

Heme Oxygenase-1 Expression in Gastric Mucosa and Liver after Burns: Preliminary Immunohistochemical Study

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Abstract

Objective: Generation of reactive oxygen metabolites (ROM) and oxidative stress are crucial in pathogenesis of thermal skin injury. Heat shock proteins such as HSP32 known as heme oxygenase-1 (HO-1) possesses antioxidant, anti-inflammatory, and vasodilative properties and play an important role in the protecting of tissues from several stresses. In this immunohistochemical study, we have assessed the constitutive expression of HO-1 in normal gastric mucosa and liver and tested the hypothesis that its expression in these tissues is upregulated in thermal skin injury characterized by increased production of ROMs and other pro-inflammatory mediators. We used malondialdehyde (MDA) as a marker of oxidative stress and tumor necrosis factor-alpha (TNF- α) as a marker of inflammatory response in burn rat model.

Methods: HO-1 expression in formalin-fixed sections was assessed by peroxidase anti-peroxidase immunohistochemistry using a polyclonal rabbit anti-HO-1 as primary antibody. TNF- α in plasma was quantified using enzyme-linked immunosorbent assay (ELISA) kit. Spectrometric method was used for plasma and tissue MDA assay.

Results: Immunohistochemical staining revealed HO-1 expression in all groups. Staining scores for HO-1 in endothelial cells in both gastric mucosa and liver were increased with 32% ($p < 0.001$) and 51 % ($p < 0.001$), compared to that of controls. The levels of MDA in the gastric mucosa were increased by 43% ($p < 0.05$) and in liver in 126% ($p < 0.001$). Plasma concentration of TNF- α was also elevated by 111 % ($p < 0.001$) and MDA level in plasma was increased by 37% ($p < 0.05$). **Conclusion:** Our data suggest that HO-1 induction following burn is an adaptive response which can protect gastric mucosal and liver against further oxidative damage. HO-1 system may represent a target and an effective and cooperative strategy to intervene in the protection against inflammatory processes and oxidative tissue injury.

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INTRODUCTION

Extensive burns lead to the production of free radicals following the ischemia/ reperfusion as well as local and systemic inflammatory response causing damage in the organ distant from burn wound including liver and gastric mucosa [1-2]. Although the exact mechanisms involved in burn-induced tissue damage is not clear yet, increasing evidence indicates decreased hepatic and gastric mucosal blood flow, local and systemic liberation of cytokines and reactive oxidant intermediaries and changes in production of nitric oxide and prostaglandins as damaging factors of liver and gastric mucosa [2-4].

When cells are exposed to various stressors they induced number of so-called stress proteins in order to protect themselves [5]. Heme oxygenase-1 (HO-1) known as heat shock protein 32 (HSP32) is one of these proteins. HO-1 may be induced by a variety of

seemingly disparate stimuli hypoxia, ischemia, inflammation, most of which are linked by their ability to provoke oxidative stress [6-8]. HO-1 degrades heme to carbon monoxide (CO), free iron and biliverdin. Bilirubin and biliverdin are potent antioxidant and CO has anti-inflammatory and anti-apoptotic effect [8]. Recent studies indicate that HO-1 may play an important role in protecting gastrointestinal tissues by ischemia reperfusion, ethanol and non-steroidal anti-inflammatory drugs (NSAID) [9-10] as well as hepatic injury induced by ischemia/reperfusion, hemorrhage/resuscitation, heat shock and hepatotoxins (endotoxin and acetaminophen) [11-14] and is one of the most sensitive and reliable indicators of cellular oxidative stress [10]. The role of HO-1 in liver and gastric mucosa after burns remains unclear.

The aim of the present study was to assess the constitutive expression of HSP32 in normal gastric

mucosa and liver and tested the hypothesis that its expression in these tissues is upregulated in thermal skin injury characterized by increased production of ROM and other mediators. Malondialdehyde (MDA) was used as marker of oxidative stress while tumor necrotic factor α (TNF- α) was applied as marker of inflammatory response.

MATERIAL AND METHODS

Experimental thermal skin injury

The experimental procedure was approved by the Home Office for Care and Use of Laboratory Animals and performed with a strong consideration for ethics of animal experimentation. Age-matched male rats weighing between 220 and 250 g fasted for 12 h were allowed free access to water before injury. Animals were housed in a 20°C and offered rat chow and water ad libitum. They were kept in dark/light cycles (DL = 12:12 h) in individual wire-bottomed cages. Thus, lights were turned off at 8:00 p.m. and turned on at 8:00 a.m. for achieving satisfactory photoperiod.

After light ether inhalation, general anesthesia was intraperitoneally performed using thiopental (30 mg/kg). In order to accomplish 30% of third degree burn hot boiling water (98°C) was applied on the back of the animals during a period of 10 sec. For those rats which were subjected to burn injury, 4 mL of physiological saline was intraperitoneally applied for immediate resuscitation following burn injury. No animals died within the first 24 h of post-burn period. Sixteen male Wistar rats were divided into two equal (n=8) groups: 1. Control, non-burned group; 2. Burned, non-treated. All the animals were intraperitoneally given buprenorphine (0.3 mg /kg b.w.) twice daily for pain control post-burn. They were re-anesthetized with thiopental and sacrificed 24 h after burns as liver and stomach was sampled.

Biochemical analysis

Membrane lipid peroxidation was assayed by MDA measured by its thiobarbituric acid (TBA) reactivity of stomach homogenate using the method of Porter et al [15]. Results were expressed as nmol MDA/g tissue and nmol MDA/mL plasma. They were determined using the extinction coefficient of MDA-TBA complex at 532 nm = $1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ solution.

Plasma TNF- α level was quantified using enzyme-linked immunosorbent assay (ELISA) kit (Diacclone Gen-Probe, France) specific for the previously mentioned rat cytokines according to the manufacturer's instructions and guidelines. Plasma TNF- α level was expressed as pg/mL.

Immunohistochemistry

Rat stomach and liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. The deparaffinized and rehydrated sections (5 μm thick) were treated with 1% hydrogen peroxide for peroxidase activity inhibition for 5 min. Then they were rinsed in 0.1 M phosphate buffered saline (PBS) (pH 7.4) and treated in normal goat serum for 20 min. Subsequently, the sections were incubated with polyclonal primary antibody for 24 h at room temperature. Rabbit anti-HO-1 antibody (Santa Cruz, USA) was used. After rinsing with PBS the sections were incubated for 20 min in goat anti-rabbit immunoglobulins at room temperature. Then they were rinsed in PBS again, treated with rabbit peroxidase-antiperoxidase complex for 20 min at room temperature and then rinsed in PBS. Finally, peroxidase activity was estimated by the diaminobenzidine-tetrachloride H_2O_2 -method.

Negative controls were incubated with non-immune sera instead of primary antibody.

Morphometric method was used to assess semi-quantitatively enzyme contents of HO-1. Enzyme content was determined as: strong=score 3; moderate=score 2; weak=score 1, and lack of staining=score 0 on the basis of the occurrence of immunodeposits [16]. HO-1 contents of the cells in gastric mucosa and liver were defined as the enzyme content of each cell was multiplied by their scoring factors and divided by total number of cells. Morphometric investigation was performed on 50 cells from each sample.

Statistics

Our data were log-transformed to satisfy the assumptions required to perform parametric tests and, therefore, presented as geometric mean and 95% confidence intervals of the mean. Orthogonal contrasts in ANOVA were used to statistically analyze the difference between any two specified groups.

RESULTS

Hepatic MDA levels in the burned group were found to be higher (by 126%, $p < 0.001$) than those of the control one at the 24 hours after burns (Table 1). Gastric mucosal MDA levels in the burned group were found to be significantly higher (by 43%, $p < 0.05$) than those of the control one (Table 1). Plasma TNF- α levels increased by 111% ($p < 0.001$) while plasma MDA level increased by 37 % ($p < 0.05$) in the burned group when compared to those of the control one at the 24th h after burns (Table 2).

Table 1. Changes in the level of hepatic and gastric mucosal MDA after burns.

Parameter/Groups	Hepatic MDA (nmol/g)	Gastric Mucosal MDA (nmol/g)
Control	1.20± 0.06	2.81 ± 0.76
Burned	2.72 ± 0.39***	4.02 ± 1.77*

***p < 0.001, *p < 0.01: Burned versus Control. Results are given as mean ± SEM

Table 2. Changes in the level of plasma TNF- α and MDA after burns.

Parameter/Groups	Plasma TNF- α (pg/ml)	Plasma MDA (nmol/ml)
Control	34.05±1.01	1.10±0.10
Burned	74.28±4.19***	1.51±0.11*

*p < 0.01, ***p < 0.001 : Burned versus Control. Results are given as mean ± SEM

HO-1 expression was found in sinusoidal cells of the liver in the control group. The staining intensity of HO-1 positive cells was weak. The mean HO-1 content in the cells was 1.35±0.23 (Figure 1A). In the burned group, induction of HO-1 was principally in the sinusoidal cells and some hepatocytes around the central vein (Figure 1B). It was moderate to strong in the individually cells and their mean content (2.05±0.81) was significantly higher (51%, p<0.0001) than this of the control rats.

HO-1 expression was found in endothelial cells of the mucous neck region of the gastric mucosa in the control group. The staining intensity of HO-1 positive cells was weak. The mean HO-1 cell content was 0.91±0.23 (Figure 2A). In the burned group, HO-1 positive cells were localized also in endothelial cells in the upper half in gastric glands (Figure 2B). It was moderate in the individually cells and their mean cell content (1.21±0.51) was significantly higher (32%, p<0.001). Expression of HO-1 in the liver is higher than this in gastric mucosa.

DISCUSSION

Generation of ROM and oxidative stress are crucial in pathogenesis of thermal skin injury. The activation of oxidative stress pathway in many organs in splanchnic region including liver and stomach is more likely due to increased production of free radicals by burned skin, activated leukocytes and xanthine/xanthine oxidase system [1-4]. The excessive free radicals generated from inflammatory cells and the activation of lipid peroxidation may cause overproduction of cytokines e.g. TNF- α , interleukin-6 (IL-6) and iNOS activation

that stimulates the inflammatory response and oxidative stress [17]. The biological effect of free radicals is controlled by wide range of antioxidant and antioxidant enzymes. It has been reported that activity of some antioxidant enzyme such as superoxide dismutase (SOD) increased after burns [3]. The present study in the first line shows that HO-1 is significantly up-regulated in the gastric mucosa and liver at the 24 h after burns.

Oxidative stress could be counterbalanced by induction of anti-oxidative sensitive signal pathway such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) which is responsible for activation of important ROS detoxifying enzymes such as HO-1, SOD, glutathione S-transferase (GST) [18]. Because HO-1 is induced by oxidative stress, it could be speculated that the increased expression of HO-1 in gastric mucosa and liver is likely related to overproduction of ROM after burns. Indeed, HO-1 expression and MDA (as marker of oxidative stress) level in liver were elevated more significantly than these in gastric mucosa at the 24 hours after burns.

HO-1 is the inducible isoform of HO, which catalyzes the first and rate-limiting step in heme degradation to produce equimolar quantities of biliverdin, CO and free iron [19]. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase and free iron. Bilirubin has antioxidant properties [6, 8]. Sequestration of free iron by ferritin lowers the pro-oxidant state of the cell [20]. Recent experimental evidence shows that HO-1-deficient cells and mice are susceptible to the accumulation of free radicals and to oxidative injury after endotoxin administration [21]. Therefore, induction of HO-1 may provide cytoprotection against oxidative stress. CO stimulates the formation of cyclic guanosine monophosphate and lead to vasodilatation and inhibition of platelet aggregation which could prevent hepatic and gastric mucosal perfusion disturbances [22]. In addition, both CO and IL-10 can inhibit the expression of pro-inflammatory genes associated with macrophage activation, e.g. TNF- α , IL-1 β [23]. The cellular functions of overproduced CO in the liver cells are unclear; however, the CO generated through the HO-1 pathway during burn-induced liver injury might be important in the antioxidant defense as well as in the inflammatory response [17]. These findings suggest that the induction of HO-1 may enhance the overall cellular antioxidant capacity and prevent oxidative stress induced cytotoxicity in liver and stomach after burns.

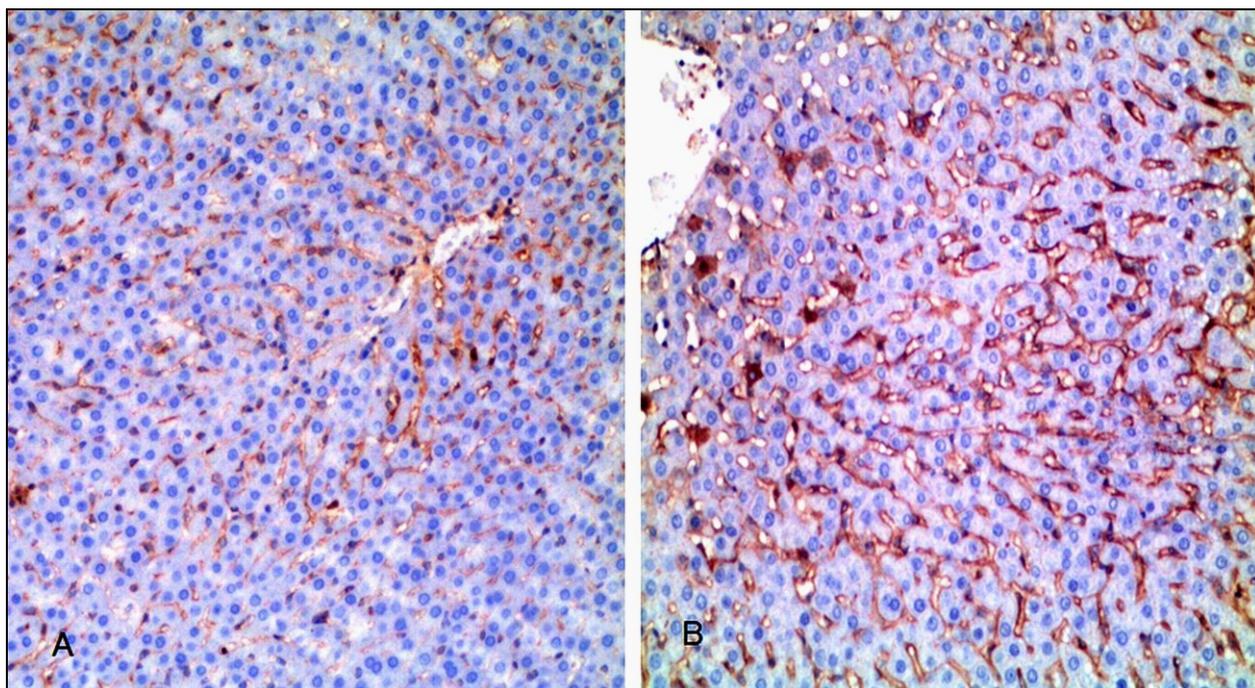


Figure 1. A: Immunohistochemistry detection for heme-oxygenase-1 (HO-1) in liver after burns. It is localized in sinusoidal cells of the liver in the control group. The staining intensity of HO-1 positive cells was weak (Immunohistochemistry, x200). **B:** In the burned group, induction of HO-1 positive cells was principally in the sinusoidal cells and some hepatocytes around the central vein. It was moderate to strong in the individually cells (Immunohistochemistry, x400).

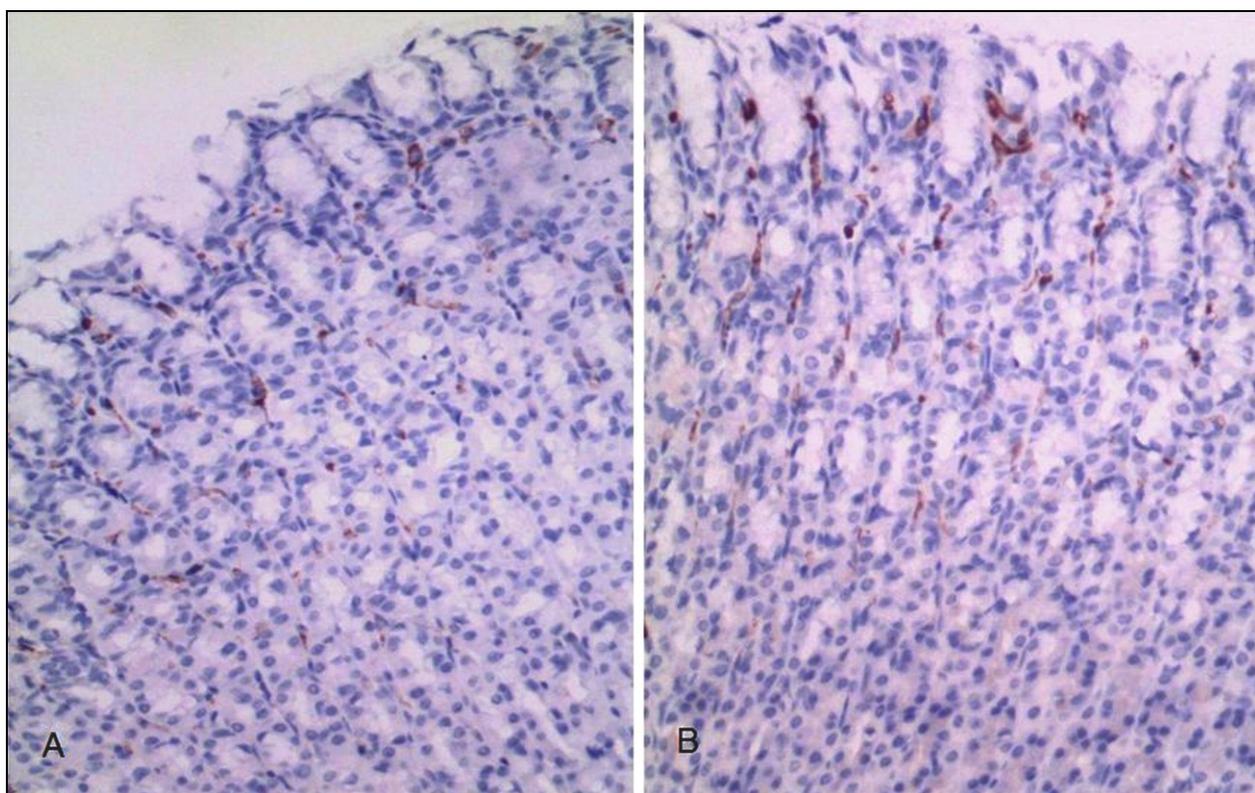


Figure 2. A: Immunohistochemistry detection for heme-oxygenase-1 (HO-1) in gastric mucosa after burns. It is localized to endothelial cells of the mucous neck region of the gastric mucosa in the control group. The staining intensity of HO-1 positive cells was weak. **B:** In the burned group, HO-1 positive cells were localized also in endothelial cells in the upper half in gastric glands. It was moderate in the individually cells (Original magnification, x200 for A and B).

Recent studies indicate that HO-1 an important enzymatic antioxidant system may play an important role in protecting gastrointestinal tissues by ischemia reperfusion, ethanol and NSAID [9-10, 23]. Both endogenous HO-1 and drugs (sofalcone and zinc-L-carnosine)-induced HO-1 may protect against gastrointestinal injury [24-25]. Severe lines of studies suggest that HO-1 prevents hepatic injury, induced by ischemia/reperfusion, hemorrhage/resuscitation, heat shock, and against hepatotoxins (endotoxin, acetaminophen) [11-14] and is one of the most sensitive and reliable indicators of cellular oxidative stress [10].

In conclusion, we found that thermal skin injury caused an increased expression of HO-1 in liver and gastric mucosa. It may be supposed that HO-1 induction may protect gastric mucosa and liver from injury by countering the effect of increased oxidative stress and inflammatory response due to thermal skin injury. We suggested that the HO-1 induction following burn is an adaptive response which can confer gastric mucosal and liver protection against further oxidative damage. HO-1 system may represent a target and an effective and cooperative strategy to intervene in protection against burn-induced oxidative tissue injury.

DISCLOSURE

The authors declare that they have no conflict of interest.

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