Rosiglitazone Regulates TLR4 and Rescues HO-1 and NRF2 Expression in Myometrial and Decidual Macrophages in Inflammation-Induced Preterm Birth

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Abstract

Introduction: Elevated inflammation accounts for approximately 30% of preterm birth (PTB) cases. We previously reported that targeting the peroxisome proliferator-activated receptor gamma (PPAR γ) pathway reduced the incidence of PTB in the mouse model of endotoxin-induced PTB. The PPAR γ has proven anti-inflammatory functions and its activation via rosiglitazone significantly downregulated the systemic inflammatory response and reduced PTB and stillbirth rate by 30% and 41%, respectively, in our model. Oxidative stress is inseparable from inflammation, and rosiglitazone has a reported antioxidative activity. In the current study, we therefore aimed to evaluate whether rosiglitazone treatment had effects outside of inflammatory pathway, specifically on the antioxidation pathway in our model. **Methods:** Pregnant C57BL/6J mice (E16.5) were treated with phosphate-buffered saline (PBS), rosiglitazone (Rosi), lipopolysaccharide (LPS; 10 μ g in 200 μ L 1XPBS), or LPS + Rosi (6 hours after the LPS injection). The myometrial and decidual tissues were collected and processed for macrophage isolation using magnetic cell sorting and F4/80+ antibody. Expression levels of antioxidative factors—*Nrf2* and *Ho-1*—along with the LPS receptor *Tlr4* were quantified by quantitative polymerase chain reaction. The protein levels were assessed by immunofluorescence staining. **Results:** Both the decidual and myometrial macrophages from the LPS-treated animals showed significantly lowered expression of *Ho-1* and *Nrf2* and higher expression of *Tlr4* when compared to the PBS control group. The macrophages from the animals in the LPS + Rosi group had significantly elevated expression of *Ho-1* and *Nrf2* and downregulated expression of *Tlr4* when compared to the LPS group. **Conclusion:** Rosiglitazone administration prevents PTB by downregulating inflammation and upregulating antioxidative response.

Keywords

preterm birth, macrophages, LPS, PPAR γ , antioxidant

Introduction

Preterm birth (PTB) is defined as delivery before 37 weeks of gestation, which affected approximately 9.62% of births in 2015.¹ Premature neonates are at risk for short- and long-term health problems, making PTB one of the leading causes of neonatal mortality and morbidity worldwide.² Approximately 70% of all PTBs are preceded by spontaneous preterm labor (PTL),³⁻⁷ a syndrome of multiple pathological processes.⁸ Of all the putative causes associated with spontaneous PTL, only intra-amniotic infection/inflammation has been causally linked to PTB.⁹⁻¹² Inflammation-associated PTB is characterized by increased expression of proinflammatory proteins and infiltration of immune cells in the maternal-fetal interface¹³ as well as elevated oxidative stress.¹⁴⁻¹⁶ Several animal models have been established in order to study the mechanisms whereby inflammation induces PTB.¹⁷⁻¹⁹ Lipopolysaccharide (LPS)-induced PTB is the most widely used model.²⁰ In

pregnant mice, administration of LPS induces an inflammatory response resulting in premature delivery.²¹ The LPS binds to Toll-like receptor 4 (TLR4), which recruits the adaptor protein myeloid differentiation factor (Myd88)²² in order to initiate the

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activation of proinflammatory proteins such as the transcription factor—nuclear factor κ B (NF- κ B).^{23,24} Activation of the NF- κ B pathway results in the expression of inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin 1 β ,²² triggering an inflammatory cascade that ultimately leads to PTL and PTB. Similar to the human syndrome, LPS-induced PTB increases oxidative stress and promotes the infiltration of neutrophils and macrophages at the maternal–fetal interface.^{25–28} Therefore, targeting proinflammatory macrophages at the maternal–fetal interface may represent a new strategy to prevent inflammation-associated PTB.

Proinflammatory macrophages are present at the maternal–fetal interface in term (ie, physiological inflammation) and preterm (ie, pathological inflammation) parturition.^{13,29–31} Such innate immunity cells have a proinflammatory or M1-like phenotype in both processes of labor, which can be attenuated by administering rosiglitazone (Rosi).³² Rosiglitazone belongs to the thiolidazone family of compounds and acts as a specific agonist for the nuclear hormone receptor, peroxisome proliferator–activated receptor γ (PPAR γ).³² The PPAR γ is known for its role in lipid metabolism, adipocyte differentiation as well as for regulating the genes involved in inflammation and oxidative stress.^{33–36} Interestingly, PPAR γ knockouts die in utero due to placental abnormalities, suggesting a pivotal role in placentation.³⁷ Altered levels of PPAR γ or its activators have been associated with pregnancy-related pathologies such as gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR), and preeclampsia (PE)^{38–40}; however, its potential role in PTB remained unexplored. Recently, we showed that treatment with Rosi (1) reduced the rate of LPS-induced PTB by 30%, (2) reduced the rate of stillbirth by 41%, and (3) significantly downregulated the systemic inflammatory response in mice.³² Therefore, by targeting the PPAR γ pathway we could reduce the inflammatory effects of LPS.

In addition to inflammation, elevated oxidative stress is a major contributor to LPS-induced PTB.^{14,41} Indeed, antioxidant supplementation has been demonstrated to improve pregnancy outcomes in different models of PTB.^{26,27,42} We therefore aimed to evaluate whether Rosi had similar actions in our model of PTB. In the study herein, we focused on nuclear factor (erythroid-derived 2)-like 2 (NRF2) and its downstream target heme oxygenase 1 (HO-1) due to their dual role as anti-inflammatory and antioxidative regulators.^{43,44} The NRF2 is a transcription factor that regulates the expression of proteins involved in the detoxification of oxygen radicals by binding to antioxidant response element in gene promoters.⁴⁵ Additionally, it has been reported to block inflammatory signaling in bone marrow–derived macrophages and reduce inflammation in a thrombin-induced PTB model.^{46,47} The HO-1 is the rate-limiting enzyme catalyzing the breakdown of heme, producing carbon monoxide and biliverdin. Biliverdin is then degraded further into bilirubin which is a strong antioxidant.⁴⁸ The HO-1 has been shown to play a critical role in placental function and its reduced levels have been associated with preeclampsia and cases of spontaneous abortions.^{49,50} The HO-1 has also been

shown to reduce myometrial contractility and may thus play a role in PTB.⁵¹

Rosiglitazone has been shown to upregulate the expression of both NRF2 and HO-1 in hepatocytes,⁵² but its effects on expression in decidual and myometrial macrophages are unknown. Herein, we hypothesized that treatment with Rosi would improve pregnancy outcomes in an LPS-induced model of PTB by indirectly affecting the inflammatory cascade by regulating TLR4 receptor and reducing oxidative stress by upregulating the antioxidant factors NRF2 and HO-1.

Materials and Methods

Animal Treatments

Pregnant C57BL/6J mice were divided into 4 groups and received intraperitoneal injections on 16.5 days post coitum (dpc): group I: LPS—received 10 μ g of LPS (*Escherichia coli* 055: B5; Sigma-Aldrich (St. Louis, Missouri) in 200 μ L of 1 $\times$ phosphate-buffered saline (PBS); group II: PBS—200 μ L of 1 $\times$ PBS as a control; group III: LPS + Rosi—received 10 μ g of LPS in 200 μ L of 1 $\times$ PBS followed by 10 mg/kg of Rosiglitazone (in 1:10 DMSO) (Selleckchem, Houston, Texas) 6 hours after the initial injection; and group IV: Rosi—10 mg/kg of Rosi as a control. All procedures were approved by the institutional animal care and use committee at Wayne State University (protocol no. A 09-08-12).

Macrophage Isolation From Murine Myometrial and Decidual Tissues

Eight hours after the first injection, the mice were euthanized and decidual and myometrial tissues were collected and processed immediately for macrophage isolation and cryosectioning.

Macrophage Isolation

Macrophages were isolated from the decidua and myometrium, as previously described.³² Briefly, the tissues were mechanically disaggregated using the Accutase cell dissociation reagent (Life Technologies, Carlsbad, California) and filtered using a 100- μ m cell strainer (Fisher Scientific, Hampton, New Hampshire) to obtain single-cell suspensions. The cells were then washed once with staining buffer (1 $\times$ PBS [Fisher Scientific Bioreagents] containing 0.1% bovine serum albumin [Sigma-Aldrich] and 0.05% sodium azide [Fisher Scientific Bioreagents]). The cells were resuspended in 96 μ L of staining buffer, with 4 μ L of an anti-mouse F4/80 antigen biotin (clone BM8; eBioscience, Santa Clara, California) and incubated at 4°C for 15 minutes. After incubation, the cells were washed by centrifugation at 1250 $\times$ *g* for 7 minutes at 4°C. The cell pellet was resuspended in 90 μ L of staining buffer containing 10 μ L of streptavidin microbeads (Miltenyi Biotec, Bergisch, Gladbach) and again incubated at 4°C for 15 minutes followed by 1 wash with 2 mL of staining buffer. The resulting pellet was resuspended in 500 μ L of magnetic cell sorting (MACS; Miltenyi Biotec) buffer and F4/80+ cells (macrophages) were separated by positive selection using MS columns

Table 1. Sequences of Primer Used for the Study.

Gene	Sequence
<i>Gapdh</i>	Primer 1 5'-AAT GGT GAA GGT CGG TGT G-3' Primer 2 5'-GTG GAG TCA TAC TGG AAC ATG TAG-3'
<i>Top1</i>	Primer 1 5'-CTT TAA TTC GTG GCG GAC TAG A-3' Primer 2 5'-AGA CAA GGA AAG ACG AAA GGA G-3'
<i>Tbp</i>	Primer 1 5'-TTC ACC AAT GAC TCC TAT GAC C-3' Primer 2 5'-CAA GTT TAC AGC CAA GAT TCA CG-3'
<i>Tlr4</i>	Primer 1 5'-GAA GCT TGA ATC CCT GCA TAG-3' Primer 2 5'-AGC TCA GAT CTA TGT TCT TGG TTG-3'
<i>Ho-1</i>	Primer 1 5'-ACA CTC TGG AGA TGA CAC CT-3' Primer 2 5'-TTG TGT TCC TCT GTC AGC ATC-3'
<i>Nrf2</i>	Primer 1 5'-CCT TGT ACT TTG AAG ACT GTA TGC-3' Primer 2 5'-GAG GGA CTG GGC CTG AT-3'

and a magnetic MACS separator. Macrophages were then washed with MACS buffer at $1250 \times g$ for 7 minutes at 4°C . The resulting cell pellet was lysed using 400 μL of RNeasy lysis buffer (Qiagen, Germantown, Maryland) and used for RNA isolation.

RNA Isolation and Complementary DNA Synthesis

Total RNA was extracted (RNeasy; Qiagen, Hilden, Germany) following the manufacturer's instructions. All of the samples were simultaneously reverse transcribed using the iScript Reverse Transcription Supermix RT synthesis kit Bio-Rad Laboratories (Hercules, California), according to the manufacturer's instructions. As the number of macrophages isolated from the murine tissues is very low, the amount of RNA extracted was also low. In order to increase the sensitivity and efficiency of quantitative polymerase chain reaction (qPCR), the target genes *Tlr4*, *Ho-1*, and *Nrf2* and housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), TATA-box binding protein (*Tbp*), and topoisomerase I (*TOP1*) were enriched by preamplification. The primers were obtained from integrated DNA technology (Coralville, Iowa), and reconstituted with Tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) to a 500- μM working stock; 2.5 μL from each primer was mixed and the final volume was adjusted to 500 μL to create the preamplification primer cocktail (0.5 μM); 10 μL of the complementary DNA (cDNA 25 ng) was mixed with 5 μL of primer cocktail, 10 μL of nuclease free water, and 25 μL of preamplification master mix (Sso – Advanced PreAmp Supermix; Bio-Rad Laboratories). The mix was then cycled for 3 minutes at 95°C , 15 seconds at 95°C , and 4 minutes at 58°C for 10 cycles.

Real-Time PCR and Data Analysis

The target genes *Tlr4*, *Ho-1*, and *Nrf2* and the housekeeping genes *Gapdh*, *Tbp*, and *Top1* were run in triplicates for each sample using 3 μL of preamplified cDNA (diluted 1:30) per PCR. The primers used are outlined in Table 1. Briefly, 3 μL of cDNA template was mixed with 1 μL primer (500 nM), 1 μL nuclease free water, and 5 μL of Sybr-green master mix (LuminoCT; Sigma-Aldrich, St Louis, Missouri). The PCR protocol used was as follows: initial 95°C for 5 minutes

followed by 38 cycles of 95°C for 15 seconds and 60°C for 20 seconds. The qPCR data were analyzed by Pfaffl method using housekeeping genes as an internal reference.^{53,54} Briefly, the mean C_T values of housekeeping genes per tissue sample was used to calculate the geometric mean C_T value (Geo-mean C_T) and used as reference for the respective sample. The relative expression of target genes was calculated by using the formula: $2^{-\Delta C_T}$, where $\Delta C_T = \text{mean } C_T (\text{target gene}) - \text{Geo-mean } C_T$. The mean relative expression (MRE) of the target gene for each treatment groups was calculated by taking an average of the relative expression of individual tissue samples from the respective groups.

Immunofluorescence Staining

Myometrial and decidual tissues ($n = 5$ each) were immediately frozen in Tissue-Tek O.C.T Compound (Sakura, Torrance, California). The tissues were sectioned into 10 μm sections and placed on Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, Waltham, Massachusetts). For staining, the tissues were fixed in 4% paraformaldehyde for 10 minutes and washed in $1 \times \text{PBS}$ containing 0.1% Tween-20. The tissues were then permeabilized with 0.25% Triton X-100 in $1 \times \text{PBS}$ for 10 minutes. Nonspecific antibody interactions were blocked using donkey serum (Jackson Immuno Research Laboratories, West Grove, Philadelphia) at room temperature for 1 hour. Slides were then incubated with the following: anti-TLR4 antibody (Abcam, Cambridge, UK), anti-HO-1 antibody (Abcam), anti-NRF2 antibody (Cell Signaling, Danvers, Massachusetts) at 4°C overnight. The following day, the slides were washed 3 times (5 min/wash) with $1 \times \text{PBS}$ containing 0.1% Tween-20. The slides were incubated with 2 mg/mL of goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Jackson Immuno Research Laboratories) at room temperature for 1 hour. The slides were washed again with $1 \times \text{PBS}$ containing 0.1% Tween-20 and incubated with 2 mg/mL of rat anti-mouse F4/80 antibody conjugated to fluorescein isothiocyanate (FITC) (1:100; Abcam) at room temperature for 2 hours. Post incubation, the slides were again washed 3 times and stained with 4',6-diamidino-2-phenylindole (DAPI) (room temperature for 20 minutes). The slides were given a final wash and mounted in Vectashield Mounting Medium. Immunofluorescence was visualized using the Leica DM IRB epifluorescence microscope, and images were captured using a Hamamatsu Orca digital camera.

Results

Macrophage Populations

Macrophages were isolated from the decidual and myometrial tissues of mice treated with LPS only, LPS + Rosi, Rosi, and PBS only (PBS). The purity of the isolated F4/80+ macrophage cells was assessed by flow cytometry. The percentage of macrophages isolated from the decidual and myometrial tissues was 84.9% and 88.6%, respectively (Figure 1A and B).

Figure 1. Purity of isolated macrophages from decidual and myometrial tissues. Decidual (A) and myometrial (B) macrophages (F4/80+ cells) were isolated by magnetic cell sorting, and purity was evaluated by flow cytometry. The red histogram represents the autofluorescence control and the blue histogram represents isolated macrophages from decidual or myometrial tissues.

Rosiglitazone downregulated LPS-induced *Tlr4* expression. Total RNA was extracted from decidual and myometrial macrophages isolated from mice from LPS, LPS + Rosi, Rosi, and PBS groups and converted to cDNA. The expression of the *Tlr4* receptor was assessed using qPCR.

The MRE of *Tlr4* in decidual macrophages isolated from mice injected with endotoxin alone was significantly higher compared to those isolated from mice injected with PBS only (1.81 ± 0.23 vs 1.23 ± 0.10 ; $P = .031$). The MRE of decidual macrophages from mice injected with Rosi only was significantly reduced compared to those isolated from mice injected with endotoxin alone (0.96 ± 0.08 vs 1.81 ± 0.23 ; $P = .007$). The MRE of decidual macrophages isolated from LPS-injected mice treated with Rosi was significantly lower compared to those isolated from mice injected with endotoxin alone (1.14 ± 0.18 vs 1.81 ± 0.23 ; $P = .048$). However, no significant differences were observed in the *Tlr4* expression of isolated macrophages from mice injected with PBS and Rosi alone (Figure 2A).

In line with the observation in decidual macrophages, the MRE of *Tlr4* in myometrial macrophages isolated from mice injected with endotoxin alone was significantly higher compared to those isolated from mice injected with PBS only (2.29 ± 0.24 vs 1.25 ± 0.11 ; $P = .007$) and Rosi only (2.29 ± 0.24 vs 1.44 ± 0.10 ; $P = .031$). The MRE of *Tlr4* in myometrial macrophages isolated from LPS-injected mice treated with Rosi was significantly lower compared to those isolated from mice injected with endotoxin alone (1.20 ± 0.19 vs 2.29 ± 0.24 ; $P = .015$; Figure 2B).

Typically, the number of isolated macrophages is too low for conventional quantitative protein assessment by Western blotting. Hence, we decided to assess TLR4 protein expression by immunohistochemistry.³² The cryo-fixed decidual and myometrial tissues from mice injected with LPS only, LPS + Rosi, Rosi only, and PBS only were sectioned and stained with F4/80+ and anti-TLR4 antibodies. The presence

of F4/80 and TLR4 was observed in both decidual and myometrial tissues. The relative expression of TLR4 was higher in myometrial and decidual tissues from mice injected with endotoxin compared to controls injected with PBS and Rosi only (Figure 2C and D).

Treatment with Rosi inhibited the LPS-mediated downregulation of *Nrf2* expression. The NRF2 is the master transcription factor that regulates oxidative stress via the expression of antioxidant factors.^{55,56} Therefore, in order to evaluate the oxidative stress signaling pathway in an endotoxin-induced model of PTB, we assessed NRF2 expression in isolated myometrial and decidual macrophages from mice injected with LPS only, LPS + Rosi, Rosi only, and PBS only.

The MRE of *Nrf2* in decidual macrophages isolated from mice injected with endotoxin was significantly lower compared to those isolated from mice injected with PBS (2.10 ± 0.22 vs 3.28 ± 0.30 ; $P = .028$) and Rosi alone (2.10 ± 0.22 vs 3.28 ± 0.37 ; $P = .028$). The MRE of *Nrf2* in decidual macrophages isolated from LPS injected mice treated with Rosi was significantly increased compared to those isolated from mice injected with endotoxin alone (3.15 ± 0.2 vs 2.10 ± 0.22 ; $P = .028$). The MRE of *Nrf2* was comparable between decidual macrophages isolated from mice treated with LPS + Rosi, PBS, and Rosi alone (Figure 3A).

A similar trend in *Nrf2* expression was observed in myometrial macrophages. The MRE of *Nrf2* in myometrial macrophages isolated from mice injected with endotoxin was significantly lower compared to those isolated from mice injected with PBS (1.37 ± 0.16 vs 2.31 ± 0.18 ; $P = .028$) and Rosi only (1.37 ± 0.16 vs 2.51 ± 0.29 ; $P = .015$). In addition, the expression of *Nrf2* in myometrial macrophages isolated from LPS injected mice treated with Rosi was significantly higher than those injected with endotoxin alone (2.80 ± 0.49 vs 1.37 ± 0.16 ; $P = .031$; Figure 3B). The *Nrf2* expression levels were comparable between myometrial macrophages

Figure 2. Rosiglitazone downregulates LPS-induced TLR4 expression: mean relative expression of *Tlr4* mRNA in decidual (A) and myometrial (B) in macrophages isolated from animals ($n = 4-7$) in the 4 treatment groups. Macrophages from the LPS-treated group showed increased expression of TLR4 which was significantly downregulated when rosiglitazone was administered. Representative images show immunostaining for TLR4 protein expression (red) in (C) decidual and (D) myometrial macrophages (green, arrows). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Data are shown as box plots (median). “*” and “#” indicate significance at $P < .05$, when compared to the LPS group and PBS group, respectively. Magnification is $\times 1000$, $n = 3$. LPS indicates lipopolysaccharide; mRNA, messenger RNA; TLR4, Toll-like receptor 4; PBS, phosphate-buffered saline.

isolated from LPS injected mice treated with Rosi and those isolated from mice injected with PBS and Rosi alone.

Visualization of the NRF2 protein was performed using immunohistochemistry. Neither decidual nor myometrial tissues from mice injected with LPS endotoxin expressed the NRF2 protein; however, a comparable expression of NRF2 was observed in decidual and myometrial tissues from LPS-injected mice treated with Rosi and those from mice injected with PBS and Rosi alone (Figure 3C and D).

Treatment with Rosi inhibited the LPS-mediated reduction in *Ho-1* expression. The messenger RNA (mRNA) expression of *Ho-1*, a known downstream target for NRF2 signaling and a critical antioxidant mediator in cells, was assessed in decidual and myometrial macrophages.⁵⁶

The MRE of *Ho-1* in decidual macrophages isolated from mice injected with endotoxin was significantly lower compared to those isolated from mice injected with PBS (0.11 ± 0.02 vs 0.21 ± 0.004 ; $P = .028$) and Rosi only (0.11 ± 0.02 vs 0.42 ± 0.09 ; $P = .015$). The HO-1 expression in decidual macrophages isolated from LPS-injected mice treated with Rosi was significantly higher compared to those isolated from mice injected

with endotoxin alone (0.50 ± 0.10 vs 0.11 ± 0.02 ; $P = .015$). Interestingly, the HO-1 expression of decidual macrophages isolated from LPS-injected mice treated with Rosi was significantly elevated compared to those isolated from PBS controls (0.50 ± 0.10 vs 0.21 ± 0.004 ; $P = .0159$). The HO-1 expression in isolated decidual macrophages from mice injected with Rosi alone was also significantly higher compared to PBS controls (0.42 ± 0.09 vs 0.21 ± 0.004 ; $P = .015$; Figure 4A).

In contrast to decidual macrophages, HO-1 expression in myometrial macrophages isolated from mice injected with endotoxin was not significantly different compared to PBS controls (0.26 ± 0.020 vs 0.22 ± 0.04 ; $P = .45$). However, HO-1 expression in isolated myometrial macrophages from LPS-injected mice treated with Rosi was comparable to those isolated from the decidua. The MRE of *Ho-1* in isolated myometrial macrophages from mice injected with LPS + Rosi was significantly higher compared to those isolated from mice injected with endotoxin (0.53 ± 0.06 vs 0.26 ± 0.020 ; $P = .03$) and PBS alone (0.53 ± 0.06 vs 0.22 ± 0.04 ; $P = .015$). The MRE of *HO-1* in myometrial macrophages isolated from mice injected with Rosi alone was elevated compared to those isolated from LPS-injected mice (0.46 ± 0.06 vs $0.26 \pm$

Figure 3. Rosiglitazone reduces LPS-mediated downregulation of NRF2 expression: mean relative expression of *Nrf2* mRNA in decidual (A) and myometrial (B) macrophages isolated from animals ($n = 4-7$) in the 4 treatment groups. Macrophages from the LPS-treated group showed a significantly lower expression of *Nrf2*, which was rescued to control levels when rosiglitazone was administered. Representative images show immunostaining for NRF2 protein expression (red) in (C) decidual and (D) myometrial macrophages (green, arrows). The nuclei were stained with DAPI (blue). Data are shown as box plots (median). “*” and “#” indicate significance at $P < .05$, when compared to the LPS group, PBS group, and Rosi group, respectively. Magnification is $\times 1000$, $n = 3$. LPS indicates lipopolysaccharide; mRNA, messenger RNA; NRF2, nuclear factor (erythroid-derived 2)-like 2; PBS, phosphate-buffered saline.

0.020) and PBS controls (0.46 ± 0.06 vs 0.22 ± 0.04); yet, these values were not statistically significant (Figure 4B).

The relative protein levels of *Ho-1* observed in myometrial tissues also supported the mRNA expression data. The *Ho-1* was expressed in myometrial and decidual macrophages isolated from LPS injected mice treated with Rosi as well as those isolated from mice injected with PBS and Rosi alone. A reduced expression of *HO-1* was observed in myometrial and decidual tissues from mice injected with endotoxin compared to those injected with LPS + Rosi and PBS and Rosi alone (Figure 4C and D).

Discussion

In the current study, we expanded the involvement of molecular pathways in response to rosiglitazone treatment in a murine model of LPS-induced PTB. Our previous study showed that administration of rosiglitazone reduced the rate of LPS-induced PTB by 30%, increased the pup viability by 41%, and lowered systemic and local inflammation.³² We also showed a downregulation of the NF- κ B pathway mediators—TNF- α and NF- κ B1—in decidual and myometrial

macrophages. These findings demonstrated that treatment with rosiglitazone contributes to reduced inflammation via reducing NF- κ B activity in local macrophages. Herein, we investigated whether treatment with rosiglitazone also regulated TLR4 expression and induced the expression of the antioxidants *HO-1* and *NRF2*, which could further support the reduction in PTB and pup mortality.

Depletion of macrophages in pregnant mice abrogates the effects of LPS and prevents PTB,⁵⁷ suggesting that these innate immune cells are involved in mediating the effects of LPS. Therefore, we focused on the role of macrophages in our LPS-induced model of PTB. Decidual and myometrial macrophages from mice injected with LPS only, LPS + Rosi, Rosi only, and PBS only were isolated and their gene expression was analyzed by qPCR. The protein expression was assessed by staining decidual and myometrial tissues with antibodies against F4/80+ and the proteins of interest. We observed a significant increase in *Tlr4* expression in both decidual and myometrial macrophages from mice injected with endotoxin compared to those isolated from mice injected with PBS and rosiglitazone alone. Administration of Rosi post LPS prevented this upregulation and macrophages from the LPS +

Figure 4. Rosiglitazone rescued LPS-mediated decrease in HO-1 expression: mean relative expression of *Ho-1* mRNA in decidual (A) and myometrial (B) macrophages isolated from animals ($n = 4-7$) in the 4 treatment groups. Macrophages from the LPS-treated group showed a lower expression of *Ho-1*, which was significantly upregulated when rosiglitazone was administered. Representative images show immunostaining for HO-1 protein expression (red) in (C) decidual and (D) myometrial tissue macrophages (green, arrows). The nuclei were stained with DAPI (blue). Data are shown as box plots (median). “#”, “*” and “ψ” indicate significance at $P < .05$, when compared to the LPS group, PBS group, and Rosi group, respectively. Magnification is $\times 1000$, $n = 3$. LPS indicates lipopolysaccharide; mRNA, messenger RNA; HO-1, heme oxygenase 1; PBS, phosphate-buffered saline.

Rosi group had TLR4 levels (protein and mRNA) comparable to those seen in control groups, suggesting an active regulation of TLR4 in our model.

Induction of *Tlr4* expression by LPS has been observed in smooth muscle cells and microglia; however, information on the expression of this protein in mouse decidual and myometrial tissues and tissue macrophages is inconclusive.^{2,58,59} Salminen et al reported *Tlr4* mRNA expression in the uteri of pregnant mice; however, its expression significantly declined post LPS treatment.⁶⁰ In vitro studies in mouse macrophages showed no alteration in TLR4 expression after LPS treatment, whereas human mononuclear cells showed an upregulation at the mRNA level with no alteration in protein expression.^{61,62} In contrast, we observed that the LPS upregulated TLR4 mRNA expression and protein in macrophages from both tissues. These results suggest that LPS regulates the expression of its own receptor in these cells. Increased expression of TLR4 is associated with increased sensitivity to LPS and increased activation of inflammatory pathways, for example, NF- κ B.⁵⁸ In return, we suggest that rosiglitazone mediated the downregulation of TLR4 expression (as observed in the LPS + Rosi group) and impaired the activation of

proinflammatory pathways, which could ultimately contribute to a reduction in PTB.⁶³ Further, in both decidual and myometrial tissues, the TLR4 protein was exclusively localized in the macrophages (F4/80+ cells; Figure 2C and D). While it is known that human decidual cells express the TLR4 protein, its expression in mouse decidual tissue remains unknown.⁶⁴ We report that in murine decidual and myometrial tissues, only the macrophages express the TLR4 receptor, suggesting an active role in pathogen recognition and clearance during pregnancy.

In addition to activation of NF- κ B, TLR4 activation has been shown to induce production of reactive oxygen species (ROS), which leads to oxidative stress.⁶⁵ Oxidative stress is an inevitable part of pregnancy and in normal circumstances, a balance between the production of ROS and the antioxidants that scavenge them is maintained. However, excess ROS—induced by inflammation or lowered antioxidant capacity—leads to oxidative stress damage which has been associated with pathologies such as PE, IUGR, and PTB (detailed review in^{66,67}). Further, elevated ROS has been shown to augment the expression of TLR4 in mice which, in turn, has been shown to downregulate the expression of antioxidant enzyme HO-1.

These data suggest that there is a mutual regulation between inflammatory and oxidative stress pathways.⁶⁸⁻⁷⁰

The transcription factor NRF2 is the master regulator of antioxidant enzyme expression and is induced under normal cellular stress conditions by various ROS.^{55,56} Activation of NRF2 leads to its release from the cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap-1) and translocation to the nucleus where it induces expression of antioxidant enzymes like HO-1, which then help in scavenging ROS and other oxidants. The NRF2 has been reported to play a role in murine placental development.⁷¹ Its activation was shown to reduce thrombin-induced PTB, suggesting its active role in inflammation-induced PTB. The HO-1 has also been implicated in playing crucial roles during pregnancy.^{72,73} It was reported to regulate recruitment and maintenance of myeloid cells in pregnant uteri and placental vasculature development.^{74,75} Additionally, HO-1 activation via statins was shown to delay myometrial contractions, cervical ripening, and inhibit pathological complement activation in the LPS-induced PTB model.⁷⁶ However, expression of both HO-1 and NRF2 in myometrial and decidual tissues and tissue macrophages has not been described.

We observed that LPS treatment downregulated the expression of both NRF2 and its downstream target HO-1. These results demonstrate, for the first time, that LPS actively inhibits the antioxidant response in the pregnant mice by downregulating NRF2. Thus, LPS-mediated inflammation contributes to the elevated oxidative stress in this model. As reported in other cell systems, Rosi treatment induced the expression of both NRF2 and HO-1. In addition, the expression of these proteins in the LPS + Rosi group was comparable to that in the PBS and Rosi groups.⁵² A cross talk between the NRF2-HO-1 signaling and TLR4-NF- κ B signaling pathway has been suggested and activation of NRF2 has been shown to rescue the effects of TLR4-mediated proinflammatory pathways in mouse liver and adipose tissue cells.⁷⁷⁻⁷⁹ We suggest activation of a similar pathway due to rosiglitazone administration in our model.

Furthermore, HO-1 expression was significantly upregulated in decidual macrophages isolated from mice injected with Rosi only, suggesting its regulation by an NRF2-independent mechanism. Similar to TLR4, the NRF2 and HO-1 proteins were predominantly localized to macrophages from decidual and myometrial tissues, suggesting their role in mediating the oxidative and inflammatory stress cross talk.

Taken together, our results demonstrate that LPS might promote inflammation and oxidative stress by upregulating TLR4 expression and downregulating the antioxidants NRF2 and HO1 in the LPS-induced model of PTB. In addition, rosiglitazone prevents PTB by downregulating TLR4-mediated proinflammatory signaling and upregulating the antioxidant response via NRF2 and HO-1.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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