Calcium Channel Blocker (Diltiazem) Inhibits Apoptosis of Vascular Smooth Muscle Cell Exposed to High Glucose Concentration Through Lectin-like Oxidized Low Density Lipoprotein Receptor-1 (LOX-1) Pathway

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ABSTRACT

Aim: to examine the role of diltiazem in the prevention of VSMC apoptosis exposed to high glucose through inhibition of LOX-1 expression.

Methods: we performed experimental study on the primary culture of VSMCs. Expression LOX-1, and Caspase-3 activity (a key regulatory protease at which many signaling pathways merge for the execution of apoptosis) were measured by Elisa. Data were expressed as mean ± SEM. The statistical significance was assessed by one-way analysis of variance (ANOVA) followed by post hock analysis by Turckey test, $p<0.05$ was considered statistically significant.

Results: chronic exposed VSMC to high glucose concentration (25 mM), increase cytosolic $Ca^{++}$ concentration (3127 ± 413.89 v.s. 2011.81 ± 410.93 unit/cell, $p<0.01$), expression of LOX-1 ((506.80 ± 10.47 v.s. 458.40 ± 36.49 ng/ml, $p<0.05$), and caspase-3 activity (129.98 ± 5.97 v.s. 114.73 ± 10.84%, $p<0.05$) respectively, compare exposed to 5mM glucose concentration. LOX-1 was related to caspase-3 activity, pre-treated with inhibitor LOX-1 activity, k-carragenan before being exposed to glucose 25 mM, prevents the increasing of caspase-3 activity (96.41 ± 5.11 v.s. 129.98 ± 5.98 %, $p<0.01$). Pre incubation with 10µM of diltiazem before being exposed to 25 mM glucose concentration significantly inhibits the elevation of cytosolic $Ca^{++}$ concentration (2149.61 ± 339.49 v.s. 3127 ± 413.89 unit/cell, p < 0.01), LOX-1 expression (468,60 ± 14.44 v.s. 506.80 ± 10.47 ng/ml, p < 0.05), and caspase-3 activity (82.50 ± 9.90 v.s. 129.98 ± 5.97%, p<0.01).

Conclusion: overall, these results demonstrate that high glucose induces VSMCs apoptosis through caspase-3 pathway. This effect appears to be inhibited by diltiazem through decreasing LOX-1 expression and activity.

Key words: hyperglycemia, CCB, LOX-1, VSMC apoptosis.

INTRODUCTION

The prevalence, incidence, and mortality from all forms of cardiovascular diseases are increased in patients with diabetes. Vascular smooth muscle cell (VSMCs) are essential cells for angiogenesis and blood vessel formation but also serve an important role in several different vascular pathologies including atherosclerosis, or vascular remodeling. Apoptosis of the VSMCs has both beneficial and detrimental consequences. VSMCs apoptosis has been detected within the atherosclerotic plaques, and may affect the size and stability of the plaque, leading to regression and destabilization of the fibromuscular lesion, resulting in plaque rupture.

In recent years, several new members of the scavenger receptor family have been identified on the basis of their ability to recognize modified LDL. These include the lectin-like oxidized LDL receptor-1 (LOX-1), which was identified in endothelial cells (EC), platelets, macrophage, VSMCs, and syncytiotrophoblast of the human placenta. LOX-1 expression was detected in EC of early atherosclerotic lesions of human carotid arteries. In the advanced lesions LOX-1 expression was also detected in macrophages and more frequently in VSMCs. LOX-1 expression was well colocalized with Bax expression in the rupture-prone shoulders areas of human atherosclerotic plaque in vivo, suggesting that LOX-1 may have an important role in the apoptosis process of VSMCs, modulating the Bax to Bel2 ratio. In the macrophage, as well as in EC, high glucose concentration enhanced LOX-1 expression. PKC plays an important role in mediating biological responses, including tumor promotion and inflammation.
Studies on the regulation of LOX-1 expression in EC, and VSMCs revealed up-regulation of LOX-1 expression after incubation with PMA, an activator of PKC. There was evidence that high glucose concentration activates PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), and PKC-\(\varepsilon\) in vascular smooth muscle cells. Inoguchi et al., have suggested that high glucose concentration leads preferentially to an increased expression of PKC-\(\beta\) isoform in cardiovascular tissue. PKC-\(\beta\)II is a member of conventional PKC, that is stimulated by diacylglycerol (DAG) and phosphatidylserine and requires Ca++ for activation. We tested the hypothesis that high glucose-induced VSMCs apoptosis through LOX-1 pathway may be prevented by CCB (diltiazem) treatment. This research was approved by Ethical Commission of Faculty of Medicine Brawijaya University.

METHODS

VSMCs Culture

Arterial VSMCs were isolated from the umbilical cord of a healthy baby, prepared by modification of the method of Maasch. A 10 cm segment of the umbilical artery was dissected from the surrounding tissues by fine hook. The artery was opened length wise, and the endothelial layer removed gently. The medial layer was then minced into small pieces, followed by enzymatic digestion with collagenase-I. Fragments of the medial layer were placed onto tissue culture plates, and implant in free calcium media, in DMEM supplemented with 10% FBS, 25 ml HEPES, 40 U/ml penicillin G, 40 µg/ml streptomycin. Cells were maintained at 37°C, in humidified 95% air atmosphere, 5% CO\(_2\). Cells were grown to >90% confluences. The media were changed every 3 days during growth. Cells were made quiescent by incubation for 48 hours in culture medium containing 0.2% FCS, before treatment. VSMCs identity was verified by immunostaining with anti-human \(\alpha\)-actin antibody and by characteristic “hill and valley” growth pattern. We used cells in passages 3-6 for all experiments. Experiments were done in groups of VSMCs exposed to D-glucose 5 mM, 25 mM, and without glucose exposure.

Assay of Caspase-3 Activity

Caspase-3 activity was measured using the method of Bio Vision for caspase-3 activity measurement (caspase-3/CPP32 colorimetric assay kit-Biovision). Briefly, count cells in pellet: 1-5 x 10\(^6\) cells. Resuspend cells in 50 µl of chilled Cell Lyses Buffer and incubates on ice for 10 minutes. Centrifuge for 1 minute in a microcentrifuge (10.000 x g). Transfer supernatant to a fresh tube and put on ice for immediately assay. For assay protein concentration, dilute 50-200 µg protein to 50 µl Cell Lyses Buffer for each assay. Add 50 µl of 2x Reaction Buffer (containing 10 mM DTT) to each sample. Add 5 µl of the 4 mM DEVD-pNA substrate (200 µM final concentration), incubate at 37°C for 1-2 hour.Read sample at 400 or 405 nm in micrometer plate reader. Fold-increase in caspase-3 activity can be determined by comparing these results with the level of the uninduced control.

LOX-1 Measurement by ELISA Method

Measurement of LOX-1 by ELISA based on the method of Kataoka, with minor modification. Cells after being fixed with 4% paraformaldehyde and PBS for 5 minutes at 4°C, cells were washed once with PBS and then were incubated with PBS and 1% BSA for 30 minutes at room temperature to block non-specific binding sites. Cells were incubated with primary monoclonal antibody LOX-1 (clone 23C11-HyCut biotechnology) for 60 minutes at 37°C, followed by washing with Phosphate Buffer Saline containing Tween-20 0.2% three times, then incubate with secondary antibody (Ig-Biotin-KPL), for 60 minutes at room temperature. After being washed by PBS three times, followed by incubation with horseradish peroxidase conjugate anti-rat immunoglobulin antibody for 60 minutes at room temperature. Antibody binding was determined by cell incubation using sure-blue TMB substrate for 30 minutes, followed by incubation with HCL 1 N for 15 minutes to stop the reaction. The absorbance was measured at 450 nm from cell ELISA for LOX-1, and was corrected by the absorbance at 450 nm from BSA.

Measurement of LOX-1 by Immunocytochemistry Method

Cells were fixed by 95% ethanol in cover slips at room temperature. Cells were subsequently processed for incubation with monoclonal antibody for Lectin-like Oxidized Receptor-1 (LOX-1; Cell Science), 1:20 dilution, for overnight at 37°C. Slides were washed three times in D-PBS, once with dionized H\(_2\)O\(_2\). FITC labeled (KPL) was used as fluorescent dye. Following staining, cells were observed with an argon convocal laser scanning microscope (Olympus FV-1000), at 488 nm excitation length-wave. The intensity of fluorescence was observed in the region of interest (ROI). Fluorescent signal from samples quantified by FV10-ASW 1.7 software embedded on Olympus FV-1000.

Measurement of Cytosolic Ca++

The intracellular Ca++ concentration was monitored in VSMCs using Fluo-3 and an argon convocal laser scanning microscope (Olympus FV-1000), at 488 nm.
excitation and 550 nm emission length-wave. In brief, viable VSMCs were incubated with Fluo-3 for 30 minutes at 37°C in the dark room with humidified 95% air atmosphere, 5% CO2-air. The intensity of fluorescence signal was observed in the region of interest (ROI). Fluorescent signal from samples quantified by FV10-ASW 1.7 software was embedded on Olympus FV-1000.

Data Analysis
Experiments were repeated at least five times to ensure reproducible results. Data were expressed as mean ± SD of the number of observations. The statistical significance was assessed by one-way analysis of variance (ANOVA) or student’s t-test. p<0.05 was considered statistically significant.

RESULTS

High Glucose Concentration Enhances LOX-1 Expression
We examined the effect of high glucose concentration on LOX-1 expression in VSMCs. Enhanced LOX-1 expression was indicated by ELISA and immunocytochemistry methods. Exposed VSMCs to glucose 25 mM within 6 hours, enhances LOX-1 expression (506.80 ± 10.47 ng/mL), which was higher than exposed to glucose 5 mM during same period (458.40 ± 36.49 ng/mL), p<0.05 (Figure 1).

Measurement by immunocytochemistry method showed that LOX-1 expression in VSMCs exposed to glucose 25 mM was higher than those VSMCs that were exposed to glucose 5 mM (Figure 2).

Caspase-3 Activity in VSMCs Exposed to High Glucose Concentration
Measurement of caspase-3 activity in VSMCs was done after being exposed to glucose 5 mM and glucose 25 mM. Fold-increase in caspase-3 activity was determined by comparing these results with the level of the uninduced control (without being exposed to glucose). The increase of caspase-3 activity after being exposed to glucose 25 mM, reached around two fold compared to the increase activity after being exposed to glucose 5 mM (29.98 v.s. 14.73).

The Role of LOX-1 on Caspase-3 Activity
To examine the role of LOX-1 activity on caspase-3 activity, VSMCs was pre-treated with k-carragenan 250 µg/mL (LOX-1 blocker) for 48 hours, followed by incubation with glucose 25 mM + k-carragenan for 6 days. Preincubation with k-carragenan inhibits caspase-3 activity significantly (Figure 4). This study proved that the activity of LOX-1 at least in part has role for caspase-3 activity, and responsible for VSMCs apoptosis exposed to high glucose concentration.

Expression of LOX-1 Identified by ELISA, Under Diltiazem Treatment
Pre-treatment with diltiazem 10 µM for 30 minutes before incubation in glucose 25 mM + diltiazem, inhibits LOX-1 expression in VSMCs. The expression of LOX-1 in the group of VSMCs exposed to glucose 25 mM + diltiazem was lower than uninduced control (without being exposed to glucose). This study demonstrated that calcium channel blocker (diltiazem) inhibits apoptosis in glucose-exposed VSMCs.
The LOX-1 imaging by argon convocal laser scanning microscope suggested that diltiazem has contributed to the LOX-1 expression. Pre-treatment with diltiazem 10 µM for 30 minutes inhibits LOX-1 expression in VSMCs exposed to glucose 25 mM. LOX-1 expression was lower in VSMCs than pre-treated with diltiazem compared to VSMCs exposed to glucose 25 mM (Figure 6).

Diltiazem Inhibits Apoptosis of VSMCs Exposed to Glucose 25 mM

Apoptosis is an evolutionary conserved pathway of biochemical and molecular events that underlie cell death processes involving the stimulation of intracellular zymogens. Evidence for involvement of Ca++ influx into the cytosol as triggering event for apoptosis has come from studies with specific Ca++ channel blockers that abrogate apoptosis of cancer cells. Caspase-3, a key regulatory protease at which many signaling pathways merge for the execution of apoptosis. The processes involving caspase activation has been examined in cancer cell by detail (Wertz, 2000). To examine whether diltiazem could affect VSMCs apoptosis, we measured caspase-3 activity of VSMCs incubated in glucose 25 mM with and without exposure to diltiazem. VSMCs were treated with diltiazem 10 µM for 30 minutes, then incubated the cells in the glucose 25 mM + diltiazem for 6 days. Pretreatment with diltiazem decreases activity of caspase-3 until under basal condition. This result confirmed that diltiazem protects apoptosis of VSMCs exposed to high glucose concentration (Figure 7).

The Role of Diltiazem on Ca++ Cytosolic Concentration

Evidences of the involvement of high glucose concentration on cytosolic Ca++ regulation have been established. The role of cytosolic Ca++ in cell death...
processes involving caspase activation has been examined in cancer cell.\textsuperscript{22} Ca\textsuperscript{++} variations play a predominant role in VSMCs activities, and there are compelling data that Ca\textsuperscript{++} channel blockers inhibit VSMCs growth or proliferation.\textsuperscript{23} Pre-treatment with diltiazem 10 µM for 30 minutes prevents the increase of cytosolic Ca\textsuperscript{++} concentration in VSMCs exposed to glucose 25 mM.

![Cytosolic Ca\textsuperscript{+} (expose to glucose 5 mM)](image)

![Cytosolic Ca\textsuperscript{+} (expose to glucose 25 mM)](image)

![Cytosolic Ca\textsuperscript{+} (expose to glucose 25 mM + diltiazem)](image)

#### DISCUSSION

Apoptosis of VSMCs may play important role in the destabilization of atherosclerotic plaques. During atherogenesis, medial VSMCs dedifferentiated, proliferate, and transmigrate into the intima, resulting in the formation of fibrous caps together with extracellular matrices (ECM).\textsuperscript{16} VSMCs produce the ECM, which can be degraded by metalloproteinases that are produced mainly by macrophages.\textsuperscript{17} Ox-LDL has been known to promote plaque instability by regulating the expression of metalloproteinases by macrophages. LOX-1 may be a key molecule in mediating the ox-LDL-induced apoptosis in VSMCs leading to plaque rupture.\textsuperscript{3}

LOX-1 is a receptor for ox-LDL expressed in vascular EC, macrophages, and VSMCs.\textsuperscript{3-5} LOX-1 expression is increased in the endothelium, and aortas of diabetes rat\textsuperscript{18} and thus may play a role in atherogenesis associated diabetes. Kataoka et al.\textsuperscript{3} revealed that the ox-LDL induced apoptosis of VSMCs through a Bax/Bcl2-dependent pathway. It can be, that ox-LDL-induced apoptosis of VSMCs involved downregulation of Bcl2 protein\textsuperscript{17}, that changed the Bax/Bcl2 ratio. Ox-LDL-induced VSMCs apoptosis also involved the activation of caspase-3.\textsuperscript{19}

The present study has established that the expression of LOX-1 was increased in VSMCs exposed to high glucose. We have further established that high glucose concentration induces activation of caspase-3, that may responsible for VSMCs apoptosis. Pre-treatment with LOX-1 blocker, k-carragenan 250 µg/mL for 48 hours before being exposed to glucose 25 mM, inhibits caspase-3 activity (Figure 4). This study proved that LOX-1 mediates the high glucose concentration-induced apoptosis in VSMCs.

Important functional properties of the vascular tissues are known to be closely coupled to change in cytosolic Ca\textsuperscript{++} concentration. In fact, elevation of Ca\textsuperscript{++} in response to several agonists is required for the synthesis of some intracellular proteins. High glucose concentration promotes increase cytosolic Ca\textsuperscript{++} concentration. There are compelling evidences that Ca\textsuperscript{++} channel blockers (CCBs) inhibit VSMCs growth and proliferation\textsuperscript{23}, but the mechanism underlying the inhibitory effect of CCB on VSMCs apoptosis in high glucose environment remain to be established.

We used free Ca\textsuperscript{++} media for this study. The increasing of Ca\textsuperscript{++} concentration in cytoplasma of VSMCs exposed to glucose 25 mM may be due to release of Ca\textsuperscript{++} from internal storage. There were three subclasses of CCB: phenylkylamine derivate (verapamil), benzothiazepin derivate (diltiazem), and dihydropyridine derivat (nifedipin, nimodipin, amlodipin, nicardipin, sisoldipin, nutrendipin, lasidipin, and lercandipin). All CCB works in L-type of voltage-dependent Ca\textsuperscript{++} channels (VDCC) lay in cell membrane. Amlodipin inhibited thrombin-induced Ca\textsuperscript{++} mobilization from a thapsigargin-sensitive pool and thapsigargin-induced Ca\textsuperscript{++} responses, including Ca\textsuperscript{++} mobilization from internal stores and store-operated Ca\textsuperscript{++} entry. The result was decreasing Ca\textsuperscript{++} concentration in the cytoplasma.\textsuperscript{28} Diltiazem which is a benzothiazepin class of CCB, has been reported to either block sarcoplasmic-reticulum leaks of Ca\textsuperscript{++} through Ryanodine-receptor (RyR), or block L-type of VDCC, making the concentration of Ca\textsuperscript{++} in the cytoplasma decrease. In contrast, the hydropyridine nifedipin is thought to block surface L-type Ca\textsuperscript{++} channels without affecting Ca\textsuperscript{++} leaks from SR Ca\textsuperscript{++} stores.\textsuperscript{30}

Pre-treatment with diltiazem 10 µM for 30 minutes, prevents the increase of cytosolic Ca\textsuperscript{++} concentration in VSMCs exposed to glucose 25 mM (Figure 8), pre-treatment also decrease LOX-1 expression (Figure 5), and caspase-3 activity (Figure 7). This study confirmed the role of cytosolic Ca\textsuperscript{++} on LOX-1 expression and caspase-3 activity.
PKC plays an important role in mediating biological responses, including tumor promotion and inflammation. Studies on the regulation of LOX-1 expression in EC, and VSMCs revealed upregulation of LOX-1 expression after incubation with PMA, an activator of PKC. This evidence proved the role of PKC on LOX-1 expression. High glucose concentration activates PKC-α, PKC-β, PKC-δ, and PKC-ε in vascular smooth muscle cells. Inoguchi et al. have suggested that high glucose concentrations lead preferentially to an increased expression of PKC-βII isoform in cardiovascular tissue. Ca++ was needed for the activity of conventional PKC, including PKC-βII isoform. It can be suggested that increasing Ca++ concentration in the cytoplasm of VSMCs exposed to high glucose concentration leads the increasing of LOX-1 expression and caspase-3 activity may through the PKC pathway. This hypothesis remains to be established.

CONCLUSION

Our studies offer the new insight for the role of diltiazem on the apoptosis of VSMC exposed to high glucose concentration. It can be stated for the first time that diltiazem inhibits apoptosis of VSMCs exposed to high glucose concentration through inhibition to LOX-1 expression.

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