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(−)-Epigallocatechin Gallate Suppresses Proliferation of Vascular Smooth Muscle Cells Induced by High Glucose by Inhibition of PKC and ERK1/2 Signalings

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ABSTRACT: Proliferation of vascular smooth muscle cells (VSMCs) plays an important role in the development and progression of diabetes-related vascular complications. (−)-Epigallocatechin gallate (EGCG), the major catechin derived from green tea, is able to exert antidiabetes effects in animal models. However, it is not known whether or not EGCG inhibits VSMC proliferation induced by high glucose. This study tested the hypothesis that EGCG might have an inhibitory effect on VSMC proliferation induced by high glucose. VSMC proliferation was determined by [^{3}H]-thymidine incorporation and uptake of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT). Extracellular signal-regulated kinase (ERK) 1/2 phosphorylation was determined by immunoblotting, and ERK 1/2 activity was detected by measuring the ability to phosphorylate its substrate Elk-1. Glucose increased VSMC proliferation in a concentration-dependent manner, which was reduced in the presence of EGCG. VSMC proliferation mediated by high glucose (30 mM) was involved in protein kinase C (PKC) and ERK1/2 signalings, because its effect was blocked by PKC inhibitor (PKC inhibitor 19−31) and ERK1/2 inhibitor (PD98059). Pretreatment of VSMCs with EGCG significantly inhibited the stimulatory effect of high glucose on PKC and ERK1/2 activation, followed by attenuation of its downstream transcription factor Elk-1 phosphorylation. Taken together, these results suggest that EGCG could suppress VSMC proliferation induced by high glucose by inhibition of PKC and ERK1/2 signalings in VSMCs, which indicates that EGCG might be a possible medicine to reduce vascular complications in diabetes.

KEYWORDS: EGCG, high glucose, proliferation, vascular smooth muscle cells

INTRODUCTION

The prevalence of diabetes has increased dramatically worldwide. Diabetes is associated with increased incidence of atherosclerotic disease, which is the major cause of morbidity and mortality in people with diabetes. Hyperglycemia is a key clinical manifestation in diabetic patients and is believed to be one of the major causes of diabetes-associated vascular complications such as atherosclerosis and restenosis.[1,2] Vascular smooth muscle cells (VSMCs) are a major constituent of blood vessel walls responsible for the maintenance of vascular tone. It is recognized that the proliferation of VSMCs is a key event in the formation of atherosclerotic lesions. High glucose increases the proliferation of VSMCs;[3,4] accelerated VSMC proliferation and accumulation in atherosclerotic lesions has also been observed in diabetic animal models,[5,6] suggesting that abnormal VSMC proliferation plays an important role in the progression of diabetes-associated vascular complications.

Numerous studies have focused on natural products derived from plant resources, which display potent therapeutic effect and have been extensively utilized in the treatment of vascular disorders such as atherosclerosis. Teas, produced from the leaves of the plant *Camellia sinensis*, are widely consumed beverages throughout the world. Among the consumed teas, green tea is the best studied for health benefits. Green tea contains many biologically active polyphenolic flavonoid, commonly known as tea polyphenols.[7] The major components of tea polyphenols are the catechins, a family that includes (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-gallate, and (−)- epigallocatechin-3-gallate (EGCG). As the principal constituent, EGCG is a major bioactive polyphenol present in green tea and has received attention as a prospective dietary intervention in cardiovascular diseases.[8,9]

In recent years, studies have paid more attention to the benefits of EGCG in diabetes and its complications. EGCG, as a natural dietary supplement with a potent antidiabetes property, delays the onset of type 1 diabetes in spontaneous nonobese diabetic mice, reduces the increase of blood glucose levels and ameliorates the decrease of islet mass induced by low doses of streptozotocin.[10,11] Additionally, experimental studies have also confirmed that EGCG alleviates the development of diabetic nephropathy and attenuates insulin signaling and expressions of pro-inflammatory cytokines induced by high glucose.[12−14] To determine if EGCG has an inhibitory effect on VSMC proliferation induced by high glucose, we studied the effect of EGCG or vehicle on VSMC proliferation in a high-glucose medium. EGCG reduced proliferation of A10 cells induced by high glucose, via inhibition of PKC, ERK1/2, and nuclear transcription factor Elk-1. Our results put forward the possibility that EGCG is a potential natural inhibitor of abnormal proliferation of VSMCs under high-glucose circumstances.

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MATERIALS AND METHODS

Cell Culture. Embryonic thoracic aortic smooth muscle cells[15,16] (passage 10−20) from normotensive Berlin-Druckrey IX (A10; CRL 1476, American Type Culture Collection, Manassas, VA) were cultured at 37 °C in a 95% air/5% CO_2 atmosphere in Dulbecco's modified eagle medium (DMEM). The confluent VSMCs were incubated for 24 h in serum-free DMEM and then were exposed to normal glucose (5.5 mM) or high glucose (30 mM) for the indicated times; VSMCs were pretreated with EGCG for 30 min prior to the treatment of glucose.

[^{3}H]-Thymidine Incorporation. Cell proliferation was determined by measuring the incorporation of [^{3}H]-thymidine into DNA of cells cultured in 24-well plates.[17,18] After induction of quiescence, the cells were stimulated with high glucose (30 mM) or normal glucose concentration (5.5 mM) in the presence or absence of EGCG for the indicated times. Thereafter, [^{3}H]-thymidine (1 μCi/mL) was added to the growth medium of each well 6 h prior to the measurements. At the end of incubation, the medium was removed and the cells were treated with 0.25 mL of 0.05% trypsin−0.53 mmol/L EDTA for 5 min and diluted to 10 mL with a balanced electrolyte solution. The cells were then treated with 10% trichloroacetic acid to precipitate acid-insoluble materials from which the DNA was extracted with 0.1 N NaOH. The DNA was collected on a Whatman GF/B filter and washed twice with 5 mL of ice-cold PBS. The filter was then cut and shaken in 3.5 mL of scintillation fluid for 24 h before counting in a liquid scintillation counter (Beckman LS6500). Data are presented as [^{3}H]-thymidine uptake per microgram of protein. The protein refers to the total protein of the cell sample.

MTT Assay. The number of viable cells in each well was also estimated by the uptake of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT). MTT, selectively taken up by mitochondria, is converted to a dark-blue product by living, but not by dead, cells. After the induction of quiescence in 96-well plastic culture dishes at the density of 1×10^3 cells/well, the cells were incubated with the indicated drugs for 24 h. Subsequently, 20 μL of MTT (2.5 g/L) was added to each well, and the incubation was continued for an additional 4 h at 37 °C. Thereafter, 150 μL of DMSO was added to each well, and an absorbance at 490 nm was read on a microplate reader (model 680, Bio-Rad).[19,20]

Immunoblotting. A10 cells were treated with vehicle (dH_2O), high glucose, and/or EGCG at the indicated concentrations and times. The cells were lysed in a lysis buffer, sonicated, placed on ice for 1 h, and centrifuged at 16000*g* for 30 min. The supernatants were stored at −70 °C until use. After the protein concentrations had been measured, the supernatants were mixed with Laemmli sample buffer, boiled for 5 min, subjected to electrophoresis, and then transferred electrophoretically onto nitrocellulose membranes. The transblots were blocked overnight with 5% nonfat dry milk in PBS-T buffer (0.05% Tween 20 in 10 mM phosphate-buffered saline) at 4 °C with constant shaking and then incubated with the ERK1/2 (1:1000) or phosphorylated ERK1/2 antibody (1:1000) in 5% nonfat dry milk in PBS-T buffer for 1 h at room temperature. The primary antibody binding was then probed by a peroxidase-labeled goat anti-rabbit IgG antiserum. The signal was detected using chemiluminescence and developed on X-ray film. The density of the bands was quantified by densitometry using Quantiscan, as reported previously.[21,22]

ERK1/2 Activity Assay. To evaluate ERK1/2 activity, we measured the ability of ERK1/2 to phosphorylate its substrate Elk-1 by using a commercially available kit (ERK1/2 Assay Kit; Cell Signaling Technology, Beverly, MA).[23] Briefly, quiescent VSMCs were preincubated for 30 min with various concentrations of EGCG and then stimulated with high glucose (30 mM) for 10 min. Cells were lysed in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 7.4; 2 mmol/L EDTA, pH 8.0; 2 mmol/L EGTA; 100 mmol/L NaCl; 10 μg/mL leupeptin; 10 μg/mL aprotinin; 2 mmol/L phenylmethanesulfonyl fluoride; 1% NP-40). Cell lysates were immunoprecipitated with immobilized phospho-ERK1/2 (Thr202/Tyr204) monoclonal antibody, and then an in vitro kinase assay was performed using Elk-1 protein as substrate peptide. Phosphorylated Elk-1 was then detected by immunoblotting using phospho-Elk-1 (Ser383) antibody.

Materials. Antibodies (ERK1/2 and phospho-ERK1/2) were rabbit anti-rat antibodies and purchased from Cell Signaling Technology. EGCG, MTT, PKC activator phorbol 12-myristate13-acetate (PMA), ERK1/2 inhibitor PD 98059, and PKC inhibitor peptide 19−31 were from Sigma (St. Louis, MO). [^{3}H]-thymidine was from Atomic Energy Research Establishment of China (Beijing, China). All other chemicals for various buffers were of the highest purity available and purchased from Sigma or Gibco (Gibco, Grand Island, NY).

Statistical Analysis. The data are expressed as the mean ± SEM. Comparison within groups was made by ANOVA for repeated measures, and comparison among groups was made by ANOVA with Duncan's test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Effect of High Glucose on the Proliferation of VSMCs. To determine the effect of high glucose on the proliferation of VSMCs, A10 cells were treated with glucose at various concentrations (15, 30, and 50 mM) for 24 h. Glucose increased proliferation of VSMCs in a concentration-dependent manner (Figure 1A). To exclude the possible role of osmolarity in the proliferation of VSMCs mediated by glucose, we treated the VSMCs with D-mannitol and L-glucose (30 mM); neither D-mannitol nor L-glucose had any effect on VSMC proliferation (Figure 1B).

Inhibitory Effect of EGCG on Proliferation of VSMCs Mediated by High Glucose. To investigate whether EGCG inhibits the proliferation of VSMCs induced by high glucose, A10 cells were incubated with high glucose (30 mM) and/or EGCG for 24 h. EGCG had a tendency to reduce proliferation of VSMCs. The significance was found in 30 and 50 μM concentration (Figure 2A). Although EGCG at 10 μM concentration had no effect on VSMC proliferation, it significantly reduced VSMC proliferation induced by high glucose, and the inhibitory effect of EGCG on VSMC proliferation mediated by high glucose was in a concentration-dependent manner (Figure 2B). As compared with the inhibitory effect in Figure 2A, EGCG at 30 and 50 μM concentration further decreased VSMC proliferation induced by high glucose (Figure 2B). The inhibitory effect of EGCG, determined by [^{3}H]-thymidine incorporation, was confirmed by MTT method (Figure 2C).

Role of PKC on the Effect of EGCG on VSMC Proliferation Induced by High Glucose. The role of PKC on the effect of high glucose-induced VSMC proliferation was examined, and it was found that PKC inhibitor peptide 19−31 (10^{-6} M), by itself, had no effect, but inhibited the stimulatory effect of high glucose (30 mM) on the proliferation of VSMCs (Figure 3A). To further confirm the stimulatory effect of PKC on VSMC proliferation, A10 cells were treated with PKC activator, PMA. PMA increased VSMC proliferation in a concentration-dependent manner (10^{-8}−10^{-6} M) (Figure 3B). As with the inhibitory effect of EGCG on VSMC proliferation induced by high glucose, EGCG also inhibited proliferation of VSMCs induced by PMA (Figure 3C).

Role of ERK1/2 on the Effect of EGCG on VSMC Proliferation Induced by High Glucose. The role of ERK1/2 on the

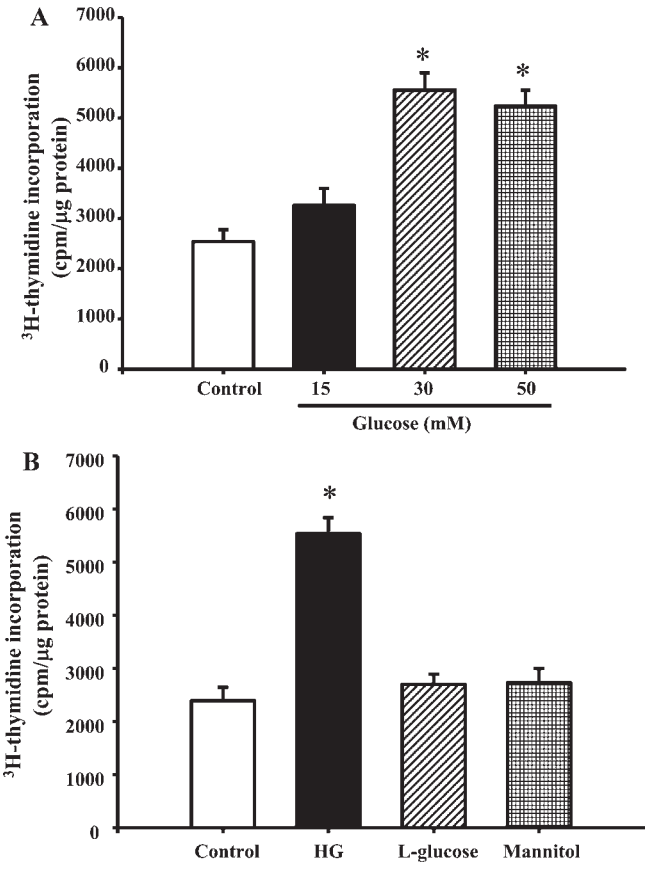

Figure 1. Effect of high glucose, D-mannitol, or L-glucose on proliferation of VSMCs. (A) Effect of high glucose on proliferation of VSMCs. A10 cells were treated with high glucose (15−50 mM) for 24 h, and VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 5; ∗,P < 0.05 vs control, ANOVA, Duncan's test). (B) Effect of high glucose, D-mannitol, or L-glucose on proliferation of VSMCs. A10 cells were treated with high glucose (30 mM), D-mannitol (30 mM), or L-glucose (30 mM) for 24 h, and VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 5; ∗, P < 0.05 vs control, ANOVA, Duncan's test).

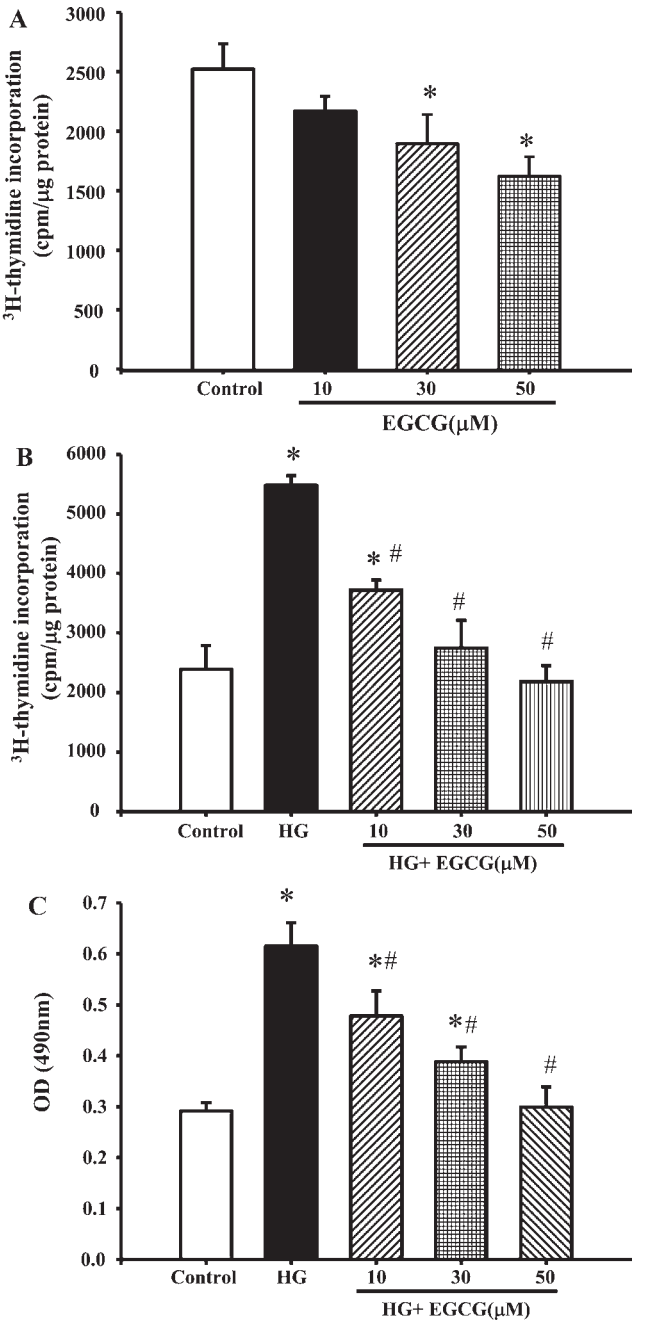

Figure 2. Effect of EGCG on proliferation of VSMCs induced by high glucose. (A) Effect of EGCG on the proliferation of VSMCs. A10 cells were treated with EGCG (10−50 μM) for 24 h. VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 8; ∗, P < 0.05 vs control, ANOVA, Duncan's test). (B_ Effect of EGCG on proliferation of VSMCs induced by high glucose. A10 cells were treated with high glucose (HG, 30 mM) with or without EGCG (10−50 μM) treatment for 24 h. VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 4; ∗, P < 0.05 vs control; #, P < 0.05 vs high glucose alone, ANOVA, Duncan's test). (C) Effect of EGCG on proliferation of VSMCs induced by high glucose. A10 cells were treated with high glucose (HG, 30 mM) with or without EGCG (10−50 μM) treatment for 24 h. Results are expressed as MTT optical density (n = 6; ∗, P < 0.01 vs control; #, P < 0.05 vs high glucose alone, ANOVA, Duncan's test).

effect of VSMC proliferation induced by high glucose was examined; it was found that PD98059 (10^{-5} M), an ERK1/2 inhibitor, by itself had no effect, but inhibited the stimulatory effect of high glucose (30 mM) on the proliferation of VSMCs (Figure 4A). High glucose increased ERK1/2 phosphorylation, which was evident at 5 min, reached peak value at 10 min, and recovered to normal level at 40 min (Figure 4B). EGCG reduced the stimulatory effect of high glucose (30 mM/10 min) on ERK1/2 phosphorylation in a concentration-dependent manner (10−50 μM) (Figure 4C).

Activated ERK1/2 has been shown to phosphorylate cytosolic proteins or translocate those proteins to the nucleus to phosphorylate transcription factors and thereby activate gene expression of cell-related proteins and regulate cell growth and differentiation.[24] Transcription factor Elk-1 phosphorylation induced by high glucose was also tested by immunoblotting. The level of Elk-1 phosphorylation was increased by high glucose (30 mM) stimulation for 10 min. However, in the presence of EGCG, the stimulatory effect of high glucose on phosphoryaltion of Elk-1 was reduced (Figure 4D).

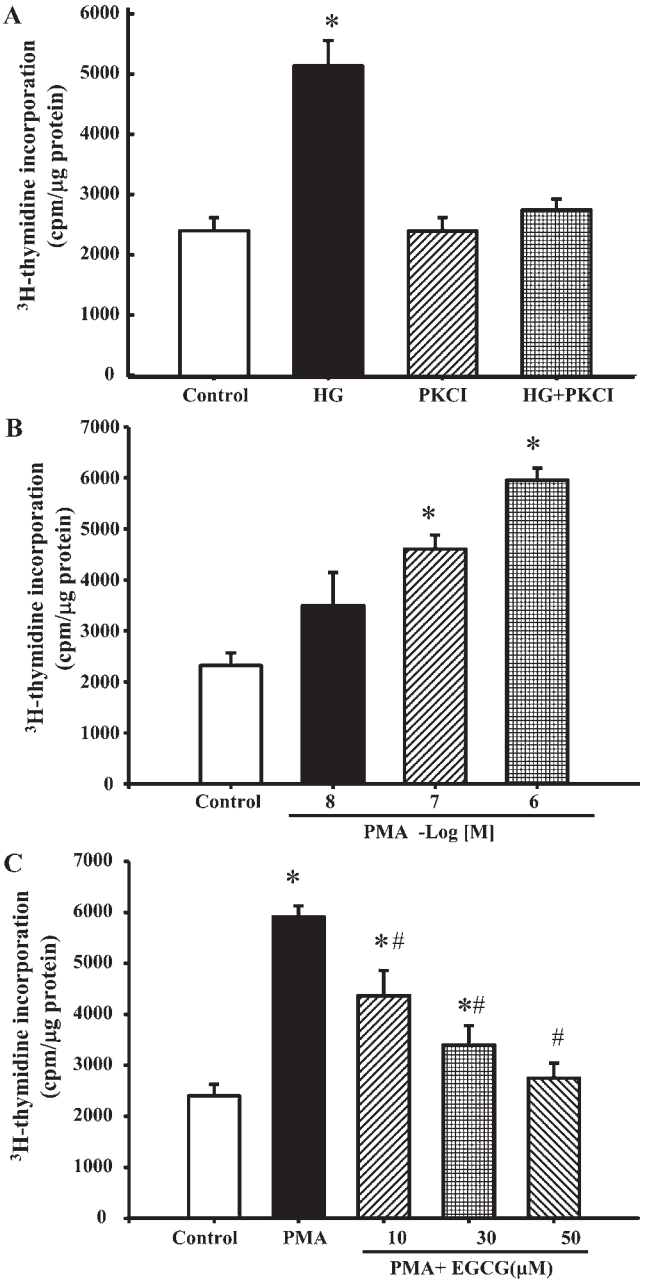

Figure 3. Role of PKC on the inhibitory effect of EGCG on proliferation of VSMCs induced by high glucose. (A) Role of PKC on the effect of VSMC proliferation induced by high-glucose. A10 cells were treated with high glucose (HG, 30 mM) for 24 h in the presence of PKC inhibitor (PKC inhibitor peptide 19−31; 10^{-6} M, PKCI). VSMC proliferation was determined by [^{3}H]-thymidine incorporation, and results are expressed as cpm/μg protein (n = 4; *, P < 0.05 vs others, ANOVA, Duncan's test). (B) Effect of PKC on the proliferation of VSMCs. A10 cells were treated with PKC activator, PMA (10^{-8}−10^{-6} M) for 24 h. VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 6; *, P < 0.05 vs control, ANOVA, Duncan's test). (C) Effect of EGCG on proliferation of VSMCs induced by PMA. A10 cells were treated with PMA (10^{-6} M) with or without EGCG (10−50 μM) treatment for 24 h. VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 3; *, P < 0.01 vs control; #, P < 0.05 vs high glucose alone, ANOVA, Duncan's test).

DISCUSSION

Vascular complications are the most common complications in diabetes, and chronic hyperglycemia seems to be an important factor in this process. Accumulating evidence has demonstrated that persistent hyperglycemia could lead to the accelerated VSMC growth and cell cycle progression, which are believed to play a central role in the development of atherosclerosis.[3−6,25] Similar to other studies,[3,4] we confirmed the stimulatory effect of high glucose on VSMC proliferation in the present study (Figure 1). How to inhibit abnormal VSMC proliferation induced by high glucose is an important issue to prevent cardiovascular complications and to improve vascular injury in patients with diabetes.

In recent years, phytochemicals have offered promising new options for the development of more effective therapeutic strategies for cardiovascular diseases and diabetes. Several flavonoids, including green tea catechins, which are ubiquitously present in foods of plant origin, are believed to play important roles in the cardiovascular system.[8,9,26] Epidemiological studies have suggested that consumption of green tea might prevent the incidence of various cardiovascular diseases, including atherosclerosis.[27] EGCG, the major constituent found in green tea leaves, is known to exert a variety of cardiovascular beneficial effects. EGCG increases the antioxidant capacity in local vascular tissue and systemic circulation, reduces progressive atherosclerotic lesions in the apolipoprotein E-null mice, restrains invasive activity of cultured human VSMC, represses VSMC adhesion and migration in rat, and inhibits angiotensin II-stimulated VSMC proliferation.[28−31] Furthermore, the protective effects of EGCG in diabetes also are evident. In vitro and in vivo studies show evidence that treatment with EGCG ameliorates vascular reactivity in diabetic rats, improves the survival rate of isolated islets, reduces the loss of functional islet mass, alleviates diabetic nephropathy, and enhances glucose tolerance in diabetic rodents.[12,32−34] In vitro studies show that EGCG attenuates some high glucose-induced harmful effects, such as the down-regulation of the cardiac gap junction, insulin signaling blockade, embryonic vasculopathy, and expression of pro-inflammatory cytokines.[13,14,35,36] Our present study shows that high glucose increases VSMC proliferation. However, in the presence of EGCG, the VSMC proliferation induced by high glucose is inhibited (Figure 2B,C). It should be noted that the direct inhibitory effect of EGCG is evident only at 30 and 50 μM concentrations; at lower concentration, EGCG has no effect on basal VSMC proliferation, but the inhibitory effect on VSMC proliferation induced by high glucose is remarkable.

In the control cells, we used a glucose concentration of 5 mM, which is within the normal blood glucose concentration in humans. Whereas we agree that a 30 mM glucose concentration is higher than the usual nonfasting glucose level in diabetic patients, this could be the glucose concentration in some uncontrolled diabetic patients. Moreover, this relatively high glucose concentration is used in many published papers, for example, 20−30 mM for vascular smooth muscle cells,[37−40] 20−40 mM for endothelial cells,[41,42] 30 mM for cardiomyocytes and hepatocytes,[43,44] and even higher levels, >100 mM, for peritoneal mesothelial cells.[45]

Although EGCG has been reported to have a potent antidiabetes property,[10,11] the plasma concentrations of EGCG were not published in those papers. Pharmacokinetic studies in humans indicate that the peak plasma concentration after a single oral dose of EGCG is <1.0 μM, although tissue concentrations may

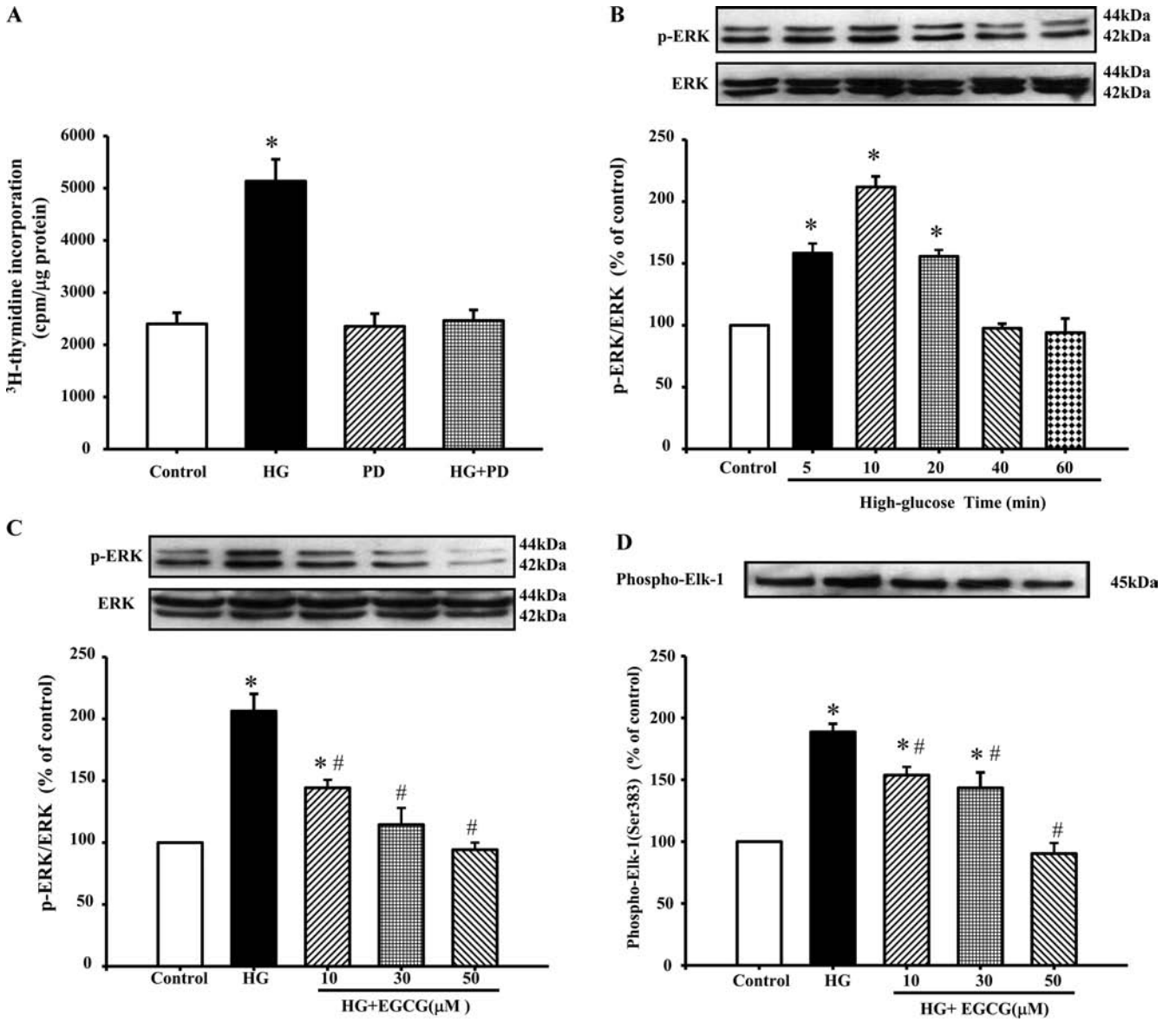

Figure 4. Role of ERK1/2 or Elk-1 on the inhibitory effect of EGCG on proliferation of VSMCs induced by high glucose. (A) Role of ERK1/2 on the effect of VSMC proliferation induced by high glucose. A10 cells were treated with high glucose (HG, 30 mM) for 24 h in the presence of ERK1/2 inhibitor, PD98059 (PD, 10^{-5} M). VSMC proliferation was determined by [^{3}H]-thymidine incorporation. Results are expressed as cpm/μg protein (n = 4; ∗. $P < 0.05$ vs others, ANOVA, Duncan's test). (B) Effect of high glucose on the ERK1/2 phosphorylation in A10 cells. A10 cells were treated with high glucose (30 mM) for different periods (5−60 min). ERK1/2 and ERK1/2 phosphorylations were determined by immunoblotting. Results are expressed as the ratio of ERK1/2 phosphorylation and ERK1/2 expression (n = 3; ∗, $P < 0.05$ vs control, ANOVA, Duncan's test). (C) Effect of EGCG on the stimulatory effect of high glucose on ERK1/2 phosphorylation in A10 cells. A10 cells were treated with high glucose (HG, 30 mM) for 10 min with or without the presence of EGCG (10−50 μM). ERK1/2 and ERK1/2 phosphorylations were determined by immunoblotting. Results are expressed as the ratio of ERK1/2 phosphorylation and ERK1/2 expression (n = 4; ∗, $P < 0.05$ vs control; #, $P < 0.05$ vs high glucose alone, ANOVA, Duncan's test). (D) Effect of EGCG on the stimulatory effect of high glucose on Elk-1 phosphorylation in A10 cells. A10 cells were treated with high glucose (HG, 30 mM) for 10 min with or without the presence of EGCG (10−50 μM). Elk-1 phosphorylation was determined by immunoblotting. Results are expressed as percent changes of control (n = 3; ∗, $P < 0.01$ vs control; #, $P < 0.05$ vs high glucose alone, ANOVA, Duncan's test).

be much higher than systemic concentrations.[46,47] In rodents, the tissue concentration of EGCG in the lung can be 3 times that of the plasma.[48] Ingestion of 1200 mg of EGCG resulted in a plasma concentration of 5.6 μM,[49] which could result in a lung concentration of 16.8 μM. Whether or not higher concentrations can be achieved remains to be determined. In a concentration response study, we found that 30 and 50 μM EGCG inhibited the proliferative effect of high glucose. The concentrations of EGCG in most published papers are within this range, for example, 20 and 50 μM in a proliferation study in VSMCs,[31] 10−50 μM in a hypertrophy study in VSMCs,[50] and two signaling study.[51,52] Some studies have used even higher concentration, >100 μM.[53,54]

PKC and ERK are two key regulatory kinases involved in the regulation of cellular proliferation. There is evidence that the PKC-ERK1/2 pathway is involved in the pathogenesis of

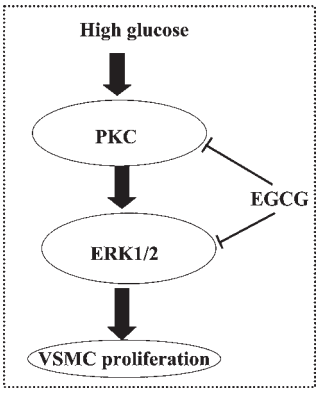

Figure 5. Schematic representation of the effect of EGCG on VSMC proliferaton. PKC, protein kinase C; ERK1/2, extracellular signal-regulated kinase 1/2.

complications in diabetes. For example, PKC inhibitors attenuate a number of vascular abnormalities associated with diabetes in animal models.[55,56] ERK1/2, activated by glucose, takes part in stimulation of rat vascular chymase activation, increasing angiotensin II and reactive oxygen species production.[57,58] Moreover, similar to our findings, there is evidence showing that high glucose stimulates VSMC proliferation via PKC-ERK1/2 pathway. Due to the importance of PKC-ERK1/2 pathway in the proliferation of VSMCs, we hypothesize that the inhibitory effect of EGCG on VSMC proliferation might be via interaction with those signalings. In the present study, we found that, in the presence of EGCG, the stimulatory effects of high glucose on ERK1/2 and Elk-1 phosphorylations are reduced (Figure 4C,D). As a nuclear transcription factor, Elk-1 phosphorylation will trigger a lot of nuclear transcription factor, such as NF-κB, and increase VSMC proliferation.[59] Therefore, reduced ERK1/2 and Elk-1 phosphorylation by EGCG leads to inhibition of VSMC proliferation induced by high glucose.

In summary, our study has identified for the first time that EGCG inhibits proliferation of VSMCs induced by high glucose through the inhibitory effect on PKC-ERK1/2 signal pathway activated by high glucose (Figure 5), which indicates that EGCG might be a possible medicine to reduce vascular complication in diabetes.

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Author Contributions

‖The first two authors contributed equally to this work.

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REFERENCES

(1) Milicevic, Z.; Raz, I.; Beattie, S. D.; Campaigne, B. N.; Sarwat, S.; Gromniak, E.; Kowalska, I.; Galic, E.; Tan, M.; Hanefeld, M. Natural history of cardiovascular disease in patients with diabetes: role of hyperglycemia. *Diabetes Care* **2008**, *31*, S155–S160.

(2) Haffner, S. J.; Cassells, H. Hyperglycemia as a cardiovascular risk factor. *Am. J. Med.* **2003**, *115*, 6S–11S.

(3) Lee, Y. J.; Kim, J. S.; Kang, D. G.; Lee, H. S. *Buddleja officinalis* suppresses high glucose-induced vascular smooth muscle cell proliferation: role of mitogen-activated protein kinases, nuclear factor-κB and matrix metalloproteinases. *Exp. Biol. Med. (Maywood)* **2010**, *235*, 247–255.

(4) Sun, J.; Xu, Y.; Dai, Z.; Sun, Y. Intermittent high glucose enhances proliferation of vascular smooth muscle cells by upregulating osteopontin. *Mol. Cell. Endocrinol.* **2009**, *313*, 64–69.

(5) Askari, B.; Renard, C. B.; Bornfeldt, K. E. Regulation of smooth muscle cell accumulation in diabetes-accelerated atherosclerosis. *Histol. Histopathol.* **2002**, *17*, 1317–1328.

(6) Alipui, C.; Ramos, K.; Tenner, T. E., Jr. Alterations of rabbit aortic smooth muscle cell proliferation in diabetes mellitus. *Cardiovasc. Res.* **1993**, *27*, 1229–1232.

(7) Carlson, J. R.; Bauer, B. A.; Vincent, A.; Limburg, P. J.; Wilson, T. Reading the tea leaves: anticarcinogenic properties of (−)-epigallocatechin-3-gallate. *Mayo Clin. Proc.* **2007**, *82*, 725–732.

(8) Stangl, V.; Dreger, H.; Stangl, K.; Lorenz, M. Molecular targets of tea polyphenols in the cardiovascular system. *Cardiovasc. Res.* **2007**, *73*, 348–358.

(9) Senthil Kumaran, V.; Arulmathi, K.; Sundarapandiyan, R.; Kalaiselvi, P. Attenuation of the inflammatory changes and lipid anomalies by epigallocatechin-3-gallate in hypercholesterolemic diet fed aged rats. *Exp. Gerontol.* **2009**, *44*, 745–751.

(10) Fu, Z.; Zhen, W.; Yuskavage, J; Liu, D. Epigallocatechin gallate delays the onset of type 1 diabetes in spontaneous non-obese diabetic mice. *Br. J. Nutr.* **2011**, *105*, 1218–1225.

(11) Song, E. K.; Hur, H.; Han, M. K. Epigallocatechin gallate prevents autoimmune diabetes induced by multiple low doses of streptozotocin in mice. *Arch. Pharm. Res.* **2003**, *26*, 559–563.

(12) Yamabe, N.; Yokozawa, T.; Oya, T.; Kim, M. Therapeutic potential of (−)-epigallocatechin 3-*O*-gallate on renal damage in diabetic nephropathy model rats. *J Pharmacol. Exp. Ther.* **2006**, *319*, 228–236.

(13) Lin, C. L.; Lin, J. K. Epigallocatechin gallate (EGCG) attenuates high glucose-induced insulin signaling blockade in human hepG2 hepatoma cells. *Mol. Nutr. Food Res.* **2008**, *52*, 930–939.

(14) Wu, C. H.; Wu, C. F.; Huang, H. W.; Jao, Y. C.; Yen, G. C. Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells. *Mol. Nutr. Food Res.* **2009**, *53*, 984–995.

(15) Zeng, C.; Wang, D.; Yang, Z.; Wang, Z.; Asico, L. D.; Wilcox, C. S.; Eisner, G. M.; Welch, W. J.; Felder, R. A.; Jose, P. A. Dopamine D_1 receptor augmentation of D_3 receptor action in rat aortic or mesenteric vascular smooth muscles. *Hypertension* **2004**, *43*, 673–679.

(16) Kimes, B. W.; Brandt, B. L. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Exp. Cell Res.* **1976**, *98*, 349–366.

(17) Nagata, D.; Takeda, R.; Sata, M.; Satonaka, H.; Suzuki, E.; Nagano, T.; Hirata, Y. AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. *Circulation* **2004**, *110*, 444–451.

(18) Chen, S.; Qiong, Y.; Gardner, D. G. A role for p38 mitogen-activated protein kinase and c-myc in endothelin-dependent rat aortic smooth muscle cell proliferation. *Hypertension* **2006**, *47*, 252–258.

(19) Lin, H.; Lee, J. L.; Hou, H. H.; Chung, C. P.; Hsu, S. P.; Juan, S. H. Molecular mechanisms of the antiproliferative effect of beraprost, a prostacyclin agonist, in murine vascular smooth muscle cells. *J. Cell Physiol.* **2008**, *214*, 434–441.

(20) Lu, S. Y.; Zhu, M. Z.; Wang, D. S.; Chen, S. Y.; Zhang, W. D.; Dong, H.; Yu, J.; Guo, H. T. Inhibition of the proliferation of smooth muscle cells from human coronary bypass vessels by vasonatrin peptide. *Physiol. Res.* **2004**, *53*, 387–393.

(21) Yang, J.; Cui, Z.; He, D.; Ren, H.; Han, Y.; Yu, C.; Fu, C.; Wang, Z.; Yang, C.; Wang, X; Zhou, L; Asico, L. D.; Villar, V. A.; Hopfer, U;

Mi, M; Zeng, C; Jose, P. A. Insulin increases D5 dopamine receptor expression and function in renal proximal tubule cells from Wistar-Kyoto rats. *Am. J. Hypertens.* **2009**, *22*, 770–776.

(22) Zeng, C.; Liu, Y.; Wang, Z.; He, D.; Huang, L.; Yu, P.; Zheng, S.; Jones, J. E.; Asico, L. D.; Hopfer, U.; Eisner, G. M.; Felder, R. A.; Jose, P. A. Activation of D_3 dopamine receptor decreases angiotensin II type 1 receptor expression in rat renal proximal tubule cells. *Circ. Res.* **2006**, *99*, 494–500.

(23) Wong, C. H.; Xia, W.; Lee, N. P.; Mruk, D. D.; Lee, W. M.; Cheng, C. Y. Regulation of ectoplasmic specialization dynamics in the seminiferous epithelium by focal adhesion-associated proteins in testosterone-suppressed rat testes. *Endocrinology* **2005**, *146*, 1192–1204.

(24) Touyz, R. M.; Schiffren, E. L. Signal transduction mechanisms mediating the physiological and pathophysiological action of angiotensin II in vascular smooth muscle cells. *Pharmacol. Rev.* **2000**, *52*, 639–672.

(25) Natarajan, R.; Gonzales, N.; Xu, L.; Nadler, J. L. Vascular smooth muscle cells exhibit increased growth in response to elevated glucose. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 552–560.

(26) Jochmann, N.; Baumann, G.; Stangl, V. Green tea and cardiovascular disease: from molecular targets towards human health. *Curr. Opin. Clin. Nutr. Metab. Care* **2008**, *11*, 758–765.

(27) Kuriyama, S. The relation between green tea consumption and cardiovascular disease as evidenced by epidemiological studies. *J. Nutr.* **2008**, *138*, 1548S–1553S.

(28) Chyu, K. Y.; Babbidge, S. M.; Zhao, X.; Dandillaya, R.; Rietveld, A. G.; Yano, J.; Dimayuga, P.; Cercek, B.; Shah, P. K. Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice. *Circulation* **2004**, *109*, 2448–2453.

(29) Cheng, X. W.; Kuzuya, M.; Nakamura, K.; Liu, Z.; Di, Q.; Hasegawa, J.; Iwata, M.; Murohara, T.; Yokota, M.; Iguchi, A. Mechanisms of the inhibitory effect of epigallocatechin-3-gallate on cultured human vascular smooth muscle cell invasion. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 1864–1870.

(30) Lo, H. M.; Hung, C. F.; Huang, Y. Y.; Wu, W. B. Tea polyphenols inhibit rat vascular smooth muscle cell adhesion and migration on collagen and laminin via interference with cell-ECM interaction. *J. Biomed. Sci.* **2007**, *14*, 637–645.

(31) Won, S. M.; Park, Y. H.; Kim, H. J.; Park, K. M.; Lee, W. J. Catechins inhibit angiotensin II-induced vascular smooth muscle cell proliferation via mitogen-activated protein kinase pathway. *Exp. Mol. Med.* **2006**, *38*, 525–534.

(32) Roghani, M.; Baluchnejadmojarad, T. Chronic epigallocatechin-gallate improves aortic reactivity of diabetic rats: underlying mechanisms. *Vascul. Pharmacol.* **2009**, *51*, 84–89.

(33) Hara, Y.; Fujino, M.; Takeuchi, M.; Li, X. K. Green-tea polyphenol (−)-epigallocatechin-3-gallate provides resistance to apoptosis in isolated islets. *J. Hepatobiliary Pancreat. Surg.* **2007**, *14*, 493–497.

(34) Wolfram, S.; Raederstorff, D.; Preller, M.; Wang, Y.; Teixeira, S. R.; Riegger, C.; Weber, P. Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J. Nutr.* **2006**, *136*, 2512–2518.

(35) Yu, L.; Zhao, Y.; Fan, Y.; Wang, M.; Xu, S.; Fu, G. Epigallocatechin-3 gallate, a green tea catechin, attenuated the downregulation of the cardiac gap junction induced by high glucose in neonatal rat cardiomyocytes. *Cell Physiol. Biochem.* **2010**, *26*, 403–412.

(36) Yang, P.; Li, H. Epigallocatechin-3-gallate ameliorates hyperglycemia-induced embryonic vasculopathy and malformation by inhibition of Foxo3a activation. *Am. J. Obstet. Gynecol.* **2010**, *203*, 75.e1–e6.

(37) Zhu, L. H.; Wang, L.; Wang, D.; Jiang, H.; Tang, Q. Z.; Yan, L.; Bian, Z. Y.; Wang, X. A.; Li, H. Puerarin attenuates high-glucose-and diabetes-induced vascular smooth muscle cell proliferation by blocking PKCβ2/Rac1-dependent signaling. *Free Radical Biol. Med.* **2010**, *48*, 471–482.

(38) Ling, S.; Little, P. J.; Williams, M. R.; Dai, A.; Hashimura, K.; Liu, J. P.; Komesaroff, P. A.; Sudhir, K. High glucose abolishes the antiproliferative effect of 17β-estradiol in human vascular smooth muscle cells. *Am. J. Physiol. Endocrinol. Metab.* **2002**, *282*, E746–E751.

(39) Bellin, C.; de Wiza, D. H.; Wiernsperger, N. F.; Rösen, P. Generation of reactive oxygen species by endothelial and smooth muscle cells: influence of hyperglycemia and metformin. *Horm. Metab. Res.* **2006**, *38*, 732–739.

(40) Popov, D.; Nemecz, M.; Dumitrescu, M.; Georgescu, A.; Böhmer, F. D. Long-term high glucose concentration influences Akt, ERK1/2, and PTP1B protein expression in human aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 51–55.

(41) Zanetti, M.; Zwacka, R.; Engelhardt, J.; Katusic, Z.; O'Brien, T. Superoxide anions and endothelial cell proliferation in normoglycemia and hyperglycemia. *Arterioscler. Thromb. Vasc. Biol.* **2001**, *21*, 195–200.

(42) Varma, S.; Lal, B. K.; Zheng, R.; Breslin, J. W.; Saito, S.; Pappas, P. J.; Hobson, R. W., 2nd; Durán, W. N. Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction. *Am. J. Physiol. Heart Circ. Physiol.* **2005**, *289*, H1744–H1751.

(43) Kui, L.; Weiwei, Z.; Ling, L.; Daikun, H.; Guoming, Z.; Linuo, Z.; Renming, H. Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway. *Regul. Pept.* **2009**, *155*, 62–69.

(44) Suh, H. N.; Lee, S. H.; Lee, M. Y.; Heo, J. S.; Lee, Y. J.; Han, H. J. High glucose induced translocation of Aquaporin8 to chicken hepatocyte plasma membrane: involvement of cAMP, PI3K/Akt, PKC, MAPKs, and microtubule. *J. Cell Biochem.* **2008**, *103*, 1089–1100.

(45) Matsuo, H.; Tamura, M.; Kabashima, N.; Serino, R.; Tokunaga, M.; Shibata, T.; Matsumoto, M.; Aijima, M.; Oikawa, S.; Anai, H.; Nakashima, Y. Prednisolone inhibits hyperosmolarity-induced expression of MCP-1 via NF-κB in peritoneal mesothelial cells. *Kidney Int.* **2006**, *69*, 736–746.

(46) Chow, H. H.; Cai, Y.; Alberts, D. S.; Hakim, I.; Dorr, R.; Shahi, F.; Crowell, J. A.; Yang, C. S.; Hara, Y. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 53–58.

(47) Lee, M. J.; Maliakal, P.; Chen, L.; Meng, X.; Bondoc, F. Y.; Prabhu, S.; Lambert, G.; Mohr, S.; Yang, C. S. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemio.l Biomarkers Prev.* **2002**, *11*, 1025–1032.

(48) Kim, S.; Lee, M. J.; Hong, J.; Li, C.; Smith, T. J.; Yang, G. Y.; Seril, D. N.; Yang, C. S. Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutr. Cancer* **2000**, *37*, 41–48.

(49) Chow, H. H.; Hakim, I. A.; Vining, D. R.; Crowell, J. A.; Ranger-Moore, J.; Chew, W. M.; Celaya, C. A.; Rodney, S. R.; Hara, Y.; Alberts, D. S. Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. *Clin. Cancer Res.* **2005**, *11*, 4627–4633.

(50) Zheng, Y.; Song, H. J.; Kim, C. H.; Kim, H. S.; Kim, E. G.; Sachinidis, A.; Ahn, H. Y. Inhibitory effect of epigallocatechin 3-*O*-gallate on vascular smooth muscle cell hypertrophy induced by angiotensin II. *J. Cardiovasc. Pharmacol.* **2004**, *43*, 200–208.

(51) Park, J. S.; Kim, M. H.; Chang, H. J.; Kim, K. M.; Kim, S. M.; Shin, B. A.; Ahn, B. W.; Jung, Y. D. Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2. *Int. J. Oncol.* **2006**, *29*, 1247–1252.

(52) Weber, A. A.; Neuhaus, T.; Skach, R. A.; Hescheler, J.; Ahn, H. Y.; Schrör, K.; Ko, Y.; Sachinidis, A. Mechanisms of the inhibitory effects of epigallocatechin-3 gallate on platelet-derived growth factor-BB-induced cell signaling and mitogenesis. *FASEB J.* **2004**, *18*, 128–130.

(53) Monteiro, R.; Calhau, C.; Martel, F.; Faria, A.; Mateus, N.; Azevedo, I. Modulation of MPP+ uptake by tea and some of its components in Caco-2 cells. *Naunyn Schmiedebergs Arch. Pharmacol.* **2005**, *372*, 147–152.

(54) Jung, J. Y.; Han, C. R.; Jeong, Y. J.; Kim, H. J.; Lim, H. S.; Lee, K. H.; Park, H. O.; Oh, W. M.; Kim, S. H.; Kim, W. J. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. *Neurosci. Lett.* **2007**, *411*, 222–227.

(55) Clarke, M.; Dodson, P. M. PKC inhibition and diabetic microvascular complications. *Best Pract. Res. Clin. Endocrinol. Metab.* **2007**, *21*, 573–586.

(56) Tuttle, K. R.; Anderson, P. W. A novel potential therapy for diabetic nephropathy and vascular complications: protein kinase C β inhibition. *Am. J. Kidney Dis.* **2003**, *42*, 456–465.

(57) Lavrentyev, E. N.; Estes, A. M.; Malik, K. U. Mechanism of high glucose induced angiotensin II production in rat vascular smooth muscle cells. *Circ. Res.* **2007**, *101*, 455–464.

(58) Yu, T.; Jhun, B. S.; Yoon, Y. High-glucose stimulation increases reactive oxygen species production through the calcium and mitogen-activated protein kinase-mediated activation of mitochondrial fission. *Antioxid. Redox Signal.* **2011**, *14*, 425–437.

(59) Hasan, R. N.; Schafer, A. I. Hemin upregulates Egr-1 expression in vascular smooth muscle cells via reactive oxygen species ERK-1/2-Elk-1 and NF-κB. *Circ. Res.* **2008**, *102*, 42–50.