Inhibition of p66ShcA redox activity in cardiac muscle cells attenuates hyperglycemia-induced oxidative stress and apoptosis.

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Abstract

Apoptotic myocyte cell death, diastolic dysfunction and progressive deterioration in LV pump function, characterize the clinical course of diabetic cardiomyopathy. A key question concerns the mechanism(s) by which hyperglycemia (HG)-transmits danger signals in cardiac muscle cells (CMC). The growth factor adapter protein, p66ShcA is a genetic determinant of longevity, which controls mitochondrial metabolism and cellular responses to oxidative stress. Here, we demonstrate interventions that attenuate or prevent HG-induced phosphorylation at critical position 36 Ser residue (phospho-Ser-36), inhibit the redox function of p66ShcA and promote the survival phenotype. Adult rat ventricular myocytes (ARVM) obtained by enzymatic dissociation were transduced with mutant 36 p66ShcA (mu-36) dominant negative expression vector and plated in serum free media (SFM) containing 5 mM or 25 mM glucose. At HG, ARVM exhibit marked increase in reactive oxygen species (ROS) production, upregulation of phospho-Ser-36, collapse of mitochondrial transmembrane potential ($\Delta \psi_m$) and increased formation of p66ShcA/cytochrome c complexes. These indices of oxidative stress were accompanied by 40% increase in apoptosis and upregulation of cleaved caspase 3 and the apoptosis related proteins p53 and Bax. To test if p66ShcA functions as a redox sensitive molecular switch in vivo, we examined hearts of male Akita diabetic nonobese (C57BL/6J) mice. Immunoblot analysis detected upregulation of phospho-Ser-36, translocation of p66ShcA to mitochondria and formation of p66ShcA/cytochrome c complexes. Conversely, correction of HG by adenoassociated viral delivery of leptin (rAAV-Lep) reversed these alterations. We conclude p66ShcA is a molecular switch
whose redox function is turned on by phospho-Ser-36 and turned off by interventions that prevent this modification.

**Keywords:** Diabetes mellitus, Hyperglycemia, Oxidative stress, Reactive Oxygen Species.
**Introduction**

Apoptotic myocyte cell death, diastolic dysfunction and progressive deterioration in left ventricular (LV) pump function characterize the clinical course of diabetic cardiomyopathy (3, 13, 20, 30). Hyperglycemia (HG) and diabetes mellitus (DM) are associated with an exponential increase in reactive oxygen species (ROS) production at the cellular level (4, 15), which may play a causal role in the development of diabetic complications (2, 4). Mitochondria are the primary source of reactive oxygen intermediates and critical determinants of cell death and cell survival (7). HG increases the generation of superoxide anion ($\text{O}_2^-\bullet$) by interfering with the flow of electrons along the mitochondrial electron transport chain (4). In recent communications, our laboratory has provided compelling evidence that signaling molecules of the IGF-1/insulin pathway can attenuate or prevent HG-induced ROS production, oxidative DNA damage and apoptosis (19, 36). Since adult cardiac muscle cells (CMC) possess a finite capacity to proliferate, myocyte cell death is a critical determinant of ventricular remodeling and progression to heart failure. It seems reasonable to infer that strategies, which interrupt or suppress initiation of the apoptosis program, may offer an innovative approach to preserve CMC number and LV pump function.

The p66ShcA protein is one of three isoforms encoded at the mammalian ShcA locus. The three overlapping Shc proteins, p66ShcA, p52ShcA and p46ShcA all share a C-terminal SH2 domain, central collagen homology region (CH) and N-terminal phosphotyrosine-binding domain. p46ShcA and p52ShcA are the products of alternative translation initiation sites within the same transcript, whereas p66ShcA is distinguished by a unique N-terminal region (CH2), generated by alternative splicing (11, 26). The
ShcA family of proteins is cytoplasmic substrates for the activated IGF-1/insulin receptors. p46 and p52 participate in mitogenesis via the recruitment of the Ras signaling pathway (26), whereas p66ShcA by virtue of its unique N-terminal region, is a genetic determinant of longevity (9,28), that controls mitochondrial metabolism (9). In the proposed scheme, phosphorylation at a critical Ser-36 (phospho-Ser-36) residue position activates p66ShcA redox function, by facilitating its translocation to mitochondria, where p66ShcA generates ROS via the oxidation of cytochrome c. The latter redox reactions result in the opening of the mitochondrial transition pore, collapse of mitochondrial transmembrane potential (Δψm) and cytochrome c release. We have proposed a model in which p66ShcA redox function is shut down by interrupting HG-induced phosphorylation at the critical Ser-36 residue, preventing translocation to mitochondria, where p66ShcA functions as a ROS producer, resulting in organelle dysfunction and cell death.

In the current study, adult rat ventricular myocytes (ARVM) were genetically engineered to express mu36 p66ShcA (mu-36) dominant negative expression vector, to test the hypothesis mu-36 inhibit p66ShcA redox function by attenuating phospho-Ser-36 and promotes the survival phenotype. To evaluate p66ShcA signaling in diabetic myocardium, studies were performed with male Akita mice. Furthermore, Akita mice were treated with recombinant adenoassociated virus (rAAV) vector containing leptin cDNA (33,35), to test if correction of HG protects CMC from p66ShcA dependent signals that target mitochondria, oxidizing cytochrome c to generate ROS and promote activation of the terminal apoptosis program. Our results indicate p66ShcA is a molecular switch whose redox function is turned on by HG-induced phospho-Ser-36 and turned off by interventions that prevent this modification.
Materials and Methods

All experiments were performed under a protocol approved by the UMDNJ Institutional Animal Care and Use Committee.

Reagents. Sodium orthovanadate, Triton X-100, EDTA, EGTA and HEPES buffers were purchased from Sigma Aldrich Inc. Aprotinin, leupeptin, phenylmethyl sulfonyl fluoride (PMSF), and protease inhibitor cocktails (Type I and III) were purchased from Calbiochem-Novabiochem Inc.

Isolation of adult rat ventricular myocytes and cell culture. Adult Rat Ventricular Myocytes were isolated from the hearts of adult male Sprague-Dawley rats (175–225 g) as described (23-25). Briefly, hearts were removed from the chest cavity, suspended and rinsed with minimum essential media (MEM; Sigma Chemical Co.). Cardiac myocytes were isolated by enzymatic dissociation with collagenase type II (Worthington). Briefly, myocytes were washed with MEM buffer (∗2) and resuspended in incubation buffer (MEM plus 0.1mM BSA) in serum-free medium (SFM) with incremental addition of CaCl₂ at concentrations of 0.25mM, 0.5mM and 1mM respectively. Myocytes were washed and resuspended in SFM. Freshly isolated myocytes were plated in laminin coated petri dishes at a density of 2×10⁴ cells/cm² and incubated in SFM at 37°C in an atmosphere containing 5% CO₂. Routinely, 80–90% of cells obtained were viable myocytes, as determined by their characteristic morphological shape, striations, and trypan blue staining.

Animals: Six weeks old WT male C57BL/6J and diabetic nonobese Akita mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were housed one mouse
per cage in a temperature (21°C) and light-controlled (10 h light: 14 h dark), specific pathogen-free environment with chow diet and water available ad libitum throughout the experiment. Institutional Animal Care and Use Committee of the University of Florida approved the experimental protocols.

Experimental design: The non-immunogenic, nonpathogenic and replicative deficient recombinant virus vector encoding either leptin (rAAV-lep) or green fluorescent protein (rAAV-GFP, control) was packaged, purified, concentrated and titred in the Vector Core Laboratory at the University of Florida as described earlier and used in the previous studies (1,33). GFP in this study was used as an internal control. These vectors were used to investigate the effects of leptin on diabetic hearts. Two weeks after arrival, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), blood samples were collected and glucose levels were determined. Thereafter, each genotype of mice was divided into subgroups and underwent the following experimental procedures.

Mice were anesthetized and one subgroup of each genotype (n = 6) was injected intracerebroventricularly (icv, 1.5 μl) rAAV-GFP (1.9 × 10^9 infectious particles), or rAAV-lep (8.6 × 10^7 infectious particles) as described (1, 33). Mice were monitored at weekly intervals after icv injection. At 8-10 weeks post icv injections, the mice were anesthetized with sodium pentobarbital and mice were sacrificed by decapitation and hearts were dissected out and stored frozen at −80 °C for various analyses.

Construction of Adenovirus (p66ShcA). Recombinant adenoviruses were constructed, propagated, and tittered as previously described by Dr. Frank Graham (10). The viruses were purified on a cesium chloride gradient followed by dialysis against 20mM Tris...
buffered saline with 2% glycerol. As a negative control, green florescence protein (GFP) virus was employed. ARVM were cultured in SFM containing 5 mM glucose (Normal Glucose, NG) and 25mM glucose (High Glucose, HG) and incubated for 4 h at 37°C. Cells were infected with adenovirus vector (5) expressing dominant negative (DN) mutant 36 p66ShcA construct (MOI, 10-20). After 16 h incubation cells were used for further studies.

**Analysis of DNA fragmentation by ELISA.** Histone-associated DNA fragments were quantified by Cell Death Detection ELISA (Roche Diagnostic, Branchburg, NJ) as previously described (19,36).

**Detection of HG-induced oxidative stress.** Glucose-mediated oxidative stress in cardiac myocytes was studied by trafficking of 2,3,4,5,6-pentafluorodihydrotetramethylrosamine (PF-H2TMRos or Redox Sensor Red CC-1; Molecular Probes) using fluorescence microscopy as previously described (6). Redox Sensor Red CC-1 is oxidized in the presence of O$_2$-• and H$_2$O$_2$. Cells were washed with PBS and visualized using Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device (CCD) camera (Nikon DXM1200). The staining was performed in quadruplicate for each group, and 30 random fields (average 500 cells) were studied in each replicate. Images were captured using Nikon ACT-1 (Version 1.12) software and combined for publishing format using Adobe Photoshop 7.0 software.

**Assessment of Δψm.** Mitochondrial Δψm was monitored with the dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) using fluorescence microscopy (19). Cardiac myocytes were seeded and infected
with adenovirus vector expressing dominant negative (DN) mutant 36p66ShcA construct as described (19). At the end of the incubation, cells were loaded with JC-1 cationic dye (0.5 µg/ml) added to respective media and incubated at 37°C for 15 min. Cells were washed three times with PBS (1x) and visualized using Nikon fluorescence microscope. JC-1 dye exhibits potential-dependent accumulation in mitochondria (J-aggregates; accumulate at high membrane potential), indicated by a fluorescence shift from green to red fluorescence. Green fluorescence reflects the monomeric form of JC-1, appearing in cytosol after mitochondrial membrane depolarization. The staining was performed in quadruplets for each group as described earlier (19).

**Immunoblotting.** ARVM were harvested from the petridishes and washed with PBS. The pellets were lysed on ice with 300 µl of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA 10% EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 0.2 mM sodium orthovanadate, 10 mg/ml aprotinin) and protease inhibitor cocktails (Type I and III). For *in vivo* (Akita mice) study, cardiac tissue samples (20-25mg) were lysed on ice with 400 µl of lysis buffer. Proteins (50µg) were separated on 4-15% gradient SDS polyacrylamide gel (Bio-Rad) and transferred to nitrocellulose membranes for immunoblotting. Blots were probed with primary rabbit polyclonal antibodies for p66shcA and phospho-anti ShcA (Ser36) mouse monoclonal antibody (1:500 dilution; Calbiochem) to determine the phosphorylation status of Ser36. ARVM cell lysates or tissue homogenates were also analyzed for the expression of phospho-p53 (1:500 dilution; Cell Signaling), superoxide dismutase (SOD) (sheep polyclonal antibody, 1:700 dilution; Calbiochem), Catalase (1:500 dilution; Calbiochem), Bax and cleaved Caspase 3 (1:500 dilution; Santa Cruz Biotechnology). Anti-actin antibody (1:500;
dilution; Santa Cruz Biotechnology) and mouse monoclonal anti-GFP (green fluorescence protein; 1:20,000 dilution) were used as internal controls. Secondary antibody was used at a dilution of 1:5000. The blots were developed by using SuperSignal West Pico Chemiluminiscence Kit (Pierce) and the bands were scanned by using Biorad-1 computerized image analysis (6, 36).

Preparation and Immunoblotting of subcellular fractions. Mouse hearts were fractionated into cytosol and mitochondria by differential centrifugation as described (22). Briefly, hearts were homogenized with a tissue grinder (Tekmar) in a buffer that contained (in mM) 250 sucrose, 10 Tris·HCl, pH 7.4, 2 EDTA, 1 Na₃VO₄, 10 NaF, and protease inhibitor cocktail I and were centrifuged at 700×g for 10 min at 4°C. The pellet was discarded, and the supernatant was further centrifuged at 16,000×g for 25 min at 4°C. The supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were separated. Protein samples (50 µg) were separated and transferred on nitrocellulose membrane as described above. Anti-cytochrome c oxidase subunit IV (COX IV) monoclonal antibody (1:1,000 dilution; Molecular Probes) was used to probe the protein COX IV as a marker in mitochondria, and monoclonal Akt1 antibody (1:500 dilution; Cell Signaling) was used as a marker of cytosol in Western blot analysis. Anti-ShcA (1:500; Cell Signaling) was used to determine the accumulation of p66-ShcA in mitochondria. Cytochrome c expression was determined in cytosolic fraction using Mouse monoclonal Anti-cytochrome c antibody (1:1000; BD Biosciences Pharmingen), and the protein was detected by horseradish peroxidase-linked secondary antibody (1:5000 dilution). The blots were developed using Super Signal West Pico Chemiluminiscence Kit (Pierce) and the bands were scanned using Bio-Rad-1 computerized image system (6, 36).
Immunoprecipitation and Immunoblotting of p66ShcA and Cytochrome c.

Mitochondrial fraction of hearts were obtained from WT, rAAV-GFP (GFP group) and rAAV-Lep (LEP group) mice as described above. Six hundred micrograms of soluble mitochondrial extracts was incubated with 6 µg of rabbit polyclonal anti-p66ShcA antibody (Cell Signaling Inc.) and 500 µL of RIPA buffer (0.15 M NaCl; 20 mM Tris, pH 7.4; 1 mM EGTA, pH 7.4; 1 mM EDTA; 1% Triton X-100; 0.5% P-40;) containing the protease inhibitors 0.2 mM PMSF, 2 µg/µL aprotinin, and 0.2 mM Na3VO4 overnight at 4°C. Subsequently, 100 µl of protein A–agarose (Pierce) was added to each sample. After several washings (×3) with a buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, and 2 mM EGTA, samples were spun at 2,500 rpm for 2 minutes. Loading buffer was added to each pellet, and immunoprecipitated proteins were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and exposed to mouse monoclonal anti-mouse cytochrome c antibodies (BD Biosciences) or rabbit polyclonal anti-mouse p66Shc antibody (Cell Signaling Technology) at a concentration of 1:500 in TBST. Samples were subjected to Western blotting as described.

Statistical analysis. Data are expressed as means±SD. For multiple comparisons among different groups of data, the significant differences were determined by the Bonferroni method (34). Significance was defined at $P \leq 0.05$. 
Results

*Generation of mu-36 ARVM.* ARVM were enzymatically dissociated from adult rat hearts (24, 25) and transduced with adenoviral vector expressing the mu-36 p66ShcA. Immunoblot analysis of Shc isoforms (Fig 1) shows the position of mu-36 (top band) and p66ShcA protein immediately below. The p52ShcA protein is also shown along with low expression level of p46ShcA protein.

*mu-36 inhibits the HG-induced apoptosis signal.* HG induces an exponential increase in the intracellular production of ROS, an event linked to the development of diabetic complications (2, 6). ARVM possess the genetic program for apoptosis (24) and the DNA double helix is a target for ROS-dependent signals, that inflict more than 100 different types of DNA lesions, ranging from base modifications, to single strand breaks and potentially lethal double strand breaks, that trigger activation of the apoptosis program (12). To test if transfection of ARVM with mu-36 is sufficient to rescue ARVM from HG-induced DNA damage, ARVM and mu-36-ARVM were cultured in SFM containing 5mM or 25mM glucose for 16 h. Apoptosis was evaluated by ELISA cell death assay, which detects histone associated DNA fragments in the cytosol. As shown in Fig 2, ARVM and mu-36 ARVM at 5 mM glucose exhibit a baseline level of apoptosis due to serum starvation. This parameter increased by 40% in ARVM at 25 mM glucose, whereas mu-36-ARVM shows no change in apoptotic death from baseline. Taken together, mu-36 prevents activation of the apoptosis program in ARVM maintained at HG.
p66ShcA dependent regulation of HG-induced oxidant stress. HG alters the redox status of cells through the overproduction of ROS by mitochondria and NADPH oxidase (18, 19). To test if mu-36 ARVM exhibit increased resistance to HG-induced intracellular ROS production, ARVM and mu-36-ARVM were loaded with the redox sensitive probes Red Sensor Red CC-1 and the mitochondria specific dye Mito-Tracker green FM. As shown in Fig. 3A, at HG ARVM exhibit bright yellow orange fluorescence in mitochondria (19), due to the co-localization of oxidized Red CC-1 and Mito-Tracker green, indicative of augmented ROS production in the mitochondria. Conversely, mu-36-ARVM at HG show barely detectable fluorescent signal, indicative mu-36 induces a strong oxidant resistant phenotype.

mu-36 inhibits HG. -induced collapse of Δψm. Mitochondria are critical determinants of cell death and cell survival (19). To determine if mu-36 prevents collapse of ΔΨm, this parameter was examined in ARVM and mu-36-ARVM under control and experimental conditions. Cells were loaded with the fluorescent probe JC-1, which exhibits potential dependent accumulation in mitochondria. Under control conditions (Fig 3B) ARVM show punctate red staining, due to the accumulation of JC-1 in mitochondria. At HG, mitochondria of ARVM (upper panel) depolarize indicated by the reduction in J-aggregates (red/orange fluorescence) and increased JC-1 monomers (green fluorescence). Conversely, mu-36-ARVM exhibit punctate red/orange staining at 5 mM and 25 mM glucose. Taken together, mu-36 inhibits HG-induced collapse of ΔΨm, a key event in organelle dysfunction and apoptosis.
mu-36 and leptin downregulate catalase and Cu/Zn SOD. The antioxidant enzymes catalase and Cu/Zn SOD contribute to the defense of cellular redox status. Catalase catalyzes the dismutation of $H_2O_2 \rightarrow O_2 + H_2O$; SOD catalyzes the dismutation of $O_2\cdot \rightarrow O_2 + H_2O_2$. Immunoblot analysis (Fig 4A) of lysates from ARVM maintained at HG, show upregulation of catalase and SOD expression, which serve as surrogate markers of oxidative stress. An identical analysis performed with lysates from mu-36ARVM did not detect an alteration in the expression levels of catalase and SOD, indicative of increased resistance to the redox stimulus of HG.

We next asked if the antioxidant enzymes catalase and Cu/Zn SOD are upregulated in the diabetic myocardium and whether correction of HG turns off the signal for catalase and Cu/Zn SOD expression. A dominant mutation in the Ins2 gene, results in maturity onset diabetes in Akita mice (14). To avoid the confounding effect of insulin on survival pathways to be studied, Akita mice were treated with leptin, which has potent blood glucose (BG) lowering effect (35). Accordingly, Akita mice received recombinant adenoassociated virus vector (rAAV) containing green fluorescent protein (GFP group) or leptin cDNA (LEP group). Aged-matched-wild type (WT) mice served as control. For this protocol and those described below, Akita mice were euthanized 8-10 weeks post-injection, hearts excised and lysates prepared for immunoblot analysis. Blood glucose levels of control and experimental groups, immediately prior to euthanasia were; WT (150 ± 20 mg/dl); LEP (120 ± 37 mg/dl); GFP (*568 ± 40 mg/dl; *p<0.05 as compared to WT or LEP groups). As shown in Fig 4B, expression of catalase increased 3-fold and SOD 1.5 fold in hearts of GFP mice, whereas LEP mice exhibit expression levels of catalase and Cu/Zn SOD comparable to WT control. Taken together, we have shown
transfection of ARVM with mu-36 or treatment of Akita mice with leptin transgene; turn off the signal for HG-induction of catalase and Cu/Zn SOD.

**mu-36 and leptin inhibit p66ShcA redox function.** We next asked if mu-36 quenches HG-induced oxidative stress by inhibiting the redox function of the p66ShcA protein. Since the redox function of the p66ShcA is activated by ROS dependent phosphorylation of the Ser-36 residue residing at the amino-terminus, we hypothesized mu-36 will inhibit p66ShcA redox function by interrupting transmission of signals that target Ser-36. To test this hypothesis, immunoblot analysis was performed with phospho- anti-ShcA/p66 (phospho-Ser-36) mouse monoclonal antibody. This antibody recognizes the 66-kDa isoform of ShcA phosphorylated at Ser-36 and does not cross react with nonphosphorylated p66ShcA, mu-36 or with unrelated phosphorylation sites (27). As shown in Fig 5A, mu-36-ARVM maintained at HG exhibit no detectable alteration in the phosphorylation status of Ser-36, whereas ARVM show an upregulation in phosphorylation at Ser-36 p66ShcA protein, indicative mu-36 suppresses transmission of ROS dependent signals that target Ser-36.

We examined the phosphorylation status of Ser-36 in hearts from Akita mice treated with rAAV-leptin (LEP group), or rAAV-GFP (GFP group) and WT controls. As shown in Fig 5B, immunoblot analysis of cardiac lysates from control and experimental mice, show increased levels of phospho-Ser-36 in GFP mice, whereas phospho-Ser-36 levels in LEP mice were comparable to WT controls. Taken together, mu-36 and leptin inhibit transmission of signals that target the critical Ser-36 residue of the p66ShcA protein.
mu-36 and leptin inhibit p66ShcA translocation to mitochondria. Phosphorylation at Ser-36 induces translocation of the p66ShcA protein to the mitochondria, where p66ShcA interacts with cytochrome c to produce H$_2$O$_2$ (9). We hypothesize mu-36 and leptin will attenuate or prevent p66ShcA/cytochrome c complexes that result in the generation of H$_2$O$_2$, leading to organelle dysfunction and apoptosis. To test this hypothesis, mitochondria enriched fractions (19) were prepared from ARVM maintained under euglycemic and hyperglycemic conditions. Mitochondrial subfractions were immunoprecipitated with anti-p66ShcA and probed with p66ShcA and cytochrome c antibodies. As shown in Fig 6A, mitochondrial subfractions of ARVM maintained at HG show increased levels of p66ShcA/cytochrome c complexes, whereas mu-36ARVM at HG, exhibit no detectable alteration from control.

Mitochondria enriched fractions were also prepared from the hearts of WT control and Akita mice, expressing rAAV-leptin (LEP group) or rAAV-GFP (GFP group); (Fig 6B). Akt was used as an internal marker for cytosol and COX4 for mitochondria. As shown in Fig 6C, LEP mice show marked reduction in the levels of p66ShcA/cytochrome c complexes, when compared with GFP mice. Taken together, mu-36 and leptin attenuate HG-induced p66ShcA/cytochrome c complexes that trigger ROS production and organelle dysfunction.

mu-36 and leptin attenuate HG-induced expression of caspase-3 and apoptosis related proteins. We next asked if inhibition of p66ShcA redox signals prevents HG-induced activation of the terminal apoptosis program. Cleaved caspase-3 expression was examined by immunoblotting in lysates from ARVM and mu-36-ARVM, maintained at euglycemic and hyperglycemic conditions. As shown in Fig 7A, at HG ARVM show 2-
fold increase in cleaved caspase-3 expression, whereas mu-36-ARVM at HG did not affect expression of this proteolytic protein. This analysis was repeated with lysates prepared from hearts of WT control and Akita diabetic mice expressing rAAV-GFP and rAAV-leptin in GFP and LEP groups (Fig 7B). A 2.5 fold increase in cleaved caspase-3 expression was detected in GFP group but LEP group show levels comparable to WT control.

The redox sensitive pro-apoptosis transcription factor p53 is activated by the stress of HG (8, 19). Ser-392 is located at the COOH terminus of p53 and phosphorylation at this site is correlated with transcriptional activation. Bax is a p53 dependent gene, whose level of expression is increased during myocyte apoptosis (21). As shown in Fig 8A, immunoblot analysis of lysates from ARVM maintained at HG, show 2-fold increase in the expression levels of phospho-Ser-392 and Bax whereas mu-36 ARVM show no detectable alteration in phospho-Ser-392 or Bax. This analysis was repeated with lysates prepared from hearts of WT control and Akita mice, expressing rAAV-GFP and rAAV-leptin in GFP and LEP groups (Fig 8B). As anticipated, expression levels of phos-Ser-392 and Bax increased by 2-fold in hearts of rAAV-GFP mice, while rAAV-leptin mice show no difference from WT control. Taken together, mu-36 and leptin prevent transmission of HG-induced stress signals that activate the terminal apoptosis program.
Discussion

The present study demonstrates that p66ShcA is necessary for HG-induced oxidative stress and that interventions which interrupt or prevent phosphorylation of the p66ShcA protein at Ser-36, turn off p66ShcA redox activity. We have shown ARVM transduced with mu-36 construct exhibit an oxidant resistant phenotype, as judged by inhibition of HG-induced ROS production, stabilization of mitochondrial energetics and expression of the survival program. To evaluate p66ShcA signaling in vivo, studies were performed with hearts of Akita diabetic mice. The results show an order of progression for phos-Ser-36 and downstream p66ShcA signaling events, identical to that detected in our in vitro studies with ARVM at HG. To explore whether correction of HG mimics the p66ShcA expression profile detected in mu-36-ARVM, Akita mice were treated with the potent blood glucose-lowering hormone, leptin. Analysis of hearts from LEP mice show an order of progression of p66ShcA signaling, identical to that detected in mu-36-ARVM, indicative HG stress signals target phos-Ser-36 in diabetic myocardium to turn on p66ShcA redox activity. Our results indicate p66ShcA is a molecular switch whose redox function is turned on by HG-induced phosphorylation at Ser-36 and turned off by interventions that prevent this modification.

The major objective of the present study was to test whether adult cardiac muscle cells exhibit increased resistance to HG-induced oxidative stress following interventions that interrupt or prevent phosphorylation at Ser-36 of the p66ShcA protein. The p66ShcA protein has emerged as a genetic determinant of longevity in mammals (29) that controls mitochondrial metabolism and cellular responses to, oxidative stress, aging and apoptosis. At the organismal level, the p66ShcA−/− mouse is the unique genetic model of
increased resistance to oxidative stress, aging and apoptosis (9, 29). We hypothesized adenoviral transduction of ARVM with position 36 mutant of the p66ShcA protein, would confer a dominant interfering phenotype, attenuating or preventing transmission of HG- danger signals. Our result show mu-36-ARVM exhibit an oxidant resistant phenotype, as judged by inhibition of HG-induced ROS production, attenuation of phos-Ser-36 levels and maintenance of \( \Delta \Psi_m \). Conversely, ARVM show marked increase in ROS production at HG, upregulation in phos-Ser-36 levels, and collapse of \( \Delta \Psi_m \).

Importantly, the HG-induced component of apoptosis was not detected in mu-36-ARVM whereas ARVM exhibit a 40% increase in this parameter. Taken together, we have identified a pivotal role for p66ShcA redox function as a precursor to HG-induced free radical injury in ARVM.

A dominant mutation in the Ins2 gene due to an amino acid change results in HG and DM in the Akita mouse. Akita mice have several advantages over inbred mouse strains that require streptozotocin treatment (16, 17), including better-defined etiology (endoplasmic reticulum stress and proteotoxicity in pancreatic \( \beta \) cells), along with more pronounced and durable HG (16, 17). For these reasons, Akita mice were used to evaluate the \textit{in vivo} consequences of p66ShcA signaling in diabetic myocardium. Our results show Akita diabetic mice develop significant HG along with upregulation in phospho-Ser36 levels that were accompanied by translocation of p66ShcA protein to mitochondria and increased formation of p66ShcA/cytochrome c complexes. Mitochondria are critical determinants of cell death and cell survival. Similar to our \textit{in vitro} results with ARVM at HG, expression of cleaved caspase 3 and the apoptosis related proteins, p53 and Bax were found to be upregulated in hearts of Akita mice. Conversely, rAAV-leptin (LEP)
Akita mice were euglycemic and hearts show no detectable alteration in phospho-Ser-36, p66ShcA/cytochrome c complexes or in the expression of terminal components of the apoptosis program. Taken together, we have shown that in hearts of Akita mice, a single injection of rAAV-leptin (LEP) reverses the effect of HG on the phosphorylation status and redox function of the p66ShcA protein.

The present study has certain limitations, including the necessity to maintain cells under serum free conditions to eliminate the confounding effects of contained growth factors on signaling pathways linked to cell survival and oxidant stress. Second, we must acknowledge the shortcomings of an in vitro system in simulating a complex metabolic disorder, such as DM. Finally, the precise mechanism(s) by which leptin corrects HG, remains to be determined.

Taken into account the above limitations, the present study clearly demonstrates that p66ShcA functions as a potentially harmful regulatory gene, which is required for the generation of HG-induced oxidative stress and apoptosis. Recently, a unifying hypothesis has been proposed for the development of diabetic complications, based on the overproduction of ROS. In support of this hypothesis, Rota et al (31) report that following induction of experimental DM, p66ShcA−/− mice express a cardioprotection phenotype, characterized by decrease markers of cell senescence and preservation of cardiac muscle cells (CMC) number and LV function, whereas WT-diabetic mice exhibit cardiac stem cell aging, myocyte apoptosis and develop heart failure. Whether gene based strategies that incorporate siRNA to silence disease causing genes (32), such as p66ShcA, can be applied in vivo to selectively target cardiac muscle cells, remains to be determined.
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Disclosures: None.
References:


Figure Legends:

Figure 1. Representative immunoblot analysis of Shc isoforms. ARVM were plated in serum free medium (SFM) containing 5mM glucose (normoglycemic; NG) or 25mM glucose (hyperglycemic; HG). ARVM (WT), negative control showing GFP empty vector (GFP) and ARVM transduced with (mu-36) mutant36 p66ShcA (upper band) in NG. Also shown are p52ShcA and p46ShcA.

Figure 2. Mu-36 p66ShcA attenuates HG-induced apoptosis. Histone-associated DNA fragments were quantified by using ELISA cell death detection kit and presented as optical density (OD) at 405nm in 4 different groups. Data represent 7 independent experiments, mean±SD; *p≤0.05.

Figure 3A. p66ShcA dependent regulation of HG-induced oxidant stress. ARVM and mu-36 ARVM were maintained in SFM containing 5mM (NG) or 25mM (HG) glucose for 16 hours. Cells were loaded redox Sensor red CC-1 and the mitochondrial-specific dye MitoTracker green FM. At HG, ARVM show reddish orange fluorescent signal due to colocalization of oxidized red CC1 and mitotracker green in mitochondria.

Figure 3B. Mu-36 p66ShcA inhibits HG-induced collapse of mitochondrial transmembrane potential (ΔΨm). ARVM and mu-36 ARVM were maintained in SFM containing 5mM (NG) or 25mM (HG) glucose for 16 hours. Cells were loaded with the fluorescent probe, JC-1 that exhibits potential dependent accumulation in mitochondria. Under control conditions (5mM glucose), ARVM and mu-36 ARVM show punctate red stain due to JC-1 accumulation in mitochondria (J-aggregates). At HG, mitochondria of
ARVM depolarize indicated by release of JC-1 into cytoplasm and shift from red to green fluorescence.

**Figure 4.** Mu-36 and Leptin inhibit expression of catalase and Cu/Zn SOD.  
**Panel A:** Representative immunoblot analysis of Cu/Zn SOD and catalase expression in lysates prepared from ARVM. Protein extracts from wild type (WT)-ARVM and ARVM transduced with GFP and mu-36 were separated by PAGE and nitrocellulose blots probed with specific antibodies for Cu/Zn SOD and catalase. Densitometric analyses for catalase and Cu/Zn SOD expression are shown below. Data represent 4-5 independent experiments for WT and mu-36 ARVM and 2-3 independent experiments for GFP negative controls; mean ± SD; *p ≤ 0.05.  
**Panel B.** Representative immunoblot analysis of Cu/Zn SOD and catalase expression in lysates prepared from hearts of WT and Akita mice expressing rAAV-GFP (GFP) or rAAV-Leptin (LEP). Protein extracts from WT, GFP and LEP were separated by PAGE and nitrocellulose blots probed with specific antibodies for Cu/Zn SOD and catalase. Densitometric analyses for catalase and Cu/Zn SOD expression are shown below. Data represent 3-4 independent experiments; mean ± SD; *p ≤ 0.05.

**Figure 5.** Mu-36 and Leptin inhibit phospho-Ser-36 in p66ShcA.  
**Panel A.** Representative immunoblot analysis showing phosphorylation status of Ser-36 in lysates prepared from ARVM. Protein extracts from wild type (WT)-ARVM and ARVM transduced with GFP and mu-36 were separated by PAGE and nitrocellulose blots probed with mouse monoclonal anti phospho-serine antibody that recognizes the
66kDa form of Shc phosphorylated at Ser-36. Densitometric analysis for phospho-Ser-36 expression is shown below. Data represent 4 independent experiments; mean ± SD; *p≤0.05.

**Panel B.** Representative immunoblot analysis showing phosphorylation status of Ser-36 in lysates prepared from hearts of WT and Akita mice expressing rAAV-GFP (GFP) or rAAV-Leptin (LEP). Protein extracts from WT, GFP and LEP were separated by PAGE and nitrocellulose blots probed with mouse monoclonal anti phospho-serine antibody as in Panel A. Densitometric analysis for phospho-Ser-36 is shown below. Data represent 3-4 independent experiments; mean ± SD; *p≤0.05.

**Figures 6.** Mu-36 and Leptin inhibit p66ShcA translocation to mitochondria.

**Panel A.** Representative immunoblot analysis showing expression levels of p66ShcA/cytochrome C complexes in mitochondria–enriched fractions prepared from WT-ARVM and mu-36 ARVM. Mitochondrial subfractions were immunoprecipitated with anti-p66ShcA and probed with p66ShcA and cytochrome c antibodies. Results shown are representative of 3 experiments.

**Panel B.** Mitochondria–enriched subfractions prepared from hearts of WT and Akita mice expressing rAAV-GFP (GFP) or rAAV-Leptin (LEP). Cox IV was used as an internal marker for mitochondria. AKT was used as an internal marker for cytosolic subfraction.

Panel C. Representative immunoblot analysis showing expression levels of p66ShcA/cytochrome C complexes in mitochondria–enriched fractions prepared from
hearts of WT and Akita mice. Lysates were probed with p66ShcA and cytochrome c antibodies. Results shown are representative of 3 experiments.

**Figures 7.** Mu-36 and Leptin attenuate HG-induced cleaved caspase-3 expression.

**Panel A.** Representative immunoblot analysis showing cleaved caspase-3 expression in lysates prepared from ARVM. Protein extracts from wild type (WT)-ARVM and ARVM transduced with GFP and mu-36 were separated by PAGE and nitrocellulose blots probed with antibodies to cleaved caspase-3. Data represent 5-6 independent experiments for WT and mu-36 at 5mM and 25mM glucose and 2-3 independent experiments for GFP negative controls; mean±SD; *p≤0.05.

**Panel B.** Representative immunoblot analysis showing cleaved caspase-3 expression in lysates prepared from hearts of WT and Akita mice expressing rAAV-GFP (GFP) or rAAV-Leptin (LEP). Protein extracts from WT, GFP and LEP were separated by PAGE and nitrocellulose blots probed with antibodies to cleaved caspase-3 as in Panel A. Densitometric analysis for phos-Ser-36 is shown below. Data represent 3-4 independent experiments; mean±SD; *p≤0.05.

**Figures 8.** Mu-36 and Leptin attenuate HG-induced p53 activity and expression of apoptosis related factors.

**Panel A.** Representative immunoblot analysis showing p53 phospho-ser392 and Bax expression in lysates prepared from ARVM. Protein extracts from wild type (WT)-ARVM and ARVM transduced with GFP and mu-36 were separated by PAGE and nitrocellulose blots probed with antibodies to cleaved caspase-3. Densitometric analysis
for phospho-Ser-36 is shown below. Data represent 5 independent experiments for WT and mu-36 at 5mM and 25mM glucose; mean±SD; *p≤0.05.

**Panel B.** Representative immunoblot analysis showing p53 phospho- Ser329 and Bax expression in lysates prepared from hearts of WT and Akita mice expressing rAAV-GFP (GFP) or rAAV-Leptin (LEP). Protein extracts from WT, GFP and LEP were separated by PAGE and nitrocellulose blots probed with antibodies to p53 phospho-Ser392 and Bax as in Panel A. Densitometric analyses for p53 phospho- Ser329 and Bax expression are shown below. Data represent 4-5 independent experiments; mean±SD; *p≤0.05.
Figure 1
Figure 4
Figure 6
Figure 7
Figure 8