Indomethacin, a Non-steroidal Anti-inflammatory Drug, Develops Gastropathy by Inducing Reactive Oxygen Species-mediated Mitochondrial Pathology and Associated Apoptosis in Gastric Mucosa

A NOVEL ROLE OF MITOCHONDRIAL ACONITASE OXIDATION*

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We have investigated the role of mitochondria on the development of indomethacin (a non-steroidal anti-inflammatory drug)-induced gastric mucosal apoptosis and associated gastropathy in rat. Transmission electron microscopic studies indicate that indomethacin damages mitochondrial ultrastructure and causes mitochondrial dysfunction as evident from decreased stage-3 respiration, dehydrogenase activity, and transmembrane potential (Δψm). Mitochondrial pathology is associated with increased generation of intra-mitochondrial reactive oxygen species, such as O2·-, H2O2 and ·OH, leading to oxidative stress. O2·- is the most effective to damage mitochondrial aconitase, leading to the release of iron from its iron-sulfur cluster. The released iron, by interacting with intra-mitochondrial H2O2 forms ·OH. Immunoprecipitation of mitochondrial aconitase and subsequent Western immunoblotting indicate carbonylation of aconitase along with the loss of activity in vivo after indomethacin treatment. The release of iron has been documented by fluorescence imaging of mucosal cells by using Phen Green SK, a specific probe for chelatable iron. Interestingly, intra-mitochondrial ·OH generation is crucial for the development of mitochondrial pathology and activation of mitochondrial death pathway by indomethacin. Scavenging of ·OH by dimethyl sulfoxide or α-phenyl-n-tert-butylnitrone, a spin-trap, prevents indomethacin-mediated mitochondrial ultrastructural changes, oxidative stress, collapse of Δψm, and mitochondrial dysfunction. The scavengers also restore indomethacin-induced activation of caspase-9 and caspase-3 to block mitochondrial pathway of apoptosis and gastric mucosal damage. This study, thus, reveals the critical role of O2·-mediated mitochondrial aconitase inactivation to release intra-mitochondrial iron, which by generating ·OH promotes gastric mucosal cell apoptosis and gastropathy during indomethacin treatment.

Non-steroidal anti-inflammatory drugs (NSAIDs)2 are one of the most commonly prescribed drugs in the world to treat pain and inflammation (1). Everyday about 30 million people consume NSAIDs (1). These drugs are gaining enormous interest for cancer therapy as well (2, 3), as they are potent inducers of apoptosis and inhibitors of cell proliferation (4). However, long term use of NSAIDs is associated with severe gastropathy (1, 5) that may arise from induction of gastric mucosal cell apoptosis (6–9). NSAIDs induce apoptosis in vitro in varieties of cancer cells such as esophageal (10) and gastric adenocarcinoma cells (11), lung carcinoma cells (12), myeloid leukemia cells (13), and prostate carcinoma cells (3). NSAIDs also induce apoptosis in normal gastric mucosal cells (7, 9), hepatocytes (14), and chondrocytes (15). Up-regulation of proapoptotic Bax, Bak, and down-regulation of antiapoptotic Bcl-2, BclXL, are found to occur in NSAID-induced gastric mucosal apoptosis (7). Up-regulation of Bax as well as down-regulation of Bcl-2 are also observed in indomethacin-induced chronic myeloid leukemic cell apoptosis (13). Indomethacin in vitro induces apoptosis in gastric mucosal cell line through the release of cytochrome c and activation of Ca2+ signaling (6, 8). Studies claim that activation of mitochondrial death pathway contributes significantly in the apoptotic death of gastric mucosal cells by NSAIDs (6–8, 16, 17). The mitochondrial death pathway is initiated by the up-regulation of Bcl-2 family of proapoptotic proteins such as Bax, Bak, and/or down-regulation of antiapoptotic Bcl-2, BclXL (18) to induce activation and mitochondrial translocation of Bax, where it oligomerizes (18, 19) to open mitochondrial permeability transition pores in the mitochondrial membrane (20). Opening of mitochondrial permeability transition pores releases some apoptosis promoting factors, such as cytochrome c, Smac/DIABLO, apoptosis-inducing factor, and endonuclease G into the cytosol (18, 20, 21). The role of mitochondria in the regulation of cell death is now well established (18, 20). The generation of reactive oxygen species (ROS)

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‡ The abbreviations used are: NSAID, non-steroidal anti-inflammatory drugs; PBN, α-phenyl-n-tert-butylnitrone; RCR, Respiratory control ratio; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TEM, transmission electron microscopy; HBSS, Hanks’ balanced salt solution.
(22) and the release of proteins from the mitochondria lead to the activation of different pathways of cell death (18–20, 22). At present, it seems that a combination of proteins released from the mitochondria and maintenance of a sizable intracellular ATP pool are required for the execution of the suicide program and that mitochondrial protein release is associated with enhanced ROS production by this organelle (22). Mitochondria are not only a major source of ROS in aerobic cells but are also a sensitive target for the damaging effects of ROS. Increased ROS generated by mitochondria can cause oxidative damage of cellular macromolecules, including nucleic acids, lipids, and proteins along with depletion of cellular antioxidants, leading to cellular injury (22).

Many aspects of mitochondrial death pathway for the initiation of gastric mucosal apoptosis during NSAID-induced gastropathy have been evident (6–8, 16). It is now fairly established that the release of cytochrome c to the cytosol initiates the execution step of apoptosis (18–22). However, no studies have been reported yet on how cytochrome c, which is strongly associated with the cardiolipin, is released from the mitochondria during indomethacin-induced gastropathy and the role of intra-mitochondrial ROS thereon. In this study we have shown that indomethacin (NSAID) in vivo induces mitochondrial pathology by promoting mitochondrial oxidative stress through intra-mitochondrial iron mobilization from the iron-sulfur cluster of aconitase and the subsequent -OH generation to activate the mitochondrial pathway of apoptosis in gastric mucosal cells. Scavenging of -OH significantly prevents indomethacin-induced mitochondrial pathology, mitochondrial pathway of gastric mucosal apoptosis, and the associated gastropathy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Indomethacin, thioribarbituric acid, 5,5′-dithiobis(nitrobenzoic acid), reduced glutathione (GSH), DMSO, α-phenyl-n-tet-butylidinitro (PBN), oligomycin, ADP, collagenase, hyaluronidase, paraformaldehyde, glutaraldehyde, mитoисolation kit, caspase-3 assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit, trichloroacetic acid, and aconitase were purchased from Sigma. Rabbit IgG against aconitase was obtained from Abgent (San Diego, CA). Horseradish peroxidase-coupled anti-rabbit IgG was procured from Santa Cruz. ECL-based chemiluminescence kit was procured from GE Healthcare. JC-1 (5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarboxyanine iodide), MitoSOX, Mitotracker Red, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), and Phen Green SK TEM analysis (7). The animals were anesthetized with ketamine hydrochloride (12 mg kg−1) and perfused with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The gastric mucosal tissue from control and indomethacin-treated (at different ulcer index) and scavenger-pretreated were dissected out, washed with sodium phosphate buffer, and then cut into small pieces (1 mm3) followed by fixation in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 4 h at room temperature (24 °C). The tissues were then postfixed in 2% osmium tetroxide in 0.1M sodium phosphate buffer (pH 7.4) for 2 h at room temperature and dehydrated in an ascending grades of ethanol followed by embedding in Epon 812 and polymerized at 60 °C for 24 h. Ultra-thin sections (50–70 nm) were obtained using an Ultra cut Ultra-microtome (Leica Microsystems GmbH, Wetzlar, Germany) and picked up onto 200 mesh copper grids. The sections were double-stained with uranyl acetate and lead citrate and analyzed under a FEI Tecnai-12 Twin Transmission Electron Microscope equipped with a SIS Mega View II CCD camera at 80kV (FEI Co., Hillsboro, OR).

**Isolation of Mitochondria**—Mitochondria from gastric mucosal cells were isolated using commercially available kit (Sigma) (26, 27). In brief, gastric mucosa was scraped, suspended in mitochondria extraction buffer, and minced finely. This was homogenized in a Ultra-Turrax T-25 homogenizer. The homogenate was subjected to differential centrifugation first at 800 × g for 10 min to remove nuclei and unbroken cells and finally at 12,000 × g for 15 min to get the mitochondrial fraction. It was finally suspended in mitochondrial storage buffer in a ratio of 40 μl per 100 mg tissue initially taken.

**Assessment of Mitochondrial Function**—Mitochondrial functional status was analyzed in terms of mitochondrial oxygen consumption and mitochondrial dehydrogenase activity using an MTT reduction assay. Mitochondrial oxygen consumption was measured by using a Clark-type electrode in a Liquid-Phase Oxygen Measurement System (Oxygraph, Hansatech, Norfolk, UK) with a thermoderegulated chamber set at 24 °C (28). Oxygen
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consumption by complex I (Stage 3) was initiated by the addition of glutamate and malate (5 mM each) to 1 ml of respiratory medium (250 mM sucrose, 5 mM KH₂PO₄, 5 mM MgCl₂, 0.1 mM EDTA, 0.1% bovine serum albumin in 20 mM HEPES (pH 7.2)). The basal respiration (state 2) was recorded after the addition of mitochondria suspension (20 μg). State 3 respiration was initiated by the addition of 1 mM ADP to the respiration medium. State 4 respiration was initiated by the addition of 15 μM oligomycin, which blocks the ATP synthase of complex V. Respiratory control ratio (RCR) was calculated from the ratio of State 3 respiration (nmol of O₂ consumed) and State 4 respiration (nmol of O₂ consumed). Mitochondrial metabolic function was studied by observing the ability of mitochondrial dehydrogenases to reduce MTT into formazan dye. Equal amounts of mitochondrial protein (25 μg) from control or experimental groups was incubated with MTT (0.1% final concentration) for 3 h at 37 °C. After the incubation, the insoluble formazan dye was solubilized with MTT solubilization solution containing 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol. The absorbance of formazan dye developed as a result of MTT reduction was measured at 570 nm.

Measurement of Mitochondrial Transmembrane Potential (Δψₘ)—Mitochondrial transmembrane potential was measured as described earlier (26). In brief, mitochondria (20 μg) isolated from gastric mucosa of different groups of rats were incubated with JC-1 (300 nM) in the dark for 10 min at 37 °C in JC-1 assay buffer. The fluorescence of each sample was measured in a PerkinElmer Life Sciences LS50B spectrofluorometer (excitation, 490 nm; slit, 5 nm; emission, 530 nm for J-aggregate and 530 nm for J-monomer; slit, 7.2 nm).

Assay of Caspase-9 and Caspase-3 Activities—Caspase-9 activity was measured from cytosolic fraction of gastric mucosal homogenate obtained by subcellular fractionation using a commercially available kit and according to the manufacturer’s protocol (Biovision). Caspase-3 activity was measured from cytosolic fraction of gastric mucosa using a commercially available kit and according to manufacturer’s protocol (Sigma) as described earlier (26).

Measurement of Mitochondrial Oxidative Stress—Mitochondrial oxidative stress was measured in terms of GSH depletion, lipid peroxidation (23, 24), and protein carbonyl formation. Mitochondrial GSH content was measured as described (23). In brief, gastric mucosal scraping was homogenized using an Ultra-Turax T25 homogenizer to get the mitochondrial fraction using a commercially available mitochondria isolation kit (Sigma) as already described. The mitochondrial fraction was again sonicated in 20 mM ice-cold EDTA. Proteins present in the mitochondrial lysate were removed by trichloroacetic acid precipitation. The protein-free lysate (1 ml) was then added to 2 ml of 0.8 M Tris-Cl (pH 9) containing 20 mM EDTA. GSH content was finally determined by its reaction with 2,4-dinitrophenylbenzoic acid to yield the yellow chromophore of thionitrobenzoic acid, which was measured at 412 nm. GSH was used as standard (23). For mitochondrial lipid peroxidation, mitochondrial fraction (20 μg) was homogenized in ice-cold 0.9% saline. 1 ml of this homogenate was mixed with 2 ml of a thiobarbituric acid-trichloroacetic acid mixture (0.375% w/v, 15% w/v, respectively) in 0.25 N HCl followed by boiling for 15 min. The solution was then cooled, and after centrifugation the absorbance of the supernatant was read at 535 nm. Tetraethoxypropane was used as standard. Mitochondrial protein carbonyl content was measured using indirect enzyme-linked immunosorbent assay-based protocol (Cell Biolabs, San Diego, CA). Proteins were first adsorbed onto microtiter plate. The protein carbonyl present in the sample were first derivatized with 2,4-dinitrophenylhydrazine. The derivatized carbonyl-dinitrophenol was then measured with anti-dinitrophenol antibody followed by horseradish peroxidase-conjugated secondary antibody and o-phenyldiamine.

Isolation of Gastric Mucosal Cells—Gastric mucosal cells from control and indomethacin-treated rats were isolated as described earlier (29). Briefly, mucosa was scraped in HBSS containing 100 units/ml penicillin and 100 μg/ml streptomycin. The scraped mucosa was then resuspended and finely minced in HBSS containing 0.1% collagenase and 0.05% hyaluronidase. The suspension was incubated in the same solution for 30 min at 37 °C and filtered through a sterile nylon mesh. The filtrate was centrifuged at 500 × g for 5 min to get the cell pellet. The pellet was washed and suspended in HBSS for using in the following studies.

Detection of Intramitochondrial-reactive Oxygen Species—The isolated cells were used for detection of intramitochondrial ROS. Intramitochondrial ROS was measured using two specific probes, MitoSOX (to detect mitochondrial O²⁻) and DCF-DA (general ROS). Cells were stained with the respective fluorescent probe in HBSS (pH 7.4) and incubated for 15 min at 37 °C in the dark as described in the manufacturer’s protocols. Mitotracker Red was used to visualize mitochondria. After the incubation, cells were washed with HBSS three times and continued for fluorescence microscopy (Leica, DM-2500, Leica Microsystems). Staining of MitoSOX and Mitotracker Red was visualized using Red filter, and DCF-DA staining was visualized with a green filter. Hydroxyl radical (•OH) generated in the mitochondria of the gastric mucosa cells during indomethacin treatment was measured using DMSO as the •OH scavenger (30). Rats from both control and indomethacin-treated groups received 500 μl of 25% DMSO/100 g intraperitoneally, 30 min before indomethacin treatment. The animals were sacrificed after 4 h of indomethacin treatment, stomachs were dissected out, and mitochondria were purified using mitosisolation kit as described earlier (26, 27). The mitochondrial pellet was sonicated in ice-cold triple-distilled water and processed for the extraction of methanesulfonic acid formed by the reaction of •OH with DMSO. The extracted methanesulfonic acid was allowed to react with Fast Blue BB salt, and the intensity of the resulting yellow chromophore was measured at 425 nm using benzene-sulfonic acid as standard.

Measurement of Intramitochondrial Chelatable Iron—Intramitochondrial free (chelatable) iron was quantified using QuantiChrom™ Iron assay kit (Bioassay Systems). Mitochondria were lysed with lysis buffer, and protein-free lysate was used for estimation of free iron. Mitochondrial protein-free lysate (50 μl) was mixed with 200 μl of reaction mixture, provided with the kit, and incubated for 40 min at room temperature, and optical density was measured at 590 nm in a microtiter plate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
The concentration of free iron was measured from a standard curve using iron standard provided with the kit.

**Intramitochondrial Free Iron Localization**—Isolated mucosal cells from both control and indomethacin-treated rat stomach was used for free iron localization using Phen Green SK, an iron-sensitive fluorescence probe using the protocol as described in the product catalogue. Cells were first incubated with Mitotracker Red (250 nM) at 37 °C for 20 min followed by washing with HBSS and further incubation with Phen Green SK (20 μM) for 15 min at 37 °C in the dark. After the incubation, cells were washed with HBSS and used for fluorescence microscopy (Leica, DM-2500, Leica Microsystems).

**Detection of Protein Carbonyl in Mitochondrial Aconitase by Immunoprecipitation and Western Immunoblotting**—The mitochondria isolated from control and indomethacin-treated mucosa were lysed in RIPA radioimmune precipitation assay lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor mixture (Sigma), and the lysate was used for immunoprecipitation using protein A-Sepharose conjugated with rabbit antibody against aconitase. To 30 μl of 50% (v/v) protein-A antibody conjugate (in phosphate-buffered saline (pH 7.4)), 5 μl of rabbit polyclonal antibody against aconitase was added. This was allowed to incubate at 4 °C for 2 h to prepare protein-A-antibody conjugate. An equal amount of (500 μg protein) mitochondrial lysate was used for immunoprecipitation. The pellet containing protein-A-antibody-Antigen conjugate was mixed with gel loading buffer and incubated at 95 °C for 5 min and then centrifuged to collect supernatant. These immunoprecipitated samples were run in 12% SDS-PAGE and transferred to nitrocellulose membrane. Electroblotted membrane was processed for the detection of protein carbonyl using protein carbonyl immunoblot kit (Cell Biolabs). The membrane was then stripped by incubating in stripping buffer for 15 min at 37 °C and washed 3 times with Tris-buffer saline (pH 7.6) containing 0.5% Tween 20. This stripped membrane was then used for the detection of total aconitase using rabbit antibody against aconitase and goat anti-rabbit horseradish peroxidase-coupled secondary antibody to demonstrate actual protein (immunoprecipitated aconitase) loading in the gel.

**Measurement of Mitochondrial Aconitase Activity**—The activity of mitochondrial aconitase was assayed by determining the rate of formation of cis-aconitate from the substrate l-citrate (31). In brief, mitochondria (25 μg) were added to 1 ml of assay buffer containing 20 mM triethanolamine-HCl (pH 7.5) and 1.0 mM l-citrate. The changes in absorbance at 240 nm were recorded for 120 s by a Shimadzu UV-1700 UV-visible spectrophotometer. Aconitase activity was expressed as cis-aconitate formed/min/mg of mitochondrial protein (32).

**Effect of ROS on Purified Aconitase**—Purified mitochondrial aconitase (Sigma) was incubated with different ROS generating systems (such as the xanthine-xanthine oxidase system for O₂⁻ and the copper-ascorbate system for ·OH and H₂O₂) for 30 min at room temperature (24 °C) followed by the measurement of aconitase activity, formation of protein carbonyl, and measurement of free iron release as described earlier. Aconitase (30 μM) in 50 mM Tris buffer (pH 7.4) was incubated with 1 mM xanthine and 0.025 units/ml xanthine oxidase for 30 min at room temperature (33). Similarly, in another set, aconitase (30 μM) in 50 mM Tris buffer (pH 7.4) was incubated with 0.2 mM CuCl₂ and 1 mM L-cysteine at 37 °C for 30 min followed by measurement of protein carbonyl and mitochondrial aconitase activity as described earlier.
Table 1

Effect of Different Doses of Indomethacin on Mitochondrial Function and Apoptosis

Indomethacin was administered orally at single doses of 6, 12, 24, and 48 mg kg⁻¹ body weight to respective groups containing 6–8 rats in each group. Only vehicle was administered to the control group. Animals were sacrificed 4 h after indomethacin treatment to measure MTT reduction, ΔΨₘ, caspase-9, and caspase-3 activity as described under "Experimental Procedures." Data are presented as the mean ± S.E.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT Reduction (absorbance 570 nm)</th>
<th>ΔΨₘ Fluorescence (nmol/mg/h)</th>
<th>Caspase 9 Activity pNA Release (nmol/mg/h)</th>
<th>Caspase 3 Activity pNA Release (nmol/mg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.45 ± 0.05</td>
<td>250.4 ± 14.8</td>
<td>21.99 ± 1.8</td>
<td>3.45 ± 0.5</td>
</tr>
<tr>
<td>Control + indomethacin (6 mg kg⁻¹)</td>
<td>0.41 ± 0.04</td>
<td>240.1 ± 13.2</td>
<td>26.31 ± 2.1</td>
<td>4.01 ± 0.5</td>
</tr>
<tr>
<td>Control + indomethacin (12 mg kg⁻¹)</td>
<td>0.36 ± 0.03</td>
<td>202.4 ± 10.3</td>
<td>29.65 ± 2.0</td>
<td>4.66 ± 0.6</td>
</tr>
<tr>
<td>Control + indomethacin (24 mg kg⁻¹)</td>
<td>0.31 ± 0.03</td>
<td>179.5 ± 9.7</td>
<td>33.82 ± 3.2</td>
<td>8.66 ± 0.6</td>
</tr>
<tr>
<td>Control + indomethacin (48 mg kg⁻¹)</td>
<td>0.25 ± 0.03</td>
<td>85.6 ± 7.8</td>
<td>49.73 ± 4.8</td>
<td>24.73 ± 0.7</td>
</tr>
</tbody>
</table>

⁎ p < 0.02 versus control, n = 6–8.
⁎⁎ p < 0.01.

Results

Indomethacin induces mitochondrial pathology in gastric mucosal cells—Mitochondrial pathway from control gastric mucosal cells appeared intact with clearly visible double membrane and structurally intact and distinct cristae (Fig. 1). Indomethacin treatment disintegrated the double membrane and distorted the structure of cristae. The disintegration of outer membrane and distortion of cristae were more pronounced with the severity of mucosal damage as measured by ulcer index (Fig. 1).

The functional integrity of mitochondria was also investigated after indomethacin treatment by measuring mitochondrial respiratory activities, dehydrogenase activity, and ΔΨₘ. Mitochondria from indomethacin-treated gastric mucosa showed severe inhibition of complex-I-mediated state 3 (in the presence of ADP) respiration and mild inhibition of state 4 (ATP synthesis inhibited by oligomycin) respiration. As a consequence, the respiratory control ratio (RCR, the ratio of state 3 and state 4 respiration) was significantly decreased (Fig. 2A), and the decrement was increased with the increase of severity of the mucosal injury, indicating uncoupling of mitochondrial respiration with ATP synthesis. The RCR of mitochondria from indomethacin-induced injured stomach was significantly decreased along with the severity of mucosal damage (Fig. 2A). Another parameter for mitochondrial dysfunction is functional loss of mitochondrial dehydrogenases, which can be assessed by following the reduction of the tetrazolium salt, MTT. MTT reduction assay showed that indomethacin significantly decreased the activity of mitochondrial dehydrogenases, and this inhibition was correlated well with the severity of gastric mucosal damage, indicating mitochondrial dysfunction (Fig. 2B). The maintenance of mitochondrial membrane potential (ΔΨₘ) is vital for electron transport and ATP formation. Therefore, the status of ΔΨₘ was checked using a lipophilic-cationic dye JC-1, which accumulates within the mitochondria depending on mitochondrial membrane potential. The data indicate that indomethacin-induced gastric damage was associated with the collapse of ΔΨₘ (Fig. 2C). At higher membrane potential JC-1 molecules mainly exist as J-aggregates, emitting red fluorescence, and at lower potential JC-1 molecules mostly present as J-monomer, emitting green fluo-
A decrease in the ratio of red (J-aggregate, 590 nm) to green (J-monomer, 530 nm) JC-1 fluorescence indicated mitochondrial depolarization. Indomethacin also significantly decreased the red to green ratio, and this decrease was again correlated well with the severity of gastric injury (Fig. 2C, inset).

It is worth mentioning that indomethacin dose-dependently induces mitochondrial dysfunction and apoptosis (Table 1), although indomethacin at 6 mg kg\(^{-1}\) did not show any significant effect but at higher doses (12–48 mg kg\(^{-1}\)) caused significant mitochondrial dysfunction and apoptosis in gastric mucosa as evident from the MTT reduction assay, fall of \(\Delta \psi_{\text{mito}}\), and caspase-9 and caspase-3 activation (Table 1). Results, thus, clearly indicate that indomethacin causes mitochondrial pathology in vivo through structural and functional impairment.

**Indomethacin Stimulates Mitochondrial Oxidative Stress and the Generation of Intramitochondrial ROS—**To understand the actual cellular events leading to mitochondrial injury and associated pathology, studies were aimed at assessing the possible role of mitochondrial oxidative stress caused by the generation of ROS. Indomethacin treatment leads to the development of mitochondrial oxidative stress as indicated by increased depletion of mitochondrial GSH content (Fig. 3A), augmentation of mitochondrial lipid peroxidation, indicated by increased thiobarbituric acid-reactive species (Fig. 3B), and increased mitochondrial total protein carbonyl formation (Fig. 3C). In all cases mitochondrial oxidative stress is gradually increased with the increase in the severity of mucosal injury (ulcer index) induced by indomethacin. Mitochondria are a major source of ROS, which can induce mitochondrial oxidative stress (22, 34). Moreover, inhibition of mitochondrial respiration or uncoupling of oxidative phosphorylation by indomethacin may lead to the enhanced generation of ROS (35).

Interestingly, indomethacin stimulates the generation of intramitochondrial ROS as measured by MitoSOX Red, a mitochondrial-specific \(\text{O}_2^\cdot\) indicator (Fig. 4A) and DCF-DA (Fig. 4B). MitoSOX is a derivative of hydroethidium, accumulates within the mitochondria and upon oxidation by \(\text{O}_2^\cdot\), produces a red fluorescence. The intensity of red fluorescence is, thus, dependent on the level of \(\text{O}_2^\cdot\) generation. Mitochondria of mucosal cells

**FIGURE 4.** Indomethacin augments intramitochondrial ROS generation. A, mitochondrial generation of \(\text{O}_2^\cdot\) as detected by MitoSOX Red staining of gastric mucosal cells from control and indomethacin-treated (ulcer index (UI) 45) rats. B, mitochondrial generation of ROS. Gastric mucosal cells from control and indomethacin-treated (ulcer index 45) rats were stained with DCF-DA for ROS and Mitotracker Red for viable mitochondria. C, indomethacin stimulates mitochondrial generation of \(-\text{OH}\). The details of the methods were described under “Experimental Procedures.” Data were presented as the mean \(\pm\) S.E. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\) versus control, \(n = 6–8\).

**FIGURE 5.** Indomethacin increases mitochondrial accumulation of free iron. A, quantitation of free iron in mitochondria using quantichrom iron assay kit. Data were presented as the mean \(\pm\) S.E. B, release of intramitochondrial iron in gastric mucosal cells of control and indomethacin-treated (ulcer index (UI) 45) rats was detected by Phen Gree SK (PG SK) fluorescence. Mitochondria were stained with Mitotracker Red. The details of the methodology were described under “Experimental Procedures.” *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\) versus control, \(n = 6–8\).
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Oxidative Damage of Mitochondrial Aconitase Result in Intramitochondrial Iron Release—To investigate the source and mechanism of intramitochondrial iron release, we looked for the possible involvement of mitochondrial aconitase containing the iron-sulfur cluster.

Mitochondrial aconitase and O$_2^-$ play a major role in mitochondrial oxidative damage (37). Oxidative damage of protein is reflected by the formation of carbonyl within the protein structure (38). Immunoprecipitation of mitochondrial aconitase and subsequent Western immunoblotting using protein carbonyl antibody and aconitase antibody clearly indicate the increased protein carbonyl formation in aconitase after indomethacin treatment, and the carbonyl content increases with the severity of gastric damage (ulcer index) caused by indomethacin (Fig. 6, A and B). Western immunoblotting of mitochondrial aconitase using aconitase antibody indicates the loading of almost equal quantity of aconitase (identical band intensity) to measure protein carbonyl (Fig. 6). The oxidative damage also significantly inhibits aconitase activity, and the inhibition increases with the severity of gastric injury induced by indomethacin (Fig. 6C). To get an idea that reactive species of oxygen is responsible for oxidative damage, the purified porcine heart mitochondrial aconitase was incubated with ROS-generating systems and H$_2$O$_2$. It is evident that mitochondrial aconitase is more susceptible to inactivation and damage by O$_2^-$ when compared with H$_2$O$_2$ or -OH (Fig. 6D). The release of iron from the aconitase in the incubation medium was also higher when incubated with the O$_2^-$-generating system compared with H$_2$O$_2$ or the -OH-generating systems (Fig. 6D, inset).

The Critical Role of Intramitochondrial -OH for Indomethacin-induced Mitochondrial Pathology, Gastric Mucosal Apoptosis, and Gastropathy—Intramitochondrial iron release and subsequent formation of -OH may be critical for indomethacin-induced mitochondrial pathology, apoptosis, and gastropathy. To test this hypothesis, we used two probes such as DMSO, a scavenger, and PBN, a spin trap (radical scavenger), to see their effects on mitochondrial pathology and oxidative stress induced by indomethacin. Interestingly, both these ROS scavengers significantly blocked indomethacin-induced mitochondrial increased lipid peroxidation and GSH depletion (Fig. 7A). These scavengers also prevented indomethacin-induced functional impairment of mitochondria by restoring mitochondrial respiratory control ratio (Fig. 7B), dehydrogenase activity (Fig. 7C), and $\Delta$$\psi$$_m$ (Fig. 7D) close to controls, indicating the protective role of these scavengers in the prevention of indomethacin-induced mitochondrial pathological changes.

from control stomach did not show any significant MitoSOX Red fluorescence, but mitochondria of indomethacin-treated gastric mucosal cells showed high MitoSOX staining (Fig. 4A), indicating increased O$_2^-$ generation. The dismutation of O$_2^-$ (by superoxide dismutase) leads to increased generation of H$_2$O$_2$ inside the mitochondria. We further detected intramitochondrial ROS generation using DCF-DA by co-localization studies after staining the mitochondria of gastric mucosal cells with Mitotracker Red, which selectively labels mitochondria and DCF-DA, an indicator for ROS (Fig. 4B). Indomethacin also stimulated mitochondrial -OH generation as measured by methanesulfonic acid production after DMSO treatment, which was progressively increased with the severity of gastric damage (Fig. 4C). In biological systems, -OH is generated from H$_2$O$_2$ in reaction with transition metal ions such as iron by the Fenton reaction (36). Interestingly, indomethacin treatment was found to increase intra-mitochondrial-free iron content significantly over the control mitochondria, and the increase correlates well with the severity of the mucosal damage (Fig. 5A). Fluorescence microscopy was further conducted to visualize iron release within the mitochondria of isolated gastric mucosal cells using Phen Green SK, an iron-sensitive fluorescence probe. Results shown in Fig. 5B indicate that the fluorescence of Phen Green SK was more abundant in the mitochondria of mucosal cell from indomethacin-induced damaged mucosa, whereas the fluorescence of Phen Green SK was negligible in the mitochondria of control mucosal cells.
control levels. Furthermore, these scavengers also inhibited the induction of mitochondrial death pathway by preventing indomethacin-induced activation of both caspase-9 and caspase-3 (Fig. 7E), which were associated with significant block of indomethacin-induced mucosal injury (Fig. 7E). TEM studies clearly indicate that -OH/ROS scavengers prevented indomethacin-induced ultrastructural alteration of mitochondria by restoring abnormalities of cristae and outer membrane (Fig. 8). TEM studies further indicated that ROS scavengers also prevent indomethacin-induced mucosal cell apoptosis as evident from the restoration of nuclear structure and subcellular architecture (Fig. 9A). ROS scavengers also protect gastric mucosa from indomethacin-induced injury as evident from actual histological images of gastric mucosa (Fig. 9B). Indomethacin damages gastric mucosa by increasing the mucosal cell shading, and treatment with ROS scavengers restored mucosal integrity (Fig. 9B). Thus, it is suggested that indomethacin induces mitochondrial pathology by stimulating the generation of intramitochondrial -OH/ROS, which may play a pathogenic role through induction of oxidative damage of the mitochondria.

**DISCUSSION**

The present study shows that indomethacin, through increased intra-mitochondrial -OH/ROS generation, induces mitochondrial pathology that ultimately activates the mitochondrial pathway of apoptosis in gastric mucosa to develop gastropathy. This is the first report showing the critical role of mitochondrial aconitase in ROS generation and subsequent gastropathy.

The induction of mitochondrial pathology by indomethacin was evident from the ultrastructural and functional abnormalities of mitochondria. TEM studies clearly demonstrated the alteration of mitochondrial structural integrity as revealed by destruction of outer membrane and deformed cristae structure. The loss of functional integrity of the mitochondria was indicated by decreased RCR and inactivation of mitochondrial dehydrogenases. Although indomethacin dose-dependently induced mitochondrial dysfunction and apoptosis in gastric mucosa, indomethacin at a dose of 48 mg kg⁻¹ was used to induce gastric injury in rats as reported earlier (7, 24, 25). The doses (24 or 48 mg kg⁻¹) used to induce gastropathy in rats when extrapolated to human of 60 kg of body weight seem to be very high. However, the dose is not directly proportional to the body weight of any species including human (39). When the single effective dose of

![FIGURE 7. Effect of -OH-specific scavengers, such as DMSO and the spin trap PBN on indomethacin-induced mitochondrial oxidative stress, mitochondrial dysfunction, mitochondrial pathway of apoptosis, and gastric injury.](http://www.jbc.org/)

A, DMSO and PBN prevent mitochondrial GSH depletion and lipid peroxidation (LP) induced by indomethacin. B, DMSO (ulcer index (UI) 8–12) and PBN (ulcer index 7–10) prevent indomethacin-mediated (ulcer index 40–55) reduction of RCR of complex I. DMSO (ulcer index 8–12) and PBN (ulcer index 7–10) block indomethacin-induced (ulcer index 40–55) inhibition of dehydrogenase activity measured by MTT reduction (C) and collapse of ΔΨm (D). E, DMSO and PBN prevent indomethacin-induced activation of caspase-9 and caspase-3 and also gastric mucosal injury. Data were presented as the mean ± S.E. ***p < 0.001 versus control; #p < 0.05; ##p < 0.01; ###p < 0.001 versus indomethacin, n = 6–8).

![FIGURE 8. DMSO and PBN restore indomethacin-induced altered mitochondrial ultrastructure.](http://www.jbc.org/)

TEM studies indicate that DMSO and PBN protect mitochondrial structure from the damaging action of indomethacin as revealed by the intact outer membrane and clearly visible cristae.
indomethacin \((24 \text{ or } 48 \text{ mg kg}^{-1})\) in rat is converted to the human equivalent dose, as per the recommendation of United States Food and Drug Administration \((40, 41)\) based on body surface area and assuming 60 kg human body weight, it comes to 211 mg \((3.5 \text{ mg kg}^{-1})\) or 423 mg \((7.05 \text{ mg kg}^{-1})\). Indomethacin at a dose of 6 mg kg\(^{-1}\) did not seem to cause any mitochondrial pathology, but from 12 mg kg\(^{-1}\) in rat (equal to human equivalent dose of 1.76 mg kg\(^{-1}\) or 105 mg), it showed significant mitochondrial pathology and gastric mucosal apoptosis. For therapeutic purposes, indomethacin is used at a dose of 25 mg 2–3 times daily or 150–200 mg daily to treat inflammatory disorders \((42)\). Frequently, this dose of indomethacin is continued for several days for therapeutic control of chronic inflammatory disorders, which may result in an even higher cumulative effective dose to cause gastric damage \((43, 44)\). Therefore, the single effective dose of indomethacin in rat \((12 \text{ or } 24 \text{ or } 48 \text{ mg kg}^{-1})\) to develop mitochondrial pathology and gastric damage is in good agreement with the therapeutic doses of indomethacin in human.

The structural and functional impairment of the mitochondria (mitochondrial pathology) induced by indomethacin leads to mitochondrial oxidative stress associated with the generation of intramitochondrial ROS. Mitochondrial structure and functional loss is common for cell damage by oxidative stress \((45)\). How ROS is generated within the mitochondria is not clear yet. Inactivation of ROS scavenging systems by indomethacin \((25, 46)\) may be one of the possible causes \((47)\). Again, indomethacin induces ischemia in the mucosal region through suppression of prostaglandin synthesis and decreased blood flow \((46, 48)\). Under ischemic conditions the mitochondrial electron transport chain remains in a relatively reduced state, favoring leakage of electrons from some specific sites of the electron transport chain, mainly from complex I and complex III, leading to partial reduction of \(O_2\) to generate \(O_2^-\), which is dismutated to form \(H_2O_2\) by superoxide dismutase \((49, 50)\). More specifically, complex-I is considered as the major site of ROS generation within the mitochondria for the development of oxidative stress \((51)\). It is generally accepted that complex-I derived \(O_2^-\) is released into the matrix, whereas \(O_2^-\) derived from complex-III is released on both sides of the inner membrane \((52)\). Thus, complex-I derived \(O_2^-\) is the major contributor of matrix \(O_2^-\) pool. Moreover, NSAIDs are also known to act as uncoupler of oxidative phosphorylation \((35)\). The present study also shows that indomethacin significantly inhibits complex-I-mediated mitochondrial respiration, which may lead to the leakage of electron to the mitochondrial matrix to reduce \(O_2\) to \(O_2^-\). Moreover, ischemia also helps in the release of some redox-active metal ions, which can catalyze generation of \(\cdot OH\) from \(H_2O_2\), thereby causing oxidative damage of the molecules along with thiol depletion \((50, 53)\). Increased lipid peroxidation, thiol depletion, and elevated protein carbonyl formation observed after indomethacin treatment are the consequence of increased ROS generation. The level of \(\cdot OH\) is mainly regulated by the level of \(H_2O_2\) and its scavenging systems, such as peroxidases and catalase \((25)\). In the event of inactivation of scavenging systems, as seen after indomethacin treatment \((25, 46)\), \(H_2O_2\) accumulates within the mitochondria. Our studies indicate that ROS especially \(O_2^-\) can induce oxidative damage of mitochondrial aconitase, as revealed by the increase of its carbonyl content. A very less amount of protein carbonyl formation was evident in control mitochondria in vivo and purified aconitase.
in vitro. This is probably due to the autoxidation of aconitase at room temperature and subsequent generation of ROS (54). O$_2^-$-mediated oxidative damage of aconitase leads to the release of ferrous iron (Fe$^{2+}$) from the iron-sulfur cluster of the enzyme. This released free iron then reacts with accumulated H$_2$O$_2$ to increase endogenous -OH as shown by our studies. Site-specifically generated -OH can also damage aconitase. Aconitase can also be oxidatively damaged and inactivated by other reactive oxygen species, such as H$_2$O$_2$ (55). In many instances, generation of ROS causes damage of mitochondrial DNA leading to abnormal expression of mitochondrially encoded proteins for the electron transport chain (34). Thus, any alteration in mitochondrial gene expression can lead to impairment of mitochondrial respiration and other functions (56, 57). However, our studies on mitochondrial transcriptome analyses indicated that indomethacin-induced mitochondrial dysfunction was not due to alteration of mitochondrial gene expression (data not shown). Indomethacin-induced generation of mitochondrial ROS, especially the -OH, is critical for the induction of mitochondrial pathology, which in turn leads to the activation of mitochondrial pathway of apoptosis and gastropathy. Pretreatment with DMSO, a specific -OH scavenger (30), and PBN, which can trap all the reactive free radicals in vivo (58), significantly prevents indomethacin-induced mitochondrial oxidative stress, mitochondrial dysfunctions, and activation of mitochondrial pathway of apoptosis in gastric mucosa. Both DMSO and PBN prevent indomethacin-induced mitochondrial oxidative stress, as revealed from decreased lipid peroxidation and near normal restoration of GSH pool. These scavengers also restore the mitochondrial respiration, dehydrogenase activity, and $\Delta\psi_m$, indicating restoration of mitochondrial function. These scavengers also prevent activation of mitochondrial pathway of apoptosis as revealed from inhibition of caspase-9 and caspase-3 activities. Furthermore, these scavengers prevent indomethacin-induced mitochondrial structural defects (as shown by TEM studies) and gastric mucosal lesions (as revealed from ulcer index and histological studies). We conclude that indomethacin induces intramitochondrial ROS generation to promote mitochondrial pathology leading to activation of mitochondrial death pathway in gastric mucosa to develop gastropathy. Therefore, the designing of non-toxic mitochondrially targeted -OH/ROS scavenger will be a novel therapeutic strategy to prevent NSAID-induced mitochondrial pathology in gastric mucosal cells and associated gastropathy.

REFERENCES

NSAID Induces Mitochondrial Pathology

Indomethacin, a Non-steroidal Anti-inflammatory Drug, Develops Gastropathy by Inducing Reactive Oxygen Species-mediated Mitochondrial Pathology and Associated Apoptosis in Gastric Mucosa: A NOVEL ROLE OF MITOCHONDRIAL ACONITASE OXIDATION

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