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Biochemical Pharmacology 67 (2004) 855–864
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Reduction of high glucose and phorbol-myristate-acetate-induced endothelial cell permeability by protein kinase C inhibitors LY379196 and hypocrellin A

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Received 20 June 2003; accepted 6 October 2003

Abstract

Endothelial barrier dysfunction plays a pivotal role in the pathogenesis of diabetic vascular complications. Although recent studies have established a link between protein kinase C (PKC) pathway and hyperglycaemic-induced vascular permeability, it is unclear which PKC isoforms involve increased endothelial cell permeability. In the present study, we investigated whether high glucose induced endothelial hyperpermeability via distinct PKC isoforms in human umbilical vein endothelial cells (HUVECs) and whether increased endothelial permeability could be substantially reversed by PKC inhibitors LY379196 and hypocrellin A (HA). High glucose (20 mM) and phorbol-myristate-acetate (PMA)-induced endothelial hyperpermeability was almost abolished by 150 nM HA and partially reduced by 30 nM PKC β inhibitor (LY379196). LY379196 and HA inhibited the membrane fraction of PKC activity in a dose-dependent manner. Western blot analysis revealed high-glucose-induced overexpression of PKC α and PKC β2 in the membrane fraction of HUVECs. LY379196 (30 and 150 nM) selectively inhibited PKC β2 with no significant effect on PKC α expression. HA (150 nM) significantly reduced PKC α expression with no inhibitory effect on PKC β2. At higher concentrations (300 nM), both LY379196 and HA were no longer selective for PKC β or α, respectively. This study showed that both PKC α and β2 contributed to endothelial hyperpermeability. Since reduction of endothelial hyperpermeability was greater with inhibition of PKC α rather than PKC β2, we conclude that PKC α may be a major isoform involved in endothelial permeability in HUVECs, and that PKC α-mediated endothelial permeability was significantly reversed by the PKC inhibitor HA.

Keywords: Endothelial permeability; Diabetic vascular complications; Protein kinase C; LY379196; Hypocrellin A; Human umbilical vein endothelial cells

1. Introduction

Diabetes mellitus is associated with an increased risk of cardiovascular disease. In the last decade, there has been increasing evidence that endothelial dysfunction plays a key role in the pathogenesis of diabetic micro- and macrovascular complications, which are the major causes of morbidity and mortality in patients with type 1 and type 2 diabetes [1,2].

The endothelial cells, which line the lumen of all the vasculature, provide not only a simple barrier but also produce vasoactive factors, which maintain vascular tone and hemostasis [3]. Thus, endothelial dysfunction may result in multiple abnormalities in the regulation of blood vessels, including decreased endothelium-dependent vasorelaxation, abnormalities in retinal and glomerular blood flow and increased permeation of circulating macromolecules [4–6]. Several studies have shown that increased endothelial permeability is an early manifestation of endothelial dysfunction in animals and humans with diabetes [7–9]. Moreover, increased endothelial permeability can lead to macular edema and proliferative diabetic retinopathy [10], as well as mesangial matrix expansion and glomerulosclerosis [11]. A current hypothesis suggests that endothelial dysfunction associated with increased permeability to plasma constituents may contribute to

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Abbreviations: PKC, protein kinase C; HA, hypocrellin A; PMA, phorbol-myristate-acetate; HG, high glucose; NG, normal glucose; HUVECs, human umbilical vein endothelial cells; LY, LY379196; IC_{50}, 50% inhibition concentration; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; FBS, foetal bovine serum; ECGP, endothelial cell growth promoter; SFM, serum-free medium; BSA, bovine serum albumin; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid.

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doi:10.1016/j.bcp.2003.10.012

pathogenesis of atherosclerosis [12], since impaired barrier function of endothelial cells can facilitate deposition of lipid molecules in the vessel wall and accelerate development of inflammation and coagulation, which are involved in the genesis of atherosclerotic plaques.

The increased endothelial permeability in diabetes is multifactorial. There are various lines of evidence demonstrating that activation of PKC, in particular overexpression of PKC β isoform significantly increases endothelial permeability, which is related to diabetic microvascular complications such as diabetic retinopathy [13] and diabetic nephropathy [14]. Hence, research has been directed towards the development of inhibition of PKC. It has been demonstrated that a selective PKC β inhibitor ruboxistaurin (LY333531) ameliorated diabetes-induced vascular dysfunction in diabetic rats [15,16]. In recent clinical studies, ruboxistaurin treatment substantially reversed the diabetes-induced retinal vascular dysfunction in patients with type 1 and type 2 diabetes [17]. The knowledge obtained from biological and pharmacological studies on endothelial dysfunction and diabetic microvascular complications has given impetus to the clarification of the role of PKC activation and isoform expression in diabetic macrovascular complications and to the search for novel PKC inhibitors. Yuan *et al.* [7] have shown that PKC β_2 and ε contribute to dysfunction of the endothelial barrier in coronary vessels in diabetic animals; but an *in vitro* study has shown that a high-glucose-induced increase in albumin permeability in endothelial cells was associated with the overexpression of PKC α [18]. Thus, which specific PKC isoforms relate to hyperglycaemia-induced endothelial permeability in larger vessels needs to be determined.

PKC inhibitors have been used extensively to investigate a variety of biological mechanisms. Most of the PKC inhibitors target the catalytic domain of PKC, which shows striking homology with ATP binding sites of tyrosine kinase and other serine/threonine kinases, such as protein kinase A. LY379196 is a new semi-synthesized PKC inhibitor which targets the catalytic domain of PKC. It has been confirmed by Lilly Research Laboratories that LY379196 has a similar PKC isoform inhibitory profile to LY333531 which shows high selectivity for β isoforms of PKC [19]. Hypocrellin A was isolated from a parasitic fungus *Hypocrella bambuase* (B.et Br) sacc which grows abundantly in the northwestern region of Yunan Province in China. HA was originally used as a phototherapeutic agent for tumours and various skin diseases [20]. A number of studies have indicated that HA possesses a high specificity for inhibiting the regulatory domain of PKC [20,21]. Chemical analysis has revealed perylene quinonoid structure of HA [22]. In view of its selective and potent inhibitory effect on PKC activity, HA has been synthesized as a pharmacological tool to study biochemical function of PKC [23]. Our previous studies have shown that HA has a potent inhibitory effect on PKC activity in the liver and skeletal muscles of obese Zucker rats [24].

Most of the previous investigations of endothelial permeability associated with high glucose have been carried out in animal models. In the present experiments we have studied human endothelial cells from the umbilical cord and demonstrated that PKC α and β2 are the isoforms which are activated by hypergycaemia. In addition, the effects of isoform-selective inhibitors suggest that PKC α may be the major isoform involved in hyperglycaemia-induced permeability in these human endothelial cells.

2. Materials and methods

Trypan blue, PMA, DMSO, EDTA and glucose were obtained from Sigma Chemical Co. L-Glutamine, Media 199 (M199), trypsin-EDTA and FBS were supplied by Trace Scientific. ECGP was supplied by Starrate Pty. Ltd. PKC α, β1, β2, ε, θ, δ, λ, ζ and η antibodies were purchased from Santa Cruz, LY379196 was generously provided by Lilly Research Laboratories. Hypocrellin A was isolated from *H. banbuase* in Botany Research Centre at Yunnan University, China. In brief, ethanol extract of dried fungus *H. bambuase* was applied to thin layer chromatography (TLC)-grade silica gel followed by crystallised twice from acetone to obtain HA compound which appeared a single spot in two TLC solvents: hexane/ethyl acetate and chloroform/methanol.

2.1. Cell culture

HUVECs were isolated from fresh human umbilical cords as described by Du *et al.* [25]. Briefly, the vein was washed with serum-free M199 medium (M199-medium containing 2 mM L-glutamine, 0.1 mg/mL streptomycin and 2 U/mL penicillin) to remove blood. Then a solution of 0.1% collagenase was injected into the vein which was then incubated at 37° for 15 min. The reaction was stopped by complete medium (M199-medium containing 20% foetal bovine serum, 0.5% Endothelial Cell Growth Promoter, 2 mM L-glutamine, 0.1 mg/mL streptomycin and 2 U/mL penicillin). Cells were washed out and collected in falcon tubes by light centrifugation at 400 *g* for 5 min then re-suspended in complete media and transferred onto 150 cm^2 gelatin coated flask (4% gelatin diluted 1:250 Dulbeccos' PBS which was incubated in 5% CO_2, 95% air incubator at 37°. Medium was changed every 2–3 days. Cells were identified as HUVECs by their typical cobalt stone morphology. Subcultures were performed by using trypsin-EDTA (1:250) after confluence, which took about 7 days. For all experiments, second–third passages were used.

2.2. PKC inhibitors

The cells were incubated in normal (5 mM) or high (20 mM) concentrations of glucose, or mannitol 20 mM (as an osmotic control) for a period of 48 hr. The PKC

inhibitors (or DMSO as a vehicle control) were then added for 60 min.

Both LY379196 and HA were first dissolved in DMSO at a concentration of 2 mg/mL. A series of concentrations of PKC β inhibitor LY379196 (30, 150, 300 nM) and HA (30, 150, 300, 600 nM) were used. Fifteen microlitres of appropriately diluted PKC inhibitors or DMSO was added into the cell culture flask containing 10 mL of serum-free medium (SFM). The final concentration of DMSO was 0.15% (v/v).

2.3. Measurement of endothelial cell permeability

For the detection of endothelial permeability, albumin diffusion across endothelial monolayers was measured as described by Chen *et al.* [26]. Falcon cell culture inserts (3.0 μm) for use with 24-well tissue culture plates were coated with 50 μg/mL of type I collagen in 20 mM acetic acid for 1 hr at room temperature. After aspirating the remaining solution from the wells, inserts were washed with PBS buffer to remove excess acid. Complete medium was added to both inserts and wells, and the membrane equilibrated for 3 hr in 5% CO_2 plus humidified air at 37°. Passage 2 of HUVECs were seeded on inserts at 2×10^5 cells/insert then incubated at 37° in 5% CO_2, 95% air incubator. Media in both inserts and lower wells were replaced every day. HUVECs monolayers grown to confluence on membranes were examined by phase-contrast microscopy to ensure that they were intact [26]. After growing cells on inserts for 4 days, cells were incubated with 5 or 20 mM glucose in ECGF-free M199 for 48 hr. Media were changed to SFM prior to addition of PKC inhibitors. A series of concentrations of LY379196 and HA were added to both inserts and lower wells and incubated for 1 hr. After washing with ice cold PBS to stop drug enzyme reaction, 750 μL of PBS was added to each lower well and 150 μL of trypan blue-labeled albumin (0.035% trypan blue and 0.8% BSA fraction V) was added to each insert. These volumes ensured similar hydrostatic pressure on either side of the HUVEC monolayer. Incubation was continued for 30 min at 37° in a 5% CO_2 incubator. At the end of incubation, inserts were carefully removed and the medium in each lower well was thoroughly mixed. To determine the amount of albumin that had passed through the cell monolayer, a 50 μL sample from both insert and lower well was collected for measuring the absorbance at 590 nm using an UV-spectrophotometer. When conducting the time course study, 50 μL of medium was gently taken out every 10 min with gel loading tip from lower well up to 40–60 min and replaced by an equal volume of PBS. HUVEC monolayer permeability was quantitated as a percentage clearance of BSA from insert to lower well as described by Quan [27]. In a separate series of experiments, PMA was used to induce endothelial permeability. Cells were incubated with the PKC inhibitors, or DMSO, for 60 min before the addition of PMA 100 nM for 30 min.

2.4. Preparation of cell fraction for western blotting and PKC activity assay

Subconfluent (80% $\sim 2.4 \times 10^6$ to 3.2×10^6 cells in 75 cm^2 flask) third passage of HUVECs was incubated with either normal (5 mM), high (20 mM) glucose or mannitol (20 mM) in 2% serum media for 48 hr. After treatment with glucose or mannitol, media were changed to SFM then a series of concentrations of LY379196 or HA were added. Cells were incubated with PKC inhibitors for 1 hr then the reaction was stopped by incubating cells with ice-cold PBS for 10 min at 4°. Cells were washed twice with ice-cold PBS, scraped off and pelleted by light centrifugation at 400 *g* for 5 min at 4°. After aspirating PBS, cells were suspended in 0.2 mL ice-cold buffer A (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.25 mM EGTA, 0.25 M sucrose, 2 μg/mL leupeptin and pepstain A, and 4 μg/mL each of aprotinin, calpain-I and calpain-II), and homogenised for 2×15 s (setting 5) using a Polytron (Ultra-Turrax T8, IKA-Labortechnik,). Samples were then spun at 100,000 *g* for 60 min at 4° in an ultracentrifuge (Beckman L7-35). This supernatant, termed the cytosol fraction, was removed and kept in ice. The pellet (membrane fraction) was resuspended in 0.15 mL ice-cold buffer B (buffer A + 0.5%, v/v, Triton X-100) and ultracentrifuged at 100,000 *g* for 30 min at 4° to get the supernatant as membrane fraction. Amount of protein from cytosol and membrane fractions were quantitated using the Bradford method [28].

2.5. PKC activity assay

Cytosol and membrane fractions of HUVECs prepared as above were applied to the DEAE columns, respectively. One millilitre of elution buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA and 0.3 M NaCl) was added to the column and the elution was collected as the enzyme fractions. Enzyme activity was assayed immediately after completion of the chromatography step by following the incorporation of [^{32}P] from [γ-^{32}P] ATP into acceptor GS peptide (H-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-NH_2), a synthetic dodecapeptide of glycogen synthase used as a PKC substrate (Auspep) as described by Azhar [29]. The standard reaction mixture contained the following components in a final volume of 50 μL: 10 μL aliquots of chromatography fractions; 25 mM HEPES–NaOH (pH 7.4), 10 mM magnesium acetate, 20 mM GS peptide and 5 mM 2-mercaptoethanol; 0.5 mM ATP and 0.1 mM [γ-^{32}P] ATP (200–300 cpm/pmol); 1.0 mM $CaCl_2$; 150 mg/mL phosphatidylserine and 10 mg/mL diolein. Basal activity was determined in the presence of 0.5 mM EGTA (instead of Ca^{2+}, phosphatidylserine and diolein). The reaction was initiated by the addition of [γ-^{32}P] ATP at 30°. After incubating for 15 min, the reaction was terminated by spotting 25 μL of the mixture onto 2 cm × 2 cm phosphocellulose strips, which were dropped

immediately into 75 mM phosphoric acid. The strips were then washed twice, including one overnight wash, in 75 mM phosphoric acid. The dried strips were counted in a liquid scintillation counter after the addition of 5 mL of scintillation solution. PKC activity was calculated by subtracting the enzyme activity observed in the presence of 0.5 mM EGTA from that measured in the presence of phosphatidylserine, diolein and calcium. One unit of PKC activity was defined as that amount catalyzing the transfer of 1 pmol of [^{32}P] phosphate from [γ-^{32}P] ATP to GS peptide per min at 30°.

2.6. Western blot analysis

Cytosol and membrane samples from the cell preparation steps were mixed with equal volumes of 2X sample-loading buffer (4.4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 16% (w/v) sucrose and 0.1 M Tris–HCl, pH 6.8). Four microgram of total protein of each sample was subjected to 7% SDS–polyacrylamide electrophoresis gel followed by transfer to a polyvinylidene difluoride membrane using semi-dry transfer system (Bio-Rad Laboratories,). The membranes containing the samples were then incubated for 2 hr at room temperature in PBS buffer containing 1% Tween-20 and 5% (w/v) nonfat dried milk to block nonspecific binding. After blocking, membranes were washed in rinsing solution (PBS-T with 1% (w/v) dried milk, pH 7.4) and then incubated overnight with isoform-specific polyclonal PKC antibodies (PKC α, β1, β2, ε, θ, δ, λ, η and ζ, diluted in 1:100 with rinsing solution). To detect the antigen-bound antibody, the blots were treated with horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG for 1 hr. After washing the membrane with PBS buffer, a chemiluminescence detection kit (Dupont) was used for revealing step and development on to Kodak-XOMAT film. Each film was exposed for 3 min. The bands obtained from immunoblotting were scanned using a Kodak Image Station (Model III) with automated software analyses of the intensity of the bands.

2.7. Statistical analysis

The data are expressed as means ± SEM. Differences between control and high glucose, PMA and PKC inhibitors were statistically assessed using a one-way ANOVA test and unpaired student's *t*-test with $P < 0.05$ considered as significant.

3. Results

3.1. Effect of PKC inhibitors on high-glucose- and PMA-induced increase in permeability

Exposure of HUVECs to 20 mM glucose media for 48 hr caused significant increase in endothelial permeability compared with normal glucose incubation (Fig. 1). An increased flux of albumin across the endothelial cell barrier was observed within minutes of incubation with Trypan blue-labeled BSA and reached the maximum at about 30 min. Thereafter, endothelial permeability slowly declined to a plateau phase (Fig. 1). Based on the findings of the time course study, the effect of high glucose, PMA and PKC inhibitors on endothelial cell permeability were measured at 30 min of incubation.

Figure 2 shows the effects of PKC inhibitors on high-glucose- and PMA-induced endothelial cell permeability to albumin. Incubation of cells for 60 min with the PKC

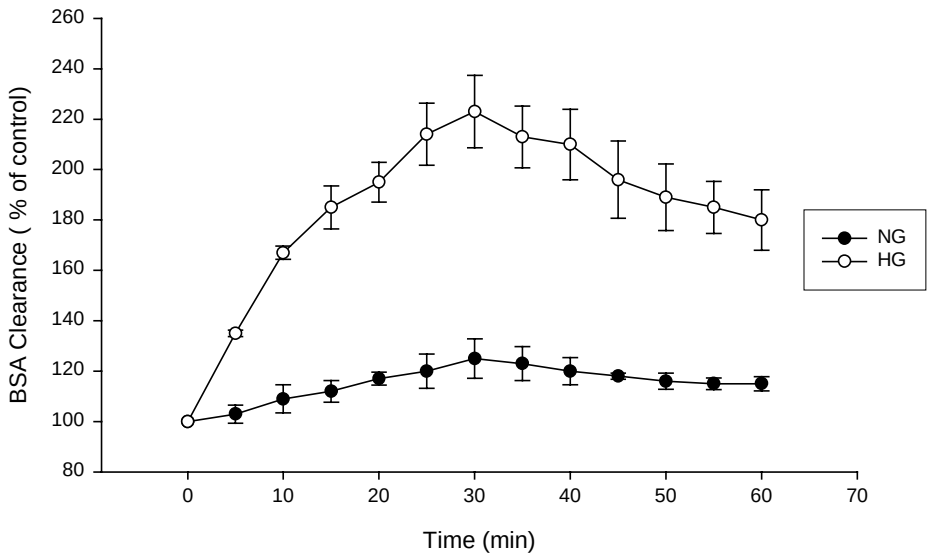

Fig. 1. Time course of glucose-induced albumin flux across HUVECs monolayers, which have been previously exposed to high or normal glucose Data are a percentage of control; 100% corresponds to cell permeability to albumin under normal glucose (NG) concentration (5 mM) at baseline (time 0). Data are mean ± SEM (N = 5 separate experiments). Significant increase in permeability in the HUVECs pre-incubated with high glucose (HG, 20 mM) was observed within minutes and reached its maximum at 30 min and remained at high levels thereafter.

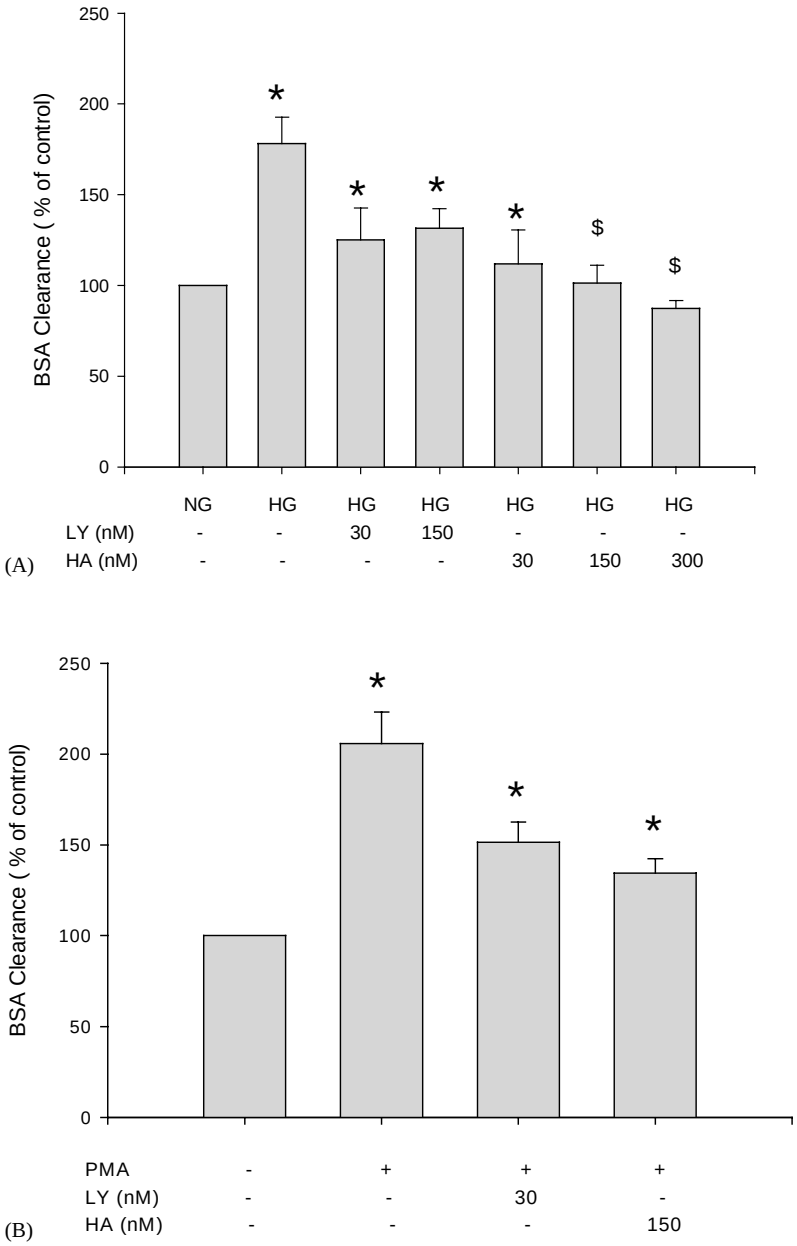

Fig. 2. Effect of PKC inhibitors on high-glucose- and PMA (100 nM)-induced hyperpermeability to albumin in HUVECs. (A) Data show 30 nM LY379196 significantly reduced endothelial permeability from 178 ± 14.4% to 125 ± 17.5% ($P < 0.05$), while 150 nM LY379196 had a similar effect to 30 nM of LY379196. 150 nM HA reduced the permeability to almost baseline (101 ± 9.6%, $P < 0.01$)). (B) 30 nM LY379196 and 150 nM HA significantly reduced the hyperpermability induced by PMA (205 ± 17.2% vs. 151 ± 11.2% and 134 ± 8.1%, respectively). N = 5 separate experiments. $^{*}P < 0.05$ and $^{\$}P < 0.01$ vs. HG without PKC inhibitors.

inhibitors, LY379196 and HA significantly attenuated the increased endothelial permeability induced by high glucose concentrations (Fig. 2A). Thirty nanomolar LY379196 reduced hyperpermeability from 178 ± 14.4% (HG control) to 125 ± 17.5% ($P < 0.05$), but higher LY379196 concentration (150 nM) caused no further inhibition. HA reduced high-glucose-induced endothelial permeability in a dose-dependent manner. The hypepermeability was almost abolished by 150 nM HA (178 ± 14.4% to 101 ± 9.6%, $P < 0.01$).

LY379296 and HA also significantly inhibited PMA-induced endothelial permeability (Fig. 2B). After pretreatment for 60 min with 30 nM LY379196 or 150 nM HA, PMA-induced hyperpermeability reduced from 205 ± 17.2% to 151 ± 11.2% ($P < 0.05$) and 134 ± 8.1% ($P < 0.01$), respectively. Thus, 150 nM HA produced a greater protection against PMA-induced endothelial hyperpermeability than 30 or 150 nM of LY379196.

3.2. Effects of LY379196 and HA on high-glucose-induced PKC activity in HUVECs

After 48 hr of incubation with 20 mM glucose, PKC activity was significantly higher in the membrane fraction

Table 1
Effect of glucose on PKC activity in the membrane and cytosol fractions of cultured human umbilical vein endothelial cells

Glucose (mM)	PKC activities (pmol/min/mg protein)		
	Membrane	Cytosol	Membrane/cytosol
5	2.48 ± 1.51	12.41 ± 3.24	0.27 ± 0.13
20	15.40 ± 5.95*	8.37 ± 3.15	1.93 ± 0.65*

Data are mean ± SEM from four separate experiments.
* $P < 0.01$ compared with 5 mM glucose-treated cells.

of HUVECs compared with membrane PKC activity from cells incubated in normal glucose (Table 1) while cytosol PKC activity was lower in high-glucose-treated cells compared with normal glucose-treated cells, but the difference was not statistically significant. PKC activation and translocation to the membrane fraction induced by high glucose was clearly shown by comparison of the ratio of membrane over cytosol PKC activity (Table 1, $P < 0.01$).

The inhibitory effects of HA and LY379196 on PKC activity are shown in Fig. 3. Treatment with a range of concentrations of LY379196 (3, 30, 300 nM and 3 μM) and HA (15, 150 nM, 1.5 and 15 μM) for 1 hr markedly attenuated high-glucose-induced PKC activity. Both LY379196 and HA inhibited PKC activity in a dose-dependent manner ($IC_{50} = 4.7 \times 10^{-8}$ M and 1.4×10^{-8} M, respectively) with HA being approximately three times more potent (Fig. 3).

3.3. PKC isoform expression and translocation in HUVECs

With immunoblotting analysis using specific antibodies seven isoforms of PKC (α, β1, β2, δ, ε, θ and ζ) were detected in HUVECs, whilst PKC λ and η were not. Of these, PKC α and β2 were the major isoforms expressed in HUVECs, and translocation of PKC α and β2 from the cytosol to membrane fraction occurred when cells were exposed to media containing 20 mM of glucose.

3.4. Effects of LY379196 and HA on PKC α and β2 protein expression in the membrane fraction of HUVECs

After screening detection of expression of PKC isoforms in HUVECs, we intensively analysed the inhibitory effects of LY379196 and HA on PKC α and β2 protein in the membrane fraction of high-glucose-treated HUVECs. Incubation with serial concentrations of LY379196 and HA significantly reduced PKC α and β2 expression in the membrane (Fig. 4). LY379196 inhibited PKC β2 expression in the membrane fraction at 30 nM (64 ± 15.3% vs. control, $P < 0.05$), but a higher concentration (300 nM) was required to significantly inhibit PKC α (55 ± 8.6% vs. control, $P < 0.05$). HA significantly inhibited PKC α at 150 nM (70 ± 9.0% vs. control, $P < 0.05$) but did not significantly affect PKC β2 expression at this concentration. Higher HA concentration (300 nM) also significantly reduced PKC β2 expression (47 ± 11.2% vs. control, $P < 0.01$).

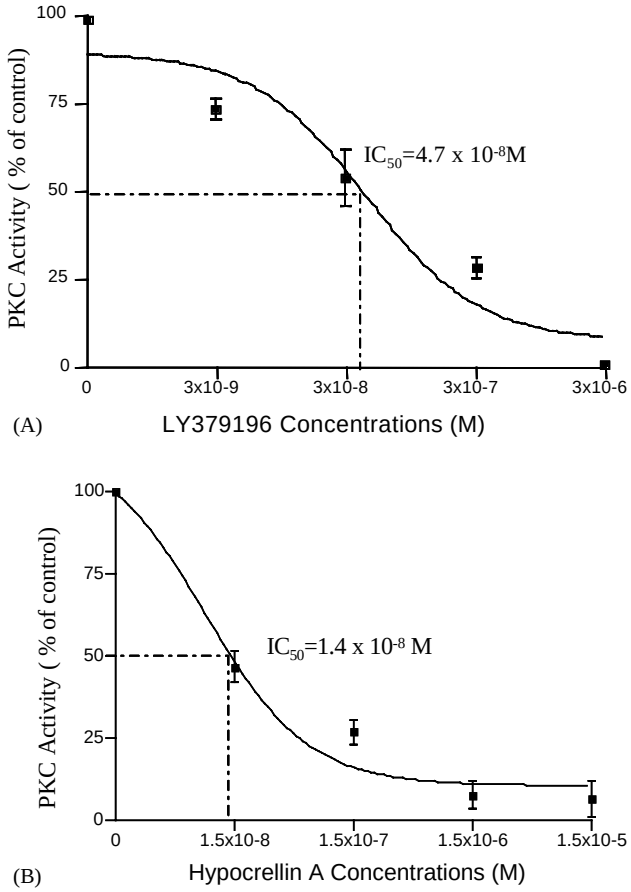

Fig. 3. The effect of PKC inhibitors on the membrane PKC activity induced by high glucose (20 mM). Results were expressed as a percent of control (activity measured in 15 μL DMSO-treated cells). (A) LY379196 dose–response curves. Thirty nanomolar LY379196 reduced about 30 ± 3.5% of PKC activity compared with control ($P < 0.05$). (B) HA dose–response curves. With 150 nM HA treatment, PKC activity decreased by 55 ± 5.0% compared with control ($P < 0.01$). N = 5 separate experiments.

4. Discussion

In the present study, endothelial dysfunction, as shown by increased endothelial permeability to albumin, was induced by high glucose concentration and PMA. These data strongly suggest that PKC was involved in increased endothelial permeability in HUVECs, as PMA is a potent PKC activator [30]. In addition, hyperglycaemia can activate PKC via enhancement of *de novo* synthesis of diacylglycerol (DAG) which activates of PKC by binding to a specific domain on its regulatory subunit [31]. The present findings agree with most previous studies, which have demonstrated a role of PKC activation in the permeability of endothelial cells obtained from porcine aorta and bovine pulmonary arteries [18,32]. Our findings with cultured endothelial cells freshly isolated from human umbilical

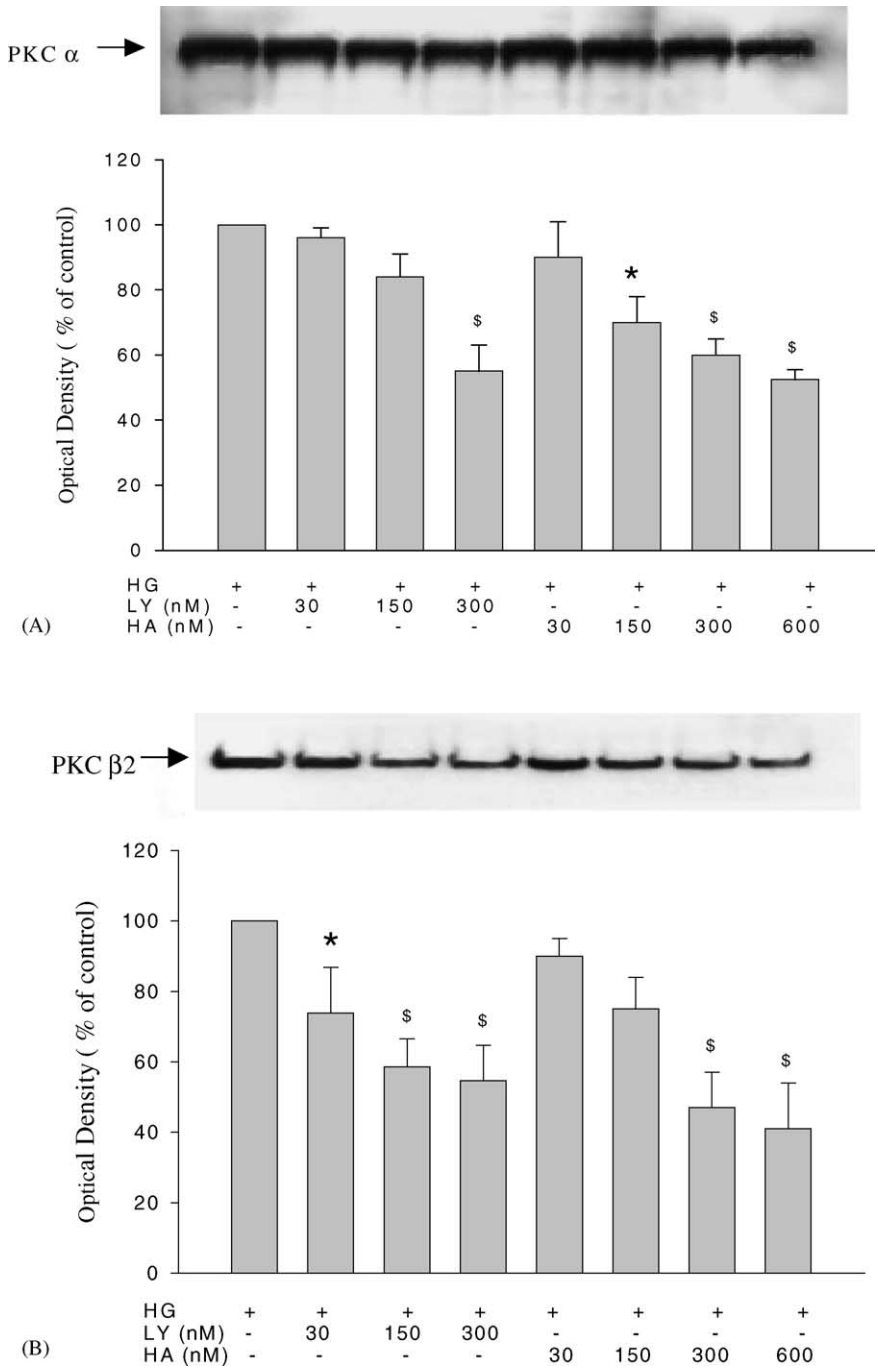

Fig. 4. Effect of LY379196 and HA on PKC α and β2 expression in the membrane fraction of HUVECs. (A) 30 and 150 nM LY379196 had no significant effect on PKC α expression, but 300 nM LY379196 inhibited PKC α expression. HA inhibited PKCα from 150 nM ($70 \pm 9\%$ vs. control, $P < 0.05$). (B) LY379196 significantly inhibited PKC β2 expression at 30 nM ($64 \pm 15.3\%$ vs. control, $P < 0.05$). Lower concentration (150 nM) HA did not significantly affect PKC β2 expression but significantly inhibited PKC β2 expression at 300 nM. N = 4 separate experiments. $^{*}P < 0.05$ and $^{\$}P < 0.01$ vs. HG without PKC inhibitor.

cords suggest that increased permeability in the presence of hyperglycaemia may be a significant pathopysiological factor in human diabetes. Furthermore, our finding that inhibition of PKC activity and PKC α and β2 expression in the membrane fraction reduced the permeability in HUVECs induced by PMA and high glucose further implicates PKC in hyperglycaemia-mediated endothelial permeability.

Aberrations of endothelial barrier function lead to an abnormal extravasation of plasma proteins (including lipoproteins) and their deposition into the blood vessel wall, resulting in tissue oedema and acceleration of atherosclerosis [33]. In the microvasculature, hyperglycaemia-induced increased endothelial permeability promotes macromolecule leakage through the endothelial barrier into the renal and retinal tissues, leading to diabetic nephropathy and

diabetic retinopathy [6,9,10]. Several studies have demonstrated the enhanced transendothelial albumin leakage and increased lipoprotein deposition in aorta of diabetic animals [34,35]. These data from animal experiments, together with our observations in HUVECs, suggest that increased endothelial permeability to macromolecules may be an important factor in the macroangiopathy in diabetic patients [36].

Increased endothelial permeability in response to high concentration of glucose and inflammatory mediators, such as bradykinin and interleukin-6 is mediated by PKC pathway [37,38]. Since PKC is a ubiquitous phospholipid-activated serine/threonine kinase comprising a family of at least 12 isoforms with different enzymatic properties, functions and distributions [39], it is important to know which PKC isoforms modulate vascular endothelial permeability. Hyperglycaemia-induced DAG production seems to predominantly activate PKC-β in retinal and renal vascular endothelial cells [13,14], which in turn mediate retinal and renal blood flow abnormalities via a reduction in the production of nitric oxide in experimental animal models of diabetes [40]. Yuan *et al.* have shown that PKC β2 and ε contribute to dysfunction of the endothelial barrier in coronary venules of diabetic pigs [7]. Their recent analysis of the PKC gene profile using a newly developed real-time RT-PCR technique indicates a preferential up-regulation of PKC genes in the porcine heart and aorta of early stages of experimental diabetes [41]. In contrast, some studies demonstrate that endothelial hyperpermeability resulting from hyperglycaemia, ischaemia, and angiogenesis secondary to inflammatory stimulation is mediated by PKC α [12,18,42,43]. A recent study suggests that loss of endothelial barrier function in HUVECs can be triggered by a PKC α-dependent activation of Rho pathway, a novel pathway in regulating endothelial contractile response [44]. In agreement with these findings, our data obtained with specific PKC antibodies revealed an overexpression/translocation of both PKC α and β2 (in particular the α isoform), in the membrane fraction of HUVECs under a high glucose condition. These observations, taken together, suggest that both PKC α and β2 may contribute to endothelial dysfunction associated with hyperglycaemia in diabetes.

The role of individual PKC isoforms in specific biological functions raises the intriguing possibility of selective inhibitors of certain isoforms preventing the complications of diabetes. Two PKC inhibitors were used in our study: LY391976, a bisindolylmaleimide derivative of staurosporine with high selectivity for β isoform of PKC [19,45], and HA, a naturally occurring perylenequinoid pigment from *H. bambuase* with a selective inhibitory effect towards the regulatory domain of PKC [20–23]. Because the regulatory domain of PKC lacks homology with other known kinases, HA acts at this region as a PKC inhibitor with little effect on other pathways, such as PKA pathway. Furthermore, amongst the three groups of PKC, the conventional isoforms (cPKC: α, β1, β2 and γ), novel isozymes (nPKC: δ, ε, η, θ and μ) and the atypical isoforms (aPKC: ζ, ι and λ), only the cPKC group possesses the full primary sequence of regulatory domain (C1, C2 and V1–V3) [46]. Thus, HA preferentially inhibits cPKC isoforms.

Our previous study has shown that HA significantly reduced PKC activity in the liver from obese Zucker rats [24]. In the present study we investigated the effects of the naturally occurring PKC inhibitor HA on the expression of individual PKC isoforms on human endothelial cell permeability. Although there is not enough evidence to prove specific inhibitory effect of HA on certain PKC isoforms, we found that HA has a preferential inhibitory effect on PKC α at the lower concentration and at the higher concentration (300 nM) it also inhibited PKC β2 expression, indicating a loss of selectivity for PKC α at this higher concentration.

The inhibitory effects of LY379196 on PKC activity and PKC β2 expression in the membrane fraction of HUVECs correlated with its reduction of endothelial permeability. Various data obtained in experimental animals with ruboxistaurin (LY333531, a selective inhibitor of PKC β), and the efficacy of ruboxistaurin in clinical trials have indicated an important role for PKC β in the endothelial abnormalities and microvascular leakage in the retina and kidney of animals and humans with diabetes [13,16,47]. However, recent studies have shown that increased permeability in porcine aortic endothelial cells and bovine pulmonary microvessel endothelial cells occurs via activation of PKC α [18,48]. We found the lower concentration of HA (150 nM) inhibited PKC α expression but not PKC β2 and this lower concentration of HA also substantially attenuated the increased endothelial permeability to albumin. These results provide evidence that PKC α has a pivotal role in albumin leakage in HUVECs, and that endothelial hyperpermeability is partially dependent on PKC β2 activation. Our study also suggests that an optimal concentration of HA (between 150 and 300 nM *in vitro*) can significantly reverse high-glucose-induced endothelial permeability probably via inhibition of both PKC α and β2. Finally, our observations in HUVECs which were obtained from larger vessels raise the possibility that PKC may be involved in the pathogenesis of macrovascular disease via similar mechanisms to those which have been proposed for the microvascular disease.

Acknowledgments

We express our thanks to Dr. Kirks Ways (Eli Lilly and Company, USA) and Professor Zhuqiang Li (Yunnan University) who provided LY379196 and hypocrellin A, respectively. This research was supported in part by the Eli Lilly 2001 Endocrinology Research Grant (Eli Lilly Australia Pty. Ltd).

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