The β-Specific Protein Kinase C Inhibitor Ruboxistaurin (LY333531) Suppresses Glucose-Induced Adhesion of Human Monocytes to Endothelial Cells in Vitro

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Abstract

Aims:
Strong evidence shows that late diabetic complications in diabetes mellitus are substantially related to an increased synthesis of diacylglycerol with a subsequent activation of protein kinase C (PKC) β. Several studies have shown that specific inhibition of the PKC isoform β by ruboxistaurin is able to attenuate the development of microvascular complications under diabetic conditions. The aim of this in vitro study was to investigate the effect of ruboxistaurin on glucose-induced adhesion of monocytes to endothelial cells, representing one of the first pivotal steps in the course of atherogenesis.

Methods:
Human umbilical venous endothelial cells were isolated and cultured to confluence in microtiter plates. After coincubation with monocytes in the presence of 0, 10, or 400 ng ruboxistaurin to achieve PKC β-specific and -unspecific PKC inhibition, cells were fixed and monocyte adhesion was determined by means of a standardized chemiluminescence assay. Expression of adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin) was also measured by chemiluminescence methods.

Results:
Adhesion of monocytes to endothelial cells cultured under hyperglycemic conditions (27.7 mM glucose) was increased by 30.9 ± 5.1% (p < 0.001) versus endothelial cells cultured under normoglycemic (NG) conditions (5.5 mM). Pretreatment of endothelial cells with 10 nM (PKC β-specific concentration) and 400 nM (PKC β-unspecific concentration) led to a significant reduction of glucose-induced adhesion of monocytes to endothelial cells that was statistically not different from endothelial adhesion under NG conditions (-7.2 ± 3.1 and -8.1 ± 2.6%, respectively; not significant vs NG). A nonsignificant tendency to lower the expression of adhesion molecules was seen with 10 ng of ruboxistaurin.

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Abbreviations: (DAG) diacylglycerol, (DMEM) Dulbecco’s modified Eagle’s medium, (HG) hyperglycemic, (HUVEC) human umbilical vein endothelial cells, (ICAM-1) intercellular adhesion molecule-1, (NG) normoglycemic, (NO) nitric oxide, (PBS) phosphate-buffered saline, (PC) personal computer, (PKC) protein kinase C, (PKC-2) protein kinase C-2, (RLU) relative light units, (VCAM-1) vascular cell adhesion molecule-1

Keywords: adhesion molecules, endothelial dysfunction, macrophage binding, protein kinase C, ruboxistaurin

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Abstract (continued)

Conclusions:
We conclude that monocyte adhesion to endothelial cells under hyperglycemic conditions is at least mediated by PKC β activation. Ruboxistaurin is able to suppress this monocyte adhesion even in a PKC β-specific concentration. Further studies should evaluate these potential effects of ruboxistaurin in vivo.

Introduction

Since the discovery of insulin, the most common cause of mortality in diabetic patients is vascular disease affecting macro- and microvasculatures. Multiple risk factors such as hypertension, hyperlipidemia, and hyperglycemia are considered to play a dominant role in the development of diabetic vascular complications. Several biochemical mechanisms have been proposed to explain the structural and functional abnormalities associated with vascular disease in diabetes mellitus.

Activation of the polyol pathway and the accumulation of diacylglycerol (DAG) have been reported in various cells and tissues in diabetic animals and humans, including the retina, aorta, heart, and glomeruli. Elevated DAG in turn activates protein kinase C (PKC) isoenzymes in different tissues such as the heart, aorta, retinal, or glomerular cells. The role of hyperglycemia in activating the DAG–PKC system has been shown in retinal endothelial cells, aortic endothelial and smooth muscle cells, and renal mesangial cells. A total of 12 isoenzymes of PKC have so far been cloned and characterized. PKC β isoforms seem to play a major role in diabetes-induced vascular damage in diabetes mellitus. PKC activation regulates a number of vascular functions, such as vascular permeability, contractility, cellular proliferation, basement membrane synthesis, and signal transduction mechanisms for hormones and growth factors.

Monocyte binding to endothelial cells is an important factor in diabetes-induced vascular disease and is one of the earliest steps in atherogenesis. Several investigators have demonstrated that monocyte binding is increased in vitro under hyperlipemic or hyperglycemic (HG) conditions. PKC is involved in the initial adhesion of monocytes to the vascular wall and fibrinogen binding and promotes their differentiation into macrophages.

An increase in the activity of the membrane PKC β2 isoform was observed in human monocytes under hyperglycemic conditions.

Quagliaro and colleagues examined the effects of stable and intermittent high glucose concentrations on intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin production, PKC activity, and PKC βI, βII, and δ isoform expression in cultured human umbilical vein endothelial cells (HUVEC). They reported that intermittent high glucose induces a greater expression of adhesion molecules than stable high glucose, which effect seems to be related to an activation of PKC β, but completely dependent from mitochondrial-free radical overproduction. PKC activity increased in fluctuating glucose compared to stable high glucose as a consequence of an overexpression of PKC βI, βII, and δ isoforms. Addition of the total PKC inhibitor bisindolylmaleimide I and LY379196, a specific inhibitor of PKC β, led to equal reduction of the adhesion molecules.

In another in vitro experiment with human arterial endothelial cells, Kouroedov and co-workers were able to demonstrate that phosphorylation of protein kinase C-2 (PKC-2) at serine/threonine residues Ser-660 represents a selective regulatory mechanism for glucose-induced upregulation of VCAM-1. They concluded that PKC-2-selective inhibitors may be promising drugs for the treatment of endothelial dysfunction during acute hyperglycemia and possibly in diabetes, which could potentially lead to a decrease in macrovascular complications.

In the context of the aforementioned study reports, this experiment was performed to determine the influence of ruboxistaurin (LY333531), a specific inhibitor of PKC...
β, which is approved for the treatment of secondary complications in the United States and Europe, on the adhesion of human monocytes to endothelial cells in vitro.

**Material and Methods**

**Isolation and Culture of Endothelial Cells**

Human umbilical vein endothelial cells were isolated according to the method of Jaffé and co-workers\(^2\) by the perfusion of human umbilical veins with 0.1% collagenase for 20 minutes. Harvested cells were washed with medium 199 and plated into 25-ml flasks coated with gelatin. Cells were cultured in medium 199 containing 20% fetal calf serum, 5 mg/ml endothelial growth supplement (Sigma Aldrich, Deisenhofen, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. The purity of endothelial cells was checked by indirect immunofluorescence microscopy using a monoclonal antibody against the von Willebrand factor. Only early passages were used for subsequent studies.

Cell growth assays were performed with a personal computer (PC)-based cell counter system (Casy 1, Schärfe Systems, Freiburg, Germany) after harvesting endothelial cells with 0.1% collagenase.

**Isolation of Monocytes**

Blood was drawn from healthy donors and anticoagulated with 5000 IU heparin. First, each blood sample was diluted with an equal volume of phosphate-buffered saline (PBS). Mononuclear cells were obtained by density gradient centrifugation using Ficoll (Pharmacia, Freiburg, Germany). The interface was collected and washed twice in DMEM/20% fetal calf serum. The amount of cells was determined in a luminometer (EG & Bertold, Freiburg, FRG) and analyzed after subtraction of photonic emission of nonspecific binding of the secondary antibody.

**Measurement of Adhesion Molecule Expression**

Secondary passages of HUVEC were cultured in microtiter plates coated with gelatin. After various stimulation steps, cells were incubated with a chemiluminescent-labeled secondary antibody (flashlight-GxM1gG; Biotrend, Köln, FRG) at a concentration of 25 ng/ml, photonic emission [relative light units (RLU)] was measured in a luminometer (EG & Bertold, Freiburg, FRG) and analyzed after subtraction of photonic emission of nonspecific binding of the secondary antibody.

**Statistical Analysis**

Data are shown as means ± SD. Statistical analysis was performed using the Mann–Whitney U test. A p value <0.05 was considered to be statistically significant.

**Results**

High levels of glucose significantly reduced endothelial cell growth in culture compared to endothelial cells kept at physiological levels of glucose (Figure 1). The presence...
of different concentrations of ruboxistaurin (10 and 400 nM), however, did not modify endothelial cell growth in vitro.

There was a significant increase of monocyte adhesion to endothelial cells in culture if stimulated with high levels of glucose (Figure 2). The percentage of attached monocytes under normoglycemic (NG) conditions averaged 13.9 ± 2.9%, whereas after stimulation of endothelial cells with 27.7 mM glucose, adhesion increased to 19.9 ± 3.5% (p < 0.01). Endothelial stimulation with 27.7 mM mannitol had no effect on monocyte adhesion.

Pretreatment of cell culture assays with ruboxistaurin at concentrations of 10 and 400 nM significantly suppressed this glucose-induced monocyte adherence to levels that were not different from baseline adherence of monocytes to endothelial cells under NG conditions (Figure 3).

Furthermore, the expression of cell adhesion molecules on the surface of endothelial cells was not altered by ruboxistaurin, neither under NG conditions (data not shown because high levels of glucose did not modify the expression of the different cell adhesion molecules in this short-term stimulation assay) nor under HG conditions (Figure 4).

Figure 1. Endothelial cells were cultured in different media containing 5.5 and 277 mM glucose representing NG and HG conditions. HG cultures reveal decreased cell growth compared to NG cultures, whereas the addition of ruboxistaurin at different concentrations (10 and 400 nM) did not affect cell growth.

Figure 2. Under normoglycemic conditions (5.5 mM glucose; NG), monocytes adhered to endothelial cells at a rate of 13.9 ± 2.9%. Stimulation of endothelial cells with 27.7 mM glucose (HG) significantly increased the adhesion rate to 19.9 ± 3.5% (p < 0.01). Stimulation of endothelial cells with 27.7 mM mannitol (serving as an isoosmolar control) had no effect on monocyte adhesion compared to NG (14.7 ± 2.6%; not significant, n.s.).

Figure 3. Compared to normoglycemia, there was a 30.9 ± 5.1% increase of monocyte adhesion after stimulation with 27.7 mM glucose. Coincubation of these hyperglycemic cultures with either a β-specific concentration (10 nM) or a nonspecific concentration (400 nM) of ruboxistaurin was able to suppress monocyte adhesion to levels that were not significantly different from those seen in NG cultures [-7.2 ± 3.1 and -8.1 ± 2.6%, respectively; each not significant (n.s.) vs NG].

Figure 4. Stimulation of endothelial cells with 27.7 mM glucose (HG) did not modify expression of the investigated adhesion molecules (VCAM-1, ICAM-1, and E-selectin) compared to endothelial cells grown in medium containing 5.5 mM glucose (data not shown). Coincubation with a β-specific (10 nM) and a nonspecific (400 nM) concentration of ruboxistaurin did not change the endothelial expression of adhesion molecules, as shown by means of a chemiluminescence assay (not significant vs each hyperglycemic control).
Discussion

The most common cause of mortality and morbidity in patients suffering from diabetes mellitus is vascular complications, affecting both micro- and macro-vascularatures. Several hypotheses have been proposed to explain the relationship between diabetes and diabetic vascular complications. Numerous studies have provided evidence that activation of PKC plays a dominant role in the pathogenesis of diabetic complications.

The diabetes-induced activation of PKC by elevated glucose levels appears to be related to the elevation of DAG deriving from de novo synthesis. DAG per se is a physiological activator of PKC enzymes in vivo and in vitro. PKC comprises a family of at least 12 isoforms of serine threonine kinases, which are classified into subfamilies on the basis of their enzymatic and structural properties. It is well established that PKC isoform β is activated predominantly in the vasculature under diabetic conditions, especially in the heart and aorta.

This isoform-selective activation of PKC by diabetes produces a number of biochemical abnormalities associated with vascular complications of diabetes, e.g., inhibition of Na+/K-ATPase and regulation of gene expression of many different proteins, including those involved in vascular contractility and the basement membrane.

Given the role of PKC β in diabetes-induced changes in vascular tissues, there has been considerable interest in developing isoform-specific PKC inhibitors. The PKC β-selective inhibitor ruboxistaurin, a macrocyclic bisindolylmaleimide compound, has been shown to be able to ameliorate microvascular damage in streptozotocin-induced diabetic rats, e.g., normalization of retinal blood flow and glomerular filtration, improvement of renal albumin excretion, and prevention of abnormalities in mRNA expression of transforming growth factor β1, type IV collagen, and fibronectin in glomeruli.

In contrast to these findings in the microvasculature, there is no clear evidence regarding the potential beneficial effect of ruboxistaurin on the development of macrovascular dysfunction and atherogenesis in diabetes mellitus. Thus, it was the aim of this study to evaluate the impact of this β-selective inhibitor of PKC on monocyte adhesion, which is known to be one of the first pivotal steps in atherogenesis. Several authors have demonstrated increased adhesion of monocytes under diabetic conditions in vitro, which is coincident with prior findings of our group and the results of this study.

High levels of glucose (27.7 mM) were able to increase the adhesion of monocytes in vitro by approximately 30% compared to adhesion assays conducted under normoglycemic (5.5 mM glucose) conditions. Based on the early studies of King and Brownlee, it has been hypothesized that this increased adhesion of monocytes in vitro is related to PKC β activation in endothelial cells, which in turn leads to the activation of unknown adhesion molecules (e.g., integrins) on the monocyte surface.

Therefore, we investigated the effect of ruboxistaurin on this in vitro model of early atherogenesis. Hyperglycemic conditions led to a decrease in cell growth in our investigation. This finding is in line with previous results, e.g., from Rojas and co-workers who identified that D-glucose-dependent inhibition of thymidine incorporation and cell proliferation is associated with increased PKC, endothelial nitric oxide (NO) synthase, and p42 and p44 mitogen-activated protein kinases, but decreased superoxide dismutase activity, and higher intracellular levels of cyclic guanosine monophosphate, cyclic adenosine monophosphate, and Ca2+ in HUVECs. They postulated that these potential cellular mechanisms may reduce endothelial cell growth in pathological conditions such as in diabetes mellitus or hyperglycemia in humans. Hyperglycemic conditions, however, did not lead to an increase in adhesion molecule concentrations in our experimental setting, which is in contradiction to the findings of Quagliaro et al. and Kouroedov et al. who reported a significant higher expression of ICM, VCAM, and E-selectin in their in vitro models. This difference may be related to the different experimental conditions. We worked with freshly prepared HUVEC with a short continuous exposition to hyperglycemia, whereas Quagliaro and colleagues applied intermittent hyperglycemia over a longer period of time and Kouroedov et al. used a different cell population and also applied a longer hyperglycemic incubation.

In any case, we demonstrated that ruboxistaurin did not modify cell growth of endothelial cells in culture or expression of endothelial adhesion molecules (VCAM-1, ICAM-1, and E-selectin), which are involved predominantly in monocyte adhesion. Moreover, we were able to demonstrate in a substudy by means of quantitative reverse transcriptase polymerase chain reaction (data not published) that ruboxistaurin did not modify the expression of the two gene products of the PKC β gene (β1 and β2) generated by alternative splicing.
The most important finding of this study was the observation that ruboxistaurin was able to totally inhibit the glucose-induced adhesion of monocytes to endothelial cells so that the monocyte–endothelial interaction was not different from normoglycemic conditions. As this effect was not linked to a decrease in adhesion molecules, it is postulated that it is mediated by an increase in NO production, as shown previously.\textsuperscript{38} Specific inhibition of PKC \( \beta \) seems to be able to prevent endothelial adhesion of monocytes, representing one of the first steps of atherogenesis.

We conclude that endothelial activation of PKC \( \beta \) may be substantially involved in monocyte adhesion in diabetes mellitus, explaining at least a major part of the pathogenesis of atherosclerosis in the face of this metabolic disease. Moreover, we were able to demonstrate that ruboxistaurin is a potent inhibitor in this \textit{in vitro} model of atherogenesis. Therefore, subsequent clinical trials with this selective inhibitor of PKC \( \beta \) activation should investigate whether this beneficial effect could be confirmed \textit{in vivo}.

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Disclosures:

Ruboxistaurin is manufactured and marketed by Eli Lilly, Indianapolis. Christof Kazda is an employee of Eli Lilly. Thomas Kunt, Thomas Forst, and Andreas Pfützner have received speaker fees and travel support from Eli Lilly.

References:

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