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Delineating the relationships among the formation of reactive oxygen species, cell membrane instability and innate autoimmunity in intestinal reperfusion injury

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Abstract

Acute intestinal ischemia is a medical emergency with a high mortality rate, attesting to the need for a better understanding of its pathogenesis and the development of effective therapies. The goal of this study was to delineate the relationships among intracellular and extracellular events in intestinal ischemia/reperfusion (I/R) injury, particularly the formation of reactive oxygen species (ROS), cell membrane instability associated with lipid peroxidation and the innate autoimmune response mediated by natural IgM and complement. A murine model of natural IgM-mediated intestinal I/R was used. Mice overexpressing anti-oxidant enzyme SOD1 were found to have significantly reduced intestinal tissue damage and complete blockage of IgM-mediated complement activation compared with WT controls. To determine if cell membrane instability was an event intermediate between ROS formation and natural IgM-mediated innate autoimmune response, the cell membrane stabilizer (trehalose) was administered to WT mice prior to the induction of intestinal ischemia. Treatment with trehalose significantly protected animals from I/R injury and inhibited IgM-mediated complement activation although it did not prevent membrane lipid peroxidation. These data indicate that in normal mice subjected to I/R injury, intracellular ROS formation is an event upstream of the lipid peroxidation which results in cell membrane instability. The membrane instability leads to an innate autoimmune response by natural IgM and complement. Trehalose, a nontoxic disaccharide tolerated well by animals and humans, has

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Author contributions

H.L. designed, performed and analyzed experiments and wrote the paper; E.H.K and Z. K. did quantification of IHC images; M.L., N.W., and J.B. assisted in experiments; M.Z. designed and analyzed experiments and wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

promise as a protective agent for patients with medical conditions related to acute intestinal ischemia.

Keywords

reactive oxygen species; cell membrane instability; natural IgM; complement; reperfusion injury; trehalose

1. Introduction

Splanchnic ischemia can occur in a wide spectrum of clinical conditions including gastrointestinal and aortic surgery, critical illness, as a consequence of hemodialysis, atherosclerosis, and the use of causative medication (e.g. alpha-adrenergic agents) (Kolkman and Geelkerken, 2005). The deadliest form is acute mesenteric ischemia, a medical emergency with a mortality rate of 70–90%, representing 30% of intestinal ischemia cases (Brandt, 2003). Immediate reperfusion is required in these cases, but may result in acute tissue injury locally and systemically (Kolkman and Geelkerken, 2005). Currently there is no clinically approved treatment. A better understanding of the pathogenesis is required if effective therapies are to be developed.

Pathogenic events occur during intestinal I/R include the production of reactive oxygen species (ROS) (Grisham et al., 1986; Li and Jackson, 2002; Watts and Kline, 2003) and damage to cell membranes (Moore et al., 1995). Ischemia also triggers an acute inflammatory response that involves natural IgM and the complement system and mimics an autoimmune response (Zhang and Carroll, 2007). In particular, a specific self-reactive IgM has been isolated which can induce intestinal I/R injury (Zhang et al., 2004). Two ischemic self-antigens (non-muscle myosin heavy chain-II, NMHC-II, isoforms A & C) were identified as targets for this pathogenic IgM (Zhang et al., 2006). In addition, autoantibodies against negatively charged phospholipids were found to induce intestinal I/R injury (Fleming et al., 2004). Thus the innate autoimmune response makes an important contribution to acute intestinal I/R injury (Zhang and Carroll, 2007). However, the relationships between the newly discovered innate autoimmunity and other known pathogenic events, i.e. ROS formation and cell membrane damage, remain unclear.

ROS are generated by multiple intracellular sources during I/R and are removed by intracellular antioxidant enzymes (Eberhardt, 2001). Specifically, superoxide dismutase-1 (SOD1) converts intracellular $\bullet\text{O}_2^-$ to H_2O_2 which is subsequently removed by glutathione peroxidase and catalase. Administering SOD1 or genetically over-expressing this enzyme can prevent intestinal I/R-induced inflammatory cell recruitment (Kurose et al., 1998; Russell et al., 2000). An important consequence of excessive ROS production is cell membrane damage via lipid peroxidation (Toledo-Pereyra et al., 2004). Our hypothesis is that during intestinal I/R, ROS cause cellular changes, particularly cell membrane damage, which results in exposure of self-antigens to natural IgM. If ROS are effectively scavenged by overexpression of antioxidant enzymes, the exposure of ischemic antigens on the cell surface will be minimized or prevented and IgM-mediated complement activation will be lowered or abolished. Further, if cell membrane damage occurs between ROS formation and the innate autoimmune response involving natural IgM, effective preservation of cell membrane integrity should block IgM-mediated complement activation but not ROS formation. We believe that determining the relationships between these intracellular and extracellular pathogenic events will allow identification of specific target(s) for therapeutic intervention in intestinal I/R injury.

2. Materials and Methods

2.1. Animals and intestinal model of I/R injury

Wild type C57BL/6J and SOD1 transgenic (tg) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in compliance with the requirements of the National Institute of Health and the Institutional Animal Care and Use committee of SUNY-Downstate Medical Center.

Intestinal I/R was induced surgically as previously described (Zhang et al., 2004). Briefly, male mice, 8–12 weeks old, were anesthetized (pentobarbital, 60 mg/kg, i.p.). A laparotomy was performed and a microclip (125 g of pressure; Roboz, MD) was applied to the superior mesenteric artery. After 40 minutes of ischemia, the microclip was removed and animals were kept warm for 3 hours of reperfusion. At the end of reperfusion, the ischemic segment of the jejunum was harvested and the central 4-cm section was excised for pathological analysis and immunohistochemistry. Sham control animals underwent laparotomy without the application of the microclip to the superior mesentery artery.

Trehalose (Sigma, MO) was dissolved in normal saline at a concentration of 100 mg/ml and administered i.p. at 1g/kg to WT mice (n=11) 30 minutes prior to intestinal ischemia surgery. Normal saline was used i.p. in the control animals (n=11).

For histopathology analyses, a semi-quantitative pathology score was assessed on sections stained with Hematoxylin and Eosin (H&E). The pathology score is based on a previously described procedure that directly inspects the injured villi over 4-cm of the jejunum (Zhang et al., 2004).

$$\text{Pathology score} = S/(V + [P \times 25]) \times 50\% + D/(V + [P \times 25]) \times 100\% + (P \times 25)/(V + [P \times 25]) \times 100\%$$

where V = total number of villi; S = number of villi which have a subepithelial space; D = number of villi which have epithelial disruption; P = the number of 10× objective power fields with complete loss of villi (each power field in unaffected intestine will have 25 villi).

2.2. Immunohistochemical analyses

Cryosections of intestinal tissues were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and blocked with 2% bovine serum albumin (BSA) buffer. To detect IgM and complement C3 deposition, the cryosections were incubated with biotin-labeled goat antimouse IgM (2.5µg/ml; SouthernBiotech, AL), followed by staining with streptavidin-Alexa-568 (4µg/ml; Molecular Probes, CA). The deposition of C3 was detected using FITC-labeled anti-C3c (Dako, CA).

2.3. Measurement of repertoire of pre-existing anti-NMHC-II IgM

The quantitative immunoassay used was a modification of a previously described method (Lee et al., 2010) to detect serum anti-NMHC II-IgM levels. A 96-well flat-bottom immulon 2HB plate (Thermo, MA) was coated with rabbit anti-NMHC-II A (1µg/ml; Covance, NJ) and incubated overnight at 4°C. The plate was washed and blocked with 1% BSA/0.05% Tween-20/PBS for 2 hours at room temperature. NMHC-II antigens in intestinal lysate from sham-treated mice diluted to a total protein concentration of 12 mg/ml were added to the coated wells and the plates incubated for 2 hours at room temperature. The wells were washed; 1:50 diluted serum from experimental mice was added and the plates were incubated for 2 hours at room temperature. To detect NMHC II-specific IgM bound to the captured Ag, alkaline phosphatase (AP)-labeled rabbit anti-mouse IgM (0.33µg/ml; Rockland, PA) was added and the plates were incubated for 1 hour at room temperature. The

unbound antibodies were removed by washing and phosphatase substrate (Sigma, MO) was added to develop color. The optical density at 405nm was determined in a Multiscan microplate reader (Thermo, MA). The absorbance of each individual sample was converted to an amount (Unit) of the IgM of interest using a standard described previously (Lee et al., 2010).

2.4. ELISA method to measure serum C3 levels in mice with or without trehalose treatment

To determine serum C3 levels in animals with or without trehalose treatment, a 96-well flat-bottom MaxiSorp plate (Nunc, NY) was coated with a monoclonal anti-C3 Ab (Cedarlane, NC) and incubated overnight at 4°C. The plate was washed and blocked with blocking buffer (1% BSA/0.05% Tween-20 in PBS) for 2 hours at room temperature. The plate was washed again. Serum (1:1,000 dilution in blocking buffer) from mice with or without trehalose treatment (from Methods, Section 2.1) was added to the plate which was incubated for 1 hour at room temperature. The plate was then washed 3 times. To detect the captured C3, a goat polyclonal anti-human C3 Ab (Comptech, TX) was added and the plate was incubated for 1 hour at room temperature. After washing, a secondary antibody, rabbit anti-goat IgG-AP (Abcam, MA) was added to the plate which was incubated for 1 hour at room temperature. The unbound antibodies were washed out and phosphatase substrate was added to develop color. The optical density at 405 nm was determined in a Multiscan microplate reader.

2.5. An ELISA method to evaluate binding between natural IgM and trehalose

To determine if there was any specific interaction between IgM and trehalose, a 96-well flat-bottom MaxiSorp plate was coated with 1mg/ml trehalose and incubated overnight at 4°C. BSA (1mg/ml) was used as a negative control to coat additional wells in the same plate. Because natural IgM is known for its polyreactivity towards self-antigens (Zhang and Carroll, 2007), the binding of IgM to intracellular proteins can serve as a positive control in an ELISA assay to test IgM binding to other factors. To provide self-antigens, a soluble cytosolic protein fraction was purified from naive WT mouse heart lysate (the purification followed a published protocol (Zhang et al., 2010)) and used at a concentration of 1 mg/ml to coat additional wells. The plate was washed once and blocked with 1% BSA/0.05% Tween-20 in PBS for 1 hour at room temperature. The plate was washed once. Purified IgM from naive WT mice at a concentration of 100 µg/ml (Millipore, CA) was added to the plate which was incubated for 1 hour at room temperature. The plate was then washed 3 times. To detect any bound IgM, AP-labeled rabbit anti-mouse IgM was added to the plate and incubated for 1 hour at room temperature. The unbound antibodies were removed by washing with 0.05% Tween-20/PBS and phosphatase substrate was added to develop color reaction. The optical density at 405 nm was determined in a Multiscan microplate reader. Experiments were performed in triplicate.

2.6. Detection of lipid peroxidation by the malondialdehyde (MDA) assay in intestinal tissue lysates

Lipid peroxidation was assessed using the LPO-586 assay kit (OxisResearch, OR), which detects MDA. MDA is produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism and considered an indicator of lipid peroxidation (Halliwell and Chirico, 1993). Lipid standards used in the assays and tissue homogenates were incubated for 1 hour in a 45°C water bath with N-methyl-2-phenylindole (NM2P, dissolved in acetonitrile). The ratio of cell lysate to the volume of NM2P solution was 1:3.25.

2.7. Detection of lipid peroxidation by assaying for 4-HNE in intestinal cryosections

4-HNE is an α,β -unsaturated hydroxyalkenal that is produced by lipid peroxidation in cell membranes (Esterbauer et al., 1991). Intestinal sections were stained with an anti-4-HNE Ab (LifeSpan Biosciences, WA) followed by a FITC-labeled secondary Ab. The numbers of 4-HNE positive villi and total villi in the section were counted under a fluorescent microscope. The percentage of 4-HNE positive villi/total villi were calculated for each mouse.

2.8. Statistical analysis

Data were entered into a Microsoft Excel database and SPSS software was used for quantitative data and statistical analyses. Data were presented as means $\pm$ standard error of the mean (SEM). Comparisons between two groups for significance were performed using the independent t-test (two-tailed, unequal variance). For multi-group analyses, Levene's test was used first to determine the homogeneity of variances. Statistical significance was then assessed by a one-way ANOVA test followed by a *post hoc* Bonferroni's test (when the equality of variances assumption held) or Dunnett T3 test (when the equality of variances was not met). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. ROS formation is a pathogenic event upstream of the natural IgM-mediated complement activation that occurs in intestinal I/R injury

To investigate the relationship between ROS formation and the natural IgM-mediated inflammatory response, we used tg mice that overexpressed the anti-oxidant enzyme SOD1 in an intestinal I/R injury model (40 minutes ischemia / 3 hours reperfusion).

Histopathological analyses of intestinal tissues showed that SOD1 tg mice had significantly reduced mucosal damage compared with WT control mice (pathology scores: SOD1 tg = 15 ± 5 , n=11; WT = 48 ± 9 , n=12; $P < 0.01$) (Figures 1a & 1b).

To investigate whether SOD1 overexpression blocked natural IgM-mediated complement activation in the intestinal villi, intestinal sections of SOD1 tg and WT mice were immunostained for IgM and complement C3. WT mice were found to have co-localized depositions of IgM and C3 in the villi (Figure 1c, panels *i*, *ii*, and *iii*). In contrast, SOD1 tg mice had neither IgM nor C3 deposition in the villi (Figure 1c, panels *iv*, *v*, and *vi*). These results demonstrated that SOD1 overexpression blocked natural IgM-dependent complement activation in intestinal I/R.

To rule out the possibility that SOD1 tg mice were protected from the I/R injury due to a potential deficiency of the pathogenic natural IgM, we developed an immunoassay to quantify serum levels of anti-NMHC-II IgM. SOD1 tg mice had levels of anti-NMHC-II IgM similar to those of WT mice (86.1 ± 13.1 Unit and 59.2 ± 12 Unit, respectively, n = 5 per group, $P > 0.05$) (Figure 1d).

3.2. Cell membrane instability is a pathogenic event upstream of the innate autoimmune response in intestinal I/R injury

Ischemic antigens NMHC II are cytoskeletal proteins that are normally shielded by the cell membrane from extracellular immune factors. We hypothesized that cell membrane disruption by intracellular ROS formation exposed these self-antigens to pathogenic natural IgM. Thus, we speculated that cell membrane instability was an event occurring between ROS formation and the innate autoimmune response of natural IgM and complement to I/R injury.

To test this hypothesis, WT mice were treated with trehalose, a cell membrane stabilizer (Beattie et al., 1997; Crowe et al., 1984; Higashiyama, 2002), prior to induction of intestinal ischemia. Saline-injected WT mice were used as controls. Histopathological analysis revealed a significant reduction in mucosal damage to the intestine in the trehalose-treated group compared with the saline control group (pathology scores: saline group = 25 ± 4 , $n=11$; trehalose group = 10 ± 2 , $n=11$; $P < 0.01$) (Figure 2a and 2b). The reduction of tissue damage was accompanied by blockage of the innate autoimmune response involving natural IgM (Figure 2c).

Immunohistochemistry analyses showed that neither IgM nor C3 were deposited in the intestinal tissues of trehalose treated mice (Figure 2c: *iv*, *v*, and *vi*). In contrast, the saline group had IgM and C3 deposition in the villi of the intestines (Figure 2c: *i*, *ii*, and *iii*). Thus, these results indicated that cell membrane instability was an event upstream of the IgM-mediated innate autoimmune response.

To rule out the possibility that trehalose might directly bind IgM and thus interfere with IgM-mediated complement activation, we developed an *in vitro* immunoassay to evaluate any interaction between trehalose and natural IgM. Our results showed no interaction between trehalose and IgM that was more specific than that between BSA and IgM (IgM binding to trehalose, 0.215 ± 0.001 absorbance units at 405 nm; IgM binding to BSA, 0.193 ± 0.004 absorbance units at 405 nm, $P > 0.05$) (Figure 2d). The positive control, based on the known reactivity of natural IgM for self-antigens (Zhang and Carrol, 2007) (soluble self-proteins from heart muscle cytosol were used) bound significantly to IgM (0.655 ± 0.01 absorbance units at 405 nm; $P < 0.05$ compared with the binding of IgM to trehalose or BSA) (Figure 2d).

To rule out the possibility that trehalose may have consumed the circulating C3 to make it less available for activation, we used an ELISA assay to test the serum C3 levels in mice with or without trehalose treatment (serum were obtained from mice in the experiment of Figure 2a). Trehalose treatment did not change the circulating C3 levels (trehalose treatment group: 0.965 ± 0.026 absorbance units at 405 nm; saline control group: 0.906 ± 0.043 absorbance units at 405 nm, $P > 0.05$) (Figure 2e).

3.3. Stabilizing the cell membrane by trehalose does not prevent lipid peroxidation in intestinal I/R

To investigate a relationship between cell membrane stabilization by trehalose and lipid peroxidation in intestinal I/R, we analyzed markers of lipid peroxidation after I/R in trehalose pretreated and saline pretreated control mice. Since a one-way ANOVA test of MDA levels with all groups showed statistical significance ($P < 0.01$) and Levene's test of the homogeneity of variances indicated that $P < 0.05$, the Dunnett T3 test was used for *post hoc* analyses. Our results showed that after I/R, MDA levels in the intestinal tissues increased significantly in both the trehalose and saline treated groups compared with the sham operated group (Figure 3a; MDA concentrations in intestinal lysates: sham group = 0.002 ± 0.0002 μM ; saline group = 1.99 ± 0.31 μM ; trehalose-treated group = 1.8 ± 0.21 μM ; $n=5$ per group; $P < 0.05$). There was no significant difference in MDA levels between the saline and trehalose groups.

To confirm that lipid peroxidation was not changed by trehalose treatment, we examined the generation of 4-HNE in intestinal tissues. A one-way ANOVA test of the percent of 4-HNE positive villi with all groups showed statistical significance ($P < 0.05$) and Levene's test of the homogeneity of variances indicated that $P < 0.05$, so the Dunnett T3 test was used for *post hoc* analyses. The percent of 4-HNE positive villi increased significantly in both the saline treated I/R group and the trehalose treated I/R group compared with the sham group (Figure

3b; % 4-HNE positive villi: sham group = 1.4 ± 0.4 %, n=8; saline group = 19.0 ± 5.8 %, n=12; trehalose treated group = 15.8 ± 3.8 %, n=11; $P < 0.05$). There was no significant difference in the percent of 4-HNE positive villi between the saline and trehalose I/R groups. These findings indicated that stabilizing the cell membrane with trehalose did not alter the lipid peroxidation present after I/R. We hypothesize that trehalose stabilizes the membrane against the instability resulting from lipid peroxidation.

4. Discussion

This study has established that ROS formation resulting from I/R is an event upstream of the innate autoimmune response by natural IgM that occurs in intestinal I/R injury (Figure 1). Cell membrane lipid peroxidation due to the formation of ROS occurs whether or not the disaccharide trehalose is present (Figure 3). In the absence of trehalose, cytoplasmic self-antigens break through the destabilized membrane and can be bound by circulating natural IgM (Figure 1c, upper panel, Figure 2c, upper panel). In the presence of trehalose, despite the existence of membrane lipid peroxidation, trehalose stabilizes (covers) the cell membrane, preventing interaction of IgM and self-antigen and subsequent binding of C3 (Figure 2, lower panel). Taken together, our results support the hypothesis that during intestinal I/R, intracellular ROS formation leads to cell membrane damage, which in turn exposes self-antigens to circulating natural IgM followed by complement activation. An important implication is the establishment of a link in the pathogenesis of intestinal I/R between a major intracellular event and the extracellular innate autoimmune response.

The mechanism of intestinal I/R injury appears to be multi-factorial. In addition to the three pathogenic events investigated in this study, ROS, an innate immune response and lipid peroxidation, other players were recently implicated in intestinal I/R injury, e.g., IL-17A (Lee et al., 2013), free fatty acids (Qin et al., 2012), and Toll-like receptor (Watanabe et al., 2012). Questions remain, however. For instance, TLR4 deficient mice sustained less tissue damage and C3 deposition after I/R than WT mice, but additional complement inhibition by CR2-Crry did not further reduce tissue damage (Pope et al., 2010). Thus, TLR4 may function upstream of complement activation in intestinal I/R. It remains unclear whether TLR4 functions upstream of lipid peroxidation related cell membrane instability. This could be investigated by administering trehalose to TLR4 deficient mice to determine if there is further reduction of tissue damage.

An implication of our findings is that stabilization of the cell membrane may provide a new way to intervene in the innate autoimmune response in I/R. In the past, inhibitors directed against terminal complement factors, e.g. C5, have been tested as a way to prevent I/R injury. However, attempts to curtail C5 activation have not had improved outcomes in clinical trials of patients with ischemic heart disease (Testa et al., 2008). A possible explanation is that C5 operates in the common pathway of complement. Activation of upstream complement factors may affect pathways traditionally thought to be separate from the complement pathway, e.g. coagulation (Amara et al., 2008; Opal and Esmon, 2003), thus influencing clinical outcomes. Our results showed that treatment with trehalose significantly blocked IgM deposition, the initiating step in complement activation in intestinal I/R injury leading to tissue damage (Figure 2). Thus, stabilizing the cell membrane with trehalose despite ROS-induced lipid peroxidation was able to effectively prevent the IgM-mediated complement response and tissue damage, providing a method upstream of C5 for preventing intestinal I/R injury.

Targeting cell membrane instability could also be an alternative approach to targeting the effects of ROS. A number of pre-clinical studies reported that SOD1 could reduce tissue damage in heart ischemia models (Ambrosio and Flaherty, 1992; Gross et al., 1986;

Hangaishi et al., 2001; Kanamasa et al., 2001; Nishikawa et al., 1991; Otani et al., 1986; Wang et al., 1998), but others found that it failed to show protection (Gallagher et al., 1986; Jones et al., 2003; Klein et al., 1988; Matsuda et al., 1991; Nejima et al., 1989; Ooiwa et al., 1991; Patel et al., 1990; Przyklenk and Kloner, 1989; Richard et al., 1988; Uraizee et al., 1987; Watanabe et al., 1993). Attempts to use ROS scavengers to treat I/R injury in clinical settings have been unsuccessful (Flaherty et al., 1994; Murohara et al., 1991). A possible explanation is that intracellular ROS are generated rapidly in I/R as demonstrated recently in our myocardial I/R model (Charlagorla et al., 2013). Thus the delivery of anti-oxidants into cells after the onset of ischemia may be either too late or provide insufficient amounts. Barriers to the delivery of anti-oxidants include timely entry into ischemia tissue, passage through the cell membrane, and achieving effective concentrations in ischemic tissue without raising systemic drug concentrations to toxic levels. Our results showed that trehalose pretreatment decreased tissue injury despite the ROS-induced membrane damage in intestinal I/R. Thus, even though tissues suffered an initial ischemic attack with ROS formation, targeting cell membrane instability using trehalose was able to offer significant protection at the local tissues level.

Trehalose, a nonreducing disaccharide comprised of two glucose molecules linked in an α , α -1, 1-glucoside bond, is synthesized by fungi, plants and invertebrate animals (Crowe et al., 1984; da Costa Morato Nery et al., 2008; Eleutherio et al., 1993; Herdeiro et al., 2006; Higashiyama, 2002). It is the storage disaccharide of fungi (including yeast) and the blood sugar of insects. Humans and mice contain the enzyme trehalase bound to the microvillous membrane of intestinal epithelial cells, where it hydrolyzes trehalose present in large amounts in foods such as mushrooms into two molecules of glucose. In our animal model, we administered trehalose i.p., thus bypassing intestinal trehalase hydrolysis. Trehalose has good water solubility (50 g/l), high thermo-stability, and a wide pH-stability (no degradation at 100 °C or at pH values from 3.5~10 for 24 h) (Higashiyama, 2002). As a nonreducing sugar, in the absence of exposure to enzymes with the function of trehalase, it is considerably more inert than other common disaccharides (Newman et al., 1993). At the molecular level, trehalose can interact with dipalmitoyl phosphatidylcholine (DPPC) by hydrogen bonding between the hydroxyl groups in trehalose and the polar head groups of DPPC (Crowe et al., 1984). A number of cell biology studies have shown that trehalose can increase the survival of cells by covering the outer cell membrane (da Costa Morato Nery et al., 2008; Eleutherio et al., 1993; Herdeiro et al., 2006). For instance, yeast cells are well known for their ability to survive complete dehydration, a phenomenon that is directly linked to the presence of trehalose on both sides of the cell membranes. Mutant yeast strains which lack the ability to translocate trehalose from the cytosol to the outer cell membrane cannot survive dehydration although they contain accumulated endogenous trehalose. When these mutants were dehydrated in the presence of exogenous trehalose, they showed increased survival. Trehalose thus appears to bind extremely well to the hydrophilic surfaces of cell membranes. The ability of trehalose to stabilize cell membranes has been used for preservation of various types of mammalian cells (Barbas and Mascarenhas, 2009; Chen et al., 2004; Cui et al., 2007; He et al., 2008; Isowa et al., 1996; Satpathy et al., 2004) as well as tissues before transplantation (Beattie et al., 1997; Fukuse et al., 1996; Yokomise et al., 1996). It has been used in a rat model of septic shock (Minutoli et al., 2007), in which trehalose administration (1g/kg, i.p.) was safe and tolerated with significant reduction in mortality. The median lethal dose (LD₅₀) for oral ingestion of trehalose in rats is 4.6g/kg according to the manufacturer (Sigma, MO). In humans, with average body weight of 51kg, ingestion of 30g trehalose was reported to be well tolerated and produced no obvious side effects (Oku and Nakamura, 2000). These properties of trehalose make it a good candidate for potential applications in acute intestinal I/R injury or intestinal surgical procedures involving intestinal ischemia. Although studies remain to be done to determine how much

trehalose enters the blood when given orally, trehalose could be given by intravenous injection which would bypass hydrolysis by intestinal trehalase.

In summary, our study demonstrated that, following induction of I/R injury in intestinal tissue, intracellular ROS formation produces membrane lipid peroxidation leading to membrane instability. The disaccharide trehalose stabilizes such membranes, despite the lipid peroxidation. In the absence of trehalose, an innate autoimmune response involving natural IgM and complement occurs resulting in gross tissue damage. Stabilizing ROS peroxidized cell membranes using trehalose holds promise as an interventional approach to minimize intestinal I/R injury.

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Highlights

- Intracellular ROS formation is an event upstream of the lipid peroxidation which results in cell membrane instability.
- Cell membrane instability leads to an innate autoimmune response by natural IgM and complement.
- Trehalose protects against I/R injury by blocking IgM-mediated complement activation.

Figure 1. SOD1 overexpression decreased tissue injury and blocked IgM-mediated complement activation in the intestinal I/R model

(a) Intestinal I/R (40 min./3 hrs.) was carried out and H&E staining performed as described in Methods. Arrows indicate typical pathology features of villi injury (200× magnification). (b) Bar graph: bars indicate mean pathology scores based on the degree of villi injury as described in Methods. Error bars indicate standard error of the mean (SEM). Asterisks indicate statistical significance ($P < 0.01$; $n = 12$ for WT, $n = 11$ for SOD1 tg). (c) SOD1 overexpression results in the blocking of IgM and C3 binding *in vivo*. Panels i–iii: WT mice; panels iv–vi: SOD1 tg mice. Representative cryosections were stained with anti-IgM-biotin/streptavidin-Alexa-568 (red fluorescence in panels i and iv) and anti-C3-FITC (green

fluorescence in panels ii and v). Sections were counterstained with DAPI (Violet/Blue). Panels iii and vi are merged images of (i + ii) and iv + v) respectively. Magnification=400×. **(d)** Serum levels of anti-NMHC-II IgM were similar in SOD1 tg and WT mice (n=5 for each group; $P>0.05$ between these groups).

Figure 2. Cell membrane stabilization reduced tissue damage and blocked IgM-mediated complement response in the intestinal I/R

(a) Trehalose or saline were administered i.p. at a dose of 1g/kg to WT mice 30 minutes prior to I/R surgery (n=11/group). H&E staining was performed as described in Methods. Arrows indicate typical pathology features of villi injury (200× magnification). (b) Bar graph: bars indicate pathology scores. Asterisks indicate statistical significance ($P<0.01$). (c) Trehalose treatment blocks the deposition of IgM/C3 in WT mice. Cryosections were prepared following I/R and analyzed for IgM (panels i & iv) and C3 deposition (panels ii & v). Panels iii and vi are merged images of (i + ii) and (iv + v), respectively. Panels i-iii: WT mice received saline; panels iv-vi: WT mice received trehalose. Sections were stained as in

Figure 1 (200 × magnification). **(d)** IgM does not bind specifically to trehalose. ELISAs were performed in triplicate as described in Methods. The binding of IgM to BSA served as a negative control; the binding of IgM to the cytosolic protein fraction of naive WT mouse served as a positive control. * indicated that IgM binding to the cytosolic proteins was significantly higher ($P < 0.05$) than IgM binding to either BSA or trehalose. **(e)** Trehalose treatment did not change the systemic C3 levels in mice. Serum complement C3 levels in mice with or without trehalose treatment (n=11/group) were measured by ELISA as described in Methods.

Figure 3. Trehalose treatment did not reduce lipid oxidation in intestinal I/R

(a) No reduction of MDA by trehalose treatment. Three groups of WT mice were studied: (i) sham-operated, (ii) I/R with saline treatment, and (iii) I/R with trehalose treatment; ($n=5$ per group). Intestinal lysates were prepared and MDA levels were measured as described in Methods. **(b)** Intestinal cryosections were stained with an anti-4 HNE Ab and a FITC-labeled secondary Ab ($n=8$ for the sham group; $n=12$ for the saline treated I/R group; $n=11$ for the trehalose treated I/R group). Bars indicate percent of 4-HNE positive villi in the intestinal sections. * indicates $P < 0.05$; N.S. indicates not significant ($P > 0.05$).