p53 Promotes Cardiac Dysfunction in Diabetic Mellitus Caused by Excessive Mitochondrial Respiration-Mediated Reactive Oxygen Species Generation and Lipid Accumulation

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Background—Diabetic cardiomyopathy is characterized by energetic dysregulation caused by glucotoxicity, lipotoxicity, and mitochondrial alterations. p53 and its downstream mitochondrial assembly protein, synthesis of cytochrome c oxidase 2 (SCO2) are important regulators of mitochondrial respiration, whereas the involvement in diabetic cardiomyopathy remains to be determined.

Methods and Results—The role of p53 and SCO2 in energy metabolism was examined in both type I (streptozotocin [STZ] administration) and type II diabetic (db/db) mice. Cardiac expressions of p53 and SCO2 in 4-week STZ diabetic mice were upregulated (185% and 152% versus controls, respectively, P<0.01), with a marked decrease in cardiac performance. Mitochondrial oxygen consumption was increased (136% versus control, P<0.01) in parallel with augmentation of mitochondrial cytochrome c oxidase (complex IV) activity. Reactive oxygen species (ROS)-damaged myocytes and lipid accumulation were increased in association with membrane-localization of fatty acid translocase protein FAT/CD36. Antioxidant tempol reduced the increased expressions of p53 and SCO2 in STZ-diabetic hearts and normalized alterations in mitochondrial oxygen consumption, lipid accumulation, and cardiac dysfunction. Similar results were observed in db/db mice, whereas in p53-deficient or SCO2-deficient diabetic mice, the cardiac and metabolic abnormalities were prevented. Overexpression of SCO2 in cardiac myocytes increased mitochondrial ROS and fatty acid accumulation, whereas knockdown of SCO2 ameliorated them.

Conclusions—Myocardial p53/SCO2 signal is activated by diabetes-mediated ROS generation to increase mitochondrial oxygen consumption, resulting in excessive generation of mitochondria-derived ROS and lipid accumulation in association with cardiac dysfunction. (Circ Heart Fail. 2012;5:106-115.)

Key Words: cardiomyopathy ■ diabetes mellitus ■ metabolism ■ heart failure ■ free radicals ■ mitochondria

Diabetic cardiomyopathy is one of the leading causes of increased morbidity and mortality in the patients with diabetes mellitus. Although the pathogenesis of this cardiac contractile dysfunction is still unclear, an involvement of increased reactive oxygen species (ROS) production and altered mitochondrial function has been reported. Mitochondrial uncoupling was shown to be a possible mechanism to reduce cardiac efficiency in type 2 diabetes models but not in type 1 diabetes models. Recent study using positron emission tomography in patients with type 1 diabetes mellitus has revealed the increased oxygen consumption and altered fatty acid (FA) metabolism. These human and animal studies have shown that increased oxidative stress correlates with lipid overload.

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suggested a role for FA in the generation of ROS,7,8 whereas an involvement of ROS generated by high glucose concentrations in metabolic abnormalities in diabetic cardiomyopathy remains to be determined.7

Tumor suppressor, p53, has a well-characterized role in regulating apoptosis and the cell cycle in response to genomic stress; however, its broader roles in organ homeostasis are just beginning to be understood.8 Especially, activation of p53 protein demonstrated critical roles in the heart. Induction of p53 protein by myocardial ischemia is associated with decreased survival rate due to cardiac rupture.9 Doxorubicin induced the expression of p53 and cardiac toxicity associated with myocyte apoptosis.10 Chronic pressure overload upregulated p53 protein to cause inhibition of Hif-1 activity, which impaired cardiac angiogenesis and contractility.11

We previously reported that p53 regulates mitochondrial respiration (oxygen consumption),12 in which we newly discovered the synthesis of cytochrome c oxidase 2 (SCO2) as a novel transcriptional target of p53 and demonstrated that p53 positively regulated mitochondrial respiration through SCO2 expression. Rescue of SCO2 in p53-deficient cells restored the impaired aerobic respiration, indicating that p53 directly regulates mitochondrial respiration through SCO2.13 The SCO2 protein is essential for the assembly of mitochondrial cytochrome c oxidase (complex IV), the metabolic center of oxygen consumption.14 Although the biological significance of the p53/SCO2 pathway remains to be clarified, p53-mediated regulation of mitochondrial respiration might suggest a new mechanism in cardiac metabolic alterations.

In the present study, we focused on the role of the cardiac p53/SCO2 system in mitochondrial respiration, lipid metabolism, and cardiac performance in the diabetic hearts. Our results demonstrate for the first time that SCO2 plays a crucial role in the enhancement of mitochondria-derived ROS generation and lipid accumulation by increasing mitochondrial respiration, suggesting a novel function of p53 and the SCO2 system in regulating cardiac energy metabolism in the diabetic hearts.

Methods

Animals

All animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The protocol was approved by the bioethics committee of Kyoto Prefectural University School of Medicine.

Each method is described in the online-only Data Supplement material.

Statistical Analysis

Data were expressed as mean±SEM. For comparison of variables of the excised hearts in the same gene model, a 1-way ANOVA test was performed. Then Bonferroni procedure was used to assess differences between individual groups. To assess group differences over time or intervention, 2-way ANOVA was used. We verified that the data were normally distributed and used parametric tests. After finding significant interaction among the data in each group and time point, differences were assessed with the Bonferroni procedure. Data between 2 groups were compared using the unpaired t test. A probability value of <0.05 was considered to indicate statistical significance.

Results

p53 Expression Is Upregulated in Diabetic Hearts to Cause Impairment of Cardiac Function

To investigate the role of p53 in the diabetic heart, wild-type p53 (+/+) and p53 (−/−) mice were treated with STZ (type 1 diabetes mellitus model). p53 protein expression was increased 4 and 8 weeks after injection of STZ (188% and 152% versus control, respectively, P<0.01; Figure 1A). Left ventricular fractional shortening was significantly decreased in p53 (+/+) mice (75.8% at 4 weeks and 67.7% at 8 weeks versus baseline), whereas these alterations were not observed in p53 (−/−) mice (95.1% at 4 weeks and 91.9% at 8 weeks versus baseline; Figure 1B and 1C and online-only Data Supplement Table 1). There were no significant differences in glucose and lipid profiles after STZ injection between p53 (+/+) and p53 (−/−) mice (online-only Data Supplement Figure 1A and 1B). BNP mRNA levels were upregulated significantly 4 and 8 weeks after STZ injection in p53 (+/+) diabetic heart (3.1- and 3.5-fold versus baseline), whereas there were no significant changes in p53 (−/−) mice (online-only Data Supplement Figure 1C).

ROS-Mediated DNA Damage and Lipid Accumulation Are Decreased in p53 (−/−) Diabetic Hearts

To study the effect of p53 deficiency on diabetic hearts, we performed histological examination of the left ventricle (interstitial fibrosis, vessel formation) at 4 weeks after STZ injection. Hematoxylin and eosin and Masson’s trichrome staining showed that there were no significant changes in myocyte size, infiltration of inflammatory cells, interstitial fibrosis, and the numbers of CD31-positive cells between p53 (+/+) and p53 (−/−) diabetes mice (online-only Data Supplement Figure 2A through 2C). Although TUNEL-positive cells were increased 3, 7, and 14 days after STZ injection, their numbers were not significantly different between p53 (+/+) and p53 (−/−) diabetes mice (online-only Data Supplement Figure 3). In contrast, we found marked increases in ROS-damaged DNA (8-OHdG immunostaining) and lipid accumulation (oil-red-O staining) in p53 (+/+) diabetic hearts, whereas these changes were barely observed in p53 (−/−) diabetic mice (Figure 2A through 2H).

Electron micrographs revealed that lipid droplets were more accumulated around mitochondria in myocytes from p53 (+/+) diabetic mice (3.4-fold versus control), whereas such change was not observed in p53 (−/−) diabetic mice (Figure 2I through 2L). We also analyzed cardiac triglyceride and mitochondrial DNA contents. Triglyceride content was increased in p53 (+/+) diabetic hearts (2.3-fold versus control, P<0.01; Figure 2N) but not in p53 (−/−) diabetic mice. Mitochondrial DNA content did not show any significant change between p53 (+/+) and p53 (−/−) mice (Figure 2O).

Mitochondrial Oxidative Respiration Is Augmented in Diabetic Heart

Because the increase of mitochondrial respiration and FA influx caused myocardial lipid accumulation,6,8,15 we evaluated the functional change in mitochondrial oxidative
respiration of diabetic hearts. Basal respiration (state 2, \(V_0\)), ADP-stimulated respiration (state 3, \(V_{ADP}\)), and oligomycin-administered respiration after ADP stimulation (state 4, Voligomycin) were compared in p53 (\(+/+\)) and p53 (\(-/-\)) mice. In the p53 (\(+/+\)) diabetic hearts, state 3 respiration (\(V_{ADP}\)) was significantly increased (136% versus control, \(P<0.01\); Figure 3A), whereas this change was not observed in p53 (\(-/-\)) diabetic mice (Figure 3A). There were no alterations in state 2 (\(V_0\)) or state 4 (Voligomycin) respirations in p53 (\(+/+\)) and p53 (\(-/-\)) diabetic hearts (Figure 3A). Considering that the state 4 respiration was reported to reflect uncoupling of mitochondrial oxidative phosphorylation,6,16 the present findings raised the possibility that mitochondrial uncoupling was not involved in STZ-induced diabetic hearts.

We also analyzed the individual activity of mitochondrial complexes I—IV, composing the mitochondrial electron transport chain. Normally, mitochondria respiration generates a proton gradient by transferring electron through complexes I, III, and IV. Alteration in each complex activity is an important functional clue to clarify diabetes-mediated mitochondrial abnormalities. The result showed that the increase in oxygen consumption in p53 (\(+/+\)) diabetic mice was mainly due to the augmentation of mitochondrial complex IV activity (135% versus control, \(P<0.01\); Figure 3B). As the mitochondrial complex IV, a final electron acceptor in this chain, has the greatest capacity of oxygen consumption, these data indicated that the elevated activity of mitochondrial complex IV is responsible for the abnormalities of mitochondrial function in the diabetic hearts.

To further examine the time-dependent changes of mitochondrial respiration, we measured mitochondrial complex activities at 0, 2, 4, and 8 weeks after STZ injection. Mitochondrial complex IV activity was significantly augmented in p53 (\(+/+\)) diabetic heart at 4 and 8 weeks after STZ injection (135% and 132% versus control, respectively, \(P<0.01\)), whereas the activity was not changed in p53 (\(-/-\)) diabetic mice, and mitochondrial complex I–III activities were not affected during 8 weeks in both p53 (\(+/+\)) and p53 (\(-/-\)) diabetic hearts (Figure 3C and 3D).

We next analyzed whether p53 induction and the increase of mitochondrial respiration are dependent on diabetes. By treating STZ-induced diabetic mice with insulin for 4 weeks (0.2 U insulin/day), the glucose levels were decreased to the control range (238±56 mg/dL at 4 weeks, \(n=6\)) and the upregulation of state 3 respiration, complex I–IV activities, and induction of p53 were all reversed to the control levels. Insulin treatment also improved cardiac dysfunction and lipid accumulation (online-only Data Supplement Figure 4).
SCO2 Is Induced in Diabetic Hearts to Augment FA Metabolism and Uptake

SCO2 protein is essential for the assembly of the mitochondrial complex IV and is transcriptionally regulated by p53. We studied SCO2 expression in STZ-induced diabetic hearts. The expression of SCO2 was markedly increased 4 and 8 weeks after STZ injection (148% versus control, respectively, \( P < 0.01 \)) in p53 (+/+/-) diabetic mice, whereas there was no significant difference in p53 (+/-/-) diabetic mice (Figure 4A).

We further examined SCO2-mediated FA metabolism by using SCO2-overexpression myocytes. Cardiac myocytes were incubated with \(^{14}\text{C}\)-oleate, and the amounts of metabolized FA (assessed by \(^{14}\text{C}\)O\(_2\) production from \(^{14}\text{C}\)-oleate) and amounts of \(^{14}\text{C}\)-oleate residual in myocytes were then measured. As shown in Figure 4B, SCO2 overexpression induced an increase (1.3-fold versus control) in \(^{14}\text{C}\)O\(_2\) production from \(^{14}\text{C}\)-oleate, whereas amounts of residual \(^{14}\text{C}\)-oleate further increased (1.5-fold versus control). Knockdown of SCO2 reduced \(^{14}\text{C}\)O\(_2\) production and amounts of residual \(^{14}\text{C}\)-oleate (Figure 4C), and accumulation of fluorescent long-chain FA analog (BODIPY-conjugated palmitate) was also augmented by SCO2 induction (Figure 4D). These findings suggest that p53/SCO2 drives increased lipid accumulation in the context of increased oleate oxidation.

SCO2 overexpression caused a marked increase in mitochondrial ROS generation assessed by MitoSox fluorescence (1.4-fold versus control, Figure 4E). Mitochondria were visualized with Mitotracker. Image merging of MitoSox with Mitotracker showed that the enhanced ROS was generated from mitochondria (Figure 4F), suggesting that SCO2 stimulates the uptake and utilization of FA and augments mitochondrial ROS generation in cardiac myocytes.

**Tempol Treatment Improves Cardiac and Metabolic Dysfunction**

To investigate the implication of oxidative stress in STZ-induced diabetes mice, we examined the effect of tempol for
As shown in online-only Data Supplement Figure 5A, tempol pretreatment normalized the elevated p53 and SCO2 protein levels on day 28. Augmented consumption of mitochondrial oxygen and ROS-mediated DNA damage were reduced by tempol (online-only Data Supplement Figure 5B and 5C). Cardiac dysfunction and BNP levels were improved toward normal levels (online-only Data Supplement Figure 5D and 5E).

To examine the effect of ROS on the myocyte contraction and viability, we compared contraction rates of pulse-stimulated myocytes after administration of oleate. We found that oleate stimulated ROS generation in myocytes (online-only Data Supplement Figure 6A), and oleate-induced ROS reduced myocyte contraction (45% versus control, P < 0.01) without a significant change in cell viability or apoptotic cell number (online-only Data Supplement Figure 6B through 6D).

SCO2 (+/−) Diabetic Mice Reproduce the Metabolic Phenotype of p53 (+/−) Diabetic Mice
Because SCO2 (−/−) mice were embryonically lethal,17 we studied the role of SCO2 using SCO2 (+/−) mice. The basal level of SCO2 protein was lower (32% versus control, P < 0.01) in diabetic SCO2 (+/−) hearts compared with SCO2 (+/+) hearts (Figure 5A). After administration of STZ, the level of SCO2 was increased in diabetic SCO2 (+/+) hearts (151% versus control, P < 0.01) but not in diabetic SCO2 (+/−) hearts (Figure 5A). Although blood glucose and lipid profile were significantly increased in diabetic SCO2 (+/+) mice (online-only Data Supplement Figure 7A), no apparent difference in the histological analyses (hematoxylin and eosin and Masson’s trichrome staining) was observed between control and diabetic SCO2 (+/−) mice (online-only Data Supplement Figure 7B). Basal mitochondrial complex IV activity was significantly decreased (64% versus control, respectively, P < 0.01) in SCO2 (+/−) hearts compared with SCO2 (+/+) hearts (Figure 5B). Although complex IV activity was increased in diabetic SCO2 (+/+) hearts (135% versus control, respectively, P < 0.05), there was no significant increase in diabetic SCO2 (+/−) hearts (Figure 5B). No apparent changes were observed in ROS damage, lipid accumulation and lipid droplet in the myocardium of SCO2 (+/−) mice (Figure 5C).

Figure 3. Mitochondrial respiration was augmented in diabetic hearts. Mitochondrial respiration was measured with Clark-type electrodes, using isolated cardiac mitochondria. A, There were no changes in state 2 or state 4 respirations. State 3 mitochondrial respiration was upregulated in p53 (+/+) diabetic mice. RC indicates respiratory control ratio. B, Complex IV activity was augmented in p53 (+/+) diabetic mice. C, Mitochondrial complex IV activity was significantly augmented in p53 (+/+) diabetic hearts at 4 and 8 weeks after streptozotocin (STZ) injection, whereas mitochondrial activity of complex I, II, and III was not changed 2–8 weeks after STZ injection (n = 10 each). D, Alteration of complex IV activity at 4 weeks was notable in p53 (+/+) mice. **P < 0.01 versus p53 (+/+) control; ##P < 0.01 versus p53 (−/−) control.
and BNP mRNA levels did not significantly change in diabetic SCO2 (\(+/+\)) mice (Figure 5D and 5E and online-only Data Supplement Table 2). These findings indicated that diabetes-induced cardiac dysfunction resulted from SCO2-mediated excessive responses in mitochondrial respiration, ROS production and lipid accumulation.

**Expression Profiles of Genes Regulating Cardiac FA Metabolism**

We next examined the expressions of genes closely involved in cardiac FA metabolism.5,18,19 Although peroxisome proliferator-activated receptor-\(\alpha\) mRNA levels were unchanged (online-only Data Supplement Figure 8A), the mRNA expression of proliferator-activated receptor-\(\gamma\) coactivator 1 (PGC-1) \(\alpha\) was elevated to a similar extent in p53 (+/+) diabetic mice (174% and 159% versus control, \(P<0.05\), online-only Data Supplement Figure 8B). The mRNA expressions of FA import regulating genes, such as fatty-acid transport protein (FATP) and carnitine palmitoyltransferase I, did not differ between p53 (+/+) and p53 (−/−) diabetic hearts, whereas FAT/CD36 mRNA was significantly upregulated in p53 (+/+) diabetic mice (online-only Data Supplement Figure 8C through 8F).

**FAT/CD36 Protein Is Localized at Membrane of Myocytes**

FA enter myocytes by passive diffusion or by protein carrier-mediated pathway.20 This mechanism for protein-mediated uptake involves binding of FA to FA-binding protein (FABP), which concentrates the FA by either passive diffusion or uptake through the FAT/CD36- or FATP1-mediated pathway.21 FAT/CD36 translocates between intracellular endosomes and sarcolemmal membrane, known to be important in the regulatory control of fatty acid uptake.22 Therefore, we examined membrane localization of FA transporter, FAT/CD36 protein, and FATP1 protein. Caveolin 3 was used as a sarcolemmal marker. The expression of FATP1 protein was not altered in diabetic p53 (+/+) mice (data not shown). Cardiac expression of sarcolemmal FAT/CD36 was significantly increased in diabetic p53 (+/+) mice (151% versus

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**Figure 4.** Synthesis of cytochrome c oxidase 2 (SCO2) protein was increased in p53 (+/+) diabetic heart and modulated lipid metabolism and reactive oxygen species (ROS) generation. A, In p53 (+/+) diabetic mice, the expressions of SCO2 was increased, whereas no increases were observed in p53 (−/−) diabetic mice (n=10 each). **\#**\(P<0.01\) versus p53 (+/+) control. B, SCO2 overexpression augmented oleate oxidation and residual oleate in myocytes (n=10 each). **\(P<0.01\) versus Ad-LacZ. C, Knockdown of SCO2 reduced oleate oxidation and residual oleate in myocytes (n=10 each). \(\#\)\(P<0.05\) versus small interfering NS. D, BODIPY-conjugated palmitate was accumulated in SCO2 overexpressing myocytes (n=10 each). **\(P<0.05\) versus Ad-LacZ. E, SCO2 protein increased the ROS generation detected by Mitosox in myocytes (n=10 each). **\(P<0.05\) versus Ad-LacZ. F, ROS stained with Mitosox (red) were colocalized with MitoTracker (green) (n=10 each).
control, $P<0.05$), whereas such upregulation of FAT/CD36 was not observed in diabetic p53 (/-) or SCO2 (/-) mice (online-only Data Supplement Figure 9A).

We also examined the regulation of FAT/CD36 in cardiac myocytes. p53 induction by nongenotoxic p53 activator Nutlin-3 upregulated FAT/CD36 mRNA expression in a time-dependent manner (online-only Data Supplement Figure 9B). Induction of p53 augmented CD36 expression (online-only Data Supplement Figure 9C). Treatment with antioxidant tempol reduced the induction of FAT/CD36 protein (online-only Data Supplement Figure 9D). Administration of $H_2O_2$ ($1\times10^{-8}\text{mol/L}$) induced CD36 expression with p53 protein, and knockdown of p53 inhibited CD36 expression (online-only Data Supplement Figure 9D). Knockdown of CD36 gene downregulated the basal expression of CD36 mRNA to the control level and inhibited nongenotoxic p53 activator Nutlin-3–mediated residual oleate to the control level (online-only Data Supplement Figure 9E), whereas overexpression of SCO2 markedly induced FAT/CD36 protein expression (2.8±0.6-fold versus control, online-only Data Supplement Figure 10A).

gp53/SCO2 Expression and Lipotoxicity Are Involved in db/db Mouse Heart

We also examined whether the induction of p53/SCO2 protein and mitochondrial function in STZ-treated mice was similarly observed in the genetic model of type 2 diabetes. Ten-week-old db/db mice showed hyperglycemia and lipid profile abnormality (online-only Data Supplement Figure 11A). We found that cardiac contractile function was impaired (63% versus db/+, $P<0.01$; online-only Data Supplement Figure 11B and online-only Data Supplement Table 3) and cardiac p53 and SCO2 expressions were increased in db/db mice (208% and 151% versus db/+, $P<0.01$; online-only Data Supplement Figure 11C). Along with the increase in SCO2 expression, state 3 respiration ($V_{ADP}$), oxygen consumption, and mitochondrial complex IV activity were upregulated in db/db hearts (156%, 142%, and 131% versus db/+; online-only Data Supplement Figure 11D). Moreover, ROS-mediated DNA damage (8OHdG) and lipid accumulation (oil-red-O) were apparently increased in db/db hearts compared with db/+ hearts (online-only Data Supplement Figure 11E).
Electron micrographic analysis showed that the number of lipid droplets around mitochondria was increased in cardiac myocytes from db/db mice compared with db/+ mice (4.9-fold versus control; online-only Data Supplement Figure 11E). Thus, cardiac lipotoxicity was observed in db/db mice, in which the expressions of p53 and SCO2 were markedly induced as observed in STZ-induced diabetic mice.

**Discussion**

We have previously reported that p53 regulates mitochondrial respiration through activation of SCO2, a transcriptional target of p53.13 The present study is the first to focus on the role p53/SCO2 on energy metabolism in the diabetic hearts; we found that (1) the expressions of p53 and SCO2 were induced with the progression of cardiac dysfunction in type 1 and type 2 diabetic mice in a ROS-dependent manner; (2) activated SCO2 enhanced the mitochondrial complex IV activity and upregulated the rate of FA oxidation and excessive generation of mitochondria-derived ROS, resulting in the lipid accumulation and DNA damage in myocytes. Inhibition of SCO2 function suppressed the augmentation of mitochondrial respiration and protected heart against the progression of diabetic cardiomyopathy; and (3) induction and translocation of FA translocase FAT/CD36 were highly associated with activation of the p53/SCO2 system, resulting in lipid over-storage in myocytes. These findings suggest that lipotoxicity with excessive augmentation of mitochondrial respiration through the p53/SCO2 system plays an important role in the progression of diabetic cardiomyopathy.

In diabetic hearts, energy production derived from FA oxidation is promoted despite the decrease in energy production from glucose oxidation.23 This alteration is accompanied with increased myocardial oxygen consumption. Because cardiac work is unchanged or reduced, energy efficiency is relatively declined in diabetic animal and human hearts.24,25 Tumor suppressor, p53, has a well-characterized role in regulating apoptosis and cell cycle in response to genomic stress; however, its role on metabolism remains to be determined.9 In cardiac myocytes, p53 is activated by high glucose, leading to myocyte death.26 In the in vivo condition, hyperglycemia causes enzymatic O-glycosylation of p53, whose activation enhances myocyte apoptosis.27 Our present findings demonstrated that diabetic condition caused excessive oxidative DNA damage with p53 induction, and this change was inhibited by administration of antioxidant (Figure 1A and online-only Data Supplement Figure 5), suggesting that diabetes-induced ROS generation upregulates the expression of p53, and the p53 increases mitochondrial respiration through the p53 downstream mitochondrial assembly protein, SCO2.

It remains to be determined how diabetes-mediated increase in oxygen consumption is associated with cardiac dysfunction. SCO2 overexpression augments oxygen consumption,13 and higher respiration activity generates more ROS.28 Oxidative stress and p53 have been suggested by several studies to underlie hyperglycemia-induced myocardial cell death.26,29,30 Despite its prime importance of cell death, mitochondrial alteration by ROS was reported to cause cardiomyocyte damages in STZ-induced diabetic mice and db/db mice.7 As shown in online-only Data Supplement Figure 3, there was no significant difference in apoptotic cell numbers between p53(+/-) and p53(-/-) mice, although the numbers tended to be decreased in p53(-/-) mice. Thus, as the present result indicated that the reduced contractile performance in the diabetic hearts probably was not due to cell death and loss of parenchymal cells from the organism, we next examined whether generation of ROS actually affects contraction activities of myocytes in vitro and found that oleate-induced ROS markedly reduced myocyte contraction rates (45% versus control, P<0.01) without a change in cell viability or apoptotic cell numbers (online-only Data Supplement Figure 6). Taken together with the result that antioxidant tempol inhibited the upregulation of p53 and SCO2 and improved cardiac dysfunction in diabetic p53 (+/-) mice to the level similar to p53 (-/-) mice (Figure 1 and online-only Data Supplement Figure 5), these findings suggest that reduced cardiac function in the diabetic hearts is due to ROS-mediated, direct inhibitory effects on contractile performance of individual myocytes rather than loss of parenchymal cells from the organ.

SCO2 protein is essential for the assembly of complex IV, the metabolic center of eukaryotic oxygen consumption. Disruption of SCO2 eliminates mitochondrial respiration,31 and overexpression of SCO2 augments mitochondrial respiration.13 We showed, using SCO2 (+/-) mice, the marked decreases in SCO2 expression and mitochondrial respiration compared with the wild-type mice (Figure 5). In the STZ-induced diabetic conditions, myocardial oxygen consumption was increased as much as 36% (Figure 3), and SCO2 expression was also markedly induced (148% versus control) (Figure 4). These alterations were also observed in db/db mice (online-only Data Supplement Figure 11), indicating the critical involvement of p53 and SCO2 in the increase of mitochondrial respiration in diabetic hearts. In the present study, we found for the first time that p53/SCO2 increased mitochondrial respiration activity to cause the uptake lipid more than its necessary consumption rate, thus leading to the lipid overstorage and ROS production. We also showed that increased CD36 expression in the membrane of myocytes was closely associated with p53, and knockdown of CD36 inhibited p53-mediated oleate uptake to the control level (online-only Data Supplement Figure 9). Loss-of-function studies using CD36-deficient mice would be necessary to further define the role of FA transporter and lipotoxicity in the diabetic heart. Considering that there are p53 consensus binding sites in the promoter regions of human and rat/mouse CD36 genes (online-only Data Supplement Figure 10B), it might be possible to speculate that the expression of the CD36 gene is transcriptionally regulated by p53. Further studies, such as luciferase assays or chromatin-p53 immobilization experiments, will be needed to define the p53-mediated transcriptional control of CD36 gene.

PGC-1α is a pivotal mediator of energy metabolism in augmenting lipid metabolism and mitochondrial biogenesis.5,19 In the insulin-resistant diabetic heart, PGC-1α was activated and PGC-1α-mediated mitochondrial biogenesis is assumed as an adaptive response to increased FA oxidation.5 Sahin et al reported that p53 also represses PGC-1α and...
damages heart mitochondria in the setting of telomere dysfunction. They described that p53 can exert varied effects in other physiological settings, citing our previous results. In our diabetic mice, PGC-1α was upregulated independent of p53 status (online-only Data Supplement Figure 8B). In contrast to the previous results by the DePinho group, p53 did not exert a repressive effect on PGC-1α. As p53 is a multifunctional protein important for the maintenance of genomic integrity, timing and duration of p53 activation affects the number of its downstream genes. Modulation of PGC-1α might be a potential avenue to protect the heart from the development of diabetic cardiac dysfunction. Senile diabetic mice models or longer exposure to high glucose of the heart might reveal another role of p53 and PGC-1α.

In conclusion, we found for the first time that diabetes-induced ROS generation upregulates p53 and SCO2, resulting in the increase of mitochondrial respiration and subsequent lipid accumulation and excessive generation of mitochondrial ROS. This hypothesis was supported in our in vitro study showing that SCO2-overexpressing myocytes accelerated lipid uptake and mitochondrial ROS generation in substrate-rich circumstances, and knockdown of SCO2 reduced FA metabolism. Thus, p53 and SCO2 may be therapeutic targets to prevent the progression of diabetes-mediated cardiac dysfunction.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

Diabetic cardiomyopathy is one of the leading causes of increased morbidity and mortality in the patients with diabetes mellitus. Although the pathogenesis of this cardiac contractile dysfunction is still not fully understood, much interest has been focused on the involvement of increased reactive oxygen species (ROS) production and altered mitochondrial function. Increased myocardial fatty acid utilization and cardiac triglyceride accumulation in diabetic patients were also demonstrated. Recently, activation of p53 and its target genes in the development of heart failure has received considerable attention. We found myocardial p53/SCO2 (synthesis of cytochrome c oxidase 2) signal is activated by diabetes-mediated ROS generation to increase mitochondrial oxygen consumption in both type I and type II diabetic (db/db) mice. The activation of p53/SCO2 results in excessive generation of mitochondria-derived ROS and lipid accumulation in association with cardiac dysfunction. Thus, p53 and SCO2 may be novel therapeutic targets to prevent the progression of diabetes-mediated cardiac dysfunction.
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In the article “p53 Promotes Cardiac Dysfunction in Diabetic Mellitus Caused by Excessive Mitochondrial Respiration-Mediated Reactive Oxygen Species Generation and Lipid Accumulation” by Nakamura et al, which published online November 9, 2011, and appeared in the January 2012 issue of the journal (Circ Heart Fail 2012;5:106–115), a correction to the supplemental material was needed.

The left panels shown in Supplemental Figure 5A and the left lower picture in Supplemental Figure 11E were incorrectly selected and labeled. The left panels in Supplemental Figure 5A were found to be from control and streptozotocin (STZ)-induced diabetic mice prepared for other experiments. The corrected left panels now show the protein levels from control and STZ-induced diabetic mice treated with vehicle for Tempol. The figure of electronmicroscopy of db/+ mice in Supplemental Figure 11E was incorrectly chosen, and we now show the correct picture. The authors wish to apologize for these errors and emphasize that the correction of these errors does not impact the article’s conclusions.

These corrections have been made to the current online version of the article, which is available at http://circheartfailure.ahajournals.org/content/5/1/106.full.
Supplemental Online Material

includes methods, 11 figures, 3 tables and Supplementary figure legends.

Materials and Methods

Induction of type 1 diabetes

p53(-/-)mice (with a C57BL/6 background) were purchased from Jackson Laboratories. SCO2(+/-) mice are provided to us from Dr. Eric A Schon [1]. Eight-week-old male p53(+/+), p53(-/-) and SCO2(+/-) C57Bl6 mice were injected intraperitoneally with 200 mg/kg of streptozotocin (STZ). Animals with blood glucose levels greater than 20 mmol/L at 48 hours after STZ injection were deemed diabetic. Vehicle-treated mice served as nondiabetic controls. Tempol (Sigma, 1 mmol/L) was dissolved in drinking water and given ad labium from 7 days before STZ injection through 4 weeks after STZ injection.

Genetic type 2 diabetes mice

db/db mice [2], defect in the leptin receptor, have obesity, insulin resistance, and diabetes. db/+ mice were analyzed as a control mouse. 10-week-old male db/db mice and db/+ mice were analyzed.

Transthoracic echocardiology

Echocardiographic analysis was performed using a commercially available echocardiograph (SONOS 5500, Hewlett-Packard) equipped with a 15-MHz linear array
ultrasound transducer as previously described [3].

**Glucose tolerance test**

After 6-hour fasting period, a bolus of glucose (2 g/kg) was delivered into the stomach by a gavage needle and 200 μL of blood was sampled at 0, 15, 30, 60, and 120 min for plasma glucose in conscious state [4].

**Treatment with Insulin**

Insulin pellets (Linshin Canada) were implanted dorsally and subcutaneously after 3 days of STZ injection. The pellets released 0.2 U insulin/day for 4 weeks.

**Tissue lipid content**

Hearts were excised after a perfusion with 3 mL of PBS from the left ventricle. Lipids were extracted to measure content of triacylglycerol (TG) using assay kits (Wako) according to product protocol.

**Mitochondrial DNA content quantification**

Mitochondrial DNA was co-purified with genomic DNA from mouse heart tissues using DNA extraction kits (Takara Bio). Ct values determined for COX2 gene encoded by the mitochondria DNA and 18S rRNA gene encoded by the nuclear DNA, and the relative mitochondria DNA copy number was calculated by normalizing to 18S rRNA gene copy number. Primer sequences are described as follows.
Mouse COX2 RT-PCR primer sequences:

Forward 5’- CCATAGGGCACCAATGATACTG
Reverse 5’- CGCTGAGCCAGTCAGTGTAG

Mouse 18S rRNA RT-PCR primer sequences:

Forward 5’- CTTAGAGGGACAAGTGGCGTTC
Reverse 5’- CGCTGAGCCAGTCAGTGTAG

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the hearts and cultured myocytes using TRIzol reagent, and reverse transcribed by Super Script III First-Strand Synthesis System (Invitrogen). First-strand cDNA was assessed by CYBR Green I real-time PCR (Light Cycler, Roche). The expression levels of target genes were normalized by the expression levels of β-actin. Primer sequences used for RT-PCR to detect the expression of each target gene are as follows.

Mouse BNP RT-PCR primer sequences:

Forward 5’- AAGGGAGAACACGGCATCAT
Reverse 5’- GATCCATGCCGCAGGAGC

Mouse CD36 RT-PCR primer sequences:

Forward 5’- ATTTGGTCAAGCCAGCT
Reverse 5’- TGTAGGCTCATCCACTAC

Mouse FATP RT-PCR primer sequences:

Forward 5’- GGCTCCTGGAGCAGGAAC
Reverse 5’- ACGGAAGTCCCAGAAACCAA

Mouse LPL RT-PCR primer sequences:
Forward 5’- TCTGTACGGCACACGTGG
Reverse 5’- CCTCTCGATGACGAAGC

Mouse CPT-1 RT-PCR primer sequences:
Forward 5’- ATCATGTATCGCCGCAAAC
Reverse 5’- GGGATGCGTGTAGTGTTGAAC

Mouse PPARα RT-PCR primer sequences:
Forward 5’- ACTACGGAGTTCACGCATGTG
Reverse 5’- TTGTCGTACACCAGCTTCAGC

Mouse PGC1α RT-PCR primer sequences:
Forward 5’- CGGAAATCATATCCAACCAG
Reverse 5’- TGAGAACCGCTAGCAAGTTTG

Rat CD36 RT-PCR primer sequences:
Forward 5’- CTCTGACATTTGCAGGTCCA
Reverse 5’- CACAGGCTTCTTTCTTTGC

Anti body and Western blot analysis
Affinity-purified rabbit polyclonal antisera were made against mouse SCO2 (amino acids 39-53,GQRQGPGLRTRLIT). Equal amounts of protein were fractionated on Tris-Glycine SDS-polyacrylamide gels and subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Chemiluminescence was detected with
ECL Western blot detection kits (Amersham) according to the supplier’s recommendations. Other antibodies: anti-actin monoclonal antibody (C-2, Santa Cruz Biotech) was used as an additional control for protein loading; anti-p53 antibody (C-19, Santa Cruz Biotech) anti-FAT/CD36 antibody (abcam ab36977-100).

**Mitochondrial respiration**

The respiratory rates of isolated cardiac mitochondria were determined with oxygen sensor probe. Basal respiration rates before the addition of ADP were defined as state 2 (V\(_0\)). Maximally ADP (1 mmol/L)–stimulated respiration rates were defined as state 3 (V\(_{ADP}\)), and respiration rates in the absence of ADP phosphorylation and measured in the presence of 1\(\mu\)g/mL oligomycin were termed state 4 (V\(_{oligomycin}\)). Respiratory control ratio (RC) was calculated from the ratio of state 3 to state 4 respirations. Cardiac mitochondria were isolated by differential centrifugation. Mitochondria (0.35 mg/ml) were resuspended in the assay buffer (containing 5mM glutamate, and 2mM malate) in a sealed, stirred chamber. Basal respiration rates before the addition of ADP were defined as state 2. Maximally ADP (1 mmol/L)–stimulated respiration rates were defined as state 3, and respiration rates in the absence of ADP phosphorylation and measured in the presence of 1\(\mu\)g/mL oligomycin were termed state 4. O\(_2\) was measured in 1 mL of buffer at 25°C with continuous stirring as previously described [5]. The solubility of oxygen in assay buffer is 237 nmol of O\(_2\) per mL at 25°C. Oxygen consumption rates were expressed as natom of O\(_2\)/min/mg protein.

The activities of complexes I, II, III and IV were measured as previously described [6]. For the measurement of complexes I, II, III and IV substrates and inhibitors were added in
the following order and concentrations: 5 mmol/L malate; 5 mmol/L pyruvate; 100 nmol/L rotenone; 5 mmol/L succinate; 50 nmol/L antimycin A; 1 mmol/L ascorbate; 0.4 mmol/L N,N,N’-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and 5 mmol/L KCN. The activity of complex IV was measured after the addition of TMPD.

**Histological studies**

Whole hearts were paraffin-embedded and sliced into 7 μm sections. These sections were stained with hematoxylin-eosin (HE) and Masson Trichrome (MTC). A midventricular slice of myocardium was snap-frozen in a cryomold containing OCT for sectioning. Frozen myocardium was sliced into 10 μm-thick sections. These sections were stained with anti-PE-labeled CD31 antibody (BD Pharmingen) and anti-FITC-labeled α-smooth muscle actin (Sigma). To detect apoptotic cells, we performed TUNEL staining with TMR red Kit (Roche) and DAPI. Using the avidin-biotin complex method with alkaline phosphatase, immunohistochemistry for 8-hydroxy-2’-deoxyguanosine (8-OHdG) was performed. To detect neutral lipid, frozen sections were stained with Oil Red O.

Electron microscopy (EM) cardiac specimens were prepared. Heart muscle was fixed in 3% glutaraldehyde with 0.1 mmol/L phosphate buffer (pH 7.2) for 3 hours at 4°C and postfixed in 2% osmium tetroxide with 0.1 mmol/L phosphate buffer (pH 7.2) for 120 min at 4°C and then serially dehydrated in ethanol and embedded in epoxy resin. Sections were cut on an LKB ultramicrotome and consecutive ultrathin sections were mounted on copper grids. Ultrathin sections were stained with 3% uranyl acetate and 0.2% lead citrate. Examinations were conducted with an electron microscope (H-7100; Hitachi, Tokyo,
Cultured neonatal rat cardiac myocytes

Primary cultures of neonatal rat cardiac myocytes were prepared from neonatal Wistar rat hearts by digestion with 0.2% collagenase as described previously [7]. Briefly, cardiac ventricles from one-day-old Wistar rats were minced and dissociated with 0.2% of type I collagenase. The dispersed cells were incubated in 100mm culture dishes for 30 minutes at 37 °C, and the nonattached cardiac myocytes were collected and seeded into 35 mm (5 x 10^5 cells per dish) culture dishes. The myocytes were incubated in Dulbecco’s modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; Bioserum Lenexa). Bromodeoxyuridine (1x10^{-4} mol/L) was added during the first 48 hours to inhibit the proliferation of nonmyocytes.

Gene over expression by adenovirus

For gene over expression, recombinant adenoviruses expressing SCO2 (Ad-SCO2), LacZ (Ad-LacZ), were generated. The cDNA encoding rat SCO2 was inserted into a pAxCAwtit cosmid vector in an adenovirus expression vector kit (Dual Version; Takara Bio Inc., Otsu, Japan). Recombinant adenoviruses expressing SCO2 (Ad-SCO2), LacZ (Ad-LacZ), were generated. Cardiomyocytes were infected with Ad-SCO2, Ad-LacZ, diluted in the culture medium at a multiplicity of infection (MOI) of 10 and incubated at 37°C for 1 hour.

Gene silencing via RNA interference
Nonspecific, SCO2-specific siRNA duplexes and CD36-specific siRNA duplexes were purchased from Invitrogen. siRNAs were transiently transfected into the myocytes using Lipofectamine RNAiMAX (Invitrogen) according to the product protocol. Myocytes were given fresh media 24 hours after transfection, and total RNA was isolated from myocytes 48 hours after transfection.

Sequences of siRNAs which were used to suppress the expression of SCO2 or CD36 in cultured cardiomyocytes are as follows:

Nonspecific: Stealth RNAi Negative Control Duplexes, Medium GC Duplexes #2

SCO2 : Sense 5’- CAGGACUAUAUUGUGGACCAUCCA
Antisense 5’- UGGAAUGGUCCACAAUAUAGUCCUG

CD36 : Sense 5’- GAGACCUCACUCAUGAGAAGACAAU
Antisense 5’- AUUGUCUUCUCAUGAGUACGUCUC

Measurement of [14C]-oleate oxidation and residual [14C]-oleate in myocytes

Oleate oxidation were assessed by measuring the production of 14CO2 from [14C]-oleate [8]. Briefly, Ad-SCO2 was infected into rat neonatal myocytes. SCO2 siRNAs were transiently transfected into the myocytes using Lipofectamine RNAiMAX (Invitrogen) according to the product protocol. Myocytes were incubated with 10 mmol/L glucose and 10% FBS. At 48 hours after transfection or siRNA, myocytes were incubated for 2 hours with serum-free medium containing 11 kBq/mL [14C]-oleate. For measuring conversion [14C]-oleate into 14CO2, a piece of Whatman paper wet with phenylethylamine/methanol (1:1) was taped onto the inside of each petri dish to trap the CO2 produced during the incubation period. Finally the pieces of Whatman paper were carefully transferred to scintillation vials for
radioactivity counting. For measuring residual $^{14}$C-oleate in myocytes, the incubation medium was aspirated at 2 hours after incubation containing 11 kBq/mL $^{14}$C-oleate. Then cells were washed with ice-cold saline and lysed with 0.2 mol/L NaOH-10% SDS solution. Cell lysates were transferred to scintillation vials for radioactivity counting.

**Measurement of FA uptake**

To determine FA uptake, we used fluorescent palmitate [9]. Myocytes were subsequently serum-starved for 4 hours, and then incubate with boron dipyrromethene difluoride (BODIPY) -conjugated palmitate (1 mmol/L) in serum-free media at 37 °C for 2 minutes.

**Detection of ROS and mitochondria**

ROS production by mitochondrial was assessed by using Mito SOX Red (Invitrogen) according to product protocol. Adenovirus were infected 48 hours before examination. Slides were labeled with Mito SOX Red (300nM) and Mito Tracker green (100 nM) for 20 min at 37°C, then observed by confocal microscopy (FV1000, Olympus) with the excitation wavelength set at 488 and 543 nm.

**Contraction of cardiac myocytes and Histochemical determination of cell viability**

Twenty -four hours after the administration of oleate (1 mM, 5 mM) or tempol (100 μM), cardiac myocytes stained by di-4-ANEPPS (1-min application at 500 nM) were electrically paced at 3 Hz by field stimulation via Ag-AgCl wires (diameter; 0.5 mm, interpolar distance; 5 mm) with the stimulation intensity of twice the threshold for synchronous
contraction under superperfusion with HEPES-buffered Tyrode’s solution at 34°C. The cell borders, visualized by di-4-ANEPPS-fluorescence (excitation by 488-nm Argon laser and emission at 530-nm wavelength) with the rapid-scanning confocal microscopy [10], were detected by MiCAM02 system (Brainvision Inc. Japan). Contractility of the myocytes was analyzed by changes in the cell lengths with Image J software. The averaged fractional cell shortening was obtained.

To monitor cell viability, myocytes were grown on Type I collagen-coated glass coverslips. Following 24 h of incubation with oleate, the relative number of living and dead cells was determined using a viability/cytotoxicity kit (Molecular Probes—Catalog number L-3224). The culture medium was replaced with 2×10⁻⁶ mol/l calcein acetoxymethyl ester and 4×10⁻⁶ mol/l ethidium homodimer-1 and the cells were incubated for 45 min at room temperature. Cells with permeabilized membranes (necrosis) take up the ethidium homodimer-1 dye and their nuclei appear red. In contrast, cells with intact membranes exclude the ethidium dye, but take up and hydrolyze the calcein ester and therefore appear green. Cell viability was quantitated by counting the number of necrotic and viable myocytes in 10 random microscopic fields per condition per experiment using a fluorescence microscope [11].

Apoptotic myocytes were identified by their distinctive condensed or fragmented nuclear morphology in cells stained with 5 × 10⁻⁴ g/L of Hoechst 33258 (Molecular Probes). A mean average of 800 to 1,000 nuclei from randomly selected fields was analyzed for each experiment, and the number of apoptotic myocytes was expressed as a percentage of the total number of nuclei counted.
References


**Supplementary Figure Legend**

**Supplemental Figure 1.** Blood glucose, lipid profile and glucose tolerance were not different between p53(+/+) mice and p53(-/-) mice.
(A) The level of blood glucose (Glu), triacylglycerol (TG), free fatty acid (FFA) and total cholesterol (Tchol) were increased at 2, 4 and 8 weeks after STZ injection in both p53(+/+) and p53(-/-) mice (n=10 each) ** p<0.01 vs control There were no significant differences between both mice. (B) No significant differences were observed in glucose tolerance. (n=6 each) (C) mRNA expression of BNP was augmented at 2, 4 and 8 weeks after STZ injection in p53(+/-) diabetic mice, while mRNA expression of BNP was not increased in p53 (-/-) diabetic mice. (n=10 each) ##p<0.01 vs. p53 (+/+ ) control

Supplemental Figure 2. Histological appearance of left ventricle and Characterization of vasculature and microcirculation

The representative HE staining (A, magnification×200) and MTC staining (B, magnification×200) depicted the histological appearance of left ventricles from p53(+/+) and p53(-/-) mice after 4 weeks of diabetes. There were no histological changes in HE and MTC staining. Representative CD31 staining demonstrated the microcirculation of myocardium. (C, magnification×200) Number of CD31 positive cells was not changed in diabetes or in p53 deficiency. (C, right panel) (n=10 each)

Supplemental Figure 3. Number of apoptosis after STZ injection

Number of TUNEL positive myocytes was markedly increased rapidly after injection of STZ, reaching the peak level on day 7 and then declining to the basal level on day 28. There was no significant difference between p53(+/-) and p53(-/- ) diabetic mice from day 3 to day 28 (n=10 each). ** p<0.01 vs day 0
Supplemental Figure 4. Treatment with insulin inhibited induction of mitochondrial respiration, p53 induction and cardiac dysfunction

(A) Insulin treatment reduced the increase of state3 respiration. (B) Insulin treatment reduced the augmentation of complex IV. (C) The level of p53 protein did not change at 4 weeks. (D) Fractional shortening of the heart did not change at 4 weeks. (E) The staining of 8OHdG, Oil red O and the number of lipid droplets did not change in 4 weeks. (n=10 each)

**p<0.01 vs. control, Ins: insulin

Supplemental Figure 5. Tempol improved heart function with the reduction of ROS damage

(A) p53 and SCO2 protein induction was less in tempol-treated group than that in untreated group. (B) Mitochondrial complex IV activity was not induced in tempol-treated diabetic mice. (C) ROS-induced DNA damage, lipid accumulation or droplets (arrowheads) were not increased in tempol-treated diabetic mice. (D) Contractile function was preserved in tempol-treated diabetic mice. (E) BNP mRNA levels were not increased in tempol-treated diabetic mice. (n=10 each) ** p<0.01 vs control tempol (-) *p<0.05 vs control tempol (+)  # p<0.05 vs. STZ 4W tempol (-)

Supplemental Figure 6. Tempol improved myocyte contraction with reduction of ROS generation
(A) Incubation with oleate increased ROS generation detected by Mitosox in myocytes. Treatment with tempol reduced ROS generation. (B) Contraction of myocytes was decreased by the administration of oleate (5mM). Oleate-induced cell alteration was prevented by tempol. (C) There were no significant differences in cell viability. (D) There were no significant differences in the number of apoptotic myocytes. (n=10 each) **p<0.01 vs control  OL:Oleate

Supplemental Figure 7. Blood chemistries and histological appearance in SCO2 (+/-) diabetic mice

(A) The blood glucose was increased in SCO2 (+/-) mice 4 weeks after STZ injection. Hyperlipidemia was documented in SCO2 (+/-) diabetic mice. (B) The representative HE staining (magnification×200) and MTC staining (magnification×200) depicted the histological appearance of left ventricles from SCO2 (+/-) mice after 4 weeks of diabetes. There were no morphological changes in HE and MTC staining. (n=10 each) **p<0.01 vs. SCO2 (+/-) control, *p<0.05 vs. SCO2(+/-) control

Supplemental Figure 8. mRNA expressions of fatty acid metabolism regulating genes

(A) mRNA expression of PPAR α was not changed in p53(+/-) and p53(-/-) mice. (B) PGC-1α mRNA levels were increased in both p53(+/-) and p53(-/-) mice in diabetic myocardium after STZ injection. (C, D, E) mRNA expressions of FATP, LPL, and CPT1
were not changed in p53(+/+) and p53(-/-) mice. (F) CD36 mRNA levels were increased in p53(+/+) but not in p53(-/-) mice in diabetic myocardium. (n=10 each) *p<0.05 vs. control

Supplemental Figure 9. Membrane expression of FAT/CD36 was increased in p53(+/+) diabetic mice.

Representative cross-sections of cardiac muscle from mice as indicated after incubation with antibodies against caveolin 3 and FAT/CD36. (A) Sarcolemmal FAT/CD36 protein expression was increased more in cardiac muscle in p53 (+/+) than those in p53 (-/-) and SCO2 (+/-) mice. (n=10 each) *p<0.05 vs p53 (+/+) control (B) CD36 mRNA was induced by p53 induction. (n=6 each) ##p<0.01 vs. control (C) CD36 protein was decreased by antioxidant tempol. (n=6 each) (D) Knock-down of p53 reduced H2O2 induced CD36 expression. (n=6 each) (E) p53 induction augmented residual oleate, while knock-down of FAT/CD36 blunted the p53-induced residual oleate. (n=6 each) ##p<0.01 vs. control

Supplemental Figure 10. SCO2-induced CD36 expression and p53 binding sites in CD36 genes

(A) SCO2 over-expression augmented CD36 expression in cardiac myocytes. (n=6 each)

(B) Schematic representation of the CD36 genomic DNA structure. p53 consensus binding sites in the CD36 genes were referenced by the gene information from NCBI (accession number Human CD36 NM_000072, Rat CD36 NM_031561, Mouse CD36 NM_001159555) E: Exon
Supplemental Figure 11. p53 and SCO2 protein were induced in db/db mice heart.

(A) Hyperglycemia and hyperlipidemia were documented in 10-week-old db/db mice. (n=10 each) * p<0.05, ** p<0.01 vs 10 weeks old db/+ mice

(B) Cardiac contractile function was declined in db/db mice. (n=12 each) ** p<0.01 vs 10 weeks old db/+ mice

(C) Protein expressions of p53 and SCO2 were increased in db/db mice. (n=10 each) ** p<0.01 vs 10 weeks old db/+ mice

(D) There were no changes in state 2 or state 4 respirations. State 3 respirations and mitochondrial complex IV activity were augmented in db/db mice. (n=10 each) ** p<0.01 vs 10 weeks old db/+ mice

(E) ROS-induced DNA damage, lipid accumulation and lipid droplets (arrowheads) were increased in db/db mice. (n=10 each) ** p<0.01 vs 10 weeks old db/+ mice

Supplemental Table 1.

(A) Cardiac functional data of p53(+/+) mice under control conditions, after STZ injection. Data are mean ± SE. ** p<0.01 as compared with control group; LVDd: LV end diastolic diameter, LVDs: LV end systolic diameter, LVFS: LV fractional shortening, LVPd: LV posterior wall thickness at end diastole, LVPs: LV posterior wall thickness at end systole.

(B) Cardiac functional data of p53(-/-) mice under control conditions, after STZ injection. LVDd: LV end diastolic diameter, LVDs: LV end systolic diameter, LVFS: LV fractional shortening, LVPd: LV posterior wall thickness at end diastole, LVPs: LV posterior wall thickness at end systole.
**Supplemental Table 2.**

Cardiac functional data of SCO2(+/−) mice under control conditions, after STZ injection. LVDd: LV end diastolic diameter, LVDs: LV end systolic diameter, LVFS: LV fractional shortening LVPWd: LV posterior wall thickness at end diastole, LVPWs: LV posterior wall thickness at end systole.

**Supplemental Table 3.**

Cardiac functional data of db/+ and db/db mice. Data are mean ± SE. ** p<0.01 as compared with db/+; LVDd: LV end diastolic diameter, LVDs: LV end systolic diameter, LVFS: LV fractional shortening, LVPWd: LV posterior wall thickness at end diastole, LVPWs: LV posterior wall thickness at end systole.
Suppl. Fig. 2

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Bar = 10 μm
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CD31 (/mm²)
Suppl. Fig. 3

TUNEL positive cell (%)

- **p53 +/+**
- **p53 -/-**

Day: 0, 3, 7, 14, 28
Suppl. Fig. 4

A

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Bar = 10μm × 200

Bar = 10μm × 400

Bar = 1μm × 5000
Suppl. Fig. 6

A

Control  | Oleate 1mM  | Oleate 5mM  | Oleate 5mM + tempol

MitoSOX

Contraction

Viable cells

Apoptotic cells
Suppl. Fig. 7

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<td>(mg/dl)</td>
</tr>
<tr>
<td>cont</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>STZ4W</td>
<td>800</td>
<td>150</td>
<td>1500</td>
<td>200</td>
</tr>
</tbody>
</table>

B

SCO2(+-) mice

<table>
<thead>
<tr>
<th></th>
<th>cont</th>
<th>STZ 4W</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = p < 0.05
** = p < 0.01
Suppl. Fig. 9

A

<table>
<thead>
<tr>
<th></th>
<th>p53(+/+)</th>
<th>p53(-/-)</th>
<th>SCO2(+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>STZ4W</td>
<td>control</td>
</tr>
<tr>
<td>CD36</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Caveolin 3</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

- **CD36 mRNA**
  - ![Image](image13.png)
  - Relative ratio
  - **p53**
    - ![Image](image14.png)
  - **Actin**
    - ![Image](image15.png)
  - **Nut3**
    - 0h, 3h, 6h, 12h

C

- ![Image](image16.png)

D

- ![Image](image17.png)

E

- ![Image](image18.png)

**[14C]-oleate residual in myocytes**

- ![Image](image19.png)
Suppl. Fig. 10

A

<table>
<thead>
<tr>
<th>Ad-Lac Z</th>
<th>Ad-SCO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCO2</td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
</tr>
</tbody>
</table>

B

**Human CD36**
-1288 -1280 -1198 -1180
GGACTTGCTCC AAACCTTGCTT

**Rat CD36**
-98 -90
AAACAAAGT

**Mouse CD36**
-1100 -1091 -1056 -1047
GGCTAGCCT AGACATGCC

E1 +1 E2

E1 +1 E2
Suppl. Fig. 11

A

<table>
<thead>
<tr>
<th></th>
<th>Glu (mg/dl)</th>
<th>TG (µEq/l)</th>
<th>FFA (mg/dl)</th>
<th>Tchol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/+</td>
<td>800</td>
<td>2000</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>db/db</td>
<td>1200</td>
<td>3000</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

** indicates p < 0.01 compared to db/+.

B

%FS

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>(%)</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

** indicates p < 0.01 compared to db/+.

C

- p53
- SCO2
- actin

Relative Protein

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Protein</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

** indicates p < 0.01 compared to db/+.

D

<table>
<thead>
<tr>
<th></th>
<th>db/+</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>%FS</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

** indicates p < 0.01 compared to db/+.

E

8OHdG

bar = 10µm × 200

Oil red O

bar = 10µm × 400

EM

bar = 1µm × 5000

<table>
<thead>
<tr>
<th></th>
<th>db/+ 10W</th>
<th>db/db 10W</th>
</tr>
</thead>
<tbody>
<tr>
<td>droplet</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

** indicates p < 0.01 compared to db/+.
## A. p53(+/+)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2week</th>
<th>4week</th>
<th>8week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>587 ± 15.3</td>
<td>582 ± 14.5</td>
<td>578 ± 11.9</td>
<td>579 ± 10.8</td>
</tr>
<tr>
<td>LVDd(mm)</td>
<td>3.30 ± 0.11</td>
<td>3.19 ± 0.19</td>
<td>2.90 ± 0.09</td>
<td>3.18 ± 0.17</td>
</tr>
<tr>
<td>LVDs(mm)</td>
<td>1.26 ± 0.19</td>
<td>1.38 ± 0.14</td>
<td>1.52 ± 0.08* *</td>
<td>1.67 ± 0.12* *</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>61.9 ± 4.64</td>
<td>56.8 ± 4.04</td>
<td>47.4 ± 3.07* *</td>
<td>45.5 ± 2.03* *</td>
</tr>
<tr>
<td>LVPWs(mm)</td>
<td>1.22 ± 0.05</td>
<td>1.17 ± 0.06</td>
<td>1.20 ± 0.05</td>
<td>1.18 ± 0.07</td>
</tr>
<tr>
<td>LVPWd(mm)</td>
<td>0.80 ± 0.03</td>
<td>0.82 ± 0.02</td>
<td>0.81 ± 0.04</td>
<td>0.81 ± 0.04</td>
</tr>
</tbody>
</table>

## B. p53(-/-)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2week</th>
<th>4week</th>
<th>8week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>566 ± 17.1</td>
<td>584 ± 12.1</td>
<td>572 ± 11.1</td>
<td>568 ± 10.9</td>
</tr>
<tr>
<td>LVDd(mm)</td>
<td>3.19 ± 0.04</td>
<td>3.01 ± 0.21</td>
<td>2.97 ± 0.03</td>
<td>3.18 ± 0.03</td>
</tr>
<tr>
<td>LVDs(mm)</td>
<td>1.23 ± 0.06</td>
<td>1.25 ± 0.15</td>
<td>1.20 ± 0.07</td>
<td>1.40 ± 0.18</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>61.0 ± 1.83</td>
<td>58.3 ± 1.58</td>
<td>59.4 ± 2.56</td>
<td>55.9 ± 2.03</td>
</tr>
<tr>
<td>LVPWs(mm)</td>
<td>1.19 ± 0.04</td>
<td>1.16 ± 0.05</td>
<td>1.20 ± 0.06</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>LVPWd(mm)</td>
<td>0.82 ± 0.03</td>
<td>0.83 ± 0.03</td>
<td>0.81 ± 0.05</td>
<td>0.80 ± 0.05</td>
</tr>
</tbody>
</table>
### Suppl. Table 2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2week</th>
<th>4week</th>
<th>8week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>581 ± 20.1</td>
<td>569 ± 14.3</td>
<td>571 ± 18.2</td>
<td>562 ± 19.8</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.32 ± 0.15</td>
<td>3.12 ± 0.12</td>
<td>2.95 ± 0.08</td>
<td>2.90 ± 0.19</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>1.27 ± 0.09</td>
<td>1.23 ± 0.14</td>
<td>1.22 ± 0.05</td>
<td>1.24 ± 0.15</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>61.6 ± 3.24</td>
<td>60.6 ± 3.21</td>
<td>57.9 ± 1.56</td>
<td>57.2 ± 4.01</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.25 ± 0.05</td>
<td>1.19 ± 0.08</td>
<td>1.19 ± 0.07</td>
<td>1.20 ± 0.09</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.82 ± 0.04</td>
<td>0.83 ± 0.05</td>
<td>0.80 ± 0.05</td>
<td>0.83 ± 0.07</td>
</tr>
</tbody>
</table>

SCO2(+-/-)
<table>
<thead>
<tr>
<th>Parameters</th>
<th>db/+</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>572 ± 14.3</td>
<td>583 ± 16.5</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.31 ± 0.16</td>
<td>3.20 ± 0.10</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>1.37 ± 0.12</td>
<td>1.69 ± 0.10*</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>58.5 ± 1.51</td>
<td>47.1 ± 2.03**</td>
</tr>
<tr>
<td>LV PWs (mm)</td>
<td>1.24 ± 0.05</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.83 ± 0.06</td>
<td>0.86 ± 0.05</td>
</tr>
</tbody>
</table>
In the article “p53 Promotes Cardiac Dysfunction in Diabetic Mellitus Caused by Excessive Mitochondrial Respiration-Mediated Reactive Oxygen Species Generation and Lipid Accumulation” by Nakamura et al, which published online November 9, 2011, and appeared in the January 2012 issue of the journal (Circ Heart Fail 2012;5:106-115), a correction to the supplemental material was needed.

The left panels shown in Supplemental Figure 5A and the left lower picture in Supplemental Figure 11E were incorrectly selected and labeled. The left panels in Supplemental Figure 5A were found to be from control and streptozotocin (STZ)-induced diabetic mice prepared for other experiments. The corrected left panels now show the protein levels from control and STZ-induced diabetic mice treated with vehicle for Tempol. The figure of electromicroscopy of db/+ mice in Supplemental Figure 11E was incorrectly chosen, and we now show the correct picture. The authors wish to apologize for these errors and emphasize that the correction of these errors does not impact the article’s conclusions.

These corrections have been made to the current online version of the article, which is available at http://circheartfailure.ahajournals.org/content/5/1/106.full.pdf+html.