Geranylgeranylacetone protects against morphine-induced hepatic and renal damage in mice

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Abstract. The acute or chronic administration of opioid drugs may induce oxidative damage and cellular apoptosis in the liver and kidney, and hence result in hepatic and renal damage. Thioredoxin-1 (Trx-1) and heat shock protein 70 (Hsp70) are emerging as important modulators of cellular functions. They have been shown to be involved in cellular protective mechanisms against a variety of toxic stressors. The present study was designed to investigate the effects of geranylgeranylacetone (GGA), a pharmacological inducer of Trx-1 and Hsp70, on morphine-induced hepatic and renal damage. Morphine induced apoptosis in the liver and kidney through the mitochondria-mediated apoptosis pathway, but not the endoplasmic reticulum-mediated pathway. The activation of caspases-9 and -3 was attenuated by pre-treatment with GGA. In addition, the morphine-induced increase of malondialdehyde (MDA) levels was suppressed by GGA. Furthermore, GGA enhanced morphine-induced expression of Trx-1 and Hsp70 in the liver and kidney. The findings of this study suggest that GGA may be a safe and novel therapeutic agent for morphine-induced hepatic and renal damage.

Introduction

Addiction to opioid drugs such as morphine is a universal social and public problem. The repeated administration of opioid drugs may lead to the development of analgesic tolerance and dependence, which limit the clinical use of opioid drugs in pain treatment (1). Furthermore, prolonged exposure to opioid drugs may result in pathological changes in the liver in almost 100% of cases (2,3). A previous study found that 44% of drug addicts suffered from acute hepatitis, 34% from chronic hepatitis and 16% from liver damage (4). Lately, clinical studies also suggest that opiate addicts are at increased risk of progressive renal failure (5-7). Renal diseases in opiate addicts are associated with nephritic syndrome, acute glomerulonephritis and interstitial nephritis (8,9). The acute or chronic administration of opioid drugs may induce oxidative damage and cellular apoptosis in the liver and kidney, and hence result in hepatic and renal damage (10-13). Considering the role of the liver and kidney in metabolism and detoxification, opioid drug-induced liver and kidney toxicity has been a crucial issue in the therapy for drug abuse.

Previous studies have shown that blocking oxidative damage is a useful strategy for the treatment of opioid drug-induced hepatic and renal damage (14). There are several molecules to counteract oxidative stress in the body, such as thioredoxin-1 (Trx-1) and heat shock protein 70 (Hsp70). Trx-1 is a small ubiquitous protein with redox-controlled cell functions (15,16). Trx-1 has radical-scavenging activity and couples with peroxiredoxin to scavenge hydrogen peroxide (17,18). Trx-1 is able to protect cells from hydrogen peroxide, UV irradiation and ischemic reperfusion (19,20). Hsp70 is a stress-inducible protein that prevents protein aggregation and facilitates refolding of dysfunctional proteins (21). Hsp70 is able to enhance the survival of cells and inhibits apoptosis induced by oxidative damage during various stress conditions. Geranylgeranylacetone (GGA) is an acyclic polyisoprenoid and widely used as an anti-ulcer drug in the clinical setting. Previous studies have suggested that GGA induced Trx-1 and Hsp70 expression in various cells to exert cytoprotection (22-24). GGA has been widely reported to exert cytoprotective effect against various stresses, such as oxidative stress in the gastric mucosa, liver, heart and brain (25-28). Our previous study also has demonstrated that GGA protected against morphine-induced hyperlocomotion, rewarding effect and withdrawal syndrome in mice (29). However, there are no studies of the role of GGA in the liver and kidney toxicity induced by opioid drugs. In the present study, we postulated that GGA induces Trx-1 and Hsp70 expression, which may in turn prevent opiate-induced hepatic and renal injury. The present study was designed to evaluate the protective effect of GGA against morphine-induced hepatic and renal damage in mice.

Materials and methods

Reagents. GGA was purchased from Eisai (Tokyo, Japan). Anti-mouse Trx-1 rabbit polyclonal antibody was obtained from Redox Bioscience (Kyoto, Japan). Antibodies

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(pro-caspase-12 and -9, caspase-3, β-actin and Hsp70) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Morphine hydrochloride was purchased from the Shenyang First Pharmaceutical Factory, Northeast Pharmaceutical Group Corporation (Shenyang, China). Mouse malondialdehyde (MDA) enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA).

Animals. Sixteen C57BL/6 mice (Chongqing Medical University, China; age, 6-7 weeks) were used in the experiments. The mice were housed in plastic cages and kept on a 12-h light-dark cycle and had free access to food and water. The animals were cared for and used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiment was approved by the local Committee on Animal Use and Protection.

GGA and morphine treatment. Granulated GGA was suspended in saline immediately before use. Mice were administered saline or GGA (800 mg/kg, orally), using a micropipette 2 h prior to morphine treatment. The mouse model of morphine dependence was created as described in our previous study (29). The mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg) for 7 days. Two hours following the last morphine injection, the mice were sacrificed using deep anesthesia, and then heart perfusion was performed using saline. The liver and kidney tissues were rapidly dissected, immediately frozen and stored in a deep freezer at -80°C until use.

Western blot analysis. Protein lysates were prepared using a solubilizing solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerolphosphate and 1 mg/ml leupeptin]. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). An equal quantity of proteins was separated by 10% (for Hsp70), 12% (for pro-caspase-12) or 15% (for pro-caspase-9, caspase-3 and Trx-1) SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was soaked in 10% skimmed milk (in PBS, pH 7.2, containing 0.1% Tween-20) overnight at 4°C, then incubated with primary antibody followed by peroxidase-conjugated anti-mouse or anti-rabbit IgG (KPL, Gaithersburg, MD, USA). The epitope was visualized using an ECL Western Blot Detection kit (Millipore Corporation). Densitometry analysis was performed using the ImageJ software.

ELISA. The liver and kidney tissues were pooled and homogenized in PBS. Samples were spun at 4,000 rpm for 15 min at 4°C. The supernatant was aliquoted and stored at -80°C for future study. The concentration of MDA was measured using specific ELISA kits (R&D, USA), according to the manufacturers' instructions.

Statistical analysis. Data are expressed as the mean ± SD values. Statistical analysis was performed using the SPSS software. The one-way ANOVA followed by a post hoc
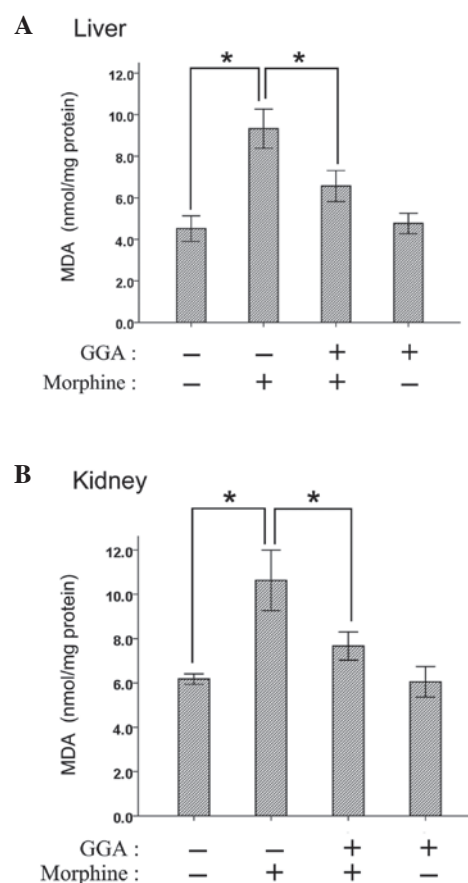

Figure 1. GGA suppressed morphine-induced lipid peroxidation in the liver and kidney. Mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg), 2 h after GGA treatment (800 mg/kg, orally) for 7 days. Two hours after the last morphine injection, the liver and kidney tissues of mice were dissected. The MDA level in (A) the liver and (B) kidney was measured using ELISA. Values are the mean ± SD; n=4. Asterisks indicate a statistically significant difference (*P<0.05). GGA, geranylgeranylacetone; MDA, malondialdehyde; ELISA, enzyme-linked immunosorbent assay.

multiple comparison test was used to compare control and treated groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of GGA on lipid peroxide formation in the liver and kidney after chronic morphine treatment. As an important marker of oxidative lipid damage, MDA was examined to estimate the extent of lipid peroxidation in the present study. The levels of MDA in the liver and kidney were significantly increased after chronic morphine treatment (Fig. 1). By contrast, the pre-treatment of GGA significantly reduced lipid peroxide content in the liver and kidney.

Effect of GGA on morphine-induced apoptosis in the liver and kidney. To clarify the manner in which GGA affects cell death pathways involved in the morphine-induced hepatic and renal damage, the alterations of pro-caspases-9 and -12 and caspase-3 were examined following chronic morphine administration with or without GGA pre-treatment using western blotting. Chronic morphine administration led to the activa-
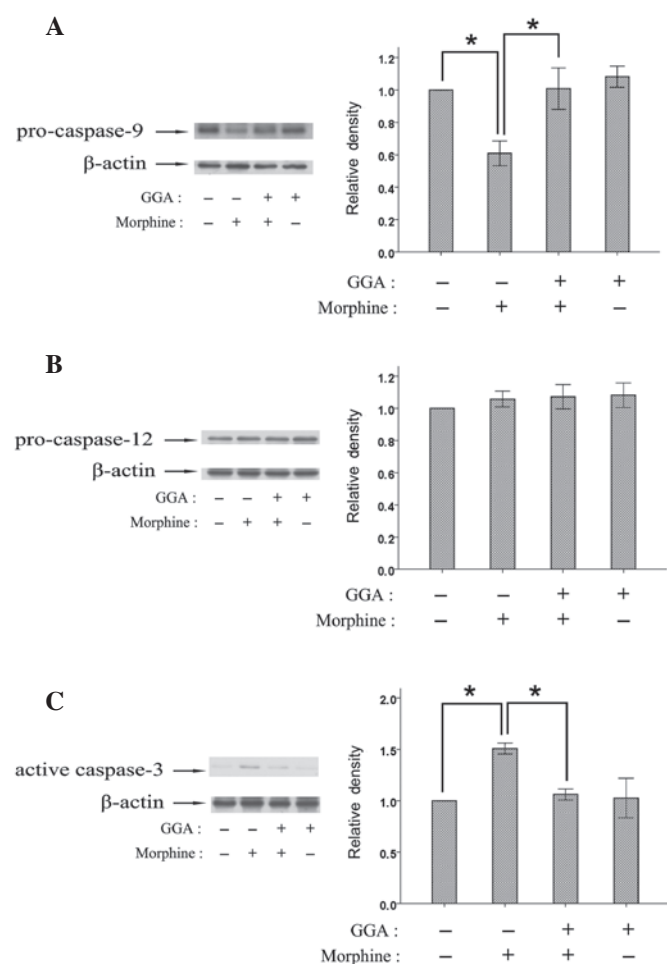
Figure 2. GGA regulated the activation of caspases induced by morphine in the liver. Mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg), 2 h after GGA treatment (800 mg/kg, orally) for 7 days. Two hours after the last morphine injection, the liver tissues of mice were dissected, and the expression of (A) pro-caspase-9, (B) -12 and (C) caspase-3 was detected using western blotting. Quantification is shown in the right panels (error bars represent the mean± SD; n=4). Density values for each band were normalized first to loading control and then to the control group. Asterisks indicate a statistically significant difference (*P<0.05). GGA, geranylgeranylacetone.
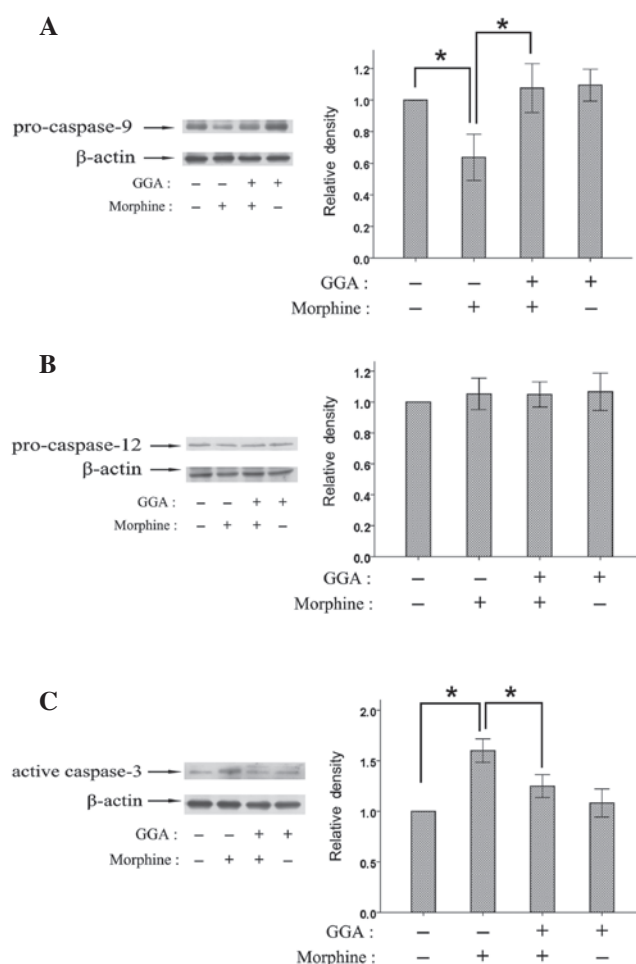
Figure 3. GGA regulated the activation of caspases induced by morphine in the kidney. Mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg), 2 h after GGA treatment (800 mg/kg, orally) for 7 days. Two hours after the last morphine injection, the kidney tissues of mice were dissected, and the expression of (A) pro-caspase-9, (B) -12 and (C) caspase-3 was detected using western blotting. Quantification is shown in the right panels (error bars represent the mean ± SD; n=4). Density values for each band were normalized first to loading control and then to the control group. Asterisks indicate a statistically significant difference (*P<0.05). GGA, geranylgeranylacetone.

tion of caspases-9 and -3 in the liver and kidney, while the level of pro-capase-12 was not affected by morphine (Figs. 2 and 3). Furthermore, the pre-treatment with GGA significantly inhibited the activation of caspases-9 and -3 induced by morphine in the liver and kidney.

Effects of GGA on Trx-1 and Hsp70 expression in the liver and kidney following chronic morphine treatment. To investigate the roles of Trx-1 and Hsp70 underlying the protective effect of GGA against morphine-induced hepatic and renal damage, the levels of Trx-1 and Hsp70 were examined following morphine treatment. Morphine significantly increased the levels of Trx-1 and Hsp70 expression in the liver and kidney (Figs. 4 and 5). GGA was also found to enhance morphine-induced expression of Trx-1 and Hsp70 in the liver and kidney. The above data indicated that GGA induced the expression of Trx-1 and Hsp70, which may be involved in the protective roles of GGA against morphine-induced hepatic and renal damage.

Discussion

GGA is an anti-ulcer drug developed in Japan and is known to protect against organ and cell damage via inducing Hsp70 or Trx-1 expression. In the present study, we found that the pre-treatment of GGA suppressed morphine-induced lipid peroxidation and activation of caspases-9 and -3 in the liver and kidney. These findings are consistent with those of previous studies, which reported the protective effect of GGA against hepatic and renal damage caused by other stimulations (30,31). GGA suppressed the H_2O_2 and ethanol-induced activation of JNK and caspases-9 and -3, leading to significant inhibition of apoptosis in rat hepatocytes (32). Oral administration of GGA protects liver damage following massive hepatectomy, warm ischemic-reperfusion injury and exposure of acetaminophen in rats (31,33,34). In addition, GGA ameliorated ischemic acute renal failure via inducing Hsp70 expression (35). The dose of GGA used in this study is 800 mg/kg. However, it did not show toxic effects in the present study. The results of our
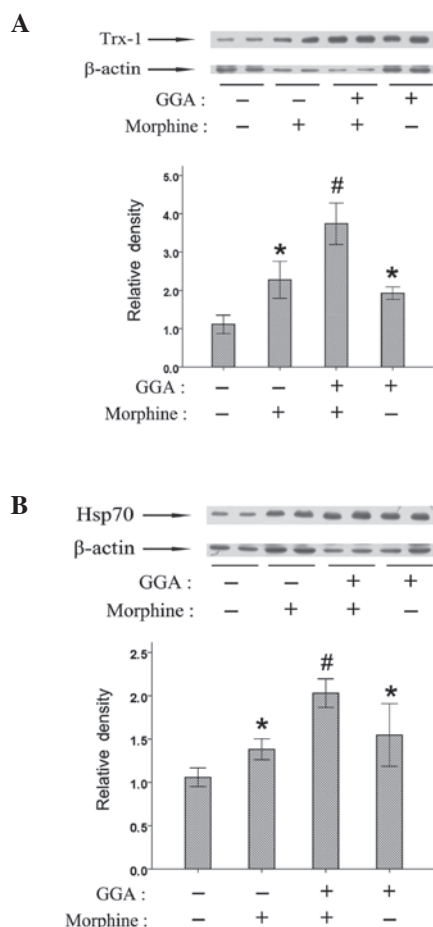
Figure 4. Changes in the expression of Trx-1 and Hsp70 after morphine treatment in the liver. Mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg), 2 h after GGA treatment (800 mg/kg, orally) for 7 days. Two hours after the last morphine injection, the liver tissues of mice were dissected, and the expression of (A) Trx-1 and (B) Hsp70 was detected using western blotting. Quantification is shown in the right panels (error bars represent the mean ± SD; n=4). Density values for each band were normalized first to loading control and then to the control group. *P<0.05 vs. the control group, and #P<0.05 vs. the chronic morphine-treated group. GGA, geranylgeranylacetone; Hsp, heat shock protein.
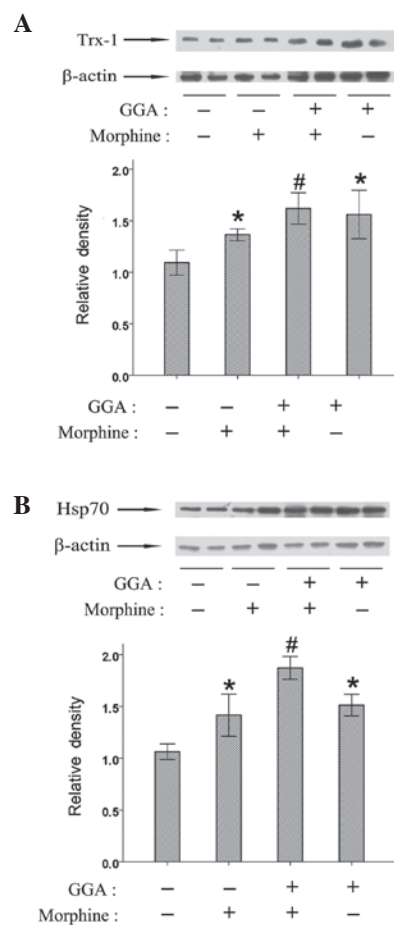

Figure 5. Changes in the expression of Trx-1 and Hsp70 after morphine treatment in the kidney. Mice were injected with escalating morphine doses twice daily (10, 20, 40, 60, 80 and 100 mg/kg), 2 h after GGA treatment (800 mg/kg, orally) for 7 days. Two hours after the last morphine injection, the kidney tissues of mice were dissected, and the expression of (A) Trx-1 and (B) Hsp70 was detected using western blotting. Quantification is shown in the right panels (error bars represent the mean ± SD; n=4). Density values for each band were normalized first to loading control and then to the control group. *P<0.05 vs. the control group, and #P<0.05 vs. the chronic morphine-treated group. GGA, geranylgeranylacetone; Hsp, heat shock protein.

previous animal experiment revealed that the pre-treatment of GGA (800 mg/kg, 1 week) protected against morphine-induced hyperlocomotion, rewarding effect and withdrawal syndrome (29). Oral GGA (600 and 1,200 mg/kg, 7 weeks) ameliorates symptomatic phenotypes of spinal and bulbar muscular atrophy mice (36). There is little information concerning any adverse drug effects of GGA. These results suggest that GGA is a promising safe therapeutic candidate for opioid drug-induced hepatic and renal damage. Besides the cytoprotection in the liver and kidney, GGA also exerts protective effects against various stresses in other organs, including the heart and brain (37-39). The toxicities of morphine in a number of tissues, including the spinal cord and brain, have also been reported (40,41). Whether or not GGA is able to protect against morphine-induced toxicity in other organs remains to be elucidated.

Liver and kidney toxicities have been reported in chronic use of morphine (10,42). Serum aspartate aminotransferase and alanine aminotransferase levels are increased following exposure to morphine in rats (13). Animal studies have

shown that morphine may have a direct effect on the glomerulus, causing oxidative stress, proliferation of fibroblasts and a decrease in the degradation of type IV collagen (43-45). Excessive free radical formation or antioxidant deficiency may result in oxidative stress, a possible mechanism of the toxicity of opioid drugs. Heroin abusers exhibited increased levels of lipoperoxides and nitric oxide. The levels of various antioxidants, including superoxide dismutase, glutathione and catalase, were decreased following heroin or morphine exposure (46). It has also been reported that morphine may result in lipid peroxidation in the liver and kidney (13). In the present study, we found that morphine increased MDA levels, demonstrating lipid peroxidation in the liver and kidney following chronic morphine treatment.

Extrinsic and intrinsic cell death pathways have been shown to be involved in morphine-induced apoptosis. The extrinsic cell death pathway has been reported to contribute to morphine-induced macrophage apoptosis. Morphine enhanced Fas and FasL expression in macrophages (47). The intrinsic cell death pathway includes the mitochondria-mediated

apoptosis pathway and endoplasmic reticulum (ER)-mediated apoptosis pathway. Although activation of several distinct upstream pathways may lead to apoptosis, caspase-3 may be a common integration point (48). In the present study, we found that caspase-3 was activated by morphine, suggesting that morphine leads to apoptosis in the liver and kidney. Mitochondrial dysfunction leads to the release of cytochrome c into the cytoplasm, and initiates the activation of the caspase cascade by triggering the activation of caspase-9, which elicits the activation of caspase-3, leading to the morphological changes associated with apoptosis (49). The results of *in vivo* and *in vitro* studies indicate that caspases-9 and -3 are activated following morphine treatment in macrophages, the spinal cord or brain (41,50). In this study, we demonstrated that morphine decreased the level of pro-caspase-9 expression, indicating that morphine induces apoptosis through the mitochondria-mediated pathway in the liver and kidney. Pro-caspase-12 is localized on the cytoplasmic side of ER and is proteolytically activated by excess ER stress. ER stress-induced cell death has been reported to be mediated by the activation of caspase-12 (51). Caspase-12-deficient mice are resistant to ER stress-induced apoptosis (52). However, in the present study, the level of pro-caspase-12 expression was not affected by morphine in the liver and kidney. These findings suggest that the ER-mediated apoptosis pathway is not involved in the morphine-induced apoptosis in the liver and kidney.

Eukaryotic cells have developed multiple mechanisms to mount a response against environmental stressors. Cellular stress responses require the activation of pro-survival pathways and the production of molecules with antioxidant and anti-apoptotic activities, allowing cells to effectively counteract diverse stresses. Among the cellular pathways providing protection against environmental stressors, a key role is played by vitagenes, such as heat shock proteins and Trx-1 (53). Trx-1 expression is upregulated by various stressors, including viral infection, mitogens, polycyclic aromatic hydrocarbons, X-ray and UV irradiation, hydrogen peroxide and ischemic reperfusion (54). Hsp70 is also induced in response to a wide variety of stressors, such as heat shock, ischemia, hydrogen peroxide and light damage (26). The induction of Trx-1 and Hsp70 contributes to cytoprotection against diverse stresses. It has been reported that morphine increased Hsp70 expression in the frontal cortex, amygdale and spinal cord (41,55,56). Our previous study demonstrated that the levels of Trx-1 and Hsp70 expression in the nucleus accumbens of mice brain are increased after chronic morphine treatment (29). In the present study, we found that Trx-1 and Hsp70 were induced by morphine in the liver and kidney. These results suggest that cellular defense mechanisms are activated in the liver and kidney to resist the cellular damage caused by morphine.

Modulation of endogenous cellular defense mechanisms via the stress response signaling is becoming an innovative approach to therapeutic intervention in diseases. Several studies have used Trx-1 or Hsp70 inducers, including GGA, sulforaphane, panaxatriol saponins and neurotropin, for therapeutic purposes (57-60). In the present study, we found that the increased level of MDA in the liver and kidney was suppressed by the pre-treatment with GGA. The morphine-induced decrease of pro-caspase-9 was suppressed by GGA. Furthermore, GGA inhibited the morphine-induced activation

of caspase-3 in the liver and kidney. These results suggest that GGA treatment may be beneficial for preventing morphine-induced hepatic and renal damage. In the present study, we also demonstrated that GGA increased Trx-1 and Hsp70 expression in the liver and kidney following morphine treatment. A previous study has shown that morphine promotes p38 MAPK phosphorylation via opiate receptors through TGF-β and iNOS-mediated downstream signaling (47). Trx-1 is known to have radical-scavenging activity and scavenge hydrogen peroxide in cooperation with peroxiredoxin. In addition, Trx-1 has been reported to act as an endogenous inhibitor of apoptosis signaling kinase 1 and p38 MAPK (61,62). Hsp70 regulates the cellular redox status by modulating glutathione-related enzyme activities (63). Hsp70 enhances the survival of cells and prevents apoptosis against oxidative damage during various stress conditions (21). The antioxidant effects of GGA were previously reported in the ethanol- and H_2O_2-induced cultured rat hepatocyte injury and in light-induced photoreceptor cell damage through inducing Trx-1 expression (58,64). Thus, the protective effects of GGA against morphine-induced lipid peroxidation and apoptosis in the liver and kidney might contribute to the increased expression of Trx-1 and Hsp70.

In conclusion, the findings of the present study indicate that morphine induces lipid peroxidation and mitochondria-mediated apoptosis, and regulates Trx-1 and Hsp70 expression in the liver and kidney. GGA induced Trx-1 and Hsp70 expression and suppressed morphine-induced lipid peroxidation and apoptosis in the liver and kidney. Our results suggest that GGA is a promising therapeutic candidate for morphine-induced hepatic and renal damage.

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