five fields/slide was determined in three independent experiments.

Western Blot Analysis

Western Blot Analysis was performed according to Sharma et al.[11] Cells were washed three times with PBS, harvested, and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing protease inhibitor mixture (Roche Diagnostics). Cells were disrupted in a syringe fitted with a 21-gauge needle, with cell debris removed by centrifugation at 13000 rpm for 15 min at 4°C. Protein samples were prepared in SDS-PAGE sample buffer, and equal amounts of protein were loaded onto the gel.

After 10% SDS-PAGE, the separated proteins were blotted onto pure nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Hercules, CA, USA). The membrane was then blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS and incubated overnight at 4°C with primary antibody.

All antibodies were obtained from commercial sources and used at the following dilutions: anti-GRP78/Bip antibody (BD Bio sciences, San Jose, CA, USA), 1:1000; anti-ATF4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:250; anti-CHOP antibody (Santa Cruz Biotechnology), 1:250; anti-procaspase-12 antibody (Cell Signaling Technology, Beverly, MA, USA), 1:1000; anti-procaspase-3 antibody (Cell Signaling Technology), 1:1000. The filters were washed three times with PBS-T and incubated with secondary antibody (Santa Cruz Biotechnology) labeled with horseradish peroxidase for 1 hr. The specific protein bands were made visible by incubating the membrane with luminol reagent (Santa Cruz Biotechnology) and exposing the membrane to x-ray film (CL-XposureTM Film; Pierce, Rockford, IL, USA). To ascertain the comparative expression and equal loading of these protein samples, the membrane was re-probed with antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Novus Biological, Littleton, CO, USA) as an internal control.

Statistical Analyses

Statistical analyses were performed using a one-way ANOVA, and a value of $p < 0.05$ was considered to be statistically significant.

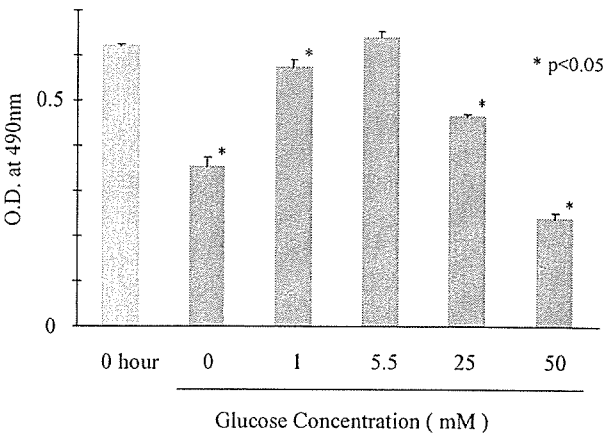

FIGURE 1 **Effect of glucose concentration on the viability of rat retinal pericytes grown for 24 hr in DMEM media containing 0 to 50 mM of glucose. Cell viability was determined by the MTS assay at 490 nm to be significantly lower at all levels higher or lower than 5.5 mM ($p < 0.05$). 0 hour represents the initial cell number. Each bar represents the average of three experiments. Mean ± SD.**

RESULTS

Glucose Concentrations Regulate Cellular Growth and Apoptosis

The percentage of living pericytes cultured for 24 hr with 0, 1, 5.5, 25, or 50 mM of glucose was determined with the MTS assay. The growth inhibition and death of pericytes cultured with 0, 1, 25, and 50 mM of glucose are summarized in Figure 1. Significant decreases in cell viability were observed at all concentrations of media glucose higher or lower than 5.5 mM glucose. After 24 hr of culture in media containing no (0 mM) or high (25 mM) glucose, approximately 15–18% of the pericytes demonstrated TUNEL positive staining (Fig. 2). These results suggested that apoptosis is induced in some of the pericytes cultured with medium containing either no or high glucose.

UPR-Specific Enzymes are Activated in Glucose-Deprived Pericytes

To determine whether a UPR is induced in pericytes subjected to glucose imbalance, protein blot analysis of the SDS-PAGE of the cell homogenates was conducted with antibodies against four enzymes specific for the UPR, viz., GRP78/Bip, CHOP, ATF4, and procaspase-12. In addition, the general apoptotic biomarker, procaspase-3, was also investigated.

GRP78/Bip and CHOP were upregulated in pericytes cultured in the absence of glucose (0 mM), but not

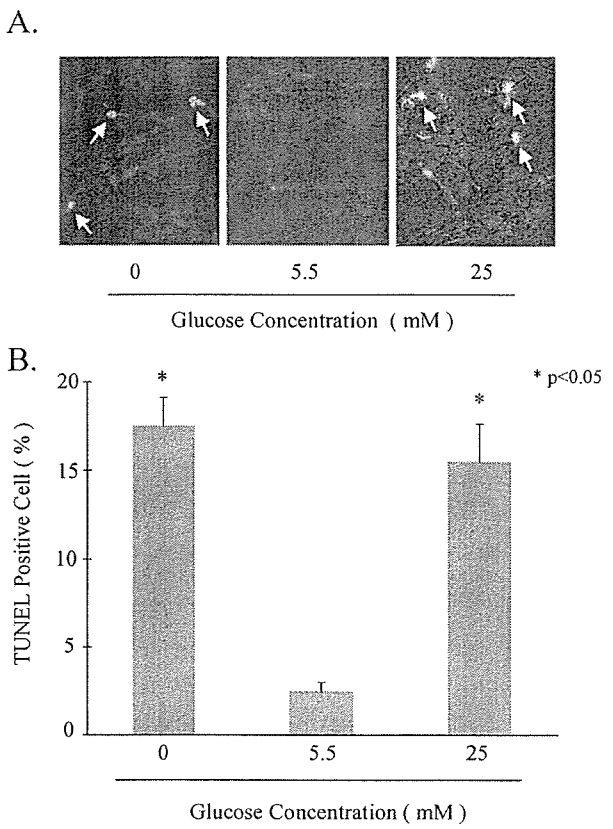

FIGURE 2 TUNEL staining of retinal pericytes cultured in different concentrations of glucose. (A) TUNEL staining of retinal pericytes cultured for 24 hr in DMEM media containing 0, 5.5, or 25 mM of glucose. Increased TUNEL staining is observed in cells cultured under glucose deprived (0 mM) and high glucose (25 mM) conditions compared with normoglycemic (5.5 mM) glucose levels. Arrows point to apoptotic cells. (B) Summary of percentage of TUNEL positive staining cells cultured for 24 hr in DMEM media containing 0, 5.5, or 25 mM of glucose. TUNEL staining was significantly increased in cells cultured at either 0 or 25 mM glucose compared with 5.5 mM glucose ($p < 0.05$). Each bar represents the average of three experiments. Mean ± SD.

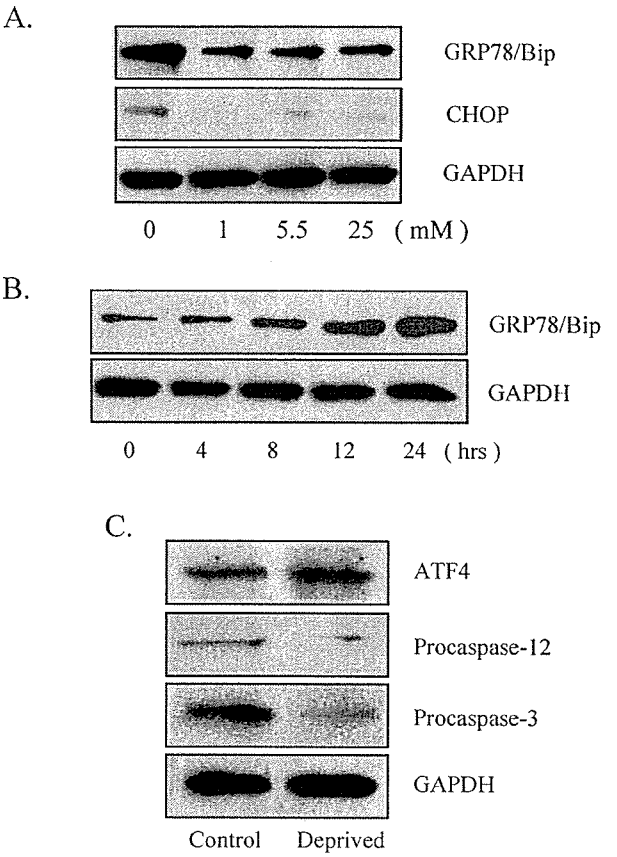

FIGURE 3 Protein blot analysis of pericytes cultured in different concentrations of glucose. (A) Protein blot of GRP78/Bip and CHOP in rat pericytes cultured for 24 hr in DMEM media containing 0, 1, 5.5, or 25 mM of glucose. (B) Protein blot illustrating time-dependent activation of GRP78/Bip in pericytes cultured up to 24 hr under glucose-deprived (0 mM) conditions. (C) Protein blot comparing the levels of the ER stress marker, ATF4, and procaspase-12 and procaspase-3 in pericytes cultured up to 24 hr under glucose-deprived (0 mM) conditions. Equal amounts of protein were loaded on each well, and the expression of GAPDH was used as an internal control. The protein blots are representative of the results obtained from three independent experiments.

in pericytes cultured with 1, 5.5, or 25 mM of glucose (Fig. 3A). The increase in GRP78/Bip levels in pericytes was time dependent (Fig. 3B). The effect of culturing pericytes in extremely high glucose medium (100 and 250 mM) for periods of 6–48 hr was also examined. Under these hyperglycemic conditions, pericyte death was observed; however, GRP78/Bip and CHOP levels remained unchanged (data not shown).

Pericytes cultured for 24 hr in the absence of glucose also showed an upregulation of ATF4, a transcriptional activator for CHOP. In addition, procaspase-12 and procapsase-3 were cleaved, indicating that caspase-12 and caspase-3 (Fig. 3C) were also activated. These biochemical data indicated that the UPR pathway was activated by the absence of glucose in retinal pericytes. In all of these experiments, a similar expression level of GAPDH was observed indicating that equal samples of pericyte homogenates were compared.

Rapid Glucose Deprivation Results in Increased Levels of UPR Activation

In uncontrolled hyperglycemic patients with diabetes mellitus, the glucose level can fluctuate rapidly with insulin treatment resulting in hypoglycemia. To investigate whether fluctuations in the glucose environment can affect pericytes, groups of pericytes were cultured for 1 day in DMEM containing 5.5 mM glucose and switched to a medium containing 25 mM glucose

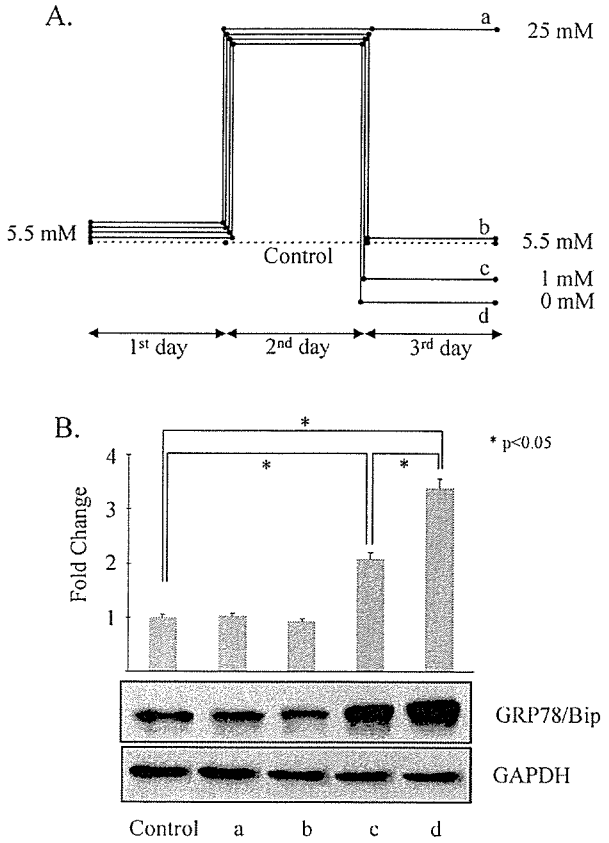

FIGURE 4 A shift from a higher to lower concentration of glucose strongly induces the UPR. (A) Diagram summarizing changes in culture time and media glucose concentrations of pericytes in groups a–d. (B) Protein blot analyses of GRP78/Bip and GAPDH levels of retinal pericytes from groups a–d. Equal amounts of protein were loaded on each well. The photographs are representative of the results obtained from three independent experiments. Bar indicates the average fold change. Mean ± SD. * Indicates significant difference ($p < 0.05$).

on the second day, and then on the third day some cells were left in the 25 mM glucose media (group a) while the other cells were placed in either 5.5 mM (group b), 1 mM (group c), or 0 mM (group d). In the normal control group, glucose levels in the DMEM medium were maintained at 5.5 mM for the entire duration of the study (Fig. 4A). On the fourth day, all cells were harvested and analyzed by protein blot using antibody against GRP78/Bip. As indicated in Figure 4B, the GRP78/Bip levels were not upregulated in pericytes maintained under high glucose conditions (25 mM glucose, group a) or those returned from high, 25 mM glucose to normal levels (5.5 mM, group b). This is consistent with observation that ER stress is not induced by either 25 mM glucose or 5.5 mM glucose. GRP78/Bip levels, however, were significantly increased when pericytes shifted from the medium containing 25 mM glucose to the medium containing only 1 or 0 mM glucose (Fig. 4B, groups c and d), suggesting that a rapid decrease from medium simulating hyperglycemia (25 mM glucose) to hypoglycemia (1 mM glucose) can induce ER stress and an UPR.

DISCUSSION

It is well established that hyperglycemia is a risk factor for diabetic complications that affect the eyes, nerves, and kidneys in patients with either insulin-dependent diabetes mellitus (IDDM, type 1 diabetes)[12,13] or noninsulin-dependent diabetes mellitus (NIDDM, type 2 diabetes).[14] Tight glycemic control can delay the onset and progression of these complications, although trying to maintain tight glycemic control through insulin or hyperglycemic agents often results in acute glucose fluctuations and hypoglycemia, especially in patients with IDDM.[13,15] Both chronic glucose fluctuations and hypoglycemia have also been linked to the onset of these complications.[15,16]

Diabetic retinopathy is one of the major microvascular complications in diabetes, and the selective degeneration of pericytes in the retinal capillary vessels is an early histopathologic feature of this disease. Studies on diabetic eye bank eyes have shown that retinal pericytes undergo apoptosis.[3,17] The apoptosis induced in medium simulating hyperglycemia is independent of UPR as high-glucose-containing media (25–250 mM) in the current study did not induce the expression of GRP78/Bip. The current studies suggest that low glucose levels or a sudden reduction of glucose levels from normal to low levels induces ER stress that subsequently results in apoptosis in cultured retinal pericytes. Glucose deprivation is known to trigger the ER stress response in a number of cell types by disrupting protein unfolding in the ER.[18–20] The current studies clearly demonstrate that glucose deprivation also induces ER stress in retinal capillary pericytes as evidenced by an increased expression of the ER chaperone, GRP78/Bip. GRP78/Bip is involved in the translocation of polypeptides across the ER membrane and can also regulate apoptosis. Transcription of GRP78/Bip is a classical marker of UPR activation in mammalian cells.[21] When unfolded or misfolded proteins accumulate within the cell, they bind to GRP78/Bip, which results in activation of the UPR pathway.[22]

Glucose deprivation also induced the expression of CHOP (C/EBP homologous protein) in pericytes. This downstream gene in the UPR pathway is induced by increased translation of ATF4 (activating transcription factor 4), which follows eIF2α phosphorylation during times of ER stress.[23–25] CHOP is known as a growth arrest and DNA damage–inducible gene 153 (GADD153)[19] and plays an essential role in ER stress–induced apoptosis. Overexpression of CHOP or microinjection of CHOP protein leads to cell cycle arrest and/or apoptosis.[26–29] In contrast, apoptosis in response to ER stress is reduced in CHOP-deficient transgenic mice.[30–32]

Caspase activation was also observed in glucose-deprived pericytes. Activation of caspase-12 from procaspase-12 is specifically induced by ER stress.[33,34] Caspase-3, one of the key executioners of apoptosis, is a downstream gene of caspase-12.[8,22]

The observed upregulation of these ER stress–mediated proteins in this study indicated that the UPR pathway that leads to apoptosis is induced by glucose deprivation in retinal pericytes. Because GRP78/Bip expression is responsive to glucose regulation, its expression in pericytes cultured with increasing levels of glucose for various time periods was also examined. Prolonged exposure of pericytes to high levels of glucose (25–250 mM) did not result in increased levels of GRP78/Bip. However, the GRP78/Bip levels increased when high glucose levels were rapidly reduced to below normoglycemic (5.5 mM) levels. Interestingly, reducing the glucose level from normoglycemic to 1 mM did not alter the expression of GRP78/Bip (Fig. 3A), but when glucose levels were reduced from hyperglycemic levels of 25 mM to 1 mM, there was the elevation of GRP78/Bip (Fig. 4B, c). These results indicate that the UPR pathway activation in pericytes only occurs when the cells are deprived of glucose (0 mM glucose in media) or when glucose levels are rapidly reduced from high (25 mM) to low (1 mM) or no (0 mM) glucose but not under normal (5.5 mM) or high glucose ($\geq$25 mM) conditions. Therefore, the mechanisms of apoptosis in retinal pericytes differ depending upon whether there is prolonged low level of media glucose that simulates hypoglycemia, rapidly decreasing glucose levels simulating decreases from hyper- to hypoglycemic levels (25 mM to $\leq$1 mM glucose), or prolonged high media levels of glucose simulating hyperglycemia (25 mM).

Clinically, the establishment of tight glycemic control in patients with retinopathy often results in an initial worsening of their disease.[13,35,36] Establishing tight glycemic control often results in acute glucose fluctuations and hypoglycemia. While the mechanism for the initial worsening of retinopathy during tight control is unknown, Li et al.[9] have observed the activation of bcl-2, bax, and other apoptosis markers in retinal pericytes during rapid glucose reduction. The current results, which indicate the increased ER stress and activation of the UPR pathway leading to pericyte apoptosis occurs with glucose deprivation and rapid glucose reduction of glucose from high to low media glucose levels, support the observations of Li et al.[9] ER stress has also been linked to pancreatic β-cell apoptosis in diabetic patients[37] and to obesity and insulin action in type 2 diabetics.[38] The current experimental results support the premise that the clinical acceleration of retinopathy as tight control glucose is established is linked to the increased incidence of rapid glucose swings and hypoglycemia that induces ER stress, a UPR, and subsequent apoptosis. The current studies, however, were conducted under extreme hypoglycemic (1 mM) or glucose deprived (0 mM) conditions. Hypoglycemia is defined to occur at glucose levels $\leq$4 mM with loss of consciousness occurring at levels below 3 mM. Although, clearly, further work is required to define the exact relationship between the levels of hypoglycemia and ER stress, the current results suggest that controlling ER stress may be an essential mechanism for controlling the progression of retinopathy as tight glycemic control is clinically initiated.

ACKNOWLEDGMENTS

This work was supported in part by funding from Research to Prevent Blindness (RPB) and funds from the Department of Ophthalmology and Visual Science, University of Nebraska Medical Center. The authors would like to thank Dr. Duco Hamasaki for critical reading of the manuscript and Mrs. Ryoko Yamamoto for her technical assistance.

REFERENCES

[1] Cogan DG, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy. *Arch Ophthalmol.* 1961;66:366–378.
[2] Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye.* 2002;16:242–660.
[3] Li W, Yanoff M, Liu X, Ye X. Retinal capillary pericyte apoptosis in early human diabetic retinopathy. *Chin Med J (Engl).* 1997;110:659–663.
[4] Mandarino LJ. Current hypotheses for the biochemical basis of diabetic retinopathy. *Diabetes Care.* 1992;15:1892–1901.

[5] Hammes HP, Lin J, Renner O, et al. Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes*. 2002;51:3107–3112.
[6] Kondo T, Hosoya K, Hori S, et al. Establishment of conditionally immortalized rat retinal pericyte cell lines (TR-rPCT) and their application in a co-culture system using retinal capillary endothelial cell line (TR-iBRB2). *Cell Struct Funct*. 2003;28:145–153.
[7] Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem*. 2005;74:739–789.
[8] Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest*. 2002;110:1389–1398.
[9] Li W, Liu X, He Z, et al. Expression of apoptosis regulatory genes by retinal pericytes after rapid glucose reduction. *Invest Ophthalmol Vis Sci*. 1998;39:1535–1543.
[10] Kikuchi S, Shinpo K, Takeuchi M, et al. Glycation—a sweet tempter for neuronal death. *Brain Res Brain Res Rev*. 2003;41:306–323.
[11] Sharma P, Fatma N, Kubo E, et al. Lens epithelium-derived growth factor relieves transforming growth factor-beta1-induced transcription repression of heat shock proteins in human lens epithelial cells. *J Biol Chem*. 2003;278:20037–20046.
[12] 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. The Diabetes Control and Complications Trial Research Group. *N Engl J Med*. 1993;329:977–986.
[13] The Diabetes Control and Complications Trial Research Group. Progression of retinopathy with intensive versus conventional treatment in the Diabetes Control and Complications Trial. *Ophthalmology*. 1995;102:647–661.
[14] UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet*. 1998;352:837–853.
[15] Dagogo-Jack S. Hypoglycemia in type 1 diabetes mellitus: pathophysiology and prevention. *Treat Endocrinol*. 2004;3:91–103.
[16] Heine RJ, Balkau B, Ceriello A, et al. What does postprandial hyperglycaemia mean?. *Diabet Med*. 2004;21:208–213.
[17] Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest*. 1996;97:2883–2890.
[18] Benavides A, Pastor D, Santos P, et al. CHOP plays a pivotal role in the astrocyte death induced by oxygen and glucose deprivation. *Glia*. 2005;52:261–275.
[19] Gao Y, Feng HC, Walder K, et al. Regulation of the selenoprotein SelS by glucose deprivation and endoplasmic reticulum stress—SelS is a novel glucose-regulated protein. *FEBS Lett*. 2004;563:185–190.
[20] Lefebvre DL, Rosen CF. Regulation of SNARK activity in response to cellular stresses. *Biochim Biophys Acta*. 2005;1724:71–85.
[21] Misra UK, Pizzo SV. Up-regulation of GRP78 and antiapoptotic signaling in murine peritoneal macrophages exposed to insulin. *J Leukoc Biol*. 2005; 78:187–194.
[22] Rao RV, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ*. 2004;11:372–380.
[23] Rutkowski DT, Kaufman RJ. All roads lead to ATF4. *Dev Cell*. 2003;4:442–444.
[24] Blais JD, Filipenko V, Bi M, et al. Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol*. 2004;24:7469–7482.
[25] Harding HP, Novoa I, Zhang Y, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell*. 2000;6:1099–1108.
[26] Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ*. 2004;11:381–389.
[27] Matsumoto M, Minami M, Takeda K, et al. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. *FEBS Lett*. 1996;395:143–147.
[28] Maytin EV, Ubeda M, Lin JC, Habener JF. Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. *Exp Cell Res*. 2001;267:193–204.
[29] Gotoh T, Oyadomari S, Mori K, Mori M. Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J Biol Chem*. 2002;277:12343–12350.
[30] Oyadomari S, Takeda K, Takiguchi M, et al. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci U S A*. 2001;98:10845–10850.
[31] Zinszner H, Kuroda M, Wang X, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev*. 1998;12:982-995.
[32] Oyadomari S, Koizumi A, Takeda K, et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest*. 2002;109:525–532.
[33] Nakagawa T, Zhu H, Morishima N, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature*. 2000;403:98-103.
[34] Morishima N, Nakanishi K, Takenouchi H, et al. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem*. 2002;277:34287–34294.
[35] Dahl-Jorgensen K, Brinchmann-Hansen O, Hanssen KF, et al. Rapid tightening of blood glucose control leads to transient deterioration of retinopathy in insulin dependent diabetes mellitus: the Oslo study. *Br Med J*. 1985;290:811–815.
[36] Funatsu H, Yamashita H, Ohashi Y, Ishigaki T. Effect of rapid glycemic control on progression of diabetic retinopathy. *Jpn J Ophthalmol*. 1992;36:356–367.
[37] Araki E, Oyadomari S, Mori M. Impact of endoplasmic reticulum stress pathway on pancreatic beta-cells and diabetes mellitus. *Exp Biol Med*. 2003;228:1213–1217.
[38] Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 2004;306:457–461.