., 1979). The amount of MDA formed was calculated from the standard curve prepared using 1, 1', 3, 3' tetramethoxy propane and value expressed as nmole/mg protein. For conjugated diene measurement, total lipids were extracted as described (Bligh and Dyer 1959) dissolved in 1ml heptane and read at 233 nm using Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated using molar absorption coefficient of 2.52 x 10^4 and expressed as μmoles/mg protein (Chan and Levett et al., 1972). Protein carbonyl content was measured using 2,4-dinitro phenyl hydrazine (DNPH) (Sohal, Agarwal et al., 1993). The amount of protein carbonyl content formed was calculated using an extinction co-efficient of 22 mM^{-1}cm^{-1}. α-Tocopherol content was measured using HPLC as described for liver microsomes (Cheeseman, Davies et al., 1987) and quantitated using Shimadzu 6A HPLC Vatassery (1989).

**Gelatin Zymography**

Zymogram of protease activity was performed as described (Heussen and Dowdle 1980), with slight modification. Polyacrylamide gel (12%) containing 0.1% gelatin were cast and 15μg of mitochondrial 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 gel was run at 100 volts for 2 hours and then soaked in 2% Triton X-100 and incubated at
20°C on a shaker for 90 minutes with three changes. Following this, the gel was soaked in reaction buffer (0.1M Tris-HCl pH 7.5) overnight at 37°C and then stained with Coomassie blue.

**Statistical analysis**

Values are expressed as mean ± SD. The students t-test was used for independent comparisons and Bonferroni correction was applied for multiple t-test where necessary. Statistical calculations were performed using SPSS for windows (version 9.0) software.

**Results**

Lipids play a crucial role in mitochondrial structure and function. Lipid analysis of the enterocyte mitochondria obtained from control and various time periods after surgical stress showed maximum alteration in the phospholipid composition, especially a decrease in phosphatidyl choline and phosphatidyl ethanolamine accompanied by an increase in lysophosphatidylcholine, lysophosphatidylethanolamine by 60min after surgical stress and the phospholipid composition came back to control level by 24 hours (Table I). An increase in total free fatty acids was also seen maximum at 60 min following surgical stress, which also reversed to control level by 24 hours (Figure 1). There was no significant change in other phospholipids and neutral lipids between stressed mitochondria and control (data not shown).

Since our earlier studies (Anup, Susama et al., 2001) and the above shown data indicated maximum alteration by 60 minutes following surgical stress, further studies to look at the protective role of nitric oxide were carried out using this time period. An increase in mitochondrial swelling, MTT dye reduction and a decrease in respiratory control ratio (RCR) were observed in surgically stressed mitochondria as compared to control. These mitochondrial functional changes were prevented by L-arginine pretreatment and L-NAME inhibited this protection by L-arginine (Table II). Uncoupling of mitochondrial respiration together with increase in MTT reduction indicates superoxide generation during surgical stress. Since free radical generation can occur within the organelle, mitochondria was further examined for oxidative stress parameters. An increase in lipid peroxidation and protein oxidation products such as malondialdehyde (MDA), conjugated diene, protein carbonyl content and a decrease in antioxidant vitamin, α-tocopherol were observed following surgical stress and this was prevented by pre-treatment with L-arginine, a NO donor. This protection by L-arginine was abolished by L-NAME administration (Figure 2).

Lipids are important constituents of membranes and to look at the role of nitric oxide on mitochondrial lipid alterations following surgical stress, lipids were analyzed with and without pre-treatment with L-arginine and L-NAME. It was found that L-arginine pretreatment offered complete protection against surgical stress-induced phospholipid alterations in the mitochondria (Figure 3). L-arginine also prevented the increase in free fatty acids seen following surgical stress and this prevention was abolished in presence of L-NAME (Figure 4). Proteases have been implicated in damage caused by oxidative stress and mitochondria are known to contain proteases which can recognize and degrade oxidatively denatured proteins. Figure-5 shows the gelatin zymography of mitochondrial proteases. An increase in protease activity, specifically 105, 88, 47, 22 Kd proteases was seen in the mitochondria isolated after surgical stress. This increase was almost completely abolished
Figure 1. (TOP ONE PANEL) Free fatty acid content of enterocyte mitochondria isolated at different time periods following surgical stress. Analysis was done as described under methods. Values are mean ± SD of four separate experiments. *p<0.01 when compared to control. #p<0.01 when compared to 60 minutes after surgical stress.

Figure 2. (BOTTOM FOUR PANELS) Peroxidation parameters- malondialdehyde (MDA) (A), conjugated diene (B), protein carbonyl content (C) and α-tocopherol (D) in enterocyte mitochondria isolated from control and 60 minutes after surgical stress with or without pretreatment with L-arginine or L-arginine + Nω-nitro-L-arginine methyl ester (L-NAME). Assays were done as described under methods. Values are mean ± SD of three separate experiments. *p<0.01 when compared to control. #p<0.01 when compared to surgical stress.
Figure 3. (TOP FOUR PANELS) Enterocyte mitochondrial phospholipids – phosphatidyl choline (PC) (A), phosphatidyl ethanolamine (PE) (B), lysophosphatidyl choline (LPC) (C), lysophosphatidyl ethanolamine (LPE) (D) of control and 60 minutes after surgical stress with or without pretreatment with L-arginine or L-arginine + N⁵-nitro-L-arginine methyl ester (L-NAME). Assays were done as described under methods. Values are mean ± SD of three separate experiments. *p<0.01 when compared to control. †p<0.01 when compared to surgical stress.

Figure 4. (BOTTOM ONE PANEL) Free fatty acid content of enterocyte mitochondria isolated from control and 60 minutes after surgical stress with or without pretreatment with L-arginine or L-arginine + N⁵-nitro-L-arginine methyl ester (L-NAME). Analysis was done as described under methods. Values are mean ± SD of four separate experiments. *p<0.01 when compared to control. †p<0.01 when compared to surgical stress.
Figure 5. Zymogram and quantitation of mitochondrial protease activity after surgical stress. Gelatin zymography was performed as described in the text. Lane 1, control; Lane 2, surgical stress; Lane 3, L-arginine + surgical stress; Lane 4, L-arginine + L-NAME + surgical stress.
Figure 6. Schematic diagram showing the relationship between nitric oxide (NO), stress and phospholipid metabolic pathways. RCR- respiratory control ratio, MDA-malondialdehyde, CD-conjugated diene, PE-phosphatidyl ethanolamine, LPE-lysophosphatidyl ethanolamine, PC-phosphatidyl choline, LPC-lysophosphatidyl choline, FFA- free fatty acids.
Table-I

Enterocyte mitochondrial phospholipid composition in control and various time periods after surgical stress:

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Control</th>
<th>30min (Time after surgical stress)</th>
<th>60min</th>
<th>120min</th>
<th>24hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline</td>
<td>39.8 ± 4.6</td>
<td>20 ± 1.33*</td>
<td>18.5 ± 0.94*</td>
<td>21 ± 1.4*</td>
<td>33.8 ± 3.15*</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>21.32 ± 1.7</td>
<td>9.6 ± 0.81*</td>
<td>10.3 ± 0.74*</td>
<td>14.8 ± 1.7</td>
<td>19.1 ± 0.47*</td>
</tr>
<tr>
<td>Lyso-phosphatidyl choline</td>
<td>3.5 ± 0.22</td>
<td>8 ± 0.61*</td>
<td>8 ± 0.3*</td>
<td>8.9 ± 0.59*</td>
<td>4.26 ± 0.44*</td>
</tr>
<tr>
<td>Lyso-phosphatidyl ethanolamine</td>
<td>3.76 ± 0.41</td>
<td>8.2 ± 0.64*</td>
<td>10.6 ± 1.4*</td>
<td>8.3 ± 0.64*</td>
<td>4.8 ± 0.5*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of four separate experiments.
*p<0.01 when compared to control.
**p<0.01 when compared to 60min after surgical stress.

Table-II

Functional parameters of enterocyte mitochondria from control and after surgical stress with and without pretreatment of L-arginine and N°-nitro-L-arginine methyl ester:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Surgical stress</th>
<th>L-arg + surgical stress</th>
<th>L-NAME + L-arg + surgical stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swelling (absorbance / min / mg protein)</td>
<td>0.009 ± 0.0006</td>
<td>0.018 ± 0.0008*</td>
<td>0.012 ± 0.0013*</td>
<td>0.017 ± 0.0005*</td>
</tr>
<tr>
<td>MTT reduction (nmoles / mg protein)</td>
<td>78.6 ± 2.8</td>
<td>128.4 ± 12.2*</td>
<td>81.5 ± 6.13*</td>
<td>130.75 ± 14*</td>
</tr>
<tr>
<td>Respiratory Control Ratio (RCR)</td>
<td>1.3 ± 0.105</td>
<td>0.708 ± 0.013*</td>
<td>1.286 ± 0.086*</td>
<td>0.687 ± 0.029*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of three separate experiments.
*p<0.01 when compared to control.
**p<0.01 when compared to surgical stress.
by pre-treating the animals with L-arginine and inclusion of NOS inhibitor L-NAME prevented this protection.

Discussion

Mitochondria are important sub-cellular organelles involved in energy production and are susceptible to oxidative stress. 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 (Kurokawa, Kobayashi et al., 1996). Alteration in the balance between 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 (Anup, Susama et al., 2001). Functional alterations in the mitochondria may be induced by structural changes and this was checked by analyzing the lipid composition, which are essential components of the mitochondrial membrane. Maximum lipid alterations were seen by 60 minutes following surgical stress, which reversed to control level by 24 hours. Phospholipids comprise the major structural component of cell membranes, and fatty acids and lysophospholipids once generated can exert toxic effects (Bonventre 1993; Humes, Nguyen 1989). The lipid profile suggested activation of phospholipase A2. Mitochondrial calcium flux is affected during surgical stress (Anup, Susama et al., 2001) and it is possible that cytosolic calcium levels are altered in the enterocytes following intestinal handling and this can activate cPLA2 (Channon and Leslie 1990), or possibly translocates them on to the mitochondria in response to oxidative stress. Earlier reports have shown that I/R injury is associated with activation of mucosal PLA2 (Otamiri and Tagesson 1989).

Our earlier studies and the above mentioned data indicated maximum alterations 60 minutes following surgical stress and hence further studies on the protective role of nitric oxide using L-arginine as NO donor were confined to this time period. Nitric oxide has been shown to act as an antioxidant in membranes (Hayashi, Noguchi et al., 1995), and in the present study L-arginine pretreatment prevented the lipid alterations seen following surgical stress. This agrees with our earlier study which has shown a protective role of nitric oxide in mitochondrial damage during ischemia reperfusion injury to the intestine (Madesh, Ramachandran et al., 2000). L-NAME is an inhibitor of nitric oxide synthase and to confirm that protection was due to synthesis of nitric oxide, experiments were also done using L-NAME together with arginine. This prevented the protection offered by L-arginine, suggesting a role for NO in protection of mitochondria during surgical stress.

Reactive oxygen species (ROS) have been implicated in damage induced by surgical stress and are capable of altering mitochondrial function (Yukhova, Tanaka et al., 1996). Decreased respiratory control ratio (RCR) might generate superoxides, which was confirmed by increased MTT reduction following surgical stress (Madesh and Balasubramanian et al., 1998). Mitochondrial function has been shown to be affected after sepsis (Taylor, Kantrow et al., 1998; Cardellach, Casademont et al., 1998) and our previous reports have shown that NO prevents the decrease in RCR during apoptosis of HT-29 cells (Madesh, Anup et al., 1999) and ischemia-reperfusion injury in the intestine (Madesh, Ramachandran et al., 2000). 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 shown that surgical stress in the intestine resulted in an increase in MDA and conjugated diene and a decrease in α-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 extra-cellular targets can produce new reactive species during the course of chain reaction resulting in damage to cells and tissues (Dargel 1992). Recent studies have indicated NO as a potent inhibitor of lipid peroxidation (Rubbo, Raddi et al., 2000), and this role of NO was also seen in surgical stress. Protein carbonyl content, a marker of protein oxidation, has been shown to be significantly increased in spinal injury (Leski, Bao et al., 2001) and in DSS-induced colitis (Blackburn, Doe et al., 1999). In this study an increase in protein carbonyl content in the mitochondria was seen following surgical stress, and L-arginine pre-treatment protected the mitochondria from protein oxidation. Proteases are important mediators of cellular damage and are known to be activated in oxidative stress (Marcillat, Zhang et al., 1988). It was observed that surgical stress induced activation of mitochondrial proteases, which was also prevented by nitric oxide. It has been shown recently that nitric oxide inhibition increases metalloproteinas expression in rat aortic smooth muscle cells (Upchurch, Ford et al., 2001). In our study pre-incubation with EDTA or PMSF did not affect the protease activity (data not shown).

Nitric oxide may play a role in reducing mitochondrial damage in surgical stress through its ability to inactivate oxidants. Superoxide is known to be generated during surgical stress, and NO has the capacity to scavenge reactive oxygen metabolites, including superoxide anion by rapidly reacting and abolishing its biological activity (Rubanyi, Ho et al., 1991). In conclusion, this study has shown oxidative stress-induced structural and functional alterations to enterocyte mitochondria during surgical stress that can be prevented by prior administration of the nitric oxide donor, L-arginine. The exact mechanism by which NO offers protection in conditions like this may involve multiple pathways and is under investigation.

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References


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