Hypoxia Inducible Factor-1 Improves the Actions of Nitric Oxide and Natriuretic Peptides after Simulated Ischemia-Reperfusion

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Key Words

Cyclic GMP • Cyclic GMP protein kinase • Cardiac myocytes • Hypoxic Inducible Factor-1 • Ischemia-reperfusion • New Zealand white rabbit

Abstract

Ischemia-reperfusion reduces the negative functional effects of cyclic GMP in cardiac myocytes. In this study, we tested the hypothesis that upregulation of hypoxic inducible factor-1 (HIF-1) would improve the actions of cyclic GMP signaling following simulated ischemia-reperfusion. HIF-1 α was increased with deferoxamine (150 mg/kg for 2 days). Rabbit cardiac myocytes were subjected to simulated ischemia [15 min 95% N₂-5% CO₂] and reperfusion [reoxygenation] to produce myocyte stunning. Cell function was measured utilizing a video-edge detector. Shortening was examined at baseline and after brain natriuretic peptide (BNP, 10⁻⁸, 10⁻⁷M) or S-nitroso-N-acetylpenicillamine (SNAP, 10⁻⁶, 10⁻⁵M) followed by KT5823 (cyclic GMP protein kinase inhibitor, 10⁻⁶M). Kinase activity was measured via a protein phosphorylation assay. Under control conditions, BNP (-30%) and SNAP (-41%) reduced percent shortening, while KT5823 partially restored function (+18%).

Deferoxamine treated control myocytes responded similarly. In stunned myocytes, BNP (-21%) and SNAP (-25%) reduced shortening less and KT5823 did not increase function (+2%). Deferoxamine increased the effects of BNP (-38%) and SNAP (-41%) in stunning and restored the effects of KT5823 (+12%). The cyclic GMP protein kinase increased phosphorylation of several proteins in control HIF-1 +/- cells. Phosphorylation was reduced in stunned cells and was restored in deferoxamine treated stunned cells. This study demonstrated that simulated ischemia-reperfusion reduced the negative functional effects of increasing cyclic GMP and this was related to reduced effects of the cyclic GMP protein kinase. Increased HIF-1 α protects the functional effects of cyclic GMP thorough maintenance of cyclic GMP protein kinase activity after ischemic-reperfusion.

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Introduction

Hypoxic inducible factor-1 (HIF-1) is a major regulator of oxygen homeostasis [1]. While the HIF-1 β

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subunit is constitutively expressed within the cell, degradation of the HIF-1 α subunit occurs, but this degradation is reduced by hypoxia. DNA binding and upregulation of gene transcription by HIF-1 requires the presence of both the α and β subunits [2-6]. Once active, 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 [1, 7-9]. It is well established that HIF-1 increases inducible nitric oxide levels [10]. Activation of HIF-1 has also been shown to upregulate brain natriuretic peptide (BNP) leading to augmented intracellular cyclic GMP production, which may serve a cytoprotective role during ischemia [10-13]. Cyclic GMP activates cyclic GMP dependent protein kinases leading to the phosphorylation of proteins and reduced myocyte function [14]. It has been shown that the nitric oxide-cyclic GMP pathway also affects HIF-1 [15, 16].

Exposure of cardiac myocytes to a period of ischemia leads to a decrement in cellular function that if sustained long enough, results in irreversible cell injury leading to myocardial necrosis and apoptosis. Even with rapid reperfusion, myocyte function is often reduced, called myocardial stunning [17]. With time, stunning reverses and function can be restored. Based upon the target genes of HIF-1, it has been suggested that increases in HIF-1 levels can protect cardiac myocytes from the short and more long-term effects of ischemia-reperfusion [4-6, 18, 19]. Agents that raise the level of cyclic GMP may also protect against myocardial stunning [20-22]. This protection by cyclic GMP occurs despite ischemia-induced damage to cyclic GMP protein kinase activity [23]. The interaction between HIF-1 and cyclic GMP signaling has not been studied after myocyte ischemia-reperfusion.

In this study, we tested the hypothesis that upregulation of HIF-1 would improve the actions of cyclic GMP signaling following simulated ischemia-reperfusion. To test this hypothesis, we pre-treated rabbits with deferoxamine (an agent that increases the level of HIF-1 α) before exposing isolated cardiac myocytes to simulated ischemia (95% N₂-5% CO₂)-reperfusion. Cellular function was then measured at baseline and in the presence of agents that raise cytosolic cyclic GMP, BNP and S-nitroso-N-acetyl-penicillamine (SNAP) as well as a cyclic GMP protein kinase inhibitor (KT5823). We demonstrated that upregulation of HIF-1 α diminished the detrimental effects of simulated ischemia-reperfusion upon the cyclic GMP protein kinase signaling pathway in cardiac myocytes.

Materials and Methods

The investigation was conducted in accordance with the Guide for the Care of Laboratory Animals (DHHS Publication 85-23, revised 1996) and was approved by our Institutional Animal Care and Use Committee.

Myocyte Isolation

Ventricular myocytes were isolated from hearts of New Zealand white rabbits ($n = 15$, 2-3 kg) as previously described [24]. Briefly, the rabbits were anesthetized with sodium pentobarbital (35 mg/kg) followed by the administration of heparin (10 unit/g body weight) intravenously using the circumflex ear vein. The heart was immediately removed after an overdose of pentobarbital (60 mg/kg) and retrograde perfused through the aorta with minimal essential medium (MEM, Sigma) supplemented with 10 mM taurine, 2 mM L-glutamic and 20 mM HEPES, pH 7.2. After 5 min of perfusion with MEM, the heart was perfused with MEM containing 0.1% type II collagenase (Worthington, Freehold, NJ) for 16 min. The perfusion buffer was maintained at 37°C and equilibrated with water-saturated gas mixture (95% O₂-5% CO₂). After collagenase digestion, the heart was removed from the apparatus, and the ventricles were cut into small pieces. The tissue suspension was further treated with MEM containing 0.1% collagenase and 0.5% bovine serum albumin (BSA) at 37°C and gently swirled at 2 cycles/sec for 5 min. The supernatant containing isolated myocytes was then transferred to 15-ml centrifuge tubes. The cells were washed three times in MEM containing 0.5% BSA and centrifuged at low speed (34 g). Cell viability was assessed by the maintenance of rod-shaped morphology and was routinely between 40-60%.

Myocyte functional measurement

Myocyte function was studied in individual ventricular myocytes. Cells were suspended in 2 ml of MEM supplemented with 2 mM Ca²⁺ and 0.5% BSA maintained at 37°C in a chamber that was fitted onto the stage of an inverted light microscope (Zeiss Axiovert 125). Two platinum wires placed in the chamber were used to pace the myocytes (1 Hz, 5-ms duration, voltage 10% above threshold, and polarity alternated with each pulse). The contraction of individual myocytes was measured with a Myotrack system containing a camera and a video-edge detector (Crystal Biotech, model VED-114, Data Sciences International). The contraction data were continuously obtained on a television monitor and a desktop computer. The parameters obtained during a single contraction included the diastolic cell length, percentage shortening, maximal rate of relaxation, maximal rate of shortening, time to peak shortening, time to 50% relaxation, and time to 90% relaxation.

Experimental Protocol

Eight groups of myocytes were used in the following protocol for cell function measurements. One group of rabbits ($n=7$) served as control animals, while the other group ($n=8$) was injected with deferoxamine (150 mg/kg/day) subcutaneous for two days prior to experimentation. Following myocyte isolation, half of the cells (stunning group) were subjected to

simulated ischemia (15 min of 95% N₂-5% CO₂ at 37°C), which reduced the PO₂ to no higher than 6 mm Hg. These myocytes were then subjected to 30 min of reoxygenation (simulated reperfusion) prior to functional measurements. In all groups, myocytes were suspended in a chamber with 2 ml of MEM containing 2 mM Ca²⁺ and 0.5% BSA. After a 5-min stabilization period paced with electrical field stimulation, baseline contraction data for an individual myocyte was recorded. A 5-min interval was allowed between reagent treatments and 10 consecutive contractions were used for analysis. In the first set of cells, B-type natriuretic peptide (BNP) was added at a concentration of 10⁻⁸ M, followed by a higher dose of BNP 10⁻⁷ M, followed by KT5823 10⁻⁶, a cyclic GMP protein kinase inhibitor. In the second set, SNAP, a NO donor, was added at a concentration of 10⁻⁶ M, followed by a higher dose of SNAP 10⁻⁵ M, followed by KT5823 10⁻⁶ M. For each protocol, at least 3 control and 3 stunned cells were measured in each animal.

Myocyte Extract Preparation

Myocytes obtained from the control and deferoxamine treated rabbits were subjected to a cell extraction procedure and in vitro phosphorylation assay. A suspension of myocytes was centrifuged at 34 g. The pellet was collected and frozen immediately at -80°C. Myocytes were homogenized for 15 sec at 20,000 rpm (Polytron homogenizer) in buffer (0.25 M sucrose, 5 mM Tris, and 1 mM MgCl₂, pH 7.4) and centrifuged at 15,500 rpm at 4°C for 20 min. The supernatant was harvested and used as the myocyte extract for the phosphorylation assay. BSA was used as the standard. The protein concentration of each sample was determined by the Bradford dye-binding procedure using a spectrometer at 595 nm and adjusted to 5 mg/ml. Cell extract aliquots were stored at -80°C.

HIF-1 Western Blotting

The protein concentration of the normoxic myocyte extracts were equalized to 1 mg/ml. Extracts were heated at 95°C for 5 min. Proteins were electrophoresed and separated by 10% SDS-PAGE using the BioRad mini-gel. The gel was transferred to nitrocellulose paper using BioRad transfer apparatus with 20% methanol, 192 mM glycine and 25 mM Tris buffer. Following electrophoretic transfer, the blot was blocked using 5% dry milk buffer for 1 hr at room temperature with agitation. The blot was incubated with mouse anti-HIF-1 α antibody and mouse anti-Ran (Novus Biologicals) at 4°C overnight. The blot was rinsed with TBST (Tris with Tween) and then incubated with a horseradish peroxidase-conjugated anti-mouse antibody for 2 hr at room temperature (Pierce Biotechnology) and developed with an enhanced chemiluminescence detection system.

Protein Phosphorylation Assay

Myocytes isolated from the rabbit ventricles were homogenized (Polytron Homogenizer, 15 sec at 20,000 rpm) in buffer (5 mM Tris HCl, pH 7.4, 1 mM MgCl₂, 0.25 M sucrose, 10x protease inhibitor, 1 mM IBMX) and centrifuged at 15,500 rpm for 20 min at 4°C. The supernatant was divided and used as the myocyte extract for the phosphorylation reactions. 8-Bromo-cGMP (8-Br-cGMP, 2.5 x 10⁻⁴ M, an activator of cyclic

Fig. 1. Effects of injection of deferoxamine subcutaneously for two days prior to myocyte isolation. Intracellular levels of HIF-1 α and the control protein Ran were determined from extracts from isolated cardiac myocytes via Western blot. Please note that deferoxamine upregulates the intracellular levels of HIF-1 α .

GMP protein kinase) and an inhibitor of cyclic GMP protein kinase (KT5823, 7.5 x 10⁻⁴ M) were added to a 10 μ l cell extract (2 mg total protein per ml). The reactants were kept at room temperature and allowed to equilibrate for 10 min. They were then cooled on ice and 0.5 μ l [γ -³³P] ATP at 10 μ Ci/ μ l was added to initiate the reaction. The reaction was terminated 15 min later by adding a volume of BioRad reducing sample buffer equal to the entire reaction volume. 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 hours, dried using a gel drier kit (Promega) and exposed to X-ray film at room temperature for 36 hours. The exposed X-ray film demonstrated phosphate labeled proteins that were then sized by comparison to molecular weight standard markers.

Statistical Analysis

A repeated measure analysis of variance was used to compare variables measured in the experiment. Duncan's multiple range test was used to compare differences between baseline and the various treatments post hoc. A value of P < 0.05 was used as the level of statistical significance. All results are expressed as mean $\pm$ S.E.M.

Results

Eight animals were injected with deferoxamine (150 mg/kg/day) for the two days immediately preceding cardiac myocyte isolation. The cardiac myocytes isolated from the deferoxamine injected animals showed a significant upregulation in the levels of HIF-1 α as compared to myocytes from the non-injected animals (Figure 1).

Upregulation of HIF-1 α in the control animals did not significantly affect baseline functional data. The percentage shortening, maximal rate of shortening,

Table 1. Baseline functional data for all groups. Note: Values are represented as mean $\pm$ S.E.M. * Significantly different from Control. + Significantly different from Stun.

	Control (n=7)	HIF-1 (n=8)	Stun (n=7)	Stun+HIF-1 (n=8)
Percent shortening (%)	5.9 $\pm$ 0.3	6.0 $\pm$ 0.3	4.6 $\pm$ 0.4*	5.6 $\pm$ 0.3 ⁺
Maximal rate of relaxation (μ m/sec)	57.8 $\pm$ 3.6	55.8 $\pm$ 3.1	49.7 $\pm$ 3.1	48.3 $\pm$ 3.1
Maximal rate of shortening (μ m/sec)	59.8 $\pm$ 3.8	58.7 $\pm$ 3.0	51.2 $\pm$ 3.3	52.0 $\pm$ 3.8
Time to peak shortening (sec)	0.27 $\pm$ 0.02	0.26 $\pm$ 0.01	0.30 $\pm$ 0.02	0.29 $\pm$ 0.01
Time to 90% relaxation (sec)	0.26 $\pm$ 0.02	0.26 $\pm$ 0.01	0.30 $\pm$ 0.01	0.29 $\pm$ 0.01

	Baseline	BNP 10 ⁻⁸ M	BNP 10 ⁻⁷ M	KT5823 10 ⁻⁶ M	Baseline	SNAP 10 ⁻⁶ M	SNAP 10 ⁻⁵ M	KT5823 10 ⁻⁶ M
Control								
Maximal Rate of Relaxation (μ m/sec)	57.8 $\pm$ 3.6	48.3 $\pm$ 3.7	44.4 $\pm$ 4.9*	51.8 $\pm$ 4.7	64.0 $\pm$ 10.7	48.2 $\pm$ 5.9	35.9 $\pm$ 3.0*	42.1 $\pm$ 5.5 [†]
Maximal Rate of Shortening (μ m/sec)	59.8 $\pm$ 3.8	50.2 $\pm$ 3.6	46.1 $\pm$ 5.0*	53.6 $\pm$ 4.8	66.2 $\pm$ 10.5	49.1 $\pm$ 6.1	36.3 $\pm$ 3.2*	42.6 $\pm$ 5.6
HIF-1								
Maximal Rate of Relaxation (μ m/sec)	55.8 $\pm$ 3.1	47.5 $\pm$ 3.9	40.7 $\pm$ 4.2*	49.3 $\pm$ 3.1 [†]	57.8 $\pm$ 4.0	48.3 $\pm$ 2.7*	38.1 $\pm$ 1.8*	43.6 $\pm$ 3.7 [†]
Maximal Rate of Shortening (μ m/sec)	58.7 $\pm$ 3.0	49.1 $\pm$ 3.7	41.4 $\pm$ 4.3*	50.1 $\pm$ 3.5 [†]	62.1 $\pm$ 4.2	50.3 $\pm$ 3.1	39.1 $\pm$ 1.8*	42.9 $\pm$ 2.5
Stun								
Maximal Rate of Relaxation (μ m/sec)	49.7 $\pm$ 3.2	41.8 $\pm$ 2.8	36.7 $\pm$ 2.9*	37.9 $\pm$ 2.3*	44.5 $\pm$ 2.6	41.1 $\pm$ 2.4	36.3 $\pm$ 2.9*	34.5 $\pm$ 3.4*
Maximal Rate of Shortening (μ m/sec)	51.2 $\pm$ 3.4	42.7 $\pm$ 3.0	37.5 $\pm$ 2.9*	39.1 $\pm$ 2.3*	47.2 $\pm$ 2.8	42.7 $\pm$ 2.7	37.2 $\pm$ 2.7*	35.3 $\pm$ 3.2*
Stun + HIF-1								
Maximal Rate of Relaxation (μ m/sec)	48.3 $\pm$ 3.1	40.1 $\pm$ 2.8	33.1 $\pm$ 2.7*	35.5 $\pm$ 1.9	54.0 $\pm$ 4.8	45.1 $\pm$ 4.7	36.3 $\pm$ 2.7*	39.4 $\pm$ 3.0
Maximal Rate of Shortening (μ m/sec)	52.0 $\pm$ 3.9	42.3 $\pm$ 2.8	34.5 $\pm$ 2.8*	36.3 $\pm$ 2.1	57.4 $\pm$ 5.3	47.8 $\pm$ 5.1	37.9 $\pm$ 2.8*	40.0 $\pm$ 3.1

Table 2. Effect of BNP, SNAP and KT5823 on maximal rate of relaxation and shortening under control conditions, deferoxamine treatment, stunning and stunning + deferoxamine. Note: values are means $\pm$ S.E.M. * Significantly different from baseline. [†] Significantly different from previous treatment.

maximal rate of relaxation, time to peak shortening and time to 90% relaxation were similar under basal conditions (Table 1). Furthermore, HIF-1 α upregulation did not significantly alter the functional responses of the control cells following the addition of BNP, SNAP, or KT5823 (Figure 2 and 3 and Table 2).

In the non-stunned cells, when BNP (10⁻⁸ and 10⁻⁷ M) was added to the myocytes, percent shortening was reduced in a dose dependent manner by 21% and 30% in the control cells and 23% and 36% in the deferoxamine treated cells (Figure 2 Top). The percentage shortening was significantly lower in the presence of 10⁻⁸ and 10⁻⁷ M BNP than baseline for all conditions. The maximal rate of shortening was depressed by 16% and 23% in control cells and 16% and 29% in deferoxamine treated cells and the maximal rate of relaxation was depressed by 17% and 23% in control cells and 15% and 27% in deferoxamine treated cells (Table 2).

When SNAP (10⁻⁶ and 10⁻⁵ M) was added to the non-stunned myocytes, percent shortening was reduced by 26% and 41% in the control cells and 23% and 42% in the deferoxamine treated cells (Figure 3 Top). The dose of 10⁻⁵ SNAP was significantly different from baseline for both groups. The maximal rate of shortening was depressed by 26% and 45% in control cells and 19% and 37% in deferoxamine treated cells and the maximal rate of relaxation was depressed by 25% and 44% in control cells and 16% and 34% in deferoxamine treated cells (Table 2). For both the maximal rate of shortening and relaxation, the effects of 10⁻⁵ SNAP were statistically significant compared to the basal levels.

The addition of the cyclic GMP protein kinase inhibitor KT5823 (10⁻⁶ M) to the non-stunned cells partially restored the decrement in cellular function that followed the addition of BNP or SNAP. This effect was statistically significant in the control and deferoxamine treated cells

Fig. 2. Ventricular myocyte percentage shortening in non-stunned (Top) and stunned (Bottom) cells. Myocyte contractions were measured in non-treated control cells (CON) and cells pre-treated with deferoxamine (HIF). Percent shortening is shown at baseline and after increasing concentrations of BNP followed by the cyclic GMP protein kinase inhibitor (KT5823). BNP reduced percent shortening and KT5823 partially restored these values. In the stunned cells, KT5823 had no significant effect. Deferoxamine restored the effects of KT5823 after stunning. * Statistically different from baseline. † Statistically different from BNP 10^{-7} M.

after BNP for percent shortening. After SNAP, KT5823 restored percent shortening in the deferoxamine treated cells. Similar effects of KT5823 were also observed in the maximal rate of shortening and relaxation data, Table 2.

Following simulated ischemia-reperfusion, baseline percent shortening was significantly reduced, Table 1. The declines in basal maximal rate of shortening and maximal rate of relaxation were not significant. The additions of BNP or SNAP were able to significantly reduce percent shortening from the stunned baseline; however these decrements were less than in control myocytes, Figures 2 and 3. Similar decrements were observed in maximal rate of shortening and relaxation after BNP and SNAP, Table 2. The addition of 10^{-6} M KT5823 was unable to significantly improve the cellular function of stunned cells for all observed parameters

Fig. 3. Ventricular myocyte percentage shortening in non-stunned (Top) and stunned (Bottom) cells. Myocyte contractions were measured in non-treated control cells (CON) and cells pre-treated with deferoxamine (HIF). Percent shortening is shown at baseline and after increasing concentrations of SNAP followed by the cyclic GMP protein kinase inhibitor (KT5823). SNAP reduced percent shortening and KT5823 partially restored these values. In the stunned cells, KT5823 had no significant effect. Deferoxamine restored the effects of KT5823 after stunning. * Statistically different from baseline. † Statistically different from SNAP 10^{-5} M.

following SNAP or BNP in the control stunned cells.

Deferoxamine treated stunned cells exhibited a basal percent shortening that was significantly elevated compared to non-treated stunned cells. The effect of HIF-1 upregulation on basal maximal rate of shortening and relaxation was not significant. In the deferoxamine treated stunned cells, increasing doses of SNAP reduced the maximal rate of shortening by 17% and 34%, maximal rate of relaxation by 16% and 33%, and percent shortening by 27% and 41%. BNP reduced the maximal rate of shortening by 19% and 34%, maximal rate of relaxation by 17% and 32%, and percent shortening by 21% and 38%. These changes were similar to those observed in the non-treated stunned cells. However, all parameters increased following the addition of KT5823.

The protein phosphorylation activity of the cyclic GMP protein kinase was also tested in ventricular

Fig. 4. Cyclic GMP protein kinase activity as measured through incorporation of γ -P³³ radiolabeled cyclic AMP into cellular proteins. Molecular weights (left) were determined by comparison to molecular weight standard markers. Protein bands increased by cyclic GMP are shown on the right. Cyclic GMP increased the activity at the reported bands in cardiac myocytes and the cyclic GMP protein kinase inhibitor KT5823 reduced this activity under all treatments except stunning alone.

myocyte extracts. Basal phosphorylation levels were similar in all four groups, Figure 4. The increases in the phosphorylation level after cyclic GMP were not significantly different when comparing HIF-1 +/- non-stunned cellular extracts. These increases could be blocked by the addition of the cyclic GMP protein kinase inhibitor KT5823. Following stunning, the phosphorylation level of several protein bands was reduced after the addition of cyclic GMP. Upregulation of HIF-1 prior to stunning resulted in a protein-banding pattern that was increased as compared to the control stunned level.

Discussion

Agents, such as BNP and SNAP, that increase the level of cyclic GMP in cardiac myocytes decrease cell function, primarily through effects of the cyclic GMP dependent protein kinase. Simulated ischemia-reperfusion decreases the negative inotropic effects of cyclic GMP through decrements in cyclic GMP protein kinase activity. The major finding of this study was that upregulation of HIF-1 served to improve the negative inotropic effects of BNP and SNAP via protection of cyclic GMP dependent protein kinase activity following simulated ischemia-reperfusion. Inhibition of cyclic GMP protein kinase activity through the introduction of KT5823 reduced the negative effects that BNP and SNAP induced within the cardiac myocytes under control and HIF-1 + stunned conditions, however, it failed to significantly alter myocyte function in stunned cells not pre-treated with deferoxamine.

Consistent with previous studies, we showed that natriuretic peptides and nitric oxide have a concentration-dependent negative inotropic effect in control cardiac myocytes [24-26]. Application of BNP and SNAP resulted in an increased activity of particulate and soluble guanylyl cyclase respectively which leads to a significant increase

in the intracellular levels cyclic GMP [27, 28]. This rise in cyclic GMP in turn leads to increased phosphorylation of target proteins by the cyclic GMP protein kinase including the L-type Ca²⁺ channel and phospholamban [29, 30]; thereby leading to a concomitant increase in the activity of SERCA as well as a reduction in the influx of calcium into the cell. This leads to a reduction in the overall level of myocyte contractile function. Cyclic GMP acts via a variety of mechanisms including: 1) protein phosphorylation through cyclic GMP-dependent protein kinases, 2) direct effects on ion channels including the inhibition of L-type calcium channels, 3) cyclic GMP-stimulated or inhibited cyclic AMP phosphodiesterases, 4) activation of sarcoplasmic reticulum Ca²⁺-ATPase [14, 31, 32]. The major mechanism for the functional effects of cyclic GMP appears to be through actions of the cyclic GMP protein kinase [30, 32].

In non-stunned cells, the upregulation of HIF-1 had minimal effect upon the cellular baseline function or the cell's ability to respond to cyclic GMP dependent negative inotropic agents [9, 26]. 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 [1, 7-9]. Activation of these genes does not seem to play a major role under baseline conditions in cardiac myocytes. The cyclic GMP system also appears unaffected by HIF-1 upregulation under basal conditions. Furthermore, our study showed that in cells not subjected to simulated ischemia-reperfusion, HIF-1 had minimal effects upon the activity of the cyclic GMP protein kinase.

Following simulated ischemia-reperfusion, cardiac myocytes showed a significant reduction in the basal level of percentage shortening. The maximal rate of shortening and maximal rate of relaxation were also decreased, but this was not significant. Previous studies have also demonstrated reduced cardiac myocyte function after

simulated or true ischemia-reperfusion [17, 20-23].

Increases in cyclic GMP may be protective after ischemia-reperfusion [33, 34]. Nitrates, which increase cyclic GMP, have long been used to treat coronary artery disease. However, the cardiac myocytes used in the present study showed a reduced capability to respond to the cyclic GMP based negative inotropic agents, BNP and SNAP, or to the cyclic GMP protein kinase inhibitor KT5823. It has previously been shown that ischemia-reperfusion reduced the importance of the cyclic GMP protein kinase in cardiac myocytes [23]. This evidence in combination with our cyclic GMP protein kinase based protein phosphorylation assay suggested that the reduced functional effects of BNP and SNAP observed following simulated ischemia-reperfusion might result from diminished cyclic GMP protein kinase activity.

Cells that were pre-treated with deferoxamine and subjected to simulated ischemia-reperfusion showed reduced signs of baseline functional decrements. Though elevated as compared to control stunned cells, these functional values still remain slightly below those measured in non-stunned cells. Thus, pre-treatment may partially, but not fully, protect cardiac myocytes from the function decrements that result from simulated ischemia-reperfusion based injuries. There is much evidence that HIF-1 upregulation can be beneficial in terms of cell protection after ischemia-reperfusion [4, 6, 11, 19]. Several cardioprotective genes are upregulated by HIF-1 after ischemia-reperfusion [1, 7-9].

Upregulation of HIF-1 also restored the cell's negative functional response to the cyclic GMP based agents, BNP and SNAP, and the cyclic GMP protein kinase inhibitor KT5823. HIF-1 protected against the loss of the ability of the cyclic GMP protein kinase to phosphorylate proteins that results from simulated ischemia-reperfusion injury. The mechanism for this protective effect is not known. HIF-1 activation does lead to significant increases in inducible nitric oxide synthase and BNP [10, 13], but there are no reported direct effects of HIF-1 on the cyclic GMP protein kinase.

Since cyclic GMP can be protective after ischemia-reperfusion [33, 34], upregulation of HIF-1 may lead to increases in this beneficial effect. Nitrates have been used

for over one-hundred years to treat coronary artery disease. Increases in the level of HIF-1 appear to activate the cyclic GMP protein kinase. However, other effects of HIF-1 pretreatment on cyclic GMP-regulated phosphodiesterases and cyclic nucleotide-gated ion channels must still be examined [35-37]. Additionally, it is not yet clear how the interaction between HIF-1 and the cyclic GMP protein kinase occurs during myocardial stunning. Interestingly, HIF-1 and the cyclic GMP protein kinase may affect the same protein SOX9 [9, 11, 38]. It is clear that 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 this effect of deferoxamine is not related to its effect on HIF-1, such as effects on free radical formation. 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 cyclic GMP protein kinase activity.

In summary, we found that agents that increased cardiac myocyte levels of cyclic GMP, BNP and SNAP, reduced myocyte function and this was primarily related to activation of the cyclic GMP protein kinase. Simulated ischemia-reperfusion reduced the negative inotropic effects of BNP and SNAP and reduced the actions of the cyclic GMP protein kinase. HIF-1 activation did not significantly alter basal shortening, the effects of cyclic GMP or the cyclic GMP protein kinase activity under resting conditions. However, after simulated ischemia-reperfusion, it led to a partial restoration of basal function and increased effects of cyclic GMP and its protein kinase. We suggest that the improved ability to respond to the negative inotropic agents BNP and SNAP following simulated ischemia-reperfusion caused by HIF-1 activation primarily resulted from protection of cyclic GMP dependent protein kinase activity.

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