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ORIGINAL PAPER

Pioglitazone Attenuates Lipopolysaccharide-Induced Oxidative Stress, Dopaminergic Neuronal Loss and Neurobehavioral Impairment by Activating Nrf2/ARE/HO-1

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Abstract

The aim of the present study was to examine the neuroprotective potential of pioglitazone via activation of Nrf2/ARE-dependent HO-1 signaling pathway in chronic neuroinflammation and progressive neurodegeneration mouse model induced by lipopolysaccharide (LPS). After assessing spatial memory, anxiety and motor-coordination, TH+ neurons in substantia nigra (SN) were counted. The oxidative stress marker carbonyl protein levels and HO-1 enzyme activity were also evaluated. RT-qPCR was conducted to detect HO-1, Nrf2 and NF-κp65 mRNA expression levels and Nrf2 transcriptional activation of antioxidant response element (ARE) of HO-1 was investigated. Pioglitazone ameliorated LPS-induced dopaminergic neuronal loss, as well as mitigated neurobehavioral impairments. It enhanced Nrf2 mRNA expression, and augmented Nrf2/ARE-dependent HO-1 pathway activation by amplifying HO-1 mRNA expression. Moreover, it induced a significant decrease in NF-κB p65 mRNA expression, while reducing carbonyl protein levels and restoring the HO-1 enzyme activity. Interestingly, LPS induced Nrf2/antioxidant response element (ARE) of HO-1 activation, ultimately resulting in slight enhanced HO-1 mRNA expression. However, LPS elicited decrease in HO-1 enzyme activity. Zinc protoporphyrin-IX (ZnPPIX) administrated with pioglitazone abolished its effects in the LPS mouse model. The study results demonstrate that coordinated activation of Nrf2/ARE-dependent HO-1 pathway defense mechanism by the PPARγ agonist pioglitazone mediated its neuroprotective effects.

Keywords Pioglitazone · Neurobehavioral impairment · Dopaminergic neurons · Oxidative stress · Nrf2/HO-1 signaling pathway · Transcriptional activation

Introduction

The interplay between neuroinflammation and oxidative stress can act as a predisposing factor for degenerating dopaminergic neurons in substantia nigra (SN), which are responsible for characteristic motoric symptoms [1]. Findings yielded by several studies have demonstrated that peripheral inflammation inducted by lipopolysaccharide (LPS) leads to chronic ongoing neurodegeneration and neuroinflammation long after peripheral inflammation has ablated. Systematic administration of 5 mg/kg LPS i.p. has been shown to cause sustained microglial activation in SN [2, 3], as well as elevation of TNF-α, among other inflammatory cytokines. In the study conducted by Qin et al., these effects persisted for up to 7 and 10 months compared to only 9 h and 1 week, for the same parameter determined in serum and liver, respectively [4]. Reinert and colleagues showed that 6- to 8-month-old mice treated with 5 mg/kg LPS i.p. exhibited microglial activation and progressive dopaminergic neuron loss 3 h post-injection, which persisted for the remaining 9 h of observation [5]. These results are in accordance with the findings yielded by studies in which different regimes of systematic LPS administration were employed,

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which was found to be associated with dopaminergic neuronal loss in nigrostriatal pathway shortly after administering the injection, and persisted for days and even weeks [6, 7].

Pioglitazone, a potent agonist of the PPARγ receptor capable of CNS penetration, has been shown to have beneficial effects in several studies investigating CNS disorders and injury such as Alzheimer's disease (AD) [8], traumatic brain injury [9], and spinal cord injury [10]. In extant studies based on different 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinsonism mouse and monkey models, pioglitazone was demonstrated to prevent motor and cognitive impairment, as well as dopaminergic neuronal loss, which was attributed to its anti-inflammatory effect and capacity to elicit antioxidant response [11–13]. In human clinical trials, pioglitazone was shown to decrease negative symptoms of schizophrenia [14], as well as incidence of dementia [15] and Parkinson's disease (PD) in diabetic patients receiving glitazone treatment [16, 17]. Moreover, pioglitazone is currently undergoing a Phase III trial for AD treatment [18]. However, in a randomized controlled trial involving 210 patients in the early stages of PD, pioglitazone failed to demonstrate statistically significant effect as a disease modifying agent [19].

Heme oxygenase-1 (HO-1), an antioxidant enzyme with established cytoprotective effects, alleviates oxidative stress injury and attenuates neuronal loss [20, 21]. HO-1 is considered a cellular sensor for noxious stimuli the expression of which, unlike that of constitutively expressed HO-2, is upregulated by oxidative challenges and other cellular stress [22]. HO-1 has been shown to modulate several pathological processes. According to the available evidence, it enhances neurotrophic factor upregulation [23], increased dopaminergic neuronal survival in SN [24, 25], and prevented exacerbation of α-synuclein aggregation [26]. In patients diagnosed with PD, it is suggested that chronic oxidative stress leads to elevated systematic HO-1 in serum [27]. In postmortem biopsies of SN overexpression of HO-1 in astrocytes, and microglia surrounding dystrophic dopaminergic neurons and co-localized with lewy bodies within affected dopaminergic neurons in PD patients, suggested as cellular defense mechanism [28].

Findings yielded by extant studies indicate that HO-1 is regulated by upstream regulators of PPARγ [29–31]. Moreover, nuclear transcription factor-erythroid 2 related factor (Nrf2) binding to antioxidant response element (ARE) present in the HO-1 promoter region [32]. Enhancing the adaptive response of Nrf2, and translocating to the nucleus coupled with transcriptionally activating gene expression of HO-1, provides substantial protection to dopaminergic neurons by counteracting oxidative stress insult [33, 34]. The decline in Nrf2 activity with aging is believed to be one of the main PD risk factors, based on the evidence of Nrf2 accumulation in the nucleus of surviving nigral neurons in PD patients which was found to be accompanied by decreased Nrf2/ARE-dependent cytoprotective enzyme levels [35].

A positive feedback transcriptional regulation between PPARγ and Nrf2 is thus suggested by several studies [36, 37]. Rosiglitazone administration increased Nrf2 and PPARγ protein levels in different PD models [38]. In their recent study, Li et al. similarly showed that luteoloside treatment increased Nrf2 nuclear translocation through PPARγ activation [39]. Pioglitazone treatment increased nuclear translocation and protein levels of Nrf2 along with HO-1. On the other hand, siRNA-mediated knockdown of Nrf2 abolished pioglitazone effects on NFE2L2 and HMOX1 increased mRNA and proteins expression in MPP+-induced PD model [40]. Against this research background, the aim of the present study was to investigate PPARγ agonist pioglitazone modulation of HO-1 through Nrf2/ARE activation, and elucidate its critical neuroprotective effects on neurobehavioral deficits and dopaminergic neuronal loss in SN.

Materials and Methods

Animals

The study sample comprised of Swiss male albino mice (*Mus musculus albinus*) aged 6–8 months and weighing 20–30 g, which were obtained from the animal house colony of the National Research Centre (Cairo, Egypt). All animals were kept at room temperature and constant humidity at a 12 h dark/night cycle and were supplied with laboratory food and water ad libitum. Animal procedures were performed in accordance with the GUC Ethical Committee guidelines, and adhered fully to the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The study sample was divided into five groups, ten animals each, denoted as the control group, an LPS (0111: B4 Sigma-Aldrich, Germany), LPS + Pio (pioglitazone, Santa Cruz Biotechnology, USA), LPS + ZnPPIX + Pio (Santa Cruz Biotechnology, USA) and Pio (Santa Cruz Biotechnology, USA). While the control group received saline solution only, the Pio group received 30 mg/kg p.o. for 7 days. The LPS group received 5 mg/kg of LPS i.p. once, which has been reported not to affect weight and 7-day survival rate [41]. The animals placed into the LPS + Pio group received Pio 30 mg/kg p.o. 30 min prior to LPS injection on the first day, after which mice continued to receive the same Pio dose for further 7 days. On the other hand, on the first day, the LPS + ZnPPIX + Pio group was treated with Pio 30 mg/kg p.o., followed by LPS 5 mg/kg i.p. 30 min later. After further 30 min, animals were injected with ZnPPIX 5 mg/

kg i.p., and continued to receive the same Pio dose for the duration of the experiment. According to previous study, ZnPPIX alone had no significant effect on dopaminergic neurons in SN in LPS-PD mouse model [42]. Thus, in the present study, ZnPPIX was administered in accordance with the experimental designs adopted by other authors [43, 44]. ZnPP i.p was reported to cross dirupted blood brain barrier [45], similarly systematic administration of ZnPP inhibit HO-1 in neuro-inflammatory mouse models [46, 47], and COX-2 in LPS treated rat brain [48]. LPS 5 mg/kg i.p used in this study was previously reported to induce blood brain barrier hyper-permeability, [49], allowing ZnPPIX reaching brain tisue. On the 7th day, mice were trained in the ladder rung test, and then on the following day, they were tested.

Behavioral Testing

Y-Maze

Spatial memory was assessed through the recognition of novel arm and animal's tendency to explore it [50]. Mice in training were placed at the center of a Y-maze with one arm blocked by a removable door and were allowed to explore freely for 10 min, after 1 h the "novel" arm was opened and the animals were allowed to move freely from the center in all three directions for 5 min. Discrimination index of novelty versus familiarity was determined by calculating the preference for the "novel" arm as a discrimination ratio, i.e., (Time spent in the novel arm/Time spent in all three arms).

Elevated Plus Maze (EPM)

The elevated plus maze was used to investigate anxiety-related behavior based on the unconditional fear of heights and open spaces. For this experiment, animals were placed at the center of the EPM facing an open arm and were allowed to explore freely for 5 min. The time spent in the open arms and the ratio of open arms/total arms entries were calculated [51].

Ladder Rung Test

Motor coordination during locomotion was assessed in training sessions by placing mice at one side of the ladder, allowing them to walk to the other side via rungs positioned at 1 cm intervals. Each mouse crossed the ladder three times. On the next day, each animal was subjected to three trials, using different irregular rung spacing patterns, while increasing the spacing distance to elevate the difficulty for higher sensitivity. Foot placement accuracy was recorded and failed or failed attempted placement was noted as an error. Average errors for three trials per each mouse were evaluated as described by Metz and Whishaw 2009 [52].

Brain Sample Collection

Upon completion of drug treatment and behavioral tests, mice were anesthetized and sacrificed by cervical dislocation before decapitation. Brains were dissected out and washed with ice-cold phosphate-buffered saline (PBS, pH 7.4). Brains from each group were cut into equal halves along the longitudinal fissure, and the midbrain was isolated on ice. Samples were stored in − 80 °C until required for further analyses.

Immunofluorescence

Paraformaldehyde-fixed sections of 6 µm thickness were cut through SN and were permeabilized and stained with [1:200] anti-TH primary antibodies (Santa Cruz Biotechnology Cat# sc-25269, RRID: AB_628422) before being diluted in 0.5% BSA in PBS overnight at 4 °C. The slides were washed three times with PBS and incubated for 1 h at room temperature with [1:400] rhodamine conjugated secondary antibodies (Santa Cruz Biotechnology Cat# sc-2492, RRID: AB_628499) as previously described [53].

Protein Carbonyl Content Assay

Homogenates required for the assay were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and all procedures were performed according to the manufacturer's instructions. Protein carbonyl content levels were determined using a Protein Carbonyl Content Assay Kit (Abcam, US) based on spectrophotometric detection of protein carbonyl groups at the wavelength of 370 nm and were expressed as nmol carbonyls/mg protein.

Heme Oxygenase Activity Assay

Heme oxygenase activity was determined by bilirubin generation following the procedure described [54] with minor modifications. Homogenates were added to a reaction mixture containing 2 mM glucose 6-phosphate (Sigma-Aldrich, Germany), 1 U glucose 6-phosphate dehydrogenase (Sigma-Aldrich, Germany), 25 µM hemin (Sigma-Aldrich, Germany), 4.5 mM NADH (Sigma-Aldrich, Germany) and 3 mg biliverdin reductase from rat liver cytosol. The thus prepared samples were incubated in the dark for 15 min at 37 °C, while the same protocol was adopted for the reference samples, with the omission of NADH. The reactions were incubated at 37 °C for 1 h in the dark and were terminated by placing the samples on ice for at least 5 min. Bilirubin formation was measured as the difference in absorbance at $\lambda = 464$ nm and 530 nm, detected by double beam spectrophotometer. The protein concentrations in each sample were determined via a BCA protein assay and

were expressed in nmoles of bilirubin formed per milligram of protein per hour.

Real-Time Quantitative PCR

Total RNA was extracted using the Trizol reagent (Invitrogen, USA). To synthesize single strand complementary DNA (cDNA) 1 µg of total RNA was used, and High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor was adopted for RT-PCR (Invitrogen, USA). RT-qPCR was performed using the QuantiTect SYBR Green kit master mix with QuantiTect primer assays for Hmox-1, Nfe2l2 (encodeNrf2), Rel A (encodeNF-κB p65) and GAPDH (Qiagen, USA) by Mx30005P real-time PCR system (Stratagene, La Jolla, USA). For each sample, reactions were set in duplicates and the expression levels of target genes were normalized to the GAPDH levels, which served as an internal standard control. Relative expression was calculated with respect to the control group, whereby gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [55].

Chromatin Immunoprecipitation (ChIP) assay

The Imprint ChIP kit (Sigma-Aldrich, Germany) was used to analyze the in vivo binding of Nrf2 to the ARE-1 and ARE-2 of HO-1 gene, respectively. The ChIP assays were performed following the manufacturer's instructions after nuclear lysates preparations from brain tissues using anti-Nrf2 antibody (Thermo Fisher Scientific Cat# PA5-27882, RRID: AB_2545358). DNA samples were analyzed by RT-qPCR using the following primer pairs:

ARE-1F, 5′-TTC CTC ACT GCT CAT TTC CTC-3′, and
ARE-1R, 5′-TTC CGGAAC CTT TTA CCAAC-3
ARE-2F, 5′-CCAGGG CAG TCT TAA GCA AT-3′, and
ARE-2R, 5′-AAG GGT TCA GTC TGG AGC AA-3.

Statistical Analyses

GraphPad Prism (version 7.04) software was used for statistical analysis and all obtained results were expressed as means ± SEM (standard error of the mean). For statistical comparisons among multiple groups, one-way ANOVA was employed followed by Tukey test, multiple comparison tests. In all comparisons, $P < 0.05$ was considered statistically significant.

Results

Effect of Pioglitazone on LPS-Induced Behavioral Impairments

LPS-injected mice exhibited substantial working memory impairment, anxiety-like behavior and a significant decline in motor coordination compared to those placed in the control group. As shown in Fig. 1a, in the LPS group, a significant decrease in the discrimination ratio score ($F_{4,45} = 2.88$, $P < 0.001$) was obtained, suggesting increased aversion to the open arms, manifested in a smaller number of entries ($F_{4,45} = 7.622$, $P = 0.0051$) and shorter time spent ($F_{4,45} = 1.247$, $P < 0.001$) in Fig. 1b, along with a greater incidence of limb placing errors ($F_{4,45} = 2.372$, $P < 0.001$) compared to the control group Fig. 1c. Pioglitazone administration to LPS-injected mice resulted in a pronounced preference for novelty arm, as indicated by a much higher discrimination ratio score ($P < 0.001$), greater number of open arm entries ($P = 0.0056$) and longer time spent exploring open arms ($P = 0.0086$). Moreover, considerable improvement in motor coordination, as indicated by a much smaller average number of stepping errors ($P < 0.001$, n = 10), was observed compared to the LPS group. However, the beneficial effects of pioglitazone were counteracted by injection of ZnPPIX, as indicated by a significant reduction in the discrimination ratio ($P < 0.001$), number of entries ($P = 0.0017$) and time spent in open arms ($P = 0.0293$), as well as a greater number of average stepping errors ($P < 0.001$) in the LPS + ZnPPIX + Pio group compared to the mice in the LPS + Pio group.

Effect of Pioglitazone Treatment on Dopaminergic Neurons in SN in LPS Mouse Model

TH+ neuron count in SN was significantly lower in LPS-injected mice compared to those in the control group ($F_{4,10} = 38.6$, $P < 0.001$) as depicted in Fig. 2, and morphological changes in SN in LPS model Supplementary Fig. 1. Moreover, administration of pioglitazone to LPS-treated mice resulted in a marked increase in the number of TH + neurons in SN compared to the LPS group ($P = 0.0135$). In contrast, following ZnPPIX administration, mice in the LPS + ZnPPIX + Pio group exhibited a marked decline in TH + neuron count relative to the LPS + Pio-injected mice ($P = 0.0202$).

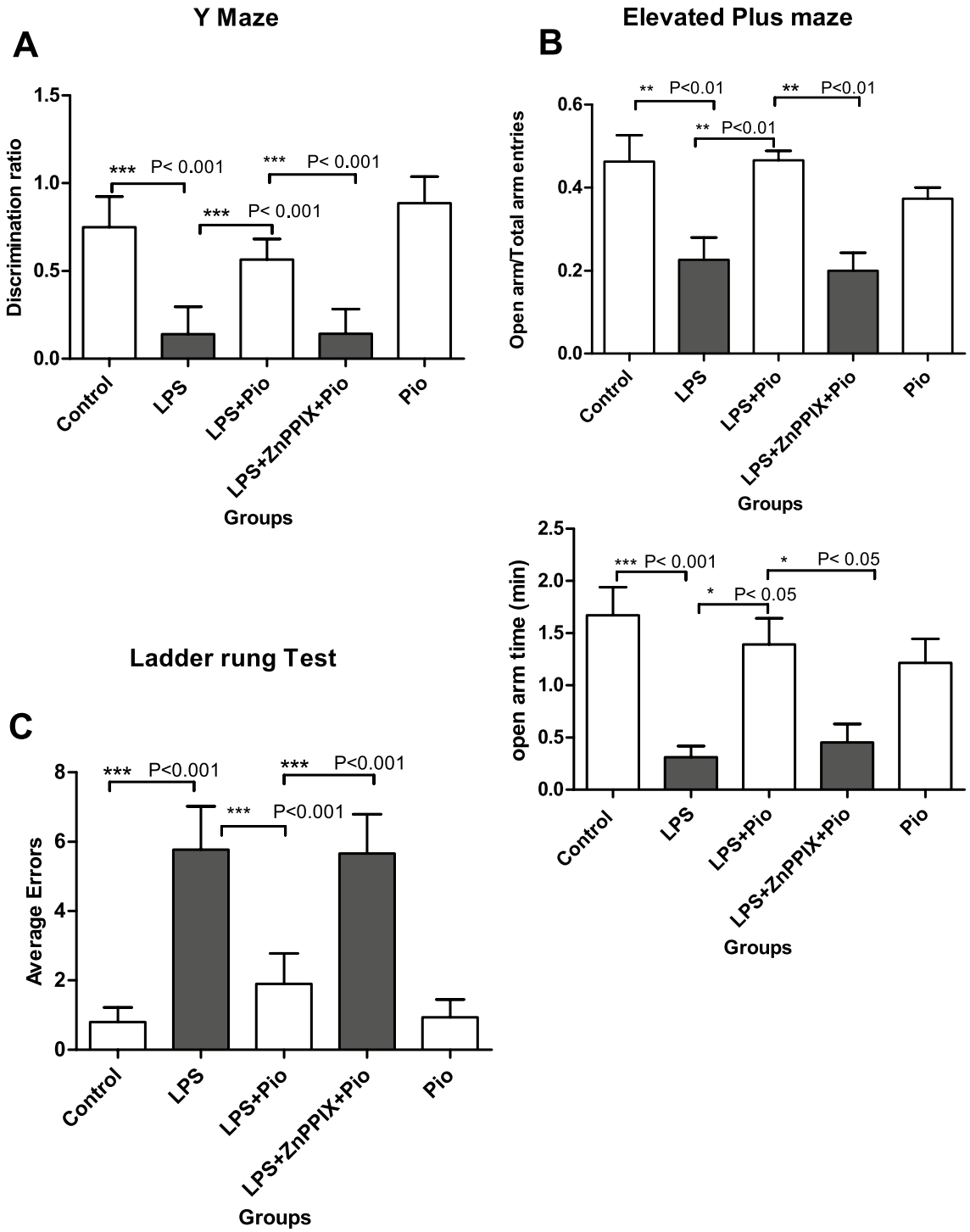

Fig. 1 Behavioral impairment in LPS-injected mice was alleviated after pioglitazone treatment. **a** Short-term working memory functioning, estimated as a preference for exploring novel arm relative to the other two arms in Y-maze as a discrimination ratio. **b** Anxiety-like behavior, measured by comparing the number of open arm and closed arm entries, and time spent in the open arm. **c** Motor coordination during locomotion, expressed as the number of stepping errors mice made while crossing the ladder rung. All data is expressed as mean ± SEM ($n = 10$)

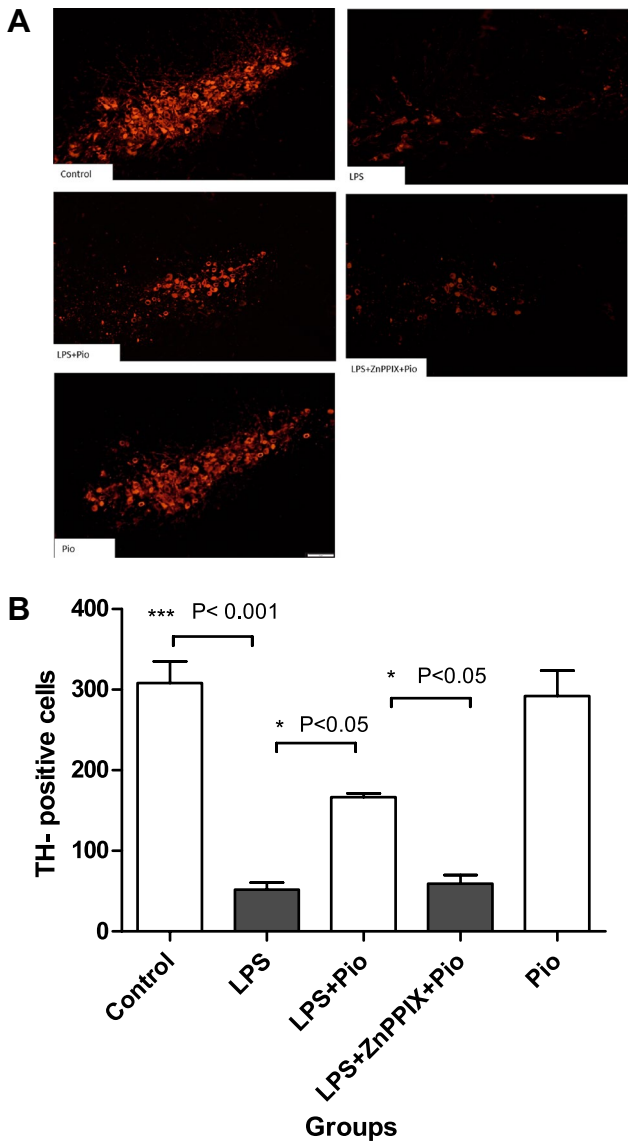

Fig. 2 Effect of pioglitazone treatment on dopaminergic neurons in SN in LPS mouse model. **a** Immunofluorescence staining of TH in the SN showed marked decrease in the number of TH+ neurons in the LPS mouse model, compared to the control group and pioglitazone-treated mice ($n = 3-4$, scale bar: 20 µm). **b** Quantification of the number of TH+ neurons in SN. All data is expressed as mean ± SEM ($n = 3$–4)

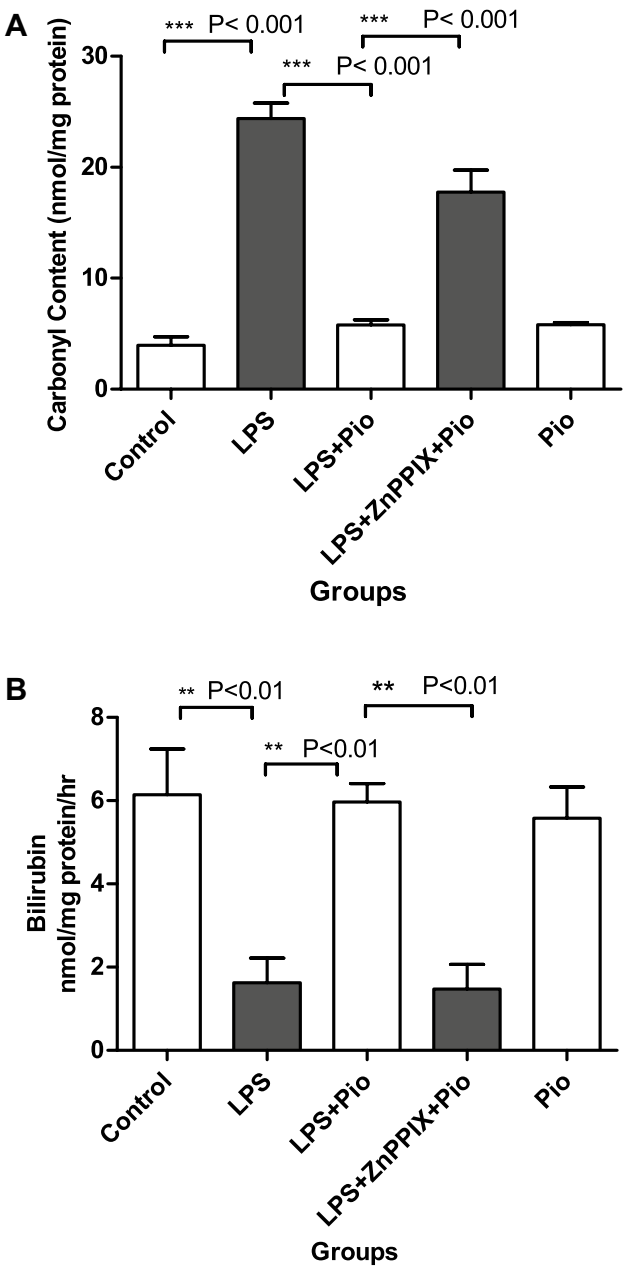

Fig. 3 **a** Effect of pioglitazone on LPS-induced oxidative stress. Pioglitazone elicited decrease in carbonyl protein levels, and this effect was inhibited by ZnPPIX administration. Data represented as mean ± SEM ($n = 5$). **b** Effects of pioglitazone on HO activity in LPS mouse model. ZnPPIX abolished the beneficial effects of pioglitazone on HO-1 enzyme activity, as indicated by bilirubin formation in LPS mouse model. Data represented as mean ± SEM ($n = 5$)

Effect of Pioglitazone on LPS-Induced Oxidative Stress

Carbonyl proteins serve as a marker of oxidative stress associated with various disorders related to aging and neurodegenerative diseases in humans, such as AD and PD [56]. Their levels were assessed in the present study, whereby a significant increase was observed 7-days post-LPS injection ($F_{4,20} = 61.78$, $P < 0.001$), as shown in Fig. 3a. The carbonyl protein levels determined for mice in the Pio group were significantly lower than in the LPS group ($P < 0.001$). However, co-administration of ZnPPIX with pioglitazone resulted in a significant increase in carbonyl protein levels compared to those measured in mice placed in the LPS + Pio group ($P < 0.001$).

Effect of Pioglitazone on HO Activity in LPS Mouse Model

Increase in HO activity in response to CNS injury is attributed to inducible isoform HO-1 [57]. Accordingly, the HO assay employed in the present study should reflect changes in HO-1 activity. As expected, HO-1 activity was significantly lower in LPS-injected mice compared to those in the control group ($F_{4,20}$= 10.72, P = 0.0024). While pioglitazone treatment significantly increased HO enzyme activity compared to that measured in LPS-injected mice (P = 0.0035), ZnPPIX administration negated the beneficial effects of pioglitazone treatment (P = 0.0026) in the LPS + ZnPPIX + Pio group, as shown in Fig. 3b.

Effect of Pioglitazone on the HO-1, Nrf2, and NF-κB p65 Expressions

The transcription levels of HO-1, its major transcription regulatory protein Nrf2, and the p65 subunit of NF-κB were also evaluated as a part of the current investigation. The findings revealed that steady-state levels of HO-1 transcripts in LPS-injected mice were significantly (approximately by twofold) higher compared to those obtained for the control group ($F_{4,20}$= 1.388, P = 0.0191), as shown in Fig. 4a. Likewise, the graph shown in Fig. 4c indicates that NF-κB p65 subunit levels were threefold higher than those measured for the control group ($F_{4,20}$=25.06, P < 0.001). On the other hand, Nrf2 expression in this group remained unchanged (Fig. 4b).

In the LPS + Pio group, the HO-1 expression levels increased by ~ 2.5-fold (P < 0.001), and were accompanied by a twofold increase in Nrf2 expression ($F_{4,20}$= 15.77, P = 0.0018) when compared to LPS-injected mice. On the other hand, NF-κB p65 subunit expression decreased significantly after LPS-injected mice received pioglitazone treatment (P < 0.001).

Administration of the HO-1 inhibitor ZnPPIX counteracted the aforementioned pioglitazone effects on HO-1 (P < 0.001) and Nrf2 expression (P < 0.001), while inducing marked increase in the NF-κB p65 transcript levels (P < 0.001).

Regulation Effects of Pioglitazone on Nrf2/ARE-1 and ARE-2 of HO-1 Pathway

The binding activity of Nrf2 to the HO-1 promoter elements ARE-1 and ARE-2 was examined by CHIP in all animal groups, aiming to elucidate the functional activity of this regulatory transcription factor. Findings yielded by real-time qPCR showed that anti-Nrf2 antibody-enriched DNA fragments (ARE-1 and ARE-2 HO-1 sequences) in LPS-injected mice were significantly higher, at (($F_{4,15}$ = 78.06, P = 0.0077 and $F_{4,15}$ = 116.2, P = 0.0141, respectively, compared to the control group (Fig. 5). Moreover, mice in the LPS + Pio group showed significant increase in binding activity to both ARE-1 (P < 0.001) and ARE-2 (P < 0.001) sequences relative to those in the LPS group. By contrast, a significant depreciation in the pioglitazone effect on Nrf2 binding activity to ARE-1 (P < 0.001) and ARE-2 (P < 0.001) was noted in ZnPPIX-treated mice.

Discussion

The benefits of PPARγ activation and its capacity for modulating Nrf2/HO-1-dependent ARE as negative regulator of oxidative stress were investigated in the present study by assessing changes in neurobehavioral impairments in a neurodegenerative mouse model. Moreover, ZnPPIX, a known selective competitive HO-1 enzyme inhibitor, was administrated to one of the study groups to evaluate pioglitazone-induced neuroprotective effect associated with HO-1 expression and activity, and its related mechanism in LPS mouse model.

The study findings revealed that, when 6- to 8-month-old mice were treated with LPS (5 mg/kg) they exhibited impaired spatial memory, as indicated by their inferior performance in the Y-maze experiment relative to the control group. This is in line with the findings reported by Cho et al. who examined short-term memory impairment due to progressive dopaminergic neuronal loss [58]. This LPS dose reported similar results in novel object recognition test after 7 days [41]. In the present study, LPS induced anxiety-like behavior when animals were subjected to the EPM test, supporting the findings of an open field test [41]. Animals in the study conducted by Anderson et al. exhibited similar performance in EPM receiving LPS (5 mg/kg) after 1 month [59]. Furthermore, LPS-injected mice showed deficit in motor coordination performance in Ladder rung test, which was attributed to dopaminergic neuronal loss induced by systematic LPS administration [6]. In the LPS + Pio group, pioglitazone (30 mg/kg) administration for seven days blocked LPS-induced neurocognitive impairment significantly, as the animals in this group exhibited short-term spatial memory motor coordination improvements, while demonstrating reduced anxiety-like behavior. However, ZnPPIX administration ameliorated the beneficial effects of pioglitazone treatment, whereby mice in the LPS + ZnPPIX + Pio group exhibited similar neurobehavioral impairments as those observed in LPS-challenged mice. These results are in line with the decrease in spatial memory and motor coordination in an induced ischemic brain damage rat model with ZnPPIX i.p. inhibition of pterostilbene-induced HO-1 [60].

The results obtained in the present study support those reported by Reinert et al., indicating that, in the LPS model,

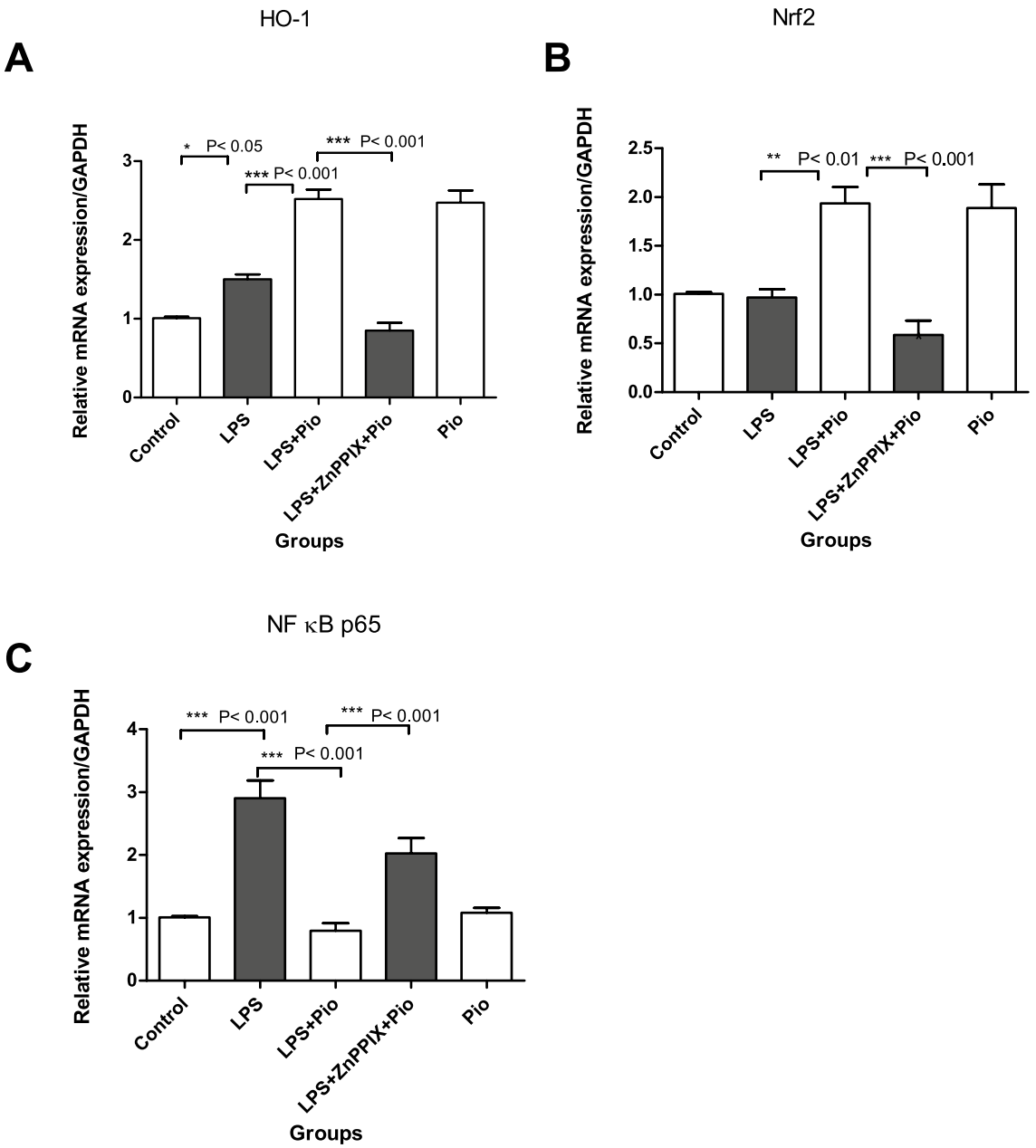

Fig. 4 Effect of pioglitazone on the expression of HO-1, Nrf2, and NF-κB p65 by RT-qPCR. **a** Pioglitazone enhanced HO-1 expression in LPS mouse model, but this beneficial effect was inhibited by ZnPPIX administration. **b** Pioglitazone in LPS group significantly elevated Nrf2 expression, whereas ZnPPIX administration counteracted this effect, resulting in the Nrf2 levels akin to those obtained for the LPS and control groups. **c** LPS and ZnPPIX induced significant elevation in NF-κB p65, which was reduced by pioglitazone treatment. All data are represented as mean ± SEM ($n = 5$)

neurobehavioral impairments coincide with dopaminergic neuronal loss in SN, assessed via the number of TH+ neurons [5]. Pioglitazone treatment was shown to significantly reduce dopaminergic neuronal loss; however, its beneficial effects were counteracted by ZnPPIX i.p. administration. These results are in line with those reported by Kurauchi et al., who found that ZnPPIX alone had no effect on dopaminergic neurons viability and number *invitro,* and neither on TH positive neurons after i.c.v injection in mice. Furthermore, ZnPPIX inhibition of caffeic acid phenethyl ester induced HO-1 in LPS mouse model of PD, which resulted in marked nigral dopaminergic neuronal loss relative to the control group [42].

Oxidative stress and damage to biomolecules, producing reactive end products such as carbonyl proteins, is linked to lower antioxidant enzyme activity as well as lower

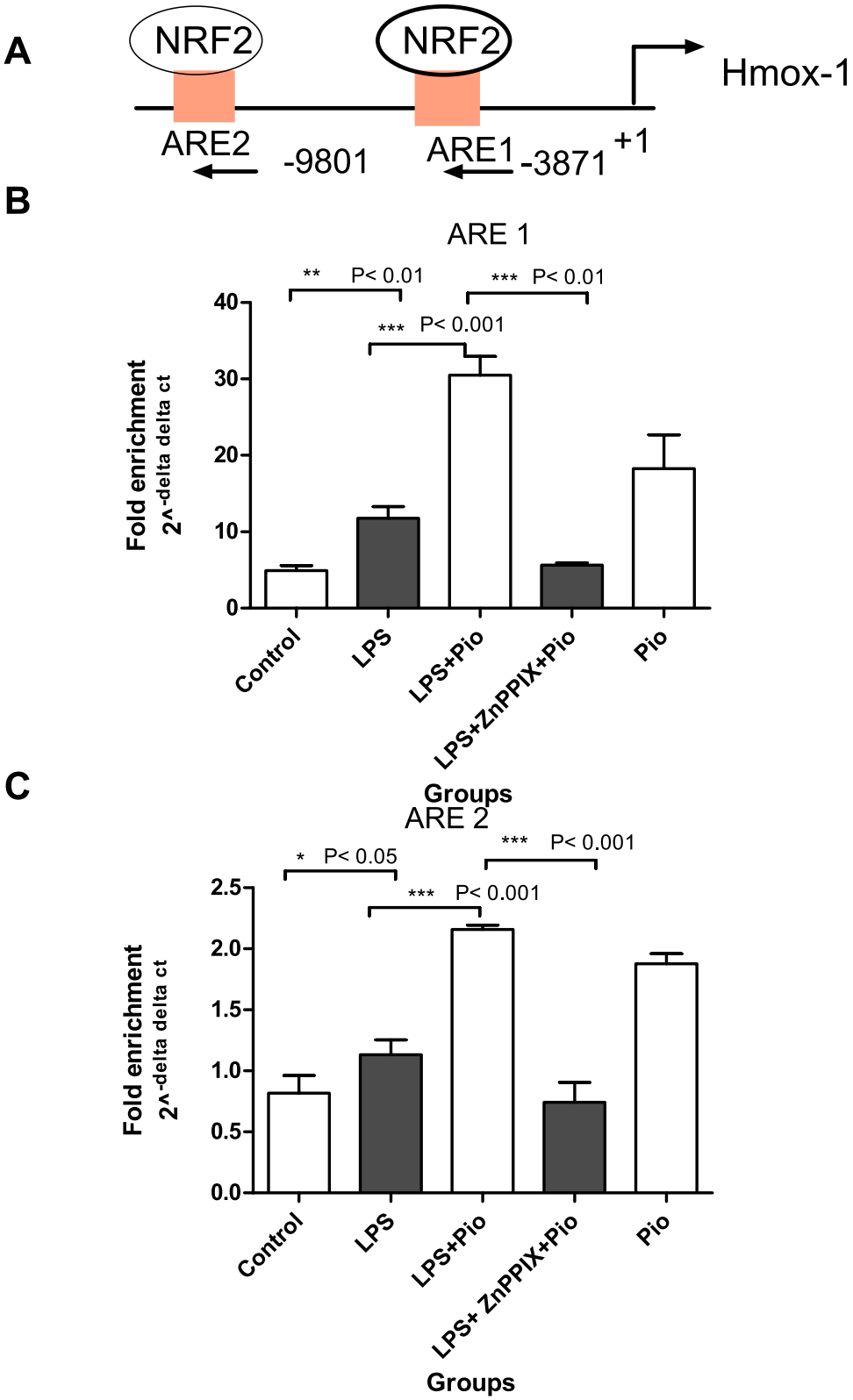

Fig. 5 Regulation effects of pioglitazone on Nrf2/ARE-1 and ARE-2 of HO-1 pathway. **a** Schematic diagram of Nrf2 binding activity to the Hmox-1 promoter region containing ARE binding sites with primer locations, where Nrf2 binding to ARE-1 is more pronounced than binding to ARE-2. **b–c** ChIP-qPCR findings revealed fold enrichment over IgG of Nrf2 binding to ARE-1 and ARE-2 of HO-1, with Anti IgG serving as negative control. Pioglitazone treatment resulted in a significant enhancement in Nrf2 binding to both ARE-1 and ARE-2, compared to LPS group while ZnPPIX abolished these beneficial effects. All reported data is represented as mean ± SEM ($n = 4$)

resistance to neurodegenerative insults [61]. As shown in Fig. 3, in the present study, HO-1 restoration of function and amelioration of oxidative stress was accompanied by a decrease in carbonyl content in mouse brain, supporting the findings previously reported [62]. Moreover, pioglitazone treatment restored HO-1 enzyme activity repressed

by LPS, while diminishing oxidative stress, as indicated by markedly reduced carbonyl protein content. In their study on 20 mg/kg p.o. pioglitazone treatment for 4 days, Hunter et al. observed partially reduced mitochondrial carbonyl protein after striatal LPS injection [63]. In extant studies, no significant effects of ZnPPIX on liver [64], or brain [65] were reported when administered in isolation under normal conditions. However, ZnPPIX reversed the beneficial pioglitazone effects on HO-1 activity and carbonyl protein levels in LPS mouse model. A similar decrease in HO-1 induced by ginsenoside Rg1 and rosiglitazone was attained by administering ZnPPIX in cerebral ischemia–reperfusion injury rat model [44].

The effect of pioglitazone on the molecular signaling pathway underlying the observed neuroprotective effect in LPS-challenged mice was also examined as a part of this work. Unexpectedly, the LPS induced increase in the steady-state levels of HO-1 transcripts, as well as in the NF-κB p65 subunit levels, as shown in Fig. 4. Moreover, enhanced Nrf2 binding to HO-1 ARE-1 and ARE-2 was detected, supporting the findings reported by Ramsey et al. who observed elevated Nrf2 nuclear translocation and increased expression of HO-1 in PD patients [35]. Moreover, the results yielded by the present study are in line with those pertaining to increased HO-1 expression in other LPS-PD mouse models [3, 42, 66]. When considered in conjunction with the previously observed decrease in the HO-1 activity, these findings suggest that activating microglia induced by neuroinflammation might trigger HO-1 expression, whereas post-translational modification is expected to decrease its activity.

PPARγ silencing or antagonist have been previously shown to inhibit Nrf2/ARE signaling in LPS stimulated microglia [67]. Empirical evidence further indicates that PPARγ and Nrf2 are crucial upstream modulators of HO-1 expression in rat brain astrocytes [68]. Zhao et al. and later Choi et al. postulated that PPARγ-mediated NF-κB anti-inflammatory effects might be associated with Nrf2 reduced oxidative molecules required for its activation [37, 67]. These findings are supported by the results obtained in the present study, as pioglitazone treatment resulted in significantly upregulated HO-1 and Nrf2 expression, along with a marked reduction in NF-κB p65 levels. Pioglitazone transcriptionally regulates HO-1 via enhanced Nrf2 binding to both ARE-1 and ARE-2 sites in HO-1 promoter. However, these beneficial pioglitazone effects on Nrf2, HO-1 and NF-κB p65 transcripts, as well as Nrf2-binding activity were counteracted by ZnPPIX i.p. Several lines of evidence support cross-regulation between PPARγ and HO-1. For example, Park et al., showed amelioration of PPARγ transcriptional activity induced by rosiglitazone and cilostazol by HO-1 inhibitor ZnPPIX, and with HO-1 siRNA in human endothelial cells [30]. Moreover, ZnPPIX i.p. inhibited sulforaphane-induced Nrf2/HO-1 proteins expression in retina ischemic/reperfusion injury rat model [43], and ginsenoside Rg1 and rosiglitazone induced PPARγ/HO-1 protein expression [44].

In summary, the current study findings support the view that the PPARγ agonist pioglitazone mediated via Nrf2/ARE dependent HO-1 pathway exhibits neuroprotective effects in LPS mouse model. Specifically, pioglitazone attenuated the neurobehavioral impairments and dopaminergic neuronal loss by promoting HO-1 enzyme and Nrf2 expression and activity, while also reducing oxidative carbonyl protein and NF-κBp65 transcript levels. However, the underlying molecular modulation and regulation between the aforementioned signaling pathway cascades should be explored further in future studies.

Author Contributions AZ conceived of the presented idea, preformed the experimental work and wrote the manuscript; MR contributed in the experimental work; LM co- supervised the study; KAA contributed in the experimental work, conceived the presented idea and supervised the study. All the authors discussed the results and contributed in the manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Ethics Approval This study was carried out in accordance with the principles and recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the ethics committee of the German University in Cairo.

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