Lack of osteopontin improves cardiac function in streptozotocin-induced diabetic mice

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Subramanian V, Krishnamurthy P, Singh K, Singh M. Lack of osteopontin improves cardiac function in streptozotocin-induced diabetic mice. Am J Physiol Heart Circ Physiol 292: H673–H683, 2007. First published September 15, 2006; doi:10.1152/ajpheart.00569.2006.—The purpose of this study was to investigate the role of osteopontin (OPN) in diabetic hearts. Diabetes was induced in wild-type (WT) and OPN knockout (KO) mice by using streptozotocin (150 mg/kg) injection. Left ventricular (LV) structural and functional remodeling was studied 30 and 60 days after induction of diabetes. Induction of diabetes increased OPN expression in cardiac myocytes. Heart weight-to-body weight ratio was increased in both diabetic (D) groups. Lung wet weight-to-dry weight ratio was increased only in the WT-D group. Peak left ventricular (LV) developed pressures measured using Langendorff perfusion analyses were reduced to a greater extent in WT-D versus KO-D group. LV end-diastolic pressure-volume curve exhibited a significant leftward shift in WT-D but not in KO-D group. LV end-diastolic diameter, percent fractional shortening, and the ratio of peak velocity of early and late filling (E/A wave) were significantly reduced in WT-D mice as analyzed by echocardiography. The increase in cardiac myocyte apoptosis and fibrosis was significantly higher in the WT-D group. Expression of atrial natriuretic peptide and transforming growth factor-β1 was significantly increased in the WT-D group. Induction of diabetes increased protein kinase C (PKC) phosphorylation in both groups. However, phosphorylation of PKC-βII was significantly higher in the WT-D group, whereas phosphorylation of PKC-ζ was significantly higher in the KO-D group. Levels of peroxisome proliferator-activated receptor-γ were significantly decreased in the WT-D group but not in the KO-D group. Thus increased expression of OPN may play a deleterious role during streptozotocin-induced diabetic cardiomyopathy with effects on cardiac fibrosis, hypertrophy, and myocyte apoptosis.

diabetes; heart; apoptosis; fibrosis; hypertrophy

DIABETIC CARDIOMYOPATHY has extensively been studied in a variety of animal models through the use of streptozotocin (STZ) to destroy the pancreatic islet β-cells and induce an insulin-dependent diabetic state (18). STZ-induced diabetic animals develop cardiomyopathy, which is characterized by decreased left ventricular (LV) contractility and by diminished ventricular compliance with markedly abnormal systolic and diastolic function (27, 36). Activation of protein kinase C (PKC) is implicated in the diabetes-induced cardiovascular complications (32). Activation of PKC is associated with biochemical changes such as increased vascular permeability, basement membrane thickening, and extracellular matrix deposition during diabetic cardiomyopathy (26, 32, 54). PKC-βII isoform is shown to be preferentially activated in the heart and aorta of diabetic animals (25, 32). Peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the nuclear receptor superfamily, is suggested to play a critical role in the regulation of a variety of biological processes within the cardiovascular system (45). PPAR-γ is expressed in cardiac myocytes, and ligand-dependent activation of PPAR-γ attenuates pressure overload-induced cardiac hypertrophy and ventricular remodeling and failure following myocardial infarction (2, 47). PPAR-γ agonist, such as thiazolidinedione anti diabetic drugs, is shown to suppress PKC-β activation and translocation (52).

Osteopontin (OPN), also called cytokine Eta-1, contains Arg-Gly-Asp-Ser cell-binding sequence and interacts with integrins (αvβ1, αvβ3, and αvβ5) and CD44 receptors (13, 51). Interaction of OPN with various extracellular matrix proteins, including fibronectin and collagen, suggests a possible role of OPN in matrix organization and stability (28, 39). Heart expresses OPN at low levels under normal conditions. Cardiac myocytes, microvascular endothelial cells, and fibroblasts are known to express OPN (3, 48, 57). Expression of OPN in the myocardium increases coincident with the development of heart failure (49). With the use of OPN knockout (KO) mice and myocardial infarction as a model of cardiac remodeling, it has been demonstrated that lack of OPN results in increased LV dilation, suggesting a role for OPN in cardiac remodeling (50). However, the role of OPN in diabetic cardiomyopathy has not yet been studied.

To investigate the role of OPN in the pathogenesis of diabetic cardiomyopathy, diabetes was induced in a group of wild-type (WT) and OPN-KO mice by using a single intraperitoneal injection of STZ. The data presented here suggest that increased expression of OPN in the heart during diabetes plays a deleterious role with increased cardiac fibrosis and apoptosis.

MATERIALS AND METHODS

Experimental animals. Mice lacking OPN (KO) and WT were of a 129black Swiss hybrid background. Genotyping was carried out by polymerase chain reaction analysis using primers suggested by Liaw et al. (34). Once genotyped, the KO and WT animals were bred and maintained as separate colonies. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and approved by the University Committee on Animal Care, which are certified by the American Association for Accreditation of Laboratory Care.

Induction of diabetes in mice. Diabetes was induced in age-matched mice (~4 mo old) by injecting a single dose of freshly
prepared STZ solution (150 mg/kg body wt ip, in citrate buffer, pH 4.2) after overnight fasting. Mice injected with the same volume of citrate buffer served as sham. Mice had unlimited access to water and standard mouse chow (Harlan, Madison, WI). Plasma glucose level was measured 7 and 60 days after STZ injection. Animals exhibiting plasma glucose levels of >16 mmol/l at day 7 were considered diabetic and used for 30- and 60-day experiments. All other physiological and biochemical parameters were measured 4-8 wk after STZ injection. Mortality among diabetic mice was ≤25% with no significant difference between WT-D and KO-D groups.

**Measurement of plasma glucose.** Blood was collected from mice under anesthesia through sino-ocular puncture using heparinized tubes. Plasma was separated by centrifuging the blood at 3,000 rpm for 20 min at 4°C. For glucose measurement, 100 µl of plasma were mixed with 5.0 ml of O-toluidine reagent (Sigma, St. Louis, MO) and boiled vigorously for 10 min in a water bath. The developed green color was measured spectrophotometrically at 640 nm. Different concentrations of glucose were used as standards (23).

**Langendorff perfusion analysis.** LV function was measured using isolated buffer-perfused heart preparation as described (16, 43). Briefly, isolated hearts were perfused retrogradely using normothermic Krebs-Henseleit buffer (in mmol/l: 118.0 NaCl, 25.0 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 12.0 glucose, 1.9 CaCl2) at a constant perfusion pressure of 70 mmHg and paced at 7 Hz. The Krebs-Henseleit buffer was gassed continuously with 95% O2-5% CO2. A small polyvinyl chloride fluid-filled balloon attached to the apex, midcavity, and base (n = 7 in each group) were visualized under a Nikon TE 2000 inverted microscope. Images were acquired with a Bioquant image acquisition and analysis system (Nashville, TN) using a Retiga 1300 color-cooled camera. For each heart, fibrosis was quantified from 30 images (10 images each from the apex, midcavity, and base). A second individual, blinded to the identity of samples, also performed the quantitative analysis in few samples with comparable results. To calculate lung wet-to-dry weight ratio, lung wet weight was obtained after the esophagus and trachea were trimmed away and the pleural surface blotted dry, whereas lung dry weight was obtained after the tissue was dried at 65°C for 72 h.

**TUNEL staining.** To measure apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining followed by Hoechst 33258 staining was carried out on 4-µm thick paraffin-embedded sections as per manufacturer’s instructions (Cell death detection assay kit, Roche). To identify apoptosis associated with cardiac myocytes, the sections were then immunostained using α-sarcromeric actin antibodies (1:50; SC5 clone, Sigma). Hoechst 33258 (10 µM; Sigma) staining was used to count the total number of nuclei. TUNEL-positive nuclei that were clearly seen within cardiac myocytes were counted. The number of apoptotic cardiac myocyte nuclei were counted and averaged by examining 30 fields/animal using the ×20 objective. Index of apoptosis was calculated as the percentage of apoptotic myocardium nuclei per total number of nuclei.

**In situ oligo ligation assay.** Apoptosis was confirmed using situ oligo ligation (ISOL) assay according to the manufacturer’s instructions (Intergen, Purchase, NY). Hoechst 33258 staining was used to count the total number of nuclei. Apoptosis is expressed as percentage of ISOL-positive myocardium nuclei per total number of nuclei.

**Reverse transcription-polymerase chain reaction.** Total RNA was isolated from LV as described (48). The RNA (1 µg) was reverse transcribed using oligo dT, and the products were amplified by PCR using the following primers: OPN, 5′-GCTGGTGCTATGAGCAG-3′/GACCCCACTAGACCAC-3′; transforming growth factor-β1 (TGF-β1), 5′-GCCGCCAGGGCTACTATG-3′/5′-CAGCACCAGC GGTATCG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TCCATGACACGTCATGC-3′/5′-TTCAGTCTTGGATGACCT-3′.

**Western analysis.** Tissue lysates were prepared in lysis buffer (1% Triton X-100, 50 mmol/l NaCl, 10 mmol/l Tris, pH 7.4, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l sodium orthovanadate and 0.5% Nonidet P-40) using a homogenizer. Equal amounts of total protein (50 µg) were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto polyvinylidene fluoride membrane. The blots were then probed with primary antibodies directed against OPN (1:1,000; Santa Cruz Bio-technology, Santa Cruz, CA), p-PKC, p-PKC-δ, p-ERK-1/2 (1:250; Santa Cruz), p21 (1:1,000; Cell Signaling Technology, Danvers, MA), PPAR-γ (1:500; Affinity Bioreagents, Nashville, TN), and α-tubulin (1:5,000; Sigma) staining was used to count the total number of nuclei. Apoptosis is expressed as percentage of ISOL-positive myocardium nuclei per total number of nuclei.

**Histology and morphometric analyses.** After the Langendorff studies, the hearts were arrested in diastole using KCl (30 mmol/l), followed by perfusion fixation with 10% buffered formalin. To measure fibrosis, trichrome-stained sections (4 µm thick) from the LV apex, midcavity, and base were visualized under Nikon TE 2000 inverted microscope. Images were acquired with a Bioquant image acquisition and analysis system (Nashville, TN) using a Retiga 1300 color-cooled camera. For each heart, fibrosis was quantified from 30 images (10 images each from the apex, midcavity, and base). A second individual, blinded to the identity of samples, also performed the quantitative analysis in few samples with comparable results. To calculate lung wet-to-dry weight ratio, lung wet weight was obtained after the esophagus and trachea were trimmed away and the pleural surface blotted dry, whereas lung dry weight was obtained after the tissue was dried at 65°C for 72 h.

**Echocardiography.** Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Apio 80 Imaging System (Tochigi, Japan) equipped with a 12-MHz linear transducer. Echocardiographic studies were performed before and after 1 mo of induction of diabetes in mice anesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 l/min). The body temperature was maintained at ~37°C using heating pad. M-mode tracings were used to measure LV wall thickness, LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percent fractional shortening (%FS) was calculated as described (17). Doppler tracings of mitral and aortic flow were used to measure peak velocity of the early ventricular filling (E wave), peak velocity of the late ventricular filling (A wave), peak E/A ratio, and isovolumic relaxation time. All echocardiographic assessments were performed by the same investigator.

**Histology and morphometric analyses.** After the Langendorff studies, the hearts were arrested in diastole using KCl (30 mmol/l), followed by perfusion fixation with 10% buffered formalin. To measure fibrosis, trichrome-stained sections (4 µm thick) from the LV apex, midcavity, and base (n = 7 in each group) were visualized under Nikon TE 2000 inverted microscope. Images were acquired with a Bioquant image acquisition and analysis system (Nashville, TN) using a Retiga 1300 color-cooled camera. For each heart, fibrosis was quantified from 30 images (10 images each from the apex, midcavity, and base). A second individual, blinded to the identity of samples, also performed the quantitative analysis in few samples with comparable results. To calculate lung wet-to-dry weight ratio, lung wet weight was obtained after the esophagus and trachea were trimmed away and the pleural surface blotted dry, whereas lung dry weight was obtained after the tissue was dried at 65°C for 72 h.

**Table 1. Morphometric measurements**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>WT</th>
<th>KO</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>37.66±1.45</td>
<td>36.36±0.97</td>
<td>32.42±1.54*</td>
<td>32.92±1.51†</td>
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<tr>
<td>Plasma glucose, mmol/l</td>
<td>8.86±0.23</td>
<td>8.48±0.20</td>
<td>30.07±1.40*</td>
<td>29.54±1.12†</td>
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<tr>
<td>HW, mg</td>
<td>205.6±4.37</td>
<td>202.74±5.56</td>
<td>241.28±4.15*</td>
<td>234.08±4.06†</td>
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<tr>
<td>HW/BW, mg/g</td>
<td>5.61±0.29</td>
<td>5.57±0.40</td>
<td>7.48±0.39*</td>
<td>7.18±0.23†</td>
</tr>
<tr>
<td>Lung wet-to-dry weight ratio, g/g</td>
<td>4.44±0.11</td>
<td>4.75±0.09</td>
<td>5.38±0.07*</td>
<td>5.18±0.13</td>
</tr>
<tr>
<td>Septal wall thickness, mm</td>
<td>1.35±0.03</td>
<td>1.33±0.02</td>
<td>1.53±0.05‡</td>
<td>1.23±0.07</td>
</tr>
<tr>
<td>Anterior wall thickness, mm</td>
<td>1.22±0.03</td>
<td>1.25±0.04</td>
<td>1.35±0.05</td>
<td>1.05±0.04‡</td>
</tr>
<tr>
<td>LV inner circumference, mm</td>
<td>9.03±0.42</td>
<td>9.12±0.58</td>
<td>8.13±0.17‡</td>
<td>9.31±0.21</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 mice for all parameters, except plasma glucose where n = 25 mice. HW, heart weight; BW, body weight; WT, wild type; KO, knock out; LV, left ventricular. *P < 0.05 vs. Sham WT; †P < 0.05 vs. Sham KO; ‡P < 0.05 vs. Sham WT, diabetic KO; §P < 0.05 vs. Sham KO, diabetic WT.
Golden, CO), and appropriate secondary antibodies. Protein loading was normalized using antibodies against actin or GAPDH.

**Immunohistochemistry.** The OPN protein expression was studied using monoclonal anti-OPN antibodies (monoclonal antibody AK2C5 provided by Aaron Kowalski and David T. Denhardt, Rutgers University, Piscataway, NJ) as described previously (49). Briefly, the sections (4-μm thick) were rehydrated, quenched with 3% hydrogen peroxide, and blocked with 1.5% goat serum for 1 h. The sections were incubated with monoclonal anti-OPN antibody (1:100 dilution) for 1 h at 37°C in a humidified chamber followed by incubation with secondary antibody for 45 min at 37°C (goat anti-mouse IgG-horseradish peroxidase conjugate, Santa Cruz Biotechnology). The antibody binding sites were visualized following reaction with DAB-peroxidase substrate solution (Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin and visualized under brightfield microscopy, and images were recorded using Retiga 1300 color-cooled camera and Bioquant software.

**Statistical analysis.** Data are expressed as means ± SE. Data were analyzed using Student’s t-test or one-way ANOVA followed by
Diabetes and increased OPN expression. Basal plasma glucose levels were not different between the WT and KO (in mmol/l: WT-sham, 8.86 ± 0.23; KO-sham, 8.48 ± 0.20; n = 25; P = not significant) groups. Plasma glucose levels were increased to 18.68 ± 0.78 and 19.44 ± 0.67 mmol/l in WT and KO mice, respectively, 7 days after the injection of STZ. Plasma glucose levels were further increased to 30.07 ± 1.40 and 29.54 ± 1.12 mmol/l in WT and KO mice, respectively, after 60 days of STZ administration with no significant difference between the two diabetic groups (Table 1).

Western blot analysis of LV tissue indicated increased levels of OPN protein (ranging from ~37 to 69 kDa) in WT-diabetic (WT-D) hearts compared with sham hearts (~1.5-fold vs. WT-sham; Fig. 1A). OPN exhibits multiple bands on SDS-PAGE due to differential glycosylation, phosphorylation, and processing (13, 55). Immunohistochemical staining using anti-OPN antibodies demonstrated increased expression of OPN in the diabetic hearts. Coimmunostaining of the sections using α-sarcomeric actin antibodies showed that the increased expression of OPN is mainly associated with myocytes (Fig. 1C). RT-PCR analysis indicated 4.19- and 2.96-fold increase in OPN gene expression 30 and 60 days after induction of diabetes, respectively (Fig. 1B).

Morphological studies. After 60 days of STZ administration, body weights were significantly decreased in both groups. Heart weight and heart-to-body weight ratios were significantly higher in both diabetic groups compared with shams. Lung wet weight-to-dry weight ratios were significantly higher in WT-D and not in KO-D mice (Table 1). The LV inner circumference decreased while the septal wall thickness was increased significantly in WT-D (Table 1), indicating hypertrophy in WT-D group. In the case of KO-D mice, the anterior wall thickness decreased significantly while the LV inner circumference and septal wall thickness remained unchanged.

LV pressure-volume relationship. LV developed pressures measured over a range of volumes were depressed in both diabetic groups after 60 days of induction of diabetes. However, LV developed pressures at 20–50 μl of volumes were significantly higher in KO-D when compared with WT-D mice (P < 0.05; Fig. 2A). The LV end-diastolic pressure-volume curve was shifted leftward in WT-D (P < 0.05 vs. KO-D) mice. In contrast, KO-D mice exhibited a nonsignificant rightward shift in the LV end-diastolic pressure-volume curve (P = 0.148 vs. control; Fig. 2B), indicating a trend toward LV dilation in KO-D mice. Similar data were obtained after 30 days of induction of diabetes (data not shown).

Echocardiographic measurements. At baseline, there was no difference in septal and posterior wall thicknesses, LVESD, LVEDD, and %FS between WT and KO groups (data not shown). After 30 days of induction of diabetes, there was a significant increase in septal wall and posterior wall thicknesses in WT-D group compared with WT-sham and KO-D groups. LVEDD was significantly decreased in the WT-D group (P < 0.05 vs. sham and KO-D). Percent FS was significantly reduced in the WT-D group but not in the KO-D group. Septal and posterior wall thicknesses, LVESD, LVEDD, and %FS remained unchanged in the KO-D group compared with the KO-sham group (Table 2). Peak E velocity was significantly decreased in WT-D group, whereas peak A velocity was significantly increased in both diabetic groups. E/A ratio, a measure of LV relaxation, was significantly reduced in both diabetic groups. However, the decrease was significantly higher in the WT-D versus the KO-D group (Table 3). Isovolumic relaxation time was not different among the groups.

Apoptosis and fibrosis. Induction of diabetes increased the number of apoptotic cardiac myocytes in both groups. However, the number of apoptotic cardiac myocytes was significantly lower in the KO-D group compared with the WT-D group (percentage of apoptotic myocyte nuclei/total number of nuclei: WT-sham, 0.045 ± 0.010; KO-sham, 0.043 ± 0.005; WT-D, 0.187 ± 0.006; KO-D, 0.119 ± 0.008. P < 0.05 vs. WT-sham and KO-sham; P < 0.05 vs. KO-D; see Fig. 3 for P value symbols). Similar to TUNEL staining, the number of ISOL-positive cardiac myocytes was significantly higher in the
WT-D group compared with the KO-D group (percent apoptosis: WT-sham, 0.034 ± 0.009; KO-sham, 0.031 ± 0.004; WT-D, 0.168 ± 0.009; KO-D, 0.101 ± 0.008; P < 0.05 vs. WT-sham and KO-sham; P < 0.05 vs. KO-D; see Fig. 3C for P value symbols).

Quantitative analysis of trichrome-stained sections showed increased fibrosis in both diabetic groups. However, the increase in fibrosis was significantly lower in the KO-D group compared with the WT-D group (percent fibrosis: WT-sham, 0.18 ± 0.03; KO-sham, 0.17 ± 0.01; WT-D, 2.24 ± 0.26; KO-D, 0.39 ± 0.10, P < 0.05 vs. WT-sham and KO-sham; P < 0.05 vs. KO-D; see Fig. 4 for P value symbols).

**ANP and TGF-β1 gene expression**. Analysis of ANP and TGF-β1 gene expression after 60 days of induction of diabetes using RT-PCR detected increased expression of ANP and TGF-β1 in the WT-D hearts but not in the KO-D hearts (Fig. 5).

**Activation of PKC**. Activation of the PKC family of serine/threonine kinases is well documented in the diabetic myocardium (25). Induction of diabetes increased phosphorylation (activation) of total PKC in diabetic hearts. However, the phosphorylation of PKC was significantly higher in the OPN KO-D group compared with the WT-D group (P < 0.05 vs. WT-D and KO-Sham; Fig. 6A). Activation of PKC-βII is reported to play an important role in the endothelial barrier dysfunction in the heart during diabetes (60), whereas activation of PKC-ζ is suggested to play an anti-apoptotic role during diabetes (8). Western blot analyses using phospho-specific antibodies indicated increased phosphorylation of PKC-βII in the WT-D group (P < 0.05 vs. WT-Sham and KO-D; Fig. 6B), whereas the PKC-ζ phosphorylation was significantly increased in the KO-D group (P < 0.05 vs. WT-D and KO-Sham; Fig. 6C). Normalization of band intensities with total PKC-ζ or GAPDH antibodies indicated similar change in densitometric values. Therefore, GAPDH was used for normalization of p-PKC and its isoforms.

**PPAR-γ expression**. PPAR-γ signaling plays a critical role in the regulation of a variety of biological processes, including cardiac hypertrophy, oxidative stress, and apoptosis, within the cardiovascular system (2, 10, 35, 47). Analysis of PPAR-γ expression using Western blot demonstrated decreased PPAR-γ levels in the WT-D group but not in the KO-D group (P < 0.05 vs. WT-sham and KO-D; Fig. 7).

**DISCUSSION**

Diabetic cardiomyopathy is a condition characterized by cardiac hypertrophy, decreased ventricular compliance, diastolic, and systolic dysfunction (27, 36). This is the first study to suggest that lack of OPN leads to improvement in function of the diabetic heart. The major findings of this study are that OPN expression increases in the heart after induction of diabetes, and mice lacking OPN exhibit improved LV function with reduced fibrosis and apoptosis compared with WT-D mice. Lack of OPN was associated with reduced ANP and TGF-β1 expression, activation of PKC-ζ, and preservation of PPAR-γ levels.

Previously, increased OPN expression was observed in the myocardium of spontaneously hypertensive rats and aortic-banded rats coincident with the development of heart failure (49). Increased OPN expression in mouse hearts was also observed following myocardial infarction, aldosterone infusion, and pressure overload-induced hypertrophy (44, 50, 58). Myocardial infarction and aldosterone infusion predominantly increased OPN expression in the interstitial cells (44, 50), whereas increased expression of OPN was mainly associated with cardiac myocytes during pressure overload-induced hypertrophy (58). Here, we show increased mRNA and protein expression of OPN in the heart after induction of diabetes. OPN exhibits multiple bands on SDS-PAGE due to differential glycosylation, phosphorylation, and processing (13, 55). In one of the diabetic samples, a band around 55 kDa for OPN was observed (Fig. 1A). The identity of the band around 55 kDa is not known. It may represent a cleaved fragment of OPN. OPN is shown to be cleaved by thrombin (22, 46) and matrix metalloproteinases (MMPs), specifically MMP-3 and MMP-7 (1). Immunohistochemical analysis indicates that diabetes-

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**Table 2. M-mode echocardiographic measurements**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT (mm)</th>
<th>KO (mm)</th>
<th>WT (cm/s)</th>
<th>KO (cm/s)</th>
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<tr>
<td>n</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>LVEDD, mm</td>
<td>4.019±0.085</td>
<td>4.172±0.101</td>
<td>3.757±0.058*</td>
<td>3.943±0.040</td>
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<td>LVESD, mm</td>
<td>2.915±0.101</td>
<td>2.995±0.073</td>
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<td>2.901±0.053</td>
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<td>LVS, mm</td>
<td>0.463±0.019</td>
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<td>0.604±0.028*</td>
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<td>LVPWd, mm</td>
<td>0.642±0.017</td>
<td>0.654±0.011</td>
<td>0.772±0.041*</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.354±0.018</td>
<td>0.591±0.020</td>
<td>0.685±0.026*</td>
<td>0.550±0.013</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.691±0.018</td>
<td>0.705±0.028</td>
<td>0.808±0.032*</td>
<td>0.698±0.020</td>
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<td>% FS</td>
<td>27.59±1.11</td>
<td>28.11±1.20</td>
<td>21.51±1.68*</td>
<td>26.41±1.09</td>
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<tr>
<td>HR, beats/min</td>
<td>354.06±9.25</td>
<td>368.35±7.91</td>
<td>397.05±11.64</td>
<td>395.16±14.54</td>
</tr>
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</table>

Data are means ± SE; n, number of mice. LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVSWd, LV posterior wall thickness in diastole; LVSWs, LV posterior wall thickness in systole; FS, fractional shortening; HR, heart rate. *P<0.05 vs. sham WT, diabetic KO.

**Table 3. Doppler echocardiographic data**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Diabetic</th>
</tr>
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<tr>
<td>n</td>
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<td>6</td>
</tr>
<tr>
<td>E, cm/s</td>
<td>60.49±1.03</td>
<td>61.97±1.52</td>
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<tr>
<td>A, cm/s</td>
<td>29.74±0.53</td>
<td>31.39±2.01</td>
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<tr>
<td>E/A ratio</td>
<td>2.044±0.062</td>
<td>2.028±0.149</td>
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<tr>
<td>IVRT, ms</td>
<td>22.68±0.53</td>
<td>23.86±1.10</td>
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</table>

Data are means ± SE; n, number of mice. E wave, peak velocity of the early ventricular filling; A wave, peak velocity of the late ventricular filling; IVRT, isovolumic relaxation time. *P<0.05 vs. sham WT, diabetic KO; †P<0.05 vs. sham KO, diabetic WT.
induced OPN expression is associated mainly with cardiac myocytes.

Diabetic cardiomyopathy is shown to be associated with depressed cardiac function (27, 36). LV developed pressure, observed by Langendorff perfusion analysis, was significantly decreased in WT mice after 60 days of diabetes induction. Interestingly, diabetic mice lacking OPN exhibited improved LV developed pressure compared with WT-D mice. Echocardiographic analyses of the hearts using M-mode echocardiography and Doppler analyses also suggested significant LV dysfunction in WT-D mice. Myocardial infarction, aldosterone infusion, and angiotensin II treatment are shown to be associated with decreased contractile function in mice lacking OPN compared with WT mice (38, 44, 50). The reasons for different cardiac response, in relation to OPN, under diabetic conditions may include different neurohormonal response, cell types involved in the expression of OPN, and different biochemical and physiological properties of various isoforms of OPN. In myocardial infarction and aldosterone infusion models, interstitial cells appeared to be the main source of OPN expression (44, 50), whereas cardiac myocytes seem to be the main source of OPN expression during STZ-induced diabetic cardiomyopathy. In diabetic hearts, hyperglycemia is known to activate the local renin-angiotensin system, which results in the formation of angiotensin II (29, 37). Angiotensin II is shown to increase OPN expression in cardiac microvascular endothelial cells and fibroblasts and not in cardiac myocytes (3, 48, 57), suggesting increased OPN expression in cardiac myocytes in the diabetic heart may involve angiotensin II-independent mechanism(s).

Multiple isoforms of OPN are secreted by cells because of differential processing and posttranslational modifications, including phosphorylation and glycosylation. The posttranslational modifications of OPN are shown to affect its interaction with other proteins, including various receptors, leading to distinct functions (14, 55).

Echocardiographic measurements demonstrated increased septal, anterior, and posterior wall thicknesses with decreased LVEDD only in WT-D mice. In addition, the expression of...
ANP was significantly higher in WT-D mice compared with that of KO-D mice. These data suggest a role for OPN in cardiac hypertrophy after induction of diabetes. Hypertrophic response is shown to be lower in KO mice in response to chronic pressure overload (58). In contrast, hypertrophic response to angiotensin II as measured by change in heart weight-to-body weight ratio was not different in OPN KO and WT mice (38). Taken together, these studies point toward the possibility that the mechanism(s) of cardiac hypertrophy induced by pressure overload or diabetes may be different from that of angiotensin II-induced hypertrophy.

The diabetic heart is histologically characterized by myocardial fibrosis with reduced myocardial elasticity and contractility (4). The common extracellular pathology during diabetes is the thickening of basement membrane as a result of the deposition of extracellular matrix (ECM) proteins including collagen and fibronectin (31). OPN is suggested to regulate the synthesis or turnover of ECM proteins, including collagen (34). Lack of OPN is shown to be associated with reduced fibrosis in various models of myocardial remodeling (12, 38, 44, 50). In the present study, we observed increased fibrosis in both WT-D and KO-D mice. However, the increase in fibrosis was significantly lower in KO-D versus WT-D mice, thus suggesting a role for OPN in diabetes-induced fibrosis. In addition, we observed an increased expression of TGF-β1 in WT-D but not in KO-D mice. Levels of TGF-β1 are shown to be increased in

![Fig. 5. Atrial natriuretic peptide (ANP) and transforming growth factor-β1 (TGF-β1) gene expression. Total RNA isolated from LV was analyzed by RT-PCR using primers specific for ANP (A) and TGF-β1 (B). GAPDH was used as internal control. C and D: gene expression levels expressed as fold change vs. WT-sham. *P < 0.05 vs. WT-sham; #P < 0.05 vs. KO-D; n = 4.](http://ajpheart.physiology.org/)
Fig. 6. Phosphorylation of PKC and its isoforms after 60 days of induction of diabetes. Phosphorylation of total PKC (A), PKC-βII (B), and PKC-ζ (C) were analyzed in LV lysates by Western blot analysis using phosphospecific antibodies. Protein loading was normalized using GAPDH immunostaining. Phosphorylation levels expressed as fold change vs. WT-sham. *P < 0.05 vs. WT-sham and KO-sham; #P < 0.05 vs. KO-D; †P < 0.05 vs. WT-D; n = 6.

Fig. 7. Peroxisome proliferator-activated receptor-γ (PPAR-γ) protein levels. PPAR-γ protein levels were measured in LV lysates after 30 days of induction of diabetes using Western blot analysis. Protein loading was normalized using GAPDH antibodies. PPAR-γ protein levels expressed as fold change vs. WT-sham. *P < 0.05 vs. WT-sham and KO-sham; #P < 0.05 vs. KO-D; n = 7.
diabetes-induced cardiac hypertrophy and fibrosis (20, 54). Expression of active TGF-β1 decreased in OPN KO mice during bleomycin-induced pulmonary fibrosis (5). Thus increased expression of OPN in the heart during diabetes may increase fibrosis by modulating the level of TGF-β1.

Myocyte apoptosis is suggested to be involved in the development of diabetic cardiomyopathy (29). Diabetes-induced cardiomyocyte apoptosis has been shown in human patients (19) and various animal models (7). Here, we observed increased cardiac myocyte apoptosis in both diabetic groups. However, the number of apoptotic cardiac myocytes was significantly lower in mice lacking OPN. Similar to TUNEL-staining, the number of ISOL-positive cardiac myocytes was significantly higher in WT-D compared with that of the KO-D group. The number of apoptotic myocytes using TUNEL staining is slightly higher compared with the ISOL assay. TUNEL staining may detect nonspecific DNA fragmentation due to necrosis or nuclei under repair (11), whereas ISOL assay more specifically identifies apoptotic nuclei using hairpin oligonucleotide probes (15). OPN is shown to play pro- and anti-apoptotic roles in different cell types. OPN KO mice exhibit increased apoptosis in postischemic acute renal failure (42). In contrast, Yumoto et al. (61) observed significant reduction in chondrocyte apoptosis in mice lacking OPN in a model of rheumatoid arthritis. Within the heart, cardiac cell apoptosis was significantly lower in mice lacking OPN in angiotensin II-induced model of cardiac hypertrophy (38). Decreased cardiac cell apoptosis was also observed in an aldosterone-induced model of myocardial remodeling in mice lacking OPN (44).

Activation of PKC is implicated in the diabetes-induced cardiovascular complications (32). Glucose-induced activation of PKC augments the production of extracellular matrix macromolecules that accumulate during atherosclerotic lesion formation (32). PKC plays an important role in intracellular signaling for modulating cardiac myocyte development, inotropic functions, and cellular growth (40). PKC family consists of at least 11 different isoforms. PKC-βII, a Ca²⁺-dependent isoform, is shown to be preferentially activated in the diabetic heart (21, 25, 32, 56). Targeted overexpression of PKC-βII in the mouse myocardium results in LV hypertrophy and fibrosis (6, 53). PKC-ζ is reported to play an important role in the regulation of hypertrophic and apoptotic events during neonatal rat heart development (9). PKC-ζ is suggested to play an anti-apoptotic role in the regulation of Fas ligand-induced apoptosis (33). Here we observed increased phosphorylation of PKC-βII in WT-D hearts, whereas increased phosphorylation of PKC-ζ was observed in KO-D hearts. In cardiac fibroblasts, purified OPN protein is shown to inhibit interleukin-1β-stimulated activation of PKC-ζ (59). These data suggest that increased expression of OPN during diabetic cardiomyopathy may affect cardiac myocyte apoptosis, hypertrophy, and fibrosis by modulating PKC activity. Lack of OPN may activate PKC-ζ during diabetes, which in turn may be responsible for reduced apoptosis and hypertrophy observed in KO-D mice.

PPAR-γ signaling is suggested to play a critical role in cardiovascular system (45). In nondiabetic models, PPAR-γ is shown to attenuate cardiac hypertrophy, apoptosis, and fibrosis (2, 10, 24, 35). PPAR-γ protein levels decrease significantly in the hearts of diabetic ZDF fa/fa rats compared with lean control animals (41). The data presented here demonstrate decreased PPAR-γ protein levels in WT-D hearts. Lack of OPN preserved levels of PPAR-γ after induction of diabetes. PPAR-γ agonists are shown to inhibit high glucose-induced translocation of PKC-β to the plasma membrane in bovine aortic endothelial cells (52). The decreased level of PPAR-γ in WT-D may, in part, be responsible for the observed increased activation of PKC-βII.

In summary, this study underlines the important role of OPN in cardiac dysfunction under diabetic conditions and indicates that lack of OPN protein can be beneficial in preserving function of the diabetic heart. Our data raise the possibility that inhibition of OPN expression during diabetic cardiomyopathy may be of value in the treatment and prevention of diabetic cardiomyopathy.

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