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Decreased Homodimerization and Increased TIMP-1 Complexation of Uteroplacental and Uterine Arterial Matrix Metalloproteinase-9 during Hypertension-in-Pregnancy

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Abstract

Preeclampsia is a complication of pregnancy manifested as hypertension-in-pregnancy (HTN-Preg) and often intrauterine growth restriction (IUGR). Placental ischemia could be an initiating event, but the molecular mechanisms are unclear. To test the hypothesis that dimerization of matrix metalloproteinases (MMPs) plays a role in HTN-Preg and IUGR, the levels/activity of MMP-9, tissue inhibitor of metalloproteinase (TIMP-1), and their dimerization forms were measured in the placenta, uterus, and uterine artery of normal pregnant (Preg) rats and a rat model of reduced uteroplacental perfusion pressure (RUPP). Consistent with our previous report, blood pressure (BP) was higher, pup weight was lower, and gelatin zymography showed different gelatinolytic activity for pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in RUPP vs Preg rats. Careful examination of the zymograms showed additional bands at 200 and 135 kDa. Western blots with MMP-9 antibody suggested that the 200 kDa band was a MMP-9 homodimer. Western blots with TIMP-1 antibody as well as reverse zymography suggested that the 135 kDa band was a MMP-9/TIMP-1 complex. The protein levels and gelatinase activity of MMP-9 homodimer were decreased while MMP-9/TIMP-1 complex was increased in placenta, uterus and uterine artery of RUPP vs Preg rats. The epidermal growth factor (EGF) receptor blocker erlotinib and protein kinase C (PKC) inhibitor bisindolylmaleimide decreased MMP-9 homodimer and increased MMP-9/TIMP-1 complex in placenta, uterus and uterine artery of Preg rats. EGF and the PKC activator phorbol-12,13-dibutyrate (PDBu) reversed the decreases in MMP-9 homodimer and the increases in MMP-9/TIMP-1 complex in tissues of RUPP rats. Thus, the increased BP and decreased pup weight in placental ischemia model of HTN-Preg are associated with a decrease in MMP-9 homodimer and an increase in MMP-9/TIMP-1 complex in placenta, uterus, and uterine artery, which together would cause a net decrease in MMP-9 activity and reduce uteroplacental and vascular remodeling in the setting of HTN-Preg and IUGR. Enhancing EGFR/PKC signaling may reverse the MMP-9 unfavorable dimerization patterns and thereby promote uteroplacental and vascular remodeling in preeclampsia.

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Keywords

Hypertension; Placenta; Preeclampsia; Remodeling; Uterine Artery; Uterus

1. Introduction

Normal pregnancy is associated with abundant placentation and extensive remodeling of the spiral arteries in the decidua basalis. The extravillous trophoblasts invade the decidua and extend into the walls of the spiral arteries, replacing the endothelium and muscular wall, and creating dilated low-resistance vessels in order to maintain adequate blood and nutrient supply to the developing fetus [1, 2]. These changes involve marked uteroplacental and vascular tissue remodeling and lead to significant alterations in the structure and function of the uterus, placenta and vasculature during pregnancy [3, 4].

Preeclampsia is a pregnancy-related disorder that complicates 5–8% of pregnancies. Preeclampsia is manifested as hypertension-in-pregnancy (HTN-Preg), with occasional proteinuria and edema [5, 6] If not adequately managed, preeclampsia could lead to eclampsia, with severe HTN and convulsions [7–9]. Preeclampsia is also often associated with fetal intrauterine growth retardation (IUGR), causing preterm pregnancies and premature labor [10, 11]. The causes of preeclampsia are unclear, but abnormal placentation and placental ischemia could be initiating events. The trophoblasts only partially invade the decidual vessels, such that the deeper myometrial arteries do not lose their endothelial lining and musculoelastic tissue, and the external diameter of spiral arteries may reach only half the size of the corresponding vessels in the normal placenta. The role of placental ischemia is supported by reports that reducing uteroplacental perfusion pressure (RUPP) in late pregnant rats shows some of the characteristics of preeclampsia including HTN-Preg and IUGR [7, 12, 13]. Also, rat models of gestational hypoxia show preeclampsia-like manifestations [14], supporting that placental ischemia/hypoxia could be an initiating event in HTN-Preg [7, 15]. The inadequate placentation and placental ischemia are likely caused by inadequate uteroplacental and vascular tissue remodelling, but the molecular targets involved have not been clearly identified.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade different components of the extracellular matrix (ECM). MMPs play a role in remodeling of various tissues and organs, and in many physiological processes and pathological conditions [16, 17]. The MMP family includes collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs [18]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play a role in endometrial tissue remodeling during the estrous and menstrual cycles and during pregnancy [19–21]. We have previously shown that MMP-2 and MMP-9 are upregulated in the uterus and aorta of Preg rats, and suggested a role of gelatinases in pregnancy-related uterine and vascular remodeling [22, 23]. However, measurements of MMP levels in preeclampsia have not been consistent, with some studies showing an increase in circulating levels of MMP-2 and MMP-9 [24], while other studies show a decrease in MMP-9 in preeclamptic vs normal Preg women [16]. Because plasma MMPs may represent the global MMP production from various maternal tissues, we have

previously measured the levels of MMP-2 and MMP-9 in maternal tissues and found that they are reduced in uterus, placenta and aorta of RUPP vs Preg rats, and suggested that downregulation of gelatinases would cause excess collagen deposition, and interfere with spiral arteries remodeling [17].

MMPs are regulated at multiple levels including gene transcription, induction, secretion, activation of the zymogen proMMP form, inhibition by tissue inhibitors of metalloproteinases (TIMPs), dimerization, and internalization by endocytosis. Inactive pro-MMPs are cleaved into their active proteolytic forms by other MMPs or other proteases [18, 25]. Also, endogenous TIMPs bind to and inhibit MMPs [26, 27]. Also, the gelatinases MMP-2 and MMP-9 are secreted from cells as monomers which could form various complexes and dimeric forms [28–34]. Of note, activation of gelatinases may require the formation of non-inhibitory complexes with other MMPs or TIMPs [35, 36]. For example, proMMP-2 through its hemopexin domain forms a complex with TIMP-2, which localizes to the cell surface and binds to membrane type MT1-MMP [29–32], and the resulting ternary proMMP-2/TIMP-2/MT1-MMP complex then facilitates cleavage and activation of its bound proMMP-2 by another “free” MT1-MMP [35, 37, 38]. MMP-9 also exists as a homodimer as well as complexes with other proteins [33, 34], and the different molecular species and dimeric forms of MMP-9 are predicted to have different physicochemical, biochemical and enzymatic properties. Both monomeric and dimeric forms of proMMP-9 have been identified in various cells [39–41], tissues [42] and biological fluids [43, 44], indicating that they are physiological forms of MMP-9. Protein dimerization may also represent one of the mechanisms underlying pathological conditions and diseases [33, 45] and both the monomeric and dimeric forms of proMMP-9 have been identified in cancer cells [46, 47]. However, little is known regarding the extent and nature of MMP-9 dimerization, their effects on MMP-9 activity, and their triggering pathways during pregnancy. Also, the relationship between MMP-9 dimerization and HTN-Preg is unclear.

The present study was designed to test the hypothesis that alterations in dimerization and protein complexation of uteroplacental and uterine arterial MMP-9 is a major molecular mechanism in the changes in MMP-9 activity associated with uteroplacental and vascular remodeling in HTN-Preg and IUGR. We used the placenta, uterus and uterine artery from Preg and RUPP rats to investigate whether: 1) In addition to MMP-9 monomers, MMP-9 forms a homodimer and MMP-9/TIMP complex with different gelatinase activity in the placenta, uterus and uterine artery of Preg rats. 2) HTN-Preg is associated with changes in MMP-9 homodimer and MMP-9/TIMP complex in placenta, uterus and uterine artery. 3) Epidermal growth factor (EGF) receptor (EGFR) and protein kinase C (PKC) have been implicated in MMP dimerization [28]. Therefore, we tested whether modulators of the EGFR/PKC pathway affect the formation and activity of MMP-9 homodimer and MMP-9/TIMP complex in Preg and RUPP rats.

2. Materials and Methods

2.1. Animals

Timed-pregnant (gestational day 11) Sprague-Dawley rats (12 week of age) from Charles River Laboratories (Wilmington, MA, USA) were maintained on *ad libitum* standard rat

chow and tap water in 12 h light-dark cycle. On gestational day 14, pregnant rats allocated to the RUPP group underwent surgical procedure to reduce uteroplacental perfusion pressure by banding the lower abdominal aorta above the iliac bifurcation and the main uterine branches of the ovarian arteries as previously described [12, 13, 48–50]. Briefly, pregnant rats were anesthetized by inhalation of isoflurane, the abdominal cavity was opened, and the abdominal aorta near the iliac bifurcation was carefully dissected free of surrounding tissue and perivascular fat and separated from the vena cava. A blunt plastic rod (OD, 0.3 mm) was placed parallel to the aorta, and a 4–0 silk braided ligature was knotted twice around both the aorta and the adjacent plastic rod. Once taut, the rod was carefully removed from the knotted ligature, thus creating a constrictive band (ID, 0.3 mm) and reducing blood flow through the aorta. This procedure has been shown to reduce uterine perfusion pressure in the gravid rat by ~40% [51]. Since compensation of blood flow to the placenta occurs through an adaptive increase in ovarian blood flow [52], a blunt plastic rod (OD, 0.1 mm) was used to place a ligature band (ID, 0.1 mm) on the main uterine branches of both the right and left ovarian arteries. Normal Preg rats were sham operated. RUPP rats in which the banding procedure resulted in maternal death or total reabsorption of the pups were excluded from the data analyses. All procedures were performed in accordance with the National Institutes of Health Guide for the Care of Laboratory Animal Welfare Act, and were approved by the Animal Care and Use Committee at the Brigham and Women's Hospital.

2.3. Tissue preparation

On gestational day 19, BP was measured via a PE-50 carotid arterial catheter connected to a pressure transducer as previously described [49]. Rats were then euthanized by inhalation of CO_2, the abdominal cavity was opened, and the gravid uterus was excised and placed in Krebs solution. The uterine artery was first dissected under microscopic visualization. The gravid uterus was then cut open, the placentae and pups were separated, gently blotted between filter papers, and the litter size and individual pup wet weight were recorded. The placenta and uterus were cut into 5 mm wide segments, and experiments were performed on 8 to 12 tissue segments from each rat, 4 to 6 rats per group. In some experiments, the placenta, uterus and uterine artery segments from Preg rats were incubated in the presence of the EGFR antagonist erlotinib HCl (10^{-5} M, Sigma) or PKC inhibitor bisindolylmaleimide I (10^{-5} M, Biomol) for 24 h in organ culture medium. In other experiments, the placenta, uterus and uterine artery segments from RUPP rats were incubated in the presence of the EGF (1 μg/ml, Sigma) or the PKC activator phorbol-12,13-dibutyrate (10^{-6} M, LC-Laboratories) for 24 h in organ culture medium.

2.4. Gelatin zymography

Segments of the placenta, uterus and uterine artery were homogenized using a 2-ml tight-fitting homogenizer (Kontes Glass, Vineland, NJ) and a homogenization buffer [without dithiothreitol (DTT)] containing 20 mM 3-[N-morpholino] propane sulfonic acid, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 1.2 mM ethylenediaminetetraacetic acid (EDTA), 0.02% bovine serum albumin (BSA), 5.5 μM leupeptin, 5.5 μM pepstatin, 2.15 μM aprotinin and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride. The homogenate was centrifuged at 10,000x g for 10 min, and the supernatant was collected. If the supernatant contained floating debris, centrifugation was repeated to obtain a clear supernatant. The

protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA). Tissue homogenate was subjected to electrophoresis on 8% SDS polyacrylamide gel containing 0.1% gelatin (Sigma, St. Louis, MO). The gel was then incubated in a zymogram renaturing buffer containing 2.5% Triton X-100 (Sigma) with gentle agitation for 30 min at room temperature. The gel was then equilibrated in a zymogram developing buffer (pH 6.7) 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 16 h. The gel was stained with 0.5% coomassie blue R-250 (Sigma) for 30 min, then destained with a coomassie R-250 destaining solution (methanol : acetic acid : water = 50 : 10 : 40). Areas corresponding to MMP gelatinolytic activity appeared as clear bands against a blue background. Actin appeared as a dark blue band at 43 kDa against a light blue background. The gelatinolytic bands were analyzed by optical densitometry and ImageJ software (National Institutes of Health, Bethesda, MD), and the integrated protease activity was measured as pixel intensity × mm^2 normalized to actin intensity as previously described [22, 23].

2.5. Reverse Zymography

The placenta, uterus and uterine artery homogenates [without dithiothreitol (DTT)] were subjected to electrophoresis on 10% SDS polyacrylamide gel containing 0.1% gelatin and MMP-2 (0.13 μg/ml). The gel was incubated in a zymogram renaturing buffer containing 2.5% Triton X-100 for 30 min, a zymogram developing buffer (pH 6.7) containing 50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij35 and 1μM $ZnCl_2$ at room temperature for 30 min, then at 37°C for 16 h. The gel was stained with 0.5% coomassie blue for 30 min, then destained with destaining solution (methanol: acetic acid: water = 50 : 10 : 40). Areas representing TIMPs appeared as dark blue bands against faint blue background and were analyzed by optical densitometry and ImageJ software, and the integrated reversed protease activity was measured as pixel intensity × mm^2 normalized to actin intensity.

2.6. Western blots

Segments of the placenta, uterus and uterine artery were homogenized using a 2-ml tight-fitting homogenizer (Kontes Glass) and a homogenization buffer containing 20 mM 3-[N-morpholino] propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol (DTT), 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. The homogenate was centrifuged at 10,000x g for 10 min, the supernatant was collected, and protein concentration was determined using a protein assay kit (Bio-Rad). 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 phosphate buffered saline (PBS)-Tween for 1 h and then overnight at 4°C with polyclonal rabbit antibodies to MMP-9 (sc-10737, 1:1000) and TIMP-1 (sc-5538, 1:500) (Santa Cruz Biotechnology, Dallas, TX) for 24 h. Negative control experiments were performed with the omission of primary antibody, and exhibited no detectable immunoreactive bands. Membranes were washed 3 times for 15 min each in PBS-Tween then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, 1:3000) for 2 h, and the

immunoreactive bands were detected using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The membranes were stripped in stripping buffer and subsequently reprobed with β-actin antibody (A1978, Sigma, 1:5000). Data were analyzed by optical densitometry and ImageJ software. The densitometry values represented the pixel intensity normalized to β-actin to correct for loading as previously described [22, 23].

2.7. 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 $MgCl_2$, and bubbled with 95% O_2 5% CO_2, pH 7.4. Stock solution of EGF (1 mg/ml, Sigma, St-Louis, MO) was prepared in distilled H_2O. Stock solutions of the PKC activator PDBu (10^{-2} M, LC-Laboratories, Woburn, MA), EGFR antagonist erlotinib HCl (10^{-2} M, Sigma) or PKC inhibitor bisindolylmaleimide (10^{-2} M, GF109203X, Biomol, Enzo Life Sciences, Farmingdale, NY) were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in experimental solution was <0.1%. The tissue culture medium used to pretreat the tissues with EGFR antagonist erlotinib or PKC inhibitor bisindolylmaleimide was composed of Minimum Essential Medium supplemented with penicillin, streptomycin, and amphotericin B (Invitrogen, Grand Island, NY). PBS contained (in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 KH_2PO_4, at pH 7.4. All other chemicals were of reagent grade or better.

2.8. Statistical analysis

Experiments were conducted on placenta, uterus and uterine artery isolated from 4 to 6 different rats per group (Preg vs RUPP rats) 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). Data were first analyzed using ANOVA. When a statistical difference was observed, data were further analyzed using Bonferroni's *post hoc* correction. Student's unpaired *t*-test was used for comparison of two means. Differences were statistically significant when $P < 0.05$.

3. Results

Maternal measurements on gestational day 19 showed that BP was significantly higher in RUPP (127.1±3.9 mmHg) than Preg rats (100.2±6.3 mmHg). Fetal measurements showed that the litter size (number of pups) was significantly less in RUPP (8±1) than Preg rats (12±0) and that the individual pup weight was significantly reduced in RUPP (1.34±0.06 g) compared with Preg rats (1.81±0.06 g) as previously described [50].

Our previous gelatin zymography experiments in uteroplacental and vascular tissues of late pregnant rats have shown protein concentration dependency of gelatinase activity not only for proMMP-9, MMP-9, proMMP-2, and MMP-2, but also for additional high molecular weight bands [23]. The high molecular weight bands were clearly discernible at 2 μg protein concentration and therefore all further gelatin zymography experiments were performed using 2 μg protein for loading. Gelatin zymography revealed prominent bands corresponding to proMMP-9, MMP-9, proMMP-2, and MMP-2 in the placenta, uterus and uterine artery of

Preg rats (Fig. 1). Consistent with our previous report [17], analysis of the bands intensity showed a decrease in the gelatinolytic activity of proMMP-9, MMP-9, proMMP-2, and MMP-2 in the placenta, uterus and uterine artery of RUPP vs Preg rats (Fig. 1). Careful examination of the zymograms showed two additional bands at ~200 and 135 kDa. The bands were more visible in the uterus compared to placenta and uterine artery. Analysis of the bands intensity showed that they were reduced in the placenta, uterus and uterine artery of RUPP vs. Preg rats (Fig. 1).

Because MMP-9 has been shown to undergo dimerization, we used Western blots to test whether the 200 and 135 kDa bands represent MMP-9 dimers. Because dimerization could be affected by reducing and denaturing conditions, we initially used proteins in the conditions of non-reduced and non-denatured just the same as those used for gelatin zymography. Tissue homogenates of the placenta, uterus and uterine artery of Preg and RUPP rats were prepared for Western blot under non-reduced (no DTT)/non-denatured (not boiled) conditions and using antibodies to MMP-9 (1:1000). Western blots revealed bands corresponding to proMMP-9 and MMP-9 that were more apparent in the uterus compared with the placenta and uterine artery, and appeared to be enhanced in RUPP vs Preg rats (Fig. 2). The MMP-9 antibody also showed immunoreactive bands at ~200 and 135 KDa, suggesting that they may represent some forms of MMP-9 dimers, with the 200 kDa likely representing MMP-9 homodimer, while the 135 kDa likely representing an MMP-9 protein complex. Further analysis of the Western blots revealed that the 200 KDa MMP-9 homodimer was reduced in the placental, uterus and uterine artery of RUPP vs Preg rats. On the other hand, the 135 KDa MMP-9 complex appeared to be increased in the uterus and to some extent in the placenta and uterine artery of RUPP vs Preg rats (Fig. 2). Interestingly, the amount of proMMP-9 and MMP-9 was significantly increased in the tissues of RUPP compared with Preg rats. This is likely due to the decrease in MMP-9 homodimerization, as it will lead to more MMP-9 in the monomer form.

Because the presence of reducing agents and denaturing of the protein could affect the mobility of the protein in the gel and its reactivity with antibody, we performed Western blots under different reducing and denaturing conditions (Fig. 3). Western blot analysis in uterine tissue homogenates under non-reducing (no DTT)/non-denatured (not-boiled) conditions revealed both the 200 kDa MMP-9 homodimer and the 135 kDa complex. The 200 kDa MMP-9 homodimer band was reduced in RUPP vs Preg rats. In contrast the 135 kDa complex band was visibly and significantly enhanced in RUPP vs Preg rats (Fig. 3A). We then used the proteins in reduced and denatured conditions, which are traditionally used in Western blots. Neither the 200 nor the 135 kDa band could be detected in reduced (DTT)/ denatured (Boiled) conditions (Fig. 3B). Of note, both the pro-MMP-9 and MMP-9 bands could be observed under these conditions and both were significantly decreased in the RUPP compared with Preg rats (Fig 3B). Under non-reduced/denatured conditions, only the 200 kDa band was observed, and was reduced in RUPP vs Preg rats (Fig. 3C). There were also bands of pro-MMP-9 and MMP-9, the amount of proMMP-9 was significantly increased in RUPP compared with Preg rats, but there was no significant difference in MMP-9 between the two groups. On the other hand, under reduced/non-denatured conditions, only the 135 kDa MMP-9 complex was detected and was enhanced in uterine tissue homogenate of RUPP vs Preg rats (Fig. 3D). There were also bands of proMMP-9 and MMP-9, the amount of

proMMP-9 was significantly increased in RUPP compared with Preg rats, but there was no significant difference in MMP-9 between the two groups. Thus, under non-reduced/non-denatured, all MMP-9 dimers and monomer could be detected, and changing the protein homogenization conditions was associated with changes in the intensity of the MMP-9 dimers and monomer. Therefore, we used these different conditions to maximize and further examine the 200 KDa MMP-9 homodimer separate form the 135 kDa MMP-9 complex.

Using non-reduced/denatured (without DTT/boiled) conditions in order to maximize the appearance of MMP-9 homodimer, the MMP-9 antibody showed the 200 kDa MMP-9 homodimer in the placenta, uterus, and uterine artery of Preg rats, and the band was significantly reduced or almost undetectable in RUPP rats (Fig. 4A). Under the same conditions, neither the MMP-9 antibody nor TIMP-1 antibody could detect the 135 kDa MMP-9 complex in the placenta, uterus and uterine artery of Preg or RUPP rats (Fig. 4B). These observations suggest that the 200 kDa band is mainly a MMP-9 homodimer, and that it is reduced in RUPP vs Preg rats.

Using reduced/non-denatured (with DTT/not boiled) conditions in order to optimize the detection of MMP-9/TIMP-1 complex, the MMP-9 antibody reacted with the 135 kDa MMP-9 complex in the placenta, uterus, and uterine artery of Preg rats, and the band was significantly enhanced in tissues of RUPP rats (Fig. 5A). Under the same conditions, TIMP-1 antibody could also detect the 135 kDa MMP-9 complex in the placenta, uterus and uterine artery of Preg rats, and the band was significantly enhanced in tissues of RUPP rats (Fig. 5B). No band could be detected at 200 kDa with either MMP-9 or TIMP-1 antibody. These observations suggest that the 135 kDa band is a complex of MMP-9/TIMP-1, and that it is increased in RUPP vs Preg rats. Of note, we couldn't detect the bands corresponding to TIMP-1 with TIMP-1 antibody. This likely because the gel concentration was 8%, and low molecular weight proteins such as TIMP-1 need higher 10% gels for better separation and visualization.

To further test if TIMP-1 is a part of MMP-9/TIMP-1 complex, we performed reverse zymography and were able to detect TIMP-1 and −2 in placenta, uterus and uterine artery of Preg rats, and the TIMP-1 bands, but not TIMP-2 bands, appeared to increase in RUPP vs Preg rats. Importantly, reverse zymography showed a 135 kDa band that was enhanced in placenta, uterus and uterine artery of RUPP vs Preg rats (Fig. 6). These observations suggest that the 135 kDa band represent a complex with TIMP activity. Other bands representing other proteins with potential MMP inhibitory effects could be detected, but we focused on the bands of MMP-9/TIMP-1, TIMP-1 and TIMP-2, and studying the other bands could be the subject of future studies.

We tested the possible role of EGFR/PKC pathway in MMP-9 activation and homodimerization. In placenta, uterus, and uterine artery of Preg rats in organ culture, treatment with EGFR inhibitor erlotinib or PKC inhibitor bisindolylmaleimide for 24 h reduced the gelatinolytic activity of 200 kDa MMP-9 homodimer, proMMP-9 and MMP-9. On the other hand, in placenta, uterus, and uterine artery of RUPP rats in organ culture, treatment with EGF or PKC activator PDBu for 24 h enhanced the gelatinolytic activity of 200 kDa MMP-9 homodimer, proMMP-9 and MMP-9 (Fig. 8).

Control experiments showed that in placenta, uterus, and uterine artery of Preg rats in organ culture, treatment with EGF or PKC activator PDBu for 24 h did not significantly change the gelatinolytic activity of 200 kDa MMP-9 homodimer, proMMP-9 or MMP-9 (Fig. 8), likely because the dimerization process was already activated in Preg rats. The gelatinolytic activity of 200 kDa MMP-9 homodimer, proMMP-9 and MMP-9 was reduced in tissues of RUPP vs Preg rats. On the other hand, in placenta, uterus, and uterine artery of RUPP rats in organ culture, treatment with EGFR inhibitor erlotinib or PKC inhibitor bisindolylmaleimide for 24 h did not significantly change the gelatinolytic activity of 200 kDa MMP-9 homodimer, proMMP-9 or MMP-9 (Fig. 8), likely because the MMP-9 homdimerization was already inhibited in RUPP rats.

We also tested the possible role of EGFR/PKC pathway on TIMPs and MMP-9/TIMP-1 complexation using reverse zymography (Fig. 9). In placenta, uterus, and uterine artery of Preg rats in organ culture, treatment with EGFR inhibitor erlotinib or PKC inhibitor bisindolylmaleimide for 24 h caused an increase 135 kDa MMP-9/TIMP-1 complex and TIMP-1, but not TIMP-2. The 135 kDa MMP-9/TIMP-1 complex and TIMP-1, but not TIMP-2, were greater in tissues of RUPP than Preg rats. On the other hand, in placenta, uterus, and uterine artery of RUPP rats in organ culture, treatment with EGF or PKC activator PDBu for 24 h reduced the 135 kDa MMP-9/TIMP-1 complex and TIMP-1, but not TIMP-2 (Fig. 9). Other mid-range bands could be visualized in the reverse zymograms, and the identity and changes of these bands could be the subject of future investigations.

4. Discussion

The main findings are: 1) In addition to MMP-9 monomers, MMP-9 forms homodimer, and its level and gelatinase activity are reduced in the placenta, uterus and uterine artery of RUPP vs Preg rats. 2) MMP-9 forms a putative MMP-9/TIMP-1 complex and its level and reverse gelatinase activity are increased in the placenta, uterus and uterine artery of RUPP vs Preg rats. 3) EGFR and PKC inhibitors decrease MMP-9 homodimer and increase MMP-9/TIMP-1 complex in tissues of Preg rats to levels close to those in RUPP rats. 4) EGF and PKC activator reverse the decrease in MMP-9 homodimer and the increase in MMP-9/TIMP-1 complex in tissues of RUPP rats. A decrease in MMP-9 homodimer and an increase in MMP-9/TIMP-1 complex through an EGF/PKC-dependent pathway may contribute to the reduction in MMP-9 activity and decreased uteroplacental and vascular remodeling in HTN-Preg and IUGR.

Preeclampsia is a major pregnancy-related disorder with unclear mechanisms. Genetic polymorphisms could alter MMP-2 and −9 transcription in preeclampsia [53]. Also, miRNA-519d-3p and miRNA-204 could target MMP-2 and −9, respectively and in turn decrease trophoblast invasiveness [54–56]. In first trimester trophoblasts, suppression of MMP-9 expression inhibits the trophoblast invasive capability, supporting a role of MMP-9 in trophoblast invasion [54]. However, while some studies showed a decrease in serum MMP-9 in preeclampsia [16], other studies showed an increase in circulating MMP-2 and −9 in preeclamptic vs. normal Preg women [24]. This is likely because plasma MMPs represent global MMP secretion from various maternal tissues, making it important to measure the localized changes in specific MMPs in different maternal tissues. Also, MMPs are often

secreted in an inactive pro-form that undergoes proteolytic activation by other MMPs and proteases to the active form [18, 25]. MMP-2 could undergo dimerization and form complexes with other MMPs or even TIMPs [28–32, 35–38]. Also, MMP-9 may undergo homodimerization as well as complexation with other proteins [33, 34]. In addition to changes in MMP expression and tissue distribution, changes in the MMP forms, dimers and complexes could affect MMP activity and in turn uteroplacental and vascular remodeling during preeclampsia.

Animal models of HTN-Preg have provided insights into the mechanisms of preeclampsia [12, 48, 57–59]. Defective placentation and inadequate uteroplacental remodeling could lead to placental ischemia; an initiating event that could trigger a cascade of biochemical events that lead to preeclampsia. In support, studies in late pregnant rat, rabbit, dog and sheep have shown that RUPP induces some of the hallmarks of preeclampsia including HTN-Preg and IUGR [12, 48]. In accordance with previous studies [17, 49, 60–62], the present study showed that BP was increased, and the litter size and pup weight were decreased in RUPP vs Preg rats, making the RUPP rat a suitable model to study the mechanisms of HTN-Preg and IUGR.

In search for the molecular mechanisms of the uteroplacental and vascular changes in HTN-Preg and IUGR, we have previously shown that MMP-2 and MMP-9 levels/activity are altered in RUPP vs Preg rats [17]. In the present study, we measured MMPs in the placenta which provides blood supply to the developing fetus, the uterus which undergoes expansive remodeling to accommodate the growing fetus, and the uterine artery which controls blood supply to the uteroplacental circulation. Consistent with our previous report [17] and using gelatin zymography, we were able to detect gelatinase bands corresponding to proMMP-9, MMP-9, proMMP-2, and MMP-2 in the placenta, uterus and uterine artery of RUPP and Preg rats. Careful examination of the zymograms showed prominent proMMP-9 (92 kDa), MMP-9 (83 kDa) and two additional bands at ~200 and 135 kDa. Based on their molecular weight, these additional bands appear to be MMP-9 dimers. This is consistent with other reports showing 220-, 135-, and 92-kDa forms characteristic of human neutrophil gelatinase [33, 39, 47]. Importantly, measuring the gelatinolytic activity of the 200 and 135 kDa bands suggests that they are altered in RUPP vs Preg rats. Using different reducing and denaturing conditions, we were able to optimize and separate the bands into a MMP-9 homodimer and a putative MMP-9/TIMP-1 complex.

MMP dimerization and complexation with other proteins is an intracellular process partly mediated by the C-terminal of the MMP hemopexin (PEX) domain, a propeller structure composed of four blades (B1-B4); each blade consists of one α-helix and four anti-parallel β-strands [63]. PEX plays a role not only in macromolecular substrate processing, but also in regulating MMP activation, localization and inhibition. MMP-9 is capable of binding to different proteins involved in these processes with high affinity at an overlapping recognition site in its PEX domain (PEX-9). Examination of the crystal structure of PEX-9 dimer has shown that blade B4 of PEX-9 mediates non-covalent and mainly hydrophobic dimerization contact and interactions of two PEX domains and a salt-bridge between the C-terminus of PEX-9 and the side chain of R677 of PEX-9. Large shifts of blades B3 and B4 accompany the dimerization, resulting in an asymmetric homodimer [63, 64]. The present study supports

the formation of MMP-9 homodimer because: 1) Gelatin zymography showed that the putative homodimer ran at 200 kDa, which is roughly in agreement with reports that proMMP-9 may exist in a disulfide-bonded homodimeric form (~220 kDa) [33, 40, 47]. 2) Western blot on homogenates of the placenta, uterus and uterine artery under non-reduced (no DTT)/non-dentaured (not boiled) conditions and using MMP-9 antibody showed prominent band at 200 kDa. 3) The 200 kDa band appeared to be reduction-sensitive, and was optimized in Western blots under non-reduced/denatured conditions, and absent in Western blots of tissues under either reduced/denatured or reduced/non-denatured conditions. 4) The optimized 200 kDa band was detected with MMP-9 antibody and did not cross-react with TIMP-1 antibody. The present study also support that the MMP-9 homodimer was decreased in RUPP vs Preg rats because: 1) Gelatin zymography showed that the 200 kDa gelatinolytic band was decreased in tissues of RUPP vs Preg rats. 2) Western blots in tissues under non-reduced/non-denatured conditions showed that MMP-9 homodimer was decreased in RUPP vs Preg rats. 3) Western blots in tissues under non-reduced/denatured conditions that optimize the 200 kDa MMP-9 homodimer, confirmed that it was decreased in RUPP vs Preg rats.

MMP-9 homodimerization is expected to generate new physico-chemical properties and function of the enzyme. Although the precise role of MMP-9 homodimerization is unclear, studies have suggested that PEX-9 is a prerequisite for enhanced cell migration. Following a series of substitution mutations within PEX-9, blade B4 was shown to be critical for homodimerization. COS-1 transfected with PEX-9 blade B4 mutant showed diminished cell migration compared with wild-type MMP-9-transfected cells. Also, peptides mimicking motifs in the outermost strands of PEX-9 blade B4 blocked MMP-9 dimer formation and inhibited motility of COS-1 cells overexpressing MMP-9 [63]. MMP-9 homodimerization could be an adaptive mechanism to maximize gelatinase activity for effective trophoblast migration and invasion of spiral arteries and tissue remodeling during pregnancy. MMP-9 homodimerization could also shield MMP-9 from rapid degradation or binding to endogenous TIMPs, and thus maintain adequate MMP-9 activity for tissue remodeling during pregnancy. The decease in MMP-9 homodimerization in RUPP vs Preg rats could cause a decrease in net MMP-9 gelatinase activity, and lead to a decrease in trophoblast migration and invasion of spiral artery and inadequate uteroplacental remodeling in the setting of HTN-Preg and IUGR.

Like MMP-2, MMP-9 can bind to and form complexes with other proteins. Pro-MMP-9 and pro-MMP-2 bind TIMP-1 and TIMP-2, respectively, through their PEX domain. Studies of PEX-9 dimer have suggested an asymmetry that may provide a mechanism of adaptive protein recognition, where different proteins such as PEX-9 and TIMP-1 can bind with high affinity to PEX-9 at an overlapping site [64]. MMP-9 could bind to TIMP-1 via the C-terminal domain of both the enzyme and the inhibitor. An internal disulfide bond in PEX-9 involving Cys704 and glycosylation of the enzyme may be necessary for its complexation with TIMP-1 [27]. The present study support the formation of MMP-9/TIMP-1 complex because: 1) Gelatin zymography showed that the putative complex ran at 135 kDa, which may roughly represent one MMP-9 molecule (~90 kDa) and one TIMP-1 molecule (28 kDa). 2) MMPs and TIMPs bind in a 1:1 stoichiometry [26, 27, 65]. 3) Western blot on homogenates of the placenta, uterus and uterine artery under non-reduced/non-denatured

conditions and using MMP-9 antibody showed a reactive band at 135 kDa. 4) The MMP-9/TIMP-1 complex appears to be denaturing-sensitive, and was optimized in Western blots under reduced/non-denatured conditions, and absent in Western blots under reduced/denatured or non-reduced/denatured conditions. 5) The optimized 135 kDa band could be detected with MMP-9 antibody and also showed prominent immunoreactivity with TIMP-1 antibody. 6) Reverse zymography showed a band of decreased gelatinase activity at 135 kDa. The present data also support that the MMP-9/TIMP-1 complex is increased in RUPP vs Preg rats because: 1) Western blots under non-reduced/non-denatured conditions showed that the 135 kDa complex was greater in RUPP vs Preg rats. 2) Western blots under reduced/non-denatured condition optimized the 135 complex and showed that it was markedly greater in RUPP vs Preg rats. 3) Western blots showed that the 135 kDa band cross-reacted with both MMP-9 and TIMP-1, and its immunoreactivity was greater in RUPP vs Preg rats. 4) Reverse zymography showed that the 135 kDa band representing decreased gelatinase activity was more prominent in RUPP vs Preg rats.

An important question is whether the MMP-9/TIMP-1 complex affects MMP-9 activity. Non-inhibitory complexes between progelatinases and TIMPs have been described between proMMP-2 and TIMP-2, −3, or −4, or between MMP-9 and TIMP-1 [35, 36]. For example, TIMP-2 first forms a complex with proMMP-2 by binding to its PEX domain, and the complex then localizes to the cell surface where it binds to the active site of a MT1-MMP molecule [29–32, 36]. This ternary proMMP-2/TIMP-2/MT1-MMP complex then facilitates the cleavage and activation of its bound proMMP-2 to active MMP-2 by another “free” MT1-MMP molecule. This non-inhibitory complex is different from the inhibitory complex of TIMP-2/active MMP-2. It is formed between the C-terminal domain of TIMP-2 and the C-terminal of the PEX domain of MMP-2, such that both molecules maintain their inhibitory and proteolytic properties, respectively [35, 37, 38]. Although our data can not rule out the formation of a non-inhibitory MMP-9/TIMP-1 complex, the present reverse zymography data showed that the 135 KDa representing decreased gelatinase activity was more prominent in RUPP vs Preg rats. The formation of MMP-9/TIMP-1 inhibitory complex may reduce the amount of MMP-9 available for ECM protein degradation and uteroplacental and vascular remodeling in HTN-Preg. Our data are consistent with reports that MMP-9 could form complexes with other proteins, which would in turn affect the enzyme activity and cell function. For instance, PEX-9 blade B1 may be required for dimerization with CD44, a process that can influence cell migration [63]. Also, studies have generated glutathione-S-transferase (GST) fusion proteins containing PEX-9 or truncated forms corresponding to its specific structural blades B1-B4. GST-PEX-9 inhibited MMP-9-dependent degradation of gelatin. Also, ELISA assays demonstrated that GST-B4 and GST-B1 specifically bound to gelatin [66]. PEX-9 may shield gelatin and specifically prevent its binding to and degradation by MMP-9 or MMP-2. Thus complexation of PEX-9 and its blades B4 and B1 with TIMP-1 may lead to inhibition of gelatin degradation [66]. Other studies have identified a 125 kDa progelatinase as a covalently linked, disulfide-bridged heterodimer formed through the interaction of proMMP-9 monomer with a 25 kDa α2-microglobulin-related protein [40]. Also, in neutrophil granules, a 125–130 kDa form of proMMP-9 has been identified as a complex of the enzyme with lipocalin, and was termed neutrophil gelatinase-associated lipocalin (NGAL) [39]. Complexation of MMPs with

endogenous proteins may affect their activity. For example, like TIMPs, α2-macroglobulin is an endogenous MMP inhibitor found in blood and tissue fluids. Human α2-macroglobulin is a glycoprotein consisting of four identical subunits that may function as a wide-spectrum proteinase inhibitor of most endopeptidases including MMPs by entrapping them within the macroglobulin. The complex is then rapidly internalized and cleared by endocytosis via low density lipoprotein receptor-related protein-1 [67]. Because MMP-9 facilitates cell growth and migration by promoting proteolysis of ECM, a decrease in MMP-9 activity in RUPP rats could impede trophoblast migration and invasion and decrease uteroplacental and vascular remodeling in the setting of HTN-Preg and IUGR. A decrease in gelatinase activity could also decrease the blood vessel's plasticity and in turn contribute to increased vascular resistance and HTN [17]. In addition to their proteolytic effects on ECM, MMPs may affect vascular and uterine function and the mechanisms of smooth muscle contraction. We have previously shown that MMP-2 and −9 cause relaxation of precontracted rat aorta [68] and uterus [22], and induce vasodilation in rat inferior vena cava via hyperpolarization and activation of K^+ channels [69, 70]. A net decrease in vascular MMP-9 could lead to increased vascular contraction and HTN-Preg, and a net decrease in uterine MMP-9 could lead to increased uterine contraction and premature labor [17, 22].

We also tested the potential pathways that could cause the observed changes in MMP-9 dimerization. Studies have suggested a role of EGF in trophoblast growth and proliferation during normal pregnancy. EGF has been suggested to stimulate MMP-9 activity and trophoblast survival, migration and invasion [71–74], and relative deficiency of EGF during placental development in early pregnancy could impair trophoblast survival, differentiation, and invasion, and lead to poor placental perfusion, HTN-Preg, and IUGR [75–77]. Some of the effects of EGFR could be mediated by PKC [78–80], a major signaling molecule that could affect uterine and vascular function during pregnancy. Normal pregnancy is associated with changes in uterine blood flow caused by changes in uterine arterial Ca^{2+}-dependent phasic contraction and maintained DAG/PKC-mediated tonic contraction [81]. PKC activity and uterine artery and aortic contraction change during the course of pregnancy, and are altered in late pregnant ewes, gilts and rats [82–84]. Also, the expression and subcellular redistribution of PKCα, δ and ζ are altered in the aorta of late pregnant rats [83, 85]. Placental ischemia/hypoxia could affect uterine vascular tone and PKC activity in pregnant sheep [86]. Also, in cultured rat cardiomyocytes, treatment with IgG obtained from preeclamptic women enhances AT_1R-mediated response, which is ameliorated with the PKC inhibitor calphostin C, further supporting a role of PKC in preeclampsia [87]. Studies have also shown that EGF and PKC could promote MMP dimerization [28]. The present study support a role of EGFR/PKC pathway in the observed MMP-9 homodimerization because: 1) In placenta, uterus, and uterine artery of Preg rats, treatment with EGFR inhibitor or PKC inhibitor reduced the gelatinolytic activity of 200 kDa MMP-9 homodimer. 2) In tissues of RUPP rats, treatment with EGF or PKC activator PDBu enhanced the gelatinolytic activity of 200 kDa MMP-9 homodimer. 3) In tissues of Preg rats, treatment with EGF or PKC activator PDBu did not change the gelatinolytic activity of 200 kDa MMP-9 homodimer, suggesting that the EGFR/PKC-dependent MMP-9 dimerization is already activated. 4) In tissues of RUPP rats, the gelatinolytic activity of 200 kDa MMP-9 homodimer was reduced when compared to the tissues of Preg rats, and treatment with EGFR blocker or PKC

inhibitor did not cause any further reduction in the gelatinolytic activity of the 200 kDa MMP-9 homodimer, suggesting that the EGFR/PKC-dependent MMP-9 dimerization pathway is already inhibited in RUPP rats. The present study also supports a role of EGFR/PKC pathway in reducing the formation of MMP-9/TIMP-1 complex during pregnancy because using reverse zymography, in placenta, uterus, and uterine artery of Preg rats, treatment with EGFR inhibitor or PKC inhibitor increased the 135 kDa MMP-9/TIMP-1 complex. Also, the 135 kDa MMP-9/TIMP-1 complex was greater in tissues of RUPP than Preg rats, while in tissues of RUPP rats, treatment with EGF or PKC activator PDBu reduced the 135 kDa MMP-9/TIMP-1 complex. These findings support a role of EGFR/PKC pathway in the regulation of MMP dimerization and activity, and in turn uteroplacental and vascular remodeling and function during pregnancy.

Other observations and considerations include: **1**) MMP activity could be influenced by other MMP activators and inhibitors. In effect, some MMPs may cleave other pro-MMPs, and MT1-MMP is a key activator of pro-MMP-2 [18, 25, 88, 89]. Also, TIMPs are important endogenous modulators of MMPs activity [16, 18, 25, 88, 89]. Some studies have shown increases or no change in TIMP-1 and −2 in the circulation and umbilical cord serum of preeclamptic vs normal Preg women [16, 24, 90]. Other studies have shown increases in TIMP-1 and −3 in preeclamptic patients [91]. In the present study, we focused on TIMP-1, and the changes in other TIMPs in HTN-Preg need to be further examined. **2**) In the present study, the changes in MMPs in placenta, uterus and uterine artery were measured on gestational day 19, and the progressive changes in MMPs during the course of pregnancy, their effects on BP and placental and fetal development, and their reversal in the postpartum period need to be examined. **3**) The present study examined the effects of treatment of uterine, placental and uterine arterial segments with modulators of EGF/PKC on MMP-9 dimerization and complexation with TIMP-1. Future studies should test whether *ex vivo* treatment of uteroplacental tissues or *in vivo* administration of modulators of EGF/PKC signaling would also affect uteroplacental remodeling and tissue morphometry and/or alter uterine function and uterine arterial diameter and uterine blood flow in hypertensive versus normal pregnancy. **4**) MMPs are released from different maternal tissues, and plasma MMPs levels often reflect global changes in multiple tissues. In the present study we examined three pregnancy-relevant tissues, and showed changes in MMP-9 dimerization in placenta, uterus and uterine artery of HTN-Preg rats. The changes in MMP dimerization and activity and their effects on the structure and function of other tissues, such as the small resistance vessels which affect BP [49, 92], need to be examined. **5**) Placental ischemia during pregnancy may cause the release of vasoactive factors such as anti-angiogenic factors, cytokines, reactive oxygen species (ROS) and hypoxia-inducible factors (HIF) [8, 13, 58, 59, 93–98]. Preeclamptic women show imbalance between pro-angiogenic factors and the anti-angiogenic factor soluble fms-like tyrosine kinase 1 (sFlt-1) [95, 99–107], and placental ischemia in RUPP rats is associated with increased serum sFlt-1 [13]. Placental ischemia may enhance the release of inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) [96–98]. Circulating TNFα levels are increased in preeclamptic vs normal Preg women [108–110], and the plasma levels and $CD4^+$T cell production of TNFα are increased in RUPP vs Preg rats [98, 111, 112]. TNF-α may modify the expression of adhesion molecules in placental vessels [98] and may contribute to abnormal MMP

production in preeclampsia. MMPs could in turn increase cytokines or promote their effects on ROS, causing a feed-forward cycle in preeclampsia [9]. The circulating levels of HIF-1α are increased in preeclamptic vs normal Preg women [113], and placental levels of HIF-1α are elevated in RUPP rats [95]. Markers of lipid peroxidation and oxidative stress are increased in serum of preeclamptic women at gestational weeks 10–14 [114], and in the plasma, aorta and placenta of RUPP vs Preg rats [111, 115]. MMPs may contribute to the increases in ROS in preeclampsia [9]. Also, sFlt-1, ROS and HIF may affect MMP expression/activity [17, 116, 117], and studying their potential interaction with cytokines in modulating MMP dimerization and activity and EGFR/PKC pathway in the setting of HTN-Preg and IUGR should be examined.

In conclusion, HTN-Preg is associated with decreased formation of MMP-9 homodimer and increased MMP-9/TIMP-1 complex. The decreased MMP-9 homodimer and increased MMP-9/TIMP-1 complex appear to involve an EGF/PKC-dependent pathway and may play a role in the decreased MMP-9 activity and uteroplacental and vascular remodeling, and thereby contribute to IUGR and increased vasoconstriction in HTN-Preg and preeclampsia. Modulators of EGFR/PKC signaling may reverse the decrease in MMP-9 homodimer and the increase in MMP-9/TIMP-1 complex and promote uteroplacental and vascular remodeling, and thereby improve fetoplacental growth and decrease BP in HTN-Preg and preeclampsia.

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List of Abbreviations

BP	blood pressure
ECM	extracellular matrix
DTT	dithiothreitol
EGF	epidermal growth factor
HIF	hypoxia-inducible factor
HTN-Preg	hypertension-in-pregnancy
IL	interleukin
IUGR	intrauterine growth retardation
MMP	matrix metalloproteinase
PDBu	phorbol-12,13-dibutyrate

PKC	protein kinase C
Preg	pregnant
PEX	hemopexin domain
ROS	reactive oxygen species
RUPP	reduced uteroplacental perfusion pressure
TIMP	tissue inhibitor of metalloproteinase
sFlt-1	soluble fms-like tyrosine kinase 1
TNFα	tumor necrosis factor-α

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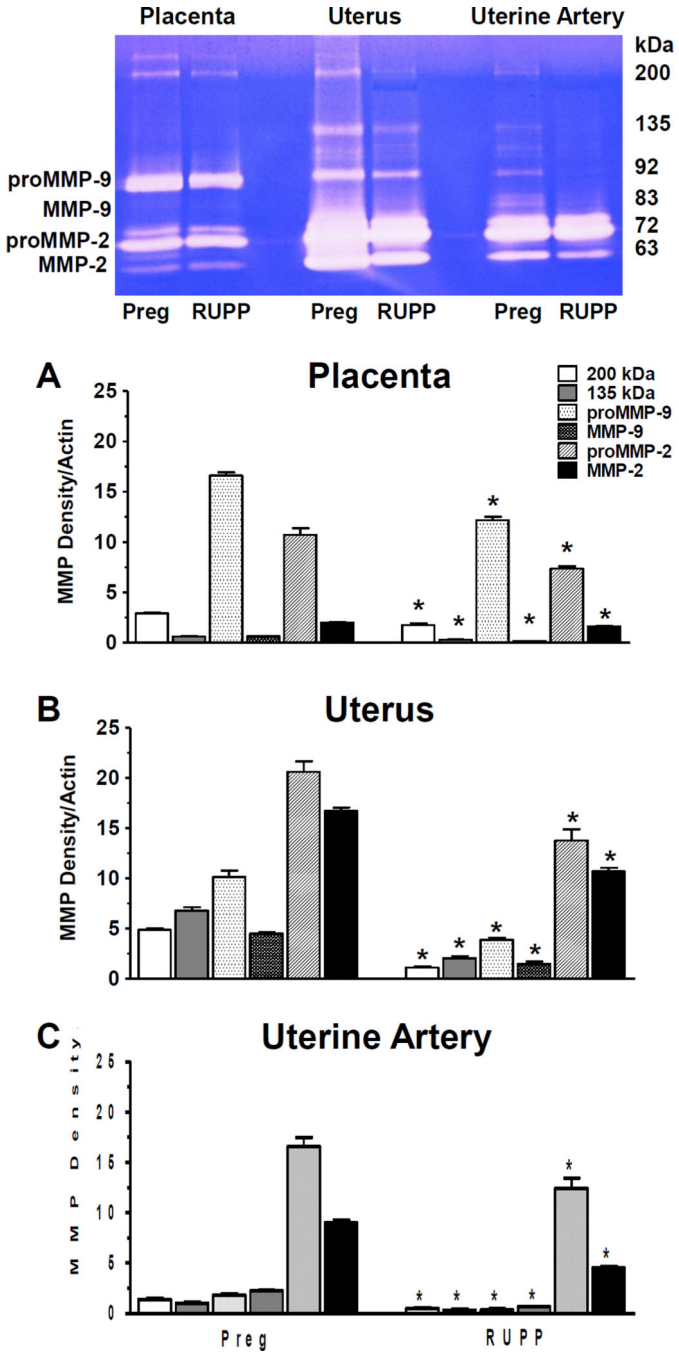

Fig. 1.

Gelatinase activity in placenta, uterus and uterine artery in Preg and RUPP rats. Tissue homogenates of the placenta (A), uterus (B) and uterine artery (C) of Preg and RUPP rats were prepared for gelatin zymography. The densitometry values of the proteolytic bands corresponding to MMP was presented as pixel intensity × mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group. * $P<0.05$, RUPP vs Preg rats.

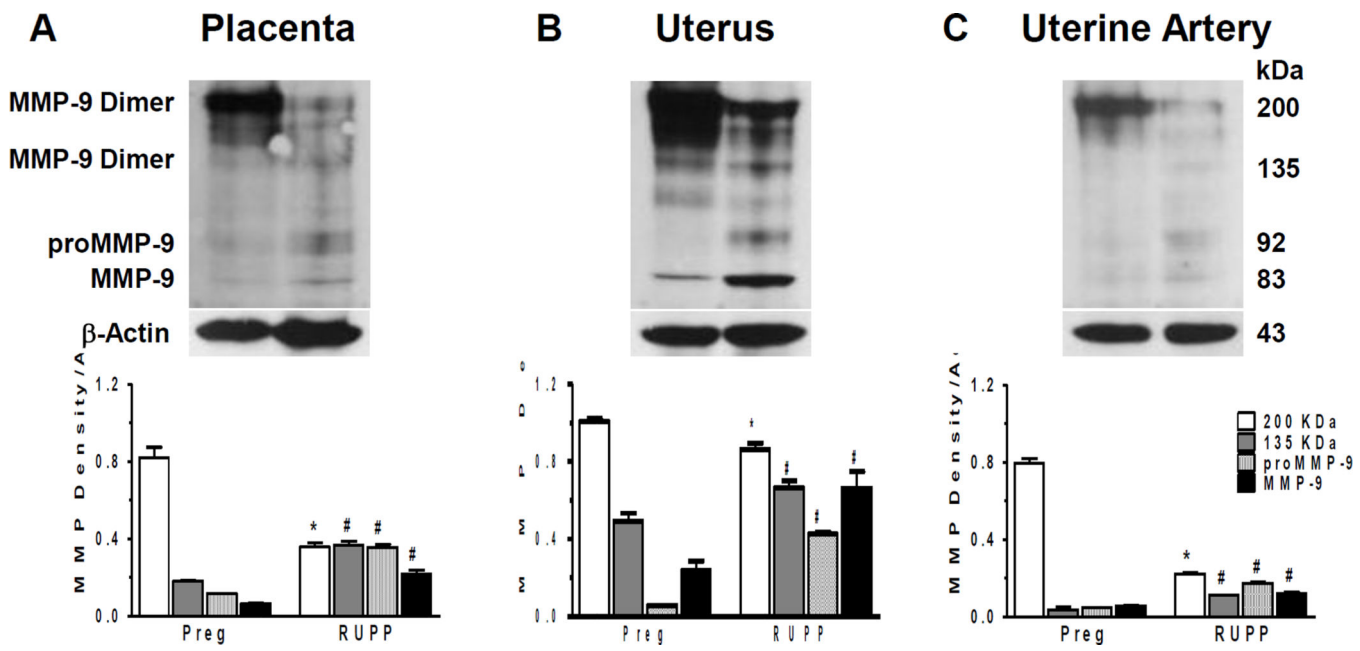

Fig. 2.

Protein amount of MMP-9, pro-MMP-9, putative 135 kDa and 200 kDa proteins in placenta (A), uterus (B) and uterine artery (C) of Preg and RUPP rats. Tissue homogenates of the placenta, uterus and uterine artery of Preg and RUPP rats were prepared for Western blot under non-reduced (no DTT)/non-denatured (not boiled) conditions and using antibodies to MMP-9 (1:1000). Immunoreactive bands corresponding to MMP were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means ±SEM, n=5/group.

* Significantly reduced ($P<0.05$) in RUPP vs Preg rats.

Significantly increased ($P<0.05$) in RUPP vs Preg rats.

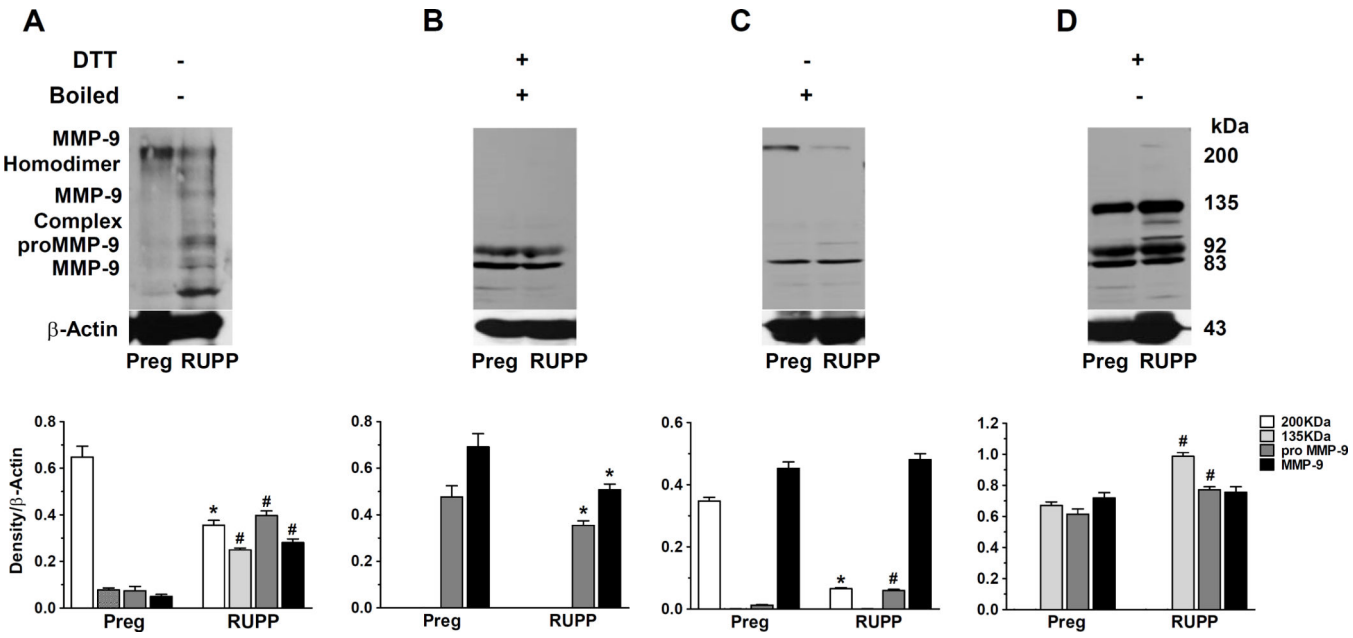

Fig. 3.

MMP-9 monomers and dimers under non-reduced (no DTT)/non-denatured (not-boiled) (A), reduced (DTT)/denatured (Boiled) (B), non-reduced/denatured (C) and reduced/non-denatured (D) conditions in uterus of Preg and RUPP rats. Uterus homogenates from Preg and RUPP rats were prepared for Western blot analysis using antibodies to MMP-9 (1:1000). Immunoreactive bands corresponding to MMP were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group.

* Significantly reduced ($P<0.05$) in RUPP vs Preg rats.

Significantly increased ($P<0.05$) in RUPP vs Preg rats.

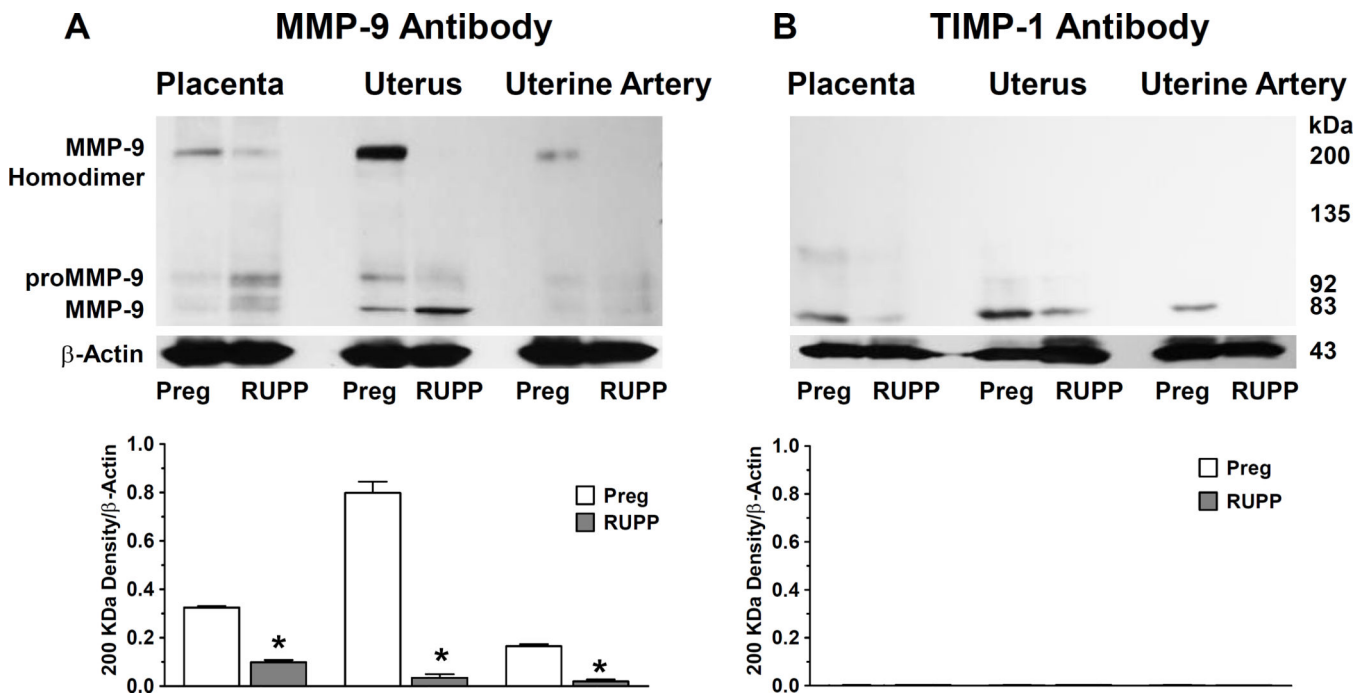

Fig. 4.

MMP-9 monomers and dimers under non-reduced/denatured (no DTT/boiled) conditions in order to maximize the detection of MMP-9 homodimer in placenta, uterus, and uterine artery of Preg and RUPP rats. Tissue homogenates of the placenta, uterus and uterine artery of Preg and RUPP rats were prepared for Western blot analysis using antibodies to MMP-9 (1:1000) (A) or TIMP-1 (1:500) (B). Immunoreactive bands were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group.

* $P<0.05$, RUPP vs Preg rats.

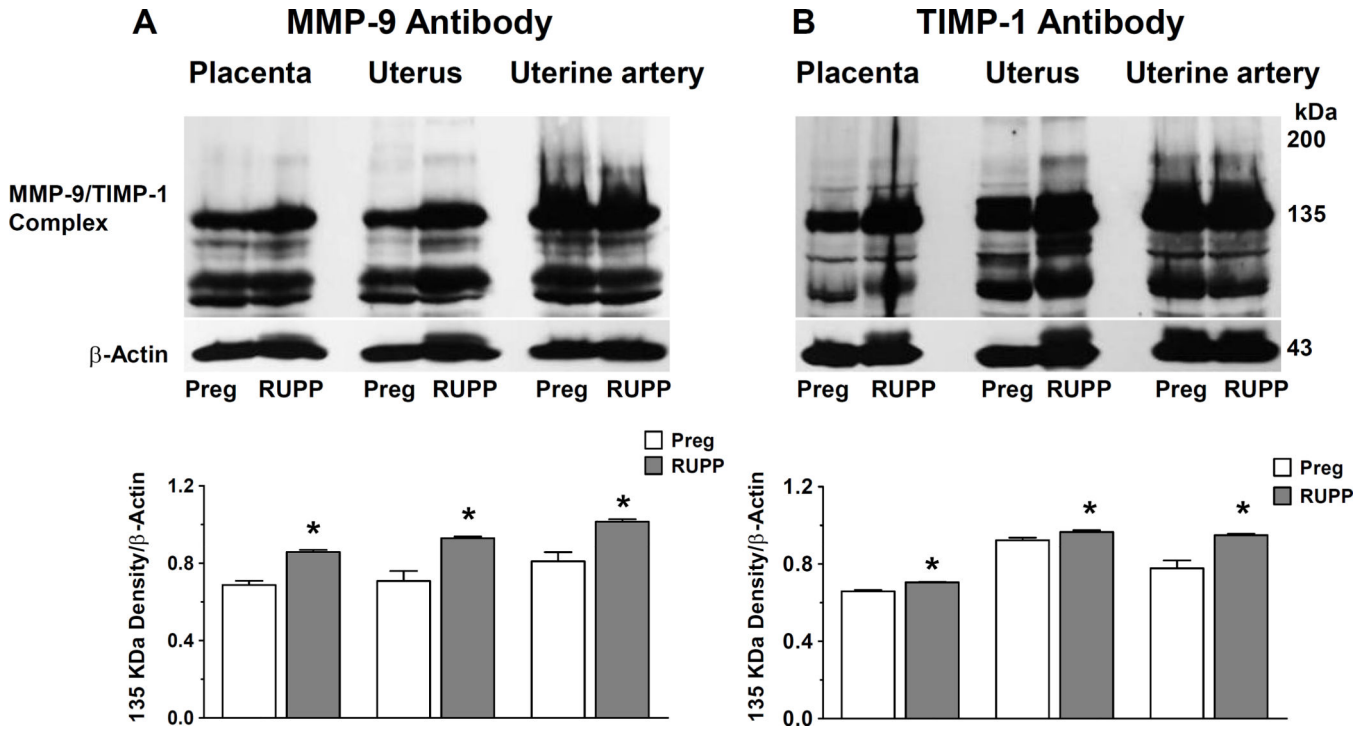

Fig. 5.

MMP-9 monomers and dimers under reduced/non-denatured (with DTT/not boiled) conditions in order to maximize the appearance of MMP-9/TIMP-1 complex in placenta, uterus and uterine artery of Preg and RUPP rats. Tissue homogenates of the placenta, uterus and uterine artery of Preg and RUPP rats were prepared for Western blot analysis using antibodies to MMP-9 (1:1000) (A) or TIMP-1 (1:500) (B). Immunoreactive bands were analyzed by optical densitometry and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group. * P<0.05, RUPP vs Preg.

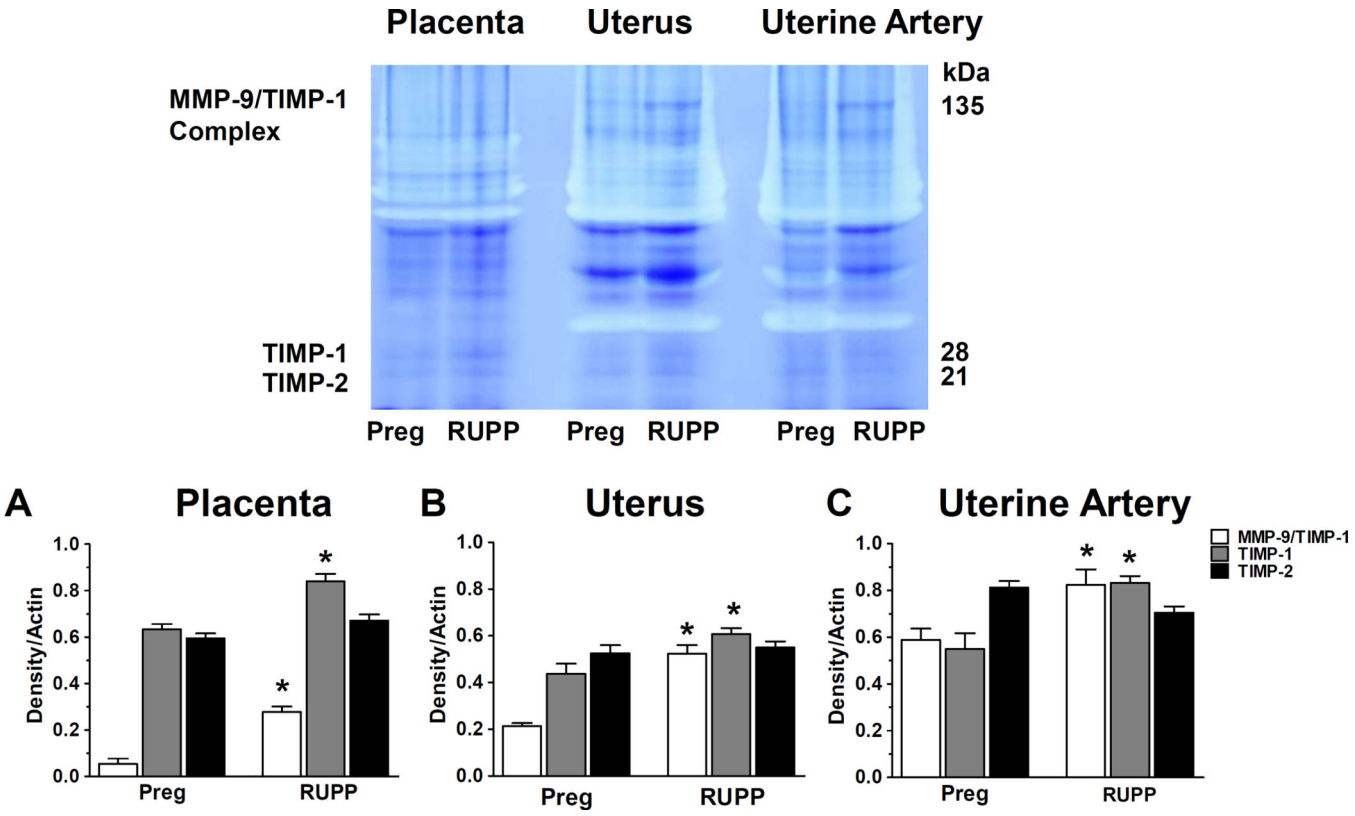

Fig. 6.

MMP-9/TIMP-1 complex and TIMPs activity in placenta (A), uterus (B) and uterine artery (C) of Preg and RUPP rats. Tissue homogenates of the placenta, uterus and uterine artery of Preg and RUPP rats were prepared for reverse gelatin zymography analysis using 10% acrylamide in order to provide better resolution of the low molecular weight TIMP-1 and TIMP-2. The densitometry values of the bands corresponding TIMP was presented as pixel intensity × mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group. * $P<0.05$, RUPP vs Preg.

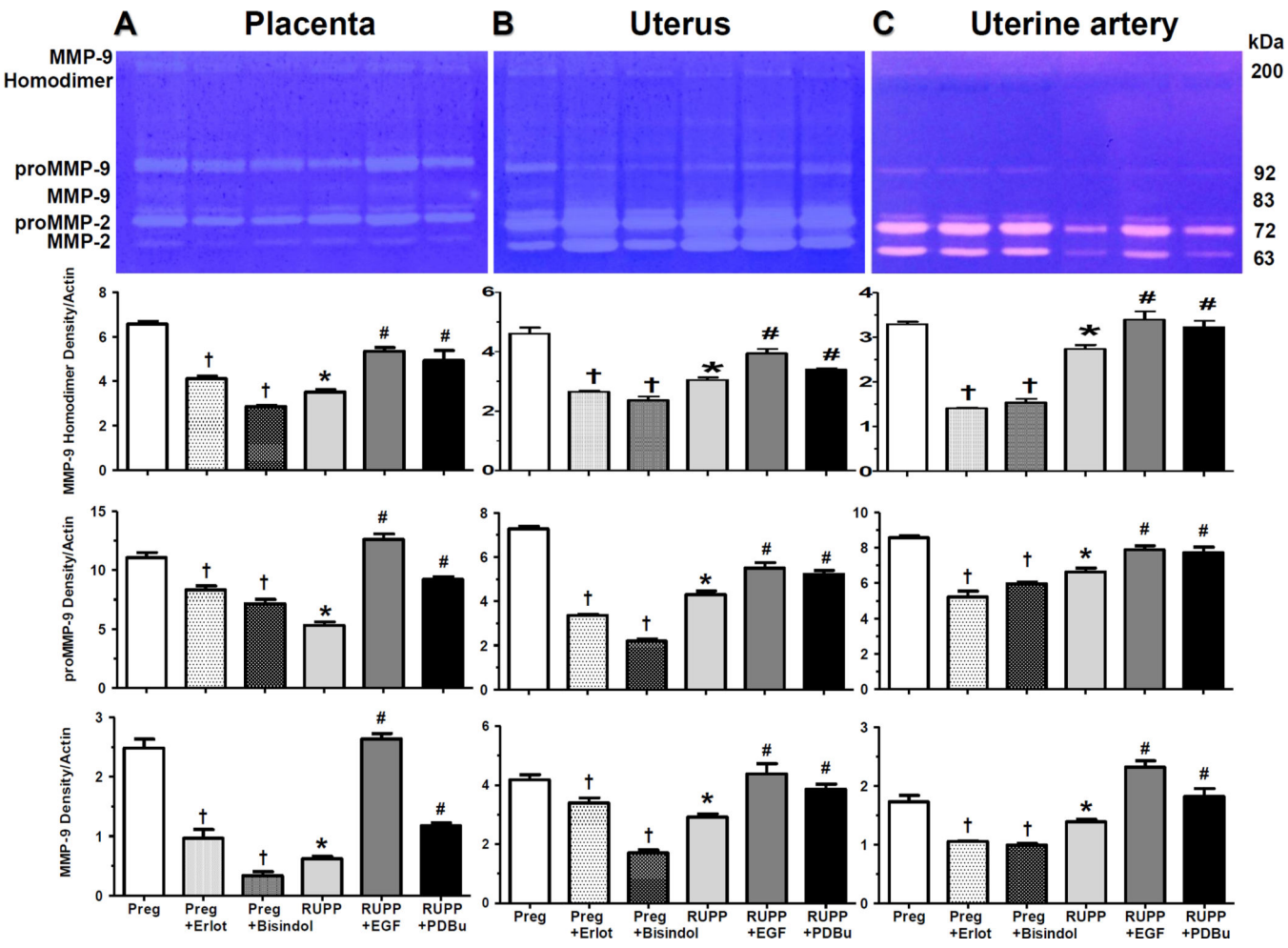

Fig. 7.

Effect of modulators of EGFR and PKC on gelatinase activity of MMP-9 homodimer and monomers in placenta (A), uterus (B), and uterine artery (C) of Preg and RUPP rats. Tissue homogenates of Preg were treated in culture with EGFR inhibitor erlotinib (10^{-5} M) or PKC inhibitor bisindolylmaleimide (10^{-5} M) and tissues of RUPP rats were cultured with EGF (1 μg/ml) or PKC activator PDBu (10^{-6} M) then prepared for gelatin zymography analysis. The densitometry values of the proteolytic bands corresponding to MMPs was presented as pixel intensity × mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group.

* P<0.05, RUPP vs Preg.

† P<0.05, EGFR inhibitor or PKC inhibitor pretreated vs. nontreated tissues of Preg rats.

# P<0.05, EGF or PKC activator pretreated vs. nontreated tissues of RUPP rats.

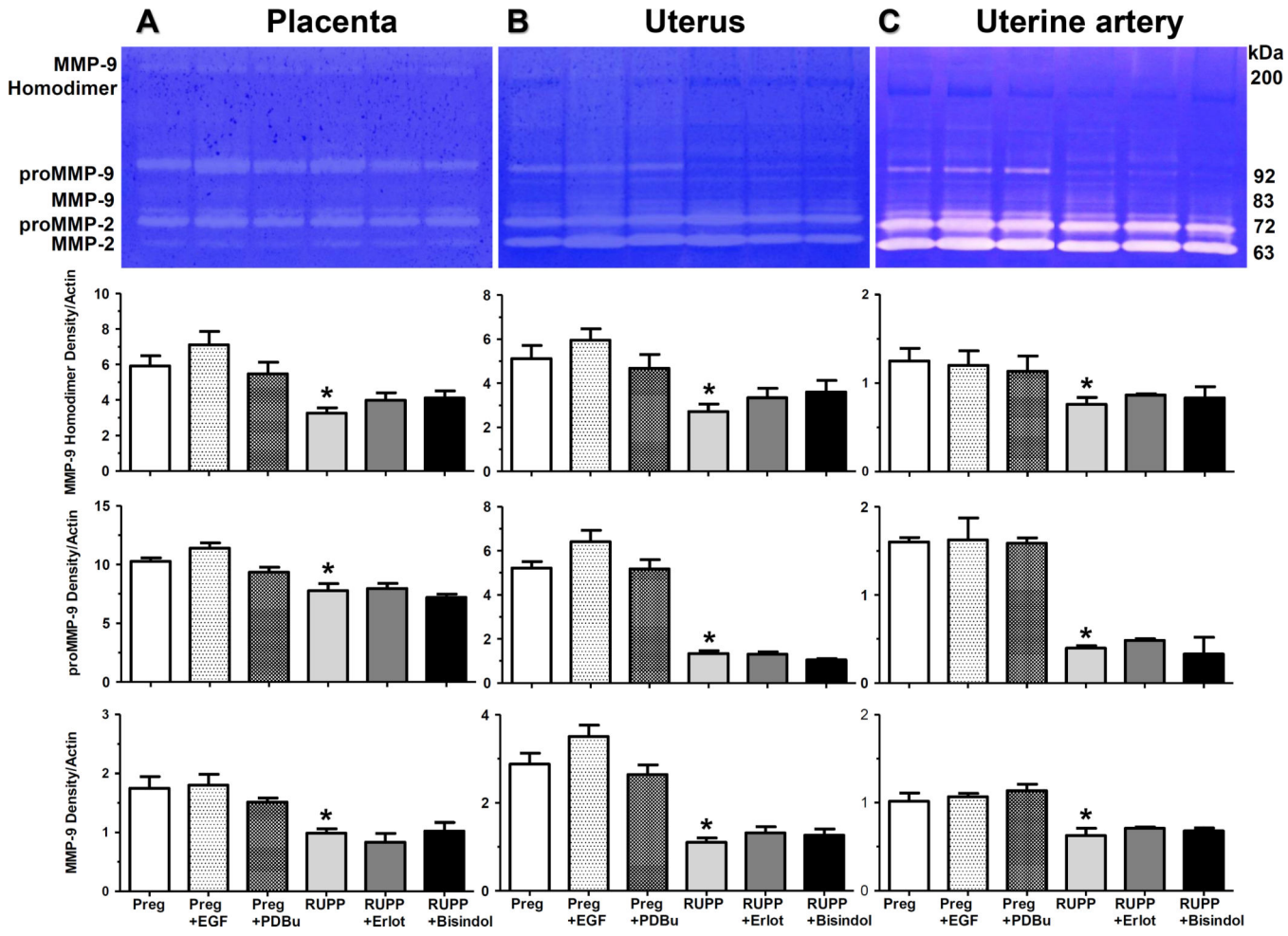

Fig. 8.

Effect of modulators of EGFR and PKC on the gelatinase activity of MMP-9 homodimer and monomers in placenta (A), uterus (B), and uterine artery (C) of Preg rats and RUPP rats. Tissue homogenates of placenta, uterus, and uterine artery of Preg were treated in culture with EGF (1 μg/ml) or PKC activator PDBu (10^{-6} M), and tissues of RUPP rats were cultured with EGFR inhibitor erlotinib (10^{-5} M) or PKC inhibitor bisindolylmaleimide (10^{-5} M) then prepared for gelatin zymography analysis. The densitometry values of the proteolytic bands corresponding to MMP was presented as pixel intensity × mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group. * $P<0.05$, RUPP vs Preg.

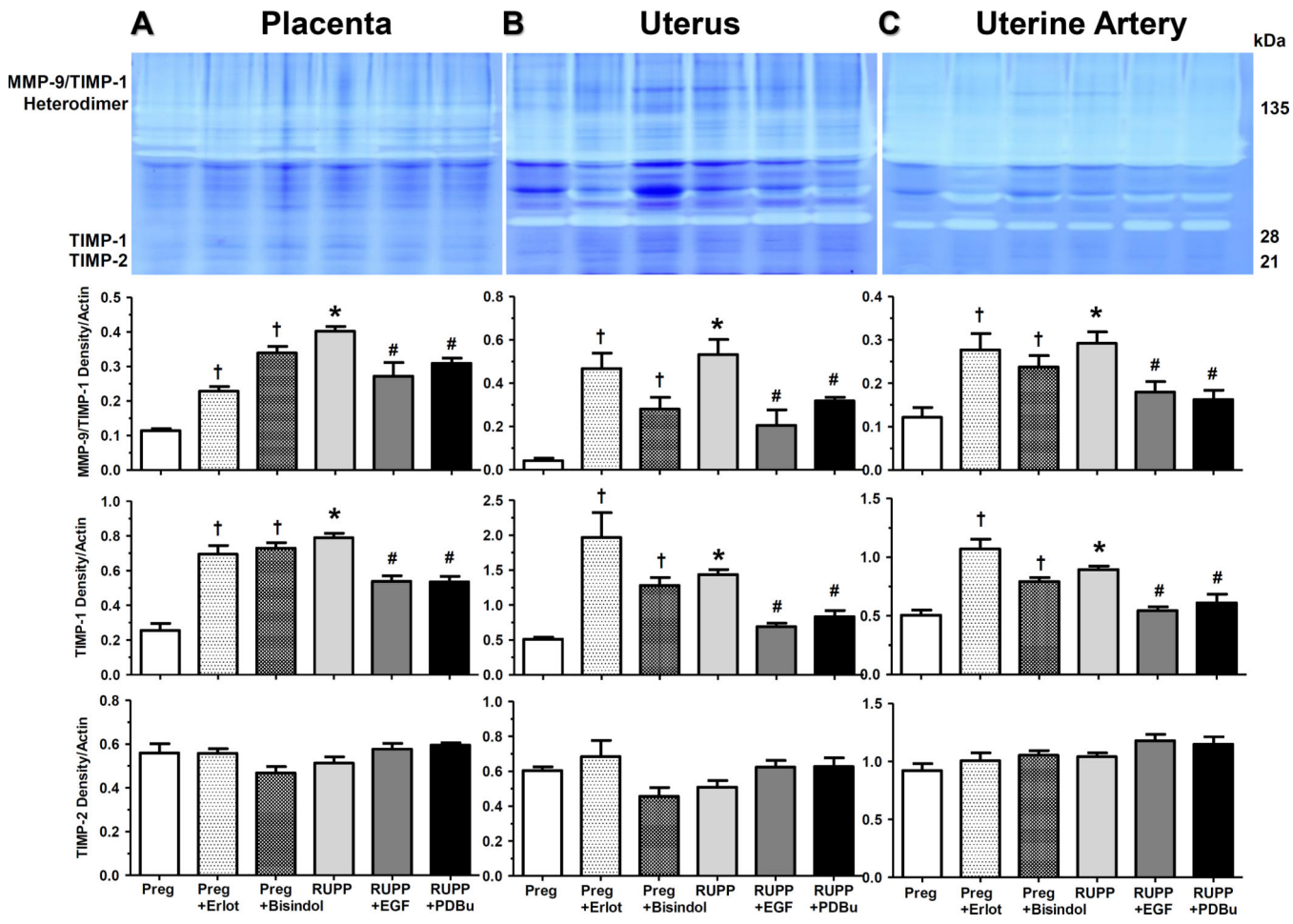

Fig. 9.

Effect of modulators of EGFR and PKC on MMP-9/TIMP-1 complex and TIMP activity in placenta (A), uterus (B), and uterine artery (C) of Preg rats and RUPP rats. Tissue homogenates of placenta, uterus, and uterine artery of Preg rats were treated in culture with EGFR inhibitor erlotinib and PKC inhibitor Bisindolylmaleimide and tissues of RUPP rats were cultured with EGF and PKC activator PDBu then prepared for reverse gelatin zymography analysis. The densitometry values of the bands corresponding to TIMP was presented as pixel intensity × mm^2 and normalized to β-actin to correct for loading. Bar graphs represent means±SEM, n=5/group.

* P<0.05, RUPP vs Preg.

† P<0.05, EGFR inhibitor or PKC inhibitor pretreated vs. nontreated tissues of Preg rats.

# P<0.05, EGF or PKC activator pretreated vs. non-treated tissues of RUPP rats.