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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Running title: Non-steroidal anti-inflammatory drug induces mitochondrial pathology

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Summary
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 ($\Delta \psi_m$). Mitochondrial pathology is associated with increased generation of intramitochondrial reactive oxygen species, such as $O_2^{\cdot-}$, $H_2O_2$ and $^\prime$OH leading to oxidative stress. $O_2^{\cdot-}$ 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 intramitochondrial $H_2O_2$ forms $^\prime$OH. Immunoprecipitation of mitochondrial aconitase and subsequent westernimmunoblotting indicate carboxylation of aconitase along with the loss of activity in vitro 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 $^\prime$OH generation is crucial for the development of mitochondrial pathology and 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 $O_2^{\cdot-}$-mediated mitochondrial aconitase inactivation to release intramitochondrial iron, which by generating $^\prime$OH promotes gastric mucosal cell apoptosis and gastropathy during indomethacin treatment.

INTRODUCTION
Non-steroidal anti-inflammatory drugs (NSAIDs) 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), since 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). Upregulation of proapoptotic Bax, Bak and downregulation of antiapoptotic Bcl-2, BclXL are found to occur in NSAID-induced gastric mucosal apoptosis (7). Upregulation of Bax as well as downregulation of Bcl-2 are also observed in indomethacin-induced chronic myeloid leukemic cell apoptosis (13). Indomethacin in vitro induces apoptosis in...
gastrointestinal mucosal cell line through the release of cytochrome c and activation of Ca\textsuperscript{2+} 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 upregulation of Bcl-2 family of proapoptotic proteins such as, Bax, Bak and/or downregulation 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 (MPTP) in the mitochondrial membrane (20). Opening of MPTP releases some apoptosis promoting factors, such as cytochrome c, Smac/DIABLO, AIF 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) (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 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 intramitochondrial ROS thereon. In this study, we have shown that indomethacin (NSAID) in vivo induces mitochondrial pathology by promoting mitochondrial oxidative stress through intramitochondrial iron mobilization from the iron-sulfur cluster of aconitase and the subsequent \( \cdot \)OH generation to activate the mitochondrial pathway of apoptosis in gastric mucosal cells. Scavenging of \( \cdot \)OH significantly prevents indomethacin-induced mitochondrial pathology, mitochondrial pathway of gastric mucosal apoptosis and associated gastropathy.

**EXPERIMENTAL PROCEDURES**

*Materials*—Indomethacin, thiobarbituric acid (TBA), 5,5'-dithiobis-nitrobenzoic acid (DTNB), reduced glutathione (GSH), dimethyl sulfoxide (DMSO), alpha-phenyl-n-tert-butyl-nitrone (PBN), oligomycin, ADP, collagenase, hyaluronidase, paraformaldehyde, glutaraldehyde, mitoiosolation kit, caspase-3 assay kit, MTT assay kit, trichloroacetic acid (TCA), aconitase were purchased from Sigma (St. Luis, MO, USA). Rabbit IgG against aconitase was obtained from Abgent (San Diego, CA, USA). Horseradish peroxidase-coupled anti-rabbit IgG was procured from Santa Cruz (CA, USA). ECL-based chemiluminescence kit was procured from GE Healthcare (Piscataway, NJ, USA). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidolcarbocyanine iodide), MitoSOX, Mitotracker Red, 2',7' dichlorodihydrofluorescein diacetate (DCF-DA) and phen green SK were purchased from Invitrogen (USA). Caspase-9 assay kit was obtained from Biovision (Biovision, Mountain View, CA, USA). Quantichrom Iron assay kit was procured from Bioassay systems (Hayward, CA, USA). All other reagents were of analytical grade purity.

*Animal Used*—Sprague-Dawley rats (180-220 gm) were used throughout the experiments. Each group (control or experimental or ROS scavenger-pretreated) of animals (n = 6-8) was maintained at 24 ± 2°C with 12 hrs light and dark cycles. The animals were fasted for 24 hrs with water *ad libitum* before the start of the experiments to avoid food-induced increased acid secretion and its aggravating effect on gastric lesions. All the in vivo studies were done in accordance with the guidelines of institute animal ethical committee.

*Indomethacin-induced Gastric Damage*—Indomethacin-induced acute gastric mucosal damage was performed as described earlier (23-25). Gastric ulcer was induced in the fasted animals (n = 6-8) with oral administration of indomethacin at the doses from 6-48 mg kg\textsuperscript{-1}
b.w. Control group received only vehicle instead of indomethacin. In the ROS scavenger-pretreated groups, the animals were administered with DMSO (500 μl of 25% dry DMSO) or PBN (200 mg kg⁻¹ b.w) intraperitoneally 30 min prior to ulcer induction with indomethacin. The optimum dose of each of the scavengers was selected from the dose response curve. After 4 hrs of indomethacin treatment, the animals were sacrificed under proper euthanasia and stomachs were dissected out. The mucosal injury was scored as ulcer index as described earlier: 0 = no pathology; 1 = one pinhead ulcer and 2-5 for thread-like lesions of 2-5 mm length (25). The sum of the total scores divided by the number of animals gave the ulcer index.

**Mitochondrial Morphology by Transmission Electron Microscopy (TEM)** - Mitochondrial morphology was detected by 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 hrs at room temperature (24°C). The tissues were then postfixed in 2% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.4) for 2 hr at room temperature and dehydrated in an ascending grades of ethanol, followed by embedding in Epon 812 and polymerized at 60°C for 24 hrs. 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 Company, Hillsboro, OR, USA).

**Isolation of Mitochondria** - Mitochondria from gastric mucosal cells were isolated using commercially available kit (Sigma) (26,27). In brief, gastric mucosa was scrapped, 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 12000 × 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 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 thermoregulated chamber set at 24°C (28). Oxygen 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% BSA 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 (nmoles of O₂ consumed) and State 4 respiration (nmoles of O₂ consumed). Mitochondrial metabolic function was studied by observing the ability of mitochondrial dehydrogenases to reduce MTT into formazan dye. Equal amount of mitochondrial protein (25 μg) from control or experimental groups was incubated with MTT (0.1% final concentration) solution for 3 hrs 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 dark for 10 min.
at 37°C in JC-1 assay buffer. The fluorescence of each sample was measured in a Perkin Elmer LS50B spectrofluorometer (excitation 490 nm, slit, 5 nm; emission 590 nm for J-aggregate, 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 commercially available kit and according to manufacturer's protocol (Biovision, Mountain View, CA, USA). Caspase-3 activity was measured from cytosolic fraction of gastric mucosa, using commercially available kit and according to manufacturer's protocol (Sigma, St. Louis, MO, USA) 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 Ultra-Turax T25 homogenizer to get mitochondrial fraction using commercially available mitochondria isolation kit (Sigma, St Louis, MO, USA) as already described. The mitochondrial fraction was again sonicated in 20 mM ice-cold EDTA. Proteins present in the mitochondrial lysate were removed by TCA 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-dithionitrobenzoic acid (DTNB) to yield the yellow chromophore of TNB (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 TBA-TCA 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 ELISA based protocol (Cell Biolabs, San Diego, CA, USA). Proteins were first adsorbed onto microtiter plate. The protein carbonyl present in the sample was first derivatized with 2,4-dinitrophenyl hydrazine. The derivatized carbonyl-dinitrophenol was then measured with anti-dinitrophenol antibody, followed by HRP-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 scrapped in HBSS containing 100 U/ml penicillin and 100 μg/ml streptomycin. The scrapped 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 (ROS)** - The isolated cells were used for detection of intramitochondrial ROS. Intramitochondrial ROS was measured using two specific probes, MitoSOX (to detect mitochondrial O$_2^•−$), 2',7' dichlorodihydrofluorescein diacetate (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 dark as described in the manufacturer's protocols. Mitotracker Red was used to visualize mitochondria. After the incubation, cells were washed with HBSS thrice and continued for fluorescence microscopy (Leica, DM-2500, Leica Microsystems GmbH, Wetzlar, Germany). Staining of MitoSOX and Miotracker Red was visualized using Red filter and DCFDA staining was visualized with Green filter. Hydroxyl radical (•OH) generated in the mitochondria of the gastric mucosa cells during indomethacin-treatment was measured using dimethyl sulfoxide (DMSO) as •OH scavenger (30). Rats from both control and indomethacin-treated groups received 500 µl of 25% DMSO/100 gm intraperitoneally, 30 min prior to indomethacin treatment. The animals were sacrificed after 4 hrs of indomethacin treatment, stomach was dissected out and mitochondria were purified using mitoisolation 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, Hayward, CA, USA). 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, 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, USA). 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 (PG 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 dark. After the incubation, cells were washed with HBSS and used for fluorescence microscopy (Leica, DM-2500, Leica Microsystems GmbH, Wetzlar, Germany).

Detection of Protein Carbonyl in Mitochondrial Aconitase by Immunoprecipitation and Westernimmunoblotting- The mitochondria isolated from control and indomethacin-treated mucosa were lysed in RIPA lysis buffer (50 mM Tris-Cl pH 7.4. 150 mM NaCl. 1% NP40. 0.25% Na-deoxycholate. 0.1% SDS) supplemented with protease inhibitor cocktail (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 slurry (in PBS, pH 7.4), 5 μl of rabbit polyclonal antibody against aconitase was added. This was allowed to incubate at 4°C for 2 hrs to prepare Protein-A-antibody conjugate. 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 were transferred to nitrocellulose membrane. Electroblickt membrane was processed for the detection of protein carbonyl using protein carbonyl immunoblot kit (Cell Biolabs, San Diego, CA, USA). The membrane was then stripped by incubating in striping buffer for 15 min at 37°C and washed three times with Tris-buffer saline (TBS, 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 HRP-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 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 sec by a Shimadzu UV-1700 UV-Vis spectrophotometer. Aconitase activity was expressed as cis-aconitate formed per minute per mg mitochondrial protein (32).

Effect of ROS on Purified Aconitase- Purified mitochondrial aconitase (Sigma) was incubated with different ROS generating systems (such as, xanthine-xanthine oxidase system for O₂•, 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 U/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 ascorbate for 30 min at room temperature (23) and in another set same amount of aconitase was incubated with 1 mM H₂O₂ under the same condition. Control consisting of aconitase (30 μM) in 50 mM Tris buffer (pH 7.4) was incubated at room temperature for 30 min. The iron released due to
aconitase inactivation was measured with the help of Quantichrom Iron Assay Kit (Bioassay Systems, Hayward, CA, USA). In brief, 50 μl of protein free reaction medium was mixed with 200 μl of reaction mixture and proceeded as described in the product manual.

Gastric Mucosal Histology- Stomachs after dissecting out from the animals (control and indomethacin-treated) were washed in PBS (pH 7.4), cut into small pieces and fixed in 10% neutral buffered formalin. The fixed tissues were dehydrated and finely embedded in paraffin. The embedded tissue was cut using a rotary microtome (5 micron), placed over a poly-L-lysine coated glass slide and stained with haematoxylin-eosin and examined under microscope (Leica, DM-2500, Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis- All the data were presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by multiple comparison t-tests for evaluation of differences between groups. P value \( \leq 0.05 \) was considered as significant.

RESULTS

Indomethacin Induces Mitochondrial Pathology in Gastric Mucosal Cells- Mitochondrial pathology is originated from the structural and functional abnormalities of the mitochondria. The alteration of the ultrastructural morphology of mitochondria was examined by transmission electron microscopic (TEM) studies of control and indomethacin-treated gastric mucosa. Mitochondria 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 \( \Delta \psi_m \). Mitochondria from indomethacin–treated gastric mucosa showed severe inhibition of complex-I mediated state 3 (in 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 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, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 (\( \Delta \psi_m \)) is vital for electron transport and ATP formation. Therefore, the status of \( \Delta \psi_m \) 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 \( \Delta \psi_m \) (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 fluorescence. 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 MTT reduction assay, fall of \( \Delta \psi_m \), 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 Reactive Oxygen Species (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 TBA-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 major source of reactive oxygen species (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 intra-mitochondrial ROS as measured by MitoSOX Red, a mitochondrial specific O$_2^•$ indicator (Fig. 4A) and DCF-DA (Fig. 4B). MitoSOX is a derivative of hydroethydium, accumulates within the mitochondria and upon oxidation by O$_2^•$, produces a red fluorescence. The intensity of red fluorescence is thus dependent on the level of O$_2^•$ generation. Mitochondria of mucosal cells 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 system, *OH is generated from H$_2$O$_2$ in reaction with transition metal ions such as iron by 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 cell using Phen Green SK (PG SK), an iron sensitive fluorescence probe. Results shown in Fig 5B indicate that the fluorescence of PG SK was more abundant in the mitochondria of mucosal cell from indomethacin-induced damaged mucosa, while the fluorescence of PG SK was negligible in the mitochondria of control mucosal cells.

Oxidative Damage of Mitochondrial Aconitase result in Intramitochondrial Iron Release - In order to investigate the source and mechanism of intramitochondrial iron release, the possible involvement of mitochondrial aconitase containing the iron-sulfur cluster was looked for. 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. 6A, 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). In order to get an idea which 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 O$_2^*$-generating system, compared to H$_2$O$_2$ or •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 specific •OH 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 ∆ψm (Fig. 7D) close to control level. 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 shedding 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 rat as reported earlier (7,24,25). The dose used in rats (24 mg kg$^{-1}$ or 48 mg kg$^{-1}$) when extrapolated to human of 60 kg body weight to show gastropathy seems 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 indomethacin (24 mg kg$^{-1}$ or 48 mg kg$^{-1}$) in rat is converted to human equivalent dose (HED) as per the recommendation of U.S. Food and Drug Administration, (40,41) based on body surface area and assuming 60 kg human body weight, it will come to 211 mg (3.5 mg kg$^{-1}$) or 423 mg (7.05 mg kg$^{-1}$). Although 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 HED of 1.76 mg kg$^{-1}$ or 105 mg), it showed significant mitochondrial pathology and gastric mucosal apoptosis. For therapeutic purpose, 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 even higher cumulative effective dose to cause gastric damage (43, 44). Therefore, the single effective dose of indomethacin in rat (12 mg kg$^{-1}$ or 24 mg kg$^{-1}$ or 48 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 to develop oxidative stress in the gastric mucosa after indomethacin treatment 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). In ischemic condition, 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 the complex I and complex III leading to partial reduction of O2 to generate O2•−, which is dismutated to form H2O2 by SOD (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 O2•− is released into the matrix, while O2•− derived from complex-III is released on both sides of the inner membrane (52). Thus complex-I derived O2•− is the major contributor of matrix O2•− 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 O2 to O2•−. Moreover, ischemia also helps in the release of some redox-active metal ions, which can catalyze generation of *OH from H2O2 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 *OH is mainly regulated by the level of H2O2 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), H2O2 accumulates within the mitochondria. Our studies indicate that ROS especially O2•− can induce oxidative damage of mitochondrial aconitase, as revealed by the increase of its carbonyl content. 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 autooxidation of aconitase at room temperature and subsequent generation of ROS (54). O2•−-mediated oxidative damage of aconitase leads to the release of ferrous iron (Fe2+) from the iron-sulfur cluster of the enzyme. This released free iron then reacts with accumulated H2O2 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 H2O2 (55). In many instances, generation of ROS causes damage of mitochondrial DNA leading to abnormal expression of mitochondrial 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 that 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 Δψm, 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 nontoxic mitochondrially targeted •OH/ROS scavenger will be a novel therapeutic strategy to prevent NSAID-induced mitochondrial pathology in gastric mucosal cells and associated gastropathy.

ACKNOWLEDGEMENTS

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REFERENCES


LEGENDS FOR FIGURES

FIGURE 1. Indomethacin causes structural distortion of mitochondria. Mitochondria from control stomach show well-defined cristae and intact membranes. Mitochondria from mild ulcerated stomach (ulcer index 10-20) show distortion of cristae and some parts of outer membrane get disintegrated. Mitochondria from moderately ulcerated stomach (ulcer index 25-35) show more disintegration of cristae and outer membrane. Mitochondria from severely ulcerated stomach (ulcer index 40-55) exhibit complete disintegration of cristae. (bar = 200 nm, UI = ulcer index). Single mitochondrion presented in the figure is the representative of maximum appeared type of mitochondria found in the ultrathin section.

FIGURE 2. Indomethacin impairs mitochondrial functions (A) Respiratory control ratio (RCR) of mitochondrial oxygen consumption mediated by complex I at different ulcer index. (B) Status of the mitochondrial dehydrogenases as measured by MTT reduction at varied ulcer index. (C) Mitochondrial membrane potential (Δψm) as measured by fluorescence intensity following JC-1 uptake. Spectrum ‘a’ represents the fluorescence of Δψm of control mitochondria, ‘b’ represents the same from of mitochondria obtained from mucosa of ulcer index 10-20, ‘c’ represents the same from ulcer index 25-35 and ‘d’ with ulcer index 45-55. Inset shows the ratio of 590 nm (red, J-aggregate)/530nm (green, J-monomer) of JC-1 fluorescence at different ulcer index. Data were presented as mean ± SEM. (*P<0.05, **P<0.02, ***P<0.01, ****P<0.001 vs control, n = 6-8).

FIGURE 3. Indomethacin induces mitochondrial oxidative stress in gastric mucosa. (A) Mitochondrial GSH level, (B) mitochondrial lipid peroxidation as measured by quantification of thiobarbituric acid reactive substances (TBARS) and (C) protein carbonyl content at different ulcer index. The details of the methods were described under “EXPERIMENTAL PROCEDURES”. Data were presented as mean ± SEM. (**P<0.01, ***P<0.001 vs control, n = 6-8).

FIGURE 4. Indomethacin augments intramitochondrial ROS generation. (A) Mitochondrial generation of O2•− as detected by MitoSOX Red staining of gastric mucosal cells from control and indomethacin-treated (ulcer index 45) rats. (B) Mitochondrial generation of ROS. Gastric mucosal cells from control and indomethacin treated (ulcer index 45) rats were stained with DCFDA for ROS and Mitotracker Red for viable mitochondria. (C) Indomethacin stimulates mitochondrial generation of •OH. The details of the methods were described under “EXPERIMENTAL PROCEDURES”. Data were presented as mean ± SEM. [UI= ulcer index] (*P<0.05, **P<0.01, ***P<0.001 vs 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 mean ± SEM. (B) Release of intramitochondrial iron in gastric mucosal cells of control and indomethacin-treated (ulcer index 45) rats was detected by Phen Green SK (PG SK) fluorescence. Mitochondria were stained with Mitotracker Red. The details of the methodology were described under “EXPERIMENTAL PROCEDURES”. [UI= ulcer index] (*P<0.05, **P<0.01, ***P<0.001 vs control, n = 6-8).

FIGURE 6. Inactivation of mitochondrial aconitase (A) Western immunoblot of mitochondrial aconitase protein carbonyl and mitochondrial aconitase in control and after indomethacin treatment at different ulcer index. The gel is the representative of three different experiments. (B) Densitometric analysis of protein carbonyl formation in mitochondrial aconitase. The densitometric value of each aconitase protein carbonyl (upper gel) was corrected with respective protein loading (aconitase, lower gel) (C) Inhibition of mitochondrial aconitase activity during indomethacin-induced gastric damage. (D) Inactivation of the purified mitochondrial aconitase by different ROS. Purified mitochondrial aconitase was incubated with different ROS generating systems (such as, xanthine-xanthine oxidase system for O2•−, copper-ascorbate system for •OH and H2O2) for 30 min at room temperature followed by measurement of aconitase activity, formation of carbonyl and measurement of iron release (inset).
The details of the methods were described under “EXPERIMENTAL PROCEDURES”. Data were presented as mean ± SEM. (*P<0.05, **P<0.01, ***P<0.001 vs control, n = 6-8).

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. (A) DMSO and PBN prevent mitochondrial GSH depletion and lipid peroxidation (LP) induced by indomethacin. (B) DMSO (ulcer index 8-12) and PBN (ulcer index 7-10) prevent indomethacin-mediated (ulcer index 40-55) reduction of respiratory control ratio (RCR) of complex I, (C) 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 and (D) collapse of Δψm. (E) DMSO and PBN prevent indomethacin-induced activation of caspase-9 and caspase-3 and also gastric mucosal injury. Data were presented as mean ± SEM. [UI= ulcer index] (**P<0.001, vs control, *P<0.02, ##P<0.01, ###P<0.001 vs indomethacin, n = 6-8).

FIGURE 8. DMSO and PBN restore indomethacin-induced altered mitochondrial ultrastructure. 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.

FIGURE 9. DMSO and PBN prevent indomethacin (NSAID)-induced gastric mucosal apoptosis and gastric mucosal damage (A) Ultrastructural analysis of gastric mucosal cells using TEM. The morphology of control cell is well-defined with intact nucleus and cell membrane (CM). Chromatins are well characterized and nucleolus distinctly visible within the nucleus. Large numbers of mitochondria in their characteristic architecture are also visible. On the other hand distorted nuclei with condensed chromatin are visible in cells from indomethacin induced damaged gastric mucosa. Nucleoli are not clearly distinguished. Cytoplasmic condensation and decrease in the number of intact mitochondria are observed. Cell membrane is damaged with the appearance of blebbing. Pretreatment of DMSO and PBN significantly protected indomethacin–induced alteration of cellular architecture. [N= Nucleus, M= Mitochondria, CM= Cell membrane, CC= Cytoplasmic condensation, ChC= Chromatin condensation] (B) Haematoxylin-eosin staining of gastric mucosal sections. Sections were observed at 20X under a microscope (Leica DM-2500,Leica Microsystems GmbH, Wetzlar, Germany) equipped with high-resolution digital camera. Arrow indicates the damages in the mucosa. The details of the methodology were described under “EXPERIMENTAL PROCEDURES”.
**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\(^{-1}\) b.w to respective group containing 6-8 rats in each group. Only vehicle was administered to the control group. Animal were sacrificed 4 hours after indomethacin treatment to measure MTT reduction, \(\Delta \psi_m\), caspase-9 and caspase-3 activation as described under ‘EXPERIMENTAL PROCEDURES’.

<table>
<thead>
<tr>
<th></th>
<th>MTT reduction (Absorbance 570 nm)</th>
<th>(\Delta \psi_m) Fluorescence unit</th>
<th>Caspase 9 activity pNA release (nmol/mg/hr)</th>
<th>Caspase 3 activity pNA release (nmol/mg/hr)</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 ± 10.2**</td>
<td>29.65 ± 2.6**</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>

Data were presented as mean ± SEM

\(^*\)p < 0.01, \(^{**}\)p < 0.02 versus control, n = 6-8
Figure 1

CONTROL

INDOMETHACIN (Ulcer Index 10-20)

INDOMETHACIN (Ulcer Index 25-35)

INDOMETHACIN (Ulcer Index 40-55)
Figure 3

A

GSH (nmol/mg)

0 10-20 25-35 40-55

Ulcer index

B

Lipid peroxidation (TBARS, nmol/mg)

0 10-20 25-35 40-55

Ulcer index

C

Total protein carbonyl (Absorbance, 450 nm)

0 10-20 25-35 40-55

Ulcer index
Figure 4

A

MitoSOX  Phase  Merged

Control

Indomethacin (1 uM)

B

DCF-DA  MitoTracker Red  Merged

Control

Indomethacin (1 uM)

C

Mitochondrial OH (nmol/mg)

Ulcer index

0  10-20  25-35  40-55

*  **  ***

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Figure 5

A

Mitochondrial free iron (nmol/mg)

Ulcer index

0 10-20 25-35 40-55

B

Control

Indomethacin (U 45)

PG SK MitoTracker Red Merged

* ** ***
Figure 8

Control  Indomethacin (Uleer Index 40-55)  DMSO+Indomethacin (Uleer index 8-12)  PBN+Indomethacin (Uleer index 7-10)
Figure 9

A  Control  Indomethacin (Ulcer Index 40-55)  DMSO+Indomethacin (Ulcer index 8-12)  PBN+Indomethacin (Ulcer index 7-10)

B
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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