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Hypoxia inducible factor-1 improves the negative functional effects of natriuretic peptide and nitric oxide signaling in hypertrophic cardiac myocytes

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Abstract

Aims—Both natriuretic peptides and nitric oxide may be protective in cardiac hypertrophy, although their functional effects are diminished in hypertrophy. Hypoxia inducible factor-1 (HIF-1) may also protect in cardiac hypertrophy. We hypothesized that upregulation of HIF-1 would protect the functional effects of cyclic GMP (cGMP) signaling in hypertrophied ventricular myocytes.

Main Methods—A cardiac hypertrophy model was created in mice by transverse aorta constriction. HIF-1 was increased by deferoxamine (150 mg/kg for 2 days). HIF-1 α protein levels were examined. Functional parameters were measured (edge-detector) on freshly isolated myocytes at baseline and after BNP (brain natriuretic peptide, 10^{-8} - 10^{-7} M) or CNP (C-type natriuretic peptide, 10^{-8} - 10^{-7} M) or SNAP (S-nitroso-N-acetyl-penicillamine, a nitric oxide donor, 10^{-6} - 10^{-5} M) followed by KT5823 (a cyclic GMP-dependent protein kinase (PKG) inhibitor, 10^{-6} M). We also determined PKG expression levels and kinase activity.

Key findings—We found that under control conditions, BNP (−24%), CNP (−22%) and SNAP (−23%) reduced myocyte shortening, while KT5823 partially restored function. Deferoxamine treated control myocytes responded similarly. Baseline function was reduced in the myocytes from hypertrophied heart. BNP, CNP, SNAP and KT5823 also had no significant effects on function in these myocytes. Deferoxamine restored the negative functional effects of BNP (−22%), CNP (−18%) and SNAP (−19%) in hypertrophic cardiac myocytes and KT5823 partially reversed this effect. Additionally, deferoxamine maintained PKG expression levels and activity in hypertrophied heart.

Significance—Our results indicated that the HIF-1 protected the functional effects of cGMP signaling in cardiac hypertrophy through preservation of PKG.

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Keywords

Cyclic GMP; Cyclic GMP-dependent protein kinase; Natriuretic peptides; Nitric oxide; Cardiac hypertrophy; Myocytes; Hypoxic inducible factor-1 (HIF-1)

Introduction

In the heart, the cyclic GMP (cGMP) signaling system induces cardiodepressive effects (Rastaldo et al., 2007). It reduces cellular function and metabolism in cardiac myocytes. This second messenger can be activated by natriuretic peptides and nitric oxide. The actions of cGMP occur primarily through the cyclic GMP-dependent protein kinase (PKG), although cGMP regulated ion channels and phosphodiesterases may also play a role (Lucas et al., 2000; Zaccolo and Movsesian, 2007).

Cardiac hypertrophy especially left ventricular hypertrophy is an adaptive response to pressure and volume overload. Pathologic cardiac hypertrophy is an independent risk factor for the development of heart failure and premature death (Rame and Dries, 2007). It is characterized by altered gene expression including ones encoding membrane receptors, second messengers, and transcription factors to modify energy metabolism and hormonal pathways in the heart (Carreno et al., 2006). Agents that increase cGMP, such as natriuretic peptides, may be beneficial in hypertrophy and failure (Abassi et al., 2004; London, 2006). This may be related to reduced myocardial metabolic needs. However with cardiac hypertrophy, reduced effects of the cGMP signaling system and attenuated effects of cGMP based agents have been demonstrated (Meyer et al., 2007; Su et al., 2005).

Hypoxia inducible factor (HIF)-1, a transcriptional regulator, is composed of an oxygen regulated subunit HIF-1 α and an oxygen-insensitive constitutively expressed subunit HIF-1 β (Semenza, 2000, 2004). Under hypoxic conditions, HIF-1 α heterodimerizes with the β -subunit. Subsequently, this heterodimeric complex can affect activation of numerous downstream genes such as erythropoietin, glucose transporter-1, glycolytic enzymes, VEGF (vascular endothelial growth factor) and p21 etc. (Dery et al., 2005; Poellinger and Johnson, 2004; Semenza, 2001). HIF-1 also interacts with cGMP signaling through upregulation of BNP (brain natriuretic peptide) and iNOS (inducible nitric oxide synthase) (Belaidi et al., 2008; Weidemann et al., 2008). These genes are involved in cardiovascular protective effects, including angiogenesis, energy metabolism, stress response, nitric oxide synthesis, extracellular matrix remodeling and vascular perfusion during myocardial ischemia and ischemia-reperfusion injury (Cai et al., 2003; Lefer, 2006; Wilhide and Jones, 2006). A recent study has reported beneficial protective effects of HIF-1 on hypertrophic hearts, in part, through angiogenesis (Sano et al., 2007). However, the question of whether the interaction of HIF-1 with cGMP signaling has beneficial effects on myocyte function during hypertrophy has not been explored.

In the present study, we hypothesized that HIF-1 would serve a protective role to preserve myocyte function during cardiac hypertrophy, in part, by protecting the functional effects of cGMP signaling. We utilized a transverse aorta constriction mouse model and isolated ventricular myocytes from control and hypertrophic mouse hearts to observe contractile changes in response to cGMP-dependent negative inotropic agents with and without HIF-1 upregulation. Our findings suggested reduced cGMP effects and cyclic GMP protein kinase activity in hypertrophy. We found that in vivo upregulation of HIF-1 prevented the functional impairment and damaged cGMP signaling through maintenance of PKG during cardiac hypertrophy.

Materials and Methods

Experimental Animals and Chemicals

Experiments were conducted in accordance with Guide for the Care of Laboratory Animals (DHHS publications No.85–23, revised 1996) and approved by our Institutional Animals Care and Committee.

All the animals used in this study were C57BL/6J mice from Jackson Laboratory (Bar Harbor, Maine). A cardiac hypertrophy model was created by transverse aorta constriction surgery on 8-week-old C57BL/6J mice (either sex) (Su et al., 2005). Specifically, in this model, animals were prepared under sterile, anesthetized conditions. The transverse aorta was constricted with 6-0 nylon suture against a 3 cm length of a blunted 27-gauge wire. The wire was removed once the suture was tied. After aortic constriction, the chest was closed and wiped by the iodine tincture, and then mice were allowed to recover from anesthesia. This procedure produces a 65–85% aortic narrowing. Animals were studied after three weeks (Jiang et al., 2007; Su et al., 2005). Only animals having a heart weight (mg)/body weight (g) ratio ≥ 8.5 (approximately 40% greater than control) were considered to have cardiac hypertrophy for this study. For both the control mice and hypertrophic mice, half the animals (7 animals) were injected subcutaneously with 150 mg/kg/day of deferoxamine (DFX, Sigma) for 2 days before the heart was removed. This protocol increases HIF-1 α and VEGF (vascular endothelial growth factor) (Chi et al., 2008; Luciano et al., 2008). All chemicals used in the current study were obtained from Sigma-Aldrich Co., St. Louis, MO, unless otherwise stated.

Myocyte isolation

Freshly isolated ventricular myocytes were prepared by a standard protocol as described previously (Zhang et al., 2007) with modifications. After the administration of heparin (10 unit/g body weight, Baxter Healthcare Corporation, Deerfield, IL, USA), the animals were anesthetized and their hearts were rapidly removed after an overdose of sodium pentobarbital (90 mg/kg, Fort Dodge Animal Health, Fort Dodge, Iowa, USA). Both the body and the heart weight were measured. The aorta was rapidly cannulated and the heart was mounted on a Langendorff apparatus. Aortic retrograde perfusion was initiated at 70 mm Hg pressure with Ca²⁺-free modified minimal essential medium (MEM) supplemented with 10 mM taurine, 2 mM L-glutamic acid, and 20 mM HEPES, pH 7.37–7.40. After 5 min, the heart was perfused with MEM containing 0.1% type II collagenase for 10 min. The ventricle was then cut into small pieces. The tissue was pipetted in the 0.1% collagenase until all the large pieces were dispersed. The supernatant was washed in MEM supplemented with 0.5% bovine serum albumin (BSA); 1 mM calcium was reintroduced in the presence of 10 mM 2, 3-butanedione monoxime. After being washed three times, the myocytes were suspended in the media with 0.5% BSA and 1 mM CaCl₂ at room temperature. Cell viability was assessed by a standard of maintenance of rod-shaped morphology and routinely was about 40–60%.

Measurements of Cardiac Myocyte Contractile Function

Details of the analysis of myocyte function have been reported previously (Su et al., 2005). Briefly, isolated mouse myocytes were put into an open chamber on the stage of an inverted microscope in MEM solution supplemented with 0.5% BSA and 2 mM CaCl₂. Two platinum wires were used to pace the myocytes (1 Hz, 5 msec duration, voltage 10% above threshold, and polarity alternated with each pulse). The chamber was maintained at 37°C. The contraction was measured with a Myotrack system containing a camera and video edge detector (Crystal Biotech, Patton Biomedical, Inning, Germany). We used at least three myocytes per protocol per experiment. The contraction data were continuously obtained.

Untreated cells would continue to contract with a constant shortening level over the time course of these experiments. We are reporting percentage shortening ((maximum length-minimum length)/maximum length), maximal rate of shortening and maximal rate of relaxation.

Experimental Protocols

There were four groups of mice used in this study: control, control + DFX, hypertrophy and hypertrophy + DFX. Seven animals per group were used in this study. Three protocols were performed and each experimental protocol was repeated in at least three cells in each animal. Myocytes were suspended in a well-oxygenated chamber with 2 mM Ca^{2+} -MEM and the cells were allowed to stabilize for 5–10 min. Then the myocytes were paced with electrical field stimulation. A stabilization period of three min was used before the contraction data for the individual ventricular myocyte was recorded. A three-min interval was allowed between treatments and at least 10 consecutive contractions were used for analysis. The protocols were: 1. baseline, BNP (brain natriuretic peptide, 10^{-8} and 10^{-7} M) followed by KT5823 (PKG inhibitor, 10^{-6} M), 2. baseline, CNP (C-type natriuretic peptide, 10^{-8} and 10^{-7} M) followed by KT5823 (10^{-6} M), 3. baseline, SNAP (S-nitroso-N-acetyl-penicillamine, a nitric oxide donor, 10^{-6} and 10^{-5} M) followed by KT5823 (10^{-6} M). Individual myocytes were required to complete their protocol for inclusion in the data. Results per protocol were averaged for analysis in each animal.

Western Blot

Protein extracts were prepared from ventricular myocytes. Western blot analysis was performed as described (Zhu et al., 2004). In brief, proteins were electrophoresed and separated by 10% SDS-PAGE using the BioRad mini-gel, and transferred to a nitrocellulose membrane. After 1 hr in 5% non-fat dried milk, the nitrocellulose membrane was incubated overnight at 4°C in mouse monoclonal anti-HIF-1 α (Novus Biologicals, Littleton, CO, USA. 1:250) or anti-PKG I α (Stressgen Biotechnology, Ann Arbor, Michigan, USA. 1:500) antibody in 5% non-fat dried milk. Ran or β -actin were used as internal standards. The blot was rinsed with TBST (TBS buffer with Tween) and then incubated with horseradish peroxidase-conjugated anti-mouse antibody for 2 hr at room temperature. The blot was rinsed and developed with the enhanced chemiluminescence immunoblotting detection system (Pierce Biotechnology, Rockford, IL, USA).

Determination of PKG expression and activity

Myocytes were homogenized in buffer: 5 mM Tris HCl, 1 mM MgCl_2 , 0.25 M sucrose, SIGMAFAST Protease Inhibitor (Sigma), 1 mM IBMX (3-isobutyl-1-methylxanthine, a non-specific inhibitor of phosphodiesterases, Biomol International. Plymouth Meeting, PA, USA), pH 7.4, then centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was collected and stored at -80°C . Protein concentrations were determined by Bio-Rad protein assay at 595 nm. The protein samples were thawed on ice and protein concentrations were equalized. An activator (8-bromo-cGMP, 2.5×10^{-4} M) and an inhibitor of PKG (KT5823, 7.5×10^{-4} M) were used. The reaction mixture included 10 μl of protein extract (2 mg protein/ml), 1 mM IBMX, 0.5 μl [$\gamma\text{-P}^{33}$] ATP at 10 $\mu\text{Ci/ml}$, 8-bromo-cGMP (2.5×10^{-4} M) and KT5823 (7.5×10^{-4} M). The reaction was carried out at room temperature for 15 min. An equal volume of BioRad reducing sample buffer was added to terminate the reaction. The samples were heated at 95°C for 5 min and electrophoresed using miniature 12% SDS-polyacrylamide slab gels. The gels were then stained with Coomassie brilliant blue for 30 min, destained for 2–3 hr, dried using gel drying kit (Promega. Madison, WI, USA) and exposed to X-ray film for 36 hr. The exposed X-ray film demonstrated phosphate labeled proteins that were then sized by comparison to standard markers.

Statistical Analysis

A repeated measures analysis of variance (ANOVA) was used to compare variables measured in these experiments. Duncan's multiple range test was used post hoc to compare differences between and within the various treatments and groups. A value of $P < 0.05$ was used as the level of statistical significance. All values are expressed as mean $\pm$ S.E.M.

Results

We measured HIF-1 α protein expression levels in the four groups of ventricular myocytes ($N=3-4$ /group). In the control animals, under the normal condition, the HIF-1 α protein expression level was low (Fig. 1, lane 1, 0.26 ± 0.03 HIF-1 α /Ran). In the hypertrophic myocytes, the Western blot showed a small increase of HIF-1 α expression level (Fig. 1, lane 2, 0.53 ± 0.06). After DFX injection for 2 days (150 mg/kg/day), HIF-1 α was significantly upregulated in both the control (0.93 ± 0.05) and cardiac hypertrophied animals (1.05 ± 0.08) (Fig. 1, lane 3 and 4).

Myocyte functional measurements from all groups of mice are presented in Figure 2 and Table 1. We did not observe that upregulation of HIF-1 had any significant effects on the basal percentage shortening, maximal rate of shortening and maximal rate of relaxation of the ventricular myocytes in the control animals. Additionally, HIF-1 induction did not significantly alter the control myocytes functional response to natriuretic peptides or nitric oxide.

In the control and control+DFX group, when BNP (10^{-8} and 10^{-7} M) was introduced to the myocytes, percent shortening was decreased in a concentration dependent manner by -15% and -24% in the control group and -15% and -22% in the control+DFX group (Fig. 2). The percentage shortening was significantly lower in the presence of the high concentration of BNP (10^{-7} M) compared to baseline. The maximal rate of shortening was reduced by -12% and -18% in the control group and -14% and -15% in the control+DFX group (Table 1). The maximal rate of relaxation was depressed by -10% and -16% in the control group and -14% and -16% in the control+DFX group (Table 1).

When CNP (10^{-8} and 10^{-7} M) or SNAP (10^{-6} and 10^{-5} M) was administered to the myocytes, the percentage shortening was decreased in a concentration dependent manner in the control and the control+DFX groups (Fig. 2). High concentrations of CNP and SNAP both significantly depressed the percentage shortening from baseline for both groups. Similarly, the maximal rates of shortening and relaxation were reduced in the control and control+DFX groups by CNP and SNAP (Table 1). High concentrations of CNP and SNAP significantly affected the maximal rate of shortening in the control group and both maximal rates of shortening and relaxation in the control+DFX group.

The introduction of KT5823 (10^{-6} M, a specific PKG inhibitor) to the control and control+DFX myocytes following BNP, CNP or SNAP partially reversed the reduced percentage shortening caused by these agents. The decrements in function caused by BNP, CNP or SNAP were no longer statistically significant after KT5823 in both non-hypertrophic groups. KT5823 had the same effects on the maximal rate of shortening and the maximal rate of relaxation following BNP, CNP or SNAP (Table 1).

Aortic stenosis led to a significant increase in heart weights and heart weight/body weight ratios for the control (170 ± 11 vs. 258 ± 10 mg heart weight, 6.02 ± 0.33 vs. 9.16 ± 0.25 mg/g heart weight/body weight) and control+DFX (171 ± 8 vs. 274 ± 16 , 6.12 ± 0.25 vs. 9.44 ± 0.33) groups. Resting cell length was also longer in hypertrophy (119 ± 7 control vs. 132 ± 8 μ m hypertrophy). In the hypertrophic group, baseline levels of percentage shortening, maximal

rate of shortening and relaxation were all depressed compared to the control myocytes. BNP, CNP and SNAP had no significant effects on percentage shortening (Fig. 2). The maximal rate of shortening and maximal rate of relaxation also exhibited a lack of response to these agents (Table 1). The addition of KT5823 also produced no significant effects on percentage shortening or maximal rate of shortening or relaxation in ventricular myocytes from the hypertrophic group of mice.

In the hypertrophic animals pretreated with DFX, the average basal percentage shortening, maximal rate of shortening and maximal rate of relaxation levels were elevated compared to hypertrophy alone group. BNP, CNP and SNAP decreased percent shortening (Fig. 2). The high concentration of BNP also reduced the maximal rate of shortening and relaxation significantly. CNP and SNAP also had similar effects. The addition of KT5823 partially restored function in the hypertrophy+DFX group. These responses were comparable to non-hypertrophic group.

We performed triplicate Western blots and triplicate *in vitro* phosphorylation assays in the four groups of myocyte extracts in order to determine the effects of HIF-1 on PKG levels and activity (Fig. 3). For the Western blot analysis, our results indicated that in the myocytes from hypertrophied hearts, the protein expression level of PKG was reduced (0.18 ± 0.07 PKG/actin) (Fig. 3 top, lane 2) comparing to the control level (0.54 ± 0.02) (lane 1). DFX had no effects on protein levels in control myocytes (0.67 ± 0.01) (lane 3). However, after pretreatment with DFX, the induction of HIF-1 restored PKG content in the hypertrophied hearts (0.62 ± 0.01) (lane 4).

Using an *in vitro* phosphorylation assay, we determined PKG activity. Before stimulation with 8-bromo-cGMP, all groups had a similar basal level of protein phosphorylation (Fig. 3 bottom, left lane in each group). The middle lane shows protein phosphorylation after addition of 8-bromo-cGMP. Except for the hypertrophy group, there were increases in five protein bands at approximate molecular masses of 130, 100, 55, 32 and 24 kDa (labeled at the right). The intensity of these bands was reduced using KT5823. In hypertrophy, activation of PKG by 8-bromo-cGMP was decreased and the effect of KT5823 was eliminated. DFX restored the effects of PKG in the hypertrophic myocytes. For the 55 kDa band, the effects of 8-bromo-cGMP were blunted in the hypertrophy group (1.71 ± 0.11 (cGMP-baseline)/baseline control, 0.18 ± 0.07 hypertrophy, 1.60 ± 0.02 control+DFX, 1.62 ± 0.04 hypertrophy+DFX). Similar quantitative changes were observed in the density measurements from the 32 kDa band (3.56 ± 0.33 control, 0.23 ± 0.11 hypertrophy, 4.10 ± 0.03 control+DFX, 3.77 ± 0.01 hypertrophy+DFX).

Discussion

In control myocytes, natriuretic peptides and nitric oxide produce negative functional effects that are partially dependent on PKG. Our study found no changes in control myocytes after *in vivo* upregulation of HIF-1 expression in terms of basal or cGMP dependent functional effects. We then performed transverse aorta constriction to establish a cardiac hypertrophy model in mice. We found reduced functional effects of cGMP producing agents in hypertrophic ventricular myocytes, which was related to reduced effects of PKG. The major finding of this study was that upregulation of HIF-1 restored the functional effects of nitric oxide and natriuretic peptides. This was related to preservation of PKG activity and level.

In the heart, the second messenger cGMP can act as a negative regulator of cardiac function (Lucas et al., 2000). Both prior work and the current study showed exogenous introduction of BNP, CNP and SNAP had concentration dependent negative inotropic effects (Moalem et al., 2006a; Su et al., 2005). There can also be positive functional effects related to cGMP

affected cAMP phosphodiesterases (Kojda and Kottenberg, 1999). Much of the negative inotropic effects of natriuretic peptides and nitric oxide are related to increased PKG. Increased PKG activity augments the phosphorylation of its substrates, in part, to inhibit Ca^{2+} entry through the L-type Ca^{2+} channel (Carabelli et al., 2002; Schroder et al., 2003), enhance the sequestration of cytosolic Ca^{2+} into the sarcoplasmic reticulum through activation of the Ca^{2+} -ATPase (SERCA) by phosphorylation of phospholamban (Zhang et al., 2007) and reduced Ca^{2+} entry and release (Schlossmann et al., 2000; Schroder et al., 2003). The overall effect of these actions of PKG is a decrease of the cytoplasmic Ca^{2+} concentration resulting in reduced contractility. KT5823, an inhibitor of PKG, partially blocked the negative inotropic effects of BNP, CNP and SNAP in the current study.

In control cells, the upregulation of HIF-1 had no significant effects on baseline function or the cell's ability to respond to cGMP dependent negative inotropic agents. Deferoxamine increases HIF-1 α protein levels by preventing its breakdown. HIF-1 has been shown to increase the transcriptional activity of several cardioprotective genes serving to modulate blood and iron metabolism, angiogenesis, cellular proliferation, energy metabolism, and vasomotor control (Wilhide and Jones, 2006; Zhou and Brune, 2006). Activation of these genes does not seem to play a major role under baseline conditions in cardiac myocytes. The cGMP system also appears unaffected by HIF-1 upregulation under basal conditions. This is despite reports that HIF-1 increases the expression of BNP and iNOS (Belaidi et al., 2008; Weidemann et al., 2008). Our study also showed that in control cells, HIF-1 had minimal effects upon PKG activity. This kinase does not appear to affect baseline myocyte function. There are controversial reports on whether nitric oxide signaling increases (Kimura et al., 2002; Sandau et al., 2001) or decreases (Hagen et al., 2003; Huang et al., 1999) expression of HIF-1. However, our administration of SNAP was too brief to affect HIF-1 levels or effects.

Cardiac hypertrophy develops as a basic response to physiological and pathological changes including persistent increases in blood pressure and a variety of diseases. In myocardial hypertrophy, cardiac myocyte function is often maintained, although it decreases as failure ensues (Carreno et al., 2006; Moalem et al., 2006b; Zaccolo and Movsesian, 2007). In the current study, basal percentage shortening and maximum rates of shortening and relaxation were generally reduced in the hypertrophic myocytes compared to non-hypertrophic myocytes.

The cGMP signaling pathway may serve a protective role in maintaining cardiac function by reducing the effects of excessive cyclic AMP signaling. High levels of catecholamines are associated with cardiac failure (Carreno et al., 2006; Rame and Dries, 2007). However, the protective effects of the cGMP pathway appear to be disrupted in acute and chronic heart failure (Lewis and Semigran, 2004; Zhang et al., 2005). Many drugs that increase cGMP such as BNP exert effects to alleviate heart failure (Abassi et al., 2004; London, 2006). However, lack of iNOS has been suggested to be protective in some but not all mouse models of cardiac hypertrophy (Sun et al., 2005; Sun et al., 2009). This might be related to compartmentalization of cGMP signaling (Su et al., 2005). In the hypertrophied mouse heart, cGMP signaling activated by natriuretic peptides and nitric oxide was impaired. Our results showed that myocytes from these hearts had a reduced response to BNP, CNP and SNAP. Reduced effects of nitric oxide and natriuretic peptides had been reported previously in several in vivo and in vitro cardiac hypertrophy and failure models (Meyer et al., 2007; Moalem et al., 2006b). Similar reduced effects of cyclic GMP signaling have been observed after ischemia-reperfusion (Luciano et al., 2008). This may limit the protective role of this signaling system in heart failure. This is similar to reduced functional effects of catecholamine in heart failure (Carreno et al., 2006; Zaccolo and Movsesian, 2007).

The effect of inhibition of PKG with KT5823 was also abrogated in the hypertrophied myocytes. These results were consistent with our previous studies demonstrating a reduced importance of PKG in a variety of hypertrophic models (Katz et al., 2006; Moalem et al., 2006a; Su et al., 2005). Reduced effects of PKG were also observed after ischemia-reperfusion (Luciano et al., 2008). We performed an in vitro phosphorylation assay and found that PKG activity was depressed. In our current study, we also found that both the protein expression level and activity of PKG were downregulated in hypertrophy.

The major finding of this study was that we found protective effects of HIF-1 α upregulation in cardiac hypertrophy and that it caused an increased effectiveness of the functional effects of cGMP signaling system. Only a few studies have examined the effects of HIF-1 on cardiac hypertrophy. Shyu et al (Shyu et al., 2005) and Kim et al (Kim et al., 2002) found the expression of HIF-1 was upregulated in the hypertrophied heart responding to hemodynamic overload stress. In our experiment, we also observed that the HIF-1 expression level had slightly increased in the control hypertrophic mouse hearts. We also observed some improvements in baseline function of the hypertrophic mouse myocyte.

After deferoxamine injection, HIF-1 α was significantly upregulated. This further upregulation of HIF-1 reversed the cardiac hypertrophic myocytes' blunted response to cGMP based agents. This improvement appeared to be related to increased PKG protein levels. The beneficial effects of deferoxamine on cGMP signaling in hypertrophy may not be due to HIF-1 alone. However, its effect must be on the myocytes, since we examined isolated ventricular myocytes. Further, deferoxamine has been routinely used to increase the levels of HIF-1 in cells (Dery et al., 2005; Poellinger and Johnson, 2004; Semenza, 2000, 2004). This may be of benefit as the effectiveness of this cGMP system is reduced in heart failure (Zhang et al., 2005). Cyclic GMP signaling may protect against excessive cyclic AMP signaling in hypertrophy and failure. We found that HIF-1 could preserve PKG expression levels and maintain its kinase activity during cardiac hypertrophy. It is not clear whether deferoxamine also affects other aspects of cell function in cardiac hypertrophy.

There are no reports of any direct effect of HIF-1 on PKG expression. It is not clear how HIF-1 affected PKG expression and activity. Nevertheless, this appears to be part of its protective mechanisms improving cGMP signaling in the hypertrophied heart. More work is necessary to elucidate the mechanism for this protective effect of HIF-1 in hypertrophy. HIF-1 acts, in part, through activation of VEGF expression and its angiogenesis effects, which may protect myocytes (Jiang et al., 2007; Sano et al., 2007). Deferoxamine helps to maintain the function and effect of the protein kinase as well as its ability to phosphorylate proteins; however the mechanism is not known. It is also possible that the effect of deferoxamine was not related to its effect on HIF-1, e.g. effects on free radical formation, iron chelation, etc. Further work is necessary to determine whether there are direct effects on this protein kinase, effects on associated proteins or changes in the internal environment that increase PKG activity.

In summary, natriuretic peptides and nitric oxide reduce myocyte function in control myocytes. This was related to activation of PKG. These changes were not affected by increases in HIF-1 α expression (deferoxamine injection). In hypertrophic ventricular myocytes, the functional effects of the cGMP signaling system were impaired. Our results suggested that HIF-1 played a protective role in cardiac hypertrophy. Baseline function was improved and the functional effects of cGMP signaling were maintained. This was related to maintenance of PKG protein levels, activity and functional effects. Although further work is necessary, these findings suggest that HIF-1 may prove to be a therapeutic target protecting heart against damage caused by cardiac hypertrophy. This suggests a potential new modality for treatment of heart failure.

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A.

B.

Figure 1.

(Top) HIF-1 α protein expression levels in isolated myocytes from four groups of mice: Control, Control plus deferoxamine (Control+DFX), Hypertrophy and Hypertrophy+DFX detected by Western blot. Lanes were normalized to equal levels of the protein Ran. Note the increased expressions in hypertrophy and after DFX. (Bottom) The corresponding densitometric analysis of the relative expression levels of HIF-1 α protein normalized to Ran (mean $\pm$ SEM). (*: $P < 0.05$).

Figure 2. Effects of BNP (top), CNP (middle) and SNAP (bottom) followed by KT5823 on percentage shortening in freshly isolated ventricular myocytes from 4 groups of mice: Control, Control +DFX, Hypertrophy and Hypertrophy+DFX. Note reduced effects of these agents in Hypertrophy and maintenance of these effects in Hypertrophy+DFX. Data are shown as mean $\pm$ SEM. (N=7). *P<0.05 vs. baseline. + Change from previous treatment significantly different from hypertrophy alone.

Figure 3.

(Top, left panel) Western blot analysis of cyclic GMP-dependent protein kinase (PKG) protein expression levels in isolated myocytes from 4 groups of mice. Lanes were normalized using β -actin. (Top, right panel) The quantitative densitometric analysis of Western blot. (Bottom) In vitro cGMP-dependent protein phosphorylation in myocytes from 4 groups of mouse hearts. The left lanes from each group show the basal activities of protein phosphorylation. The middle lanes show protein phosphorylation after addition of 8-bromo-cGMP, 2.5×10^{-4} M. The right lane from each group shows phosphorylation in the presence of both 8-bromo-cGMP and the PKG inhibitor KT5823 7.5×10^{-4} M. Molecular weight standards (kDa) are shown on the left. 8-Bromo-cGMP activated PKG to phosphorylate 5 protein bands (selected for their response to PKG) at approximate molecular weights of 130, 100, 55, 32 and 24 kDa and KT5823 reduced this effect (labeled at the right). The activity and expression of the PKG was reduced in the hypertrophy alone group.

Table 1

Effects of BNP, CNP and SNAP on maximal rate of shortening and maximal rate of relaxation in ventricular myocytes from four groups of mouse hearts.

Maximal Rate of Shortening (μm/s)				
	Control	Control+DFX	Hypertrophy	Hypertrophy+DFX
Baseline	56.2±4.4	62.0±4.6	38.6±3.1 [#]	50.0±4.4
BNP				
10 ⁻⁸ M	49.7±2.9	53.5±3.9	35.3±2.4 [#]	44.0±3.7
10 ⁻⁷ M	46.0±1.7 [*]	52.8±4.3 [*]	36.4±3.2 [#]	41.9±4.4 [*]
+KT5823, 10 ⁻⁶	49.0±3.0	55.6±4.2	33.1±2.4 [#]	46.3±6.0 ⁺
Baseline	59.8±4.5	58.6±6.8	40.2±3.0 [#]	48.2±2.2
CNP				
10 ⁻⁸ M	49.6±3.2 [*]	51.3±7.4	38.8±3.3 [#]	43.8±2.6
10 ⁻⁷ M	51.5±2.0	46.6±7.6 [*]	37.1±3.0 [#]	42.0±1.6
+KT5823, 10 ⁻⁶	52.4±3.6	54.8±8.8	35.7±2.1 [#]	44.5±2.6
Baseline	47.7±2.7	65.9±4.9	44.0±3.9	47.3±4.6
SNAP				
10 ⁻⁶ M	43.2±2.8	54.9±4.5	40.8±3.2	43.1±4.7
10 ⁻⁵ M	39.4±3.4 [*]	51.3±3.7 [*]	41.1±4.0	40.8±4.7
+KT5823, 10 ⁻⁶	44.9±4.3	59.0±2.5	40.1±4.2	46.9±5.7 ⁺
Maximal Rate of Relaxation (μm/s)				
	Control	Control+DFX	Hypertrophy	Hypertrophy+DFX
Baseline	53.4±3.9	60.2±5.2	36.3±2.9 [#]	48.7±4.4
BNP				
10 ⁻⁸ M	48.2±3.1	52.0±4.2	34.6±2.5 [#]	42.7±4.0
10 ⁻⁷ M	44.7±1.4 [*]	50.5±4.5 [*]	37.4±3.1	41.4±4.6 [*]
+KT5823, 10 ⁻⁶ M	47.0±3.1	53.8±4.4	32.6±2.7 [#]	44.6±6.0
Baseline	59.0±4.2	55.5±6.5	37.9±2.9 [#]	46.7±2.5
CNP				
10 ⁻⁸ M	50.2±2.5	49.1±6.9	36.0±3.1 [#]	42.2±2.7
10 ⁻⁷ M	52.4±2.1	44.6±7.2 [*]	34.6±3.0 [#]	40.2±1.7
+KT5823, 10 ⁻⁶ M	51.8±3.5	52.5±8.5	33.8±2.4 [#]	43.7±3.0
Baseline	46.4±2.8	65.6±5.2	41.5±4.1	44.6±4.6
SNAP				
10 ⁻⁶ M	41.7±2.8	54.0±4.3	39.4±3.3	42.7±4.6
10 ⁻⁵ M	38.3±3.3	50.7±3.8 [*]	40.0±4.2	39.6±5.1

Maximal Rate of Relaxation ($\mu\text{m/s}$)				
	Control	Control+DFX	Hypertrophy	Hypertrophy+DFX
+KT5823, 10^{-6} M	43.7 $\pm$ 4.3	56.5 $\pm$ 2.4	39.0 $\pm$ 4.6 [#]	45.6 $\pm$ 6.3 ⁺

Note: values are means $\pm$ SEM.

* Significantly different from baseline.

[#] Significantly different from control group.

⁺ Significantly different from previous treatment.