Anti-Inflammatory and Anti-Apoptotic Effects of Fumonisin B1, an Inhibitor of Ceramide Synthase, in a Rodent Model of Splanchnic Ischemia and Reperfusion Injury

Salvatore Cuzzocrea, Rosanna Di Paola, Tiziana Genovese, Emanuela Mazzon, Emanuela Esposito, Concetta Crisafulli, Placido Bramanti, and Daniela Salvemini

Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Italy (S.C., C.C.); IRCCS Centro Neurolesi “Bonino-Pulejo”, Messina, Italy (S.C., R.D.P., T.G., E.M., E.E., P.B.); Department of Experimental Pharmacology, University of Naples “Federico II”, Naples, Italy (E.E.); and Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, St. Louis University School of Medicine, St. Louis, Missouri (D.S.)

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ABSTRACT

Ceramide is a sphingolipid with potent proinflammatory and proapoptotic properties. This study sought to determine whether pharmacological inhibition of ceramide biosynthesis in the intestine attenuates pathophysiological sequelae of shock induced by splanchnic artery occlusion and reperfusion. Ischemia and reperfusion injury was induced in anesthetized rats by clamping both the superior mesenteric artery and the celiac artery for 45 min followed by reperfusion. Within 6 min after reperfusion, animals developed significant systemic hypotension with 100% of the animals dying during the 4-h period of reperfusion. In parallel experiments, animals were necropsied after 60 min of reperfusion, and the ileum was harvested for histological examination and assessment of biochemical changes. Administration of fumonisin B1 (FB1), a competitive and reversible inhibitor of ceramide synthase (3 mg/kg, 15 min before reperfusion), significantly reduced i) the increased ceramide expression as detected by immunohistochemistry; ii) peroxynitrite-mediated protein nitration; iii) infiltration of the reperfused intestine with polymorphonuclear neutrophils following a decrease in intercellular adhesion molecule-1 expression; iv) production of the proinflammatory cytokine tumor necrosis factor-α; and v) apoptosis in the ileum. Overall, tissue-protective effects were clearly observed upon histological examination of the ileum. These beneficial events were ultimately linked to decreases in both the development of hypotension and overall mortality. These results implicate ceramide as a key signaling molecule in splanchnic arterial ischemia and reperfusion-induced shock. The broader implications of our results provide a pharmacological rationale for the development of inhibitors of ceramide biosynthesis as novel therapeutics for ischemia and reperfusion-induced shock of several etiologies.

Occlusion of the splanchnic circulation (SAO) followed by reperfusion (SAO/R) is a well established model of ischemia and reperfusion characterized by severe hypotension, multiple organ injury/dysfunction, and high mortality (Landow and Andersen, 1994; Homer-Vanniasinkam et al., 1997; Suliburk et al., 2008). Endothelial dysfunction plays a key role in SAO/R-induced shock by predisposing to vasoospasm, platelet activation, and increased neutrophil adherence, which exacerbates the local bowel injury as well as the general cardiocirculatory failure (Haglund, 1994; Jakob, 2002; Boros, 2003; Cerqueira et al., 2005). Considering these data and the lack of uniformly effective treatments for ischemia and reperfusion-induced shock, there is a compelling need for novel mechanism-based therapies. We propose that enzymes involved in the biosynthesis of the sphingolipid ceramide represent attractive targets for such novel therapies. Ceramide is generated by enzymatic hydrolysis of sphingomyelin by sphingomyelinases (SMases) (sphingomyelin pathway) and/or synthesized by serine palmitoyltransferase and ceramide synthase (de novo pathway) (Kolesnick, 2002). The steady-state availability of ceramide is further regulated by ceramidases that convert ceramide to sphingosine by catalyzing hydrolysis of its amide group (Kolesnick, 2002). Cer-

ABBREVIATIONS: SAO, occlusion of the splanchnic circulation; SAO/R, occlusion of the splanchnic circulation followed by reperfusion; FB1, fumonisin B1; DBA, biotin and avidin; IL, interleukin; NT, 3-nitrotyrosine; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor; ICAM-1, intercellular adhesion molecule 1; MAP, mitogen-activated protein; MPO, myeloperoxidase; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; PBS, phosphate-buffered saline; NB6, (3-carbazol-9-yl-propyl)-[2-(3,4-dimethoxy-phenyl)-ethyl]-methyl-amine.
amide serves as a second messenger to activate downstream effectors, including ceramide-activated protein kinase and ceramide-activated protein phosphatase, and generates other second messengers, such as sphingosine-1-phosphate (Kolesnick, 2002).

The important role of ceramide as an inflammatory mediator has been documented through inhibition of its biosynthesis with pharmacological inhibitors of sphingomyelinases or enzymes of the de novo pathway that inhibit the synthesis of proinflammatory cytokines, such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6 and reactive nitro-oxidative species, such as nitric oxide (\(\cdot\)NO), superoxide (\(\cdot\)O\(_2\)), and the product of their interaction, peroxynitrite (ONOO\(^-\)) (Claus et al., 2000; Won et al., 2004). In addition, ceramide activates the transcription factor NF-kB, as well as mitogen-activated protein kinase kinases, such as \(\alpha\) p38 kinase that amplify the inflammatory response (Claus et al., 2000; Won et al., 2004). Besides its role in inflammation, ceramide is also a well established proapoptotic mediator following radiation exposure, chemotherapeutics, cytokine administration, and nitro-oxidative stressors (Huwiler et al., 1999a,b, 2000; Kolesnick, 2002; Kolesnick and Fuks, 2003). Preclinical and clinical evidence supports a causal role for ceramide in the development of radiation-induced injury (Kolesnick and Fuks, 2003), acute lung injury (Göggel et al., 2004), emphysema (Petrache et al., 2005), asthma (Masini et al., 2008), and sepsis (Haimovitz-Friedman et al., 1997; Delogu et al., 1999; Dobrowsky and Kolesnick, 2001; Claus et al., 2005), all of which share inflammation, nitro-oxidative stress, and apoptosis in their pathogenesis. In animal models of septic shock, increased plasma levels of ceramide correlated with multiple organ dysfunction and death (Haimovitz-Friedman et al., 1997; Claus et al., 2005). Inhibition of ceramide synthesis with NB6, an inhibitor of the acid form of sphingomyelinase, reduced the severity of multiple organ dysfunction while improving survival rate (Claus et al., 2005). Confirmatory mechanistic results were obtained in acid sphingomyelinase-deficient mice (Haimovitz-Friedman et al., 1997). Thus, mortality rates after endotoxin administration in sphingomyelinase knockout mice were significantly reduced compared with those in wild-type mice (Haimovitz-Friedman et al., 1997). Furthermore, clinical studies have demonstrated a strong correlation between increased concentrations of plasma ceramide and the increased incidence of patients with sepsis who develop progressive and lethal multiple organ failure (Delogu et al., 1999; Dobrowsky and Kolesnick, 2001; Claus et al., 2005). It is clear that ceramide is a key signaling molecule in septic shock, al-

![Fig. 1](image1.png)

**Fig. 1.** a and b, effect of fumonisin B1 on SAO shock-induced alteration of MAP (a) and mortality (b). No significant alteration of MAP was observed in sham-operated rats. Fall in MAP in SAO rats \((n = 10)\) is blocked by pretreatment with fumonisin B1 (FB1). Survival was monitored for 4 h after SAO shock. Data are means \(\pm\) S.D. of 15 rats for each group. *, \(P < 0.01\) versus sham; °, \(P < 0.01\) versus I/R.

![Fig. 2](image2.png)

**Fig. 2.** Immunohistochemical localization of ceramide in the rat intestine. a, no positive staining for ceramide was found in the ileum from sham-treated rat. b, on the contrary, 60 min after reperfusion, positive ceramide staining was found in the intestine (arrows). c, there was no detectable immunostaining in the intestine (arrows) of SAO-shocked rats when rats were treated with FB1. Figures are representative of at least three experiments performed on different experimental days.
though its mechanisms remain ill-defined. Whether ceramide is also involved in pathophysiological events during the evolution of nonseptic shock, such as ischemia/reperfusion injury, has not been determined. Accordingly, and using a well established rat model of SAO/R-induced shock previously used in our laboratories (Salvemini et al., 1999), our goals are to determine whether ceramide is involved in the pathophysiology of nonseptic shock and, if so, identify the molecular and biochemical pathways involved.

Combined with other reports (Haimovitz-Friedman et al., 1997; Delogu et al., 1999; Dobrowsky and Kolesnick, 2001; Claus et al., 2005), our results support the proposition that therapeutic manipulations of ceramide and its downstream consequences are mechanistically grounded targets for the development of novel agents for the management of shock of several etiologies and strengthens the concept that ceramide is a clinically relevant biomarker to predict clinical outcomes in critically ill subjects with post-traumatic septic and nonseptic shock.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats (250–300 g; Harlan Nossan, Correzzana, Italy) were housed in a controlled environment and provided with ad libitum standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

**Surgical Procedures.** Rats were anesthetized with sodium pentobarbital (45 mg/kg i.p.), and catheters were placed in the carotid artery and jugular vein. Blood pressure was monitored continuously by a Maclab A/D converter (Ugo Basile, Varese, Italy) and displayed on a Macintosh personal computer. After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution. Rats were observed for a 30-min stabilization period before either SAO or sham ischemia. SAO shock was induced by clamping both the superior mesenteric artery and the
celiac trunk, resulting in a total occlusion of these arteries for 45 min. After this period of occlusion, the clamps were removed.

**Experimental Groups.** In this study, the various groups of rats were sacrificed at 60 min for histological examination of the bowel and for biochemical studies, as described below. In the treated groups of animals (n = 10), fumonisin B1, a competitive and reversible inhibitor of ceramide synthase (Delgado et al., 2006), or corresponding volume of vehicle (saline solution) was given as an intravenous bolus 3 mg/kg 15 min before reperfusion (SAO + fumonisin B1 groups). In separate groups of rats, surgery was performed identically to the SAO group, with the exception that the blood vessels were not occluded (time-controlled sham group, Sham). FB1 was used at a dose (3 mg/kg) previously shown to block ceramide biosynthesis in vivo and to exert anti-inflammatory and antiapoptotic actions (Masini et al., 2008).

**Immunohistochemical Localization of Fas-Ligand, Ceramide, Nitrotyrosine, MPO, ICAM-1, Bax, and Bcl-2.** At 60 min after reperfusion, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA), respectively. Sections were incubated overnight with anti-FAS-l (1:500 v/v in PBS), anticeramide antibody (1:500 v/v in PBS), antinitrotyrosine rabbit polyclonal antibody (1:500 v/v in PBS), anti-MPO rat antibody (1:500 v/v in PBS), anti-ICAM-1 antibody (1:500 in...
PBS), anti-Bax rabbit polyclonal antibody (1:500 v/v in PBS), or with anti-Bcl-2 polyclonal rat antibody. Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). To verify the binding specificity for nitrotyrosine, ceramide, MPO, FAS-1, Bax, and Bcl-2, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all of the experiments carried out. Immunocytochemistry photographs (n = 5) were assessed by densitometry by using Axiovision software (Zeiss, Milan, Italy) on an IBM personal computer (IBM, White Plains, NY) and evaluated by two independent observers blinded to the experimental protocol.

**Myeloperoxidase Activity.** Myeloperoxidase activity, a biochemical index of neutrophil accumulation in tissues, was determined as described previously (Mullane et al., 1985). Ileal segments, collected 60 min after reperfusion, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. Aliquots of supernatants were reacted at 37°C with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H2O2. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min⁻¹ at 37°C and was expressed in microunits per gram weight of wet tissue.

**Measurement of Cytokines.** TNF-α levels were evaluated in clarified supernatants of homogenized ileum samples at 60 min after reperfusion. The assay was carried out by using a colorimetric commercial kit (Calbiochem, San Diego, CA).

**Light Microscopy.** Ileum biopsies were taken 60 min after reperfusion, fixed for 1 week in buffered formaldehyde solution (10% in phosphate-buffered saline) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast tissue embedding medium (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness, 7 μm) were deparaffinized with xylene, stained with hematoxylin/eosin, and studied using light microscopy (Dialux 22; Esselte Leitz GmbH & Co KG, Stuttgart, Germany). The following morphological criteria were used for scoring: 0, no visible damage; 1, slight injury with focal epithelial edema and necrosis; 2, moderate injury with diffuse swelling and necrosis of the villi; 3, severe injury with necrosis and evidence of neutrophil infiltration in the submucosa; and 4, severe injury with widespread necrosis and massive neutrophil infiltration plus evidence of hemorrhage. All histological sections were examined by the same investigator without knowledge of their treatment group.

**Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling Assay.** TUNEL assays were conducted by using a detection kit according to the manufacturer’s instruction (horseradish peroxidase kit; D.B.A., Milan, Italy). In brief, deparaffinized sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by immersing sections in 3% H2O2 for 5 min at room temperature and then washing them with PBS. Sections were then immersed in terminal deoxynucleotidyltransferase buffer containing deoxynucleotidyl transferase and biotinylated dUTP in terminal deoxynucleotidyltransferase buffer, incubated in at 100% relative humidity at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL-positive cells/high-power field was counted in 5 to 10 fields for each coded slide.

**Total Protein Extraction and Western Blot Analysis for Bax and Bcl-2.** Ileal tissues were disrupted by homogenization with a Ultra-Turrax T8 homogenizer on ice in a buffer containing: 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μg/ml trypsin inhibitor, 3 μg/ml pepstatin, 2 μg/ml leupeptin, 40 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenates were centrifuged at 100,000g for 30 min at 4°C. Aliquots of supernatants were reacted at 37°C in 1 at 37°C and

![](fig5.png)

**Fig. 5.** Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all rats in each experimental group) for ceramide, nitrotyrosine, MPO, ICAM-1, FAS-ligand, Bax, and Bcl-2 from ileum tissues was assessed. The assay was carried out by using Axiovision software (Zeiss) on an IBM personal computer. Data are expressed as percentage of total tissue area. *P < 0.01 versus Sham; **P < 0.01 versus SCI. ND, not detectable.
washed with PBS, developed using the SuperSignal West Pico chemiluminescence substrate (Pierce, Milan, Italy) according to the manufacturer’s instructions, and exposed to Kodak X-Omat AR film. The protein bands of Bax (~29 kDa), Bcl-2 (~26 kDa) on x-ray film were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad).

**Reagents.** Biotin-blocking kit, biotin-conjugated goat anti-rabbit IgG, and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA). Primary anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Saranac Lake, NY). The primary antibodies directed at Bax and Bcl-2 were obtained from Santa Cruz Biotechnology, Inc. The secondary antibody was obtained from Jackson ImmunoResearch Laboratories Inc. Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter, McGaw Park, IL).

**Data Analysis.** All values in the figures and text are expressed as means ± S.E.M. of the number of animals or ileal segments. In the experiments involving histology or immunohistochemistry, the figures are representative of at least three experiments, each performed on different days. Data sets were examined by one- and two-way analyses of variance, and individual group means were then compared with Student’s unpaired t test. Nonparametric data were analyzed with the Fisher’s exact test. A P value less than 0.05 was considered significant.

**Results**

**Protective Effects of Fumonisin B1 in Splanchnic Artery Occlusion Shock.** Occlusion of the splanchnic arteries produced an increase in MAP, which then decreased until death (Fig. 1a). The mean survival time was found to be 75 ± 9 min (n = 10), whereas control sham animal survived for the entire observation period of 4 h (n = 10) (Fig. 1b). Having established the mean survival time, in subsequent experiments, animals were sacrificed 60 min after reperfusion to collect blood and tissues for biochemical analysis. Reperfusion of the ischemic splanchnic circulation was associated with the appearance of ceramide in the ileum by immunohistochemistry (Figs. 2b and 5, densitometry analysis). Histological examinations of the small intestine at 60 min of reperfusion (see representative sections in Fig. 3) revealed significant pathologic changes that were not observed in ileal tissues from sham-operated rats (Fig. 3a). Indeed profound inflammatory cellular infiltration was observed that extended through the wall and concentrated below the epithelial layer (Fig. 3, b, arrows, and D, histological score), as well as extensive epithelial exfoliation at the tips of the villi. The administration of fumonisin B1 (3 mg/kg, n = 10), a competitive and reversible inhibitor of ceramide synthase (Delgado et al., 2006) given intravenously 15 min before reperfusion attenuated the appearance of ceramide (Figs. 2c and 5, densitometry analysis) and exerted a significant tissue protective of ileal tissues (Fig. 3, c and d, histological score). This dose of FB1 also prevented the fall in blood pressure (Fig. 1a) that was otherwise noted after reperfusion, and it increased survival time from 75 ± 9 min without FB1 to 220 ± 12 min with the inhibitor (n = 10, P < 0.01; Fig. 1b). We next sought to unravel the molecular and biochemical pathways by which ceramide caused these pathophysiological alternations after reperfusion of the ischemic splanchnic circulation. To this end, we focused on nitro-oxidative stress, inflammation, and apoptosis since these events contribute to shock of various etiologies (Cerqueira et al., 2005; Fink et al., 2007).
Inhibition of Ceramide Biosynthesis Attenuates Peroxynitrite Formation and Nitro-Oxidative Stress. Formation of 3-nitrotyrosine (NT) (Figs. 4b, arrows, and 5, densitometry analysis) in the ileum after reperfusion of the ischemic splanchnic circulation originates from the production of ONOO⁻ (Cuzzocrea et al., 2000). Here, such NT staining was blocked by FB1 (3 mg/kg, n = 10) (Figs. 4c and 5, densitometry analysis), suggesting the critical role of ceramide in the production of ONOO⁻ within the ileum after reperfusion. It is important to note that such NT staining was not noted in ileal tissues that were fixed after the ischemic phase alone (data not shown).

Anti-Inflammatory Effects of Fumonisin B1. Ischemia/reperfusion injury of the gut results in the up-regulation of proinflammatory mediators in the intestine, as well as other organs such as the lung (Esposito et al., 2005). Ileal TNF-α levels were significantly increased in SAO-shocked rats in comparison with sham animals but were significantly reduced in SAO-shocked rats treated with FB1 (Fig. 6a). Likewise, neutrophil-derived myeloperoxidase activity was significantly elevated after splanchnic ischemia/reperfusion in control rats and was significantly reduced by FB1 treatment (Fig. 6b). In addition, tissue sections obtained from vehicle-treated animals after SAO-shock demonstrated positive staining for MPO (Figs. 4e and 5, densitometry analysis) mainly localized in the infiltrated inflammatory cells. In SAO-shocked rats treated with FB1, the staining for MPO (Figs. 4f and 5, densitometry analysis) was visibly and significantly reduced. No positive staining for MPO was found in intestine tissue section from sham-operated rats (Fig. 4d). The elevation of the MPO activity was associated with increased immunohistochemical staining for ICAM-1.
Anti-Apoptotic Effects Fumonisin B1

TUNEL. As measured by TUNEL staining, almost no apoptotic cells were detectable in the ileal tissues of sham-operated rats (Fig. 8a). At 60 min after reperfusion, ileum tissues obtained from vehicle-treated SAO-shocked rats showed numerous dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 8, b, b1, and e, TUNEL + cells) in the injured area. In contrast, tissues obtained from FB1-treated rats (Fig. 8, c and e, TUNEL + cells) demonstrated almost no apoptotic cells.

Fas Ligand Expression. The potential effect of FB1 on apoptosis in ischemia and reperfusion was evaluated by immunohistochemical detection of Fas ligand. No positive staining for Fas ligand was observed in the ileum tissues collected from sham-operated rats (Fig. 9a). At 60 min after reperfusion, positive staining for Fas ligand are readily detected in the ileum tissues from vehicle-treated SAO-shocked rats (Fig. 9b, arrows) mainly localized within infiltrating inflammatory cells (Fig. 9b1, arrows) in injured area. Such positive staining for Fas ligand of infiltrating inflammatory cells (Fig. 9c) was significantly reduced in the FB1-treated rats at 60 min after reperfusion.

Bax and Bcl-2 Expression. The appearance of Bax in homogenates of ileum tissues was investigated by Western blot at 60 min after reperfusion. Basal levels of Bax that were barely detectable in the homogenized ileum tissues from sham-operated animals were substantially increased in the ileal tissues of SAO-shocked rats, whereas FB1 treatment prevented the SAO-mediated Bax expression (Fig. 10, a and a1). Basal Bcl-2 expression was readily detected by Western blots of ileal tissue from sham-operated rats but was significantly diminished in ileal tissues of vehicle-treated rats 60 min after reperfusion (Fig. 10, b and b1). Treatment of rats with FB1 significantly reduced this SAO-induced inhibition of Bcl-2 expression (Fig. 10, b and b1). Moreover, ileum tissues were taken at 60 min after reperfusion and immunohistologically stained for Bax and Bcl-2. Although ileal tissues from sham-operated rats did not stain for Bax (Figs. 5, densitometry analysis, and 11a), those from SAO-shocked rats exhibited positive staining for Bax (Fig. 11b, arrows). FB1 treatment (15 min before reperfusion) reduced the de-
gree of positive staining for Bax in the ileum section of SAO-shocked rats (Figs. 5, densitometry analysis, and 11c). In addition, sections of ileum from sham-operated rats demonstrated positive staining for Bcl-2 (Fig. 11d, arrows). Ileum sections obtained from vehicle-treated SAO-shocked rats exhibited significantly less staining for Bcl-2 (Figs. 5, densitometry analysis, and 11e). FB1 treatment (15 min before reperfusion) reduced the loss of positive staining for Bcl-2 in the ileum section of SAO-shocked rats (Figs. 5, densitometry analysis, and 11f).

**Discussion**

Despite improvements in the early resuscitation and stabilization of traumatic injuries, the development of shock following sepsis or ischemia and reperfusion injuries that culminate in progressive multiple organ dysfunction continues to be a major factor limiting survival. Our studies implicate for the first time ceramide as a key signaling mediator in biochemical events associated with splanchnic artery occlusion and reperfusion-induced shock and ultimately death.

Shock induced by splanchnic artery occlusion and reperfusion increased both immunoreactive ceramide within the epithelium of ileal tissues and mortality rates. Such SAO/R-induced shock is also associated with overt production of nitro-oxidative species, including superoxide (Salvemini et al., 1998, 1999), nitric oxide (Cuzzocrea et al., 1998, 2002), and peroxynitrite (Cuzzocrea et al., 2000). Ceramide has been implicated in nitro-oxidative stress. Indeed, ceramide
stimulates formation of O$_2^-$ and NO (Huwiler et al., 2000; Goldkorn et al., 2005), effects that are amplified by ceramide transcriptional up-regulation of constitutive and inducible nitric-oxide synthase (Pahan et al., 1998; Li et al., 2002). In turn, elevated levels of O$_2^-$ and NO (and thus ONOO$^-$) increase steady-state concentrations of ceramide by activating sphingomyelinases and by increasing the degradation of ceramidases, the enzymes responsible for the degradation of ceramide. In addition, NO can increase the levels of ceramide via ceramide synthase (Franzen et al., 2001; Huwiler et al., 2001; Pautz et al., 2002; Castillo et al., 2007a,b). We have previously shown that nitric oxide-derived ONOO$^-$ promotes the post-translational nitration of proteins in the ileum following SAO/R, whereas inhibitors of ONOO$^-$ block NT formation, attenuate tissue damage, and reduce mortality. As shown in Fig. 3b, the increase in ceramide was associated with the appearance of peroxynitrite-derived NT formation in the ileum, further underscoring the link between ceramide and nitro-oxidative stress. Systemic inhibition of ceramide synthase by FB1 attenuated the formation of ceramide, reduced development of nitro-oxidative stress, prevented the development of hypotension, and ultimately improved survival.

Important roles of the ceramide metabolic pathway in inflammation have been documented. It is noteworthy that ceramide activates NF-$\kappa$B (Claus et al., 2000; Won et al., 2004), which regulates various proinflammatory cytokines genes, such as TNF-$\alpha$, IL-1$\beta$, and IL-6 and adhesion molecules to recruit neutrophils at sites of inflammation (Muller-Ladner et al., 2002; Gwinn and Vallyathan, 2006). Pharmacological manipulation of ceramide accumulation by inhibiting sphingomyelinase or the de novo pathways attenuates the synthesis of several cytokines and reactive oxygen species (Claus et al., 2000; Won et al., 2004), leading to beneficial effects in several animal models (Haimovitz-Friedman et al., 1997; Delogu et al., 1999; Drobnik et al., 2003; Kolesnick and Fuks, 2003; Goggel et al., 2004; Claus et al., 2005; Petrache et al., 2005; Masini et al., 2008). The critical roles of neutrophils and proinflammatory cytokines in the pathogenesis of shock associated with ischemia and reperfusion injuries are well documented. For instance, steroids (Cuzzocrea et al., 1997) or anti-TNF-$\alpha$ antibodies (Esposito et al., 2007) exert significant anti-inflammatory and tissue-protective effects in several models of septic and nonseptic models of shock associated with ischemia and reperfusion. Our results suggest that the protective effects of FB1 may be secondary to attenuation of the inflammatory response that accompanies tissue damage after reperfusion of the ischemic splanchnic circulation given that FB1 clearly attenuated TNF-$\alpha$ expression in the ileum as well as subsequent neutrophil infiltration of that tissue. Hypoxic or injured endothelial cells synthesize TNF-$\alpha$, which can up-regulate the expression of ICAM-1 on their luminal surfaces, corresponding to the induction of neutrophil recruitment that is maximum within 60 min of reperfusion and persists at a lower rate in the late phase of reperfusion (Esposito et al., 2007). In accordance with these findings, we observed the expression of ICAM-1 on vascular endothelia in the ileal tissues from SAO-shocked rats and that this was blocked by FB1. These results suggest that inhibition of ceramide may interfere with the interaction of neutrophils and endothelial cells at the firm adhesion phase mediated by ICAM-1.

We propose that ceramide modulation of the inflammatory response may involve peroxynitrite as an intermediary link.

![Fig. 10. a, a1, b, and b1, representative Western blots showing the effects of fumonisins B1 administration on Bax (a and a1) and Bcl-2 (b and b1) expression at 60 min of reperfusion. a and b, representative blot of lysates obtained from five animals per group is shown, and densitometry analysis of all animals is reported. The results in b and b1 are expressed as mean ± S.E.M. from n = 5/6 ileum tissues for each group. *, P < 0.01 versus Sham; †, P < 0.01 versus SAO-shocked rats.](image-url)
for several reasons. First, as shown in this work, inhibition of ceramide biosynthesis blocked ONOO$^-$. Second, nitro-oxidative species, such as ONOO$^-$, can induce endothelial cell damage (Salvemini et al., 2002, 2006) and activate redox-sensitive NF-$\kappa$B and AP-1 (Gwinn and Vallyathan, 2006) that in turn regulate proinflammatory and pronociceptive cytokine genes, including TNF-$\alpha$ (Gius et al., 1999; Bowie and O'Neill, 2000; Salvemini et al., 2001; Haddad and Land, 2002; Matata and Galinanes, 2002; McInnis et al., 2002; Ndengele et al., 2005; Gwinn and Vallyathan, 2006). These nitro-oxidative species also up-regulate adhesion molecules on endothelial cells (ICAM) and P-selectin on neutrophils to recruit neutrophils to sites of inflammation (Salvemini et al., 2002, 2006). Thus, therapeutic reduction of nitro-oxidative species is anti-inflammatory and tissue-protective in several animal models of shock and reperfusion injuries (Salvemini and Riley, 2000; Cuzzocrea et al., 2001, 2004; Salvemini and Cuzzocrea, 2002).

Here we also implicate that apoptosis also contributes to the actions of ceramide in SAO/R-induced shock of intestinal epithelial cells. The potent proapoptotic roles of ceramide were recognized earlier than its proinflammatory ones. We now know that ceramide is well established as a major proapoptotic mediator after exposures to radiation, chemotherapeutics, cytokines, and nitro-oxidative species (Huwiler et al., 1999a,b, 2000, 2001; Kolesnick, 2002; Kolesnick and Fuks, 2003). Kolesnick and colleagues reported that injection of endotoxin or TNF-$\alpha$ in mice increased levels of ceramide in

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**Fig. 11.** a, no positive staining for Bax was observed in ileum section taken from sham-operated rats. b, ileum sections taken from ischemia/reperfusion-injured rats showed positive staining for Bax localized mainly in the inflammatory cells (arrows). c, the degree of positive staining for Bax was markedly reduced in the ileum section obtained from SAO-shocked rats that had received fumonisin B1 at the dose of 3 mg/kg. d, in addition, positive staining for Bcl-2 was observed in the ileum sections taken from sham-operated rats (arrows). e, the degree of positive staining for Bcl-2 was significantly attenuated in ileum section from SAO-shocked rats that had received fumonisin B1 at the dose of 3 mg/kg (arrows). Figure is representative of at least three experiments performed on different experimental days.
endothelium of intestine, lung, fat, and thymus that were followed by severe apoptosis in these tissues within 6 h (Haimovitz-Friedman et al., 1997). These biochemical changes preceded nonendothelial tissue damage and death, were blocked by inhibitors of ceramide biosynthesis (acid sphingomyelinase), and were not observed in acid sphingomyelinase knockout mice (Haimovitz-Friedman et al., 1997). These results suggest that, in the presence of TNF-α or other proinflammatory cytokines released by stimuli like endotoxin, the generation of ceramide in critical organs such as liver and lung leads to endothelial cell apoptosis, which contribute to overall tissue injury and death (Haimovitz-Friedman et al., 1997). Furthermore, ischemia and reperfusion induce apoptosis in several tissues, including brain, myocardium (Itoh et al., 1995), intestine (Noda et al., 1998), and liver (Fukuda et al., 1993). Previous studies have also suggested that the Fas/FasL interaction regulates apoptosis (Mor et al., 2003). Moreover, the Bel-2 family of apoptotic regulators is—in addition to caspases—another important functional component of the apoptotic pathway. As shown in Fig. 9, a and a1, splanchnic artery occlusion and reperfusion led to apoptosis in the ileum but was blocked by F81B, thereby linking ceramide to apoptosis in this setting. Because TNF-α causes apoptosis through ceramide (Haimovitz-Friedman et al., 1997) and because SAO increases levels of this cytkine, it is likely that TNF-α further contributes to apoptosis in this setting by stimulating the production of additional ceramide to propagate the cycle of inflammation, tissue injury, and death.

In summary, our results reveal that ceramide contributes to the development of shock and intestinal injury after splanchnic artery occlusion and reperfusion through ONOO−, which in turn engages downstream pathways of inflammation and apoptosis collectively contributing to high mortality rates. In addition, our findings support a proximate relationship between the ceramide metabolic and the nitro-oxidative pathways as observed in other pathological settings (Delogu et al., 1999; Kolesnick and Fuks, 2003; Göggl et al., 2004; Petrache et al., 2005; Masini et al., 2008). Strategies that reduce in situ levels of the enzymes regulating their metabolism in response to cell stress. (1999) Splanchnic ischemia and reperfusion injury is reduced by genetic or pharmacological inhibition of TNF-α. J Leukoc Biol 61:1023–1042.

References


Dobrovsky and Kolesnick, 2001), should stimulate in-depth shock (Haimovitz-Friedman et al., 1997; Delogu et al., 1999; Masini et al., 2008). Strategies that reduce in situ levels of apoptosis collectively contributing to high mortality rates. In addition, our findings support a proximate relationship between the ceramide metabolic and the nitro-oxidative pathways as observed in other pathological settings (Delogu et al., 1999; Kolesnick and Fuks, 2003; Göggl et al., 2004; Petrache et al., 2005; Masini et al., 2008). Strategies that reduce in situ levels of ceramide may represent novel and promising therapeutic strategies for shock and ischemia-reperfusion injuries. Our findings, coupled with those of others implicating ceramide in preclinical animal models of septic shock as well as in humans with septic shock (Haimovitz-Friedman et al., 1997; Delogu et al., 1999; Dobrowsky and Kolesnick, 2001), should stimulate in-depth investigation of the importance of this pathway in the pathogenesis of shock of multiple origins.


Address correspondence to: Dr. Daniela Salvemini, Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, St. Louis University School of Medicine, 3635 Vista Avenue, DeSoto Towers, St Louis, MO 63110. E-mail: salvemd@slu.edu