Progesterone-induced activation of membrane-bound progesterone receptors in murine macrophage cells

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Abstract

Parturition is an inflammatory process mediated to a significant extent by macrophages. Progesterone (P₄) maintains uterine quiescence in pregnancy, and a proposed functional withdrawal of P₄ classically regulated by nuclear progesterone receptors (nPRs) leads to labor. P₄ can affect the functions of macrophages despite the reported lack of expression of nPRs in these immune cells. Therefore, in this study we investigated the effects of the activation of the putative membrane-associated PR on the function of macrophages (a key cell for parturition) and discuss the implications of these findings for pregnancy and parturition. In murine macrophage cells (RAW 264.7), activation of mPRs by P₄ modified to be active only extracellularly by conjugation to BSA (P4BSA, 1.0×10^{-7} mol/l) caused a pro-inflammatory shift in the mRNA expression profile, with significant upregulation of the expression of cyclooxygenase 2 (COX2 (*Ptgs2*)), *Il1B*, and *Tnf* and downregulation of membrane progesterone receptor alpha (*Pagr7*) and oxytocin receptor (*Oxtr*). Pretreatment with PD98059, a MEK1/2 inhibitor, significantly reduced P4BSA-induced expression of mRNA of *Il1B*, *Tnf*, and *Ptgs2*. Inhibition of protein kinase A (PKA) by H89 blocked P4BSA-induced expression of *Il1B* and *Tnf* mRNA. P4BSA induced rapid phosphorylation of MEK1/2 and CREB (a downstream target of PKA). This phosphorylation was inhibited by pretreatment with PD98059 and H89, respectively, revealing that MEK1/2 and PKA are two of the components involved in mPR signaling. Taken together, these results indicate that changes in membrane progesterone receptor alpha expression and signaling in macrophages are associated with the inflammatory responses; and that these changes might contribute to the functional withdrawal of P₄ related to labor.

Key Words

- ▶ membrane progesterone receptor
- ▶ progesterone
- ▶ macrophages
- ▶ inflammatory response

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Introduction

Parturition is an inflammatory process observed at term and preterm. Evidence from human and animal studies has demonstrated that leukocytes infiltrate myometrium, cervix, and decidua during and before the process of

labor, playing a critical role in parturition (Mackler *et al.* 1999, Thomson *et al.* 1999, Hamilton *et al.* 2012, Care *et al.* 2013, Shynlova *et al.* 2013). In mice, numbers of uterine macrophages increase during the period of early pregnancy

and then decline near term (Mackler *et al.* 1999). In contrast, numbers of macrophages in the cervix increase at term and peak on the day before delivery. Therefore, it has been suggested that macrophage trafficking between uterus and cervix, and associated cytokine production, contribute to the termination of pregnancy (Mackler *et al.* 1999). Decidual macrophage infiltration has also been shown in both term labor and idiopathic preterm labor in humans and rats before parturition, indicating an initiating role of inflammatory events in labor (Hamilton *et al.* 2012). Taken together, the results of these studies highlight the importance of inflammatory cell infiltration into reproductive tissues as a physiological mechanism regulating pregnancy maintenance and parturition.

17-Hydroxyprogesterone caproate injections have been shown to prevent preterm delivery in high-risk women (Meis *et al.* 2003). Progesterone (P_4) maintains pregnancy by promoting uterine quiescence until parturition is initiated by certain forms of withdrawal of this ' P_4 block' (Csapo 1956). In humans, maternal levels of circulating P_4 do not change during spontaneous labor or in the weeks preceding labor (Pieber *et al.* 2001). Therefore, alternative mechanisms of functional P_4 withdrawal have been proposed (Zakar & Hertelendy 2007, Mesiano *et al.* 2011). P_4 exerts its actions through the classical intracellular nuclear progesterone receptors (nPRs; Mulac-Jericevic *et al.* 2000, Conneely *et al.* 2003, Merlino *et al.* 2007), leading to the translocation of hormone-receptor complexes into the nucleus, where they bind to hormone-responsive elements of DNA to regulate gene transcription (Webster *et al.* 2002). However, some of the effects of P_4 are not related to its transcriptional activity (Gellersen *et al.* 2009). In 2003, putative mPR receptors (mPR α , β , and γ) were cloned, shedding new light on PR (Zhu *et al.* 2003).

P_4 elicits a variety of functional effects on immune cell types, including dendritic cells (DCs), monocytes, lymphocytes, and macrophages. P_4 shifts the proinflammatory activity of DCs toward a more tolerogenic state (Kammerer *et al.* 2000, Liang *et al.* 2006) and promotes a T helper 2 (Th2)-biased profile that is a prerequisite for fetal survival and the maintenance of pregnancy (Piccinni *et al.* 1995, Raghupathy 1997, Szekeres-Bartho *et al.* 2009, Sykes *et al.* 2012). Functional P_4 withdrawal may contribute to the switch from a Th2- to a Th1-dominant phenotype via the actions of P_4 -induced blocking factor in lymphocytes toward the end of pregnancy (Szekeres-Bartho & Chaouat 1990, Szekeres-Bartho *et al.* 1990, Druckmann & Druckmann 2005, Raghupathy *et al.* 2009). Interestingly,

classical nPRs are undetectable or expressed at very low levels in these immune cells. For instance, results from several studies have demonstrated the absence of nPRs in peripheral blood leukocytes, T lymphocytes, immortalized T cells (Jurkat cells), and the murine RAW 264.7 macrophage cell line, as well as in mouse bone marrow-derived macrophages (Mansour *et al.* 1994, Mulac-Jericevic *et al.* 2000, Merlino *et al.* 2007, Dosiou *et al.* 2008, Ndiaye *et al.* 2012). Since then, results from several studies have provided evidence for the activation of mPRs in reproductive tissues and immune cells, and indicated that these mPRs act as G-protein-coupled receptors (GPCRs) in fish oocytes (Zhu *et al.* 2003), human myocytes (Karteris *et al.* 2006), human T lymphocytes, and Jurkat T cells (Dosiou *et al.* 2008, Ndiaye *et al.* 2012). As P_4 elicits a variety of functional effects on immune cell types, even in those lacking nPRs (Dressing *et al.* 2011), the functions of P_4 -mediated mPR activation and signaling are of great interest. As mPRs are putative GPCRs (Dosiou & Giudice 2005, Karteris *et al.* 2006, Thomas *et al.* 2007, Dosiou *et al.* 2008, Dressing *et al.* 2011) and activation of GPCRs leads to downstream activation of cAMP-dependent protein kinase A (PKA), MAPK kinase (MEK), and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways, we investigated the potential involvement of mPR, PKA, MEK1/2, and PI3K/AKT in murine macrophage responses to P_4 .

Materials and methods

Reagents and antibodies

P_4 3-(O-carboxymethyl)oxime: BSA-FITC conjugate (P4BSA; a cell-impermeable form of P_4 ; Gaetjens & Pertschuk 1980), lipopolysaccharide (LPS, cat #L2262, a MEK1/2 activation positive control), forskolin (cat #F3917, a PKA activation positive control), and dihydrochloride hydrate (H89; cat #B1427, a PKA inhibitor) were purchased from Sigma Chemical Co. PD98059 (cat #9900, a MEK1/2 inhibitor) and LY294002 (cat #99901, a PI3K inhibitor) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-IL1B (cat #ab9722) was from Abcam (Cambridge, MA, USA). Antibodies to GAPDH (cat #5174), CREB (cat #9197), phospho-CREB (cat #9198), MEK (cat #9126), phospho-MEK (cat #9154), p38 (cat #9212), and phospho-p38 (cat #9211) were from Cell Signaling Technology. Antibodies to ERK (cat #sc-135900), phospho-ERK (cat #sc-7383), and HRP-conjugated anti-rabbit (cat #sc-2030) and anti-mouse (cat #sc-2031) secondary antibodies were from Santa Cruz Biotechnology.

Cell culture

Mouse RAW 267.4 macrophage cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were maintained from passages 5 to 25 in DMEM (Life Technologies) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin, and streptomycin in a humidified incubator with 5% CO₂ at 37 °C to 80–90% confluence. For treatment, RAW 264.7 cells were cultured in duplicate in 12-well plates (for RNA and signal transduction experiments) or 100 mm Petri-dishes for protein extraction for 24 h before being subjected to the serum-free medium with 1% L-glutamine and 1% penicillin and streptomycin overnight. The cells were then pre-treated with either a PKA inhibitor H89 (3.0×10^{-5} mol/l), a MEK1/2 inhibitor PD98059 (2.0×10^{-5} mol/l), or a PI3K/AKT inhibitor LY294002 (1.0×10^{-5} mol/l) for 1 h in a fresh serum-free medium followed by incubation with control (medium or medium plus vehicle) or P4BSA (1×10^{-7} mol/l) for 2, 15, or 30 min for signal transduction experiments and 4 h for RNA and protein isolation.

Real-time PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies). RNA concentrations were measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) and 500 ng of RNA from each sample was used to generate cDNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). mRNA expression was determined by semiquantitative real-time PCR (RT-PCR) using an ABI StepOnePlus RT-PCR instrument (Applied Biosystems). The reactions were performed in duplicate in 10 µl volumes using 1 µl diluted cDNA ($5 \times$) and 9 µl of a mixture of TaqMan Universal Master Mix Reagents (Roche) and TaqMan Gene Expression Assay for mPR α (*Paqr7*, Mm00510958_m1), tumor necrosis factor (TNF (*Tnf*), Mm00443260_g1), interleukin 1B (*Il1B*), Mm00434228_m1), cyclooxygenase 2 (*COX2* (*Ptgs2*), Mm00478374_m1), iNOS (*Nos2*, Mm00440502_m1), oxytocin receptor (*Oxtr*, Mm01182684_m1), *Creb3* (Mm00457268_m1), and steroid receptor coactivator 2 (*SRC2* (*Ncoa2*), Mm00500749_m1) (Life Technologies). The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The mRNA level of each gene of interest was normalized against GAPDH (*Gapdh*, Mm99999915_g1), as the levels of the reference gene (*Gapdh*) did not differ among treatment groups in our study (data not shown). Gene expression is presented as fold change relative to respective controls and is plotted on a logarithmic scale with base 2.

Western blottings

To extract total cell lysates for detection of protein expression, at the indicated time points cells were washed with $1 \times$ ice-cold PBS twice in dishes and scraped into conical tubes and again washed with PBS. The cells were then solubilized in cold lysis buffer, containing 0.02 mol/l HEPES at pH 7.4, 0.15 mol/l NaCl, 1.0×10^{-9} mol/l EDTA, 1% Nonident P-40 (IGEPAL-CA-630) supplemented with cOmplete ULTRA Protease Inhibitors (Roche Applied Science) on ice for 20 min. The supernatants were collected after centrifugation at 12 000 *g* for 10 min at 4 °C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) and a total of 30 µg of protein lysate was subjected to electrophoresis. For signal transduction studies, cells were washed with $1 \times$ PBS twice and then immediately lysed by adding 200 µl of $2 \times$ SDS sample buffer with 10% 2-mercaptoethanol and kept on ice for 10 min. The suspension was sonicated for 20 s to shear DNA and to reduce the sample viscosity. The samples were heated at 95–100 °C for 5 min. After being cooled on ice for 2 min, the samples were centrifuged for 2 min at 12 000 *g* before 10 µl of supernatant was loaded into each well of 4–12% precast SDS–PAGE gels (Life Technologies) and transferred to PVDF membranes using a semi-dry transfer system (Bio-Rad). The membranes were blocked with 5% nonfat milk (NFM, Bio-Rad) in Tris-buffered saline (TBS, 0.02 mol/l Tris–HCl, 0.137 mol/l NaCl, pH = 7.5) with 0.1% Tween-20 (TBST) for an hour on a shaker at room temperature and then probed with appropriate primary antibodies in 5% NFM in TBST overnight at 4 °C. The membranes were washed four times for 10 min each time with TBST and then incubated with secondary antibodies for 1 h at room temperature. The chemiluminescent signal was detected using ECL Plus from Amersham (GE Healthcare Life Science, Piscataway, NJ, USA) and captured using a STORM phosphor imager (Molecular Dynamics, Piscataway, NJ, USA). The density of each band was quantified with ImageJ (NIH, Bethesda, MD, USA) and normalized to GAPDH or the respective total protein and presented as fold change relative to the control.

Statistical analyses

Kruskal–Wallis ANOVA was used to test overall heterogeneity and differences among groups. If significant differences were identified, *post hoc* tests were performed by multiple comparisons of means, allowing for non-normality in the data. Adjusted *P* values were computed

using a bootstrap re-sampling method with step-down tests. Statistical analysis was performed on the SAS 9.3 (Cary, NC, USA) platform, and *P* values <0.05 were considered statistically significant.

Results

RAW 264.7 cells express mPR α but do not express nPRs

To verify reports that RAW 264.7 cells lack classical nPRs, protein expression of PR-A and PR-B, the two isoforms of PR, was evaluated by western blotting. As shown in Fig. 1, total cell extracts were used to detect protein levels of PRs in several cell lines. While MCF7 (a human breast adenocarcinoma cell line) and T47D (a human ductal breast epithelial tumor cell line) cells are known to highly express nPRs (Horwitz *et al.* 1982, Cho *et al.* 1994), MDA-MB-231 (a human breast adenocarcinoma cell line) cells are known not to express nPRs (Dressing *et al.* 2011). We detected PR-A and PR-B protein in both MCF7 and T47D cells, but neither was present in MDA-MB-231 or RAW 264.7 cells, confirming the previous reports that RAW 264.7 cells lack nPRs.

Although we were able to detect the expression of *Pagr7* mRNA in RAW 264.7 cells (see data below), attempts to detect mPR α protein by western blotting were not fully confirmed, probably due to the nonspecificity of commercially available antibodies (see Supplementary Fig. 1, see section on supplementary data given at the end of this article). Other groups, using a custom-made mPR α antibody generated by Dr Peter Thomas at University of Texas (Thomas 2008), have demonstrated that RAW 264.7 cells express mPR α protein (Dressing *et al.* 2011).

Figure 1

Expression of nuclear progesterone receptors (nPRs) in RAW 264.7 cells, MCF7 cells, MDA-MB-231 cells, and T47D WT cells. To detect the expression of PR-A and PR-B, whole-cell lysates were subjected for western blotting analysis.

P4BSA-induced mPR activation induces MEK-dependent increases in *Il1B* and *Ptgs2* mRNA expression

To study the functions of membrane-bound PRs specifically, we used P4BSA, which is cell-impermeable. Cellular responses to P4BSA were assessed, along with whether these effects are dependent on MEK1/2, signaling components of GPCR pathways. RAW 264.7 murine macrophages were pretreated without or with 2.0×10^{-5} mol/l PD98059 (a specific inhibitor of MEK1/2) for 1 h and then incubated with P4BSA (1.0×10^{-7} mol/l) for 4 h. We found that stimulation of mPRs with P4BSA resulted in significant increases of *Il1B*, *Tnf*, *Ptgs2*, and *Nos2* mRNA transcripts (expressed as fold change relative to untreated controls; Fig. 2a, b, c and d), indicating a pro-inflammatory role for P₄ in RAW 264.7 macrophage cells. PD98059 alone did not change the expression of *Il1B*, *Ptgs2*, and *Nos2* mRNA (Fig. 2a, c and d), but significantly reduced the basal expression of *Tnf* (Fig. 2b). Pre-incubation with PD98059 followed by stimulation with P4BSA significantly reduced P4BSA-induced *Il1B*, *Tnf*, and *Ptgs2* mRNA expression (Fig. 2a, b and c), but not *Nos2* mRNA expression (Fig. 2d). These results indicate that MEK1/2 activity contributes to P4BSA-induced expression of *Il1B* and *Ptgs2* and is partially responsible for P4BSA-induced *Tnf* expression.

P4BSA-induced upregulation of *Il1B* and *Tnf*, but not *Ptgs2* or *Nos2*, is PKA-dependent

As PKA is one of the main kinases in the cAMP-related signal transduction pathway upon GPCR activation, we evaluated the potential involvement of PKA in P4BSA-induced mPR activation. RAW 264.7 cells were pretreated without or with 3.0×10^{-5} mol/l H89 (a PKA inhibitor) for 1 h and then stimulated with P4BSA (1.0×10^{-7} mol/l) for 4 h. Inhibition of the activity of PKA by H89 completely eliminated the P4BSA-induced increase in *Il1B* mRNA expression (Fig. 2e). P4BSA-induced *Tnf* mRNA was significantly reduced, but not completely blocked, by H89 treatment (Fig. 2f). In contrast, inhibition of PKA had no effect on P4BSA-induced *Ptgs2* or *Nos2* expression (Fig. 2g and h), although it increased baseline expression of these mRNAs. These results indicate that PKA is an important regulator of mPR-mediated *Il1B* and *Tnf* and baseline *Ptgs2* and *Nos2* expression.

P4BSA-induced upregulation of *Il1B*, *Tnf*, and *Ptgs2* mRNA is not dependent on PI3K/AKT signaling

RAW 264.7 cells were pretreated without or with 1.0×10^{-5} mol/l LY294002, a specific inhibitor of PI3K/AKT, for

Figure 2

Effects of MEK1/2 inhibition, PKA inhibition, or PI3K/AKT inhibition on P4BSA-induced increases in *Il1B*, *Tnf*, *Ptgs2*, and *Nos2* gene transcripts. RAW 264.7 cells were pretreated in the absence or presence of the MEK1/2 inhibitor PD98059 (2.0×10^{-5} mol/l) (a, b, c and d), the PKA inhibitor H89 (3.0×10^{-5} mol/l) (e, f, g and h) or the PI3K/AKT inhibitor LY294002 (1.0×10^{-5} mol/l) (i, j and k) for 1 h and then stimulated with or without

1.0×10^{-7} mol/l P4BSA for 4 h. *Il1B*, *Tnf*, *Ptgs2*, and *Nos2* mRNA expression was evaluated by RT-PCR and normalized to *Gapdh* mRNA levels. The experiment was repeated six to nine times and the results were expressed as fold changes relative to untreated controls. Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above display significant differences at $P < 0.05$.

1 h followed by stimulation with P4BSA (1.0×10^{-7} mol/l) for 4 h. Pharmacological blockade of the PI3K/AKT pathway did not normalize P4BSA-induced *Il1B*, *Tnf*, and *Ptgs2* mRNA expression (Fig. 2i, j and k), indicating that PI3K/AKT signaling does not mediate the mPR-induced upregulation of these pro-inflammatory mediators.

P4BSA-induced IL1B protein expression is dependent on PKA and MEK1/2

To further characterize the involvement of PKA and MEK1/2 in the P4BSA-induced *Il1B* upregulation seen in RAW 264.7 cells, IL1B protein expression was measured by western blotting analysis using whole-cell extracts after treatments. P4BSA administration at 1.0×10^{-7} mol/l for 4 h caused a significant increase in IL1B protein expression (Fig. 3a, b and c); Fig. 3a is a representative western blot, and Fig. 3b and c contain the relevant densitometric analyses ($n = 4$). Pretreatment with either

3.0×10^{-5} mol/l H89 or 2.0×10^{-5} mol/l PD98059 for 1 h before P4BSA treatment significantly diminished P4BSA-induced IL1B protein expression, confirming the involvement of PKA and MEK1/2 in this pathway.

Potential downstream targets of MEK1/2 involved in mPR signaling

As MEK1/2 are activated by serine phosphorylation and, in turn, phosphorylate downstream kinases to initiate the signal cascade (Wortzel & Seger 2011), we examined the effects of MEK1/2 inhibition on P4BSA-induced phosphorylation of MEK1/2, ERK1/2, and p38. Figures 4a, 5a and 6a are representative blots demonstrating that stimulation with 1.0×10^{-7} mol/l P4BSA for 2 min significantly increases phosphorylation of MEK1/2 at Ser217/221, but not ERK1/2 at Tyr202/204 or p38 at Tyr180/182. Two minutes of stimulation with 10 ng/ml LPS induced phosphorylation of MEK, but not ERK1/2 or

Figure 3

Effect of PKA or MEK1/2 inhibition on P4BSA-induced increases in IL1B protein expression. RAW 264.7 cells were pretreated in the absence or presence of the PKA inhibitor H89 (3.0×10^{-5} mol/l) or the MEK1/2 inhibitor PD98059 (2.0×10^{-5} mol/l) for 1 h and then stimulated with or without 1.0×10^{-7} mol/l P4BSA for 4 h. IL1B expression was examined by western blotting using an anti-IL1B antibody (1:1000 dilution) and normalized to GAPDH levels. The experiment was repeated four times. A representative blot is shown (a). The chemiluminescent signals were quantified using ImageJ (NIH) and the results were expressed as fold changes relative to untreated controls (b and c). Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above display significant differences at $P < 0.05$.

p38 phosphorylation. Stimulation with P4BSA for 15 min did not increase the phosphorylation levels of either MEK1/2 at Ser217/221 (Fig. 4c), ERK1/2 (Fig. 5c) at Tyr202/204 or p38 at Tyr180/182 (Fig. 6c). The observations after 15 min treatment were similar to those after 30 min (data not shown). At all three time points P4BSA treatment also did not alter MEK1/2, ERK1/2, or p38 total protein levels. These experiments were repeated five to ten times and the results of optical densitometry analyses are summarized in Figs 4c, d, 5c, d, and 6c, d. Pretreatment with the MEK1/2 inhibitor 2.0×10^{-5} mol/l PD98059 significantly suppressed basal phosphorylation of MEK (Fig. 4a, b, c and d) and ERK1/2 (Fig. 5a, b, c and d), but it had no effect on p38 (Fig. 6a, b, c and d) phosphorylation in the absence of a stimulus. The increase in phosphorylation of MEK1/2 at Ser217/221 after treatment with P4BSA for 2 min was prevented by pretreatment with PD98059 (Fig. 4a and b). These data together with results displayed in Figs 2 and 3 indicated that P4BSA-induced phosphorylation of MEK1/2 is involved in P4BSA-induced inflammatory responses.

Potential targets of PKA in the mPR-activated pathway

As PKA catalytic subunit α ($C\alpha$) phosphorylates CREB on Ser133 (Gonzalez & Montminy 1989) and CREB is an essential component of the cAMP signaling pathway in

the regulation of *Il1B* and *Ptgs2* transcription (Chandra *et al.* 1995, Ghosh *et al.* 2007), we tested whether the inhibition of PKA affects phosphorylation of CREB in macrophages stimulated with P4BSA. RAW 264.7 cells were pretreated with 3.0×10^{-5} mol/l H89 for 1 h before treatment with 1.0×10^{-7} mol/l P4BSA for 2, 15, or 30 min. The cell lysates were collected and subjected to western blotting to determine the phosphorylation levels of CREB at Ser133 using total CREB as a loading control. P4BSA significantly increased phosphorylation of CREB at Ser133 after stimulation for 2 min (Fig. 7a and b), but not after stimulation for 15 min (Fig. 7c and d). Inhibition of PKA by H89 did not affect phosphorylation of CREB at any time point. The rapid and transient P4BSA-induced CREB phosphorylation was blocked by pretreatment with H89 (Fig. 7a and b). The observations after 15 min of treatment were similar to those after 30 min (data not shown). These results indicate that the signaling of PKA through CREB might be responsible for the effects of P4BSA on the gene expression of the targets studied.

P4BSA did not affect the mRNA levels of the transcription factors *Creb3* and *Ncoa2*

As studies have demonstrated the involvement of CREB in the regulation of transcription of *Il1B* and *Ptgs2* (Chandra

Figure 4

Effects of PD98059 on P4BSA-stimulated activation of MEK1/2 in macrophages. RAW 264.7 cells were pretreated in the absence or presence of the MEK1/2 inhibitor PD98059 (2.0×10^{-5} mol/l) for 1 h and then stimulated with or without 1.0×10^{-7} mol/l P4BSA for 2 min (a and b) or 15 min (c and d). Phosphorylation levels of MEK1/2 were examined by western blotting and normalized to total MEK1/2. Representative blots are shown (a and c). The experiments were repeated six times. The chemiluminescent signals were quantified using ImageJ (NIH) and the results are expressed as fold change relative to untreated controls (b and d). Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above display significant differences at $P < 0.05$.

Figure 5

Effects of PD98059 on ERK1/2 phosphorylation in P4BSA-stimulated macrophages. The treatment was similar to that described in Fig. 4. Phosphorylation levels of ERK1/2 were examined by western blotting and normalized to total ERK1/2. Representative blots are shown (a and c). The experiments were repeated six times. The chemiluminescent signals were quantified using ImageJ (NIH) and the results are expressed as fold changes relative to untreated controls (b and d). Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above displayed significant differences at $P < 0.05$.

et al. 1995, Ghosh *et al.* 2007) and downregulation of SRC2 (*Ncoa2*) has been implicated in the effects of mPRs activation in human myometrial cells (Karteris *et al.* 2006), we determined the effects of activation of mPR via P4BSA treatment on expression of *Creb3* and *Ncoa2* in RAW 264.7 cells. As shown in Fig. 8a and c, pretreatment with 2.0×10^{-5} mol/l PD98059 did not affect the basal levels of *Creb3* and *Ncoa2* mRNA expression. Subsequent stimulation with 1.0×10^{-7} mol/l P4BSA also did not affect the transcriptional levels of both genes. Pretreatment with 3.0×10^{-5} mol/l H89 did not change the expression levels of *Creb3* (Fig. 8b), but did significantly decrease the mRNA level of *Ncoa2* (Fig. 8d). There was no additional downregulation of *Ncoa2* transcription when PKA inhibition was followed by P4BSA stimulation (Fig. 8d). These results indicate that H89 regulates basal mRNA level of *Ncoa2*.

P4BSA downregulates mPRa (*Paqr7*) and *Oxtr* mRNA expression in RAW 264.7 cells

As ligand-induced downregulation of receptor mRNA levels is a mechanism for diminishing receptor signaling (Hadcock & Malbon 1988), and OXTR is reportedly downregulated by P_4 through miR-200 (Renthal *et al.* 2010), we explored the mechanisms through which P_4 might regulate cellular responses in murine macrophages. Expression of *Paqr7* and *Oxtr* was evaluated by RT-PCR

after the administration of the treatments described earlier. We observed that P4BSA treatment significantly reduced the mRNA expression levels of *Paqr7* (Fig. 8e and f) and *Oxtr* (Fig. 8g and h). Interestingly, MEK1/2 inhibition (Fig. 8e) did not affect either baseline *Paqr7* or P4BSA-induced downregulation of *Paqr7*, while PKA blockade (Fig. 8f) resulted in significant downregulation of *Paqr7* expression at baseline. Pretreatment with H89 did not affect P4BSA's ability to downregulate *Paqr7*. In comparison, inhibition of either MEK or PKA alone reduced the baseline mRNA levels of *Oxtr* (Fig. 8g and h). *Oxtr* downregulation by P4BSA stimulation was not affected by pretreatment with the MEK inhibitor or PKA inhibitor. These results indicate that P4BSA induced downregulation of expression of both *Paqr7* and *Oxtr*. PKA regulates steady-state expression of *Paqr7* whereas both PKA and MEK regulate steady-state *Oxtr* expression in murine macrophages.

Discussion

During pregnancy, decidual macrophages exhibit an immunosuppressive phenotype that is required for maintaining immunological homeostasis and supporting immune tolerance of the fetus. As the placenta develops, macrophages are recruited around spiral arteries to support vascular remodeling by producing pro-angiogenic factors. At the end of the pregnancy, classically activated

Figure 6

Effects of PD98059 on p38 phosphorylation in P4BSA-stimulated macrophages. The treatment was similar to that described in Fig. 4. Phosphorylation levels of p38 were examined by western blotting and normalized to total p38. Representative blots are shown (a and c). The experiments were repeated four to six times. The chemiluminescent signals were quantified using ImageJ (NIH) and the results are expressed as fold changes relative to untreated controls (b and d). Kruskal–Wallis ANOVA with *post hoc* test was performed and no significant differences were detected between groups.

Figure 7

Effect of PKA inhibition on CREB phosphorylation in P4BSA-activated macrophages. RAW 264.7 cells were pretreated in the absence or presence of the PKA inhibitor H89 (3.0×10^{-5} mol/l) for 1 h and then stimulated with or without 1.0×10^{-7} mol/l P4BSA for 2 or 15 min. Phosphorylation levels of CREB were examined by western blotting using anti-phospho-CREB and normalized to total CREB levels. Representative blots are shown (a and c). These experiments were repeated five to six times. The chemiluminescent signals were quantified using ImageJ (NIH) and the results were expressed as fold changes relative to untreated controls (b and d). Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above display significant differences at $P < 0.05$.

macrophages engage in the cervical remodeling process toward the onset of labor (Lee *et al.* 2012). Therefore, macrophages, among other immune cells, are pivotal for the maintenance of pregnancy and initiation of labor. P_4 has both immunosuppressive and immunostimulatory effects on macrophages (Miller & Hunt 1996), demonstrating the plasticity and versatility of these cells, depending on the biological environment. Despite the reported various effects of P_4 on macrophages, most studies have failed to detect the expression of the so-called classical nPRs in macrophages (Miller & Hunt 1996, Dressing *et al.* 2011). On the other hand, recent advances in this area have revealed the existence of mPRs in macrophages (Dressing *et al.* 2011). This indicates that they might be responsible for P_4 's action in these immune cells.

In this study, we demonstrate that activation of a putative family of mPRs by a cell-impermeable form of P_4 resulted in a pro-inflammatory profile in murine macrophages. This cell-impermeable form of P_4 induced robust increases in the expression of mRNA for pro-inflammatory markers such as *Il1B*, *Tnf*, *Ptgs2*, and *Nos2*. Both PKA and MEK1/2 are involved in the regulation of the mRNA and protein expression of *Il1B*. MEK1/2 also regulates transcription of *Tnf* and *Ptgs2*. P4BSA-stimulated production of *Tnf* mRNA is also mediated by PKA. Furthermore, P_4 regulates mRNA expression of one of its own receptors, *Paqr7*, and of *Oxtr*, thus providing potential mechanisms by which P_4 affects cellular events in macrophages.

The roles of membrane PRs in pregnancy and labor are diverse and complex. These roles are almost certainly tissue-, cell type-, and gestational age-specific. Pro-inflammatory actions of P_4 have been shown in other cell types (i.e. other than the macrophages we studied). For example, exposure to P_4 activates a wide array of genes involved in several biological processes, including cell adhesion, cell survival, and inflammation, in the mammary gland (Santos *et al.* 2009). Feng *et al.* (2014) demonstrated diminished progesterone receptor membrane component 1 (PGRMC1) at the rupture site among preterm prelabor rupture of membranes (PPROM) subjects compared with preterm nonlabor or term-nonlabor subjects. They deduced from the data that PGRMC1 functions to maintain fetal membrane integrity on the basis of results from the previous studies, which indicated that nPR expression is negative in the chorion and amnion. Our results were in agreement with the results from previous studies (Mackler *et al.* 1999, Thomson *et al.* 1999, Shynlova *et al.* 2013), which demonstrate that infiltration of macrophages to decidua and cervix and the inflammatory responses associated with the infiltration might be the keys for the initiation of labor. The uterus also undergoes a switch from quiescence to contraction near term due to a proposed functional withdrawal of P_4 (Tan *et al.* 2012). In this model, classical anti-inflammatory nPR-B is downregulated and PR-A (pro-inflammatory) is increased at the onset of labor. The net results of this altered expression of PR-A/PR-B in the uterus are thought to increase the expression of pro-inflammatory factors and contraction-associated proteins to promote labor. Therefore our results on macrophages stimulated with P4BSA are in agreement with the overall concept that labor is an inflammatory event in which macrophages play an active role. Furthermore, we provide evidence that not the classical nPRs, but mPRs are responsible for the action of P_4 on macrophages.

The actions of mPRs activated specifically by P4BSA have been described in recent years. Blackmore and colleagues demonstrated that P_4 covalently linked to BSA is capable of eliciting Ca^{2+} influx in human sperm. The conclusion drawn from the study is that there is a receptor that is most probably present in the plasma membrane of the spermatozoa and that the P_4 -binding site resides in the extracellular portion of the receptor (Blackmore *et al.* 1991). In a cell model system of human parafollicular cells, P4BSA increases the release of calcitonin through activation of adenylyl cyclase and PKA (Lu & Tsai 2007). Flock *et al.* (2013) reported that activation of mPRs by P4BSA is responsible for the increased secretion of

Figure 8

Effects of MEK1/2 inhibition or PKA inhibition on *Creb3*, *Ncoa2*, *Paqr7*, and *Oxt* gene expression in P4BSA-treated macrophages. RAW 264.7 cells were pretreated in the absence or presence of 2.0×10^{-5} mol/l MEK1/2 inhibitor PD98059 (a, c, e and g) or 3.0×10^{-5} mol/l PKA inhibitor H89 (b, d, f and h) for 1 h and then stimulated with or without 1.0×10^{-7} mol/l P4BSA for 4 h.

Gene transcripts were evaluated by RT-PCR and normalized to *Gapdh* mRNA levels. The experiment was repeated nine times and the results were expressed as fold changes relative to untreated controls. Kruskal–Wallis ANOVA with *post hoc* test was performed and groups with different letters above display significant differences at $P < 0.05$.

glucagon-like peptide 1 in enteroendocrine cells *in vitro* and improvement in glucose tolerance *in vivo*. The results from our study, using a murine macrophage cell line expressing at least one of the mPRs, mPR α , but not expressing classical nPRs, are consistent with the results of the above-mentioned studies and indicate that the site of P₄'s action in macrophages is the plasma membrane.

Several lines of evidence indicate that mPRs have the functional characteristics of GPCRs in fish oocytes (Zhu *et al.* 2003), MDA-MB-231 (human breast adenocarcinoma) cells transfected with mPR α (Zhu *et al.* 2003, Kelder *et al.* 2010), human myocytes (Sulke *et al.* 1985), human T lymphocytes, and Jurkat T cells (Dosiou *et al.* 2008, Ndiaye *et al.* 2012). However, others question the subcellular localization and signal coupling of this family of receptors (Fernandes *et al.* 2008, Smith *et al.* 2008). To date, the seven-transmembrane-domain topology, a hallmark of the GPCR, has never been conclusively demonstrated in mPRs, but has been deduced from the amino acid sequence of mPRs. Furthermore, there is a large deviation from canonical G-protein-induced signaling pathways downstream of activation of mPRs. Although early reports indicated that P₄ can promote phosphorylation of ERK1/2 in fish oocytes and MDA-MB-231 cells transfected with mPR α (Zhu *et al.* 2003), in human myometrial cells both P₄ and P4BSA did not increase the levels of phosphorylation of ERK1/2 (Karteris *et al.* 2006). P4BSA was also reported to induce phosphorylation of p38 MAPK in human myometrial cells (Karteris *et al.*

2006), but when Krietsch *et al.* (2006) stably expressed human, sea trout, and Fugu mPR α in HEK293 and MDA-MB-231 cells, administration of P₄ did not decrease cAMP levels or increase levels of phosphorylation of ERK and p38. In this study, P4BSA induces a rapid and transient phosphorylation of MEK1/2 and CREB, but not ERK and p38, demonstrating that MEK1/2 and CREB/PKA are two of the signaling components involved in the pathways triggered by the activation of mPRs in macrophages.

Downregulation of GPCRs through ligand binding (a form of negative feedback) is one of the distinctive characteristics of this large family of receptors (Haddock & Malbon 1988). The diminished steady-state level of GPCR expression following prolonged treatment with an agonist is the combined result of increased degradation and decreased synthesis as a consequence of a decrease in the level of its mRNA (Drake *et al.* 2006). Our results indicate that reduced expression of *Paqr7* at 4 h may be one of the adaptive mechanisms that function to desensitize macrophages to the inflammatory environment, representing a potential negative feedback mechanism for immune cells to withstand further inflammatory insults. Further study using prolonged stimulation with P4BSA is warranted to test whether the downregulation of *Paqr7* parallels diminishing pro-inflammatory responses in these macrophages and whether this downregulation is in concert with a global functional withdrawal of P₄ in myometrium before labor.

Oxytocin induces uterine contraction in labor (Fuchs *et al.* 1984), and increased expression of *Oxtr* mRNA in the uterus occurs before delivery in many species (Murata *et al.* 2000). P₄ blocks both preterm labor and the associated increase in myometrial expression of *Oxtr* in ovariectomized rats (Ou *et al.* 1998), indicating a role for P₄ in the regulation of the expression of *Oxtr* mRNA. In fact, Renthal *et al.* (2010) demonstrated that P₄, through binding to its nPRs, directly upregulated zinc finger E-box-binding homebox protein 1 and suppression of miR-200b/429, which in turn resulted in the downregulation of contraction-associated genes such as connexin 43 and *Oxtr* in immortalized human myocytes. Other studies also revealed that P₄ suppressed expression of *Oxtr* mRNA in endometrial epithelial cells (Kombe *et al.* 2003) and bovine lymphocytes (Ndiaye *et al.* 2008). RAW 264.7 macrophages express OXTR (Szeto *et al.* 2008) and in our study we demonstrated that extracellular P₄ downregulates the expression of *Oxtr* in these murine macrophages. Further studies are needed to examine the physiological significance of hormonal regulation of expression of *Oxtr* in macrophages within the context of reproductive systems.

In summary, a non-cell-permeable form of P₄ elicits pro-inflammatory responses and downregulates gene transcripts of *Pagr7* and *Oxtr* in macrophages, most probably through binding to mPRs in a process dependent on PKA and MEK1/2. Changes in expression of mPR or of its activation by P₄ may represent a novel pathway that contributes to the regulation of inflammatory responses in macrophages and overall regulation of parturition by P₄.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0470>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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