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Research Article

Metformin attenuates susceptibility to inflammation-induced preterm birth in mice with higher endocannabinoid levels†

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Abstract

Premature decidual senescence is a contributing factor to preterm birth. Fatty acid amide hydrolase mutant females (*Faah*$^{-/-}$) with higher endocannabinoid levels are also more susceptible to preterm birth upon lipopolysaccharide (LPS) challenge due to enhanced decidual senescence; this is associated with mitogen-activated protein kinase p38 activation. Previous studies have shown that mechanistic target of rapamycin complex 1 (mTORC1) contributes to decidual senescence and promotes the incidence of preterm birth. In this study, we sought to attenuate premature decidual aging in *Faah*$^{-/-}$ females by targeting mTORC1 and p38 signaling pathways. Because metformin is known to inhibit mTOR and p38 signaling pathways, *Faah*$^{-/-}$ females were treated with metformin. These mice had a significantly lower preterm birth incidence with a higher rate of live birth after an LPS challenge on day 16 of pregnancy; metformin treatment did not affect placentation or neonatal birth weight. These results were associated with decreased levels of p38, as well as pS6, a downstream mediator of mTORC1 activity, in day 16 *Faah*$^{-/-}$ decidual tissues. Since metformin treatment attenuates premature decidual senescence with limited side effects during pregnancy, careful use of this drug may be effective in ameliorating specific adverse pregnancy events.

Summary Sentence

Metformin treatment attenuates premature decidual senescence caused by higher endocannabinoid levels and susceptibility to inflammation-induced preterm birth in mice.

Key words: metformin, FAAH, endocannabinoids, preterm birth, decidual aging.

Abbreviations

2-AG 2-arachidonoyl glycerol
AEA *N*-arachidonoyl ethanolamine, also known as anandamide
CB1 cannabinoid receptor 1, coded by *Cnr1*
CB2 cannabinoid receptor 2, coded by *Cnr2*
DIG digoxigenin
E_2 estradiol-17β
FAAH fatty acid amide hydrolase
LEA *N*-linoleoylethanolamine
LPS lipopolysaccharide
MAPK mitogen-activated protein kinase
mTORC1 mechanistic target of rapamycin complex 1
NAE *N*-acylethanolamine
OEA *N*-oleoylethanolamine
P_4 progesterone
PEA *N*-palmitoylethanolamine

PECAM platelet/endothelial cell adhesion molecule 1
PGE_2 prostaglandin E_2
$PGF_{2\alpha}$ prostaglandin $F_{2\alpha}$
Prlr prolactin receptor
Ptgs2 prostaglandin-endoperoxide synthase 2
S6 ribosomal protein S6
SA-β-gal senescence-associated-β-galactosidase
SEA *N*-stearoylethanolamine
Tpbpa trophoblast specific protein alpha
WT wild type
γH2AX phosphorylated histone-2AX

Introduction

Preterm birth is associated with low birth weight and immaturity of multiple organ systems, particularly the respiratory tree. In humans, preterm birth accounts for 12% of all births and is the leading cause of neonatal death. Preterm birth affects around 13 million babies worldwide, resulting in 1 million neonatal deaths per year [1]. Limited prevention and treatment of preterm birth is attributed to the varied etiology of this pregnancy disorder, including genetic factors, infection/inflammation, stress, decidual senescence, and environmental insults [2, 3].

Proliferation and differentiation of uterine stromal cells give rise to decidual cells; the process is termed decidualization. In mice, this process begins with the initiation of embryo implantation and peaks on day 8 of pregnancy followed by allantois-chorion fusion to initiate the process of placentation. With the progress of placentation, the maternal decidua in the mesometrial domain functions as an anchoring dock for the placenta and fetus, and as a buffer zone between the maternal myometrium and placental trophoblasts. The decidua regulates orderly invasion of trophoblasts, which remodel maternal blood vessels for the delivery of nutrition and oxygen from the mother to the fetus. While approaching parturition, maternal decidual cells undergo senescence and thinning of the decidual layer occurs. Our recent study showed that excessive exposure to elevated endocannabinoid signaling during the midgestational stage causes premature decidual senescence [4].

Research on cannabinoid signaling was greatly advanced by the discovery of several endogenous cannabis-like compounds and their target receptors in the early 1990s. *N*-arachidonoyl ethanolamine (known as anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) are two most studied endocannabinoids [5–7]. Both compounds primarily target two G-protein coupled cannabinoid receptors: cannabinoid receptor 1 (CB1) encoded by *Cnr1* [8, 9] and cannabinoid receptor 2 (CB_2) encoded by *Cnr2* [10]. Although AEA (anandamide) is known to be produced primarily from *N*-arachidonoyl phosphatidylethanolamine [11], complete suppression of AEA synthesis in vivo has not been successful due to the presence of multiple pathways for AEA synthesis [11–14]. AEA is degraded to ethanolamine and arachidonic acid by a membrane-bound fatty acid amide hydrolase (FAAH) [15, 16]. Although FAAH can hydrolyze a panel of endocannabinoids including 2-AG [17] and other bioactive lipids [18], studies in *Faah*$^{-/-}$ mice suggest that the magnitude and duration of AEA signaling is mainly regulated by FAAH [13, 19]. Activation of CB_1 and CB_2 exerts different biological effects in a cell-type specific manner. Coupling with $G_{i/o}$ or G_q proteins, CB_1 and CB_2 can regulate Ca^{2+} channels [20, 21], inhibit adenylyl cyclase activity [9, 22], and stimulate mitogen-activated protein kinases (MAPKs) including ERK, JNK, and p38 [21, 23–25].

Several studies demonstrate that metformin inhibits both mechanistic target of rapamycin (mTOR) and p38 signaling pathways

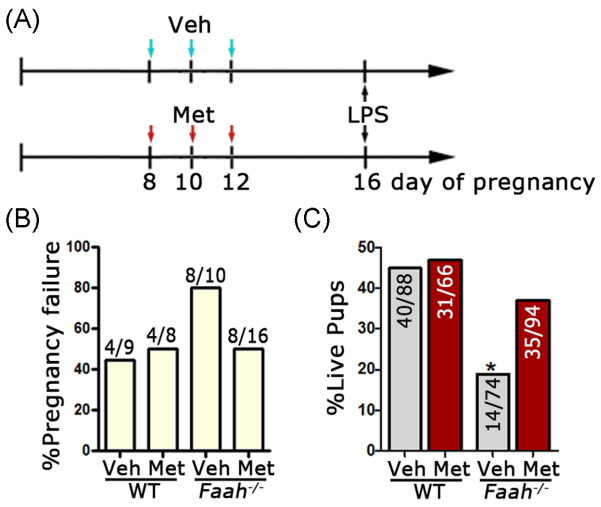

Figure 1. Metformin attenuates inflammation-induced preterm birth in *Faah*$^{-/-}$ mice. (A) Scheme of metformin and LPS administration. (B) Rates of pregnancy failure in WT and *Faah*$^{-/-}$ mice after LPS injection. (C) Rate of live pups over all pups generated by LPS-challenged WT and *Faah*$^{-/-}$ mice at full term. Numbers on bars are no. of live pups/no. of total pups. ($*P < 0.05$, Chi-square tests). Veh, vehicle; Met, metformin.

[26–28]. Metformin is the first line of medication for the treatment of type 2 diabetes and is also used to treat polycystic ovary syndrome (PCOS), and it has also been used during pregnancy to treat gestational diabetes mellitus [29]. In a double-blind, placebo-controlled trial, pregnant women without diabetes who had a body mass index (BMI) greater than 35 experienced reduced maternal weight gain and incidence of pre-eclampsia after Metformin treatment [30]. Both studies showed that metformin apparently has no adverse effects on neonatal outcomes.

Previously, we found that *Faah*$^{-/-}$ decidual cells show increased senescence-associated-β-galactosidase (SA-β-gal) staining on days 12 and 16 of pregnancy, suggesting that decidual cells undergo some premature senescence under elevated endocannabinoid signaling. In addition, *Faah*$^{-/-}$ mice are more prone to preterm birth in response to lipopolysaccharide (LPS) challenge [4]. Since metformin can inhibit p38 activation and mTOR signaling which influence decidual senescence, we speculated that metformin treatment would reduce the incidence of inflammation-induced preterm birth in *Faah*$^{-/-}$ mice. We administered metformin by oral gavage (1 mg kg^{-1} body weight) on days 8, 10, and 12 followed by an LPS injection (10 μg) on day 16 of pregnancy (Figure 1A). We found that *Faah*$^{-/-}$ females treated with metformin had a significantly lower preterm birth rate and a higher rate of live birth as compared to vehicle-treated *Faah*$^{-/-}$ after LPS challenge. Indeed, Metformin administration decelerated the decidual ageing process in *Faah*$^{-/-}$ females which was accompanied by reduced p38 and mTOR signaling.

Materials and methods

Animals and treatments

Faah$^{-/-}$ mice were generated as described [19] and maintained on a C57BL6/CD-1 mixed background. *Faah*$^{-/-}$ mice and their wild type (WT) littermates were housed in the animal care facility at the Cincinnati Children's Hospital Medical Center according to National Institutes of Health and institutional guidelines for laboratory animals. All protocols of this study were approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care

and Use Committee. All mice were housed in wall-mount negative airflow polycarbonate cages with corn cob bedding. They were provided ad libitum with double distilled autoclaved water and rodent diet (LabDiet 5010).

Female mice were mated with WT fertile males to induce pregnancy (vaginal plug = day 1 of pregnancy). Mice were euthanized by cervical dislocation right before tissue collection under deep anesthesia. Pregnant WT and *Faah*$^{-/-}$ females were treated with metformin by oral gavages (1 mg kg^{-1} body weight) on days 8, 10, and 12 of pregnancy, and tissue was collected on day 16. Highly purified LPS (*Escherichia coli* 0111:B4, tlrl-3pelps, Invivogen) was dissolved in saline and given intraperitoneally (10 μg) in the morning of day 16. The dose and treatment regimen of metformin are based on our previous work [26]. We lowered the dose of LPS compared to our previous study [4], since the potency of LPS acquired from the same vendor varies from batch to batch. We tested different doses of LPS in WT and *Faah*$^{-/-}$ pregnant mice. We found that about 80% of *Faah*$^{-/-}$ pregnant females gave preterm birth at 10 μg of LPS, which is comparable to our previous study [4]. Parturition events were monitored from days 17 through 21 by observing mice daily in the morning and evening. Preterm birth was defined as birth occurring earlier than day 19.

In situ hybridization

In situ hybridization was performed as previously described [31]. Both radioactive (^{35}S GTP) and Digoxigenin (DIG) labeling methods were used. In brief, frozen sections (12 μm) were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS). The sections were prehybridized and hybridized at 45°C for 4 h in 50% (vol/vol) formamide hybridization buffer containing ^{35}S-labeled antisense RNA probes (Perkin Elmer). RNase A-resistant hybrids were detected by autoradiography. For DIG labeling, sections were hybridized at 65°C overnight in 50% (vol/vol) formamide hybridization buffer containing DIG-labeled probes (Roche). All sections were poststained with hematoxylin and eosin. prostaglandin-endoperoxide synthase 2 (*Ptgs2*), prolactin receptor (*Prlr*), and trophoblast specific protein alpha (*Tpbpa*) probes were synthesized as previously described [32].

Measurement of estradiol-17β and progesterone levels

Serum levels of estradiol-17β (E_2) and progesterone (P_4) were measured by enzyme immunoassay kits (Cayman).

Senescence-associated-β-galactosidase staining

Staining of SA-β-gal activity was performed as described previously [33]. The staining was performed at pH 5.5. To compare the intensity of SA-β-gal staining, sections from different genotypes and on different days of pregnancy were processed on the same slide.

Measurement of *N*-acylethanolamines, 2-acyl-glycerols, and prostaglandins profiles

Maternal decidual tissues of implantation sites were collected on day 16 of pregnancy. These tissues were flash frozen and stored at –80°C until used for extractions. Methanolic extracts of tissues were partially purified using C18 solid-phase extraction columns (Agilent), and lipids including AEA, 2-AG, and prostaglandins (PGs) were quantified by high-performance liquid chromatography-tandem mass spectrometry as previously described [33].

Immunofluorescence

Immunofluorescence for platelet/endothelial cell adhesion molecule 1 (PECAM; BD Pharmingen) and phosphorylated histone-2AX (γH2AX; Millipore) was performed using secondary antibodies Cy3-conjugated donkey anti-Rat and Cy3-conjugated donkey anti-mouse (Jackson Immunoresearch), respectively. Nuclear staining was performed using Hoechst 33342 (H1399, Molecular Probes, 2 μg/ml). Immunofluorescence was performed on fresh-frozen sections. Sections were fixed in 10% neutral buffered formalin and incubated with primary antibody at 4°C overnight, followed by incubation in secondary antibody for 1 h in PBS. Immunofluorescence was visualized under a confocal microscope (Nikon Eclipse TE2000).

Western blotting

Protein extraction and western blotting were performed as previously described [33]. Antibodies to p38, phospho-p38 (p-p38), S6, p-S6, and Tubulin were obtained from cell signaling. Bands were visualized by using an ECL Prime Western blotting detection system (GE Healthcare), and band intensities were quantified by ImageJ. Experiments were repeated three times, and a set of representative gel images is presented. Band intensities of p-p38 and p-S6 were normalized against p38 and S6, respectively. Antibodies to Tubulin were also used as a loading control. Values are mean ± SEM.

Statistical analysis

Data were analyzed by the Student *t*-tests, Mann–Whitney tests, or Chi-square tests as indicated in figure legends. Data of *N*-acylethanolamines (NAEs), 2-acyl-glycerols, and PGs levels were analyzed by analysis of variance including three-way interaction to test the effect of genotype (WT VS *Faah*$^{-/-}$), LPS treatment, and metformin treatment, and their interaction on the outcomes. Posthoc analysis was conducted to compare the pairwise difference in the eight group combinations. Tukey method was used to control for multiple comparisons. $P < 0.05$ was considered significant. Values are mean ± SEM.

Results

Metformin attenuates inflammation-induced preterm birth in *Faah*$^{-/-}$ mice

After LPS injection on day 16 of pregnancy, 44% of WT and 80% of *Faah*$^{-/-}$ pregnant females had pregnancy failure (Figure 1B), confirming our previous observation that *Faah*$^{-/-}$ mothers are more vulnerable to preterm birth and/or resorptions after an LPS challenge [4]. Metformin treatment reduced the rate of pregnancy failure from 80% to 50% in *Faah*$^{-/-}$ females. Among all pregnancy failures, preterm birth rates decreased from 40% (4/10) to 19% (3/16) in *Faah*$^{-/-}$ females and resorption rates decreased from 40% (4/10) to 31% (5/16) (Supplemental Table S1). The percentage of metformin-treated *Faah*$^{-/-}$ females that had term births (50%) is higher than that of vehicle-treated *Faah*$^{-/-}$ females (20%), whereas the percentage of WT females with term birth did not change significantly after metformin or vehicle treatment (Supplemental Table S1). The rate of live pups delivered from metformin-treated *Faah*$^{-/-}$ females (37%) significantly increased compared to those vehicle-treated *Faah*$^{-/-}$ females (19%) (Figure 1C); metformin at the same dose had limited effects on WT animals. These results suggest that metformin reduces inflammation-induced preterm birth in mice with higher endocannabinoid levels.

Metformin treatment does not compromise embryonic growth

Metformin has been used in treating gestational diabetes mellitus and PCOS during pregnancy [30, 34, 35]. In the current study, we tested the effects of metformin exposure during the midgestational stage on placentation and embryo development in mice. Following the same metformin treatment regime on days 8, 10, and 12 as shown in Figure 1A, we examined the placental development on day 16. Weights of implantation sites in both WT and *Faah*$^{-/-}$ females are comparable after Metformin treatment (Supplemental Figure S1). Since the placental labyrinth zone consists mainly of blood vessels, we also examined the vascular density and morphology in this zone by immunostaining of PECAM, an endothelial cell marker. The morphology of vasculature in the labyrinth layer and decidua is comparable in metformin- or vehicle-treated WT and *Faah*$^{-/-}$ females (Figure 2A and B). We also examined the spongy layer of the placenta by its marker *Tpbpa* expression. The morphology of the spongy layer in metformin-treated animals appeared similar to that of vehicle-treated WT and *Faah*$^{-/-}$ females (Figure 2A and B). The results suggest that exposure to metformin during midgestation has little or no adverse effects on placentation. The effect of metformin on embryo development was further monitored by examining litter size and neonatal birth weight after metformin treatment. Without LPS and metformin treatment, WT and *Faah*$^{-/-}$ females produced healthy offspring with normal litter sizes, birth weights, and neonatal survival rates, and metformin treatment apparently has no adverse effects on offspring health (Figure 2C–E). In sum, the results showed that females receiving metformin during pregnancy have normal placentation and embryo development.

Metformin treatment elevates *N*-acylethanolamines in pregnant *Faah*$^{-/-}$ females exposed to lipopolysaccharide

To study the impact of metformin on maternal decidual endocannabinoids and structurally similar bioactive lipids in *Faah*$^{-/-}$ females, levels of 2-acyl-glycerols and NAEs, including 2-AG and AEA, in decidual tissues were measured 12 h after vehicle or LPS (10 μg) injections. We found that levels of the endocannabinoid 2-AG, as well as other 2-acyl-glycerol lipids, showed no significant changes in *Faah*$^{-/-}$ decidua as compared to those in WT mice with or without LPS injection, and no significant differences in tissues treated with metformin compared to corresponding vehicle-treated groups were noted in WT or *Faah*$^{-/-}$ decidua except for 2-palmitoylglycerol levels in LPS-challenged *Faah*$^{-/-}$ mice (Figure 3). As expected, levels of AEA and other NAEs were significantly elevated in *Faah*$^{-/-}$ decidua as compared to those in WT mice (Figure 4), and NAE levels were also elevated after LPS stimulation in both WT and *Faah*$^{-/-}$ deciduae (Figure 4). Interestingly, metformin increased the levels of NAEs only in LPS-challenged *Faah*$^{-/-}$ decidua, but not in WT decidua (Figure 4). The results suggest that metformin augments NAEs in *Faah*$^{-/-}$ mice after an injection of LPS. Collectively, the results show that the increased rate of LPS-induced preterm birth occurs in *Faah*$^{-/-}$ mice with higher levels of NAEs.

Metformin treatment tempers premature decidual senescence in *Faah*$^{-/-}$ females

Our previous studies have shown that increased endocannabinoid signaling causes premature decidual senescence [4]. To investigate the effect of metformin on decidual cells, we examined SA-β-gal staining in vehicle- and metformin-treated WT and *Faah*$^{-/-}$ females.

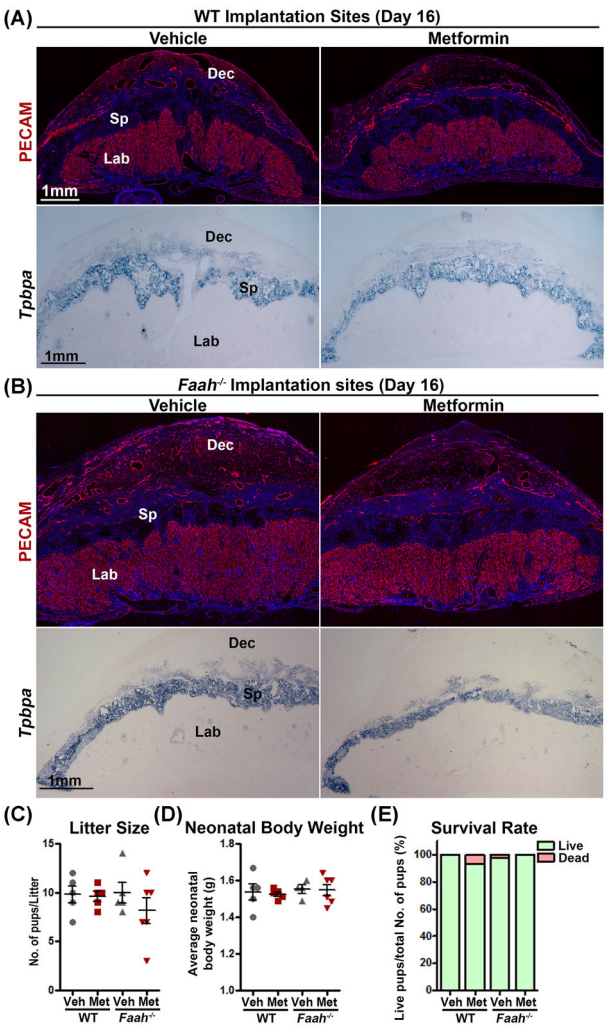

Figure 2. Metformin treatment does not compromise the development of placenta and embryos (A and B) Immunostaining of PECAM and in situ hybridization of *Tpbpa* on day 16 in WT and *Faah*$^{-/-}$ implantation sites. Positive PECAM signals highlighted all endothelial cells, whereas *Tpbpa* signals are localized on spongy trophoblast cells. Three implantation sites from different females were examined, and a representative image for each staining is shown in the figure. (C) Litter size of WT and *Faah*$^{-/-}$ mice with or without metformin treatment. (D and E) Body weights and survival rates of neonatal pups of WT and *Faah*$^{-/-}$ mice with or without metformin treatment. All neonatal pups were derived from litters shown in panel C. Dec, decidua; Sp, spongy layer; Lab, Labyrinth zone; Veh, vehicle; Met, metformin.

We found that metformin treatment decreased SA-β-gal staining in both WT and *Faah*$^{-/-}$ decidual cells on day 16 of pregnancy (Figures 5A and 6A), suggesting that metformin effectively lessened decidual senescence. In further support of this finding, we examined γH2AX expression, another marker of senescence associated with DNA damage response [36]. The number of γH2AX positive decidual cells was substantially reduced in metformin-treated WT or *Faah*$^{-/-}$ females when compared to vehicle-treated groups (Figure 5A and B; Figure 6A and B). These results were associated with reduced decidual senescence in *Faah*$^{-/-}$ females after metformin treatment as evident by the increased height of the decidual zone, demarcated by *Prlr* expression compared with those in vehicle-treated *Faah*$^{-/-}$ implantation sites (Figure 6). Collectively, these results show that metformin attenuates premature decidual aging in *Faah*$^{-/-}$ females.

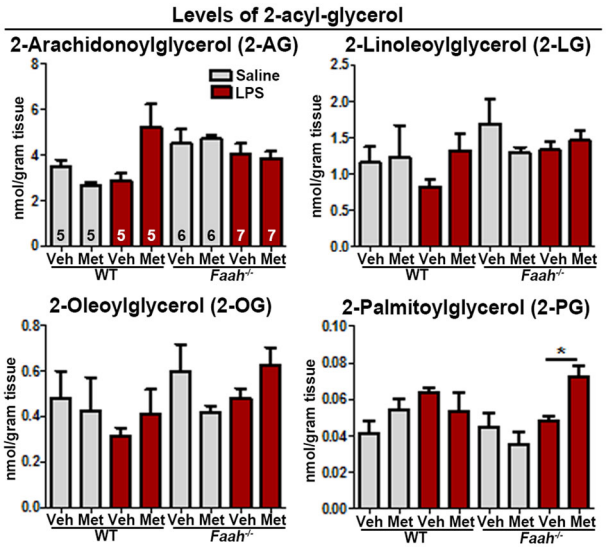

Figure 3. Levels of 2-acyl-glycerols with or without LPS challenge in maternal decidua with or without metformin treatment on day 16 of pregnancy. Levels of 2-acyl-glycerols in maternal decidua on day 16. Sample sizes (n) are indicated in the first bar diagrams, and the sample sizes are the same throughout all graphs. Veh, vehicle; Met, metformin. ($*P < 0.05$, Mann–Whitney tests).

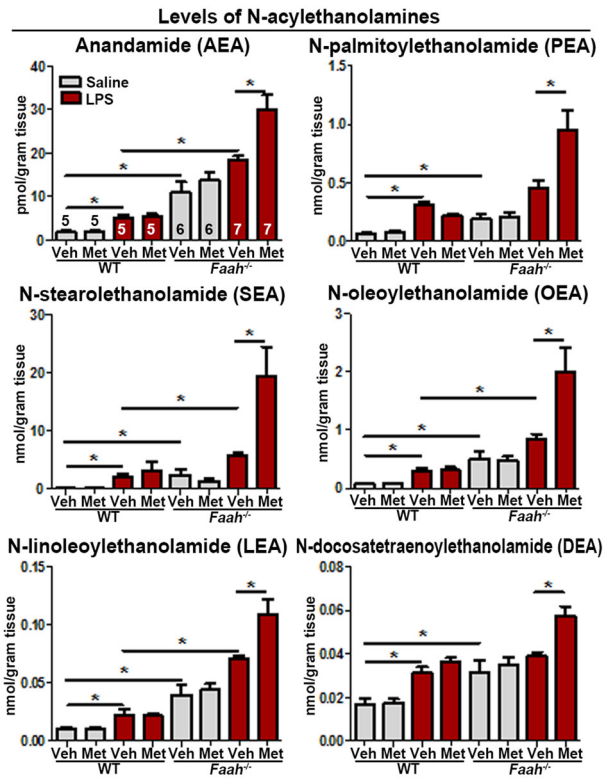

Figure 4. Levels of NAEs with or without LPS challenge in maternal decidua with or without metformin treatment on day 16 of pregnancy. Levels of NAEs in maternal decidua on day 16. Sample sizes (n) are indicated in the first bar diagrams, and the sample sizes are the same throughout all graphs. Veh, vehicle; Met, metformin ($*P < 0.05$, Mann–Whitney tests).

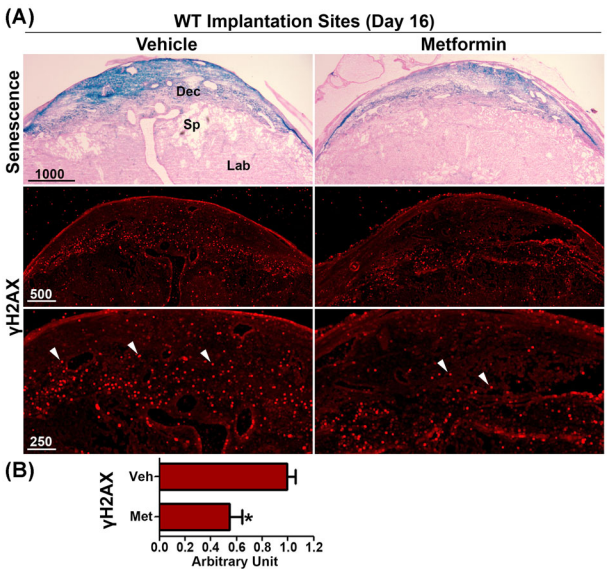

Figure 5. Metformin treatment reduces decidual senescence in WT females. (A) SA-β-gal staining (blue) and immunostaining of γH2AX in sections of WT implantation sites 12 h after LPS injection on day 16 of pregnancy. White arrowheads indicate signals of γH2AX. Three implantation sites from different pregnant females were examined, and a representative image for each staining is shown in the figure. (B) Quantification of γH2AX positive signals in WT implantation sites. γH2AX positive signals were counted in three randomly selected fields in each of three implantation sites examined. Veh, vehicle; Met, metformin. ($*P < 0.05$, Mann–Whitney tests).

Higher serum P_4 levels maintain myometrial quiescence during pregnancy. LPS-induced inflammation promptly diminishes P_4 levels [37], predisposing pregnant females to preterm birth [38]. To examine whether the effect of metformin in *Faah*$^{-/-}$ mice correlated with ovarian hormone levels, we quantified serum levels of estradiol-17β (E_2) and P_4 12 h after LPS injection on day 16 by enzyme immunoassays. Although an LPS injection decreased P_4 levels in both metformin- and vehicle-treated *Faah*$^{-/-}$ and WT mice, the levels were comparable between the two groups (Figure 7A and B). E_2 levels were also comparable in all groups examined in WT and *Faah*$^{-/-}$ females (Figure 7C and D).

Prostaglandins play a major role in parturition. There is evidence that prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and PGI_2 levels are upregulated in the uterus and decidua at the onset of parturition [39], which enhance contractile response/activity in the myometrium [40, 41]. Cyclooxygenases *Ptgs1* (Cox1) and *Ptgs2* (Cox2) are two major enzymes that catalyze PG biosynthesis. *Ptgs1* is constitutively expressed in many tissues, while *Ptgs2* is induced by growth factors, cytokines, and various inflammatory stimuli [42]. Therefore, we examined the expression of *Ptgs2* on day 16 by in situ hybridization in vehicle- and metformin-treated *Faah*$^{-/-}$ implantation sites 12 h after LPS challenge. We found that *Ptgs2* expression appeared at the maternal–conceptus interface, primarily in the decidual cells, but the expression pattern and signal intensity were comparable in vehicle- or metformin-treated *Faah*$^{-/-}$ implantation sites (Figure 7E). This observation was reflected in levels of PGE_2, $PGF_{2\alpha}$, and 6-keto prostaglandin F1α (the stable metabolite of PGI2) as measured by High-performance liquid chromatography-tandem mass spectrometry [43] (Figure 7F–H). These results implicate that PG levels were not affected by metformin treatment in *Faah*$^{-/-}$ mice.

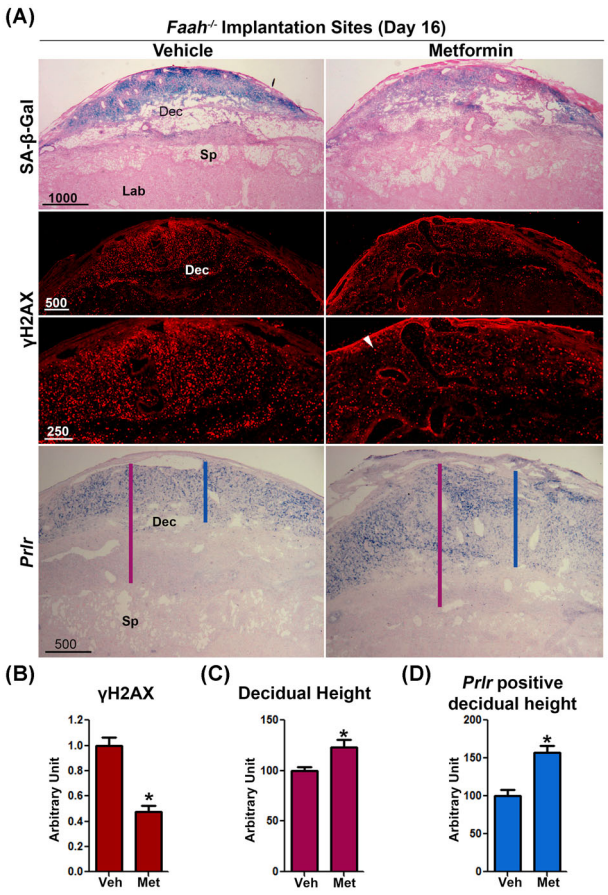

Figure 6. Metformin treatment tempers premature decidual senescence in *Faah*$^{-/-}$ female. (A) SA-β-gal staining, immunostaining of γH2AX, and in situ hybridization of *Prlr* in sections of *Faah*$^{-/-}$ implantation sites 12 h after LPS injection on day 16 of pregnancy. White arrowheads indicate signals of γH2AX. Presented pictures are representative pictures from three different implantation sites. Decreased signals for γH2AX, a senescence, and DNA damage response marker, was observed in metformin-treated samples, which is quantitated in panel (B). ($*P < 0.05$, Mann–Whitney tests). The height of the whole decidual zone and *Prlr* positive decidual zone are marked by red and blue lines respectively. (C and D) Quantitation of heights of the whole decidual zone and *Prlr* positive decidual zone. Decidual heights increased in metformin-treated *Faah*$^{-/-}$ implantation sites. Dec, decidua; Sp, spongy layer; Lab, Labyrinth zone; Veh, vehicle; Met, metformin. (n = 9; $*P < 0.05$, Student *t* tests).

Metformin regulates decidual senescence by targeting mechanistic target of rapamycin and p38 pathway

Our recent studies have shown that elevated endocannabinoid signaling advances decidual senescence by activating p38 MAPK signaling [4], and our present results show that metformin reverses premature decidual aging in *Faah*$^{-/-}$ females (Figure 4A). Thus, we further investigated the signaling pathways by which metformin regulates decidual senescence in tissues collected from vehicle or metformin-treated WT and *Faah*$^{-/-}$ females on day 16 of pregnancy. Western blotting results of p38 showed that levels of p-p38 were higher in *Faah*$^{-/-}$ decidua than those in WT decidua on day 16, and that metformin significantly decreased p38 activation in both WT and *Faah*$^{-/-}$ decidua (Figure 8). In addition, the levels of pS6, a downstream effector of mechanistic target of rapamycin complex 1 (mTORC1) signaling, were also decreased by metformin treatment in WT and *Faah*$^{-/-}$ decidua (Figure 8). The results suggest that metformin suppresses both p38 and mTORC1 signaling pathways in decidua.

Discussion

In this study, we show that decidua in *Faah*$^{-/-}$ mice have increased levels of AEA and related NAEs, which are associated with premature decidual senescence and increased susceptibility to LPS-induced preterm birth. Metformin rescues *Faah*$^{-/-}$ mice from inflammation-induced preterm birth by suppressing p38 and mTORC1 signaling in decidual tissues without altering levels of ovarian hormones and PGs, suggesting that targeting MAPK and mTORC1 signaling is an alternative approach to protect pregnancy under increased endocannabinoid tone and inflammation.

Placentation is initiated after day 8 of pregnancy, and decidual cells in the mesometrial domain connect the placenta with the maternal system throughout pregnancy until parturition occurs. Our previous research showed that a higher amount of senescent cells could be detected in the decidua as early as day 12 of pregnancy in *Faah*$^{-/-}$ females [4]. Therefore, we speculated that metformin administered during the midgestational stage and LPS given on day 16 of pregnancy would provide meaningful information. Metformin has been used in humans to treat PCOS and gestational diabetes [30, 34, 35]. Our observation of normal litter size and neonatal birth weight following metformin treatment in WT and *Faah*$^{-/-}$ suggests that metformin has little, if any, adverse side effects on fetal health. However, we cannot exclude the possibility that fetal exposure to metformin may have adverse effects in adulthood. A previous study showed compromised fetal testis development if males are exposed to metformin during pregnancy [44].

Endocannabinoid signaling is generally considered to offer protection against aging (reviewed in [45]). Mice with genomic *Cnr1* deletion or conditional deletion in hippocampal GABAergic neurons show accelerated cognitive decline, an indicator of brain aging [46]. *Cnr1*$^{-/-}$ mice were also shown to have deficits in social memory starting from 3 months of age with early onset of aging-like histological changes in skin, but not in other organs [47]. *Cnr2*$^{-/-}$ mice show accelerated aging in bone [48]. These results suggest that lack of either CB_1 or CB_2 induces accelerated aging in specific tissues. Interestingly, studies on *Caenorhabditis elegans* show that *N*-eicosapentaenoylethanolamine (EPEA), the most abundant NAE in worms, inhibits lifespan extension induced by dietary restriction [49], implicating NAE signaling in the accelerated aging process in worms. Our current study together with our previous report [4] demonstrates that CB_1-mediated cannabinoid signaling advances the decidual aging process, which again reinforces the concept that the role of endocannabinoid in aging is tissue specific.

Decidual tissues produced more NAEs in response to LPS challenge in WT and *Faah*$^{-/-}$ females, including AEA, *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA), *N*-linoleoylethanolamine (LEA), and *N*-stearoylethanolamine (SEA). A recent study showed increases in plasma levels of NAEs after LPS injection in pregnant mice [43]. The significance of these increases is not clearly understood at this time. However, endocannabinoids are generally produced in response to injury, aiming to decrease proinflammatory mediators. This role may extend to nonendocannabinoid NAEs. For example, PEA is well known for its anti-inflammatory effects mediated by PPARα [50]. PEA was shown to reduce microglia activation and proinflammatory cytokine production in the spinal cord [51] and suppress rat mast cell activation in vitro [52]. OEA also plays an inflammatory role, though studies mostly focus

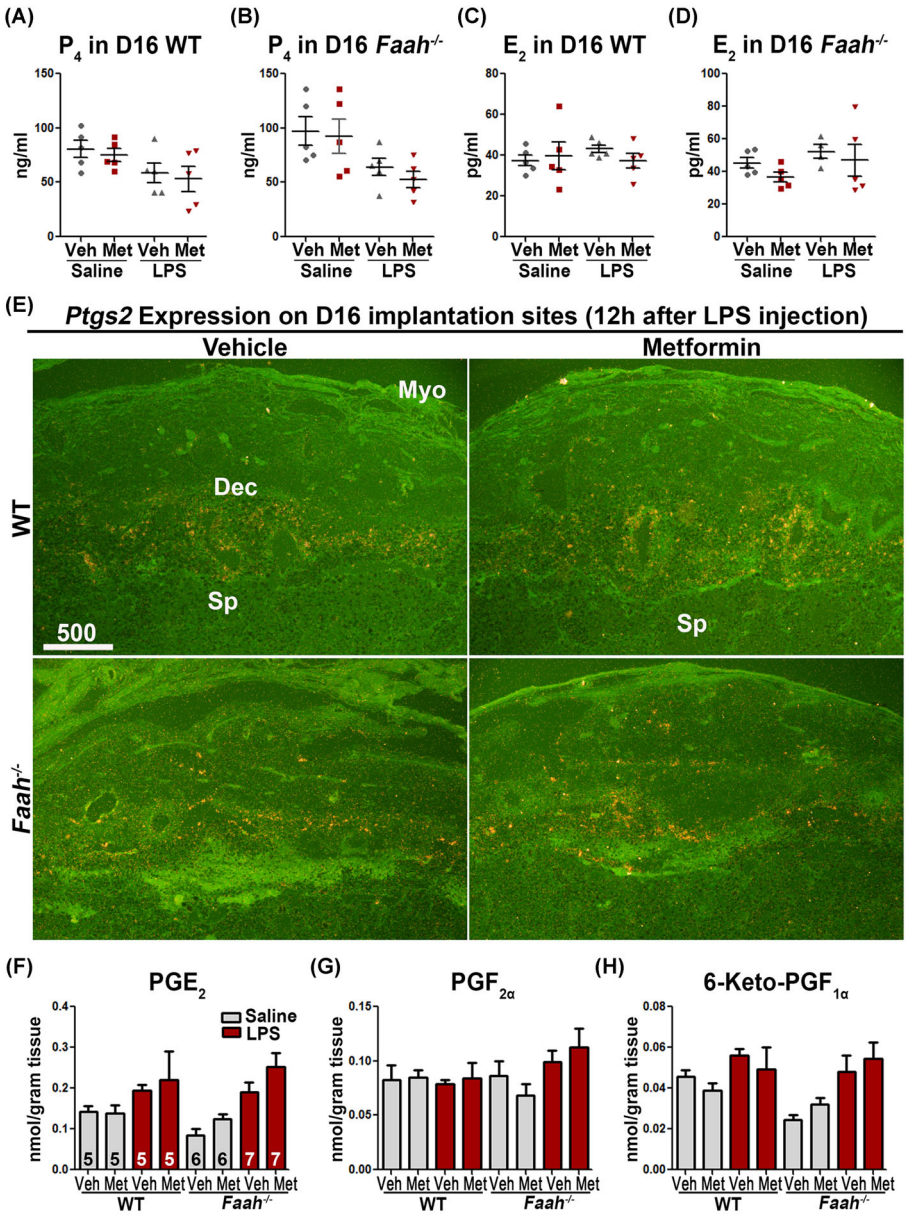

Figure 7. Levels of ovarian hormones and PGs are not affected by metformin treatment. (A–D) Serum P_4 and E_2 levels 12 h after LPS or saline injection on day 16 of pregnancy. *Faah*$^{-/-}$ and WT mice had comparable P_4 and E_2 levels in metformin or vehicle-treated groups. (n = 5 in each group; mean ± SEM). (E) In situ hybridization of *Ptgs2* on day 16 implantation sites 12 h after LPS injection. Metformin-treated WT and *Faah*$^{-/-}$ mice had comparable *Ptgs2* signals as compared to those treated with vehicle. Dec, decidua; Sp, spongy layer; Lab, Labyrinth zone. (F–H) Profiles of PGs in maternal decidua 12 h after LPS injection on day 16. Sample sizes (n) are indicated in bar diagrams. Veh, vehicle; Met, metformin.

on its anorexigenic effects [53]. OEA suppresses the expression of interleukin-6, interleukin-8, vascular adhesion molecule-1, and intercellular adhesion molecule-1 in TNFα-treated human umbilical vein endothelial cells [54]. LEA and SEA are another set of NAEs with anti-inflammatory roles. In mouse RAW264.7 macrophages, LEA suppressed LPS-induced expression of proinflammatory cytokines, and in a contact dermatitis animal model, applying LEA to affected ear skin ameliorated dermatitis and proinflammatory cytokine expression at inflamed sites [55]. SEA downregulates allergic inflammation in mouse skin [56]. Our observations together with these reports suggest that increased levels of NAEs in response to LPS challenge may suppress inflammatory decidual responses when exposed to an environmental insult. However, we cannot rule out the possibility that NAEs also have proinflammatory roles. Recent reports showed that plasma AEA levels increase with active labor in humans [57], and endocannabinoids promote production of PGs in gestational tissues in humans and mice [58, 59].

In the absence of LPS, metformin treatment had minimal impact on levels of NAEs and 2-monoacylglycerols. Metformin influenced NAE levels in LPS-injected *Faah*$^{-/-}$ decidual tissues. Normally, NAE levels are higher due to inefficient degradation in the absence of FAAH [18, 19]. The result that LPS injection further increased the levels of NAEs in *Faah*$^{-/-}$ tissues suggests that LPS may suppress alternative pathway(s) for NAE degradation or increase NAE

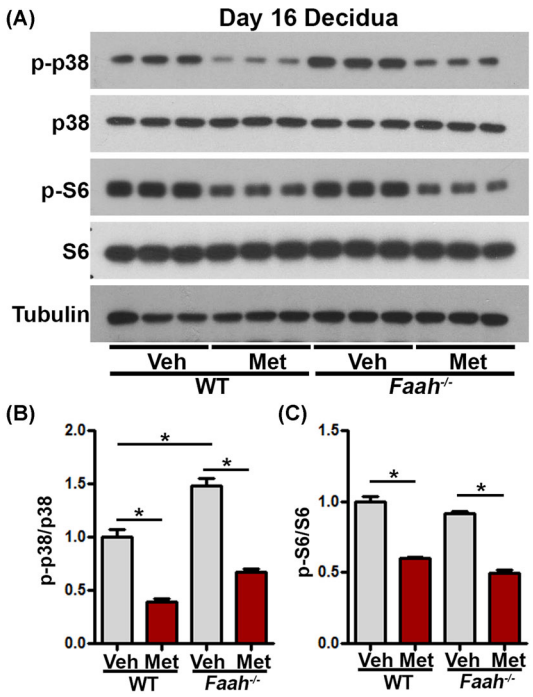

Figure 8. Metformin regulates decidual senescence by targeting mTORC1 and p38 pathways. (A) Western blotting of p-p38 and p-S6 in WT and *Faah*$^{-/-}$ decidual tissues on day 16. Band intensities quantification were shown in bar diagrams in panel (B). Western blotting results showed that p-p38 and p-S6 levels were decreased in metformin-treated WT and *Faah*$^{-/-}$ decidual tissues on day 16 of pregnancy. Veh, vehicle; Met, metformin. ($*P < 0.05$, Student *t*-tests).

synthesis. Under this condition, metformin can further increase NAEs levels, suggesting that metformin augments LPS's effects on NAE degradation or synthesis. However, metformin has limited impact on NAEs metabolism in the presence of FAAH.

Our results suggest that metformin prevents preterm birth without affecting PGs. There are two critical factors in preterm labor: (1) the strength and frequency of muscle contractions and (2) the strength of the placenta/fetus attachment with the maternal decidua. Prostaglandins are major players in determining the strength and frequency of myometrial contractions [60, 61]. Metformin treatment makes the anchoring of placenta/fetus attachment with maternal decidua much stronger by slowing the premature decidual senescence and thus ameliorates preterm birth in response to LPS.

On day 16 of pregnancy, *Prlr* is expressed in maternal decidual cells close to the myometrium (Figure 6); decidual cells in the same domain showed the highest intensity of SA-β-Gal staining, suggesting that decidual cells close to the myometrium undergo premature decidual senescence. Interestingly, our previous study also showed that p-P38 positive signals are present in the same domain [4]. All these results suggest that cells in the decidual basalis did not behave uniformly and could be classified into two layers: the decidual zone close to the placenta, which is penetrated by fetal trophoblasts, has little or no *Prlr* expression and reduced SA-β-Gal staining; the decidual zone close to the myometrium, which expresses *Prlr*, has a higher likelihood of undergoing cellular senescence. *Prlr*$^{-/-}$ females are infertile due to compromised ovulation, impaired fertilization, and implantation failure [62]. Since the formation of corpus luteum in *Prlr*$^{-/-}$ females is defective, we and others previously showed that implantation failure in *Prlr*$^{-/-}$ females is rescued by P_4 supplement, suggesting its critical role for ovarian function [63]. Our current study and our previous work [32] show that *Prlr* is also expressed in the decidua. *Prlr*$^{-/-}$ mice with continuous P_4 supplement have an increased number of resorption sites, emphasizing the significance of decidual *Prlr* in pregnancy. Although the function of decidual PRLR is not clear yet, the expression pattern of *Prlr* is a useful marker in outlining decidual senescent cells.

Decidual health is critical to parturition timing. The current study suggests a possible approach to strengthen pregnant females' resistance to environmental insults during pregnancy by restraining the decidual aging process. Metformin treatment suppressing both mTORC1 and p38 signaling is an effective way to rescue premature decidual aging in *Faah*$^{-/-}$ decidua. These findings provide new insights to improve maternal and fetal health during pregnancy and encourage further investigation in the field.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Weights of implantation sites of WT and *Faah*$^{-/-}$ mice with or without metformin treatment on day 16 of pregnancy (mean ± SEM). Veh, vehicle; Met, metformin.
Supplemental Table S1. LPS-induced pregnancy failure is alleviated by metformin treatment.

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Authors' contribution

XS, AT, WD, EL, and HBB have contributed to the experimental design and acquisition, and analysis of data. XS, HBB, and SKD have contributed to the interpretation of data and manuscript writing.

Conflict of Interest: The authors have declared that no conflict of interest exists.

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