Thioredoxin-Interacting Protein Controls Cardiac Hypertrophy Through Regulation of Thioredoxin Activity

Jun Yoshioka, MD, PhD; P. Christian Schulze, MD, PhD; Mihaela Cupesi, MD; Jeremy D. Sylvan, BS; Catherine MacGillivray, AD; Joseph Gannon; Hayden Huang, PhD; Richard T. Lee, MD

**Background**—Although cellular redox balance plays an important role in mechanically induced cardiac hypertrophy, the mechanisms of regulation are incompletely defined. Because thioredoxin is a major intracellular antioxidant and can also regulate redox-dependent transcription, we explored the role of thioredoxin activity in mechanically overloaded cardiomyocytes in vitro and in vivo.

**Methods and Results**—Overexpression of thioredoxin induced protein synthesis in cardiomyocytes (127±5% of controls, P<0.01). Overexpression of thioredoxin-interacting protein (Txnip), an endogenous thioredoxin inhibitor, reduced protein synthesis in response to mechanical strain (89±5% reduction, P<0.01), phenylephrine (80±3% reduction, P<0.01), or angiotensin II (80±4% reduction, P<0.01). In vivo, myocardial thioredoxin activity increased 3.5-fold compared with sham controls after transverse aortic constriction (P<0.01). Aortic constriction did not change thioredoxin expression but reduced Txnip expression by 40% (P<0.05). Gene transfer studies showed that cells that overexpress Txnip develop less hypertrophy after aortic constriction than control cells in the same animals (28.1±5.2% reduction versus noninfected cells, P<0.01).

**Conclusions**—Thus, even though thioredoxin is an antioxidant, activation of thioredoxin participates in the development of pressure-overload cardiac hypertrophy, demonstrating the dual function of thioredoxin as both an antioxidant and a signaling protein. These results also support the emerging concept that the thioredoxin inhibitor Txnip is a critical regulator of biomechanical signaling. (Circulation. 2004;109:2581-2586.)

**Key Words:** hypertrophy ■ mechanics ■ stress

Epidemiological studies implicate cardiac hypertrophy as an independent risk factor for cardiac morbidity and mortality.1 Cardiomyocyte hypertrophy occurs in response to various stimuli, including mechanical stress and inborn mutations in sarcomeric proteins. Increasing evidence suggests that reactive oxygen species (ROS) play an important role in the pathogenesis of cardiac hypertrophy. ROS participate in signal transduction during cellular growth and differentiation.2 For example, mechanical strain,3 angiotensin II,4 and α-adrenergic receptor stimulation4 increase the formation of ROS and cause cardiomyocyte hypertrophy through ROS-dependent pathways. Thus, redox-sensitive mechanisms participate in the development and progression of cardiac hypertrophy.

ROS elicit a wide spectrum of responses, and these responses depend on dose and duration of the initiating stimulus. Low levels of ROS can promote cellular growth, and intermediate levels can result in growth arrest.6 High levels of ROS cause oxidative damage that can lead to cell death via either apoptotic or necrotic mechanisms.6 Thus, the regulation of internal redox balance critically controls signaling pathways and ultimately the cellular response to stresses. The 2 primary intracellular thiol-reducing systems that maintain a reduced intracellular state are the interacting glutathione and thioredoxin systems. The thioredoxin system is a highly conserved and ubiquitously expressed thiolducing system that functions through the reversible oxidation of 2 cysteine residues at Cys32 and Cys35 of the thioredoxin protein. Although thioredoxin and its reducing enzyme thioredoxin reductase function as powerful antioxidants,7 thioredoxin activation paradoxically can also enhance cellular growth.8 This paradox may be explained by thioredoxin’s redox-dependent regulation of several transcription factors after activation and nuclear translocation.9

Thioredoxin-interacting protein (Txnip), also known as vitamin D3 upregulated protein 1 or thioredoxin binding protein 2, is an endogenous inhibitor of thioredoxin function.10 Txnip is suppressed by mechanical strain, leading to increased thioredoxin activity that promotes cardiomyocyte viability.11 Because mechanical strain causes cardiomyocyte hypertrophy through the formation of ROS,12 this raises the hypothesis that biomechanical regulation of Txnip promotes redox-sensitive growth of cardiomyocytes.

In this study, we demonstrate that activation of thioredoxin is a central mediator of cardiomyocyte growth. This activa-
tion of thioredoxin can occur through biomechanical suppression of its inhibitor, Txnip. Thioredoxin promotes cardiomyocyte hypertrophy despite its antioxidant activity. These experiments highlight the dual function of thioredoxin as both an antioxidant and a potent regulator of cellular growth.

Methods

Culure and Biomechanical Strain of Myocytes

The Harvard Medical School Standing Committee on Animal Research approved the study protocols. Neonatal rat cardiomyocytes from 1-day-old Sprague-Dawley rats (Charles River, Boston, Mass) were isolated and cultured on transparent membranes coated with 3 μg/mL of fibronectin. After serum starvation for 48 hours, mechanical deformation was applied to membranes as previously described.12

Adenoviral Vectors for Gene Transfer

Recombinant adenoviral constructs expressing thioredoxin (AdTrx) or Txnip (AdTxnip) in tandem with constitutive green fluorescent protein (GFP) were generated using the Ad-Easy system and the pAdTrack-CMV vector.11 For controls, we used the identical virus expressing GFP alone (AdGFP).

[^H]Leucine Incorporation

Cardiomyocytes were subjected to cyclic mechanical strain (4%, 1 Hz, 12 hours), phenylephrine (50 μM, 24 hours), or angiotensin II (0.1 μM, 24 hours) with 1.0 μCi/mL[^H]leucine (New England Nuclear-Life Science Products). Leucine incorporation was detected as previously described.13,14

ROS Measurements

Intracellular generation of ROS was quantified using 2′7′-dichlorofluorescein diacetate (DCFDA). Cells were incubated for 45 minutes with 10 μM DCFDA (Molecular Probes Inc.), and fluorescence intensity was measured at 595 nm by the VICTOR2 multibluel counter (PerkinElmer Life and Analytical Sciences, Inc).

Real-Time PCR

Gene expression was analyzed by real-time polymerase chain reaction (PCR) (LightCycler, Roche) using specific oligonucleotides: mouse txnip sense, 5′-ATGGCCAGACAAAAGTGTTC-3′ and antisense, 5′-GGCTGTCCTTGAAGTGGTC-3′; mouse trx sense, 5′-CAACAGC-CAAAATGTGTGAAGCTG-3′ and antisense, 5′-AGGTTTTAACA-GCTG-3′; mouse β-tubulin sense, 5′-CTGGGCTAAAGGCAAC-3′ and antisense, 5′-AGACACCTTTGGCCCGAG-3′; rat brain natriuretic peptide (BNP) sense, 5′-GGAAATGGCTCAGAGACAGC-3′ and antisense, 5′-CGATCCCCGTCTATCTTCTG-3′; rat β-tubulin sense, 5′-CATCCAGGAGCTCTTCAAGC-3′ and antisense, 5′-CGCCTTAC-GGCCTTCTCTTCT-3′.

Thioredoxin Activity Assay

Total cellular protein was extracted by lysis buffer (9.8 mol/L urea, 2% [wt/vol] NP40, and 100 mmol/L DTT). Thioredoxin activity was measured by the insulin disulfide reduction assay.11

Western Analysis

Western analysis was performed using a specific monoclonal mouse anti-Txnip antibody and polyclonal rabbit antisera against thioredoxin or Txnip as previously described.11

Myocyte In Vivo Gene Transfer

We developed a method to evaluate the functional effects of a gene on pressure-overload cardiomyocyte growth in vivo by comparing cells infected with adenovirus to overexpress the gene with cells that were not infected in the same hearts. When adenoviruses were injected into the left ventricle (2×109 pfu in 50 μL), only a subset of cells were infected, as assessed by GFP fluorescence. By performing gene transfer at the same time as pressure overload induced by transverse aortic constriction (TAC), we can assess the effects of a gene on the pressure-overload response. In addition to the noninfected cells in the same heart, control studies also included infection with the identical virus expressing only GFP as well as sham-operated mice. Male FVB mice (age 8 to 10 weeks) were anesthetized with pentobarbital (45 μg/g IP), and the aorta was constricted by tying a 7-0 silk suture around the vessel against a blunted 27-gauge needle. Sham-operated animals were treated identically, with the exception that the suture was not tied. All surgeries were performed in a blinded, randomized manner with respect to treatment. At 7 days after TAC, echocardiography was performed under light anesthesia with pentobarbital (20 μg/g) to assess cardiac hypertrophy. All images were taken at a heart rate >400 bpm using a Sonos 4500 (Philips).

One week after TAC, hearts were harvested and fixed with 4% paraformaldehyde. Paraffin-mounted histological sections were prepared with Evan’s blue stain, which does not quench GFP fluorescence. The sections were imaged with both light and fluorescence microscopy. This approach allowed identification of cell size and determination of whether that individual cell was infected with the gene transfer vector. We wrote an image analysis software using Matlab (The MathWorks, Inc) that automatically identifies and measures cardiomyocytes in the bright-field image. The program identified pixels associated with each cell by means of a threshold-assisted watershed segmentation method. Cells with a ratio of major axis to minor axis >1.5 were excluded from analysis to minimize the effects of eccentricity. Myocytes were then grouped as infected or noninfected by comparing the bright-field images with the fluorescent images.

Statistical Analysis

All data are presented as mean±SEM. Comparison of groups was performed with ANOVA followed by post hoc analysis with the Tukey-Kramer test. Statistical significance was achieved at a value of P<0.05.

Results

Thioredoxin, Txnip, and Cardiomyocyte Growth

To assess the effects of thioredoxin and Txnip on cardiomyocyte hypertrophy, cells were infected with adenoviral vectors expressing GFP (AdGFP), thioredoxin and GFP (AdTrx), or Txnip and GFP (AdTxnip) at a multiplicity of infection of 20 for 3 hours in serum-free conditions. Western analysis confirmed successful gene transfer of thioredoxin (13±1-fold, P<0.01) or Txnip (14±2-fold, P<0.01) relative to the level of endogenous protein expression in cardiomyocytes. Overexpression of thioredoxin increased[^H]leucine uptake (127±5% of AdGFP, n=13, P<0.05) (Figure 1A), whereas overexpression of Txnip resulted in a nonsignificant decrease (90±6% of AdGFP, n=11, P=NS). These results demonstrate that thioredoxin can promote cardiomyocyte growth in the absence of hypertrophic stimuli.

Next, we assessed the effects of thioredoxin and Txnip on cardiomyocyte growth in response to established hypertrophic stimuli (Figure 1B).[^H]leucine incorporation was increased by mechanical strain (124±7% of AdGFP baseline, n=14, P<0.05), phenylephrine (160±8% of AdGFP baseline, n=6, P<0.01), or angiotensin II (133±4% of AdGFP baseline, n=6, P<0.05). Stimulation of cardiomyocytes with phenylephrine (185±10% of AdTrx baseline, n=6, P<0.01) or angiotensin II (153±4% of AdTxnip baseline, n=6, P<0.05) increased[^H]leucine uptake in AdTrx-infected cells, indicating that overexpression of thioredoxin does not block the
effects of these growth factors despite its antioxidant activity. Overexpression of the endogenous thioredoxin inhibitor Txnip reduced protein synthesis in response to mechanical strain (5% reduction, \( n=11, P<0.01 \)).

We also investigated the effects of thioredoxin and Txnip on the expression of BNP mRNA, a molecular marker for cardiac hypertrophy induced by mechanical strain. Real-time PCR showed that mechanical strain (8%, 1 Hz, 6 hours) induced BNP mRNA expression in AdGFP-infected cells (2.6-fold, \( n=4 \) to 5 normalized to \( \beta \)-tubulin expression, \( P<0.05 \), Figure 1C). There was a trend toward an increase in BNP gene expression with mechanical strain when cells overexpressed thioredoxin, although this did not reach statistical significance (\( P=0.09 \)). However, overexpression of Txnip attenuated strain-induced BNP expression, consistent with the ability of Txnip to inhibit strain-induced hypertrophy. These results suggest that Txnip is a central inhibitor of the cardiomyocyte growth response.

**Figure 1.** Effects of gene transfer of thioredoxin and Txnip on cardiomyocyte growth. A, \([3H]\)leucine uptake in cardiomyocytes infected with adenoviral vectors expressing GFP (AdGFP), thioredoxin and GFP (AdTrx), or Txnip and GFP (AdTxnip).

**Figure 2.** A and B, Effects of gene transfer of thioredoxin (AdTrx) and Txnip (AdTxnip) on formation of ROS. Intracellular generation of ROS was quantified by DCFDA fluorescence. Raw data were expressed as percent increase vs baseline control. *\( P<0.05 \) and **\( P<0.01 \) vs baseline control for each experiment. ¶\( P<0.05 \) and ††\( P<0.01 \) vs AdGFP at same time point. C, Effects of mechanical strain (6%, 1 Hz, 3 hours) on thioredoxin activity. *\( P<0.01 \) vs AdGFP baseline, ††\( P<0.01 \) vs AdGFP mechanical strain.
caused significant increases in ROS in control (AdGFP-infected) cells. Because the increases in ROS with phenylephrine (50 μmol/L, 3 hours) were less apparent than with angiotensin II, we used angiotensin II as the positive control in subsequent experiments. The effects of mechanical strain (1 hour) and angiotensin II (1 and 3 hours) were abolished by overexpression of thioredoxin. Overexpression of Txnip dramatically enhanced cellular levels of ROS in cardiomyocytes undergoing mechanical strain (4 hours, 274±60% of AdGFP, n=9, P<0.01) or angiotensin II stimulation (3 hours, 502±61% of AdGFP, n=6, P<0.01). These findings indicate a profound pro-oxidant shift in cellular redox balance by Txnip.

To determine whether the increase in cellular levels of ROS by Txnip accompanies the change of thioredoxin activity in cardiomyocytes, thioredoxin-specific insulin-reducing assays were performed. Mechanical strain (8%, 1 Hz, 4 hours) or overexpression of thioredoxin increased thioredoxin activity compared with the AdGFP baseline value (n=8, P<0.01, Figure 2C). However, overexpression of Txnip suppressed the increase in thioredoxin activity by mechanical strain (77±4% of AdGFP mechanical strain, n=7, P<0.01). These data confirmed the effects of Txnip on cellular redox balance through biomechanical regulation of thioredoxin activity.

Thioredoxin Activity and Expression of Txnip in Pressure-Overload Hypertrophy

We investigated the activation of the thioredoxin system in pressure-overload hypertrophy in vivo. As expected, transaortic constriction increased left ventricular weight/body weight (0.34±0.01, n=6) compared with sham surgery (0.30±0.02, n=4, P<0.05). Fractional shortening and end-systolic dimensions were similar between TAC (65±3%, 0.9±0.1 mm, respectively) and sham (63±4%, 1.0±0.1 mm, respectively) mice, indicating that systolic function was preserved. Pressure-overload cardiac hypertrophy caused a 3.5-fold increase in myocardial thioredoxin activity (n=6) compared with sham (n=4, P<0.01) (Figure 3A). Furthermore, TAC (n=6) led to a marked decrease in gene expression of Txnip in the hypertrophied ventricle (40±6% expression of sham hearts, n=4, P<0.05) (Figure 3B), whereas expression of thioredoxin was similar between sham and TAC hearts (114±68% expression of sham hearts, P=NS). The suppression of Txnip mRNA by pressure overload was accompanied by changes at the protein level of Txnip (Figure 3, C and D). Compared with sham-operated mice (n=4), left ventricular Txnip protein decreased by 30±6% in TAC mice (n=6, P<0.05). These data are consistent with the hypothesis that pressure overload suppresses Txnip expression and that this leads to an increase in thioredoxin activity.

Effects of Txnip Gene Transfer on Pressure-Overload Hypertrophy

To explore whether thioredoxin may play a critical role in the development of pressure-overload cellular hypertrophy in vivo, adenoviral vectors (AdGFP or AdTxnip) were injected into the left ventricular free wall concomitantly with TAC (GFP-TAC, n=10; Txnip-TAC, n=11) and sham (GFP-sham, n=9; Txnip-sham, n=11) surgery in mice. Gene expression was confirmed as positive cellular fluorescence that allowed determination of which cells were infected at 7 days (Figure 4A), and controls included sham-operated animals, AdGFP-infected cells, and noninfected cells from the same animals that received Txnip. GFP-TAC (n=134) cells showed the anticipated cellular hypertrophy compared with GFP-sham (n=156) cells (cross-sectional area, 229±2 versus 158±2 μm²; P<0.01), whereas no difference in cell size was detectable between AdGFP-infected and noninfected cells of both sham and TAC animals. These controls showed that expression of GFP in these conditions does not alter the development of cellular hypertrophy in response to pressure overload. No difference of cell size was observed between AdTxnip noninfected (n=121) and infected (n=120) cells in

Figure 3. Thioredoxin activity and expression of Txnip in pressure-overload hypertrophy. A, Thioredoxin activity was measured in mouse ventricle 7 days after TAC (n=6) or sham (n=4) surgery. B, Txnip gene expression was analyzed by real-time PCR in same animals. C and D, Western analysis using anti-Txnip monoclonal antibody (1:500) or anti-actin antibody (1:1000). **P<0.01 and *P<0.05 vs sham.
animals of the Txnip-sham group. However, cellular hypertrophy in response to pressure-overload was inhibited by overexpression of Txnip (28.1±5.2% reduction versus noninfected cells, P<0.01). AdTxnip-infected cells (n=108) were significantly smaller than AdTxnip noninfected cells (n=140) (cross sectional area, 227±8 μm² in AdTxnip-infected cells versus 262±8 μm² in noninfected cells; P<0.05) (Figure 4B). Thus, overexpression of Txnip inhibited cardiomyocyte growth in response to pressure overload.

**Discussion**

The ubiquitously expressed thioredoxin system participates in the regulation of cellular redox potential and is functionally regulated by the endogenous inhibitor Txnip. In this study, we demonstrated that biomechanical overload can induce cellular growth through activation of the thioredoxin system. Functional inhibition of the thioredoxin system by Txnip prevents cardiomyocyte growth both in vitro and in vivo. These studies therefore identify Txnip as a central growth control mechanism in the stress response.

ROS play an important role in the development of acute pressure-overload cardiac hypertrophy. Previous reports demonstrated that antioxidants attenuate pressure-overload cardiac hypertrophy. Transgenic cardiac overexpression of thioredoxin decreases the myocardial infarct size after ischemia/reperfusion injury and protects against adriamycin-induced cardiotoxicity by reducing oxidative stress. Therefore, thioredoxin may play a pivotal role in the myocardial defense against oxidative stress. It is reasonable to anticipate that thioredoxin would inhibit oxidative bursts that are necessary for growth signals and that activation of thioredoxin would inhibit cellular growth. However, as shown by this study, thioredoxin can be a growth-promoting protein even while it functions in its antioxidative capacity.

We previously showed that mechanical strain induces thioredoxin activity in vitro. Here, we demonstrate that pressure-overload induces thioredoxin activity in vivo. In addition, overexpression of thioredoxin reduced strain- and angiotensin II–induced levels of ROS but did not prevent hypertrophic effects of these stimuli. These data support the concept that the biomechanically induced increase in thioredoxin activity participates in stress-induced cardiac hypertrophy. Moreover, overexpression of Txnip, the endogenous inhibitor of thioredoxin, further increased mechanical strain- and angiotensin II–induced levels of ROS. However, overexpression of Txnip reduced the hypertrophic growth of cardiomyocytes in response to phenylephrine, angiotensin II, and mechanical strain.

Given the known interaction between ROS and its cohypertrophic effects in cardiomyocytes, our findings initially seem counterintuitive. Oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest, senescence, and cell death, depending on its amplitude and the duration of exposure. It has been demonstrated that ROS produce either cell proliferation or death of vascular smooth muscle cells, depending on the dose and frequency of exposure to ROS. Therefore, the functional role of ROS as mediators of cellular signaling critically depends on the regulation of cellular redox balance by pro-oxidative and antioxidative systems. Our findings that thioredoxin induced cellular growth and that Txnip inhibited cellular growth are consistent with previous reports on vascular smooth muscle cells and tumor cells. Recently, Yamamoto et al demonstrated that transgenic mice with cardiac-specific overexpression of a dominant negative mutant of thioredoxin exhibited enhancement of cardiac hypertrophy in response to pressure overload. In contrast, adenovirus-mediated transduction as used in our study might lead to the shorter-term expression of the endogenous thioredoxin inhibitor Txnip.

Thioredoxin can serve as a transcriptional coactivator after nuclear translocation. Well-known examples of biomechanically or redox-sensitive transcription factors in cardiomyocyte hypertrophy are nuclear factor-κB (NF-κB) and activator protein-1. Thioredoxin translocates from the cytoplasm into the nucleus to potentiate several transcription factors.
factors, including NF-κB and activator protein-1.\(^\text{9,31}\) Our preliminary experiments on this issue suggest that overexpression of thioredoxin enhances mechanical strain–induced NF-κB activation (data not shown). Therefore, thioredoxin may participate in cardiomyocyte hypertrophy by regulating the transcriptional activity of NF-κB. Txnip is a stress-regulatory molecular switch mechanism that controls the thioredoxin system by interfering with 2 important aspects of thioredoxin function. The interaction of thioredoxin with Txnip explains the apparent paradox of thioredoxin function, first, leading to enhanced levels of ROS by inhibiting thioredoxin antioxidative function, and second, inhibiting thioredoxin-dependent activation of transcriptional processes. Additional studies on the role of thioredoxin activation in known molecular pathways of hypertrophy could lead to improved understanding of how redox balance participates in growth control.

Finally, in this study, the DCFDFA assay was used as a quantitative index of ROS. Although this assay has been widely used as an indicator of cellular levels of ROS, DCFDFA is not specific, and fluorescence can depend on a variety of reactive oxygen species as well as intracellular peroxidase levels.\(^\text{32}\) More specific methods of assaying reactive oxygen species could better characterize the nature of the oxidative stress changes.

In conclusion, we describe the regulation of thioredoxin function by Txnip as a novel stress-induced mechanism in cardiomyocytes that controls pressure-overload cardiac hypertrophy. These results are consistent with the emerging concept that Txnip is a critical regulator of biomechanical signaling. Our findings provide evidence for the dual function of thioredoxin as both an antioxidant and a growth-promoting signaling protein.

Acknowledgments
This work was supported by an American Heart Association New England Affiliate Fellowship Award (to Dr Yoshioka) and grants from the National Institutes of Health (to Drs Lee and Huang).

References
Thioredoxin-Interacting Protein Controls Cardiac Hypertrophy Through Regulation of Thioredoxin Activity

Jun Yoshioka, P. Christian Schulze, Mihaela Cupesi, Jeremy D. Sylvan, Catherine MacGillivray, Joseph Gannon, Hayden Huang and Richard T. Lee

_Circulation_. 2004;109:2581-2586; originally published online May 3, 2004; doi: 10.1161/01.CIR.0000129771.32215.44

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/21/2581

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/