

High Glucose Increases Acetylcholine-Induced Ca^{2+} Entry and Protein Expression of STIM1

Hong Ding, Fatiha Benslimane, Isra Marei, Chris R. Triggles

Abstract—Hyperglycaemia is a key factor that contributes to the development of diabetes-related microvascular disease and a major risk factor for endothelial dysfunction. In the current study, we have explored glucose-induced abnormal intracellular calcium (Ca^{2+}_i) homeostasis in mouse microvessel endothelial cells (MMECs) in high glucose (HG) (40mmol/L) versus control (low glucose, LG) (11 mmol/L). We demonstrated that the exposure of MMECs to HG for 3 days did not change basal Ca^{2+}_i , however, there was a significant increase of acetylcholine-induced Ca^{2+} entry. Western blots illustrated that exposure to HG also increased STIM1 (Stromal Interaction Molecule 1), but not Orai1 (the pore forming subunit), protein expression levels. Although the link between HG-induced changes in STIM1 expression, enhanced Ca^{2+} entry and endothelial dysfunction requires further study, the current data are suggestive that targeting these pathways may reduce the impact of HG on endothelial function.

Keywords—store-operated calcium entry, hyperglycaemia, STIM1, endothelial dysfunction

I. INTRODUCTION

ENDOTHELIAL dysfunction, which can be defined from a functional view as the loss or reduction of endothelium-dependent vasodilatation to an endothelium-dependent vasodilator, such as acetylcholine is a very early indicator of the vascular disease that is closely associated with diabetes [1]. Although there is a strong association between endothelial dysfunction and the development of cardiovascular disease the cellular mechanism(s) remain poorly defined [2]-[4].

A variety of stimuli that includes circulating hormones, certain autacoids, shear stress, thrombin and platelet derived products activate endothelial nitric oxide synthase (eNOS) as a result of an increase in intracellular calcium (Ca^{2+}_i) [5]. Endothelial cells do not express voltage-dependent calcium channels [6] and agonist-stimulated Ca^{2+} influx depends on the Ca^{2+} driving force and the opening of store-operated cation channels (SOCs) by a process referred to as capacitative calcium entry or store-operated calcium entry (SOCE) [7]-[10].

H. Ding* is with Departments of Pharmacology & Medical Education, Weill Cornell Medical College in Qatar, P.O. Box 24144, Education City, Doha, Qatar (phone: 974-4492-8362; fax: 974-4492-8333; e-mail: hod2005@qatar-med.cornell.edu). *Author for correspondence

F. Benslimane is with Departments of Pharmacology & Medical Education, Weill Cornell Medical College in Qatar, P.O. Box 24144, Education City, Doha, Qatar (e-mail: fatiha.b.86@hotmail.com).

I. Marei is with Departments of Pharmacology & Medical Education, Weill Cornell Medical College in Qatar, P.O. Box 24144, Education City, Doha, Qatar (e-mail: iym200@qatar-med.cornell.edu).

C. R. Triggles is with Departments of Pharmacology & Medical Education, Weill Cornell Medical College in Qatar, P.O. Box 24144, Education City, Doha, Qatar (e-mail: cht2011@qatar-med.cornell.edu).

Advances in our knowledge of SOCs indicate that STIM1 (Stromal Interaction Molecule 1) is the endoplasmic reticulum Ca^{2+} sensor and the Ca^{2+} release-activated channel protein 1, Orai1, is the pore forming subunit of the highly selective CRAC (Ca^{2+} release-activated Ca^{2+} current) in endothelial cells [11]. However, it has been previously argued that mammalian homologues of the transient receptor potential, TRP, gene family and notably the canonical TRP (TRPC) sub-family of channels function as SOCs [12]. Indeed, TRPC1 has been shown to co-localize with Orai1 and STIM1 and studies with exogenously expressed TRPC1 and STIM1 in endothelial cells indicate that TRPC1 is regulated by STIM1 [13],[14]. In addition to TRPC1, STIM1 and Orai1, have been identified as the key regulators of SOCE with TRPC1 forming a ternary complex [14] and preliminary data from our laboratory indicates that exposure of mouse microvascular endothelial cells (MMECs) to high glucose (HG) also increases the expression of STIM1 protein. The current study was designed to further investigate the effects of HG on STIM1 and SOCE in microvascular endothelial cells.

II. METHODS

A. Cell Culture

Mouse microvessel endothelial cells (MMECs) were purchased from the American Type Culture Collection (Cat # CRL-2279). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 0.02% N-2 hydroxyethylpiperazine-N'_2_ethane sulfonic acid (HEPES) and 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. MMECS grown in low glucose (LG) DMEM (11mM – equivalent to that observed for normal glucose levels in mice) and HG DMEM, 40mM, the equivalent to that observed in db/db type 2 diabetic mice and thus simulating hyperglycaemia in diabetic mice [15,16]. MMECs were cultured in 25 cm^2 flasks coated with Extra-cellular Matrix (ECM). Equimolar substitution of mannitol was used as an osmotic control. All experiments were standardized by seeding 5×10^5 cells for subculture and performed on cells, which underwent 7-18 passages. Cell cultures were maintained in an incubator at 37 °C, with saturating humidity and an atmosphere of 5% carbon dioxide to 95% air.

B. Intracellular Calcium (Ca^{2+}_i) Measurement

MMECs were removed from tissue culture flasks by treatment with Ca^{2+} -free solution containing 0.02% EDTA and 0.25% trypsin and plated (10^5 cells /mL) on glass coverslips that had been coated with ECM. Cells were then allowed to adhere for 16 h under conditions of 37°C, 5% CO_2 prior to

loading with Fura-2-AM and changes in Ca^{2+}_i determined using an inverted microscope. Cells were excited with alternating 340 and 380 nm wavelength light by use of a spinning filter wheel while emission intensity was monitored after passing through a 510-nm dichroic filter. Changes in intracellular Ca^{2+}_i were expressed in terms of changes in the 340/380 nm fluorescence intensity ratio. Values obtained are typically averages of fields of groups of 10-12 single cells; however, preliminary and earlier studies [17] have shown that this is representative of single cell behaviour and is not altered by the presence of cell-cell contacts.

C. Western Blots

80-90% confluent cells were washed 3-5 times with DPBS; then 300 μ l of cell lysis buffer was added into each flask, and kept on ice with continuous shaking for 30 minutes. Cell lysates were then centrifuged at 14,000RPM (10,000 g) for 30 minutes. The supernatant was transferred into Eppendorf tubes and kept at -80°C until required. 50 μ g of protein samples were loaded onto and separated by an SDS-PAGE gel. The gel was then transferred onto a nitrocellulose membrane. The membrane was blocked by 5% skim milk, and then incubated with a primary antibody overnight on a shaker at 4°C , and then washed with tween-20 buffer for 45 minutes (3 x 5 minutes and 3 x 10 minutes washing intervals) followed by incubation of the membrane in an horseradish peroxidase-conjugated secondary antibody for 1 hour, then 45 minutes of washing. Proteins were then visualized using enhanced chemiluminescence (BIO-RAD) and quantitated with Chemidoc software and normalized to the corresponding band density of β -actin.

D. Superoxide Measurement

MMECs grown on cover slips were washed to remove DMEM. Cells were incubated with dihydroethidium (DHE, 5 μ M) for 30 min at room temperature and imaged by fluorescence microscopy [18]. Values were obtained from the mean fluorescent intensity of multiple cells (MMECs) from multiple cells captured within each section.

E. Statistics

Data are expressed as means \pm S.E.M. with statistical comparisons performed using Excel software. Differences between two groups were evaluated with paired or unpaired Student's t test as appropriate and differences between groups by one-way analysis of variance. Differences were considered to be significant when $P < 0.05$.

III. RESULTS

A. Acetylcholine-Induced Ca^{2+} Entry Is Increased In MMECs Treated With HG

Application of the endothelium-dependent vasodilator and muscarinic receptor agonist, acetylcholine (ACh), when administered, elicited an immediate rise in Ca^{2+}_i . ACh-mediated Ca^{2+}_i signaling was studied in MMECs treated with HG for 72 h. After 72 h treatment (Figures 1A,B), there was a

significant increase ($P < 0.05$) of intracellular Ca^{2+}_i in HG. Under comparable conditions 40mmol/L mannitol did not affect ACh-induced changes in Ca^{2+}_i .

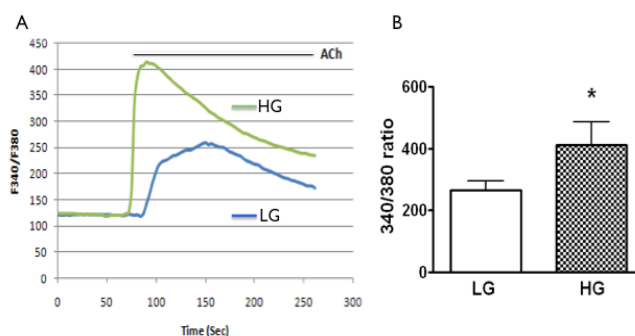


Fig. 1 Ca^{2+}_i changes in MMECs treated with 11mmol/L glucose (LG) or 40mmol/L glucose (HG) for 72 h and stimulated with ACh, induced a sustained increase in (Ca^{2+}_i) due to (Ca^{2+}_i) released activated Ca^{2+} entry (1A). A summary comparison of mean (Ca^{2+}_i) during the peak of (Ca^{2+}_i) phase for LG and HG are shown in 1B. Data are means \pm SEM. *** indicates $P < 0.05$ as compared to control

B. STIM1 Protein Expression

The effect of glucose concentration on protein expression of STIM1 in cultured MMECs exposed to low or high (LG; 11 or HG; 40 mM) glucose was examined by Western blot. STIM1 antibody recognized the 80kDa protein bands (Figure 2A). Equal amount of proteins was used to measure and determine the levels of STIM1 protein. β -actin was used as a loading control. Exposure to HG for 72 h resulted in a significant increase (7.2 fold increase) ($P < 0.05$) in the expression of STIM1 protein compared to LG (Figure 2A,B).

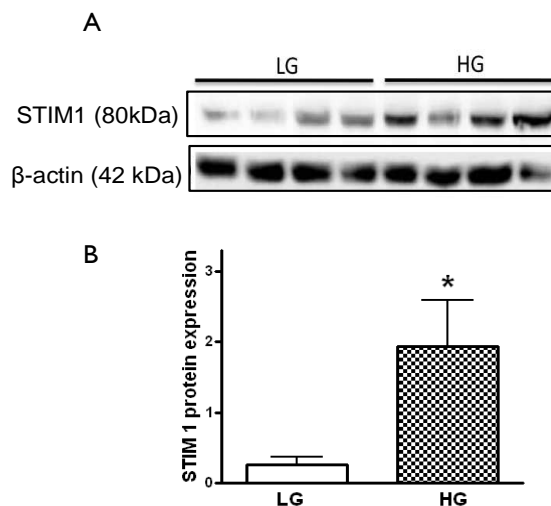


Fig. 2 (A) Representative gels for STIM1 expression in MMECs cultured for 72hr with 11 mM (LG) and 40mM glucose (HG) by Western blot method. (B) Densitometric analysis of detected protein expression of STIM1 calculated as % of β -actin is summarized in the bar graph.*** indicates $P < 0.05$ as compared to control

C. Orai-1 Protein Expression

Protein expression of Orai 1 was also examined using Western blot technique. Orai 1 antibody recognized the 32

KDa protein bands (Figure 3A). There was no significant difference in protein expression of Orai 1 in MMECs exposure to HG for 72 h comparing to that in MMECs exposure to LG ($P>0.05$) (Figure 3A,B).

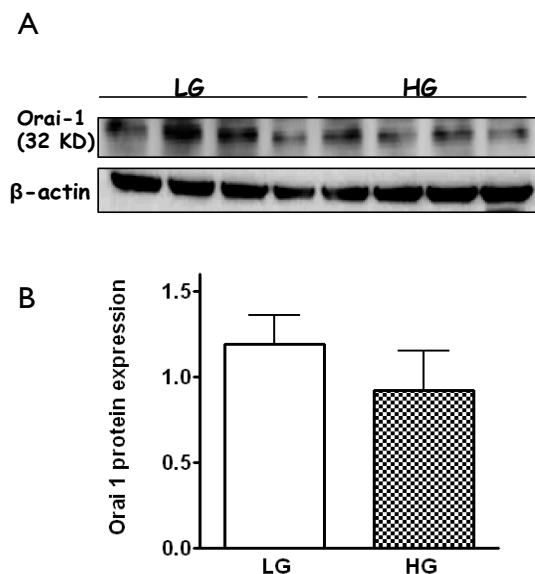


Fig. 3 (A) Representative gels for Orai-1 expression in MMECs cultured for 72hr with 11 mM (LG) and 40mM glucose (HG) by Western blot method. (B) Densitometric analysis of detected protein expression of Orai-1 calculated as % of β -actin is summarized in the bar graph

D. Superoxide Generation in MMECs

Superoxide generation was measured by comparing DHE fluorescence intensity of MMECs cultured for 72h with 11 mM (LG) and 40 mM glucose (HG). Representative fluorescence intensity images of MMECs suggested that there is increased superoxide generation in MMECs grown in HG compared to that in MMECs grown in LG media for a 72h period (Figure 4).

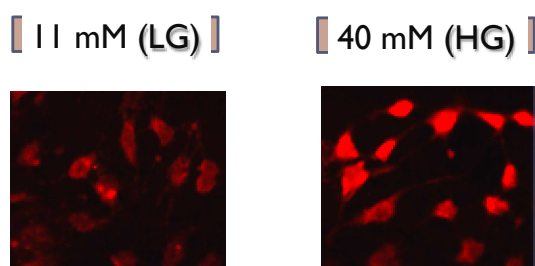


Fig. 4 Representative fluorescence intensity images of MMECs treated with DHE for 30min and cultured for 72h with 11 mM (LG) and 40 mM glucose (HG) were compared

IV. DISCUSSIONS

The key findings from the present study are the increase in the expression of STIM1 protein and a heightened SOCE response following the 72-hour exposure of MMECs to HG. As previously discussed we chose 40mM glucose to simulate the level of hyperglycaemia that has been reported in the db/db leptin receptor mutant mouse model of type 2 diabetes

[15],[16]. We had previously reported that, following a comparable experimental protocol as for the current study, there was a heightened expression of TRPC1, a putative component of SOCs in some cells, as well a heightened SOCE in bovine aortic endothelial cells (BAECs) exposed to HG [19]. A sustained increase in Ca^{2+}_i via SOCE has been linked to an increase in TRPC1 expression and the development of neointimal hyperplasia and vascular disease [20],[21]. An association between metabolic syndrome and vascular disease in coronary arteries from Ossabaw pigs has also been linked to increased SOCE and TRPC1 and another protein associated with the regulation of SOCE, STIM1 [22]. Interestingly changes in SOCE and the associated regulatory proteins are normalised in Ossabaw pigs that are subjected to an exercise regimen [22]. Data from the 1991 United Kingdom Prospective Diabetes Study, UKPDS, with type 2 and the 1993 Diabetes Control and Complications Trial, DCCT with type 1 diabetes patients conclude that tight glycaemic control is necessary to reduce the progression of vascular disease, notably microvascular disease [23],[24]. Although therapeutic strategies that lower glucose concentrations are a priority in the management of diabetes how best to protect the endothelium against glucose toxicity remains unclear [25]. Studies of the effects of relatively short periods, up to 72 hours, of exposure to high glucose suggest that the changes that occur in expression of proteins that regulate endothelial function may have long-term consequences [26]-[28]. Hyperglycaemia-induced endothelial cell apoptosis has been linked to an increase in SOCE in human umbilical vein endothelial cells [26]. In addition, the exposure of MMECs to HG for 72 hours results in a significant increase in oxidative stress plus changes in the expression of a number of proteins, including eNOS, superoxide dismutase and NADPH oxidase that are involved in the regulation of the bioavailability of nitric oxide as well as oxidative stress [18],[27]. There is also an upregulation of the nox2 and nox4 subunits of NADPH oxidases in resistance arteries from streptozotocin-induced type 1 diabetic mice thus demonstrating that the data from cell culture studies are comparable to that seen in animal models of diabetes [28]. It is therefore of interest to note the important role that Ca^{2+}_i plays in the regulation of NADPH oxidase in macrophages and that STIM1 is an essential regulator of NADPH oxidase expression although the contribution of a non-SOCE pathway involving TRPC3 has also been identified [29]-[31]. Thus, collectively these data indicate an important trigger for hyperglycaemia-induced vascular disease is an increase in SOCE and the associated increase in activity of NADPH oxidase and resultant increase in oxidative stress.

The link between hyperglycaemia, SOCE and enhanced NADPH oxidase activity may be important in determining the epigenetic changes that are associated with diabetes and result in "hyperglycaemic memory" wherein sustained effects of a prior exposure to hyperglycaemia continue to progress in animal models of diabetes as well as in patients unless subjected to a tight control of blood glucose levels [32],[33].

High glucose and oxidative stress are thought to be associated factors in initiating the changes in chromatin remodeling that result in hyperglycaemic memory and have been linked to a reduction in the bioavailability of nitric oxide and an increase in the expression of the transcription factor, NF κ B [34]-[36]. Thus, in the current study, we have identified that an enhancement in SOCE is an early change induced by sustained high glucose levels and therefore a potential target for limiting the effects of glucose toxicity on vascular function.

ACKNOWLEDGMENT

Support from a Qatar Foundation NPRP grant (08-165-3-054) is gratefully acknowledged.

REFERENCES

- [1] Andrews KL, Pannirselvam M, Anderson TJ, Jenkins AJ, Triggle CR, Hill MA. The vascular endothelium in diabetes: a practical target for drug treatment? *Expert Opin Ther Targets* 9: 101-117, 2005.
- [2] De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM. Endothelial dysfunction in diabetes. *Br J Pharmacol*. 130: 963-274, 2000.
- [3] Feletou M, Vanhoutte PM. Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol*. 291: H985-H1002, 2006.
- [4] Pannirselvam M, Simon V, Verma S, Anderson T, Triggle CR. Chronic oral supplementation with sepiapterin prevents endothelial dysfunction and oxidative stress in small mesenteric arteries from diabetic (db/db) mice. *Br J Pharmacol* 140: 701-706, 2003.
- [5] Vanhoutte PM. Endothelium and control of vascular function. *State of the Art lecture. Hypertension* 13: 658-667, 1998.
- [6] Tsien RW, Tsien RY. Calcium channels, stores, and oscillations. *Ann Rev Cell Biol* 6: 715-760, 1990.
- [7] Luckhoff A, Busse R. Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pflugers Arch* 416: 305-311, 1990.
- [8] Nilius B, Viana F, Droogmans G. Ion channels in vascular endothelium. *Ann Rev Physiol* 59: 145-170, 1997.
- [9] Parekh AB, Penner R. Store depletion and calcium influx. *Physiol Rev* 77: 901-930, 1997.
- [10] Parekh AB, Putney JW Jr. Store-operated calcium channels. *Physiol Rev*. 85: 757-810, 2005. Review
- [11] Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. *Circ Res* 103:1289-1299, 2008.
- [12] Pedersen SF, Owsianik G, Nilius B. TRP channels: an overview. *Cell Calcium* 38: 233-252, 2005.
- [13] Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF. STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 8: 1003-1010; 2006.
- [14] Ong HL, Cheng KT, Liu X, Bandyopadhyay BC, Paria BC, Soboloff J, Pani B, Gwack Y, Srikanth S, Singh BB, Gill DL, Ambudkar IS. Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem*. 282: 9105-9116, 2007.
- [15] Pannirselvam M, Wiehler WB, Anderson T and Triggle CR. Enhanced vascular reactivity of small mesenteric arteries from diabetic mice is associated with enhanced oxidative stress and cyclooxygenase products. *Br J Pharmacol*. 2005; 144: 953-960.
- [16] Aasum, E., Cooper, M., Severson, DL., and Larsen, TS. Effect of BM 17,0744, a PPARalpha ligand, on the metabolism of perfused hearts from control and diabetic mice. *Can J Physiol Pharmacol* 2005; 83:183-190.
- [17] Bishara NB, Murphy TV, Hill MA. Capacitative Ca(2+) entry in vascular endothelial cells is mediated via pathways sensitive to 2-aminoethoxydiphenyl borate and xestospongine C. *Br J Pharmacol* 135: 119-128, 2002.
- [18] Ding H, Aljofan M, Triggle CR. Oxidative stress and increased eNOS and NADPH oxidase expression in mouse microvessel endothelial cells. *J Cell Physiol* 212: 682-689, 2007
- [19] Bishara NB, Ding H. Glucose enhances expression of TRPC1 and calcium entry in endothelial cells. *Am. J. Physiol. Heart Circ Physiol* 298: H171-H178, 2010.
- [20] Kumar B, Dreja K, Shah SS, Cheong A, Xu SZ, Sukumar P, Naylor J, Forte A, Cipollaro M, McHugh D, Kingston PA, Heagerty AM, Munsch CM, Bergdahl A, Hultg rdh-Nilsson A, Gomez MF, Porter KE, Hellstrand P, Beech DJ. Upregulated TRPC1 channel in vascular injury in vivo and its role in human neointimal hyperplasia. *Circ Res*. 98: 557-663, 2006.
- [21] van Breemen C, Poburko D, Okon EB. TRP proteins: a new dimension in the treatment of occlusive vascular disease. *Circ Res*. 98: 446-447, 2006.
- [22] Edwards, JM., Neeb, ZP., Alloosh, MA., Long, X., Bratz, IN, Peller, CR., Byrd, JP., Kumar, S., Obukhov, AG., Sturek M. Exercise training decreases store-operated Ca²⁺ entry associated with metabolic syndrome and coronary atherosclerosis. *Cardiovasc Res*. 85: 631-640, 2010.
- [23] UKPDS: UK Prospective Diabetes Study (UKPDS) Group: UK Prospective Diabetes Study (UKPDS). VIII. Study design, progress and performance. *Diabetologia* 34: 877-890, 1991.
- [24] DCCT: (1993). The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329: 977-986, 1993.
- [25] Ding, H, Triggle, CR. Glycaemic control and protection of the vasculature against glucose toxicity. In *Medical Complications of Diabetes*. Edited by Collen Croniger, Chapter 6, pp 87-108. INTECH Open Access Publisher ISBN: 978-953-307-363-7. 2011.
- [26] Tamarelle S, Mignen O, Capiod T, R cker-Martin C, Feuvray D. High glucose-induced apoptosis through store-operated calcium entry and calcineurin in human umbilical vein endothelial cells. *Cell Calcium*. 39: 47-55, 2006.
- [27] Aljofan M, Ding H. High glucose increases expression of cyclooxygenase-2, increases oxidative stress and decreases the generation of nitric oxide in mouse microvessel endothelial cells. *J Cell Physiol* 222: 669-675, 2010.
- [28] Ding H, Hashem M, Triggle C. Increased oxidative stress in the streptozotocin-induced diabetic apoE-deficient mouse: changes in expression of NADPH oxidase subunits and eNOS. *Eur J Pharmacol*. 561: 121-128, 2007.
- [29] Br chard S, Plan on S, Melchior C, Tschirhart EJ. STIM1 but not STIM2 is an essential regulator of Ca²⁺ influx-mediated NADPH oxidase activity in neutrophil-like HL-60 cells. *Biochem Pharmacol*. 78:504-513, 2009.
- [30] Br chard S, Tschirhart EJ. Regulation of superoxide production in neutrophils: role of calcium influx. *J Leukocyte Biol* 84: 1223-1237, 2008.
- [31] Br chard S, Melchior C, Plan on S, Schenten V, Tschirhart EJ. Store-operated Ca²⁺ channels formed by TRPC1, TRPC6 and Orai1 and non-store-operated channels formed by TRPC3 are involved in the regulation of NADPH oxidase in HL-60 granulocytes. *Cell Calcium*. 44: 492-506, 2008.
- [32] Engerman RL, Kern TS. Progression of incipient diabetic retinopathy during good glycemic control. *Diabetes*. 36: 808-812, 1987.
- [33] Cooper ME. Metabolic memory: implications for diabetic vascular complications. *Pediatr Diabetes*. 10: 343-346, 2009.
- [34] Peng HB, Libby P, Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem*. 270: 14214-14219, 1995.
- [35] Li N, Karin M. Is NF-kappaB the sensor of oxidative stress? *FASEB J*. 13:1137-1143, 1999.
- [36] Bierhaus A, Schiekhofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J, Hong M, Luther T, Henle T, Kl tting I, Morcos M, Hofmann M, Tritschler H, Weigle B, Kasper M, Smith M, Perry G, Schmidt AM, Stern DM, H ring HU, Schleicher E, Nawroth PP. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. *Diabetes*. 50: 2792-2808, 2001.