Osteopontin Modulates Myocardial Hypertrophy in Response to Chronic Pressure Overload in Mice

Zhonglin Xie, Mahipal Singh, Krishna Singh

Abstract—Osteopontin (OPN) expression increases in the heart during hypertrophy and heart failure. Here, we studied the role of OPN in pressure overload–induced hypertrophy and analyzed the signaling pathways involved in hypertrophy. Aortic banding (AB) was performed in a group of wild-type (WT) and OPN knockout (KO) mice to induce pressure overload. Left ventricular (LV) structural and functional remodeling was studied 1 month after AB. AB increased OPN and β1 integrin (a receptor for OPN) protein expression in WT-AB group. Hypertrophic response as measured by increased heart weight-to-body weight ratio and myocyte cross-sectional area was significantly increased in WT-AB and KO-AB groups when compared with their respective shams. However, the increase was significantly higher in WT-AB. Re-expression of atrial natriuretic factor was only detected in WT-AB group. LV end-diastolic pressure-volume curve obtained using Langendorff perfusion analysis exhibited a leftward shift in WT-AB group, not in KO-AB. LV-developed pressures measured over a range of LV volumes were significantly increased in WT-AB, not in KO-AB mice. Increased phosphorylation of c-Jun N-terminal kinases, p38 kinase, Akt, and glycogen synthase kinase-3β was significantly higher in WT-AB when compared with KO-AB group. Increased OPN expression may play an essential role in modulating compensatory cardiac hypertrophy in response to chronic pressure overload. (Hypertension. 2004;44:826-831.)

Key Words: heart ■ hypertrophy ■ kinase

Cardiac hypertrophy, which occurs in response to an increased mechanical load on the heart in the form of pressure or volume overload, is characterized by increased cell size, enhanced protein synthesis, and re-expression of fetal genes, including atrial natriuretic factor (ANF). Initially, hypertrophy is considered compensatory; however, it may activate signaling events leading to heart failure. Integrins are suggested as a primary mechanical link between the extracellular matrix (ECM) and cytoskeleton, leading to induction of intracellular signaling events responsible for cardiac hypertrophy. Cardiac myocytes predominantly express β1 integrins. Dominant-negative disruption of β1 integrin and myocyte-specific excision of β1 integrin gene induced myocardial fibrosis and cardiac failure. A crucial role for melusin, a muscle-specific β1 integrin interacting protein, has also been demonstrated in cardiac hypertrophy in response to pressure overload. The signaling pathways leading to induction of cardiac hypertrophy include activation of members of the mitogen-activated protein kinase (MAPK; extracellular signal–regulated kinases 1/2 [ERK1/2], c-Jun N-terminal kinases [JNKs], and p38 kinase) superfamily, Akt/glycogen synthase kinase-3β (GSK-3β) pathway, calcineurin/NF-AT3 pathway, and protein kinase C acting downstream of G-coupled receptors.

Osteopontin (OPN), also called cytokine Eta-1, contains Arg-Gly-Asp-Ser cell-binding sequence and interacts with αvβ1, αvβ3, and αvβ5 integrins and CD44 receptors. OPN is synthesized by cardiac myocytes, microvascular endothelial cells, and fibroblasts. OPN appears to be capable of mediating diverse biological functions including cell adhesion, migration, and signaling. Heart expresses OPN at low levels under basal conditions. However, expression of OPN increases markedly under several pathological states. Using OPN knockout (OPN KO) mice and myocardial infarction (MI) as a model of myocardial remodeling, we have demonstrated that increased expression of OPN plays an important role in post-MI remodeling. However, the role of OPN in the induction of cardiac hypertrophy in response to chronic pressure overload has not yet been studied. Here, we tested the hypothesis that increased expression of OPN in response to chronic pressure overload plays an essential role in cardiac hypertrophy and functional remodeling. To gain an insight into the mechanism by which OPN may play a role in cardiac hypertrophy, we studied activation of signaling pathways involved in hypertrophy including MAPKs and Akt/GSK-3β.

Materials and Methods

Vertebrate Animals

All experiments were performed in accordance with protocols approved by the institutional animal care and use committee. Mice...
lacking OPN (KO) and wild-type (WT) were of a 129×black Swiss hybrid background. Genotyping was performed by polymerase chain reaction (PCR) as described. Once genotyped, the KO and WT animals were bred and maintained as separate colonies.

Aortic Banding
Aortic banding (AB) was performed on age-matched (∼3 months) WT and KO mice as described. Male and female animals were included in the experiments. All the measurements were made 1 month after AB. Systolic blood pressures of right carotid artery in anesthetized animals were measured using Millar Micro-tip catheter (Millar Instruments). The expanded method is provided in the online data supplement (available at http://www.hypertensionaha.org).

Langendorff Preparation
Left ventricular (LV) function was measured using the isolated buffer-perfused heart preparation as described. The expanded method is provided in the online data supplement.

Morphometric Analyses
After Langendorff studies, hearts were arrested in diastole with KCl (30 mmol/L) and fixed with 10% buffered formalin. Cross-sections (4-μm thick) stained with Masson’s trichrome were used for the measurement of fibrosis and myocyte cross-sectional area. To measure cross-sectional myocyte area, suitable area of the sections was defined as the one with nearly circular capillary profiles and nuclei. To calculate lung wet/dry weight ratio, lung wet weight was obtained after drying the tissue at 65°C for 72 hours.

Apoptosis
TUNEL and propidium iodide staining was performed to calculate the number of cardiac myocyte apoptosis versus total number of nuclei. The expanded method is provided in the online data supplement.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was isolated from LV as described. The RNA (1 μg) was reverse transcribed using oligo dT, and the products were amplified by PCR using the following primers: ANF, 5'-GCTCCTTCTCCATCACC-3'; β-actin, 5'-TTATCTTCGTACTACG-3'. B, β1 integrin expression 30 days after AB. Protein loading was verified using anti-β actin antibodies (bottom panels).

Western Analysis
Tissue lysates were analyzed by Western blot. The expanded method is provided in the online data supplement.

Statistical Analysis
Data are reported as mean±SEM. Statistical analyses were performed using Student t test or 1-way ANOVA and post hoc Tukey test. A P value of <0.05 was considered significant.

Results
Pressure Overload Increases OPN and β1 Integrins Expression
Western blot analysis of LV samples using monoclonal anti-OPN antibodies (Santa Cruz Biotechnology) showed 4 prominent bands ranging from ∼32 to 72 kDa. OPN exhibits multiple bands on SDS-PAGE because of its highly acidic nature and differential glycosylation and phosphorylation. AB increased the intensity of all 4 bands recognized by anti-OPN antibodies (Figure 1A). Densitometric analysis demonstrated 2.6- and 3.3-fold increase in OPN protein expression (P<0.001 versus WT-sham) 3 and 30 days after AB, respectively. AB also increased β1 integrin expression in WT, not in OPN KO hearts (fold increase versus WT-sham; WT-AB 1.9±0.05 [P<0.05 versus WT-Sham]; KO-sham 1.08±0.07; KO-AB 1.0±0.11 [P<0.05 versus WT-AB]; Figure 1B).

Morphological Data
No differences in body weight (BW) were observed among the 4 groups 1 month after AB (Table). AB significantly increased heart weight (HW)-to-BW and LV weight (LVW)-to-BW ratios in WT and KO mice. However, this increase was significantly higher in WT-AB mice (P<0.05; Table). Systolic blood pressures of right carotid artery were increased to a similar degree in both AB groups, indicating increased mechanical stress. Lung wet weight-to-dry weight ratio was significantly increased in KO-AB, not in WT-AB mice, when compared with their respective shams (Table).

Reduced Hypertrophic Response in KO Mice After Pressure Overload
Analysis of ANF gene expression using RT-PCR detected re-expression of ANF in the WT-AB hearts, not in KO-AB (Figure 2A). These data together with HW/BW and LVW/BW ratios demonstrate that the hypertrophic changes are significantly lower in mice lacking OPN. To further confirm these observations, we measured cross-sectional area of cardiac myocytes in all 4 groups. This analysis demonstrated increased myocyte cross-sectional area in both AB groups. However, the increase in cross-sectional area was significantly lower in KO-AB group when compared with WT-AB (WT-sham...
191.4±3.7; WT-AB 432±14.2 [P<0.001 versus WT-sham]; KO-sham 202.5±10.9; KO-AB 335.9±32.8 (P<0.001 versus KO-sham and P<0.05 versus WT-AB; Figure 2B).

**Fibrosis and Apoptosis**

Quantitative analysis of trichrome-stained sections indicated increased fibrosis in both AB groups compared with their respective shams. However, the increase in fibrosis was not significantly different between the 2 AB groups (WT-sham 0.3±0.2; WT-AB 1.4±0.4 [P<0.05 versus WT-sham]; KO-sham 0.4±0.2; KO-AB 1.9±0.2 [P<0.05 versus KO-sham]). The number of apoptotic myocytes was not significantly different between the 2 AB groups (WT-AB 0.41±0.02; KO-AB 0.38±0.16; P=NS).

**LV Pressure-Volume Relationships After Pressure Overload**

The LV end-diastolic pressure-volume curve was shifted leftward in WT-AB (P=0.002 versus sham; P<0.001 versus KO-AB). In contrast, LV end-diastolic pressure-volume relationship exhibited a nonsignificant rightward shift in KO-AB group (P=0.129 versus sham; Figure 3A). LV-developed pressures measured over a range of volumes were increased in WT-AB (P<0.05 versus sham; Figure 3B), not in KO-AB group. In fact, LV-developed pressures at 30 and 35 μL were significantly higher in WT-AB versus KO-AB group (P<0.05).

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**Morphometric and SBP Measurements 1 Month After AB**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT-Sham (n=5)</th>
<th>KO-Sham (n=10)</th>
<th>WT-AB (n=6)</th>
<th>KO-AB (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>30.4±1.4</td>
<td>32.4±1.4</td>
<td>30.2±1.0</td>
<td>31.9±1.1</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>87.8±7.6</td>
<td>90.7±6.8</td>
<td>122.4±6.6*</td>
<td>115.9±4.7*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.9±0.2</td>
<td>4.7±0.1</td>
<td>6.8±0.4*</td>
<td>5.5±0.1†</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>3.9±0.2</td>
<td>3.6±0.1</td>
<td>5.4±0.3*</td>
<td>4.4±0.1†</td>
</tr>
<tr>
<td>Lung wet/dry weight ratio (g/g)</td>
<td>4.26±0.03</td>
<td>4.27±0.04</td>
<td>4.47±0.04</td>
<td>4.50±0.05*</td>
</tr>
</tbody>
</table>

Values are mean±SEM; SBP indicates systolic blood pressure. *P<0.05 vs sham; †P<0.05 vs WT-AB.

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**Figure 2.** A, AB induces ANF gene expression in WT-AB group. Total RNA isolated from LV was analyzed by RT-PCR using primers specific for ANF. GAPDH was used as internal control. B, AB increases myocyte cross-sectional area. Mean values of myocyte cross-sectional area. Each bar represents a mean value for 900 myocytes. *P<0.01 vs WT-sham and KO-sham; #P<0.01 vs WT-AB; n=3.

**Figure 3.** A, Analysis of LV end-diastolic pressure-volume relationships determined by isovolumic Langendorf technique. One month after AB, LV end-diastolic pressure-volume relationship curve was shifted leftward when compared with WT-sham (P<0.01) and KO-AB (P<0.001) groups. B, Analysis of LV developed pressure vs volume. One month after AB, the developed pressure was significantly higher in WT-AB compared with WT-sham (*P<0.05) and KO-AB mice (#P<0.05). n=5 WT-sham; n=6 WT-AB; n=10 KO-sham; n=11 KO-AB.
Akt is the principal kinase involved in phosphorylation of the inhibitory site of GSK-3β. Therefore, we next studied phosphorylation of GSK-3β. This analysis showed strong phosphorylation of GSK-3β in WT-AB, not in KO-AB group (fold increase versus WT-sham: WT-AB 3.2 ± 0.4 [P<0.05 versus WT-sham]; KO-sham 1.0 ± 0.1; KO-AB 0.6 ± 0.3 [P<0.05 versus WT-AB]; Figure 5B).

Discussion

We have shown previously that increased expression of OPN plays an important role in post-MI remodeling. Expression of OPN is increased in the hypertrophied myocardium. This is the first study to suggest that OPN is required to sustain compensatory cardiac hypertrophy in response to chronic pressure overload. The major new findings of this study are that mice lacking OPN exhibit impaired hypertrophic response after chronic pressure overload (compared with WT), and that the lack of OPN is associated with decreased phosphorylation of p38 kinase, JNKs, Akt, and GSK-3β.

Compensatory hypertrophic response, as measured by increased HW-to-BW ratio, re-expression of ANF, and increased myocyte cross-sectional area, was higher in WT-AB compared with OPN KO-AB mice. LV pressure-volume relationships suggested increased LV developed pressure with reduced LV chamber volume in WT-AB hearts. OPN KO-AB group exhibited an impaired compensatory hypertrophic response. We have shown previously that lack of OPN results in increased LV dilation with reduced fibrosis after MI. In angiotensin II–induced model of cardiac hypertrophy and remodeling, lack of OPN resulted in reduced cardiac fibrosis. In contrast, analysis of cardiac fibrosis using Masson’s trichrome-stained sections indicated that lack of OPN does not affect cardiac fibrosis in pressure overload–induced hypertrophy. The reasons for these contrasting findings are not yet clear. The findings in OPN KO mice are similar to melusin (a muscle-specific β1 integrin–interacting protein) null mice, in which lack of melusin resulted in blunted hypertrophic response to pressure overload. Interestingly, hypertrophic response to angiotensin II was not different in WT and melusin-null mice. Hypertrophic response to angiotensin II is also not different in OPN KO and WT mice. Together, these studies suggest that the mechanism(s) of cardiac hypertrophy induced by biomechanical stress attributable to pressure overload may be different from that of neurohumoral-induced hypertrophy. Integrin-mediated signaling is proposed to play a critical role in development of biomechanically induced cardiac hypertrophy. We observed increased β1 integrin expression in WT-AB mice, not in OPN KO-AB mice. Mechanical stretch is also shown to increase β1 integrin expression in isolated cardiac myocytes. Therefore, based on our data and previously published reports, we propose that OPN signaling mediated via β1 integrins, at least in part, is a possible mechanism by which OPN modulates cardiac hypertrophy in response to pressure overload. It should also be noted that chronic pressure overload increases OPN expression in cardiac myocytes, whereas angiotensin II increases OPN expression in cardiac fibroblasts and endothelial cells, not in cardiac myocytes. OPN is secreted from cells in multiple isoforms because of post-
translational modifications including phosphorylation and glycosylation. The post-translational modifications of OPN are suggested to affect its interaction with different receptors leading to distinct functions.\textsuperscript{25,29} Therefore, it is possible that OPN isoforms synthesized and secreted by cardiac myocytes are different from those synthesized and secreted by interstitial cells, thereby leading to distinct phenotype in response to pressure overload.

Our analysis of signaling events indicated increased phosphorylation of p38 kinase and JNKs, not ERK1/2, in WT-AB when compared with OPN KO hearts. MAPKs are activated after ligation of integrins in vitro,\textsuperscript{5,30} suggesting that integrin stimulation may be responsible for their activation in the heart after biomechanical stress. MAPKs are activated in the heart after acute pressure overload and are implicated in development of cardiac hypertrophy.\textsuperscript{31-33} An increase in ERK1/2 activity was detectable within 15 minutes after AB. However, maximal activation of JNKs was observed 30 minutes after AB, whereas activation of p38 kinase was observed throughout the 1-hour observation period.\textsuperscript{34} Pretreatment with SB202190, an inhibitor of p38 kinase, inhibits mechanical stretch–induced phenylalanine incorporation in cardiac myocytes in vitro.\textsuperscript{30} Together, these data suggest that the activation of ERK1/2 may be an early and transient event, whereas sustained activation of p38 kinase and JNKs plays a critical role in development of cardiac hypertrophy in response to chronic pressure overload.

Overexpression of constitutively active Akt in the mouse heart induces cardiac hypertrophy and increases myocyte contractility and relaxation through acceleration of intracellular calcium transients.\textsuperscript{11} Akt phosphorylates the inhibitory site of GSK-3β. GSK-3β is a negative regulator of pressure overload–induced hypertrophy. In contrast to other kinases, GSK-3β is normally active in unstimulated cells and is inactivated in response to growth factors, such as insulin and insulin-like growth factor 1. In animal models, pressure overload in response to AB is shown to inhibit GSK-3β activity. Inhibition of GSK-3β increases binding of c-Jun to its consensus sequence and promotes NF-AT and GATA-4 translocation to nucleus, thereby stimulating the gene expression.\textsuperscript{11} Our results that phosphorylation of Akt and GSK-3β is lower in OPN KO-AB hearts suggest that increased expression of OPN during chronic pressure overload–induced hypertrophy promotes hypertrophy, at least in part, via the involvement of Akt/GSK-3β pathway.

Interaction of ECM proteins with integrins is proposed to play an important role in cardiac myocyte growth and myocardial hypertrophy.\textsuperscript{2} Our data suggest that lack of OPN during chronic pressure overload may affect integrin-associated signaling leading to impaired cardiac hypertrophy. However, it should be emphasized that our data on hypertrophy and signaling are obtained 1 month after initiation of pressure overload. It is possible that OPN KO mice may exhibit reduced fibrosis with LV dilation if the observation time is extended beyond 1 month.

Acknowledgments

This work is supported in part by National Heart, Lung, and Blood Institute grants HL-071519, a merit review grant from the Department of Veterans Affairs (K.S.), and a fellowship from the American Heart Association, Southeast Affiliate (Z.X.).

References


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Hypertension. 2004;44:826-831; originally published online November 8, 2004; doi: 10.1161/01.HYP.0000148458.03202.48
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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