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A PKC-beta inhibitor prompts the HUVECs apoptosis-induced by advanced glycation end products

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The accumulation of advanced glycation end products (AGEs) on micro-vasculature has been demonstrated to be a key factor in diabetes mellitus development. Evidence suggests that AGEs triggered apoptotic changes in human umbilical vein endothelial cells (HUVECs) and protein kinase C (PKC)-beta plays a pivotal role in AGEs-induced micro-vascular dysfunction. Thus the effect of the selective PKC-beta inhibitor (LY333531) on AGEs-induced HUVEC apoptosis and proliferation was investigated. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to determine the cells viability after being incubated with AGEs and LY333531. Acridine orange/ethidium bromide (AO/EB) fluorescence detection was applied to observe the pro-apoptosis effects of AGEs and LY333531. Bcl-2, Bax and Bad proteins' expression were determined by StreptAvidin-Biotin-enzyme Complex (SABC) immunocytochenistry. The results showed that pretreatment with LY333531 strikingly decreased the chance of HUVEC survival and the effect of LY333531 on apoptotic cell death in HUVEC significantly increased compared with the AGEs group. Blockade of PKC-beta up-regulated the expression of Bax and Bad proteins and down- regulated the expression of Bcl-2 protein. Moreover, LY333531 reduced the ratio of Bcl-2/Bax. The results indicate that the selective PKC-beta inhibitor, LY333531, can further prompt AGEs-induced endothelial cells apoptosis. The increased expression of Bax, Bad and decreased expression of Bcl-2 and Bcl-2/Bax ratio are associated with the apoptotic process.

1. Introduction

Vascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus, which include large vessel obstructions and micro-vascular diseases. Endothelial cells (ECs) are the critical guardians of vascular barrier function. ECs apoptosis is considered to play an important role in the pathogenesis of microangiopathies, including retinopathy, nephropathy and neuropathy (Bonetti et al. 2003). Advanced glycation end products (AGEs), the products of non-enzymatic glycation and oxidation of proteins, have been widely studied in their key role in promoting vascular dysfunction and the progress of diabetes. In addition, AGEs are pro-apoptotic for retinal fibroblasts, capillary cells, and renal mesangial cells (Yamagishi et al. 2002; Kowluru 2005; Zoubin et al. 2005). A study has shown that AGEs can exert a deleterious effect on endothelial cells through the induction of apoptosis (Zhou et al. 2006).

Abbreviations: AGEs advanced glycation end products; AO/EB Acridine orange/ethidium bromide; BSA bovine serum albumin; DAB diaminobenzidine; DAG diacylglycerol; DMSO dimethyl sulphoxide; ECs endothelial cells; FBS fetal bovine serum; HUVECs human umbilical vein endothelial cells; MAPK mitogen-activated protein kinase; MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD optical density; PBS phosphate-buffer solution; PI3 phosphoinositide-3; PKC protein kinase C; SABC StreptAvidin-Biotin-enzyme Complex.

Increased activation of protein kinase C (PKC) isoforms have been demonstrated to play a pivotal role in AGEs-modulated diabetic complications. PKC, composed of a family of serine-threonine kinases, participated in cell signal transduction in diabetic micro-vascular damage. The beta isoform of PKC plays a significant role in diabetic micro-vascular complications both *in vitro* and *in vivo* (Idris and Donnelly 2006). We and others have demonstrated that PKC-beta participates in AGEs–induced cell oxidative stress, which contributes to abnormalities associated with diabetic nephropathy (Scivittaro et al. 2000; Xu et al. 2010). Furthermore, its activation also modulates vascular endothelial permeability and neovascularization via the expression of growth factors (Geraldes and King 2010). On the other hand, PKC isoforms take part in the regulation of the cell proliferation. It has been demonstrated that activation of PKC plays a critical role in promoting endothelial cell proliferation (Xia et al. 1996), while inhibition of PKC may inhibit cell proliferation and induce cell apoptosis. LY333531 was recently developed as a selective PKC-beta inhibitor, and was found to reduce retinal endothelial cell proliferation.

The object of the present study was to evaluate the influence of PKC-beta inhibitor, LY333531 (Frank 2002), on AGEs-induced endothelial cell apoptosis. Furthermore, the potential mechanism was discussed.

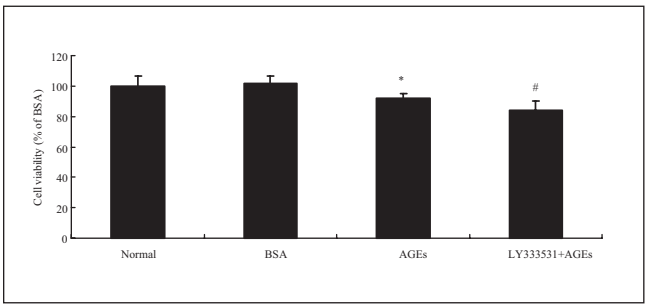


Fig. 1: The viability of HUVEC was decreased after treatment with the selective PKC inhibitor, LY333531. HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 200 nM LY333531 + 200 μg/ml AGEs for 48 h and the MTT assay was done to determine the cell viability. The formazan resolved by DMSO was calculated by Microplate spectrophotometer at 580 nm. Results were expressed as means ± S.D. $^{*}p < 0.05$, vs. BSA group. $^{\#}p < 0.05$, vs. AGEs group

2. Investigations and results

2.1. LY333531 prompted AGEs-induced apoptosis in HUVECs

The anti-proliferation activity of AGEs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As can be seen in Fig. 1, the optical density (OD) value of human umbilical vein endothelial cells (HUVECs) decreased from 101.50 ± 4.85% to 92.15 ± 2.76% after being treated with AGEs (200 μg/ml) ($p < 0.05$, vs. BSA). Furthermore, the OD value of the cells was further decreased from 92.15 ± 2.76% to 84.10 ± 6.09% on pretreatment of HUVECs with 200 nM selective PKC-beta inhibitor, LY333531 ($p < 0.05$, vs. AGEs).

2.2. Blockade of PKC-beta increased the cells apoptosis induced by AGEs

Dysfunction of endothelium in diabetes mellitus is characterized by changes in proliferation and sensitivity to apoptosis (Van den Oever et al. 2010). In the present study, acridine orange/ethidium bromide (AO/EB) fluorescence staining was used to visualize nuclear changes and apoptotic body formation. HUVECs were observed under a fluorescence microscope and counted to quantify apoptosis. As shown in Fig. 2, AGEs incubation increased the cell apoptosis from 5.54 ± 0.27 to 6.85 ± 0.59 (Fig. 2B, $p < 0.05$, vs. BSA) as compared with the bovine serum albumin (BSA) group. In addition, pretreatment with LY333531 induced more red fluorescence emission in HUVECs (Fig. 2Ad), and the cell apoptosis was increased from 6.85 ± 0.59 to 18.27 ± 1.03 (Fig. 2B, $p < 0.01$, vs. AGEs), indicating a higher apoptosis rate.

2.3. Effects of LY333531 on expression of Bcl-2, Bax and Bad in HUVECs

The Bcl-2 family proteins include Bcl-2, which is proved to be a critical apoptosis inhibitor; Bax and Bad, which function as pro-apoptotic factors (Li et al. 1998; Susnow et al. 2009).

In order to clarify if the effect of LY333531 on AGEs-induced endothelial cell apoptosis was modulated via Bcl-2 family proteins, their expressions were determined by Immunocytochemistry. As shown in Fig. 3 to Fig. 5, AGEs up-regulated the Bax and Bad proteins positive staining and down-regulated Bcl-2 protein expression as compared with the BSA group. After LY333531 pre-incubation, we observed that the expression of Bax and Bad elevated to the level even higher than that of AGEs group, while got lower level of Bcl-2 vs. AGEs group.

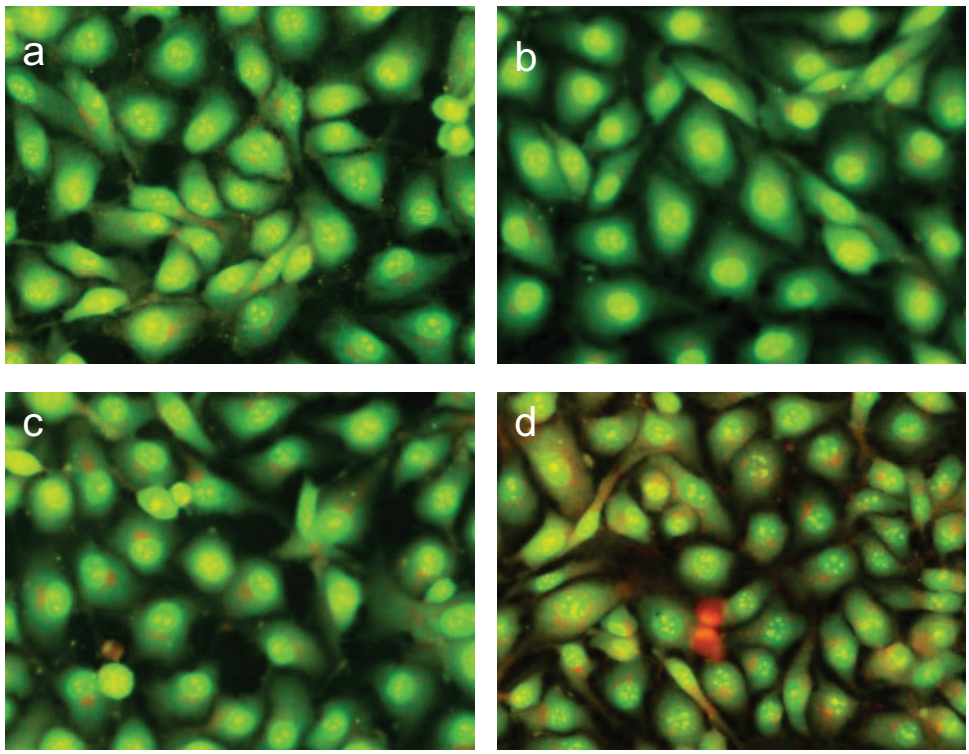
To asses the cell proliferation in whole, the ratio of Bcl-2/Bax was evaluated. As depicted in Fig. 6, the Bcl-2/Bax ratio was decreased from 17.19 ± 0.62 to 2.82 ± 0.78 ($p < 0.01$, vs. BSA) and was further decreased to 0.82 ± 0.33 ($p < 0.05$, vs. AGEs) after pretreatment with LY333531.

3. Discussion

Diabetes mellitus is associated with severe atherosclerosis and micro-vascular diseases that account for a high mobility and mortality (King 1998). There is now increasing evidence of a causal role for AGEs in the development of diabetic vasculopathy, including microangiopathy as well as atherosclerosis (Wautier et al. 1996). In the present study, we found that the selective PKC-beta inhibitor, LY333531, decreased cell viability and enhanced cell apoptosis resulting from treatment of HUVECs with AGEs. To our knowledge, this is the first report showing the pro-apoptotic effect induced by the PKC inhibitor on AGEs-induced HUVECs.

Several candidate mechanisms contributing to perturbation of micro-vascular properties in diabetes have been proposed, including increased formation of diacylglycerol (DAG), activation of PKC and accelerated formation of AGEs (Das Evcimen and King 2007). AGEs, the products of nonenzymatic glycation and oxidation of proteins and lipids, formed at an accelerated rate in diabetes (Yamagishi 2008). There is a report indicating that AGEs significantly improved the p53 and Bax protein expression and afterward induced apoptotic cell death in human mesangial cells (Yamagishi et al. 2002). In the present study we observed that in HUVECs incubated with 200 μg/ml AGEs, a significant number of apoptotic cells were detected by fluorescence microscopic analysis. Furthermore, the levels of Bax and Bad, well known proteins involved in apoptosis, were evaluated, while Bcl-2 was decreased.

Through the formation of AGEs, glucose can activate and up-regulate PKC expression (Scivittaro et al. 2000). Activation of the PKC pathway is related with many vascular abnormalities in the renal, retinal, and cardiovascular tissues in diabetic patients (Noh and King 2007). PKC activation have been associated with extracellular matrix expansion, increasing in vascular permeability, abnormal angiogenesis, excessive apoptosis, cytokine activation and the changes in enzymatic activity alterations such as phosphoinositide-3 (PI3) kinase and mitogen-activated protein kinase (MAPK) (Geraldes and King 2010). PKC activation can also contribute to tumor cell survival and proliferation. On the other hand, the inhibition of PKC-beta has been demonstrated to induce cell apoptosis. The selective PKC-beta inhibitor, enzastaurin, has a direct effect on human tumor cells, and it significantly induces human colon cancer cells apoptosis (Graff et al. 2005). Yoshiji et al. (1999) reported that the PKC-beta inhibitor could elicit cell death in hepatocellular carcinoma tissues. The PKC-beta isoform appears to be a prominent mediator of changes in cell proliferation, vascular endothelial function and micro-vascular permeability. Recently, an original compound has been developed, LY333531, a specific inhibitor of PKC-beta activated by hyperglycemia in the retina, heart, and aorta of diabetic rats (Gutterman 2002). Studies have shown that LY333531 can prevent or normalize vascular dysfunction and treatment with LY333531 for one month can normalize retinal blood flow in patients with diabetes (Bursell and King 1999). However, as discussed above, the inhibition of PKC-beta may inhibit cell proliferation. Theoretically, application of a PKC-beta inhibitor



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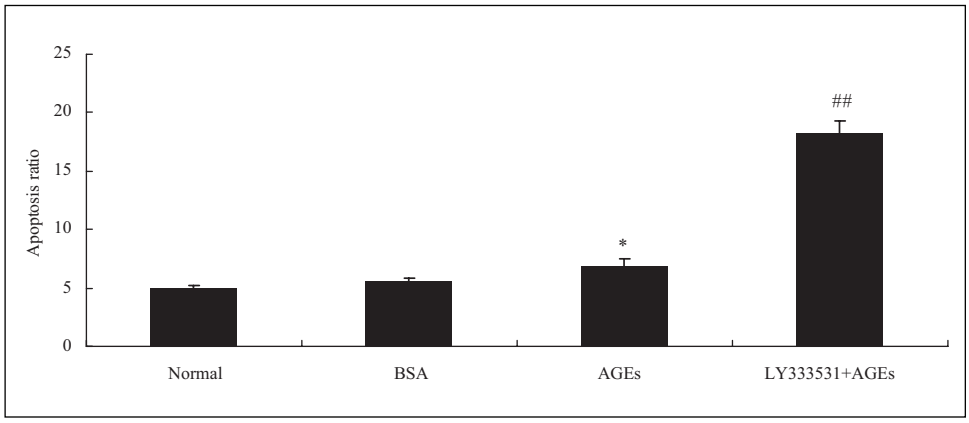


(B)

Fig. 2: Blockade of PKC-beta with LY333531 promoted HUVECs apoptosis induced by AGEs. HUVECs were incubated with 200 μg/ml BSA (Ab), 200 μg/ml AGEs (Ac), or 200 nM LY333531 + 200 μg/ml AGEs (Ad) for 48 h and HUVECs were stained with AO/EB solution. The cell viability was evaluated according to green (normal) or jacinth (apoptosis) color area as calculated by Image-Pro Plus picture analysis software and the difference between groups was compared (B). Results were expressed as means ± S.D. $^{*}p < 0.05$, vs. BSA group. $^{\#\#}p < 0.01$, vs. AGEs group. Representatives were shown (magnification, 200×)

will also influence the endothelial cell proliferation in patients with diabetes, and long-term treatment with the agent may influence the angiogenesis and vascular function. To demonstrate our hypothesis, the present study was performed. As expected, pretreatment of HUVECs with LY333531, a selective PKC-beta inhibitor, further increased AGEs-induced HUVECs apoptosis.

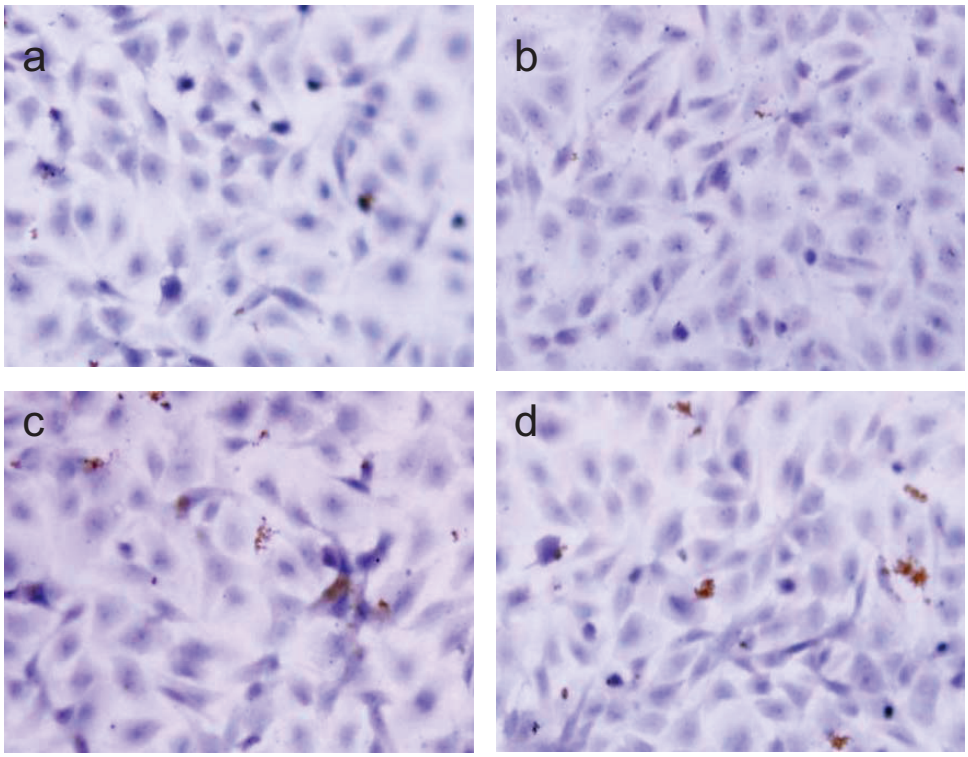
The Bcl-2 family proteins could modulate cell cycle. Differential expression of Bcl-2 family members, which include anti-apoptotic, Bcl-2, and pro-apoptotic, Bax and Bad proteins, regulate apoptosis (Antonsson and Martinou 2000; Li et al. 2003). The ratio of Bcl-2/Bax can reflect enhanced endothelial cell survival ability (Pecci et al. 1997). In the present study, Immunocytochemistry was applied to determine Bcl-2 family proteins expression. We observed that blockade of PKC-beta down-regulated Bcl-2 while up-regulated Bax and Bad expression, and reduced the ratio of Bcl-2/Bax. Thus further supposts our hypothesis that inhibition of PKC-beta will induce endothelial cell apoptosis.

In conclusion, in the present study, we demonstrated that inhibition of PKC-beta would further enhance AGEs-induced endothelial cell apoptosis. The down-regulation of Bcl-2 and up-regulation of Bax & Bad may be the potential mechanism involved in PKC-beta inhibitor-mediated cell apoptosis.

4. Experimental

4.1. Materials

The selective PKC-beta inhibitor, LY333531, was purchased from Alexis Biotechnologies (Nottingham,UK). Bcl-2, Bax and Bad antibodies were from Cell Signaling Technology (Boston, USA). 3-(4,5-Dimethylthiazol-



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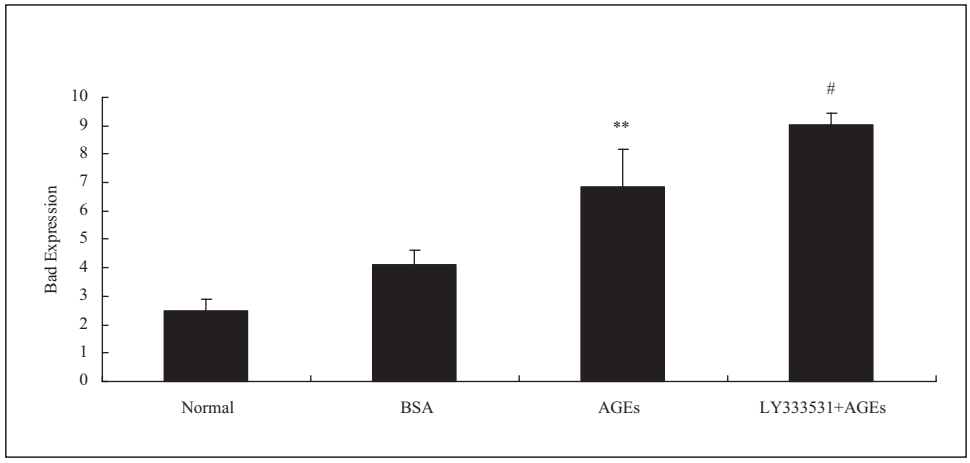


(B)

Fig. 3: LY333531 elevated AGEs-induced Bad expression. HUVECs were incubated with 200 μg/ml BSA (Ab), 200 μg/ml AGEs (Ac), or 200 nM LY333531 + 200 μg/ml AGEs (Ad). Bad expression was detected by immunocytochemistry SABC method. HUVECs were counterstained with hematoxylin and Bad expression appeared brown due to DAB colorimetric reaction. In negative control (Aa), primary antibody was replaced with PBS. The positive staining was quantitated by Image-Pro Plus software and the difference between groups was compared (B). Primary antibody was replaced with PBS in negative control (Aa). Results were expressed as means ± S.D. $^{**}p < 0.01$, vs. BSA group. $^{\#}p < 0.05$, vs. AGEs group. Representatives were shown (magnification, 200×)

2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly-L-lysine, ethidium bromide (EB) and acridine orange (AO) were obtained from Sigma (Aldrich, France). All other materials were obtained from commercial sources.

4.2. Preparation of advanced glycation end products

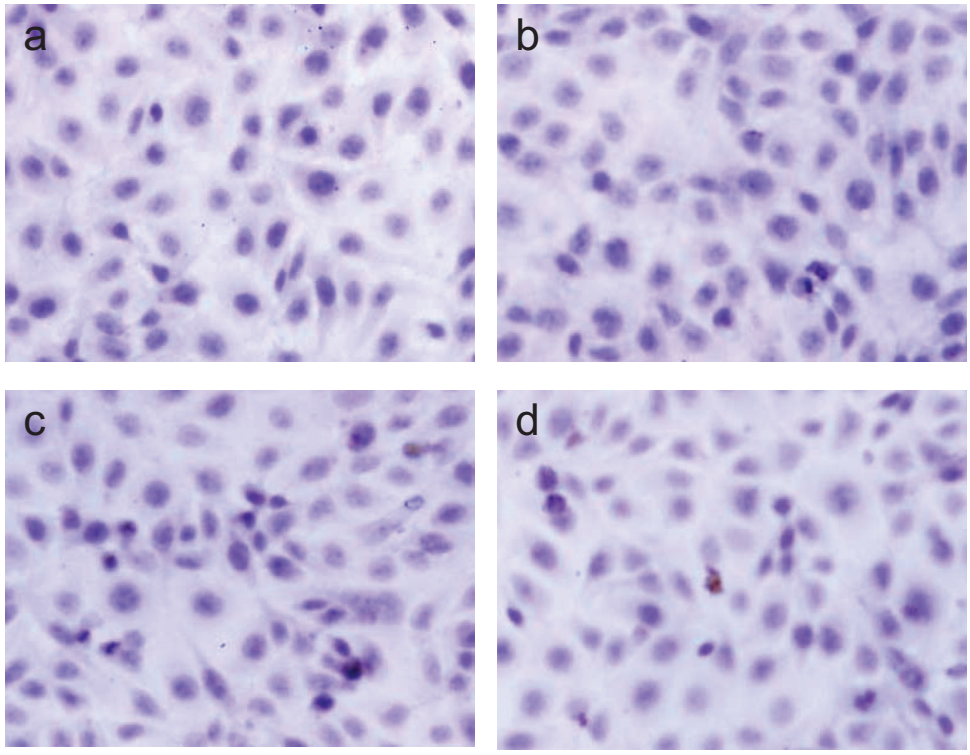
The advanced glycation end products (AGEs) were prepared as previously reported (Basta et al. 2002). Generally, bovine serum albumin (BSA) was incubated with D-glucose (50 mM/L) in PBS (pH = 7.4) under sterile conditions at 37 °C for 12 weeks. Finally, AGEs were dialyzed against PBS overnight to remove unincorporated glucose, sterile-filtered through 0.2 μm filters (Sartorius), stored at −20 °C until use.

4.3. Cell culture

Human umbilical vein endothelial cell (HUVEC) line was purchased from ATCC (Manassas, VA, USA). Cells were cultured in low glucose DMEM (Gibco) supplemented with 10% FBS (fetal bovine serum) containing 1% penicillin-streptomycin, at 37 °C in humidified 5% CO_2 in air.

4.4. Determination of cell viability by MTT assay

To evaluate the influence of PKC-beta inhibitor on cell viability of HUVEC, a total of 1×10^4 HUVECs/well were seeded into 96-well plates in low glucose DMEM with 10% FBS for 12 h. Afterwards, HUVECs were starved for 12 h and incubated with BSA (200 μg/ml), AGEs (200 μg/ml) (Cai W et al. 2006) and LY333531 (200 nM) (Koya et al. 1997; Xia et al. 2007) +AGEs (200 μg/ml) for 48 h. Then, the medium was replaced



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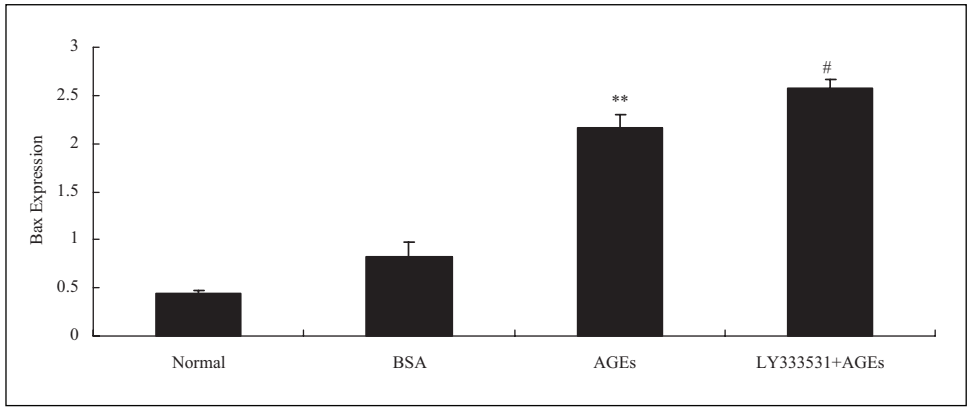


(B)

Fig. 4: Pretreatment with LY333531 increased Bax expression. HUVECs were incubated with 200 μg/ml BSA (Ab), 200 μg/ml AGEs (Ac), or 200 nM LY333531 + 200 μg/ml AGEs (Ad). Bax expression was detected by immunocytochemistry SABC method. HUVECs were counterstained with hematoxylin and Bax expression appeared brown as a result of DAB colorimetric reaction. The positive staining was quantitated by Image-Pro Plus software and the difference between groups was compared (B). In negative control (Aa), primary antibody was replaced with PBS. Results were expressed as means ± S.D. $^{**}p < 0.01$, vs. BSA group. $^{\#}p < 0.05$, vs. AGEs group. Representatives were shown (magnification, 200×)

with 0.5 mg/ml MTT and at 37 °C in a 95% air/5% CO_2 incubator for 4 h. Finally, the medium containing MTT was aspirated and replaced by dimethyl sulphoxide (DMSO). OD was measured with a Microplate spectrophotometer (SPECTRAmax190, Moleculor Devices, USA) at 580 nm.

4.5. Fluorescence microscopic analysis of cell death

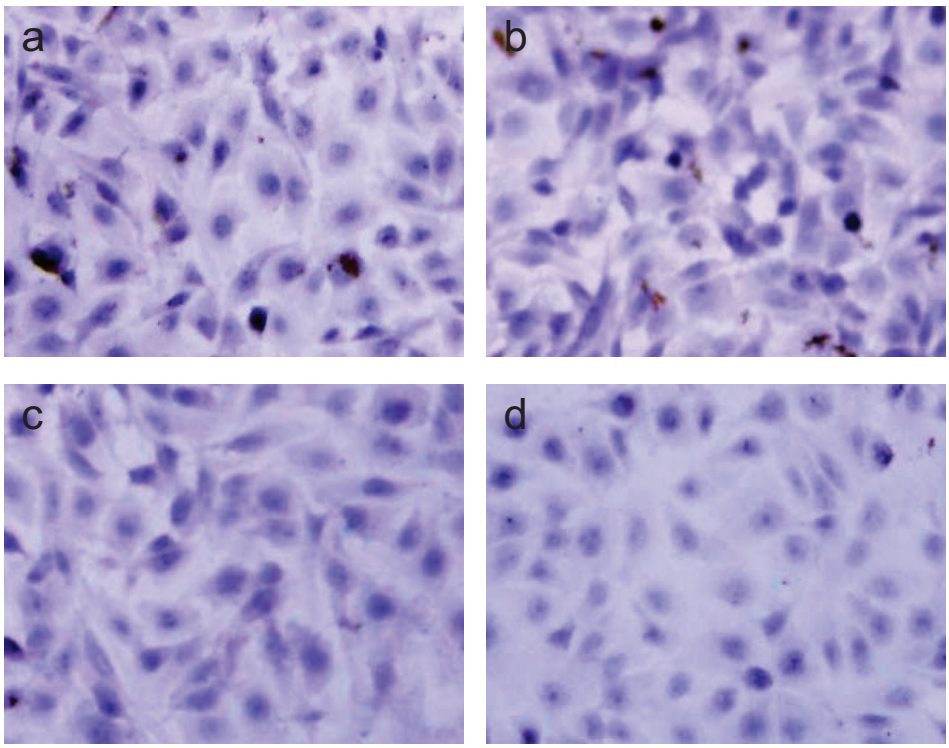
HUVECs were inoculated on coverlips in 24-well plates in DMEM (containing 2% FBS) and incubated with BSA (200 μg/ml), AGEs (200 μg/ml), or LY333531 (200 nM) +AGEs (200 μg/ml) for 48 h. After that, HUVECs were stained with AO/EB solution. The cells were observed and photographed under fluorescence microscope (Olympus IX71 inversion microscope, Olympus BX51 fluorescence microscope, Japan). To quantify apoptosis, cells were evaluated by Image-Pro Plus.

4.6. Immunocytochemistry analysis

Bcl-2, Bax and Bad proteins expression in HUVECs were analyzed by SABC immunocytochemistry method. Briefly, cells were plated onto cover glasses. After being incubated with drugs (section 2.5) for 48 h, the cells were washed with phosphate-buffer solution (PBS, pH 7.4), fixed with 4% paraformaldehyde for 90 min and incubated with a blocking solution containing 10% primary antibody-origin serum for 20 min at room temperature. The cells were further incubated with Bcl-2, Bax and Bad antibodies (1:200) at 37 °C for 120 min, and then incubated with consecutive biotin-conjugated secondary antibody and SABC for 20 min. For colorization, coverslips were exposed to 3,3′-diaminobenzidine (DAB, 0.3 mg/ml), and counterstained with hematoxylin. The coverslips were photographed under microscope. The brown precipitate indicated a positive reaction. The primary antibody was replaced with PBS for negative control.

4.7. Statistical analysis

Data were expressed as means ± SD. The statistical significance was determined by one-way ANOVA with SPSS 16.0 software. A value of $p < 0.05$ was considered to be statistically significant.



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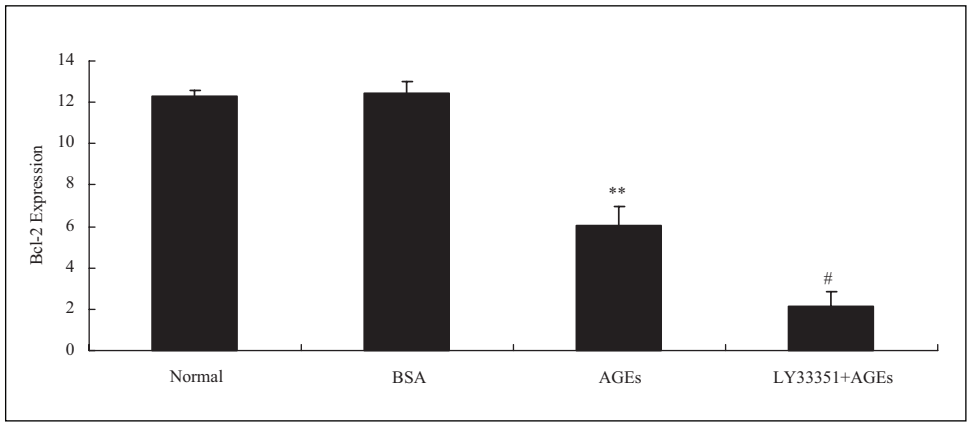

(B)

Fig. 5: LY333531 decreased Bcl-2 expression upon AGEs treatment in HUVECs. HUVECs were incubated with 200 μg/ml BSA (Ab), 200 μg/ml AGEs (Ac), or 200 nM LY333531 + 200 μg/ml AGEs (Ad). Bcl-2 expression was detected by immunocytochemistry SABC method. The cells were counterstained with hematoxylin and Bcl-2 expression appeared brown due to DAB colorimetric reaction. The positive staining was quantitated by Image-Pro Plus software and the difference between groups was compared (B). Primary antibody was replaced with PBS in negative control (Aa). Results were expressed as means ± S.D. $^{**}p < 0.01$, vs. BSA group. $^{\#}p < 0.05$, vs. AGEs group. Representatives were shown (magnification, 200×)

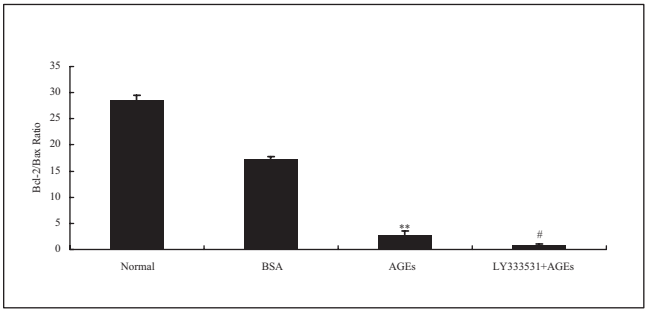

Fig. 6: LY333531 enhanced HUVEC apoptosis activity, induced by AGEs. HUVECs were incubated with 200 μg/ml BSA, 200 μg/ml AGEs, or 200 nM LY333531 + 200 μg/ml AGEs for 48 h. Levels of Bcl-2 and Bax were detected by immunocytochemistry SABC method. The ratio of Bcl-2/Bax was to evaluate cell apoptosis activity. Results were expressed as means ± S.D. $^{**}p < 0.01$, vs. BSA group. $^{\#}p < 0.05$, vs. AGEs group

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