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Antioxidative effects of azelnidipine on mesangial cell proliferation induced by highly concentrated insulin

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Abstract

Insulin resistance combined with hyperinsulinemia is involved in the generation of oxidative stress. There is known to be a relationship between increased production of reactive oxygen species and the diverse pathogenic mechanisms involved in diabetic vascular complications including nephropathy. The present study found that high doses of insulin affect mesangial cell proliferation through the generation of intracellular reactive oxygen species and the activation of cell signaling pathways. We also examined whether azelnidipine, a dihydropyridine-based calcium antagonist with established antioxidant activity, has the potential to inhibit mesangial cell proliferation. Cell proliferation was increased in a dose-dependent manner by high doses of insulin (0.1–10 μM), but was inhibited by 0.1 μM azelnidipine. Phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 was found to be increased by insulin in a dose-dependent manner (0.1–10 μM). This increased phosphorylation of ERK-1/2 was inhibited by treatment with 0.1 μM azelnidipine. Intracellular oxidative stress was also increased by insulin stimulation in a dose-dependent manner (0.01–10 μM), and 0.1 μM azelnidipine was found to block intracellular reactive oxygen species production more effectively than 0.1 μM nifedipine. The NAD(P)H oxidase inhibitor, apocynin (0.01–0.1 μM), prevented insulin-induced mesangial cell proliferation. Taken together, these results suggest that azelnidipine inhibits insulin-induced mesangial cell proliferation by inhibiting the production of reactive oxygen species. Given these pharmacological characteristics, azelnidipine may have the potential to protect against the onset of diabetic nephropathy and slow its progression.

Keywords: Insulin; Mesangial cell; Extracellular signal-regulated kinase; Azelnidipine; Oxidative stress

1. Introduction

Diabetic nephropathy is the leading cause of end-stage renal failure, accounting for 35–40% of all new cases requiring dialysis therapy worldwide (Locatelli et al., 2004; Mogensen and Cooper, 2004). Patients with early-stage type 2 diabetes mellitus have insulin resistance, which in combination with compensatory hyperinsulinemia accelerates the clustering of coronary risk factors and the development of coronary artery disease (Despres et al., 1996; Pyorala et al., 1998; Reaven, 2003).

One of the causes of diabetic organopathy associated with hyperinsulinemia is oxidative stress. In diabetes mellitus, the generation of oxidative stress is increased by hyperglycemia (Baynes and Thorpe, 1999) and hyperinsulinemia (Sarafidis and Ruilope, 2006). Oxidative stress has also been reported to be a potent mediator of diabetic kidney disease because it activates protein kinase C-mitogen activated protein kinase (MAPK) (Brownlee, 2001; Ha and Kim, 1999). Extracellular signal-regulated kinase (ERK)-1/2, a MAPK, is activated in a variety of cell types by a diverse range of extracellular stimuli, and functions by connecting the nucleus to signals derived from cell membrane receptors. The ERK-1/2 pathway is activated primarily by growth factors and participates significantly in cell proliferation (Robinson and Cobb, 1997; Widmann et al., 1999).

Glomerulosclerosis associated with mesangial cell proliferation and excessive accumulation of extracellular matrix proteins is a central pathophysiological feature of diabetic nephropathy. Glomerulosclerosis ultimately leads to renal

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failure (Wesson, 1988), and therefore, the clinical suppression of mesangial overgrowth is critical to prevent renal failure.

Dihydropyridine-based calcium channel blockers are a class of drugs used widely in the treatment of hypertension and whose dihydropyridine ring can donate electrons to a propagating radical and reduce it to a non-reactive form. Azelnidipine is a dihydropyridine-based calcium channel blocker which was developed recently in Japan and which is a highly lipid soluble drug with enhanced vascular affinity (Yoram and Ahuva, 1995). We hypothesized that it may inhibit insulin-induced mesangial cell proliferation by directly decreasing oxidative stress. In the present study, we examined the antioxidative effect of azelnidipine on the insulin-induced proliferation of mesangial cells.

2. Materials and methods

2.1. Materials

Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Azelnidipine was generously provided by Sankyo Co., Ltd. (Tokyo, Japan), and tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate) was purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). A cell proliferation enzyme-linked immunosorbent assay (ELISA) 5-bromo-2′-deoxyuridine (BrdU) colorimetric kit (Biotrak Kit) was purchased from GE Healthcare Bio-Sciences Corporation (Little Chalfont, Buckinghamshire, UK). Phospho p44/42 MAPK (Thr202/Thr204) antibody and p44/42 MAPK antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) Western blotting reagent was purchased from GE Healthcare Bio-Sciences KK (Fairfield, CT, USA). 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen (Eugene, Oregon, USA).

2.2. Cell culture and treatment

Primary mesangial cells were isolated from Sprague–Dawley rats using a sieving method (Misra, 1972) and then cultured in RPMI 1640 containing 20% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and insulin–transferrin–selenium G supplement (Invitrogen Co., Carlsbad, CA, USA). The mesangial cells were maintained at 37 °C in an atmosphere of air and 5% CO_2. Subconfluent cells were used in the experiments at passages 4 to 10. Before each experiment, the cells were serum-starved in RPMI 1640 containing 0.1% fetal calf serum. The quiescent mesangial cells were treated with azelnidipine (0.01–0.1 μM), apocynin (0.01–0.1 μM) or nifedipine (0.1 μM) (Sigma Chemical Co., St. Louis, MO, USA) for 24 h, after which they were treated with 0.001–10 μM insulin solution (Sigma) containing one of these drugs. All animal experiments were approved by our University Animal Care Committee and were carried out following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23; revised 1985).

2.3. Cell proliferation

A colorimetric assay was performed based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells as described in our previous report (Okura et al., 1998). The cells were seeded in 96-well plates and treated with azelnidipine or apocynin for 24 h before stimulation with insulin for 24 h. A 10-μl aliquot of WST-1 solution was added to each well, followed by incubation for 2 h at 37 °C. The plate was read on an ELISA reader (Multiskan Bichromatic; Labsystems, Helsinki, Finland) at 450 nm, with a reference wavelength of 650 nm. Data were expressed as the percentage absorbance relative to untreated controls.

2.4. DNA synthesis

DNA synthesis was measured using a cell proliferation ELISA BrdU colorimetric kit following the protocol described by Yang et al. (2001). The cells were seeded in 96-well plates, treated with azelnidipine for 24 h, and stimulated with insulin for 24 h. They were then labeled with BrdU for 3 h at 37 °C and, after washing, the cells were fixed and stained with anti-BrdU antibody for 90 min at 37 °C. After three washes, the substrate, tetramethylbenzidine, was added, followed by incubation for 30 min. A blocking solution (1 M H_2SO_4) was then added, and the absorbance of the samples was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

2.5. Western blotting

Western blotting analysis was performed following the protocol described by Fukuoka et al. (1999). Briefly, the cells were seeded and treated with azelnidipine for 24 h, followed by treatment with insulin for 10 min. The cells were then snap-frozen in liquid nitrogen, lysed in ice-cold lysis buffer and dissolved in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein content was assayed by the Bradford method. Ten μg of protein was added in each lane and resolved by SDS-PAGE, then blotted on polyvinylidene difluoride (PVDF) membranes incubated with phospho p44/42 MAPK (Thr202/Thr204) antibody or total p44/42 MAPK antibody. The membranes were subsequently washed three times and incubated in horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody. Blots were detected using ECL Western blotting reagents.

2.6. Oxidative stress

Oxidative stress was evaluated by visualizing the intracellular generation of H_2O_2. The cells were seeded in 96-well plates. After treatment with azelnidipine or nifedipine for 24 h, the cells were stimulated with insulin for 10 min, washed with PBS and then incubated in the dark for 30 min with 20 μM of DCF-DA. DCF-DA diffuses into cells and is hydrolyzed into

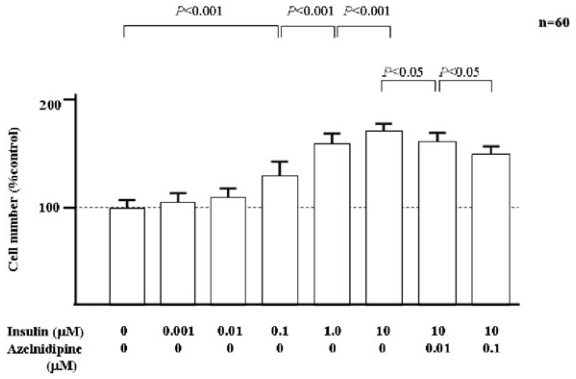

Fig. 1. Effects of insulin on mesangial cell proliferation and the inhibitory effect of azelnidipine. Cell proliferation was estimated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate). Cell proliferation was increased by treatment with insulin in a dose-dependent manner (0.1–10 μM), and was inhibited by treatment with azelnidipine. Values are expressed as mean±S.D. (n=60 for each group).

non-fluorescent 2′,7′-dichlorofluorescein (DCFH). The reactive oxygen species, including H_2O_2, superoxide and .OH, oxidized non-fluorescent intracellular DCFH into highly fluorescent dichlorofluorescein (DCF). DCF fluorescence was quantified in a multiplate fluorometer (Wallac ARVO SX; PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Furthermore, to evaluate the *in situ* production of reactive oxygen species, the cells were seeded in chamber slides and treated with azelnidipine or nifedipine for 24 h and then stimulated with insulin for 10 min. The slides were incubated with 20 μM of DCF-DA in a light-protected humidified chamber at 37 °C for 30 min. The DCF image was obtained by fluorescence microscopy (PROVIS AX; Olympus, Tokyo, Japan).

2.7. Statistical analysis

Analysis of variance with the Bonferroni–Dunn post hoc test was used to analyze differences between the 2 experimental groups. All values are expressed as mean±S.D., and statistical significance was defined as $P<0.05$.

3. Results

3.1. Effects of azelnidipine on cell proliferation and DNA synthesis induced by insulin

Insulin was found to increase mesangial cell proliferation, as estimated by WST-1, in a dose-dependent manner over the concentration range of 0.1–10 μM (Fig. 1). Doses of 0.1 and 1 μM azelnidipine inhibited by 8% and 18%, respectively, the mesangial cell proliferation induced by 10 μM insulin (Fig. 1). Insulin also caused a concurrent increase in DNA synthesis, as estimated by BrdU incorporation, with this effect being dose-dependent at concentrations above 0.1 μM. Furthermore, this effect with 10 μM insulin was attenuated by 32% under 0.1 μM of azelnidipine (Fig. 2).

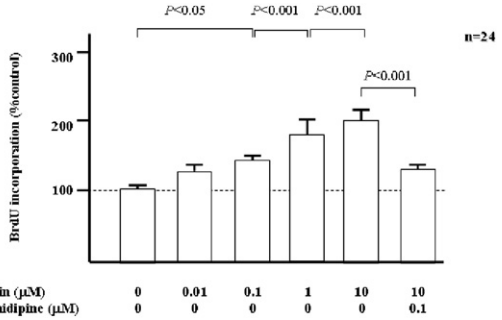

Fig. 2. Effects of insulin on DNA synthesis and the inhibitory effect of azelnidipine. DNA synthesis estimated by BrdU incorporation was increased by insulin treatment in a dose-dependent manner, and 0.1 μM of azelnidipine treatment inhibited this increment. Values are expressed as mean±S.D. (n=24 for each group).

3.2. Effects of azelnidipine on p44/42 MAPK phosphorylation induced by insulin

Since p44/42 MAPK activation is a key step in the proliferation of a variety of cells, we assessed the activation status of p44/42 MAPK on insulin-treated mesangial cells using a phosphor-specific antibody of p44/42 MAPK that reacted only with the phosphorylated (Thr202 and Tyr204) and activated forms of p44/42 MAPK. We then examined p44/42 MAPK activity after 10 min stimulation with insulin over the concentration range of 0.001–10 μM. P44/42 MAPK phosphorylation was found to increase gradually in proportion to the concentration of insulin, and increased significantly at concentrations above 0.1 μM compared to control levels (Fig. 3). We then examined the effects of azelnidipine on p44/42 MAPK phosphorylation induced by 10 μM of insulin. Azelnidipine (0.1 μM) was found to block p44/42 MAPK phosphorylation, maintaining it at almost non-stimulated control level, but did not affect total p44/42 MAPK expression (Fig. 3).

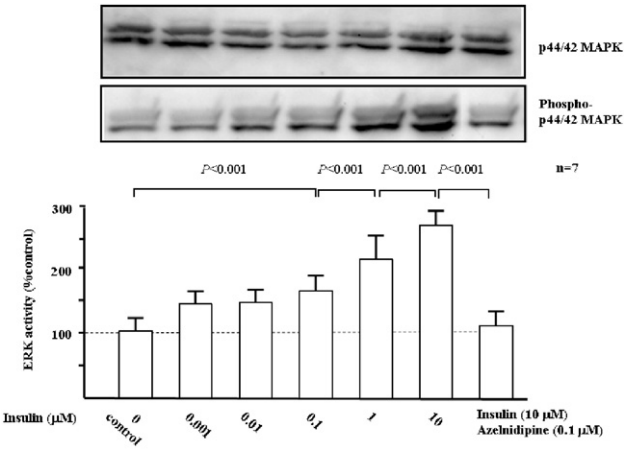

Fig. 3. Effect of insulin on ERK-1/2 phosphorylation and the inhibitory effect of the calcium channel blocker azelnidipine in mesangial cells. ERK-1/2 phosphorylation was increased by insulin in a dose-dependent manner (0.01–10 μM). Azelnidipine (0.1 μM) attenuated this effect, almost returning the samples to control values. Values are expressed as mean±S.D. (n=7 for each group).

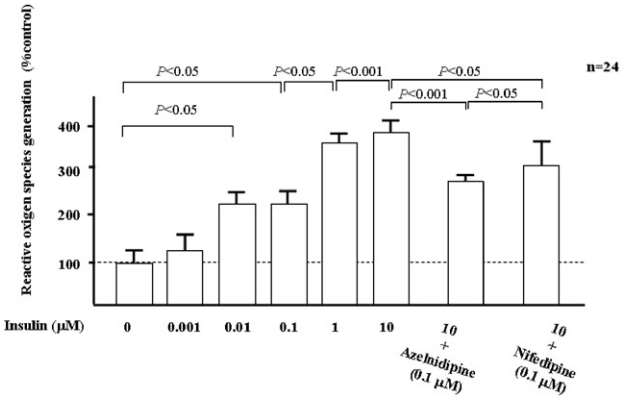

Fig. 4. Effect of insulin on the induction of reactive oxygen species and the inhibitory effect of the calcium channel blocker azelnidipine in mesangial cells. Intracellular oxidative stress was detected with DCF-DA. The production of reactive oxygen species was increased by insulin stimulation in a dose-dependent manner. Treatment with 0.1 μM of azelnidipine or 0.1 μM of nifedipine for 24 h inhibited the induction of DCF fluorescence produced by 10 μM of insulin in mesangial cells, compared with untreated controls. Values are expressed as mean ± S.D. (n = 24 for each group).

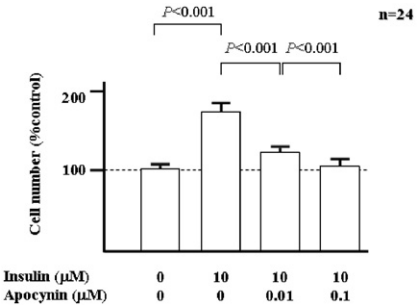

Fig. 6. The inhibitory effects of apocynin on cell proliferation induced by insulin. Cell proliferation was examined by WST-1 and was found to increase under insulin treatment; this increase was inhibited treatment in a dose-dependent manner by apocynin (0.01−0.1 μM). Values are expressed as mean ± S.D. (n = 24 for each group).

3.3. Effect of azelnidipine on reactive oxygen species induced by insulin

In order to examine whether insulin affects the production of reactive oxygen species, mesangial cells were incubated with 0.001–10 μM insulin for 10 min. Increasing concentrations (0.01–10 μM) of insulin were shown to produce more reactive oxygen species in a dose-dependent manner, as estimated by the fluorescence levels of DCF (Fig. 4). We next examined whether the calcium channel blockers azelnidipine and nifedipine inhibited the production of reactive oxygen species following insulin stimulation. The cells were incubated with 0.1 μM azelnidipine or 0.1 μM nifedipine for 24 h, and then with 10 μM of insulin for 10 min, before incubation with DCF-DA. Treatment with either azelnidipine or nifedipine reduced reactive oxygen species levels by 22% and 11%, respectively. The antioxidant effect of azelnidipine was significantly greater than that of nifedipine (P < 0.05) (Figs. 4 and 5).

3.4. Effects of apocynin on mesangial proliferation

In order to assess the effect of the reactive oxygen species blocker on insulin-induced mesangial cell proliferation, mesangial cells were pre-treated with the antioxidant apocynin, and then stimulated with 10 μM insulin for 10 min. As shown in Fig. 6, apocynin (0.01–0.1 μM) prevented insulin-induced mesangial cell proliferation in a dose-dependent manner.

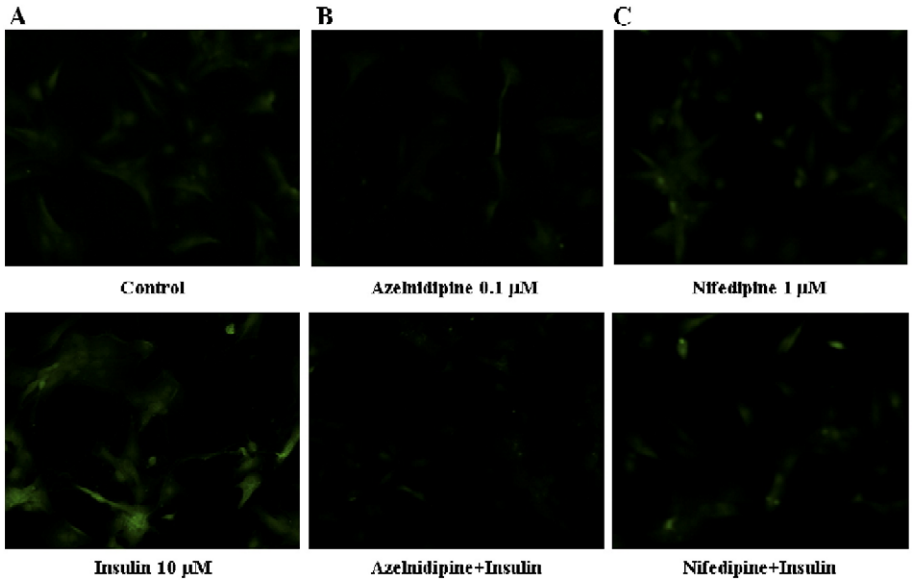

Fig. 5. *In situ* labeling of the production of reactive oxygen species induced by insulin and the inhibitory effect of the calcium channel blockers, azelnidipine and nifedipine. Representative fluorescent images of mesangial cells in the presence of DCF-DA for 30 min after stimulation with 10 μM insulin for 10 min. Insulin stimulation increased the level of intracellular reactive oxygen species, which was then decreased by azelnidipine or nifedipine treatment; azelnidipine was found to be more effective than nifedipine.

4. Discussion

The present study found that highly concentrated insulin stimulates the production of reactive oxygen species, and activates p44/42 MAPK (ERK-1/2) and cell proliferation in mesangial cells. We also demonstrated that azelnidipine, a calcium channel blocker, inhibits the production of insulin-stimulated reactive oxygen species more effectively than nifedipine.

In the present study, doses of more than 0.1 μM insulin induced mesangial cell proliferation. Under normal fasting conditions, the physiological concentration of insulin in the blood is 0.001 μM or less. On the other hand, postprandial insulin concentrations found in patients with insulin resistance is between 0.01 and 0.1 μM, the levels which were adopted for the present study. It is well known that insulin administered at the appropriate concentrations stimulates nitric oxide (NO) production in endothelial cells (Baron, 1994). NO functions not only as a potent vasodilator but also as an antiatherogenic factor by inhibiting platelet adhesion and aggregation (Cockcroft, 2005). However, in the present study, doses greater than 0.01 μM insulin increased the production of intracellular reactive oxygen species in mesangial cells, indicating that high concentrations of insulin are harmful to the cells.

Mahadev et al. (2004) report the potential role of Nox 4, a homologue of gp91phox and a subunit of NAD(P)H oxidase complex, in the generation of reactive oxygen species due to stimulation with 0.1 μM insulin in 3T3-L1 preadipocytes. We also found that apocynin, a NAD(P)H oxidase inhibitor, prevents the mesangial cell proliferation induced by highly concentrated insulin. Apocynin is a methoxy-substituted catechol obtained from the medicinal herb *Picroria kurroa*. It inhibits NAD(P)H oxidase by impeding the assembly of p47phox and p67phox subunits within the membrane NAD(P)H oxidase complex (Meyer and Schmitt, 2000). The present results suggest that highly concentrated insulin induces NAD(P)H oxidase and increases the production of intracellular superoxide. However, the present study did not examine the mechanism of NAD(P)H oxidase activation by highly concentrated insulin in mesangial cells. Further study is needed to clarify this issue.

NAD(P)H oxidase activation leads in turn to the activation of ERK-1/2 (Goldstein et al., 2005). Lin et al. (2006) report that NAD(P)H oxidase activation induced Ras phosphorylation, which acts as a crucial regulator in the transmission of ERK-1/2 signaling by high glucose and advances glycation products in mesangial cells. We also confirmed that doses greater than 0.01 μM of insulin activate ERK-1/2 in mesangial cells through NAD(P)H activation. However, we cannot exclude the possibility that insulin activation of ERK-1/2 is directly or indirectly regulated by other membrane-bound signaling molecules.

Recently, several studies have reported that azelnidipine reduces the intracellular production of reactive oxygen species. Matsui et al. (2005) demonstrated that azelnidipine inhibited the generation of angiotensin II-induced reactive oxygen species in human adult skin microvascular endothelial cells. Moreover, azelnidipine reduced the intensity of 1,1′-diphenyl-2-picrylhydrazyl (DPPH) free radicals as determined by an electron spin resonance (ESR) spectrometer, and inhibited superoxide and hydroxyl radical-scavenging activity measured by an ESR assay using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap (Naito et al., 2006). Although the mechanism by which azelnidipine acts and scavenges radicals is not fully understood, it is generally accepted that oxidation of the dihydropyridine ring can donate electrons to the radicals, reducing them to a non-reactive form (Cominacini et al., 2003).

In the present study, we showed that azelnidipine blocked the production of intracellular reactive oxygen species more effectively than nifedipine. We attribute this difference in the antioxidative effect to variations in the chemical structure of the two drugs. The reason for the stronger antioxidative action of azelnidipine may be due to its higher lipid solubility and vascular membrane affinity (Yoram and Ahuva, 1995).

In conclusion, high concentrations (over 0.01 μM) of insulin induced the production of reactive oxygen species, leading to the activation of ERK-1/2 and mesangial cell proliferation. Azelnidipine, a highly lipid soluble dihydropyridine-based calcium channel blocker, preferentially blocked the insulin-induced production of reactive oxygen species, resulting in an attenuation of mesangial cell proliferation. These results suggest that azelnidipine may have the potential to protect against the onset of diabetic nephropathy and delay its progression.

References

Baron, A.D., 1994. Hemodynamic actions of insulin. Am. J. Physiol. 267, E187–E202.

Baynes, J.W., Thorpe, S.R., 1999. Role of oxidative stress in diabetic complications. Diabetes 48, 1–9.

Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813–820.

Cockcroft, J.R., 2005. Exploring vascular benefits of endothelium-derived nitric oxide. Am. J. Hypertens. 18, 177S–183S.

Cominacini, L., Pasini, A.F., Garbin, U., Pastorino, A.M., Davoli, A., Nava, C., Campagnole, M., Rossato, P., Cascio, V.L., 2003. Antioxidant activity of different dihydropyridines. Biochem. Biophys. Res. Commun. 302, 679–684.

Despres, J.P., Lamarche, B., Mauriege, P., Cantin, B., Dagenais, G.R., Moorjani, S., Jupien, P.J., 1996. Hyperinsulinemia as an independent risk factor for ischemic heart disease. N. Engl. J. Med. 334, 952–957.

Fukuoka, T., Kitami, Y., Okura, T., Hiwada, K., 1999. Transcriptional regulation of platelet-derived growth factor α receptor gene via CCAAT/enhancer-binding protein-δ in vascular smooth muscle cells. J. Biol. Chem. 274, 25576–25582.

Goldstein, B.J., Mahadev, K., Wu, X., Zhu, L., Motoshima, H., 2005. Role of insulin-induced reactive oxygen species in the insulin signal pathway. Antioxid. Redox Signal. 7, 1021–1031.

Ha, H., Kim, K.H., 1999. Pathogenesis of diabetic nephropathy: the role of oxidative stress and protein kinase C. Diabetes Res. Clin. Pract. 45, 147–151.

Lin, C.L., Wang, F.S., Kuo, Y.R., Huang, Y.H., Huang, H.C., Sun, Y.C., Kuo, Y.H., 2006. Ras modulation of superoxide activates ERK-dependent fibronectin expression in diabetes-induced renal injuries. Kidney Int. 69, 1593–1600.

Locatelli, F., Pozzoni, P., Del Vecchio, L., 2004. Renal replacement therapy in patients with diabetes and end-stage renal disease. J. Am. Soc. Nephrol. 15 (Suppl. 1), S25–S29.

Mahadev, K., Motoshima, H., Wu, X., Ruddy, J.M., Arnold, R.S., Cheng, G., Lambeth, J.D., Goldstein, B.J., 2004. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H_2O_2 and plays an integral role in insulin signaling transduction. Mol. Cell. Biol. 24, 1844–1854.

Matsui, T., Yamagishi, S., Nakamura, K., Kikuchi, S., Inoue, H., 2005. Azelnidipine, a dihydropyridine-based calcium antagonist, inhibits angiotensin II-induced oxidative stress generation and downregulation of pigment epithelium-derived factor mRNA levels in microvascular endothelial cells. Drug. Exp. Clin. Res. 31, 215–219.

Meyer, J.W., Schmitt, M.E., 2000. A central role for the endothelial NADPH oxidase in atherosclerosis. FEBS Lett. 472, 1–4.

Misra, R.P., 1972. Isolation of glomeruli from mammalian kidneys by graded sieving. Am. J. Clin. Pathol. 58, 135–139.

Mogensen, C.E., Cooper, M.E., 2004. Diabetic renal disease: from recent studies to improved clinical practice. Diabet. Med. 21, 4–17.

Naito, Y., Shimozawa, M., Manabe, H., Nakabe, N., Katada, K., Kokura, S., Yoshida, N., Ichikawa, H., Kon, T., Yoshikawa, T., 2006. Azelnidipine, a new calcium channel blocker, inhibits endothelial inflammatory response by reducing intracellular levels of reactive oxygen species. Eur. J. Pharmacol. 28, 11–18.

Okura, T., Igase, M., Kitami, Y., Fukuoka, T., Maguchi, M., Kohara, K., Hiwada, K., 1998. Platelet-derived growth factor induces apoptosis in vascular smooth muscle cells: roles of the Bcl-2 family. Biochim. Biophys. Acta 1403, 245–253.

Pyorala, M., Miettinen, H., Laakso, M., Pyorala, K., 1998. Hyperinsulinemia predicts coronary heart disease risk in healthy middle-aged men: the 22-year follow-up results of the Helsinki Policemen Study. Circulation 98, 398–404.

Reaven, G.M., 2003. Insulin resistance/compensatory hyperinsulinemia, essential hypertension, and cardiovascular disease. J. Clin. Endocrinol. Metab. 88, 2399–2403.

Robinson, M.J., Cobb, M.H., 1997. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol. 9, 180–186.

Sarafidis, P.A., Ruilope, L.M., 2006. Insulin resistance, hyperinsulinemia, and renal injury: mechanisms and implications. Am. J. Nephrol. 26, 232–244.

Wesson, L.G., 1988. Physical factors and glomerulosclerosis. Cause or coincidence? Nephron 78, 125–130.

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

Yang, Z.H., Kitami, Y., Takata, Y., Okura, T., Hiwada, K., 2001. Targeted overexpression of CCAAT/enhancer-binding protein-δ evokes enhanced gene transcription of platelet-derived growth factor α-receptor in vascular smooth muscle cells. Circ. Res. 89, 503–508.

Yoram, Y., Ahuva, L., 1995. Azelnidipine (CS-905), a novel dihydropyridine calcium channel blocker with gradual onset and prolonged duration of action. Cardiovasc. Drug. Rev. 13, 137–148.