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Sulfonylurea as well as elevated glucose levels stimulate reactive oxygen species production in the pancreatic β-cell line, MIN6—a role of NAD(P)H oxidase in β-cells

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Abstract

Increased oxidative stress may play a key role in the progressive deterioration of pancreatic β-cells and the development of diabetes. However, the underlying mechanism is not well understood. Exposure of pancreatic β-cell line, MIN6 cells, to elevated glucose level for 2 h induced an increase in reactive oxygen species (ROS) production, as evaluated by the staining of 2′,7′-dichlorofluorescein diacetate. This effect was completely blocked by NAD(P)H oxidase inhibitor (diphenylene iodonium) and protein kinase C (PKC) inhibitor (calphostin C), but not affected by other flavoprotein inhibitors (rotenone, oxypurinol, or L-*N*-monomethyl arginine). Glibenclamide also stimulated ROS production in a dose-dependent manner. This effect was again blocked by diphenylene iodonium and calphostin C. In conclusion, insulin secretagogues, both glibenclamide and elevated glucose level, stimulated ROS production in β-cells through a PKC-dependent activation of NAD(P)H oxidase. This mechanism may be a novel therapeutic target for preventing the progression of β-cell deterioration.

Keywords: Oxidative stress; β-cell; Glucose toxicity; Sulfonylurea; NAD(P)H oxidase

Chronic exposure of pancreatic β-cells to elevated glucose levels results in β-cell dysfunction and ultimately β-cell death, a phenomenon termed β-cell glucose toxicity. Glucose toxicity is likely an important contributor of progressive β-cell deterioration and the development of overt diabetes. The mechanisms whereby chronic elevated glucose levels might damage β-cells are the subject of clinical and laboratory investigation. Among various elevated glucose level-induced adverse consequences, oxidative stress is considered to be a candidate for the mechanism underlying tissue damage that accompanies chronic elevated glucose levels. Recently, accumulating evidence suggests that oxidative stress is increased in pancreatic β-cells in diabetic animal models and diabetic patients [1–3]. Since the islet has the lowest intrinsic antioxidant capacity compared with other tissues, oxidative stress may contribute to β-cell glucose toxicity [4]. However, the mechanism responsible for increased oxidative stress in pancreatic β-cells is not well understood.

Oxidative stress is also believed to contribute to the development of microangiopathy and atherosclerosis associated with diabetes. In vascular cells, various mechanisms have been postulated, such as increased formation of advanced glycosylation end products [5], an

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enhanced polyol pathway [6], increased xanthine oxidase activity [7], increased release of superoxide from mitochondria [8], and activation of NAD(P)H oxidase [9]. Previously, we reported that high glucose levels stimulated reactive oxygen species (ROS) production through protein-kinase C (PKC)-dependent activation of NAD(P)H oxidase in cultured vascular cells [9]. Furthermore, using the in vivo electron spin resonance method, we showed in vivo evidence that increased oxidative stress in diabetes was attributed, at least in part, to a PKC-dependent activation of NAD(P)H oxidase [10]. Interestingly, Oliveira et al. [11] recently reported that pancreatic β-cells expressed phagocyte-like NAD(P)H oxidase. We therefore speculated that NAD(P)H oxidase might be an important source of oxidative stress in pancreatic β-cells as well as in vascular cells. In this report, we show that PKC-dependent activation of NAD(P)H oxidase may be a major source of oxidative stress induced by elevated glucose levels in the pancreatic β-cell line, MIN6. Furthermore, we show for the first time that not only elevated glucose levels but also the insulin secretagogue, glibenclamide, stimulates ROS production in MIN6 through the same mechanism. These findings may in part account for the mechanism for glucose toxicity and secondary sulfonylurea failure in β-cells.

Materials and methods

Materials and chemicals. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR). Pancreatic β-cell line MIN6 cells were kindly provided by Dr. S. Seino (Division of Cell Biology and Neurophysiology, Department of Neuroscience, Faculty of Medicine, Kobe University) and Dr. E. Araki (Department of Metabolic Medicine, School of Medicine, Kumamoto University). Rotenone, oxypurinol, L-*N*-monomethyl arginine, diphenylene iodonium, and calphostin C were purchased from Sigma (St. Louis, MO). Glibenclamide was kindly provided by Aventis Pharma.

Cell culture. Pancreatic β-cell line MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum (FCS), 100 mU/ml penicillin, 100 mg/ml streptomycin, and 450 mg/dl glucose at 37 °C in an atmosphere of 95% O_2/5% CO_2.

Evaluation of ROS production in MIN6. DCF-DA was used to evaluate intracellular oxidant formation. For experiments, the cells were placed into glass-bottomed dishes (MatTek, Ashland, MA) and cultured in DMEM containing 15% FCS and 450 mg/dl glucose. When the cells reached the loose confluent layer, the medium was replaced with DMEM containing 1% FBS and 100 mg/dl glucose for 24 h. The cells were then replaced with test media for the indicated intervals, washed with phenol-red free Hank, and loaded with 2 μmol/L DCF-DA; a nonfluorescent compound that freely permeates cells and interacts with intracellular oxidants to form fluorescence compound DCF. After 20 min, digital images of DCF fluorescence were obtained with a fluorescence microscope system (Olympus, Tokyo, Japan) at an excitation wavelength of 488 nm (argon laser) using a 515 nm long-pass emission filter. The obtained fluorescence images were converted to gray scale images using Photoshop software (Adobe Systems, San Jose, CA), and the fluorescence intensities were quantitatively analyzed using NIH image software.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed by ANOVA followed by post hoc comparison test.

Results

Exposure of the MIN6 cells to increasing concentrations of glucose (100–450 mg/dl) for 2 h increased ROS production in a concentration-dependent manner (Figs. 1A and B). This effect was completely blocked by treatment of NAD(P)H oxidase inhibitor, diphenylene iodonium (10^{-6} M) or the PKC inhibitor, calphostin C (5×10^{-7} M), for 2 h (Figs. 1A and B). In contrast, other flavoprotein inhibitors such as rotenone (10^{-4} M), oxypurinol (10^{-4} M), or L-*N*-monomethyl arginine (10^{-5} M) did not significantly affect ROS production (Figs. 1A and B).

Exposure of the cells to increasing concentrations of glibenclamide (10^{-7}–10^{-5} M) stimulated ROS production in a concentration-dependent manner (Figs. 2A and B). This effect was also completely blocked by treatment of diphenylene iodonium or calphostin C (Figs. 2A and B). Glibenclamide at submaximal concentration (10^{-6} M) and elevated glucose levels (200 mg/dl) additively stimulated ROS production, while there was no additive effect between glibenclamide (10^{-5} M) and elevated glucose levels (450 mg/dl) at the maximal concentration (Fig. 3).

Discussion

Increased oxidative stress has been implicated as a potential mechanism of action for glucose toxicity on pancreatic β-cells [1–3,12,13]. Indeed, several reports have shown that exposure of isolated islets to elevated glucose levels induces an increase in ROS production [12,13]. Increased oxidative stress was also shown in pancreatic β-cells in animal models of type 2 diabetes [1,2,12,13], and in patients with type 2 diabetes [3] as evaluated by various oxidative stress markers such as 8-hydroxy-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins. In addition, the islet is supposed to be one of the most vulnerable tissues to oxidative stress since the islet contains relatively small activities for the major antioxidant enzymes Cu/Zn superoxide dismutase, Mn superoxide dismutase, catalase, and glutathione peroxidase compared with other tissues such as liver, kidney, skeletal muscle, and fat [4]. Taken together, these data suggest that elevated glucose level-induced oxidative stress may play a key role in the progression of β-cell deterioration and the development of overt diabetes.

The mechanism underlying elevated glucose level-induced oxidative stress in β-cells is not well understood.

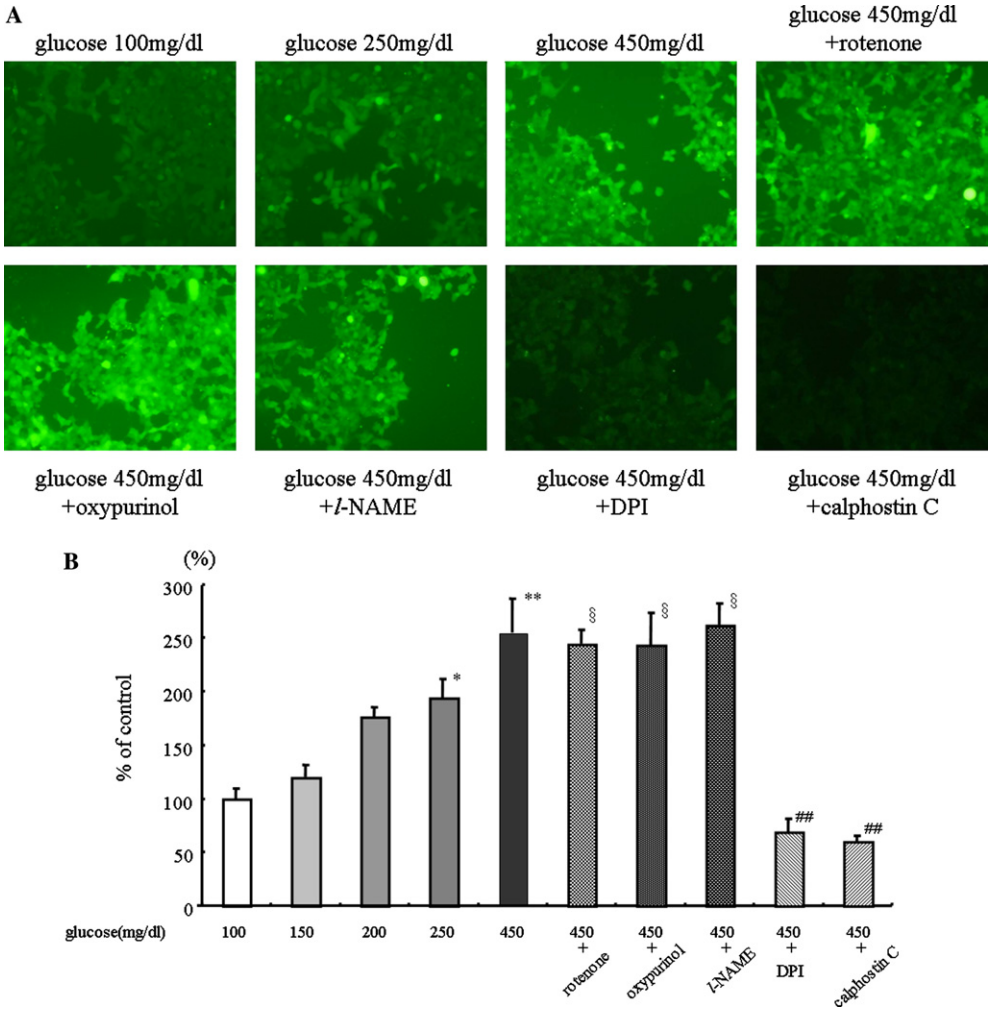

Fig. 1. Effect of high glucose levels on reactive oxygen species production in the cultured pancreatic cell line, MIN6. The MIN6 cells were cultured in test media containing 100, 150, 200, 250, and 450 mg/dl glucose, 450 mg/dl glucose plus rotenone, oxypurinol, L-*N*-monomethyl arginine (L-NAME) diphenylene iodonium (DPI), or calphostin C. After 2 h incubation, 2′,7′-dichlorofluorescein diacetate (DCF-DA) at the concentration of 2 μmol/L was added. After 20 min, digital images of DCF fluorescence were obtained with a fluorescence microscope system. (A) Representative fluorescent images. (B) Quantitative analysis of the fluorescence intensities on the images. $^{*}p < 0.05$ vs 100 mg/dl glucose, $^{**}p < 0.01$ vs 100 mg/dl glucose, $^{\#\#}p < 0.01$ vs 450 mg/dl glucose, and §not significant vs 450 mg/dl glucose. Data are expressed as means ± SE.

It has been suggested that one of the important sources of ROS production may be the mitochondria [8,14]. It is generally established that the mitochondrial respiratory chain is an important site of ROS production within the cells. Superoxide is produced continually as a byproduct of normal respiration through the one-electron reduction of molecular oxygen. A recent report showed that elevated glucose levels induced an increase in mitochondrial ROS production in cultured endothelial cells [8]. It seems probable that mitochondria may be a major source of ROS production in pancreatic β-cells. In contrast, in vascular cells NAD(P)H oxidase has received increasing attention as the most important source of ROS production. Previously, we reported that high glucose levels stimulate ROS production in cultured vascular cells through a PKC-dependent activation of NAD(P)H oxidase [9]. We and other investigators have also shown that the activity and/or expression of vascular NAD(P)H oxidase are increased in the vascular tissues of animal models of diabetes and diabetic patients [10,15]. Recently, Oliveira et al. [11] showed the presence of NAD(P)H oxidase components in rat pancreatic islet cells by RT-PCR (gp91phox, p22phox, and p47phox) and Western blotting (p47phox and p67phox). The present study confirmed that elevated glucose levels stimulated ROS production in MIN6 as evaluated by the DCF-DA staining. This stimulatory effect was completely blocked by treatment with an NAD(P)H oxidase inhibitor (diphenylene iodonium) or PKC inhibitor (calphostin C). Treatment with inhibitors of other flavoproteins such as xanthine oxidase (oxypurinol), nitric oxide synthase (L-*N*-monomethyl arginine), or the mitochondria electron transport chain (rotenone), did not affect ROS production induced by elevated glucose levels.

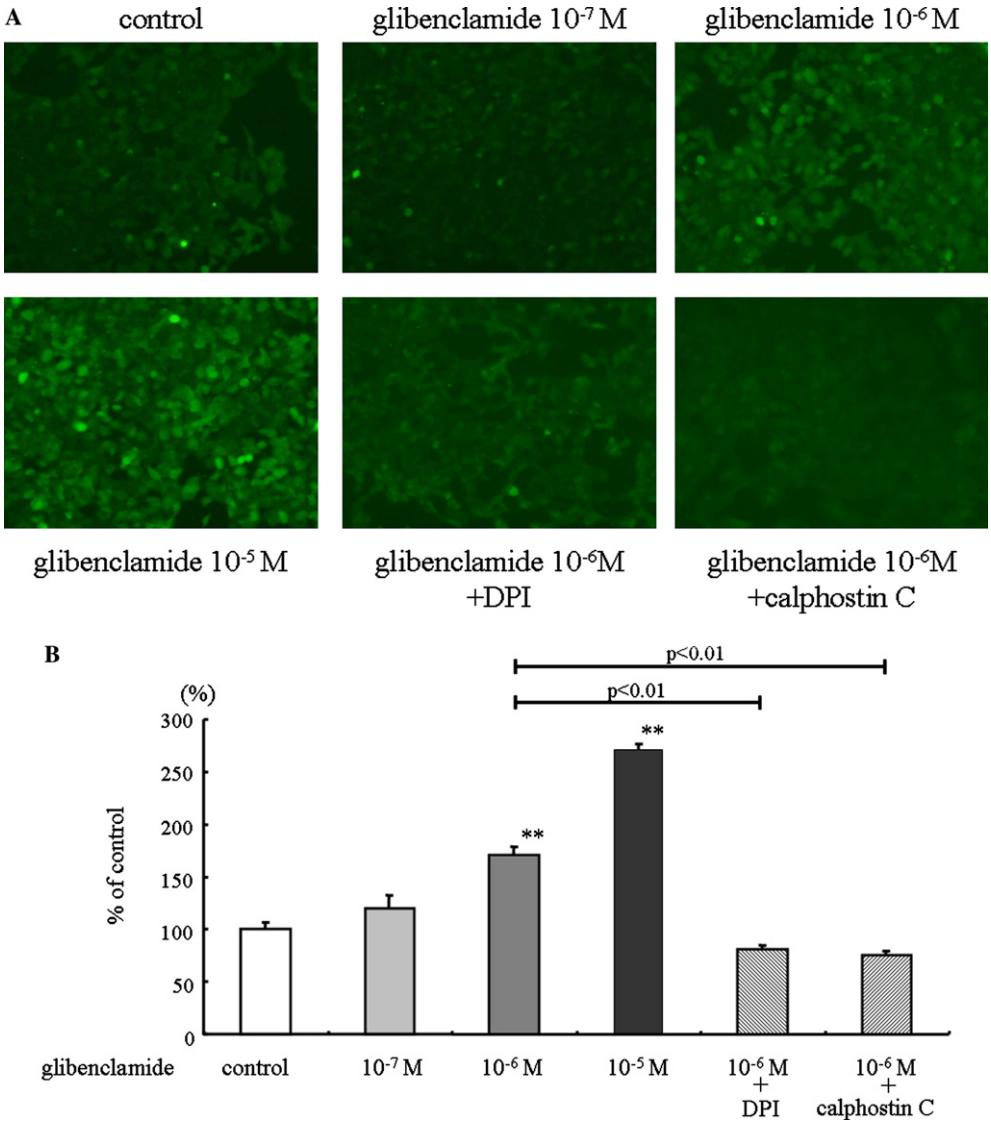

Fig. 2. Effect of glibenclamide on reactive oxygen species production in the cultured pancreatic cell line, MIN6. The MIN6 cells were cultured in test media containing 100 mg/dl glucose with or without (control) various concentrations of glibenclamide (10^{-7}, 10^{-6} or 10^{-5} M). After 2 h incubation, 2′,7′-dichlorofluorescein diacetate (DCF-DA) at the concentration of 2 μmol/L was added. After 20 min, digital images of DCF fluorescence were obtained with a fluorescence microscope system. (A) Representative fluorescent images. (B) Quantitative analysis of the fluorescence intensities on the images. $^{**}p < 0.01$ vs control. Data are expressed as means ± SE.

These results suggest that a PKC-dependent activation of NAD(P)H oxidase may be the major source of elevated glucose level-induced increased ROS production in MIN6. Although the role of PKC in glucose-stimulated insulin secretion is controversial, it is widely accepted that elevated glucose levels activate PKC in pancreatic β-cells probably through two different mechanisms; a rise in intracellular Ca^{2+} concentration and an increase in diacylglycerol (DAG) levels synthesized by the de novo pathway [16].

The present study revealed that the insulin secretagogue, glibenclamide, stimulated ROS production in MIN6 in a dose-dependent manner. This effect was also completely blocked by treatment with an NAD(P)H oxidase inhibitor or PKC inhibitor, suggesting the role of PKC-dependent activation of NAD(P)H oxidase. There has been no report with regard to the effect of sulfonylurea on ROS production. This is the first report to show the stimulatory effect of sulfonylurea on ROS production. It is known that sulfonylurea activates PKC in pancreatic β-cells. Sulfonylurea binds to a regulatory subunit of membrane ATP-sensitive K^+ (K-ATP) channels. The consequent closure of the K-ATP channels leads to depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and a rise in intracellular Ca^{2+} concentration. A rise in intracellular Ca^{2+} concentration could lead to PKC activation. However, another mechanism is also speculated. Recently, Lehtihet et al.

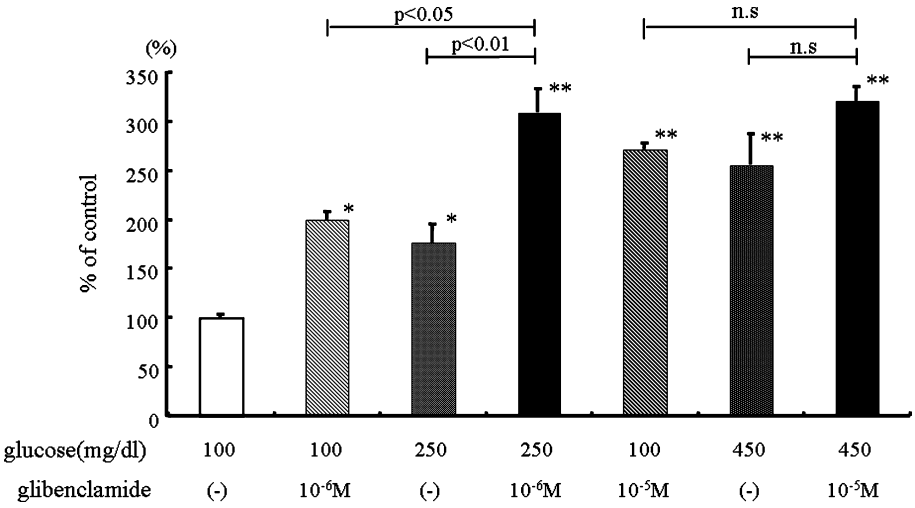

Fig. 3. Additive effects of high glucose levels and glibenclamide on reactive oxygen species production in the cultured pancreatic cell line, MIN6. The MIN6 cells were cultured in test media containing 100, 250 or 450 mg/dl glucose with or without glibenclamide at concentrations of 10^{-6} or 10^{-5} M. After 2 h incubation, 2′,7′-dichlorofluorescein diacetate (DCF-DA) at the concentration of 2 μmol/L was added. After 20 min, digital images of DCF fluorescence were obtained with a fluorescence microscope system. $*p < 0.05$, $**p < 0.01$ vs 100 mg/dl glucose without glibenclamide. n.s, not significant. Data are expressed as means ± SE.

[17] reported that glibenclamide inhibited carnitine palmitoyltransferase 1 (CPT-1) activity, consequently suppressing free fatty acid oxidation. This is associated with enhanced formation of DAG, PKC activation, and K-ATP-independent insulin exocytosis. In the present study, we showed that glibenclamide at submaximal concentration (10^{-6} M), which is a physiological concentration, stimulated ROS production additively in combination with elevated glucose levels, but did not stimulate ROS production at maximal concentration (10^{-5} M). This suggests that the mechanism for glibenclamide's effect on ROS production may be the same as that of elevated glucose levels. In any case, the finding of the additive effect found at the physiological concentration provides an important clinical insight into the use of sulfonylurea. Chronic use of high doses of sulfonylurea under poor glucose control may induce an increase in ROS production in pancreatic β-cells. This may in part contribute to secondary sulfonylurea failure.

In conclusion, the insulin secretagogues, both elevated glucose levels and sulfonylurea, may stimulate ROS production through a PKC-dependent activation of NAD(P)H oxidase in the pancreatic β-cell line, MIN6. This mechanism may be a novel therapeutic target for preventing the progression of β-cell deterioration and the development of diabetes.

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References

[1] Y. Ihara, S. Toyokuni, K. Uchida, H. Odaka, T. Tanaka, H. Ikeda, H. Hiai, Y. Seino, Y. Yamada, Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes, Diabetes 48 (1999) 927–932.

[2] Y. Tanaka, C.E. Gleason, P.O. Tran, J.S. Harmon, R.P. Robertson, Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants, Proc. Natl. Acad. Sci. USA 96 (1999) 10857–10862.

[3] H. Sakuraba, H. Mizukami, N. Yagihashi, R. Wada, C. Hanyu, S. Yagihashi, Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients, Diabetologia 45 (2002) 85–96.

[4] K. Grankvist, S.L. Marklund, I.B. Taljedal, CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse, Biochem. J. 199 (1981) 393–398.

[5] M. Brownlee, A. Cerami, H. Vlassara, Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications, N. Engl. J. Med. 318 (1988) 1315–1321.

[6] J.R. Williamson, K. Chang, M. Frangos, K.S. Hasan, Y. Ido, T. Kawamura, J.R. Nyengaard, M. van den Enden, C. Kilo, R.G. Tilton, Hyperglycemic pseudohypoxia and diabetic complications, Diabetes 42 (1993) 801–813.

[7] M.C. Desco, M. Asensi, R. Marquez, J. Martinez-Valls, M. Vento, F.V. Pallardo, J. Sastre, J. Vina, Xanthine oxidase is involved in free radical production in type 1 diabetes: protection by allopurinol, Diabetes 51 (2002) 1118–1124.

[8] T. Nishikawa, D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, M. Brownlee, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage, Nature 404 (2000) 787–790.

[9] T. Inoguchi, P. Li, F. Umeda, H.Y. Yu, M. Kakimoto, M. Imamura, T. Aoki, T. Etoh, T. Hashimoto, M. Naruse, H. Sano, H. Utsumi, H. Nawata, High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells, Diabetes 49 (2000) 1939–1945.

[10] T. Sonta, T. Inoguchi, H. Tsubouchi, N. Sekiguchi, K. Kobayashi, S. Matsumoto, H. Utsumi, H. Nawata, Evidence for contribution of vascular NAD(P)H oxidase to increased oxidative stress in animal models of diabetes and obesity, Free Radic. Biol. Med. 37 (2004) 115–123.

[11] H.R. Oliveira, R. Verlengia, C.R. Carvalho, L.R. Britto, R. Curi, A.R. Carpinelli, Pancreatic beta-cells express phagocyte-like NAD(P)H oxidase, Diabetes 52 (2003) 1457–1463.

[12] L. Wu, W. Nicholson, S.M. Knobel, R.J. Steffner, J.M. May, D.W. Piston, A.C. Powers, Oxidative stress is a mediator of glucose toxicity in insulin-secreting pancreatic islet cell lines, J. Biol. Chem. 279 (2004) 12126–12134.

[13] V.P. Bindokas, A. Kuznetsov, S. Sreenan, K.S. Polonsky, M.W. Roe, L.H. Philipson, Visualizing superoxide production in normal and diabetic rat islets of Langerhans, J. Biol. Chem. 278 (2003) 9796–9801.

[14] K. Green, M.D. Brand, M.P. Murphy, Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes, Diabetes 53 (Suppl. 1) (2004) S110–S118.

[15] Y.K. Kim, M.S. Lee, S.M. Son, I.J. Kim, W.S. Lee, B.Y. Rhim, K.W. Hong, C.D. Kim, Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes, Diabetes 51 (2002) 522–527.

[16] W.S. Zawalich, H. Rasmussen, Control of insulin secretion: a model involving Ca^{2+}, cAMP and diacylglycerol, Mol. Cell. Endocrinol. 70 (1990) 119–137.

[17] M. Lehtihet, N. Welsh, P.O. Berggren, G.A. Cook, A. Sjoholm, Glibenclamide inhibits islet carnitine palmitoyltransferase 1 activity, leading to PKC-dependent insulin exocytosis, Am. J. Physiol. Endocrinol. Metab. 285 (2003) E438–446.