Loss of parietal cell superoxide dismutase leads to gastric oxidative stress and increased injury susceptibility in mice

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Submitted 5 May 2011; accepted in final form 23 June 2011

A primary function of the vertebrate gastric mucosa in the initiation of digestion is acid secretion. In mammals, including humans, this function is provided by parietal cells (18). Acid secretion into the gastric lumen comes at the expense of abundant energy in the form of ATP. Because of this energy requirement, gastric parietal cells are rich in mitochondria, which generate ATP via oxidative phosphorylation by a proton-motive force ending in the joining of oxygen and protons to form water (10, 15). Nevertheless, this process also results in the generation of superoxide, which can result in the release of reactive oxygen species (ROS) that can lead to mitochondrial dysfunction and cell death. Antioxidant enzymes counter this threat by detoxifying ROS. In mammals, three isoforms of superoxide dismutase (SOD1, SOD2, and SOD3) catalyze the conversion of superoxide anions to hydrogen peroxide (21). Cu,Zn-SOD (SOD1) is a 32-kDa homodimer that is present in the cytoplasm, nucleus, lysosomes, peroxisomes, and mitochondrial intermembrane space (19). Extracellular SOD (SOD3) is a 135-kDa tetramer that is synthesized in the endoplasmic reticulum and secreted into the interstitial space (21). SOD3 also binds to the outer cell surface through an interaction with polyanions such as heparan sulfate (19). Mitochondrial Mn-SOD (SOD2) is an 89-kDa tetramer present in the mitochondrial matrix that normally limits the potential toxicity of superoxide generation by catalyzing its conversion to hydrogen peroxide, which is subsequently converted to water by glutathione peroxidase (19, 43).

The importance of SOD2 is borne out by the developmental lethality caused by homozygous gene deletion in mice, primarily as a result of neuronal and cardiac defects (34). In fact, reduced SOD2 activity and the resultant mitochondrial dysfunction have been linked to numerous human degenerative diseases, including Friedreich’s ataxia (33), Alzheimer’s disease (8), Parkinson’s disease (50), and diabetes (49). Increased oxidative stress and reduced antioxidant function have also been characterized in portal hypertensive gastropathy (42). Indeed, parietal cell loss and impaired acid secretion have been reported as consequences of portal hypertensive gastropathy (1, 24). Moreover, in portal hypertensive gastric mucosa, tyrosine nitration has been reported as a result of peroxynitrite formation from elevated superoxide and nitric oxide production (26, 37). Enzymes integral to mitochondrial oxidative phosphorylation (e.g., aconitate and ATP synthase), as well as SOD2 itself, have been demonstrated to be targets of inactivation by tyrosine nitration (13, 14, 35).

Certainly, impaired acid secretion would be expected to have ramifications on gastric mucosal defense, possibly resulting in enhanced pathological bacterial colonization. Gastric parietal loss, itself, has been reported to lead to dysplastic changes resulting in invasive gastric adenocarcinoma, metastasis, and glandular hyperplasia (20, 36, 44). However, the effects of gastric parietal cell loss have generally been explored by genetic manipulations to prevent parietal cell differentiation (29) or mediate parietal cell ablation (30).
Here, we have used the Cre-lox system of conditional sod2 gene deletion in the gastric parietal cells of transgenic mice to examine the consequences of SOD2 deficiency on mitochondrial enzymatic function, energy production, acid secretion, oxidative stress, and gastric injury susceptibility.

MATERIALS AND METHODS

Gastric-specific SOD2 knockout mice. All animal study protocols were reviewed and approved by the Institutional Animal Care and Usage Committee of the Veterans Affairs Long Beach Healthcare System, which maintains accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice having gastric parietal cell sod2 deficiency were generated by crossing C57BL/6 mice homozygous for the loxp site-flanked sod2 (SOD2
tfl) gene (17) with hemizygous FVB/N transgenic (Atp4b-Cre) mice expressing Cre recombinase under the control of the Atp4b gene promoter (44). Mice were housed in a specific pathogen-free facility (-CAG CGG AGG GCA GAT AGC AAG CAA G-3'), with a 12:12-h light-dark cycle and maintained at a constant temperature of 22°C. Mice were fed a standard rodent diet and received water ad libitum. For alcohol-induced gastric injury studies, as well as fundic stomach was washed several times in changes of cold PBS. The antrum and forestomach were discarded, and the curvature. The antrum and forestomach were discarded, and the

Specificity of staining was confirmed by omitting the primary antibody diluent (Dako) was added, and the sections were incubated in a humidified chamber at 4°C overnight. The following antibodies were used: rabbit anti-SOD2 (catalog no. 06-984, Millipore, Billerica, MA), rabbit anti-malondialdehyde (catalog no. MDA11-S, Alpha Diagnostic, San Antonio, TX), and rabbit anti-nitrotyrosine (catalog no. 06-284, Millipore). Secondary antibodies were conjugated with Alexa Flour 568 or Alexa Flour 488 (Invitrogen). Parietal cells were visualized with FITC-labeled Dolichos biflorus agglutinin (catalog no. L9142, Sigma Chemical), a parietal cell lineage marker (25). Fluorescence-stained sections were counterstained with 4',6-diamidino-2-phenylindole hydrochloride (DAPI). Staining was imaged using an epifluorescence microscope (Eclipse 50i, Nikon, Melville, NY) with CFL-2/CF FITC/Texas Red/DAPI filters and NIS-Elements imaging software. A monoclonal antibody (model DS-Q11, Nikon) was used to capture fluorescence images, and a color C-mouse camera (model DS-FL1 5-Meg, Nikon) was used to capture bright-field images. Specificity of staining was confirmed by omitting the primary antibody and using an appropriate blocking peptide. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed using an ApopTag in situ detection kit (Serologicals, Norcross, GA) according to the manufacturer’s instructions. Sections were counterstained with DAPI, and the total number of cells in a given field was accurately quantified by counting the number of nuclei using the NIS-Elements imaging software. Staining specificity was confirmed by using normal (preimmune) IgG in place.
Fig. 1. Gastric parietal cell-specific SOD2 deficiency. A: schematic representation of generation of parietal cell-specific SOD2-deficient mice. A targeting vector in which exon 3 (EX3) of both alleles encoding the sod2 gene was flanked by loxP sites was used to generate mice. A neomycin (Neo) resistance cassette (also flanked by loxP sites) enabled selection of the embryonic stem cells used to generate germline chimeras (17). HSV T, human simian virus 40 T. B, left: genotyping progeny mice using primers PA, PB, and PC allowed identification of homozygous inheritance of both sod2 alleles flanked by loxP sites via an expected 358-bp PCR product (fl/fl), heterozygous inheritance of 1 sod2 allele flanked by loxP sites via expected 501- and 358-bp PCR products [fl/wild-type (wt)], or homozygous inheritance of both wild-type sod2 alleles via an expected 501-bp PCR product (wt/wt). B, right: crosses of progeny mice homozygous for both sod2 alleles flanked by loxP sites (SOD2 fl/fl mice) with Atp4b-Cre mice, followed by backcrossing with SOD2 fl/fl mice, resulted in gastric parietal cell-specific excision of the sod2 gene, as verified by PCR analysis of DNA extracted from isolates enriched in parietal cells using the PA-PD primer pair that generated a 648-bp product, indicating excision of exon 3 of sod2, the neomycin resistance cassette, and the 3 loxP sites, as depicted in A. Progeny mice that did not inherit the Cre allele gave rise to a 2,500-bp product representing intact exon 3 of sod2, neomycin resistance cassette, and 3 loxP sites. These Cre littermates were used throughout the studies as age-matched controls. C: immunoblot analysis demonstrating parietal cell-specific SOD2 protein deficiency in parietal cell-enriched isolates from Cre/SOD2 fl/fl mice compared with wild-type (WT) mice of the same genetic background (C57BL/6) and Cre/SOD2 fl/fl age-matched littermate controls. Some of the Cre/SOD2 fl/fl mice exhibited low Cre expression and, correspondingly, SOD2 protein levels similar to those of wild-type mice. Therefore, only results from experimental mice having both verified gastric parietal cell-specific Cre expression and commensurate parietal cell SOD2 protein deficiency were used in evaluations in the present study. D: histological assessment of SOD2 protein expression levels and localization within gastric mucosa of Cre/SOD2 fl/fl and age-matched Cre/SOD2 fl/fl littermate control mice. Formalin-fixed, paraffin-embedded tissue sections were double-stained with the FITC-labeled gastric parietal cell-specific marker Dolichos biflorus agglutinin (DBA) (9) and rabbit anti-SOD2 polyclonal antibody. Merged images were used to assess SOD2 colocalization with parietal cells. Scale bars, 50 μm. Original magnification, ×400. E: total SOD activity (SOD1 + SOD2 + SOD3) determined by colorimetric assay. *P < 0.05. F: SOD2 activity was determined with the inclusion of KCN to inhibit SOD1 and SOD3 activity. *P < 0.001. In E and F, results (means ± SD) are expressed as units of SOD2 activity per milligram of protein of parietal cell-enriched isolates. Activity from each isolate/animal was determined independently (n = 6/group).
of primary antibody or by omitting the primary antibody and using appropriate blocking peptide.

**Immunoblot analysis.** Tissues were homogenized with a Polytron tissue homogenizer (Kinematica, Littau, Switzerland) in a lysis buffer containing 62.5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.4% deoxycholic acid, 1% Nonidet P-40, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 0.05 mM aminothiol benzene sulfonyl fluoride, and 0.1 mM sodium vanadate. The homogenates were centrifuged at 14,000 rpm for 10 min at 4°C. The protein concentrations of the homogenates were determined by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Equal amounts of protein were subjected to SDS-PAGE and transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Piscataway, NJ). Antibodies used for signal detection were as follows: rabbit anti-SOD2 (catalog no. 06-984, Millipore), rabbit anti-Cre recombinase (catalog no. 69050-3, EMD Chemicals, Gibbstown, NJ), mouse anti-ATP synthase subunit-α (catalog no. MS5070, Mitosciences, Eugene, OR), and mouse anti-heat shock protein 60 (LK-2; catalog no. ADI-SPA-807-E, EMD Chemicals). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were used as secondary antibodies, and signals were visualized by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). All membranes were also stripped and reprobed with mouse monoclonal anti-β-actin antibody (AC-15; catalog no. A1978, Sigma Chemical) to control for protein loading and membrane transfer. Quantification of the data was performed using a video image analysis system (Image-1/FL, Universal Imaging, Westchester, PA) after normalization for the corresponding total protein and/or β-actin signal.

**SOD activity.** SOD activity was determined by quantifying the dismutation of superoxide generated by xanthine oxidase in the presence of a fixed amount of hypoxanthine using a kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions. Superoxide was detected spectrophotometrically by a tetrazolium salt chromagen, and a standard curve of SOD activity was generated by serial dilutions of a stock solution of bovine erythrocyte SOD1. Known amounts of total gastric protein lysate were assayed, and activity is expressed as SOD units per milligram of total protein, where 1 SOD unit is defined as the amount of enzyme needed to catalyze 50% dismutation of total superoxide radical generated. SOD2 activity was assayed separately for each experimental sample by inclusion of 3 mM KCN to inhibit the activities of SOD1 and SOD3.

**Aconitase activity.** Aconitase activity was determined spectrophotometrically at 340 nm in a buffer containing 30 mM citrate, 0.2 mM NADP+, 0.6 mM MnCl2, 50 mM Tris-HCl (pH 7.4), and 2 U of isocitrate dehydrogenase. After addition of 25 μg of mitochondrial extract, absorbance readings were taken every min for 60 min at 22°C. The linear rates during the latter 30 min were used to determine aconitase activity; 1 μU of aconitase activity catalyzed the formation of 1 nmol of isocitrate per minute. Duplicate reactions were performed in the presence of 18.7 mM oxalomalate (aconitase inhibitor; Cayman Chemicals, Ann Arbor, MI) to control for nonspecific NADP+-dependent reactions.

**ATP synthase activity.** Mitochondrial ATP synthase activity was determined by ATP hydrolysis, which was measured spectrophotometrically by coupling the reaction to pyruvate kinase and lactate dehydrogenase (both from Sigma Chemical) and monitoring NADH oxidation at 340 nm. Isolated mitochondrial fractions (100 μg) were added to a buffer containing 20 mM HEPES, 5 mM MgCl2, 100 mM KCl, 5 mM KCN, 2.5 mM phosphoenolpyruvate, 200 μM NADH, ~8 U of pyruvate kinase, and ~12 U of lactate dehydrogenase (pH 7.5–8.0). The reaction temperature was 30°C. The enzymatic reaction was initiated by addition of ATP to 1.0 mM final concentration, and the decrease in NADH absorbance at 340 nm was monitored for 30 min. Mitochondrial ATPase activity was inhibited by addition of oligomycin (2 μg) to duplicate reactions. Mitochondria-specific ATPase activity was estimated by subtracting the oligomycin-insensitive values from the total values obtained. One unit of ATP synthase activity catalyzed the coupled oxidation of 1 μmol of NADH per minute. Addition of ADP to 100 μM was also used to test the sufficiency of pyruvate kinase and lactate dehydrogenase activities in the reactions.

**Determination of gastric mucosal ATP content.** Whole gastric tissue was homogenized with a Polytron tissue homogenizer in 10% (vol/vol) perchloric acid and then neutralized with potassium hydroxide. The homogenates were centrifuged at 14,000 rpm for 10 min at 4°C and diluted 20-fold in Tris-acetate-EDTA. The protein concentrations of the homogenates were determined by the bicinchoninic acid protein assay (Pierce Chemical) and diluted/normalized with Tris-acetate-EDTA to 1 μg/μl. One hundred microliters were assayed for ATP content in a 96-well plate using a luminescence-based assay kit (Invitrogen) according to the manufacturer’s instructions, with room temperature measurements read at 560 nm using a NOVOSTAR microplate reader (BMG Labtech, Cary, NC). A standard curve was generated from known ATP concentrations.

**Determination of gastric acid secretion.** Gastric acid secretion was determined from the method of Friis-Hansen et al. (11). Briefly, mice were fasted for 16 h with free access to water and then anesthetized with isoflurane while body temperature was maintained at 37°C by a heating pad. A midline abdominal incision was made, and a ligature was placed at the esophagogastric junction, with care taken not to injure the vagus nerve trunks. A flared segment of PE-50 tubing (0.58 mm ID, 0.965 mm OD) was placed into the stomach through the wall of the fundus along the greater curvature for infusion of normal saline at a rate of 0.2 ml/min using an infusion/withdraw syringe pump (model PHD2000, Harvard Apparatus, Holliston, MA). A segment of PE-240 tubing (1.67 mm ID, 2.42 mm OD) was placed via duodenotomy just proximal to the pylorus and secured in place with a 6-0 silk suture. After initial flushing with warm saline, samples were collected over 10-min intervals, and acid output (expressed as μmol H+ equivalents/10 min) was determined by titration. Where indicated, saline (control) or histamine (20 μg/g body wt; Sigma-Aldrich, St. Louis, MO) was injected subcutaneously.

**Superoxide anion formation.** Gastric tissue superoxide anion content was evaluated by the oxidation of dihydroethidium (DHE), which is a cell-permeable dye that, when oxidized by superoxide, is converted to the fluorescent products 2-hydroxyethidium and ethidium+ (48). Harvested gastric tissue was immediately frozen unfixed in optimum cutting temperature embedding compound (OCT, TissueTek), and cryostat sections were cut onto glass slides. The slides were subsequently thawed to room temperature for 30 min, and the tissue was incubated in PBS containing 2 mM DHE for 20 min at 37°C in a humidified incubator. The slides were then washed with room temperature PBS and analyzed with an epifluorescence microscope (Eclipse 50i, Nikon, Melville, NY) using a Chroma tetramethylrhodamine isothiocyanate filter set with excitation at 510–560 nm and emission at 570–650 nm, a range that detects primarily DNA-bound ethidium+ and not the 2-OH-ethidium product of superoxide and DHE (48). Images were captured using a DS-Q11 monochrome camera, and image intensity was analyzed using NIS-Elements imaging software. Average integrated intensities of three sections per stomach were averaged from six Cre−/SOD2fl/fl mice and six Cre+/SOD2fl/fl mice.

**Evaluation of gastric mucosal erosion (gastric injury).** Standardized specimens of fundic gastric mucosa were fixed in 10% buffered formalin and processed for histology. Extent of gastric injury was evaluated from thin mucosal sections (5 μm) stained with hematoxylin-eosin. To determine extent of injury, coded hematoxylin-eosin-stained sections of mucosal specimens harvested at 3 h following alcohol administration were evaluated under light microscopy by two investigators unaware of the codes. The extent of histological necrosis was quantified orthometrically with the aid of an ocular micrometer by measuring the length of mucosal strips and the total length of necrotic lesions for each strip. Data are expressed as percentage of total mucosal strip length (2).
Statistical analysis. Results are expressed as means ± SD. Student’s 2-tailed t-test was used to determine statistical significance between control and experimental groups. P < 0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with ANOVA followed by correction using Bonferroni’s multiple comparison test.

RESULTS

Gastric parietal cell-specific SOD2-deficient mice. The SOD2 floxed parental mice (17) and Atp4b-Cre parental mice (44) used to generate the gastric parietal cell-specific conditional SOD2-deficient mice are described elsewhere. Age- and sex-matched sibling mice of the genotype Atp4b-Cre+/−/SOD2fl/fl (control) or Atp4b-Cre+/−/SOD2fl/fl experimental mice were used in the studies. Deficiency of SOD2 protein expression in the gastric mucosa of experimental Cre+/−/SOD2fl/fl mice was confirmed by immunoblot analysis (Fig. 1C). Not all mice harboring the Atp4b-Cre allele, as determined by PCR genotyping, showed significantly reduced SOD2 protein expression compared with wild-type mice or SOD2 floxed littermates lacking the Atp4b-Cre allele. However, Cre protein expression correlated well with the reduction in gastric SOD2 protein expression (Fig. 1C). Therefore, subsequent comparative functional analyses were made using data only from Cre+/−/SOD2fl/fl control mice having confirmed “wild-type” levels of SOD2 protein expression and age/sex-matched sibling Cre+/−/SOD2fl/fl experimental mice having confirmed Cre expression and corresponding deficiency of gastric SOD2 protein expression.

We also confirmed SOD2 deficiency in the experimental Cre+/−/SOD2fl/fl mice and the extent to which SOD2 colocalized with parietal cells by immunofluorescence staining. As shown in Fig. 1D, the SOD2 signal was predominantly confined to parietal cells of Cre+/−/SOD2fl/fl control mice, whereas very little SOD2 signal was obtained for Cre+/−/SOD2fl/fl experimental mice that had confirmed deletion of the SOD2 floxed allele by PCR analysis and SOD2 protein deficiency by immunoblot analysis. Moreover, the SOD2 signal obtained for the Cre+/−/SOD2fl/fl experimental mice did not localize to parietal cells. Assessment of total gastric mucosal SOD activity levels showed a 20% (P < 0.05) reduction in total SOD (SOD1 + SOD2 + SOD3) activity in the Cre+/−/SOD2fl/fl experimental mice compared with wild-type and Cre+/−/SOD2fl/fl control mice (Fig. 1E). However, consistent with confirmed SOD2 protein deficiency, Cre+/−/SOD2fl/fl experimental mice had a >93% (P < 0.001) reduction in gastric mucosal SOD2 activity compared with wild-type and Cre+/−/SOD2fl/fl control mice (Fig. 1F).

Gastric parietal cell-specific SOD2-deficient mice have elevated mucosal superoxide levels and increased oxidative stress. Because SOD2 is responsible for the initial step in the clearance of superoxide that is generated as a by-product of mitochondrial oxidative phosphorylation needed to drive gastric parietal cell function, we examined relative superoxide anion levels in the gastric mucosa of the SOD2-deficient mice and their phenotypically wild-type littermates. Relative superoxide levels were determined by evaluating the extent of DHE conversion to ethidium+ using fluorescence microscopy (48). As shown in Fig. 2A, the superoxide anion level was increased 130% (P < 0.0001) in SOD2-deficient mice compared with Cre+/−/SOD2fl/fl control mice. A marked increase in oxidative stress to the gastric mucosa of SOD2-deficient mice was further demonstrated by increased tyrosine nitration (Fig. 2B) and lipid peroxidation (Fig. 2C). Tyrosine nitration, in particular, was very prominent within the parietal cells of the Cre+/−/SOD2fl/fl experimental mice.

Gastric parietal cell-specific SOD2-deficient mice have impaired mitochondrial aconitase and ATP synthase activities with a corresponding reduction in mucosal ATP content. We next examined the effect of gastric parietal cell SOD2 deficiency on mitochondrial dysfunction. Mitochondria were isolated from gastric tissue of wild-type, Cre+/−/SOD2fl/fl control, and Cre+/−/SOD2fl/fl experimental mice as assessed by marker enzyme enrichment (data not shown). Mitochondrial aconitase is highly susceptible to inactivation by superoxide formation as a result of oxidation of the [4Fe-4S]2+ cluster and posttranslational modification by tyrosine nitration and carbonylation (13, 12, 47). As shown in Fig. 3A, gastric mucosal mitochondrial aconitase activity was reduced 36% (P < 0.0001) in parietal cell SOD2-deficient mice compared with wild-type and Cre+/−/SOD2fl/fl control mice.

The increased susceptibility of mitochondrial aconitase, compared with cytosolic aconitase, to reversible inactivation by superoxide and/or irreversible inactivation by posttranslational modification has led to the possibility that mitochondrial aconitase acts as a sensor of the mitochondrial redox state in preventing ROS-induced apoptosis (3). In such a scenario, inactivation of aconitase would limit the production of NADH needed to fuel oxidative phosphorylation and, in doing so, would reduce the amount of superoxide that is generated as a consequence of the inherent leakiness of the electron transport chain. Nevertheless, this regulatory sensing process would be expected to result in reduced ATP production. We therefore examined ATP synthase activity and determined gastric mucosal ATP content. As shown in Fig. 3B, gastric mucosal mitochondrial ATP synthase activity was reduced by 44% (P < 0.005) in the SOD2-deficient mice. Moreover, as shown in Fig. 3C, gastric mucosal ATP content was significantly reduced by 34% (P < 0.002) in parietal cell SOD2-deficient mice compared with wild-type and Cre+/−/SOD2fl/fl control mice.

Gastric parietal cell-specific SOD2-deficient mice have impaired basal and stimulated gastric acid secretion. A primary function of gastric parietal cells is acid secretion via the H+−K+−ATPase complexes that translocate from basolateral tubulovesicles in the resting state to apical canalicular membranes, facilitating acid release into the glandular lumen following stimulatory signals from the acid secretagogues gastrin, histamine, and acetylcholine. We therefore examined basal and histaminergic acid secretion. As shown in Fig. 4, basal gastric acid secretion was reduced by 43% (P < 0.0001) in the parietal cell SOD2-deficient mice compared with Cre+/−/SOD2fl/fl control mice, while histamine-induced gastric acid output was reduced by 40% (P < 0.0005) in the parietal cell SOD2-deficient mice compared with Cre+/−/SOD2fl/fl control mice.

Gastric parietal cell-specific SOD2-deficient mice have increased apoptosis and enhanced susceptibility to mucosal injury. Oxidative stress and mitochondrial dysfunction result in increased gastric mucosal apoptosis and constitute major contributing factors to gastric mucosal injury induced by noxious stimuli such as alcohol and nonsteroidal anti-inflammatory drugs (32, 39). We therefore assessed the relative extent of apoptosis in the gastric mucosa of the parietal cell SOD2-deficient mice by TUNEL assay. As shown in Fig. 5A, the
Fig. 2. Gastric parietal cell SOD2-deficient mice have elevated superoxide anion levels, resulting in increased oxidative stress to gastric mucosa. A: superoxide anion levels determined by dihydroethidium (DHE) oxidation in unfixed frozen tissue sections. Left: representative section fields depicting relative DHE oxidation. Scale bars, 100 μm. Original magnification, ×200. Right: quantification of fluorescence intensity. Ethidium⁺ signal intensity was analyzed using NIS-Elements imaging software and is expressed as average units. Values are means ± SD; integrated intensities of 3 section fields/stomach were averaged from 6 mice/group. *P < 0.0001. B: tyrosine nitration visualized in formalin-fixed paraffin tissue sections using rabbit polyclonal antibody specific for nitrotyrosine. Sections were counterstained with fluorescein-conjugated DBA for visualization of parietal cells. Merged images depict extent of tyrosine nitration within parietal cells. Scale bars, 50 μm. Original magnification, ×400. C: lipid peroxidation visualized in formalin-fixed paraffin tissue sections using rabbit polyclonal antibody specific for malondialdehyde (MDA), a marker of lipid peroxidation. Sections were counterstained with fluorescein-conjugated DBA for visualization of parietal cells. Merged images depict extent of lipid peroxidation within parietal cells. Scale bars, 100 μm. Original magnification, ×200.
percentage of apoptotic cells increased fourfold ($P < 0.02$) in the gastric mucosa of the parietal cell SOD2-deficient mice compared with phenotypically wild-type littermate mice. Next, we examined the effect of SOD2 deficiency on a model of acute gastric injury induced by administration of a physiologically relevant concentration of ethanol approximating the upper limit found in distilled spirits consumed by humans [e.g., 50% (vol/vol)]. As shown in Fig. 5B, the extent of histologically assessed erosion, as a measurement of total tissue section length, was increased by 41% ($P < 0.001$) in the gastric parietal cell SOD2-deficient mice (31 ± 3%) compared with phenotypically wild-type littermate mice (22 ± 4%).

**DISCUSSION**

Reduced endogenous antioxidant function has been causally linked to tissue injury susceptibility as well as to several human diseases characterized by dysregulated inflammatory and immune responses (40). Because ROS play a central role in protection (e.g., phagocyte surveillance/eradication of microbial pathogens) and organ tissue injury (e.g., DNA damage, lipid peroxidation, and protein adduction), endogenous antioxidants possess a certain degree of redundancy that can confound dissection of the specific contribution/importance of any single antioxidant when studied using experimental models of diseases and/or injury.

In the present study, we therefore used the Cre-loxP system to examine the consequences of SOD2 deficiency on gastric parietal cell function in the absence of possible unknown contributing variables that might arise when more complex experimental models are utilized. Given that gastric parietal cells contain more mitochondria than any mammalian cell type other than left cardiac ventricular myocytes (15), it might seem intuitive that SOD2 deficiency would be detrimental to parietal cell function, if not parietal cell viability, the latter being a determinant of glandular hyperplasia (22). Nevertheless, the consequences of organ-specific sod2 gene deletion have been found to be quite variable. For example, liver-specific SOD2 deficiency was not associated with overt phenotypic abnormalities (17), although one study reported that hepatocyte-specific SOD2 deficiency correlated with the disruption of zonated glutamine synthase, glucokinase, and phosphoenolpyruvate carboxykinase gene expression in the liver (28). Conversely, heart muscle-specific SOD2 deficiency led to cardiomyopathy with cardiomyocyte mitochondrial respiratory defects, while brain-specific SOD2 deficiency resulted in a spongiform encephalopathy-like pathology that was associated with gliosis and led to death within 3 wk of birth (43).

Our present study demonstrates that gastric parietal cell-specific SOD2 deficiency results in 1) increased superoxide anion generation with increased lipid peroxidation and tyrosine nitration, indicating an enhanced state of oxidative stress, 2) reduced mitochondrial aconitase activity, 3) reduced ATP synthase activity with a concomitant reduction in gastric mucosal ATP content, 4) reduced basal and stimulated gastric acid secretion, and 5) increased gastric mucosal apoptosis. The
tyrosine nitration. In the present study, increased tyrosine nitration localized appreciably to parietal cells of the Cre+/SOD2fl/fl experimental mice. Mitochondrial enzymes, including SOD2, aconitase, and ATP synthase, are particularly susceptible to inactivation by tyrosine nitration (13, 14, 35). We observed a significant reduction in mitochondrial aconitase activity in the parietal cell SOD2-deficient mice, possibly as a direct result of tyrosine nitration (13) or modulation of the aconitase iron cluster (12). We observed an even greater reduction in mitochondrial ATP synthase activity in the Cre+/SOD2fl/fl experimental mice. Inactivation of mitochondrial aconitase would be expected to limit the production of NADH required to fuel oxidative phosphorylation and drive ATP synthase (3). Thus the reduction in mitochondrial ATP synthase activity in the parietal cell SOD2-deficient mice may have been an indirect result of aconitase inactivation, a direct result of ATP synthase inactivation, or a combination of both events, the resulting consequence being reduced mitochondrial ATP production. It is likely that parietal cell ATP production via mitochondrial oxidative phosphorylation was impaired to a greater extent in the SOD2-deficient mice than the reduction in total gastric tissue ATP content suggests. We were unable to obtain sufficient extract from parietal cell-enriched isolates to generate a statistically reliable parietal cell-specific ATP content assessment of the SOD2-deficient mice. However, the reductions in basal and histaminergenic gastric acid secretion of the SOD2-deficient mice were well in agreement with the extent to which mitochondrial ATP synthase was impaired. Gastric acid secretion is not only strictly dependent on ATP but is also almost completely shut down by inhibition of oxidative phosphorylation in response to chemical anoxia (5, 16). ATP depletion is a prominent feature underlying necrosis (51), so it is reasonable to infer that a compromised metabolic state would adversely impact the susceptibility to gastric mucosal injury. Moreover, ATP depletion and reduced mitochondrial ATP synthase expression have been implicated in gastric mucosal injury induced by exposure to concentrated alcohol (7, 39). By contrast, gastric mucosal restitution following injury appears to rely predominantly on glycolysis (5). This is likely to be true for the restitution of alcohol-induced gastric injury in which the tissue is rendered hypoxic as a result of ischemia due to microvascular damage.

Alcohol-induced gastric injury involves necrosis and apoptosis, which are also processes integral to normal gastric mucosal cell turnover, albeit in considerably less severity. In the present study, we found that apoptosis was increased fourfold in the gastric mucosa of the SOD2-deficient mice. Although this increase was significant, the increase in absolute percentage of apoptosis (<1%) was not as substantial as might have been expected given the increased oxidative state apparent in the gastric mucosa of the SOD2-deficient mice. A possible explanation for the relatively moderate increase in apoptosis is that, unlike necrosis, apoptosis is strictly energy-dependent (27). Our present study indicates a significant reduction in mitochondrial ATP output as a consequence of gastric SOD2 deficiency. It is possible that, despite the enhanced generation of superoxide and accompanying increased oxidative state and mitochondrial dysfunction observed in the gastric mucosa of the Cre+/SOD2fl/fl experimental mice, the extent of apoptosis was attenuated by an unmet energy requirement.
Mitochondrial dysfunction is increasingly being recognized as playing an important causal role in the pathogenesis of human diseases and tissue injury (31, 40). Within the gastric mucosa, mitochondrial dysfunction is a prominent feature of damage caused by nonsteroidal anti-inflammatory drugs and alcohol consumption (32, 39). Moreover, loss of mitochondrial function underlies the increased gastric mucosal apoptosis associated with Helicobacter pylori infection (4). Mitochondria, however, are the major intracellular source of ROS, and mitochondrial dysfunction is a contributing causal factor in increased oxidative stress and a severe consequence of increased oxidative stress (38). In addition to their roles in the gastric injury caused by the ingestion of noxious agents and by H. pylori infection, ROS also play a causal role in the gastropathy that results from portal hypertension as a result of impaired antioxidant function, including SOD (42). Studies including our own indicate that the increased oxidative state of the portal hypertensive gastric mucosa predisposes it to injury induced by noxious agents, including, notably, alcohol (25, 26).

In summary, our present study indicates that gastric mucosal SOD2 deficiency predisposes to injury as a consequence of bioenergetic impairment, mitochondrial dysfunction, and the resulting enhanced oxidative state. However, our study also raises the following question: Why does parietal cell-specific SOD2 deficiency, resulting in impaired parietal cell function, manifest such a broad increase in injury susceptibility encompassing the entire gastric mucosa? In the adult mouse, parietal cell number comprised ~13% of the total gastric mucosal cell population (23). Nevertheless, in portal hypertensive gastric mucosa, parietal cell loss and the resulting reduction in gastric acid secretion have been linked to increased injury susceptibility due to a decrease in acid-regulated expression of the cytoprotective factor heat shock protein 72, which was not limited to parietal cells but occurred in all cells (46). It is possible that the impaired acid output of the parietal cell SOD2-deficient mice may have contributed to the increased gastric injury susceptibility by an impaired induction of one or more acid-responsive factors(s) involved in cytoprotection. Moreover, dying necrotic cells have been shown to emit signals triggering the death of neighboring cells (41). It is possible that enhanced parietal cell necrosis of the SOD2-deficient mice adversely impacted injury to surrounding cells by the release of toxic and/or proinflammatory factors or other mediators of injury.

ACKNOWLEDGMENTS

The Atp4b-Cre transgenic mouse strain was generously provided by Dr. Jeffrey I. Gordon (Washington University School of Medicine, St. Louis, MO).

GRANTS

This study was supported by the Department of Veterans Affairs Biomedical Research and Development Service and by a Department of Veterans Affairs Merit Review Award (to M. K. Jones).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


