Involvement of Subcellular Organelles in Inflammatory Pain-Induced Oxidative Stress and Apoptosis in the Rat Hepatocytes

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Background: Subcutaneous injection of formalin in the plantar surface of rat’s hind paw is frequently used as an animal model to study pain associated with inflammation. The pain produced by formalin test differs significantly from that of acute nociceptive tests. In this study, we first investigated the cellular and molecular mechanisms responsible for chronic inflammatory pain-induced damage in the rat hepatocytes and finally we tried to figure out whether both selective (celecoxib) and nonselective (acetylsalicylic acid) cyclo-oxygenase inhibitors could protect hepatocytes against pain-induced damage.

Methods: The male Wistar rats were divided in one-, four-, and seven-day pain groups. Twenty-four hours prior to pain induction with subcutaneous injection of 5% formalin into the hind paw, acetylsalicylic acid or celecoxib was administered to the animals of similar one-, four-, and seven-day pain groups. A no-pain (control) group was also considered for each of the experiments.

Results: Our results showed a significant rise in both formation of reactive oxygen species and collapse in the mitochondrial membrane potential (%∆Ψm) in all pain groups (P<0.05). Significant lysosomal membrane damage and hepatocyte proteolysis were only seen in one-and four-day pain groups (P<0.05). Caspase 3 activity also showed a significant (P<0.05) rise in all three pain groups.

Conclusion: Formation of reactive oxygen species and mitochondrial/lysosomal damages were significantly inhibited by both acetylsalicylic acid and celecoxib in hepatocytes of all pain-suffering animals. Nonetheless, celecoxib’s tendency to raise caspase 3 activities, suggested that it accelerates the apoptosis in hepatocytes of pain-suffering animals. Our results showed that the pain per se, could initiate some harmful signals that affect other cells other than neurons; these malicious signals could be magnified by use of some analgesics particularly selective cyclo-oxygenase inhibitors.

Keywords: Hepatocyte • inflammation • lysosome • mitochondria • pain

Introduction

Subcutaneous (SC) injection of formalin in the plantar surface of rat’s hind paw is frequently used as an animal model to study pain associated with inflammation.1,2 The pain produced by formalin test differs significantly from that of acute nociceptive tests. Formalin creates a tonic pain secondary to tissue injury, inflammation, and central sensitization and is thought to resemble clinical pain due to its tonic nature.1,3 SC injection of this chemical irritant activates peripheral nerves, leading to activation of dorsal horn neurons and induces rapid and prolonged hyperalgesia.4

Previous studies have provided association between inflammatory pain states (carageenan/kaolin or formalin-induced) and release of spinal mediators, especially excitatory amino acids (EAAs), substance P, nitric oxide (NO), and cyclo-oxygenase (COX) products that are crucial causes of neural transmission and hyperexcitability, central sensitization, hyperalgesia, and allodynia.4,5

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Following subcutaneous formalin injection and tissue damage, inflammatory cells (e.g., macrophages) are recruited; there are upregulated production and release of a broad range of neuroactive compounds such as protons, serotonin (5HT), histamine, adenosine, bradykinin, prostaglandin E2 (PGE2), NO, interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nerve growth factor (NGF), and reactive oxygen species (ROS) by inflammatory immune cells which could also be distributed via the circulation and cause damage to the sensitive organs.6

TNF-α induces oxidative stress by the generation of ROS via leakage from the mitochondrial electron transport chain and therefore depletes glutathione (GSH) in human body.7 ROS can regulate the activities of several kinases, transcription factors, cell death machinery, and proteins such as COX-2 and inducible macrophage-type nitric oxide synthase (iNOS). On the other hand, they are important mediators of programmed cell death induced by TNF-α.8 In inflammatory pain states, we have two major sources for ROS production—immune cells and cytokines.

All of the substances that are released during inflammatory processes such as cytokines, EAAs, and substance P, have been shown to be associated with enhanced production of NO. IL-1 seems not only to enhance iNOS synthesis, but it also enhances the transport of arginine, the precursor of NO, particularly into the white blood cells and hepatocytes.9,10 Studies show that NO may be a physiologic regulator of mitochondrial respiration. Upon exposure to oxidizing species, mitochondria may undergo a permeability transition. Mitochondrial permeability transition (MPT) pore opening leads to the release of mitochondrial cytochrome c, which acts as a proapoptotic signal. MPT pore opening has been reported upon exposure of liver mitochondria to ROS and ONOO−.11

Fresh hepatocytes were the cells we preferred for our study because the largest mass of macrophages in the body, the Kupffer cells, are present in the liver and could easily be influenced by the inflammatory soup and spinal mediators released into the circulation by both macrophages and spinal glial cells.12 Activation of Kupffer cells could lead to massive release of H2O2 and pro-inflammatory cytokines which could consequently cause a serious damage to the neighboring hepatocytes.

Hepatocytes can express and release inflammatory mediators after challenge with pro-inflammatory cytokines in the circulation and also those released by the Kupffer cells during the inflammatory pain. Nitric oxide synthase-2 (NOS-2) is expressed under these conditions and the high NO synthesis that follows, contributes to the inflammatory response in the liver tissue and can be a cause for several hepatopathies.13 In this study, we used the accelerated cytotoxic mechanism screening (ACMS) technique to assess the cellular pathologic alterations and biochemical changes associated with inflammatory/tonic pain induction in hepatocytes. We also investigated the possibility of protection of hepatocytes against chronic inflammatory pain-induced cellular damage by preadministration of both selective and non-selective COX inhibitors to the pain suffering groups.

**Materials and Methods**

**Chemicals**

Rhodamine 123, collagenase, bovine serum albumin (BSA), N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), o-phthalaldehyde (OPT), N-ethylmaleimide (NEM), oxidized and reduced glutathione (GSSG and GSH), caspase 3 assay kit (CASP-3-C), and apoptosis detection kit Annexin V-CY3 were obtained from Sigma-Aldrich Co. (Taufkrichen, Germany). Acridine orange (AO), 2’,7’-dichlorofluorescein (DCFH) diacetate, trichloroacetic acid, and Trypan blue were purchased from Merck Co. (Darmestadt, Germany). Heparin was purchased from Rotexmedica, Germany. All other chemicals were of the highest commercial grade available.

**Animals**

Male Wistar rats weighing 200 to 300 g were used in the study. All rats were housed in a room at a constant temperature of 25°C on a 12/12 hr light/dark cycle with food and water available *ad libitum*. All experiments were conducted according to ethical standards and protocols approved by the Committee of Animal Experimentation of Shaheed Beheshti University of Medical Sciences, Tehran, Iran. The ethical standards were based on “European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes” Acts of 1986, and the “Guiding Principles in the Use of Animals in
Toxicology,” adopted by the Society of Toxicology in 1989, for the acceptable use of experimental animals. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86 – 23, revised 1985). This study was performed from September 2003 through July 2006 in the Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences.

Pain induction and grouping
An SC injection of 50 µL of 5% formalin into one of the animal’s hind paws was used for induction of continuous pain.14,15 Pain behavior was immediately recorded following the injection using a four-point scale for the next 60 min and a biphasic reaction (phase 1, from 0 – 10 min; and phase 2, from 10 – 60 min) of nociceptive behavior was observed in test groups.

Animal groups
Animals were grouped as:
   a) Animals suffered inflammatory pain for one day. This group received a single injection into a one side of hind paw.
   b) Animals suffered inflammatory pain for four days. For this group, the procedure mentioned for group one was repeated for four consecutive days. Every day, the formalin injection was given into a different paw site (day 1: right-dorsal, day 2: left-ventral, day 3: right-ventral, and day 4: left-dorsal).
   c) Animals suffered inflammatory pain for seven days. In this group, the formalin injection was given every other day for four times. So, like group b animals, each side of the hind paws received one formalin injection but the procedure was carried out within seven days.
   d) Animals received acetylsalicylic acid (ASA) (100 mg/kg) 24 hr prior to pain induction orally.16-18 In this group of animals, we had three main groups: one-, four- and seven-day pain groups that received ASA 24 hr prior to each pain induction session (e.g., four times for four- and seven-day pain groups).
   e) Animals received celecoxib (40 mg/kg) 24 hr prior to pain induction.19-21 In this group of animals, we had three main groups: one-, four-, and seven-day pain groups that received celecoxib 24 hr prior to each pain induction session.
   f) A control group was considered for each of the aforementioned groups in which animals experienced no pain. Moreover, we had considered control groups that received ASA and celecoxib alone without inducing tonic pain.

Note: All groups consisted of three animals.

Preparation of fresh hepatocytes
Hepatocytes were isolated from adult male Wistar rats by collagenase perfusion of the liver as described by Pourahmad and O'Brien.22 Cell viability was measured by Trypan blue (0.1% w/v) exclusion method and the viability considered in this study was at least 85 – 90%. Cells were suspended at a density of 106 cells/mL in round bottom flasks, rotating in a water bath maintained at 37°C in Krebs-Hensleit buffer at pH 7.4, supplemented with 12.5 mM HEPES under an atmosphere of 10% O₂, 85% N₂, and 5% CO₂. Each flask contained 10 mL of hepatocyte suspension. At least 80 – 90% of the control cells were still viable after three hr of incubation.

Determination of ROS
To determine the amount of ROS generation, DCFH diacetate was used as it penetrates the cells and becomes hydrolyzed by an intracellular esterase to form DCFH (nonfluorescent). The latter reacts with intracellular ROS to form the highly fluorescent DCFH, which effluxes the cell. Three mL of cell suspension was centrifuged at 1000 rpm for one min. The supernatant was removed and then three mL of fresh incubation buffer containing 1.6 µM dichlorofluorescein diacetate was added to the cell pellet. After incubating at 37°C in a thermostatic bath for 10 min with gentle shaking, the fluorescence intensity of the DCFH formed was determined at 470 nm (emission) and at 540 nm (excitation) using a Jenway 6200 (England) fluorescence spectrophotometer. The results were expressed as fluorescent intensity per 106 cells.23

Determination of mitochondrial membrane potential decline (%ΔΨm)
The cationic fluorescent probe, rhodamine, was used for determination of mitochondrial membrane potential decline.24 One-half mL of cell suspension picked up and centrifuged for one min at 1000 rpm. Cell pellet was then resuspended in two mL of fresh incubation medium containing 1.5 µM rhodamine 123 and incubated at 37°C in a thermostatic bath for 10 min with gentle shaking. After centrifugation, the amount of remained rhodamine 123 in the supernatant was measured using a Jenway 6200 (England) fluorescence spectropho-
meter set at 470 nm excitation and 540 nm emission. The capacity of mitochondria to take up rhodamine 123 was calculated as the fluorescence difference between the control and test cells.

Determination of lysosomal membrane stability
Redistribution of the fluorescent probe, acridine orange, was used to measure the lysosomal membrane damage (adapted from Refs 25 and 26). One-half mL of cell suspension that was previously stained with five µM of acridine orange, was picked up from the incubation flask and centrifuged at 1000 rpm for one min. The supernatant was removed and the cell pellet was then resuspended in two mL of fresh incubation buffer. After centrifugation, the pellet was washed out again and the acridine orange redistribution in the cell suspension was measured using a Jenway 6200 (England) fluorescence spectrophotometer set at 470 nm excitation and 540 nm emission.

Determination of proteolysis
Proteolysis was monitored using a fluorescence assay for tyrosine release (adapted from Ref 27). An aliquot of the hepatocyte suspension was precipitated with an equal volume of 20% trichloroacetic acid and allowed to stand overnight at 4°C. The sample was vortexed and centrifuged in a benchtop clinical centrifuge (at 13250 rpm for 15 min). One mL of the supernatant was removed and placed in a test tube to which was added one mL of 0.2% solution of 1-nitroso-2-naphthol and one mL acid nitrite reagent (10 mg/mL NaNO2 in 20% HNO3). The solution was vortexed, covered with parafilm, and incubated at 37°C for 30 min. Five mL of ethylene dichloride was added to the test tube. The mixture was vortexed vigorously and the sample was centrifuged for 10 min at high speed. The fluorescence of the aqueous phase was read in a Shimadzu RF5000U spectrophotometer (excitation at 460 nm and emission at 570 nm). The tyrosine content of the sample was determined from a standard curve constructed from known concentrations of tyrosine (0 – 100 µM).

Determination of caspase 3 activity
Caspase 3 activity was determined in cell lysate of hepatocytes from different groups using “Sigma’s caspase 3 assay kit (CASP-3-C).” In brief, this colorimetric assay is based on the hydrolysis of substrate peptide, Ac-DEVD-pNA, by caspase 3. The released moiety (p-nitroaniline) has a high absorbance at 405 nm. The concentration of the p-nitroaniline (µM) released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined p-nitroaniline solutions.

Detection of apoptosis
Apoptosis was detected using “Sigma’s apoptosis detection kit, Annexin V-CY3.” Briefly, in this kit two labels were used: 6-carboxyfluorescein (6-CF) was observed as green and Annexin V-Cy3 (AnnCy3) was observed as red fluorescence. After labeling at room temperature, the cells were observed by fluorescence microscopy. Live cells were stained only with 6-CF (green), while necrotic cells stained only with AnnCy3 (red). Cells starting the apoptotic process were stained with both AnnCy3 and 6-CF.

Statistical analysis
Levene’s test was used to check the homogeneity of variances. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s HSD as the post hoc test. Results were presented as mean±SD of triplicate samples. The minimal level of significance chosen was P<0.05.

Results
The ROS formation in one-, four-, and 7-day pain groups was significantly higher than the corresponding control groups (P<0.05) (Table 1). The maximum ROS formation occurred in four-day pain group. Celecoxib and ASA significantly prevented the ROS formation (P<0.05) in all pain groups.

Damage to lysosomes and mitochondria has a central role to driving the cell to death. So, we studied these two toxicity markers following the inflammatory/tonic pain induction. In four- and seven-day pain groups, the damage to lysosomal membrane was significantly higher than that observed in the one-day pain and the control group (P<0.05) (Table 2). While the lysosomes in one-day pain group are undamaged, the ROS level showed nearly two-fold increase compared to the control group. In four-day pain group, lysosomes showed the highest damage; the same event was observed for ROS formation in this group. Both ASA (100 mg/kg) and celecoxib (40 mg/kg) completely prevented the lysosomal membrane damage.
day pain group—quite similar to what was seen in four- and seven-day pain groups but not in one-
extracellular medium, was also markedly increased release of the amino acid tyrosine into the
(Table 3). The inflammatory pain-induced tyrosine decrease in the mitochondrial membrane
potential was observed in all one-, four-, and seven-day pain groups (P<0.05) (Table 4); the maximum damage was observed in seven-day pain group. Here again, ASA (100 mg/kg) and celecoxib (40 mg/kg) could completely prevent the decrease in the mitochondrial membrane potential.

Table 1. Comparison of reactive oxygen species (ROS) formation (DCFH fluorescence intensity per 10^6 cells) in 1-, 4-, and 7-day pain groups with or without celecoxib (40 mg/kg) or acetylsalicylic acid (ASA) (100 mg/kg) administration.

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain</td>
<td>72 ± 4</td>
<td>75 ± 5</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>Pain</td>
<td>154 ± 6^a</td>
<td>235 ± 12^a</td>
<td>168 ± 9^a</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain ASA</td>
<td>75 ± 4^b</td>
<td>106 ± 6^b</td>
<td>75 ± 4^b</td>
</tr>
<tr>
<td>(100 mg/kg) +</td>
<td>70 ± 4^b</td>
<td>110 ± 5^b</td>
<td>75 ± 4^b</td>
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</table>

Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. ROS formation was expressed as fluorescent intensity units. Significant difference (P<0.05) in comparison with a: “no pain” groups; b: pain groups.

Table 2. Comparison of lysosomal membrane instability in 1-, 4-, and 7-day pain groups with or without celecoxib (40 mg/kg) or acetylsalicylic acid (ASA) (100 mg/kg).

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Pain</td>
<td>4 ± 1</td>
<td>40 ± 5^a</td>
<td>15 ± 1^a</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain ASA</td>
<td>3 ± 1</td>
<td>3 ± 1^b</td>
<td>4 ± 1^b</td>
</tr>
<tr>
<td>(100 mg/kg) +</td>
<td>3 ± 1</td>
<td>3 ± 1^b</td>
<td>3 ± 1^b</td>
</tr>
</tbody>
</table>

Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. Lysosomal membrane damage was determined as intensity unit of redistributed acridine orange from lysosome to cytosol. Significant difference (P<0.05) in comparison with a: “no pain” groups; b: pain groups.

Table 3. Comparison of hepatocyte proteolysis in 1-, 4-, and 7-day pain groups with or without celecoxib (40 mg/kg) or acetylsalicylic acid (ASA) (100 mg/kg).

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Pain</td>
<td>4 ± 1</td>
<td>28 ± 2^a</td>
<td>18 ± 2^a</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain ASA</td>
<td>3 ± 1</td>
<td>4 ± 1^b</td>
<td>3 ± 1^b</td>
</tr>
<tr>
<td>(100 mg/kg) +</td>
<td>3 ± 1</td>
<td>3 ± 1^b</td>
<td>3 ± 1^b</td>
</tr>
</tbody>
</table>

Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. Hepatocyte proteolysis determined by the release of the amino acid tyrosine (µM) into the extracellular medium. Significant difference (P<0.05) in comparison with a: “no pain” groups; b: pain groups.

Table 4. Comparison of decline ion the mitochondrial membrane potential (∆Ψ_m) in 1-, 4-, and 7-day pain groups with or without celecoxib (40 mg/kg) or acetylsalicylic acid (ASA) (100 mg/kg) administration.

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain</td>
<td>2 ± 0</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Pain</td>
<td>17 ± 1^a</td>
<td>17 ± 1^a</td>
<td>28 ± 1^a</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain ASA</td>
<td>2 ± 0^b</td>
<td>3 ± 1^b</td>
<td>4 ± 1^b</td>
</tr>
<tr>
<td>(100 mg/kg) +</td>
<td>3 ± 1^b</td>
<td>3 ± 1^b</td>
<td>3 ± 1^b</td>
</tr>
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</table>

Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. ∆Ψ_m was determined as the difference in rhodamine 123 uptake by control and test cells and expressed as fluorescence intensity unit. Significant difference (P<0.05) in comparison with a: “no pain” groups; b: pain groups.

Significant decline in mitochondrial membrane potential was observed in all one-, four-, and seven-day pain groups (P<0.05) (Table 4); the maximum damage was observed in seven-day pain group. Here again, ASA (100 mg/kg) and celecoxib (40 mg/kg) could completely prevent the decrease in the mitochondrial membrane potential.

Activation of cascade of caspases, especially, inhibitors (ASA and celecoxib).
the caspase 3 which is the final mediator of apoptosis is a consequence of decline in mitochondrial membrane potential and MPT pore opening. Therefore, caspase 3 activity was measured using the “caspase 3 assay kit.” The activity of caspase 3 in one-day pain group did not have any significant changes comparing to the control group (Tables 5 and 6); in four- and seven-day pain groups, however, its activity was increased significantly ($P < 0.05$). The maximum activity was recorded in four-day pain group as it was shown for ROS formation and lysosomal membrane damage. The alteration patterns of caspase 3 activity when ASA (100 mg/kg) and celecoxib (40 mg/kg) were administered to pain groups were not identical. In all three groups which received celecoxib (one-, four-, and seven-day “celecoxib+pain” groups), caspase 3 activity was significantly higher than the corresponding pain groups. On the other hand, ASA significantly decreased the caspase 3 activity in one-, four-, and seven-day pain groups ($P<0.05$). In four-day “ASA+pain” group, the caspase 3 activity was however a little bit higher than that in the control group. The control group (no pain) which received celecoxib (40 mg/kg), had a significant higher caspase 3 activities (especially, in four- and seven-day groups) comparing to the corresponding control groups (no pain+no celecoxib) ($P<0.05$). The “no pain” group which received ASA (100 mg/kg) showed significant lower caspase 3 activity only in one-day group.

Apoptotic phenotypes were also detected in hepatocytes obtained from different pain groups, mostly in four-day pain group whereas all other toxicity markers were at their highest level (Figure 1).

### Table 5. Comparison of caspase 3 activity in 1-, 4-, and 7-day pain groups with or without celecoxib (40 mg/kg) administration.

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
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</thead>
<tbody>
<tr>
<td>No pain</td>
<td>78 ± 4</td>
<td>78 ± 4</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>Pain</td>
<td>95 ± 6$^a$</td>
<td>152 ± 8$^a$</td>
<td>134 ± 6$^a$</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain</td>
<td>171 ± 9$^{b,c}$</td>
<td>259 ± 9$^{b,c}$</td>
<td>255 ± 11$^{b,c}$</td>
</tr>
<tr>
<td>ASA (100 mg/kg) + pain</td>
<td>79 ± 4$^{b,c}$</td>
<td>190 ± 10$^{b,c}$</td>
<td>190 ± 8$^{b,c}$</td>
</tr>
</tbody>
</table>

Hepatocytes ($10^6$ cells/mL) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. Caspase 3 activity was measured by colorimetric assay, using “Sigma’s caspase 3 assay kit (CASP-3-C).” Significant difference ($P<0.05$) in comparison with a: “no pain” groups; b: pain groups; c: “celecoxib (40 mg/kg) + pain” groups. Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey’s HSD test.

### Table 6. Comparison of caspase 3 activity in 1-, 4, and 7-day pain groups with or without acetylsalicylic acid (ASA) (100 mg/kg) administration.

<table>
<thead>
<tr>
<th>Groups/Treatment</th>
<th>1-day</th>
<th>4-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain</td>
<td>78 ± 4</td>
<td>78 ± 4</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>Pain</td>
<td>95 ± 6$^a$</td>
<td>152 ± 8$^a$</td>
<td>134 ± 6$^a$</td>
</tr>
<tr>
<td>Celecoxib (40 mg/kg) + pain</td>
<td>63 ± 4$^{b,c}$</td>
<td>135 ± 6$^{b,c}$</td>
<td>84 ± 5$^c$</td>
</tr>
<tr>
<td>ASA (100 mg/kg) + pain</td>
<td>64 ± 4$^{b,c}$</td>
<td>73 ± 6$^{b,c}$</td>
<td>75 ± 5$^b$</td>
</tr>
</tbody>
</table>

Hepatocytes ($10^6$ cells/mL.) were incubated in Krebs-Henseleit buffer pH 7.4 at 37°C. Caspase 3 activity was measured by colorimetric assay, using “Sigma’s caspase 3 assay kit (CASP-3-C).” Significant difference ($P<0.05$) in comparison with a: “no pain” groups; b: pain groups; c: “ASA (100 mg/kg) + pain” groups. Values are expressed as mean±SD of three separate experiments (n=3) and analyzed using ANOVA followed by Tukey’s HSD test.

**Discussion**

In this study, the accelerated screening tests were used to assess the cellular redox status, source of pathologic alterations, and biochemical changes associated with inflammatory pain induction in hepatocytes.

Several studies have shown that in inflammatory/tonic pain states, reactive oxygen and nitrogen species (ROS and RNS) are produced that have important roles in inflammation and tissue degeneration.28

In our study, inflammatory pain increased the ROS formation in one-, four-, and seven-day pain groups (Table 1). ROS formation is expected in many inflammatory states. Pro-inflammatory cytokines, COX-2, and NOS are important sources for ROS production.10 In pathologic amounts, ROS can target intracellular organelles such as lysosomes29–32 and mitochondria.33–35 As our results showed (Tables 3 and 4), lysosomal membrane damage was significant in the four- and seven-day pain groups, although, the decline in mitochondrial membrane potential was seen in all three pain groups. So, it seems that damage to mitochondrial and lysosomal membrane could be a consequence of ROS formation.

Moreover, the phenomenon “ROS-induced ROS secretion” has already been suggested for mitochondrion34 that may perhaps propagate ROS-
induced injuries in cell. We found that the maximum ROS generation was in four-day pain group and that it was expected that this group showed the maximum lysosomal damage too. ROS-induced mitochondrial damage could spread; then autophagocytosis of the damaged mitochondria with high superoxid output by lysosomes and consequently intralysosomal Haber-Weiss reaction and hydroxyl radical formation could destabilize the lysosomal membrane integrity. Afterward, enormous digestive proteases and free radicals could be released into the cytosol. One of these important proteases is cathepsin B which can influence the mitochondria by opening the MPT pore; the released cytochrome c could activate the chain of caspases and trigger apoptosis.32, 35–37

ROS could potentially be an apoptosis inducer because 1) GSH depletion could induce apoptosis via altering the expression of the Bcl-2 family proteins and MPT pore opening38–40; 2) caspases are sensitive to redox state of the cell;40 and 3) it was shown that antioxidants could prevent apoptosis.38

As mentioned previously, mitochondria and lysosomes are among the most important sites for ROS action—a process that ends to cellular death.30,31,33,38 At the core of the apoptotic pathways, caspases exist. Caspase 3 is the most notable among the “executioner” caspases.40 In view of that, we measured the caspase 3 activity (Tables 5 and 6) that was significantly different in four- and seven-day pain groups comparing to the control group (P<0.05). The major pathway for caspase 3 activation is the release of cytochrome c from mitochondria following the MPT pore opening; the other pathways such as GSH depletion and lysosomal membrane leakage also could accelerate and exacerbate the MPT pore opening and cytochrome c release. We have already reported that inflammatory pain induction could deplete the rat hepatocyte GSH down to 50%.42

In one-day pain group, similar to ROS elevation and mitochondrial damage, caspase 3 activity was also elevated. Apparently, the antioxidant defense system is overridden in this group (Tables 1 and 4). In addition to what we found, COX activation and its products, prostaglandins, could potentially damage cells during inflammatory states. Inflammatory prostaglandin levels increase along the entire central nervous system and other body organs including hepatocytes in response to local injury or inflammation which may be due to a systemic increase in cytokines originating from the local site of inflammation, or perhaps a response of neural intermediary and glial activation. IL-1 induces transcription of COX-2, thereby increasing production of prostaglandins that sensitize the entire central nervous system within six to 12 hr of the onset of local injury or inflammation.43,44

Prostaglandins along with other mediators of exaggerated pain have a central role in induction of
Figure 2. Proposed mechanism for mitochondrial/lysosomal involvement in inflammatory pain-induced hepatocyte damage. Upon mitochondrial damage by pro-inflammatory soup (Interleukine1 [IL1], tumor necrosis factor [TNF], ROS, NO, and prostaglandins [PGs]) resulted from inflammatory pain induction, hydrogen peroxide (H₂O₂) originated in mitochondria diffused into lysosomes (or damaged mitochondria with high H₂O₂ output autophagocyted by lysosomes) and Fenton reaction catalyzed by intra-lysosomal redox-active iron occurred. This intra-lysosomal reaction led to hydroxyl radical generation. Hydroxyl radicals caused damage to lysosomal membranes and led to leaking of lysosomal proteases such as cathepsins (cathep) into the cytosol. These proteases and released hydroxyl radicals could either directly target the mitochondrial outer membranes or indirectly activate Bid or Bax and other lytic enzymes including phospholipase A₂ (PLA₂) to open the MPT pore and release cytochrome c and smac/Diablo to initiate downstream events that trigger apoptosis. This MPT pore opening could also potentiate the oxidative stress induction via the mitochondrial H₂O₂ diffusion into the lysosomes and generation of lysosomal hydroxyl radicals. Thin arrows indicate chemical transformations; while thick arrows show activation of biomolecules or diffusion of substances. Dotted arrows and black dots symbolize membrane peroxidation/modification by ROS or active biomolecules (Note: the figure is prepared by the authors).
peripheral and central sensitization, hyperalgesia, and allodynia. COX-1 involves in cytokine-induced ROS production.\textsuperscript{33,38,45} So, COX inhibitors may be of value in preventing cellular damages during inflammatory/tonic pain. In our study, we chose ASA (100 mg/kg) as a prominent non-selective COX inhibitor and celecoxib (40 mg/kg) as a specific COX-2 inhibitor. Interestingly, both drugs turned down the increased ROS formation in all pain groups (Table 1). The amount of ROS formation in one- and seven-day pain groups following administration of both drugs was returned to the control level. In four-day “drug+pain” groups although a significant decrease in ROS generation compared to four-day pain groups was observed, the amount of ROS generated was still higher than that in the control groups. We, therefore, concluded that severity of pain induction (four-day) is more critical than extending the pain (seven-day) in generating oxidative stress.

Beside the reducing of ROS formation, COX inhibitors also prevented mitochondrial and lysosomal membrane damage; however, results of caspase 3 activity showed that apoptosis would be still expected (Tables 5 and 6). In one- and seven-day “ASA+pain,” caspase 3 activities were decreased and nearly equaled to that of the control (no pain) group. In the presence of ASA, caspase 3 activity of four-day pain group was not returned to the control level and therefore cellular death could still be expected.

In conclusion, our research had three very important outcomes:

1) In addition to previously published reports regarding the cellular and molecular damages of pain on neurons and glial cells of the nervous system, persistent pain could also cause serious toxic cellular damages to some organs like liver which are not normally involved in neural routes responsible for conception or feeling of the pain.

2) The mitochondria and lysosomes cross-talk in oxidative stress and apoptosis follows the inflammatory pain induction. Upon mitochondrial damage by pro-inflammatory soup, $\text{H}_2\text{O}_2$ originated in mitochondria diffuses into lysosomes (or the damaged mitochondria with high $\text{H}_2\text{O}_2$ output is autophagocyted by lysosomes) and Fenton reaction catalyzed by intra-lysosomal redox-active iron occurs. This intra-lysosomal reaction leads to hydroxyl radical generation. Hydroxyl radicals cause damage to lysosomal membranes and lead to leaking of lysosomal enzymes into the cytosol (Figure 2). These proteases and released hydroxyl radical can either directly target the mitochondrial outer membranes or indirectly activate Bid or Bax and other lytic enzymes to open the MPT pore and release of cytochrome c which initiates down stream events that trigger apoptosis. This MPT pore opening can also potentiate the oxidative stress induction via the mitochondrial $\text{H}_2\text{O}_2$ diffusion into the lysosomes and generation of lysosomal hydroxyl radicals (Figure 2).

3) The third outcome was the unexpected results for caspase 3 activity in all “celecoxib+pain” groups, where their levels were even higher than all corresponding pain groups. Recently, many studies have shown that COX-2 inhibitors in addition to the anti-inflammatory action, have potential effects as chemopreventive and anti-tumor agents. Furthermore, selective COX-2 inhibitors when compared with nonselective COX inhibitors, further reduce the colorectal cancer progression; this antitumor activity has been attributed to the ability of these molecules to induce apoptosis in cancer cells.\textsuperscript{46,47} Also, induction of apoptosis by these agents has found to be correlated to the caspase 3 activation.\textsuperscript{48,49} Apparently, different COX-2 inhibitors induce apoptosis by different mechanisms. Albeit, the mechanism by which COX-2 inhibitors mediate apoptosis in cancer cells still remains elusive.\textsuperscript{50,51} We found that, the four- and seven-day groups which received celecoxib without pain induction, had a caspase 3 activity higher than the corresponding control and pain groups (Tables 5 and 6); therefore, apoptosis occurred in these groups.

In colon cancer as in inflammatory pain, the expression of COX-2 increases. However, previous studies suggested that COX-2 inhibitors could mediate apoptosis via a large number of signaling targets rather than the COX-2 enzyme itself.\textsuperscript{51,52} Thus, celecoxib has a pro-apoptotic activity that was shown to be COX-2-dependent as well as independent.\textsuperscript{8} As appeared in our results (caspase 3 activity in four- and seven-day “celecoxib+pain” groups), the celecoxib-induced caspase 3 activity had an additive effect to inflammation-mediated caspase 3 activity and made a higher value than it was solely expected from the inflammation.

References

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