Maternal obesity induces fibrosis in fetal myocardium of sheep

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Submitted 21 July 2010; accepted in final form 26 September 2010

Maternal obesity induces fibrosis in fetal myocardium of sheep. Am J Physiol Endocrinol Metab 299: E968–E975, 2010. First published September 28, 2010; doi:10.1152/ajpendo.00434.2010.—Maternal obesity (MO) has harmful effects on both fetal development and subsequent offspring health. The impact of MO on fetal myocardium development has received little attention. Fibrogenesis is regulated by the transforming growth factor-β (TGF-β)/p38 signaling pathway. Using the well-established model of MO in pregnant sheep, we evaluated the effect of MO on TGF-β/p38 and collagen accumulation in fetal myocardium. Nonpregnant ewes were assigned to a control diet [Con, fed 100% of National Research Council (NRC) nutrient recommendations] or obesogenic diet (OB, fed 150% of NRC recommendations) from 60 days before conception. Fetal ventricular muscle was sampled at 75 and 135 days of gestation (dG). At 75 dG, the expression of precursor TGF-β was 39.9 ± 9.9% higher (P < 0.05) in OB compared with Con fetal myocardium, consistent with the higher content of phosphorylated Smad3 in OB myocardium. The phosphorylation of p38 tended to be higher in OB myocardium (P = 0.08). In addition, enhanced Smad complexes were bound to Smad-binding elements in 75 dG OB fetal myocardium measured by DNA mobility shift assay (130.2 ± 26.0% higher, P < 0.05). Similar elevation of TGF-β signaling was observed in OB fetal myocardium at 135 dG. Total collagen concentration in OB was greater than Con fetal myocardium (2.42 ± 0.16 vs. 1.87 ± 0.04%, P < 0.05). Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases (TIMPs) (9). Inflammation is known to trigger an array of cardiovascular diseases, such as atherosclerosis (3), cardiac remodeling, and myocardial fibrosis in hypertension (16). MO may induce similar changes in fetal myocardium; thus, we hypothesized that MO would increase TGF-β signaling and result in fibrogenesis in fetal myocardium.

MATERIALS AND METHODS

Care and use of animals. All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Multiparous Rambouillet/Columbia ewes were used. Ewes were bred to a single ram. Beginning 60 days before conception and continuing to the date of necropsy (first day of mating = day 0), ewes were individually fed a highly palatable diet either 100% (Con) of National Research Council recommendations for energy (26) (OB, fed 150% of NRC recommendations) or 150% (OB) of recommended energy requirements for early gestation (n = 6) as previously reported. Ewes were housed in individual pens in a temperature-controlled room (~20°C). All ewes were weighed at weekly intervals, and rations were adjusted for weekly changes in metabolic body weight. Body condition was scored at monthly intervals to evaluate changes in fatness. A body condition score of one (emaciated) to nine (obese) was assigned by two trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L3 through L5) and the region around the tail head (16). The maternal body weight and condition scores were reported previously (38).

Immediately before necropsy on day 75 and 135 of gestation, ewes were weighed and sedated with intravenous ketamine (10 mg/kg), and anesthesia was induced and maintained by isoflurane inhalation (1–2%). Ewes were exsanguinated while under general anesthesia, and the whole left ventricular myocardium was sampled. Surface tissues were trimmed, and a piece at the center of the ventricular muscle was fixed in fresh paraformaldehyde before being embedded in paraffin. The remaining muscle was minced and snap-frozen in liquid nitrogen for biological analyses.

Antibodies. Antibodies against tubulin (no. 2128), TGF-β (no. 3711), Smad2/3 (no. 3102), Smad2/3 (no. 3102), and phospho-Smad2/3 at Ser 423/425 (no. 3102) were purchased from Cell Signaling (Danvers, MA).
**Histological examination.** Ventricular samples were fixed in 4% (wt/vol) paraformaldehyde in phosphate buffer (0.12 M; pH 7.4), embedded in paraffin, and sectioned (10 μm thickness). Paraffin was removed via a series of incubations in xylene and ethanol solutions, and sections were stained with Masson trichrome; muscle fibers stain red, nuclei black, and collagen blue.

**Western blot analyses.** Myocardium samples were washed with PBS and lysed in a buffer containing 50 mM HEPES (pH 7.4), 2% SDS, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM Na₃VO₃, and 100 mM NaF. Soluble proteins were recovered after a 10-min centrifugation (10,000 g), and their concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Proteins in cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated in a blocking solution with 1:1 Odyssey Blocking Buffer and PBS/T. Membranes were then incubated with IRDye 800CW Goat Anti-Rabbit Secondary Antibody and IRDye 680 Goat Anti-Mouse Secondary Antibody from LI-COR Biosciences (Lincoln, NE) at a 1:10,000 dilution for 1 h in 1:1 Odyssey Blocking Buffer and PBS/T with gentle agitation protecting from light. Membranes were visualized by an Odyssey Infrared Imaging System. Density of bands was quantified and then normalized according to the tubulin content.

**Collagen concentration analysis.** Myocardium samples were ground and dried in a convection oven at 60°C, and the samples were weighed and hydrolyzed in 6 N HCl at 105°C for 16 h. An aliquot was removed for hydroxyproline determination using the method of Woessner (37). Collagen concentration (% dry wt) was calculated as follows: 20 s at 95°C, 20 s at 56°C, and 20 s at 72°C for 35 cycles. After amplification, a melting curve was used to confirm product purity, and the PCR products were electrophoresed to confirm the targeted sizes. Results are expressed relative to tubulin.

**Electrophoretic mobility shift assay.** Nuclear proteins were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions. The following oligonucleotide sequences were used as probes in gel-shift assays: Smad-binding element (SBE) forward, 5'-GGAGTATGTCTAGACTGACAATGTAC-3' and reverse 5'-GGAGTATGTCTAGACTGACAATGTAC-3'; Smad2/3, forward 5'-GGAGTATGTCTAGACTGACAATGTAC-3' and reverse 5'-GGAGTATGTCTAGACTGACAATGTAC-3'; and TGF-β signaling pathway.

**Results**

**TGF-β signaling pathway.** TGF-β protein concentration was 39.9 ± 9.9% higher (P < 0.05) at 75 days gestation (dG) in OB fetal myocardium than in Con myocardium and 80.5 ± 15.5% (P < 0.05) higher at 135 dG (Fig. 1). Smad2/3 are the key downstream mediators of the TGF-β signaling pathway. At 75

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**Fig. 1.** Transforming growth factor-β (TGFβ) content in fetal ventricular myocardium of sheep fed a control (Con, open bars) and obese (OB, filled bars) diet. A: 75 days gestation (dG); B: 135 dG. *P < 0.05; mean ± SE; n = 5 sheep.
dG, Smad2/3 and phosphorylated Smad3 showed a trend toward increase \((P = 0.08\) and \(P = 0.09\), respectively) in myocardium of fetuses of MO ewes compared with Con myocardium. At 135 dG, no difference in Smad2/3 or in their phosphorylation was observed between Con and OB groups (Fig. 2).

**p38 phosphorylation.** p38 phosphorylates Smad3 at Ser\(^{203}\) and Ser\(^{207}\) residues, which dramatically enhances its transactivation potential (17). No difference in total p38 protein expression was observed between OB and Con fetal groups at either 75 and 135 dG (Fig. 3). However, phosphorylation of p38 tended to be higher in OB samples \((P = 0.08)\) at 75 dG. At 135 dG, phosphorylation of p38 was 34.7 \pm 11.2\% higher \((P < 0.05)\) in the 135 dG OB group. The ratio of phospho-p38 to p38 in 135 dG samples was also higher \((37.6 \pm 10.8\%, P < 0.05)\) in OB compared with Con fetal myocardium (Fig. 3).

To confirm the effect of p38 in Smad signaling, phosphorylation of Smad3 at Ser\(^{208}\) was measured (Fig. 4). An increase \((99.6 \pm 30.8\%, P < 0.05)\) in phospho-Smad3 \((\text{Ser}^{208})\) was observed in OB compared with Con fetal myocardium at 135 dG.

**Smads binding to SBE in OB fetal myocardium.** The TGF-\(\beta\) and p38 signaling phosphorylate Smad2/3, which enhances their binding to SBE of targeted genes. To analyze whether there was a difference in protein interaction with the SBE, we extracted nucleoproteins and conducted electrophoretic mobility shift assays using a DNA fragment corresponding to the SBE. Samples from 75 dG OB fetal myocardium showed \(130.2 \pm 26.0\% (P < 0.05)\) more DNA shift than that from Con samples (Fig. 5). Consistently, the 135 dG OB samples had \(88.3 \pm 27.8\% (P < 0.05)\) more DNA shift than Con fetal myocardium samples (Fig. 5).

**Collagen concentration in OB and Con fetal ventricular muscle.** The upregulated TGF-\(\beta\) and p38 signaling induces fibrosis as evidenced by accumulation of collagen. To test whether there is a difference in collagen accumulation, we analyzed the collagen content in 135 dG fetal myocardium. Our data from the Masson trichrome staining revealed overt

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**Fig. 2.** Smad signaling in fetal ventricular myocardium of Con (open bars) and OB (filled bars) sheep. A: Smad2/3 and phospho-Smad2/3 in fetal myocardium tended to increase in OB compared with Con fetal myocardium at 75 dG; B: no difference of Smad2/3 and phospho-Smad2/3 was detected in fetal myocardium between OB and Con fetal myocardium at 135 dG. Data are means \pm SE; \(n = 5\).
myocardial fibrosis in OB fetal myocardium compared with the Con group (Fig. 6, A and B).

The amino acid hydroxyproline is present only in significant amounts in collagen. Using hydroxyproline content of myocardium as an indicator, collagen concentration was greater in the OB (P < 0.05) than the Con fetal myocardium (2.42 ± 0.16 vs. 1.87 ± 0.04%) (Fig. 6C).

The ratio of collagen types III/I is often considered as an important marker for myocardial stiffness and is altered in some models of heart disease. However, the ratio in collagen III/I mRNA expression did not differ between the two groups examined here (1.26 ± 0.19 for Con vs. 1.32 ± 0.10 for OB).

Relative mRNA expression of MMPs and TIMPs in day 75 fetal myocardium samples. MMPs and TIMPs are important regulators in collagen metabolism. MMP9 (2.28 ± 0.45 vs. 1.28 ± 0.28, P < 0.05) and TIMP3 (1.88 ± 0.23 vs. 1.18 ± 0.08, P < 0.05) were lower in OB sheep compared with Con sheep. TIMP1 in the OB group tended to increase compared with Con (P = 0.09) (Fig. 7). No difference in mRNA expression of MMP1, MMP2, MMP13, and TIMP2 was noted between OB and Con groups.

DISCUSSION

Sheep and humans process many similarities in fetal development; therefore, the sheep is frequently employed for studying normal and abnormal pregnancy (1, 2, 6, 12, 23, 31). Humans and sheep differ in many aspects from common laboratory rodents in pregnancy and fetal development. Rodents are polytocous and generate a larger biomass of concep-
Phosho-Smad3 (Ser208) → Con OB Con OB

Fig. 4. Phospho-Smad3 (Ser208) in fetal ventricular myocardium of Con (open bar) and OB (filled bar) sheep at 135 dG. An increase in phospho-Smad3 (Ser208) was observed in OB compared with Con fetal myocardium. *\(P < 0.05\); mean ± SE; \(n = 5\).

Phosho-Smad3 (Ser208)

Fig. 5. Electrophoretic mobility shift assay (EMSA) of nucleoproteins extracted from fetal myocardium of Con (open bars) and OB (filled bars) sheep. A: EMSA for Smad-binding element (SBE) showing an increased shift of DNA in OB compared with Con fetal myocardium at 75 dG; B: EMSA for SBE showing an increased shift of DNA in OB compared with Con fetal myocardium at 135 dG. *\(P < 0.05\); mean ± SE; \(n = 5\).
p38 is a kinase essential for Smad-dependent gene transcription (8). p38 phosphorylates Smad3 at Ser^{203} and Ser^{207} residues, which are required for the full transactivation potential of Smad3 (17). Inhibition of p38 abolishes the TGF-β response (21). In this study, enhanced p38 phosphorylation was detected in OB compared with Con fetal myocardium, and the phosphorylation of Smad3 at Ser^{203}, a site phosphorylated by p38, was also increased. The enhancement of both Smad and p38 phosphorylation would be expected to enhance the transcription activity of promoters containing SBE. We used an electrophoretic mobility shift assay to analyze the binding of Smads to the conserved SBE (GTCTAGAC) (39). As expected, an enhanced interaction of Smads with SBE DNA fragment was detected in OB compared with Con fetal myocardium. Correspondingly, a higher accumulation of collagen was observed in OB fetal myocardium. To our knowledge, this is the first report demonstrating enhanced accumulation of collagen in fetal myocardium induced by MO via a mechanism of TGF-β/p38-mediated gene expression.

MMPs are crucial for cardiac remodeling (33). The activity of MMPs is inhibited by the TIMPs (22). The balance between MMP and TIMP is essential for numerous physiological processes (24). In the current study, the overall difference in the mRNA expression of MMPs and TIMPs was minor between

Fig. 6. Collagen content in fetal myocardium of Con (open bars) and OB (filled bars) sheep. A: representative images of fetal myocardium from Con sheep at different magnifications; B: representative images of fetal myocardium from OB sheep at different magnifications; C: collagen content calculated based on the concentration of hydroxyproline showing an increase in collagen concentration in the myocardium of 135 dG fetuses of MO ewes. *P < 0.05; mean ± SE; n = 5.
Con and OB groups, with a decrease in both MMP9 and TIMP3 mRNA expression in OB compared with the Con group. The rationale for such decrease is unclear at this time. Nonetheless, the lack of difference in MMPs and TIMPs indicates that the enhanced fibrosis in OB fetal myocardium is mainly the result of the increased collagen synthesis instead of collagen degradation and remodeling, which are closely associated with fibrosis in adult myocardium (33). Therefore, the increase in collagen accumulation in fetal myocardium appears to be distinctly different from cardiac remodeling in adult myocardium.

Because of their different mechanical properties, the myocardial collagen III/I ratio affects myocardial stiffness (19), which is associated with pathological, hypertension-induced cardiac hypertrophy in rats (4). In this study, myocardial collagen III/I ratio in fetal myocardium was not altered because of MO, which again indicates that fetal fibrosis as a result of MO might be different from cardiac fibrosis and remodeling occurring in the aged heart.

Cardiac fibrosis is associated with many cardiomyopathies, including conditions induced by obesity, diabetes, and aging (28, 36). In particular, myocardial extracellular matrix remodeling is considered an important factor in the development of ventricular hypertrophy and is essential for the adaptive and maladaptive changes associated with the various myopathic anomalies (13). Our observations indicate that MO induces changes in myocardium at an early developmental stage, which typically is not observed until later in life. Such changes would be expected to impair cardiac function of offspring. An earlier study from our group using the same cohort of sheep revealed impairment of cardiac function in OB fetal heart at late gestation (35). Nonetheless, the mechanism of action contributing to impaired cardiac contractility in the offspring was essentially unknown at the time. Given that MO is known to increase cardiovascular diseases in offspring, our observation that MO induces collagen accumulation in fetal myocardium should shed some light on the missing link between MO and compromised cardiac function in offspring as measured by the Langendorff preparation (35).

In conclusion, our findings demonstrate that MO activates TGF-β and p38, which enhances Smad-mediated signaling and collagen accumulation in fetal myocardium. Such a change would tend to impair the contractile function of offspring hearts and predisposes them to cardiac dysfunction.

GRANTS
This work was supported by National Institutes of Health Grants P20-RR-016474 and R03-HD-057506.

DISCLOSURES
No conflicts of interest are declared by the authors.

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