High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1

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Sakuma, Hiroya, Mayumi Yamamoto, Mie Okumura, Toshihiro Kojima, Takako Maruyama, and Keigo Yasuda. High glucose inhibits apoptosis in human coronary artery smooth muscle cells by increasing bcl-xL and bfl-1/A1. Am J Physiol Cell Physiol 283: C422–C428, 2002.—Cardiovascular disease is a serious complication in diabetic patients. To elucidate the precise mechanisms of atherosclerosis in diabetic patients, the effects of high glucose concentration (25 mM) on apoptosis regulation and bcl-2 family protein expression in human coronary artery smooth muscle cells (CASM) were examined. Treatment with high glucose concentration markedly increased mRNA expression of bcl-xL and bfl-1/A1 compared with cells treated with normal glucose. High glucose induced phosphorylation of phosphatidylinositol 3-kinase (PI 3-K) and extracellular signal-regulated kinase (ERK)1/2 along with bcl-xL and bfl-1/A1 upregulation. These results suggest that high glucose suppresses apoptosis via upregulation of bcl-xL and bfl-1/A1 levels through PI 3-K and ERK1/2 pathways in CASM. High glucose-induced increase in the expression of antiapoptotic proteins may be important in the development of atherosclerosis in diabetic patients.

diabetes mellitus; atherosclerosis; apoptosis; vascular smooth muscle cells

VASCULAR DISEASE is one of the most serious complications in diabetic patients. It is widely accepted that atherosclerosis is accelerated by the coexistence of diabetes mellitus (11, 31). Hyperglycemia is an important etiologic factor in the development of vascular complications (47).

Increased proliferation of vascular smooth muscle cells (VSMC) is a key feature in the atherosclerotic lesion (15, 35, 38, 41). It is well established that cell growth is a fundamental feature of intimal hyperplasia (34), and it is becoming clear that perturbations in the regulation of apoptosis are equally important (16, 24). Furthermore, apoptosis of VSMC is critically involved in the formation of the fibrous cap and fatty streak that is the lipid-rich core of the atheroma and may therefore contribute to the instability of advanced atherosclerotic plaques (3, 7, 12, 14, 21, 23). Excessive accumulation of VSMC in atherosclerosis suggests reduced apoptosis and excessive cell proliferation in the lesions, because apoptosis and cell proliferation are intimately coupled (10). Although many studies have focused on the mechanisms of VSMC proliferation (34), the regulatory mechanisms of VSMC apoptosis have not been fully elucidated (30).

The role of sugar in atherosclerosis has been investigated in recent studies. Several findings support the concept that hyperglycemia accelerates the development of atherosclerosis (46, 47). Although glucose concentration enhances growth rate in cultured VSMC (17, 33, 49), little is known about the effect of glucose on the regulation of apoptosis in VSMC. Apoptosis is regulated by a genetic program involving the activation and inactivation of specific genes. In particular, dominant protooncogenes such as bcl-2 and tumor suppressor genes such as p53 are potent regulators of apoptosis. Members of the bcl-2 protein family regulate the response of cells to a wide variety of apoptotic signals. At least 15 bcl-2 family members have been identified, and apoptosis is determined by the relative balance of proapoptotic and antiapoptotic members of the bcl-2 protein family (40). bcl-2 proteins were expressed in VSMC (8, 20, 29, 36, 39), and expression of several family members such as bcl-2, bcl-xL, bcl-xS, and bax were modified by apoptosis induction. For example, platelet-derived growth factor (PDGF) reduces bcl-xL and induces bcl-xs (36) and balloon injury induces bcl-xs (20). Recent reports demonstrated that p53 (3), phosphatidylinositol 3-kinase (PI 3-K; Ref. 1), and mitogen-activated protein kinase (MAPK; Refs. 19, 48) are also involved in apoptosis regulation of VSMC.

MAPKs, which constitute an ubiquitous group of serine/threonine kinases, are thought to play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis. However, the effect of glucose on the signal transduction

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pathway that regulates the apoptosis regulatory genes, MAPKs, and PI 3-K remains to be further elucidated. We have shown that acute extracellular high glucose concentration conditions stimulate cell proliferation through alteration of cell cycle regulation (49). In the present study, we investigated acute effects of extracellular high glucose concentration on apoptosis, expression of bcl-2 family proteins, and activation of PI 3-K and MAPKs.

METHODS

Chemicals. Smooth muscle basal medium (SMBM) and smooth muscle growth medium-2 (SMGM-2) Bullet Kits were purchased from BioWhittaker (Walkersville, MD). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Gibco-BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). The annexin V-FITC kit was purchased from Bender Med Systems (Vienna, Austria) for flow cytometry analysis. All other chemicals used in the present study were of reagent grade.

Cells and cell culture. Primary cultured human coronary artery smooth muscle cells (CASMC) were purchased from BioWhittaker. The cells were grown to confluence in SMGM-2 containing 5.5 mM glucose, 5% FBS, 50 ng/ml gentamicin, 50 ng/ml amphotericin-B, 5 ug/ml insulin, 2 ng/ml human recombinant fibroblast growth factor (hFGF), and 0.5 ng/ml human recombinant epidermal growth factor (hEGF) at 37°C in a humidified 5% CO₂-95% air atmosphere according to the company's recommendations. Cells were grown to ~80% confluence and used within five passages.

DNA fragmentation assay. Cells in secondary cultures maintained in SMGM-2 containing 5% FBS were trypsinized and seeded on six-well plates. When cells reached 80% confluence, culture medium was replaced with medium containing 5% FBS and 5.5 mM glucose (normal), 25 mM glucose (high glucose), or 5.5 mM glucose plus 19.5 mM mannitol (osmotic control corresponding to high-glucose medium) for 72 h. Glucose concentration of culture medium was switched to SMBM with normal glucose concentration (5.5 mM), high glucose concentration (25 mM), or osmotic control (5.5 mM glucose + 19.5 mM mannitol) for 24 h. Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 145 mM NaCl, 10% (w/v) glycerol, 5 mM EDTA, 0.5% NP-40 (nonylphenoxy polyethoxyethanol), 0.2 mM Na₂VO₄, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 10 µg/ml aprotinin. The suspension was sonicated for 5 s and centrifuged at 110,000 g for 5 min, and the resultant supernatant was used as the whole cell lysate fraction. Protein concentrations were determined by the method of Bradford (4), with bovine serum albumin (BSA) as a standard, using a protein assay kit (Bio-Rad). All procedures were performed at 4°C. The indicated amounts of the proteins were dissolved in Laemmli's sample buffer containing 1% sodium dodecyl sulfate (SDS) (26). The proteins (25 µg/lane) were subjected to SDS-polyacrylamide resolving gel electrophoresis (PAGE) on 10% (wt/vol) polyacrylamide resolving gels in a Rapidas mini slab (Atto) and electrochemically transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Life Science, Arlington Heights, IL). Membranes were equilibrated in Tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.4) and 500 mM NaCl), and nonspecific binding sites were blocked by 5% dried milk in TBS containing 0.05% (wt/vol) Tween-20 (TBS-T) at room temperature for 1 h. After being washed with TBS-T, membranes were incubated with TBS-T containing the antibodies to bcl-xL, bcl-2, bax, PI 3-K, phosphospecific PI 3-K, ERK, and phosphospecific ERK (Santa Cruz Biotechnology), Santa Cruz, CA) as primary antibody at room temperature for 1 h. The blots were developed with the enhanced chemiluminescence (ECL) system (Amersham Life Science) and visualized by exposure to Hyperfilm-ECL (Amersham Life Science). Specificity of protein bands was examined by blocking with antigens. Bands were quantitated with Imagequant software (National Institutes of Health, Bethesda, MD).

Reverse transcription-polymerase chain reaction. Cells in secondary cultures maintained in SMGM-2 containing 5% FBS were trypsinized and seeded on 100-mm plates at ~1 × 10⁷ cells/well in 10 ml of the same medium. When cells reached 80% confluence, the medium was switched to SMBM containing normal glucose concentration (5.5 mM), high glucose concentration (25 mM), or osmotic control (5.5 mM glucose + 19.5 mM mannitol) for 72 h. Apoptosis was determined with a Becton-Dickinson fluorescence-activated cell analyzer (Becton-Dickinson, San Jose, CA) and the annexin V-FITC kit according to the company's recommended procedure. Cells were washed with ice-cold DPBS twice and resuspended in 195 µl of binding buffer. Five micrograms of FITC-labeled purified recombinant annexin V was then added, gently vortexed, and incubated for 15 min at room temperature. Cells were washed with 1 ml of binding buffer. Dilute propidium iodide (PI) in 200 µl of binding buffer was added to the cell pellet. Four hundred microliters of binding buffer were added to each tube and analyzed with the Becton-Dickinson fluorescence-activated cell analyzer within 5 min.

Western blot analysis. Cells in secondary cultures maintained in SMGM-2 containing 5% FBS were trypsinized and seeded on 100-mm plates at a density of 1 × 10⁶ cells/well in 10 ml of the same medium. When cells reached 80% confluence, the medium was switched to SMBM with normal glucose concentration (5.5 mM), high glucose concentration (25 mM), or osmotic control (5.5 mM glucose + 19.5 mM mannitol) for 24 h. Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 145 mM NaCl, 10% (w/v) glycerol, 5 mM EDTA, 0.5% NP-40 (nonylphenoxy polyethoxyethanol), 0.2 mM Na₂VO₄, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 10 µg/ml aprotinin. The suspension was sonicated for 5 s and centrifuged at 110,000 g for 5 min, and the resultant supernatant was used as the whole cell lysate fraction. Protein concentrations were determined by the method of Bradford (4), with bovine serum albumin (BSA) as a standard, using a protein assay kit (Bio-Rad). All procedures were performed at 4°C. The indicated amounts of the proteins were dissolved in Laemmli's sample buffer containing 1% sodium dodecyl sulfate (SDS) (26). The proteins (25 µg/lane) were subjected to SDS-polyacrylamide resolving gel electrophoresis (PAGE) on 10% (wt/vol) polyacrylamide resolving gels in a Rapidas mini slab (Atto) and electrochemically transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Life Science, Arlington Heights, IL). Membranes were equilibrated in Tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.4) and 500 mM NaCl), and nonspecific binding sites were blocked by 5% dried milk in TBS containing 0.05% (wt/vol) Tween-20 (TBS-T) at room temperature for 1 h. After being washed with TBS-T, membranes were incubated with TBS-T containing the antibodies to bcl-xL, bcl-2, bfl-1/A1, bax, PI 3-K, phosphospecific PI 3-K, ERK, and phosphospecific ERK (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibody at room temperature for 1 h. After being washed with TBS-T, the membrane was incubated in TBS-T containing a horseradish peroxidase-linked IgG (Santa Cruz Biotechnology) at room temperature for 1 h. The blots were developed with the enhanced chemiluminescence (ECL) system (Amersham Life Science) and visualized by exposure to Hyperfilm-ECL (Amersham Life Science). Specificity of protein bands was examined by blocking with antigens. Bands were quantitated with Imagequant software (National Institutes of Health, Bethesda, MD).
glucose concentration (72-h exposure; 23.9 ± 6.7%). However, osmotic control medium containing 5.5 mM glucose and 19.5 mM mannitol (57.9 ± 6.0%) had no significant effect on DNA fragmentation, whereas osmotic control (5.5 mM glucose); 8.0%) had no significant effect on DNA fragmentation ratio (5.5 mM glucose 23.9 ± 2.4%; 25 mM glucose 16.5 ± 1.8%). However, osmotic control medium containing 5.5 mM glucose and 19.5 mM mannitol (21.0 ± 3.0%) had no significant effect on DNA fragmentation ratio. **P < 0.01 vs. control (normal glucose, 5.5 mM); n = 3.

**Effect of high glucose on expression of bcl-2 family members:** upregulation of bcl-xL and bfl-1/A1 followed by high glucose exposure in CASMC. To determine whether upregulation of bcl-2 family protein contributed to inhibition of human CASMC apoptosis, we used Western blot and reverse transcription (RT)-PCR. Western blot demonstrated that high glucose upregulates bcl-xL and bfl-1/A1 but not bax. However, bcl-2 was not detected in CASMC (Fig. 3). Treatment of human CASMC with a high glucose concentration for 24 h markedly increased mRNA expression of bcl-xL and bfl-1/A1 compared with treatment with normal glucose (Fig. 4). However, bfl-1/A1 and bcl-xL mRNA levels were not altered by mannitol treatment.

**Effect of high glucose on phosphorylation of ERK1/2 and PI 3-K: activation of ERK1/2 and PI 3-K followed by high-glucose exposure in CASMC.** Western blot analysis demonstrated that high glucose (25 mM) 16.5 ± 1.8%, P < 0.01). Mannitol, an osmotic control (20.8 ± 1.78%), had no significant effect on DNA fragmentation in CASMC (Fig. 1).

DNA fragmentation, a process that results from the activation of endonucleases, is one of the later steps during the apoptotic program. By contrast, flow cytometric analysis with FITC-conjugated annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Because annexin V is a Ca2⁺-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS), it binds to cells with exposed PS. In apoptotic cells, the membrane PS is translocated from the inner to the outer leaflet of the plasma membrane, which is one of the earliest apoptotic morphological features. Flow cytometric analysis data are shown in Fig. 2. An exposure to high glucose concentration suppressed apoptosis in a time-dependent manner relative to normal glucose treatment (72-h exposure; 55.4 ± 6.0% vs. 39.9 ± 6.7%, P < 0.01).

![Fig. 1. Effect of high glucose concentration on the apoptosis measured by DNA fragmentation ratio in human coronary artery smooth muscle cells (CASMC). Cells were plated in 6-well plates in smooth muscle growth medium (SMGM) containing 3% fetal bovine serum (FBS) with normal glucose (5.5 mM), high glucose (25 mM), or osmotic control (5.5 mM glucose + 19.5 mM mannitol). After 72 h, the DNA fragmentation assay was performed according to the method of Sellins and Cohen (43) as described in METHODS. Data are shown as mean ± SD % DNA fragmentation ratio of the fragmented DNA to the total DNA for triplicate determinations repeated in 3 separate experiments. High glucose concentration decreased the DNA fragmentation ratio (5.5 mM glucose 23.9 ± 2.4%; 25 mM glucose 16.5 ± 1.8%). However, osmotic control medium containing 5.5 mM glucose and 19.5 mM mannitol (21.0 ± 3.0%) had no significant effect on DNA fragmentation ratio. **P < 0.01 vs. control (normal glucose, 5.5 mM); n = 3.](http://www.ajpcell.org)

![Fig. 2. Effect of high glucose concentration on apoptosis expression determined by flow cytometry analysis in human CASMC. Cells were plated in 6-well plates with normal glucose (5.5 mM), high glucose (25 mM), or osmotic control (5.5 mM glucose + 19.5 mM mannitol). After 72 h, apoptosis expression was measured by flow cytometry analysis as described in METHODS. Data are shown as mean ± SD % of the number of observations. The statistical significance was assessed by ANOVA. A P value <0.05 was considered statistically significant. Significance was determined after three or more separate experiments](http://www.ajpcell.org)
treatment for 24 h increased phosphorylation status of ERK1/2 and PI 3-K (Fig. 5). However, the osmotic control (19.5 mM mannitol/5.5 mM glucose) did not increase the phosphorylation status of ERK1/2 and PI 3-K.

**DISCUSSION**

The present study provides evidence that expression of apoptosis in human CASMC is significantly suppressed by high glucose exposure. Apoptosis, or pro-
grammed cell death, consists of a distinct form of cell
death that displays characteristic alterations in cell
morphology and cell fate. From the standpoint of tissue
structural remodeling, apoptosis might be considered
to be one of the most important mechanisms that
counterbalance the effect of cell proliferation by mitotic
division (24). Because normal VSMC possess the ma-
achinery to undergo apoptosis, apoptosis may be a
means of cell number regulation in the vessel wall.
Vascular lesions, such as atherosclerosis, arise in part
from excessive accumulation of VSMC, which may sug-
gest reduced apoptosis in the lesions. Although the
processes of vessel remodeling and cell death appear to
be opposing, apoptosis and cell proliferation are inti-
ately coupled (10). For example, Diéz et al. (8) docu-
mented that VSMC of spontaneously hypertensive ani-
imals show an increased expression of bcl-2 that acts as
antiapoptotic protein. In hypertension, VSMC replica-
tion is increased but it is not counterbalanced by in-
creased apoptosis, resulting in thickening of the media
of arteries and arterioles. This indicates that the fac-
tors responsible for increased DNA synthesis also ac-
count for the relative resistance against apoptosis.
Yamamoto et al. reported (49) that acute high glucose
exposure increases VSMC proliferation through cell
cycle progression, which may contribute to abnormal
wall thickening in the hyperglycemic condition. How-
ever, it is not clear whether the counterbalance of
VSMC proliferation and apoptosis is altered by high
glucose at the early stage of atherosclerosis, when
abnormal VSMC proliferation is accelerated by high
glucose.

The significance and roles of apoptosis in atheroscle-
rosis may depend on the stage of the plaque, its local-
ization, and the cell types involved. Apoptosis has been
demonstrated to occur with increased frequency in
human atherosclerotic plaques and may contribute to
plaque rupture and thrombosis (3, 12, 14, 21). How-
ever, the frequency of apoptosis in the early stage of
atherosclerosis and the thickening of arterial wall with
accelerated VSMC proliferation are mostly unknown.
We hypothesize that the extent of apoptosis at early-
stage (committed phase) atherosclerosis might be low
compared with advanced-stage (executive phase) ath-
erosclerosis, because apoptosis expression in human
atherosclerotic plaques was much different from apop-
tosis in adjacent nonatherosclerotic intima (23). In
cultured endothelial cells (EC), high glucose concen-
tration was found to trigger apoptosis (2). Although dif-
ferent mechanisms protect EC and VSMC against apop-
tosis in the normal artery, whether VSMC apoptosis is
altered by high glucose is not fully elucidated. There-
fore, in this report we investigated the acute effect of
high glucose concentration on apoptosis regulation in
human CASMC. High-glucose-induced suppression of
CASMC apoptosis might inversely link with high-glu-
cose-induced cell proliferation and result in the abnor-
mal balance between cell replication and cell death to
accelerate cardiovascular disease in diabetes after hy-
perglycemia-induced EC apoptosis. Glucose-induced
alteration of VSMC apoptosis should be important as
an initial event of vascular injury and thickening of the
media of arteries and arterioles as demonstrated in the
abnormal VSMC growth in hypertension (8).

Another finding of the present study is that high-
glucose treatment stimulates expression of bcl-xL and
bfl-1/A1, members of the bcl-2 gene family, in human
CASMC. It is now well established that members of
the bcl-2 family are critical regulators of apoptosis in a
variety of cell types (40). Although the expression pat-
tern and role of different members of the family appear
to be cell specific, precise regulation of the family
members in VSMC has not been fully elucidated. In rat
VSMC, alterations in bcl-2 expression are accompanied
by apoptosis induction (29). When VSMC apoptosis
was induced by PDGF (36), bcl-xL expression was
reduced and bcl-xs expression was increased, suggest-
ing the antiapoptotic role of bcl-xL in VSMC. Pollman
et al. (39) demonstrated that vascular lesion formation
is associated with an upregulation of antiapoptotic
gene bcl-xL within intimal VSMC in animal models
and human specimens and that downregulation of
bcl-xL with antisense oligonucleotides induces VSMC
apoptosis and regression of vascular lesions. Igase et al. (20)
demonstrated that bcl-xL was not detected in rat carotid
artery and balloon injury-induced bcl-xs mRNA expres-
sion. Although they concluded that the selective induction
of bcl-xs expression is a key regulator of rat VSMC apoptosis, the
specific function of bcl-2 family members may be different in
each cell species. Our data indicated that high glucose
concentration led to specific induction of bcl-xL in hu-
man CASMC, and glucose-induced alteration of bcl-xL is
a key step for glucose-induced apoptosis suppression.
On the other hand, the mechanism and the role of
bfl-1/A1 expression in VSMC are unknown. bfl-1/A1 is
expressed in the bone marrow and at a low level in
some other tissues, and the expression level of bfl-1/A1
gene and the development of stomach cancer were
correlated (6), suggesting the involvement of bfl-1/A1 in
the regulation of cell survival. In epithelial cells,
bfl-1/A1 suppresses p53-induced apoptosis (9) and may
play an important role in cell survival of lymphocytes
(28). In our observations, high glucose also stimulates
phosphorylation of PI 3-K and ERK1/2 in CASMC. The
signaling pathways involved in the antiapoptotic effect
of growth factor with intrinsic tyrosine kinase activi-
ties are known, such as activation of PI 3-K and ERK1/2
by insulin-like growth factor-1 in PC12 cells (37). Because
the PI 3-K and ERK1/2 pathways converge at some point
before activation of MAPK kinase, a MAPK activator
(22), the mechanisms activated by exposure to high glu-
cose levels remain unclear.

Our observations indicate that apoptosis in human
CASMC may be suppressed by high glucose via alter-
ations of high glucose-induced bcl-xL and bfl-1/A1 expres-
sion. Recently, it was reported that nuclear factor
(NF)-κB is a downstream target of the MAPK cascade
(42) and that high glucose increased NF-κB activity
and modified tumor necrosis factor (TNF)-α-induced
transcriptional factor NF-κB activation in porcine
VSMC (50). Glucose-derived advanced glycation end-
products (AGE) have been shown to accumulate in diabetic tissues and have an effect of acceleration of atherosclerosis induced by glucose toxicity (44). Lander et al. (27) demonstrated that AGE activates NF-κB in rat VSMC. Recently, it was demonstrated that bfl-1/A1 is a direct transcriptional target of NF-κB in B lymphocytes (51) and that NF-κB induces bfl-1/A1 expression and suppress apoptosis (45). It is known that the high glucose effect activates NF-κB, which induces its direct transcriptional target, bfl-1/A1, and results in apoptosis suppression induced by alteration of bfl-1/A1 function in apoptosis regulation in human CASMC. On the other hand, several lines of evidence have implicated increased protein kinase C (PKC) activity initiated by hyperglycemia as a key player in the pathologi- cal effects of high glucose in diabetic cardiovascular tissues (25). Recently, Hall et al. (13) demonstrated that hyperglycemia inhibits VSMC apoptosis through a PKC pathway. PKC mediates high-glucose-induced inhibition of synthesis of nitric oxide (NO) (32), which induces apoptosis in VSMC (19), and hence glucose-induced PKC elevation might inhibit apopto- sis through suppression of NO production. Because bcl-2 regulates an antioxidant pathway for preventing apoptosis (18), it is acceptable that glucose-modified NO production may alter bcl-2 family functions, including bcl-xL and bfl-1/A1, enough to alter apoptosis expression in human CASMC. These signal pathways involving PKC, AGE, NF-κB, or NO synthesis may act in both cell proliferation and apoptosis. Further precise mechanisms in regulation of apoptosis and cell prolifi-eration modified by high glucose, however, should be elucidated.

In conclusion, these results showed that high glucose concentration mediates an increase of abnormal cell proliferation, and this may be regulated by suppression of apoptosis related to upregulation of bcl-xL and bfl-1/A1 expression through PI 3-K and MAPK pathways in human CASMC. This suggests that glucose-modified expression of antapoptotic members of the bcl-2 family proteins may play an important role in the development of atherosclerotic lesion leading to cardiovascular disease in diabetic patients. Identification of mechanisms that regulate high glucose concentra- tion-induced apoptosis inhibition might provide a new target for further therapeutic intervention to pre- vent vascular complications in diabetic patients.

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