Human umbilical vein and dermal microvascular endothelial cells show heterogeneity in response to PKC activation

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Mason, Justin C., Helen Yarwood, Katharine Sugars, and Dorian O. Haskard. Human umbilical vein and dermal microvascular endothelial cells show heterogeneity in response to PKC activation. Am. J. Physiol. 273 (Cell Physiol. 42): C1233–C1240, 1997.—Changes in endothelial cell (EC) phenotype are central to the function of endothelium in inflammation. Although these events mainly occur in the microvasculature, previous studies have predominantly used large-vessel EC. Using enzyme-linked immunosorbent and flow cytometric assays, we compared the responses of human umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells (DMEC) to the activation of protein kinase C (PKC). Stimulation with phorbol 12,13-dibutyrate and more selective PKC agonists, including 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), induced morphological changes and proliferation in both EC types. PKC activation induced a marked increase in Thy-1 expression on DMEC and only a moderate rise on HUVEC. Furthermore, heterogeneity in the induction of the adhesion molecules intercellular adhesion molecule 1, vascular cell adhesion molecule 1 (VCAM-1), and E-selectin between the two EC types following activation of PKC was demonstrated. In particular, E-selectin and VCAM-1 were significantly upregulated on HUVEC but not DMEC. The data indicate that the PKC pathway is unlikely to be important for E-selectin and VCAM-1 expression in the microvasculature but are consistent with a role for PKC in angiogenesis. This diversity in signaling in response to PKC activation may depend on differential utilization of PKC isozymes and may facilitate specialized endothelial responses.

Thy-1; adhesion molecules; protein kinase C isozymes

CHANGES IN ENDOTHELIAL cell (EC) surface antigen expression are central to the various roles of endothelium in inflammatory responses. Cytokines, such as tumor necrosis factor (TNF) and interleukin 1 (IL-1), stimulate a coordinated series of events with expression of cell surface leukocyte adhesion molecules, including E-selectin (CD62E), vascular cell adhesion molecule 1 (VCAM-1; CD106), and intercellular adhesion molecule 1 (ICAM-1; CD54) (25). These molecules have been shown to orchestrate leukocyte tethering, rolling, adhesion, and subsequent transmigration across endothelium, thereby directing leukocyte traffic into inflamed tissues (30). On the other hand, distinct changes in EC activation occur in response to angiogenic stimuli such as the vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), or integrin-mediated adhesive interactions with the extracellular matrix (31). The different signaling pathways involved in the regulation of these different EC functions are not well understood but are likely to be complex and controlled at multiple levels (2).

The protein kinase C (PKC) family consists of at least 12 isozymes, which are divided on the basis of structure and response to phorbol esters into the classical PKC (α, βI, βII, and γ), novel PKC (δ, ε, η, ζ, and ι), and atypical PKC (λ and ξ) (7, 23). This family of enzymes has been implicated in a variety of biological processes, such as cellular differentiation, gene regulation, and proliferation (7, 23). Most studies investigating PKC have used phorbol esters such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), which are thought to activate all PKC isozymes except PKC-λ and ξ (23). The availability of more specific agonists now allows the dissection of cellular responses following activation of a more limited number of isozymes. Thus thymeleatoxin and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA) are both more selective than PMA and PDBu, with thymeleatoxin demonstrating maximal potency on the PKC-α, -βI, and -γ isozymes and dPPA on the βI isozyme, respectively (14, 16, 28).

The exact contribution that PKC activation makes in EC proliferation and adhesion molecule expression remains to be determined. Activation of PKC by phorbol esters has been shown to induce angiogenesis both in vivo and in vitro (20, 21). In addition, VEGF and bFGF induce EC proliferation, at least in part, via the activation of PKC (15, 36). These effects may, however, be dependent on the EC type, since phorbol esters have been shown to inhibit the proliferation of bovine microvascular EC in vitro (8, 21). Although phorbol esters are able to stimulate expression of E-selectin, VCAM-1, and ICAM-1 on human umbilical vein endothelial cells (HUVEC), differing conclusions have been drawn on the degree to which activation of PKC mediates the effects of cytokines such as TNF and IL-1 on EC leukocyte adhesion molecule expression (6, 22, 26). Thus the physiological role of PKC in EC leukocyte adhesion molecule expression remains unclear.

The glycosyl-phosphatidylinositol-anchored protein Thy-1 (CD90) has recently been found to be expressed by rat and human EC (12, 17). Moreover, this molecule is strongly induced on human EC by phorbol esters and can act as a surface reporter of PKC activation (17). Although the function of Thy-1 is not known, data to date suggest that it may have an important role in inflammatory responses (12, 17). We have recently reported that Thy-1 expression on the EC surface is upregulated by the proinflammatory cytokine TNF and that ligating EC Thy-1 with antibodies results in a...
transient calcium flux (17). Furthermore, it has been demonstrated that rat Thy-1 may play a role in the modulation of endothelial permeability in inflammation (12). Studies with other cell types have suggested that Thy-1 may also have an important role in the regulation of cell adhesion, growth, and differentiation (11, 34).

Most in vitro studies on EC activation have utilized EC cultured from human umbilical veins. However, these cells are isolated from large vessels and derived from fetal tissue and may not accurately represent microvascular endothelium that is particularly involved in inflammation and angiogenesis in vivo. Furthermore, HUVEC are known to differ from microvascular EC in terms of surface antigen expression (19, 32), prostanoid production (3), and responsiveness to cytokines (1, 9, 24, 33). Because we are specifically interested in the role of EC in cutaneous inflammation, we isolated dermal microvascular endothelial cells (DMEC) to use as a more appropriate in vitro model of endothelium. In this study, we looked for direct evidence that HUVEC and DMEC differ with respect to their response to activation of PKC, using both broadly specific and isoenzyme-restricted pharmacological agonists. The data support the view that EC may behave differently in response to agonists that act through activation of PKC, with potentially important physiological and pathological consequences.

**METHODS**

EC cultures. The DMEC were isolated and cultured using a previously described method (17). Human foreskins, from routine circumcisions of children aged 3–9 years, were collected in Hanks’ balanced salt solution (HBSS) supplemented with 1,000 IU/ml penicillin, 1 mg/ml streptomycin, and 20 µg/ml amphotericin (GIBCO). After incubation for 1 h at 4°C, the tissue was cut into strips, washed twice in HBSS, and incubated overnight in 2 µg/ml dispase (Boehringer Mannheim, Lewes, UK) at 4°C. Subsequently, the epidermis was removed and microvascular segments were expressed from the edges of the tissue into medium 199 (M199) (ICN Biomedicals), which was then passed through a 100-µm filter. After centrifugation at 200 g for 5 min, the pellet was resuspended in DMEC growth medium consisting of M199 supplemented with 30% heat-inactivated pooled human serum (HHS), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 5 µg/ml amphotericin, 2 mM l-glutamine (ICN), 10 U/ml heparin, and 75 µg/ml endothelial cell growth factor (ECGF; Sigma). The initial isolates consisted of ∼70% DMEC and were further purified using magnetic beads (Dynal, Skogn, Norway) coated with ulex europaeus agglutinin-I (UEA-I) (Vector Labs, Peterborough, UK) (17). The cells were mixed with the UEA-I-coated magnetic beads at a ratio of 1:5 and incubated with end-over-end rotation for 30 min at 4°C. After four washes in HBSS with 5% fetal bovine serum (FBS), the beads were incubated with 0.1 M fucose (Sigma) for 1 h at 4°C to detach DMEC from the beads. The supernatant was then harvested and centrifuged at 200 g for 5 min. The DMEC pellet was resuspended in growth medium, placed into fibronectin-coated 25-cm² tissue culture flasks, and grown to confluence. DMEC were also isolated from adult human skin with this method, and no differences in responsiveness to the activation of PKC between the two sources of DMEC were found. All DMEC used were from passages 3–6.

HUVEC were obtained by collagenase type II (Boehringer Mannheim) digestion of umbilical veins as previously described (35). The isolated HUVEC were resuspended in growth medium consisting of M199 supplemented with 20% FBS (HyClone Laboratories, Logan, UT), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM l-glutamine, 10 U/ml heparin, and 30 µg/ml ECGF. EC were assayed as ≥98% pure by uptake of acetylated low-density lipoproteins, staining for factor VIII-related antigen, CD31, and binding of UEA-I (data not shown). Described differences in responsiveness between HUVEC and DMEC could not be accounted for by the different culture media used to grow the two cell types, since similar observations were made when HUVEC were grown in DMEC culture medium.

Monoclonal antibodies, cytokines, and other reagents. The anti-human Thy-1 monoclonal antibody (MAb) B7.1 (immunoglobulin G1 (IgG1)) was a kind gift from Professor M. Ritter, Royal Postgraduate Medical School (London, UK); this antibody binds specifically to Thy-1 on human EC (17). Other mouse MAb (all IgG1) used in this study were anti-ICAM-1 (CD54) 6.5BS, anti-VCAM-1 (CD106) 1.4C3, anti-E-selectin (CD62E) 1.2B6 (35), EN4 (Sanbio, Uden, Holland), and anti-CD31 9G11, a kind gift from Dr. Rod Pigott, British Biotechnology (Oxford, UK). MOPC-21 (IgG1), used as an isozyme matched negative control, and human recombinant TNF-α were both a kind gift from Dr. Martyn Robinson, Celltech (Slough, UK). The following PKC agonists were used: PDBu (Sigma), DPPA, and thymeleatoxin (Calbiochem-Novabiochem, Nottingham, UK). The specific PKC antagonist Ro-31–8220 (5) was a kind gift from Dr. Trevor Hallam, Roche Research Centre (Weyts Garden City, UK). Cycloheximide, the biologically inactive phorbol ester phorbol 12,13-didecanoate (PDD), and sodium butyrate were purchased from Sigma.

Analysis by flow cytometry. EC were plated at confluence onto 35-mm petri dishes (7 × 10⁵ cells/dish) and cultured overnight at 37°C before stimulation with PKC agonists or TNF-α. Unstimulated or activated EC were harvested by treatment with trypsin-EDTA (ICN) for 1 min at 37°C. After repeated pipetting to ensure single cell suspensions, the EC were stained with the appropriate primary MAb for 30 min at 4°C. After they were washed twice in phosphate-buffered saline (PBS)-2.5% FBS, EC were resuspended in a rabbit polyclonal anti-CD14 fluorescein isothiocyanate antibody (DAKO, Glostrup, Denmark) for 30 min at 4°C, followed by another washing as above and fixation in 1% paraformaldehyde. Samples were analyzed on an Epics XL-MCL flow cytometer (Coulter, Hialeah, FL). For PKC inhibition studies, Ro-31–8220 (1–2.5 µM) was added to cell culture supernatants 30 min before addition of activating agents and remained throughout the experiment. In all experiments, cell viability was assessed by examination of monolayers with phase-contrast microscopy, cell counting, and estimation of trypan blue exclusion in the EC populations before staining.

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay (ELISA) to detect MAb binding to the surface of EC was performed as previously described (35). Briefly, EC were plated at confluence (4 × 10⁶ cells/well) on 96-well microtiter plates (Costar) and cultured for 24 h in the absence of ECGF. The EC were then stimulated with test agents before fixation with 2% paraformaldehyde/lysine periodate for 10 min at 4°C. After fixation, EC were incubated with primary MAb for 1 h, washed twice in PBS, and incubated with biotinylated rabbit anti-mouse immunoglobulin (DAKO) for 1 h at 37°C. After two further washes in PBS, binding of the biotinylated antibody was detected by incubation with a high-molecular-weight complex of streptavidin-biotin-peroxidase (DAKO). The ELISA was developed with 0.5 mg/ml o-phenylenediamine and 0.03% hydrogen peroxide.
(vol/vol) in a pH 5 citrate-phosphate buffer, and color development was stopped with 2 M sulfuric acid. The optical density was then measured at 491 nm with a Titertek ELISA plate reader (ICN-Flow). Specific MAb binding was calculated by subtracting the background as represented by the mean optical density of triplicate wells incubated with PBS in place of primary MAb. To control for differences in cell numbers between wells, either as a consequence of EC proliferation or cell loss due to cytotoxicity, uptake of crystal violet was measured. At the end of the ELISA assay, the wells of the microtiter plates were washed with PBS, and 100 µl of crystal violet (Sigma) (0.1% wt/vol in distilled water) were added. The stain was eluted after 10 min with 33% (vol/vol) acetic acid, and the optical density was measured at 620 nm. With the use of this approach, no differences in cell numbers between wells were detected.

EC proliferation assay. EC of passage 4 were plated in 35-mm gelatin-coated petri dishes at a concentration of $1 \times 10^5$ cells/dish and cultured overnight in reduced DMEC growth medium consisting of M199 supplemented with 5% HIHS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 5 µg/ml amphotericin B, and 2 mM L-glutamine. The supernatant was then aspirated and replaced with reduced DMEC growth medium containing the substances to be tested for proliferative activity. After 4 days of culture at 37°C, EC were washed twice in HBSS, harvested, and counted with a hemocytometer, using trypan blue exclusion to identify dead cells. In each experiment, samples were tested in triplicate and the

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Fig. 1. Activation of protein kinase C (PKC) induces changes in endothelial cell (EC) morphology. Dermal microvascular endothelial cells (DMEC; A, C, E) and human umbilical vein endothelial cells (HUVEC; B, D, F) were plated at confluence in 35-mm petri dishes and stimulated for 48 h with plain growth medium alone (A and B) or containing phorbol 12,13-dibutyrate (PDBu; 0.1 µM; C and D) or dPPA (1 µM; E and F). Morphological analysis of cell monolayers was performed by phase-contrast microscopy, and photomicrographs were obtained of representative areas. Similar morphological changes were seen with thymeleatoxin (data not shown).
results were expressed as the percent cell increase from the initial plating concentration. Phase-contrast microscopy was performed for morphological analysis of adherent cell monolayers and to exclude cytotoxicity, using a Leitz Labovert microscope (Leica, Milton Keynes, UK). In addition, photomicrographs were obtained with an Olympus C35AD camera (Olympus, London, UK).

Analysis of E-selectin mRNA by reverse transcription polymerase chain reaction. The semiquantitative reverse transcription polymerase chain reaction (RT-PCR) used to detect changes in HUVEC E-selectin mRNA was as previously described (18). HUVEC were removed from tissue culture flasks (Costar) with trypsin-EDTA and plated at confluence in gelatinized 24-well plates and cultured overnight at 37°C. The EC were then stimulated with either PDBu (0.2 µM) or dPPA (1.0 µM) for 4 h with or without pretreatment for 30 min with 3 µg/ml cycloheximide (Sigma) at 37°C. After stimulation, EC were lysed in guanidium isothiocyanate (Sigma) and RNA was extracted by the method of Chomcyznski and Sacchi as described in Sambrook et al. (29) and stored at -70°C until use. For RT-PCR, RNA was diluted 1:1 with diethyl pyrocarbonate (DEPC)-treated water and 1 µl was mixed with 1 µl of mutant E-selectin mRNA at a known concentration. The tubes were then made up to 12.5 µl with DEPC-treated water, and two drops of mineral oil were layered on the surface to prevent evaporation. The procedures for both RT-PCR and heminested PCR were as previously described in detail (18). Products of RT-PCR were analyzed and quantified by electrophoresis in 2% agarose gels (29), and bands were visualized under ultraviolet light and photographed. The quantity of wild-type mRNA that could be amplified by RT-PCR in a given sample could be obtained from the amount of mutant mRNA necessary to give wild-type and mutant bands of equal intensity, and the data are expressed in these terms.

Statistical analysis. Differences between the results of experimental treatments were evaluated by the Mann-Whitney U-test. Differences were considered significant if P values were <0.05.

RESULTS

Activation of PKC induces morphological changes and proliferation in DMEC and HUVEC. EC were stimulated with the PKC agonists PDBu, dPPA, or thymeleatoxin for 48 h, after which the degree of shape change was recorded by photographic analysis of representative areas of each culture. As shown in Fig. 1, A and B, unstimulated DMEC and HUVEC formed characteristic cobblestone monolayers. Changes in EC morphology were seen in both cell types after treatment with the PKC agonists, with the cells adopting an

Fig. 2. Activation of PKC induces EC proliferation. EC were plated in 35-mm gelatin-coated petri dishes at a concentration of 1 x 10^5 cells/dish and incubated at 37°C overnight. EC were then incubated with PDBu (0.1 µM), dPPA (1 µM), endothelial cell growth factor (ECGF; 50 µg/ml), tumor necrosis factor-α (TNF-α; 10 ng/ml), or reduced growth medium with 5% heat-inactivated pooled human serum (PM) alone for 4 days before cell counting. Results show means ± SD of triplicate samples for DMEC (A) and HUVEC (B) and is representative of 3 similar experiments. Statistical analysis was performed with the Mann-Whitney U-test (*P < 0.05).

Fig. 3. Kinetics of Thy-1 expression on EC stimulated with PKC agonists. HUVEC and DMEC were plated at confluence in 96-well microtiter plates and stimulated with PDBu (0.1 µM; solid bars) or dPPA (1.0 µM; hatched bars) for the times shown. Thy-1 expression was measured by enzymelinked immunosorbent assay (ELISA) using monoclonal antibody (MAb) B7.81 on HUVEC (A) and DMEC (B). Results are representative of 3 similar experiments and show means ± SD of triplicate wells. OD, optical density.
elongated irregular shape (Fig. 1, C–F). This change in morphology was present by 3–6 h after stimulation and was maximal by 24–48 h.

For assessment of EC proliferation in response to PKC activation, EC were cultured for 4 days in reduced DMEC growth medium with 5% HIHS, either alone or supplemented with ECGF, TNF-α, PDBu, or dPPA. In addition to the morphological changes, there was a significantly increased proliferation of both cell types in the presence of PDBu or dPPA compared with unstimulated EC (P < 0.05 for both agonists) (Fig. 2). However, no significant differences were observed when PDBu and dPPA were compared. In contrast, treatment of EC with TNF-α had an inhibitory effect on EC, which was significant compared with the basal proliferation of DMEC (P < 0.05) and HUVEC (P < 0.05) (Fig. 2). In addition to demonstrating EC proliferation, these experiments (Figs. 1 and 2) indicated that the agonists were not toxic to EC during the incubation periods used.

Thy-1 expression in response to PKC activation is greater on DMEC than HUVEC. With the use of flow cytometric analysis, which was found to be more sensitive than the ELISA, the constitutive expression of Thy-1 was compared on EC from passage 4 and found to be slightly higher on HUVEC [mean fluorescence intensity (MFI) = 1.463 ± 0.775 (SD)] than on DMEC (0.556 ± 0.115). In contrast, the expression of Thy-1 on EC following treatment for 48 h with PDBu was found to be greater on DMEC than on HUVEC. Thus, in four
experiments that used separate EC cultures, Thy-1 expression, as represented by MFI ± SD, was increased to 17.36 ± 12.29, whereas on HUVEC Thy-1 expression was increased to 3.055 ± 1.597 (P < 0.05) (data not shown).

To explore which PKC isozymes were involved in the regulation of Thy-1 on EC, both DMEC and HUVEC were stimulated with PDBu, thymeleatoxin, and dPPA for up to 48 h. PDBu and thymeleatoxin had identical effects on Thy-1 expression, and therefore only the PDBu data are shown. Upregulation of Thy-1 in response to activation of PKC was detectable by 24 h and maximal at 48 h on both HUVEC (Fig. 3A) and DMEC (Fig. 3B) (17). Moreover, these experiments confirmed the observations with flow cytometry that the induction of Thy-1 after activation of EC PKC is greater on DMEC than HUVEC. In addition, dPPA was as effective as PDBu in inducing the upregulation of Thy-1 by these cells. Finally, pretreatment of DMEC with the PKC antagonist Ro-31–8220 completely abrogated the induction of Thy-1 by PDBu and dPPA, consistent with the effects being mediated by PKC activation (data not shown).

Heterogeneity in expression of EC adhesion molecules following activation of PKC. Whereas treatment of HUVEC or DMEC monolayers with the phorbol ester PDBu led to morphological changes and stimulated proliferation and Thy-1 expression in both EC types, measurement of the surface expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 following stimulation with PDBu revealed clear differences in responsiveness (Fig. 4). ICAM-1 showed a high level of constitutive expression on both cell types and only a minimal increase following PDBu stimulation (Fig. 4A and B). E-selectin expression was markedly upregulated on HUVEC by PDBu (Fig. 4C) but showed only a small increase in expression on DMEC (Fig. 4D). Furthermore, although PDBu led to a marked increase in expression of VCAM-1 by HUVEC (Fig. 4E), it failed to affect VCAM-1 expression by DMEC (Fig. 4F). These results were confirmed in at least four ELISA and three flow cytometric experiments, using EC isolated from different donors on each occasion. It should be noted that all three molecules were upregulated to a similar degree on both cell types by TNF-α in all these experiments (data not shown). Finally, to control for nonspecific effects on Thy-1 and adhesion molecule expression, both sodium butyrate and the biologically inactive phorbol ester PDD were included in the ELISA assays. In contrast to PDBu, no effect on surface antigen expression was seen with either agent (data not shown).

To demonstrate that the increased surface expression of adhesion molecules observed on EC in response to PDBu was dependent on PKC activation, EC were preincubated with the specific PKC antagonist Ro-31–8220 before stimulation. Flow cytometric analysis confirmed that preincubation with Ro-31–8220 prevented the PDBu-induced upregulation of E-selectin, ICAM-1, and VCAM-1 on HUVEC and E-selectin and ICAM-1 on DMEC (data not shown). The inhibitory effects of Ro-31–8220 were not due to cytotoxicity, as determined by cell morphology and cell counting and by trypan blue exclusion (>98% viability).

To investigate further the effect of PKC activation on EC adhesion molecule expression, we compared the PKC agonists PDBu, thymeleatoxin, and dPPA (28). The activity of thymeleatoxin was found to be the same as for PDBu, and therefore the data are not shown. In initial dose-response experiments, both PDBu and dPPA were found to stimulate adhesion molecule expression on HUVEC, with optimal effects at 0.5–1.0 µM for dPPA and 0.1 µM for PDBu (data not shown). As shown in Fig. 4, dPPA and PDBu differed in two ways in their actions on leukocyte adhesion molecule expression. First, dPPA was unable to stimulate expression of ICAM-1, E-selectin, or VCAM-1 on DMEC (Fig. 4B, D, and F). Second, whereas dPPA was able to stimulate E-selectin and VCAM-1 expression on HUVEC, this action was relatively delayed compared with PDBu, with peak expression of these two molecules at 6 rather than at 3 h (Fig. 4, C and E). The failure of dPPA to induce adhesion molecule expression on DMEC in the ELISA was also confirmed in separate experiments, on three different EC preparations, using flow cytometric analysis (data not shown).

It was established that expression of the three adhesion molecules is to a large extent regulated at the level of gene transcription (4). To investigate whether the differences between the kinetics of PDBu- and dPPA-induced E-selectin expression were due to an indirect effect of dPPA, we used semiquantitative RTPCR analysis to measure E-selectin mRNA (18). As shown in Table 1, E-selectin mRNA was barely detectable in unstimulated HUVEC, whereas stimulation for 4 h with PDBu, dPPA, or TNF-α resulted in a detectable increase, as measured by semiquantitative RT-PCR. Pretreatment of HUVEC with cycloheximide before stimulation with dPPA or PDBu did not inhibit the increase in E-selectin mRNA, instead resulting in
increased E-selectin mRNA compared with the PKC agonists alone. This result, together with the observation that treatment of HUVEC with cycloheximide alone also resulted in increased E-selectin mRNA (Table 1), is consistent with cycloheximide stabilizing E-selectin mRNA by inhibiting nucleases, a mechanism that has previously been proposed (10). The failure of cycloheximide to reduce E-selectin mRNA levels stimulated by dPPA argues against the delayed action of dPPA on HUVEC (relative to PDBu) being related to a requirement for a de novo-synthesized intermediary protein.

**DISCUSSION**

PKC is a family of serine/threonine-specific kinases that may be involved in regulating a number of EC functions, including the expression of leukocyte adhesion molecules, cell morphology, and proliferation. In this study, we have found evidence for heterogeneity between two different types of EC with respect to responsiveness to PKC activation.

The two EC types responded to the different PKC agonists in a very similar manner with respect to morphological changes and enhanced proliferation, consistent with the view that PKC activation is important for endothelial growth during angiogenesis (15, 20, 21, 29, 36). Likewise, both HUVEC and DMEC showed an upregulation of surface Thy-1 in response to each of the PKC agonists, although this was more marked on DMEC. Although the function of Thy-1 on EC is unknown, cross-linking this antigen with MAbs induces a transient calcium flux (17). Moreover, ligating Thy-1 on lymphocytes can provide accessory signals for proliferation (27), and it is tempting to speculate that it might also have a role in EC proliferation during angiogenesis.

In support of this hypothesis, we have recently demonstrated the specific expression of Thy-1 on EC of new vessels within the base of atheromatous plaques (D. V. Parums, M. D. Ashton, J. C. Mason, and D. O. Haskard, unpublished observations).

Although PKC agonists had similar effects on the morphology and proliferation of DMEC and HUVEC, we observed marked differences in responsiveness of the two EC types in terms of leukocyte adhesion molecule expression. In particular, the expression of the adhesion molecules E-selectin and VCAM-1 was stimulated by PDBu on HUVEC in a manner similar to that which is seen with IL-1 and TNF. Previous studies have suggested that activation of PKC is probably not involved in TNF- or IL-1-induced adhesion.

Whereas PDBu and thymeleatoxin had very similar actions in all the assays, the effects of dPPA differed in several ways. First, whereas dPPA was fully effective in stimulating morphological changes, proliferation, and Thy-1 expression, it did not stimulate leukocyte adhesion molecule expression on DMEC. Furthermore, dPPA stimulated expression of E-selectin and VCAM-1 on HUVEC with kinetics that were delayed compared with those for PDBu. This delay could not be accounted for by a synthesis requirement of an intermediary protein, since inclusion of cycloheximide in the assay system had no effect on accumulation of E-selectin mRNA in response to dPPA. Although the isozymes involved remain unknown, the differences we have observed in responses to dPPA and PDBu suggest that there is heterogeneity in PKC isoenzyme distribution and/or function between the two EC types.

In conclusion, this study provides the first evidence for differential effects of PKC activation in the regulation of the function of different types of EC and provides a baseline for future work in this area. In view of the limitations of a pharmacological approach in terms of specificity of agonists and antagonists for individual isozymes (13), it will be important to investigate the effects of overexpression of individual PKC isozymes using transfection techniques and, eventually, to study the effects of their specific inhibition with anti-sense or dominant-negative mutants. The further elucidation of PKC-mediated pathways of activation will help in understanding the complex regulation of endothelial responses in inflammation, so helping to define novel and specific therapeutic targets in inflammatory diseases.

We thank P. Kiely, P. Singh, Dr. L. Lovat, and M. McNamara for help with collection of foreskin and the staff of the Maternity Unit, Hammersmith Hospital, for collection of umbilical cords.

This work was performed while J. C. Mason was a Medical Research Council Training Fellow. D. O. Haskard is the recipient of a British Heart Foundation professorial discretionary award. Address for reprint requests: D. O. Haskard, Cardiovascular Medicine Unit, Dept. of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Rd., London W12 ONN, UK.

Received 20 February 1997; accepted in final form 25 June 1997.

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