Statins prevent cervical remodeling, myometrial contractions and preterm labor through a mechanism that involves heme oxygenase-1 and complement inhibition

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Abstract: Preterm birth (PTB) is a major public health problem, with a global prevalence of 9.6% and over a million annual neonatal deaths. In a mouse model of preterm labor (PTL) induced by intravaginal administration of a subclinical dose of lipopolysaccharide (LPS), we previously demonstrated that LPS ascends to the cervix, inducing complement activation, cervical remodeling and PTL. Here we show that complement activation also plays a role in myometrial contractions during PTL in this model. Increased levels of C5a were detected in the myometrium of LPS-treated mice but not in age-matched control or term myometrium. Human and mouse myometrium incubated with C5a showed increased frequency of contractions and expression of connexin 43, suggesting that C5a is an uterotonic molecule. Statins, which showed beneficial effects in preventing complement-mediated pregnancy complications, prevented cervical remodeling, myometrial contractions and PTL in the LPS model. The protective effects of statins in PTL were associated with increased synthesis, expression and activity of heme oxygenase (HO-1) in myometrium and cervix. Coadministration of HO-1 inhibitor tin-protoporphyrin-IX with pravastatin abrogated the protective effects of pravastatin on cervical remodeling and myometrial contractions leading to PTL. In addition, pravastatin inhibited complement activation in the cervix by increasing the synthesis and expression of complement inhibitor decay-accelerating factor. This study in mice suggests that statins might be useful to prevent PTL in humans. Clinical trials in humans are needed and if these results are confirmed, they may form the basis for a new clinical approach to prevent PTB.

Key words: cervix / complement / myometrium / preterm labor / statins

Introduction

Preterm birth (PTB) is a major public health problem, with a global prevalence of 9.6% and over a million annual neonatal deaths (Beck et al., 2010). PTB is a syndrome with many causes and phenotypes (Villar et al., 2012). However, in most clinical situations, PTB follows spontaneous preterm labor (PTL), without a known underlying maternal, fetal or placental condition. Unfortunately, in the majority of cases, there are few applicable preventative and therapeutic solutions for spontaneous PTL. While spontaneous PTL culminates in a common pathway with term parturition, characterized by cervical dilation and myometrial contractions, the initiating factors are still unknown. The identification of mechanisms underlying PTL holds the promise of new, safer and better treatments. Numerous lines of evidence support a role for a subclinical genital tract infection in the etiology of spontaneous PTL (Gibbs et al., 1992; Andrews et al., 1995). Despite the growing association between infection/inflammation and activation of innate immunity with PTL and PTB (Andrews et al., 1995; Goldenberg et al., 2000; Romero et al., 2002), the mediators, receptors and cellular participants in PTL remain unclear. Activation of the complement system plays a crucial role in the pathogenesis of infection and inflammation. In particular, complement activation product C5a is a potent proinflammatory mediator. Using a mouse model of PTL that resembles the most common clinical scenario of subclinical vaginal infection (Gonzalez et al., 2011a, b), we
demonstrated that C5a plays a critical role in the cervical remodeling process that ripens and dilates the cervix in PTL but not in term labor in mice (Gonzalez et al., 2011a, b).

In this study we hypothesized that C5a—generated by a localized infection/inflammation in the cervix—would also play a role in the premature myometrial contractions that lead to PTB. We also hypothesized that statins, which prevented complement-mediated pregnancy complications in several animal models (Redecha et al., 2008; Kumasawa et al., 2011; Singh et al., 2011), would also prevent PTB. A role for HO-1/carbon monoxide (CO) in the maintenance of uterine quiescence and its potential use as a tocolytic agent has been suggested (Acevedo and Ahmed, 1998; Smith et al., 1999; Bainbridge and Smith, 2005). Given that statins have been shown to modulate HO-1 synthesis and activity (Chen et al., 2006), we hypothesized that statins might prevent PTL and PTB by increasing HO-1 activity.

**Materials and Methods**

**Animals**

All studies involving animals were subjected to the University’s ethical review process and authorized by the Home Office. C57BL/6 mice from commercial vendors were used in all experiments. A mouse model of PTB, which resembles most clinical scenarios, was used (Gonzalez et al., 2011a, b). In this model, mice were treated with lipopolysaccharide (LPS) [E. coli serotype 055:B5 Sigma-Aldrich, St Louis, MO, USA (250 μg/mouse, intravaginally)] on Day 15 of pregnancy (Gonzalez et al., 2011a, b). We previously demonstrated that LPS ascends to the cervix, inducing complement activation and cervical remodeling (Gonzalez et al., 2011a, b; Pedroni et al., 2014). A group of mice received pravastatin (10 μg/mouse, i.p.) or simvastatin (20 μg/mouse, i.p.) 24 h before and 2 h after LPS intravaginal administration. The dose and administration schedule were selected from prior studies in mice showing protective effects of statins in inflammation-related pregnancy complications (Redecha et al., 2008; Kumasawa et al., 2011; Singh et al., 2011; Pedroni et al., 2014). Another group of mice only received pravastatin or simvastatin. LPS-treated mice gave birth within 12–24 h after LPS administration. Delivery was considered preterm if it occurred before gestational Day 17. Pregnant mice treated with LPS were euthanized during delivery (intrapartum, by direct observation after the passage of 1 or 2 pups). Age-matched untreated control mice and mice treated with statins and LPS plus LPS were sacrificed at the same time points. Cervical tissue and myometrial tissue were collected in all groups.

Another group of mice was treated with HO-1 inhibitor SnPP-IX (6 mg/kg of body weight, intraperitoneally on Days 14 and 15, 8 h before the administration of pravastatin (10 μg/mouse, i.p.). On Day 16 the mice were sacrificed, the myometrium harvested and contractile response to C5a was studied in the organ bath system.

**Simvastatin, pravastatin and Tin-protoporphyrin IX solutions**

Simvastatin (Sigma-Aldrich, St Louis, MO, USA) was prepared as a 4-mg/ml stock. Briefly, 4 mg of simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH and incubated at 50 °C for 2 h, then the pH was adjusted to 7, and the total volume was corrected to 1 ml. The stock solution was diluted to the appropriate concentration in sterile phosphate-buffered saline (PBS). Pravastatin (Sigma Aldrich, St Louis, MO, USA) was directly dissolved in sterile PBS. Tin-protoporphyrin-IX (SnPP-IX; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), an inhibitor of HO-1, was dissolved in 8.4% sodium-bicarbonate and PBS to achieve a final concentration of 5 μmol/ml. The solution was stored at 4 °C in the dark and used within the next hour.

**Complement activation products**

C5adesArg in the myometrium was measured by sandwich ELISA as previously described (Gonzalez et al., 2011a, b) using rat anti-mouse C5a and biotin rat anti-mouse C5a (BD Biosciences, Oxford, UK). Myometrium tissue was homogenized in ice-cold PBS (0.02 mol/l, pH 7.0) using a glass homogenizer. The resulting suspension was then subjected to two freeze–thaw cycles to break the cell membranes. Homogenates were then centrifuged for 5 min at 5000 g and the supernatants were assayed immediately or stored at −20 °C.

**Organ bath studies**

**Human tissue**

The myometrium was obtained through the Edinburgh Reproductive Tissue Bio Bank, with informed written consent, from women undergoing elective Cesarean section at term, not in labor (mean gestational age, 39 + 3 weeks; mean maternal age, 33; range 20–43 years, n = 15). Indications for Cesarean section included maternal request, previous Cesarean section or breech presentation. None of the women included in this study presented signs of underlying disease (hypertension, diabetes, pre-eclampsia, intrapartum growth retardation, etc.). Biopsies were obtained from the midline of the upper lip of the uterine incision at Cesarean section. The tissue specimens were immediately placed in a buffered Krebs solution stored at 4 °C and used within 12 h of collection. In the laboratory, strips of myometrium 1.5 cm long, 0.2 cm wide and 0.2 cm deep were cut. Some strips were incubated overnight in Krebs buffer containing pravastatin (20 μg/ml) or simvastatin (40 μg/ml) before contractility studies were performed. Control tissue was incubated in Krebs buffer only.

**Mouse tissue**

Uterine horns from pregnant mice untreated and treated with pravastatin or simvastatin were isolated 18 h after treatment. Excessive fat and connective tissue were removed. After removing the endometrium, longitudinal pieces (1.0 cm long, 0.2 cm wide and 0.2 cm deep) of uterine horns were cut.

**Contractile analysis**

Each mouse and human myometrium strip was mounted vertically into a separate 10-ml organ bath containing oxygenated Krebs solution, gassed with 95% O2/5% CO2 solution at 37 °C under isometric conditions with 20-mN resting tension. After a stabilization period (90 min to 3 h) spontaneous contractions were observed. Once the amplitude and frequency became stable (showing <5% variation between contractions), the relevant drugs for each experiment were added. The response to drug was defined as the effect on contraction at the time point of interest and recorded in milli-Newtons. Frequency (contractions/hour) was calculated by measuring the interval between the peaks of two consecutive contractions occurring at the time point of interest. Contraction frequency was recorded after the tissue present a regular rhythmic activity, immediately prior to the addition of the relevant drug (time 0) and immediately after for a 60-min period.

To study the role of C5a on myometrial contractility, recombinant active mouse/human C5a (100 nM, Sigma Chemicals, St Louis, MO, USA) was added to the Krebs buffer. To confirm that C5a-induced myometrial contractions via C5a–C5a receptor (C5aR) interaction, C5aR antagonist peptide F[OPdChaWR] (Phe-[Orn-Pro-d-cyclohexylalanine-Trp-Arg]) (1 μM) was used (Finch et al., 1999).

To study the effects of CO on myometrial contractions, CO-releasing molecule tricarbonylchloro(glycinato)ruthenium (II) (CORM-2, Sigma-Aldrich, St Louis, MO, USA; 30 μM) was used. Tissue responsiveness was assessed
before and after all treatments with the addition of KCl (45 mM) that produces regular phasic and tonic myometrial contractions.

Contractility was recorded via a tension transducer (FT03, Grass Technologies, Slough, UK) attached to one end of the strip, which was connected to a data acquisition system (ML186/c-v, AD Instruments, Oxford, UK). Data were analysed using LabChart 7 data acquisition software (AD Instruments Ltd, Oxford, UK). Changes in contractility were expressed as % increase in frequency compared with the frequency of spontaneous contractions.

**Immunohistochemistry**

Mouse and human myometrial tissue and mouse cervical tissue were frozen in O.C.T. compound, and cut into 10-μm sections. A list of the antibodies and their respective dilutions used for IHC is shown in Table I.

**In situ zymography**

Metalloproteinases (MMP)-2 and MMP-9 activity against collagen I, abundant in the cervix, was measured by in situ zymography as previously described (Gonzalez et al., 2011a, b). Briefly, 10-μm-cervix sections were washed in PBS and then incubated for 4 h with DQ-collagen I (Invitrogen, Carlsbad, CA, USA). The enzyme-driven hydrolysis of this substrate results in an increase in fluorescence signal. Increased fluorescence indicates increased collagen I degradation by MMPs. In parallel, control sections were preincubated with buffer containing the MMP inhibitor EDTA to indicate the contribution of MMPs. The reaction was stopped by a 10-min incubation in 4% paraformaldehyde−PBS. Finally, mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA, USA) was applied. Sections were observed under a fluorescence microscope.

**HO-1 activity assay**

HO-1 activity was determined in the myometrium from statins-treated mice and in human myometrium incubated with statins as described previously (Huber et al., 2009). Briefly, microsomes from harvested myometrium were added to a reaction mixture containing nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase, mouse liver as the source of biliverdin reductase and the substrate hemin. The reaction mixture was incubated in the dark at 37 °C for 1 h and terminated by the addition of 1 ml of chloroform. The amount of extracted bilirubin in the chloroform layer was determined by measuring the difference in absorbance of 1 ml of chloroform. The amount of extracted bilirubin in the chloroform was determined by measuring the difference in absorbance of 1 ml of chloroform.

**Immunoblotting**

Mouse and human myometrium tissue were homogenized in ice-cold lysis buffer (50 mmol/l Tris, pH 7.4, 0.27 mol/l sucrose, 1 mmol/l sodium orthovanadate, pH 10, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l sodium β-glycerophosphate, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 1% [v/v] Triton X-100, 0.1% [v/v] 2-mercaptoethanol, one tablet of complete TM protease inhibitor (Roche, Burgess Hill, UK) and 50 μg of protein were run on 4−12% Bis-Tris gels for western blotting. Protein signals were visualized using anti-HO-1 antibody generated in rabbit (Abcam, Cambridge, UK) and enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) by exposure to Amersham Hyperfilm ECL film (GE Healthcare Life Sciences, Buckinghamshire, UK).

**Quantitative qRT−PCR**

To determine whether statins differentially regulated the expression of HO-1 in human and mouse myometrium, and complement delay-accelerating factor (DAF) and HO-1 in mouse cervix, qRT−PCR was performed. RNA was prepared using Qiagen RNeasy Mini kits (Qiagen, Crawley, West Sussex, UK) and its integrity was calculated using RNA 6000 Nano chip on Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). By using cDNA Synthesis Kit (4368813, Applied Biosystems, Carlsbad, CA, USA), 600 ng of total RNA was reverse transcribed. Expression of mRNA was quantified by ABI 7900HT (Applied Biosystems, Hill, UK) with inventoried probes and primers sets (Applied Biosystems, Hill, UK), using standard curve methods normalizing data against actin B mRNA levels.

**Statistical analysis**

Data are expressed as mean ± SD. Statistical differences between groups were determined using one-way ANOVA with subsequent two-tailed Student’s t-test.

**Results**

**Complement activation in the myometrium during PTL**

To test our hypothesis that complement activation plays a role not only in cervical remodeling but also in myometrial contractility in PTL, we measured complement activation split product C5a concentration in myometrium harvested intrapartum during PTL and term labor in mice. Myometrial samples from age-matched controls were also studied. Increased levels of C5a were detected in the myometrium of LPS-treated mice (PTL mice) (Table II) but not in age-matched control or term myometrium (Table II). Increased expression of contraction-associated protein connexin 43 (Cx43) was also observed in the myometrium collected intrapartum from PTL mice and mice that gave birth at term.

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**Table I  List of antibodies used for IHC.**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>Manufactured by</th>
<th>Dilution</th>
<th>Human myo</th>
<th>Mouse myo</th>
<th>Mouse cervix</th>
<th>Developed with</th>
</tr>
</thead>
<tbody>
<tr>
<td>ab11370</td>
<td>Connexin 43/GJA1</td>
<td>Abcam</td>
<td>1/800</td>
<td>x</td>
<td>x</td>
<td></td>
<td>Anti-rabbit HRP/DAB</td>
</tr>
<tr>
<td>mAb SS/1</td>
<td>Human C5aR</td>
<td>Hycult biotech</td>
<td>1/20</td>
<td>x</td>
<td></td>
<td></td>
<td>Anti-mouse HRP/DAB</td>
</tr>
<tr>
<td>mAb 10/92</td>
<td>Mouse C5aR</td>
<td>Hycult biotech</td>
<td>1/50</td>
<td>x</td>
<td></td>
<td></td>
<td>Anti-rat HRP/DAB</td>
</tr>
<tr>
<td>Ab25377</td>
<td>Anti-Ly6G on mouse neutrophils</td>
<td>Abcam</td>
<td>1/300</td>
<td>x</td>
<td></td>
<td></td>
<td>Anti-rat HRP/DAB</td>
</tr>
<tr>
<td>Cl-A3-1</td>
<td>F4/80 on mouse macrophages</td>
<td>Novus Biological</td>
<td>1/100</td>
<td></td>
<td>x</td>
<td></td>
<td>Anti-rat HRP/DAB</td>
</tr>
<tr>
<td>ab13423</td>
<td>HO-1</td>
<td>Abcam</td>
<td>1/200</td>
<td>x</td>
<td></td>
<td></td>
<td>Anti-rabbit HRP/DAB</td>
</tr>
<tr>
<td>mAb 3D5</td>
<td>DAF (CD55)</td>
<td>Hycult biotech</td>
<td>1/50</td>
<td>x</td>
<td></td>
<td></td>
<td>Anti-rat HRP/DAB</td>
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<tr>
<td>R1038</td>
<td>Collagen I</td>
<td>Acris GmbH</td>
<td>1/100</td>
<td></td>
<td></td>
<td></td>
<td>Anti-rabbit HRP/DAB</td>
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</table>
Increased levels of Cx43 detected in intrapartum term samples were not associated with increased C5a levels (Table I).

We then hypothesized that C5a could be a uterotonic molecule in PTL. Following this hypothesis, we investigated whether C5a can directly affect myometrium contractility. Human myometrium, collected at term from non-laboring women, incubated with C5a showed increased Cx43 expression (Fig. 1B), suggesting that C5a might trigger myometrial contractility. We then looked for the presence of C5aR on mouse and human myometrial tissue. Positive staining for C5aR was observed both in the mouse and human non-laboring myometrium (Fig. 2A). Because macrophages and neutrophils express C5aR and can release uterotonic molecules and thus affect myometrial contractility, we looked for the presence of these inflammatory cells in the myometrium during PTL in mice (Fig. 2B). Immunohistochemical (IHC) studies showed very few neutrophils and macrophages infiltrating the myometrium during PTL compared with term parturition, suggesting that it is unlikely that myometrial contractions during PTL are mediated by inflammatory cells. These studies suggest that myometrial contractions during PTL might result from the direct effect of C5a with its receptor on myometrial fibers.

To demonstrate that C5a affects myometrial contractility, we performed experiments using the organ bath system. In these studies, addition of C5a increased the frequency of contractions in both human (Fig. 2C and D) and mouse (Fig. 2C) term non-laboring myometrium. Blockade of C5aR with antagonist peptide (C5aR-AP)—prior to the addition of C5a—prevented the increase in frequency of contractions induced by C5a in mouse and human myometrium (Fig. 2C and D).

Statins prevent PTB in mice

Given that statins have beneficial effects in preventing pregnancy complications that are mediated by inflammation and in particular by complement activation (Redecha et al., 2008; Kumasawa et al., 2011; Singh et al., 2011), we investigated if statins prevent PTL and PTB in the LPS model. Mice treated with LPS that received simvastatin (S) or pravastatin

<table>
<thead>
<tr>
<th>Table II C5a levels (measured as C5adesarg) in myometrium homogenates in LPS-treated mice, age-matched controls and mice that delivered at term</th>
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<tbody>
<tr>
<td>C5adesArg Mouse Myometrium (ng/mg)</td>
</tr>
<tr>
<td>LPS (PTL) (n = 7)</td>
</tr>
<tr>
<td>934 ± 256*</td>
</tr>
</tbody>
</table>

*Different from control, P < 0.04.

Figure 1 Expression of Connexin 43 (Cx43) in mouse and human myometrium. (A) Immunohistochemical detection of Cx43 in LPS-treated mice, age-matched controls and mice that delivered at term. (B) Cx43 staining in human myometrium (sample from a non-laboring woman at term) incubated with and without C5a. Microphotographs (A and B) represent one of four to five similar experiments.
Statins prevent cervical remodeling in mice
Based on the findings that statins prevent PTB, we hypothesized that statins inhibit cervical remodeling and myometrial contractions. As we previously described (Gonzalez et al., 2011a,b) decreased collagen type I content and increased MMPs activity against collagen I—signs of cervical remodeling—were observed in the cervix of PTL mice (Fig. 3A). Mice pretreated with statins showed a significant reduction in MMPs activity and an increase in collagen I content in the cervix comparable to the age-matched control group (Fig. 3A), suggesting that LPS-induced cervical remodeling does not occur in these mice.

Statins prevent myometrial contractility in mice and human tissue
To study the effects of statins on C5a-induced contractility, human myometrial tissue was incubated overnight with simvastatin or pravastatin

Table III Pravastatin and simvastatin prevent PTB.

<table>
<thead>
<tr>
<th></th>
<th>Delivery (days)</th>
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<tbody>
<tr>
<td>Control (n = 5)</td>
<td>19.9 ± 0.5</td>
</tr>
<tr>
<td>LPS (n = 7)</td>
<td>15.8 ± 0.3*</td>
</tr>
<tr>
<td>P + LPS (n = 6)</td>
<td>19.8 ± 0.8</td>
</tr>
<tr>
<td>S + LPS (n = 6)</td>
<td>19.9 ± 0.6</td>
</tr>
</tbody>
</table>

*Mice pretreated with pravastatin (S) or simvastatin (S) do not deliver preterm in response to LPS. P + LPS and S + LPS mice deliver at term comparable to control untreated mice. Values are expressed as mean ± SD.

*Different from control, P < 0.01.

(P) 24 h prior to intravaginal administration of LPS did not deliver preterm (Table III).
and then stimulated with C5a in the organ bath system. The control group was incubated with only media. Myometrial tissue from mice treated with simvastatin or pravastatin were also stimulated with C5a in the organ bath system. Both, in vitro incubation of human tissue with statins and in vivo administration of statins to mice diminished the frequency of C5a-induced contractions (Fig. 3B). Figure 3C illustrates tracings for myometrial contractions in human myometrium preincubated with statins and stimulated with C5a. Incubation of human myometrium with statins or treatment of mice with statins also diminished the frequency of myometrial spontaneous contractions [contractions/10 min period: mouse (n = 5/group), untreated: 6.2 ± 1.6, pravastatin: 2.9 ± 0.6*, simvastatin: 2.5 ± 0.6; human (n = 5/group): control: 7.5 ± 1.2; simvastatin: 3.3 ± 1.4*, pravastatin: 2.3 ± 1.8*; *different from control P < 0.05].

**Role of HO-1 in the protective effects of statins in PTL**

It has been suggested that statins activate the HO-1 enzyme that generates CO by degradation of heme and that the HO-1/CO pathway is associated with decreased myometrial contractility (Acevedo and Ahmed, 1998; Bainbridge and Smith, 2005). Thus, we investigated if HO-1 activity was involved in the inhibitory effects of statins on myometrial contractility in mice. Increased expression of HO-1 was detected by IHC in myometrium from statins-treated mice compared with control mice (Fig. 4A). Increased HO-1 expression was also observed in human myometrium incubated with statins compared with untreated tissue (Fig. 4A). Western blot studies confirmed the results obtained by IHC. Increased HO-1 expression was observed in human myometrium incubated with P compared with respective controls (Fig. 4B).
In addition, significant increase in HO-1 gene expression was observed in human myometrium incubated with pravastatin and in the myometrium of mice treated with pravastatin (Fig. 4C). HO-1 activity was also increased in myometrium from statins-treated mice and human myometrium incubated with statins (Fig. 4D).

Based on the findings that statins increase HO-1 synthesis, expression and activity, and that HO-1 generates CO that can affect myometrial contractility, we then investigated the effects of CO on C5a-induced myometrial contractility in the organ bath system. As source of CO we used CO-releasing molecule CORM-2. CORM-2 significantly diminished C5a-induced contractility (Fig. 4E). In addition, CORM-2 completely inhibited spontaneous contractility in mouse and human myometrium in the organ bath (Fig. 4F), whereas the contractile response remained constant in control myometrial strips incubated with vehicle.
[dimethylsulphoxide (DMSO); contractions/10 min period: mouse (n = 4), before DMSO: 5.1 ± 0.7 after DMSO: 5.4 ± 0.6; human (n = 5): before DMSO: 7.2 ± 1 after DMSO: 6.9 ± 0.8].

To confirm that the tocolytic effects of statins are mediated by HO-1/CO, we performed experiments using HO-1 inhibitor SnPP-IX. While myometrium harvested from mice treated with pravastatin—that showed increased HO-1 synthesis, expression and activity—exhibited diminished contractility in response to C5a (Figs 3C and 5A), mice that received SnPP-IX and pravastatin responded to C5a in a similar manner to myometrium from control untreated mice (Fig. 5A). C5a increased the frequency of contractions in myometrium harvested from mice treated with SnPP-IX plus pravastatin similarly to control tissue. That SnPP-IX treatment blocks the tocolytic effect of pravastatin in C5a-stimulated myometrium indicates that HO-1 activity may be involved in the inhibitory effect of pravastatin on myometrial contractions.

Increased HO-1 synthesis was also observed in the cervix of statins-treated mice (Fig. 5B). LPS-treated mice co-treated with pravastatin

Figure 5 HO-1 inhibitor SnPP-IX abrogated the protective effects of statins in myometrium and cervix in LPS-treated mice. (A) C5a induces contractions in myometrium isolated from mice treated with SnPP-IX and pravastatin in a comparable way to LPS-alone-treated mice. *Different from control untreated myometrial strips, P < 0.05. (B) Quantitative RT–PCR analysis of HO-1 gene expression. Increased HO-1 gene expression in cervical tissue harvested from statins-treated mice compared with LPS-treated mice and age-matched control mice (for LPS-treated mice) 12–18 h after treatment. *Different from control, P < 0.05. (C) Collagen I staining and MMP activity in the cervix of LPS + SnPP-IX + P-treated mice. Signs of cervical remodeling (decreased collagen I expression and increased MMP activity) are present in this experimental group in contrast with P + LPS- and S + LPS-treated mice (Fig. 3B). LPS + SnPP-IX + P-treated mice and LPS + statins-treated mice were euthanized at the same time that LPS-treated mice gave birth (12–18 h after LPS administration). The microphotographs represent one of four to five similar experiments. (D) Increased DAF gene expression in cervical tissue from mice treated with statins + LPS compared with LPS-treated and control mice (age-matched control for LPS-treated mice). n = 4 in each experiment. *Different from control, P < 0.05. (E) Mice treated with P + LPS and S + LPS showed decreased deposition of C3 and increased cervical expression of the complement inhibitor DAF compared with mice treated with LPS alone. P + LPS- and S + LPS-treated mice were euthanized at the same time that LPS-alone-treated mice gave birth (12–18 h after LPS administration). Mice treated with pravastatin, SnPP-IX and LPS showed increased complement deposition and decreased expression of DAF, comparable to tissue from LPS-alone-treated mice. The microphotographs represent one of five to six similar experiments.
and HO-1 inhibitor SnPP-IX showed increased MMPs activity and diminished collagen I staining—indicative of active cervical remodeling—in the cervix compared with LPS-treated mice that received pravastatin only. These data suggest that HO-1 also plays a role in the protective effects of statins in cervical remodeling (Fig. 5C) in PTL mice.

**Role of DAF in the protective effects of statins in the cervix in PTL**

Complement DAF is a membrane protein widely expressed on essentially all cell types and prevents C3 deposition and formation of C5a (Hourcade et al., 1999). Given that statins and HO-1 increase the expression of complement inhibitor DAF (Mason et al., 2002; Kinderlerer et al., 2009), we hypothesized that statins—through HO-1—increase DAF expression in the cervix, inhibiting complement activation and cervical remodeling. In accordance with the literature, we found increased DAF synthesis and expression in the cervix of mice treated with simvastatin or pravastatin plus LPS when compared with mice treated with LPS alone or controls (Fig. 5D and E). In addition, increased DAF expression in the cervix in statins + LPS-treated mice was associated with diminished C3 deposition (Fig. 5E). qRT–PCR experiments showed increased synthesis of DAF in the cervix in mice that received statins + LPS compared with control and LPS-treated mice (Fig. 5D). Interestingly, HO-1 inhibitor SnPP-IX abrogated the stimulatory effects of statins on cervical expression of complement inhibitor DAF (Fig. 5E), suggesting a role for HO-1 in statins-induced increase in DAF synthesis and expression.

Co-administration of SnPP-IX with pravastatin abrogated the protective effects of pravastatin on PTL and mice treated with SnPP-IX + pravastatin delivered preterm in response to LPS comparable to LPS-alone-treated mice [delivery time (days): control (n = 5) = 19.8 ± 0.5; LPS (n = 7): 19.8 ± 0.3*; P + LPS (n = 6): 19.8 ± 0.8; SnPP-IX + P + LPS (n = 4): 16.5 ± 0.4*]. Mice treated with SnPP-IX alone delivered at term as control mice (SnPP-IX (n = 4): 20.3 ± 0.4 days) *Different from control, P < 0.05.

**Discussion**

Prematurity is the main cause of neonatal mortality and morbidity. Unfortunately, the mechanisms behind spontaneous PTL are still unclear, restricting, therefore, the development of preventive strategies and therapies. In this study we identified statins as a potential treatment to prevent PTL.

In pregnant women, subclinical genital tract infection has been associated with spontaneous PTL. Using a mouse model of PTL that resembles closely this clinical scenario (Gonzalez et al., 2011a, b), we found that a subclinical vaginal inflammation/infection can ascend through the vagina to the cervix causing an inflammatory response that leads to cervical remodeling, myometrial contractions and PTB. We identified complement component C5a as a crucial mediator in this inflammatory response that leads to PTB. Increased levels of C5a were found in mouse myometrium during PTB. Interestingly, no increase in C5a levels was observed in myometrial samples collected intrapartum at term, this observation is in agreement with our previous studies showing that complement activation does not play a role in term parturition (Gonzalez et al., 2011a, b).

Complement activation has been implicated in smooth muscle (Drouin et al., 2001) and cardiac muscle contraction (Berger et al., 1993). However, there is no available data on the role of complement on myometrial contractility. In this study we describe a previously unrecognized role for C5a as an uterotonic molecule in PTL. The low number of inflammatory cells infiltrating the myometrium during PTL and the presence of C5aR in the myometrium suggest that C5a contractile effects are mediated by the interaction of C5a with its receptor on myometrial fibers. We also need to consider that C5a may also contribute indirectly to myometrial contractility during PTL. In response to C5a, macrophages that infiltrate the cervix during PTL (Gonzalez et al., 2011a, b) in mice could release prostaglandin E2 (Brecht et al., 2011) that could also increase myometrial contractility. It has been described that incubation of human placental and fetal membranes with C5a up-regulates prolabor mediators, such as proinflammatory cytokines (IL6 and IL8), cyclooxygenase (COX)-2, prostaglandins (PGE2 and PGF2α) and MMP-9 among others (Lappas et al., 2012).

In an attempt to find a therapeutic strategy to prevent PTL and PTB, statins were administered to LPS-treated mice prior to LPS intravaginal administration to induce PTL. We tested statins because these compounds have shown to prevent complement-mediated pregnancy complications in mice (Redecha et al., 2008; Kumasawa et al., 2011; Singh et al., 2011). In this study we showed that statins prevented myometrial contractions, cervical remodeling and PTB. A previous study showed that pravastatin and simvastatin reduced the levels of proinflammatory cytokines that are related to PTL in the cervix and uterus of mice (Basraon et al., 2012). Interestingly, some of these cytokines can be released downstream of C5a–C5aR interaction. However, these authors did not show that statins prevent PTB in their mouse model (Basraon et al., 2012).

Statins exert numerous pleiotropic effects (Wang et al., 2008). Many of these effects may contribute to the protective effects of statins in preventing cervical remodeling, myometrial contractions and premature birth.

Our study shows an important role of HO-1 activity in the protective effects of statins in PTL. Statins induce the expression of HO-1 in many different cells (Ali et al., 2009; Leung et al., 2011). Here we found that statins increased the synthesis and expression of HO-1 in cervical and myometrial tissue. We also have shown increased HO-1 activity in human myometrium incubated with statins and myometrium from statins-treated mice.

It was reported that HO-1 diminished myometrial contractility through the production of CO and that progesterone up-regulated HO-1 synthesis (Acevedo and Ahmed, 1998). The up-regulation of HO-1 by progesterone may also explain the protective effects of progesterone against PTB observed in this mouse model (Gonzalez et al., 2011a, b) and in women (Conde-Agudelo et al., 2013).

Several studies demonstrated there is a correlation between statins, HO-1/CO pathway and complement inhibition (Kinderlerer et al., 2009; El-Mousleh et al., 2012). The first report, more than 10 years ago demonstrated that statins protect vascular endothelium against complement-mediated injury by inducing DAF expression (Mason et al., 2002). In addition, HO-1 also stimulates DAF expression (Kinderlerer et al., 2009), suggesting that statins induction of DAF may be mediated by the HO-1 pathway. Interestingly, recent data indicated that there is a relationship between CO and complement activation. Low levels of CO exhibited anti-inflammatory effects and significantly diminished complement activation and rescued pregnancies in a mouse model of intrauterine growth restriction (El-Mousleh et al., 2012).
Furthermore, our studies showed that statins increased DAF synthesis and expression in cervical tissue. The increased synthesis of HO-1 and the overexpression of DAF in the cervix induced by statins suggest that statins might prevent cervical remodeling by inhibiting complement activation through a mechanism involving the HO-1/CO pathway.

It has been shown that macrophages exposed to CO show a suppressed inflammatory response in response to LPS (Sawle et al., 2005). Therefore, it is also tempting to speculate that statins by increasing HO-1 and CO may inhibit C5a-induced macrophage activation in the cervical tissue and thus arrest cervical remodeling and PTB. Inhibition of MMP-9 by statins is another mechanism that can contribute to the protective effects of statins on cervical remodeling (Massaro et al., 2010). It has been reported that simvastatin and atorvastatin inhibit MMP-9 and COX-2. Interestingly, COX-2 inhibitors have been described as tocolytic agents in a mouse model of PTB induced by intraperitoneal administration of LPS (Sakai et al., 2001).

Our studies demonstrated that HO-1/CO might also be involved in the tocolytic effects of statins. Increased HO-1 expression and synthesis was found in human myometrial tissue incubated with statins and myometrium from statins-treated mice. In addition, CO-releasing molecule CORM-2 inhibited spontaneous and C5a-induced myometrial contractions. That SnPP-IX abrogated the inhibitory effects of pravastatin on C5a-induced myometrial contractions is indicative that the HO-1/CO pathway also plays a role in the inhibitory effects of statins on uterine contractility. Finally, the in vivo studies showing that mice co-treated with HO-1 inhibitor SnPP-IX and pravastatin delivered preterm in response to LPS, confirms that the protective effects of pravastatin in preventing PTB are mediated by HO-1 activity.

In conclusion, statins prevented cervical remodeling, myometrial contractions and PTB. Activation of the HO-1/CO pathway and inhibition of complement activation by induction of DAF expression are involved in the protective effects of statins in preventing PTB. In our studies, statins were administered prior the induction of PTL and were beneficial in preventing PTL and PTB. This suggests that statins might be a good prophylactic measure in women who are at risk of PTL (short cervix, smokers, previous PTB, black race among others) (Goldenberg et al., 1998).

Premature babies are particularly vulnerable to cortical brain damage associated with long-term cognitive, behavioural, attentional or socialization deficits. We recently published that statins also prevent fetal brain cortical abnormalities in this mouse model of PTB (Pedroni et al., 2014). Statins are already approved for therapeutic use and are unlikely to be harmful to the fetus (Kusters et al., 2012). In addition, a clinical trial to collect safety data for statins when used in pregnant women at high risk of pre-eclampsia recently started in the USA (Costantine et al., 2013). If these results are confirmed in humans they can eventually lead to an effective clinical treatment to prevent PTB.

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Authors’ roles

J.M.G. and S.M.A.P. performed experiments, contributed to drafting the article and to final approval of the version to be published, G.G. contributed to design of the study, performed experiments, analyzed and interpreted data and wrote the paper.

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Conflict of interest

No authors declare any financial or other relationships that might lead to a conflict of interest.

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Statins to prevent preterm labor


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