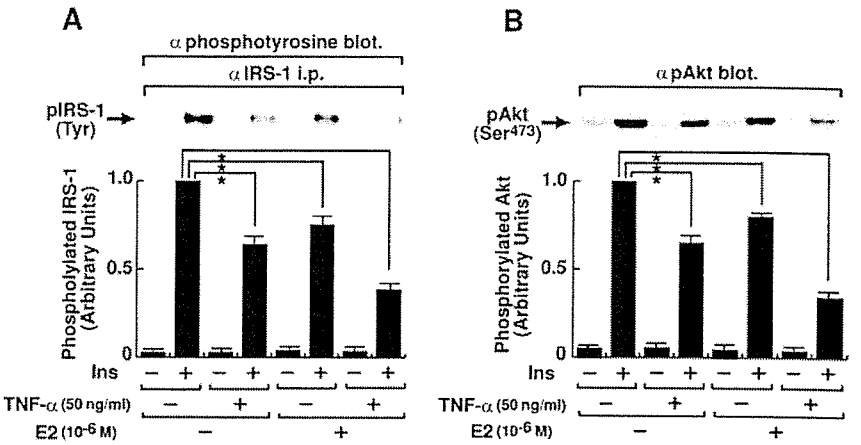

FIG. 4. Effect of the combined presence of E2 and TNF-α on insulin signaling. Serum-starved cells treated with 10^{-6} M E2 and/or 50 ng/ml TNF-α were stimulated with 17 nM insulin for 5 min. A, The cell lysates were immunoprecipitated with anti-IRS-1 antibody. The precipitates were immunoblotted with antiphosphotyrosine antibody. B, Total cell lysates were immunoblotted with antiphospho-Ser^{473}-specific Akt antibody. Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* insulin-induced phosphorylation of IRS-1 and Akt without E2 and TNF-α treatment, by ANOVA and Scheffé's test.

SP600125, on the effect of E2. The enhancement of the insulin-induced phosphorylation at Ser^{307} caused by 10^{-5} M E2 was abrogated by treatment with the JNK inhibitor (Fig. 6A). As a result, inhibition of the insulin-induced tyrosine phosphorylation of IRS-1 (Fig. 6B) and serine phosphorylation of Akt (Fig. 6C) caused by E2 treatment was restored by pretreatment with the JNK inhibitor.

Effects of ICI182,780 and E2-BSA on E2-induced alteration of insulin signaling

We employed a specific estrogen receptor antagonist, ICI182,780, to examine whether the stimulatory effect of E2 on insulin signaling seen at 10^{-8} M is mediated by estrogen receptor (Fig. 7A). Pretreatment with 10 μM ICI 182,780 abrogated the enhancement of insulin-induced tyrosine phosphorylation of IRS-1 (data not shown) and serine phosphorylation of Akt caused by 10^{-8} M E2. Pretreatment with 1 μM ICI182,780 had the same effect. Similarly, we examined the effect of ICI182,780 to clarify whether the inhibitory effect of E2 at 10^{-5} M on insulin signaling is mediated by the estrogen receptor. Pretreatment with ICI182,780 at 10 μM, but not at 1 μM, restored the inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 (data not shown) and serine phosphorylation of Akt caused by 10^{-5} M E2. These results indicate that the estrogen receptor is implicated in the effects of both concentrations of E2 on insulin signaling.

The estrogen receptor is located on the plasma membrane in addition to the cytosol and nucleus (21). To examine the impact of membrane-based estrogen receptors, a membrane-impermeable E2, E2-BSA, was employed. Insulin-induced tyrosine phosphorylation of IRS-1 (data not shown) and serine phosphorylation of Akt were not enhanced by 10^{-8} M E2-BSA in contrast to E2 (Fig. 7B). However, higher concentrations of E2-BSA inhibited these actions, similar to the results obtained with E2. Thus, 10^{-5} M E2-BSA inhibited the insulin-induced tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt by 54.9 ± 3.5% (data not shown) and 49.4 ± 4.1%, respectively (Fig. 7B). These results indicate that estrogen receptors at the plasma membrane are implicated in the inhibitory effect of E2 at high concentrations, but are not involved in the enhancing effect of E2 at low concentrations.

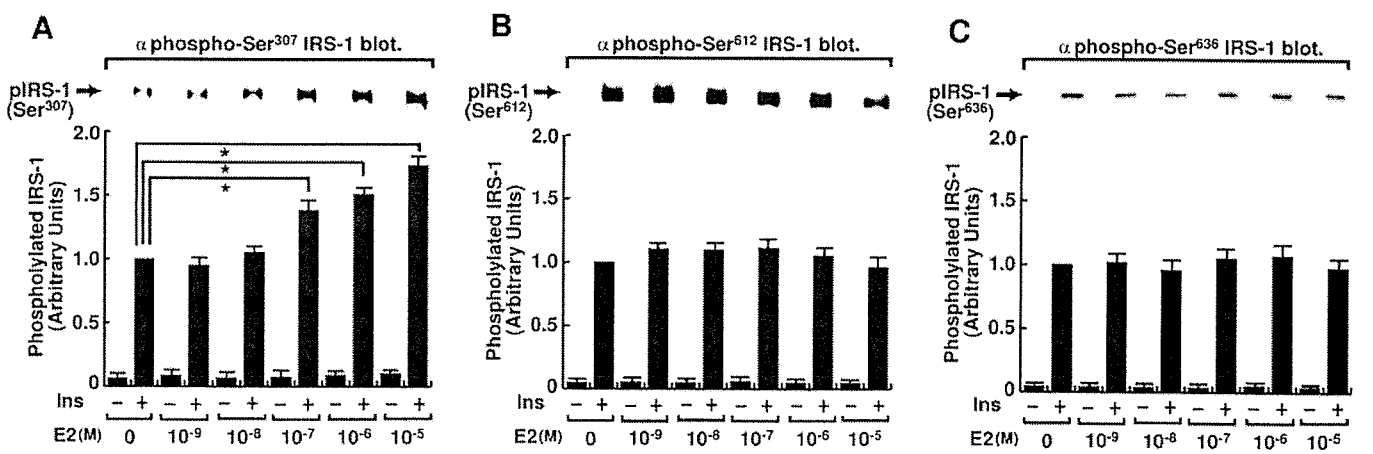

FIG. 5. Effect of E2 on insulin-induced serine phosphorylation of IRS-1. Serum-starved cells treated with various concentrations of E2 were stimulated with 17 nM insulin for 10 min. The cell lysates were immunoprecipitated with anti-IRS-1 antibody. The precipitates were immunoblotted with antiphospho-Ser^{307}-specific IRS-1 antibody (A), antiphospho-Ser^{612}-specific IRS-1 antibody (B), or antiphospho-Ser^{636}-specific IRS-1 antibody (C). Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* insulin-induced serine phosphorylation of IRS-1 without E2 treatment, by ANOVA and Scheffé's test.

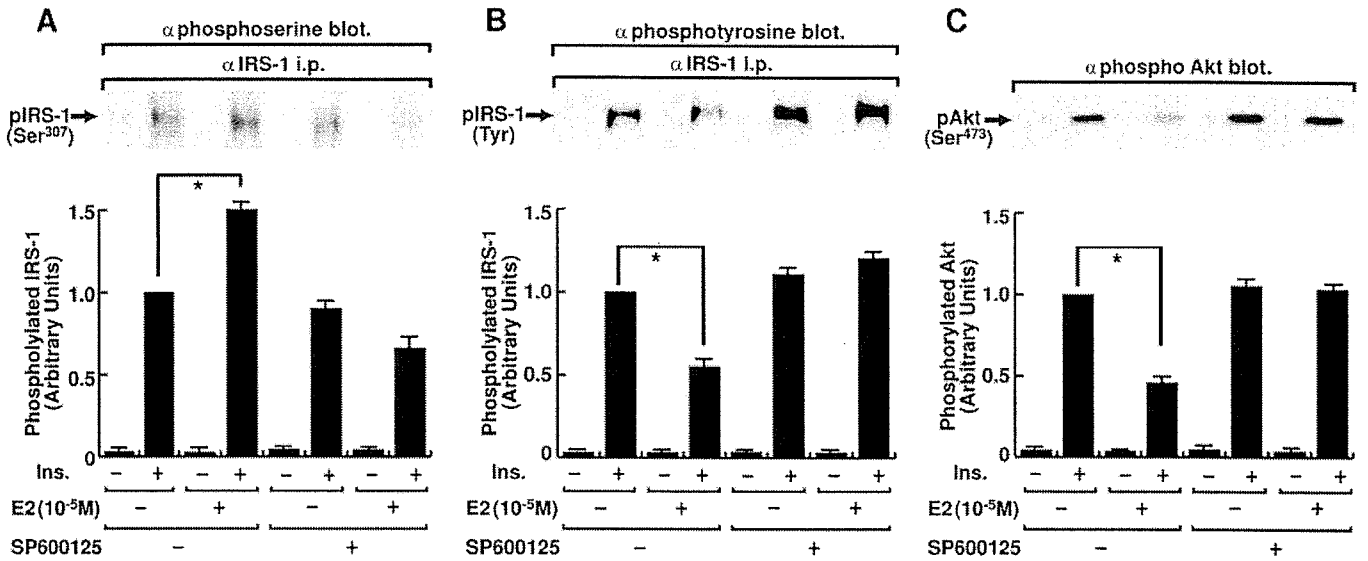

FIG. 6. Effect of a JNK inhibitor on the alteration of insulin signaling caused by E2. Serum-starved cells treated with 10^{-5} M E2 and 20 μM of the JNK inhibitor SP600125 for 16 h were stimulated with 17 nM insulin for 10 min. The cell lysates were immunoprecipitated with anti-IRS-1 antibody. The precipitates were immunoblotted with antiphospho-Ser^{307}-specific IRS-1 antibody (A) or antiphosphotyrosine antibody (B). Whole-cell lysates were immunoblotted with antiphospho-Ser^{473}-specific Akt antibody (C). Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* insulin-induced serine phosphorylation of IRS-1, tyrosine phosphorylation of IRS-1, or serine phosphorylation of Akt without E2 and SP600125 treatment, by ANOVA and Scheffé's test.

Effect of E2 treatment on subcellular distribution of estrogen receptors α and β

We also investigated the subcellular redistribution of the estrogen receptor in response to E2. Treatment with 10^{-8} M E2 reduced the amount of estrogen receptor α at the plasma membrane by 17.1 ± 2.9%, but increased the amount in the nucleus by 90.7 ± 4.4%. The amount of estrogen receptor α in the cytosol was not apparently changed by E2 treatment. In contrast, treatment with 10^{-5} M E2 increased the amount of estrogen receptor α at the plasma membrane by 71.3 ± 4.1%, but decreased it in the cytosol and nucleus by 36.3 ± 3.8% and 47.3 ± 3.4%, respectively (Fig. 8, A–C). In contrast, the amounts of estrogen receptor β at the plasma membrane, in the cytosol, and in the nucleus were not apparently altered by treatment with any concentration of E2 (Fig. 8, D–F). In addition, insulin treatment did not appear to alter the subcellular distribution of estrogen receptors.

Discussion

Our results demonstrated that treatment with a physiological concentration of E2 (10^{-8} M) enhanced the insulin-induced uptake of glucose, whereas treatment with a high concentration of E2 (10^{-5} M) inhibited it. These results clearly indicate that E2 controls glucose metabolism in a concentration-specific manner. These results are consistent with clinical reports that the incidence of type 2 diabetes increases in women after menopause, and that insulin resistance occurs in women during late pregnancy (4, 7, 8). Taken together, the present results strongly indicate that physiological concentrations of E2 enhance insulin sensitivity, whereas high concentrations of E2 inhibit insulin-induced glucose metabolism. Part of the E2 may be converted to estrone by 17β-hydroxysteroid dehydrogenase (33). We cannot rule out the possibility that the effect of E2 is at least in part derived from estrone in 3T3-L1 adipocytes.

Treatment with E2 at any concentration did not affect the insulin-induced tyrosine phosphorylation of the insulin receptor. In contrast, the insulin-induced tyrosine phosphorylation of IRS-1, the association of IRS-1 with p85, and the phosphorylation of Akt were modulated by E2. Along with the insulin-induced glucose uptake, they were enhanced by 10^{-8} M E2 treatment and inhibited by 10^{-5} M E2 treatment. These results clearly indicate that E2 affects insulin signaling, leading to glucose uptake at least in part at the step of insulin-induced tyrosine phosphorylation of IRS-1. Although a previous report identified the decrease as the cause of 10^{-7} M E2-induced insulin resistance in 3T3-L1 adipocytes (5), 10^{-7} M E2 did not significantly affect insulin signaling, and the amount of IRS-1 was not changed by the treatment in our experiment. The difference may arise from the experimental conditions employed. In this regard, we employed fully differentiated 3T3-L1 adipocytes 14–16 d after differentiation, whereas the previous study used the cells 8–12 d after the induction of differentiation (5). By contrast, our results are consistent with reports indicating the possible mechanisms of insulin resistance in pregnant women (2, 34). Insulin-induced tyrosine phosphorylation of IRS-1 is reported to be decreased in the skeletal muscle of pregnant women compared with that in nonpregnant women (2). The reduction was even greater in pregnant women with type 2 diabetes (2). Therefore, it is possible that the effect of E2 at the level of tyrosine phosphorylation of IRS-1 is involved at least in part in the insulin resistance seen in pregnant women. It is worth noting that the cause of insulin resistance in pregnancy appears to be complex. The role of E2 in other target tissues of insulin in addition to fat cells and the roles

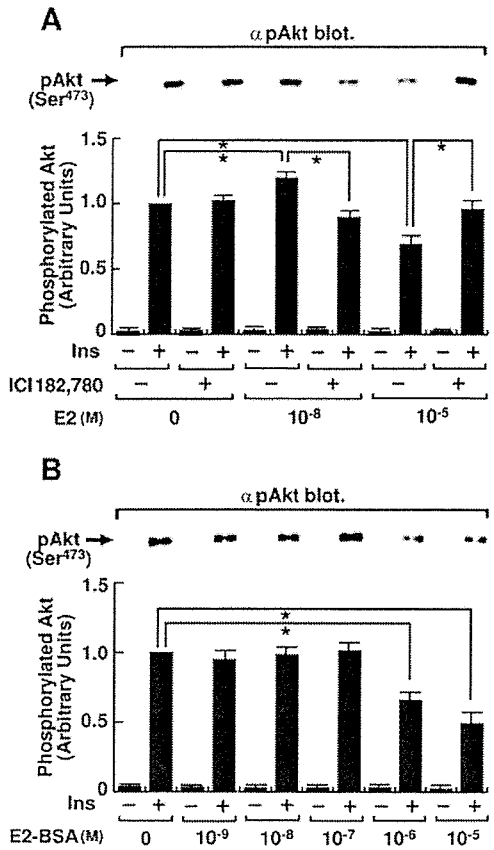

FIG. 7. Effects of ICI182,780 and E2-BSA on E2-induced alteration of insulin signaling. A, Serum-starved cells treated with 10 μM ICI182,780 and the indicated concentrations of E2 for 16 h were stimulated with 17 nM insulin for 5 min. The cell lysates were immunoblotted with antiphospho-Ser^{473}-specific Akt antibody. Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* insulin-induced serine phosphorylation of Akt without ICI182,780 treatment, by ANOVA and Scheffé's test. B, Serum-starved cells treated with various concentrations of E2-BSA for 16 h were stimulated with 17 nM insulin for 5 min. The cell lysates were immunoblotted with antiphospho-Ser^{473}-specific Akt antibody. Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* insulin-induced phosphorylation of Akt without E2-BSA treatment, by ANOVA and Scheffé's test.

of other hormones including progesterone need to be clarified.

Serine phosphorylation of IRS-1 is known to be a mechanism in the attenuation of insulin signaling, resulting in inhibition of tyrosine phosphorylation of IRS-1 (31). Phosphorylation of IRS-1 at Ser^{307}, Ser^{612}, and Ser^{636} is known to cause insulin resistance in 3T3-L1 adipocytes (35, 36). Interestingly, phosphorylation of IRS-1 at Ser^{307}, but not at Ser^{612} or Ser^{636}, is induced by treatment with E2 in a concentration-dependent manner. These results indicate that high concentrations of E2 inhibit insulin signaling at least in part via phosphorylation of IRS-1 at Ser^{307}. Serine 307 of IRS-1 is known to be the site of phosphorylation caused by JNK, which is a crucial mediator of insulin resistance (32). JNK1 activity is abnormally elevated in the state of insulin resistance, and an absence of JNK1 results in enhanced insulin signaling in mice and cultured cells (37). In the present study, inhibition of JNK activity by a JNK inhibitor abrogated the E2-induced inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt. These results strengthen our idea that E2-induced JNK activation is implicated in the phosphorylation of IRS-1 at Ser^{307}, followed by inhibition of the insulin-induced tyrosine phosphorylation of IRS-1 and phosphorylation of Akt.

TNF-α is known to be a key factor causing insulin resistance by inhibiting insulin-induced tyrosine phosphorylation of IRS-1 (38, 39). Because TNF-α is also secreted from the placenta and has been implicated in insulin resistance in pregnancy, we examined the combined effects of E2 and TNF-α on insulin signaling. Because the data indicated that TNF-α combined with a submaximal concentration of E2 further inhibited insulin signaling, both agents appear to be involved in insulin resistance during late pregnancy.

ICI182,780 is known to be an antagonist of the estrogen receptor (40). Because our results showed that pretreatment with ICI182,780 abrogated the modulation of insulin-stimulated Akt phosphorylation caused by both 10^{-8} and 10^{-5} M E2, the effect of E2 on insulin signaling appears to be mediated by the estrogen receptor. Studies have revealed the existence of two receptor subtypes, estrogen receptors α and β (15, 16). Although several functional differences have been reported between these subtypes, a number of studies have demonstrated that estrogen binds to the estrogen receptor in the cytosol, and that its effect is elicited via estrogen receptors translocated to the nucleus, resulting in transcriptional activation (19–21). In addition to being located in the cytosol and, to a lesser extent, the nucleus, the estrogen receptor is known to exist on the plasma membrane (41, 42). Recent studies indicate a nontranscriptional role for the estrogen receptor in the mediation of estrogen signaling (20, 43). However, the role of estrogen receptors in the specific cellular localization during insulin signaling is totally unknown. The present study showed that membrane-impermeable E2 (E2-BSA) at 10^{-5} M inhibited the insulin-induced tyrosine phosphorylation of IRS-1 (data not shown) and serine phosphorylation of Akt, whereas they were not enhanced by E2-BSA at 10^{-8} M. These results indicate that E2's effect at high concentrations, but not at physiological concentrations, appears to be mediated by the estrogen receptors on the plasma membrane. Importantly, estrogen receptor α, but not estrogen receptor β, was redistributed upon treatment with E2. Thus, E2 at a physiological concentration (10^{-8} M) elicited the redistribution of estrogen receptor α from plasma membrane to nucleus, whereas a high concentration of E2 (10^{-5} M) induced redistribution from cytosol and nucleus to plasma membrane. Taken together, estrogen receptor α at the plasma membrane appears to be involved in the inhibitory effect of 10^{-5} M E2 on insulin signaling. The possible importance of estrogen receptor α at the plasma membrane is strengthened by the fact that E2 affects the membrane-proximal signaling of the insulin receptor located at the plasma membrane. These results are consistent with recent reports that the PI3-kinase/Akt signaling cascade is a downstream target of nonnuclear estrogenic signaling (44, 45). The lack of an effect of E2 on the cellular distribution of estrogen receptor β may be due to the low levels of estrogen receptor β in 3T3-L1 adipocytes (46).

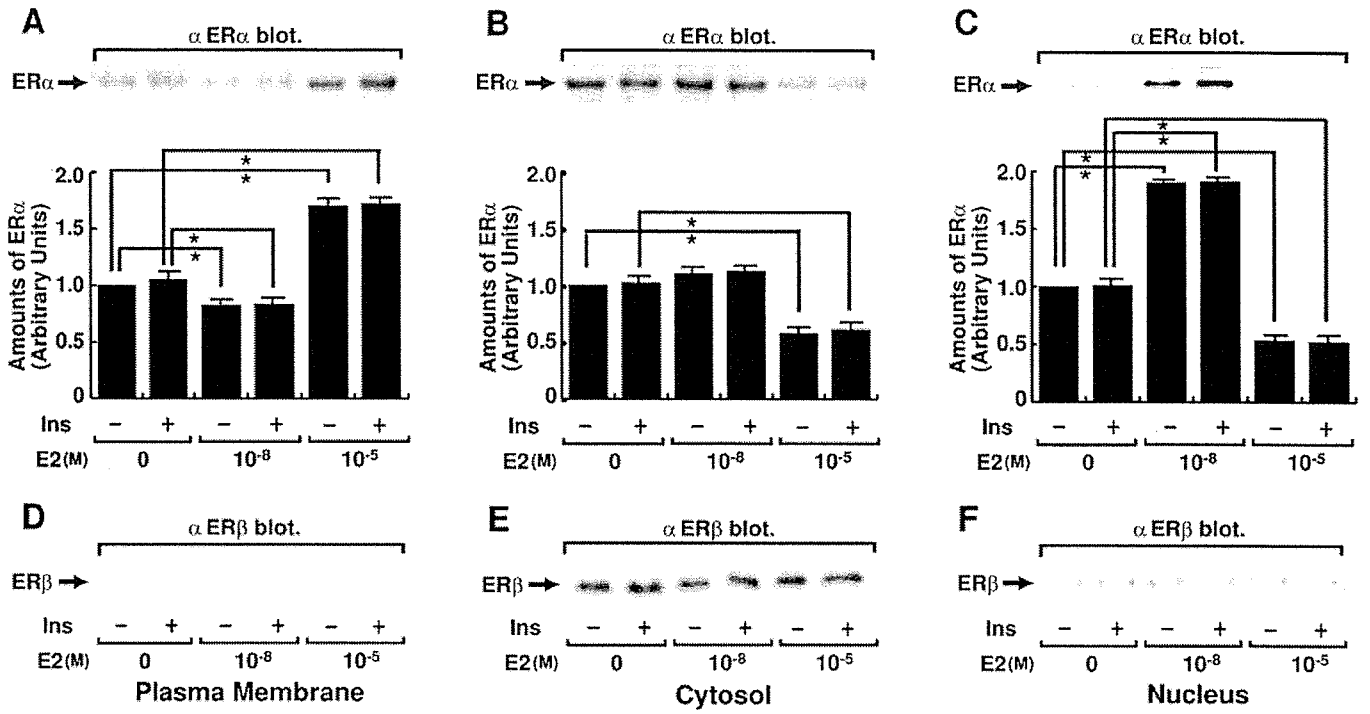

FIG. 8. Effect of E2 treatment on subcellular distribution of estrogen receptor. Serum-starved cells treated with the indicated concentrations of E2 for 16 h were stimulated without or with 17 nM insulin for 5 min. The cell lysates were fractionated into plasma membranous, cytosolic, and nuclear preparations. The samples were separated by SDS-PAGE and immunoblotted with antiestrogen receptor α antibody (αERα; A–C) or antiestrogen receptor β antibody (αERβ; D–F). Results are the mean ± SE of four separate experiments. *, $P < 0.05$ *vs.* amount of estrogen receptor α without E2 treatment, by ANOVA and Scheffé's test.

In summary, our results indicate that 1) E2 affects the metabolic action of insulin in a concentration-specific manner; 2) high concentrations of E2 inhibit insulin signaling at the level of IRS-1 by modulating the phosphorylation of IRS-1 at Ser^{307} via a JNK-dependent pathway; and 3) estrogen receptor α translocated to the plasma membrane in response to E2 is implicated in E2's inhibitory effect in 3T3-L1 adipocytes.

Acknowledgments

Received July 5, 2005. Accepted October 21, 2005.

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This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to T.S., M.K., and S.S.); the 21st Century Center of Excellence (COE) program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to S.S.); and a grant for Research on Sensory and Communicative Disorders from the Ministry of Health, Labor, and Welfare, Japan (to T.S.).

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Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Current Eye Research, 31:947–953, 2006

ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.1080/02713680600966785

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Induction of Endoplasmic Reticulum Stress in Retinal Pericytes by Glucose Deprivation

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Received 24 October 2005
Accepted 15 August 2006

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ABSTRACT Diabetic retinopathy is one of the major microvascular complications associated with diabetes mellitus, and the selective degeneration of retinal capillary pericytes is considered to be a hallmark of early retinopathy. Because glucose fluctuations commonly occur in diabetes, we hypothesized that these fluctuations will increase the endoplasmic reticulum (ER) stress and induce the unfolded protein response (UPR) in retinal pericytes. To study whether ER stress and the UPR can be induced in retinal pericytes, rat retinal capillary pericytes were cultured in different concentrations of glucose. Hypoglycemia but not hyperglycemia was found to activate UPR-specific enzymes in pericytes. Strong UPR activation leading to apoptosis was also observed when pericytes were cultured in glucose concentrations that were reduced from high to low or no glucose. These results indicate that induction of UPR is related not only to absolute concentrations but also to a shifting from higher to lower concentrations of glucose.

KEYWORDS apoptosis; diabetic retinopathy; endoplasmic reticulum (ER) stress; retinal pericytes; unfolded protein response (UPR)

INTRODUCTION

The incidence of diabetes mellitus has significantly increased in recent years, and poor control of this disease leads to a number of systemic and ocular complications. Diabetic retinopathy is a major microvascular complication of diabetes, and its proliferative stage is a leading cause of blindness.

Retinal capillaries are composed of pericytes and endothelial cells. Pericytes provide vascular stability, and their selective loss is a hallmark of early microvascular lesions.[1–3] Pericytes do not replicate in the adult retina, and their degeneration leads to basement membrane thickening, increased vascular permeability, and retinal edema.[4] The loss of pericytes can also result in focal retinal capillary endothelial cell proliferation leading to microaneurysms[5] or to degeneration of endothelial cells to form acellular capillaries, which can lead to a subsequent formation of areas of nonperfusion.

Animal models have played a critical role in elucidating the pathologic mechanisms associated with the clinical lesions of diabetic retinopathy. Extensive studies have been conducted using diabetic rats. Absent to date have been *in vitro* studies using cultures of rat retinal capillary cells. Studies have mainly

used bovine pericytes, presumably because of difficulties in preparing rat retinal pericytes in adequate amounts and purity. Recently, however, a cell line of rat retinal capillary pericytes (TR-rPCT) has been established from a transgenic rat harboring the temperature-sensitive simian virus 40 (SV 40) large T-antigen gene (Tg rat).[6] Use of this cell line allows for direct biochemical comparisons and correlations between *in vitro* and *in vivo* results.

Recently, the endoplasmic reticulum (ER) has been recognized to play a pivotal role in the progression of many human diseases.[7] Unfolded protein aggregates in the ER induce ER stress, which then generates the unfolded protein response (UPR). Upon activation, the UPR initiates diverse signaling responses. Under normal conditions, this coordinated response halts the buildup of proteins, allows time for the elimination of unfolded proteins, and reestablishes cellular homeostasis. However, a prolongation of the UPR induces apoptosis.[7] The UPR was initially observed in diseases such as Alzheimer disease and Parkinson disease, which are characterized by the accumulation of misfolded mutant protein aggregates.[8] More recently, obesity has been reported to induce UPR, and this may be associated with a diabetes-induced imbalance of glucose.[9,10]

Because UPR signaling has been shown to be essential for the maintenance of glucose homeostasis, it is hypothesized that the ER stress–UPR system may also affect retinal pericyte viability and function. Glucose fluctuations in patients with diabetes mellitus could initiate ER stress that generates a UPR that, if unalleviated, could lead to apoptosis of retinal pericytes. In this report, we present evidence that glucose deprivation, a known ER stressor, and rapid reduction of glucose levels induce the UPR in cultured retinal pericytes. This suggests a link between ER stress and apoptosis of retinal pericytes. Moreover, these findings may provide insight into the clinical observation that establishment of tight control in diabetics, which is associated with increased incidences of hypoglycemia, is initially accompanied by an acceleration of retinopathy.

MATERIALS AND METHODS

Cell Culture

Conditionally immortalized rat retinal pericytes (TR-rPCT)[6] were cultured on collagen type 1 coated plates or dishes in a normal glucose (5.5 mM) Dulbecco'sModified Eagle's Medium (DMEM; Invitrogen-Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin in a 6% CO_2 atmosphere at 33°C. The cells were released from the plates with trypsin in phosphate-buffered saline (PBS) containing 1 mM EDTA-Na and plated at a density of 2×10^4 in 96-well plates, 1×10^5 in 24-well plates, or 2×10^6 in 100-mm dishes depending on the experiment. After an overnight incubation, the attached pericytes were washed twice and incubated with serum-free DMEM containing specified concentrations of glucose for specific times.

Cell Viability Assay

A colorimetric assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. This colorimetric assay of cellular proliferation uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2 to 4-sulfophenyl)-2H-tetrazolium salt (MTS). When MTS is added to a medium containing living cells, it is reduced to a water-soluble formazan salt, and the quantity of formazan is directly proportional to the number of living cells. The absorbance of formazan was measured at 490 nm with an ELISA plate reader 1 hr after the addition of MTS.

TUNEL Staining

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was carried out using a fluorescein *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were washed with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS. This was followed by a 2-min incubation with ice-cold permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). The cells were then rinsed twice with PBS and incubated for 1 hr at 37°C with the TUNEL reaction mixture. After incubation, the cells were rinsed three times with PBS, mounted on microscope slides, and photographed using a fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA). The percentage of apoptotic cells/total cells (100 cells) in

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% nonfat 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.

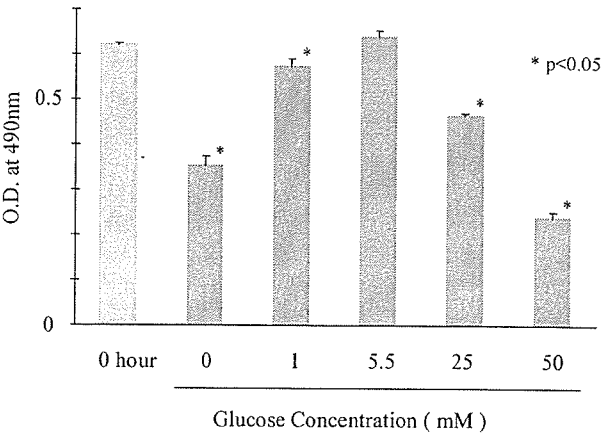

FIGURE 1 **Effect of glucose concentration on the viability of rat retinal pericytes grown for 24 hr in DMEM media containing 0 to 50 mM of glucose. Cell viability was determined by the MTS assay at 490 nm to be significantly lower at all levels higher or lower than 5.5 mM ($p < 0.05$). 0 hour represents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

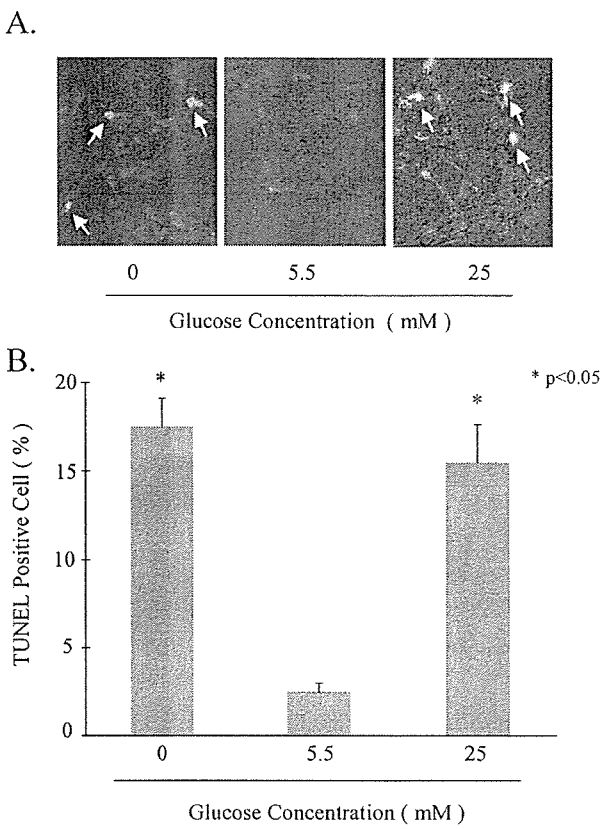

FIGURE 2 TUNEL staining of retinal pericytes cultured in different concentrations of glucose. (A) TUNEL staining of retinal pericytes cultured for 24 hr in DMEM media containing 0, 5.5, or 25 mM of glucose. Increased TUNEL staining is observed in cells cultured under glucose deprived (0 mM) and high 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.

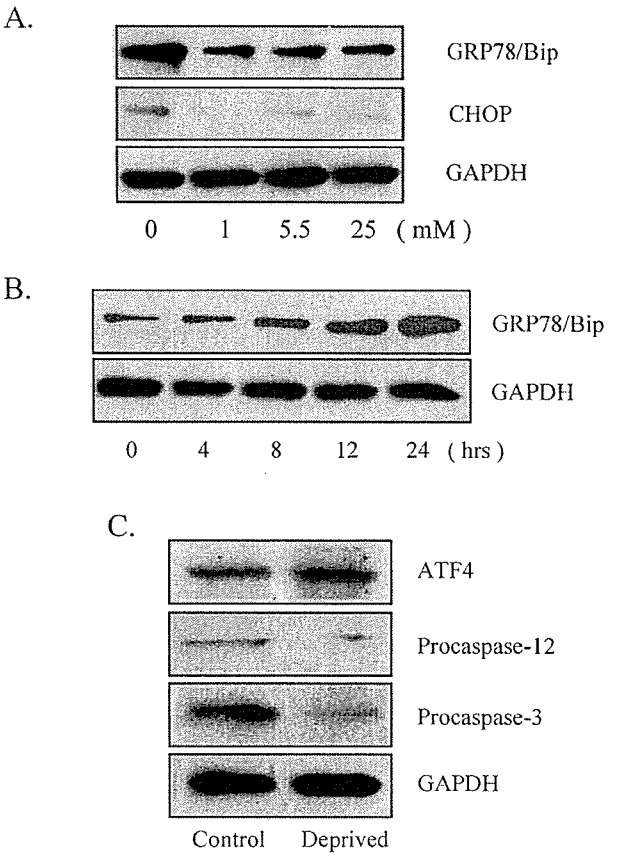

FIGURE 3 Protein blot analysis of pericytes cultured in different concentrations of glucose. (A) Protein blot of GRP78/Bip and CHOP in rat pericytes cultured for 24 hr in DMEM media containing 0, 1, 5.5, or 25 mM of glucose. (B) Protein blot illustrating time-dependent activation of GRP78/Bip in pericytes 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

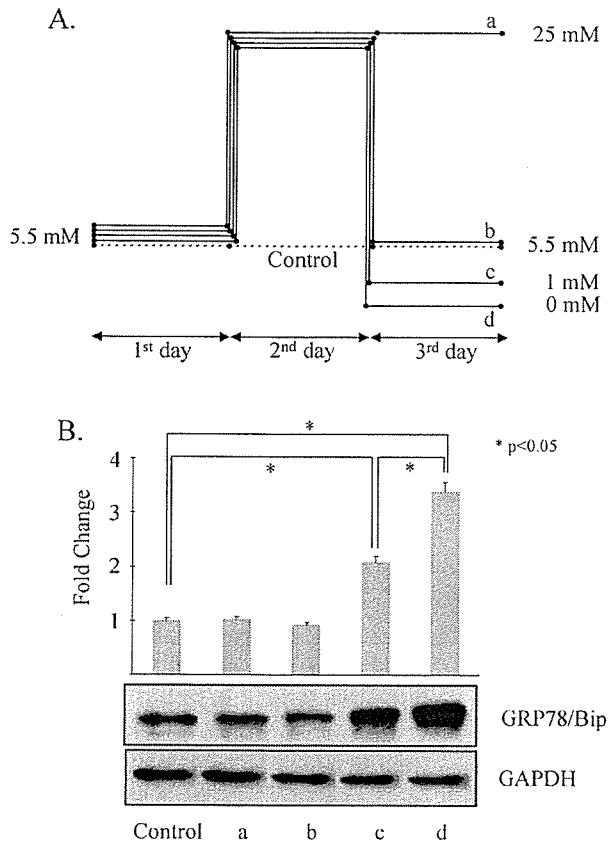

FIGURE 4 A shift from a higher to lower concentration of glucose strongly induces the UPR. (A) Diagram summarizing changes in culture time and media glucose concentrations of pericytes in groups a–d. (B) Protein blot analyses of GRP78/Bip and GAPDH levels of retinal pericytes from groups a–d. Equal amounts 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.

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