Role for complement in mediating intestinal nitric oxide synthase-2 and superoxide dismutase expression

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Montalto, Michael C., Melanie L. Hart, James E. Jordan, Koichiro Wada, and Gregory L. Stahl. Role for complement in mediating intestinal nitric oxide synthase-2 and superoxide dismutase expression. Am J Physiol Gastrointest Liver Physiol 285: G197–G206, 2003.—Inducible nitric oxide synthase (iNOS) and superoxide dismutase (SOD) play an important role in the pathology of ischemia-reperfusion. This study sought to determine if the proinflammatory effects of complement modulate iNOS and SOD in the rat after gastrointestinal ischemia and reperfusion (GI/R). An inhibitory or noninhibitory anti-complement component 5 (C5) monoclonal antibody (18A or 16C, respectively) was administered before GI/R. RT-PCR revealed a significant increase in intestinal iNOS mRNA compared with sham after GI/R that was attenuated significantly by 18A. Immunohistochemistry demonstrated increased iNOS protein expression within the intestinal crypts after GI/R. Cu/Zn SOD (mRNA and protein) was unaffected by GI/R, whereas Cu/Zn SOD activity was reduced significantly. Mn SOD protein expression was decreased significantly by GI/R. Anti-C5 preserved Cu/Zn SOD activity and Mn SOD protein expression. Staining for nitrotyrosine showed that anti-C5 treatment reduced protein nitration in the reperfused intestine. Immunohistochemistry demonstrated prominent phosphorylated (p) inhibitory factor-κB (IκB)-α staining of intestinal tissue after GI/R, whereas anti-C5 reduced p-IκB-α expression. These data indicate that complement may mediate tissue damage during GI/R by increasing intestinal iNOS and decreasing the activity and protein levels of Cu/Zn SOD and Mn SOD, respectively.

ischemia-reperfusion; inhibitory factor-κB; interleukin-1β

GASTROINTESTINAL ISCHEMIA-reperfusion (GI/R) is a common clinical problem in the settings of sepsis, hemorrhagic shock, vascular surgery, and small bowel transplantation (17, 21). GI/R causes gut dysfunction characterized by histological evidence of impaired gut motility, increased intestinal permeability, and mucosal injury (18). Numerous mediators have been implicated in GI/R injury, including cytokines (8, 15, 43, 54, 55), reactive oxygen species (ROS; see Ref. 13), nitric oxide (NO; see Refs. 45 and 46), inducible nitric oxide synthase (iNOS; see Refs. 38 and 44), and cell adhesion molecules (34, 35, 54). The expression of these inflammatory mediator genes is controlled, in part, by nuclear factor-κB (NF-κB).

NF-κB is maintained in a latent form in the cytoplasm of cells where it is complexed to inhibitory factor-κB (IκB) proteins (24). Upon activation of NF-κB, IκB is phosphorylated (p) by IκB kinases at two conserved serine residues in the NH2 terminus, which targets the protein for ubiquination and degradation by the proteosome. This rapidly frees NF-κB to translocate to the nucleus, where it upregulates the transcription of a variety of adhesion molecules (ICAM-1 and VCAM-1), cytokines (TNF, IL-1, and IL-6), and enzymes (iNOS; see Refs. 24 and 37).

NO has been implicated as a mediator of tissue damage in several models of intestinal disease, including reperfusion injury (26, 47, 48). NO can be produced by three nitric oxide synthase (NOS) isoforms (neuronal NOS, iNOS, and endothelial NOS). Specific inhibition of iNOS has been shown to attenuate NO production significantly and decrease intestinal injury, indicating that iNOS mediates the excessive production of NO and plays a deleterious role in GI/R (5, 46). Additionally, iNOS knockout mice exhibited significant resistance to barrier dysfunction and bacterial translocation after GI/R, further supporting a role for iNOS as an important mediator of reperfusion injury in the intestine (45).

During reperfusion of the intestine, there is an increase in superoxide production that is likely derived from neutrophils and other various sources, such as endothelial or epithelial cells (3, 14, 46). The absence of effective dismutation of superoxide anion allows for its rapid reaction with NO to form the potent reactive nitrogen species peroxynitrite, which can react with cellular proteins and induce general organ dysfunction (31, 36). Several reports indicate that the overexpression of superoxide dismutase (SOD) can attenuate GI/R injury (11, 22). The exogenous administration of SOD in a rat model of gastric ischemia-reperfusion increases luminal NO levels, suggesting that SOD-mediated protection is afforded by decreasing the local concentra-

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tion of superoxide that is available to react with NO (53). Because endogenous SOD levels are not adequate to quench the sudden surge in superoxide levels after reperfusion, attention has been directed toward the delivery of exogenous SOD (21, 39). To date, factors mediating the regulation of endogenous SOD after ischemia-reperfusion have not been identified.

It is well established that the complement system plays a significant role in the pathogenesis of ischemia-reperfusion injury (36, 42). Specifically, complement depletion or inhibition can attenuate several of the known mediators of reperfusion injury, including neutrophil trafficking, cytokine production, and adhesion molecule expression (32, 42, 49). Recently, our laboratory has demonstrated that inhibition of the late complement components significantly attenuates neutrophil infiltration and subsequent reperfusion injury after gastrointestinal ischemia (54, 58). Furthermore, it was shown that activation of complement component 5 (C5) mediates intestinal damage via the upregulation of ICAM-1, TNF-α, and IL-1α. Although it is clear that blocking complement can protect tissue from reperfusion injury, the specific molecular mechanism(s) of complement-induced damage at the cellular level remains a topic of investigation. In models of myocardial ischemia-reperfusion, it has been postulated that complement can potentiate the production of oxygen free radicals by recruiting and stimulating neutrophils to produce various ROS (36). However, a definitive link between complement and known regulators of ROS, such as SOD and iNOS, has not been demonstrated in any model of ischemia-reperfusion. Therefore, the goal of this study was to determine how the late complement components affect the regulation iNOS and SOD in an established model of GI/R and to elucidate further the molecular mechanisms of protection afforded by anti-complement treatment.

MATERIALS AND METHODS

Antibodies and reagents. Anti-rat C5 monoclonal antibodies (MAbs) 18A10.62 (18A) and 16C9.10 (16C) have been characterized previously (49). 18A functionally blocks C5b-9 and C5a formation, whereas 16C binds C5 but does not inhibit C5 cleavage (49). Commercially available antibodies specific for rat iNOS (KAP-NO001), rat Cu/Zn SOD (SOD-101), and rat Mn SOD (SOD-110) were purchased from Stressgen (Victoria, BC, Canada), and a mouse monoclonal anti-nitrotyrosine (clone no. HM11; Zymed Laboratories, San Francisco, CA) was used for histochemistry and Western blot. A mouse monoclonal anti-p-IKB-α (B-9; Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with phosphorylated serine-32 of IκB-α was also used.

Intestinal ischemia-reperfusion. Intestinal ischemia-reperfusion injury was induced as previously described (54). Briefly, Sprague-Dawley rats (280–340 g) were anesthetized with pentobarbital sodium (50 mg/kg) and ketamine (20 mg/kg). PE-50 catheters filled with heparinized saline (i.e., 10 U/ml in 0.9% NaCl) were inserted in the left carotid artery and jugular vein for mean arterial pressure (MAP) and anti-body injection, respectively. MAP was monitored continuously via a pressure transducer and an Astromed MT95K2 recorder. In pilot experiments, there was no significant difference in MAP between 18A (n = 7) and 16C (n = 8)-treated ischemia-reperfusion groups (data not shown). A laparotomy was performed, and the superior mesenteric artery was occluded with a microaneurysm clamp for 90 min. This degree of ischemia is comparable with that observed in patients undergoing supraceliac aortic cross-clamping during thoracoabdominal aortic aneurysm repair (16). Rats were killed after 60 min of reperfusion, and intestinal tissue was excised 20 cm from the cecum and irrigated with normal saline. Sham-operated animals underwent the same procedure without clamping. MAB treatment (20 mg/kg iv) was delivered 60 min before ischemia, as previously described (54).

Semiquantitative RT-PCR. Total RNA was extracted from frozen tissue using the acid guanidinium thiocyanate extraction procedure, as previously described (6). DNase-treated RNA concentrations were determined by optical density and confirmed by agarose gel electrophoresis. cDNA was synthesized with random primers using the Reverse Transcription system (Promega, Madison, WI) according to the manufacturer's instructions and 2 μg total RNA. CDNA was amplified in 50-μl reactions containing 2 μl of the cDNA reaction mix, 1× PCR buffer (20 mmol/l Tris-HCl, pH 8.4, and 50 mmol/l KCl), 1.5 mmol/l MgCl2, 200 μmol/l of each dNTP, and 2.5 units of Taq DNA polymerase (GIBCO Life Technologies, Gaithersburg, MD). Reactions were heated to 94°C for 1 min before adding 20 pmol of each primer. iNOS was amplified at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for a total of 27 cycles followed by a 10-min extension at 72°C using the following primers: 5'-tggtcaccaggagttgtt-3' and 5'-tgagcagctctcttcggca-3'. GAPDH was amplified as previously described. (54) Cu/Zn SOD and Mn SOD were amplified at 94°C for 45 s, 56°C for 30 s, and 72°C for 45 s for a total of 23 cycles followed by a 10-min extension at 72°C using the following primers: Cu/Zn SOD, 5'-ctcagagactgcggcctc-3' and 5'-caggtagggegcaagt-3'; Mn SOD, 5'-gctgtcggttctaaag-3' and 5'-ctcagagactgcggcctc-3'. The same conditions were used to amplify IL-1β for a total of 30 cycles using the following primers: 5'-ctcctggctgtgatgaag-3' and 5'-cctctgaaaaaacaataa-3'. Amplification products were resolved by electrophoresis on a 1.8% agarose gel containing 0.06 μg/ml ethidium bromide. Bands were digitized using an electrophoresis documentation and analysis system 120 and were analyzed by one-dimensional (1-D) analysis software (Kodak Digital Science). Net band intensity (background subtracted intensity) was normalized to values for GAPDH and plotted as arbitrary units. To ensure that amplification was in the linear range, the number of PCR cycles for each gene was titrated, and the optimal cycle numbers were chosen. The linearity of amplification was confirmed by real-time PCR analysis using an iCycler detection system (Bio-Rad, Hercules, CA; data not shown). Water samples or RNA samples containing no RT were amplified in parallel to ensure that no contaminating DNA was present during PCR.

Immunohistochemistry. Tissue for histological analysis was rinsed in normal saline and fixed in 3.7% formaldehyde-PBS for 18–24 h and embedded in paraffin. Thin (7-μm) sections were cut and stained by the Rodent Histopathology Core (Dana-Farber/Harvard Cancer Pathology Core Facility) using a horseradish peroxidase-labeled detection antibody and 3,3’-diaminobenzidine as a substrate. All samples were counterstained with hematoxylin. Individual primary antibodies were used at the same concentration for each tissue sample. Controls using the detection antibody only were used to confirm that staining was dependent on the primary antibody. Ischemic intestinal tissue samples used for p-IκB-α histological analysis were taken after 0, 5, 15, and 60 min of reperfusion.
**SOD assay.** The molecular mass and activity of SOD were assessed simultaneously by an in-gel assay, as previously described (1, 4). Tissue lysates were prepared as described, and protein was measured using a Bio-Rad protein assay (30). Briefly, 5 μg of total cell lysate were diluted in sample buffer containing 30% glycerol-0.5 mol/l Tris-HCl, pH 6.8, and resolved on a native 10% polyacrylamide gel. Gels were rinsed in PBS and soaked for 20 min in 1.22 mmol/l nitro blue tetrazolium (NBT) (Sigma, St. Louis, MO) and rinsed and soaked for an additional 20 min in 28 μmol/l riboflavin containing 28 mmol/l N,N,N′,N′-tetramethylthelene diamine (Sigma). Gels were rinsed and monitored for the reduction of NBT. Achromatic bands of the predicted molecular mass of CuZn SOD were observed within 30 min. Decreased achromatic band intensity indicates a decrease in the ability of SOD to inhibit the reduction of NBT. The inclusion of 2 mmol/l potassium cyanide in the photoreduction reaction completely inhibited the development of the achromatic bands, confirming that these bands contained CuZn SOD (data not shown; see Ref. 4). Gels were photographed, and achromatic zone intensity was recorded and expressed in arbitrary units using an electrophoresis documentation and analysis system 120 and analyzed by 1-D analysis software (Kodak Digital Science). Serial dilutions of tissue extract confirmed this assay to be within the linear range (data not shown).

**Western blot.** Tissue samples were homogenized in phosphate buffer containing 0.5% Nonidet P-40, 0.5% Triton X, 1 μg/ml aprotinin, and 2 mmol/l phenylmethylsulfonfyl fluoride. Homogenates were sonicated for three 15-s pulses at 30% power using a Sonic Dismembrator 550 (Fisher Scientific). Cellular debris was pelleted by centrifugation at 1,700 g, the supernatant was collected, and protein concentration was determined. Lysates (5 μg) were boiled for 5 min in reducing buffer and were separated by SDS-PAGE. Gels were electroblotted to nitrocellulose and blocked with 10% nonfat dry milk overnight at 4°C. Primary antibodies were diluted in PBS-Tween-BSA according to the manufacturer’s instructions and were incubated for 1 h at 4°C (Stressgen). After being washed, the primary antibody was detected with a horseradish peroxidase-conjugated anti-rabbit antibody (1:3,000) for 1 h at 4°C. The membrane was developed with the enhanced chemiluminescence Super Signal System (Pierce, Rockford, IL).

**Statistical analysis.** All data were analyzed by a one-way ANOVA with pairwise multiple comparisons using the Tukey test. Sigma Stat (Jandel Scientific, San Rafael, CA) was used for statistical analysis.

**RESULTS**

**Intestinal iNOS expression after ischemia-reperfusion.** Histological analysis of intestinal tissue from rats subjected to intestinal ischemia-reperfusion showed significant tissue damage and loss of villi height that was attenuated by blocking C5, confirming our previous observations (Fig. 1, A–C; see Ref. 54). Immunohistochernistry of intestinal tissue from rats treated with a nonfunctional anti-C5 MAb (16C) demonstrated an increase in iNOS protein expression compared with sham-operated controls (Fig. 1, E and D, respectively). The majority of iNOS staining was observed within the crypts of the intestinal mucosa and minimally in the intestinal vasculature (Fig. 1, B, E, and H). Blockade of C5 with a functionally inhibitory MAb (18A) attenuated iNOS protein expression in the reperfused intestine compared with 16C-treated animals (Fig. 1, F and E, respectively). The increase of iNOS protein after GI/R was accompanied by an increase in iNOS mRNA expression compared with sham-operated controls (Fig. 2), indicating that ischemia-reperfusion significantly induces iNOS gene expression in the rat intestine. Additionally, inhibition of the terminal complement cascade significantly attenuated the increase in iNOS mRNA and protein expression.

**IL-1β mRNA expression.** Because it is known that IL-1β can mediate the transcription of iNOS, we examined the effects of GI/R on IL-1β expression (27, 28, 50). GI/R significantly increased IL-1β mRNA expression compared with sham-operated controls (Fig. 3). Inhibition of complement C5 attenuated the transcription of IL-1β to levels comparable with sham-operated controls (Fig. 3), demonstrating that GI/R-mediated iNOS or IL-1β expression may be mediated by complement C5 activation.

**Complement-mediated dysregulation of SOD after ischemia-reperfusion.** We further analyzed the mRNA and protein expression profile of CuZn SOD and Mn SOD in the rat model of GI/R. The CuZn SOD mRNA and protein levels were unaffected by GI/R compared with sham-operated controls (Fig. 4, A and B). Furthermore, anti-C5 treatment had no affect on the basal levels of SOD mRNA (Fig. 4). The enzymatic activity of Cu/Zn SOD was assessed by an in-gel assay that measures the ability of SOD to inhibit the oxygen-dependent reduction of NBT (1, 4). Rats subjected to GI/R demonstrated a modest but significant decrease in intestinal Cu/Zn SOD activity compared with sham-operated controls (8,759 ± 554 vs. 12,406 ± 416 optical density units, respectively; P < 0.05, n = 3). Anti-C5 treatment preserved Cu/Zn SOD activity to levels indistinguishable from sham (12,817 ± 1,315 optical density units; P < 0.05 vs. 16C, n = 3).

In contrast to CuZn SOD levels, Mn SOD protein levels were decreased significantly after GI/R compared with sham-operated control animals (Fig. 5A). Interestingly, the Mn SOD mRNA levels were increased significantly in the reperfused intestine (Fig. 5B), suggesting that the decrease in protein levels was not mediated transcriptionally. The alterations in Mn SOD protein and mRNA levels were attenuated significantly in 18A-treated animals, indicating that inhibition of C5 activation during GI/R can preserve Mn SOD levels to that of nonischemic animals.

**Analysis of tyrosine nitration in the rat intestine.** The level of tyrosine-nitrated proteins generated by GI/R was assessed by immunohistochemistry as an indication of reactive nitrogen products. Animals treated with 16C MAb showed intense staining for nitrotyrosine residues compared with sham-operated controls (Fig. 6). Inhibition of C5 greatly attenuated nitrotyrosine staining (Fig. 6), indicating a diminished production of reactive nitrogen species in anti-C5-treated animals.

**Intestinal p-1KB-α expression after ischemia and reperfusion.** Recent reports have demonstrated activation of NF-κB in postischemic rat intestine (19, 20, 56).
The intensity of p-IκB-α immunohistochemical staining was used to assess NF-κB activation indirectly in intestinal tissue from rats undergoing GI/R in our model. We observed a time-dependent (i.e., 0, 5, 15, and 60 min of reperfusion) increase in p-IκB-α staining after gastrointestinal ischemia (Fig. 7, A1–A4). p-IκB-α staining peaked after 15 min reperfusion (Fig. 7A3). Staining for p-IκB-α was increased after 15 min of reperfusion in ischemic intestine (Fig. 7, B6 and B9) compared with sham-operated controls (Fig. 7, B5 and B8). Inhibition of C5 (Fig. 7, B7 and B10) attenuated p-IκB-α staining compared with PBS-treated rats (Fig. 7, B6 and B9). Cytoplasmic staining for p-IκB-α was observed (Fig. 7C).

DISCUSSION

iNOS and SOD have been implicated as potent modulators of injury after intestinal ischemia-reperfusion. Although it has been shown that ischemia-reperfusion leads to an increase in intestinal NO and superoxide anion, endogenous pathways that regulate the produc-
tion of these oxygen metabolites have not been defined clearly (3, 14, 46). We have previously demonstrated that the activation of complement induces a series of potent inflammatory events, including the induction of endothelial ICAM-1 gene expression and the upregulation of proinflammatory cytokines such as TNF-α and IL-1β (54). The present report extends our earlier findings to include evidence that complement mediates p-IκB-α expression and upregulation of intestinal iNOS and diminishes protein levels of mitochondrial SOD (Mn SOD) and the activity of Cu/Zn SOD. Furthermore, blocking complement C5 activation significantly attenuates tyrosine nitration observed in the intestinal mucosa, indicating that complement initiates a series of potent inflammatory events leading to oxygen radical-mediated damage after GI/R.

Our study is consistent with the observations of others that demonstrated that specific inhibition of iNOS protects the gut from injury after GI/R (5, 45, 46). However, the exact source of iNOS-generated NO has not been identified previously in a model of GI/R. We show that iNOS protein expression is confined mainly

![Fig. 2. Intestinal iNOS mRNA after gastrointestinal ischemia-reperfusion (GI/R). Semiquantitative RT-PCR was performed on sham-operated and ischemic-reperfused rats pretreated with 16C or 18A MAbs. iNOS PCR products were digitized and normalized to GAPDH (GAP) levels. Inset shows representative data. *P < 0.05 vs. sham and 18A (n = 5–6).](image)

![Fig. 3. IL-1β mRNA levels after GI/R. Semiquantitative RT-PCR was performed on nonischemic (sham) and ischemic-reperfused rats pretreated with 16C or 18A MAbs. RT-PCR products were digitized and normalized to GAPDH. Inset shows representative data. *P < 0.05 vs. sham and/or 18A (n = 3).](image)

![Fig. 4. Cu/Zn superoxide dismutase (SOD) protein and mRNA levels in the reperfused intestine. A: mRNA levels for intestinal Cu/Zn SOD after GI/R. RT-PCR products were normalized to GAPDH levels (n = 5–6). B: Western blot of Cu/Zn SOD (sham) after intestinal reperfusion. The blot is representative of 4 independent experiments. C: Cu/Zn SOD activity.](image)
to the intestinal crypt cells in the present study. This observation is consistent with a previous report (38) that localized iNOS within the rat mucosa during inflammation. Considering the large surface area of the intestinal mucosa, it is likely that the epithelium contributes significantly to the observed increase in NO after reperfusion. Furthermore, our data indicate that complement plays an important role in inducing epithelial-specific iNOS expression after GI/R.

It is not clear how complement affects the induction of iNOS mRNA within the intestinal crypt cells. Others have shown that iNOS mRNA expression can be augmented significantly by IL-1β or TNF-α (27, 28, 50). We have previously shown that complement augments intestinal TNF-α expression after GI/R, and our present study shows complement-dependent increases in IL-1β transcription, suggesting that iNOS may be mediated by IL-1β and TNF-α induction (54). It has been shown that iNOS mRNA can be regulated transcriptionally via NF-κB in the rat intestine (37). We have previously shown that the terminal complement cascade can directly regulate the translocation of NF-κB in an in vitro model (7). Our present findings extend our in vitro findings to an animal model and demonstrate that NF-κB activation (e.g., increased p-IκB-α expression) can be inhibited by anti-C5 treatment. Previous studies have shown that signal-induced phosphorylation of IκB-α leads to its proteolysis, which results in NF-κB activation (29, 33, 56). Furthermore, our data are supported by a study showing that, after stimulation with TNF-α or IL-1, IκB-α undergoes rapid phosphorylation at serine residues 32 and 36 (2). Therefore, our data suggest that C5 activation may lead to translocation of NF-κB and an increase in the transcription of iNOS.

Fig. 6. Immunohistochemical analysis of nitrotyrosine after intestinal ischemia and reperfusion. Sections (7 μm) of intestine from nonischemic (sham), noninhibitory anti-C5 MAb (16A)-treated, or inhibitory anti-C5 MAb (18A)-treated animals were immunostained for nitrotyrosine and counterstained with hematoxylin. Original magnification is ×200. Each micrograph is representative of 3 separate experiments.

*P < 0.05 vs. sham (n = 4–7).
either directly or via complement-induced cytokine production.

This study demonstrates that complement activation not only mediates the upregulation of iNOS but also effects the activity/protein levels of SOD. The supply and/or overexpression of exogenous SOD in rat models of intestinal ischemia significantly reduce injury (11, 39, 53). This finding suggests that maintenance of endogenous SOD may provide protection in GI/R. Data presented in this manuscript show that complement inhibition can maintain endogenous Cu/Zn activity and Mn SOD protein expression. Furthermore, complement activation may contribute to the surge in total superoxide anion production not only by recruiting and activating neutrophils but also by diminishing SOD levels after reperfusion.

Mn SOD mRNA and protein levels are influenced significantly by ischemia-reperfusion. Interestingly, Mn SOD protein levels were decreased in a complement-dependent fashion, despite an increase in mRNA expression. A difference between mRNA and protein levels has been observed previously in renal ischemia-reperfusion (15). This phenomenon suggests that there is an attempt to compensate for increased superoxide

![Image](image-url)
anion production within the mitochondria by increasing the transcription of Mn SOD, possibly via NF-κB-mediated transcription. However, our study suggests that there are posttranscriptional events leading to a decrease in Mn SOD protein levels. Decreased Mn SOD expression and/or activity have been demonstrated previously in several models of inflammation (31). Furthermore, decreased Mn SOD protein levels and increased mRNA levels in a rat model of colitis have also been observed in the presence of increased NOS expression (41). Our data confirm and extend these observations to suggest that Mn SOD dysregulation may be partly dependent on complement activation after ischemia-reperfusion.

The specific mechanism leading to a reduction in Mn SOD protein after C5 activation is not known. Mn SOD is susceptible to peroxynitrite-dependent tyrosine nitration and, furthermore, it is known that the nitration of proteins can lead to an increase in the rate of protein degradation (23, 30). However, coimmunoprecipitation experiments showed that there was no change in Mn SOD nitration after GI/R even after normalizing to Mn SOD protein levels (data not shown). Because there is a significant increase in tyrosine nitration after GI/R, the possibility remains that unidentified proteins are nitrated, leading to the eventual decrease in Mn SOD. Further investigation in this area is warranted. The mechanism(s) of cell injury beyond the decrease in Mn SOD is not completely understood. It is possible that reduced levels of Mn SOD in the mitochondria may contribute to the dysregulation of mitochondrial electron transport processes and may promote total cellular dysfunction and potentially cell death (12, 23). This theory is consistent with published reports demonstrating iNOS-dependent peroxynitrite formation in rat colonic epithelia cells is associated with apoptosis (57). Our data indicate that inhibition of the late complement cascade preserves Mn SOD possibly by decreasing nitration at tyrosine residues and supports the notion that complement is an early event that has a profound impact on tissue damage at the molecular and cellular level.

Unlike Mn SOD, Cu/Zn SOD protein and mRNA expression were unaffected by GI/R. However, we did observe a reduction in Cu/Zn SOD activity after GI/R that was attenuated with anticomplement treatment. The specific mechanism by which complement mediates the observed reduction of Cu/Zn SOD activity is not clear. Immunoprecipitations of rat Cu/Zn SOD and probing for nitrotyrosine residues revealed that Cu/Zn SOD is not nitrated (data not shown). It is possible that some Cu/Zn SOD is inactivated via peroxidation and/or oxidation of the active site histidine (25). It is logical to speculate that the rise in peroxide levels after reperfusion could modify Cu/Zn SOD in this manner. It should be noted that the observed decrease in Cu/Zn SOD activity was modest (~30% compared with sham). It is unclear if such a decrease would have an impact on the total levels of superoxide anion. Regardless, it is clear that complement activation does not affect Cu/Zn SOD transcription or translation, supporting the notion that endogenous levels of Cu/Zn SOD are overwhelmed by the large increase in superoxide anion levels. Furthermore, if the decrease in Cu/Zn SOD activity is physiologically relevant, it is possible that complement contributes to the inability of endogenous Cu/Zn SOD to quench ROS generated during reperfusion.

It is known that peroxynitrite, formed by the rapid reaction between NO and superoxide, can nitrate proteins at tyrosine residues in vivo (30, 31, 37). In this study, we used tyrosine nitration as a marker of protein nitration formation after intestinal ischemia-reperfusion. Our data demonstrate that anti-C5 treatment decreases nitration formation likely via decreased phosphorylation of IκB-α, decreased expression of iNOS, decreased NO production, and preservation of functional SOD levels. It should be noted that other nitrigen-containing compounds, such as nitrogen dioxide or nitryl chloride, could nitrate tyrosine residues in the absence of NO or superoxide (23, 51). However, it has been demonstrated that such compounds are inefficient nitrating compounds under physiological conditions and are required to be present at relatively high concentrations compared with peroxynitrite (23, 40, 52). Additionally, the role of NO and superoxide in tyrosine nitration has been demonstrated in recent studies. For example, the intensity and degree of nitrotyrosine staining are markedly reduced in tissue sections from iNOS-deficient mice after complement activation with zymosan (9). Furthermore, the novel SOD mimic, M-40401, significantly reduces nitrotyrosine staining in a similar model of rat GI/R (9, 10). Thus there is a definite link between iNOS, SOD, and nitrotyrosine staining.

In summary, our data demonstrate that complement not only contributes to the dysregulation of Cu/Zn SOD and Mn SOD but also induces the expression of intestinal iNOS. Because peroxynitrite formation is dependent on the presence of both NO and superoxide anion, complement inhibition may provide a degree of protection that cannot be afforded by the exogenous supply of SOD or iNOS inhibitors alone. Furthermore, we report here for the first time that ischemia-reperfusion leads to complement-dependent protein nitration and attenuation of SOD, which likely has a severe impact on normal cellular functions, confirming that complement is an important early mediator of the pathology associated with intestinal reperfusion injury.

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