Pressure-Mediated Hypertrophy and Mechanical Stretch Induces IL-1 Release and Subsequent IGF-1 Generation to Maintain Compensative Hypertrophy by Affecting Akt and JNK Pathways

Shoken Honsho,* Susumu Nishikawa,* Katsuya Amano, Kan Zen, Yasushi Adachi, Eigo Kishita, Akihiro Matsui, Asako Katsume, Shinichiro Yamaguchi, Kenichiro Nishikawa, Kikuo Isoda, David W.H. Riches, Satoaki Matoba, Mitsuhiko Okigaki, Hiroaki Matsubara

Rationale: It has been reported that interleukin (IL)-1 is associated with pathological cardiac remodeling and LV dilatation, whereas IL-1β has also been shown to induce cardiomyocyte hypertrophy. Thus, the role of IL-1 in the heart remains to be determined.

Objective: We studied the role of hypertrophy signal-mediated IL-1β/insulin-like growth factor (IGF)-1 production in regulating the progression from compensatory pressure-mediated hypertrophy to heart failure.

Methods and Results: Pressure overload was performed by aortic banding in IL-1β-deficient mice. Primarily cultured cardiac fibroblasts (CFs) and cardiac myocytes (CMs) were exposed to cyclic stretch. Heart weight, myocyte size, and left ventricular ejection fraction were significantly lower in IL-1β-deficient mice (20%, 23% and 27%, respectively) than in the wild type 30 days after aortic banding, whereas interstitial fibrosis was markedly augmented. DNA microarray analysis revealed that IGF-1 mRNA level was markedly (~50%) decreased in the IL-1β-deficient hypertrophied heart. Stretch of CFs, rather than CMs, abundantly induced the generation of IL-1β and IGF-1, whereas such IGF-1 induction was markedly decreased in IL-1β-deficient CFs. IL-1β released by stretch is at a low level unable to induce IL-6 but sufficient to stimulate IGF-1 production. Promoter analysis showed that stretch-mediated IL-1β activates JAK/STAT to transcriptionally regulate the IGF-1 gene. IL-1β deficiency markedly increased c-Jun N-terminal kinase (JNK) and caspase-3 activities and enhanced myocyte apoptosis and fibrosis, whereas replacement of IGF-1 or JNK inhibitor restored them.

Conclusions: We demonstrate for the first time that pressure-mediated hypertrophy and mechanical stretch generates a subinflammatory low level of IL-1β, which constitutively causes IGF-1 production to maintain adaptable compensation hypertrophy and inhibit interstitial fibrosis. (Circ Res. 2009;105:1149-1158.)

Key Words: interleukin-1 □ insulin-like growth factor-1 □ Akt □ JNK □ hypertrophy

Cardiac hypertrophy is defined by augmentation of the ventricular mass against hemodynamic loads and up-regulates contractile capacity and reduces ventricular wall stress, whereas the capacity of this compensation is limited, and stronger and longer pressure overload induces pathological cardiac remodeling with left ventricular (LV) dilatation. Pathological cardiac remodeling is associated with production of the extracellular matrix and causes increased signals of myocyte apoptosis. Receptor tyrosine kinase, such as insulin-like growth factor (IGF)-1 receptor is involved in not only physiological hypertrophy but also compensated hypertrophy after pressure overload. IGF-1 promotes myocardial hypertrophy by activating phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt. In addition, mitogen-activated protein kinase (MAPK) acts as downstream molecules to promote hypertrophy. Overexpression of G protein–coupled 7-transmembrane receptors in the heart induced cardiac remodeling, resulting in heart failure with increased propensity toward apoptosis and fibrosis. Stress-activated mitogen-activated protein kinase, JNK (c-Jun N-terminal kinase), was reported to transmit this...
action and transgenic mice of JNK induced cardiac fibrosis and apoptosis. JNK inhibited NFATc3 (nuclear translocation of the transcription factor), which is involved in myocardial hypertrophy, leading to impaired LV hypertrophy. Thus, the balance between the IGF-1/Akt system and JNK activation seems to be important to determine the fate of pressure-overloaded heart, ie, compensated hypertrophy or pathological cardiac remodeling; however, the interaction between these pathways has not been fully clarified.

Numerous data demonstrated that a proinflammatory cytokine, interleukin (IL)-1, is associated with inhibition of IGF-1 production and IGF-1-mediated protein synthesis. IL-1β has a negative inotropic effect on the heart and produces extracellular matrix to promote pathological cardiac remodeling and LV dilatation. There are reports showing that IL-1β promotes myocyte hypertrophy and that cardiac-targeted IL-1α overexpression mice exhibit concentric LV hypertrophy with preserved LV systolic function. Thus, the role of IL-1β in the heart remains to be determined.

In this study, we found that compensated LV hypertrophy during pressure overload was attenuated in IL-1β–deficient mice. Further analysis revealed that a low level of IL-1β is constitutively produced by mechanical stretch of cardiac fibroblasts (CFs), as well as cardiac myocytes (CMs). Unexpectedly, DNA microarray and gene promoter analysis showed that the stretch-mediated IL-1β release promotes IGF synthesis transcriptionally through the JAK2/STAT5 (Janus kinase 2/signal transducer and activator of transcription 5) pathway. IGF-1 inhibited stretch-induced JNK activation, myocyte apoptosis, or interstitial fibrosis and stimulated Akt-mediated signals, contributing to compensated LV hypertrophy in the initial phase after pressure overload.

## Methods
Materials and Methods for animal models, transthoracic echocardiography, pathology, Western Blotting, cell culture, mechanical stretch, microarray analysis, RNA isolation and real-time polymerase chain reaction (PCR), Luciferase assay, small interfering RNA knockdown of IL-1α, immunostaining, quantifications for IL-1β and IGF-1 protein, oxidative stress, apoptosis, and data analysis are described in the Online Data Supplement.

## Results
### IL-1β Maintains Pressure-Mediated LV Hypertrophy
LV hypertrophy was induced by aortic banding. The ratio of heart weight/body weight ratio (mg/g) and the myocyte size in a cross-sectional area were significantly smaller (20.2% and 23.3%, respectively, at day 30) in IL-1β–deficient mice compared with the wild type (WT) (Figure 1A), whereas the ratio of lung weight/body weight was similar between WT and IL-1β–deficient mice (6.8±0.3 versus 6.1±0.1 mg/g). Echocardiographic evaluation showed that the LV diastolic diameters in IL-1β–deficient mice at day 30 and day 60 were significantly larger (19.5% and 25.1% versus WT, respectively) and percentage fractional shortening (%FS) was lower (17.4% and 26.5%) (Figure 1B), although these baseline parameters were similar between groups.

Posterior and anterior wall diameters at day 60 were ~38% smaller in IL-1β–deficient LV than the WT (posterior wall diameter: 0.8±0.04 versus 1.1±0.08 mm; anterior wall diameter: 0.8±0.06 versus 1.1±0.07 mm; each P<0.05), although there was no significant difference in the baseline (0.7±0.04 mm in both groups). The interstitial fibrosis in IL-1β–deficient LV was markedly increased (3.1-fold at day 60) compared with the WT (Figure 1C). Atrial natriuretic peptide and β-myosin heavy chain mRNA levels were increased in hypertrophied hearts of WT (3.7- and 3.5-fold, respectively), whereas, in IL-1β–deficient mice, mRNA levels were increased to a significantly less extent (38.2% and 32.2% versus WT) (Figure 1D).

### Hypertrophy Stimulus Causes IGF-1 Production via IL-1β Release
To identify the molecules involved in IL-1β–mediated cardiac hypertrophy, we analyzed the transcripts from LV samples 3 days after aortic banding using a DNA oligonucleotide microarray approach. Interestingly, we found that IGF-1 was one of the molecules in which the mRNA level was markedly (~38%) reduced in IL-1β–deficient mice. Indeed, the baseline mRNA level of IGF-1 was 51% lower in IL-1β–deficient LV. Pressure overload induced a 1.9-fold increase in IGF-1 mRNA in WT left ventricles (day 14 versus baseline), whereas, in IL-1β–deficient mice, the increase was markedly attenuated (Figure 2A, left). Cardiac IGF-1 protein levels at day 0 (baseline), day 14 and day 60 after aortic banding were ~60% lower in IL-1β–deficient mice compared with the WT. When Histidine (His)-tagged IGF-1 (50 μg/kg per day, once per day) was subcutaneously injected into IL-1β–deficient mice after aortic banding, we found that His-IGF-1 levels increased to the plateau at day 14 and total (exogenous His-IGF-1 plus endogenous) IGF-1 amounts were elevated to the levels similar to the WT (Figure 2A, middle). Immunostaining revealed that IGF-1 protein is expressed in both myocytes and interstitial cells, the level of which appeared to be lower in IL-1β–deficient mice (Figure 2A, right). Phosphorylation levels of cardiac IGF-1 receptor were significantly lower (55% to 50% versus WT) in IL-1β–

### Non-standard Abbreviations and Acronyms
- **CF**: cardiac fibroblast
- **CM**: cardiomyocyte
- **ERK**: extracellular signal-regulated kinase
- **FS**: fractional shortening
- **IGF**: insulin-like growth factor
- **IL**: interleukin
- **JAK**: Janus kinase
- **JNK**: c-Jun N-terminal kinase
- **LV**: left ventricular
- **PCR**: polymerase chain reaction
- **PI3K**: phosphatidylinositol 3-kinase
- **ROS**: reactive oxygen species
- **STAT5**: signal transducer and activator of transcription 5
- **TNF**: tumor necrosis factor
- **WT**: wild type

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deficient mice after aortic banding, and treatment with 50 μg/kg per day of IGF-1 for 14 days normalized the decreased phosphorylation, whereas more than 250 μg/kg per day of IGF-1 caused the superphosphorylation over the normal level (Figure 2C).

We further studied whether exogenous IGF-1 reverses cardiac phenotype in IL-1β-deficient mice. Subcutaneous injection of IGF-1 (50 μg/kg per day, once per day) for 14 days restored the phosphorylation level of cardiac IGF-1 receptor to the WT level, whereas more than 250 μg/kg per day of IGF-1 caused the superphosphorylation over the normal level (Figure 2C).

To study the involvement of IGF-1 in the hypertrophy process, we examined whether cyclic stretch (60 cycle/min at 12% extension) can affect IGF-1 expression. As we found that stretch-mediated IGF-1 generation is much greater in CFs than CMs (Figure 2D), the primarily cultured CFs was used in the following stretch experiment. Stretch induced IGF-1 mRNA accumulation (4.1-fold versus baseline) and IGF-1 protein synthesis (3.6-fold) in WT CFs, and this increase in IGF-1 protein level was abolished by anti–IL-1β neutralization antibody, whereas in IL-1β-deficient CFs, the stretch-mediated increase was markedly lower (49% versus WT CFs) (Figure 2D). Stretch caused a phosphorylation of

Figure 1. Impaired compensative LV hypertrophy in IL-1β-deficient mice. Abdominal aorta of WT or IL-1β-deficient mice (KO) was banded (day 0), and thereafter mice were subcutaneously injected with or without IGF-1 (50 μg/kg per day, once per day for 14 days). A, Hearts were removed and the ratio of heart weight/body weight ratio (Hw/Bw) (mg/g) was evaluated. Paraffin-sectioned left ventricles were stained with hematoxylin/eosin (H-E), and cross-sectional areas of cardiomyocytes were evaluated. *P<0.05, **P<0.01 vs WT (n=15). B, Echocardiography. Dd indicates end-diastolic diameter; Ds, end-systolic diameter. %FS was calculated as described in the Online Data Supplement. *P<0.05, **P<0.01 vs WT (n=10). C, Paraffin-sectioned left ventricles were stained with Masson’s trichrome to evaluate the fibrotic area (arrows). *P<0.01 vs WT (n=7). D, Total RNAs were extracted from left ventricles. Atrial natriuretic peptide (ANP), α-myosin heavy chain (αMHC), β-myosin heavy chain (βMHC), and GAPDH mRNA levels were evaluated by quantitative RT-PCR. *P<0.05, **P<0.01 vs baseline (day 0) (n=5).
IGF-1 receptor, the extent of which was significantly greater in WT CFs than IL-1β–deficient CFs (Figure 2E).

IL-1β Is Produced by Stretch to Stimulate IGF-1 Generation

We next studied whether IL-1β expression is actually induced by aortic banding or cyclic stretch. IL-1β mRNA was expressed in LV and the level was increased 2.0-fold after aortic banding, and immunohistochemistry revealed that IL-1β protein is detectable in both myocytes and interstitial cells (Figure 3A). Stretch also induced IL-1β mRNA accumulation in both CFs and CMs in parallel with percentage extension and duration of stretch, whereas the amount of IL-1β mRNA produced by CFs was 2.2-fold higher than CMs (Figure 3B). The significant induction of IL-1β mRNA was observed 3 hours after stretch and the level reached to a peak around 6 hours (Figure 3B, right).

We also measured the concentration of IL-1β released by stretch and found that a substantial amount of IL-1β (≈1.2 ng/mL) was detected in the condition medium derived from 24-hour stretched CFs, the level of which was much lower than that in the condition medium (≈5.1 ng/mL) from a sepsis-associated inflammatory cytokine tumor necrosis factor (TNFα)-treated (1 ng/mL, 24-hour exposure) CFs (Figure 3C). Because plasma TNFα was shown to increase to ≈1 ng/mL in patients with sepsis,20 we examined the effects of 1 and 10 ng/mL TNFα in this experiment (Figure 3C). We found that incubation medium containing IL-1β released by stretch barely induced an inflammatory cytokine IL-6 expression from CFs, whereas the condition medium from TNFα-treated (1 ng/mL) CFs abundantly stimulated IL-6 synthesis and this action was abolished by neutralization with anti–IL-1β antibody (Figure 3D).

We further studied whether IL-1β actually stimulates IGF-1. IGF-1 synthesis in WT CFs and WT CMs was maximally induced by 10 ng/mL IL-1β, but downregulated with 20 ng/mL IL-1β, suggesting that CFs and CMs were
responsive to IL-1β in a similar dose-responsive manner (Figure 3E). IL-1β-mediated (10 ng/mL) induction of IGF-1 mRNA was much greater (>2-fold) in WT CFs compared with WT CMs and no induction was observed in endothelial cells (Figure 3E). IL-1β (10 ng/mL) stimulated the release of IGF-1 protein with a peak at 6-hour exposure and thereafter decreased, whereas stretch-mediated IGF-1 protein release was sustained over 72 hours (Figure 3F). CFs produced 1.2 ng/mL IL-1β under stretch (Figure 3C), whereas nonstretched CFs did not respond to 1 ng/mL IL-1β and required >5 ng/mL IL-1β to induce a significant increase in IGF-1 mRNA (Figure 3E), suggesting that there is a differential sensitivity to IL-1β between stretched and nonstretched CFs and mechanical stretch elevates the sensitivity to IL-1β. We therefore stimulated the nonstretched CFs with 10 ng/mL IL-1β in the following experiments.

We also examined an involvement of IL-1α in IL-1β deficiency. After 24-hour stretch of IL-1β-deficient CFs, IL-1α mRNA levels were increased 1.8-fold compared with WT CFs (Figure 3G). Knockdown of IL-1α by small interfering RNA in IL-1β-deficient CFs completely abolished the stretch-induced increase in IGF-1 mRNA (Figure 3G), indicating that IL-1α partially compensates for IGF-1 induction in IL-1β deficiency.

**Reactive Oxygen Species Is Needed for Stretch-Mediated IL-1β Induction**

Reactive oxygen species (ROS) has been shown to be involved in stretch-mediated cytokine induction in the heart. Intracellular ROS levels evaluated by the production of 2′,7′-dichlorofluorescein (DCF) were markedly increased (4.2-fold versus nonstretched baseline) after 24-hour stretch
of WT CFs (Figure 4A, left). A low dose of diethyldithiocarbamate (1 μmol/L), which increases the intracellular ROS level by inhibiting Cu/Zn-superoxide dismutase, augmented stretch-mediated IL-1β mRNA levels (2.0-fold versus control), whereas 1 to 10 μmol/L diethyldithiocarbamate without stretch did not affect these levels and scavenging ROS by Tempol (1 mmol/L) markedly reduced stretch-mediated IL-1β mRNA accumulation (58% versus control) (Figure 4B, right). These findings indicate that intracellular ROS is closely involved in stretch-mediated IL-1β production.

**IL-1β Induces IGF-1 Through JAK2/STAT5 Pathway**

IGF-1 release by growth hormone is reportedly activated by the JAK2/STAT5 pathway. Stimulation of WT CFs with IL-1β significantly induced the phosphorylation of JAK2 and STAT5 by 2.1- and 2.3-fold, respectively, and JAK inhibitor (AG490, 10 μmol/L) abolished the phosphorylation of STAT5 (Figure 4C). We also found that the stretch-mediated induction of IL-1β and IGF-1 was abolished by pretreatment of Tempol and AG490 (data not shown), suggesting that the induction of IL-1β/IGF-1 by mechanical stretch is regulated with the same signaling between CFs and CMs.

Transcription of IGF-I is positively regulated by the 5′ noncoding region flanking IGF-1 exon 1, and STAT5-binding sites are identified in this region. To study the role of STAT5 in IL-1-mediated IGF-1 transcription, a luciferase reporter gene construct containing mouse IGF-1 gene promoter and exon 1 (1711 to 329) was transfected into WT CFs with or without STAT5 decoy or scramble oligonucleotides. Stimulation of WT CFs with IL-1β for 6 hours induced luciferase activity (Figure 4D).
transcriptional activity (3.2-fold versus baseline), whereas JAK inhibitor and STAT5 decoy abolished this IL-1β-mediated action (Figure 4D).

Furthermore, stretch-induced phosphorylations of JAK2 and STAT5 was severely inhibited in IL-1β-deficient CFs (JAK2, 51.2%; STAT5, 59.3% reduction versus WT CFs at 6-hour stretch) or the addition of neutralizing anti–IL-1β antibody (JAK2, 49.7%; STAT5, 52.4% reduction) (Figure 4E).

**IL-1α Receptor Signaling Augments IGF-1 Synthesis and LV Hypertrophy**

We further studied the hypertrophy-mediated IGF-1 expression using CM-specific IL-1α overexpression transgenic mice. Although cardiac size and function in IL-1α transgenic mice at baseline did not differ from the WT, the heart/body weight ratio and myocyte area were more increased (P<0.05 versus WT) 60 days after aortic banding. %FS was 18% better (P<0.05 versus WT), and LV dilatation was reduced 14% (Online Figure I, A and B). IGF-1 mRNA levels in LV were significantly increased at the baseline and after aortic banding (2.2-fold at day 7) (Online Figure I, C), and Tyr1135/1136 phosphorylation levels of IGF-1 receptor were also elevated (2.3-fold) (Online Figure I, D). Considering that IL-1α binds to the receptor in common with IL-1β, these findings suggest that hypertrophy stimuli and IL-1 signals are closely involved. Therefore, we next examined the effect of IGF-1 on the Akt/Erk pathway using the CMs. Phosphorylation levels of Akt and ERK after aortic banding and stretch stimuli were markedly decreased in IL-1β-deficient LV (59% and 79% at day 14, respectively) and IL-1β-deficient CMs (62% and 65% at 6 hours of stretch, respectively), whereas addition of IGF-1 normalized the decrease (Online Figure II, A and B). Treatment of WT CMs with anti–IL-1β antibody significantly diminished the phosphorylation levels of ERK and Akt-1 (48% and 53%, respectively) (Online Figure II, B). Thus, cardiac Akt and ERK activities in response to hypertrophy stimulus are attenuated in IL-1β-deficient mice, in which the lack of IGF-1 attributable to IL-1β deficiency is closely involved.

**IL-1β Deficiency Activates JNK**

JNK is closely involved in the development of myocyte apoptosis and cardiac fibrosis. Basal JNK activities in CFs increased to the peak level 1 hour after stretch and, thereafter, decreased to the baseline, whereas the level was higher and sustained for 6 hours in IL-1β-deficient CFs, and this induction was abolished by addition of IGF-1 or IL-1β or Tempol (Figure 5B). Consistent with these findings, subcutaneous injection of JNK inhibitor (SP600125, 30 mg/kg per day) to IL-1β-deficient mice after aortic banding showed that JNK inhibitor restored cardiac dysfunction, such as LV dilatation and decreased %FS, to the WT levels at day 60 (Figure 1B), accompanied by normalization of heart/body weight ratio and myocyte area (Figure 1A).

**IL-1β-Mediated IGF-1 Generation and Cardiac Akt and ERK Activities**

IGF-1 is known to promote LV hypertrophy through the extracellular signal-regulated kinase (ERK) or PI3K/Akt-1 pathway. CFs is a main source for IL-1β-mediated IGF-1 generation, which has an effect on CMs in a paracrine fashion. Therefore, we examined the effect of IGF-1 on the Akt/Erk pathway using the CMs. Phosphorylation levels of Akt and ERK after aortic banding and stretch stimuli were markedly decreased in IL-1β-deficient LV (59% and 79% at day 14, respectively) and IL-1β-deficient CMs (62% and 65% at 6 hours of stretch, respectively), whereas addition of IGF-1 normalized the decrease (Online Figure II, A and B). Treatment of WT CMs with anti–IL-1β antibody significantly diminished the phosphorylation levels of ERK and Akt-1 (48% and 53%, respectively) (Online Figure II, B). Thus, cardiac Akt and ERK activities in response to hypertrophy stimulus are attenuated in IL-1β-deficient mice, in which the lack of IGF-1 attributable to IL-1β deficiency is closely involved.

**IL-1β Deficiency Causes JNK-Mediated Myocyte Apoptosis and Fibrosis**

Inhibition of PI3K/Akt-1 and activation of JNK in the heart induces myocyte apoptosis and fibrosis. The number of TUNEL-positive cells in IL-1β-deficient LV was 3.1-fold greater (versus WT left ventricles) 14 days after aortic banding, and the subcutaneous administration of IGF-1 or JNK inhibitor (SP600125) normalized them (Figure 6A). JNK activates the mitochondrial apoptosis pathway through the caspase cascade. Total and cleaved caspase-3 levels in IL-1β-deficient LV were markedly increased 7.2-fold and 2.7-fold 14 days after aortic banding, respectively, and the increase was abolished by administration of IGF-1 (Figure 6B).

**Discussion**

The present study demonstrates that pressure-mediated hypertrophy and mechanical stretch induce a low level of IL-1β...
generation from both CFs and CMs to efficiently stimulate the synthesis and release of IGF-1 in an autocrine and/or paracrine fashion, leading to Akt-mediated compensative hypertrophic response of myocytes, as well as antiapoptotic and antifibrosis effects. Furthermore, we found that stretch-mediated ROS generation stimulated IL-1β generation and that IL-1β–mediated JAK/STAT activation transcriptionally induced IGF-I gene expression. Simultaneously, stretch stimulus markedly activated JNK, and IGF-1 generated by stretch negatively regulated JNK signals, resulting in the prevention of myocyte apoptosis and interstitial fibrosis, suggesting that the balance between hypertrophy stimulus-induced IL-1β/IGF-1 and subsequent JNK activation plays a key role to regulate the progression from adaptable compensation hypertrophy to heart failure (Figure 7).

IL-1β generated by sepsis or tissue inflammation has been reported to inhibit IGF-1 production. What is the mechanism regulating the dual effect of IL-1β on IGF-1 generation? In this study, a physiological range of IL-1β generated by hypertrophic stimulus that caused IGF-1 synthesis was defined to be a low level of IL-1β (≈1 ng/mL), and a much higher level (over 10 fold) of IL-1β, such as generated by tissue inflammation, that negatively regulated IGF-1 synthesis (Figure 3E) was defined to be a high level of IL-1. This low level of IL-1β was unable to induce proinflammatory cytokine IL-6 (Figure 3C and 3D), the amount of which can actively stimulate IGF-1 production sufficient to promote myocyte hypertrophy during pressure overload, as well as negatively regulate JNK activation. The study using STAT5 knockout mice indicated that STAT5 was required for both basal and growth hormone–induced expression of hepatic IGF-1. We found that JAK2/STAT5 system is involved in IL-1β–mediated IGF-1 induction (Figure 4), whereas the higher level of IL-1β induced by endotoxin in sepsis was reported to rather inhibit STAT5-mediated IGF-1 production.
through the activation of JNK. The present study demonstrates that an appropriate level of ROS generation also plays an important role for stretch-mediated IL-1β production (Figure 4B).

IL-1β deficiency causes a decrease in IGF-1 synthesis, leading to reduction in pressure-induced LV hypertrophy associated with myocyte apoptosis and interstitial fibrosis (Figure 1) and JNK hyperactivity (Figure 5), whereas JNK inhibition restored cardiac function by decreasing cardiac apoptosis and fibrosis (Figure 6). Thus, JNK hyperactivity is closely involved in cardiac phenotype of IL-1β-deficient mice. The magnitude and duration of JNK activity are determined by the balance between activating kinases and inhibitory phosphatases for JNK. Catalytic cysteine of phosphatase is sensitive to oxidation. JNK phosphatase oxidation by ROS converts the catalytic cysteine to sulfenic acid and inhibits its activity, leading to sustained JNK activation. We here showed that pulsating stretch induced ROS (Figure 4A), and ROS scavenger inhibits JNK activation (Figure 5), suggesting that ROS activates JNK in the heart. Considering that cardiac IGF-1 levels in IL-1β-deficient mice are >40% lower at baseline and after aortic banding than the WT (Figure 5), it is possible that IGF-1 prevents JNK hyperactivation under normal condition, as well as pressure-overload.

Which comes first to determine the cardiac phenotype in IL-1β-deficient mice, stimulation of JNK or downregulation of IL-1β? We examined how JNK is affected after IGF-1 treatment. The result showed that exogenous IGF-1 normalized cardiac JNK hyperactivation in IL-1β-deficient mice (Figure 5). It was reported that IGF-1 receptor/Akt signaling inhibits JNK pathway. Thus, it is considered that the downregulation of IL-1β/IGF-1 system is the upstream and subsequent hyperactivation of JNK is involved in the cardiac phenotype in IL-1β-deficient mice.

Calcineurin-dependent transcription factor, NFATc, promotes cardiac hypertrophy, and phosphorylation of NFATc by JNK inhibits its nuclear translocation and cardiac growth. We found that stretch induced nuclear translocation of NFATc4, which was attenuated in IL-1β-deficient mice and JNK inhibition restored nuclear translocation of NFATc4 (Figure 6D), suggesting that JNK hyperactivation is closely involved in inhibition of pressure-overloaded hypertrophy in IL-1β-deficient mice.

The mechanism of striking increase of total caspase-3 has not been fully clarified in this study. Receptor tyrosine kinase, such as IGF-1 receptor, and its downstream Akt downregulates X-linked inhibitor of apoptosis protein (XIAP), a ubiquitin–protein ligase, which promotes proteasomal degradation of caspase-3. Indeed, IGF-1 attenuates caspase-3 activation and inhibits myocyte apoptosis, suggesting that inhibition of IGF-1 receptor/Akt signaling in IL-1β-deficient mice leads to a decrease in degradation of caspase-3 followed by striking accumulation.

In conclusion, mechanical stretch constitutively induces a low level of IL-1β in the heart. This level of IL-1β is sufficient to induce IGF-1 production that negatively regulates JNK signals, affecting the progression of myocyte hypertrophy and subsequent transition from the compensated state to heart failure. The counterbalance of hypertrophy-induced IL-1/IGF-1 activation and the JNK pathway determines the fate of the pressure-overloaded heart: compensative hypertrophy or heart failure.

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Disclosures

None.

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Supplement Materials and Methods

Materials

IL-1β-/ mice were obtained from Dr. Sudo and Dr. Iwakura in University of Tokyo, Japan\(^1\). Cardiac specifically overexpressed mice of IL-1α were obtained from Dr. Ohsuzu in National Defense Medical College, Japan\(^2\).

Animal model

The descending aorta was banded in wild type (WT) and IL-1β-deficient male mice (C57Bl/6 strain, 10 weeks old, ~25g). Mice were anesthetized with intra-peritoneal injection of pentobarbital and a longitudinal skin incision was made on the left lateral side of the abdomen. The abdominal aorta was snared with a silk suture at the proximal region of the renal artery. A 27-gauge needle was then placed along the aorta, and the suture was tied snugly around the needle and the aorta. After ligation, the needle was quickly removed, the skin was closed, and the mice were allowed to recover. JNK inhibitor (SP60012530 mg/kg) was subcutaneously injected in PPCES buffer: 30% PEG400, 20% polypropylene glycol, 15% Chremophor EL, 5% ethanol, 30% saline. All
experimental protocols were approved by our university’s Animal Committee.

**Transthoracic echocardiography**

Urethane was injected intraperitoneally at 1g/kg of body weight for mild sedation and placed on controlled heating pads. Echocardiographic analysis was performed using a commercially available echocardiograph (SONOS 5500, Hewlett-Packard) equipped with a 15-MHz linear array ultrasound transducer. The left ventricle (LV) was assessed in the parasternal short-axis view. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV was obtained, respectively. Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured from the LV M-mode tracing (with a sweep speed of 50 mm/s) at the papillary muscle level. Percentage fractional shortening (%FS) was calculated using a standard formula: %FS = [(LVEDD - LVESD)/LVEDD] ×100.

**Pathology**

The whole heart was removed, weighed, and subsequently, fixed with 4% paraformaldehyde (4°C) for 12 hours. Paraffin-embedded samples were sliced (7 µm in
thickness). These sections were stained with hematoxylin-eosin to evaluate the area of cardiomyocyte. Also, LV sections were stained with Masson Trichrome. The lesions were examined in the microscopic fields (n=12) obtained by 300 μm intervals from 4 different LV sections, and the fibrotic lesions stained by blue were shown relative to the non-fibrotic lesions (n=12) using scion image software.

**Western blotting**

Lysates of myocardium, CM and CF were extracted with buffer containing 0.5% SDS, 1% NP-40, 0.1% deoxycholic acid, 1mmol/l DTT, 1mmol/l PMSF, 1mmol/l Na$_3$VO$_4$, 1μg/ml leupeptin and 1μg/ml aprotinin. Transferred membranes were incubated with primary antibodies against Caspase-3, Collagen type-I, α-tubulin, and IGF-1 (Santa Cruz Biotechnology), $^{202}$Thr/$^{204}$Tyr-phospho-ERK, ERK, $^{183}$Thr/$^{185}$Tyr-phosphorylated JNK, JNK-1, $^{180}$Thr/$^{182}$Tyr-phospho-p38-MAPK, p38-MAPK, $^{473}$Ser-phosphorylated Akt-1, Akt-1, $^{1135/1136}$Tyr-phosphorylated IGF-1-receptor, type I or IGF-1 receptor type I and His-tag (Cell Signaling Technology).

Relative densities in bands were analyzed by Adobe Photoshop.
Culture and condition medium

Hearts were removed from 1- to 2-day-old C57BL6 mice anesthetized by diethyl ether under aseptic conditions and placed in Ca$^{2+}$- and Mg$^{2+}$-free PBS. They were, then, washed with PBS, and the atria and aorta were discarded. The ventricles were minced and enzymatically digested with PBS containing 0.2% collagenase (Sigma type I). The liberated cells were collected by centrifugation and incubated in 100-mm culture dishes (Falcon) for 60 minutes at 37°C in a humidified incubator with 5% CO$_2$ air. Nonadherent cells were harvested as cardiomyocytes and seeded into culture dishes or stretch chambers. They were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 36~48 hours and used for all the experiments. Cells were characterized as CM by immunostaining using anti-α myosine heavy chain (αMHC) antibody (Santa Cruz). More than 90% of the cells were positive for αMHC. The adherent cells were cultured in the DMEM with 10% FBS. Cells were characterized as CF staining with anti-vimentin antibody (Dako Cytomation, Denmark). More than 90% of cells were positive for vimentin. Second passaged cells were used for experiments.

Condition media were prepared from CF (1x10$^6$ cells) exposed to cyclic stretch (60 cycle/min, 12% extension) for 24 hours in DMEM with 10% FBS or pre-incubated in
DMEM with 10% FBS and TNFα (1 ng/ml) for 24 hours and subsequently incubated in DMEM without TNFα for next 24 hours. These condition media were added to CFs to induce IL-6 mRNA.

**Mechanical stretch**

Cells were plated in a stretch chamber coated with fibronectin (STREX Corp., Japan) and cultured for 2 days. They were, then, incubated in starved medium (DMEM with 0.5% FBS) for 24 hours. Cells in the stretch chamber were subjected to a repetitive cyclic stretch with 60 cycles/minute and 4-12% prolongation.

**Microarray analysis**

Total RNA was isolated from LVs of WT and IL-1β-deficient mice 3 days after aortic banding using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany). RNA was processed and hybridized with DNA oligonucleotide Array (Microarrays Inc., TN, USA). Two independent experiments were performed, and gene expression was compared between WT and IL-1β-deficient samples. Genes were considered up-regulated/down-regulated if they showed a >2-fold change in two independent experiments. Only probe sets with a signal intensity of >100 were considered as
expressed.

**RNA isolation and real-time PCR**

Total RNAs were isolated using an RNA extraction kit (QIAGEN K.K., Tokyo, Japan) and cDNAs were generated with the SuperScript III First-Strand Synthesis System (Invitrogen). After initial denaturation at 95°C for 10 min, reactions were cycled 30 times, followed by annealing at 58°C and primer extension at 72°C for 30 sec. For real time PCR analysis, first strand cDNA was assessed by CYBR Green I real-time polymerase chain reaction (PCR) (Light Cycler, Roche). After initial denaturation at 95 °C for 10 min, reactions were cycled 40 times, annealing at 58°C and primer extension at 72°C for 10 sec. Oligonucleotide primer sequences were as follows. For mouse αMHC, forward: 5’-GTGCTGTACACCTCAAGG-3’, reverse: 5’-CGTTTTGTGTTTCAGTCTTC-3’ For mouse βMHC, forward: 5’-TTGAGAATCAGGCTCCTATC-3’, reverse: 5’-TGAGGTCAAGGTCCTCCTC-3’ For mouse ANP: forward: 5’-CCAGCATGGCTCCTTCTCCA-3’ reverse: 5’-GGCAGTGAGGAGAGTGAAG AG-3’ For mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward: 5’-GGCAGTGAGGAGAGTGAAG AG-3’ reverse: 5’-TGAGTTAGACTGAGTGAAG AG-3’ For mouse IL-1β, forward: 5’-GGCAGTGAGGAGAGTGAAG AG-3’
TGATGATAACCTG-3’, reverse: 5’- TGGTCGTTGCTTGTTTC TCCT-3’ For mouse IL-1α, forward: 5’-ACGTCAGG CAGAAGTTTGCTCA-3’, reverse: 5’-TGATGAGTTTTGGTTTTCTGG-3’ For mouse IGF-1, forward: 5’-CTCTGC TTGCTCACCTTC-3’, reverse: 5’-CCTTCTCC- TTTGCAGCTTC-3’, For mouse IL-6, forward: 5’-ACAACCACGGCCTCCCTAC-3’, reverse: 5’-ACAATCAGA ATTGCCATTGCAC-3’, For mouse α-actin: forward: 5’-GTGGGGCGGCCCGCCCA GGCACCA-3’, reverse: 5’-CTCCTTTATTGTACG CACGATTTC-3’, For mouse TNFα, forward: 5’-TTCTGTCTACTGAATTCGGGGTG- GATGATCGGTCC-3’, reverse: 5’-GTATGAGATAGCAATCGGTGACGCGGTG- GG-3’

Luciferase assay

Luciferase reporter constructs (pGL3) inserted by the IGF-1 promoter or empty pGL3 vectors (each 1μg/ml) were transfected into CFs with STAT5-decoy oligonucleotide or control oligonucleotide (each 1μg/ml) using the lipofection method with 10 μl of Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours after transfection, the cells were washed in PBS and starved in DMEM supplemented with 0.5% FCS for 12 hours. Subsequently, the cells were stimulated by IL-1β (10 ng/ml) for the next 24 hours. Cells were then lysed, and luciferase activities were measured using
the luciferase reporter assay system (PicaGene). STAT5 decoy and control oligonucleotide were thiol-modified at the 5’ end and their sequence was as follows:

STAT5 decoy: 5’-GATCGCATTTCG- GAGAAGACG-3’, control oligonucleotide: 5’-GATCGCATTA- CGGAGTAGACG-3’

**SiRNA knockdown of IL-1α**

SiRNA against mouse IL-1α (sc 39614, Santa Cruz) and non-silencing RNA (sc 37007, Santa Cruz) (each 50 nM) were transfected into CFs (5x10^5, 75%-85% confluent) with lipofectamine™ RNAi MAX kit (Invitrogen). 48 hours after transfection, total RNA was subjected to RT-PCR analysis to evaluate the knockdown efficiency of IL-1α.

**Measurement of IL-1β and IGF-1**

In cardiac fibroblasts or myocytes, IGF-1 released into incubation medium was quantified by ELISA, since it was reported that IGF-1 in cardiac fibroblasts is rapidly released after synthesis into culture medium under cyclic stretch^3. 1x10^6 cells were plated in each well of a 24-well tissue culture plate. The cells were washed twice with PBS, pH 7.4, and then incubated for 24 hours in 500 ml of serum-free RPMI 1640 medium containing TNFα (R&D Systems). IL-1β and IGF-1 was measured in the
supernatant of cultured CF by ELISA (Quantikine; R&D Systems for IL-1β and Murine IGF-I ELISA Development Kit; Peprotech for IGF-1). The detection threshold of the assay was 1 pg/ml for IL-1β and 62pg/ml for IGF-1. These assays were performed in accordance with the manufacturer’s instructions.

**Measurement of oxidative stress**

CF were stretched for 24 hours, washed with PBS, and 2,7-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, U.S.A) was added at a final concentration of 10 µmol/L, incubated for 20 minutes, and then washed twice with PBS. Fluorescence was monitored using a Nikon fluorescence microscope equipped with a FITC filter. Dichlorodihydrofluorescein diacetate (DCF) was excited at 498 nm and the emission was measured at 522 nm in a quartz cuvette with a 10-mm light-path. Fluorescence micrographs were captured with a Nikon fluorescence microscope equipped with FITC filter settings. Mean fluorescence intensity values from three different fields of view were obtained using Metamorph software. ROS production levels (% control) were arbitrarily shown as ratios relative to the control WT cells stretched for 24 hours (set as 100%).
**Immunostaining**

Paraffin-embedded LV samples 3 days after and before banding the aorta were immunostained with antibodies against IL-1β and IGF-1 (Santa Cruz). CFs were exposed to cyclic stretch for 24 hours and immunostained with anti-NFATc4 antibody (Santa Cruz). The number of cells in which NFATc4 was translocated into the nucleus was counted.

**Apoptosis**

Deparaffinized tissue section was treated with 0.3% H₂O₂ in 100% methanol to inhibit endogenous peroxidase activity and then incubated with normal goat serum for 20 min at room temperature. The terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to detect apoptotic cells (ApopTag kit, Chemicon International, Temecula, USA), as previously described.

**Data analysis and statistics**

All visualized data quantification was performed using ImageJ 1.32j software (NIH).
All data were transformed by the natural logarithm prior to analysis of variance (ANOVA) corresponding to each experiment. Repeated measures ANOVA was used to analyze the time course experiment. Scheffe’s test was used as a multiple comparison test. For comparisons between two groups, the two-sample t-test was performed.

Data are presented as means ± SE. P value < 0.05 (two-tail) was considered statistically significant

Supplemental Reference


2. Nishikawa K, Yoshida M, Kusuhara M, Isoda K, Miyazaki K, Ohsuzu F Left ventricular hypertrophy in mice with a cardiac-specific overexpression of interleukin-1

Supplemental Figure Legend

Online Fig. 1: Analysis of cardiomyocyte specific IL-1α overexpressed mice after banding aorta

Abdominal aorta of wild-type (WT) or IL-1α-overexpressed mice (Tg) was banded (day 0). (A) Hearts were removed at day-7 and day-60 after operation and the ratio of heart to body weight (Hw/Bw, mg/g) was evaluated. Paraffin-sectioned LVs were stained with hematoxylin-eosin (H-E) and cross sectional areas of cardiomyocytes were evaluated. (n=10 each). (B) Echocardiography. %FS: Fractioning shortening, Dd: end-diastolic diameter. Ds: end-systolic diameter. %FS was calculated as described in Materials and Methods. (n=10 each). (C) Total RNAs were extracted from LV and analyzed by real-time PCR to quantify IGF-1-mRNA. (n=7 each) (D) Total lysates were analyzed by Western Blotting using antibodies against IGF-1-R or 1135/Tyr-phosphorylated-IGF-1-R. (n=5 each). *p<0.05, **p<0.01 vs. WT

Online Fig. 2: Defect of IL-1β/IGF-1 abolishes stretch-mediated activation of Akt, ERK and synthesis of βMHC

(A) LV were removed at 7 or 14 days after banding the aorta with or without
administration of IGF-1 (50 μg/kg/day for 14 days). *p<0.05, **p<0.01 vs. WT (n=4 each). (B) CM were exposed to stretch for 0-6 hours with or without IGF-1 (5 ng/ml) or anti-IL-1β-antibody (1 μg/ml). Lysates from of LV or cells were analyzed by Western blotting using antibodies against 473Ser-phosphorylated Akt-1, 202Thr204Tyr phosphorylated ERK, Akt-1 or ERK. *p<0.05, **p<0.01 vs. baseline (n=4 each).
Online Fig. 1

**A**

Hw/Bw

Cross sectional myocyte area

**B**

LV-Dd

%FS

**C**

IGF-1 mRNA

**D**

1135p Yp-IGF-1-R

IGF-1-R

p-IGF-1-R

p<0.05
Online Fig. 2

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**p<0.05**, ***p<0.01**