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Original Article

Heme oxygenase in diabetes-induced oxidative stress in the heart

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Abstract

Diabetic cardiomyopathy is responsible for substantial morbidity and mortality in the diabetic population. Increased oxidative stress has been associated with the pathogenesis of chronic diabetic complications including cardiomyopathy. Multiple biochemical mechanisms have been proposed to increase oxidative stress in diabetes. The present study was aimed at elucidating the role of a potent oxidative and cellular stress-responsive system, the heme oxygenase (HO) system, in the heart in diabetes. Streptozotocin-induced diabetic rats were treated with a potent inhibitor of HO system, tin protoporphyrin IX (SnPPIX, 50 μmol/kg/d), and were compared with untreated diabetic and non-diabetic animals. All treatments began at the onset of diabetes, 48 h after injection of streptozotocin along with the confirmation of hyperglycemia. Animals were euthanized after 1 week and 1 month of treatment, and heart tissues were harvested. Frozen tissues were subjected to HO-1 and HO-2 mRNA expression by real-time RT-PCR and HO activity determination. Paraffin-embedded tissue sections were used for immunohistochemical analysis of HO-1 and HO-2. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) stain, a sensitive and specific marker of DNA damage, was preformed to assess damage induced by oxidative stress. In addition, tissue sections were subjected to histochemical analysis for iron. We further examined non-diabetic animals treated with a direct HO agonist, hemin (50 mg/kg/d). A possible relationship between the HO and the nitric oxide (NO) pathways was also considered by studying the mRNA levels of endothelial nitric oxide synthase (NOS) and inducible NOS, and by measuring the amount of NOS products. Our results demonstrate no significant alterations of the HO system following 1 week of diabetes. However, 1 month of diabetes caused increased oxidative stress as demonstrated by higher levels of 8-OHdG-positive cardiomyocytes (80% positive as compared to 11.25% in controls), in association with increased HO isozyme mRNA (2.7-fold increase as compared to controls) and protein expression, and augmented HO activity (759.3 as compared to 312.3 pmol BR/h/mg protein in controls). Diabetic rats further demonstrated increased number of cardiomyocytes with stainable iron. SnPPIX treatment resulted in reduced number of 8-OHdG-positive cardiomyocytes (19.5% as compared to 80% in diabetics) in parallel with reduced HO activity (569.7 as compared to 759.3 pmol BR/h/mg protein in diabetics). Non-diabetic rats treated with HO-agonist hemin exhibited abnormalities similar to diabetic rats. Our results provide the first direct demonstration that diabetes-induced oxidative stress in the heart is, in part, due to upregulated HO expression and activity. These results provide evidence of pro-oxidant activity of HO in the heart in diabetes, which could be mediated by increased redox-active iron.

Keywords: Cardiomyopathy; Hyperglycemia; 8-OHdG; Heme; Iron

1. Introduction

As diabetes reaches epidemic magnitudes worldwide, more diabetics face the problems of morbidity and mortality due to chronic diabetic complications [1,2]. Nearly three-fourths of the deaths associated with diabetes are due to cardiovascular complications [3]. Among the complications associated with long-standing diabetes, cardiomyopathy is clearly prominent and detrimental to the diabetic population [4,5]. Oxidative stress has been suggested to be involved in the pathogenesis of several diabetic complications including cardiomyopathy [6,7].

Sustained hyperglycemia has been identified as a principle mediator of increased reactive oxygen species (ROS) generation in diabetes [8]. In addition, there is considerable evi-

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dence suggesting that hyperglycemia-induced oxidative stress occurs before overt chronic diabetic complications develop [6,7]. As a defense mechanism against such toxic insults, cells induce various stress proteins. One oxidative stress-response mechanism is the induction of the heme oxygenase (HO) enzyme system. HO enzymes catalyze the oxidation of heme into the biologically active components, biliverdin, carbon monoxide (CO), and iron [9]. Three isozymes of HO have been identified to date: the inducible HO-1, also known as heat-shock protein 32, and the constitutive HO-2 and HO-3 [10]. Heme and various non-heme agents, which increase oxidative stress have been shown to induce the HO system providing evidence that HO may play a pivotal role in cellular homeostasis [9–11].

Induction of HO system by agents (such as hydrogen peroxide, UV irradiation, endotoxin, and hypoxia) suggests that HO may represent an adaptive defense mechanism [9–11]. The mechanistic basis of such anti-oxidant activity remains to be fully elucidated. Biliverdin, produced by the HO-catalyzed reaction, is reduced to a potent anti-oxidant, bilirubin, by the action of biliverdin reductase [9–11]. Bilirubin has also been shown to protect isolated perfused rat hearts [12]. In addition, CO is suggested to be cytoprotective due to potent anti-fibrinolytic activity [13]. It should be noted that most studies, which indicate a protective role of HO are devoted to two products of the enzymatic reaction, namely biliverdin and CO. Other by-product, redox-active iron from heme, has not been thoroughly investigated. Ferrous iron is a potent cytotoxic element by virtue of its ability to participate in Fenton reaction giving rise to hydroxyl radicals. Recent findings indicate a pro-oxidant role of HO depending on tissue microenvironment and disease context [14].

One enzyme system, which has been suggested to interact with HO is the nitric oxide (NO) system [15]. NO is synthesized from L-arginine in an enzymatic reaction catalyzed by nitric oxide synthase (NOS) [16]. Three enzymes comprise the NOS family: the constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS), and the inducible NOS (iNOS) [17,18]. It has been shown that increased NO levels upregulate HO expression and inhibitors of the HO system inhibit NOS activity [15]. Such parallel interactions between these enzymes could be important in the context of diabetic complications, which entail alterations of the NO pathway.

In the present study, we have evaluated the role of HO system in the heart of streptozotocin-induced diabetic rats. Previous studies in this well-established model of chronic diabetic complications have shown increased expression of HO in glomerular cells of diabetic rat kidneys [19]. However, no study to date has demonstrated how diabetes affects the expression and function of HO in the heart and whether any HO alteration is associated with anti- or pro-oxidant activity. Furthermore, we have studied the regulation of NO pathway to elucidate any interactive relationship between NO and HO systems.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River Canada Ltd., St. Constant, Que., Canada) weighing 200–250 g were made diabetic by single intravenous injection of streptozotocin (65 mg/kg, in citrate buffer, pH = 5.6) [20]. Age- and sex-matched rats were used as controls and were given equal volume of citrate buffer. Animals from each group were randomized into two subgroups: controls and controls treated with the HO agonist, hemin; and diabetics and diabetics treated with a potent competitor inhibitor of HO, tin protoporphyrin IX (SnPPIX). SnPPIX treatment for the diabetic animals began at the onset of diabetes, specifically 48 h after injection of streptozotocin along with the confirmation of hyperglycemia, and the treatments continued until euthanasia. Hemin treatment in the control rats started on the same day as the treatment for the diabetic animals and continued for the same time period. Hemin, was dispensed subcutaneously (50 mg/kg daily) [21,22], and SnPPIX was administered by intraperitoneal injection (50 μmol/kg daily) [22,23]. The treatment dosage has been established in previous animal studies [21–23]. Blood glucose levels were measured to confirm hyperglycemia (Surestep™ blood glucose meter, Lifescan, Burnaby, BC, Canada). To maintain the blood glucose levels between 15 and 25 mmol/l and to prevent ketosis, diabetic rats were injected with one slow-releasing insulin implant (LinShin, Scarborough, Ont., Canada) that liberated 2 U/d [20].

Rats were placed in individual air-filtered metabolic cages where they received rat chow and water *ad libitum*, and were monitored for glucosuria and ketonuria (Uriscan Gluketo™, Yeong Dong Co., Seoul, South Korea). Measurements of glycated hemoglobin (Glycotest™, Pierce, Rockford, IL, USA) were preformed at sacrifice. Before sacrificing the rats, systolic blood pressure was recorded by tail plethysmography as previously described [24]. The animals were sacrificed after 1 week and 1 month of treatment to elucidate the temporal alterations of the HO system. The tissues were snap-frozen in liquid nitrogen for gene expression analysis and HO activity assay. In addition, part of harvested tissues was also fixed in formalin and embedded in paraffin for immunohistochemical analysis of HO-1, HO-2, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and histochemical analysis of iron. All experiments were conducted in a masked fashion.

The animals were cared for according to the *Guiding Principles in the Care and Use of Animals*. All the experiments were approved by the University of Western Ontario Council on Animal Care Committee.

2.2. RNA isolation and cDNA synthesis

To isolate total RNA, TRIZOL™ reagent (Invitrogen Inc., Burlington, Ont., Canada) was used as described previously [20]. Briefly, aqueous and organic phases were separated by addition of chloroform and centrifugation. RNA was isolated

from the aqueous phase using isopropyl alcohol and was suspended in DEPC-treated water. Total RNA was measured by determining UV absorbance at 260 nm. Purity of samples was assessed by measuring OD 260:280 nm.

cDNA was synthesized using the Superscript-II™ system (Invitrogen Inc., Canada). Oligo (dT) primers were added to 4 μg of total RNA and the samples were denatured at 70 °C. MMLV-reverse transcriptase and dNTPs were added to the samples, which were further incubated at 42 °C for 50 min. The reaction was stopped by incubating the samples at 70 °C for 15 min, and the cDNA product was stored at –20 °C [20].

2.3. Real-time quantitative RT-PCR

Real-time RT-PCR for HO (HO-1 and HO-2) and NOS (eNOS and iNOS) was performed using the LightCycler™ (Roche Diagnostic Canada, Laval, Que., Canada). This technique allows amplification and kinetic detection in a single microcapillary tube (Roche Diagnostic Canada). For a final reaction volume of 20 μl, the following reagents were added: 2.5 μl of 10× PCR buffer, 1.25 μl of 5 mM dNTP, 1.2 μl of 50 mM $MgCl_2$, 1.0 μl of each forward and reverse 10 μM primers (Table 1), 0.5 μl of 5 U/μl platinum Taq polymerase (Invitrogen Inc., Canada), 0.75 μl of SYBR Green I (Molecular Probes, Eugene, OR, USA), 10.8 μl of H_2O, and 1.0 μl of cDNA template.

As the PCR protocols for the genes were optimized, melting curve analysis (MCA) was used to determine melting temperature (T_m) of specific amplification products and primer–dimers. For each gene, the specific T_m values were used to create a signal acquisition step (2–3 °C below T_m), which was added onto each elongation period. This phase allows for signal acquisition from specific products without signal interference from primer–dimers and non-specific amplification products. The levels of mRNA were quantified by using the standard curve method. Standard curves for all target genes were constructed by using serially diluted standard template. All PCR reactions were subjected to MCA to determine specificity of amplification. In addition, PCR products were subjected to gel electrophoresis (2% agarose) to determine size of the amplified products. The data were normalized to β-actin to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures.

2.4. HO activity

For HO activity assay, microsomal fractions from heart tissue were prepared as previously described [25]. The following reagents were added to the reaction mixture: potassium phosphate buffered saline (KPBS) (0.1 M, pH = 7.2), hemin (25 μM), and mouse liver cytosol prepared from 105,000 × *g* and used as a source of biliverdin reductase. The reaction was initiated by adding reduced nicotinamide adenine dinucleotide phosphate (NADPH), while the blanks received the same amount of 0.1 KPBS. The assay was

Table 1
Primer sequences and real-time RT-PCR temperature profiles

Primers (5′→3′)	Product (bp)	PCR parameters [a]	
		Phase	Temperature in °C (time in s)
HO-1	200	Denaturation	95 (0)
(+) ACAACCCCACCAAGTTCAAA		Annealing	55 (5)
(–) CCTCTGGCGAAGAAACTCTGA		Extension	72 (10)
		Signal	83 (1)
HO-2	554	Denaturation	95 (0)
(+) ATGGCAGACCTTTCTGAGCTC		Annealing	59 (5)
(–) CTTCATACTCAGGTCCAAGGC		Extension	72 (24)
		Signal	85 (1)
eNOS	207	Denaturation	95 (0)
(+) GCAAGACCGATTACACGACA		Annealing	57 (5)
(–) GTCCTCAGGAGGTCTTGCAC		Extension	72 (10)
		Signal	85 (1)
iNOS	220	Denaturation	95 (0)
(+) ATGGAACAGTATAAGCGAAACACC		Annealing	57 (5)
(–) GTTTCCGGTCGATGTCATGAGCAAAGG		Extension	72 (10)
		Signal	83 (1)
β-Actin	214	Denaturation	95 (0)
(+) CCTCTATGCCAACACAGTGC		Annealing	58 (5)
(–) CATCGTACTCCTGCTTGCTG		Extension	72 (8)
		Signal	83 (1)

[a] Initial denaturation was 1 min. Ramp rate was 20 °C/s.

preformed in triplicates. Bilirubin concentration was calculated by the difference between 470 and 530 nm readings and an extinction coefficient of 40 mM^{-1} cm^{-2} was used. HO activity was expressed as bilirubin (BR) produced per hour per milligram of protein.

2.5. Nitrate and nitrite measurements

The amount of NO produced was measured first by assaying the total NOS products (nitrate and nitrite) and finally by assaying the amount of nitrite in accordance to the guidelines provided in the nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA).

2.6. Immunohistochemistry

Heart tissues were stained immunohistochemically for HO-1, HO-2, and 8-OHdG. Five micrometer sections were transferred to positively charged slides. Monoclonal rabbit anti-rat HO-1 antibody (1:250) (Stressgen Biotechnologies Corp. Victoria, BC, Canada), polyclonal rabbit anti-rat HO-2 antibody (1:200) (Stressgen), and monoclonal mouse 8-OHdG antibody (1:150) (Chemicon Lab, Temecula, CA, USA) were used in conjunction with the streptavidin biotin reaction described in the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA). The chromogen, diaminobenzidine, was used to detect the staining pattern of HO-1 and HO-2, and hematoxylin was used to counterstain the slides. The negative controls were produced by replacing the primary antibody with non-immune rabbit serum. After three repetitions of the experiment, the slides were read by two investigators unaware of the particular treatment and were arbitrarily scored. 8-OHdG staining was quantified by the number of positive cardiomyocytes in 10 random fields (magnification 400×) containing approximately 100 cells per field, and the data were expressed as percentage total cells.

2.7. Histochemical analysis of iron

Paraffin-embedded tissues were used for Perl's Prussian blue staining modified with 3′,3′-diaminobenzidine (DAB) (Sigma-Aldrich Canada Ltd., Oakville, Canada) enhancement and localization of iron in liver tissues using standard procedures [26]. Briefly, 5-μm tissue sections were treated with HCl (5%) to liberate ferric ions, followed by H_2O_2 blockade. The samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. DAB enhancement was performed by incubating the slides with

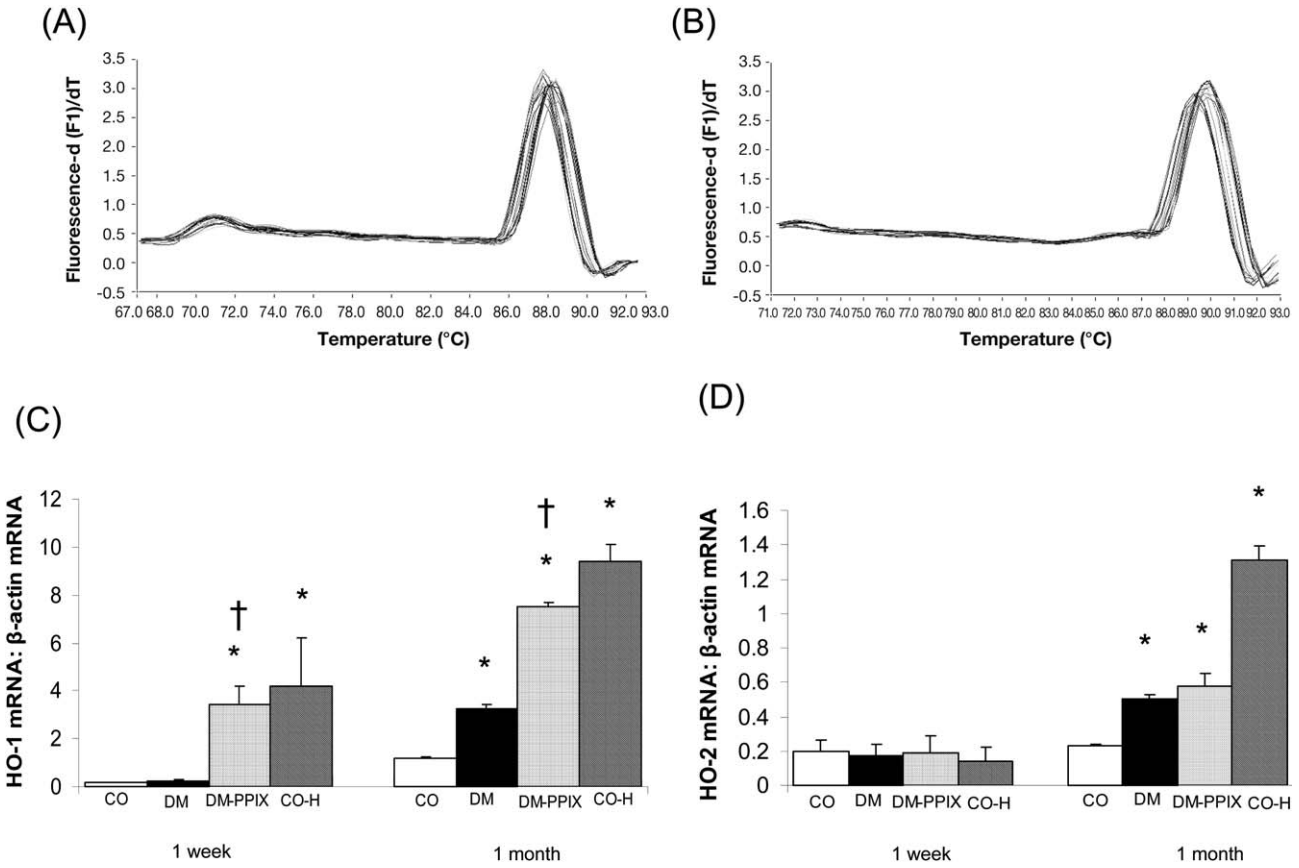

Fig. 1. Real-time RT-PCR amplification of HO-1 and HO-2 mRNA from heart tissues, showing (a) MCA of HO-1 PCR products, (b) MCA of HO-2 PCR products, (c) quantification of HO-1 mRNA levels, and (d) quantification of HO-2 mRNA levels. HO isozyme transcripts were expressed as ratio of target mRNA to β-actin mRNA levels (panels (A) and (B) represent MCA of post-PCR products used to determine specificity of PCR amplification, and each colored line represents individual PCR products; CO, controls, DM, diabetics, DM-PPIX, diabetics treated with SnPPIX, and CO-H, non-diabetic controls treated with hemin; * $P < 0.05$ as compared to controls, † $P < 0.05$ as compared to diabetics).

DAB (reconstituted with 5 ml H_2O) and 10% H_2O_2. The slides were washed and counterstained with methyl green.

2.8. Statistical analysis

The data are expressed as mean ± S.E.M. and were analyzed by ANOVA followed by Student's *t*-test. Differences were considered significant at values of $P < 0.05$.

3. Results

3.1. Clinical monitoring of animals

Diabetes was confirmed by blood glucose estimation, body weight gain, and glycated hemoglobin levels in rats. Diabetic animals exhibited hyperglycemia (18.67 ± 0.47 mmol/l after 1 week; 21.76 ± 1.83 mmol/l after 1 month) as compared to non-diabetic controls (4.87 ± 0.42 mmol/l after 1 week; 4.73 ± 0.65 mmol/l after 1 month). In addition, diabetic animals demonstrated elevated glycated hemoglobin levels (12.9 ± 1.0% vs. 6.3 ± 0.7%; $P < 0.05$ after 1 month) and reduced body weight gain (460 ± 18 g vs. 543 ± 19 g; $P < 0.05$ after 1 month). Hemin and SnPPIX treatments had no effects on these parameters. In addition, systolic blood pressure, recorded by tail plethysmography, did not differ among the animal groups (range 103 ± 9 mmHg).

3.2. Diabetes-induced upregulation of HO-1 and HO-2 mRNA, protein, and activity in the heart

We have used a sensitive and accurate real-time RT-PCR-based assay to quantify mRNA levels of HO-1 and HO-2. Although short-term (1 week) diabetes had no effect on HO-1 and HO-2 heart mRNA levels, our data show upregulation of both isozymes in diabetic rats after 1 month (Fig. 1). Heart tissues were also analyzed immunohistochemically for HO-1 and HO-2 protein expression and localization. Increased HO-1 and HO-2 immunoreactivity were seen in the cardiomyocytes of diabetic rats only after 1 month of diabetes (Figs. 2 and 3). In order to determine whether alterations of mRNA and protein levels coincide with altered function, we performed HO activity assay according to well-established methodologies [25]. An augmented HO activity in heart tissues of untreated diabetics was observed after 1 month compared to non-diabetic animals (Fig. 4).

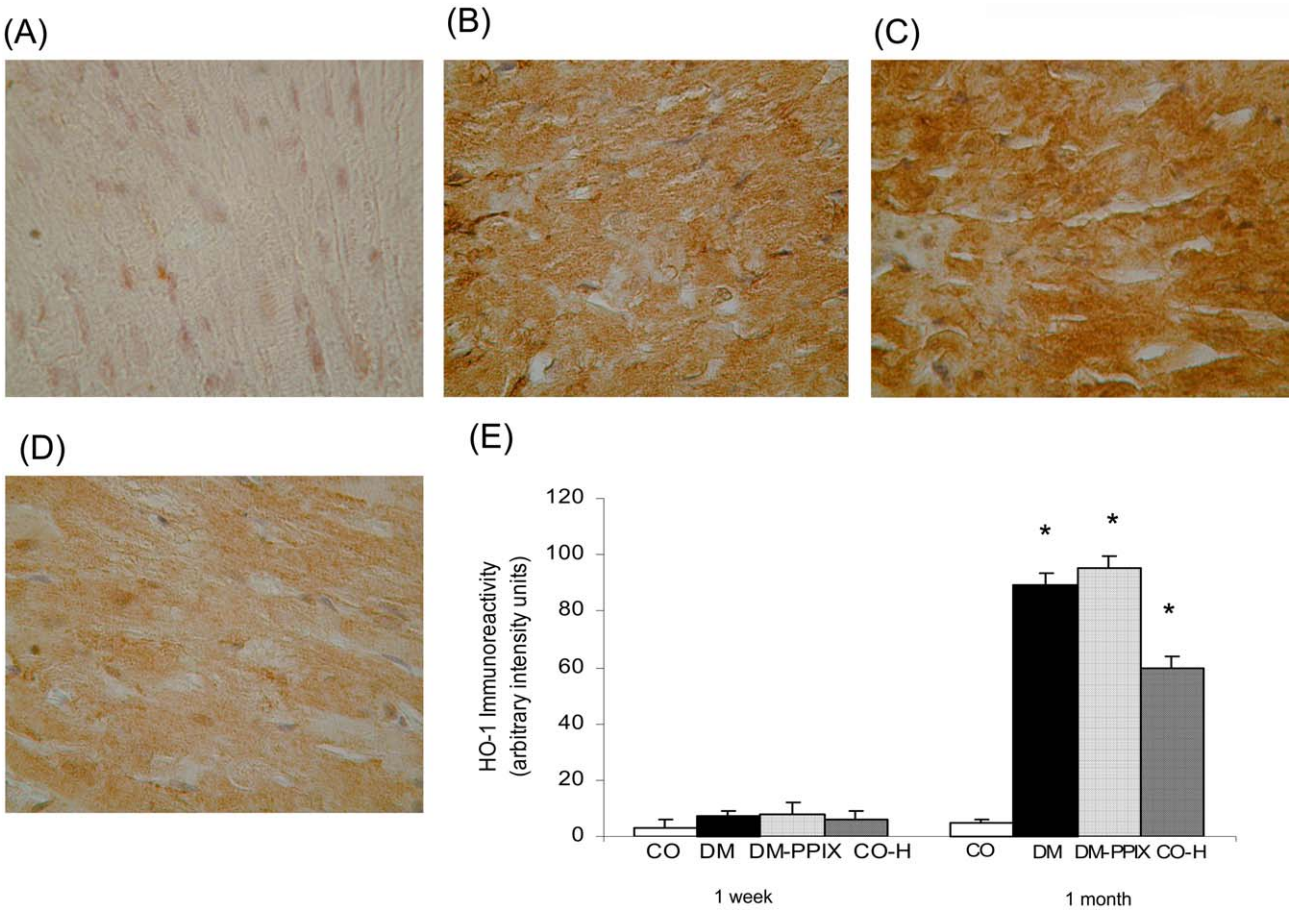

Fig. 2. Immunohistochemical analysis of HO-1 protein in heart tissues after 1 month of follow-up in (a) controls, (b) diabetic rats, (c) diabetic rats treated with SnPPIX, (d) non-diabetic control rats treated with hemin, and (e) semi-quantitative analysis of HO-1 protein levels in 1 week and 1 month of treatment groups (magnification 400×).

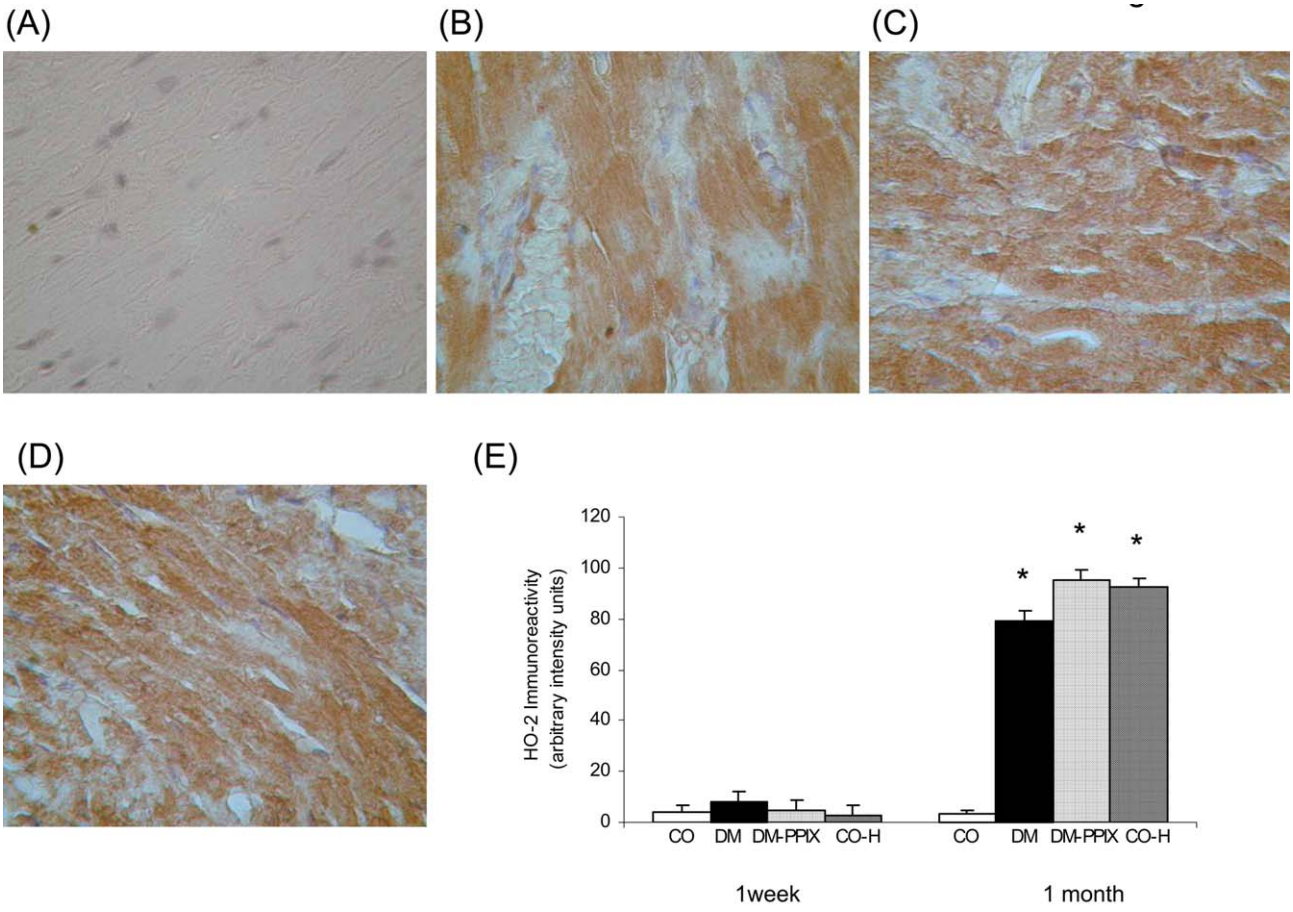

Fig. 3. Immunohistochemical analysis of HO-2 protein in heart tissues after 1 month of follow-up in (a) controls, (b) diabetic rats, (c) diabetic rats treated with SnPPIX, (d) non-diabetic control rats treated with hemin, and (e) semi-quantitative analysis of HO-2 protein levels in 1 week and 1 month of treatment groups (magnification 400×).

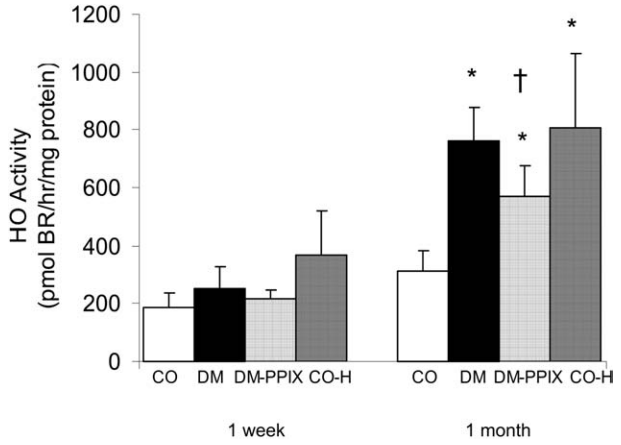

Fig. 4. HO activity following 1 week and 1 month of follow-up as assessed by bilirubin production from rat heart microsomal fractions and expressed as bilirubin produced per hour per milligram of protein. For abbreviations see caption of Fig. 1.

3.3. HO-mediated increased oxidative stress in the heart in diabetes

We have used 8-OHdG as a marker of oxidative stress. Previous studies using 8-OHdG have shown that this marker is the most sensitive and specific for DNA damage induced by ROS [27]. Our results indicate that although no measured difference in the 8-OHdG stains were seen after 1 week of diabetes, after 1 month of diabetes there was an increase in 8-OHdG immunoreactivity in heart tissues of diabetic animals (Fig. 5). These changes were also associated with the presence of stainable iron in cardiomyocytes (Fig. 6). SnPPIX treatment prevented diabetes-induced oxidative stress as detected by 8-OHdG stains (Fig. 5) and prevented iron accumulation in cardiomyocytes (Fig. 6).

3.4. Effects of an HO inhibitor on HO mRNA, protein, and activity in the heart in diabetes

Although SnPPIX prevented oxidative stress in diabetes (Fig. 5), treatment of diabetic rats with SnPPIX resulted in HO mRNA upregulation, both after 1 week (HO-1 only) and 1 month of diabetes (HO-1 and HO-2), and protein augmentation after 1 month of diabetes (Figs. 1–3). This finding is in accordance with previous studies which indicate a dual role of competitive HO inhibitors of the protoporphyrin family [28]. It has been demonstrated that protoporphyrins, including SnPPIX, upregulate mRNA and protein levels of HO system while inhibiting the activity of both preformed and newly synthesized HO [28]. Our data confirm that SnPPIX

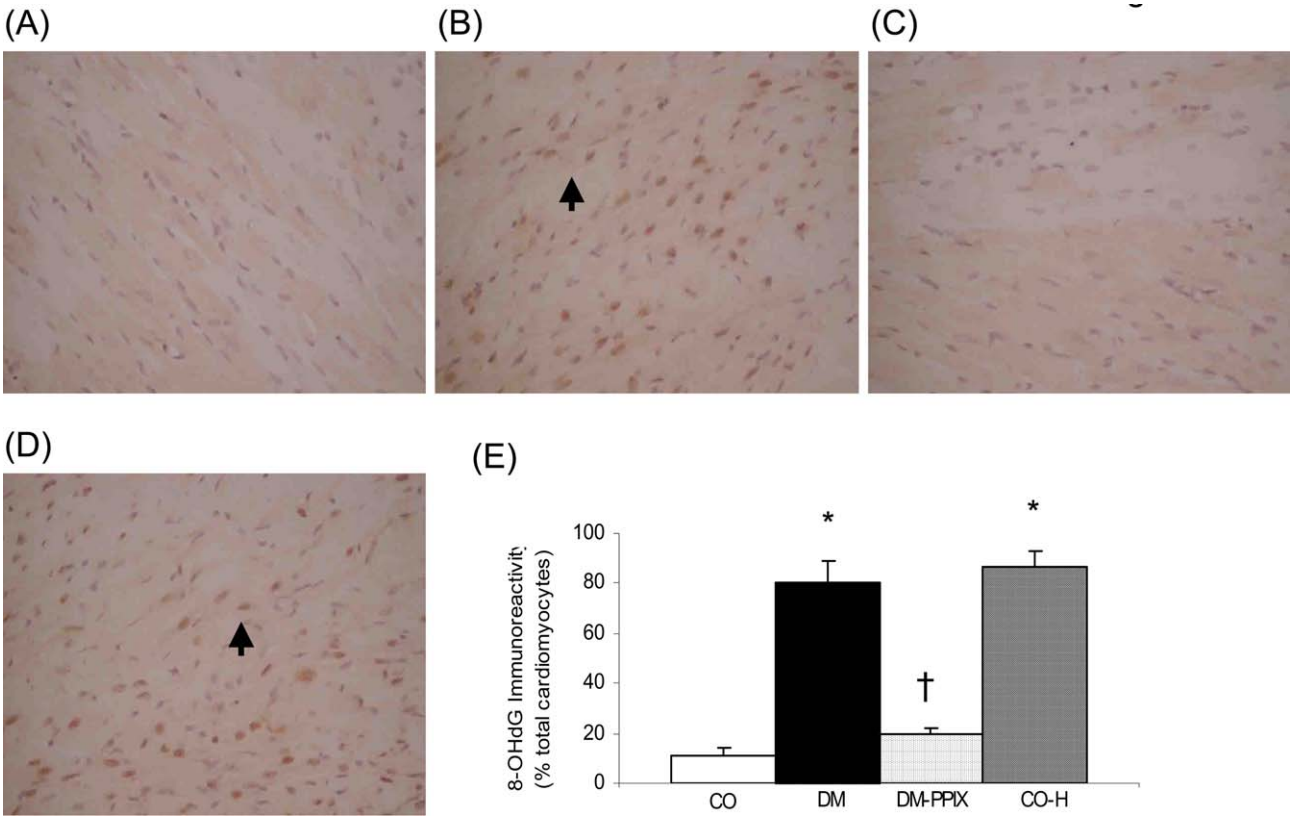

Fig. 5. 8-OHdG immunoreactivity in heart tissues after 1 month of follow-up in (a) control rats, (b) diabetic rats, (c) diabetic rats treated with SnPPIX, (d) non-diabetic controls rats treated with hemin, and (e) semi-quantitative analysis of 8-OHdG positive cardiomyocytes in 1 month treatment groups (positivity is demonstrated as brown nuclear stain (arrow) (magnification 400×). For abbreviations see caption of Fig. 1.

does upregulate mRNA and protein levels while inhibiting HO activity (Figs. 1–4).

3.5. HO agonist produces diabetes-like changes in the heart

To further elucidate the role of HO in the heart in diabetes, we treated a group of non-diabetic animals with an HO agonist, hemin, for the same time periods. Hemin treatment in non-diabetic animals demonstrated increased HO mRNA levels after 1 week (HO-1 only) and 1 month of hemin treatment (HO-1 and HO-2), and augmented HO protein levels after 1 month of treatment (Figs. 1–3). In association with increased mRNA and protein levels, hemin treatment after 1 month increased HO activity in the heart of non-diabetic animals (Fig. 4). Although hemin increased HO-1 mRNA levels after 1 week of treatment, no significant alterations were observed with the amount and activity of the protein. Furthermore, hemin treatment for 1 month also increased 8-OHdG immunoreactivity in the cardiomyocytes (Fig. 5), as well as mediating iron accumulation in the cardiomyocytes (Fig. 6).

3.6. Relationship of HO with NO

Analysis of a possible NO system alteration revealed no significant differences among any of the groups after 1 week of diabetes (data not shown). However, following 1 month of diabetes, heart tissues exhibited upregulation of both eNOS and iNOS mRNA levels (Fig. 7a,b). Although diabetes showed a slight increase in the amount of total NOS products (nitrate and nitrite) produced, these changes did not reach a level of significance (Fig. 7c,d). SnPPIX treatment of diabetic rats had no effect at the mRNA level, as compared to diabetic animals, and had no effect with regard to the amount of NOS products formed. Hemin treatment of the non-diabetic animals also showed an upregulation of NOS mRNA levels. However, such changes did not parallel alterations of activity as assessed by NOS product levels.

4. Discussion

In the present study, we have demonstrated that short-term diabetes (1 month) leads to an upregulation of HO system at both the mRNA and protein level. Such upregulation was found to be associated with increased HO activity and oxidative stress as evident by 8-OHdG immunoreactivity. In parallel, our results indicate reduced HO activity and oxidative stress in diabetic rats treated with a potent HO inhibitor SnPPIX. We have also demonstrated diabetes-like changes in regards to aforementioned parameters by treating non-diabetic animals with HO agonist, hemin. These results provide the first evidence of a pro-oxidant activity of HO in the heart in diabetes.

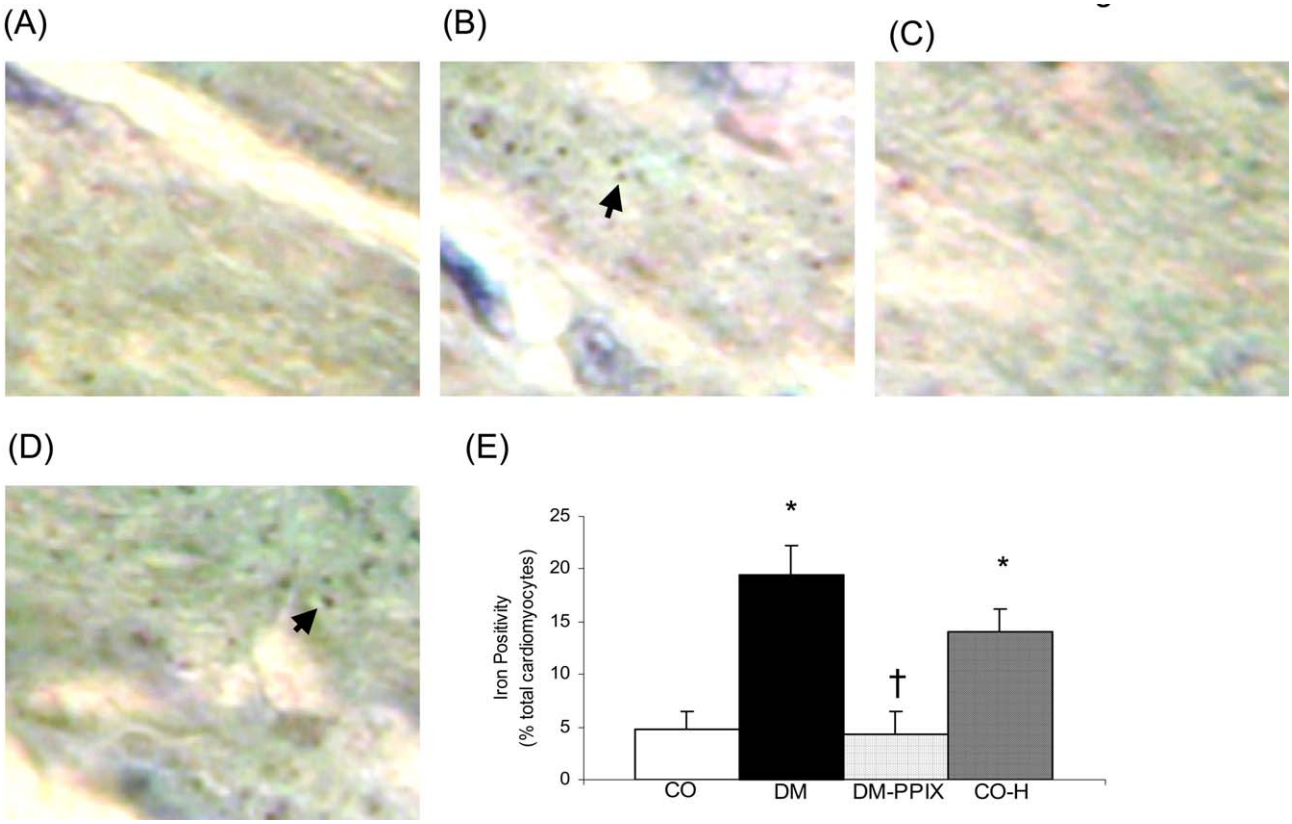

Fig. 6. Histochemical analysis of iron in heart tissues as assessed by DAB-enhanced Perl's Prussian blue staining after 1 month of follow-up in (a) control rats, (b) diabetic rats, (c) diabetic rats treated with SnPPIX, (d) non-diabetic control rats treated with hemin, and (e) semi-quantitative analysis of stainable iron in cardiomyocytes of 1 month treatment groups (positive staining is observed as dark granules (arrow) within the cytoplasm; magnification 1000× (oil immersion)).

It is believed that the HO represents a more generalized anti-oxidant response system [29–33]. A number of in vitro and in vivo studies indicate induction of the HO system in response to a wide array of oxidative and cellular stresses [29–33]. The mechanism of such cytoprotection is not fully known. It is speculated that oxidative degradation of heme, production of anti-oxidant bilirubin, and sequestration of redox-active iron by ferritin could arbitrate such HO-mediated cytoprotection [34–36]. However, such notion of a generalized protective role of HO should be approached with caution. In the present study, we have confirmed recent evidence, which suggests that HO induction might not be beneficial depending on the particular context [14,37–39]. We have demonstrated that diabetes-induced oxidative stress in the heart is, in part, due to increased expression as well as increased activity of the HO system. These findings suggest that HO induction could act as a pro-oxidant mechanism depending on the disease milieu. HO-mediated reaction releases iron directly into the endoplasmic reticulum. Little is known about the immediate consequences of such iron release. However, in vivo effects of pro-oxidant iron have been well documented and include lipid peroxidation and oxidative stress-induced DNA damage [40,41]. These effects are mediated by hydroxyl radical production through Fenton reaction. In addition to iron release, HO could also produce H_2O_2 as a by-product, which could potentiate HO-mediated oxidative stress [42]. It is interesting to note that although in this study HO acted as a cytotoxic factor, it may also function as an anti-oxidant. Such dual roles of HO as well as other compounds have been demonstrated [43,44].

We have performed these experiments in a well-established model of chronic diabetic complications. Using this model, we and others have previously demonstrated diabetes-induced pathogenetic changes in the heart such as focal scarring and activation of redox-sensitive transcription factors, nuclear factor-kappa B (NFκB) and activating protein-1 (AP-1) [45–47]. NFκB and AP-1 are common targets for activation by hyperglycemia, oxidative stress, cytokines, and vasoactive factors, all of which are implicated in the development of diabetic complications. NFκB activation in diabetes, possibly in part through augmented HO activity, could alter NO formation and action [48,49]. In addition, NO may react with superoxide anion to produce reactive peroxyl nitrite radicals and potentiate oxidant toxicity. In our study, we did find eNOS and iNOS mRNA levels to be upregulated in diabetic as well as hemin-treated non-diabetic animals. However, such upregulation was not found to coincide with increased NO formation as assessed by nitrite and nitrate levels in heart tissues. Furthermore, no alterations of the NOS–NO pathway was observed following 1 week of treatment. It is plausible that an interrelationship exists between HO and NO systems in mediating oxidative stress. Diabetes-induced impairment of the NO system, a well-documented event, could be responsible, in part, for the

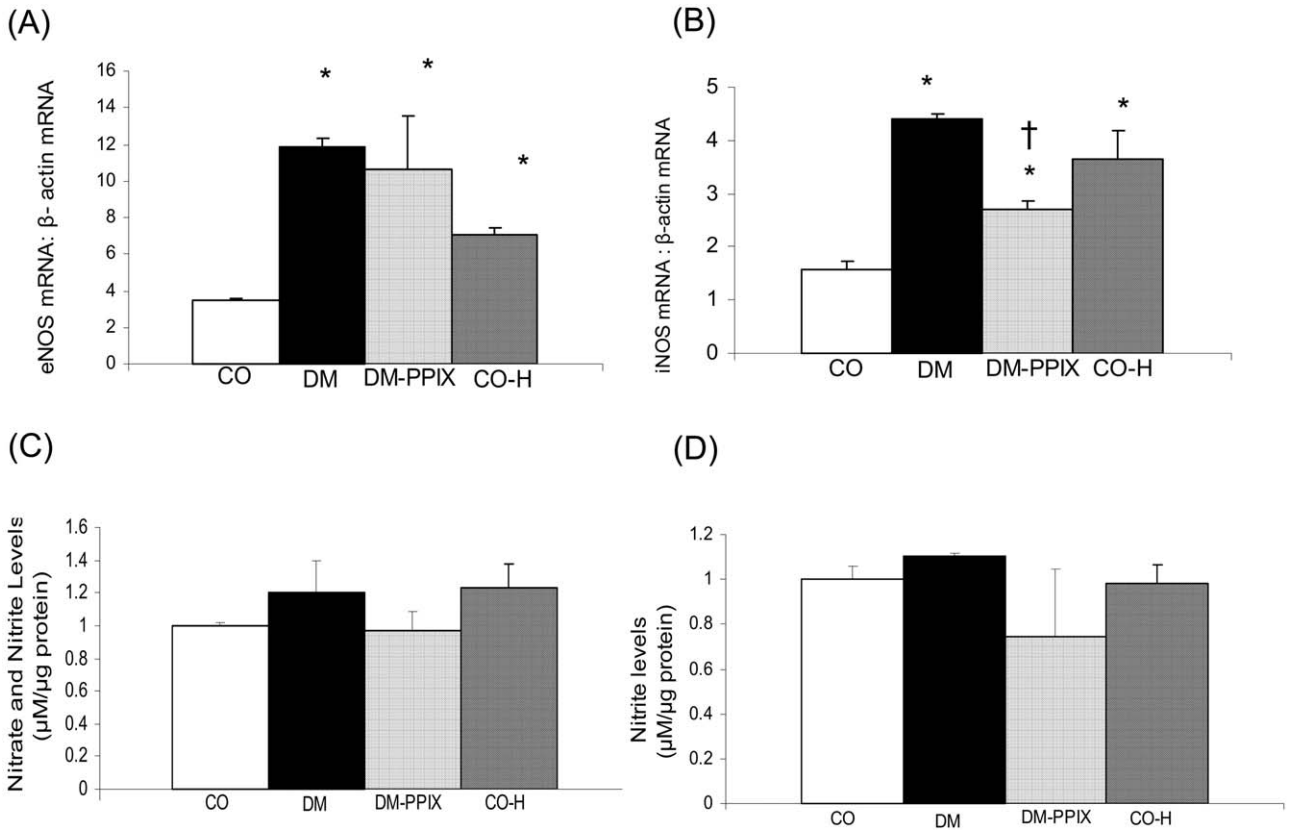

Fig. 7. NOS mRNA expression and the amount of NOS products produced in various groups after 1 month of follow-up. Figure illustrates (a) eNOS mRNA levels, (b) iNOS mRNA levels, (c) total NOS products (nitrate and nitrite levels), and (d) nitrite levels after 1 month of treatment. NOS isozyme transcripts were expressed as ratio of target mRNA to β-actin mRNA levels. NOS products were measured as μM/μg protein, relative to control. For abbreviations see caption of Fig. 1.

induction of HO system. However, our study revealed no changes of NOS activity suggesting an NO-independent mechanism for HO upregulation.

In summary, we have demonstrated that 1 month of diabetes leads to alteration of the HO system in the heart which is, in part, responsible for increased oxidative stress. Furthermore, such HO-mediated oxidative stress could be involved in cellular damage and diabetic cardiomyopathy.

Acknowledgements

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