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**gp130 Plays a Critical Role in Pressure Overload-Induced Cardiac Hypertrophy**

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**RUNNING TITLE:** gp130 in pressure overload-induced cardiac hypertrophy
gp130, a common receptor for the interleukin 6 family, plays pivotal roles in growth and survival of cardiac myocytes. In the present study, we examined the role of gp130 in pressure overload-induced cardiac hypertrophy using transgenic (TG) mice which express a dominant negative mutant of gp130 in the heart under the control of α myosin heavy chain promoter. TG mice were apparently healthy and fertile. There were no differences in body weight and heart weight between TG mice and littermate wild type (WT) mice. Pressure overload-induced increases in the heart weight/body weight ratio, ventricular wall thickness and cross sectional areas of cardiac myocytes were significantly smaller in TG mice than in WT mice. Northern blot analysis revealed that pressure overload-induced upregulation of BNP gene and downregulation of SERCA2 gene were attenuated in TG mice. Pressure overload activated extracellular signal-regulated kinases (ERKs) and STAT3 in the heart of WT mice, whereas pressure overload-induced activation of STAT3, but not of ERKs, was suppressed in TG mice. These results suggest that gp130 plays a critical role in pressure overload-induced cardiac hypertrophy possibly through the STAT3 pathway.
INTRODUCTION

Since recent clinical studies have suggested that cardiac hypertrophy is an independent risk factor of cardiac morbidity and mortality (1), it has become even more important to clarify the mechanism of how cardiac hypertrophy is developed. Cardiomyocyte hypertrophy can be induced by a variety of factors such as mechanical stress (2), catecholamines (3), angiotensin II (4), endothelin-1 (5) and cytokines (6). Among them, hemodynamic overload, namely mechanical stress, is clinically most important. We and others have reported that mechanical stress induces cardiomyocyte hypertrophy through vasoactive peptides such as angiotensin II and endothelin-1 (7-9).

Cardiotrophin-1 (CT-1), a member of the interleukin 6 (IL6) family, was isolated and found to have a potent hypertrophic effect on cultured cardiomyocytes (10). The IL6 family of cytokines promotes cell type-specific pleiotropic effects by engaging multimeric receptor complexes that share the common affinity converter/signal transducing subunit gp130 (11-13). CT-1 has been reported to induce hypertrophy of cardiac myocytes in vitro (14). It has been reported that transgenic mice expressing both IL6 and soluble IL6 receptor, in which the gp130 is continuously activated, showed marked hypertrophy of the ventricular myocardium (15), and that targeted disruption of gp130 leads to severe anemia and a hypoplastic ventricular myocardium in the embryo (16). These results suggest that activation of gp130 induces cardiac hypertrophy, and it is still unknown whether gp130 mediates load-induced cardiac hypertrophy. CT-1 has been reported to promote survival of cardiac myocytes (17). Ventricular restricted gp130 knockout mice showed marked ventricular wall dilatation with marked cardiomyocyte apoptosis and died in a week by pressure overload (18). These results suggest that gp130 signalings prevent cardiomyocytes from apoptotic cell death during the pressure overload.

In the present study, to determine the physiological significance of gp130 in load-induced cardiac hypertrophy, we generated transgenic (TG) mice which express a dominant negative form of gp130 specifically in the heart and examined hypertrophic responses by
pressure overload produced by constriction of the abdominal aorta.
MATERIALS AND METHODS

Transgene construction and generation of TG mice

A dominant negative mutant of gp130 (D.N.gp130) was constructed by converting cysteine at 702 to a stop codon as described previously (19). D.N.gp130 cDNA was inserted into the unique \textit{Kpn}I site of \textit{p\alpha}MHCSA which carries the mouse \textit{\alpha} myosin heavy chain (\textit{\alpha}MHC) promoter (20). \textit{\alpha}MHC promoter-D.N.gp130-polyA DNA was excised by \textit{Xho}I and \textit{Not}I, and microinjected into the pronuclei of fertilized BDF1 mouse eggs. Offsprings from eggs microinjected with the DNA were selected by Southern blot analysis and PCR.

Pressure-overload model

Male TG and littermate wild type (WT) mice of 20 weeks old were used in the present study. Mice were housed under climate-controlled conditions with a 12-hour light/dark cycle and were provided with standard food and water ad libitum. All protocols were approved by local institutional guidelines. Pressure overload was produced by constriction of the abdominal aorta as described previously in our laboratory (21, 22). Briefly, mice were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (30 mg/kg). The abdominal aorta was constricted at the suprarenal level with 7-0 nylon strings by ligation of the aorta with a blunted 27-gauge needle, which was pulled out thereafter.

Echocardiographic measurement

Transthoracic echocardiography was performed with HP Sonos 100 (Hewlett-Packard Co) with a 10 MHz imaging transducer as described previously (22, 23). Mice were anesthetized with ketamine (10 mg/kg IP) and xylazine (15 mg/kg IP). After a good-quality two-dimensional image was obtained, M-mode images of the left ventricle were recorded. Left ventricular end-diastolic internal diameter (LVEDD), left ventricular end-systolic internal diameter (LVESD), intraventricular septum thickness (IVST) and left ventricular posterior wall thickness (LVPWT) were measured. All measurements were performed by use
of the leading edge-to-leading edge convention adopted by the American Society of Echocardiography (24). Fractional shortening (FS) was calculated as FS=\[(LVEDD-LVESD)/LVEDD\]x100. Ejection fraction (EF) was calculated by the cubed method as follows: EF=\[(LVEDD^3-(LVESD)^3)/LVEDD^3\].

Histological analysis

For histological analysis, hearts were fixed with 10% formalin by perfusion fixation. Fixed hearts embedded in paraffin were sectioned at 4-µm thickness, and stained with hematoxylin-eosin. Cross sectional areas of cardiac myocytes were measured from 10 sections. Suitable cross sections were defined as having nearly circular capillary profiles and nuclei.

RNA preparation and Northern blot analysis

The left ventricle was excised, and total RNA (10 µg) was prepared using ZolB (Biotecx Laboratories, Inc.), fractionated in 1% formaldehyde agarose gel, and transferred to nylon membrane. The blots were hybridized with the cDNA fragments of gp130, brain natriuretic factor (BNP) and sarcoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) genes.

Western blot analysis of STAT3 phosphorylation

Polyclonal antibody to STAT3 (C-20) was purchased from Santa Cruz Biotechnology, Inc. The left ventricle was excised and lysed in lysis buffer (25 mM Tris-HCl, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EGTA and 1 mM phenylmethlysulfonyl fluoride), and equal amounts (100µg) of protein were incubated with 1 µg of anti-STAT3 for 1 hour at 4 °C. The immune complexes were precipitated with protein A Sepharose, and the immuneprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were then blocked and incubated with anti-phosphotyrosine antibody (4G10), and the phosphotyrosine was detected by enhanced chemiluminescence, ECL, (Amersham).
Assay of extracellular signal-regulated kinases (ERKs)

The activities of ERKs were measured using myelin basic protein (MBP)-containing gel (25). In brief, lysates of the left ventricles were subjected to electrophoresis on a SDS-polyacrylamide gel containing 0.5 mg/ml MBP. ERKs in the gel were denatured in guanidine HCl and renatured in Tris-HCl (pH 8.0) containing 0.04% Triton X-100 and 2-mercaptoethanol (5 mM). Phosphorylation activities of ERKs were assayed by incubating the gel with [γ-32P]ATP.

Terminal deoxynucleotidyl transferase assay (TUNEL)

The 4-µm thickness paraffin sections were deparaffinized by immersing in xylene, rehydrated, and incubated with proteinase K (20 µg/ml). Next, the sections were incubated in methanol with 3% H2O2 to inactivate endogenous peroxidases, washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase and FITC-dUTP for 90 min, and horseradish peroxidase-conjugated anti-FITC for 30 min at 37 °C using an apoptosis detection kit (Takara Biochemicals). The sections were stained with diaminobenzine and hematoxylin, and mounted for light microscopic observations.

Statistics

Differences within groups were compared by the one-way ANOVA and Dunnett’s t test. The accepted level of significance was $P<.05$. 
RESULTS

Generation of TG mice

The carboxyl-terminal region of gp130 containing box3 is considered to play a critical role in gp130-mediated biological responses (26). D.N.gp130 TG mice were generated by overexpression of a box3-deleted form of gp130 under the control of αMHC promoter (Fig. 1). Six founders containing the transgene were identified, and three transgenic lines, in particular #3, were used in this study. TG mice were apparently healthy and fertile, and there was no difference in the heart weight to body weight ratio between TG mice and WT mice, at baseline (Fig. 2).

Cardiac hypertrophy induced by pressure overload

Constriction of the abdominal aorta is a established method to produce pressure overload-induced cardiac hypertrophy (21, 22). Four weeks after the operation, all mice were healthy, and the blood pressure monitored at right carotid artery was elevated as reported before in our laboratory (22) without any differences between TG mice and WT mice (data not shown). Echocardiography at 4 weeks after the operation revealed that left ventricular wall thickness was markedly increased (IVST, sham 0.50±0.03 vs constriction 0.81±0.03; LVPWT, sham 0.54±0.04 vs constriction 0.83±0.08) in WT mice, however, TG mice showed less increase in left ventricular wall thickness (IVST, sham 0.48±0.03 vs constriction 0.59±0.00; LVPWT, sham 0.52±0.00 vs constriction 0.64±0.04) (TABLE 1). Furthermore, constriction of the abdominal aorta for 4 weeks increased the heart weight/body weight ratio by 40±4% in WT mice and 15±3% in TG mice (Fig. 2). The left ventricle of WT mice showed more marked hypertrophy than of TG mice (Fig. 3A). Microscopic analysis showed that cross sectional areas of cardiac myocytes of WT mice were more enlarged by pressure overload than those of TG mice (Fig. 3B). These results suggest that cardiac hypertrophy induced by pressure overload was attenuated in D.N.gp130 TG mice.
Expression of cardiac specific genes

Pressure overload by the constriction of the abdominal aorta upregulates fetal-type cardiac genes and downregulates SERCA2 gene (27). We therefore examined the expression of BNP and SERCA2 in the hearts of WT and TG mice at the early and late phase after constriction of the abdominal aorta. BNP gene was slightly upregulated at 2 days after pressure overload and markedly at 28 days in WT mice (Fig. 4). In TG mice, although BNP gene was also upregulated by pressure overload, the expression levels were quite low compared with those of WT mice. In WT mice, SERCA2 gene was downregulated from 2 days by pressure overload (Fig. 4). The downregulation of SERCA2 gene by pressure overload was also attenuated in TG mice.

Activation of STAT3 and ERKs

Activation of gp130 evokes two distinct pathways, JAK-STAT pathway and Ras-ERKs pathway (28). We examined which pathway is important in the pressure overload-induced cardiac hypertrophy (Fig. 5). Pressure overload activated both STAT3 and ERKs in the heart of WT mice. However, in TG mice, activation of STAT3 was barely detectable. In contrast, there was no difference in activation of ERKs between TG and WT mice. These results collectively suggest that pressure overload-induced activation of STAT3, but not of ERKs, is dependent on gp130 and that STAT3 may play a critical role in pressure overload-induced cardiac hypertrophy.

No increase in a number of TUNEL-positive cardiomyocytes

It has been reported that activation of gp130 promotes survival of cardiac myocytes (17) and that ventricular restricted gp130 knockout mice showed marked cardiomyocyte apoptosis and marked ventricular wall dilatation by pressure overload (18). We therefore examined whether TG mice showed cardiomyocyte apoptosis during pressure overload. As shown in Fig. 6, there was no increase in the number of TUNEL-positive cardiomyocytes in the heart of TG mice at the basal and by pressure overload compared with those of WT mice.
DISCUSSION

It has been reported that gp130 is implicated in regulating cell growth, differentiation and cell death in response to external stimuli in various tissues. In the present study, the TG mice were apparently healthy with no cardiac abnormalities at basal condition, suggesting that gp130 is not necessary for the development and the physiological function of the heart after birth. In contrast, gp130 plays a critical role in pressure overload-induced cardiac hypertrophy. By constriction of the abdominal aorta, TG mice showed less increase in the heart weight/body weight ratio and less changes in expression of BNP and SERCA2 genes compared with WT mice. We used #3 line of transgenic mice, which expressed D.N.gp130 most abundantly. Mice of #1 and #2 lines also showed similar results with mild degree (data not shown). These results suggest that D.N.gp130 dose-dependently suppresses cardiac hypertrophy.

The intracellular signaling pathways evoked by gp130 activation include the JAK-induced STAT pathway and the Ras-ERKs pathway (28). Activated STAT3 has been reported to form a homodimer which subsequently forms cis-inducing factor complexes and induces hypertrophy of cardiac myocytes (29). It has been reported that STAT3 plays a critical role in generating the hypertrophic signal (30), and that mice overexpressing STAT3 showed marked cardiac hypertrophy (31). In the present study, pressure overload activated ERKs and STAT3 in the heart of WT mice, whereas pressure overload-induced activation of STAT3, but not of ERKs, was suppressed in TG mice. These results suggest that pressure overload-induced activation of STAT3, but not of ERKs, is dependent on gp130 and that STAT3 may play a critical role in pressure overload-induced cardiac hypertrophy.

Hirota et al. have reported that ventricular restricted knockout mice of gp130 showed dilatation of left ventricular wall and died within a week by constriction of transverse aorta without progression of adaptive hypertrophy of left ventricle (18). Although cardiac hypertrophy was attenuated, there was no sign of heart failure such as dilatation of left ventricular wall and reduced cardiac function nor death by constriction of the abdominal aorta
in the D.N.gp130 TG mice. There are several possibilities for this discrepancy. First, the methods applied to produce the pressure overload were different. Transverse aortic constriction, applied in their experiment, induces stronger pressure overload than constriction of the abdominal aorta. It is possible that if the pressure overload is too strong, heart failure but not adaptive cardiac hypertrophy would be induced. To determine the role of gp130 in pressure overload-induced cardiac hypertrophy, we used mild pressure overload model of abdominal aortic constriction. Second, they used gp130 null mice, while we used D.N.gp130-overexpressing mice. Since pressure overload-induced activation of STAT3 was abolished in the heart of D.N.gp130 TG mice, endogenous gp130 activity is thought to be strongly suppressed by overexpression of D.N.gp130. However, there is a possibility that the two mice have different gp130 activity. The transgene lacks box3 but has box1 and box2 regions of the cytoplasmic domain of gp130. Although box3 is indispensable for the JAK-induced activation of STAT3 (26), there is a possibility that box1 and box2 have some biological functions. There is a possibility that the two mice have different ERK activities, which they did not examine (18). It has been reported that the ERK signaling pathway is important for the gp130-dependent cell survival of cardiac myocytes (17) and that tyrosine-containing motif, Y116XXV, of the cytoplasmic domain of gp130 is indispensable for the activation of SHP-2, a key molecule for gp130-mediated signaling pathway leading to ERKs (26, 32). Although the transgene lacks this motif, pressure overload induced activation of ERKs also in TG mice. These results suggest that some other factors such as angiotensin II and endothelin-1, which exert their effects through G protein coupled receptors, play a predominant role in the activation of ERKs and cardiomyocyte survival in the TG mice. Although it remains to be determined how pressure overload induces production of the IL6 family of cytokines in the heart, the present study suggests that gp130 plays a critical role in pressure overload-induced cardiac hypertrophy possibly through the STAT3 pathway.
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FIGURE LEGENDS

Fig. 1. Diagram of the transgene construct and expression in the heart. A, Schematic structure of the αMHC promoter-D.N.gp130-polyA transgene construct. A 2.4 kb of truncated murine gp130 cDNA was subcloned between the murine αMHC promoter and polyA. The truncated form of gp130 contains the membrane proximal 63-aa part of the cytoplasmic region of gp130 but lacks box3. B, Expression of the truncated form of gp130. 10 µg of total RNA from the hearts were hybridized with labeled gp130 cDNA.

Fig. 2. Heart weight/body weight ratio. Pressure overload was produced by constriction of the abdominal aorta for 4 weeks. Mean±SE of the heart weight/body weight ratio was shown (n=3 in sham operated mice, n=4 in constricted mice). *P<.05; N.S., not significant.

Fig. 3. Histological analysis. A, Transverse sections of the hearts at papillary muscle level at 4 weeks after constriction of the abdominal aorta. Data are representative of three or four independent experiments with nearly identical results. B, Cross sectional areas of cardiac myocytes from 10 sections. Normalized values in WT mice without constriction are arbitrarily expressed as 1.0. *P<.05.

Fig. 4. Expression of cardiac genes. Hearts were excised from WT and TG mice at 2 and 28 days after constriction of the abdominal aorta. Expression of cardiac specific genes were examined by Northern blot analysis. A, A representative autoradiogram was shown. Ethidium bromide staining of 18S RNA was shown below. B, The band intensity of BNP, SERCA2 and 18S RNA were quantified by densitometer. Each histogram represents the fold of BNP and SERCA2 compared with 18S RNA from three independent experiments (mean ± S.E.). *P<.05 vs control.
FIG. 5. Activation of ERKs and STAT3 by pressure overload. The abdominal aorta
was constricted for indicated periods of time, and the activities of ERKs and STAT3 were
measured as described in "Materials and Methods." Each histogram represents the fold of
controls from three independent experiments (mean ± S.E.). *P<.05.

FIG. 6. TUNEL assay of the heart. The abdominal aorta was constricted for 4 weeks,
and cardiomyocyte apoptosis was examined by TUNEL as described in "Materials and
Methods". 500 cardiomyocytes were counted from each sides. Each histogram represents the
number of TUNEL-positive cells from five independent experiments (mean ± S.E.).
Pretreatment with micrococcal DNase I (1 mg/ml) to induce DNA strand breaks was carried
out as positive controls.
**TABLE 1**

|                | WT mice      | TG mice      |
|----------------|--------------|--------------|
|                | sham (n=3)   | constriction (n=4) | sham (n=3) | constriction (n=4) |
| LVEDD (mm)     | 2.87±0.04    | 3.06±0.20    | 2.93±0.01  | 2.97±0.04           |
| LVESD (mm)     | 1.56±0.05    | 1.53±0.08    | 1.53±0.08  | 1.42±0.03           |
| IVST (mm)      | 0.50±0.03    | 0.81±0.03*   | 0.48±0.03  | 0.59±0.00†          |
| LVPWT (mm)     | 0.54±0.04    | 0.83±0.08*   | 0.52±0.00  | 0.64±0.04†          |
| FS (%)         | 46±3         | 50±3         | 48±4       | 52±1                |
| EF             | 0.84±0.03    | 0.87±0.03    | 0.86±0.03  | 0.89±0.01           |

Analysis of cardiac size and function. LVEDD, left ventricular end-diastolic internal diameter; LVESD, left ventricular end-systolic internal diameter; IVST, intraventricular septum thickness; LVPWT, left ventricular posterior wall thickness; FS, fractional shortening; EF, ejection fraction. *P<.05 vs WT mice without constriction. †P<.05 TG mice after constriction vs WT mice after constriction.
Heart weight (mg)/Body weight (g) ratio

- WT mice
- TG mice
- Sham
- Constriction

N.S. - Not Significant

* Significant difference
A 28 days after constriction
WT mice  TG mice

B

Cross sectional areas of cardiac myocytes

|               | sham | constriction | sham | constriction |
|---------------|------|--------------|------|--------------|
| WT mice       | 1.0  | 1.4          | 1.2  | 1.3          |
| TG mice       | 1.0  | 1.4          | 1.2  | 1.3          |

* indicates significance
A

| Time after constriction | WT mice | TG mice |
|-------------------------|---------|---------|
| 0                      |         |         |
| 2                      |         |         |
| 28 day                 |         |         |

**BNP**

**SERCA2**

**18S**

B

| Time after constriction | WT mice | TG mice |
|-------------------------|---------|---------|
| 0                      |         |         |
| 2                      |         |         |
| 28 day                 |         |         |

\[ \frac{BNP}{18S} \]

\[ \frac{SERCA2}{18S} \]
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