Gene Silencing of the Mitochondrial Adaptor p66Shc Suppresses Vascular Hyperglycemic Memory in Diabetes

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Rationale: Hyperglycemic memory may explain why intensive glucose control has failed to improve cardiovascular outcomes in patients with diabetes. Indeed, hyperglycemia promotes vascular dysfunction even after glucose normalization. However, the molecular mechanisms of this phenomenon remain to be elucidated.

Objective: The present study investigated the role of mitochondrial adaptor p66Shc in this setting.

Methods and Results: In human aortic endothelial cells (HAECs) exposed to high glucose and aortas of diabetic mice, activation of p66Shc by protein kinase C βII (PKCβII) persisted after returning to normoglycemia. Persistent p66Shc upregulation and mitochondrial translocation were associated with continued reactive oxygen species (ROS) production, reduced nitric oxide bioavailability, and apoptosis. We show that p66Shc gene overexpression was epigenetically regulated by promoter CpG hypomethylation and general control nonderepressible 5’-induced histone 3 acetylation. Furthermore, p66Shc-derived ROS production maintained PKCβII upregulation and PKCβII-dependent inhibitory phosphorylation of endothelial nitric oxide synthase at Thr-495, leading to a detrimental vicious cycle despite restoration of normoglycemia. Moreover, p66Shc activation accounted for the persistent elevation of the advanced glycated end product precursor methylglyoxal. In vitro and in vivo gene silencing of p66Shc, performed at the time of glucose normalization, blunted ROS production, restored endothelium-dependent vasorelaxation, and attenuated apoptosis by limiting cytochrome c release, caspase 3 activity, and cleavage of poly (ADP-ribose) polymerase.

Conclusions: p66Shc is the key effector driving vascular hyperglycemic memory in diabetes. Our study provides molecular insights for the progression of diabetic vascular complications despite glycemic control and may help to define novel therapeutic targets. (Circ Res. 2012;111:278-289.)

Key Words: vascular disease ■ diabetes mellitus ■ free radicals ■ endothelium

The prevalence of diabetes has dramatically increased worldwide, with a further rise anticipated in the next decades.1,2 Morbidity and mortality from cardiovascular disease is 2- to 8-fold higher in subjects with than in those without diabetes.3

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Recent prospective clinical trials have failed to confirm unequivocal benefits from normalization of glycemia on cardiovascular outcomes.4-8 In these trials, intensive glucose-lowering therapy was started after a median duration of diabetes ranging from 8 to 11 years.4-8 By contrast, early treatment of diabetes-related deaths, and all-cause mortality.9-11 These observations support the concept that hyperglycemic environment may be remembered in the vasculature.12 Reactive oxygen species (ROS) are probably involved in this phenomenon defined “hyperglycemic memory,” but the underlying molecular mechanisms remain unknown.13-15 Overproduction of ROS by mitochondria is considered as a causal link between elevated glucose and the major biochemical pathways involved in the development of vascular complications in diabetes.16 Furthermore, mitochondria are intimately associated with the initiation of apoptosis.17,18 The p66Shc adaptor protein functions as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into...
apoptosis. Several chronic stimuli activate protein kinase C βII (PKCβII) isomor to induce Ser-36 phosphorylation of p66Shc, allowing transfer of the protein from the cytosol to the mitochondrion where it catalyzes ROS production via cytochrome c oxidation. This latter event leads to mitochondrial disruption and cell death. Indeed, increased ROS generation alters mitochondrial permeability facilitating the release of intermembrane space proapoptotic proteins such as cytochrome c. Once released in the cytosol, cytochrome c is responsible for activation of the apoptosis execution enzyme caspase 3. On the other hand, mice lacking p66Shc gene responsible for activation of the apoptosis execution enzyme release of intermembrane space proapoptotic proteins such as p66Shc (supervised by the notion that p66 Shc gene expression is supported by the notion that p66 Shc gene expression is involved in vascular hyperglycemic memory. Our findings indicate that p66Shc is a crucial mediator of sustained vascular hyperglycemic stress and endothelial dysfunction and oxidative stress. Unlike diabetic wild-type littermates, p66Shc−/− diabetic mice did not develop any impairment of acetylcholine-induced vasorelaxation by virtue of an unaltered nitric oxide (NO) bioavailability. Of note, the expression of p66Shc protein was increased in aortas from wild-type diabetic mice as compared with normoglycemic controls, thus underlining a causal link between high glucose and p66Shc. The relevance of p66Shc in the clinical setting of diabetes is underlined a single high dose of streptozotocin (180 mg/kg, via intraperitoneal injection) dissolved in sterile 0.025 mol/L citrate buffer (pH 4.5) and injected within 10 minutes. Control animals received an equal volume of citrate buffer. Hyperglycemia was defined as 3 random blood glucose levels >13.9 mmol/L after streptozotocin injection (Online Figure I). Mice were housed in temperature-controlled cages (20° to 22°C), fed ad libitum, and maintained on a 12:12-hour light/dark cycle. All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal Care Committee (Kommission für Tierversuche des Kantons Zürich, Switzerland).

Methods
A detailed description of the methods used in this study is provided in the Online Data Supplement.

Cell Culture and Reagents
Human aortic endothelial cells (HAECs, passages 5–7) were exposed for 6 days either to normal glucose (5 mmol/L) or high glucose concentration (25 mmol/L) as well as to high glucose for 3 days followed by normal glucose for the remaining 3 days.

Western Blotting
This method is reported in the Online Data Supplement.

Real-Time PCR
All PCR experiments were performed using TaqMan Gene Expression Assays kit and TaqMan Gene Expression Master Mix both provided by Applied Biosystems.

Measurements of O2−, NO, and ONOO− by Electron Spin Resonance (ESR) Spectroscopy
O2− generation, NO release, and ONOO− production in intact cells was assessed by ESR spectroscopy.

Small Interfering RNA Transfection
HAECs were transfected with PKCβII, p66Shc, and general control nonderepressible 5 (GCN5) siRNAs or scrambled siRNA by using electroporation.

Annexin V Staining and Caspase 3 Activity Assay
Annexin V staining and Caspase 3 activity were determined by using commercially available kits.

p66Shc and Promoter Methylation In Vitro
For each analyzed genomic DNA sample, 30 ng of genomic DNA was digested with methylation sensitive enzyme (Ms) or with methylation dependent enzyme (Md) and also with both methylation sensitive and dependent enzymes (Ms+Md) in 15 µL total volume including 5× digestion buffer overnight at 37°C.

Streptozotocin-Induced Diabetic Mice
Four- to 6-month-old male 129/Sv background mice were administered a single high dose of streptozotocin (180 mg/kg, via intraperitoneal injection) dissolved in sterile 0.025 mol/L citrate buffer (pH 4.5) and injected within 10 minutes. Control animals received an equal volume of citrate buffer. Hyperglycemia was defined as 3 random blood glucose levels >13.9 mmol/L after streptozotocin injection (Online Figure I). Mice were housed in temperature-controlled cages (20° to 22°C), fed ad libitum, and maintained on a 12:12-hour light/dark cycle. All animal studies were conducted in accordance with the guidelines approved by the Institutional Animal Care Committee (Kommission für Tierversuche des Kantons Zürich, Switzerland).

Animals were divided into 5 experimental groups: (1) control; (2) diabetic; (3) diabetic mice treated with insulin after 3 weeks from the induction of diabetes; (4) diabetic mice treated with insulin plus p66Shc siRNA; and (5) diabetic mice treated with insulin plus scrambled siRNA. Insulin administration was started 3 weeks after the induction of diabetes and continued for a period of 3 weeks (groups 3–5).

In Vivo Knockdown of p66Shc
In vivo knockdown of p66Shc was performed by intravenous injection of 2 predesigned siRNAs specifically targeting p66Shc (Online Figure II). Alexa 546–tagged p66Shc siRNA was used to assess siRNA distribution in mouse aorta. Immunofluorescence for the endothelial marker CD31 was performed to demonstrate the endothelial uptake of fluorescent p66Shc siRNA.

Tissue Harvesting and Organ Chamber
The description of these methods is provided in the Online Data Supplement.
Measurement of Superoxide Anion Production in Mouse Aorta
Superoxide production was determined in mouse thoracic aorta sections by ESR spectroscopy using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidine and an e-scan ESR spectrometer (Bruker BioSpin).

In Situ Measurement of Superoxide Anion
Dihydroethidium (DHE) staining was carried out for superoxide determination in mouse aorta.

Isolation of Mitochondrial and Cytosolic Fractions
Mitochondria were isolated from HAECs and mouse aortas by centrifugation, as previously described.31

p66Shc Mitochondrial Translocation and Cytochrome c Release
A description of the method is provided in the Online Data Supplement.

In Situ Detection of Vascular Apoptosis by TUNEL Assay
DNA fragmentation was detected in situ, using the TUNEL assay, by use of a commercially available kit (Roche, Basel, Switzerland).

Statistical Analysis
All data are presented as mean±SEM. Statistical comparison were made by using the Student t test for unpaired data and 1-way ANOVA, followed by Bonferroni post hoc test when appropriate. Probability values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism Software (version 5.0).

Results
Persistent Oxidative Stress and Apoptosis Despite Glucose Normalization
In HAECs exposed to high glucose, superoxide anion (O$_2^-$) production was elevated even after glucose normalization.
(Figure 1A). Mannitol did not affect ROS production (Online Figure IIIA). In accordance with increased $O_2^-$ generation, high glucose impaired NO release and restoration of normal glucose levels did not improve the bioavailability of NO (Figure 1B). Peroxynitrite (ONOO$^-$) levels, a marker of NO breakdown, remained elevated in those cells exposed initially to high and then to normal glucose (Figure 1C). The persistence of increased $O_2^-$ and ONOO$^-$ levels after normalization of glucose concentrations suggests that oxidative stress plays a key role in this setting (Figure 1A through 1C). Furthermore, glucose-induced cellular apoptosis, assessed by caspase 3 activity and annexin V staining, did not change and remained high even after restoration of normal glucose levels (Figure 1D and 1E).

**Sustained Upregulation of PKCβII and p66$^{Shc}$**

After exposure to high glucose, we found a selective upregulation and membrane translocation of PKCβII (Figure 1F). Other PKC isoforms were not affected (Figure 1G and Online Figure IV). mRNA and protein expression of p66$^{Shc}$ were also significantly increased on glucose exposure (Figure 1H). Mannitol used as an osmotic control did not affect PKCβII or p66$^{Shc}$ expression (Online Figure IIIB). Interestingly, the increased expression of PKCβII and p66$^{Shc}$ persisted despite 3 days of glucose normalization (Figure 1F through 1H). As shown by dose-response experiments, sustained upregulation of p66$^{Shc}$ was already found at glucose concentrations of 15 mmol/L (Online Figure V).

**PKCβII-Mediated Activation of p66$^{Shc}$**

p66$^{Shc}$-activating phosphorylation at Ser-36 residue was enhanced by high glucose and did not change after glucose normalization (Figure 2A). To investigate whether persistent upregulation of PKCβII was linked to sustained p66$^{Shc}$ activation, we added the PKCβII selective inhibitor CGP 53353 (10$^{-6}$ mol/L) at the time of glucose normalization.

Interestingly, CGP 53353 abolished p66$^{Shc}$ phosphorylation, suggesting that PKCβII is responsible for continued activation of p66$^{Shc}$ despite normoglycemia restoration (Figure 2A). Moreover, blunting of p66$^{Shc}$ phosphorylation by selective PKCβII inhibition abolished the persistent translocation of p66$^{Shc}$ to the mitochondria (Figure 2B). Since p66$^{Shc}$ is a crucial mediator of oxidative stress, 19 we hypothesized that inhibition of PKCβII might affect p66$^{Shc}$-dependent $O_2^-$ production. Indeed, only in the presence of PKCβII pharmacological inhibition or specific RNA interference (Online Figure VIA) glucose normalization led to a significant decrease of $O_2^-$ production (Figure 2C). This finding was reproduced by using a range of different selective and nonselective PKCβ inhibitors (Online Figure VIIA). The decrease in $O_2^-$ generation, as a result of PKCβII inhibition, was associated with restored NO release (Figure 2D) and blunted ONOO$^-$ levels (Online Figure VIIB). We also found that high glucose led to inhibitory phosphorylation of endothelial nitric oxide synthase (eNOS) at the Thr-495 residue, and restoration of normal glucose concentrations did not revert this effect (Figure 2E). However, PKCβII inhibition together with glucose normalization attenuated eNOS inhibiting Thr-495 phosphorylation, thus contributing to the restoration of NO availability in this setting (Figure 2D and 2E).

By contrast, our experimental conditions did not exert any effect on activating eNOS phosphorylation at Ser-1177 (data not shown). Given the pivotal role of p66$^{Shc}$ in triggering mitochondrial disruption, we investigated whether inhibition of PKCβII could affect glucose-induced cytochrome c release and hence cellular apoptosis. Interestingly, high glucose exposure caused a significant release of cytochrome c from
expression after p66Shc knockdown at the time of glucose normalization. Results are presented as mean ± SEM; n = 4 to 7 per group. NG indicates normal glucose; HG, high glucose; HN, high to normal glucose; PKC, protein kinase C; PEG-SOD, polyethylene glycol-superoxide dismutase.

the mitochondria to the cytosol and normalization of glucose levels did not interrupt such event (Figure 3A). By contrast, selective PKCβII inhibition at the time of glucose normalization restored mitochondrial localization of cytochrome c. This latter finding was paralleled by a reduction of apoptosis, as suggested by reduced caspase 3 activity and annexin V staining (Figure 3B and 3C).

**p66Shc Drives Persistent ROS-Mediated Hyperglycemic Stress**

To further elucidate the relevance of p66Shc in maintaining ROS-mediated hyperglycemic stress, we selectively targeted p66Shc, using siRNA technology. p66Shc-specific siRNA resulted in a significant reduction of protein expression, whereas scrambled siRNA did not exert any significant effect (Online Figure VIB). Since glucose-induced upregulation of p66Shc persisted despite subsequent normalization of glucose concentrations (Figure 1H), RNA interference was performed at the time of glucose normalization to address whether p66Shc was involved in the sustained ROS generation. Whereas glucose normalization alone did not influence excess superoxide production induced by prior hyperglycemia, p66Shc knockdown restored NO availability by attenuating O2- levels (Figure 2C and 2D). In addition, p66Shc silencing significantly reduced cytochrome c release, annexin V staining, caspase 3 activity, and subsequent cleavage of the proapoptotic protein poly (ADP-ribose) polymerase (PARP) (Figure 3A through 3C and Online Figure VIII).

Based on the knowledge that oxidative stress triggers de novo transcription of p66Shc is involved (Figure 4A). Nomycin D abolished p66Shc gene expression, suggesting that de novo transcription of p66Shc is involved (Figure 4A). Interestingly, quantitative analysis of p66Shc promoter methylation showed that high glucose resulted in a significant hypomethylation of CpG dinucleotides and that restoration of normoglycemia did not change this pattern (Figure 4B). Methylation of CpG dinucleotides is closely linked with histone acetylation, leading to an open chromatin and gene transcription. Therefore, we determined whether p66Shc

**Figure 3. p66Shc knockdown attenuates persistent cellular apoptosis.** HAECs were cultured in normal (5 mmol/L), high glucose (25 mmol/L), or high glucose followed by normal glucose in the absence and in the presence of selective PKCβII inhibitor GCP 53353 (10⁻⁶ mol/L), PKCβII, or p66Shc siRNAs. Scrambled siRNA (scr siRNA) was used as a control. A, Western blot (WB) and densitometric quantification of cytochrome c release from mitochondria in the presence and in the absence of selective PKCβII blockade and p66Shc knockdown. Bar graphs represent percentage change of cytochrome c in the cytosol versus mitochondria. B, Caspase 3 activity. C, Annexin V staining (green, original magnification ×20) and relative quantification. Nuclei are stained with DAPI (blue). D, Representative WB of PKCβII and eNOS Thr-495 protein expression after p66Shc knockdown at the time of glucose normalization.
expression might also be regulated by histone 3 (H3) acetylation in our experimental setting. We found that high glucose increased acetylation of H3, and this modification was not reverted after glucose normalization (Figure 4C). This finding was explained by an increased expression of the H3 acetyltransferase GCN5. Interestingly enough, pharmacological inhibition as well as siRNA-mediated knockdown of GCN5 significantly suppressed p66Shc upregulation during subsequent normoglycemia, suggesting that H3 acetylation may augment transcription of the p66Shc gene (Figure 4E and Online Figure XI).

### Persistent Endothelial Dysfunction Despite Restoration of Normoglycemia in Diabetic Mice

To explore the relevance of our in vitro findings, 129/Sv mice were made diabetic by a single intraperitoneal injection of streptozotocin and followed for 6 weeks. Control mice received citrate buffer alone (Figure 5A). After 3 weeks of diabetes, subgroups of diabetic mice were randomized to treatment with insulin alone, insulin plus p66Shc, or scrambled siRNA for other 3 weeks (Figure 5A). Age, sex, and body weight did not differ across the experimental groups (data not shown). As expected, blood glucose was significantly higher in diabetic mice when compared with controls (Figure 5B). After 3 weeks of hyperglycemia, optimal glycemic control was obtained in all groups receiving insulin (Figure 5B). Indeed, glucose levels did not differ among control and insulin-treated diabetic mice, as shown by weekly monitoring from weeks 4 to 6 as well as over a 24-hour period (Figure 5B and Online Figure XII).

Endothelium-dependent relaxation to acetylcholine (10−9 to 10−6 mol/L) was impaired in aortic rings from diabetic mice when compared with controls (Figure 5C). Interestingly, despite 3 weeks of normoglycemia restoration with insulin, impairment of acetylcholine-induced relaxation persisted (Figure 5C). Notably, an approach to reduce ROS accumulation, PEG-SOD (150 U/mL) restored endothelial dysfunction in insulin-treated diabetic mice, confirming a link between persistent endothelial dysfunction and ROS generation in vivo (Figure 5C). By contrast, the cyclooxygenase inhibitor indomethacin (10−5 mol/L) did not affect relaxation to acetylcholine (data not shown). Furthermore, endothelium-independent relaxation to sodium nitroprusside (10−10 to 10−5 mol/L) was identical in all experimental groups (Online Figure XIII).

### In Vivo Knockdown of p66Shc Blunts Vascular Hyperglycemic Memory

p66Shc protein expression was significantly increased in diabetic mice, and this upregulation was sustained despite 3 weeks of normoglycemia with insulin treatment (Figure 5D). Moreover, activating p66Shc phosphorylation at Ser-36 was also increased and this finding was associated with increased translocation of active p66Shc into the mitochondria (Figure 5E and 5F). Interestingly enough, restoration of normoglycemia did not reduce p66Shc activation or mitochondrial translocation (Figure 5E and 5F). In view of our in vitro findings linking p66Shc to sustained ROS-mediated impairment of NO bioavailability, we explored the effects of p66Shc knockdown during restoration of glycemic control. Aortic expression of p66Shc was blunted by specific RNA interference as compared with scrambled siRNA (Figure 6A). Assessment of a p66Shc-tagged siRNA delivery in the vasculature showed an uptake by the endothelium, as confirmed by CD31 immuno-staining (Figure 6B). Importantly, in the presence of p66Shc silencing, insulin treatment restored endothelium-dependent...
The effect of p66Shc knockdown in suppressing persistent insulin alone or insulin plus scrambled siRNA (Figure 6E). The present study demonstrates for the first time that vascular hyperglycemic memory is driven by p66Shc adaptor protein. Several lines of evidence support our conclusion. First, in endothelial cells glucose normalization did not revert p66Shc activation or the concomitant increase in ROS. Second, on high glucose exposure, PKCβII activated p66Shc by Ser-36 phosphorylation, which, in turn, via ROS production led to a persistent PKCβII-dependent eNOS inhibiting Thr-495 phosphorylation, thus creating a detrimental vicious cycle despite restoration of normoglycemia. Third, gene silencing of p66Shc, performed at the time of glucose normalization alone, silencing of p66Shc also rescued vessel relaxations as well as diabetics treated with insulin, despite 3 weeks of normoglycemia restoration. In the latter group, pretreatment of aortic rings with PEG-SOD (150 U/mL) restored impaired endothelium-dependent agonist acetylcholine (Ach) relaxations to the endothelium-dependent agonist acetylcholine (Ach) were normal in controls. Diabetic mice exhibited impaired endothelial-dependent relaxations as well as diabetics treated with insulin, despite 3 weeks of normoglycemia restoration. In the latter group, pretreatment of aortic rings with PEG-SOD (150 U/mL) restored impaired responses. Results are presented as mean±SEM; n=6 to 8 per group. C, Isometric tension studies in aortic rings isolated from controls and diabetic mice with and without insulin treatment. Vessel relaxations to acetylcholine whereas concomitant administration of PEG-SOD (150 U/mL) restored impaired responses. Results are presented as mean±SEM; n=6 to 8 per group.

Figure 5. Restoration of normoglycemia does not revert p66Shc activation and endothelial dysfunction in diabetic mice treated with insulin. A. Experimental groups of the in vivo study. B, Blood glucose levels across the different groups with insulin treatment started after 3 weeks from the induction of diabetes. Results are presented as mean±SEM; *P<0.001 versus control, #P<0.001 versus diabetes; n=6 to 8 per group. C, Isometric tension studies in aortic rings isolated from controls and diabetic mice with and without insulin treatment. Vessel relaxations to endothelium-dependent agonist acetylcholine (Ach) were normal in controls. Diabetic mice exhibited impaired endothelium-dependent relaxations as well as diabetics treated with insulin, despite 3 weeks of normoglycemia restoration. In the latter group, pretreatment of aortic rings with PEG-SOD (150 U/mL) restored impaired responses. Results are presented as mean±SEM; n=6 to 8 per group. *P<0.05 versus diabetes and diabetes treated with insulin. PEG-SOD indicates polyethylene glycol-superoxide dismutase; NE, norepinephrine. D and E, Representative Western blot with densitometric quantification of p66Shc protein expression and Ser-36 activating phosphorylation. F, p66Shc mitochondrial translocation in aortic lysates of controls and diabetic mice with or without insulin treatment. Results are presented as mean±SEM; n=3 to 4 per group.

relaxation to acetylcholine (Figure 6C). The effect of PEG-SOD on maximal relaxation to acetylcholine was abolished in mice receiving p66Shc siRNA (Figure 6D). Furthermore, in the same group, ESR-determined aortic superoxide production was blunted as compared with diabetic mice receiving insulin alone or insulin plus scrambled siRNA (Figure 6E). The effect of p66Shc knockdown in suppressing persistent vascular superoxide accumulation was confirmed by fluorescence microscopy of DHE-labeled aortas (Figure 6F). Such inhibition of p66Shc-derived ROS generation also abolished aortic PKCβII upregulation and Thr-495 eNOS inhibitory phosphorylation (Figure 6G and 6H).

**Discussion**

The present study demonstrates for the first time that vascular hyperglycemic memory is driven by p66Shc adaptor protein. Several lines of evidence support our conclusion. First, in endothelial cells glucose normalization did not revert p66Shc activation or the concomitant increase in ROS. Second, on high glucose exposure, PKCβII activated p66Shc by Ser-36 phosphorylation, which, in turn, via ROS production led to a persistent PKCβII-dependent eNOS inhibiting Thr-495 phosphorylation, thus creating a detrimental vicious cycle despite restoration of normoglycemia. Third, gene silencing of p66Shc, performed at the time of glucose normalization, blunted PKCβII upregulation, restored NO bioavailability, decreased superoxide anion and oxidant-induced apoptosis mostly through the suppression of cytochrome c release, caspase 3 activation, and cleavage of the proapoptotic protein PARP. Moreover, the levels of glucose metabolite methylglyoxal, an important mediator of oxidative stress involved in AGEs synthesis, were almost abolished by p66Shc knockdown in HAECs. Similarly, p66Shc was upregulated in the aorta of streptozotocin-induced diabetic mice and restoration of normoglycemia by insulin did not reduce its expression and consequent production of ROS. Indeed, insulin treatment alone did not restore impaired endothelium-dependent relaxation to acetylcholine whereas concomitant in vivo knockdown of p66Shc normalized vascular relaxation by abolishing the persistent aortic superoxide generation. As compared with glucose normalization alone, silencing of p66Shc also rescued vascular apoptosis by limiting cytochrome c release and caspase 3 activation.

Long-lasting hyperglycemic stress, even when glucose level is normalized, has been reported in various contexts, but the molecular mechanisms thus far remain unknown. In human endothelial cells, sustained overproduction of ROS was attenuated by antioxidant treatment together with glucose normalization. Consistently, in patients with type 1 diabetes, flow-mediated vasodilatation was improved by simultaneous control of hyperglycemia and oxidative stress with insulin plus intravenous infusion of the antioxidant vitamin.
However, it should be noted that available antioxidants only partially scavenge cellular ROS but do not target intracellular redox signaling. This notion is confirmed by the negative results of major trials with oral supplementation of high-dose vitamins. Taken together, these observations support the concept that ROS-driven hyperglycemic stress is remembered in the vasculature. In line with this interpretation, intensive glucose-lowering therapy has failed to reduce cardiovascular events in diabetes.

An increasing body of evidence links p66Shc to oxidative stress as the adaptor protein plays a pivotal role in modulating the intracellular redox state. A recent study clearly showed that hyperglycemia activates p66Shc via a PKC-dependent pathway, leading to ROS generation, reduced mitochondrial membrane potential, cytochrome c release, and apoptosis in renal tubular cells in vitro and in vivo. Of note, mice carrying a targeted mutation of p66Shc (p66Shc−/−) are protected from oxidative stress. This may be clinically
relevant as p66Shc gene expression is increased in blood mononuclear cells of patients with diabetes and correlates with oxidative stress. Our in vitro experiments showed that persistent upregulation of p66Shc and PKCβII results in ROS production despite restoration of normal glucose conditions. We found that PKCβII upregulation maintained p66Shc activation as a result of Ser-36 phosphorylation of this protein. Indeed, selective inhibition of PKCβII interrupted mitochondrial translocation of p66Shc, confirming the molecular link between these proteins even after normalization of glucose levels. In turn, knockdown of p66Shc blunted PKCβII upregulation, suggesting that p66Shc-dependent ROS production is required for the maintenance of PKCβII activation despite glucose normalization (Figure 8). This latter finding builds on seminal studies connecting ROS generation with PKC activation in states of hyperglycemia. In line with this notion, we found that treatment with ascorbic acid as well as with the free radical scavenger PEG-SOD blunted PKCβII overexpression, thus strengthening the role of ROS in hyperglycemic memory. Furthermore, glucose normalization alone did not restore endothelial NO release with the levels of superoxide anion remaining elevated. The short half-life of superoxide anion (10^{-9} to 10^{-11} seconds) cannot explain such persistent oxidative burden. This would suggest that there are key mediators of ROS generation that remain activated after restoration of normal glucose levels. Consistent with this postulate, p66Shc knockdown in the context of glucose normalization restored the NO/O2- balance, limited cytochrome c release and reported cellular apoptosis to control levels via blunting caspase 3 and PARP activation. Indeed, on DNA cleavage by caspases, activated PARP (PAR) plays a crucial role in cellular death by depleting cells from ATP in the attempt to repair the DNA damage. Our in vitro experiments also showed that p66Shc silencing concomitant with glucose normalization interrupted the synthesis of the glucose metabolite methylglyoxal. This AGE compound is a major contributor of hyperglycemia-induced oxidative stress. A recent study has shown that p66Shc is critically involved in methylglyoxal-dependent oxidative stress since the use of p66Shc mutants abolished ROS production in human kidney cells. Interestingly, our results suggest that p66Shc controls methylglyoxal levels in HAECs and may contribute to the persistent increase of this detrimental metabolite. However, further investigation is required to better explore the mechanistic link between p66Shc and the methylglyoxal/AGEs pathway.

Rapid changes in the phosphorylation state of endothelial nitric oxide synthase (eNOS) on 2 separate residues (Thr-495 and Ser-1177) precede its activation in response to the stimulation of endothelial cells with Ca^{2+}-elevating agonists. Although Ser-1177 phosphorylation increases the specific activity of eNOS at any given Ca^{2+} concentration, the initiation of NO production is regulated by the Ca^{2+}-dependent dephosphorylation of eNOS at Thr-495. In endothelial cells, high glucose leads to a phosphorylation of eNOS at the Thr-495 residue and, in turn, inhibits NO production. This prompted us to determine whether such a post-translational modification was persistent in our experimental setting. Indeed, phosphorylation of eNOS at the inhibitory Thr-495 residue occurred in response to hyperglycemia and remained so despite normalization of glucose.
We found that selective pharmacological inhibition of PKC_II/H9252 blunted eNOS Thr-495 phosphorylation. Interestingly, p66Shc knockdown, by reducing PKC_II/H9252 upregulation, exerted similar effects. Hence, the increased NO availability after p66Shc silencing might result not only from decreased breakdown but also from Thr-495 dephosphorylation favoring NO synthesis. By contrast, we did not observe any increase of eNOS activating Ser-1177 phosphorylation. This finding is in line with previous report showing that in HAECs chronic exposure to high glucose does not induce any significant change of p-eNOS at Ser-1177.44 To translate our in vitro findings to an in vivo context, we extended the investigation to a model of insulin-deficient diabetes. Endothelium-dependent relaxation to acetylcholine was impaired in diabetic mice, and 3 weeks of normoglycemia with insulin treatment did not restore vascular function. By contrast, impairment of relaxation was abrogated by the free-radical scavenger PEG-SOD, confirming a ROS-dependent persistence of endothelial dysfunction. In line with these findings, p66Shc expression was increased in the aorta of diabetic mice and remained so despite glucose normalization with insulin. Moreover, phosphorylation of p66Shc and its translocation into the mitochondria were not abolished by restoration of normoglycemia. Consistently, p66Shc silencing blunted aortic superoxide production, as shown by ESR analysis and DHE fluorescence. Such inhibition of p66Shc expression abolished aortic PKC_II upregulation and eNOS Thr-495 inhibitory phosphorylation. All together, these events may contribute to the restoration of endothelium-dependent relaxation observed in the diabetic mice treated with insulin plus p66Shc-specific siRNA. In this regard, immunofluorescence analysis of p66Shc-tagged siRNA delivery in the vasculature showed an endothelial uptake as confirmed by costaining with endothelial marker CD31. This finding is in line with the improvement of endothelium-dependent vascular relaxations and apoptosis observed in mice treated with insulin plus p66Shc siRNA. Restored NO availability, which in our setting results from blunted ROS generation, may exert its antiapoptotic effect throughout the vessel wall. Indeed, endothelial NO pathway plays a crucial role in preventing vascular remodeling and apoptosis of smooth muscle cells.45,46

Figure 8. Role of p66Shc adaptor protein in vascular hyperglycemic memory. On exposure to hyperglycemia, PKC_II induces activation of p66Shc via Ser-36 phosphorylation (+) as well as eNOS inhibitory phosphorylation at Thr-495 residue (−) (left). Together with posttranslational modifications, high glucose also triggers continued p66Shc gene transcription via increased histone 3 acetylation (AcH3+) and decreased promoter methylation (Met−). In turn, p66Shc-derived ROS production maintains PKC_II upregulation and eNOS inhibition, creating a vicious cycle that is not interrupted by restoration of normoglycemia with insulin treatment (middle). Indeed, persistent ROS production leads to vascular apoptosis (cytochrome c release and caspase 3 activation) and endothelial dysfunction (reduced NO availability). By contrast, p66Shc siRNA plus insulin treatment rescues vascular apoptosis and endothelial function by reducing ROS generation, PKC_II upregulation, and eNOS Thr-495 phosphorylation (right).
Recent work has shown that in addition to posttranslational modifications of the p66Shc protein, transcriptional regulation could also modulate its biological functions.\(^4\) Indeed, treatment with an inhibitor of histone deacetylases or a demethylating agent resulted in induction of p66Shc expression in cells that normally do not express this isoform, suggesting that epigenetic changes might modulate p66Shc levels in different settings.\(^4\) Consistently, another recent work found that homocysteine stimulates p66Shc transcription in human endothelial cells via specific CpG dinucleotides demethylation in the human p66Shc promoter.\(^4\) Of note, in this study p66Shc promoter CpG methylation was significantly reduced in peripheral blood leukocytes of patients with coronary artery disease and high plasma homocysteine levels, thus strengthening the relevance of p66Shc-related epigenetic changes in the context of cardiovascular diseases.\(^4\) Therefore, we investigated whether persistent p66Shc upregulation observed after glucose normalization was transcriptionally regulated. The transcription inhibitor actinomycin D blunted p66Shc mRNA upregulation, whereas normalization of glucose levels alone did not exert any effect. We then investigated whether the observed de novo transcription was induced by epigenetic changes that may modulate gene expression under hyperglycemic conditions.\(^32,49,50\) Interestingly, we found that promoter hypomethylation as well as enhanced H3 acetylation are the epigenetic markers that trigger persistent p66Shc overexpression despite glucose normalization. Hence, transcriptional and post-translational modifications suggest that p66Shc is the upstream signaling molecule driving vascular hyperglycemic memory in diabetes (Figure 8). In conclusion, these findings help to explain why diabetic vascular complications progress also in the presence of improved glycemic control and may assist in defining novel therapeutic targets to reduce the deleterious effect of hyperglycemic memory on the vasculature.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is Known?

- Recent prospective clinical trials have failed to confirm unequivocal benefits of glycemic control on cardiovascular outcomes.
- A long-term persistence of hyperglycemic stress even after blood glucose normalization has been recently defined as “hyperglycemic memory.”
- Reactive oxygen species (ROS) are likely involved in this phenomenon but the underlying molecular mechanisms remain unknown.

What New Information Does This Article Contribute?

- Mitochondrial adaptor p66Shc is the key effector driving vascular hyperglycemic memory in diabetes.
- Persistent p66Shc upregulation is associated with continued ROS production, reduced nitric oxide (NO) availability, and apoptosis.
- p66Shc-derived ROS maintain protein kinase CβII (PKCβII) upregulation as well as inhibitory phosphorylation of endothelial NO synthase (eNOS) atThr-495, leading to a detrimental vicious cycle despite restoration of normoglycemia.

- In vivo gene silencing of p66Shc, performed at the time of glucose normalization, suppresses persistent endothelial dysfunction and vascular apoptosis.

Hyperglycemic memory may explain why intensive glycemic control has failed to improve cardiovascular morbidity and mortality in patients with diabetes. The present study investigated the role of mitochondrial adaptor p66Shc in this setting. In human endothelial cells and diabetic mice, glucose normalization did not revert p66Shc upregulation or the concomitant increase of ROS. Interestingly, we found that promoter hypomethylation as well as enhanced histone 3 acetylation are the epigenetic markers that trigger persistent p66Shc overexpression despite glucose control. As compared with insulin alone, in vivo selective targeting of p66Shc in the context of glucose normalization restored endothelial function, blunted oxidative stress, and vascular apoptosis. Thus, p66Shc is the upstream signaling molecule whereby sustained hyperglycemic stress occurs. In conclusion, these findings could explain why diabetic vascular complications progress even in the presence of improved glycemic control and may assist in identifying novel therapeutic targets.
Gene Silencing of the Mitochondrial Adaptor p66^{Shc} Suppresses Vascular Hyperglycemic Memory in Diabetes
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SUPPLEMENTAL MATERIAL

Gene Silencing of the Mitochondrial Adaptor p66\textsuperscript{Shc} Suppresses Vascular Hyperglycemic Memory in Diabetes

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Detailed Methods

Cell culture
Human aortic endothelial cells (HAECs, passages 5 to 7) were purchased from Clonetics (Allschwil, Switzerland) and grown in fibronectin-coated 75cm² flasks in optimized endothelial growth medium-2 (EGM-2, Clonetics, Walkersville, USA) supplemented with 10% FCS. The cells were detached by using Tripsin/EDTA for 2 minutes and reseeded in fibronectin-coated 3 cm-cell culture dishes or 12 multiwell plates. HAECs were cultured in EGM-2 containing 2% FCS and exposed for 6 days either to normal glucose (5mmol/L) or high glucose concentration (25 mmol/L) as well as to high glucose for 3 days followed by normal glucose for the remaining 3 days. Mannitol was purchased from Sigma Aldrich (St Louis, USA) and used at the final concentration of 25 mmol/L as osmotic control.

Reagents
The non-selective PKC inhibitor Calphostin C, the selective PKCβ inhibitors 539654 (specific for βI and βII isoforms) and PKC myristoylated cell-permeable peptide (myr-ψ) were purchased from Calbiochem (Darmstadt, Germany). The selective PKCβII inhibitor CGP 53353, actinomycin D, ascorbic acid, cyclopentylidene-[4-(4-chlorophenyl)thiazol-2-yl]hydrazone (CPTH2), urate, streptozotocin (STZ), citrate buffer, norepinephrine (NE), acetylcholine (Ach), sodium nitroprusside (SNP), polyethylene glycol-superoxide dismutase (PEG-SOD), dihydroethidium and indometacin were purchased from Sigma Aldrich (St Louis, USA). To exclude cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase in cell supernatant was performed according to the manufacturer’s recommendations (Roche, Basel, Switzerland).

Western Blotting
After incubation cells were washed twice with PBS and lysed for immunoblotting (150 mmol/L sodium chloride, 50 mmol/L Tris, 1 mmol/L sodium fluoride, 1 mmol/L DTT, 1 mmol/L EDTA, 10 µg/µL leupeptin, 10 µg/µL aprotinin, 0.1 mmol/L sodium vanadate, 1 mmol/L PMSF, and 0.5% NP-40). Equal amounts (40 µg) of samples were subjected to SDS-PAGE gel for electrophoresis followed by semidry transfer onto Immobilon-P filter papers (Millipore, Billerica, USA). The membranes were blocked with 5% dry milk or 5% BSA in PBS-Tween buffer (0.1% Tween 20; pH 7.5) for 60 minutes and incubated with anti- human Shc, anti phospho-p66Shc (Ser-36), anti-PARP (Cell Signaling Technology, Danvers, USA), anti-PKCα, PKC β1, PKC βII, PKCγ, PKCδ, cytochrome c, GCN5 (Santa Cruz Biotechnology, Nunningen, Switzerland), total eNOS, phospho-eNOS Thr-495, phospho-eNOS Ser-1177 (Transduction Laboratories, Lexington, USA), and anti-acetylated Histone 3 (Millipore, Billerica, USA). GAPDH and alpha-tubulin antibodies were used as loading controls and purchased from Millipore (Billerica, USA) and Sigma Aldrich (St Louis, USA), respectively. Anti-rabbit and anti-mouse secondary antibodies were bought from GE Healthcare (Buckinghamshire, United Kingdom). The immunoreactive bands were detected by an enhanced chemiluminescence system (Millipore, Billerica, USA). Related signals were quantified using a Scion image software (Scion Corp, Frederick, USA).

Detection of membrane translocation of PKCβII
HAECs were incubated in a lysis buffer [50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 1 mmol/L MgCl₂, 2 mmol/L EDTA, 1 mmol/L PMSF, 10 mg/ml leupeptin, 1 mmol/L NaVO₃, and 0.1% Triton X-100] for 5 minutes on ice. The cell lysates were centrifuged at 15 000 rpm and 4°C for 15 minutes. After the supernatant had been collected as the cytosol fraction, the pellet was resuspended in 1% Triton X-100 in the lysis buffer and centrifuged at 15 000 rpm and 4°C for 15 minutes. The supernatant was then collected as the membrane fraction. Equal amounts (25 µg) of protein from each fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-PKCβII antibody (Santa Cruz Biotechnology, Nunningen, Switzerland).
Real time-PCR

Conversion of the total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamers (Amersham Bioscience, Piscataway, USA) in a final volume of 33 µl, using 4 µg of cDNA according to the manufacturer’s recommendations. Real time PCR was performed in a ABI 7500 real time PCR cycler (Applied Biosystems, Foster City, USA) according to the manufacturer’s instructions. All PCR experiments were performed using TaqMan Gene Expression Assay kit and TaqMan Gene Expression Master Mix, both provided by Applied Biosystems (Foster City, USA). Each reaction (20 µL) contained 2 µl cDNA, 1 µl of mixture containing primers and TaqMan gene-specific probe, 3 µl of water and 10 µl of TaqMan Gene Expression Master Mix (containing buffer, dNTPs, and Taq polymerase). A p66Shc predesigned primer was provided by Applied Biosystems (Foster City, USA). The 18S rRNA was used as endogenous control for normalizing RNA concentration. Real time PCR was performed in 96-well plates with the ABI 7500 Fast Real-Time PCR System. Standard thermal cycling conditions (20 sec at 95°C, 40 cycles for 3 sec at 95°C, 30 sec at 60°C) were used for all genes. Cycle threshold (Ct) values for each gene were obtained for each sample using the SDS1. Differences in Ct values between test gene and endogenous controls (18S rRNA) (∆Ct) were calculated and used for statistical analysis.

Measurements of \( \text{O}_2^- \), NO and ONOO⁻ by ESR spectroscopy

O\(_2^-\) generation in intact cells was assessed by electron spin resonance (ESR) spectroscopy analysis using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH). O\(_2^-\) production was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP) (1). Endothelial NO release was examined by ESR spectroscopy analysis with the use of the spin-trap colloid Fe(DETC)$_2$. Briefly, cells were washed and resuspended in 900 µL of Krebs-HEPES buffer (37°C), 300 µL of colloid Fe(DETC)$_2$ (final concentration 285 µmol/L) was added to each sample and incubated at 37°C for 60 minutes. Samples were scraped on ice, cells were collected from each sample and tubes were centrifuged at 4°C at 5000 rpm for 10 minutes. Cells were resuspended and aspirated into 1 mL syringes which were frozen immediately in liquid nitrogen (2).

Peroxynitrite (ONOO⁻) release from HAECs was determined by using the spin probe 1-hydroxy-3-carboxy-2, 2,5-tetramethyl-pyrrolidine (CPH) at a final concentration of 1 mM. Urate was used at the concentration of 50 mM for the reaction with ONOO⁻ (3). All ESR spectra relative to \( \text{O}_2^- \), NO and ONOO⁻ measurements were recorded with the use of NOX-E.5-ESR spectrometer (Bruker, Bremen, Germany). Signals were quantified by measuring the total amplitude after correction of baseline and subtraction of background signals.

Small interfering RNA transfection

Commercially available human PKC\( \beta \)II and GCN5 siRNAs (Santa Cruz Biotechnology, Switzerland ) and two siRNAs specifically targeting both human and mouse p66\( ^{\text{Shc}} \) predesigned from Microsynth (p66\( ^{\text{Shc}} \) 5’-UGA GUC UCU GUC AUC GCU G [dT][dT]-3’ and p66\( ^{\text{Shc}} \) 5’-CAG CGA UGA CAG AGA CUC A [dT][dT]-3’) were used. As a control, predesigned scrambled siRNA was used (Microsynth, 5’ UAC ACA CUC UCG UCU CU dTdT 3’). Briefly, cells were resuspended in the transfection buffer DMEM (Clonetics, Walkersville, USA) and placed in 4 mm cuvettes (400.000/each) for electroporation. For each cuvette, siRNA was diluted into 100µL of transfection buffer in order to obtain a final concentration of 33 nmol/L. Cells were subjected to: 250V, 150µF, 10-13 s, single pulse. Cellular viability and transfection efficiency were 70 and 60%, respectively, as determined by trypan blue exclusion and FACS analysis. Protein expression was assessed by Western blot.

Annexin V staining in human endothelial cells

Quantitative determination of percentage apoptotic cells was determined using Annexin V-FLUOS Staining (Roche, Basel, Switzerland), according to manufacturer’s protocol. Briefly, HAECs were stained with fluorescein isothiocyanate (FITC)-annexin and viewed under a fluorescence microscope (Olympus). DAPI and FITC signal images were merged (original magnification 20x). Cells not binding FITC-annexin V were classified as annexin V negative.
Caspase 3 activity in endothelial cells and mouse aorta

Caspase 3 activity was determined in both cellular and aortic lysates by using a colorimetric assay kit (Sigma Aldrich, USA), following the manufacturer’s protocol.

Detection of Methylglyoxal

Methylglyoxal levels were assessed by using a commercially available sandwich ELISA kit, according to the manufacturer’s instructions (OxiSelect™ methylglyoxal ELISA kit, Cell Biolabs, Inc., CA, USA).

p66Shc promoter methylation in vitro

For each analyzed genomic DNA sample, 30 ng of genomic DNA was digested with methylation sensitive enzyme (Ms) or with methylation dependent enzyme (Md) and also with both methylation sensitive and dependent enzymes (Msd) in 15µl total volume including 5X digestion buffer overnight at 37°C. In parallel, 30 ng of each genomic DNA sample was mock-treated (Mo) under identical conditions with the exception that water was substituted for digestion enzyme. Following digestion, enzyme activity was heat inactivated at 65°C for 20 min. A total of 6 ng digested DNA was analyzed by quantitative real-time PCR. A pre-designed primer for human p66Shc promoter was obtained from SA Biosciences (Frederick, MD, USA). Standard quantitative PCR cycling conditions were used with a ‘hot’ plate read of 72°C for 1 min. The melt curve of each amplicon was calculated within a temperature gradient from 60 to 95°C at 1°C increments with a 15 s hold time for each read. The cycle number at which the Ms and Md digested sample crossed the threshold was subtracted from the cycle number at which the Mo-treated sample crossed the threshold to determine the ∆Ct of the locus. Since Ms/Md digests only DNA including purine-5mC, thereby decreasing the amplifiable copies of loci containing DNA methylation and increasing the Ct relative to the mock-treated sample, increasing ΔCt values reflect increasing levels of local DNA methylation.

Insulin implants

Insulin or placebo were administered (1 implant/10g of body weight) as slow-release implants placed subcutaneously under the mid dorsal skin (LinBit, LinShin, Canada) after anesthesia with isofluorane (FORENE, Baxter Healthcare Corporation, USA). Glycemic control was assessed by measuring blood glucose from the tail vein with a sip-in sampling glucose meter (Glucometer Elite XL, Bayer, Laubach, Germany).

In vivo knockdown of p66Shc

In vivo knockdown of p66Shc was performed by injecting a mix of 2 pre-designed siRNAs specifically targeting p66Shc (Microsynth, 5’-UGA GUC UCU GUC AUC GCU G dTdT-3’ and 5’-CAG CGA UGA CAG AGA CUC A dTdT-3’). A scrambled-siRNA was used as a negative control (Microsynth, 5’-UAC ACA CUC UCG UCU CU dTdT-3’). Amount of p66Shc siRNA was selected based on dose optimization studies (data not shown). The siRNA mix at the final dose of 1.6 mg/kg was incubated with the in vivo-jetPEI delivery reagent (Polyplus Transfection) for 15 min at room temperature, in a final volume of 100 µL following the manufacturer’s instructions. According to time course studies, p66Shc siRNA was injected intravenously every 5 days for 3 weeks (Supplemental Figure II). Successful knockdown of p66Shc was assessed by Western blot in aortic lysates (anti-Shc antibody, Upstate Biotechnology, USA).

In order to verify p66Shc siRNA distribution in mouse aorta, the same siRNA sequences (5’-UGA GUC UCU GUC AUC GCU G dTdT-3’ and 5’-CAG CGA UGA CAG AGA CUC A dTdT-3’) were newly designed and labelled with a red fluorescent tag (Alexa fluor 546, Qiagen, USA). Tag-siRNA (4µM) was incubated with in vivo-jetPEI for 15 min at room temperature, in a final volume of 200 µL. After 48 hours from i.v siRNA injection, mice were harvested and thoracic aorta segments were embedded in optimum cutting temperature and stored in liquid nitrogen. 5µm-thick slices were then cut, fixed in cold acetone for 10 min, permeabilized with Triton 0.1%, and blocked with 1% BSA for 1 hour. Slides were then incubated with anti-mouse CD31 (PECAM-1) antibody (BD Biosciences, CA, USA) for 1 hour. A rat anti-mouse IgG secondary antibody was applied for 1 hour (Alexa fluor 488, Molecular Probes, USA). Finally, slides were incubated with 4,6 diamidino-2-phenyldiode
hydrochloride (DAPI) solution (Vector laboratories, CA, USA) for 10 minutes and observed using fluorescence microscopy (Olympus). Signals were then merged in a single image to demonstrate endothelial uptake of Tag-p66Shc siRNA (Figure 6B).

Tissue harvesting and organ chamber experiments
Mice were euthanized by intraperitoneal administration of 50 mg/Kg sodium pentobarbital. The entire aorta from the heart to the iliac bifurcation was excised and placed immediately in cold modified Krebs-Ringer bicarbonate solution (pH 7.4, 37°C, 95% O₂; 5% CO₂) of the following composition (mmol/L): NaCl (118.6), KCl (4.7), CaCl₂ (2.5), KH₂PO₄ (1.2), MgSO₄ (1.2), NaHCO₃ (25.1), glucose (11.1), and calcium EDTA (0.026). The aorta was cleaned from adhering connective tissue under a dissection microscope, and either snap-frozen in liquid nitrogen and stored at -80°C or used immediately for organ chamber experiments. For isometric tension studies, aorta was cut into 2 mm rings. Each ring was then connected to an isometric force transducer (Multi-Myograph 610M, Danish Myo Technology), suspended in an organ chamber filled with 6 mL Krebs-Ringer bicarbonate solution (37°C, pH 7.4), and bubbled with 95% O₂, 5% CO₂. After a 30 minute equilibration period, rings were gradually stretched to the optimal point of their length–tension curve, as determined by the contraction in response to potassium chloride (80 mmol/L). Several rings from the same aorta were studied in parallel. Responses to Ach (10⁻⁹ to 10⁻⁶ mol/L) in the presence or absence of PEG-SOD (150 U/mL) were recorded during submaximal contractions to NE (10⁻⁷ mol/L). The cyclooxygenase inhibitor indomethacin was used at the concentration of 10⁻⁵ mol/L. The NO donor SNP (10⁻¹⁰ to 10⁻⁵ mol/L) was added to test endothelium-independent responses. Relaxations were expressed as a percentage of the precontracted tension.

Measurement of superoxide anion production in mouse aorta
Superoxide production was determined in mouse thoracic aortas by ESR spectroscopy using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidine and an e-scan ESR spectrometer (Bruker BioSpin). Time-dependent formation of superoxide was analysed using the following instrumental settings: centre field 1.99 g, microwave power 20 mW, modulation amplitude 2 G, sweep time 60 s, field sweep 60 G. The intensity of ESR spectra was quantified after calibration of ESR signals with the free radical 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy.

In situ measurement of superoxide anion
Dihydroethidium (DHE) staining was carried out for superoxide determination in mouse aorta. Aortas were harvested, placed in cold PBS, and embedded for cryosectioning in optimal cutting temperature compound (Tissue-Tek). Unfixed frozen ring sections were cut into 5µ-thick sections and DHE (25 µM) was topically applied to each tissue section and coverslipped. Slices were incubated in a light-protected humidified chamber at 37°C for 1 hour. Images were obtained with a Leica fluorescent microscope. Superoxide signal specificity was confirmed by incubating sections with PEG-SOD (150 U/mL) for 30 minutes at 37°C.

Isolation of mitochondrial and cytosolic fraction
Mitochondria were isolated by centrifugation (4). HAECs and thoracic aortas were suspended in the mitochondrial buffer containing 10mM MOPS (pH 7.2), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 0.25 M sucrose, and gently homogenized with a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 750g for 10 minutes at 4°C to remove nuclei and unbroken cells, and the supernatant was centrifuged at 10,000g for 15 minutes. The resultant mitochondrial pellet was resuspended in 40 µL of standard lysis buffer (see Western blot for details) while the supernatant was collected as the cytosolic fraction.

p66Shc mitochondrial translocation and cytochrome c release
Mitochondrial and cytosolic proteins (30 µg) were separated on 10% and 15% reducing polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked in 5% milk, followed by incubation with antibodies against p66Shc (Upstate, USA) and cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membranes were incubated with anti-rabbit and anti-mouse IgG horseradish peroxidase–conjugated antibodies in blocking buffer for 1
hour, washed, and developed using a Western blot chemiluminescence detection kit (Millipore, Billerica, USA). Cytochrome c release and mitochondrial translocation of p66Shc were shown as percentage change of protein content in the cytosol vs. mitochondria and vice versa, respectively.

**In situ detection of vascular apoptosis by TUNEL assay**

DNA fragmentation was detected *in situ* using the TUNEL assay by using a commercially available kit (Roche, Basel, Switzerland). Briefly, 5 µm sections were fixed with 4% paraformaldehyde and permealized with 0.1% Triton X for 20 minutes. Solutions A (450ul) and B (50ul) were gently mixed in the dark and a volume of 100 ul was applied on each slice for 1 hour at 37°C in the dark. Solution A alone (100 ul) was used as a negative control. Nuclei were stained with DAPI (blue). TUNEL-positive nuclei (green) were counted under an Olympus fluorescent microscope.
Online Figure-I: Induction of diabetes with streptozotocin. Blood glucose levels in mice after a single high dose intraperitoneal injection of streptozotocin (STZ, 180 mg/Kg) dissolved in sterile 0.025 M citrate buffer (PH 4.5). Control animals received an equal volume of citrate buffer. Results are presented as mean ± SEM, n=15 per group.

Online Figure II: Time-course of in vivo siRNA-mediated p66Shc knockdown. (A-B) Representative Western blots and densitometric quantification of protein expression from aortic lysates of mice injected intravenously with transfection reagent JetPEI alone (control) and with scrambled or p66Shc-specific siRNA. Results are presented as mean ± SEM; n=4 per group. *, p<0.01; #, p<0.05.
Online Figure III: Mannitol does not affect neither ROS production nor PKC\(\beta\)II and p66\(^{Shc}\) expression. (A) ESR spectroscopy analysis of superoxide anion (O\(_2^\cdot\)) production in HAECs exposed to normal glucose (5 mmol/L) or mannitol (25 mmol/L). (B) Representative Western blots and densitometric quantification of PKC\(\beta\)II and p66\(^{Shc}\) expression under the same experimental conditions. Results are presented as mean ± SEM; n=4. NG, normal glucose; M, mannitol.

Online Figure IV: Protein expression of other PKC isoforms. HAECs were cultured for 6 days in normal (5 mmol/L), high glucose (25 mmol/L) or high glucose for 3 days followed by normal glucose for the remaining 3 days. Densitometric quantification of PKC\(\alpha\), \(\beta\)I, \(\gamma\) and \(\delta\) protein expression. Results are presented as mean ± SEM; n=5 per group. PKC, protein kinase C; NG, normal glucose; HG, high glucose; HN, high to normal glucose.
Online Figure V: p66<sub>Shc</sub> expression in response to increasing glucose concentrations. HAECs were cultured for 6 days in normal (5 mmol/L), high glucose (10-25 mmol/L) or high glucose for 3 days followed by normal glucose for the remaining 3 days. Representative Western blots and densitometric quantification of p66<sub>Shc</sub> expression. Results are presented as mean ± SEM; n=4-6 per group. NG, normal glucose; HG, high glucose; HN, high to normal glucose.

Online Figure VI: siRNA-mediated knockdown of PKCβ<sub>II</sub> and p66<sub>Shc</sub>. Representative Western blots and densitometric quantification of PKCβ<sub>II</sub> and p66<sub>Shc</sub> expression in HAECs after transfection with scrambled, PKCβ<sub>II</sub> and p66<sub>Shc</sub> specific siRNA. Results are presented as mean ± SEM; n=6 per group.
Online Figure VII: Selective and nonselective inhibition of PKCβ at the time of glucose normalization blunts $O_2^-$ and ONOO$^-$ production. (A) ESR spectroscopy analysis of superoxide anion ($O_2^-$) production in HAECs exposed to normal (5 mmol/L), high glucose (25 mmol/L) or high glucose by glucose normalization in the absence or in the presence of nonselective PKC inhibitor Calphostin c (3x10^{-7} mol/L), selective PKCβ inhibitors 539654 (1.25x10^{-9} mol/L) and ψ cell-permeable myristilated peptide (ψ-myr, 10^{-4} mol/L) as well as selective PKCβII inhibitor CGP 53353 (10^{-6} mol/L); (B) ESR spectroscopy analysis of peroxynitrite (ONOO$^-$) production in HAECs exposed to high glucose followed by glucose normalization in the absence or in the presence of selective PKCβII inhibition (CGP 53353, 10^{-6} mol/L) or PKCβII-specific siRNA. Results are presented as mean ± SEM; n=4-6 per group. PKC, protein kinase C; NG, normal glucose; HG, high glucose; HN, high to normal glucose.

Online Figure VIII: p66Shc silencing suppresses persistent activation of poly (ADP-ribose) polymerase (PARP). Representative Western blot and densitometric quantification of activated PARP (PAR) in HAECs cultured for 6 days in normal (5 mmol/L), high glucose (25 mmol/L) or high glucose for 3 days followed by glucose normalization in the presence and in the absence of p66Shc or scrambled siRNA. Results are presented as mean ± SEM; n=4 per group. NG, normal glucose; HG, high glucose; HN, high to normal glucose.
Online Figure IX: ROS are critically involved in maintaining upregulation of PKCβ despite restoration of normal glucose levels. (A) ESR spectroscopy analysis of superoxide anion (O$_2^-$) production in HAECs exposed to normal, high glucose or high followed by normal glucose in the absence or in the presence of the antioxidant ascorbic acid (100 µmol/L), free radical scavenger PEG-SOD (150 U/ml), scrambled or p66$^{Shc}$ siRNA; (B) Representative Western blot and densitometric quantification of PKCβII expression under the same experimental conditions. Results are presented as mean ± SEM; n=4-5 per group. NG, normal glucose; HG, high glucose; HN, high to normal glucose.

Online Figure X: Levels of glucose metabolite methylglyoxal persist elevated despite glucose normalization and are abolished by knockdown of p66$^{Shc}$. ELISA-based determination of methylglyoxal levels in HAECs cultured for 6 days in normal (5 mmol/L), high glucose (25 mmol/L) or high glucose for 3 days followed by glucose normalization in the presence and in the absence of p66$^{Shc}$ or scrambled siRNA. Results are presented as mean ± SEM; n=5-7 per group. NG, normal glucose; HG, high glucose; HN, high to normal glucose.
Online Figure XI: siRNA-mediated knockdown of GCN5. Representative Western blots and densitometric quantification of GCN5 expression in HAECs after transfection with scrambled and GCN5 siRNA. Results are presented as mean ± SEM; n=4 per group.

Online Figure XII: Glucose control in diabetic mice treated with insulin. Random 24h blood glucose monitoring in controls and diabetic mice treated with insulin after 3 weeks of diabetes. Results are presented as mean ± SEM, n=4 per group.
Online Figure XIII: Endothelium-independent relaxation across the experimental groups. Vascular relaxations to the NO donor sodium nitroprusside (SNP). Results are presented as mean ± SEM, n=6 per group.
Supplemental References


