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Short communication

Protein kinase C-β inhibition and diabetic microangiopathy: effects on endothelial permeability responses in vitro

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Abstract

Protein kinase C (PKC)-β and other PKC isozymes have been implicated in the loss of endothelial barrier function in diabetic microangiopathy. The effects of a PKC-β-specific inhibitor, LY379196, on hyperpermeability responses to high-glucose, angiotensin II, α-thrombin and endothelin-1 were evaluated using an in vitro model of human pulmonary artery endothelial cell monolayers. LY379196 attenuated the increase in transendothelial albumin flux induced by glucose 40 mM (e.g. 411 ± 160% [high-glucose] vs. 167 + 37% [high-glucose + LY379196], $P < 0.001$) and angiotensin II 10 μM (e.g. 121 ± 12% vs. 246 ± 35%, $P < 0.01$); endothelin-1 had no significant effect on monolayer permeability. LY379196 had no significant effect on the marked hyperpermeability response to α-thrombin 1 μM. Thus, two major pathways involved in vascular leakage in diabetic microangiopathy are amenable to therapeutic blockade by PKC-β inhibition.

Keywords: Protein kinase C-β (PKC-β) inhibitor; Protein kinase C (PKC); Endothelial permeability; Angiotensin II; Diabetic retinopathy

1. Introduction

Leakage of macromolecules across the endothelial barrier is an early feature of diabetic vascular disease and one that has important prognostic implications (e.g. proteinuria and macular oedema affect survival and visual outcome, respectively). Various pathophysiological mechanisms contribute to the loss of endothelial barrier function in diabetes but hyperglycaemia (Yamashita et al., 1995), activation of the renin–angiotensin system (Gilbert et al., 2000) and local release of humoral mediators such as thrombin (Garcia et al., 1986) and endothelin (Chen et al., 2000) have been implicated in diabetic nephropathy and retinopathy. The signal transduction of hyperpermeability responses is complex, but diacylglycerol-mediated activation of protein kinase C (PKC) is important in regulating endothelial cell contraction, shape-change and intercellular adhesion (Song et al., 2001). In particular, the Ca^{2+}-dependent PKC isozymes, e.g. PKC-α (Hempel et al., 1997) and PKC-β (Nagpala et al., 1996), have been strongly implicated in endothelial barrier function, but in vivo studies are difficult to interpret because systemic changes in haemodynamics and blood flow, independent of endothelial barrier function, affect vascular permeability.

The extent to which PKC inhibitors attenuate hyperpermeability responses in diabetic microangiopathy is unclear, in part because several PKC isoforms may be involved, but a selective PKC-β inhibitor, ruboxistaurin, is currently undergoing clinical trials in diabetic retinopathy and neuropathy (Aiello et al., 1997; Jirousek et al., 1996). Thus, the purpose of the present study was to evaluate the direct effects of a selective PKC-β inhibitor on endothelial permeability responses to high-glucose, vasoactive hormones (angiotensin II and endothelin-1) and thrombin using an established in vitro model of endothelial monolayers (Idris et al., 2002; Morel et al., 1990).

2. Methods

2.1. Materials

A specific PKC-β inhibitor, LY379196, was obtained from Eli Lilly & Co. (Indianapolis, USA). LY379196 is a

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highly specific inhibitor of PKC-β (Jirousek et al., 1996), and closely related to the PKC-β inhibitor ruboxistaurin (LY333531) presently in clinical development (Aiello et al., 1997). Thus, the IC_{50} values for LY379196-induced inhibition of PKC-β_I and PKC-β_{II} are 50 and 30 nM, respectively, which are at least 100-fold lower than the IC_{50} values for all other PKC isoforms and other protein kinases (Jirousek et al., 1996). Angiotensin II, α-thrombin and endothelin-1 were obtained from Sigma (UK).

2.2. In vitro measurement of human endothelial monolayer permeability

Using the method of Garcia et al. (1986), human pulmonary artery endothelial cells (Clonetics, Biowhitaker, UK) were trypsinised from tissue culture flasks and seeded onto 0.4-μm filter inserts (Becton Dickinson, New Jersey, USA) precoated with collagen in 12-well plates (3×10^5 cells/insert). The cells were then grown in modified Endothelial Growth Media (EGM-2, Biowhitaker) at 37 °C in 5% CO_2 until a tight monolayer was achieved (approximately 2–3 days). The integrity of the monolayer was assessed daily by direct visualisation and measurement of transendothelial electrical resistance using an Endohm chamber (World Precision Instruments, Stevenage, UK). A confluent monolayer produces a transendothelial electrical resistance measurement of 9–11 Ω/cm^2 (Garcia et al., 1986), and changes in human pulmonary artery endothelial permeability in vitro have been correlated with in vivo measurements of endothelial barrier function (Morel et al., 1990).

Confluent monolayers were incubated with the PKC-β inhibitor (LY379196 30 nM), or vehicle, for 24 h prior to measurement of the endothelial permeability responses to high-glucose, angiotensin II 10 μM, endothelin-1 1 μM and α-thrombin 10^{-8}–10^{-6} M. The growth media (with test substances) bathing the monolayer was removed immediately before the start of the permeability assay.

The monolayers were then covered with HEPES-buffered saline solution (HBSS) containing an Evans blue (0.67 g/l)–bovine serum albumin (40 g/l) complex and incubated for 30 min. Thereafter, the fluid in the lower wells was agitated and sampled to measure the light absorbance at 610 nm. Measurements of transendothelial albumin flux are expressed as the percentage of clearance of bovine serum albumin, compared with untreated controls (Idris et al., 2002, 2003).

2.3. Statistical analysis

Measurements of transendothelial albumin flux are expressed as the mean percentage of albumin flux across the endothelial monolayer (compared with control) ± S.E.M. The data were analysed using a Student's *t*-test with unequal variance. Statistical significance was at the level of $P < 0.05$.

3. Results

3.1. Glucose-induced endothelial hyperpermeability

Increasing the glucose concentration had no immediate effect on endothelial permeability, but after 72 h exposure to glucose 40 mM there was a significant increase in transendothelial albumin flux (Fig. 1A). Co-incubation with LY379196 for the last 24 h of high-glucose stimulation significantly attenuated the hyperpermeability response: measurements of transendothelial albumin flux (rel. to a control value of 100%, i.e. glucose 5 mM) were 411 ± 160% (glucose 40 mM + vehicle) vs. 167 ± 37% (glucose 40 mM + LY379196 30 nM) ($P < 0.001$).

3.2. Angiotensin II-induced endothelial hyperpermeability

There was an immediate increase in endothelial permeability following exposure to angiotensin II for 1 h. This

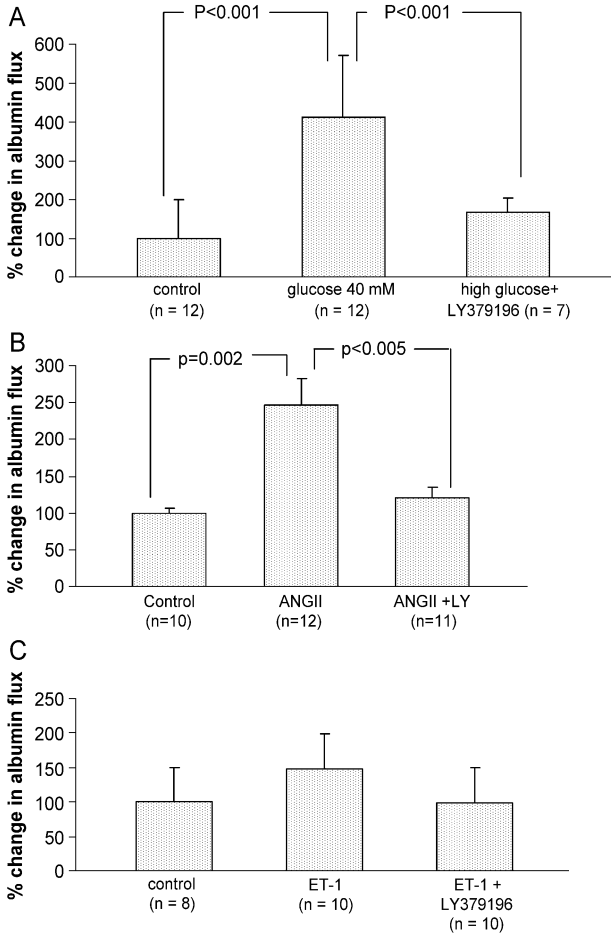

Fig. 1. (A) Effects of exposure to high-glucose (40 mM for 72 h) on endothelial permeability, and the effect of coincubation with a specific PKC-β inhibitor (LY379196) for 24 h. (B) Effects of angiotensin II (10 μM for 1 h) on endothelial permeability are attenuated by the PKC-β inhibitor LY379196. (C) Exposing the human pulmonary artery endothelial cell monolayer to endothelin-1 1 μM for 1 h and endothelin-1 + LY379196 had no significant effect on transendothelial albumin flux.

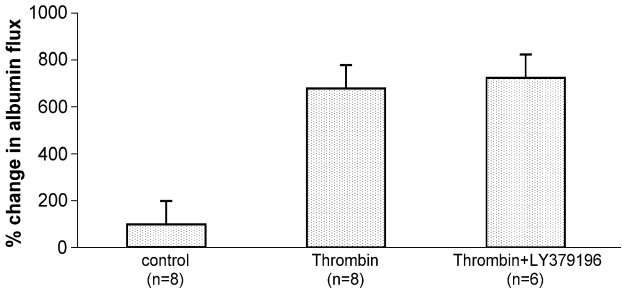

Fig. 2. Effects of α-thrombin 1 μM on endothelial permeability were unaffected by the PKC-β-specific inhibitor LY379196.

effect was markedly attenuated by preincubation with LY379196 for 24 h: e.g. measurements of transendothelial albumin flux (rel. to control value of 100%) were 246 ± 35% (angiotensin II 10 μM) vs. 121 ± 12% (angiotensin II 10 μM + LY379196 30 nM) ($P < 0.01$, Fig. 1B).

3.3. Effects of ET-1 on endothelial permeability

Acute exposure (1 h) and 24 h exposure to endothelin-1 1 μM had no significant effect on endothelial permeability: e.g. transendothelial albumin flux (rel. to control 100%) was 148 ± 45% (endothelin-1 1 h) (Fig. 1C) and 97 ± 17% (endothelin-1 24 h).

3.4. Thrombin-induced endothelial hyperpermeability

Exposure to α-thrombin for 1 h produced concentration-dependent increases in endothelial permeability which were maximal at 1 μM and reversible by washing the monolayers. The nonspecific protein kinase inhibitor staurosporine 1 μM partly but significantly attenuated the hyperpermeability response to α-thrombin, but LY379196 had no effect (Fig. 2).

4. Discussion

Loss of endothelial barrier function is an early feature of diabetic microangiopathy, especially in the retina and kidney where clinical outcomes (morbidity and mortality) correlate with the degree of proteinuria and macular oedema. Hyperglycaemia (Yamashita et al., 1995), renin–angiotensin system activation (Gilbert et al., 2000) and thrombin formation (Garcia et al., 1986) are known to impair endothelial function, but changes in vascular permeability are difficult to interpret from animal and in vivo pharmacology studies where complex simultaneous changes in haemodynamics and blood flow are difficult to distinguish from direct effects on intercellular adhesion and endothelial barrier function. For example, although endothelins contribute to microangiopathy (Hopfner and Gopalakrishnan, 1999), there are conflicting results about whether endothelin-1 and endothelin ET_A receptor antagonists affect the permeability of the endothelium independently of changes in blood flow and vessel tone (Hele et al., 2000; Victorino et al., 2001). Our own group and others have previously shown that measurement of albumin flux across human pulmonary artery endothelial cell monolayers provides a useful in vitro system for evaluating the direct effects of individual agonists and pathway-specific inhibitors (Idris et al., 2002, 2003). Furthermore, there is evidence that changes in the permeability of these monolayers correlate with in vivo changes in endothelial barrier function (Morel et al., 1990).

The results of this study, using a highly specific inhibitor of PKC-β, provide important new observations about the signal transduction and attenuation of endothelial hyperpermeability responses in experimental conditions that mimic diabetic microangiopathy. Firstly, the hyperpermeability response to high-glucose was almost completely abolished by preincubation with a β-specific PKC inhibitor. Activation of several PKC isoforms, via increased de novo synthesis of diacylglycerol, has been implicated in different aspects of endothelial dysfunction in diabetes (Song et al., 2001), including PKC-α in hyperglycaemia-induced vascular leakage (Hempel et al., 1997), but the relative contribution of different PKC isozymes has not been clearly established. The present study demonstrates a major reversible effect of PKC-β activation in the loss of endothelial barrier function in response to high-glucose. From these experimental data, it would appear that oral treatment with a PKC-β inhibitor might be particularly effective in reversing exudative diabetic microangiopathy, which in the eyes is often sight-threatening and closely related to poor glycaemic control (Idris et al., 2001).

The mechanism of angiotensin II-induced endothelial permeability is unclear. We have previously demonstrated that this effect is not blocked by the angiotensin AT_1-receptor antagonist losartan (Idris et al., 2002), thus implicating the AT_2-receptor pathway which couples to PKC (Rabkin, 1996). Angiotensin II-induced vascular endothelial growth factor formation is also PKC-dependent (Chua et al., 1998). The specific PKC isozyme(s) involved are not entirely clear, but PKC-α, -β and -δ have been implicated in pathways of vascular endothelial growth factor formation and action (Idris et al., 2001) and PKC-ζ may be involved in downstream angiotensin II signalling (Liao et al., 1997). Thus, the present study is particularly informative. Angiotensin II had an immediate, marked effect on endothelial permeability which was completely blocked by the PKC-β inhibitor, unlike losartan which had no effect on angiotensin II-induced permeability in our previous study (Idris et al., 2002). Attenuating the permeability effects of renin–angiotensin system activation is likely to be an important additional mode of action of PKC-β inhibitors in clinical practice.

Thrombin had the most powerful effect on endothelial monolayer permeability, as reported previously (Garcia et al., 1986). Although the nonspecific PKC inhibitor staur-

osporine partially attenuated this hyperpermeability response, the PKC-β inhibitor had no effect. These results are somewhat different to those reported in dermal microvascular endothelial cells transfected with an antisense cDNA for PKC-β_I (Vuong et al., 1998). In microvascular cells, PKC-β_I may exert negative feedback control on thrombin-induced intracellular signals (Vuong et al., 1998). These differences probably highlight functional heterogeneity among endothelial cells in different vascular beds. In our human pulmonary artery endothelial cell system, thrombin-induced changes in vascular permeability seem to be at least in part PKC-dependent, though apparently not involving PKC-β_I and -β_{II} isozymes to any significant extent. Previous studies have implicated Ca^{2+}-dependent PKC isoforms in thrombin-induced permeability (Patterson et al., 1994), and PKC-α is the most likely isozyme involved which is not inhibited by the LY compound.

In summary, a specific PKC-β inhibitor markedly attenuates high-glucose and angiotensin II-induced vascular permeability, two major pathways of endothelial dysfunction in diabetic microangiopathy. In contrast, thrombin-induced permeability, an important mechanism in diabetic microangiopathy, is mediated by PKC isozymes other than PKC-β.

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