Maternal hypoxia alters matrix metalloproteinase expression patterns and causes cardiac remodeling in fetal and neonatal rats

by

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Running title: Fetal hypoxia and heart remodeling

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ABSTRACT

Fetal hypoxia leads to progressive cardiac remodeling in rat offspring. The present study tested the hypothesis that maternal hypoxia results in reprogramming of matrix metalloproteinase (MMP) expression patterns and fibrillar collagen matrix in the developing heart. Pregnant rats were treated with normoxia or hypoxia (10.5% O₂) from day 15 to 21 of gestation. Hearts were isolated from 21-day fetuses (E21) and postnatal day 7 pups (PD7). Maternal hypoxia caused a decrease in the body weight of both E21 and PD7. The heart to body weight ratio was increased in E21 but not in PD7. Left ventricular myocardium wall thickness and cardiomyocyte proliferation were significantly decreased in both fetal and neonatal hearts. Hypoxia had no effect on fibrillar collagen content in the fetal heart, but significantly increased the collagen content in the neonatal heart. Western blotting revealed that maternal hypoxia significantly increased collagen I, but not collagen III, levels in the neonatal heart. Maternal hypoxia decreased MMP-1 but increased MMP-13 and MT1-MMP in the fetal heart. In the neonatal heart, MMP-1 and MMP-13 were significantly increased. Active MMP-2 and MMP-9 levels and activities were not altered in either fetal or neonatal hearts. Hypoxia significantly increased tissue inhibitors of metalloproteinase (TIMP)-3 and TIMP-4 in both fetal and neonatal hearts. In contrast, TIMP-1 and TIMP-2 were not affected. The results demonstrate that in utero hypoxia reprograms the expression patterns of MMPs and TIMPs and causes cardiac tissue remodeling with the increased collagen deposition in the developing heart.

Keywords: tissue inhibitor of metalloproteinases; collagen deposition; cardiomyocyte proliferation; hypertrophy
INTRODUCTION

Substantial evidence has shown a clear association of adverse intrauterine environment with an increased incidence of cardiovascular disease and hypertension later in life (4, 5, 14, 32), suggesting that the prenatal environment can change the postnatal physiology, namely "programming". Programming is a result of adaptive alterations in gene expression patterns and phenotype in response to the in utero stresses, which modify the growth of specific organs during the critical period of development in early life (30). This may predispose the organism to a heightened susceptibility of cardiovascular disease in its adult life. Multiple stimuli have been identified as being capable of inducing fetal programming in animal models, including malnourishment, exposure to hypoxia, cocaine, nicotine or glucocorticoid during the pregnancy (2, 14, 25, 32, 37, 48, 50). Recent animal studies have demonstrated that fetal hypoxia is linked to early changes in the developing cardiovascular system (6, 37, 39). In fact, "physiological hypoxia" is a normal part of fetal life for all vertebrates and it plays an active role in vasculogenesis, angiogenesis, hematopoiesis and chondrogenesis during the fetal development (39). The partial oxygen tension of embryo is below 10 mmHg, which is regarded as being hypoxic compared with normal adult tissues with the oxygen tension of 20-40 mmHg (45), indicating that the fetus is persistently hypoxic during the organ formation, growth and maturation, and that fetal tissues have a lower threshold to reach a state of oxygen insufficiency (38). Although a restricted oxygen supply is essential for intrauterine growth, excessive or severe hypoxia may compromise the normal fetal or neonatal development. The fetus may experience prolonged hypoxia under various conditions, such as pregnancy at high altitude, pregnancy with smoke, drug abuse, anemia, pulmonary disease, hypertension, etc. Fetal exposure to
pathophysiological hypoxia results in the redistribution of blood flow to facilitate oxygen
delivery to the vital organs, such as the brain and heart (41). In rodents, the heart is particularly
vulnerable to stressors, such as hypoxia, during the late fetal development and early postnatal life
when it undergoes rapid growth and maturation (30). The maturation process of cardiomyocytes
in rats occurs over postnatal day 4 to 12, which is marked by binucleation and escape from the
cell cycle (51). Nonetheless, little is known about cardiac remodeling and related genes
expression patterns in the heart during the critical developmental stages of the fetus and neonate
in response to fetal hypoxia.

It has been demonstrated that the timely breakdown and restructure of extracellular
matrix (ECM) are critical for the normal fetal organ development (33). ECM is a complicated
microenvironment including numerous matrix proteins (such as collagens), signaling molecules,
proteases, and all of them contribute to the tissue remodeling process (40). The functional
integrity of myocardium depends largely on the extracellular collagenous matrix (31). Aberrant
amount, distribution or organization of fibrillar collagens in the myocardium is associated with
various pathophysiological changes in the heart (18). Many factors that participate in the
cardiac tissue remodeling have been revealed and matrix metalloproteinases (MMPs) are the one
of the most significant mediators in the ECM turnover. MMPs are a family of zinc-dependent
proteases that consist of at least 25 different MMPs in vertebrate (12). Although the most studied
MMPs in the heart are gelatinases, MMP-2 and MMP-9 that are capable of degrading type I and
IV collagens, the major types of fibrillar collagens in the heart are type I and III collagens that
are digested mainly by collagenase-1 (MMP-1) and collagenase-3 (MMP-13), respectively (40,
42, 44). In addition to the secreted MMPs, there is a unique subfamily of MMPs, namely
membrane type- (MT-) MMPs. MT-MMPs (MT1-MT6 MMPs) have been identified to be fully
active enzymes once inserted into the cell membrane, rather than being secreted in the proenzyme form. Among the six types of MT-MMPs, MT1-MMP is highly expressed and is a primary enzyme that digests fibrillar collagens in the heart in addition to collagenases (10, 36, 40). Together with the four types of tissue inhibitors of metalloproteinases (TIMPs) identified, MMPs have been implicated in a variety of physiological and pathological processes in the cardiovascular system, ranging from fibrillar collagen digesting in the heart formation and growth to cardiac ischemia-reperfusion injury (23). Yet whether and to what extent MMPs and TIMPs expression patterns in the fetal heart are altered by in utero hypoxia remain to be elusive. Herein, we present evidence in a rat model that maternal hypoxia during gestation alters MMPs and TIMPs expression patterns in the developing heart and results in the abnormal cardiac growth pattern and cardiomyocyte proliferation with the aberrant content of fibrillar collagen network in fetal and neonatal hearts.
METHODS

Experimental animals. Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into two groups: 1) normoxic control, and 2) hypoxic treatment of 10.5% oxygen from gestational day 15 to day 21, as described previously (50). Hearts were obtained from day 21 fetal and day 7 neonatal rats of mixed sex. The sample size was 4 or 5 pups per group. For Western immunoblots, hearts were flash frozen in liquid nitrogen and stored at -80°C until analysis. For the tissue slide preparation, hearts were fixed in 10% buffered formalin and embedded in paraffin. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee, and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Myocardial morphometry. Transverse sections of 5 μm prepared from the middle portion of each heart were mounted and stained with hematoxylin and eosin. Sections were viewed at 20× or 40× magnification. The images were digitized and analyzed by the Image-Pro Plus image analysis software. The left ventricular wall thickness was determined in the anterior wall (AW), posterior wall (PW), septal wall (SW), and free wall (FW).

Collagen measurement. Fibrillar collagen structure and composition in fetal and neonatal hearts were examined by scanning electron microscopy (SEM), as described previously (35). Briefly, the fetal and neonatal hearts were fixed in 2.5% glutaraldehyde and stored at 4°C until processing. Hearts were immersed in 10% NaOH for 2-4 days at room temperature, and then rinsed in distilled water for several times until the heart became transparent. Hearts were then treated with 1% aqueous solution of tannic acid for 2-3 h, rinsed in distilled water for overnight,
and postfixed in 1% aqueous solution of OsO₄ for 1-2 h. The specimens were dehydrated in a series of increasing ethanol concentrations (50, 80, and 100%) and further dehydrated by critical point drying at 31°C for 5–10 min. The samples were then mounted on a specimen holder for drying overnight in a desiccator, coated with gold and examined with a Philips XL-20 SEM. High-resolution digital images were acquired directly to a computer. The collagen deposition in the heart was also determined by collagen staining using Picrosirius Red that binds specifically to collagens. Paraffin sections were first dewaxed, then further deparaffinized by xylene and rehydrated sequentially in ethanol. Rehydrated sections were hematoxylin stained and washed for 10 min in water. The sections were stained in 0.1% picrosirius red for 1 h followed by washing in two changes of acidified water (5 ml glacial acetic acid in 1 liter of water). Slides were then dehydrated again in increasing concentrations of ethanol up to 100%, followed by washing in xylene and mounted. Sections were viewed at 20 or 40× magnification, and the images were digitized and analyzed by the Image-Pro Plus image analysis software. Soluble collagen content was determined using the QuickZyme collagen assay kit (QuickZyme Biosciences, Netherlands) according to the instructions of the manufacturer. Briefly, hearts were homogenized in 0.5 M acetic acid and pepsin (1:10 weight/tissue wet weight) and incubated overnight at 4 °C. The samples were then centrifuged (10 min at 14,000 rpm), the supernatant was collected and total protein was quantified. Samples (200 µg) and the collagen standard were added to assigned wells in a 96-well plate, and the dilution buffer was added to each well to a final volume of 140 µL/well. The Sirius Red dye solution was added to each well, and the plate was sealed and incubated on ice with gently shaking for 20 min. The plate was then centrifuged at 3000 ×g at 4 °C. The collagen fiber that binds to Sirius Red dye forms a pellet at the bottom of the well. The pellets were washed 3 times with the washing buffer. The detection buffer was
added to the pellets and mixed thoroughly and the signal was read at 540 nm. A standard curve was generated using the collagen standard provided by the kit and collagen content (µg) per well was determined. All steps were performed on ice to avoid degradation of the collagen fibers.

**Ki-67 staining.** The application of the nuclear protein, Ki-67, was used to determine the cell proliferation of cardiomyocytes, as described previously (49). Hearts were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical detection of proliferation marker Ki-67 was performed using BD Pharningen anti-Ig HRP detection kit. Briefly, transverse slices of hearts were first deparaffinized in xylene and rehydrated with a series of decreased concentrations of alcohol (100%, 95%, 90%, 75%). To block the endogenous peroxidase activity, the slices were incubated with 0.3% H₂O₂ for 10 min. Nonspecific binding sites was blocked for 1 h at room temperature in a Tris-buffered saline solution containing 5% bovine serum albumin. The slices were then incubated with mouse monoclonal antibody against Ki-67 (1:50, Abcam Inc, Cambridge, MA) for overnight at 4 °C. The slices were rinsed three times in phosphate-buffered saline for 5 min each time, followed by incubation with biotinylated goat anti-mouse IgG (1:50, BD Pharningen) for 60 min at room temperature. The samples were then exposed to streptavidin-HRP and reacted with diaminobenzidine substrate solution according to the manufacture's recommendations. The slices were viewed with a Zeiss microscope, and images were captured with an attached SPOT digital camera imaging system.

**Western blot analysis.** Hearts were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween-20, 1% Triton, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml leupeptin, and 5 µg/ml aprotinin, pH 7.4 and allowed to incubate for 1 h on ice. Homogenates were then centrifuged at 4 °C for 10 min at 10,000 ×g, and supernatants collected. Protein concentrations were measured using a
protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 10% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 90 min. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites was blocked for 1 h at room temperature in a Tris-buffered saline solution containing 5% dry-milk. The membranes were then probed with primary antibodies against collagen I, III, MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:300 dilution), MMP-1 (Calbiochem, San Diego, CA; 1:1000 dilution), MMP-9, TIMP-1, -2, -3 (Millipore, Temecula, CA; 1:1000 dilution) and TIMP-4 (Abcam Inc, Cambridge, MA). To assure equal loading, band intensities were normalized to beta2-microglobulin (B2M) determined by its antibody (Abcam Inc, Cambridge, MA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image analysis software.

**Gelatin zymography.** Hearts were homogenized in a lysis buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 1% protease inhibitor, 0.5% PMSF. Homogenates were then centrifuged at 4 °C for 20 min at 15,000 ×g, and supernatants collected. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (60 μg) were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. The gel was renatured by renaturation buffer (Bio-Rad, Hercules, CA) for 1 h and then incubated with a development buffer (Bio-Rad, Hercules, CA) at 37 °C for 48 h. The gel was stained with 0.5% Coomassie blue R-250 (Bio-Rad, Hercules, CA) for 1 h and then destained with destaining buffer (Bio-Rad, Hercules, CA) till the bands became clear. Data were analyzed with the Kodak ID image analysis software.
Gelatinolytic activity was determined as clear zones or bands at the appropriate molecular weights. Mouse active MMP-9 (Chemicon, Temecular, CA) was used as a positive control.

**Statistical analysis.** Data are expressed as mean ± SEM. Experimental number (n) represents the hearts of fetuses or neonates from different dams. Statistical significance (p < 0.05) was determined by analysis of variance (ANOVA) or Student's t test, where appropriate.
RESULTS

Effect of hypoxia on fetal and neonatal heart weight and ventricular wall thickness.

Maternal hypoxia significantly decreased fetal (3.9 ± 0.1 g vs. 3.2 ± 0.1 g, p < 0.05) and neonatal (15.0 ± 0.3 g vs. 9.2 ± 0.6 g, p < 0.05) body weight. There was no significant difference in fetal heart weight between the control and hypoxic animals (23.9 ± 0.9 mg vs. 22.6 ± 0.7 mg, p > 0.05). However, hypoxia significantly increased the heart to body weight ratio in fetal rats (6.1 ± 0.1 mg/g vs. 7.0 ± 0.2 mg/g, p < 0.05). Neonatal heart weight was significantly decreased in hypoxic animals (119.5 ± 3.6 mg vs. 75.8 ± 6.0 mg, p < 0.05), while the heart to body weight ratio in neonatal rats was not changed by hypoxia (8.0 ± 0.2 mg/g vs. 8.2 ± 0.5 mg/g, p > 0.05).

To determine the effect of hypoxia on left ventricular wall thickness, hematoxylin and eosin-stained tissue slides were examined. As shown in Fig. 1, the thickness of anterior wall, septal wall and free wall was decreased in the fetal heart by hypoxia. As expected, the wall thickness of left ventricle was significantly increased in the neonatal heart, as compared with that of fetal heart. However, fetal hypoxia resulted in significant decreases in the thickness of anterior wall, posterior wall, septal wall and free wall of left ventricle in the neonatal heart (Fig. 1). Additionally, the epicardial detachment from underlying myocardium was seen in all samples of fetal hearts treated with maternal hypoxia (Fig. 1).

Effect of hypoxia on fibrillar collagen structure and composition in fetal and neonatal hearts. Fibrillar collagen structure and composition were assessed by scanning electron microscope in the heart from fetal and neonatal rats. As shown in Fig. 2, fibrillar collagen fibers were cross-linked randomly to form a complicated matrix network in both fetal and neonatal hearts. Maternal hypoxia did not change collagen matrix in the fetal heart, but increased fibrillar
collagen weave matrix in the neonatal heart (Fig. 2). The collagen content and distribution in the left ventricle were examined further by the collagen staining using picrosirius red. As shown in Fig. 3A, collagen forms the fiber bundles in the interstitial space. Maternal hypoxia did not change the total collagen content in the fetal heart but increased it in the neonatal heart (Fig. 3A). The soluble collagen content in the heart was determined using a collagen assay kit. Fig. 3B shows that maternal hypoxia had no significant effect on soluble collagen content in the fetal heart. The collagen content in the heart showed a development-dependent increase from the fetus to the neonate, and maternal hypoxia resulted in a significantly greater increase in fibrillar collagens in the neonatal heart (Fig. 3B). The expression of major types of collagens in the heart, collagen I and collagen III, were determined by Western blots (Fig. 3C). Maternal hypoxia caused a reduction in collagen I in the fetal heart but a significant increase in collagen I in the neonatal heart. In contrast, collagen III levels were not significantly altered in either fetal or neonatal hearts.

**Effect of hypoxia on cell proliferation in fetal and neonatal hearts.** Cardiomyocyte proliferation in the fetal and neonatal hearts was determined by examining the immunostaining of the nuclear protein, Ki-67. The Ki-67 expression occurs throughout all phases of the cell cycle, except for the G0 phase. As shown in Fig. 4, the immunostaining of Ki-67 revealed the dark brown dots within the cells. Maternal hypoxia significantly decreased Ki-67 positive nuclei in both the fetal and neonatal hearts, suggesting a reduced proliferative activity of cardiomyocytes (Fig. 4). Additionally, cardiomyocyte proliferation showed a development-dependent decrease from the fetal to the neonatal heart (Fig. 4).

**Effect of hypoxia on MMPs and TIMPs in fetal and neonatal hearts.** To elucidate the potential mechanisms underlying the hypoxia-induced cardiac remodeling, we determined the
effect of maternal hypoxia on the expression of active MMP-1, -2, -9, -13, MT1-MMP and the expression of TIMP-1, -2, -3, -4 in fetal and neonatal hearts. As shown in Fig. 5, maternal hypoxia resulted in differential expression patterns of MMPs in the fetal and neonatal hearts. The MMP-1 expression was decreased in the fetal heart, but was increased in the neonatal heart. Although it may be debating whether rodents express MMP-1, previous studies clearly demonstrated the expression of MMP-1 in rat hearts (8, 25). MMP-13 was increased in both the fetal and neonatal hearts. MT1-MMP was increased only in the fetal heart. In contrast, the expression of active MMP-2 and MMP-9 were not altered in either fetal or neonatal hearts. Additionally, the proteolytic activities of MMP-2 & -9 were measured with gelatin zymography. As shown in Fig. 6, there was a lack of activity of active MMP-9 at 82 kDa in fetal or neonatal hearts. MMP-2 activities at 62 kDa and 72 kDa were detected, but were not significantly altered by maternal hypoxia in either fetal or neonatal hearts. Although maternal hypoxia had no significant effect on TIMP-1 and TIMP-2 expressions, it significantly increased TIMP-3 and TIMP-4 levels in both the fetal and neonatal hearts (Fig. 7). Table 1 summarizes the relative changes of MMPs, TIMPs and collagens in fetal and neonatal hearts in response to maternal hypoxia.
DISCUSSION

The present study demonstrates in a rat model that maternal chronic hypoxia causes remodeling of the developing heart in the fetus and neonate by decreasing cardiomyocyte proliferation and increasing the collagen deposition. Previous studies demonstrated that maternal hypoxia increased hypoxia-inducible factor 1α (HIF-1α) protein levels in rodent fetal hearts (3, 39), indicating tissue hypoxia of the fetal heart in response to maternal hypoxia. In the present study, the finding that maternal hypoxia significantly decreased immunostaining of the proliferation marker Ki-67 in fetal and neonatal hearts is intriguing. Although Ki-67 is a nonspecific cell proliferation marker and may stain both cardiomyocytes and cardiac fibroblasts, the previous finding that hypoxia promoted cardiac fibroblast proliferation in rodents and humans (1, 22) suggests that the maternal hypoxia-mediated decrease in the Ki-67 staining in fetal and neonatal hearts in the present study is primarily due to the reduced cardiomyocyte proliferation. In rat heart development, the transition of proliferative and hyperplastic growth of mononucleated cells to hypertrophic growth of binucleated cells and terminal differentiation of cardiomyocytes take place within the first two weeks after birth (30). Consistent with the present finding, previous studies demonstrated an increase in the percentage and cell size of binucleated myocytes in fetal rat heart in response to maternal hypoxia (3), an early morphologic indicator of cardiomyocyte hypertrophy (9). Taken together, these studies suggest that fetal hypoxia causes a premature exit of the cell cycle in the fetal heart leading to a fewer but larger cardiomyocytes in offspring. Indeed, enlarged myocytes have been demonstrated in the heart of adult offspring rats that had been exposed to hypoxia before birth (28, 47). The finding of maternal hypoxia-mediated remodeling of the heart during its critical developmental stages of the fetus and neonate
suggests programming of aberrant heart function. Indeed, the functional impact of remodeling on postnatal development in the adult heart has been demonstrated in an essentially same animal model of maternal hypoxia, in which reduced MMP-2 and enhanced collagen accumulation were found along with left ventricular hypertrophy and stiffening, diastolic dysfunction, and increased ischemic injury in 4 and 7 month old offspring (47). Similar findings that maternal hypoxia causes fetal programming of ischemia-sensitive phenotype in the adult heart have been demonstrated in our previous studies (28, 29, 37, 48, 50).

Whereas the mechanisms underlying the hypoxia-mediated reduction of myocyte proliferation in the fetal heart remain elusive, the present finding of the epicardial detachment in the hypoxic fetus provides a possible mechanism by which hypoxia inhibits proliferative and hyperplasic growth of the fetal heart. During the fetal development, the epicardium provides the precursor cells that give rise to various cell types in the heart, and it also supplies multiple growth factors to stimulate cardiac myocyte proliferation, including fibroblast growth factor (FGF)-2, Wingless-type MMTV integration site (Wnt)-9b, FGF-9 and other epicardially-derived factors (34, 39). The detachment of epicardium from myocardium is likely to reduce the availability of mitogenic factors and growth factors to the myocardium and subsequently to inhibit myocardial proliferation (39). Additionally, the present study demonstrates that fetal hypoxia increases TIMP-3 and TIMP-4 expression levels in both fetal and neonatal hearts, suggesting another possible mechanism in the hypoxia-mediated down-regulation of myocyte proliferation. In addition to their roles in modulating MMPs, it has been demonstrated that both TIMP-3 and TIMP-4 play a key role in inhibiting cardiomyocyte proliferation in rat hearts possibly in a MMP-independent and receptor-mediated manner (13, 16, 17, 42).
In the present study, the finding that maternal hypoxia causes an increased fibrillar collagen content in neonatal, but not fetal, hearts is intriguing, and suggests a critical window in compensatory remodeling of the collagen matrix in the neonatal heart resulting from hypoxia-mediated premature exit of cell cycle in the fetal heart. In the heart, collagens are the primary extracellular proteins supporting the myocardium and determining the tissue stiffness. The present study demonstrates that hypoxia differentially regulates collagen I and collagen III expression in the developing heart. The increased collagen content found in the neonatal heart is mainly due to an increase in collagen I. During the postnatal development, collagen I represents more than 85% of the total collagen in the heart (7, 11). Unlike the finding in the neonatal heart, collagen I was decreased in the fetal heart by hypoxia. Although it remains unclear what is the major type of collagens in the fetal heart, collagen III is suggested to be one of the most important fibrillar collagens in the heart during the fetal development (21). The finding that hypoxia did not significantly affect collagen III levels in the fetal heart consists with the lack of apparent changes in the total fibrillar collagen content in the fetal heart. Although the lack of changes in collagen III was also demonstrated in neonatal hearts in the present study, a previous study showed that maternal hypoxia increased the deposition of both collagen I and III in the heart of adult offspring (47), suggesting a continuous remodeling process in the heart during the postnatal development.

The present findings of decreased MMP-1 in the fetal heart and increased MMP-1 in the neonatal heart are somewhat surprising, given that MMP-1 as collagenase 1 is a primary enzyme that digests collagen I. This suggests a complex pattern of the interaction between collagens and MMPs in the developing heart. It is possible that fetal hypoxia caused an imbalance of collagen synthesis and degradation in the developing heart with the effect of synthesis predominant.
Multiple pathways have been identified in the hypoxia-enhanced synthesis of collagen I, including reactive oxygen species, mitogen-activated protein kinase, and transforming growth factor-beta 1 (1, 19, 20). Although it is not known at present, the possibility that the collagen I and III genes have different HIF-1 promoter sites that may regulate differentially expressions of collagen I and III in the heart remains an intriguing area for the further investigation. The changes in MMP-1 levels in the same direction of those in collagen I observed in the hypoxic hearts may reflect a compensatory response to the synthesis of collagen I. On the other hand, hypoxia-mediated changes in MMP-1 expression levels may in turn lead to a compensatory response of collagen I synthesis that exceeds its degradation. Similar findings of the apparent paradoxical changes of collagens and MMPs in the same direction resulting from the hypoxia treatment were also obtained in mice (20), supporting the notion of feedback regulation of collagens on MMPs levels. It has been shown that the increased collagens can activate the discoidin domain receptor (DDR) and therefore up-regulate MMPs (43, 46). In the heart, DDR 2 is primarily expressed in fibroblasts that are the major source of ECM components and MMPs (15, 24). Similarly, MMP-13 (collagenase 3) was significantly increased by hypoxia in both fetal and neonatal hearts, whereas collagen III remained unchanged. This further supports the notion that fibrillar collagens in the developing heart are regulated primarily through their synthesis rather than their degradation via collagenases. In addition to collagenases, MT1-MMP is another primary enzyme that digests fibrillar collagens in the heart (10, 36). The increased MT1-MMP levels in the hypoxic fetal heart may contribute to the decreased collagen I, and the lack of MT1-MMP increment in the neonatal heart may enhance the accumulation of collagen I synthesis resulting from fetal hypoxia. While collagenases (MMP-1 and MMP-13) possess high substrate specificity for fibrillar collagens, MT1-MMP has been shown to degrade nonmatrix substrates
including cytokines, bioactive peptides, and growth factors in the myocardium (40). It is possible
that the elevated MT1-MMP also contributes to the reduced cardiomyocyte proliferation
observed in the hypoxic fetal heart. The lack of effect of fetal hypoxia on MMP-2 and MMP-9
expression levels and activities and their endogenous inhibitors TIMP-2 and TIMP-1 in the heart
indicates the minimal role of the gelatinases in hypoxia-mediated remodeling of fetal and
neonatal hearts. Nonetheless, the active MMP-2 levels were found decreased in the heart of adult
rats that had been exposed to hypoxia before birth (47), suggesting a continuous programming of
MMPs expression patterns in the heart during the postnatal development.

The present study provides new insights in the maternal hypoxia-mediated heart
remodeling during its critical developmental stages of the fetus and neonate, and suggests a role
of altered MMP-TIMP expressing patterns in the developing heart. Although it is difficult to
demonstrate a true cause-effect relation of MMPs-TIMPs and cardiac remodeling in living
animals particularly in a pregnant animal model at present due to the lack of selective inhibitors
and the difficulty of their use in a pregnant animal model, as well as the difficulty of transgenic
approach in a rat model, the present findings provide important physiological information and
basis for future more mechanistic investigations possibly using an approach of siRNA in cultured
organ/heart or cardiomyocytes. Given that hypoxia is one of the most important and clinically
relevant stresses to the fetus, and that large epidemiological studies indicate a link between in
utero adverse stimuli during gestation and an increased risk of heart disease in the adulthood, the
possibility that fetal hypoxia may result in programming of a heightened vulnerability of
remodeling and failing heart later in life provides a mechanistic understanding worthy of
investigation in humans. Indeed, it has been shown in rats that antenatal hypoxia enhances
collagen accumulation and left ventricular hypertrophy along with stiffening, diastolic
dysfunction, and the increased heart susceptibility to ischemia and reperfusion injury in adult offspring (28, 29, 37, 47, 48, 50).
Funding

This work was supported by the National Institutes of Health [HL82779 (LZ), HL83966 (LZ), HL89012 (LZ) and HD31226 (LZ)].

Conflict of Interest: none declared.


Figure Legends

**Figure 1. The effect of maternal hypoxia on ventricular morphology.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Arrows show the detachment of epicardium in the hypoxic fetal heart. The left ventricular wall thickness was determined at anterior wall (AW), posterior wall (PW), septal wall (SW) and free wall (FW). LV: left ventricle; RV: right ventricle. Data are means ± SEM. Data were analyzed by two-way ANOVA. * P < 0.05, hypoxia vs. control; † P < 0.05, PD7 vs. E21. n = 3-5 per group.

**Figure 2. The effect of maternal hypoxia on fibrillar collagen structure and composition.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Fibrillar collagen structure and composition were examined by scanning electron microscope. Hypoxia increased the collagen matrix in PD7 but not E21 hearts. The images were representatives of the heart samples from 4 control E21, 4 hypoxic E21, 5 control PD7, and 6 hypoxic PD7 rats.

**Figure 3. The effect of maternal hypoxia on the fibrillar collagen content.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control (C) and hypoxic (H) groups. The collagen content in tissue sections (A) and the soluble fraction of collagen content in the heart (B) were determined by picrosirius red staining. Arrows show the collagen deposition in the anterior wall of left ventricle. Protein levels of collagen I (C I) and collagen III (C III) were determined by Western blots (C). Data are means ± SEM. Data in panel B were analyzed
by two-way ANOVA. Data in panels C were analyzed by *t*-test. * P < 0.05, hypoxia vs. control;
† P < 0.05, PD7 vs. E21. n = 5 per group.

**Figure 4. The effect of maternal hypoxia on cardiomyocyte proliferation.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Cell proliferation was examined by the Ki-67 staining. Arrows show Ki-67 positive nuclei. Data are means ± SEM. Data were analyzed by two-way ANOVA. * P < 0.05, hypoxia vs. control; † P < 0.05, PD7 vs. E21. n = 4 per group.

**Figure 5. The effect of maternal hypoxia on MMPs expression.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Active MMPs protein levels were determined by Western blots. Data are means ± SEM. Data were analyzed by *t*-test. * P < 0.05, hypoxia vs. control. n = 5 per group.

**Figure 6. The effect of maternal hypoxia on the activity of MMP-2 & -9.** Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. The activities of MMP-2 & -9 were determined by gelatin zymography. Clear bands indicate positive MMP activity. MMP-2 activities are indicated by clear bands associated with molecular weights of 62 kDa and 72 kDa. No clear bands associated with active MMP-9 at 82 kDa were present in the hearts. PC: positive control of mouse active MMP-9 at 82 kDa. The bottom graph illustrates the mean values of densitometric analysis of MMP-2 activities in fetal and neonatal hearts. Data are means ± SEM. Data were analyzed by *t*-test. n = 4-5 per group.
Figure 7. The effect of maternal hypoxia on TIMPs levels. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. TIMPs protein levels were determined by Western blots. Data are means ± SEM. Data were analyzed by $t$-test. * $P < 0.05$, hypoxia vs. control. $n = 5$ per group.
Table 1: Maternal hypoxia-induced changes in the expression of MMPs, TIMPs and collagens.

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Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups, and expression levels of MMPs, TIMPs and collagens were determined by Western blot. ↑: increase by hypoxia; ↓: decrease by hypoxia; —: no change by hypoxia.
Figure 1
Figure 2
Figure 3

**A**

Control | Hypoxia
---|---
E21 |  |  | +
PD7 |  |  | +

**B**

![Graph showing collagen content](image)

- **Control** vs **Hypoxia**
  - E21
  - PD7

* indicates statistical significance.
Figure 3
Figure 4
Figure 5

E21
Control Hypoxia
Active MMP-1 42 kDa
Active MMP-2 62 kDa
Active MMP-9 82 kDa
Active MMP-13 48 kDa
MT1-MMP 65 kDa
B2M 14 kDa

PD7
Control Hypoxia
Active MMP-1 42 kDa
Active MMP-2 62 kDa
Active MMP-9 82 kDa
Active MMP-13 48 kDa
MT1-MMP 65 kDa
B2M 14 kDa

Fold of Control

- MMP-1
- MMP-2
- MMP-9
- MMP-13
- MT1-MMP

E21 PD7
**Figure 6**

The figure shows the comparison of MMP-2 activity between control and hypoxia conditions at E21 and PD7. The gel images display bands at 62 kDa and 72 kDa, indicating the presence of MMP-9 and MMP-2 under both conditions. The bar graph below the gel images quantifies the MMP-2 activity as a fold of control, with no significant difference observed in MMP-2 activity under hypoxia compared to control conditions at both time points. The data suggests that hypoxia does not significantly affect MMP-2 activity at the protein levels measured.
Figure 7

ELISA assay showing the fold change of TIMP in control and hypoxia conditions at E21 and PD7.

- **E21**
  - TIMP-1: 28 kDa
  - TIMP-2: 21 kDa
  - TIMP-3: 24 kDa
  - TIMP-4: 26 kDa
  - B2M: 14 kDa

- **PD7**
  - TIMP-1: 28 kDa
  - TIMP-2: 21 kDa
  - TIMP-3: 24 kDa
  - TIMP-4: 26 kDa
  - B2M: 14 kDa