Mitogen-activated protein kinase activation and regulation in the pressure-loaded fetal ovine heart

Aaron K. Olson, Kristin N. Protheroe, Jeffrey L. Segar, and Thomas D. Scholz

Department of Pediatrics, University of Iowa, Iowa City, Iowa

Submitted 14 September 2005; accepted in final form 10 November 2005

Mitogen-activated protein kinase activation and regulation in the pressure-loaded fetal ovine heart. Am J Physiol Heart Circ Physiol 290: H1587–H1595, 2006. First published November 18, 2005; doi:10.1152/ajpheart.00984.2005.—The mitogen-activated protein (MAP) kinase signaling pathways help to mediate the hypertrophic response of the pressure-loaded adult heart, although their importance in fetal myocardium is less known. The goal of this study was to determine the role the MAP kinase signaling pathways play in regulating the response of the fetal heart to a pressure load. Aortic (Ao) and pulmonary artery (PA) bands were placed in 132-day fetal sheep for 7 days. Protein levels of the total and active (phosphorylated) terminal MAP kinases extracellular signal-regulated kinase (ERK/P-ERK), c-Jun NH2-terminal kinase (JNK/P-JNK), and p38/P-p38 and the MAP kinase phosphatases MKP-1, MKP-2, and MKP-3 were made in the right and left ventricular (RV and LV) free walls. In both Ao- and PA-banded animals, total heart weight normalized to body weight was significantly increased, largely due to an increase in RV free wall mass in the Ao-banded animals and an increase in septal mass in the PA-banded fetuses. Total protein levels of the three terminal kinases and of P-ERK and P-JNK remained stable in both groups of banded animals. However, P-p38 was significantly increased in RV and LV of Ao- and PA-banded fetuses. Whereas MKP-1 and MKP-2 protein levels were unchanged following Ao- and PA-banding, MKP-3 protein levels were significantly increased in the RV of the PA-banded animals. These findings indicate that the MAP kinase signaling pathways are active in the fetal heart and help to modulate the response of prenatal myocardium to a pressure load.

hypothesis: hyperplasia; extracellular signal regulated kinase; c-Jun NH2-terminal kinase; p38

WHEREAS MORPHOGENESIS of the embryonic heart is largely under genetic control, fetal cardiac development during the latter three-fourths of gestation is influenced by blood flow and load placed on the cardiac chambers. The fetal heart grows through a combination of both cell division and enlargement of myocytes that have become terminally differentiated, binucleated cells. When the myocytes are subjected to increased load, such as occurs with various types of congenital heart disease that obstruct ventricular outflow, cardiac mass increases. Signaling pathways that regulate the increase in mass of the fetal heart, which can occur through either an increase in cell number or hypertrophy of myocytes, are largely unknown.

The signal transduction pathways that control cardiac hypertrophy in the adult heart continue to be elucidated. The mitogen-activated protein (MAP) kinases, the terminal kinases of which include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, are a cascade of reactions that contribute to the regulation of the hypertrophic response (3). Activation of the terminal kinases (ERK, JNK, and p38) by phosphorylation of specific serine, threonine, and/or tyrosine residues results in translocation to the nucleus and ultimately activation of gene transcription. Whereas activation of ERK appears to be consistently associated with myocyte hypertrophy, the roles of activated JNK and p38 are less defined (19). A recent review by Liang and Molkentin (19) highlighted the discrepancy between studies in isolated myocytes, which consistently identify a prohypertrophic role for JNK and p38, and in vivo studies that generally demonstrate decreased hypertrophy in hearts overexpressing active JNK and p38. Measurement of terminal kinase activation from diseased human heart samples has added to the uncertainty of the in vivo role of the MAP kinase signaling pathways. These studies have variably found an increase or decrease in myocardial MAP kinase activation (11, 14).

In addition to disease processes regulating the expression and activation of the MAP kinases in vivo, a number of protein phosphatases have been defined that dephosphorylate and inactivate the terminal kinases (16, 38). These phosphatases, which include the MAP kinase phosphatases (MKPs)-1 through -6, dephosphorylate specific Ser/Thr residues and inactivate the terminal kinases (38). In addition, specific MKPs are often active only against specific MAP kinases, although MKP-1 and MKP-4 are able to dephosphorylate ERK, JNK, and p38 in cell culture (9, 24). A feedback mechanism has also been well defined between MKP-3 and ERK2, whereby phosphorylation and activation of ERK2 result in activation of MKP-3 and subsequent dephosphorylation and inactivation of ERK2 (6, 23). Active ERK also has been shown to induce expression of Mkp3 in some models (35).

The majority of the studies that have defined the role of the MAP kinases in regulating the cardiac hypertrophic response have been performed in isolated neonatal myocytes or adult murine hearts. Expression and activation of the terminal MAP kinases and the MKPs in the fetal heart have not been extensively studied. This study uses aortic and pulmonary artery banding in fetal sheep to explore the association between MAP kinase activation and the development of increased mass of the stressed fetal heart. These interventions respectively simulate coarctation of the aorta, in which the fetus is subjected to both a right (RV) and left ventricular (LV) pressure load, and pulmonary stenosis, which results in a pressure load to the RV and a volume load to the LV. We hypothesize that the MAP kinase signaling pathways and MKPs play a role in regulating the response of the fetal heart to a stress such as pressure load.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Animal Preparation

All studies were performed using pregnant ewes of Dorset and Suffolk mixed breeding, obtained from a local source. The gestational ages were based on the induced ovulation technique as described elsewhere (15).

Anesthesia and surgical techniques for 132-day fetuses (term = 145 day) have been described previously (31). Briefly, following a 24-h fast of the pregnant ewe, general anesthesia was induced with a mixture of halothane (1%), oxygen (33%), and nitrous oxide (66%). Under sterile conditions, a uterine incision was made and fetal head and thorax were exposed. A thoracotomy in the third intercostal space was performed to expose the heart and great vessels in both the pulmonary artery (PA) (n = 6) and aortic banded (n = 11) animals. To band the main PA, the pericardium was opened and the vessel was exposed and isolated proximal to the branch pulmonary arteries. PA banding was performed by placing an inflatable occluder around the main PA. At the time of surgery, the volume of glycerol-saline (a 50/50 mixture was used) needed to be infused into the occluder to decrease cross-sectional area of the PA by 50% was determined. Aortic banding was performed in a similar fashion by placing an inflatable occluder on the proximal descending aorta just downstream of the ductus arteriosus and before the takeoff of the brachiocephalic artery. After a 72-h recovery period, the predetermined volume of the glycerol-saline mixture was then infused into the occluder to constrict the PA or aorta by 50%, resulting in a 75% decrease in cross-sectional area. Control fetuses underwent identical procedures, except the bands were not tightened and occluders were not inflated.

Catheters for myocardial blood flow and substrate utilization measurements were secured in the left atrial appendage and placed in the azygous vein. The latter catheter was advanced into the coronary sinus and the azygous sutured tightly around the catheter. A catheter was also placed in the carotid artery and the tip advanced to the proximal ascending aorta. This latter catheter was used to obtain reference blood samples during microsphere injection and arterial oxygen content and glucose and lactate concentrations.

At the end of surgery, ampicillin was directly infused into the amniotic cavity (2 g) and administered intramuscularly (2 g) to the ewe. Antibiotic administration was continued for 3 days postoperatively. Butorphenol (0.1 mg/kg iv; Torbugesic, Fort Dodge Animal Health, Fort Dodge, IA) was administered for 24 h postoperatively for analgesia.

During each experiment, fetal mean arterial blood pressure (MABP) and amniotic pressure were recorded daily for 30 min by using Statham P23 Db pressure transducers (Spectramed, Critical Care Division, Oxnard, CA), MacLab hardware and software (ADInstruments, Colorado Springs, CO), and a G4 Powerbook (Apple Computers, Cupertino CA). Fetal MABP was corrected relative to concomitant amniotic pressure. Heart rate was monitored with a cardiotachometer triggered from the arterial pressure pulse wave. Arterial blood for pH, PCO₂, PO₂, and hemoglobin was collected anaerobically in a heparinized syringe, and measurements were immediately determined at 39.5°C using a 1302 BGM pH/blood gas analyzer and an IL 682 CO-oximeter system (Instrumentation Laboratory; Lexington MA). During PA banding studies, fluorescent microspheres were injected into the left atrium before the PA occluders and before fetal tissues were harvested to measure myocardial blood flow (see Myocardial Blood Flow Measurement). Myocardial blood flow measurements were not made in the aortic banding studies. After the 7-day study period, the ewe was again anesthetized as discussed earlier, and the fetuses were removed. Fetal tissues were weighed and flash frozen in liquid nitrogen. Animals of either sex were used.

All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Myocardial Blood Flow Measurement

Myocardial blood flow was determined by using fluorescent microspheres as described previously (12). Briefly, for each blood flow determination, 1.2 × 10⁶ 15-μm fluorescent-labeled polystyrene microspheres (Triton Technology, San Diego, CA) suspended in 1 ml 0.9% NaCl with 0.02% Tween and 0.02% thimerosal were injected into the left atrium and flushed with 2 ml 0.9% NaCl over a 30-s period. Beginning 25 s before the microsphere injection, reference blood samples were withdrawn from the carotid catheter (tip in the ascending aorta) into heparinized glass syringes for 2.5 min at a rate of 2 ml/min. Samples of blood and known weights of epicardial and endocardial halves of myocardium were digested and filtered to recover microspheres using previously published methodologies ("Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion", Fluorescent Microsphere Resource Center, University of Washington; http://fmrc.pulmcc.washington.edu/fmrc/fmrc.html). After digestion was completed, the microspheres were filtered from the solutions by using 10-μm filter membranes (Triton Technology, San Diego, CA). Microspheres were dissolved with 1,000 μl of Cellosolve acetate (Fisher Scientific, Fair Lawn, NJ), 200 μl of solution were transferred to a wellplate, and fluorescence was determined. Fluorescent measurements of experimental and "standard curve" samples were determined with a luminescence spectrophotometer (LS-50B, Perkin-Elmer, Wellesley, MA) by using the appropriate excitation/emission wavelengths and slit widths for the given colored sphere. Tissue blood flow (using ascending aorta reference samples) was calculated using the formula:

\[ Q_{\text{sample}} = (Q_{\text{ref}} \times F_{\text{sample}})/F_{\text{ref}} \]

where \( Q_{\text{sample}} \), and \( Q_{\text{ref}} \) are the blood flow (in ml/min) in the specific sample and reference sample, respectively, and \( F_{\text{sample}} \) and \( F_{\text{ref}} \) are the fluorescent intensities in the specific sample and reference sample, respectively.

To correct for heart growth over the study period, the fetal weight at baseline was estimated using the equation \( W = W_e^{0.027 \times \text{days}} \), where \( W \) is the weight at autopsy, \( W_e \) is the weight on the first day of study, \( \text{days} \) is the number of days of the study, and 0.027 is the normal rate of fetal growth between 120 and 138 days of gestation that we have previously found in our sheep population (29).

Myocardial Substrate Utilization Determination

As described above, catheters positioned in the ascending aorta and coronary sinus were used to measure LV oxygen, glucose, and lactate uptake. Oxygen content measurements were made using a CO-oximeter (Instrumentation Laboratories), whereas glucose and lactate measurements were made using a YSI glucose/lactate analyzer (YSI, Yellow Springs, OH). Arterial-venous differences were determined and multiplied by the average of the LV and RV endocardial and epicardial blood flows determined from the fluorescent microsphere measurements.

Quantitative Immunoblot Studies

Immunoblots were performed as described previously, although a different detection system was used (32). Briefly, myocardial samples were homogenized in the presence of protease inhibitors, including soybean trypsin inhibitor, leupeptin, and PMSF in 50 mM Tris-10 mM EDTA-150 mM NaCl-0.1% mercaptoethanol and then sonicated for 20 s. After centrifugation, total protein of the supernatant was quantitated spectrophotometrically. Protein (20 μg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk protein for 1 h and then incubated in primary antibody overnight at 5°C. Bound primary antibody was
detected by incubation with infrared-labeled secondary antibodies (IRDye 800 or IRDye 700 700DX, Li-Cor Biotechnology, Lincoln, NE), read, and quantitated with a Li-Cor Odyssey Imaging System (Li-Cor Biotechnology, Lincoln, NE).

Primary antibodies that were utilized included antibodies specific to total and phosphorylated terminal MAP kinases (ERK1/2-sc-93 used at a dilution of 1:1,000, P-ERK1/2-sc-7383 used at a dilution of 1:1,000, JNK1/2-sc-1648 used at a dilution of 1:1,000, and P-JNK1/2-sc-6254 used at a dilution of 1:1,000 from Santa Cruz, Biotechnology, Santa Cruz, CA, and P-p38–9212 used at a dilution of 1:1,000 and P-p38–9211 used at a dilution of 1:250 from Cell Signaling Technology, Beverly, MA) and the MAP kinase phosphatases MKP-1, MKP-2, and MKP-3 (MKP-1-sc-1199 used at a dilution of 1:1,000 and MKP-2-sc-6254 used at a dilution of 1:1,000 from Santa Cruz, Biotechnology; MKP-3-AP8445a used at a dilution of 1:1,000 from Abgent, San Diego, CA).

Statistics

All data are expressed as means ± SE. Comparisons between control and study animals at baseline and at the end of the study period were performed using repeated-measures ANOVA with pairwise comparisons done by Tukey’s test. Comparisons between banded groups at baseline and at the end of the banding period were made using a paired t-test, whereas protein levels measured by immunoblot were compared between the banded and control groups by unpaired t-test. Significant differences were identified at the P < 0.05 level.

RESULTS

Fetal Sheep Morphometric and Hemodynamics

Aortic banding studies. Banding of the transverse aorta resulted in a small, but significant, decline in fetal heart rate after 7 days compared with control animals and baseline values (Table 1). No significant changes in arterial PCO2 and PO2, after 7 days compared with control animals and baseline values resulted in a small, but significant, decline in fetal heart rate (Table 1). No significant changes in arterial PCO2 and PO2, after 7 days compared with control animals and baseline values resulted in a small, but significant, decline in fetal heart rate.

Table 1. Arterial blood gas and hemodynamic data for sham-operated control and aortic-banded fetal sheep

<table>
<thead>
<tr>
<th></th>
<th>Control Baseline</th>
<th>Control Day 7</th>
<th>Aortic Banded Baseline</th>
<th>Aortic Banded Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.37 ± 0.02</td>
<td>7.36 ± 0.02</td>
<td>7.37 ± 0.01</td>
<td>7.34 ± 0.01</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>52 ± 2</td>
<td>52 ± 2</td>
<td>54 ± 2</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>PO2, mmHg</td>
<td>23 ± 1</td>
<td>23 ± 1</td>
<td>22 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>11.3 ± 0.7</td>
<td>11.1 ± 0.9</td>
<td>11.2 ± 0.6</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>45.8 ± 1.2</td>
<td>46.4 ± 1.1</td>
<td>46.7 ± 0.9</td>
<td>48.0 ± 3.4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>165 ± 77</td>
<td>165 ± 8</td>
<td>179 ± 5</td>
<td>153 ± 2*</td>
</tr>
</tbody>
</table>

All values are means ± SE; n = 7 for control and aortic-banded animal data. Hb, hemoglobin; MABP, mean arterial blood pressure; HR, heart rate. *P < 0.05 vs. controls and baseline by ANOVA.

Table 2. Effect of aortic and pulmonary artery banding on fetal and cardiac mass

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Banded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, kg</td>
<td>3.72 ± 0.26</td>
<td>3.38 ± 0.30</td>
</tr>
<tr>
<td>RV mass, g</td>
<td>7.93 ± 0.90</td>
<td>9.33 ± 1.09</td>
</tr>
<tr>
<td>LV mass, g</td>
<td>7.25 ± 0.71</td>
<td>7.44 ± 0.79</td>
</tr>
<tr>
<td>Septal mass, g</td>
<td>4.01 ± 0.48</td>
<td>3.19 ± 0.44</td>
</tr>
<tr>
<td>RV/BW, g/kg</td>
<td>2.09 ± 0.15</td>
<td>2.74 ± 0.20*</td>
</tr>
<tr>
<td>LV/BW, g/kg</td>
<td>1.93 ± 0.10</td>
<td>2.20 ± 0.13</td>
</tr>
<tr>
<td>Septum/BW, g/kg</td>
<td>1.11 ± 0.14</td>
<td>0.93 ± 0.09</td>
</tr>
<tr>
<td>RV+LV+septum/BW, g/kg</td>
<td>5.13 ± 0.10</td>
<td>5.86 ± 0.29*</td>
</tr>
</tbody>
</table>

All values are means ± SE; n = 7 for aortic-banded animal data; n = 6 for pulmonary artery (PA)-banded animal data. RV, right ventricle; LV, left ventricle; BW, fetal body weight. *P < 0.05 vs. baseline by unpaired t-test.

in arterial pH, PCO2, or PO2 (Table 3). There was a small decline in fetal heart rate in the banded animals at 7 days but no change in heart rate, mean arterial blood pressure, or serum hemoglobin over the 7-day study period (Table 3).

Placement of aortic, coronary sinus, and left atrial appendage catheters in the PA-banded animals allowed determination of myocardial blood flow, oxygen consumption, and substrate utilization. At the end of the 7-day study period, PA-banded animals had no significant change in myocardial blood flow to the epicardium or endocardium of either the LV or RV free walls compared with baseline (Table 3). Myocardial oxygen consumption, however, was more than doubled after 7 days of PA banding. This significant increase in myocardial oxygen consumption was accompanied by a trend toward an increase in utilization of both glucose and lactate (Table 3). In all animals, lactate consumption increased with banding, indicating that the PA-banded hearts continued to operate aerobically.

Body weight of the fetus was unaffected by placement of the PA band (Table 2). Whereas RV free wall mass was somewhat increased, although not significantly, in the PA-banded animals compared with controls, the most striking increase was seen in the mass of the interventricular septum, which was increased 65% in the PA-banded animals compared with controls. Because of the increased RV and septal masses, total heart weight (the sum of RV and LV free walls and septum) was significantly increased in the banded animals (Table 2).

Activation of MAP Kinase Signaling Pathways in the Loaded Fetal Heart

Aortic banding studies. Steady-state protein levels of the three primary groups of terminal kinases that are present in myocardium were measured in the RV and LV free walls of the fetal hearts. Constricting the transverse aorta had no effect on total LV protein levels of ERK1/2, JNK, or p38 when banded animals were compared with sham-operated controls (Fig. 1 and Table 4). Similarly, steady-state protein levels of the phosphorylated, active forms of ERK1/2 and JNK (P-ERK1/2 and P-JNK, respectively) were also unchanged. RV protein of total and active ERK1/2 and P-JNK and total p38 were also measured and found to be similar between banded animals and controls (Table 4). Although it is generally believed that only JNK1 and JNK2 are present in myocardium (18), the presence
Table 3. Arterial blood gas, hemodynamic, blood flow, and substrate utilization data in control and pulmonary artery-banded fetal sheep

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>PA Banded</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.38 ± 0.01</td>
<td>7.37 ± 0.01</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>53 ± 1</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>P02, mmHg</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>12.2 ± 1.0</td>
<td>11.5 ± 0.7</td>
</tr>
<tr>
<td>MABP</td>
<td>44.5 ± 2.9</td>
<td>46.7 ± 0.8</td>
</tr>
<tr>
<td>RV Endo, ml/min ^-1^100 g ^-1^</td>
<td>168 ± 7</td>
<td>170 ± 4</td>
</tr>
<tr>
<td>RV Epi, ml/min ^-1^100 g ^-1^</td>
<td>474 ± 50</td>
<td>414 ± 78</td>
</tr>
<tr>
<td>LV Endo, ml/min ^-1^100 g ^-1^</td>
<td>371 ± 48</td>
<td>321 ± 92</td>
</tr>
<tr>
<td>LV Epi, ml/min ^-1^100 g ^-1^</td>
<td>322 ± 70</td>
<td>366 ± 55</td>
</tr>
<tr>
<td>MVO2, μmol/min ^-1^100 g ^-1^</td>
<td>4.01 ± 0.77</td>
<td>8.27 ± 1.24</td>
</tr>
<tr>
<td>Glucose uptake, mg/min ^-1^100 g ^-1^</td>
<td>5.05 ± 0.76</td>
<td>8.44 ± 2.90</td>
</tr>
<tr>
<td>Lactate uptake, mmol/min ^-1^100 g ^-1^</td>
<td>8.56 ± 3.11</td>
<td>17.80 ± 5.16</td>
</tr>
</tbody>
</table>

All values are means ± SE; n = 5 for control and PA-banded animal (PA) data. RV Endo, RV endocardial blood flow; RV Epi, RV epicardial blood flow; LV Endo, LV endocardial blood flow; LV Epi, LV epicardial blood flow; MVO2, myocardial oxygen consumption. *P < 0.05 vs. controls and baseline by ANOVA; †P < 0.05 vs. sham-operated controls.

of a faint upper band on membranes probed with the JNK antibody and the identification of three distinct bands seen on membranes probed with the P-JNK-specific antibody suggested that JNK3 may be present in fetal myocardium (Fig. 1). When multiple isoforms were seen, the sum of the bands was used for the analyses in Table 4 and Fig. 1. The most prominent change in active MAP kinase signaling protein levels was found in the LV of PA-banded animals compared with controls (Fig. 2 and Table 5). Protein levels of active ERK1/2 (P-ERK1/2) and JNK (P-JNK) were unchanged in the PA-banded hearts compared with controls (Fig. 2 and Table 5). Protein levels of active ERK1/2 (P-ERK1/2) and JNK (P-JNK) were unchanged in the PA-banded hearts compared with controls (Fig. 2 and Table 5). As in the aortic-banded hearts, a significant increase in the active, phosphorylated form of p38 (P-p38) was found in both PA-banding studies. Changes in total and active MAP kinase signaling protein levels in the PA-banded fetal heart were similar to those found in aortic-banded animals. Total protein levels of ERK1/2, JNK, and p38 were similar between control and PA-banded animals in both the RV and LV, except a small, but statistically significant, 20% decline in total JNK protein levels was found in the LV of PA-banded animals compared with controls (Fig. 2 and Table 5). Protein levels of active ERK1/2 (P-ERK1/2) and JNK (P-JNK) were unchanged in the PA-banded hearts compared with controls (Fig. 2 and Table 5). As in the aortic-banded hearts, a significant increase in the active, phosphorylated form of p38 (P-p38) was found in both
the RV and LV free walls of the PA-banded animals (Fig. 2 and Table 5).

**Regulation of MAP Kinase Signaling Pathways in the Loaded Fetal Heart**

**Aortic banding studies.** A variety of regulatory points, both upstream and downstream from the terminal MAP kinases, can regulate the level of active protein levels found in myocardium. An important set of inactivators of the MAP kinases is the dual-specificity protein phosphatases that include the MAP kinase phosphatases 1, 2, and 3 (MKP-1, MKP-2, and MKP-3, respectively). Immunoblot studies detected each of these proteins in fetal ovine myocardium. No significant difference in protein levels of MKP-1, MKP-2, or MKP-3 were found between control and aortic-banded animals in the RV or LV free walls (Fig. 3 and Table 4).

**PA banding studies.** With an increased load on the right ventricle of the fetal heart, protein levels of both MKP-1 and MKP-2 were unchanged in the RV free wall of the PA-banded animals compared with controls (Fig. 4 and Table 5). LV free wall protein levels of MKP-1 and MKP-2 were also no different between the two groups (Table 5). Interestingly, levels of MKP-3 protein, which is thought to regulate ERK1/2 phosphorylation, were significantly increased in the RV free wall of the PA-banded animals (Fig. 4 and Table 5).

**DISCUSSION**

The fetal sheep model has been used for decades to explore cardiac physiology and adaptive responses to a variety of pathophysiological stimuli. The two models utilized in this study provided a load on the developing heart that would...
mimic the congenital cardiac abnormalities of coarctation of the aorta (aortic banding) and pulmonary valve or supravalvar stenosis (PA banding). Not only were the fetal myocardial morphometric responses to these loads measured, but the molecular response of the MAP kinase signaling pathways was determined. Importantly, in both models, the increase in fetal cardiac load resulted in significant activation of p38 MAP kinase, suggesting similar signaling within fetal myocytes that are faced with performing increased work in utero.

**Morphometric Response of the Loaded Fetal Heart**

A constriction in the transverse aortic arch in the periductal region places a pressure load on both ventricles. RV outflow that usually results in right-to-left ductal shunting due to the high in utero pulmonary vascular resistance is obstructed, and the relatively low systemic vascular resistance the LV typically faces is increased. In humans, early fetal echocardiography studies identified an in utero increase in RV free wall thickness in association with coarctation of the aorta (1). Similar results were found in the fetal sheep following aortic banding in this study. A significant increase in RV mass-to-body weight ratio was identified, which likely reflects the RV dominant circulation that exists in utero (30, 36). Whereas the degree of load placed on the ventricles was not directly measured in this study, it was sufficient to provide a stimulus to increase heart weight and to double myocardial oxygen consumption in the PA-banded animals. It is important to note that in the aortic-banded animals, placement of the band between the ductus arteriosus and brachiocephalic artery (which gives rise to the bicarotid artery in sheep) would result in transient hypotension.

**Fig. 3.** Steady-state protein levels of the mitogen-activated protein (MAP) kinase phosphatases (MKP)-1, MKP-2, and MKP-3 in ovine left ventricular myocardium from control and Ao banded 142-day fetuses. Myocardial protein was isolated from ovine left ventricular free wall and separated by SDS-PAGE. Membranes were probed with specific antibodies followed by detection of bound antibody with infrared-labeled secondary antibodies. MKP-3 immunoblots demonstrated a nonspecific band at 80 kDa and the appropriate band at 40 kDa, which was quantitated. Sizes of the observed proteins based on simultaneously run molecular weight markers were the following (in kDa): 80 MKP-1, 45 MKP-2, 40 MKP-3. All graphs are mean with error bars indicating SE (n = 7 for each age group).

**Morphometric Response of the Loaded Fetal Heart**

A constriction in the transverse aortic arch in the periductal region places a pressure load on both ventricles. RV outflow that usually results in right-to-left ductal shunting due to the high in utero pulmonary vascular resistance is obstructed, and the relatively low systemic vascular resistance the LV typically faces is increased. In humans, early fetal echocardiography studies identified an in utero increase in RV free wall thickness in association with coarctation of the aorta (1). Similar results were found in the fetal sheep following aortic banding in this study. A significant increase in RV mass-to-body weight ratio was identified, which likely reflects the RV dominant circulation that exists in utero (30, 36). Whereas the degree of load placed on the ventricles was not directly measured in this study, it was sufficient to provide a stimulus to increase heart weight and to double myocardial oxygen consumption in the PA-banded animals. It is important to note that in the aortic-banded animals, placement of the band between the ductus arteriosus and brachiocephalic artery (which gives rise to the bicarotid artery in sheep) would result in transient hypotension.

**Fig. 4.** Steady-state protein levels of MKP-1, MKP-2, and MKP-3 in ovine right ventricular myocardium from control and PA banded 142-day fetuses. Myocardial protein was isolated from ovine right ventricular free wall and separated by SDS-PAGE. Membranes were probed with specific antibodies followed by detection of bound antibody with infrared-labeled secondary antibodies. MKP-3 immunoblots demonstrated a nonspecific band at 80 kDa and the appropriate band at 40 kDa, which was quantitated. Sizes of the observed proteins based on simultaneously run molecular weight markers were the following (in kDa): 80 MKP-1, 45 MKP-2, 40 MKP-3. All graphs are mean with error bars indicating SE (n = 7 for each age group). *P < 0.05 vs. sham-operated controls.
at the carotid baroreceptors, which would cause a reflex tachycardia. Interaction of baroreceptors within the aortic arch may, however, result in a bradycardia (see Table 1).

Pulmonary artery banding of fetal sheep has been used by several investigators to examine the response of the fetal heart to acute and chronic increases in afterload (2, 7, 22, 28, 32, 33). In addition to causing an increase in RV afterload, PA banding likely also results in a volume load on the LV due to increased right-to-left shunting across the foramen ovale. Consistent with our previous studies using this model (32, 33), a significant increase in heart weight-to-body weight ratio was identified in this study. However, when separating the RV and LV free walls from the interventricular septum, only a significant increase in septal mass-to-body weight ratio was found. This differs from the previous studies where RV free wall-to-body weight ratio increased and no change was found in septal mass (28, 32, 33). These differences are difficult to explain but may reflect the degree of PA banding in this study or variability between studies in dissecting out the septum. However, they emphasize the need to include septal mass in studies of fetal cardiac hypertrophy. It is clear, however, that workload in the PA-banded heart increased significantly. Myocardial oxygen consumption more than doubled while both glucose and lactate consumption tended to increase in the hearts of the PA-banded animals.

MAP Kinase Signaling Pathways

The MAP kinase pathways consist of a sequence of kinase reactions that ultimately result in dual phosphorylation and activation of the terminal kinases of these signaling pathways: ERK, JNK, and p38 (3). Activated MAP kinases translocate to the nucleus where they activate a number of transcription factors that then play a key role in the response of the cell to physiological and pathological stimulation.

Studies in cultured myocytes have given conflicting results regarding the role of activated ERK in cardiac hypertrophy, whereas some studies indicated that active (phosphorylated) ERK (P-ERK) promoted myocyte hypertrophy, whereas others found P-ERK to prevent the hypertrophic response (8, 10, 34, 39, 42). More recently, transgenic and knockout mouse studies have given important insights into the in vivo role of the MAP kinase pathways. Transgenic mice overexpressing active MAP kinase kinase-1 (MEK1), which phosphorylates ERK to generate P-ERK (5), develop significant compensated cardiac hypertrophy such that systolic function is preserved. Changes in fetal cardiac morphometry and function were not investigated in these studies, leaving the role active ERK plays in fetal cardiac development uncertain.

The roles of JNK and p38 (often referred to as the stress-activated protein kinases) in cardiac hypertrophy are also controversial (19). In vitro studies, primarily using cultured neonatal myocytes, have consistently demonstrated that activation of both JNK and p38 is prohypertrophic. Results in vivo are, however, quite different. Transgenic mice overexpressing upstream activators of both p38 and JNK were found to develop a dilated cardiomyopathy and die at a young age (20, 26, 27). However, mice expressing dominant negative JNK, p38Δ, and MEK3 and 6 (upstream activators of p38) that have reduced active JNK and p38 levels showed increased cardiac hypertrophy in vivo in response to pressure overload due to aortic banding, suggesting a role for these proteins in preventing the development of cardiac hypertrophy (4).

Most of these studies have examined the role of the MAP kinases in regulating the hypertrophic response, either in isolated neonatal myocytes or in adult animal models. The MAP kinase signaling pathways appear to have unique regulatory effects on fetal cardiac myocytes, depending on the maturational state of the cell. In cultured fetal sheep cardiomyocytes, Sundgren et al. (37) found that ERK activation resulted in hyperplasia of uninucleated cells and hypertrophy of binucleated cells. A similar role for activated p38 in vivo regulating fetal cardiac myocyte proliferation has recently been demonstrated in knockout and transgenic mouse studies (13). Growth of the fetal heart was inversely related to activation of p38 (13). These same investigators also demonstrated that inhibition of p38 activation in isolated neonatal myocytes resulted in a 2.8-fold increase in cell division, whereas cardiac-specific activation of p38 in vivo decreased fetal BrdU incorporation by nearly 18% compared with control animals (13). Thus it is clear that both active ERK and p38 are capable of regulating fetal cardiac myocyte proliferation that can contribute to an increase in ventricular mass with P-ERK stimulating and P-p38 inhibiting myocyte cell division.

In this study, we found that P-p38 levels increased significantly in both the RV and LV free walls, regardless of whether the pressure load was imposed on both ventricles (transverse aortic arch banding) or the right ventricle alone (PA banding). The similar response of both ventricles in the aortic banding group would be expected. The similar response of the LV and RV in the PA-banded animals was somewhat surprising and may reflect paracrine effects within the heart or an adaptive response of the LV to the volume load it likely receives from increased right-to-left foramen ovale shunting following PA band placement.

The significance of the increased active p38 levels in the loaded fetal sheep ventricles remains speculative at this time. A unique feature of the response of the fetal, compared with the adult, heart is that the fetal heart can increase its mass by both hyperplasia of mononucleated cells and hypertrophy of terminally differentiated binucleated myocytes. Studies by Barbera et al. (2) addressed this complex adaptive response of the fetal heart by examining cellular and subcellular changes in the fetal ovine RV free wall after 10 days of PA banding. Accompanying an increase in RV free wall mass was a small, but significant, increase in myocyte length and an unchanged myocyte width. The small increase in cell volume due to the increase in width was insufficient to account for the increase in RV free wall mass, and these authors estimated that myocyte number increased by 56%. In addition, this same study demonstrated a nearly 30% increase in terminally differentiated, binucleated cardiac myocytes in the RV free wall (2). Thus despite an increase in active p38, which has been shown in vivo to decrease myocyte proliferation, myocyte hyperplasia occurred in the loaded fetal ovine RV free wall using a model similar to this study. This apparent discrepancy may reflect the time course of the adaptive response of the loaded fetal RV. An early proproliferative response following band placement may be followed by an increase in counterregulatory pathways such as activation of p38 and dephosphorylation of ERK by MAP kinase phosphatases (see MAP Kinase Phosphatase Regulation of MAP Kinase Activity) as was observed in this study. In adult
mice subjected to transverse aortic arch banding, a biphasic response of ERK and p-38 activation was found (40). Initial activation of ERK and p-38 was seen from 15 min to 3 h after the banding procedure that correlated with increased transcription of immediate early genes such as c-fos and c-jun. A later peak in ERK and p-38 activation at 2 to 7 days corresponded to the development of hypertrophy of the adult mouse hearts. It is possible that in our fetal sheep models, early activation of any of the three terminal kinases may have occurred and impacted myocyte proliferation or terminal differentiation. Additional investigations at intermediate time points following PA banding should help to define the activation and inactivation of the MAP kinase signaling pathways and their roles in regulating the proliferative response in the loaded fetal heart.

MAP Kinase Phosphatase Regulation of MAP Kinase Activity

To achieve activation of the terminal MAP kinases, phosphorylation of critical threonine and tyrosine amino acid residues in the proteins must take place. Conversely, specific or dual-specificity phosphatases that dephosphorylate the proteins at either residue inactivate the MAP kinases. More than a dozen phosphatases have been described that serve to regulate MAP kinase signaling in mammalian cells (16, 38). In this study, we focused on protein levels of three well-described dual-specificity phosphatases: MKP-1, MKP-2, and MKP-3. Each of these phosphatases shares a wide tissue distribution (21, 23), but each has a different profile of terminal MAP kinase proteins against which they are active. MKP-1 has the broadest phosphatase activity of the three and is capable of deactivating phosphorylated ERK, JNK, and p38 (9, 17). MKP-2 has primary specificity for ERK and JNK (9). The most specific of the three phosphatases is MKP-3, which mainly dephosphorylates ERK (23).

In this study, we found a significant increase in the ERK-specific phosphatase MKP-3 in the RV free wall of the PA-banded animals. The mechanism of action of MKP-3 has been extensively studied with the specific binding domains on each protein being well defined (25, 41). The association of MKP-3 to phosphorylated ERK results in a two- to threefold activation of MKP-3 phosphatase activity (6). In addition to ERK activating MKP-3, increased protein levels of active ERK also induce expression of Mkp3 (35). In this way, a simple feedback mechanism is established whereby increased levels of active ERK are downregulated by both greater MKP-3 protein production and enhanced dephosphorylation of active ERK upon MKP-3 binding to P-ERK. The increase in MKP-3 protein levels found in right ventricular myocardium of the PA-banded animals suggests that an initial increase in P-ERK occurred at a time point before 7 days. As discussed above, cyclic variation in P-ERK levels has been demonstrated in the adult mouse following banding of the transverse aortic arch (40). Additional studies at earlier time points are needed to better define the relationship between ERK activation and upregulation of MKP-3 expression.

In conclusion, as the number of signaling pathways that regulate normal and pathological cardiac development continues to grow, the influence of these pathways in utero is beginning to be explored. The MAP kinase signaling pathways are among the pathways with an important influence in post-natal cardiac growth and hypertrophy. Because the ovine fetus can be readily manipulated to monitor physiological data as well as provide ample tissue to explore expression of these signaling pathways, this model was used to explore the in vivo importance of these signaling pathways. In this study, banding of the aorta and pulmonary artery of the fetal sheep was used to replicate the increased load placed on the heart by common congenital heart diseases. The increase in cardiac load that resulted from these interventions resulted in a similar significant increase in active p38 and, in the PA-banded model, an increase in the ERK-specific phosphatase MKP-3. From these studies, it is clear that the MAP kinase signaling pathways are instrumental in helping the fetal heart adapt, regardless of the pathological load to which the heart is exposed.

GRANTS

This work was supported by the following awards from the National Heart, Lung, and Blood Institute Grants T32-HL-07413 (to A. K. Olson); R01-HL-64770 (to J. L. Segar); K02-HL-04495 (to T. D. Scholz).

REFERENCES


