Mitochondrial Targeted Antioxidant Peptide Ameliorates Hypertensive Cardiomyopathy

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Objectives
We investigated the effect of reducing mitochondrial oxidative stress by the mitochondrial-targeted antioxidant peptide SS-31 in hypertensive cardiomyopathy.

Background
Oxidative stress has been implicated in hypertensive cardiovascular diseases. Mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase have been proposed as primary sites of reactive oxygen species (ROS) generation.

Methods
The mitochondrial targeted antioxidant peptide SS-31 was used to determine the role of mitochondrial oxidative stress in angiotensin II (Ang)-induced cardiomyopathy as well as in Gαq overexpressing mice with heart failure.

Results
Ang induces mitochondrial ROS in neonatal cardiomyocytes, which is prevented by SS-31, but not the nontargeted antioxidant N-acetyl cysteine (NAC). Continuous administration of Ang for 4 weeks in mice significantly increased both systolic and diastolic blood pressure, and this was not affected by SS-31 treatment. Ang was associated with up-regulation of NADPH oxidase 4 (NOX4) expression and increased cardiac mitochondrial protein oxidative damage, and induced the signaling for mitochondrial biogenesis. Reducing mitochondrial ROS by SS-31 substantially attenuated Ang-induced NOX4 up-regulation, mitochondrial oxidative damage, up-regulation of mitochondrial biogenesis, and phosphorylation of p38 mitogen-activated protein kinase and prevented apoptosis, concomitant with amelioration of Ang-induced cardiac hypertrophy, diastolic dysfunction, and fibrosis, despite the absence of blood pressure-lowering effect. The NAC did not show any beneficial effect. The SS-31 administration for 4 weeks also partially rescued the heart failure phenotype of Gαq overexpressing mice.

Conclusions
Mitochondrial targeted peptide SS-31 ameliorates cardiomyopathy resulting from prolonged Ang stimulation as well as Gαq overexpression, suggesting its potential clinical application for target organ protection in hypertensive cardiovascular diseases. (J Am Coll Cardiol 2011;58:73–82) © 2011 by the American College of Cardiology Foundation

Hypertension is a major global health issue, accounting for approximately one-half of the cases of stroke and ischemic heart disease and approximately 13% of total death worldwide (1). Systemic hypertension induces left ventricular hypertrophy (LVH), fibrosis, and diastolic dysfunction and increases the risk of coronary artery disease, which leads to congestive heart failure (2). The renin-angiotensin aldosterone system is the central regulator of hypertensive cardiovascular diseases. Angiotensin II (Ang) induces LVH, cardiac fibrosis, and diastolic dysfunction. At the molecular level, Ang binds to the angiotensin receptor-1 (ATR1), a guanine nucleotide-binding protein type q alpha subunit (Gαq)-coupled receptor, then stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidases to produce reactive oxygen species (ROS) (3). Recent studies have reported that the nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) isoform of NADPH oxidase is present in mitochondria. The ROS generated by NADPH oxidase was shown to stimulate mitochondrial ROS production and induce mitochondrial dysfunction (4–7). Furthermore, we previously demonstrated that mitochondrial ROS plays a key role in aging and lifespan regulation, as...
shown by approximately 20% extension of lifespan in mice overexpressing catalase targeted to mitochondria (mCAT) (8). We further showed that mCAT attenuated age-dependent LVH and diastolic dysfunction, concomitant with attenuation of age-dependent increases in cardiac mitochondrial oxidative damage, without any effect on the increase of cardiac Ang observed in aged mice (9). Therefore, we hypothesized that Ang might induce mitochondrial ROS in cardiomyocytes and that scavenging mitochondrial ROS by a mitochondrial-targeted antioxidant peptide might be beneficial in the setting of hypertensive cardiomyopathy.

The Szeto-Schiller (SS)-31 peptide (D-Arg-2′,6′-dimethyl-tyrosine-Lys-Phe-NH₂) belongs to a family of aromatic cationic peptides that selectively target to mitochondrial inner membrane and can scavenge superoxide, hydrogen peroxide, peroxynitrite, and hydroxyl radicals (10,11). The SS-31 has been shown to reduce mitochondrial ROS in epithelial, endothelial, and neuronal cells exposed to electron-transport-chain inhibitors as well as pro-oxidants including t-butyl-hydroperoxide and hypochlorous acid (12,13). The SS-31 also inhibits apoptosis mediated by mitochondrial release of cytochrome c elicited by mitochondrial permeability transition (11). In vivo administration of SS-31 has demonstrated efficacy in several animal models associated with mitochondrial oxidative stress, including reduction of ischemia-reperfusion injury (14), protection against neurodegeneration (15), and prevention of insulin resistance induced by high-fat diet (16). In this study, we demonstrated that Ang induced mitochondrial ROS in cardiomyocytes and increased cardiac mitochondrial protein oxidative damage. Reduction of mitochondrial ROS by SS-31 ameliorated Ang-induced cardiac hypertrophy, fibrosis, and apoptosis, concomitant with reduced activation of p38 mitogen-activated protein kinase (MAPK). The SS-31 administration also partially rescued the heart failure phenotype of mice with Gqq overexpression as a model of chronic catecholamine/Ang stimulation (17).

**Methods**

Detailed methods are provided in the Online Appendix. Neonatal mouse cardiomyocytes were stimulated with Ang (1 μmol/l), with or without simultaneous treatment of the following: SS-31 (1 nmol/l, kindly provided by Stealth Peptides, Inc., Newton Centre, Massachusetts), N-acetyl cysteine (NAC) (0.5 mmol/l), 4-chlorodiazepam (25 μmol/l), diazoxide (200 μmol/l), cyclopentolate (0.5 μmol/l), and/or 5-hydroxydecanoate (100 μmol/l). Cardiomyocytes were then loaded with MitoSOX (5 μmol/l) and CM-DCFDA (5 μmol/l) to measure mitochondrial superoxide and total cellular ROS, respectively.

The FVBxC57BL/6J F1 hybrid mice (6 to 10/group, approximately 12 weeks of age) and wild-type littermates were treated with saline, Ang (1.1 mg/kg/day), Ang + SS-31 (3 mg/kg/day), Ang + NAC (approximately 500 mg/kg/day in drinking water) (18), Gqq (FVBxC57BL/6 F2), or Gqq + SS-31 (3 mg/kg/day). Ang and SS-31 were continuously administered for 4 weeks with subcutaneous Alzet 1004 osmotic minipumps (Alzet, Cupertino, California). Echocardiography was performed at baseline and 4 weeks after pump. As a reference for SS-31 effects, we included a genetic mouse model of Rosa-26 inducible-mCAT (C57BL/6), in which mitochondrial catalase was expressed 2 weeks before Ang treatment. Blood pressure (BP) was measured in a separate group of mice by telemetry with an intravascular catheter PA-C10 (DSI, St. Paul, Minnesota). After 4 weeks of treatment, mouse ventricles were harvested. Quantitative image analysis of trichrome staining was performed to evaluate the severity of fibrosis.

Cardiac mitochondrial protein carbonyl content was analyzed with OxiSelect Protein Carbonyl ELISA Kit (Cell Bioslabs, San Diego, California). Quantitative polymerase chain reaction (qPCR) was performed with Applied Biosystems 7900 thermocycler with Taqman Gene Expression Assays on Demand (Applied Biosystems, Foster City, California). Western blotting was used to analyze NOX4, cleaved caspase-3, phosphorylated, and total p38 MAPK.

**Statistical analysis.** All data are presented as mean ± SEM. Comparisons between 2 groups are performed with Student t tests. One-way analysis of variance was used to compare differences among multiple groups, followed by Tukey post hoc test for significance. All p values <0.05 were considered significant.

**Results**

Ang increased mitochondrial and total cellular ROS in neonatal cardiomyocytes, which was alleviated by SS-31. Flow cytometric analysis demonstrated that Ang increased MitoSOX fluorescence (an indicator of mitochondrial superoxide) (Fig. 1A) and DCFDA fluorescence (an indicator of total cellular ROS) (Fig. 1B) in neonatal cardiomyocytes by approximately 60% and 45%, respectively (p < 0.01 for both) (Fig. 1C). Treatment with NAC, a nontargeted antioxidant drug, did not show any significant effect on mitochondrial or total cellular ROS after Ang. In contrast, SS-31 significantly reduced Ang-induced MitoSOX and DCFDA fluorescence to
Figure 1  
**Ang-Increased Mitochondrial and Total Cellular Oxidative Stress in Neonatal Cardiomyocytes Is Prevented by SS-31 But Not NAC**

Flow cytometry of neonatal cardiomyocytes stimulated with Angiotensin II (Ang) (1 μmol/l) and loaded with MitoSOX (A) or CM-DCFDA (B). (C) Quantitative analysis (presented as mean ± SEM of histogram medians, n = 3 to 5) revealed a significant increase in MitoSOX and DCFDA fluorescence in Ang-treated cardiomyocytes (red) compared with saline treatment (black). The nontargeted antioxidant N-acetyl cysteine (NAC) (0.5 mmol/l) (blue line) had no significant effect on Ang-induced mitochondrial reactive oxygen species (ROS) (A, C) and total cellular ROS (B, C). Simultaneous treatment with SS-31 (1 nmol/l) (green line) substantially reduced Ang-induced mitochondrial ROS (A, C) and total cellular ROS (B, C). *p < 0.01 versus saline; #p < 0.01 versus Ang.

Figure 2  
**No Effect of SS-31 on BP Increase After Pressor Dose of Ang**

(A) Representative blood pressure (BP) tracings of mice at baseline and after angiotensin II (Ang) (1.1 mg/kg/day) administered with a subcutaneous pump. (B) Ang significantly increased systolic and diastolic BP. Administration of SS-31 (3 mg/kg/day) did not show any significant effect on BP when added to Ang treatment (n = 3).
near control levels (Fig. 1). Ang-induced MitoSOX and DCFDA fluorescence could also be attenuated by 4-Clbenzodiazepam, an inhibitor of the inner mitochondrial anion channel, or cyclosporine A, an inhibitor of the mitochondrial permeability transition pore or diazoxide, an activator of the mitochondrial K-ATP channel (Online Figs. 1A to 1D and 1G)—further supporting mitochondrial mechanisms of ROS induction (see Discussion). In contrast, 5-hydroxydecanoate, an inhibitor of the mitochondrial K-ATP channel, did not significantly affect Ang-induced ROS (Online Figs. 1E and 1F).

SS-31 ameliorates Ang-induced cardiomyopathy despite the absence of pressor effects. To recapitulate hypertensive cardiomyopathy, we administered a pressor dose of Ang (1.1 mg/kg/day) for 4 weeks via subcutaneous pumps. Intravascular telemetry (Fig. 2) revealed Ang-induced systolic and diastolic BP increases of 25 to 28 mm Hg above baseline (BP: 118.8 ± 4.0/94.5 ± 3.5 mm Hg at baseline vs. 146.0 ± 5.6/119.3 ± 4.0 mm Hg after Ang, p < 0.001). Simultaneous administration of SS-31 did not show any effect on BP.

After 4 weeks of Ang, echocardiography revealed an approximately 2-fold increase in left ventricular (LV) mass index compared with baseline (Fig. 3A), no change in LV end diastolic diameter (data not shown) or systolic function as measured by fractional shortening (FS) (Fig. 3B), and an approximately 35% decline in Ea/Aa, an indicator of diastolic function (Fig. 3C). Simultaneous administration of SS-31 significantly ameliorated Ang-induced cardiac hypertrophy and diastolic dysfunction, with a 33% reduction of LV mass index (Ang: 6.32 ± 0.39 vs. Ang + SS-31: 4.21 ± 0.17 mg/g, p = 0.001) (Fig. 3A, left panel) and better preservation of Ea/Aa (Ang: 0.723 ± 0.15 vs. Ang + SS-31: 1.17 ± 0.11, p = 0.04) (Fig. 3C, left panel). These effects

Figure 3 SS-31 Ameliorates Ang-Induced Cardiac Hypertrophy and Diastolic Dysfunction

(A) Angiotensin II (Ang) for 4 weeks substantially increased left ventricular (LV) mass index in control mice. Simultaneous administration of SS-31 significantly attenuated this increase in LV mass index (left). This was to an extent similar to that observed in mice with inducible overexpression of mitochondrial catalase (i-mCAT) (right). (B) Fractional shortening (FS) was not significantly changed after 4 weeks of Ang in the presence or absence of mitochondrial antioxidants. (C) Diastolic function measured by tissue Doppler imaging of Ea/Aa was significantly reduced after Ang but was significantly ameliorated by SS-31 or genetic overexpression of mCAT. Administration of N-acetyl cysteine (NAC) for 4 weeks did not confer any significant protection for Ang-induced hypertrophy and diastolic dysfunction (light blue bar on left panel, A to C), n = 6 to 7.
were comparable to those of catalase targeted to mitochondria (inducible mCAT), in which induction of mitochondrial catalase 2 weeks before Ang also conferred protection against Ang-induced cardiac hypertrophy and diastolic dysfunction (Figs. 3A to 3D, right panels). To investigate whether the protective effect is specific to a mitochondrial antioxidant, we administered the nontargeted antioxidant NAC in drinking water for 4 weeks simultaneously with the Ang pump. This treatment has been shown to ameliorate cardiomyopathy after acute aortic banding in mice (18); however, it did not show any beneficial effect on Ang-induced cardiomyopathy (Figs. 3A to 3C).

Ang increased heart weights by 45% above those of saline-treated control subjects (Fig. 4A) (5.3 ± 0.18 in saline vs. 7.69 ± 0.20 in Ang, p < 0.001), and SS-31 protected heart weights (6.05 ± 0.14 mg/mm, p < 0.01 vs. Ang alone), whereas oral NAC did not show any significant protection of heart weights (7.13 ± 0.7 in Ang+NAC vs. 7.69 ± 0.20 in Ang, p = 0.47). The cardiac hypertrophy phenotype was confirmed by qPCR for atrial natriuretic peptide, a fetal gene reactivated during hypertrophy. Ang induced an approximately 15-fold increased atrial natriuretic peptide gene expression, which was almost completely protected by SS-31 but not by NAC. Consistent with studies of ROS in cardiomyocytes (Fig. 1), chronic administration of Ang for 4 weeks significantly increased mitochondrial protein oxidative damage and signaling for mitochondrial biogenesis. Ang-induced mitochondrial protein oxidative damage and signaling for mitochondrial biogenesis. Consistent with studies of ROS in cardiomyocytes (Fig. 1), chronic administration of Ang for 4 weeks significantly increased
ventricular mitochondrial protein carbonyl content, an indicator of protein oxidative damage (p = 0.03) (Fig. 5A). This was significantly reduced by SS-31 (p = 0.02) (Fig. 5A), whereas nontargeted NAC did not show any significant reduction of mitochondrial protein oxidative damage (1.27 ± 0.1 in Ang + NAC vs. 1.44 ± 0.1 in Ang, p = 0.19) (Fig. 5A).

Peroxisome proliferator-activated receptor gamma co-activator (PGC-1α) is known as a master regulator of mitochondrial biogenesis, regulating nuclear respiratory factors (NRFs) and mitochondrial transcription factor A, which transcribe nuclear deoxyribonucleic acid and mitochondrial deoxyribonucleic acid-encoded mitochondrial proteins, respectively (19). Ang induced the expression of PGC-1α and its downstream target genes, including mitochondrial transcription factor A, NRF-1, and NRF-2 (Fig. 5B). The SS-31 fully prevented this up-regulation of PGC-1α and target genes (p < 0.05 for all) (Fig. 5B), whereas NAC did not show significant attenuation of any of these transcription factors.

SS-31 attenuates Ang-induced up-regulation of NOX4 and reduces activation of p38 MAPK and apoptosis. Cardiac NOX4 protein expression was significantly increased in Ang-treated and Gaq overexpressing hearts (p = 0.01) (Fig. 6A), consistent with a recent report by Ago et al. (6). The SS-31 significantly reduced NOX4 up-regulation in Ang-treated hearts (p = 0.05) and trended toward reduction of NOX4 up-regulation in Gaq hearts (p = 0.06) (Fig. 6A), whereas NOX4 up-regulation was not attenuated by NAC (p = 0.19) (Online Fig. 2A).

Excessive mitochondrial ROS is well-known to induce apoptosis. Activated (cleaved) caspase 3, a marker of apoptosis, was increased in Ang-treated ventricular tissue (p = 0.006), and this was almost completely attenuated by SS-31 (p = 0.004) (Fig. 6C). In contrast, NAC treatment did not reduce Ang-induced cleaved caspase-3 (Online Fig. 2B). Ang treatment increased phosphorylation of p38 MAPK, and this was significantly attenuated by both SS-31 and NAC (Fig. 6B, Online Fig 2C), suggesting that p38 phosphorylation depends on total ROS, regardless of the location in mitochondria or cytosol.

SS-31 partially rescues Gaq overexpression-induced heart failure. To extend our observations to a model of chronic catecholamine/Ang stimulation, we applied the genetic mouse model of cardiac-specific overexpression of Gaq (a coupling protein to catecholamine and Ang receptors), which causes heart failure in mice by 14 to 16 weeks of age, despite the absence of increased BP (17). We confirmed that Gaq mice had impaired systolic function at 16 weeks old, as shown by a substantial decline in FS (Fig. 7A), with LV chamber enlargement (Fig. 7B); diastolic dysfunction, indicated by Ea/Aa <1 (Fig. 7C); and worsening of the myocardial performance index (Fig. 7D).

SS-31 administered from 12 to 16 weeks of age significantly reduced cardiac enlargement (p = 0.001) (Fig. 7E), ameliorated systolic function (p < 0.001 vs. untreated Gaq) (Fig. 7A), and improved myocardial performance (p = 0.04) (Fig. 7D). There were trends toward amelioration of LV chamber enlargement (p = 0.08) (Fig. 7B) and preservation of Ea/Aa (p = 0.06) (Fig. 7C) in SS-31–treated Gaq mice. There was a trend toward partial attenuation of increased lung weights by SS-31 (p = 0.09) (Fig. 7E). Ventricular fibrosis increased by approximately 2-fold in Gaq mice, which was not changed in SS-31–treated mice (Online Fig. 3A), and this was confirmed by pro-collagen 1a2 qPCR (Online Fig. 3B). Mitochondrial protein oxidative damage was evident in Gaq hearts (p = 0.01) (Online Fig. 3C), and SS-31–treated mice displayed significant reduction of cardiac mitochondrial protein carbonyls (p = 0.05) (Online Fig. 3C). There was no evidence of increased cleaved-caspase 3 in Gaq mouse hearts (data not shown).

Discussion

We found that mitochondrial ROS plays an essential role in hypertensive cardiomyopathies downstream of Ang and protection from mitochondrial ROS by a mitochondrial-targeted antioxidant confers resistance to cardiomyopathy, whereas nontargeted antioxidant NAC did not. Our study provides direct evidence that Ang induces mitochondrial
ROS, which is prevented by SS-31 but not by NAC (Fig. 1). We suggest that ROS amplified in mitochondria subsequently activates downstream ROS-sensitive signaling pathways implicated in pathological cardiac hypertrophy, including p38 MAPK (Fig. 6C), apoptosis signal-regulating kinase (20), other MAPKs (21), NF-κB (22), and calcineurin–nuclear factor of activated T-cells (23).

Exposure to Ang for 4 weeks increased cardiac mitochondrial protein oxidative damage and induced the signaling for mitochondrial biogenesis (Fig. 5), consistent with the previous report that hydrogen peroxide directly activates transcription of PGC-1α, the master regulator of mitochondrial biogenesis (24). SS-31 significantly attenuated Ang-induced mitochondrial oxidative stress, reduced up-regulation of mitochondrial biogenesis, and reduced ROS-mediated p38 MAPK signaling (Fig. 8). Because inhibition of p38 has been shown to improve cardiac remodeling and inflammation and preserve cardiac function in heart failure (25,26), the attenuation of Ang-induced p38 phosphorylation by SS-31 might be one of the downstream mechanisms underlying protection by SS-31. Furthermore, mitochondrial ROS can lead to apoptosis, and our results confirm that SS-31 prevented caspase-3 activation (Fig. 6), concomitant with amelioration of Ang-induced cardiomyopathy (Figs. 3 and 4).

It has been proposed that Ang mediates its effects via Goq. However, Goq transgenic overexpression involves a persistent lifelong stimulation of Goq, starting from developmental stage, and is likely a stronger stimulus than obtained with Ang. Thus, transgenic overexpression of Goq displayed a much stronger phenotype than 4-week Ang, including systolic heart failure in mice by 14 to 16 weeks of age (17). The SS-31 for 4 weeks partially rescued the heart failure phenotype in mice overexpressing Goq, including a significant amelioration of systolic dysfunction, cardiac hypertrophy, and myocardial performance (Figs. 7A, 7D, and 7E).

Central to the scheme hypothesized in Figure 8 is the amplification of mitochondrial ROS, which can occur by multiple mechanisms. Ang is a key mediator of hypertension that binds to ATR1, a Goq coupled-receptor, then activates NADPH oxidase through a protein kinase
C-dependent mechanism. Although the NOX2 isoform of NADPH oxidase has been shown to mediate Ang effects, the NOX4 isoform has recently been reported to increase in response to various hypertrophic stimuli (6) and is localized in mitochondria (7), and activation of this isoform increases mitochondrial ROS (6). Although our hypothesis indicates that ROS produced directly from NOX4 and/or indirectly by NOX2 isoform is amplified within mitochondria, the relative contribution of these isoforms in elevating ROS in mitochondria is not yet clear, and it is possible that NOX-independent mechanism(s) might also contribute to the Ang and Goq phenotypes.

Because escape of electrons from the mitochondrial electron transport complexes is a principal source of cellular ROS, oxidative damage to these complexes has been proposed to lead to a vicious cycle (27). Another potential mechanisms of Ang-induced mitochondrial ROS could be the previously reported ROS-induced ROS release from mitochondria, which might be mediated by activation of the inner mitochondrial anion channel (inhibited by 4-chlorodiazeapem) (28), or the mitochondrial permeability transition pore (inhibited by cyclosporine) (29), or mitochondrial K_{ATP} channels (activated by diazoxide) (4). The involvement of ROS-induced ROS release from mitochondria should be considered as a primary mechanism of Ang-induced ROS signaling (30), as confirmed by observations that Ang-induced mitochondrial-ROS was reduced by simultaneous treatment with diazoxide, 4-chlorodiazeapem, or cyclosporine A (Online Fig. 1). This places mitochondria in a central position for signal amplification and, conversely, for therapeutic targeting. Although most antihypertensive medications act upstream at the receptor level (beta-blockers, calcium channel blockers, angiotensin receptor blockers) or even at remote sites of action (diuretics, angiotensin-converting enzyme inhibitor), antioxidants provide an alternate intervention strategy in cardiac hypertrophy and failure. Several antioxidant studies have demonstrated some protective effect in cardiovascular diseases. For instance, Euk-8, a superoxide dismutase and catalase mimetic, ameliorated heart failure in ROS-sensitive mouse models subjected to pressure overload (31). Zhou et al. (32) reported that overexpression of metallothionein suppressed oxidative and nitrosative stress, apoptosis, and pathological remodeling in response to a short-term subpressor dose of Ang. Our study, however, points to the rationale of targeting antioxidants to mitochondria. The mitochondrial antioxidant MitoQ has been shown to reduce BP in the spontaneous hypertensive rat, concomitant with improvement of endothelial function and reduction of heart weight; however, the mechanism of cardioprotection was unclear (33). Our study found no effect of SS-31 on BP but showed evidence of direct cardioprotective mechanisms involv-
application for the treatment or prevention of hypertensive cardiovascular diseases.

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APPENDIX

For supplementary figures and Methods, please see the online version of this article.