Heat shock protein 70-dependent protective effect of polaprezinc on acetylsalicylic acid-induced apoptosis of rat intestinal epithelial cells

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Protection of the small intestine from mucosal injury induced by nonsteroidal anti-inflammatory drugs including acetylsalicylic acid is a critical issue in the field of gastroenterology. Polaprezinc, an anti-ulcer drug, consisting of zinc and L-carnosine, provides gastric mucosal protection against various irritants. In this study, we investigated the protective effect of polaprezinc on acetylsalicylic acid-induced apoptosis of the RIE1 rat intestinal epithelial cell line. Confluent rat intestinal epithelial cells were incubated with 70 µM polaprezinc for 24 h, and then stimulated with or without 15 mM acetylsalicylic acid for a further 15 h. Subsequent cellular viability was quantified by fluorometric assay based on cell lysis and staining. Acetylsalicylic acid-induced cell death was also qualified by fluorescent microscopy of Hoechst33342 and propidium iodide. Heat shock proteins 70 protein expression after adding polaprezinc or acetylsalicylic acid was assessed by western blotting. To investigate the role of Heat shock protein 70, Heat shock protein 70-specific small interfering RNA was applied. Cell viability was quantified by fluorometric assay based on cell lysis and staining and apoptosis was analyzed by fluorescence-activated cell sorting. We found that acetylsalicylic acid significantly induced apoptosis of rat intestinal epithelial cells in a dose- and time-dependent manner. Polaprezinc significantly suppressed acetylsalicylic acid-induced apoptosis of rat intestinal epithelial cells at its late phase. At the same time, polaprezinc increased Heat shock protein 70 expressions of rat intestinal epithelial cells in a time-dependent manner. However, in Heat shock protein 70-silenced rat intestinal epithelial cells, polaprezinc could not suppress acetylsalicylic acid-induced apoptosis at its late phase. We conclude that polaprezinc-increased Heat shock protein 70 expression might be an important mechanism by which polaprezinc suppresses acetylsalicylic acid-induced small intestinal apoptosis, a hallmark of acetylsalicylic acid-induced enteropathy.

Key Words: NSAIDs, acetylsalicylic acid, apoptosis, polaprezinc, heat shock protein 70

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA) and indomethacin, are widely used for the treatment of various inflammatory diseases due to their analgesic, anti-inflammatory and antipyretic effects. On the other hand, NSAIDs are also well known to induce frequent and severe gastrointestinal mucosal injuries via multiple mechanisms including reduction of prostaglandins, activation of neutrophils, microcirculatory disturbances, and production of oxygen free radicals. Although ASA can cause gastric ulcers, it is generally believed that ASA does not cause any damage to the small bowel based on intestinal permeability and faecal inflammatory marker studies. However, since the application of capsule endoscopes, it has become clear that ASA-related small intestinal mucosal injury is not impossible. Moreover, the pathogenesis of gastric mucosal damage by ASA has been extensively investigated, while that of the small intestine has not been fully explained.

Heat shock proteins (HSP) are a type of protective protein involved in diverse biological activities, such as apoptosis, carcinogenesis, and cytoprotection of various cells from cytotoxic damage. The type of induced HSP and its level of expression can determine the fate of cells in response to various stresses or stimuli. Therefore, drugs or treatments that induce HSP expression may positively contribute to gastric mucosal defense systems and participate in gastric mucosal cytoprotection. HSP has been classified into four families: HSP90, HSP70, HSP60, and the low molecular weight HSP family, all defined by their different molecular weights. Of these families, the HSP70 family represents one of the most conserved groups, with functional counterparts identified from the most primitive bacteria through to humans. HSP70 is a molecular chaperone that is rapidly induced by physical and chemical stresses such as heat, oxidative stress, and drug exposure. The mechanisms by which HSP70 exerts its anti-apoptotic function encompass the inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway, caspase activation, mitochondrial cytochrome c release, and apoptosome formation. It is suggested that overexpressed HSP70 can reduce oxidative stress and cell injury, and has potent cytoprotective effects. Polaprezinc (PZ), a chelate compound consisting of zinc and L-carnosine, has been widely used as an anti-ulcer drug. Mucosal protection by PZ is attributed to its stimulation of mucus production, antioxidant activity, membrane-stabilizing action, and induction of HSP70. Consequently, several studies have indicated that PZ can protect gastric mucosa against various stimuli in vivo and in vitro. In this study, we examined whether PZ can protect small intestinal epithelial cells from ASA-induced apoptosis, and determined the involvement of HSP70, induced by PZ, in this process.
Materials and Methods

Cell lines and culture medium. Rat intestinal epithelial cells (RIE1) were kindly provided by Professor Tsujii (Osaka University, Osaka, Japan), and were used as a model of the small intestine in vivo. The cells were grown in 1:1 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. The experiments were performed when the cells were confluent.

Reagents. The Cytotoxic Fluoro-test was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and propidium iodide (PI) and Hoechst33422 (H3422) were purchased from Sigma-Aldrich Co. (St. Louis, MO). PZ was from ZERIA Pharmaceutical Co. Ltd. (Tokyo, Japan). FBS and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). PZ was dissolved in 0.2 M HCl and adjusted to pH 6.8 with 0.2 M NaOH just before use.35

Cell viability. Cells were plated at a density of 10⁵ cells/well in 96-well plates. When confluent, the cells were treated with ASA (15 mM) for 15 h. In some of the experiments, PZ (70 µM) was added to the cells prior to ASA addition and incubated for 24 h. Cell viability was measured using a assay which based on fluorometric assay on cell lysis and screening (FACLS) (Wako, Osaka, Japan). This test was composed of fluorescence solution and lysis solution. Cells were incubated in 96-well assay plates that had black walls with a flat-transparent bottom (Becton Dickinson Bioscience, Franklin lakes, NJ) at a concentration of 10⁵ cells/well. Dispense 200 µL of the culture medium to be assayed after culture or treatment of drug to each of well and add to 10 µL of fluorochrome working solution. And set the plate to fluorescence microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA) and measure the fluorescence intensity at a wavelength of Ex/Em = 420/460 nm. Then draw the plate from the reader and add 10 µL of lysis solution to each of the well assayed, gently mixing the suspension by finger tapping, and measure the fluorescence intensity.

Microscopic analysis of apoptotic cells. Cells (10⁴) were cultured in 30-mm tissue culture dishes until confluent. Cells were treated with ASA (15 mM) for 15 h. In some experiments, PZ (70 µM) was added to the cells prior to ASA addition and incubated for 24 h. Treated cells were stained with propidium iodide (PI; final concentration of 10 µg/ml) and Hoechst33422 (H3422; final concentration of 10 µg/ml) for 15 min at 37°C.52 Analysis was performed using an inverted fluorescence microscope (IX70-23FL/DIC-SP; Olympus, Tokyo, Japan). H3422 is a cell permeable fluorescent dye with affinity to DNA, allowing for analysis of nuclear morphology for the evaluation of cell death. H3422 enters the cells with intact membranes and stains the DNA blue, whereas PI, a polar compound, only enters dead or dying cells with damaged cell membranes and stains the DNA red. Therefore, live, viable and early-stage apoptotic cells, which have cell membrane function, take up blue dye (H3O3422). Red-stained cells (PI) are considered late apoptotic (condensed chromatin) or necrotic.25 Fluorescent cell nuclei were visualized with an Olympus microscope at ×40 magnification using an inverted fluorescence microscope (IX70-23FL/DIC-SP; Olympus, Tokyo, Japan). Photographic images (MPEG format) were taken from four random fields.

Antibodies and western blotting. Primary antibodies specific for HSP70 (SPA-812) were obtained from Stressgen (Ann Arbor, MI), and the anti-actin antibody (sc-17031) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Total proteins were extracted from RIE1 cells after the confluent cells had been treated with 70 µM PZ for 24 h and with or without ASA (15 mM) for an additional 15 h. In some experiments, RIE1 cells were first incubated at 43°C for 30 min and then at 37°C for 90 min. Cells were washed twice with ice-cold PBS, retrieved with a cell scraper and collected by centrifugation at 2,500 rpm/min at 4°C for 5 min. After removing upper PBS, cell pellets were lysed in Lysis Buffer (CellLytic M; Sigma-Aldrich Co.), stirred and incubated on the ice for 15 min. Cellular debris was removed by centrifugation at 12,000 rpm/min at 4°C for 15 min. The supernatant was stored at −80°C until use. The protein amount in each sample was determined by a protein assay (Bio-Rad Laboratories, CA) using bovine serum albumin as a standard. Thirty micrograms of protein from each sample were electrophoresed on 10% SDS-PAGE gels for 30 min at 250 V, and transferred to PVDF membranes using a semidry transfer system (Invitrogen Japan K.K., Tokyo, Japan). The membranes were then incubated with 10% EzBlock (ATTO corporation, Tokyo, Japan) in TBS-T (10 mM Tris-Cl, pH 8.0; 150 mM NaCl, 0.1% Tween-20/V) for 15 min at room temperature, and washed with TBS-T three times. The membranes were incubated for 1 h at room temperature with the primary antibody in TBS-T (diluted 1:1000), and then incubated with the secondary anti-rabbit IgG antibody in TBS-T (diluted 1:1000) for 1 h at room temperature. Immunocomplexes were detected by western blotting (ECL plus; GE Healthcare Bio-Sciences K.K., Tokyo, Japan).

Transfection using small interfering RNA. RIE1 cells (5 × 10⁴/ml) were plated in antibiotic-free DMEM/F12 with 5% FBS in 60-mm dishes overnight. The next day, RNAi duplex-Lipofectamine RNAiMAX complexes consisting of a mixture of Opti-MEM reduced serum medium (Invitrogen), HSP70-specific small interfering RNA (siRNA), a nucleic acid-transferring agent and lipofectamine 2000 (Invitrogen) were mixed and incubated together for 10–20 min at room temperature. Following this, the siRNA-lipofectamine complex-containing medium was added to RIE1 cells at a final siRNA concentration of 10 nM. Three kinds of HSP70-siRNA were used: (1) Rat hspa1b-1230, for forward 5'-CCUCCUCUGGUCAUUCCAAAGUUU-3' and reverse 3'-GGAGGACCAGACUAAGGGUUUACA-5'; (2) Rat hspa1b-1467, for forward 5'-CCAGAGAAGACUCUAACAGACCAGAA-3', and reverse 3'-GUCUCAUCUUGAGAGUC-5'; (3) Rat hspa1b-2868, for forward 5'-CAGAACACUGUGAUCUGACUCUU-3', and reverse 3'-GUCUUCUGAGACCGUGCUU-5' (Invitrogen). After 8 h, the cells were washed twice with PBS, and the complex-containing medium was exchanged for DMEM/F12 medium with 5% FBS and 1% anti-biotics. For the negative control (Mock-RIE1), 10 nM control siRNA of medium GC content (Invitrogen) was used instead of HSP70-siRNA.

Assessment of cell viability and apoptosis. Apoptosis was quantified with the annexin V/PI double staining assay that uses an annexin V-FITC apoptosis detection kit I (BD Biosciences, Tampa, FL). RIE1 cells were harvested at the end of the treatment and washed twice with cold PBS. Cells were then resuspended in 500 µl of annexin V binding buffer and incubated with 5 µl of annexin V and 10 µl of PI for 15 min at room temperature in the dark. Analysis was performed on the FACS Calibur using CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

Statistical analysis. All Data are presented as the mean ± standard error of the mean (SEM) from at least three separate experiments. Data was analyzed by the Student’s t-test or one-way ANOVA. Differences were considered to be significant at p<0.05.

Results

ASA induces cell death of RIE1 in a dose- and time-dependent manner. To determine the cytotoxicity of ASA on RIE1 cells, confluent RIE1 cells were incubated with 0–15 mM ASA for 24 h. We found that ASA was able to induce death of RIE1 cells in a concentration-dependent manner (Fig. 1A). In
addition, 15 mM of ASA induced cell death of RIE1 in a time-dependent manner (Fig. 1B). Based on this result, we selected 15 mM as the concentration for ASA and 15 h as the incubation time for the RIE1 cells for the following experiments.

PZ suppresses ASA-induced cell death of RIE1. Since we previously reported that PZ protects RIE1 from indomethacin-induced apoptosis via anti-oxidative stress mechanisms,(17) we decided to investigate whether PZ also suppresses ASA-induced cell death of RIE1. We found that pre-administration of PZ (70 µM) for 24 h significantly suppressed ASA (15 mM)-induced RIE1 cell death (Fig. 2). PZ itself did not affect cell viability.

PZ suppresses ASA-induced apoptosis-dominant cell death. To evaluate the type of cell death in RIE1 cells, we employed a double-staining method using HO342 and PI (Fig. 3). We found a small amount of apoptotic cells, including early apoptotic cells (blue condensed nuclei) and late apoptotic cells (red condensed nuclei) in the control group, which are considered to be basal apoptotic cells. Upon addition of ASA (15 mM), late apoptotic cells were clearly increased and were accompanied with a slight increase of early apoptotic cells. Although PZ (70 µM) itself did not affect cell viability, it did reduce the number of ASA-increased apoptotic cells. Very few necrotic cells were found in each group.

Both PZ and ASA affect HSP70 expression in a time-dependent manner. Since the protective role of PZ has been reported to be dependent on the induction of HSP70 expression in gastric(26) and colonic(27) epithelial cells as well as in hepatocytes,(28) we examined the expression of HSP70 in RIE1 after incubation with PZ, ASA, and PZ with ASA, respectively. We found that unstimulated RIE1 cells expressed small amounts of HSP70. After treatment with ASA (15 mM), PZ (70 µM), or ASA with PZ for various periods of time (Fig. 4 A and B), the expression of HSP70 in the cells was significantly decreased only after administration of ASA, and this progressed in a time-dependent manner. However, PZ significantly restored the expression of HSP70 at 15, 24 and 39 h. PZ when added alone strongly increased the expression of HSP70 in RIE1 in a time-dependent manner as assessed by densitometry.

Selection of siRNA specific to HSP70 and confirmation of HSP70 silencing in transfected RIE1. To further investigate the role of PZ-induced HSP70 on ASA-induced RIE1 cell death, we silenced HSP70 in RIE1 by using siRNA. At first, we tried three types of HSP70-siRNA. Among the three types of HSP70-siRNA, only one siRNA (Rat hspa1b-2868) was able to completely silence HSP70 (Fig. 5A), as assessed by WB. Therefore, we
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selected this most effective HSP70-siRNA for all of the subsequent RIE1 transfection experiments.

We also assessed how long HSP70-siRNA would be able to silence HSP70 expression after the transfection, and found that after 8 h transfection, the cells could not express HSP70 for up to 39 h after heat shock at 43°C for 30 min (Fig. 5B). This method of heat shock stimulation significantly induced HSP70 expression in naïve RIE1 and Mock-RIE1 cells (negative control) at 24 h.

**PZ is unable to induce HSP70 expression in HSP70-silenced RIE1.** With our three types of cells ( naïve RIE1, HSP70-silenced RIE1 and Mock-RIE1), we wanted to confirm the effect of HSP70 silencing by using PZ instead of heat shock. As shown in Fig. 6A, expression of HSP70 in naïve RIE1 and Mock-RIE1 significantly increased after administration of PZ for up to 39 h. However, PZ was unable to induce HSP70 expression in HSP70-silenced RIE1.

To further confirm the efficiency of our HSP70 silencing, we examined HSP70 expression after treatment with ASA and ASA with PZ. We found that unstimulated naïve RIE1 and Mock-RIE1 cells expressed small amounts of HSP70, which was significantly increased by PZ pre-treatment. However, after the treatment of these cells with ASA (15 mM) for 15 h, HSP70 significantly decreased. In contrast, unstimulated HSP-silenced RIE1 did not express HSP70, and PZ or ASA also did not increase HSP70 expression in these cells (Fig. 6B).

**Silencing of HSP70 in RIE1 results in loss of the protective effect of PZ on ASA-induced cell death.** As shown in Fig. 7, ASA significantly induced cell death in naïve RIE1 and Mock-RIE1, which was significantly suppressed by pre-treatment of cells with PZ. However, the silencing of HSP70 in RIE1 resulted in a significant loss of the protective effect of PZ on ASA-induced cell death, suggesting the active role of HSP70 in this process.

**PZ mainly suppresses ASA-induced late apoptosis in an HSP70-dependent manner.** Annexin V-FITC binding analysis and PI staining were performed to identify the type of ASA-induced cell death (Fig. 8). In each experiment, PI-negative/annexin V-negative cells represented viable cells, PI-positive/annexin V-negative cells represented necrotic cells, PI-negative/annexin V-positive cells represented early apoptotic cells, and PI-positive/annexin V-positive cells represented late apoptotic cells. As shown in Fig. 8, PZ mainly suppressed ASA-induced late apoptosis in an HSP70-dependent manner.

**Discussion**

As our society ages, the number of elderly individuals and patients frequently requiring NSAIDs, including ASA is increasing. However, aside from its preventive effects on the relapse of cerebral and cardiovascular events, (29) ASA has been reported to cause gastrointestinal injury. Therefore, establishing a method for preventing or treating ASA-induced small intestinal injury is urgently required. In this study, we investigated the protective effect and underlying mechanism of PZ on ASA-induced apoptosis of RIE1 cells.

At first, we found dose-and time-dependent cytotoxic effects of ASA on RIE1 cells. Studies on the oral administration of ASA in rats have shown that the concentration of ASA in the stomach causes gastric injury between 10 and 100 mM. (30) Thus, the *in vitro*
concentrations of ASA used in this study (15 mM) might be physiologically relevant to those in vivo (Fig. 1).

Next, we evaluated the protective effect of PZ on ASA-induced apoptosis of RIE1. PZ has been widely used as an anti-ulcer drug as it has been reported to prevent gastric mucosal damage in many experimental models.\(^\text{31}\) It is also reported that the mucosal protective effect of PZ is independent of the inhibition of acid secretion thought to contribute to gastric mucosal protection.\(^\text{16}\) Therefore, it is possible that PZ plays a role in the small intestine where the mucosa is free of gastric acid. In addition to its anti-ulcer effects, PZ has also been shown to have various effects such as quenching the production of reactive oxygen species,\(^\text{17}\) inducing the expression of HSP-27, HSP-72,\(^\text{27}\) and heme oxygenase-1 (HO-1),\(^\text{32}\) which are molecules that exhibit cytoprotective effects. PZ also demonstrates anti-inflammatory effects by inhibiting adhesion molecules on polymorphonuclear leukocytes and by inhibiting the production of cytokines by gastric epithelial cells.\(^\text{25}\) In the present study, we found that PZ significantly suppressed ASA-induced cell death of RIE1 (Fig. 2), especially ASA-induced apoptosis at its late phase (Fig. 3).

Many studies have indicated that HSPs, which function as molecular chaperones, play an important role in cell responses to stress-related events.\(^\text{33–35}\) HSPs consist of a variety of families defined by their different molecular weights. Of these families, the HSP70 family represents one of the most conserved groups, with functional counterparts identified from the most primitive bacteria through to humans. Cytoprotective effects of HSP70 in the stomach have been shown in vivo\(^\text{36}\) and in vitro,\(^\text{7}\) but these effects have not been demonstrated in the small intestine. Therefore, we examined the role of HSP70 expression induced by PZ on ASA-induced apoptosis of RIE1.

First, we measured HSP70 expression in RIE1 cells treated with PZ, ASA, and PZ + ASA. We found that, as seen in gastric epithelial cells, PZ significantly increased HSP70 expression also

\[\text{Fig. 5. Selection of siRNA specific to HSP70 and confirmation of HSP70 silencing in transfected RIE1.} \]

(A) Naive RIE1 cells were transfected with three different siRNA (rat hspa1b-1230, hspa1b-1467, and hspa1b-2868) specific to HSP70 and a stealth RNAi (Mock-RIE1). The cells were incubated at 43°C for 30 min, and then incubated at 37°C for 90 min, and WB was performed to assess the expression of HSP70. The WB result is a representative image of three separate experiments. (B) Assessment of the persistence of HSP70 silencing. Naive RIE1 and Mock-RIE1 were incubated at 43°C for 30 min, followed by incubation at 37°C for 39 h, and WB was performed to assess HSP70 expression. In addition, HSP70-silenced RIE1 (by siRNA hspa1b-2868) were incubated at 43°C for 30 min, and then incubated at 37°C for 0, 24 and 39 h, and WB and densitometry were performed to assess HSP70 expression. The WB result is a representative image of three separate experiments. The value of densitometry indicates relative HSP70 expression with actin as a control. The value is the mean ± SEM of three separate WB. *p<0.001 compared with naive RIE1.
in small intestinal epithelial cells (RIE1) in a time-dependent manner. Moreover, PZ restored the ASA-induced decrease of HSP70 expression in RIE1 (Fig. 4 A and B). We also found that PZ and zinc but not L-carnosine induces HSP70 in RIE1 (data not shown), suggesting the pivotal role of zinc in the cytoprotective action of PZ.

To further investigate the role of HSP70 expression induced by PZ on ASA-induced apoptosis of RIE1, we silenced HSP70 in RIE1 by using siRNA specific to HSP70. Among three available siRNAs specific for HSP70, only one siRNA was able to silence HSP70 in RIE1 (Fig. 5A) and this siRNA was able to silence HSP70 for more than 39 h under conditions either with heat shock (Fig. 5B) or PZ (Fig. 6A). In HSP70-silenced RIE1, we found that PZ was unable to induce HSP70 in these cells (Fig. 6B), and this correlated with the loss of the protective function of PZ on ASA-induced cell death of RIE1 (Fig. 7). These results suggest that PZ can protect small intestinal epithelial cells from ASA-induced cell death partly because of its HSP70-inducing effect.

In our final experiment, we examined the type of cell death by which PZ-induced HSP70 suppresses cell death in ASA-stimulated RIE1. We found that HSP70 induced by PZ mainly suppresses ASA-induced apoptosis at its late stage (Fig. 8). It has been reported that PZ suppresses ASA-induced cell death of the rat gastric epithelial cell line (RGM1) at its late phase of apoptosis.17

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**Fig. 6.** Confirmation of HSP70 silencing in an experimental setting. (A) Naive RIE1, HSP70-silenced RIE1 and mock-RIE1 cells were incubated with PZ for 0, 24, and 39 h, and WB and its densitometry was performed to assess HSP70 expression. The WB result is a representative image of three separate experiments. The value of densitometry indicates relative HSP70 expression with actin as a control. The value is the mean ± SEM of three separate WB. *p<0.001 compared with naive RIE1 without treatment. **p<0.001 compared with naive RIE1 at 39 h. (B) Confluent naive RIE1, HSP70-silenced RIE1 and Mock-RIE1 were incubated with 70 µM of PZ for 24 h prior to ASA stimulation (15 mM) for 24 h. WB and densitometry were performed to assess HSP70 expression. The WB result is a representative image of three separate experiments. The value of densitometry indicates relative HSP70 expression with actin as a control. The value is the mean ± SEM of three separate WB. *p<0.005 compared with control. **p<0.001 compared with ASA-stimulated cells.
This is consistent with results in our study. However, further studies should be performed to confirm this issue.

In conclusion, PZ appears to protect small intestinal epithelial cells from ASA-induced cytotoxicity through its HSP70-inducing effect without affecting cell viability. From a therapeutic point of view, PZ might be useful not only for protecting the stomach but also the small intestine from ASA-induced gastroenteropathy.

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**Abbreviations**

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<tr>
<th>Acronym</th>
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<tr>
<td>ASA</td>
<td>acetylsalicylic acid</td>
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<td>FBS</td>
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<td>HO-1</td>
<td>heme oxygenase-1</td>
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<td>JNK</td>
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<td>NSAIDs</td>
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**References**