Macrophage migration inhibitory factor contributes to development of nonsteroidal anti-inflammatory drugs-induced gastric injury in mice

Short title: Role of MIF in indomethacin-induced gastritis

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Abstract

Macrophage migration inhibitory factor (MIF) plays an important role in the development of inflammation. In this study, we evaluated the role of MIF in gastric injury induced by non-steroidal anti-inflammatory drugs (NSAIDs) in mice. To induce gastric injury, mice were intraperitnoneally injected with 35 mg/kg of indomethacin. The level of MIF protein was up-regulated and severe gastric injury with inflammatory infiltrate was observed in the stomach of wild-type (WT) mice treated with indomethacin. The severity of gastric injury in MIF-deficient mice was less than that in WT mice. Increase in TNF-α in gastric tissue of mice treated with indomethacin were suppressed in MIF-deficient mice. The expression of HSP70, which has a cytoprotective role, was remarkably up-regulated in the stomach of MIF-deficient mice compared with WT mice after indomethacin treatment. Our results suggest that MIF is essential for the development of gastric injury-induced by NSAIDs.

Keywords: Macrophage migration inhibitory factor; Gastric injury; Non-steroidal anti-inflammatory drugs

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for treatment of many kinds of diseases such as inflammatory and autoimmune diseases. NSAIDs affect the function of cyclooxygenases and strongly inhibit gastric prostaglandin (PG) production [1,2]. Nevertheless, there are serious harmful side effects of NSAIDs in the stomach. One of major harmful effects of NSAIDs is gastric mucosal injury, and this side effect affects clinical applications of NSAIDs. Although the mechanisms of NSAID-induced gastric injury have not been fully elucidated, proinflammatory cytokines such as TNF-α are also important in the development of gastric injury induced by NSAIDs [3-5].

Macrophage migration inhibitory factor (MIF) was first discovered as an activated T cell-derived factor that inhibits the random migration of macrophages [6]. Since the cloning of MIF cDNA and protein, subsequent studies have shown the expression and role of MIF in various cells and tissues [7]. Recently, it has been reported that MIF contributes to the development of inflammatory diseases as a proinflammatory cytokine and that treatment with an anti-MIF antibody improves the severity of inflammation in several disease models such as rheumatoid arthritis and colitis [8,9]. In addition, the results of studies using MIF-deficient mice support the evidence of treatment with

anti-MIF antibody [9-11]. Up-regulation of MIF is observed in the stomach of patients with gastric inflammation [12]. Additionally, Huang *et al.* have demonstrated that the expression of MIF is up-regulated in gastric mucosa of rats with acetic acid-induced gastritis [13]. However, the characteristics of NSAID-induced gastritis are different from those of acetic acid-induced gastritis. Thus, the role of MIF in NSAID-induced gastric injury has not been evaluated. In this study, we investigated the role of MIF in the development of indomethacin-induced gastric inflammation using MIF-deficient mice.

2. Materials and Methods

2.1. Animals

Eight-week-old male BALB/c mice weighing 22-25 g were purchased from Japan SLC Co. (Shizuoka, Japan). MIF-deficient mice (bred onto a BALB/c background) were established as described previously [14]. All mice were maintained on standard laboratory chow and given food and water *ad libitum*. All animal experiments adhered to the principles of the Declaration of Helsinki and were approved by the Animal Experiment Ethics Committee of the Graduate School of Medicine of Hokkaido University.

2.2. Indomethacine-induced gastric injury

For induction of gastric injury, mice were intraperitoneally injected with 35 mg/kg of indomethacin (Sigma, St Louis, MO). Mice were euthanized by intraperitoneal injection of a sufficient dose of thiopental 24 h after the treatment with indomethacin, and the stomach of each mouse was removed and stored at -80°C until use for the assay.

2.3. Histological examination

Removed stomachs were opened along the greater curvature, fixed in 10% neutral buffered formalin, and embedded in paraffin. After deparaffinizing of thin tissue sections on glass slides, the gastric tissues were stained with hematoxylin and eosin (H&E). Histological evaluation for tissue damage was performed as previously reported by a investigator in a blind fashion [15]. Briefly, the damage scores were classified as follows: 0, normal mucosa; 1, edema and/or vacuolation but minimal changes in crypt architecture; 2, epithelial disruption; 3 erosion extending to the muscularis mucosa. Inflammatory scores were as follows: 0, no inflammatory cells; 1, minimal number of inflammatory cells; 2, moderate number of inflammatory cells; 3, large number of inflammatory cells.

2.4. Enzyme-linked immunosorbent assay for MIF

Gastric samples kept at -80°C were weighed and homogenated in PBS solution with protein cocktail inhibitor (Sigma, St Louis, MO). The level of MIF in the supernatants of samples was measured with an ELISA kit specific for MIF (Sapporo IDL, Sapporo, Japan). This assay was performed in accordance with the procedure described previously [11]. The protein concentrations in the supernatants were measured with a Micro BCA protein assay reagent kit (Pierce, Rockford, IL) in accordance with protocols.

2.5. Measurement of gastric acid secretion

Basal and stimulated gastric acid secretion levels were determined as previously described by Farrell et al. [16]. In brief, mice were anesthetized with thiopental by intraperitoneal injection. An incision was made through the abdominal cavity, and the duodenum was ligated distal to the pyloric outlet. Gastric contents were removed through an incision at the gastroesophageal junction immediately after pyloric ligation. The liquid component of the gastric content was collected after centrifugation at 9.3 g for 5 min, and then the volume was measured. The acidity of the gastric contents was measured by titration with 0.01 N NaOH and expressed as microequivalents of H^+

(μEqH^+). For measurement of stimulated gastric acid secretion, mice were intraperitoneally injected with 2.5 mg/kg of bethanecol (Sigma, St Louis, MO) 1h before sacrifice, and then gastric acid contents were measured in a similar manner.

2.6. Measurement of TNF-α in gastric tissue

The sample of gastric tissue in phosphate buffered saline (PBS) with protease inhibitor cocktail (Sigma, St. Louis, MO) was homogenized, and supernatant was collected. The levels of TNF-α in the supernatant were measured using a multiplex bead array (Upstate Biotechnology) and analyzed with the Bioplex workstation and associated software according to manufacturer's procedure**.**

2.7. Western blot analysis for heat shock proteins

Gastric tissues were disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Equal amounts of homogenates were dissolved in 20 μl of Tris-HCL, 50 mM (pH 6.8), containing 2-mercaptoethanol (1%), SDS (2%), glycerol (20%) and bromophenol blue (0.04%), and the samples were heated at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto nitrocellulose membranes. The membranes

were blocked with 1% nonfat dry milk in phosphate-buffered saline (PBS), probed with anti-HSP 25, 32, 40 and 70 antibodies (diluted 1000 : 1, Stressgen, Victoria, BC, Canada), and reacted with a goat anti-rabbit IgG antibody coupled with horseradish peroxidase (HRP). The resultant complexes were processed for the detection system according to the manufacturer's protocol. The protein concentrations of the homogenates were quantified using a Micro BCA protein assay reagent kit. Quantitative analysis was carried out using an MICD image analyzer on relative intensity of the expression of HSP compared to β-actin. Average of relative intensity was obtained from three complete experiments in the other mice and assessed.

2.8. Statistics

All data are presented as means ± standard error (SE). The results were statistically analyzed using Student's unpaired *t*-test (StatView, SAS Institute, Cary, NC). $P < 0.05$ was considered as a significant difference.

3. Results

3.1 MIF protein level is increased in the stomach of mice

The levels of MIF protein in the stomach of BALB/c mice were more than 3-fold

increased at 24 h after treatment with indomethacin compared with the levels in non-treated BALB/c mice ($P<0.05$, 324.8 ± 105.8 and 89.7 ± 23.2 μg/ mg protein, respectively, Fig.1).

3.2. MIF-deficient mice are resistant to indomethacin-induced gastric injury

Histological findings were normal in the stomach of wild-type (WT) and MIF-deficient mice (Fig. 2A). Indomethacin treatment induced severe destruction of gastric mucosa with leukocyte infiltration in WT mice (Fig. 2A). On the other hand, these histological findings were mild in the stomach of MIF-deficient mice (Fig. 2A). Both the damage and inflammatory scores for indomethcin-induced gastric injury were considerably lower in the stomach of MIF-deficient mice than in that of WT mice (damage scores: 0.74 ± 0.21 and 2.00 ± 0.26, respectively, $P<0.01$; inflammatory scores: 0.60 ± 0.27 and 2.3 ± 0.26, respectively, $P<0.01$, Fig. 2B and 2C).

3.3. No difference in gastric acid secretion between MIF-deficient and wild type mice

Basal and stimulated gastric acid secretion levels were measured in WT and MIF-deficient mice. There was no significant difference in the basal gastric acid secretion levels between WT and MIF-deficient mice (0.772 ± 0.033 and 0.774 ± 0.033

μEqH^+, respectively, Fig. 3A). Moreover, there was no difference in the gastric secretion levels between WT and MIF-deficient mice under betanechol-stimulated conditions (0.742 ± 0.063 and 0.802 ± 0.032 μEqH^+, respectively, Fig. 3B).

3.4. Increase in TNF-α is inhibited in the stomach of MIF-deficient mice treated with indomethacin.

There is no difference in gastric TNF-α levels in non-treated WT and MIF-deficient mice (2.25 ± 0.42 and 1.93 ± 0.32 pg/mg protein, respectively). When mice were treated with indomethacin, gastric TNF-α was increased in WT mice (31.30 ± 7.64 and pg/mg protein), whereas the levels of TNF-α were significantly lower in MIF-deficient mice compared with WT mice (3.24 ± 0.91 pg/mg protein) (Fig.4).

3.5. Heat shock protein 70 is enhanced in the stomach of MIF-deficient mice treated with indomethacin

Western blot analysis for HSP25, 32 40 and 70 was performed in the stomach of mice. There was no difference in the levels of these HSPs in the stomach between WT and MIF-deficient mice under normal conditions (Fig. 5A, B). At 24 h after treatment with indomethacin, MIF-deficient mice showed marked up-regulation of HSP70 in the

stomach compared with the level in WT mice (Fig. 5A,B). In contrast, the expression levels of HSP25, 32 and 40 were essentially unchanged in the stomach of WT and MIF-deficient mice after treatment with indomethacin, and there was no difference in the expression levels of HSP25, 32 and 40 between WT and MIF-deficient mice (Fig. 5A,B).

4. Discussion

MIF plays a critical role in the development of inflammation [7-13,17-20]. At the site of the lesion, the level of MIF is up-regulated and neutralization of MIF bioactivity with an anti-MIF antibody remarkably suppresses inflammation and tissue destruction [7,8,11,13]. Huang *et al.* have shown up-regulation of MIF in acetic acid-induced gastritis in rats and inhibitory effects of anti-MIF antibody on the experimental gastritis [13]; however, the role of MIF in NSAID-induced gastric injury remained unclear. In this study, consistent with the model of acetic acid-induced gastritis, NSAIDs remarkably up-regulated the level of MIF in the stomach of mice. Furthermore, the severity of indomethacin-induced gastric injury was suppressed in MIF-deficient mice compared with WT mice. Our results suggested that MIF plays a critical role in the development of gastric injury induced by NSAIDs.

Gastric secretion including acid plays an important role in the development of gastric injury induced by NSAIDs [21]. Proton pump inhibitor and histamine 2 receptor antagonist are clinically used for inhibition of gastric acid secretion and treatment of gastric injury [22]. Thus, we focused on the level of gastric acid secretion in MIF-deficient mice. However, we did not find any change in the gastric acid outputs in MIF-deficient mice compared with WT mice. Moreover, in this study, bethanecol did not increased acid secretion in both WT and MIF-deficient mice. Ballabeni et al. have demonstrated that bethanecol increases the acid secretion of rats [23]. On the other hand, other study shows that bethanecol increased a few in acid secretion of mice [16]. Our results of gastric acid secretion were different from the results of previous studies. It is suggested that different species of animal and timing of sampling influence the level of gastric acid secretion. Further studies are needed to clarify the role of MIF on gastric acid secretion.

Imflammatory cytokines enhance the development of gastric injury induced by indomethacin. Especially, TNF-α mediates indomethacin-induced gastric damage [24]. In this study, we demonstrated that increasing gastric TNF-α level was inhibited in MIF-deficient mice when mice were given indomethacin. These results suggest that one of mechanisms by which MIF affects indomethacin-induced gastritis is via regulation of

TNF-α.

To further clarify the mechanism of the protective effect on NSAID-induced gastric injury in MIF-deficient mice, we focused on the expressions of heat shock proteins (HSPs) in the stomach of mice. HSPs have been found to be cytoprotective proteins induced by chemicals and stresses [25]. It has been reported that HSPs play a critical role in protection of cells and tissues from stresses, including chemical toxicity [26]. In the stomach, up-regulation of HSP ameliorates the severity of gastric injury [27-29]. Rokutan has described the essential role of HSP in the protection of gastric mucosa against stress and injury [27]. Shichijo *et al.* have reported overexpression of HSP70 in the stomach of stress-induced gastric ulcer-resistant rats [28]. Ebert *et al.* also have shown the protective role of HSP27 in gastric mucosal injury induced by indomethacin using HSP27 transgenic mice [29]. In this study, the expression of HSP70 was up-regulated in the stomach of MIF-deficient mice after treatment with indomethacin. Our results suggest that one of the mechanisms is the protective role of HSP70 in MIF-deficient mice. Interestingly, these findings are consistent with results of our previous study on the expression of HSP70 in the colon of MIF-deficient mice [30]. We found in our previous study that MIF-deficient mice were resistant to dextran sulfate sodium (DSS)-induced colitis and showed the marked up-regulation of HSP70 in the

colon. Our previous and current studies provide the evidence that MIF affects the expression of HSP. However, there has been no investigation of the mechanism by which MIF affects the expression of HSP. Besides our previous study, Bourdi *et al.* have demonstrated that MIF-deficient mice are resistant to drug-induced hepatitis and that the expressions of some HSPs are up-regulated in the liver of MIF-deficient mice [31]. Bourdi *et al.* speculated that the change in glucocorticoid activity caused by MIF affects the expressions of HSPs. On the other hand, MIF influences the activity and expression level of GC [32,33]. We investigated the serum level of corticosterone as the mouse glucocorticoid homologue. Unfortunately, there was no difference in the serum level of glucocorticoid (GC) between MIF-deficient and WT mice (data not shown). However, we could not reach a conclusion regarding the effect of GC on the expression of HSP in MIF-deficient mice because we did not precisely evaluate the GC function in MIF-deficient mice. Further study is needed to clarify the mechanism by which MIF affects the expression of HSP.

On the other hand, in this study, we did not confirm the up-regulation of HSP40 in gastric tissue in MIF-deficient mice. Our previous study showed the up-regulation of HSP40 in the colon of MIF-deficient mice [30]. There is a discrepancy between these and our previous studies. Although further study is needed to clarify the discrepancy,

different stimulants, such as indomethacin and dextran sulfate sodium, and different tissues, such as stomach and colon, may induce the different response of HSP expression.

Recently, several studies have shown the receptors against MIF such as CD74 [34, 35]. In gastric injury, the expression of CD74mRNA is up-regulated in *Helicobacter pylori*-induced gastritis in mice [36]. On the other hand, the up-regulation of CD74mRNA is inhibited in the stomach of MIF-deficient mice treated with *Helicobacter pylor.* In this study, we did not assess the expression of receptors against MIF in murine gastric injury induced by indomethacin. Although further study is needed, similar results may be found in indomethacin-induced gastric injury.

In conclusion, we demonstrated that NSAIDs up-regulated the expression of MIF in the stomach of mice and that NSAIDs-induced gastritis was inhibited in MIF-deficient mice. Our results suggest that MIF is critical in the development of NSAIDs-induced gastric injury and that reduction of MIF has a therapeutic potential in refractory NSAID-induced gastritis.

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Figure legends

Fig. 1. Treatment with indomethacin increased the level of MIF in the stomach of BALB/c mice. The level of MIF in the stomach was measured with an ELISA kit specific for MIF. Results are means ± SE (n = 5 for each group). *$P < 0.01$ vs. non-treated BALB/c mice.

Fig. 2. A. Microscopic photographs of gastric tissues. There is no difference in gastic mucosa both wild type (WT) and MIF-deficient mice under normal condition. Deep ulcer and marked leukocyte infiltrating were observed in the gastric tissue in indomethacin-treated WT mice. In indomethacin-treated MIF-deficient mice, submucosal edema was observed but tissue damage was minimal in the gastric mucosa. Representative pictures are shown. Similar appearances were observed in the gastric tissues of the other mice. Original magnification ×100. KO; MIF-deficient mice, WT; wild-type mice.

B. Histological scores of the gastric tissues of mice. Histological scores were assessed in the gastric tissues as described in Materials and Methods. Results are means ± SE (n = 5 for each group). KO; MIF-deficient mice, WT; wild type mice.

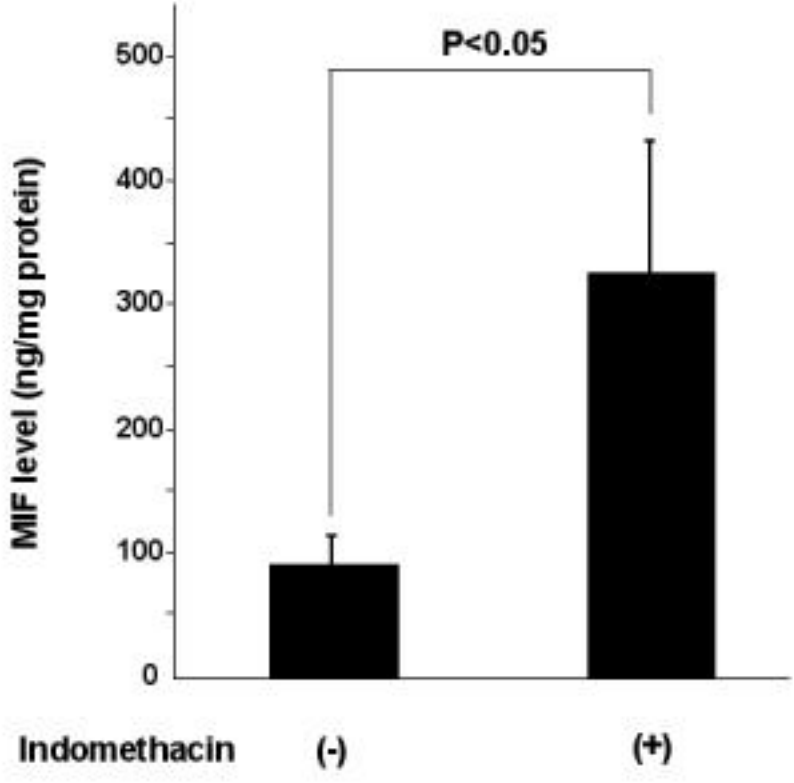
Fig. 3. No effect of MIF on gastric acid secretion in mice. **(A)** Basal gastric acid secretion in wild-type (WT) and MIF-deficient mice. **(B)** Stimulated gastric acid secretion in WT and MIF-deficient mice. Basal acid output was caluculated and expressed as micro equivalent of H^+ (μEq H^+) as described in Materials and Methods. Results are means ± SE (n = 4-5 for each group). KO; MIF-deficient mice, WT; wild type mice.

Fig. 4. Local TNF-α level in stomach of mice. Results are given as means ± SE. Statistical significance was found between WT and MIF-deficient mice treated with indomethacin (*$p<0.01$). n = 4-5 in each group. KO; MIF-deficient mice, WT; wild type mice.

Fig. 5. The up-regulation of HSP70 in the stomach of MIF-deficient mice treated with indomethacin. **A.** The expressions of HSP25, 32 40 and 70 in the stomach was detected by western blot analysis. Representative results are shown. Similar results were obtained from the other experiments. KO; MIF-deficient mice, WT; wild type mice.

B. Average of relative intensity of the expression of HSPs in stomach. Results are given as means ± SE. Average of relative intensity was obtained from three complete

experiments in the other mice. KO; MIF-deficient mice, WT; wild type mice.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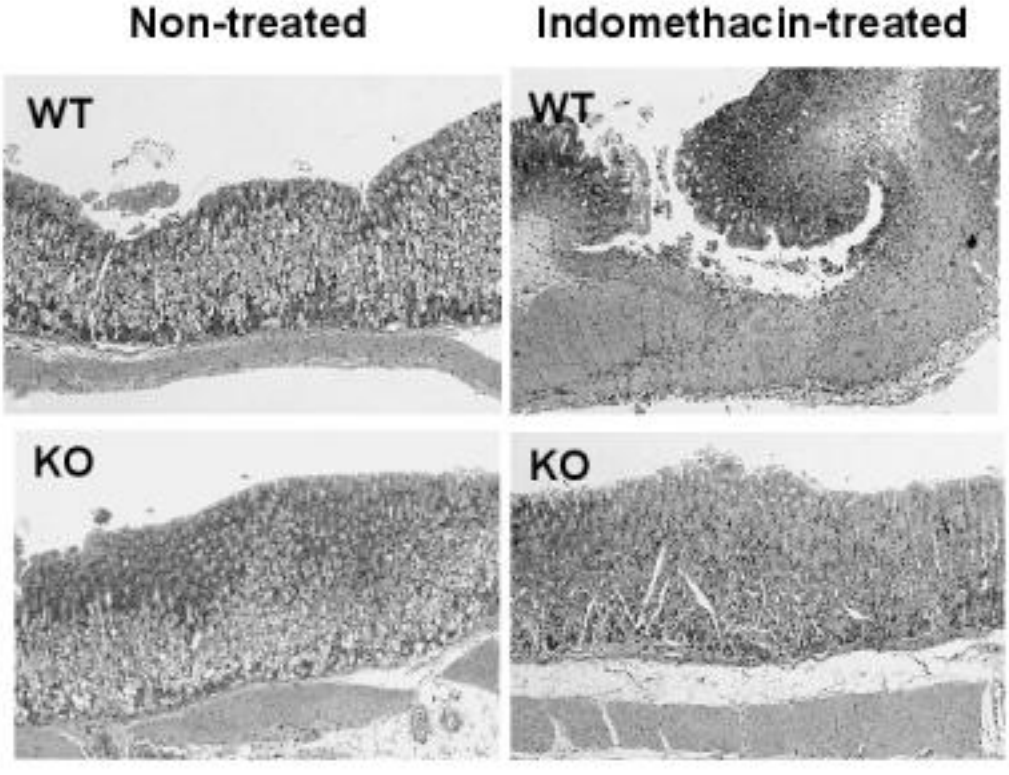
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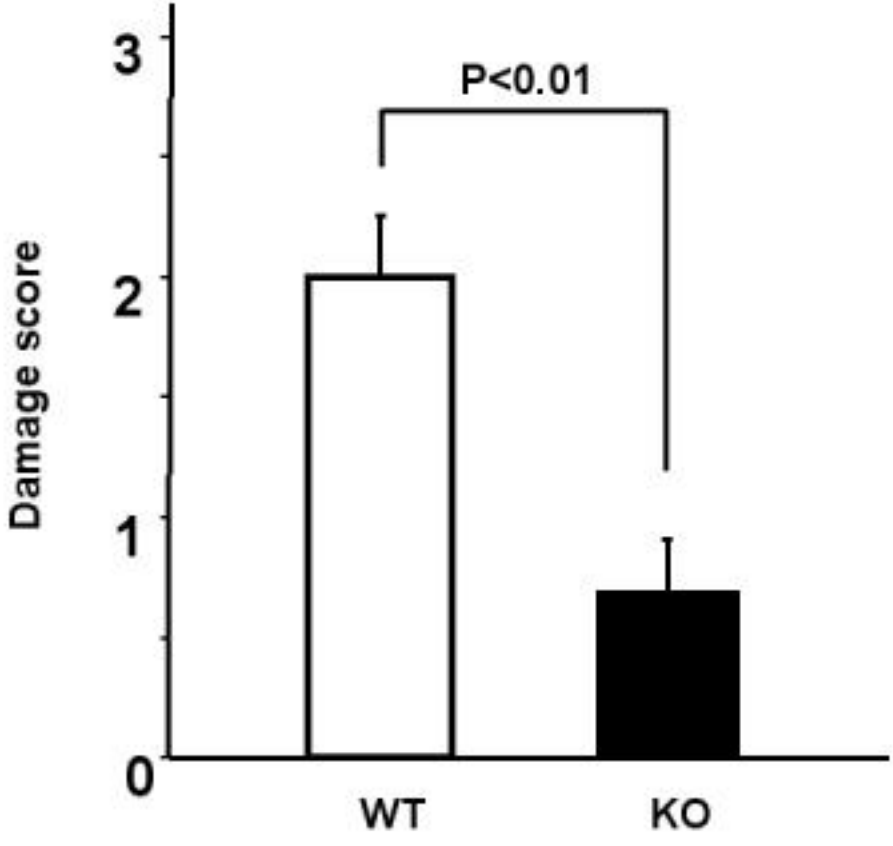
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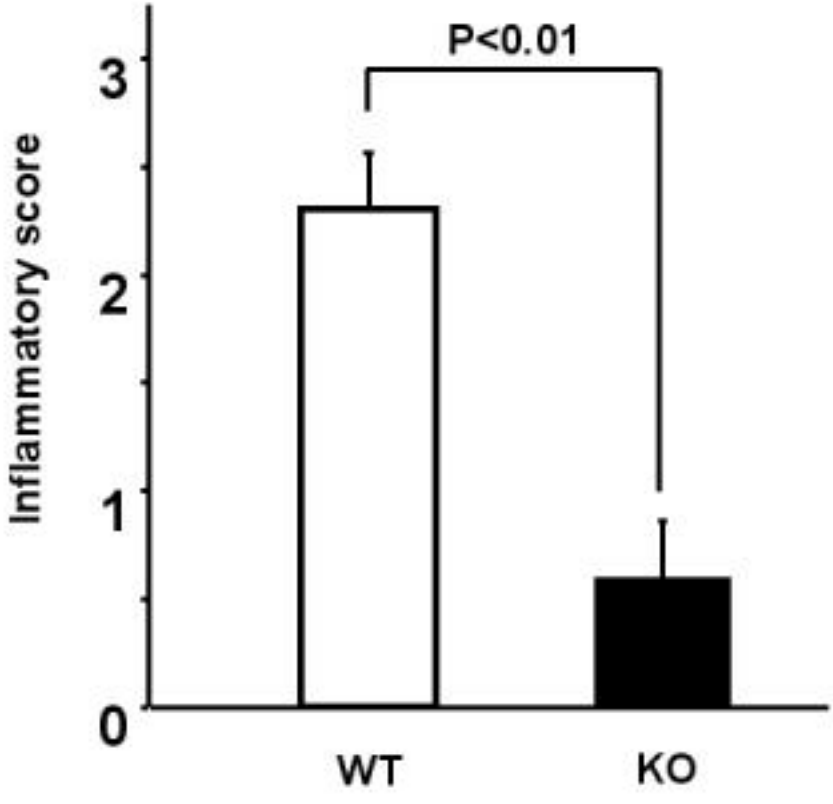
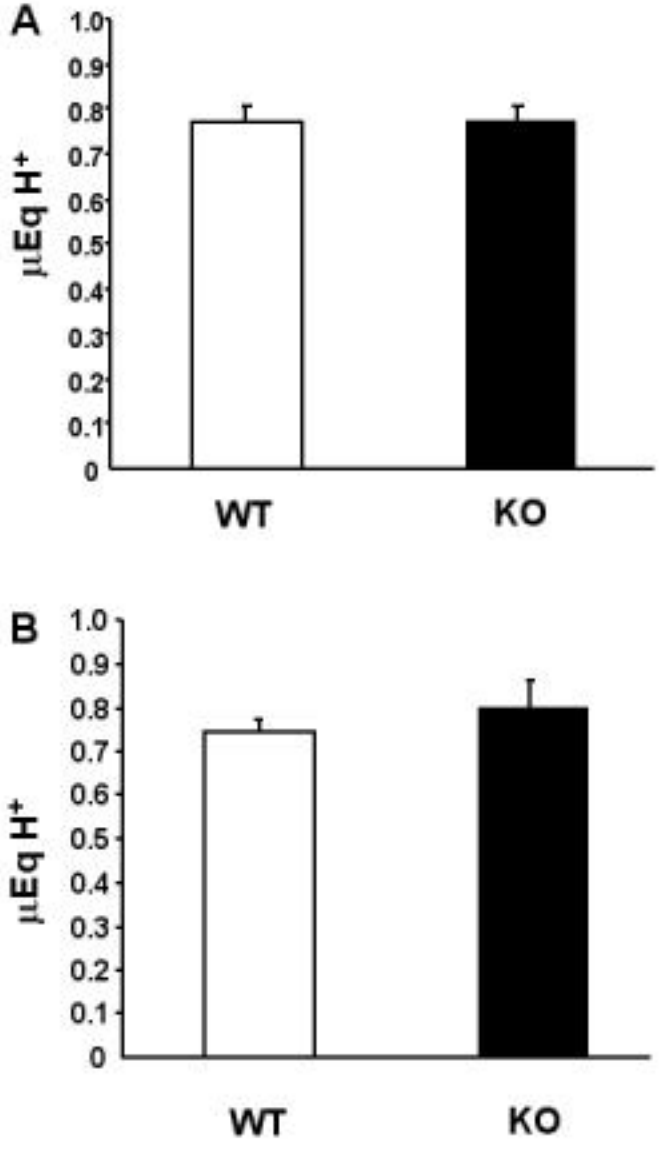
Ohkawara et al. Figure 2C

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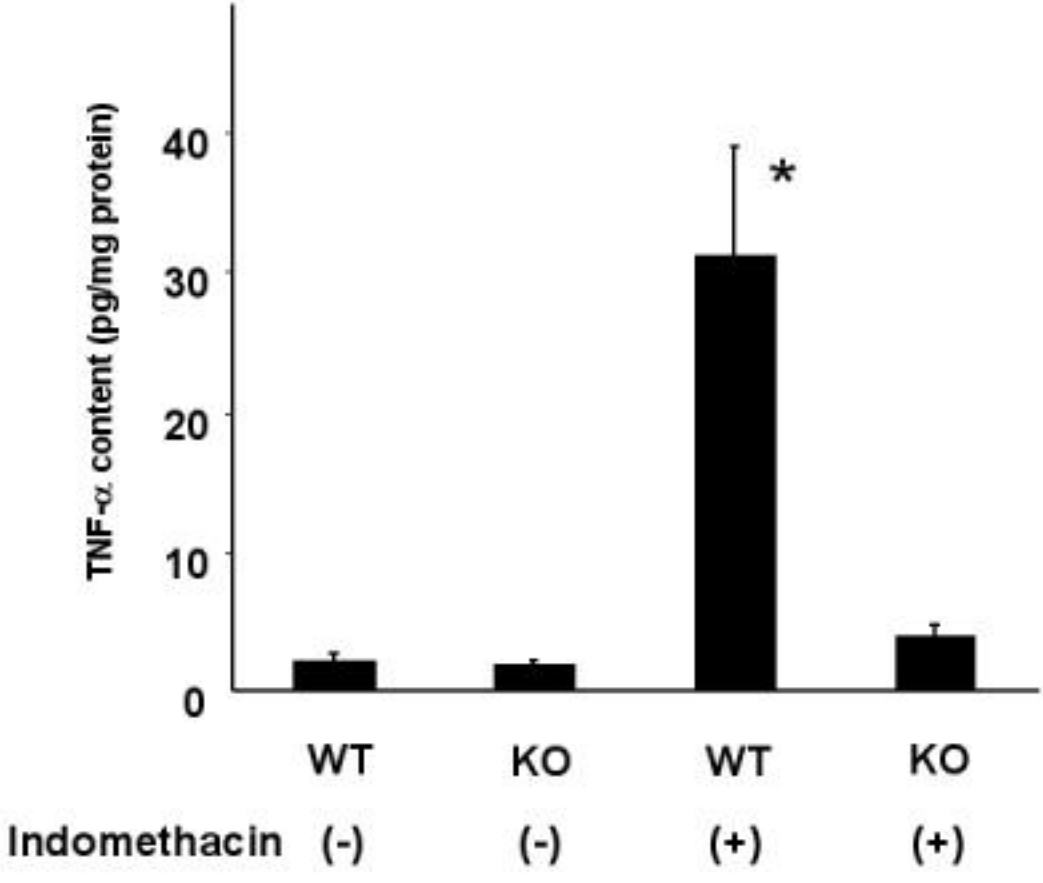
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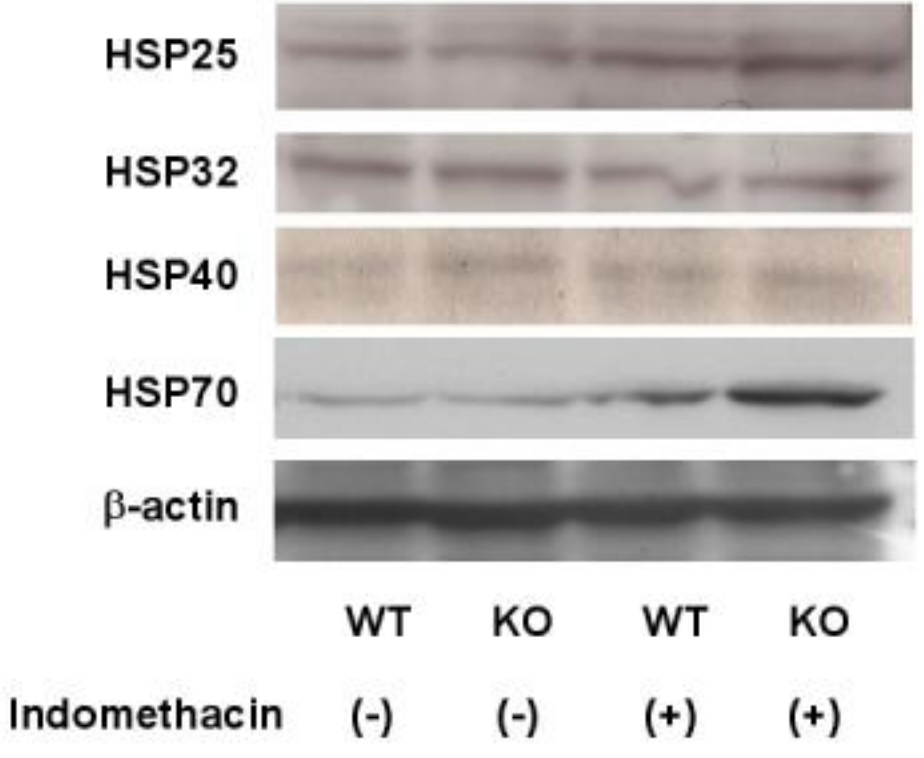
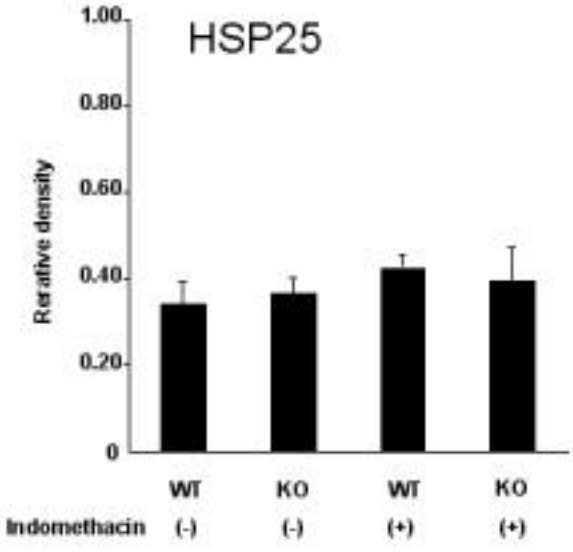
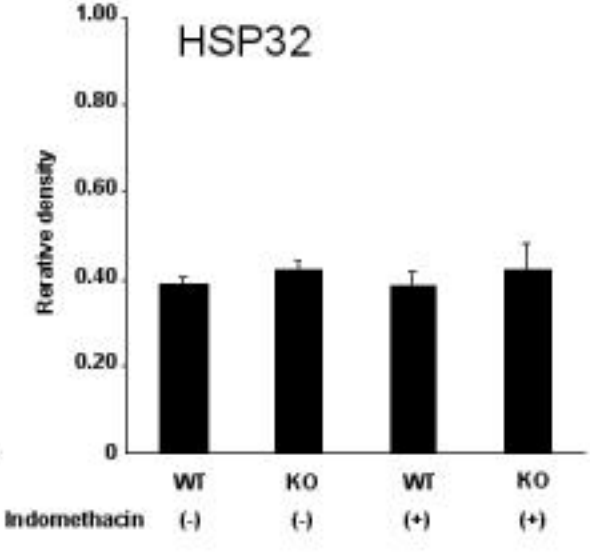
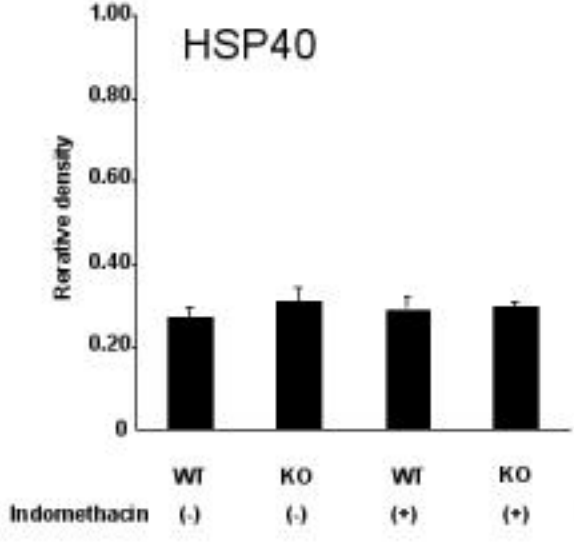
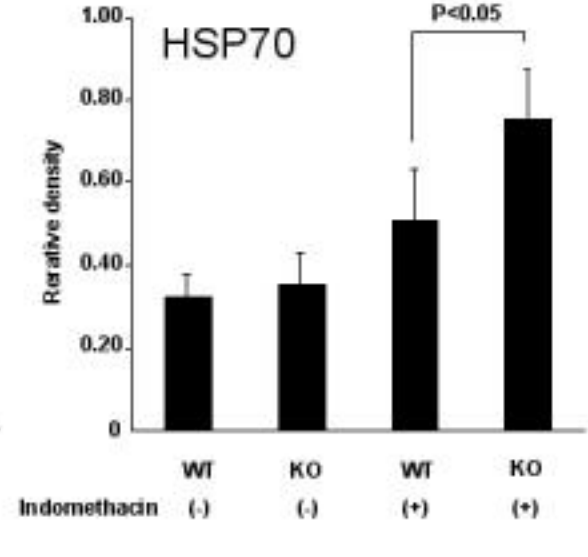
KO
Indomethacin (-) (-) (+) (+)


HSP25
HSP32
HSP40
HSP70
β-actin
WT
KO
WT
KO
Indomethacin
(-)
(-)
(+)
(+)


HSP25
Rerative density
1.00
0.80
0.60
0.40
0.20
0
WT
KO
WT
KO
Indomethacin
(-)
(-)
(+)
(+)


HSP32
Rerative density
1.00
0.80
0.60
0.40
0.20
0
WT
KO
WT
KO
Indomethacin
(-)
(-)
(+)
(+)


HSP40
Rerative density
1.00
0.80
0.60
0.40
0.20
0
WT
KO
WT
KO
Indomethacin
(-)
(-)
(+)
(+)


HSP70
P<0.05
Rerative density
1.00
0.80
0.60
0.40
0.20
0
WT
KO
WT
KO
Indomethacin
(-)
(-)
(+)
(+)