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PPAR-γ agonist, pioglitazone, reduced oxidative and endoplasmic reticulum stress associated with L-NAME-induced hypertension in rats

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ABSTRACT

Peroxisome proliferator-activated receptor γ (PPAR-γ) agonist, pioglitazone, is used clinically to improve the glycemic state in patients with type-2 diabetes mellitus. Independent of its blood glucose-lowering properties, pioglitazone ameliorates different cardiovascular disorders. The aim of the present study was to investigate the effect of pioglitazone on cardiovascular complications of N-nitro-L-arginine methyl ester (L-NAME)-induced hypertension and to determine the role of oxidative and endoplasmic reticulum (ER) stress in its activity. Nitric oxide (NO) deficiency induced by chronic L-NAME administration was associated with high blood pressure (BP) and cardiac hypertrophy. L-NAME induced oxidative stress as indicated by reduced glutathione (GSH) levels, superoxide dismutase (SOD) and catalase activities as well as increased malondialdehyde (MDA) levels. Furthermore, L-NAME increased the expression of ER stress markers, activating transcription factor-4 (ATF-4) and C/EPBα-homologous protein-10 (CHOP-10) in both heart and aorta of hypertensive rats. Activation of PPAR-γ by pioglitazone reduced BP, restored the blunted NO levels, increased endothelial NO synthase (eNOS) expression, and restored the antioxidant status of L-NAME-induced hypertensive rats. Moreover, the anti-hypertensive activity of pioglitazone was associated with a reduction in ER stress and this effect was PPAR-γ dependent. Interestingly, the effect of ER stress inhibitor, 4-phenylbutyric acid (4-PBA) and antioxidant, N-acetylcysteine (NAC), on BP, NO availability, oxidative stress and ER stress mimics the activity of pioglitazone. Taken together, our data suggests that PPAR-γ is a potential target to inhibit vascular complications and cardiac damage associated with NO-deficient HTN and puts more emphasis on the importance of ER stress in regulating PPAR-γ activity.

1. Introduction

Uncontrolled hypertension (HTN) is a global health condition which results in cardiovascular complications and end organ damage [1]. Endothelial dysfunction, cardiac remodeling and cardiac hypertrophy are the common remarks of uncontrolled HTN [2,3]. Clinical trials on patients with HTN revealed that non-antihypertensive agents such as anti-obesity and insulin sensitizing drugs improve endothelial function and reduce blood pressure and may provide important insights on HTN management [4].

Disruption of nitric oxide synthase (NOS) system and reduction in nitric oxide (NO) levels are associated with endothelial dysfunction and progression of HTN [5]. NO synthesis can be inhibited by L-arginine analogues such as N-nitro-L-arginine methyl ester (L-NAME) [6]. Several studies have reported that chronic administration of L-NAME inhibits the activity of NO synthases (NOSs) including inducible NOS (iNOS) and endothelial NOS (eNOS) in rats, causes structural changes in blood vessels, and contributes to systemic arterial HTN [7,8].

Oxidative stress occurs due to intracellular accumulation of reactive oxygen species (ROS) and/or reduction in their elimination [9]. Oxidative stress is a key mediator in the pathogenesis of HTN [10]. In vascular cells, superoxide radical ($O2^{\bullet -}$) is generated from oxygen (O_2) by NADPH oxidase, xanthine oxidase and uncoupled NOS. $O2^{\bullet}-$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) enzyme and then eliminated by catalase which converts H_2O_2 to H_2O and O_2 [11]. Previous studies have reported that HTN is associated with elevation of NADPH oxidase and reduction of SOD activities [12]. Furthermore, $O2^{\bullet}-$ produced by NADPH oxidase interacts with NO to form highly reactive peroxynitrite ($ONOO^-$) resulting in impaired endothelium-dependent vasodilation and uncoupling of NOS. Uncoupled

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List of abbreviations

15d-PGJ_2	15-deoxy-Delta(12,14)-prostaglandinJ_2
4-PBA	4-Phenylbutyric Acid
AAP	4-aminophenazone
ANOVA	One-way analysis of variance
ATF-4	Activating transcription factor-4
BADGE	Bisphenol A Diglycidyl Ether
CHOP-10	C/EPBα-homologous protein-10
DHBS	3,5-Dichloro -2-hydroxybenzene sulfonic acid
eIF2-α	Eukaryotic initiation factor 2
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic Reticulum
GMP	Guanosine Monophosphate
GSH	Reduced Glutathione
H_2O_2	Hydrogen peroxide,
HE	Hematoxylin and eosin
HTN	Hypertension
iNOS	Inducible nitric oxide synthase
L-NAME	N-Nitro-L-Arginine Methyl Ester
MAP	Mean arterial pressure
MDA	Malondialdehyde
NAC	N-acetylcysteine,
NIBP	Non-invasive blood pressure
NO	Nitric oxide,
NOS	Nitric Oxide Synthase
NOSs	Nitric Oxide Synthases
Nrf2	Nuclear factor erythroid 2-related factor 2
PERK	Protein kinase RNA-like ER kinase
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
qRT- PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive Oxygen Species
S.E	Standard error of mean
SOD	Superoxide dismutase
UPR	Unfolded protein response

NOS produce more vasoconstrictor $O2^{\bullet}-$ and less vasodilator NO [13].

Endoplasmic reticulum (ER) is a cellular organelle in which proteins are folded into their final 3-dimentional structures [14]. In various conditions, such as ischemia, hypoxia, heart shock, gene mutation, and elevated protein synthesis, ER functions are disturbed resulting in ER stress and initiation of unfolded protein response (UPR) to help cell cope with this stress [15]. However, prolonged ER stress induces signaling pathway which causes apoptotic cell death. Accumulation of unfolded protein in ER results in increased expression activating transcription factor 4 (ATF-4) which increases the transcription of the pro-apoptotic protein, C/EPBα-homologous protein-10 (CHOP-10) [16]. Previous studies have reported that ER stress signaling is implicated in HTN and cardiovascular remodeling and the use of ER stress inhibitors such as taurine-conjugated ursodeoxycholic acid and 4-phenylbutyric acid (4-PBA) reduces cardiac damage and improves vascular function in HTN [17]. Furthermore, genetic inhibition of ER stress reduced the blood pressure and inhibited ROS production in angiotensin II–induced HTN model [18].

Peroxisome proliferator activated receptor gamma (PPAR-ɣ) is ligand activated nuclear receptors which regulate the transcription of several genes involved in lipid metabolism and energy expenditure [19]. PPAR-ɣ receptors are constitutively expressed in endothelial cells, vascular smooth muscles, kidney, and heart suggesting their roles in cardiovascular diseases [20]. In addition, activation of PPAR-ɣ receptors by antidiabetics, thiazolidinediones (TZDs) has been shown to possess a protective role on the vasculature of diabetic subjects which is distinct from any beneficial vascular consequences of their glucose lowering effect [21]. Therefore, more studies shed the light on the possible mechanisms of the cardioprotective effect of PPAR-ɣ agonists. It has been reported that activation of PPAR-ɣ improves endothelial functions, elicits antioxidant activity and inhibits ER stress [22–24]. PPAR-ɣ agonist, 15-deoxy-Delta (12, 14)-prostaglandin J2 (15d-PGJ_2), increased NO availability as well as NOSs expression and activity in endothelial cells [25]. Furthermore, another PPAR-ɣ agonist, pioglitazone, increased the expression of antioxidant enzymes such as nuclear factor erythroid 2-related factor 2 (Nrf2), SOD and catalase in renal cortex of spontaneous and borderline hypertensive rats resulting in reduction in systolic blood pressure [26]. Interestingly, pioglitazone attenuated palmitate-induced ER stress in macrophages and this effect was reversed in the presence of GW9662, a PPARγ antagonist [27]. However, to the extent of the authors' knowledge, the crosstalk between PPAR-γ activation and ER stress signaling in HTN has not been previously studied.

In the present study we hypothesized that the antihypertensive effect of PPAR-γ-agonist, pioglitazone, is mediated by reduction of oxidative stress and ER stress which in turn restore NO levels. Using L-NAME-induced hypertensive rats, pioglitazone reduced mean arterial pressure (MAP) and increased NO levels in PPAR-γ-dependent manner. Moreover, pioglitazone activity was associated with reduction of both oxidative stress and ER stress. Supporting our hypothesis, the use of ER stress inhibitor, 4-Phenylbutyric acid (4-PBA), and antioxidant, N-acetylcysteine (NAC), restored NO levels in L-NAME-induced hypertensive rats.

2. Materials and methods

2.1. Animals

Fifty-six adult male Wistar rats (35–42 days old, 200–250 g) were obtained from the Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were housed 4 rats/cage and kept on a light-dark cycle (12 h/12 h) at 23 ± 2 °C. Food and water were available ad libitum. All animal procedures were approved by Ethical Committee for Animal Handling at Zagazig University.

2.2. Drugs and chemicals

L-NAME, 4-Phenylbutyric acid (4-PBA), and Kolliphor® EL were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). 4-PBA is a low molecular weight chemical chaperone prevents the aggregation of misfolded proteins and alleviates ER stress and is used in the present study as positive control [28]. Antioxidant NAC were obtained from Sedico, Egypt. Pioglitazone (PIOG) was purchased from Takeda chemical industries, Osaka, Japan. Bisphenol A diglycidyl ether (BADGE) was purchased from Alfa Aesar, Karlsruhe, Germany. BADGE is a selective PPAR-ɣ antagonist used in the present study to determine the role of PPAR-ɣ in pioglitazone activity [29]. Rabbit ATF-4 and rabbit CHOP-10 antibodies were obtained from Santa Cruz Biotechnology, Dallas, TX, USA. Microemulsion of pioglitazone, BADGE, and 4-PBA were freshly prepared using Kolliphor ® EL as surfactant, ethanol as co-surfactant, and saline (1:1:9) [30].

2.3. Induction of HTN

Hypertension was induced by inhibition of NOS activity using L-NAME [31]. L-NAME-induced inhibition of NOS is a well-established model of experimental hypertension and cardiovascular disorders which was previously validated by several studies [32,33]. In the present study, all animals except for the control (CTR) group (8 animals) were received L-NAME (75 mg/kg/day, orally) for six weeks. After that,

animals with HTN were randomly divided into six groups (8 animals/group) to receive different treatments.

2.4. Experimental groups

Animals were divided into six groups (8 rats/group), CTR group (animals received vehicle, Kolliphor ® EL: ethanol: saline (1:1:9)), HTN group (animals received L-NAME for six weeks and then were administered with vehicle until the end of the study), BADGE group (animals received L-NAME for six weeks and then treated with BADGE 15 mg/kg/day, i.p., for 30 days [34], PIOG group (animals received L-NAME for six weeks and then treated with pioglitazone, 10 mg/kg/day, orally, for 30 days [35], BADGE/PIOG group (animals received L-NAME for six weeks and then pretreated with BADGE 30 min before pioglitazone treatment for 30 days), PBA group (animals received L-NAME for six weeks and then treated with PBA, 150 mg/kg/each other day, i.p., for 30 days) [36], and NAC group (animals received L-NAME for six weeks and then treated with NAC, 500 mg/kg/day, orally, for 30 days). Several studies have reported that PIOG, BADGE, PBA and NAC have no effect on blood pressure of normotensive rats [35,37–39]. Hence, the current study is focusing on the activity of these compounds on L-NAME induced hypertensive rats.

2.5. Blood pressure measurement

Systolic blood pressure and mean arterial pressure (MAP) were measured using rat non-invasive blood pressure (NIBP) system (Harvard Apparatus Ltd, Edenbridge, Kent, England). MAP was calculated using the equation, diastolic pressure + 1/3* [systolic pressure-diastolic pressure] [40]. The average of three blood pressure readings was calculated for each animal.

2.6. Sampling

At the end of the experiment, animals were euthanized by isoflurane inhalation and then blood samples were collected from the retro-orbital plexus in heparinized tubes. Portions of heparinized blood samples were used immediately for reduced glutathione (GSH) measurement, other portions were centrifuged at 4000 rpm for 10 min for separation of plasma and blood cells (erythrocytes). Erythrocytes were used for measurement of SOD activity and plasma samples were aliquoted and stored at − 80 °C for other biochemical analyses.

Heart and aorta from each animal were immediately excised after blood sampling. Heart was used for determination of cardiac index and then fixed in formalin solution for immunohistochemical analysis. Part of aorta was flash frozen and preserved at − 80 °C for quantitative reverse transcription polymerase chain reaction (qRT- PCR). The other part of aorta was immediately fixed in formalin solution for histopathological examination.

2.7. Heart weight index determination

Heart weight index was determined as described by Refs. [41,41]. Heart excised from each animal was dotted free of blood and weighed. The heart weight index was calculated using the formula (heart weight ÷ body weight).

2.8. Measurement of NO levels

NO levels were measured in plasma samples using colorimetric NO assay kit (Bio-diagnostic, Egyptian company of biotechnology, Egypt) according to manufacturer protocol. The principle of this assay is based on the chemical reaction between nitrite in plasma samples and sulfanilamide in acidic conditions to form a product that is coupled with N-(l-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish - purple color which can be measured at 540 nm [42].

Table 1
Sequence of primers used in qRT-PCR experiment.

Target gene	Primer sequence: 5`- 3`	Gene bank accession number
iNOS	F: CTGTCACCGAGATCAATGCA R: CATGAGCAAAGGCACAGAAC	NM_012611.3
eNOS	F: GGGCAACTTGAAGAGTGTGG R: AAGAGTTCTGGGGGCTCATC	NM_021838.2
B-actin	F: ATGGATGACGATATCGCTGC R: CTTCTGACCCATACCCACCA	NM_031144.3
CHOP10	F: AGGTCCTGTCCTCAGATGAAA R: TAGGGATGCAGGGTCAAGAGT	NM_024134.2
ATF-4	F: TCCTCGATACCAGCAAATCC R: ACCCATGAGGTTTGAAGTGC	NM_024403.2

2.9. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Relative mRNA expression of iNOS, eNOS, CHOP-10 and ATF-4 was measured using qRT-PCR. Frozen aorta tissues were lysed using Qiazol reagent and total RNA was extracted using RNAeasy Mini Kit (Qiagen, USA). 10 ng of the total RNA from each sample were used for cDNA synthesis using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified using Syber Green I PCR Master Kit (Fermentas, USA). The specific primer sequences are listed in Table 1. Cycle threshold (CT) values were normalized to the internal B-actin control and ratios was detected using ΔΔCT method.

2.10. Biochemical analysis of oxidative stress markers

2.10.1. Reduced glutathione (GSH) measurement

GSH was measured in heparinized blood samples using colorimetric assay kit (Biodiagnostic, Egyptian company of biotechnology, Egypt). Erythrocytes were lysed in 4 times their volume of ice-cold distilled water, centrifuged at 4,000 rpm for 15 min at 4 °C, and then GSH was measured in the supernatant according to manufacturer protocol.

2.10.2. Malondialdehyde (MDA) analysis

Levels of lipid peroxidation was detected in blood plasma by measuring the concentration of MDA using colorimetric assay kit (Bio diagnostic kits, Egyptian company of biotechnology, Egypt) as instructed by the manufacturer.

2.10.3. Catalase activity analysis

Catalase activity was measured in blood plasma samples using colorimetric assay kit (Bio diagnostic kits, Egyptian company of biotechnology, Egypt). 0.5 mM Hydrogen peroxide, H_2O_2, (substrate) was added to plasma samples and allowed to interact with plasma catalase for 1 min. The reaction was stopped by the addition of chromogen inhibitor and 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) as well as 4-aminophenazone (AAP) were added to interact with the remaining H_2O_2 in the presence of peroxidase to form chromophore. The catalase activity in the original samples was then calculated as indicated by manufacturer instructions.

2.10.4. Superoxide dismutase (SOD) activity analysis

SOD activity was measured in erythrocyte lysate using colorimetric assay kit (Bio diagnostic kits, Egyptian company of biotechnology, Egypt). Erythrocytes separated from heparinized blood samples were washed four times with 0.9% NaCl solution, centrifuged for 10 min at 4000 rpm after each wash, and then lysed with cold deionized water (1:10). Erythrocyte lysate samples were used for measurement of SOD activity as instructed by manufacturer protocol.

2.11. Immunohistochemical (IHC) analysis

Protein expression of ER stress markers, ATF-4 and CHOP-10, was measured in heart and thoracic aorta using IHC. 5 μm thick sections of heart and aorta tissues were deparaffinized, gradually hydrated and then stained for ATF-4 or CHOP-10 (2 sections/organ/animal for each protein) using UltraVision LP detection system HRP DAB (Thermoscientific, WA7 1 TA, UK) according to manufacturer protocol. Relative integrated intensity was measured in 6 random fields/section using Fiji version of Image J software as previously described [43,44].

2.12. Histopathological examination

Histological analysis was performed on the heart and thoracic aorta (N = 6 per group). Formalin fixed tissues were embedded in paraffin and sectioned at 5 μm thickness using a microtome (Leica RM 2155, England). The paraffin sections were dewaxed in xylene, gradually hydrated, and then stained with hematoxylin and eosin (H&E). Histopathological changes were blindly analyzed and scored by expert pathologists who screened the entire section and captured the most representative images for each group. The following score system was used: 0 = no alteration, 1 = mild (weak) alterations, 2 = moderate alterations, 3 = severe alterations.

2.13. Statistical analysis

All data were presented as mean ± standard error of mean (S.E). Statistical analysis was performed using (Graph Pad software, Inc. La Jolla, CA, USA). The inter group variation was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

3. Results

3.1. Activation of PPAR-γ by pioglitazone reduced blood pressure and cardiac hypertrophy in L-NAME-induced hypertensive rats

The effects of pioglitazone on systolic blood pressure and mean arterial pressure (MAP) in L-NAME-induced hypertensive rats are graphically illustrated in Fig. 1A and B. Administration of L-NAME caused a significant increase in systolic blood pressure and MAP when compared to control group. Animals treated with pioglitazone showed reduction in systolic blood pressure and MAP when compared to HTN group. To determine if the antihypertensive activity of pioglitazone was PPAR-γ-dependent, animals with HTN pretreated with PPAR-γ antagonist, BADGE, and then treated with pioglitazone (BADGE/PIOG group). BADGE significantly diminished the effect of pioglitazone on blood pressure. Animals treated with ER stress inhibitor, 4-PBA, or antioxidant, NAC, showed reduced systolic blood pressure and MAP.

The heart weight to body weight ratio was significantly higher in L-NAME-induced hypertensive rats compared to control group. Pioglitazone treatment significantly reduced cardiac hypertrophy when compared to control group. The effect of pioglitazone on cardiac hypertrophy was inhibited in rats pretreated with BADGE (BADGE/PIOG group). Both 4-PBA and NAC reduced L-NAME-induced cardiac hypertrophy (Fig. 1C). There was no significant difference in the initial body weight (at the beginning of experiment) or final body weight (at the end of experiment) among the experimental groups (Fig. 1D).

3.2. Activation of PPAR-γ by pioglitazone increased NO levels and upregulated eNOS expression in L-NAME-induced hypertensive rats

Chronic administration of L-NAME caused a significant reduction in plasma NO levels (Fig. 2A) as well as eNOS (Fig. 2B) and iNOS (Fig. 2C) expression in HTN group when compared to control group. Animals

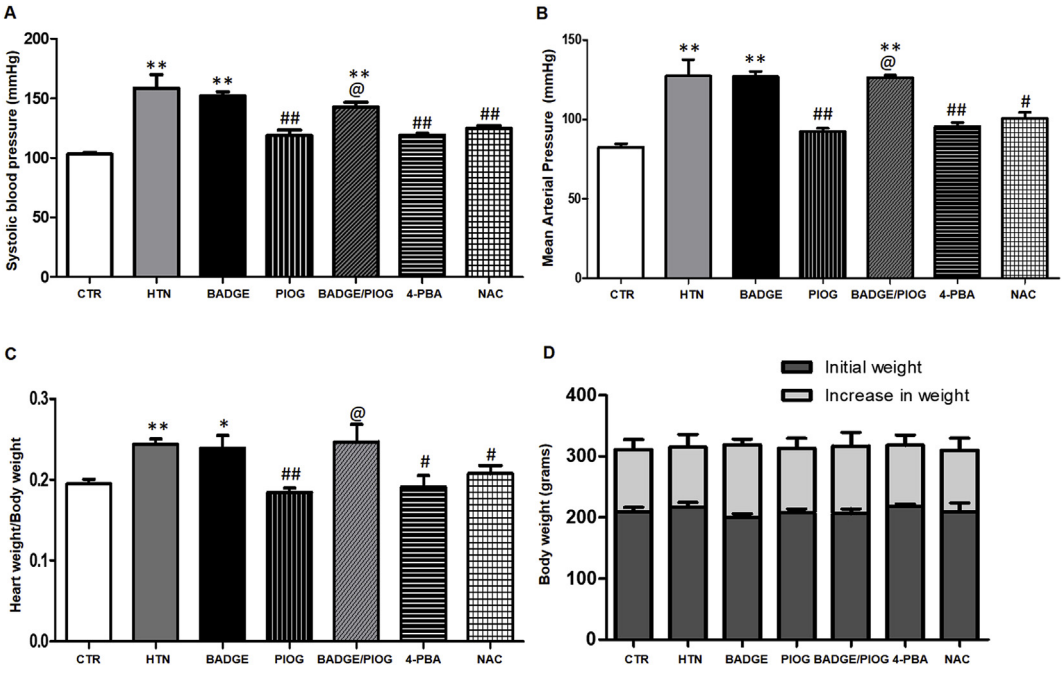

Fig. 1. Pioglitazone reduced blood pressure and heart weight index in L-NAME induced-hypertensive rats.
HTN was induced by oral administration of L-NAME (75 mg/kg/day) for 6 weeks). Animals with HTN were treated with vehicle (HTN group), BADGE (15 mg/kg/day, i.p.), pioglitazone (PIOG) (10 mg/kg/day, orally), BADGE and then pioglitazone after 30 min (BADGE/PIOG), 4-PBA (150 mg/kg/day, i.p.), or NAC (500 mg/kg/day, orally) for 30 days. Systolic blood pressure (A) and mean arterial pressure (MAP) (B) of different groups was measured using rat non-invasive blood pressure. (C) Heart weight index was calculated and represented as heart weight/body weight. (D) Initial weight of the animals (at the start of experiment), final body weight (at the end of experiment), and the increases in body weight of different groups. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, $P < 0.05$ compared to CTR group. **, $P < 0.01$ compared to control group (CTR). #, $P < 0.05$ compared to HTN group. ##$P < 0.01$ compared to HTN group. @, $P < 0.05$ compared to PIOG group. @@, $P < 0.01$ compared to PIOG group. $N \geq 6$).

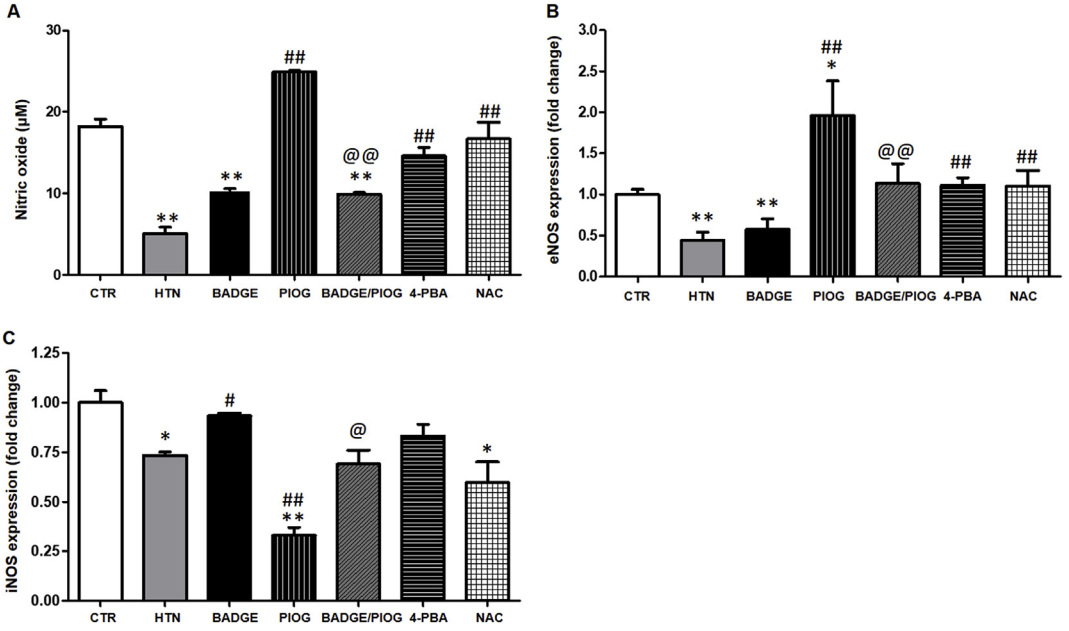

Fig. 2. Pioglitazone increased NO levels and upregulated eNOS expression in L-NAME-induced hypertensive rats.
L-NAME-induced hypertensive rats were treated as described in Fig. 1 legend. (A) NO level was measured in plasma. (B) eNOS and (C) iNOS expression were measured using qRT-PCR and expressed as fold change from control (CTR) group. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, P < 0.05 compared to CTR group. **, P < 0.01 compared to control group (CTR). #, P < 0.05 compared to HTN group. ##P < 0.01 compared to HTN group. @, P < 0.05 compared to PIOG group. @@, P < 0.01 compared to PIOG group. N ≥ 6).

treated with pioglitazone showed significant elevation in NO levels and eNOS expression when compared to HTN group (Fig. 2A and B, respectively). Further reduction in iNOS expression was observed in PIOG group when compared with HTN group (Fig. 2C). The effect of pioglitazone on plasma levels of NO and the expression of iNOS and eNOS was mediated by PPAR-γ activation. Animals pretreated with BADGE and treated with pioglitazone (BADGE/PIOG group) showed reduced levels of NO and eNOS expression as well as increased iNOS expression when compared with PIOG group (Fig. 2A–C). Furthermore, 4-PBA and NAC increased NO levels and eNOS expression with minimal effect on iNOS expression.

3.3. Pioglitazone reduced oxidative stress in the L-NAME-induced hypertensive rats

HTN induced by L-NAME was associated with oxidative stress which was manifested by suppression of GSH levels, reduction of SOD and catalase activities, and increase in MDA levels in HTN group when compared with control group (Fig. 3A–D). Pioglitazone treatment improved GSH levels, SOD and catalase activities, and MDA levels in PIOG

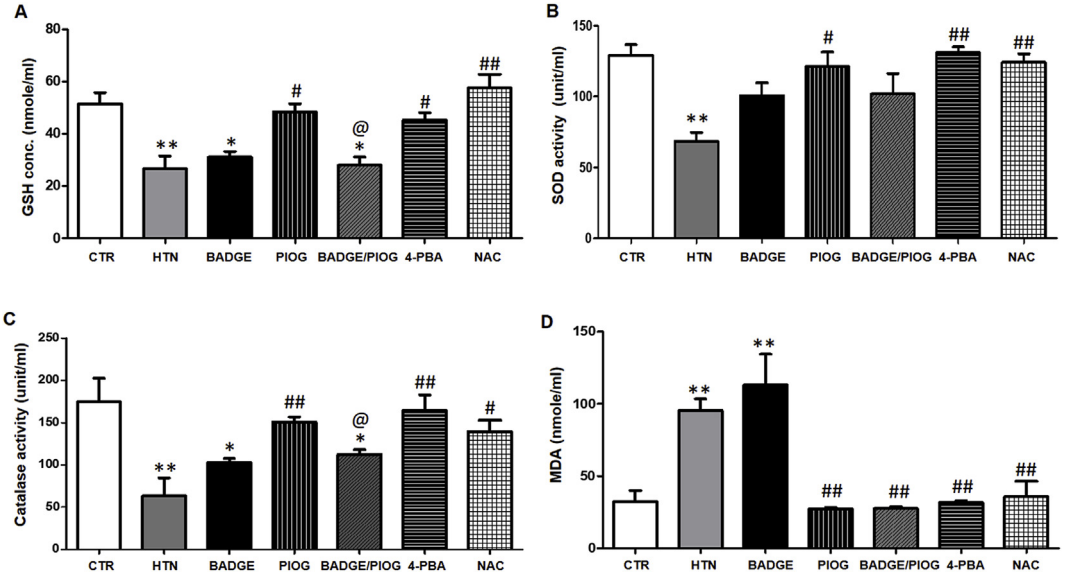

Fig. 3. Pioglitazone reduced oxidative stress in the L-NAME-induced hypertensive rats.
L-NAME-induced hypertensive rats were treated as described in Fig. 1 legend. GSH level (A), SOD activity (B), catalase activity(C), and MDA level (D) were measured as described in materials and methods section. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, P < 0.05 compared to CTR group. **, P < 0.01 compared to control group (CTR). #, P < 0.05 compared to HTN group. ##P < 0.01 compared to HTN group. @, P < 0.05 compared to PIOG group. @@, P < 0.01 compared to PIOG group. N ≥ 6).

group when compared with HTN group. The antioxidant activity of pioglitazone was partially mediated by PPAR-γ. Administration of BADGE significantly reduced pioglitazone-mediated elevation of GSH levels and catalase activity (Fig. 3A and C, respectively). However, slight reduction in pioglitazone-induced SOD activity (Fig. 3B) and minimal effect on MDA levels (Fig. 3D) were observed in animals treated with BADGE prior to pioglitazone administration (BADGE/PIOG group) when compared with PIOG group. ER stress inhibitor, 4-PBA, and antioxidant, NAC, restored GSH levels (Fig. 3A), increased catalase and SOD activities (Fig. 3B and C, respectively), and reduced MDA levels (Fig. 3D) when compared with HTN.

3.4. Activation of PPAR-γ by pioglitazone reduced ER stress in L-NAME-induced hypertensive rats

Previous studies have reported that ER stress is implicated in several cardiovascular disorders including HTN. To determine if the anti-hypertensive activity of pioglitazone is associated with reduction of ER stress, the levels of protein and mRNA expression of ER stress markers, ATF-4 and CHOP-10, were detected in heart and thoracic aorta. IHC showed that L-NAME significantly increased the expression of ATF-4 and CHOP-10 proteins in both heart (Figs. 4A and 5A) and thoracic aorta (Figs. 4B and 5B) tissues of HTN group when compared with control group. Pioglitazone treatment inhibited L-NAME-induced ER stress in PPAR-γ-dependent manner. Pioglitazone reduced ATF-4 and CHOP-10 protein expression in heart and aorta of animals in PIOG group when compared with HTN group. Pretreating animals with BADGE reduced the inhibitory effect of pioglitazone on L-NAME-induced ATF-4 and CHOP-10 protein expression. Both 4-PBA and NAC reduced the expression of ATF-4 and CHOP-10 proteins in heart and aorta when compared with HTN group (Figs. 4 and 5).

Fig. 6 shows relative mRNA expression of ATF-4 in heart (6A) and

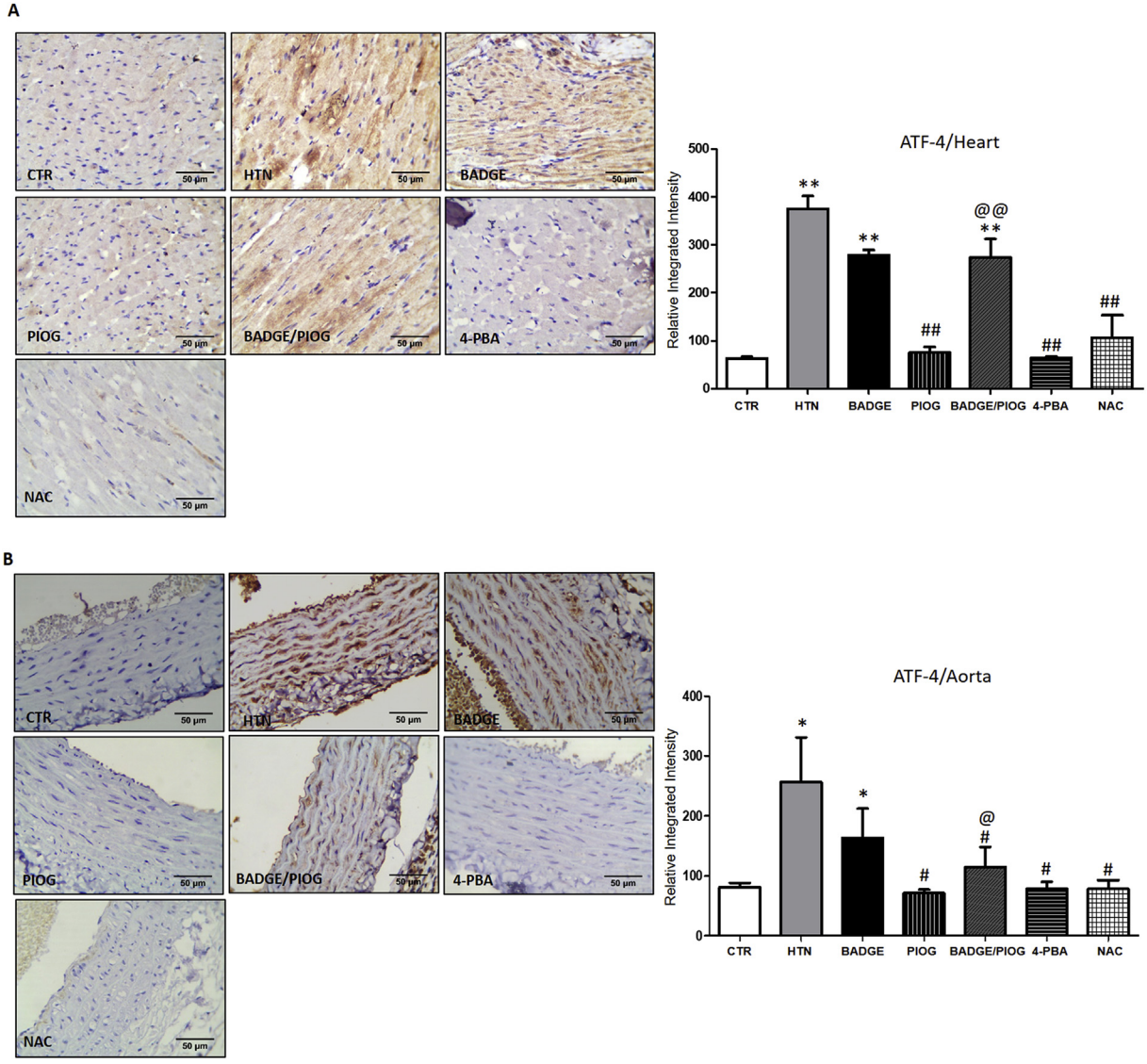

Fig. 4. Pioglitazone reduced ATF-4 protein expression in L-NAME-induced hypertensive rats.
ATF-4 expression was measured in heart (A) and thoracic aorta (B) using immunohistochemistry. Left panels show photomicrograph (X400) of ATF-4 expression in cardiac (A) and aortic (B) sections of control group (CTR), L-NAME-induced hypertensive rats (HTN), BADGE treated hypertensive rats (BADGE), pioglitazone treated hypertensive rats (PIOG), BADGE and pioglitazone treated hypertensive rats (BADGE/PIOG), 4-phenylbutyric acid treated hypertensive rats (4-PBA), and N-acetylcysteine treated hypertensive rats (NAC). Right panels are histograms showing the relative integrated intensity of ATF-4 expression using Fiji software. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, $P < 0.05$ compared to CTR group. **, $P < 0.01$ compared to control group (CTR). #, $P < 0.05$ compared to HTN group. ##$P < 0.01$ compared to HTN group. @, $P < 0.05$ compared to PIOG group. @@, $P < 0.01$ compared to PIOG group. $N \geq 6$).

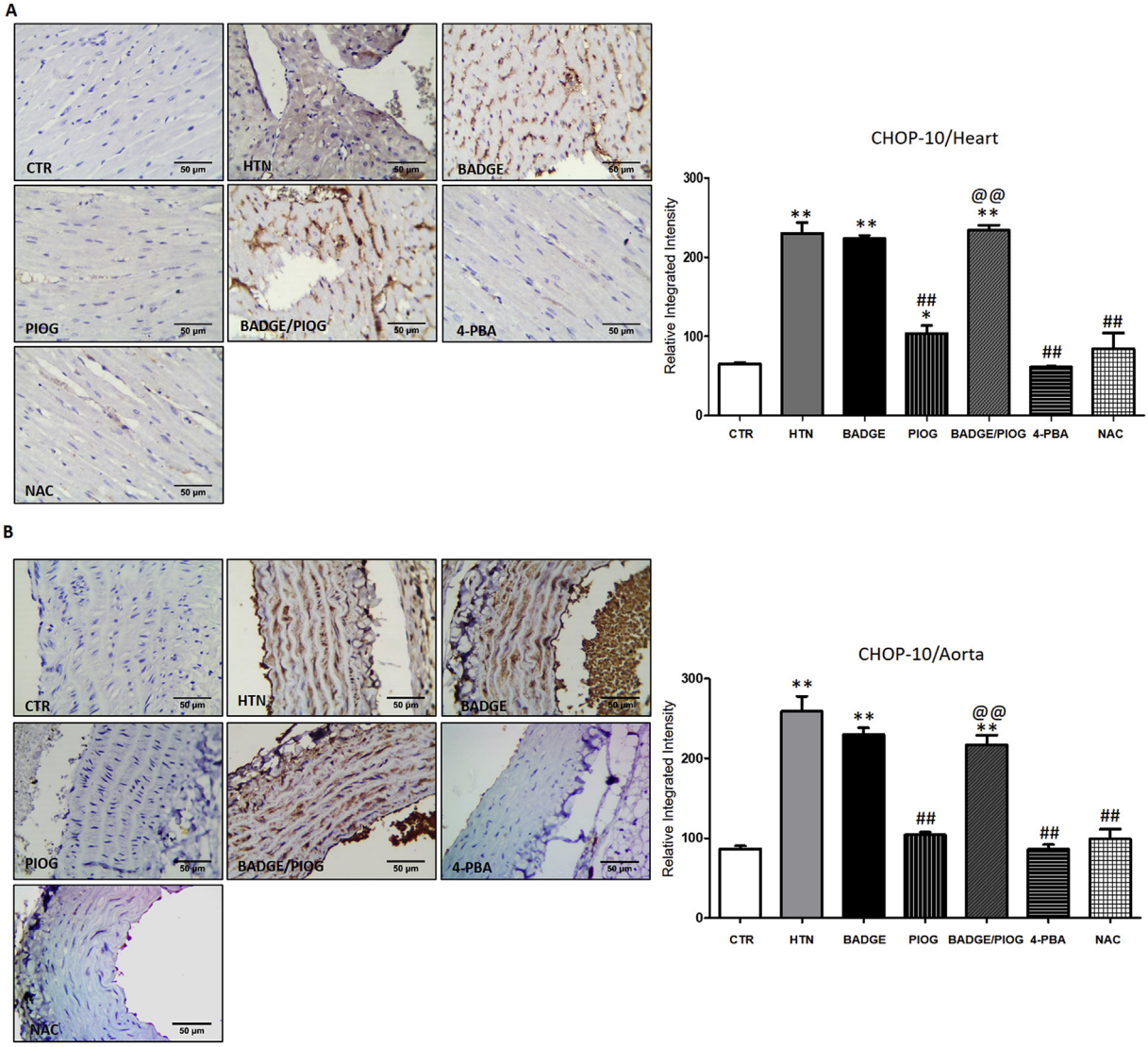

Fig. 5. Pioglitazone reduced CHOP-10 protein expression in L-NAME-induced hypertensive rats.
CHOP-10 expression was measured in heart (A) and thoracic aorta (B) using immunohistochemistry. Left panels show photomicrograph (X400) of CHOP-10 expression in cardiac (A) and aortic (B) sections of control group (CTR), L-NAME-induced hypertensive rats (HTN), BADGE treated hypertensive rats (BADGE), pioglitazone treated hypertensive rats (PIOG), BADGE and pioglitazone treated hypertensive rats (BADGE/PIOG), 4-phenylbutyric acid treated hypertensive rats (4-PBA), and N-acetylcysteine treated hypertensive rats (NAC). Right panels are histograms showing the relative integrated intensity of CHOP-10 expression using Fiji software. Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, $P < 0.05$ compared to CTR group. **, $P < 0.01$ compared to control group (CTR). #, $P < 0.05$ compared to HTN group. ##$P < 0.01$ compared to HTN group. @, $P < 0.05$ compared to PIOG group. @@, $P < 0.01$ compared to PIOG group. $N \geq 6$).

aorta (6B) and CHOP-10 in heart (6C) and aorta (6D). L-NAME increased ATF-4 and CHOP-10 expression in heart and aorta of HTN group when compared to control group. Pioglitazone significantly reduced mRNA expression of the two genes in heart and aorta. The effect of pioglitazone was inhibited in rats receiving BADGE (BADGE/PIOG group). Both 4-PBA and NAC reduced mRNA expression of ATF-4 and CHOP-10 in heart and aorta when compared to HTN group.

3.5. Histopathological findings

Histological changes of rat's heart and aorta of different groups are shown in Fig. 7A and B, respectively. Histological sections of heart of control group revealed normal cardiomyocytes, however, L-NAME-induced hypertensive rats showed multifocal and/or diffuse myocardial necrosis. BADGE treated rats showed severe hemorrhages in intramuscular spaces. Cardiac tissue of pioglitazone treated rats showed apparently normal myocardial muscles with small multifocal hyaline degeneration with or without intramuscular edema. BADGE/PIOG treated rats showed diffuse intramuscular inflammatory cell infiltrations. In addition, animals treated with 4-PBA and NAC showed normal myocardial muscles with some remodeling in intramuscular blood vessels (Fig. 7A and Table 2).

Histological sections of aorta of control group showed normal histomorphological aortic layers including intima, media and adventitia. Aorta of L-NAME-treated rats showed noticeable separation of intimal layer, focal necrosis and/or completely degenerations in media wall with apparent loss of structured elastic lamina. BADGE treated rats showed partial necrotic and/or hyalinized wall. Treatment with pioglitazone (PIOG) caused refinement in aortic wall structure with mild sub-intima inflammatory aggregation. 4-PBA treated groups showed disorganized media wall with loss of structured elastic lamina and NAC treated groups showed vascular smooth muscle shrinkage/apoptosis with appearance of vacuolization (Fig. 7B and Table 2).

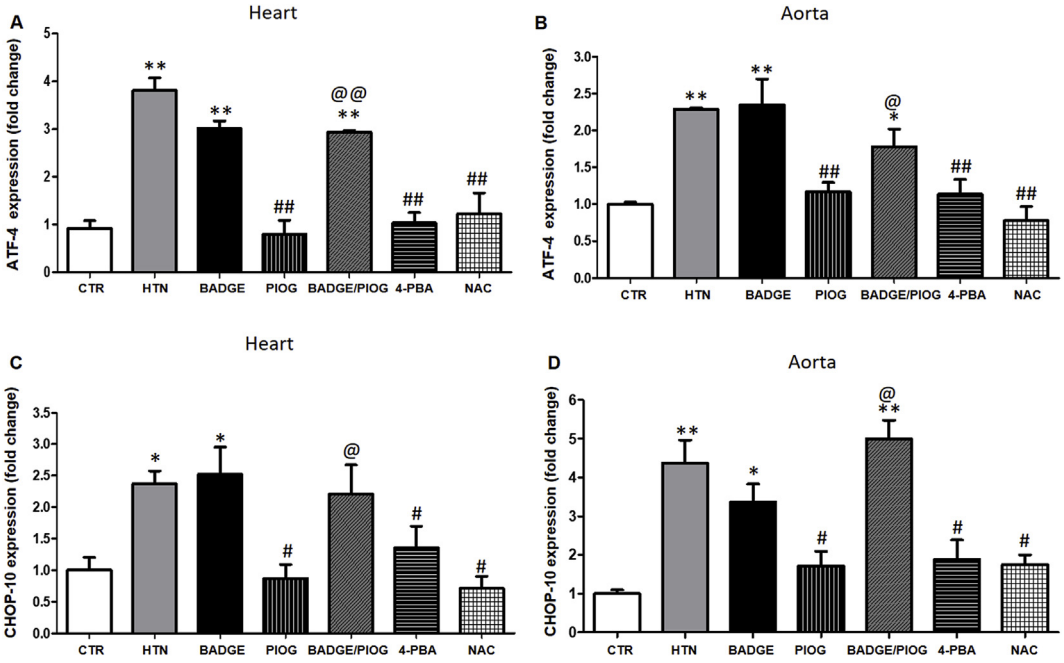

Fig. 6. Pioglitazone reduced mRNA expression of ATF-4 and CHOP-10 in heart and aorta of L-NAME-induced hypertensive rats.
Relative mRNA expression of ATF-4 in heart (A) and aorta (B) and CHOP-10 in heart (C) and aorta (D) was measured using qRT-PCR in control group (CTR), L-NAME-induced hypertensive rats (HTN), BADGE treated hypertensive rats (BADGE), pioglitazone treated hypertensive rats (PIOG), BADGE and pioglitazone treated hypertensive rats (BADGE/PIOG), 4-phenylbutyric acid treated hypertensive rats (4-PBA), and N-acetylcysteine treated hypertensive rats (NAC). Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are represented as mean ± SE (*, $P < 0.05$ compared to CTR group. **, $P < 0.01$ compared to control group (CTR). #, $P < 0.05$ compared to HTN group. ##$P < 0.01$ compared to HTN group. @, $P < 0.05$ compared to PIOG group. @@, $P < 0.01$ compared to PIOG group. $N \geq 6$).

4. Discussion

Thiazolidinediones (TZD) are antidiabetic PPAR-γ agonists. The clinical use of TZD for treatment of diabetes mellitus was restricted due to the increased risk of fluid retention and cardiac ischemic events especially in patients with congestive heart failure [45]. However, several studies have reported that activation of PPAR-γ is associated with improvement of renal handling of salt and water [46], enhancement of endothelial function, and reduction of blood pressure [47,48]. The present study provides additional insights into the effects of PPAR-γ agonist, pioglitazone, on HTN induced by L-NAME and sheds some light on the role of oxidative stress and ER stress in pioglitazone activity. As expected, L-NAME-induced hypertensive rats showed reduction in NO synthesis, elevation of oxidative stress levels, and upregulation of ER stress markers in heart and aorta. Administration of pioglitazone reduced L-NAME-induced elevation in blood pressure, increased NO production, and restored the antioxidant status of hypertensive rats. Furthermore, the present study manifested, for the first time, that the antihypertensive activity of pioglitazone was associated with reduction in ER stress. Moreover, this activity of pioglitazone was PPAR-γ-dependent. The pre-administration of PPAR-γ blocker, BADGE, diminished the corrective effect of pioglitazone on HTN, oxidative and ER stress.

NO is an endothelium-derived vasodilator which maintains vascular tone and blood pressure [49]. NO is biosynthesized endogenously from the amino acid L-arginine, oxygen and NADPH by various NOS enzymes. Endothelial NOS (eNOS) is the major source of NO in endothelial cells. eNOS activity and eNOS-derived NO levels are tightly regulated by intracellular Ca^{2+} and other regulatory proteins such as heat shock protein 90 (hsp 90) and caveolin-1. On the other hand, inducible NOS (iNOS) is not usually expressed in cells but induced in macrophages to produce high levels of NO. iNOS is always active and iNOS-derived NO levels is not regulated by intracellular Ca^{2+}. Excessive NO production by iNOS interacts with $O2^{\bullet}-$ to form peroxynitrite ($ONOO^{-}$) which plays role in inflammation and microvascular damage. Reduced eNOS and/or increased iNOS activities contribute to pathophysiology of HTN [50]. L-NAME is a competitive antagonist for eNOS and iNOS which reduces NO availability [51], and causes vasoconstriction and elevation in blood pressure with chronic administration [33]. Consistently, the current study showed that L-NAME-induced HTN is associated with reduction in NO levels and downregulation of both eNOS and iNOS. Since iNOS is not constitutively expressed, the pathogenesis of HTN in L-NAME model is probably mediated by the inhibition of eNOS. Furthermore, PPAR-γ activation by pioglitazone reduced blood pressure and restored the blunted NO levels in L-NAME-induced hypertensive rats. This effect was accompanied by elevated eNOS expression and reduced iNOS expression supporting the notion that eNOS is a key player in L-NAME model of HTN.

Several studies have reported that activation of PPAR-γ reduced iNOS expression and increased eNOS expression and/or activity. The reduction of iNOS expression by PPAR-γ agonists is mediated by inhibition of nuclear factor kappa B (NF-kB) and may contribute to their cardioprotective effect in HTN and other cardiovascular disorders [52]. Herein, we did not find increase in iNOS expression in L-NAME-induced hypertensive rats, therefore, pioglitazone-induced reduction of iNOS may not be involved in its antihypertensive effect in this model. However, pioglitazone-induced eNOS expression seems to be critical for its antihypertensive activity. In accordance with our observations, studies conducted by Polikandriotis et al. showed that PPAR-γ activation by 15d-PGJ_2 or rosiglitazone stimulates eNOS activity via HSP90-dependent mechanism [53]. In a different study, rosiglitazone also modulated eNOS phosphorylation and enhanced NO production in aorta by activation of AMPK/eNOS and cAMP/PKA pathways in adiponectin dependent manner [54]. Furthermore, pioglitazone increased eNOS activity and improved NO availability through inactivation of Rho-kinase [55]. In the present study we found that pioglitazone also increased the expression of eNOS and this effect was inhibited by PPAR-

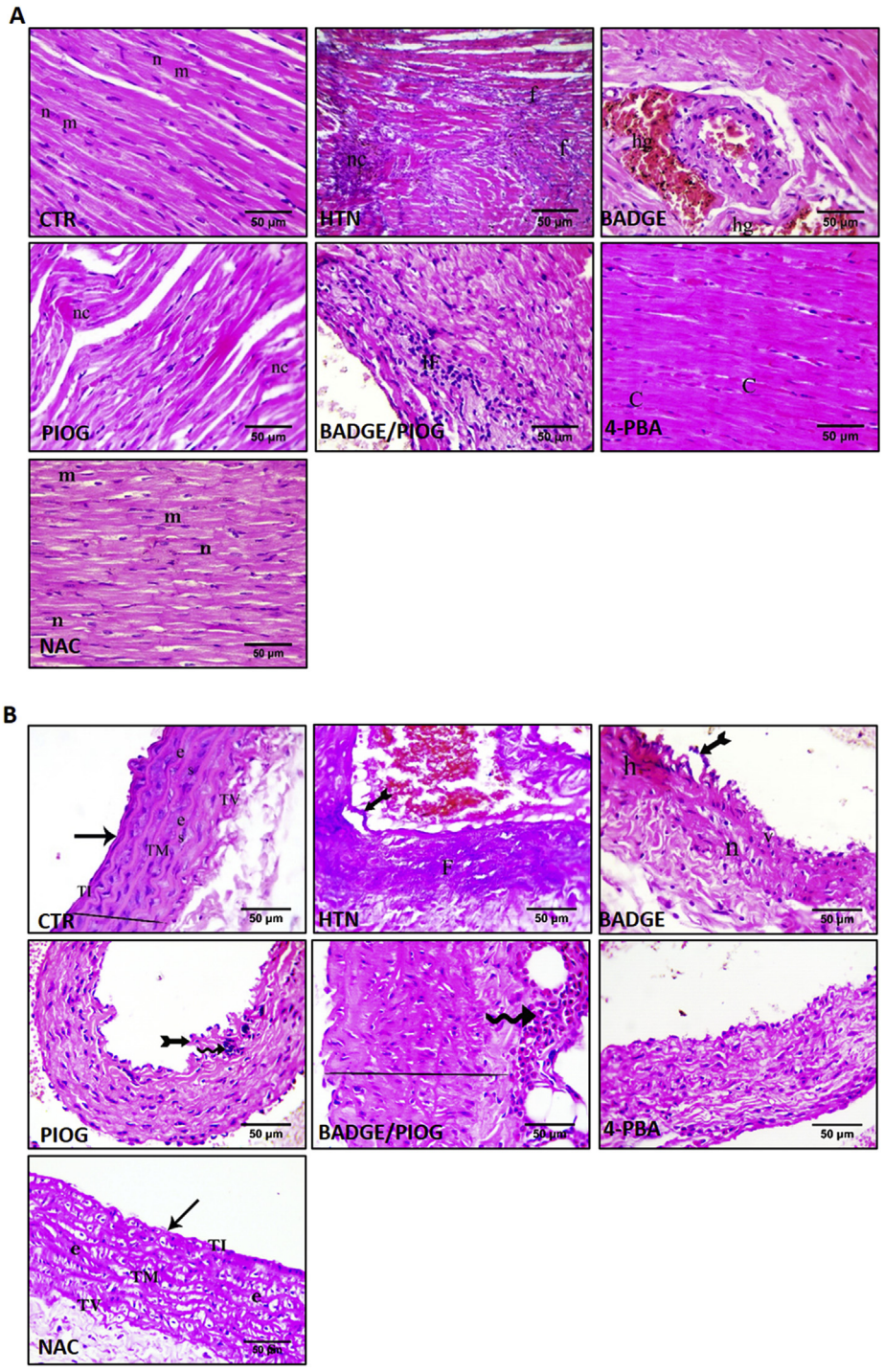

Fig. 7. Histopathological changes of rat's heart and aorta in different experimental groups.
(A) Representative photomicrograph of sections of cardiac muscle (H&E × 400). Control group (CTR) is showing normal cardiomyocytes (elongated cardiac myofibers (m) with central spherical single nuclei (n). L-NAME-induced hypertension group (HTN) is multifocal diffusemyocardial necrosis (nc) of myocardium. BADGE treated hypertensive animals (BADGE) are showing severe hemorrhages in intramuscular spaces (hg). Pioglitazone treated hypertensive animals (PIOG) are showing multifocal degeneration of some myocardial fibers (nc) and the myocardial muscles appeared apparently normal. Hypertensive animals treated with BADGE and pioglitazone (BADGE/PIOG) are showing diffuse intramuscular lymphocytic aggregations (IF). Hypertensive animals treated with 4-PBA and NAC are showing nearly normal myocardial muscles with normal intramuscular capillaries (C) with some remodeling in intramuscular blood vessels.
(B) Representative photomicrograph of sections of the wall of aorta (H&E × 400). (1) Control group (CTR) is showing normal thickness of the wall (line) and normal histomorphological aortic layers including tunica intima (TI) which lined by endothelial layer (arrow), tunica media (TM) which has elastic lamina (e), and smooth muscle cell nuclei (s) and tunica adventitia (TV). L-NAME-induced hypertension group (HTN) is showing focal necrosis and/or completely degenerations of the aortic wall and loss of structured elastic lamina (F) with partial sloughed endothelial lining of intima (forked arrow). BADGE treated hypertensive animals (BADGE) are showing disorganized media wall and partial necrotic (n) and/or hyalinized wall (h), subintimal vacuolization (v) and partial sloughed and destructed endothelial cells (forked arrow). Pioglitazone treated hypertensive animals (PIOG) are showing aortic wall remodeling with still presence of irregular and protruded of intima (forked arrow) with multifocal sub intima inflammatory aggregation (wavy arrow). Hypertensive animals treated with BADGE and pioglitazone (BADGE/PIOG) are showing thickening of aortic wall (line) with marked inflammatory cell infiltration in the adventitia (wavy arrow), and loss of structured elastic lamina. Hypertensive animals treated with 4-PBA is showing disorganized media wall with loss of structured elastic lamina and some vascular smooth muscle apoptosis. NAC treated hypertensive animals are showing vascular smooth muscle shrinkage/apoptosis with appearance of vacuolization.

γ antagonist, BADGE. The direct effect of PPAR-γ activation on eNOS expression is debatable. Calnek et al. demonstrated that 15d-PGJ_2 and ciglitazone stimulated the endothelial release of NO without alteration in eNOS mRNA expression [25]. However, Yuen et al. reported that telmisartan, AT1 receptor blocker which also has PPAR-γ agonistic activity, increased the expression of eNOS in PPAR-γ-dependent, AT1 receptor-independent manner. These findings collectively suggest that activation of PPAR-γ increases NO availability by increasing eNOS expression (direct effect) or by enhancing eNOS activity (indirect effect).

Previous studies have reported that NO-deficient HTN is associated with left ventricular hypertrophy as a result of elevation of renin and aldosterone levels, activation of angiotensin converting enzyme activity (ACE), and increased expression of angiotensin II receptors (ATI) [56,57]. Moreover, decreased NO production results in altered responsiveness and structural remodeling in aorta as a result of reduction in cGMP-mediated inhibition of protein synthesis and proliferation of vascular smooth muscles (VSMCs) [58,59]. In this regard, the present study showed that restoration of NO levels by pioglitazone in L-NAME-induced hypertensive rats was associated with reduced cardiac hypertrophy and improved the histological score of cardiac and aortic lesions.

Oxidative stress is an imbalance between the production of ROS and the ability of the cell to neutralize them by antioxidants. Oxidative stress plays a fundamental role in the development of HTN. Compared to normotensive volunteers, hypertensive patients were reported to have higher levels of oxidized/reduced glutathione ratio and MDA and lower levels of SOD, catalase, and glutathione peroxidase activities

Table 2
Scores for histopathological findings of Heart and Aortas in the different experimental groups.

Histopathological findings	CTR	HTN	BADGE	PIOG	BADGE/PIOG	4-PBA	NAC
Heart Myocardial (muscle fibers)							
Necrosis	0.00 ± 0.00	2.83 ± 0.17*	1.667 ± 0.21*#	0.33 ± 0.21#	1.5 ± 0.0.22*#@	0.5 ± 0.22#	0.33 ± 0.21#
Inflammatory cell infiltrations	0.00 ± 0.00	2.67 ± 0.21*	1.83 ± 0.31*	1.17 ± 0.31#	2.67 ± 0.21*@	0.67 ± 0.33#	0.67 ± 0.33#
Hyalinization	0.00 ± 0.00	2.67 ± 0.21*	2.67 ± 0.21*	2.17 ± 0.31*	2.67 ± 0.21*	1.33 ± 0.21*#	1.33 ± 0.21*#
Degeneration	0.00 ± 0.00	2.66 ± 0.21*	2.00 ± 0.36*	1.16 ± .0.31#	2.5 ± 0.22*@	1.00 ± 0.26#	0.66 ± 0.33#
Hemorrhage	0.00 ± 0.00	2.66 ± 0.21*	2.33 ± 0.21*	1.16 ± 0.17*#	2.33 ± 0.21*@	0.17 ± 0.17#	0.17 ± 0.17#
Edema	0.00 ± 0.00	2.33 ± 0.21*	1.667 ± 0.21*	0.67 ± 0.21#	2.00 ± 0.37*@	1.33 ± 0.21*#	1.33 ± 0.21*#
Thoracic Aorta							
Loss of structured elastic lamina	0.00 ± 0.00	2.67 ± 0.21*	2.17 ± 0.31*	1.33 ± 0.33	2.5 ± 0.34*	1.83 ± 0.4*	1.0 ± 0.37
Vascular smooth muscle shrinkage/apoptosis in media wall	0.00 ± 0.00	1.33 ± 0.21*	1.83 ± 0.31*	1.17 ± 0.31	1.33 ± 0.21*	1.83 ± 0.3*	2.16 ± 0.4*
Separation of intimal layer	0.00 ± 0.00	2.83 ± 1.67*	2.0 ± 0.37*	1.33 ± 0.21*#	2.67 ± 0.21*@	0.5 ± 0.22#	0.33 ± 0.21#
Thickening	0.00 ± 0.00	2.625 ± 0.18*	2.12 ± 0.29*	0.875 ± 0.23#	2.25 ± 0.25*@	0.625 ± 0.26#	0.5 ± 0.27#
Necrosis/degeneration	0.00 ± 0.00	2.67 ± 0.21*	1.83 ± 0.31*	0.5 ± 0.22#	1.5 ± 0.22*#@	1.33 ± 0.21*#	0.83 ± 0.3#
Vacuolization	0.00 ± 0.00	1.67 ± 0.21*	2 ± 0.36*	1.33 ± 0.21*	1.67 ± 0.21*	1.5 ± 0.22*	1.83 ± 0.4*
Inflammatory cells aggregations	0.00 ± 0.00	2.33 ± 0.21*	1.66 ± 0.21*	1.33 ± 0.21*	1.667 ± 0.21*	0.33 ± 0.21#	0.33 ± 0.21#

Histopathological scores were designed as follows: (0, negative; 1, weak; 2, moderate; 3, severe). Data were analyzed using one way ANOVA followed by Tukey's multiple comparison test and are presented as mean score ± SE (*, compared to CTR group. #, compared to HTN group. @, compared to PIOG group, $P < 0.05$, $N = 6$). CTR = Control, HTN = hypertension, PIOG = Pioglitazone, 4-PBA = 4-Phenylbutyric acid, NAC = N-acetylcysteine.

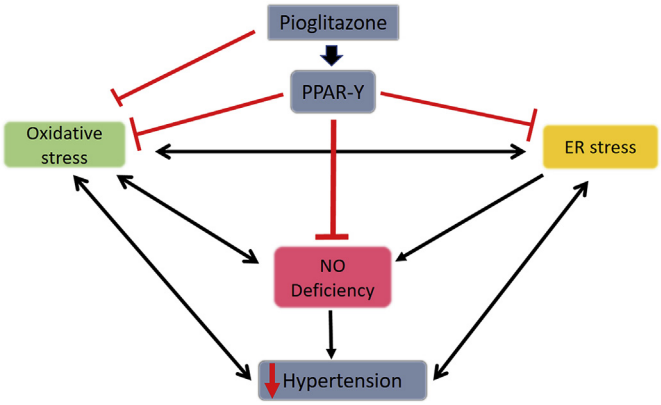

Fig. 8. Proposed mechanism of antihypertensive activity of pioglitazone in NO-deficient HTN model in rats. Reduction of NO induced by NOS inhibitor results in oxidative stress, ER stress, and eventually HTN (black arrows). Activation of PPAR-γ by pioglitazone increases NO availability and lowers ER stress which consecutively decreases oxidative stress. Pioglitazone inhibits oxidative stress by both PPAR-γ-dependent and -independent pathways. Elevation of NO levels as well as inhibition of oxidative and ER stress contribute to reduction of HTN (red arrows). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[60]. Increased ROS, especially $O2^{\bullet\ -}$, leads to a reduction in the availability of NO, destabilization eNOS, and development of HTN [61,62]. In the current study, oxidative stress associated with NO deficiency was indicated by a reduction in the activity of antioxidant enzymes, SOD and catalase, reduction in the level of non-enzymatic antioxidant GSH and elevation of MDA levels in L-NAME-induced hypertensive rats. Pioglitazone treatment decreased MDA levels and increased the activity levels of catalase, SOD and GSH. These results are consistent with the notion that activation of PPAR-γ reduced oxidative stress [63,64]. However, the use of PPAR-γ antagonist, BADGE, showed only partial inhibition of the antioxidant activity of pioglitazone. BADGE, reversed pioglitazone-mediated elevation of GSH levels and catalase activity but did not significantly cause reduction in SOD activity and had no effect on MDA levels. This is not surprising because PPAR-γ-independent activity of pioglitazone and other TZDs has been previously reported and reviewed by Feinstein et al. [65]. For example, in renal tubule-derived cell lines, pioglitazone inhibited mitochondrial membrane potential and activated 5′-AMP-activated protein kinase (AMPK) in PPAR-γ-independent manner [66]. Likewise, both troglitazone and pioglitazone, caused rapid increases in pAMPK in fibroblasts with and without enough PPAR-γ to activate target gene expression [67]. Taken together, the antihypertensive activity of pioglitazone is probably mediated by reduction of oxidative stress due to both its PPAR-γ-dependent and independent effects.

ER stress has been highlighted as an essential regulator of cardiovascular conditions including HTN [18]. The initial ER stress response is a defensive mechanism to detect unfolded and misfolded proteins and maintain ER homeostasis. However, prolonged perturbation of the ER, trigger activation of adaptive signaling pathway called the UPR [68]. Conversely, long-term UPR activation initiates apoptotic programmed cell death [69]. ER stress is involved in vascular endothelial dysfunction, cardiac damage, and pathogenesis of HTN. Angiotensin II-induced HTN was associated with upregulation of several ER stress markers including GRP78 and CHOP. The use of ER stress inhibitors such as tauroursodeoxycholic acid (TUDCA) or 4-PBA reduced the systolic blood pressure, enhanced endothelial NO synthase phosphorylation, and improved the endothelium-dependent relaxation in aorta [18]. Similarly, a study conducted by Yum et al., demonstrated that oral administration of 4-PBA partially reduced arterial blood pressure in high-salt diet-induced HTN model using salt-sensitive rat [70]. In the present study, we demonstrated for the first time that ER stress plays role in L-NAME-induced HTN model. Chronic L-NAME administration increased ATF-4 and CHOP-10 expression in heart and aorta. Oral administration of 4-PBA decreased the expression of ATF-4 and CHOP-10, lowered blood pressure, decreased cardiac hypertrophy and alleviated cardiac and aortic histopathological changes. Furthermore, ER stress inhibition by 4-PBA was associated with increased NO availability and upregulated eNOS expression. These observations reflect the importance of the ER stress in development of NO-deficient HTN.

Previous studies have reported that ER stress and oxidative stress occur concurrently in several health conditions [71]. Accumulation of ROS activates ER stress signaling. The use of antioxidants reduced ER stress-induced apoptosis in different cells [72]. In addition, scavenging ROS reduced the ER stress in RVLM of spontaneously hypertensive rats [73]. In the present study, we investigated that the relationship between oxidative stress and ER stress is mutual in L-NAME model of HTN. Oxidative stress might lead to ER stress since the use of antioxidant, NAC, reduced the expression of ER stress markers in heart and aorta of hypertensive rats. In addition, ER stress could also lead to oxidative stress. Administration of ER stress inhibitor, 4-PBA, reduced L-NAME-induced oxidative stress as indicated by reduction in MDA concentration and increased the levels of GSH and the activities of SOD and catalase. ER stress-mediated oxidative stress may be explained by

the association between the ER and the mitochondria via ER-associated mitochondria membranes (MAMs). MEM is an exchange site of Ca^{2+}, lipids and other metabolites between the two organelles. ATP depletion by protein folding process during ER stress may stimulate the mitochondria to produce more ATP by oxidative phosphorylation and eventually production of ROS. In addition, accumulation of unfolded proteins during ER stress increases the release of Ca^{2+} from ER stimulating ROS production from mitochondria [74].

As pointed out in the results of the present study, PPAR-γ activation reduced ER stress associated with L-NAME-induced HTN. Pioglitazone reduced ATF-4 and CHOP-10 expression in heart and aorta of rats received L-NAME and this effect was attenuated in animals received PPAR-γ antagonist, BADGE. In accordance of our findings, previous in-vitro studies performed on pancreatic β-cells reported that pioglitazone improved pancreatic islet function through reduction of ER stress [75]. Using murine macrophage cell line, Ikeda et al. reported that PPAR-γ agonists attenuated palmitate-induced macrophage ER stress and apoptosis [27]. Furthermore, in-vivo study performed on ER stress-activated indicator transgenic mice reported that pioglitazone reduced ER stress in the liver under diabetic condition [76]. Since the activity of pioglitazone on ER stress, oxidative stress, and NO availability in the present study mimics the activity of 4-PBA, our data suggest that the antihypertensive activity of pioglitazone is probably mediated by ER stress inhibition.

In conclusion, the present study expands the understanding of the antihypertensive activity of pioglitazone in NO-deficient HTN model in rats (Fig. 8). Pioglitazone activates PPAR-γ which increases the expression of eNOS elevating NO levels. PPAR-γ activation also reduces ER stress which in turn diminishes oxidative stress. Pioglitazone may also inhibit oxidative stress independently of PPAR-γ. Inhibition of oxidative stress and ER stress results in an increase in NO availability and eventually reduction of HTN.

The limitation of our study was that using L-NAME as a model for NO-deficient HTN downregulated the expression of both e-NOS and i-NOS. Our data suggests that e-NOS inhibition is likely the key player in L-NAME-induced HTN and the downstream target of PPAR-γ which restores NO availability. However, future studies using e-NOS knock out mice are required to explore the role of e-NOS in the proposed mechanism.

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Declaration of competing interest

None declared.

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