Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 334 (2005) 218–222
BBRC
www.elsevier.com/locate/ybbrc

Erythropoietin attenuated high glucose-induced apoptosis in cultured human aortic endothelial cells

Naotaka Sekiguchi, Toyoshi Inoguchi *, Kunihisa Kobayashi, Noriyuki Sonoda, Hajime Nawata

Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Received 15 June 2005
Available online 27 June 2005

Abstract

High glucose-induced apoptosis in vascular endothelial cells may contribute to the acceleration of atherosclerosis associated with diabetes. Here, we show that erythropoietin attenuates high glucose-induced apoptosis in cultured human aortic endothelial cells (HAECs). Exposure of HAECs to high glucose level for 72 h significantly increased the number of apoptotic cells compared with normal glucose level, as evaluated by TUNEL assay. Simultaneous addition of erythropoietin (100 U/ml) significantly attenuated high glucose-induced apoptosis. In parallel, exposure to high glucose level induced caspase-3 activation and erythropoietin also prevented it. Erythropoietin stimulated Akt phosphorylation in a dose-dependent manner (1–100 U/ml). PI3 kinase inhibitor, wortmannin or LY294002 eliminated erythropoietin's inhibitory effect on caspase-3 activity. In conclusion, erythropoietin may attenuate high glucose-induced endothelial cell apoptosis via PI-3 kinase pathway. Replacing therapy with erythropoietin is often used for correction of renal anemia, but may have potential in preventing atherosclerosis in diabetic patients with end-stage renal failure.

Keywords: Erythropoietin; Apoptosis; Endothelial cell; Diabetic nephropathy

High glucose level has been shown to induce cell apoptosis in retina [1–3], kidney [4], neuron [5], and vascular endothelial cells [6–9], which are supposed to be related to the acceleration of diabetic complications and atherosclerosis.

Erythropoietin is produced mainly in the kidney and regulates erythrocyte production. It binds to a cell surface receptor and leads to intracellular activation of several kinase pathways. Recently, erythropoietin has been reported to inhibit apoptosis of neurons [10,11] and cardiomyocytes [12] induced by ischemia and metabolic abnormalities as well as erythroid cells [13,14]. Clinically, it is well known that cardiovascular disease dramatically accelerates in diabetic patients with end-stage renal failure in which the production of erythropoietin is reduced. It appears that such a high prevalence of cardiovascular disease could not be explained by traditional risk factors such as hypertension, lipid abnormalities, and coagulation alterations which are prevalent in those patients. Since erythropoietin's receptors are present on the membrane of vascular endothelial cells [15], we speculated that erythropoietin might have an anti-apoptotic effect on vascular endothelial cells. Reduced production of erythropoietin may in part account for the acceleration of atherosclerosis in diabetic patients with end-stage renal failure.

Therefore, in this study, we examined whether erythropoietin attenuated high glucose-induced apoptosis in cultured human aortic endothelial cells and then explored the mechanisms for its anti-apoptotic effect.

* Corresponding author. Fax: +81 92 642 5287.
E-mail address: toyoshi@intmed3.med.kyushu-u.ac.jp (T. Inoguchi).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.06.072

Materials and methods

Cell culture and treatments. Human aortic endothelial cells were purchased from Clonetics (Walkersville, MD). The endothelial cells were cultured in endothelial cell basal medium (EBM; Cambrex, Walkersville, MD) supplemented with hFGF-B, VEGF, IGF-1, ascorbic acid, hEGF, hydrocortisone, and 20% fetal bovine serum (FBS). For the experiments, the cells from 2nd to 7th passages were transferred on chamber slides or culture dishes and then incubated with test media. Human recombinant erythropoietin was kindly provided by Chugai Pharmacological, Tokyo, Japan. At the indicated time, apoptosis and caspase-3 activities in the cells were evaluated as follows.

Evaluation of apoptotic cells: TUNEL assay. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. Human aortic endothelial cells were cultured with test media containing 2% FBS and normal (100 mg/dl) or high (450 mg/dl) glucose with or without erythropoitin (100 U/ml) on chamber slides. After incubation for 72 h, the cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS). Then, the slides were processed for a TUNEL assay. An ApopTag in situ detection kit from Serologicals (Norcross, GA) was used according to the manufacturer's instructions. Briefly, the slides were treated with H_2O_2 and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37 °C. Labeled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody using 3,3′-diaminobenzidine (DAB) as the chromogen. After washing in dH_2O, the cells were counterstained in 0.5% methyl green and the specimens were mounted with coverslips. Then, one hundreds endothelial cells were counted under a microscope, and the percentages of brown colored TUNEL-positive cells were quantified.

Measurement of caspase-3 activity. Caspase-3 activity assay kit from Oncogene Research Products (Boston, MA) was used according to the manufacturer's instructions. Briefly, after reaching confluent on 96-well plate, the endothelial cells were incubated with test media containing 2% FBS and normal (100 mg/dl) or high (450 mg/dl) glucose with or without erythropoietin (100 U/ml) for 72 h in accordance with TUNEL assay. Assay buffer containing 10 mM DTT was applied on the cells, followed by the addition of caspase-3 fluorescent substrate conjugate, which is DEVD (Asp-Glu-Val-Asp) peptide labeled with fluorescent 7-amino-4-trifluoromethyl coumarin. The plate was covered and incubated at 37 °C for 2 h, and fluorescence of 400 nm excitation and 505 nm emission was read using a fluorescent plate reader.

Detection of phosphorylated Akt. To investigate a possible mechanism of erythropoietin's action, Akt phosphorylation was evaluated by Western blot analysis. Endothelial cells were plated on six-well plate and incubated until the cells reached confluent on the well. The cells were exposed to erythropoietin (1–100 U/ml) for 0–30 min. After incubation with erythropoietin, the cells were lysed using RIPA buffer containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate (Sigma, St. Louis, MI). The homogenates were centrifuged at 10,000*g* for 20 min to precipitate cell debris. For Western blot analysis, the samples were denatured by boiling in Laemmli sample buffer and 40 μg of extracted protein were subjected to SDS–PAGE, and subsequently transferred to nitrocellulose filters. Filters were probed using anti-phosphoAkt antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed by exposing to HRP-conjugated anti-rabbit secondary antibodies (Amersham BioSciences, Piscataway, NJ). Antigen–antibody complexes were then visualized by a chemiluminescent reaction (ECL plus, Amersham BioSciences). The intensity was measured and quantitated.

Inhibition of phosphatidylinositol-3 kinase pathway. To determine whether erythropoietin plays an anti-apoptotic effect via PI-3 kinase pathway, we examined the effect of phosphatidylinositol-3 (PI-3) kinase inhibitor on erythropoietin's anti-apoptotic effect. The cells were incubated with test media containing high glucose and erythropoietin (100 U/ml) with or without wortmannin (200 nM) (Sigma) or LY294002 (20 μM) (Sigma). The effects of PI-3 kinase inhibitor on caspase-3 activities were evaluated.

Statistical analysis. All values were presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by comparisons using Bonferroni's method. Differences were considered to be significant at $p < 0.05$.

Results

TUNEL assay

We determined the effect of high glucose level on apoptosis in cultured endothelial cells by TUNEL

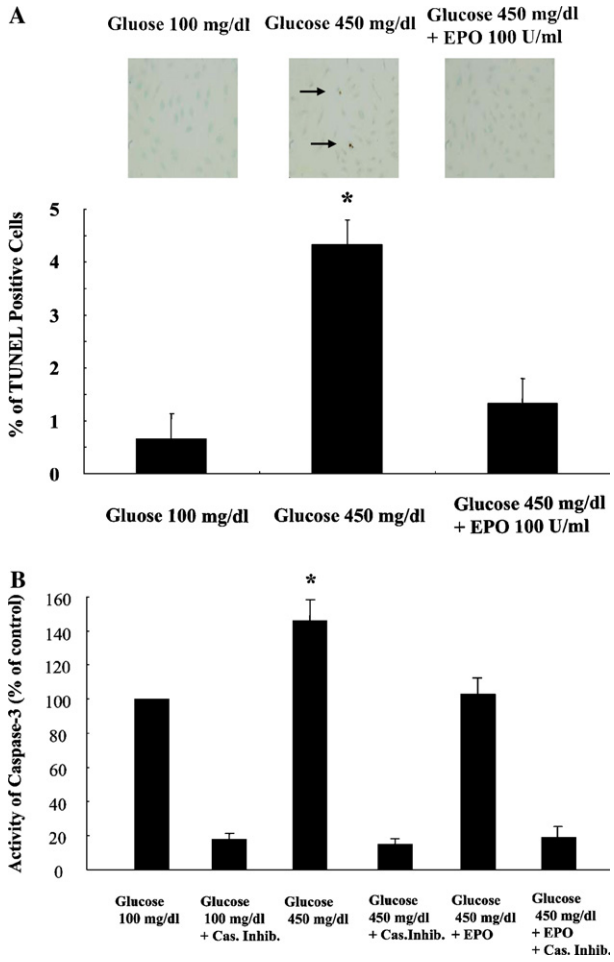

Fig. 1. Effect of erythropoietin on high glucose-induced apoptosis (A) and caspase-3 activity (B) in cultured human aortic endothelial cells. Apoptosis was evaluated by TUNEL assay. Human aortic endothelial cells were cultured with test media containing 2% FBS and normal (100 mg/dl) or high (450 mg/dl) glucose with or without erythropoietin (EPO) (100 U/ml). Apoptosis was expressed as percentage of brown colored TUNEL-positive cells. Caspase-3 activity was expressed as percentage of control (glucose 100 mg/dl). As negative control, Ac-DEVD-CHO, caspase-3 inhibitor (Cas.Inhib), was used. Results are expressed as means ± SEM from four independent experiments. *$p < 0.05$ vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

assay. The number of apoptotic cells was expressed quantitatively as a percentage of TUNEL positive cells. As shown in Fig. 1A, exposure of the cells to high glucose level (450 mg/dl) for 72 h induced a significant increase in the number of brown colored TUNEL-positive cells compared with normal glucose culture (100 mg/dl) (glucose 100 mg/dl; $0.7 \pm 0.5\%$ vs. glucose 450 mg/dl; $4.3 \pm 0.5\%$, $p < 0.05$). However, when erythropoietin (100 U/ml) was simultaneously added to high glucose media, the number of the apoptotic cells was significantly decreased (glucose 450 mg/dl + erythropoietin 100 U/ml; $1.3 \pm 0.5\%$, $p < 0.05$).

Caspase-3 activity

Since caspase-3 is considered as a key enzyme in the process of cell apoptosis, caspase-3 activities was evaluated in parallel with TUNEL assay. In consistency with the result of TUNEL assay, exposure to high glucose level for 72 h caused caspase-3 activation in endothelial cells (Fig. 1B). As compared with the activity in the cells incubated with normal glucose level, caspase-3 activity was significantly ($p < 0.05$) increased by 1.46-fold in the cells incubated with high glucose level. Simultaneous addition of erythropoietin (100 U/ml) to high glucose media significantly ($p < 0.05$) attenuated the caspase-3 activity to the control level.

Phosphorylation of Akt

To explore the mechanisms for anti-apoptotic effect of erythropoietin, we examined the effect of erythropoietin on Akt phosphorylation in endothelial cells. Erythropoietin stimulated Akt phosphorylation in a dose-dependent manner (1–100 U/ml) (Fig. 2A). The

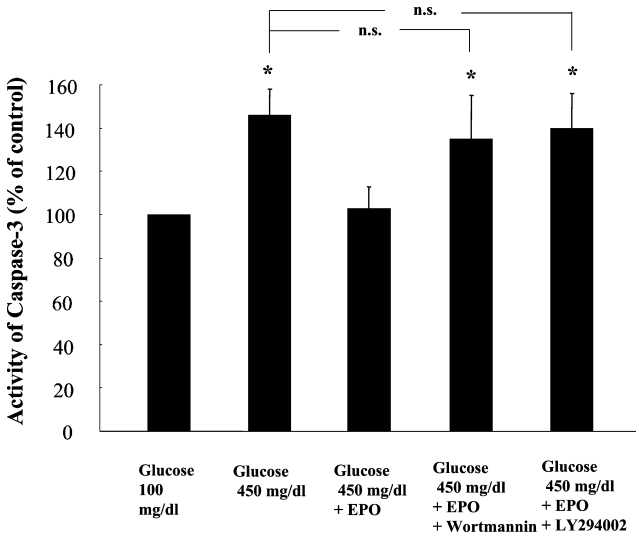

Fig. 3. Effect of PI-3 kinase inhibitor on erythropoietin's anti-apoptotic effect. Human aortic endothelial cells were cultured with test media containing 2% FBS and normal (100 mg/dl) or high (450 mg/dl) glucose with or without erythropoietin (EPO) (100 U/ml). PI-3 kinase inhibitor, wortmannin (200 nM) or LY294002 (20 μM), was simultaneously added to test media containing high glucose level (450 mg/dl) and erythropoietin (EPO) (100 U/ml). Caspase-3 activity was expresses as percentage of control (glucose 100 mg/dl). Results are expressed as means ± SEM from four independent experiments. *$p < 0.05$ vs. control.

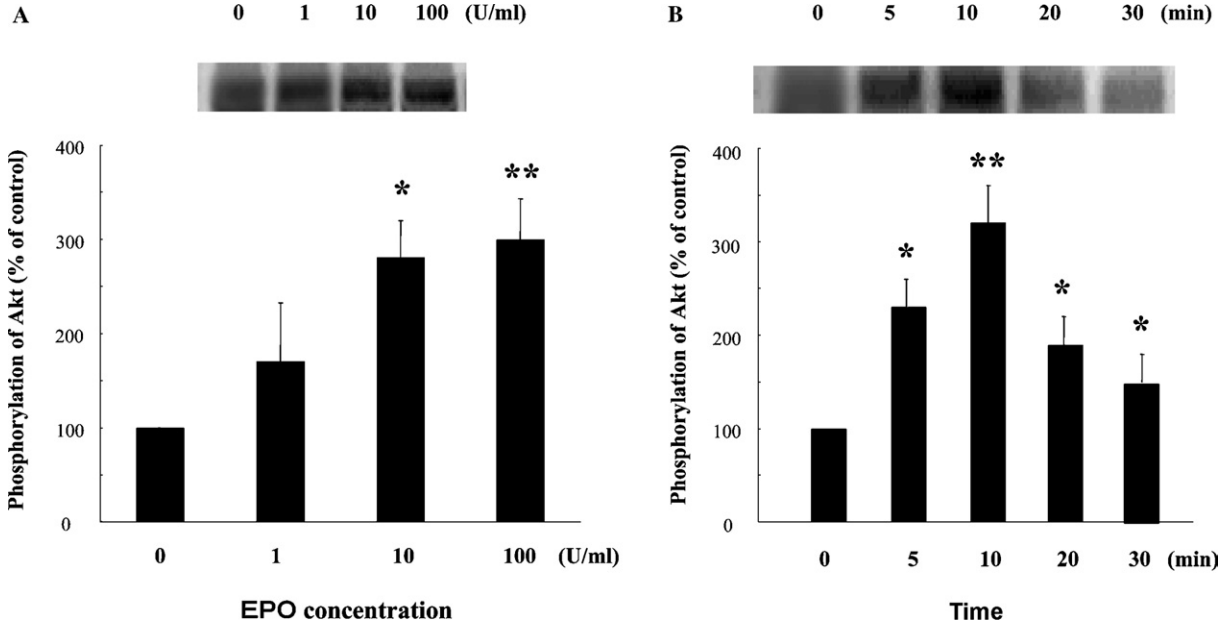

Fig. 2. Stimulatory effect of erythropoietin on Akt phosphorylation. (A) Dose–response: human aortic endothelial cells were exposed to erythropoietin (EPO) at concentrations of 0, 1, 10, and 100 U/ml for 10 min. Akt phosphorylation was evaluated by Western blot analysis using anti-phosphoAkt antibody. (B) Time course: the cells were exposed to erythropoietin (100 U/ml) for 0, 5, 10, 20, and 30 min. Results are expressed as mean percentage of control ± SEM from three independent experiments. *$p < 0.05$; **$p < 0.01$ vs. control.

stimulatory effect reached the peak at 10 min after addition of erythropoietin and lasted up to 30 min (Fig. 2B).

Inhibition of PI-3 kinase pathway

According to the result that erythropoietin induced Akt phosphorylation, it is suggested that erythropoietin may have an anti-apoptotic effect through PI-3 kinase pathway. To confirm this notion, we examined the effect of PI-3 kinase inhibitor, wortmannin (200 nM) or LY294002 (20 μM) on the inhibitory effect of erythropoietin on caspase-3 activity. Erythropoietin's effect on high glucose-induced caspase-3 activity was completely eliminated by PI-3 kinase inhibitor and the caspase-3 activity was returned to the level of high glucose culture (Fig. 3).

Discussion

A growing body of evidence has shown that high glucose induces various vascular endothelial cell dysfunctions in diabetes. Among them, high glucose-induced endothelial cell apoptosis has been noted in the pathogenesis of the acceleration of atherosclerosis associated with diabetes [6,7]. In the present study, we also showed that high glucose-induced apoptosis and activation of caspase-3, which is a central component of the proteolytic cascade, plays a pivotal role in the execution of apoptosis [16,17] in human aortic endothelial cells. As the possible mechanism for high glucose-induced endothelial cell apoptosis, increased reactive oxygen species (ROS) production has been indicated [18–20,7]. ROS is thought to be capable of activate c-Jun NH_2-terminal kinases (JNK)/stress-activated protein kinases (SAPK), which can activate caspase-3 and regulate apoptosis in certain cells [21,22]. In fact, one report showed that addition of antioxidants to cultured endothelial cells suppressed JNK activity, caspase-3 activity and the subsequent apoptosis induced by high glucose [7]. These results suggest that high glucose-induced endothelial cell apoptosis may be mediated by activation of JNK/SAPK induced by ROS and subsequent activation of caspase-3.

Recently, erythropoietin has been identified as an important mediator of the adaptive responses to metabolic stress. It is also noted that erythropoietin possesses potent neuro-protective properties in experimental cerebral, retinal, and motor neuron ischemia [10,11,23,24] and ischemic cardiomyocytes [12]. Since erythropoietin receptors are expressed on vascular endothelial cells, erythropoietin might possess vascular-protective effect. In the present study, we showed for the first time that erythropoietin attenuated high glucose-induced apoptosis in cultured human aortic endothelial cells. Concerning the regulatory mechanisms for apoptosis, several signal transduction pathways are involved. Akt, which is a downstream of PI-3 kinase, are thought to be an important factor of cell survival [25], because Akt activation ultimately results in up-regulation of the bcl family of anti-apoptotic genes [26,27]. Based on this conception, we examined the contribution of PI-3 kinase pathway to the anti-apoptotic effect of erythropoietin. Erythropoietin phosphorylated Akt in a dose-dependent manner, suggesting the PI-3 kinase activation by erythropoietin. In addition, blockade of PI-3 kinase by wortmannin or LY294002 eliminated the erythropoietin's effect on caspase-3 activity and apoptosis in endothelia cells. Taken together, these results suggest that erythropoietin may inhibit high glucose-induced endothelial cell apoptosis via PI-3 kinase pathway.

Cardiovascular disease remains the main cause of morbidity and mortality in diabetic patients with end-stage renal failure. Although traditional risk factors such as hypertension, lipid abnormalities, and coagulation alterations are prevalent in diabetic patients with end-stage renal failure, they may not be sufficient to account for the high prevalence. The present results imply that reduced production of erythropoietin which is seen in diabetic patients with end-stage renal failure may enhance high glucose-induced apoptosis of endothelial cells and this may in part account for the acceleration of atherosclerosis. Although replacing therapy with erythropoietin is often used for correction of renal anemia in diabetic patients with end-stage renal failure, this therapy may also have potential in preventing the acceleration of atherosclerosis. Clinical studies should be done to confirm the anti-atherosclerotic effect of replacing therapy with erythropoietin in diabetic patients with end-stage renal failure.

In conclusion, erythropoietin can inhibit high glucose-induced apoptosis of endothelial cells via PI-3 kinase pathway. These results suggest that replacing therapy with erythropoietin may inhibit endothelial cell apoptosis and prevent the acceleration of atherosclerosis in diabetic patients with end-stage renal failure.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research (No. 16590888) from the Ministry of Education, Science and Culture, Japan.

References

[1] M. Mizutani, T.S. Kern, M. Lorenzi, Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy, J. Clin. Invest. 97 (1996) 2883–2890.

[2] F. Podesta, G. Romeo, W.H. Liu, S. Krajewski, J.C. Reed, C. Gerhardinger, M. Lorenzi, Bax is increased in the retina of diabetic subjects and is associated with pericyte apoptosis in vivo and in vitro, Am. J. Pathol. 156 (2000) 1025–1032.

[3] S. Mohr, X. Xi, J. Tang, T.S. Kern, Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients, Diabetes 51 (2002) 1172–1179.
[4] S. Yamagishi, Y. Inagaki, T. Okamoto, S. Amano, K. Koga, M. Takeuchi, Z. Makita, Advanced glycation end product-induced apoptosis and overexpression of vascular endothelial growth factor and monocyte chemoattractant protein-1 in human-cultured mesangial cells, J. Biol. Chem. 277 (2002) 20309–20315.
[5] A.M. Schmeichel, J.D. Schmelzer, P.A. Low, Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy, Diabetes 52 (2003) 165–171.
[6] S.M. Baumgartner-Parzer, L. Wagner, M. Pettermann, J. Grillari, A. Gessl, W, Waldhausl, High-glucose-triggered apoptosis in cultured endothelial cells, Diabetes 44 (1995) 1323–1327.
[7] F.M. Ho, S.H. Liu, C.S. Liau, P.J. Huang, S.Y. Lin-Shiau, High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3, Circulation 101 (2000) 2618–2624.
[8] C. Min, E. Kang, S.H. Yu, S.H. Shinn, Y.S. Kim, Advanced glycation end products induce apoptosis and procoagulant activity in cultured human umbilical vein endothelial cells, Diabetes Res. Clin. Pract. 46 (1999) 197–202.
[9] M. Artwohl, W.F. Graier, M. Roden, M. Bischof, A. Freudenthaler, W. Waldhausl, S.M. Baumgartner-Parzer, Diabetic LDL triggers apoptosis in vascular endothelial cells, Diabetes 52 (2003) 1240–1247.
[10] M. Celik, N. Gokmen, S. Erbayraktar, M. Akhisaroglu, S. Konakc, C. Ulukus, S. Genc, K. Genc, E. Sagiroglu, A. Cerami, M. Brines, Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury, Proc. Natl. Acad. Sci. USA 99 (2002) 2258–2263.
[11] A.L. Siren, M. Fratelli, M. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, T. Mennini, R. Heumann, A. Cerami, H. Ehrenreich, P. Ghezzi, Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress, Proc. Natl. Acad. Sci. USA 98 (2001) 4044–4049.
[12] C.J. Parsa, A. Matsumoto, J. Kim, R.U. Riel, L.S. Pascal, G.B. Walton, R.B. Thompson, J.A. Petrofski, B.H. Annex, J.S. Stamler, W.J. Koch, A novel protective effect of erythropoietin in the infarcted heart, J. Clin. Invest. 112 (2003) 999–1007.
[13] M.J. Koury, M.C. Bondurant, Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progentior cells, Science 248 (1990) 378–381.
[14] K. Muta, S.B. Krantz, Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin, J. Cell Physiol. 156 (1993) 264–271.
[15] A. Anagnostou, Z. Liu, M. Steiner, K. Chin, E.S. Lee, N. Kessimian, C.T. Noguchi, Erythropoietin receptor mRNA expression in human endothelial cells, Proc. Natl. Acad. Sci. USA 91 (1994) 3974–3978.
[16] X. Wang, N.G. Zelenski, J. Yang, J. Sakai, M.S. Brown, J.L. Goldstein, Cleavage of sterol regulatory element binding protein (REBPs) by CPP32 during apoptosis, EMBO J. 15 (1996) 1012–1020.
[17] S. Kumar, The apoptotic cysteine protease CPP32, Int. J. Biochem. Cell Biol. 29 (1997) 393–396.
[18] F. Cosentino, K. Hishikawa, Z.S. Katusic, T.F. Luscher, High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells, Circulation 96 (1997) 25–28.
[19] T. Inoguchi, P. Li, F. Umeda, H.Y. Yu, M. Kakimoto, M. Imamura, T. Aoki, T. Etoh, T. Hashomoto, M. Naruse, H. Sano, H. Utsumi, H. Nawata, High glucose and 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.
[20] X.L. Du, G.Z. Sui, K. Stockklauser-Farber, et al., Introduction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells is mediated by reactive oxygen species, Diabetologia 41 (1998) 249–256.
[21] X. Wang, J.L. Martindale, Y. Liu, N.J. Holbrook, The cellular response to oxidative stress: influences of mitogen-activated protein kinase signaling pathways on cell survival, Biochem. J. 333 (1998) 291–300.
[22] N.R. Bhat, P. Zhag, Hydrogen peroxide activation of multiple mitogen-activated protein kinase in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death, J. Neurochem. 72 (1999) 112–119.
[23] M.L. Brines, P. Ghezzi, S. Keenan, D. Agnello, N.C. de Lanerolle, C. Cerami, L.M. Itri, A. Cerami, Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury, Proc. Natl. Acad. Sci. USA 97 (2000) 10526–10531.
[24] C. Grimm, A. Wenzel, M. Groszer, H. Mayser, M. Seeliger, M. Samardzija, C. Bauer, M. Gassmann, C.E. Reme, HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration, Nat. Med. 8 (2002) 718–724.
[25] W. Wu, W.L. Lee, Y.Y. Wu, D. Chen, T.J. Liu, A. Jang, P.M. Sharma, P.H. Wang, Expression of constitutively active phosphatidylinositol 3-kinase inhibits activation of caspase 3 and apoptosis of cardiac muscle cells, J. Biol. Chem. 275 (2000) 40113–40119.
[26] J.C. Reed, Cytochrome *c*: can't live with it–can't live without it, Cell 91 (1997) 559–562.
[27] J.C. Reed, Double identity for proteins of the Bcl-2 family, Nature 387 (1997) 773–776.