Research Article

Protective effect of heme oxygenase-1 induction in rat models of acute pancreatitis

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Abstract
Background: Hemin, a heme-oxygenase-1 (HO-1) inducer has a profound role in treatment of acute pancreatitis as acute pancreatitis (AP) is associated with oxidative injury and inflammation. **Methods and materials:** The effect of both hemin and pioglitazone were examined in a rat model of AP induced by L-arginine. Zinc protoporphyrin (ZnPP) was used as an HO-1 inhibitor. **Results:** The rats in the AP group showed a severe AP evident by rising in many parameters such as HO-1, and this was associated with immunohistological alterations. Treatment with hemin significantly attenuated these changes and further significantly increased pancreatic HO-1 protein level. However, these effects of hemin is opposed by ZnPP pre-treatment. Furthermore, immunohistological analysis confirmed the protective role of hemin by prevention of the pancreatic injury in the rats who received these treatments compared to those animals that received only L-arginine. **Conclusion:** The present study shows that hemin treatment significantly ameliorated the L-arginine-induced AP, by inducing HO-1which has anti-inflammatory and antioxidant activities.
Key Words: hemin, heme-oxygenase-1, acute pancreatitis, pioglitazone

Introduction
Acute pancreatitis (AP) is prevalent inflammatory disease. It causes many complications[1-3]. Which associated with Reactive oxygen species (ROS), and there is a correlation between the production of ROS and the degree of severity of AP[1]. Oxidative stress caused by ROS has a significant role in lipid peroxidation which leads to pancreatic injury caused by premature activation of digestive enzymes of the pancreas[2].

The oxidative stress in addition to inflammatory cytokines are substantially contributory to the pathogenesis of AP and this can progress to systemic morbidities such as multi-organ failure and shock[3,6,7,8,9].

Hemeoxygenase (HO)-1 is an oxidative stress enzyme. It is inducibly-expressed enzyme. However, its isoform, HO-2 is a constitutive enzyme[3]. HOs are essential for the metabolism of heme into iron, biliverdin, and carbon monoxide (CO)[4,5]. It has been previously shown that pretreatment with hemin, a hemoglobin prosthetic moiety that increases HO-1 level, has a prophylactic property against AP in mice model[6].

Materials and methods
Animals:
Adult male Sprague-Dawley rats (200–240 g, 10–12 weeks old) were dwelled with light/dark cycles of 12:12 h in room temperature and were provided by food and water ad libitum. Experimental procedures were performed according to the international ethical guidelines for animal care of the United States Naval Medical Research Centre, and the Unit No. 3, Abbaseya, Cairo, Egypt, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC international), and also comply with "Principles of Laboratory Animals Care" (NIH publication No. 85-23, revised 1985). The Ethics Committee of the Faculty of Pharmacy, Minia University, Egypt, approved the research protocol.

Chemicals:
L-arginine was sourced from Euromedex, souffel weyersheim, France. Hemin and Zinc protoporphyrin (ZnPP) were procured

from Sigma-Aldrich, USA. Other supportive reagents and chemicals were of high analytic grade.

Experimental design

The study was conducted on 6 groups of rats as follow:

Group 1; (C) Control group, rats were normal, received only solvent with the same volume.

Group 2; (AP), rats treated with a 750 mg/kg single dose intraperitoneal (*i.p.*) injection of L-arginine to induce AP[7].

Group 3; (AP + hemin), rats with induced AP were given hemin (10 mg/kg, *i.p.*) for 3 successive days from the day of induction till the sacrifice day[6].

Group 4; (AP + hemin + ZnPP), rats with induced AP were pretreated with ZnPP; (6.25 mg/kg, *i.p.*) and hemin (10 mg/kg, *i.p.*) for 3 successive days from the day of induction till the sacrifice day.

Group 5; (AP + pioglitazone), Rats with induced AP were given pioglitazone (50 mg/kg, *p.o.*) for 3 successive days from the day of induction till the sacrifice day[8].

Group 6; (AP + pioglitazone + ZnPP) Rats with induced AP were pretreated with ZnPP and (6.25 mg/kg, *i.p.*) and pioglitazone (50 mg/kg, *p.o.*) for 3 successive days from the day of induction till the sacrifice day[8].

Western blot analysis

Pancreatic tissue samples from normal and rats with induced acute pancreatitis were collected in ice-cold lysis buffer that contains the following: 20 mmol/L Tris-HCl, 1 mmol/L ethylene diamine tetra acetic acid (EDTA), 140 mmol/L sodium chloride, 1% sodium deoxycholate, 0.1% Sodium Dodecyl Sulphate (SDS), 1% Triton X-100, 1 mmol/L sodium fluoride, 1 mmol/L orthovanadate, and complete miniprotease inhibitor cocktail, pH 7.8. SDS–Polyacrylamide Gel Electrophoresis (PAGE) was used to separate equal amounts of protein (20µg/lane) and the resultant protein bands were blotted to polyvinylidene difluoride membranes (PVDF). After blocking by (4% skim milk, Sigma), PVDF membranes were probed by the primary antibodies; polyclonal anti-HO-1 antibodies (Santa Cruz, Biotechnology Inc., USA) for two hours at room temperature. After washing PVDF membranes in washing buffer, they were incubated in 1:2500 dilution of secondary antibody (Amersham Life Science, USA) for 50 min. Using enhanced chemiluminescence system (Amersham Life Science. USA) the membranes were detected. For protein loading difference correction, the membranes were washed and incubated with 1:1000 dilution of ß-actin antibody (Abcam, Cambridge, UK). Protein bands were scanned and their densities were measured by AIDA Image Analyzer Software.

Reverse transcriptase polymerase chain reaction (RT-PCR)

From 250 ng of total RNA, cDNA was obtained by iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as described by the protocol of the manufacturer. Using SYBR green PCR Master Mix (Bio-Rad, Hercules, CA), real-time PCR was carried out on the iCycler (Bio-Rad, Hercules, CA) in 20-µl reaction volume as described by the manufacturer. Both agarose gel electrophoresis and melting curve analysis were used to confirm PCR amplification specificity. The sequences of PCR primers (Sigma-Aldrich, Egypt) used in the present study were as follows: β-actin: sense, 5'-TTCTACAATGAGCTGCGTGTG-3'; antisense,5'-GGGGTGTTGAAGGTCTCAAA-3'PPARγ:sense, 5′:CTATGGAGTTCATGCTTGTG-3′;antisense,5′-GTACTGACATTTATTT -3′; have been assured to have amplification capacity equivalent to that of β-actin gene, which was also used as an internal reference for normalizing the relative expression of the target genes.

Immunohistochemistry

Sections of pancreatic tissue were deparaffinized, rehydrated and incubated in hydrogen peroxide for 5 min. To avoid non-specific staining, ultra V Block was added. The pancreatic tissue sections were probed overnight with 1:500 dilution of the primary antibody; anti-TGFβ antibody (ThermoFisher Scientific, USA) at 4ºC. For visualization, poly HRP DAB kit (Genemed Biotechnologies, USA) was applied to the sections, followed by the secondary chromogenic antibody. The stained sections were analyzed under an ordinary light microscope.

Statistical analysis

GraphPad Prism 5.0 software was used for statistical analysis. The results are shown as means ± standard error of the mean (SEM). The one-way ANOVA followed by the Tukey–Kramer post analysis test were used for statistical comparisons between means. Statistical significance level was set as probability (*P*) value of equal or below 0.05.

Results

Pancreatic HO-1 level

Effect of pioglitazone and Hemin, and their combination with ZnPP on HO-1 protein expression level in L-argnine induced AP rats were examined. Treatment with either pioglitazone or hemin caused an increase in the level of HO-1 relative to that in rats with AP. However, co-administration of pioglitazone or hemin with ZnPP inhibited this effect to some extent (Fig. 1).

Expression of PPARγ in pancreatic tissue by RT-PCR and immunohistochemistry

Expression of PPARγ gene in L-arginine-induced AP model (Fig. 2) showed that PPARγ was decreased in AP group compared with control group ($P<0.05$). Administration of pioglitazone or hemin significantly increased the levels of PPARγ ($P<0.05$), and this suggests that pioglitazone and hemin treatment could alleviate the inflammatory process of AP *via* activetion of PPARγ expression. However, ZnPP attenuated this mitigating effect (Fig. 2).

Discussion

The present study showed the anti-oxidant and anti-inflammatory properties of hemin due to HO-1 induction. However, this protective effect was attenuated by ZnPP.[13,14].

hemin significantly reduced the inflamematory markers such as TGFβ which play an essential role in tissue inflammation[25}.
It was reported that HO-1 mediates anti-oxidant and anti-inflammatory defense mechanisms in the cell[26]. HO-1 induction has also contributed to attenuation of inflammatory processes[27]. The anti-inflammatory property of HO-1 showed correlation with the anti-oxidant properties of both CO and biliverdin, both which are the final products of heme metabolism *via* HO-1-mediated catalysis[28]. Several *in vitro* and *in vivo* experiments have demonstrated that the increase of HO-1 expression levels has an essential cytoprotective property against oxidative stresses[29-31].

Furthermore, hemin has been shown to markedly increased HO-1 expression in two models of pancreatitis induced by caerulein and choline-deficient diet, resulting in a protective effect[6]. Hence, the finding of the current study showing that, pioglitazone and hemin markedly upregulated HO-1 is significant. This effect is consistent with the anti-oxidant, and anti-inflammatory effects of both pioglitazone and hemin.

Pioglitazone and hemin decreased the damage following AP in both the pancreatic tissue and the systemic response. The ameliorative effects of pioglitazone on oxidative stress and inflammation were confirmed by biochemical and immune-histochemical evaluations of the pancreatic tissue.

We conclude from these studies that both pioglitazone and hemin given during induction of experimental AP resulted in amelioration of injury. This response is probably mediated by their anti-oxidant and anti-inflammatory properties, and partially due to HO-1 expression activation.

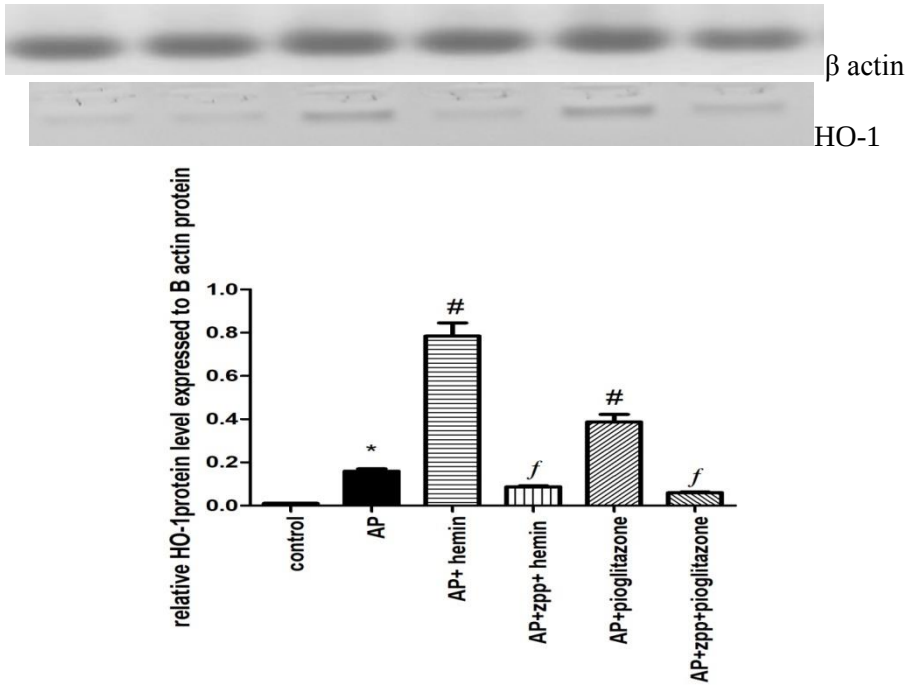

Fig. 1 Western blot analysis of HO-1 in the studied groups
Western blot for representative samples for the effect of PIO or hemin and their blockers on HO-1 protein expression in the pancreatic tissues (top panel). Bar charts quantitatively show the differences in HO-1 protein expression. Values are represented as the mean ± SEM Data represent the mean ± SEM, $^{*}P<0.05$ AP vs. control group; $^{\#}P<0.05$ AP vs. treated groups $^{\#}P<0.05$ AP vs. blocker groups, $f\,P<0.05$ treated groups vs. blocker groups

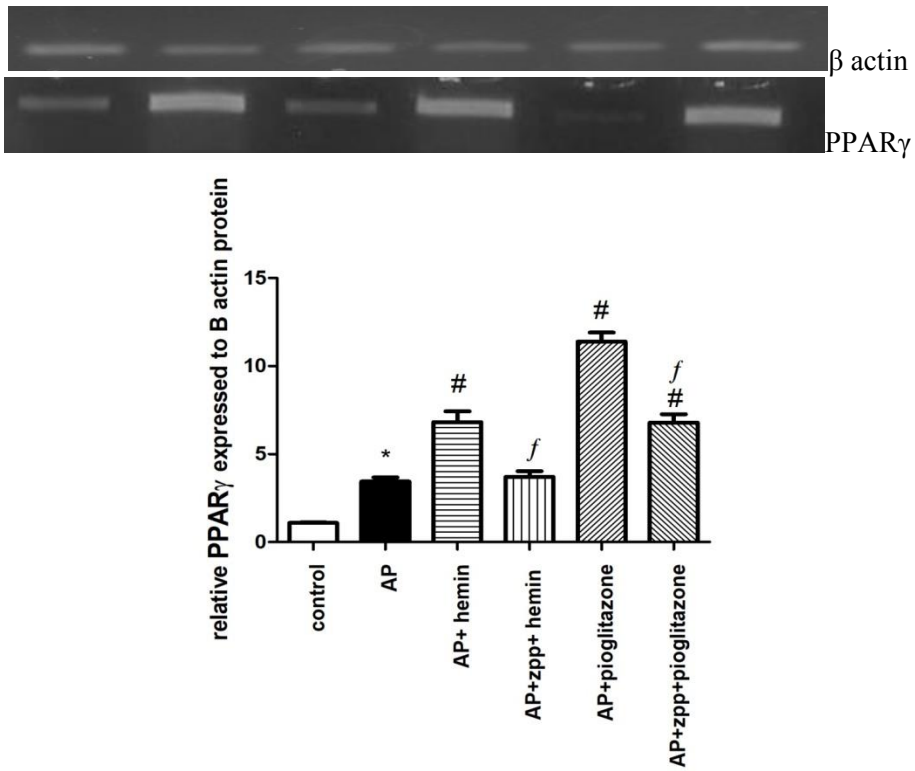

Fig. 2 RT-PCR of PPARγ in the studied groups.
Representative blots displaying influence of pioglitazone or hemin with and without ZnPP on PPARγ expression in the pancreatic tissue samples. Bar charts show the levels of PPARγ expression. Values for each bar show the mean ± SEM (bottom panel)

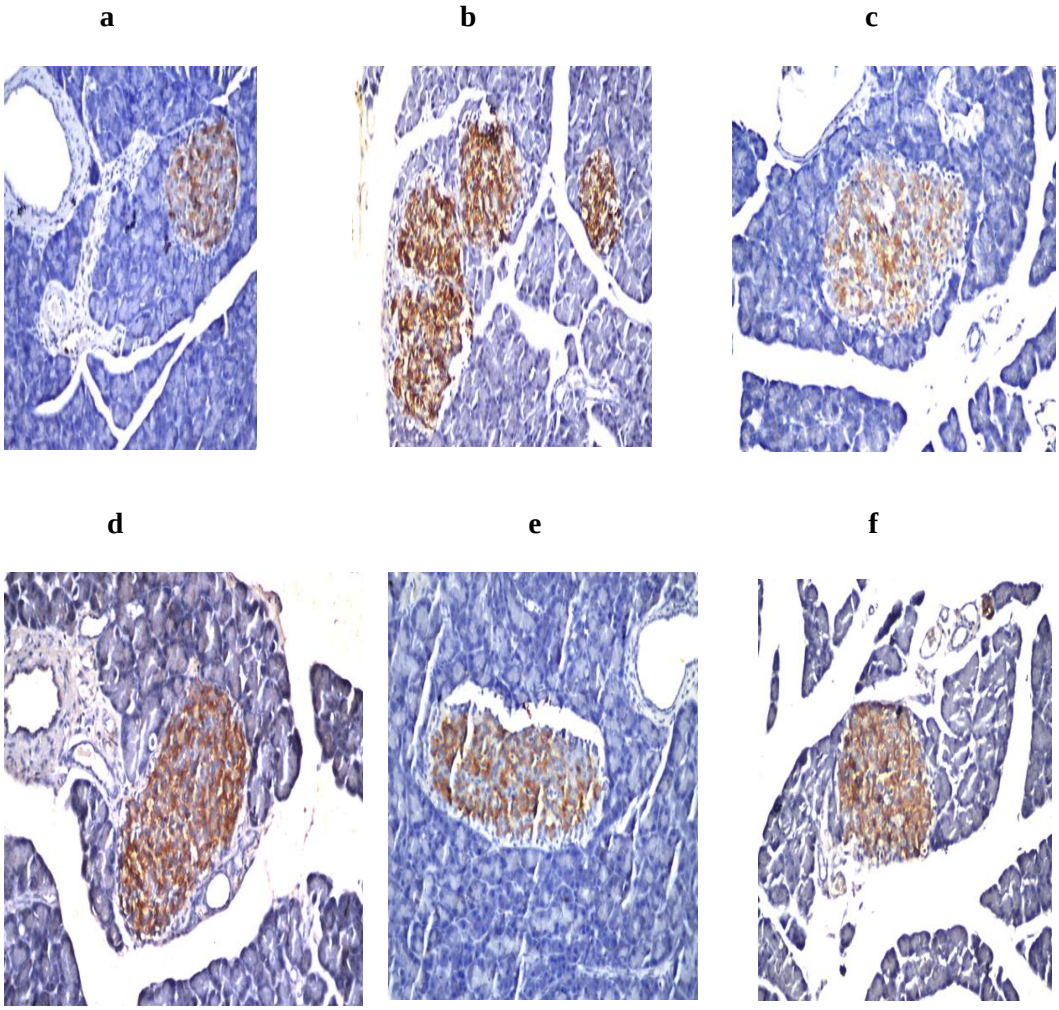

Fig. 3 Effect of pioglitazone, hemin and ZnPP on COX-2 pancreatic expression in L-Arginine-induced AP in rats using immunohistochemical staining (IHC) (×200).
(control) Pancreas of rats in showing immunoreaction in the lining epithelium of the acini
(AP) Pancreas of rats in group showing immunoreaction in the lining epithelium of the acini and the inflammatory cells
(AP + hemin) Pancreas of rats in showing immunoreaction in the lining epithelium of the acini
(AP + hemin + ZnPP) Pancreas of rats showing immunoreaction in the lining epithelium of the acini
(AP + pioglitazone) Pancreas of rats showing immunoreaction in the lining epithelium of the acini
(AP + pioglitazone + ZnPP) Pancreas of rat in showing immunoreaction in the lining epithelium of the acini

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