Protective Effect of Heme Oxygenase-1 Against Isoprenaline-induced Acute Myocardial Infarction in Rat in vivo

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Abstract

Background: Heme oxygenase-1 (HO-1) is known to have anti-inflammatory, antiapoptotic and antioxidant effects in various diseases. In this study, we aimed to investigate whether HO-1 has a protective effect on isoprenaline (IP)-induced acute myocardial infarction (AMI) in rat.

Materials and methods: Rats were received 200 mg/kg IP, alone or together with cobalt protoporphyrin (CoPP) and zinc protoporphyrin (ZnPP), an inducer and an inhibitor of HO-1, respectively. Four hours after the IP application, rats were killed. Blood and hearts were removed. Troponin-T (TnT) levels were assayed in serum as an indicator of AMI. Dien conjugate levels, protein carbonyl levels and antioxidant defense system are assayed in heart tissues by spectrophotometric methods. HO-1 protein levels were quantitated by western blot analysis in heart tissues. The results were compared with those obtained from control groups.

Results: We confirmed AMI formation with higher serum TnT levels in IP-treated groups comparing to control groups. We found a significant elevation in HO-1 protein levels in IP plus CoPP-treated rats as compared to IP-treated rats, while serum TnT levels were observed to decrease at the same time. Protein carbonyl levels in heart tissues increased significantly in IP-treated groups compared with control groups. But, dien conjugate and antioxidant defense system remained unchanged.

Conclusions: As a conclusion, we may suggest that HO-1 induction produces a protective effect against to myocardial damage occurred by IP application in rat.

Introduction

The intrinsic defense mechanisms of the body are important in protecting organs from tissue damage in response to various stress conditions. Heme oxygenase-1 (HO-1) upregulation is one of such intrinsic defense systems. HO-1, also known as Hsp 32, is a member of HO protein family comprising three isoforms, HO-1, HO-2, HO-3 [1–3]. It catalyzes the first and rate-limiting step of heme degradation into ferrous iron, carbon monoxide and biliverdin, the latter being finally converted to bilirubin. These breakdown products have been reported to have antioxidant, anti-inflammatory, antiapoptotic effects which would be important in protecting the tissues from several stress conditions [4–6].

Cardiovascular diseases are the major health problem in the developed countries of the world. Growing evidence indicate that reactive oxygen species (ROS) and ROS-induced oxidative stress are essential in the processes underlying cardiovascular diseases [7, 8]. It has been suggested that the overproduction of ROS lead to cardiomyocyte death by triggering many atherogenic events such as LDL oxidation, endothel dysfunction, vascular smooth muscle proliferation and migration [7]. Both invitro and in vivo experimental studies have shown that myocyte loss is generated by oxidative stress induced in I/R injury, myocard infarction and hypertension [8, 9]. Furthermore, oxidative stress hypothesis in cardiovascular diseases is also supported by clinical investigations [10]. On the other hand, several studies have shown that overexpression of stress proteins may protect myocardium against postischemic injury [11]. Since

HO-1 has been shown to be upregulated in several stress conditions including diabetes [12], hypoxic lung disease [13], intestinal ischemia reperfusion [14], pancreatitis [15], its role has been also implicated in cardiovascular diseases [6, 16]. However, there are limited experimental studies investigating the effect of HO-1 overexpression on cardiovascular diseases in *in vivo* conditions.

Induction of AMI by isoprenaline (IP), a synthetic β -adrenoreceptor agonist which is a widely used agent to generate AMI in rat, is a well-established experimental model to study the protective role of both exogenous agents and endogenous stress-inducible proteins [17]. For this reason, we aimed to investigate whether HO-1 overexpression has a protective effect on AMI induced by IP model. Furthermore we also wanted to search if oxidative stress is relevant to the presumed overexpression of HO-1 caused by AMI in our experimental conditions.

Materials And Methods

Male Sprague Dawley rats weighing between 180–220 g were procured from the Experimental Medical Research Institute of Istanbul University in the year of 2013. Rats were fed a standard laboratory chow and had free access to water. They were kept in wire-bottomed stainless steel cages. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of Istanbul University (ethical approval is also given by the same institution's local ethical committee) and ARRIVE guidelines. All chemicals were purchased from Sigma-Aldrich (San Diego, USA).

Experimental design

Rats those divided into 6 groups, were pretreated with HO-1 mRNA inducers such as cobalt protoporphyrine (CoPP) and zinc protoporphyrine (ZnPP). CoPP (5mg/kg; i.p) and ZnPP (20 mg/kg; i.p) were given two times with a 12-h interval. Myocardial infarction was induced by subcutaneous (sc) injection of IP (200 mg/kg body weight) dissolved in 0.9% NaCl. IP (200 mg/kg; s.c) was injected to rats 30 minutes after the last metalloporphyrine administrations. The rats were killed after 4 hours of IP administration. All experiments were performed during the years of 2013–2014.

1. **Control group** (n = 7): % 0.9 NaCl (i.p) was injected twice at interval of 12 hours. 0.9% NaCl injection was repeated 30 minutes after the last saline application.
2. **CoPP group** (n = 4): CoPP (5 mg/kg; ip) was injected twice at interval 12 hours. % 0.9 NaCl (s.c) was given to rats 30 minutes after the last CoPP application.
3. **ZnPP group** (n = 4): ZnPP (20 mg/kg; ip) was injected twice at interval 12 hours. % 0.9 NaCl (s.c) was given to rats 30 minutes after the last ZnPP application.
4. **IP group** (n = 8): % 0.9 NaCl (i.p) was injected twice at interval of 12 hours. IP (200 mg/kg, s.c) was given 30 minutes after the last saline application.
5. **CoPP + IP group** (n = 7): CoPP (5 mg/kg; ip) was injected twice at interval 12 hours. IP (200 mg/kg, s.c) was given 30 minutes after the last CoPP application.

6. **ZnPP + IP group** (n = 10): ZnPP (20 mg/kg; ip) was injected twice at interval 12 hours. IP (200 mg/kg, s.c) was given 30 minutes after the ZnPP application.

After treatment, the animals were anesthetized by phenobarbital (50 mg/kg body weight), and sacrificed by cervical dislocation. The blood was obtained from vena cava inferior (v.c.i) and centrifuged at 2000 rpm to separate serum. The hearts were removed immediately, washed with ice-cold saline and kept in – 80 °C until use.

Biochemical assays

Serum TnT levels was assayed by immunochemiluminometric assay at Cardiology Institute of Istanbul University.

Dien conjugate (DC) levels was measured in tissue lipid extract at 233 nm spectrophotometrically and calculated using a molar extinction coefficient of $2.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [18]. Protein carbonyl (PC) levels were measured according to method described by Saji K *et al* [19]. This method is based on spectrophotometric detection of the reaction between 2,4-dinitrophenylhydrazine and protein carbonyl groups to form protein hydrazones. GSH levels were measured with 5,5ditiobis-(2-nitrobenzoate) at 412 nm in tissue homogenates [20].

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione transferase (GST) activities were determined in postmitochondrial fraction of tissues, which were separated by sequential centrifugation. SOD activity was assayed by its ability to increase the effect of riboflavin-sensitized photooxidation of o-dianisidine [21]. GSH-Px activity was measured using the method of Paglia and Valentine with cumene hydroperoxide as substrate [22]. GST activity was assayed by the spectrophotometric method using 1-chloro-2,4-dinitrobenzene as the substrate [23].

HO-1 expression by western blot analysis

Tissue homogenates (10%) were prepared in a buffer containing 50 mM Tris-HCl, 1% Ipegal 630, 150 mM NaCl, 0.5% sodium deoxycholate, 1% sodium dodesyl sulphate, PMSF, aprotinin and soy bean trypsin inhibitor (SBTI) by a hand homogenizator [24]. Homogenates were centrifuged at 4 °C, 13000xg 20 min and supernatants were separated and protein content was determined. Samples containing 50 µg protein per lane were electrophorized on a 12% gel in Tris-glycine buffer. SDS polyacrylamide gel electrophoresis was performed using Bio Rad's mini gel system according to Laemmli's method [25]. After electrophoresis, separated proteins were transferred onto Immobilon-PVDF membrane (Millipore), at 20V for 2 hr 15 min. [26]. The proteins on the membrane were blocked with 3% low-fat dried milk. After washing in phosphate buffer saline containing 1% Tween 20 (PBS-T) three times, blots were treated with a 1: 3000 dilution of monoclonal primer antibody which recognizes HO-1 (W 27: sc-10789; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and then washed with PBS-T for three times. Resulting immunocomplexes were exposed to 1:30000 horseradish peroxidase-conjugated goat antirabbit IgG (sc-2004; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 hours. After washing with PBS-T, labeled-antigen bands were visualized by an ECL western blotting detection reagent (sc-2048; Santa Cruz

Biotechnology Inc., Santa Cruz, CA). The intensity of bands was semiquantified densitometrically by using Vilber Lourmat gel documentation system and Bio-1D V.97 software, an image analysing programme (Vilber Lourmat, Biotechnology Division, Torcy, France). The blots were stripped and subsequently reprobed with an anti-actin rabbit polyclonal with 1:1000 dilution (sc-1616-R; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for normalization of loading. One protein sample from control group was applied onto lane 1 in each assay performed and its value was taken as 100% arbitrarily to compare with other samples. Results were expressed as relative densitometric unit (RDU).

Protein determination was carried out by a method using bicinchoninic acid [27].

Statistical analysis

The results were expressed as mean $\pm$ S.D. Experimental groups were compared using Kruskal-Wallis variance analysis test. Where significant effects were found, post hoc analysis using Mann-Whitney U test was performed, and $p < 0.05$ was considered to be statistically significant. The correlation coefficients were determined by Pearson's method.

Results

- a. IP treatment caused significant increases in serum TnT levels as compared to control group. However, a significant decrease was found in serum TnT levels of CoPP + IP group when comparing this group with the IP group (Fig. 1, Table 1).
- b. In heart tissue, HO-1 expression was observed to increase significantly in all groups compared to control. In addition, significant increases were also detected in CoPP and CoPP + IP groups compared to IP group (Fig. 2, Table1).

Table 1
Biochemical parameters in serum and heart tissues of rats.

	Control (n:7)	CoPP (n:4)	ZnPP (n:4)	IP (n:8)	CoPP + IP (n:7)	ZnPP + IP (n:10)
HO-1 (RDU)*	100.0 ± 16.7	780.5 ± 61.2 ^{a,b}	597.8 ± 49.4 ^a	469.6 ± 43.2 ^a	1165.2 ± 69.9 ^{a,b}	617.4 ± 93.1 ^a
TnT (ng/ml)	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	5.63 ± 1.31 ^a	2.81 ± 0.52 ^{a,b}	4.86 ± 1.73 ^a
PC (µmol/g tissue)	1.65 ± 0.44	1.84 ±0.14	1.56 ± 0.35	5.37 ± 1.54 ^a	4.90 ± 0.71 ^a	3.96 ± 1.41 ^a
DK (µmol/g tissue)	1.42 ± 0.33	1.29 ± 0.26	1.38 ± 0.06	1.37 ± 0.17	1.24 ± 0.29	1.37 ±0.24
GSH (µmol/g tissue)	2.36 ± 0.40	2.18 ± 0.07	2.12 ± 0.12	2.55 ± 0.31	2.58 ± 0.56	2.40 ± 0.26
SOD (U/mg protein)	10.7 ± 1.1	10.6 ± 1.4	10.9 ± 1.6	11.9 ± 1.7	11.0 ± 1.3	12.0 ± 2.7
GSH-Px (nmol NADPH/ min/mg protein)	247.0 ± 45.8	272.0 ± 16.8	267.8 ± 58.1	214.0 ± 49.8	216.3 ± 40.1	212.6 ± 45.0
GST (nmol/min/mg protein)	53.6 ± 6.4	45.2 ± 5.4	54.1 ± 7.3	54.7 ± 7.6	46.4 ± 10.8	58.8 ± 7.9

* Relative densitometric units.

^a p < 0.05: compared to control

^b p < 0.05: compared to IP

Furthermore, there was a significant inverse correlation between HO-1 expression and serum TnT levels ($r = -0.814$; $p < 0.01$) (Fig. 3).

c. Significant increases in protein carbonyl levels were observed in IP, CoPP + IP and ZnPP + IP groups compared to control groups (Control, CoPP and ZnPP groups). But, intergroup significance was not detected (Fig. 4, Table 1). On the other hand dien conjugate levels remained unchanged (Table 1).

d. Neither GSH levels nor antioxidant enzyme activities as antioxidant defense system changed comparing with control. In addition, intergroup significance was not found (Table 1).

Discussion

HO-1 induction is suggested to enhance cellular tolerance and resistance capacity against tissue damage in various pathological states. Studies have demonstrated that induction of HO-1 by chemical inducers provides protection in cardiac transplant rejection [28], hypoxic lung disease [13], intestinal ischemia reperfusion injury. In addition, HO-1 overexpression by gene transfer has been reported to protect against hypoxic lung diseases [29]. HO-1 has also been shown to have cytoprotective effects in CCl₄- [30] and ethanol-induced [31], liver damage, pancreatitis [32] and diabetes [33]. The role of HO-1 in cardiac homeostasis was first implicated in a study showing the increase in HO-1 expression in heart in response to hyperthermia [34]. There are studies, it was demonstrating the absence of HO-1 which exacerbated remodelling in hypercholesterolemic mouse model [35] and ischemia/R- induced myocardial damage in diabetic mice [36]. On the other hand studies using cardiac-specific HO-1 transgenic mice revealed that HO-1 overexpression played a protective role against myocardial damage in I/R model [37] and also in heart failure model [38]. Lakkisto et al. have shown that HO-1 has antiapoptotic and antiproliferative effects on rat myocytes after AMI produced by coronary artery ligation [39–41]. In one of these studies, they have suggested that HO-1 induction might increase the life span of cardiomyocytes by enhancing the vascularization. In a recent study, luteolin has been reported to have a protective effect on myocardial infarction produced by IP administration which might be related to HO-1 induction in rats with AMI [42]. In our study, by a different approach, we aimed to investigate the protective role of HO-1 in *in vivo* experimental IP-induced acute AMI model by using exogenous HO-1 mRNA inducers such as CoPP and ZnPP. Isoprotrenol, a potent synthetic catecholamine when given to animals at toxic doses, produces “infarct like” lesions in the heart similar to those found in human after AMI [43–45]. Investigators have used different doses of IP such as 110 mg/kg [44], 150 mg/kg [45], 200 mg/kg [46] to produce AMI in rats. In these studies, the presence of AMI has been confirmed by assessing plasma CK, AST and LDH activities at different time periods in plasma. However, the assessment of cardiac TnT and troponin-I (TnI) are currently used as the most sensitive and specific markers of AMI.

In the present study, we administered IP at the dose of 200 mg/kg to rats and showed the presence of cardiomyocyte necrosis by detecting significant elevations in plasma TnT levels in all IP-given groups (IP, CoPP + IP, ZnPP + IP) compared to their matching control groups (control, CoPP and ZnPP) after 4 hours. But we determined a significant decrease in plasma TnT levels of CoPP + IP group when we compared it to IP group. We think that CoPP, the inducer of HO-1, might be responsible for this decrease seen in CoPP + IP group.

Regarding to the HO-1 protein levels in heart tissue, significant increases were found in all groups as compared to control group. This shows that both CoPP and ZnPP cause induction of HO-1 in the heart of rats. The metalloporphyrins are extensively used for HO-1 induction or inhibition. Although CoPP and ZnPP are known to induce HO-1 mRNA induction, ZnPP is generally used for inhibition of enzyme activity

[47–50]. In our study, we found that, HO-1 expression increased in the groups given ZnPP (ZnPP and ZnPP + IP) as compared to control, too. But this increase was not as high as those seen in the groups given CoPP (CoPP and CoPP + IP). In addition, HO-1 levels were also found to elevate in both CoPP and CoPP + IP groups as compared to IP group. This increases seen in HO-1 expression, concomitant with the decline in serum TnT levels in CoPP + IP group confirmed that HO-1 induction created a protection against cardiomyocyte loss after AMI. But, we did not observe any change in both HO-1 expression in heart tissue and serum TnT levels in ZnPP + IP group as compared to IP group. Although ZnPP induces HO-1, it didn't show protective effect on myocardium after AMI. We think that ZnPP might have an inhibitory effect on HO-1 enzyme activity.

On the other hand, it has been reported that the increased lipid oxidation [51–53] and decreased antioxidant system [52, 53] contribute to oxidative stress and affect the pathogenesis of AMI. In this study, we did not find any change either in diene conjugate levels or in antioxidative system in heart tissues of rats after AMI. However, tissue protein carbonyl levels were found to be higher in all groups with AMI (IP, CoPP + IP, ZnPP + IP) than their matching control groups (control, CoPP, ZnPP). Although there was not a statistical significance among groups, the increases observed in PK levels together with the increases in HO-1 which is a stress-inducible protein, may be suggested to reflect the oxidative stress condition in the cell. Indeed, there are studies showing that the increases in HO-1 mRNA expression might be an indicator of oxidative stress [54].

In summary, HO-1 induction together with decrease in serum TnT levels exhibits a protection against cardiomyocyte loss after AMI generated by IP. Recent studies have suggested that HO-1 up-regulation by pharmacological modulation or gene transfer might have a promising role in planning a new therapeutic strategies in AMI in the future [55, 56]. Taken together, our finding suggest that, the induction of HO-1 may be an important tool for enhancing the recovery of myocardial infarction and protection against pathological changes in myocardium.

Declarations

Ethics Approval

The ethical approval has gained from the local ethical board of Istanbul University (No:2012/10) and the trial has begun after the ethical approval. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of Istanbul University (ethical approval is also given by the same institution's local ethical committee) and ARRIVE guidelines. In the nature of this is being an animal study, informed consent could not be obtained.

Consent for Publication

By submitting the article, all authors are agreeing the publication.

Availability of Data and Materials

All the datas collected during the study is available and accessible.

Conflict of Interest

All authors declare there is no conflict of interest.

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Authors' Contribution

Our study has demonstrated that heme-oxygenase-1 has a protective effect in the rats with isoprenaline induced myocardial infarction. There are still a need for human studies and this results are hopeful for the future treatment algorhytms.

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Figures

Figure 1

Serum Troponin-T (TnT) levels in rats those HO-1 was induced by CoPP or ZnPP.

^a $p < 0.05$: compared to control

^b $p < 0.05$: compared to IP

Figure 2

Western blot profiles for HO-1 induction in heart tissues of rats those HO-1 was induced by CoPP or ZnPP.

Figure 3

Inverse correlation between HO-1 expression and serum TnT levels.

Figure 4

Protein carbonyl levels in heart tissues of rats those HO-1 was induced by CoPP or ZnPP.

^a p < 0.05: compared to control

^b p < 0.05: compared to IP