

Activation of Sphingosine kinase-1 Mediates Inhibition of Vascular Smooth Muscle Cell  
Apoptosis by Hyperglycemia

Received for publication 6 October 2006 and accepted in revised form 13 February 2007.

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Running Title: Sphingosine Kinase 1 and Apoptosis

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Word count: 3547

Figures: 7

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## Abstract

Vascular smooth muscle cell (VSMC) apoptosis plays an essential role in vascular development and atherosclerosis. Hyperglycemia inhibits VSMC apoptosis, which may contribute to the development of diabetic vasculopathy. In the present study, we analyzed the mechanism of high glucose induced anti-apoptotic effect in cultured human aortic smooth muscle cells (HASMCs). Compared with normoglycemia, exposure of HASMCs to hyperglycemia, but not mannitol, significantly increased SK1 activity, but not SK2 activity. This increase was inhibited by a PKC inhibitor, GF109203X, the antioxidant N-acetylcysteine (NAC), and the reduced form of glutathione (GSH). The mechanism of SK1 activation by high glucose involves plasma membrane translocation. In addition, hyperglycemia markedly inhibited serum withdrawal induced apoptosis in HASMCs. Importantly, inhibition of SK1 by either a competitive inhibitor N',N'-dimethylsphingosine (DMS) or expression of dominant negative mutant of SK1(G82D), or specific siRNA knockdown, substantially attenuated hyperglycemia induced anti-apoptotic effect and anti-apoptotic protein Bcl-2 expression in HASMCs. Moreover, SK1 mediated anti-apoptotic effect requires the intracellular effects of S1P. We conclude that hyperglycemia stimulates SK1 activity via PKC and oxidative stress-dependent pathways, leading to decreased apoptosis in HASMCs. Taken together, these observations have important implications for understanding the roles of the SK1 signaling pathway in the pathogenesis of diabetic vasculopathy.

Hyperglycemia is considered one of the main factors in the development of vascular complications in diabetes leading to vascular disease (1) (2). An important feature of vascular disease is exuberant vascular smooth muscle cell (VSMC) proliferation. However, the precise molecular events linking hyperglycemia with abnormal VSMC functions are incompletely understood. The pathogenesis of occlusive macrovascular disease involves an excessive accumulation of cells within the intima and media (3). Enhanced cell proliferation is believed to be a key feature of diabetic vasculopathy (4) (5). However, recent studies clearly suggest that dysregulated cell apoptosis also contributes to the pathogenesis and progression of vascular disease (6) (7). Indeed, under hyperglycemia conditions, VSMC apoptosis has been shown to be markedly inhibited (8) (9) (10). In addition, the expression of antiapoptotic proteins such as Bcl-2 and Bcl-xl is also upregulated in VSMCs under high glucose conditions (10) (9) (11).

Sphingosine kinase (SK) is a key enzyme catalyzing the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P or SPP) (12). S1P, by directly acting on G protein-coupled S1P receptors and/or functioning as an intracellular second messenger, plays important and diverse roles in biological processes such as cell growth, proliferation, calcium homeostasis and survival (13). To date, Two different isoforms of SK have been identified, namely, SK1 and SK2 (14) (15). Overexpression of SK1 promotes cell survival and protects cells from apoptotic insults, such as serum withdrawal (16) (17). Indeed, various growth factors, cytokines, antigens, and G-protein-coupled receptor agonists, have

been shown to activate SK1 and increase the intracellular levels of S1P (18). In contrast, overexpression of SK2 suppresses cell growth and enhances apoptosis in cultured cells (19). Thus, SK may function as an internal sensor that can decide the fate of cells to either undergo apoptosis or to survive.

SK activation has also been implicated in the pathogenesis of vascular disease. Oxidized LDL, a major risk factor of atherosclerosis, has been shown to activate SK in VSMCs, resulting in S1P production and enhanced cell proliferation (20). Basic fibroblast growth factor (bFGF) also induces hyperproliferation in VSMCs via SK activation (21). Importantly, a recent clinical trial has shown that the SK product S1P is more predictive of obstructive coronary artery disease than other well established risk factors, including age, sex, family history, diabetes mellitus, lipid profile, and hypertension (22). Recently, Wang et al. (23) demonstrated that SK1 activity is significantly increased in aorta of streptozotocin induced diabetic rats. Increased SK1 activity mediates high glucose induced the proinflammatory phenotype in endothelial cells. Diabetes is one of the major risk factors for vascular disease. However, the role, particularly the isoform-specific role, of SK in mediating the vascular complications of diabetes is unknown. The aim of the present study was to test the hypothesis that SK mediates the hyperglycemia-induced anti-apoptotic effects in VSMCs

## RESEARCH DESIGN AND METHODS

### Cell Culture and treatment

Human aortic vascular SMCs were isolated as described (24). To study the effects of hyperglycemia on SK activity,

confluent cells were initially cultured in Dulbecco's modified Eagle's medium (DMEM) containing 0.4% serum for 48 h. Medium was then changed to DMEM containing 0.4% serum in the presence of 5.5 mmol/l glucose (normal glucose), 22 mmol/l glucose (high glucose), or 5.5 mmol/l glucose plus 16.5 mmol/l mannitol (mannitol) as indicated. For apoptosis studies, forty-eight hours after plating, the culture medium was replaced with DMEM deprived of fetal bovine serum and supplemented with glucose, mannitol, or S1P for an additional 48 h to induce apoptosis.

#### Construction of Adenoviruses

Adenoviruses harboring GFP, WT-SK, and DN-SK1 (G82D) were made using AdMax (Microbix). Briefly, pBHGlox $\Delta$ E1,3Cre, including the  $\Delta$ E1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into Ad293 cells using Fugene 6 (Roche). The viruses were propagated on Ad293 cells and purified using CsCl<sub>2</sub> banding followed by dialysis against 20 mM Tris-buffered saline with 2% glycerol. Titering was performed on Ad293 cells using Adeno-X<sup>TM</sup> Rapid Titer kit (Clontech) according to the instructions of the manufacturer.

#### RT-PCR

Specific primers used for RT-PCR analysis of S1P receptors and SK isoforms were designed essentially as previously described (25). The expected PCR products for S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, S1P<sub>4</sub>, S1P<sub>5</sub>, SK1, and SK2 were 647bp, 321bp, 321bp, 278bp, 306bp, 500bp, and 319bp, respectively. RT-PCR was performed by using one-step RT-PCR kit (Invitrogen). Briefly, total RNA (1 $\mu$ g) isolated from

cultured smooth muscle cells was reversibly transcribed at 55°C for 30min and amplified by PCR for 35 cycles under standard conditions: denaturation for 15s at 94°C, annealing for 30s at 55°C and elongation for 1min at 68°C. The last elongation period was 5min at 68°C. The PCR products were separated by electrophoresis in 1.5 % agarose gel in the presence of ethidium bromide.

#### Western Blotting

Proteins were prepared and separated on SDS-PAGE as described (26) (27). Immunoblotting was performed using antibodies to SK1 (1:1000 dilution, Abgent), to Bcl-2 (1:200 dilution, Santa Cruz), and to  $\alpha$ -tubulin (DM1A) (1:5000 dilution, Sigma). Immunodetection was accomplished using a sheep anti-mouse secondary antibody (1:2000 dilution) or donkey anti-rabbit secondary antibody (1:2000 dilution) and the enhanced chemiluminescence (ECL) kit (Amersham Corp)

#### Sphingosine Kinase Activity Assay and measurement of S1P

Sphingosine kinase activity assay was performed essentially according to previously described procedure (28) (29). Briefly, cells were washed with ice-cold PBS and homogenized in ice-cold 0.1M phosphate buffer (pH 7.4) containing 20% glycerol, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 15 mmol/L NaF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L 4-deoxy pyridoxine. After ultracentrifugation at 100,000  $\times$  g for 30 min, lysates were assayed for sphingosine kinase 1 activity in the reaction buffer favored SK1 activity (50 mmol/L HEPES, pH 7.4, 0.5% Triton, 15 mmol/L MgCl<sub>2</sub>, 10% glycerol, 10 mmol/L NaF, 1.5

mmol/L semicarbazide). In addition, SK2 activity was determined in the SK2 reaction buffer (50 mmol/L Hepes, pH 7.4, 15 mmol/L MgCl<sub>2</sub>, 1 mol/L KCl, 10% glycerol, 10 mmol/L NaF, 1.5 mmol/L semicarbazide). The lipid products were separated by TLC on Silica Gel G60 (Whatman) using chloroform/methanol/acetic acid/water (90:90:15:6). The radioactive spots corresponding to sphingosine phosphate were scraped and counted in a scintillation counter. For quantification of S1P, VSMCs were labeled for 7 h in serum-free medium with 0.3 μCi/ml D-erythro-[3-<sup>3</sup>H]sphingosine (American Radiolabeled Chemicals). After incubating with different concentrations of glucose, cellular lipids were extracted and resolved by TLC as previously described (30) and counted by liquid scintillation.

Gene Silencing with SiRNA double-stranded SK1 specific siRNA (sense: 5'-GGGCAAGGCCUUGCAGCUCdTdT-3', antisense: 5'-GAGCUGCAAGGCCUUGCCdTdT-3') were synthesized and transfected to the cells with Oligofectamine (Invitrogen) according to the manufacturer's protocol. The control cells were transfected with a control siRNA duplex (sense 5'-UUCUCCGAACGUGUCACGUDdTdT-3', antisense: 5'-ACGUGACACGUUCGGAGAAdTdT-3'), this control siRNA has no known target in mammalian genomes. All siRNAs were purchased from Qiagen.

Assays for Apoptosis Histone-associated DNA fragments were

quantitated by the Cell Death Detection ELISA (Roche) (28). Briefly, the cytoplasmic fractions were added to the 96-well ELISA plates precoated with the anti-histone monoclonal antibody and incubated for 90 minutes at room temperature. After washing, bound nucleosomes were detected by the addition of anti-DNA-peroxidase monoclonal antibody and reacted for 60 minutes at room temperature. After the addition of substrate, optical density was read with an ELISA reader at 405 nm. TUNEL staining was performed using the In Situ Cell Death Detection kit (Roche). DNA ladder assays were performed as previously described (31).

#### Statistical Analyses

All data were analyzed by means of 1 or 2-way ANOVA and Fisher's exact test for post hoc analyses. A value of P<0.05 was considered statistically significant.

## RESULTS

### Hyperglycemia Stimulates SK1 Activity

HASMCs were treated with 5.5 mmol/L glucose (normal glucose), 22 mmol/L glucose (high glucose), or 5.5 mmol/L glucose plus 16.5 mmol/L mannitol for 48 hrs. As shown in Figure 1A, treatment with high glucose significantly increased SK1 activity by 83% in HASMCs, whereas the SK2 activity was unaltered. In contrast, mannitol, used as an osmotic control, had no effect on SK1 activity. Consistent with the increases in enzyme activity, intracellular S1P levels were also increased by 92% in HASMCs exposed to high glucose (Figure 1B). In addition, hyperglycemia didn't affect the expression of SK1, as detected by immunoblot analysis, suggesting

hyperglycemia induced SK1 activation occurred at the post-translational level (Figure 1C). The time course of SK1 activation when incubated with 22 mmol/L glucose showed increases of 43% at 24 hours, 87% at 48 hours and 76% at 72 hours, reaching a maximum after 48 hours incubation (Figure. 1D). Glucose concentrations in the media were not significantly changed during the entire course of treatment (data not shown), suggesting that HASMCs did not metabolize glucose under our experimental conditions.

#### PKC and ROS Mediate Hyperglycemia Induced SK1 Activation

Because hyperglycemia induces PKC activation and stimulates ROS production in a variety of cells, including vascular smooth muscle cells (32) (33), we investigated whether PKC and ROS are involved in hyperglycemia-stimulated SK1 activation. Interestingly, activation of SK1 by hyperglycemia was completely blocked by a nonselective PKC inhibitor, GF109203X (5  $\mu\text{mol/L}$ ), but not by dimethyl sulfoxide (Figure 2A). The antioxidants NAC and GSH, which scavenge ROS, also inhibited hyperglycemia-induced SK1 activation (Figure 2B). To further investigate the mechanism by which SK1 is activated by hyperglycemia, the plasma membrane translocation of SK1, as assayed by immunoblot analysis, was investigated. Treatment of HASMCs with high glucose caused a significant translocation of SK1 to the plasma membrane that was robustly inhibited in the presence of GF109203X and NAC (Figure 2C). Together, these results suggest that PKC and ROS mediate hyperglycemia-induced SK1 activation in HASMCs via translocation of SK1 to the membrane.

#### SK1 Mediates Hyperglycemia Induced Anti-apoptotic Effects in HASMCs.

Hyperglycemia has been shown to protect VSMC from undergoing apoptosis (8) (9) (10). Since SK1 and its product S1P play a critical role in the regulation of cell apoptosis, we investigated whether SK1 can mediate some of the antiapoptotic effects of hyperglycemia. We found that treatment of HASMCs with hyperglycemia significantly inhibited the serum withdrawal induced cell apoptosis, as determined by DNA fragmentation (Figure 3A) and TUNEL staining (Figure 3B). In the presence of a SK competitive inhibitor N,N'-dimethylsphingosine (DMS) (2.5  $\mu\text{mol/L}$ ), the antiapoptotic effects of hyperglycemia were completely abolished. To further establish whether the increase in SK1 activity by hyperglycemia contributes to its anti-apoptotic effects in HASMCs, we investigated the effects of hyperglycemia on serum withdrawal induced VSMC apoptosis by using adenovirus harboring HA tagged dominant negative DNSK1(G82D) (Ad-DNSK1) (28). Indeed, overexpression of DNSK1(G82D) almost completely abolished the hyperglycemia induced SK1 activation in HASMCs, while GFP was without effect (Figure 3C). Accordingly, the anti-apoptotic effect of hyperglycemia was also substantially attenuated in HASMCs overexpressing DNSK1(G82D) (Figure 3D).

It's reported that Bcl-2 plays a critical role in the anti-apoptotic effect of high glucose in animals or in human VSMCs (9) (11). However, the mechanism mediating upregulation of Bcl-2 under high glucose conditions is not well understood. To examine whether SK1 mediates Bcl-2 expression in HASMCs, we treated the

cells with either a SK competitive inhibitor DMS or overexpression of DNSK1(G82D). As expected, treatment of HASMCs with hyperglycemia markedly augmented the Bcl-2 expression that was suppressed in the presence of DMS (2.5  $\mu\text{mol/L}$ ) (Figure 4A) or by overexpression of DNSK1(G82D)(Figure 4B). Collectively, these findings indicate that the antiapoptotic effects of hyperglycemia in HASMCs are mediated by the upregulation of SK1 activity.

Overexpression of SK1 inhibits VSMC apoptosis.

To further substantiate the correlation between SK1 activity and VSMC apoptosis, we investigated the effect of overexpression of wild-type SK1(WTSK1) on serum withdrawal induced apoptosis in HASMCs. To this end, we generated a recombinant adenovirus for delivery of the HA tagged WTSK1 cDNA (Ad-WTSK1) into HASMCs. Transduction of SMCs with Ad-WTSK1 at a MOI of 50 resulted in robust expression of WTSK1 in HASMCs, as demonstrated by immunoblot analysis using anti-HA antibody (Figure 5A). Overexpression of WTSK1 substantially inhibited serum withdrawal induced apoptosis in HASMCs, as measured by both DNA fragmentation and DNA laddering assays, whereas overexpression of either GFP or DNSK1(G82D) was without effect (Figure 5A and 5B). Likewise, expression of anti-apoptotic protein Bcl-2 was also significantly increased in HASMCs transduced with Ad-WTSK1(Figure 5C). Exogenous addition of S1P also significantly inhibited serum withdrawal induced apoptosis in HASMCs in a dose dependent manner. In the presence of a high concentration of S1P (5  $\mu\text{mol/L}$ ), the

percentage of TUNEL positive cells was substantially reduced, from 20.1% to 7.6% (Figure 5D). To determine whether Gi coupled S1P receptors are involved in the S1P protective response, HASMCs were treated with pertussis toxin (100 ng/ml) to inhibit Gi activity. Interestingly, PTX only partially attenuated the anti-apoptotic effects of S1P (Figure 5D), suggesting that some of the antiapoptotic effects of exogenously added S1P are mediated by signaling pathways other than Gi coupled S1P receptors. Together, these results further suggest that SK1/S1P is an important mediator for VSMC apoptosis.

Knock-down of SK1 Abolishes Hyperglycemia Induced Anti-apoptosis

To further investigate whether activation of SK1 is indeed responsible for the hyperglycemia induced anti-apoptotic effects in HASMCs, we employed siRNA to knock-down the expression of SK1. Transfection of SK1 siRNA substantially reduced SK1 expression by 80% in HASMCs, as determined by both RT-PCR and immunoblot analysis (Figure 6A). In addition, hyperglycemia induced activation of SK1 was almost completely blocked by SK1 specific siRNA (Figure 6B). Likewise, the antiapoptotic effects of hyperglycemia in HASMCs were also markedly attenuated in the SK1 specific siRNA transfected HASMCs (Figure 6C). In addition, high glucose induced expression of Bcl-2 was also substantially inhibited in the SK1 siRNA transfected cells (Figure 6D). Taken together, these results further indicate that SK1 is responsible for the hyperglycemia induced anti-apoptotic effects and Bcl-2 expression in HASMCs.

## Effects of S1P Receptors on SK1 Mediated Anti-apoptosis in HASMCs

The biological consequences of SK1 activation depend on the production of S1P, which exerts biological effects by directly acting on G protein-coupled S1P receptors and/or functioning as an intracellular second messenger (13). RT-PCR analysis indicated that of five S1P receptors, only S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> are detected in HASMCs (Figure 7A). S1P<sub>1</sub> is known to activate only members of the G<sub>i</sub> family, while S1P<sub>2</sub> and S1P<sub>3</sub> can communicate with several G proteins, including G<sub>i</sub>, G<sub>q</sub>, and G<sub>12/13</sub>. (34). To verify whether S1P receptors are involved in the SK1 mediated anti-apoptosis induced by hyperglycemia in HASMCs, we treated HASMCs with pertussis toxin (PTX), an inhibitor of G<sub>i</sub> proteins. As shown in Figure 7B, treatment of HASMCs with PTX (100 ng/ml) had no effects on hyperglycemia induced anti-apoptotic effects. Moreover, treatment of HASMCs with VPC23019 (10 μmol/L), a selective S1P<sub>1</sub>/S1P<sub>3</sub> receptor antagonist (35), also did not impact hyperglycemia induced anti-apoptotic effects. In contrast to S1P, dihydro-S1P, which is structurally similar to S1P and binds to and activates S1P<sub>2</sub> and S1P<sub>5</sub>, had no effects on the serum withdrawal induced apoptosis in HASMCs (Figure 7C). Moreover, treatment of cells with either PTX or VPC23019 did not affect high glucose induced expression of Bcl-2 (Figure 7D). Taken together, these results suggest that the intracellular actions of S1P may account for the SK1 mediated anti-apoptosis induced by hyperglycemia in HASMCs.

## DISCUSSION

This study demonstrates that exposure of HASMCs to high-glucose conditions

specifically increases SK1 activity leading to inhibition of VSMC apoptosis induced by serum withdrawal. Hyperglycemia induced SK1 activation involves PKC dependent membrane translocation. These findings are consistent with previous studies showing that high glucose activates SK1 activity in vascular endothelial cells (23). Most importantly, SK1 activity is significantly increased in aorta and heart of streptozotocin-induced diabetic rats (23), further establishing the physiological significance of the findings reported in this study. At present, the mechanism underlying the activation of SK is not well understood, but certainly involves PKC mediated phosphorylation and translocation (36) (37). For example, phosphorylation of SK1 by protein kinase C was shown to activate SK1 in response to VEGF stimulation in endothelial cells (36). Protein kinase C-dependent activation of SK1 leads to SK1 translocation from cytosol to plasma membrane in HEK293 cells (37). Importantly, the association between diabetes mellitus and activation of PKC has been well documented. Activation of the diacylglycerol-PKC pathway occurs predominantly in vascular tissue. Indeed, it was reported that high glucose increases intracellular diacylglycerol levels, leading to PKC activation in vascular smooth muscle cells (38) (39). However, it remains to be determined which PKC isoform(s) activates SK1 in HASMCs under high-glucose conditions.

Our study also indicated that hyperglycemia induced SK1 membrane translocation and activation in HASMCs is ROS dependent. Both ROS and the PKC pathway have been implicated in diabetes complications, including vascular disease (39), but the exact interaction between these two pathways has not been

fully defined. Indeed, high glucose has been shown to induce intracellular ROS in various cell types including VSMCs. It is possible that PKC lies downstream of ROS. Indeed, incubation of cultured cells with high glucose increases mitochondrial ROS production, leading to subsequent PKC activation (40) (41).<http://circ.ahajournals.org/cgi/content/full/111/24/3261> - R34-166201#R34-166201 However, other studies have shown that PKC inhibitors block high glucose-induced ROS production in vascular endothelial and smooth muscle cells (42) (43). Thus, it is unclear whether PKC lies upstream of ROS production or vice versa. Interestingly, we found that neither PKC activity nor ROS production was affected by exogenously added S1P in HASMCs (data not shown), suggesting that the mechanisms mediating the antiapoptotic effects of exogenously added S1P and intracellular SK1/S1P activated by hyperglycemia may be different in HASMCs. High glucose activated SK1/S1P pathway may function as a second messenger in HASMCs. In this case, our results are consistent with previous observations (44) (23) (30). Nevertheless, our present study suggested that SK1 may function as a common downstream target of PKC and ROS and mediate high glucose induced anti-apoptotic effects in HASMCs.

Diabetes is one of the major risk factors for cardiovascular disease (45) (46). Diabetes accelerates atherosclerotic lesion formation, and it is an important predictor of restenosis after percutaneous transluminal coronary angioplasty (1) (47). The balance between proliferation and apoptosis of VSMCs is one of the critical determinants of atherosclerotic lesion formation and the vascular remodeling process (48)

(6)<http://atvb.ahajournals.org/cgi/content/full/25/9/1871> - R8-094672#R8-094672<http://atvb.ahajournals.org/cgi/content/full/25/9/1871> - R9-094672#R9-094672. Indeed, Treatment of VSMC with high glucose markedly enhanced the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl, thereby inhibiting VSMC apoptosis (9) (10; 11). However, the cell signaling pathways underlying the hyperglycemia induced anti-apoptotic effects in VSMCs are not completely understood. In the present study we demonstrated that high glucose significantly activated SK1 in HASMCs and that the high glucose induced expression of Bcl-2 is SK1 dependent. Furthermore, it's interesting to note that overexpression of WTSK1, but not DNSK1(G82D) in HASMCs elicited a similar anti-apoptotic effect. SK1 activation results in an increased production of its product, S1P, which functions by directly acting on G protein-coupled S1P receptors and/or functioning as an intracellular second messenger (13). To date, five receptors, EDG-1/S1P1, EDG-5/S1P2, EDG-3/S1P3, EDG-6/S1P4, and EDG-8/S1P5 have been characterized to bind to both S1P and dihydro-S1P with high specificity (13). Indeed, our study indicated that HASMCs express mainly three S1P receptors, including S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub>. S1P<sub>1</sub> mainly couples to G<sub>i</sub>, whereas S1P<sub>2</sub> and S1P<sub>3</sub> activate G<sub>i</sub>, G<sub>q</sub>, and G<sub>12/13</sub> (34). Unfortunately, there are no non-selective S1P receptor antagonists currently available to potentially inhibit all five S1P receptor subtypes. Thus, in the present study, we employed two inhibitors: an inhibitor of G<sub>i</sub> proteins PTX and a selective S1P1/S1P3 antagonist VPC23019 to initially evaluate the role of S1P receptor subtypes in hyperglycemia induced anti-apoptotic effect in HASMCs. Interestingly, in the presence of PTX, high

glucose induced either anti-apoptotic effects or Bcl-2 expression were not affected, suggesting that the SK1 mediated anti-apoptosis in HASMCs is independent of Gi protein coupled S1P receptors. In addition, a selective S1P1/S1P3 antagonist VPC23019 also did not impact high glucose induced anti-apoptotic effects. Furthermore, effects of S1P on HASMCs were compared with dihydro-S1P that is structurally similar to S1P and binds to S1P2 and S1P5 but lacks the intracellular effects (44). Importantly, S1P significantly inhibited serum withdrawal induced HASMC apoptosis, whereas dihydro-S1P had no effects. Taken together, these results suggest that the intracellular actions of S1P are primarily responsible for the SK1 mediated anti-apoptotic effects in VSMCs under high glucose conditions, albeit the intracellular targets need to be identified. Further studies are needed to shed light on the mechanism by which Bcl-2 expression is induced by SK1 activation.

It should be noted that the biological actions of S1P may vary depending on the cell types, cell conditions, specific microenvironments, and distinct receptors in individual tissues (18). In addition, the mechanisms mediating the biological effects of exogenous S1P and intracellular SK1/S1P could be different and may be concentration dependent (49) (17) (16). For example, at the concentration of 100 nmol/L, exogenously added S1P strongly

prevented monocyte/endothelial interactions; however, at concentrations of S1P greater than 5  $\mu\text{mol/L}$ , S1P increased monocyte adhesion to endothelial cells (23) (49). In human serum, estimates of S1P concentration range from 0.4 to 1.0  $\mu\text{mol/L}$ (22) . At present, little is known about S1P levels in blood and in vascular tissues under diabetic conditions. In this regard, more studies are needed to precisely elucidate the roles of SK1/S1P in the development of diabetic vascular complications.

In conclusion, our results demonstrate that SK1, but not SK2, is an important mediator of the hyperglycemia induced anti-apoptotic effects in human VSMCs. Targeting SK1 for inhibition by either SK1 specific inhibitors, expression of dominant negative SK1 mutant, or small interference RNA strongly attenuated the hyperglycemia induced antiapoptotic effects in VSMCs. Thus, SK1 may represent a novel therapeutic modality for treatment of diabetic vascular complications such as atherosclerosis and restenosis.

#### ACKNOWLEDGEMENTS

This work was supported by AHA Scientist Development Grant 0630047N to J. Sun. The authors thank Dr. Samuel J. Leibovich for his critical discussions and comments.

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Figure legends:

Figure 1. Effect of high glucose on SK activity in HASMCs. A, both SK1 and SK2 activity were determined in HASMCs after exposure to media containing 5.5 mmol/L glucose (NG), 22 mmol/L glucose (HG), or 5.5 mmol/L glucose plus 16.5 mmol/L mannitol (Mtol) for 48 hrs. n=4. \*P < 0.05 vs NG or Mtol. B, measurements of S1P levels after exposure to media containing NG, HG or Mtol for 48 hours. n=4. \*P < 0.05 vs NG or Mtol. C, immunoblot analysis of SK1 protein expression in HASMCs after exposure to media containing NG, HG or Mtol for 48 hours. n=3. D, activity assay showing the time dependent effect of high glucose on SK1 activity. n=4. \*P < 0.05 vs 0 hour.

Figure 2. Roles of PKC and ROS in high glucose induced SK1 Activation. A, HASMCs were cultured in media containing NG, HG or Mtol for 24 hours. Cells were treated with vehicle (Veh) or GF109203X (GFX) for the last 24 hours. SK1 activity was then measured. n=4. \*P < 0.05 vs NG or HG plus GFX. B, HASMCs were cultured in media containing NG, HG or Mtol for 24 hours. Cells were treated with NAC or GSH for the last 24 hours. SK1 activity assay was performed. n=3. \*P < 0.05 vs NG or HG/NAC or HG/GSH. C, immunoblot analysis of SK1 expression and membrane translocation in HASMCs under high glucose conditions. n=3.

Figure 3. SK1 mediates the hyperglycemia induced anti-apoptotic effects in HASMCs. Effects of high glucose on apoptosis in HASMCs were determined by the Cell Death Detection ELISA (A) and TUNEL staining (B). VSMC apoptosis was induced in the serum free media containing NG, HG or Mtol for 48 hours in the absence and presence of a SK1 competitive inhibitor DMS (2.5  $\mu$ mol/L). n=3. \*P < 0.05 compared with media containing FBS; †P < 0.05 vs HG/-FBS. C, SK1 activity was determined in HASMCs with overexpression of HA tagged dominant negative mutant DNSK1. n=3. \*P < 0.05 vs NG or HG+DNSK1. D, effect of overexpression of DNSK1 on high glucose induced anti-apoptosis in HASMCs as assayed by DNA fragmentation. n=3. \*P < 0.05 compared with NG+FBS; †P < 0.05 vs HG/-FBS.

Figure 4. Immunoblot analysis (40  $\mu$ g of total protein/lane) showing effect of treatment of cells with either DMS (2.5  $\mu$ mol/L) (A) or DNSK1 (B) on HG induced Bcl-2 levels in HASMCs. n=3.

Figure 5. Overexpression of wild-type SK1 (WTSK1) inhibits apoptosis. A, HASMCs were transduced with either Ad-GFP, Ad-WTSK 1, or Ad-DNSK1. 24 hr after transduction, apoptosis was induced by serum withdrawal and cell apoptosis was measured by the Cell Death Detection ELISA. n=4. \*P < 0.05 vs GFP or DNSK1. B. DNA laddering assay showing decreased apoptosis in HASMCs transduced with Ad-WTSK1. n=3. C, effect of SK1 overexpression on Bcl-2 expression. HASMCs were transduced with either Ad-GFP, Ad-WTSK 1, or Ad-DNSK1. 48 hr after transduction, expression of Bcl-2 was determined by immunoblot analysis. n=3. D, VSMC apoptosis was induced by serum withdrawal in the absence and presence of different concentrations

of exogenously added S1P for 48 hr as determined by TUNEL staining. n=3. \*P < 0.05 vs NG/-S1P; †P < 0.05 vs NG or NG+5  $\mu$ mol/L S1P.

Figure 6. SK1 knockdown attenuates high glucose induced anti-apoptosis. A, RT-PCR and western blot showing expression of SK1 in HASMCs transfected with SK1 specific siRNA. B, SK1 activity was determined in HASMCs transfected with either control siRNA or SK1 specific siRNA. n=3. \*P < 0.05 vs NG or HG/SK1 siRNA. C, effects of SK1 specific siRNA on cell apoptosis in HASMCs. 36 hr after transfected with control or SK1 specific siRNA, HASMC apoptosis was induced as described above and measured by the Cell Death Detection ELISA. n=4. \*P<0.05 vs NG+FBS or HG/CTL siRNA; †P < 0.05 vs HG/CTL siRNA. D, immunoblot analysis showing the expression of Bcl-2 protein in HASMCs transfected with control or SK1 specific siRNA under both normal and high glucose conditions. n=3.

Figure 7. Effect of S1P receptors on high glucose induced anti-apoptosis in HASMCs. A, RT-PCR showing the expression of S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> in HASMCs. B, VSMC apoptosis was induced by serum withdrawal in the presence and absence of either G<sub>i</sub> inhibitor PTX (100ng/ml) or S1P<sub>1</sub>/S1P<sub>3</sub> antagonist VPC23019 (10  $\mu$ mol/L) for 48hr under normal or high glucose conditions. Cell apoptosis was measured by the Cell Death Detection ELISA. n=3. \*P < 0.05 vs HG or HG/PTX, or HG/VPC. C, effect of S1P on cell apoptosis in HASMCs. VSMC apoptosis was induced by serum withdrawal in the presence of 5  $\mu$ mol/L S1P, dihydro-S1P, or vehicle for 48 hr. Cell apoptosis was measured by the Cell Death Detection ELISA. n=3. \*P < 0.05 vs HG or S1P. D, immunoblot analysis showing Bcl-2 protein expression in HASMCs treated either PTX (100ng/ml) or VPC23019 (10  $\mu$ mol/L) under both normal and high glucose conditions. n=3.

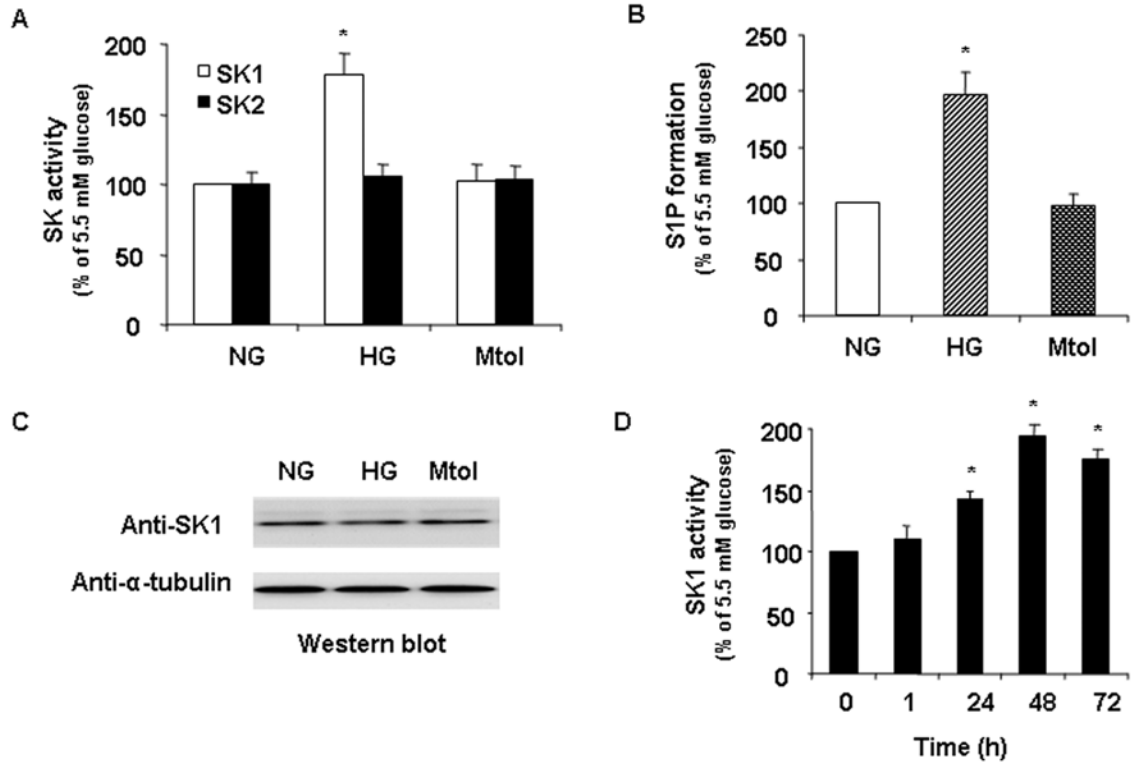


Figure 1

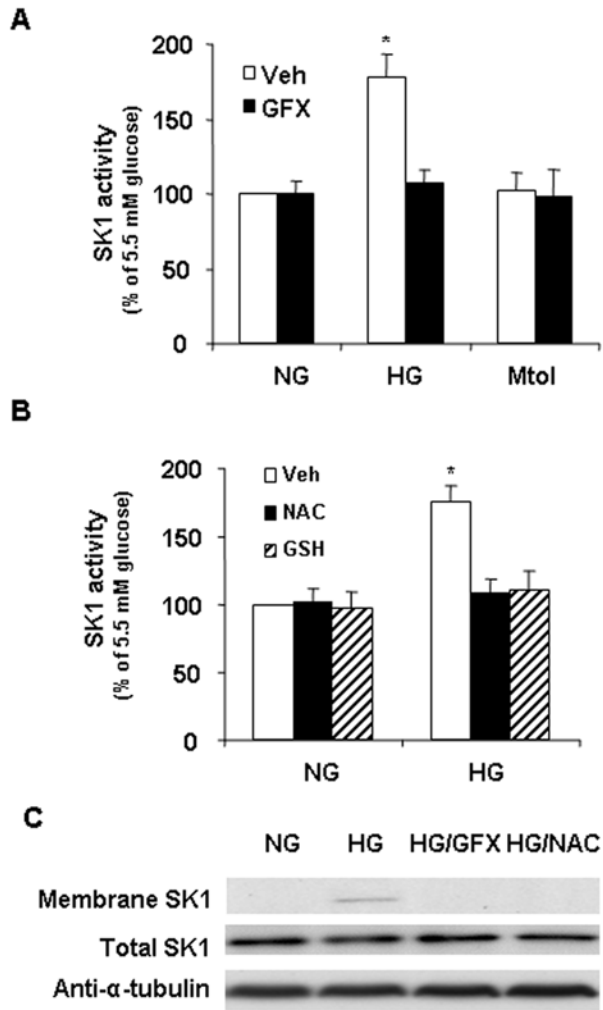


Figure 2

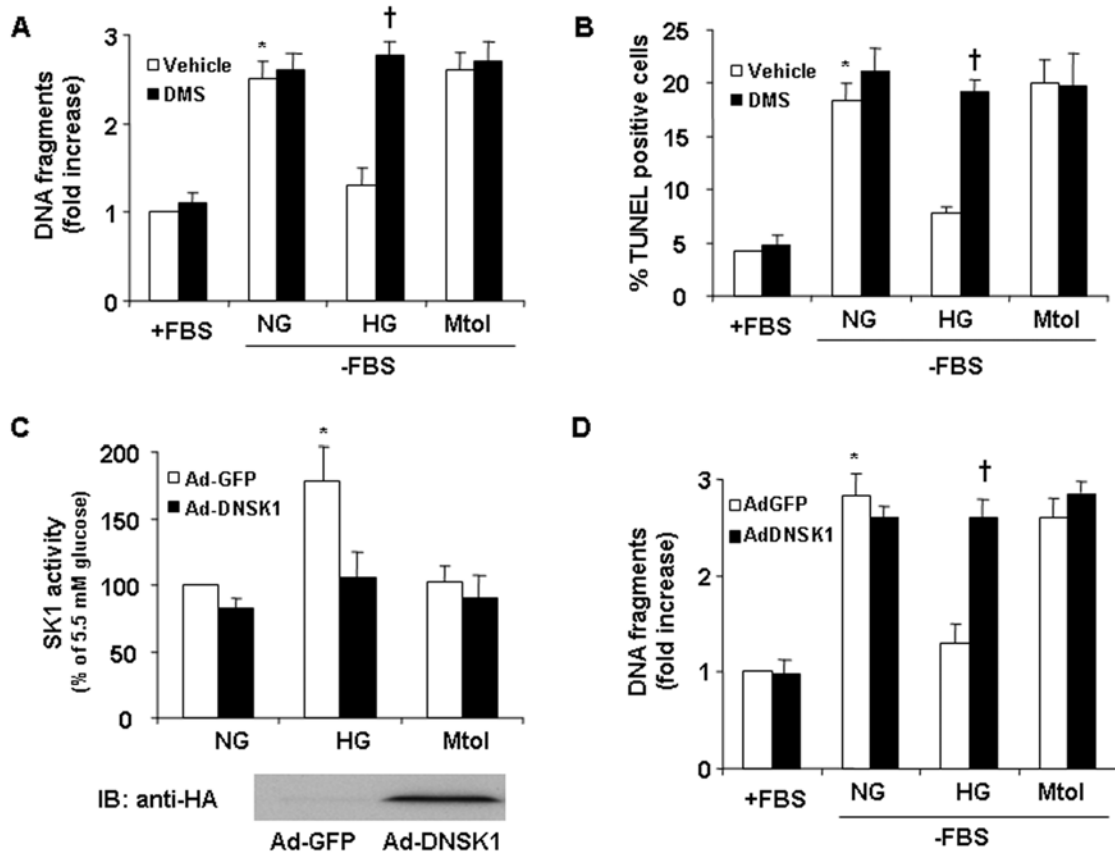


Figure 3

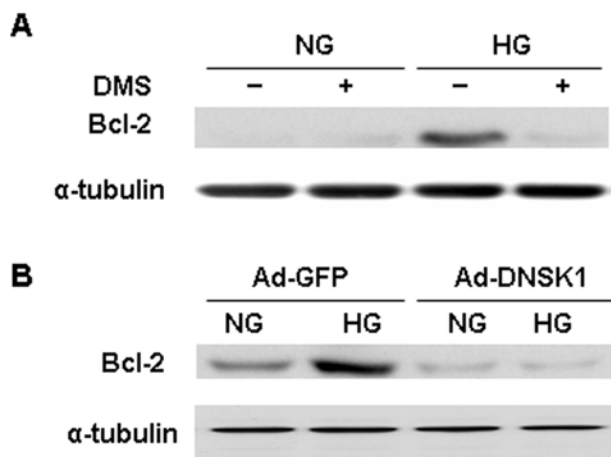


Figure 4

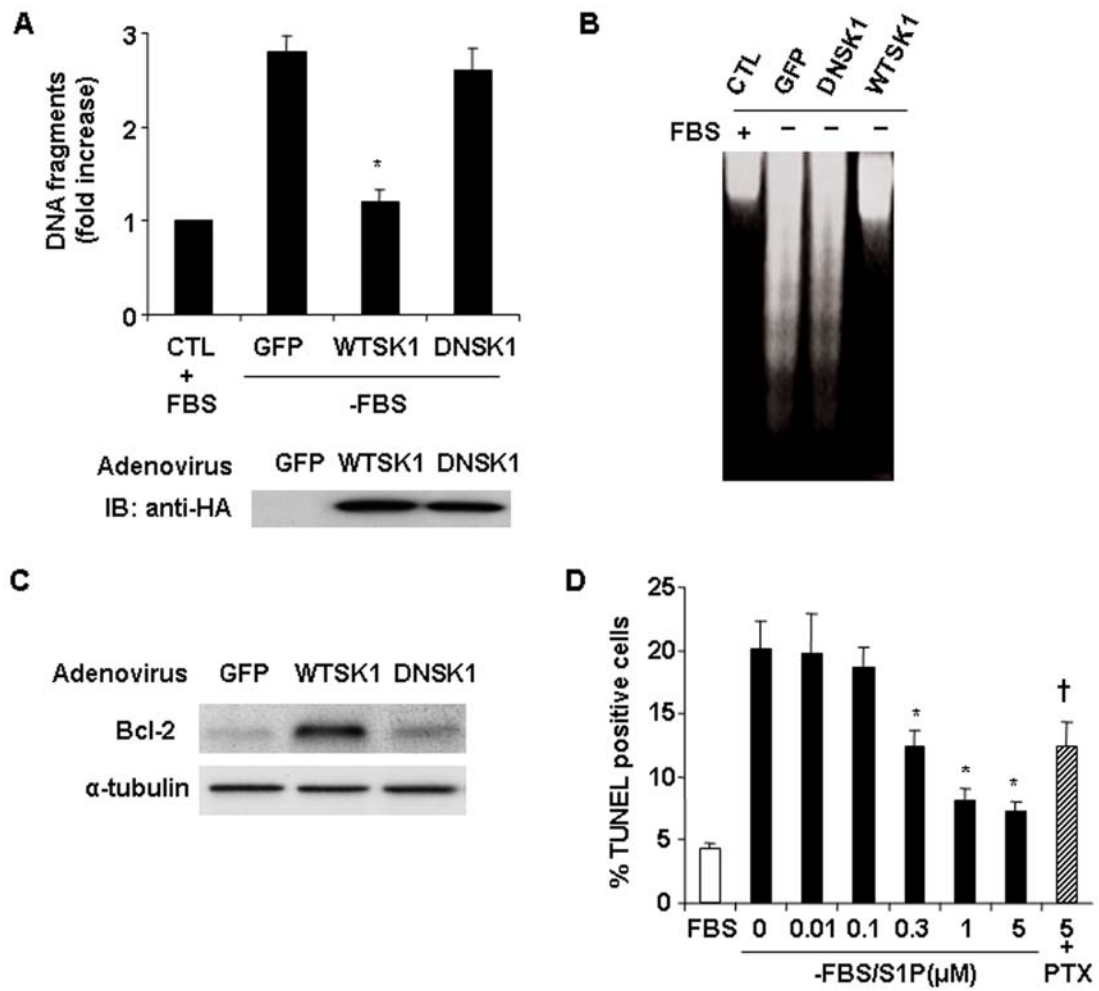


Figure 5

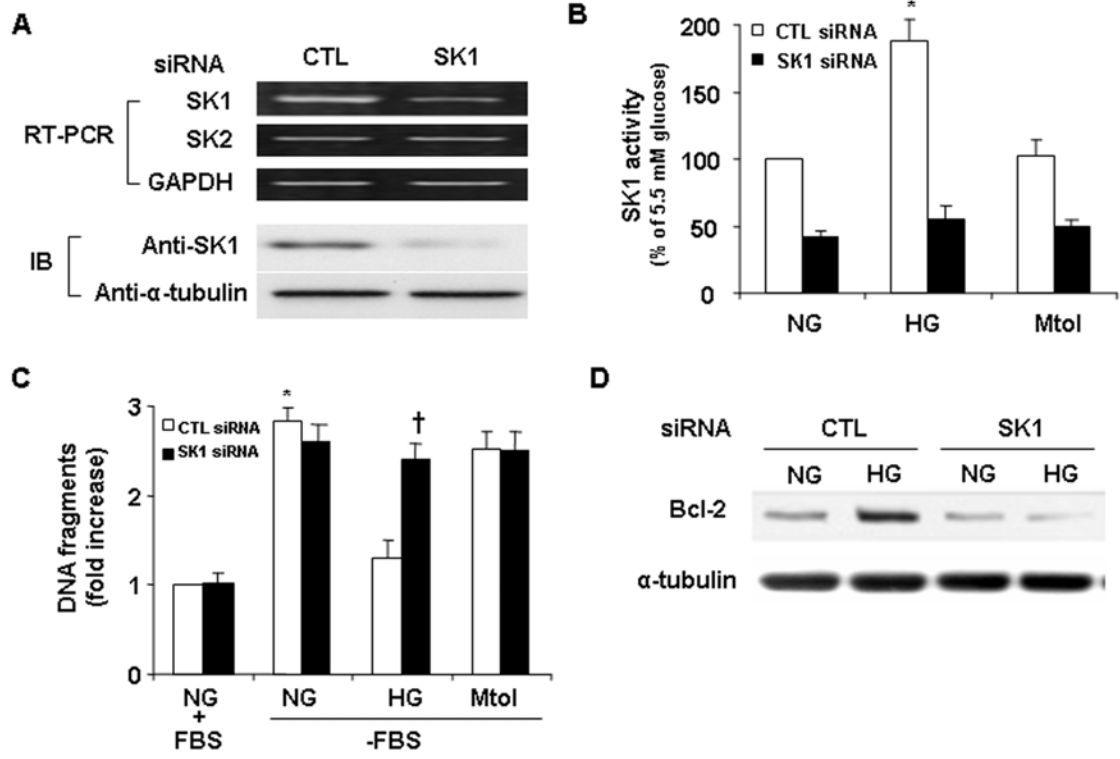


Figure 6

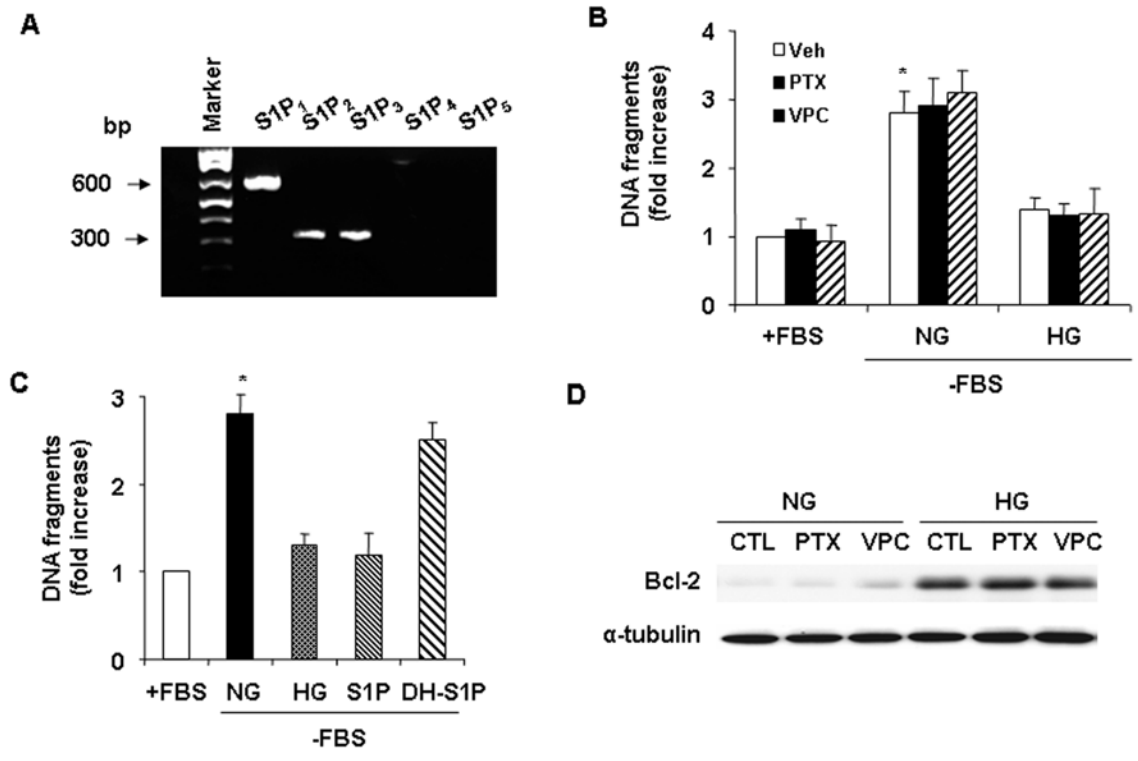


Figure 7