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EMMPRIN-Mediated Induction of Uterine and Vascular Matrix Metalloproteinases during Pregnancy and in Response to Estrogen and Progesterone

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Abstract

Pregnancy is associated with uteroplacental and vascular remodeling in order to adapt for the growing fetus and the hemodynamic changes in the maternal circulation. We have previously shown upregulation of uterine matrix metalloproteinases (MMPs) during pregnancy. Whether pregnancy-associated changes in MMPs are localized to the uterus or are generalized in feto-placental and maternal circulation is unclear. Also, the mechanisms causing the changes in uteroplacental and vascular MMPs during pregnancy are unclear. MMPs expression, activity and tissue distribution were measured in uterus, placenta and aorta of virgin, mid-pregnant (mid-Preg) and late pregnant (late-Preg) rats. Western blots and gelatin zymography revealed increases in MMP-2 and -9 in uterus and aorta of late-Preg compared with virgin and mid-Preg rats. In contrast, MMP-2 and -9 were decreased in placenta of late-Preg versus mid-Preg rats. Extracellular MMP inducer (EMMPRIN) was increased in uterus and aorta of pregnant rats, but was less in placenta of late-Preg than mid-Preg rats. Prolonged treatment of uterus or aorta of virgin rats with 17β-estradiol and progesterone increased the amount of EMMPRIN, MMP-2 and -9, and the sex hormone-induced increases in MMPs were prevented by EMMPRIN neutralizing antibody. Immunohistochemistry revealed that MMP-2 and -9 and EMMPRIN increased in uterus and aorta of pregnant rats, but decreased in placenta of late-Preg versus mid-Preg rats. Thus pregnancy-associated upregulation of uterine MMPs is paralleled by increased vascular MMPs, and both are mediated by EMMPRIN and induced by estrogen and progesterone, suggesting similar role of MMPs in uterine and vascular tissue remodeling and function during pregnancy. The decreased MMPs and EMMPRIN in placenta of late-Preg rats suggests reduced role of MMPs in feto-placental circulation during late pregnancy.

Keywords

myometrium; placenta; blood vessels; sex hormones

INTRODUCTION

Normal pregnancy is associated with physiological changes in the uterus, placenta and the maternal circulation. The pregnant uterus undergoes hypertrophy and distension in order to provide sufficient space for the developing fetus. The placenta and spiral arterioles undergo remodeling in order to maintain sufficient blood and nutrient supply to the growing fetus. Also, during pregnancy, significant hemodynamic changes occur in the maternal circulation

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including increased heart rate, plasma volume and cardiac output, and concomitant decrease in vascular resistance in order to maintain sufficient blood supply to different organs and to relieve the burden on the maternal heart [1, 2]. These pregnancy-associated changes in the uteroplacental tissues and the maternal circulation involve significant structural remodeling and functional changes in the uterus, placenta and the maternal vasculature [3, 4].

Matrix metalloproteinases (MMPs) are a family of structurally-related proteases that degrade the extracellular matrix (ECM) and connective tissue proteins [5, 6]. Pro-MMPs are cleaved into active forms that promote degradation of ECM proteins [5, 6]. MMPs include collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs with different tissue expression, distribution and substrate specificity [6]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are expressed in the bovine uterus [7, 8] and play a role in the endometrial tissue remodeling during normal estrous and menstrual cycles and pregnancy [9–11]. Also, we have previously shown an upregulation of MMP-2 and -9 in the myometrium of late pregnant rats, and suggested a role of MMPs in uterine tissue remodeling [12]. Plasma levels of MMPs also increase during pregnancy [13], suggesting additional sources of MMPs, and a role for MMPs in other maternal tissues. However, whether the pregnancy-associated changes in MMPs are localized to the uterus or are part of generalized changes in MMPs in the uteroplacental and vascular tissues is unclear. Also, the mechanisms causing the MMPs changes during pregnancy are not understood.

Hormonal changes during pregnancy could affect the uterine structure and function. Plasma estrogen (E2) and progesterone (P4) markedly increase [14, 15], and could contribute to the changes in uterine contractility during pregnancy [12, 16]. Also, uterine tissue remodeling and endometrium shedding during menstruation could involve E2-induced changes in MMPs activity [17–19]. P4 may also control the formation and maintenance of endometrial lesions partly by affecting MMPs expression [20]. In addition to its effects on the uterus, E2 promotes endothelium-dependent vascular relaxation by increasing the release of nitric oxide, prostacyclin and hyperpolarizing factor [21]. E2 also causes relaxation of endothelium-denuded vessels by inhibiting the mechanisms of vascular smooth muscle (VSM) contraction including $[Ca^{2+}]_i$ and protein kinase C [22–25]. E2 may have additional effects on the vascular cytoskeleton, ECM, lipid profile and inflammatory response [21]. P4 can also cause vasodilation by mechanisms similar to E2 [21, 25, 26]. Some of the vascular effect of E2 could involve MMPs. In cultured human coronary artery and umbilical artery VSM cells, E2 causes dose-dependent increases in MMP-2 levels in culture media [27]. Also, the activity of MMP-2 and -9 is higher in pregnant than non-pregnant bitches, and serum MMP activity correlates with the serum levels of E2 [28], supporting a relationship between sex hormones and MMPs expression/activity.

Another factor that could affect MMPs expression/activity is extracellular MMP inducer (EMMPRIN, also known as CD147, Basigin, BSG). EMMPRIN is a widely expressed membrane protein of the immunoglobulin superfamily [29], and has been implicated in tissue remodeling [30] and various pathological processes including cancer [29], rheumatoid arthritis [31], heart failure [32], and atherosclerosis [33]. EMMPRIN stimulates the production of MMP-1, -2, -3, and -9 [34], and may mediate MMP regulation in tumor and endothelial cells [35].

The present study was designed to test the hypothesis that pregnancy is associated with parallel changes in uteroplacental and vascular MMPs expression/activity and to investigate the role of sex hormones and EMMPRIN in the pregnancy-related changes in MMPs. We used the uterus, placenta and aorta from virgin and pregnant rats to investigate whether: 1) Pregnancy is associated with parallel changes in MMPs expression/activity in uteroplacental and vascular tissues, 2) Pregnancy is associated with parallel changes in uteroplacental and

vascular EMMPRIN, and 3) The pregnancy-related changes in uteroplacental and vascular MMPs and EMMPRIN are induced by the sex hormones E2 and P4.

METHODS

Animals and Tissue Preparation

Virgin, mid-pregnant (day-12 of gestation, mid-Preg) and late-pregnant (day-19 of gestation, late-Preg) Sprague-Dawley rats (12 wk of age, 250 to 350g weight) were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed in the animal facility and maintained on *ad libitum* standard rat chow and tap water in 12-hr light-dark cycle. All experiments on virgin rats were conducted during estrus in order to control for reproductive cycle and endocrine confounders. The estrous cycle was determined by taking a vaginal smear with a pasteur pipette daily in the morning [36]. An estrus smear primarily consisted of anucleated cornified squamous cells, and this was confirmed prior to all experimentations. Virgin, mid-Preg and late-Preg rats were euthanized by inhalation of CO_2. The abdominal and thoracic cavities were opened, and the uterus and thoracic aorta were rapidly excised, and placed in Krebs solution. With the aid of a dissection microscope, the virgin uterus was cut into 3 mm wide rings. The pregnant uterus was cut open and the placentae and pups were removed. The uterus was then portioned along its longitudinal axis into 5 mm long 5 mm wide strips. We did not attempt to separate the circular muscle layer from the longitudinal muscle or to remove the endometrium lining from the uterine strip. The placenta was cut into 5 mm × 5 mm strips. The aorta was cleaned of connective and adipose tissue, and portioned into 5 mm × 5 mm rings. Experiments were performed on 8 to 12 uterine, placental or aortic segments from each rat, and cumulative data from 4 to 12 rats were collected. We previously examined the effects of 17β-estradiol (E2) and progesterone (P4), both separately and combined, on MMP-2 and-9 expression in rat uterus [12]. We found that E2 alone or P4 alone caused similar increases in the amount of MMP-2 and MMP-9, and that the effects of combined E2+P4 were not significantly different form those of E2 or P4 alone. Therefore, in order to examine the effects of sex hormones, in some of the present experiments uterine and aortic strips from virgin rats were incubated with E2+P4 (10^{-7} M; Sigma-Aldrich, St. Louis, MO, USA) for 24 hr in tissue culture medium. Control sex hormones experiments included treatment of virgin uterus with E2 alone, P4 alone or the inactive 17α-estradiol (10^{-7} M) for 24 hr. Also, to test if the effects of sex hormones involve activation of EMMPRIN, the experiments were performed in the absence or presence of neutralizing EMMPRIN antibody (1:200, Santa Cruz Biotechnology, Dallas, TX). Control antibody experiments included testing the effects of treatment of virgin uterus for 24 hr with EMMPRIN antibody alone or with E2+P4 plus heat-inactivated EMMPRIN antibody (Repeated 10 times heating at 75°C for 30 sec and cooling at 4°C for 1 min) [37]. All procedures followed the guidelines of the Institutional Animal Care and Use Committee at Harvard Medical School.

Western Blots

Uterine, placental and aortic strips were homogenized in a homogenization buffer containing 20 mM 3-[N-morpholino] propane sulfonic acid (MOPS), 4% SDS, 10% glycerol, 10 mM dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 μM leupeptin, 5.5 μM pepstatin, 2.15 μM aprotinin and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, pH 7.4, using a 2-ml tight-fitting homogenizer (Kontes Glass Co., Vineland, NJ, USA). The homogenate was centrifuged at 10,000 g for 5 min. The supernatant was collected, and protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein extracts (20 μg) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 min, and size fractionated by electrophoresis on 8% SDS-polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane by electroblotting. Membranes were

incubated in 5% nonfat dry milk in TBS-Tween for 1 h and then overnight at 4°C with polyclonal rabbit anti-MMP-2 (SC-10736, 1:1000), anti-MMP-9 (SC-10737, 1:1000) or anti-EMMPRIN antibody (SC-25531, 1:200) (Santa Cruz Biotechnology). Negative control experiments were performed with the omission of primary antibody, and exhibited no detectable immunoreactive bands. Membranes were washed 5 times for 15 min each in TBS-Tween then incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 1.5 hr, and the immunoreactive bands were detected using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The blots were subsequently reprobed for β-actin (1:2000). Data were analyzed by optical densitometry and ImageJ software (National Institutes of Health, Bethesda, MD, USA). The densitometry values represented the pixel intensity normalized to β-actin to correct for loading as previously described [12].

Gelatin Zymography

Uterine, placental and aortic tissue homogenate (without dithiothreitol) was subjected to electrophoresis on 8% SDS polyacrylamide gel containing 0.1% gelatin (Sigma Aldrich, St. Louis, MO). The gel was then incubated in a zymogram renaturing buffer containing Triton X-100 2.5% (v/v) in deionized water with gentle agitation for 30 min at room temperature. The gel was then equilibrated in a zymogram developing buffer containing 50 mM Tris-base, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij35 (Fisher Scientific, Pittsburgh, PA), and 1 μM $ZnCl_2$ (Sigma) for 30 min at room temperature, then incubated in the zymogram developing buffer at 37°C for 24 hr. The gel was stained with 0.5% coomassie blue R-250 (Sigma) for 30 min, then destained with an appropriate coomassie R-250 destaining solution (methanol : acetic acid : water = 50 : 10 : 40). Areas corresponding to MMP-2 and MMP-9 activity appeared as clear bands against a dark blue background. The clear bands were analyzed by optical densitometry and ImageJ software, and the integrated protease activity density was recorded as pixel intensity x mm^2, and then normalized to actin intensity as previously described [12].

Immunohistochemistry

To determine the tissue distribution of MMP-2, MMP-9 and EMMPRIN in the uterus, placenta and aorta of virgin, mid-Preg and late-Preg rats cryosections of the uterus, placenta and aorta (6 μm thick) were thawed and fixed in ice-cold acetone for 10 min. Endogenous peroxidase was quenched in 1.5% H_2O_2 solution for 10 min, and nonspecific binding was blocked in 10% horse serum. Tissue sections were incubated with polyclonal MMP-2, MMP-9 or EMMPRIN antibody (1:500, Santa Cruz Biotechnology). After being rinsed with PBS, tissue sections were incubated with biotinylated anti-rabbit secondary antibody, rinsed with PBS, and then incubated with avidin-labeled peroxidase (VectaStain Elite ABC Kit, Vector Laboratories, Burlingame, CA). Positive labeling was visualized using diaminobenzadine and appeared as brown spots. Negative control slides were run in the absence of primary antibody, and showed no detectable immunostaining. Specimens were counterstained with hematoxylin for 30 seconds, rinsed with PBS, topped with cytoseal 60, then covered with slide coverslips. Images were acquired on a Nikon microscope with digital camera mount and analyzed using ImageJ software (NIH). The total number of pixels in the tissue section image was defined, then the number of brown spots (pixels) was counted and presented as % of total pixels [38].

Solutions and Drugs

Krebs' solution was used for tissue dissection and contained (in mM): 120 NaCl, 5.9 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 dextrose, 2.5 $CaCl_2$, 1.2 $MgCl_2$, at pH 7.4, and bubbled with 95% O_2 and 5% CO_2. Tissue culture medium was used to pretreat the tissues with sex hormones overnight and was composed of Minimum Essential Medium supplemented with

penicillin, streptomycin, and amphotericin B (Gibco/Invitrogen, Grand Island, NY). Phosphate buffered saline (PBS) was used to rinse the slides in the immunohistochemistry experiments and contained (in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 KH_2PO_4, at pH 7.4. Stock solutions of E2 and P4 (10^{-3} M; Sigma) were prepared in ethanol. All other chemicals were of reagent grade or better.

Statistical Analysis

Experiments were conducted on uterus, placenta and aorta isolated from 4 to 12 different rats per group (virgin, mid-Preg, late-Preg), and cumulative data were presented as means ±SEM, with the "n" value representing the number of rats per group. Data were analyzed and plotted using Prism (v.5.01; GraphPad software, San Diego, CA, USA). Data were first analyzed using a one way ANOVA. When a statistical difference was observed, data were further analyzed using Bonferroni's *post hoc* correction for multiple comparisons. Student's unpaired *t-test* was used for comparison of two means. Differences were statistically significant when $P < 0.05$.

RESULTS

We first measured the changes in the protein amount of uterine, placental and vascular MMPs during pregnancy. Western blot analysis revealed immunoreactive bands corresponding to pro-MMP-2 (72 kDa), MMP-2 (63 kDa), pro-MMP-9 (92 kDa) and MMP-9 (82 kDa) in the uterus and aorta of virgin, mid-Preg and late-Preg rats and the placenta of mid-Preg and late-Preg rats (Fig. 1). The protein amount of pro-MMP-2 and pro-MMP-9 were enhanced in the uterus of mid-Preg rats, and the amount of pro-MMP-2, MMP-2, pro-MMP9 and MMP-9 were significantly enhanced in the uterus of late-Preg compared with virgin rats (Fig. 1A). Along the same lines, the amount of pro-MMP-2, MMP-2 and pro-MMP-9 were greater in the aorta of mid-Preg rats, and the amount of pro-MMP-2, MMP-2, pro-MMP9 and MMP-9 were significantly enhanced in the aorta of late-Preg compared with virgin rats (Fig. 1C). In contrast, the amount of pro-MMP-2 and MMP-9 were reduced, and only the amount of pro-MMP-9 was increased in the placenta of late-Preg compared with mid-Preg rats (Fig. 1B).

Gelatin zymography analysis using uterine tissue homogenate of late-Preg rats revealed proteolytic bands corresponding to pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9 (Fig. 2). The intensity of the bands was dependent on the amount of loaded protein and showed concentration-dependent increases from 0.1 to 0.5 μg protein, and clearly discernable bands at 1 μg protein. Further increases in the loaded protein to 2, 5, and 10 μg showed further proteolysis, but the specific MMP bands became difficult to distinguish (Fig. 2A). Similarly, gelatin zymography using tissue homogenate from the placenta (Fig. 2B) and aorta (Fig. 2C) of late-Preg rats showed proteolytic bands corresponding to pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9, with the intensity of the bands dependent on the amount of loaded protein and clearly discernable bands at 1 μg protein. Therefore, all further gelatin zymography experiments comparing uterine, placental and aortic tissue homogenate of virgin and pregnant rats were performed using 1 μg protein for loading.

Gelatin zymography analysis revealed that MMP-2 gelatinase activity was enhanced in the uterus of mid-Preg rats, and that MMP-2, pro-MMP-9 and MMP-9 activity was enhanced in the uterus of late-Preg compared with virgin rats (Fig. 3A). Similarly, pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 protease activity was enhanced in the aorta of late-Preg compared with virgin rats (Fig. 3C). In contrast, pro-MMP-2 and pro-MMP-9 activity was reduced in the placenta of late-Preg compared with mid-Preg rats (Fig. 3B).

To test the effects of sex hormones, the protein amount of pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 was significantly greater in virgin uterus pretreated with E2+P4 for 24 hr compared with control nontreated uterus (Fig. 4A). In virgin uterus pretreated with E2+P4 in the presence of neutralizing EMMPRIN antibody the increases in the protein amount of pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 were reversed (Fig. 4A). Similarly, pretreatment of the aorta of virgin rat with E2+P4 was associated with increases the amount of pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9, and these increases were reversed in tissues pretreated with E2+P4 plus neutralizing EMMPRIN antibody (Fig. 4B). Sex hormone control immunoblot experiments demonstrated that treatment of virgin uterus with E2 alone or P4 alone for 24 hr caused increases in the amount of MMP-2 and MMP-9 that were not significantly different from those in virgin uterus treated with E2+P4 (Fig. 5A). On the other hand, treatment of virgin uterus with inactive 17α-estradiol did not cause any significant changes in the amount of MMP-2 or MMP-9 (Fig. 5B). EMMPRIN antibody control immunoblot experiments demonstrated that treatment of virgin uterus with EMMPRIN antibody alone for 24 hr did not cause any significant changes in the amount of MMP-2 or MMP-9 (Fig. 5B). Also, in virgin uterus treated for 24 hr with E2+P4 plus heat-inactivated EMMPRIN antibody, the increases in the amount of MMP-2 and MMP-9 were not significantly different from those observed in tissues treated with E2+P4 (Fig. 5A).

In some Western blots, particularly those of the uterus, additional protein bands for MMP-9 (Fig. 1A), pro-MMP-9 (Fig. 5A) and MMP-2 (Fig. 4A, 5A, 5B) were observed, possibly due to some endogenous proteolytic activity that is highly resistant to the anti-protease cocktail used in the specimen homogenization buffer. Another possibility is that these MMPs may undergo post-translational modification (acetylation, methylation, myristoylation, phosphorylation, glycosylation, etc) [39–41] in the uterus, which could be manifested as different forms of the same protein.

Gelatin zymography revealed an increase in pro-MMP-2, MMP-2 and MMP-9 activity in virgin uterus pretreated with E2+P4 compared to control nontreated uterus (Fig. 6A). The increase in pro-MMP-2, MMP-2 and MMP-9 gelatinase activity was reversed in virgin uterus pretreated with E2+P4 in the presence of EMMPRIN antibody (Fig. 6A). Similarly, pretreatment of the aorta of virgin rat with E2+P4 was associated with increases in pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 gelatinase activity, and these increases were reversed in tissues pretreated with E2+P4 plus neutralizing EMMPRIN antibody (Fig. 6B).

Western blot analysis revealed an immunoreactive band corresponding to EMMPRIN at 55 kDa in the uterus and aorta of virgin, mid-Preg and late-Preg rats and in the placenta of mid-Preg and late-Preg rats (Fig. 7). The protein amount of EMMPRIN was greater in the uterus of late-Preg compared with virgin rats (Fig. 7A). The protein amount of EMMPRIN was also enhanced in virgin uterus pretreated with E2+P4 for 24 hr compared with non-treated virgin uterus (Fig. 7A). Similarly, the amount of EMMPRIN was enhanced in the aorta of mid-Preg, and further enhanced in the aorta of late-Preg compared with virgin rats (Fig. 7C). Also, the protein amount of EMMPRIN was enhanced in the aorta of virgin rats pretreated with E2+P4 for 24 hr compared with the non-treated control aorta of virgin rats (Fig. 7C). In contrast, the amount of EMMPRIN was reduced in the placenta of late-Preg compared with mid-Preg rats (Fig. 7B).

Immunohistochemical analysis revealed detectable MMP-2, MMP-9 and EMMPRIN immunostaining in uterine tissue sections from virgin and pregnant rats (Fig. 8). Quantitative image analysis demonstrated that the brown staining for MMP-2, MMP-9 and EMMPRIN was greater in the uterus of mid-Preg rats and further enhanced in late-Preg compared with virgin rats. Similarly, the brown immunostaining for MMP-2, MMP-9 and EMMPRIN was greater in aortic tissue sections of mid-Preg rats and further enhanced in

late-Preg compared with virgin rats (Fig. 9). In contrast, immunohistochemistry experiments revealed a decrease in MMP-2, MMP-9 and EMMPRIN immunostaining in the placenta of late-Preg compared with mid-Preg rats (Fig. 10).

DISCUSSION

The main findings of the present study are: 1) MMP-2 and -9 expression/activity are upregulated in the uterus and aorta during mid and late pregnancy, 2) The sex hormones E2 and P4 enhance MMP-2 and -9 expression/activity in the uterus and aorta, 3) The sex hormone-induced increases in MMPs expression/activity are blocked by EMMPRIN antibody, 4) EMMPRIN is upregulated in the uterus and aorta of pregnant rats, and in uterus and aorta of virgin rats treated with sex hormones, and 5) MMP-2 and -9 expression/activity and EMMPRIN expression are downregulated in the placenta of late-Preg compared with mid-Preg rats.

During normal pregnancy the balance between uterine contraction and relaxation is tightly-regulated in order to maintain healthy and full-term pregnancy. The mechanisms responsible for maintaining the uterus in a quiescent relaxed state are important because changes in these mechanisms could cause preterm uterine contraction with untoward outcome to the pregnancy and premature newborn. MMP-2 and -9 are expressed in the uterus, and could play a role in the endometrial tissue remodeling during normal estrous and menstrual cycles and pregnancy [9, 19, 42], as well as in the endometrial changes associated with menstrual disorders and endometriosis [43, 44]. MMP-2 and -9 have also been localized in the bovine endometrium and myometrium during pregnancy [8, 9, 42] and in uterine natural killer cells in early human pregnancy [45]. MMP-2 and -9 could also play a role in degradation of uterine proteins and remodeling of the cervical ECM [46]. We have recently shown that pregnancy is associated with decreased uterine contraction and increased expression and activity of MMP-2 and -9 [12]. Also, we have previously tested if MMPs are involved in the reduced uterine contraction during pregnancy and reported that MMP-2 and -9 cause relaxation of precontracted uterus, and that MMP inhibitors enhance contraction of the pregnant uterus [12]. The present study supports that uterine MMP-2 and -9 are upregulated during the course of pregnancy because: 1) The protein amount of MMP-2 and -9 as measured by Western blots were enhanced in mid-Preg rats and further enhanced in late-Preg compared with virgin rats, 2) MMP-2 and -9 immunostaining was greater in tissue sections of late-Preg compared with virgin and mid-Preg rats, and 3) MMP-2 and -9 gelatinase activity was enhanced in late-Preg compared with virgin rats. These findings are consistent with our previous report [12] and support an increase in MMP-2 and -9 expression/activity during pregnancy. Together with our previous observation that MMP-2 and -9 cause relaxation of precontracted uterus, the present observations supports a role of MMP-2 and -9 in maintaining the uterus in a quiescent stage to accommodate fetal growth.

We examined the possible factors and signaling mechanisms that could cause the pregnancy-associated changes in uterine MMPs. During pregnancy the uterus is exposed to hormonal changes that could influence the myometrium structure and mechanical properties. Pregnancy is associated with increased plasma levels of E2 and P4 [14]. Also, the endometrial cellular concentrations of E2 and P4 are positively correlated with the plasma levels of E2 during the proliferative phase of the menstrual cycle, and a large concentration of P4 receptors characterizes the early pregnancy endometrium [47]. E2 and P4 are important regulators of myometrial growth and contractility, and these effects may involve both genomic and non-genomic mechanisms. It is generally thought that E2 augments myometrial contractility and excitability, while P4 sustains the pregnant state and promotes myometrial relaxation [48]. While E2 may have a stimulatory effect on uterine contractility at the time of parturition, these effects appear to be neutralized during the course of

pregnancy, partly due to decreased uterine expression of estrogen receptors [48], or could involve E2-induced expression of other factors that decrease myometrial contractility such as MMPs [12]. For instance, the last phase of pregnancy in rats shows a correlation between the plasma level of E2 and an increase in uterine contractility and the density of α1-adrenergic receptors [15]. On the other hand, uterine contraction is reduced in day-12 and day-19 of gestation in rats [12]. Also, E2 causes rapid non-genomic relaxation of spontaneous and depolarization-induced contraction of uterine-rings of non-pregnant rat [49]. We have also shown that prolonged treatment of virgin rat uterus with E2 is associated with decreased uterine contraction [12]. With regard to P4, in addition to its relaxing effects during pregnancy, it also decreases uterine contractility and promotes relaxation during the luteal phase, a phenomenon crucial for maximizing uterine receptivity to embryo implantation during *in vitro* fertilization cycles [50]. For example, in women undergoing *in vitro* fertilization, administration of vaginal P4 gel starting 2 days before embryo transfer induces a significant reduction in uterine contraction frequency at the time of embryo transfer, and this uterine relaxation may propitiate embryo permanence in the endometrial cavity and therefore assist implantation [51]. Other studies have examined the long-term effects of E2 and P4 replacement on uterine contractility in women aged 25 to 41. Following 2 weeks of transdermal E2 to duplicate the pattern of E2 production normally seen in the late follicular phase, vaginal administration of sustained release P4 gel every two days from cycle day 15, was associated with time-dependent decrease in uterine contractility over the course of 5 days (Cycle days 15–20) [52]. MMPs may also play a major role in uterine tissue remodeling, and some of the effects of sex hormones on the uterus may involve MMPs. In the pregnant bitches, significant correlations are observed between the elevated serum MMP-2 and -9 activity and the elevated serum levels of E2 and P4 [28]. Also, uterine tissue remodeling and endometrium shedding during menstruation may involve E2-induced changes in MMPs activity [17–19]. P4 may also regulate important factors in the formation and maintenance of endometrial lesions partly by affecting MMPs expression [20]. In mouse uterus, E2 alone or in combination with P4 increases MMP-9 activity [19]. The present study demonstrates that treatment of virgin uterus with E2+P4 causes increases in MMP-2 and -9 protein amount and gelatinase activity. These findings are consistent with reports that E2 increases MMP-2 expression in the immature rat uterus [17], and suggest that E2 and P4 regulate uterine MMPs expression/activity during uterine tissue remodeling. We should note that the effects of sex hormones were tested on tissues from virgin female rats in estrus. Testing the effects of sex hormones on tissues isolated from ovariectomized virgin rats could provide even a sharper and clearer response and should be examined in future experiments.

The mechanism via which sex hormones enhance uterine MMP expression/activity may involve one of different pathways. E2 may stimulate MMP production and activity via estrogen receptor-mediated MEK/ERK (mitogen-activated protein kinase, MAPK) pathway [53]. Also, while E2 may have anti-inflammatory effects [54–56], some studies suggest E2-mediated increase in inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin 6 (IL-6), which could in turn increase MMPs expression/activity [19, 53, 57–59]. Sex hormones may also enhance MMPs expression via EMMPRIN, a known inducer of MMPs [8, 10, 60, 61]. The present study suggests a role of EMMPRIN in the pregnancy-associated and sex hormones-induced increase in MMP-2 and -9 expression/activity because: 1) Uterine EMMPRIN expression as indicated by Western blots and immunohistochemistry was increased during the course of pregnancy, 2) EMMPRIN expression was increased in virgin uterus pretreated with E2+P4, and 3) The E2+P4 induced increase in MMP-2 and -9 expression and activity were blocked by EMMPRIN antibody. These findings are consistent with reports that endometrial expression of EMMPRIN and MMPs is regulated by ovarian sex hormones in cycling baboons and that their expression patterns are dysregulated in endometriotic baboons [62]. Our findings are also in agreement with reports that EMMPRIN expression increases in the bovine endometrium during estrous

cycle and early gestation [10]. Together these findings support a role of EMMPRIN in inducing the changes in uterine MMPs expression during pregnancy.

Because pregnancy is associated with significant changes in the maternal hemodynamics, we tested whether the changes in MMPs in the uterus are a part of a global change in MMPs in the maternal tissues and the systemic vessels. Similar to our observations in the uterus, our Western blots, immunohistochemistry and gelatinase activity experiments showed upregulation of MMP-2 and -9 expression and activity in the aorta during the course pregnancy. Our data also supports a role of EMMPRIN as an inducer of the changes in aortic MMPs during pregnancy because: 1) EMMPRIN expression was increased in the aorta of late-Preg compared with virgin and mid-Preg rats as well as in the aorta of virgin rats pretreated with E2+P4, 2) Consistent with the report that E2 enhances the release of MMP-2 from human VSM cells [27], we found that E2+P4 enhanced MMP-2 and -9 expression and activity in the aorta of virgin rats, and 3) The sex hormone-induced increases in aortic MMP-2 and -9 expression and activity were blocked by EMMPRIN neutralizing antibody.

Normal pregnancy is associated with marked vasodilation of the maternal renal and systemic vessels [63], and reduced vascular contraction mechanisms [64, 65]. The pregnancy-associated reduction in vascular contraction could be related to increased plasma levels of the sex hormones E2 and P4 [14]. E2 causes VSM relaxation in rat aorta and uterine artery [22, 66]. Also, P4 inhibits contraction of rat blood vessels [22]. The pregnancy-associated increases in vascular MMPs may play a role in vascular remodeling, angiogenesis, and the systemic changes in blood vessels. MMPs can enhance angiogenesis by helping to detach pericytes from the vessels undergoing angiogenesis, releasing ECM-bound angiogenic growth factors, exposing cryptic proangiogenic integrin binding sites in the ECM, generating promigratory ECM component fragments, and cleaving endothelial cell-cell adhesions [67]. Also, trophoblast- and VSM-derived MMP-12 mediates elastolysis and uterine spiral artery remodeling during pregnancy [68]. We have previously shown that MMP-2 and -9 cause relaxation of precontracted rat aorta [69] and inferior vena cava [70, 71]. The present observation of increased expression and activity of MMPs in the aorta together with previous reports of MMPs effects on vascular remodeling and contraction mechanisms are consistent with a possible role of increased MMPs in the reduced vascular contraction and systemic vasodilation observed during pregnancy.

In contrast with our observations in the uterus and aorta, MMP-2 and -9 expression did not increase in the placenta during the course of pregnancy. Instead, placental MMP-2 and -9 showed a decrease in expression and activity in late compared with mid-pregnancy. MMPs are involved in placental remodeling during pregnancy [72], and the expression of MMP-2, MMP-14 and EMMPRIN has been shown to be increased in the bovine placenta during late gestation [60]. This is different from our observed decrease in MMP-2 and -9 in rat placenta during late pregnancy, and could be due to species differences in the MMPs regulation mechanisms. Other studies have shown an increase in MMP-2 and -9 in the placenta of diabetic rats at mid-gestation [72], but did not follow the changes in MMPs during late-gestation. Importantly, our observed pregnancy-associated decrease in placental MMPs expression was paralleled by a decrease in placental EMMPRIN expression in late-Preg compared with mid-Preg rats, supporting a role of EMMPRIN as a critical inducer of MMPs in various tissues during pregnancy. The causes of the observed decrease in placental MMPs and EMMPRIN in late pregnancy are unclear at the present time, but could be related to the capacity of the placenta to produce MMPs or the role of MMPs during the various stages of pregnancy. Plasma MMP-2 and -9 levels have been shown to increase in pregnant bitches [28], and the placenta could be a potential source of MMPs with finite capacity particularly during the late stages of pregnancy. Also, most of the placental remodeling takes place

during the peri-implantation period and during fetal and organ development in early and mid-gestation, and further placental remodeling may not be needed during late pregnancy. In this respect, it will be useful to test the effects of E2 and P4 treatment of mid-Preg placenta on the expression of MMPs and EMMPRIN, and see if this mimics their expression during late pregnancy and should be examined in future experiments.

One limitation of the present study is that it focused on the changes in MMP-2 and -9 in the uterus, placenta and aorta during pregnancy, and the results should not minimize possible involvement of other members of the MMPs family. Also, the changes in uterine, placental and aortic MMPs and EMMPRIN were measured at two time points, namely day 12 and day 19 of gestation, and the progressive changes during the course of pregnancy and reversal of these changes in the postpartum period need to be examined. We should note that the observed decrease in uterine MMPs expression has been correlated with our previously reported decrease in uterine contraction on day-19 of pregnancy [12]. However, other studies have shown that the contractility of rat uterus and the mRNA expression of contractile agonists such as oxytocin, prostaglandin 2α, endothelin-1 and their receptors are induced after gestational day 20 [73], and that the last phase of pregnancy in rats shows an increase in uterine contractility and the density of α1-adrenergic receptors [15], further highlighting the importance of measuring MMPs and EMMPRIN during the different stages of pregnancy. Finally, we measured MMPs and EMMPRIN during normal pregnancy and the changes in the expression/activity of MMPs and EMMPRIN and their role in pathological uterine, placental and vascular remodeling in pregnancy-associated disorders such as hypertension-in-pregnancy and preeclampsia need to be examined.

In conclusion, the pregnancy-associated upregulation of uterine MMPs is paralleled by increased expression and activity of vascular MMPs, and both appear to be mediated by EMMPRIN and induced by the female sex hormones E2 and P4. The pregnancy-associated parallel changes in uterine and aortic MMPs in expression/activity are consistent with the previously reported similar role of MMPs in uterine and vascular tissue relaxation and remodeling. The decreased MMPs expression and activity and reduced EMMPRIN expression in the placenta of late-Preg rats suggest reduced role of MMPs in the feto-placental circulation during late pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

E2	17β-estradiol
ECM	extracellular matrix
EMMPRIN	extracellular matrix metalloproteinase inducer
late-Preg	late pregnant

mid-Preg	mid-pregnant
MMP	matrix metalloproteinase
P4	progesterone
VSM	vascular smooth muscle

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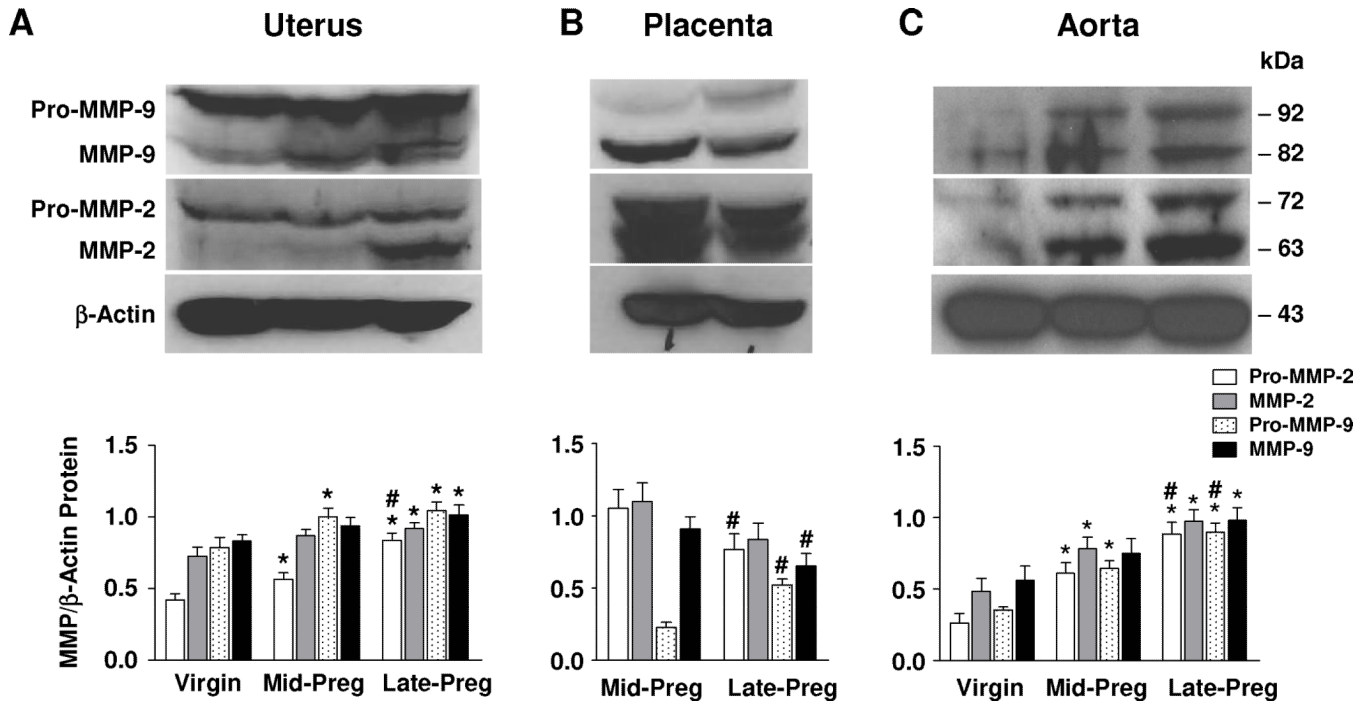

Fig. 1.

Protein amount of uterine, placental and aortic MMP-2 and MMP-9 during pregnancy. Tissue homogenates of the uterus, placenta, and aorta of virgin (A), mid-Preg (B) and late-Preg rats (C) were prepared for Western blot analysis using antibodies to MMP-2 (1:1000) and MMP-9 (1:1000). Immunoreactive bands corresponding to pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n = 4–12/group. * $P<0.05$ from corresponding measurements in virgin rats. # $P<0.05$ from corresponding measurements in mid-Preg rats.

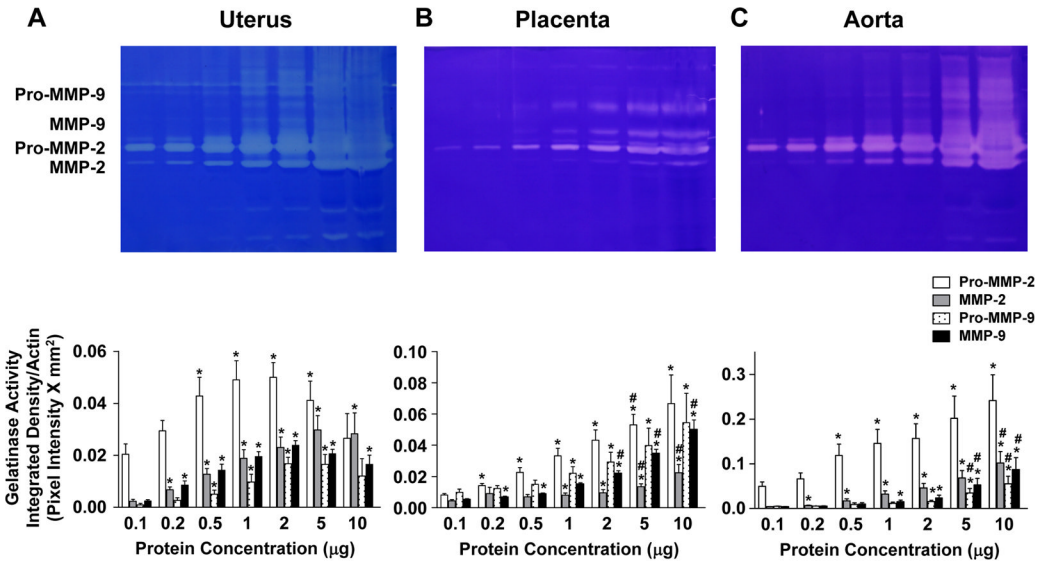

Fig. 2.

Concentration-dependent MMP-2 and MMP-9 gelatinase activity in uterus, placenta and aorta of late-Preg rats. Uterine (A), placental (B) and aortic tissue strips (C) from late-Preg rats were homogenized and prepared for gelatin zymography analysis using different concentrations of loaded protein (0.1 – 10 μg). The densitometry values of the proteolytic bands was presented as pixel intensity x mm^2. Bar graphs represent means±SEM, n = 4–12/group. * $P<0.05$ compared with corresponding measurements at 0.1 μg concentration. # $P<0.05$ compared with corresponding measurements at 1 μg concentration.

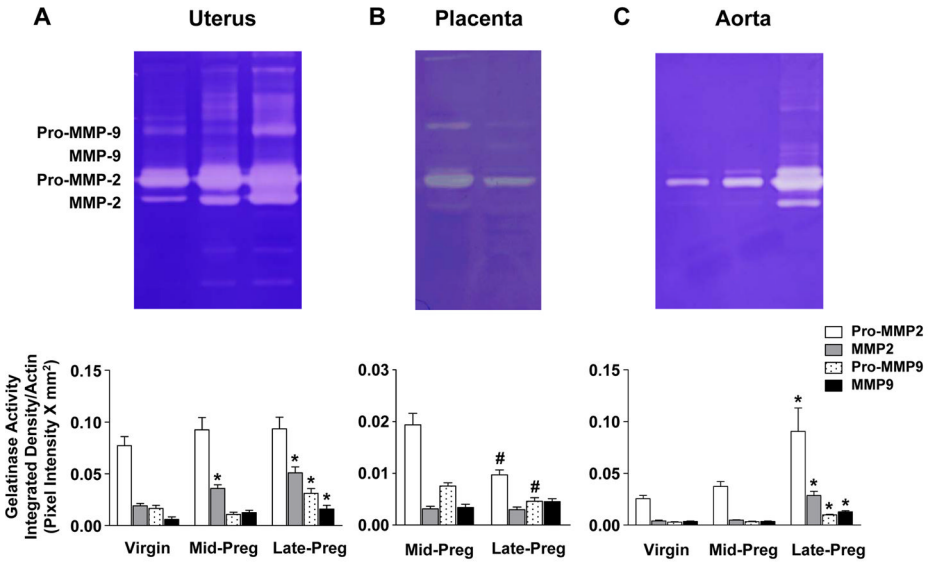

Fig. 3.

Uterine, placental and aortic MMP-2 and MMP-9 gelatinase activity during pregnancy

Tissue homogenates from the uterus (A), placenta (B) and aorta (C) of virgin, mid-Preg and late-Preg rats were prepared for gelatin zymography analysis. The densitometry values of the proteolytic bands was presented as pixel intensity x mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n = 4–12/group. * $P<0.05$ from corresponding measurements in virgin rats. # $P<0.05$ from corresponding measurements in mid-Preg rats.

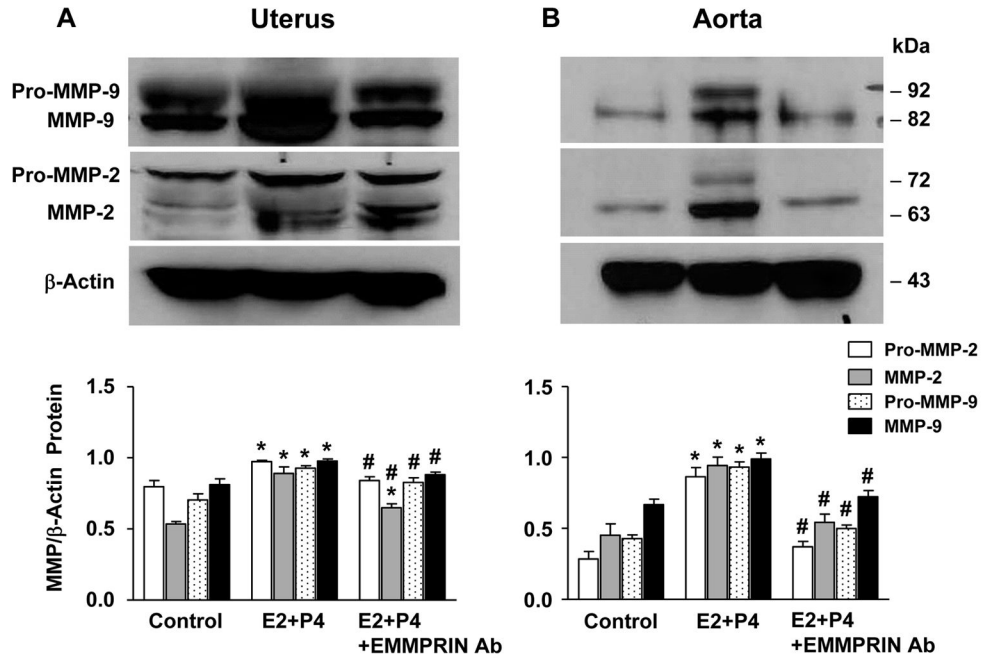

Fig. 4.

Effect of sex hormones on MMP-2 and MMP-9 protein expression in uterus and aorta of virgin rats. Uterine (A) and aortic strips (B) were either non-treated (control) or pretreated with 17β-estradiol (E2, 10^{-7} M) plus progesterone (P4, 10^{-7} M), or E2+P4 plus neutralizing EMMPRIN antibody (1:200) in culture medium for 24 hr. The tissues were then homogenized and prepared for Western blot analysis using MMP-2 and MMP-9 antibodies. The intensity of the immunoreactive bands was analyzed using optical densitometry, and normalized to the house keeping protein β-actin. Bar graphs represent means±SEM, n = 4–12/group. * $P<0.05$ compared with corresponding measurements in control nontreated tissues. # $P<0.05$ compared with corresponding measurements in tissues treated with E2+P4 only.

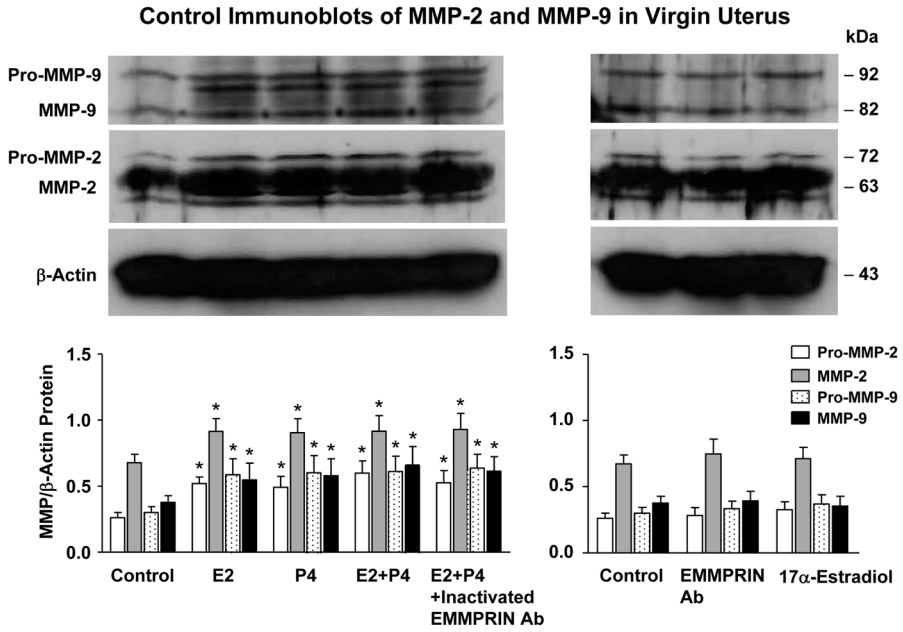

Fig. 5.

Effect of E2 alone, P4 alone, E2+P4, inactive 17α-estradiol, EMMPRIN antibody alone, and E2+P4 plus heat-inactivated EMMPRIN antibody on protein amount of MMP-2 and MMP-9 in uterus of virgin rats. Sex hormones control immunoblot experiments were conducted to compare the effects of treatment of virgin uterus for 24 hr with E2 alone, P4 alone, E2+P4 **(A)** or the inactive 17α-estradiol (10^{-7} M) **(B)** on the amount of MMP-2 and MMP-9. EMMPRIN antibody control immunoblot experiments were also conducted to test the effects of treatment of virgin uterus for 24 with EMMPRIN antibody alone **(B)** or with E2+P4 plus heat-inactivated EMMPRIN antibody **(A)**. The tissues were then homogenized and prepared for Western blot analysis using MMP-2 and MMP-9 antibodies. The intensity of the immunoreactive bands was analyzed using optical densitometry, and normalized to the house keeping protein β-actin. Bar graphs represent means±SEM, n = 5/group. * $P<0.05$ compared with corresponding measurements in control nontreated uterus.

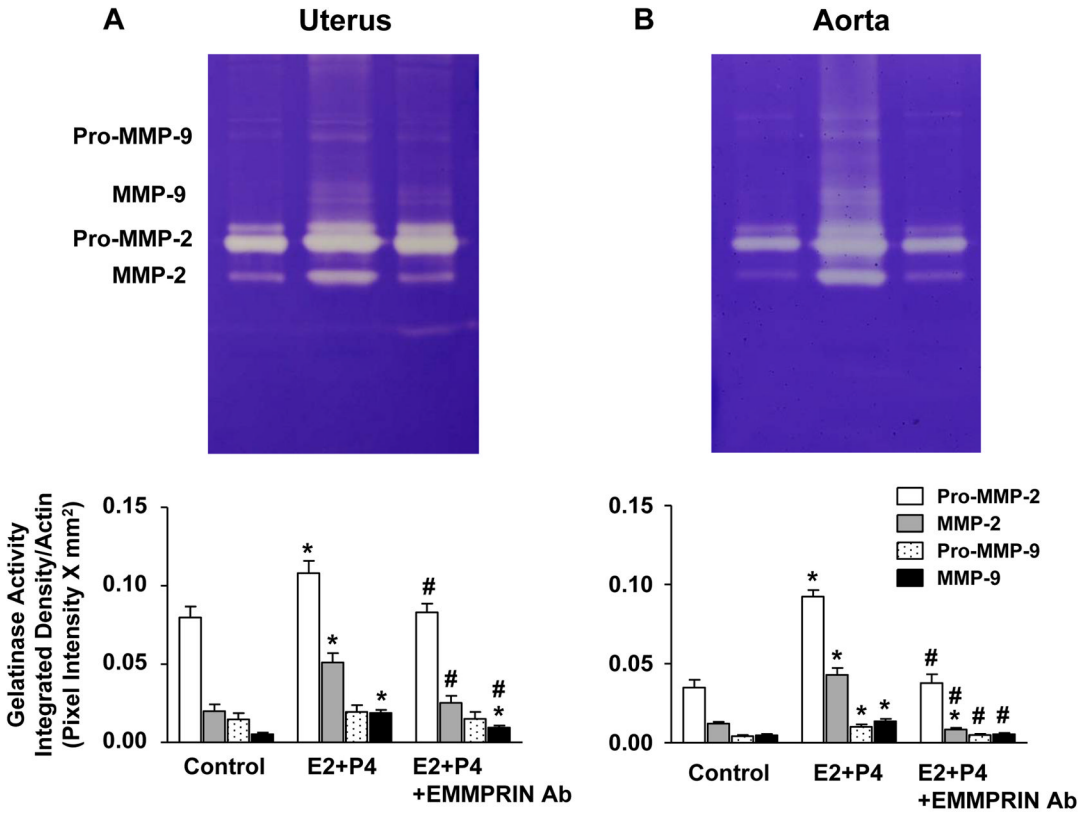

Fig. 6.

Effect of sex hormones on MMP-2 and MMP-9 gelatinase activity in uterus and aorta of virgin rats. Uterine (A) and aortic strips (B) were either non-treated (control) or pretreated with 17β-estradiol (E2, 10^{-7} M) plus progesterone (P4, 10^{-7} M), or E2+P4 plus neutralizing EMMPRIN antibody (1:200) in culture medium for 24 hr. The tissues were then homogenized and prepared for gelatin zymography analysis. The densitometry values of the proteolytic bands was presented as pixel intensity x mm^2 and normalized to actin to correct for loading. Bar graphs represent means±SEM, n=4–12/group. * $P<0.05$ compared with corresponding measurements in control nontreated tissues. # $P<0.05$ compared with corresponding measurements in tissues treated with E2+P4 only.

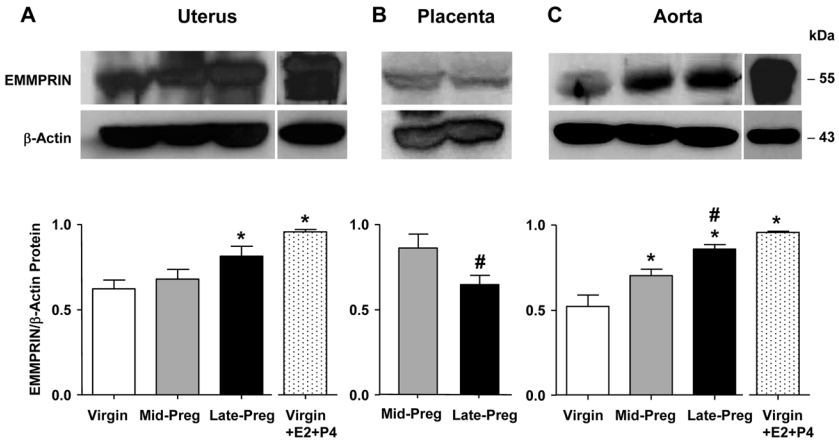

Fig. 7.

Protein amount of uterine, placental and aortic EMMPRIN during pregnancy. Uterine (A), placental (B) and aortic tissue strips (C) from virgin, mid-Preg and late-Preg rats as well as uterine (A) and aortic strips (B) from virgin rats pretreated for 24 hr with E2+P4 (10^{-7} M) were homogenized and prepared for Western blot analysis using EMMPRIN antibody (1:200). The immunoreactive bands were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n = 4–12/group. *P<0.05 from corresponding measurements in virgin rats. # P<0.05 from corresponding measurements in mid-Preg rats.

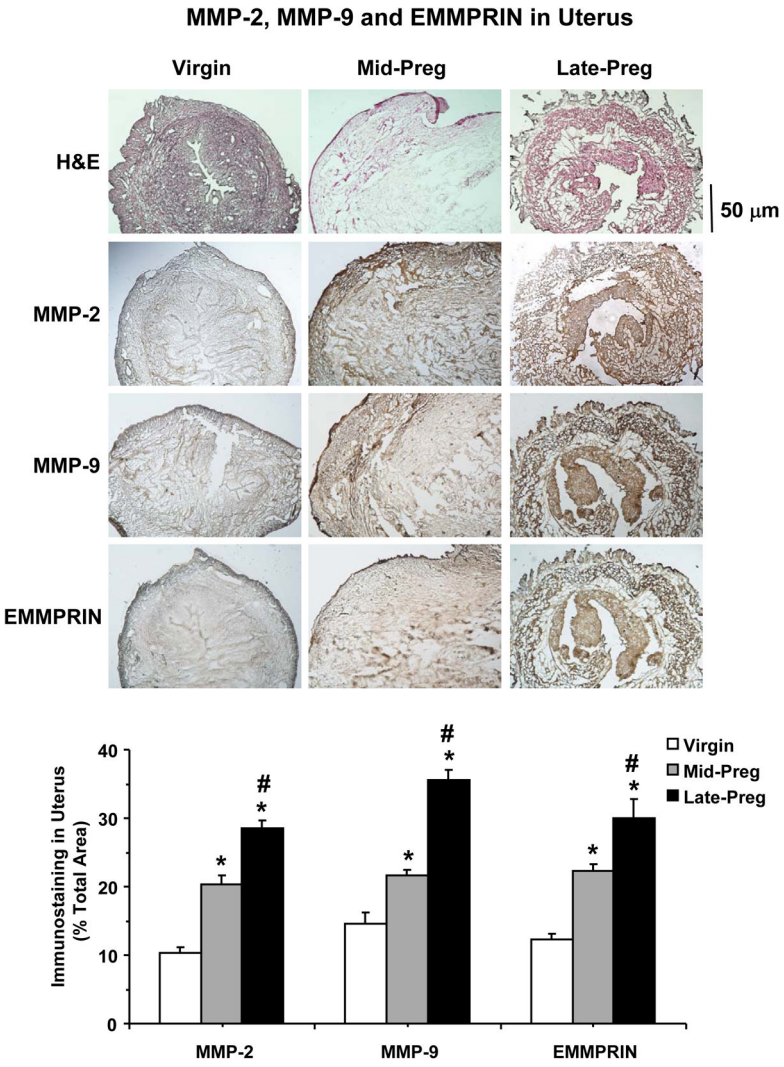

Fig. 8.

Distribution of uterine MMP-2, MMP-9 and EMMPRIN during pregnancy. Cryosections (6 μm) of the uterus of virgin, mid-Preg and late-Preg rats were prepared for hematoxylin and eosin staining (H&E). Other tissue sections were prepared for immunohistochemical analysis using MMP-2, MMP-9 and EMMPRIN antibodies, immunostained with ABC Elite Kit, and counterstained with hematoxylin. Images of tissue sections were acquired and analyzed using ImageJ software. The total number of pixels in the tissue section image was first defined, then the number of brown spots (pixels) was counted and presented as % of total pixels. Total magnification = 40. Bar graphs represent means±SEM, n = 5/group. *P<0.05 from corresponding measurements in virgin rats. # P<0.05 from corresponding measurements in mid-Preg rats.

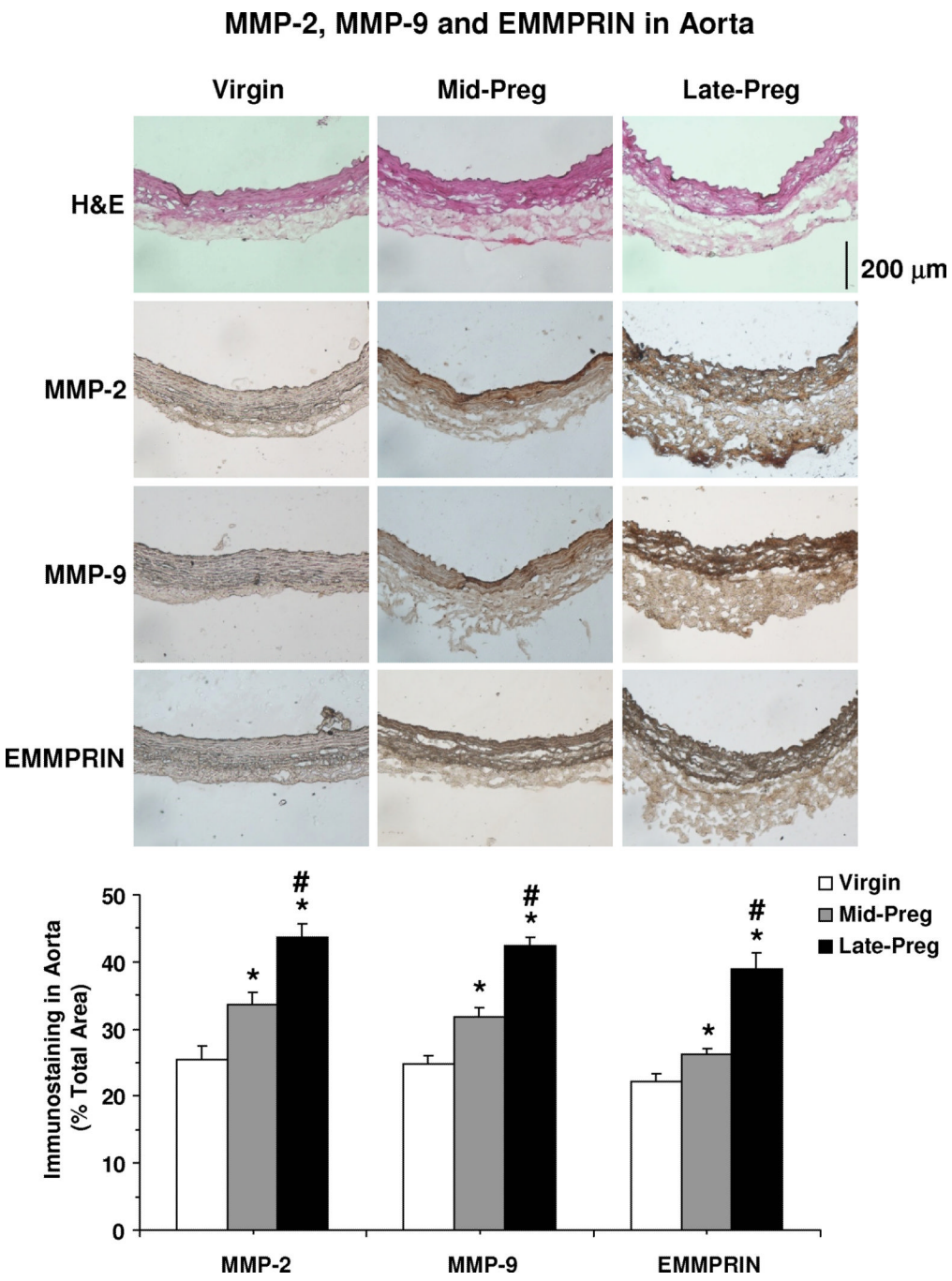

Fig. 9.

Distribution of aortic MMP-2, MMP-9 and EMMPRIN during pregnancy. Cryosections (6 μm) of the aorta of virgin, mid-Preg and late-Preg rats were prepared for hematoxylin and eosin staining (H&E). Other tissue sections were prepared for immunohistochemical analysis using MMP-2, MMP-9 and EMMPRIN antibodies, immunostained with ABC Elite Kit, and counterstained with hematoxylin. Images of tissue sections were acquired and analyzed using ImageJ software. The total number of pixels in the tissue section image was first defined, then the number of brown spots (pixels) was counted and presented as % of total pixels. Total magnification = 200. Bar graphs represent means±SEM, n = 5–6/group.

*P<0.05 from corresponding measurements in virgin rats. # P<0.05 from corresponding measurements in mid-Preg rats.

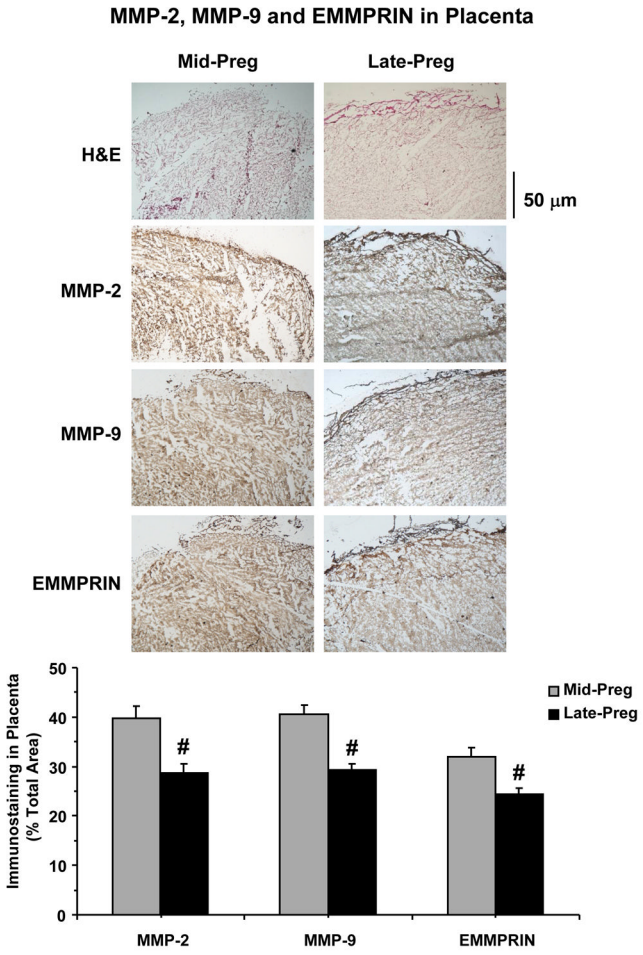

Fig. 10.

Distribution of placental MMP-2, MMP-9 and EMMPRIN during pregnancy. Cryosections (6 μm) of the placenta of mid-Preg and late-Preg rats were prepared for hematoxylin and eosin staining (H&E). Other tissue sections were prepared for immunohistochemical analysis using MMP-2, MMP-9 and EMMPRIN antibodies, immunostained with ABC Elite Kit, and counterstained with hematoxylin. Images of tissue sections were acquired and analyzed using ImageJ software. The total number of pixels in the tissue section image was first defined, then the number of brown spots (pixels) was counted and presented as % of total pixels. Total magnification = 40. Bar graphs represent means±SEM, n = 4–5/group. # $P<0.05$ from corresponding measurements in mid-Preg rats.