Hibernation confers resistance to intestinal ischemia-reperfusion injury

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Kurtz, Courtney C., Susanne L. Lindell, Martin J. Mangino, and Hannah V. Carey. Hibernation confers resistance to intestinal ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 291: G895–G901, 2006. First published June 1, 2006; doi:10.1152/ajpgi.00155.2006.—The damaging effects of intestinal ischemia-reperfusion (I/R) on the gut and remote organs can be attenuated by subjecting the intestine to a prior, less severe I/R insult, a process known as preconditioning. Because intestines of hibernating ground squirrels experience repeated cycles of hypoperfusion and reperfusion, we examined whether hibernation serves as a model for natural preconditioning against I/R-induced injury. We induced intestinal I/R in either the entire gut or in isolated intestinal loops using rats, summer ground squirrels, and hibernating squirrels during natural interbout arousals (IBA; body temperature 37–39°C). In both models, I/R induced less mucosal damage in IBA squirrels than in summer squirrels or rats. Superior mesenteric artery I/R increased MPO activity in the gut mucosa and lung of rats and summer squirrels and the liver of rats but had no effect in IBA squirrels. I/R in isolated loops increased luminal albumin levels, suggesting increased gut permeability in rats and summer squirrels but not IBA squirrels. The results suggest that the hibernation phenotype is associated with natural protection against intestinal I/R injury.

INTESTINAL ISCHEMIA-REPERFUSION (I/R) is a potentially serious consequence of several clinical and pathophysiological conditions including small bowel transplantation, cardiopulmonary bypass, trauma, and hemorrhage (22, 25). Reperfusion of the gut after an ischemic episode is associated with a progressive loss of mucosal structure with denuded and shrunken villi and reduced epithelial function (22, 36) and can eventually lead to a loss of barrier integrity and translocation of luminal antigens and bacteria. Reactive oxygen species (ROS) released during reperfusion stimulate inflammatory responses, which can increase recruitment and activation of neutrophils in the blood and eventually remote organs (22). The liver and lung are particularly susceptible to the damaging effects of increased neutrophil activation following intestinal I/R (25). Consequently, many cases of intestinal I/R end in shock, multiple organ failure, and death (25, 28, 36).

Short periods of ischemia and reperfusion can induce resistance to a subsequent, more severe insult, a phenomenon known as ischemic preconditioning. Preconditioning has been found to be protective against I/R damage for several organs including the intestine (18, 35, 39, 40). Mechanisms that have been proposed to mediate preconditioning-induced protection include prevention of apoptosis (39, 40) and the reduction of ROS formation due to increased antioxidant defenses (18, 35).

The hibernation phenotype is a seasonal adaptation to periods of high energy demand coupled with low food availability in the environment (7). In lieu of feeding, many hibernators rely solely on stored lipids for fuel and drastically reduce body temperature (Tb) and metabolism to conserve energy. During bouts of metabolic depression (torpor), blood is shunted away from the splanchnic organs toward the heart, lungs, brain, and brown adipose tissue (2, 13). Mammalian hibernators arouse periodically from the torpid state to experience short periods (<12 h) of normothermia (~37–39°C). During the arousal process, blood flow is differentially distributed throughout the body, and the intestine is one of the last major organs to be reperfused before arousal is completed (2). We hypothesized that these naturally occurring cycles of splanchnic hypoperfusion followed by reperfusion may precondition the hibernator’s intestine to an imposed I/R stress. We therefore experimentally induced intestinal I/R in two groups of ground squirrels: hibernating squirrels during a natural interbout arousal (IBA) period and summer (nonhibernating) squirrels. We used IBA hibernators to separate the putative preconditioning effects of the hibernation phenotype from the protective effects of hypothermia on the response to intestinal I/R (38). We also studied Sprague-Dawley rats as a nonhibernating species control. We used two models of intestinal I/R: a superior mesenteric artery (SMA) occlusion-reperfusion model that simulates I/R injury that might occur in clinical situations and an isolated intestinal loop model that induced a local ischemia and allowed us to compare responses of control and I/R loops within the same animal.

MATERIALS AND METHODS

Animals. Adult 13-lined ground squirrels (Spermophilus tridecemlineatus) of both sexes were trapped around Madison, WI, in summer (July–September). Squirrels were housed individually with free access to water and food (Purina rodent chow 5001, supplemented with sunflower seeds) at 22°C with a 12:12-h light-dark cycle. All squirrels were fasted overnight prior to the experiments. Hibernators displayed torpor-arousal cycles for ~11 wk prior to use. All animal protocols were approved by the University of Wisconsin Animal Care and Use Committee.

SMA I/R protocol. Rats and ground squirrels (summer and IBA hibernators; Tb 37–39°C) were anesthetized with isoflurane and main-
tained at Tb, 37–39°C throughout the experiment with external heating. The abdominal cavity was opened with a midline incision, the abdominal aorta was cannulated with polyethylene (PE)-50 catheters attached to a PowerLab system (ADInstruments, Colorado Springs) to monitor blood pressure, and the SMA was isolated. The abdomen was then closed and, after a short (<30 min) stabilization period, was opened briefly to occlude the SMA with an atraumatic vascular clamp for 20 min followed by 60 min of reperfusion. After the reperfusion period, the lung and liver were removed and frozen in liquid nitrogen. The entire small intestine was removed, and a 1-cm segment was fixed in 10% formalin for histological analysis. The remaining intestine was opened longitudinally, and the mucosa was scraped with glass slides and frozen in liquid nitrogen. Frozen tissues were stored at −80°C. Sham surgeries were identical to I/R surgeries, but the SMA was not clamped.

Loop I/R protocol. Rats and ground squirrels were anesthetized, and the abdominal cavity was exposed as above. Two 8- to 10-cm segments of ileum were isolated, and short PE-200 catheters were attached at both ends. Prior to catheter insertion, a 1-cm portion of each loop was fixed in 10% formalin for basal (“pretreatment”) histology. Both loops were flushed with 5 ml of PBS prewarmed to 37°C. Excess PBS was removed with air. The loops were then filled with a measured amount (~1–1.5 ml) of prewarmed PBS for a 30-min pretreatment period. At the end of this period, any PBS remaining in the loops was collected, and the loops were filled with fresh PBS. The portion of mesenteric leading to the I/R loop was then clamped for 20 min followed by a 60-min reperfusion period. The blood supply to the control loop was intact for the duration of the experiment. At the end of the experiment, the remaining PBS was collected, and the loops were removed and weighed. Another 1-cm portion of each loop was fixed for histological analysis (“posttreatment”). The remainder of each loop was opened longitudinally, and the mucosa was scraped and frozen in liquid nitrogen. The PBS collected from the loops was centrifuged (2,500 g, 15 min, 4°C) to pellet cellular debris, and the remaining fluid was frozen. All animals were maintained at a Tb of 37–39°C throughout the experiment. We used albumin levels in the luminal fluid as an estimate of leakage of plasma proteins into the lumen. Twelve microliters of diluted PBS (1:100 for squirrels, 1:500 for rats) from control and I/R loops were separated by SDS-PAGE and albumin levels were determined by Western blot analysis (anti-albumin, no. A110-125-2, Bethyl Laboratories). Protein bands were quantified using ImageQuant software (Amersham). Densitometric values of posttreatment samples were divided by the respective pretreatment value to obtain the fold change in albumin in control and I/R loops.

Histological analysis. Formalin-fixed intestinal tissues were blocked in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were blinded and scored as described previously (31). In brief, a score of 0 indicates no apparent damage, 1 indicates mild damage (some loss of epithelium at villus tips), 2 indicates moderate damage (increased loss of epithelium, infiltration of immune cells, and congestion of blood vessels), and 3 indicates severe damage (loss of villus structure and increased granulomatous tissue).

MPO assay. MPO activity was used as a marker for neutrophil infiltration into tissues. Fifty milligrams of the frozen mucosa or 100 mg of the frozen lung or liver were homogenized in 0.4 ml of 20 mM K2HPO4 (pH 7.4) on ice. A portion of the homogenate was diluted 10-fold in the same buffer and centrifuged (11,600 g, 15 min, 4°C). The pellet was sonicated in 50 mM K2HPO4 containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10 mM EDTA (pH 6.0). The sonicated suspension was frozen, thawed twice, and centrifuged (11,600 g, 1 min, 22°C). The supernatant was combined with 80 mM K2HPO4 (pH 5.4), 10% HETAB, and 3,3',5,5'-tetramethyl benzidine in a glass tube. H2O2 was added to start the reaction, which ran for 3 min at 37°C. Catalase and 200 mM sodium acetate buffer (pH 3.0) were added to stop the reaction, and the samples were put on ice prior to being read on a spectrophotometer at 555 nm. MPO activities for the mucosa, lung, and liver in the SMA I/R model were calculated based on a standard curve of purified enzyme (Sigma) and normalized to protein concentration. In place of a standard curve, mucosal MPO values for the loop I/R model were standardized using a control sample of 0.1 units of purified MPO and normalized to protein concentration of each sample.

TUNEL assay. The in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN) was used for terminal deoxyxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) detection of DNA fragmentation. Deparaffinized and rehydrated sections were incubated with 3% H2O2 to block endogenous peroxidases, washed, and treated with proteinase K (20 μg/ml, 15 min, 37°C). Sections were incubated with fluorescein-conjugated nucleotides and terminal deoxyxynucleotidyl transferase (1 h, 37°C). After the application of anti-fluorescein antibody, sections were treated with dianisobenzidine and counterstained with methyl green. We reported previously that TUNEL staining of epithelial cells increases as the hibernation season progresses (21). Hibernators in this study were used <3 mo into the hibernation season when baseline TUNEL staining was minimal.

Statistics. For SMA I/R experiments, sham vs. I/R comparisons were made using an unpaired t-test. Mucosal MPO activities were log transformed to equalize variances. For the loop I/R model, paired t-tests were used to compare loops within the same animal. A P value of ≤0.05 was considered statistically significant. For all groups analyzed, values are means ± SE.

RESULTS

SMA I/R model. Intestinal tissues of sham-treated animals had similar histology with little to no damage (Fig. 1, A, C, and E). After SMA I/R, the intestines of rats and summer squirrels were severely damaged with loss of villus-crypt architecture and infiltration of blood and immune cells (Fig. 1, B and D). IBA hibernators exhibited no obvious histological damage after SMA I/R (Fig. 1F). Histological scoring revealed significant increases in damage scores of the rat and summer squirrel I/R groups vs. their respective sham groups but no difference between the hibernator I/R and sham groups (Fig. 2).

Compared with sham values, mucosal MPO activity increased in rats and summer squirrels following I/R but was unchanged in IBA hibernators (Fig. 3A). MPO activity in lungs of IBA hibernators subjected to I/R was not different from sham, but, in rats and summer squirrels, MPO activity was significantly greater than the respective sham groups (Fig. 3B). Liver MPO activity after SMA I/R was greater than that in the sham group in the rats but was not altered in summer or IBA hibernators (Fig. 3C).

Loop I/R model. The pattern of histological damage induced by I/R in the loop model was similar to that in the SMA I/R model, with increased damage scores in the I/R loop of rats and summer squirrels but not IBA hibernators (Fig. 4A). No significant differences were found between pretreatment and posttreatment scores of control loops in any groups. Mucosal MPO activity increased in the I/R loop of rats and summer squirrels compared with control loops but not in IBA hibernators (Fig. 4B).

The change in luminal albumin from pretreatment to posttreatment periods was used as an indicator of intestinal permeability and mucosal damage following I/R. The fold change in luminal albumin levels from pretreatment to posttreatment periods was significantly greater in I/R loops compared with control loops for rats and summer squirrels but not IBA hibernators (Fig. 4C).

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TUNEL was used as an indicator of apoptotic cells in I/R and control loops. Increased TUNEL staining was observed in posttreatment I/R loops (Fig. 5, D and E) compared with posttreatment control loops (Fig. 5, A and B) for rats and summer squirrels, but there were few TUNEL-positive cells in any loops of IBA hibernators (Fig. 5, C and F). This pattern was consistent for the 8–10 tissue sections that were examined per animal group. A similar pattern was observed in the SMA I/R model (data not shown).

**Discussion**

Compared with the homeothermic active season, the hibernation phenotype is accompanied by profound physiological changes in multiple organ systems. To survive these changes that would be lethal to nonhibernating species, hibernators utilize endogenous defense mechanisms that minimize tissue damage and preserve function. In this study, we determined whether the natural protection associated with the hibernation phenotype translates to increased protection against an imposed stress of biomedical significance. The intestine is a useful model in this regard, because it is particularly vulnerable to stress caused by changes in nutrition, temperature, blood flow, and the production of ROS (1, 19), all conditions that are altered by hibernation (7). Moreover, although the gut undergoes substantial atrophy during the hibernation season (4, 24) and displays evidence of oxidative stress (8) and DNA damage (21), intestinal gene expression is preserved (9) and epithelial function is maintained when tissues are warmed to normothermic temperatures (4–6, 11). We chose a surgical model of intestinal stress induced by I/R because it is a severe manifestation of circulatory changes that occur naturally in hibernators, when splanchnic vessels are hypoperfused and then reperfused during torpor-arousal cycles (2). Intestinal I/R can result from a variety of traumatic events such as hemorrhage and sepsis as well as some clinical procedures, including cardiopulmonary bypass, liver surgery, and intestinal transplantation. Our results support the hypothesis that the hibernation phenotype attenuates mucosal and remote organ injury induced by intestinal I/R. Furthermore, hibernation-induced protection is not dependent on hypothermia, because we used hibernating squirrels during their natural arousals to normothermia, and all animals were maintained at high Tb (37–39°C) during the experiments.

SMA I/R caused extensive mucosal damage in rats and summer squirrels, including shedding of villus epithelial cells and infiltration of blood and immune cells. The extent of mucosal damage was consistent with that seen in other studies of intestinal I/R in rats (22, 27, 32, 36). In contrast, the intestine of IBA hibernators was less affected by I/R, with intact villi and no obvious change in cell infiltration compared with sham controls. A similar pattern of tissue damage was observed in the loop model of localized ischemia, with increased histological damage in rats and summer squirrels following I/R but minimal change in IBA hibernators.

The mucosal damage induced by I/R in the intestinal loop model appeared to alter intestinal permeability in rats and summer squirrels, as indicated by the increase in luminal albumin in I/R loops compared with controls. Increased luminal albumin in I/R loops compared with controls...
nal albumin was likely due to damage of the vascular endothelium and mucosal epithelium and subsequent leakage of plasma proteins. As was expected based on their well-preserved histology, there was no change in luminal albumin levels of hibernator I/R loops compared with control loops.

Epithelial damage induced by intestinal I/R is thought to be due to activation of the mitochondrial pathway of apoptosis subsequent to ROS production (32, 40). In the isolated loop model, TUNEL staining suggested that I/R increased epithelial cell apoptosis in the rats and summer squirrels but had no effect in the IBA hibernators. Inhibition of I/R-induced apoptosis in hibernating squirrels may result from increased expression of prosurvival proteins that inhibit the mitochondrial pathway (21). Expression of the antiapoptotic protein Bcl-xL increases in the intestinal mucosa during hibernation, and, although the proapoptotic protein Bax is also increased, the Bcl-xL-to-Bax ratio is significantly higher in hibernators, suggesting a balance in favor of antiapoptotic signaling (21). Other studies have demonstrated protective effects of antiapoptotic Bcl-2 proteins in models of intestinal I/R (15, 17). Thus the maintenance of mucosal structure after intestinal I/R in hibernating squirrels may result from their naturally induced resistance to apoptosis.

Breakdown of the intestinal barrier by I/R can lead to translocation of luminal contents, including bacteria and bacterial products, across the epithelium (36). This stimulates a systemic inflammatory response and the activation and accumulation of large numbers of neutrophils in splanchnic organs (22). Neutrophil infiltration into tissues is commonly assessed by changes in activity of MPO, an enzyme found almost exclusively in neutrophils. The increased MPO activity in the intestinal mucosa of rats and summer squirrels after I/R in the SMA and loop models suggested activation of an inflammatory response. However, no changes in MPO activity were detected in the IBA hibernators, consistent with the lack of histological damage. Neutrophil activation not only exacerbates mucosal damage but also leads to remote organ injury due to adherence of circulating neutrophils to microvasculature in susceptible organs, especially liver and lung (23, 25, 41). These tissues are thought to be particularly vulnerable to injury after intestinal I/R, because their vasculature is coupled in series with the intestinal circulation (23). SMA I/R induced neutrophil infiltration into remote organs in rats and summer squirrels, as indicated by increased lung MPO activity in both groups and increased liver MPO activity in rats. In contrast, no changes in MPO activity in lung or liver of IBA hibernators were detected. Inhibition of apoptosis and maintenance of the intestinal barrier likely contribute to the lack of systemic effects in the IBA hibernators after I/R.

In addition to maintenance of barrier function, the lack of systemic inflammation subsequent to intestinal I/R in IBA hibernators may result from suppression of immune activation within the gut during the hibernation season. For example, the anti-inflammatory cytokine IL-10 is sustained at high levels in intestinal mucosa throughout the hibernation season (28a). IL-10 has several effects on neutrophils including stimulation of apoptosis, inhibition of TNF-α and IL-1β release, and reduction of recruitment and accumulation in tissues (12). Exogenously administered IL-10 has been shown to be protective against intestinal I/R, because IL-10 given to mice prior to or after reperfusion attenuated gut mucosal damage, plasma TNF-α and IL-6 levels, and lung MPO activity (29).

Oxidative stress is one proposed mechanism for the development of I/R-induced damage. The reduction in splanchnic blood flow during ischemia forces cells to rely on anaerobic metabolism, leading to the accumulation of hypoxanthine (3). Upon reperfusion, hypoxanthine is converted to urate with the release of superoxide anions and the subsequent production of hydrogen peroxide, both of which can damage cellular proteins, lipids, and DNA (3). Hibernation itself appears to induce some degree of oxidative stress to intestinal mucosa, as indicated by increased lipid peroxidation (8) and a reduction in the ratio of reduced to oxidized glutathione (GSH) compared with controls.
Fig. 4. Effects of I/R in intestinal loop model. A: histological damage. Asterisks indicate significant differences between pretreatment and posttreatment values in control (C) and I/R loops (**P < 0.01; ***P < 0.001). n = 10 animals/group. B: mucosal MPO activity. Asterisks indicate significant differences between C and I/R loops (posttreatment) (*P < 0.05). n = 9–10 animals/group. C: luminal albumin levels (fold increase in posttreatment vs. pretreatment values for each loop). *P < 0.05. n = 10 animals/group. Representative immunoblots are shown; lane 1, pretreatment control loop; lane 2, posttreatment control loop; lane 3, pretreatment I/R loop; lane 4, posttreatment I/R loop.

Fig. 5. TUNEL staining of control and I/R loops posttreatment. A–C: control loops had minimal TUNEL staining in all groups. D and E: I/R loops of rats and summer squirrels had extensive TUNEL staining in cells of the epithelium and lamina propria. F: I/R loops of IBA hibernators had few TUNEL-positive cells.
the summer active season (10). However, the total mucosal GSH pool is significantly greater during the hibernation season, which may help minimize naturally-induced oxidative damage to the intestine (10). This may also contribute to the protection against I/R-induced injury in IBA hibernators, because administration of GSH to rats in which endogenous mucosal GSH was depleted attenuated apoptosis and ensuing damage following I/R (27).

NF-κB is constitutively activated in ground squirrel intestinal mucosa during hibernation compared with summer (8). This redox-sensitive transcription factor can be activated by oxidative stress and either contribute to stress-induced damage or provide protection, depending on the time point after initiation of the insult and the cell types involved (33). Although NF-κB appears to play a role in initiation of intestinal I/R-induced injury (43), it also inhibits epithelial cell apoptosis (14) and may be required for resolution of inflammation (43). In our previous study (8), we found that NF-κB activated in the intestine during hibernation was composed primarily of p50 subunits. Because p50/p50 homodimers lack transactivation domains, it has been suggested that the p50 homodimer acts as a transcriptional repressor of specific target genes associated with the proinflammatory activities of NF-κB-p50/p65 heterodimers (26, 42). Prolonged activation of NF-κB p50/p50 homodimers was reported in a rat model of intestinal I/R and was suggested to regulate time-dependent activation of damage repair genes (42). Thus the constitutive activation of NF-κB during the hibernation season may provide endogenous protection that inhibits epithelial cell apoptosis and suppresses inflammation in our I/R models.

The protective effects of the hibernation phenotype against intestinal I/R damage are similar in some respects to the phenomenon of preconditioning in which injury is attenuated by subjecting tissues to brief periods of I/R prior to a more severe I/R event (34). Preconditioning is thought to involve generation of a mild level of stress that is nondamaging, yet substantial enough to increase expression of defense mechanisms that are then in place during the subsequent stress. Intestinal preconditioning provides protection from I/R injury by reduction of oxidative damage (18), enhanced barrier function (34), inhibition of apoptosis (16, 39, 40), and suppression of inflammatory responses (39, 40). During torpor-arousal cycles associated with hibernation, the intestine is exposed to dramatic fluctuations in splanchnic blood flow (2, 13) and shows evidence of oxidative stress in the absence of mucosal damage (8, 10). Some of the defense mechanisms upregulated during hibernation (8, 10, 21) are also induced by preconditioning (16, 18, 40), and the idea that hibernation represents a natural model for ischemic preconditioning has been proposed for other organs (30, 37). In addition to the putative preconditioning effects of torpor-arousal cycles, there are likely other protective mechanisms that develop seasonally and protect the gut from stress during hibernation. Importantly, these mechanisms are not dependent on low Tb, as they are evident during natural arousals to normothermia. Further investigation of the molecular basis for hibernation-induced protection against ischemic injury and other trauma states will facilitate translation of this remarkable phenotype to the clinical setting.

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