Activation of PKC modulates blood-brain barrier endothelial cell permeability changes induced by hypoxia and posthypoxic reoxygenation

Melissa A. Fleegal,1 Sharon Hom,1,2 Lindsay K. Borg,1 and Thomas P. Davis1,2
1Department of Medical Pharmacology and 2Program in Physiological Sciences, The University of Arizona, Tucson, Arizona

Submitted 13 May 2005; accepted in final form 28 June 2005

The blood-brain barrier (BBB) is a metabolic and physiological barrier important for maintaining cerebral homeostasis. Brain microvascular endothelial cells (RMECs) exposed to hypoxia (1% O2–99% N2; 24 h), a significant increase in total PKC activity was observed, and this was reduced by posthypoxic reoxygenation (95% room air-5% CO2) for 2 h. The expression of PKC-βII, PKC-γ, PKC-η, PKC-μ, and PKC-λ also increased following hypoxia (1% O2–99% N2; 24 h), and these protein levels remained elevated following posthypoxic reoxygenation (95% room air-5% CO2; 2 h). Increases in the expression of PKC-c and PKC-ζ were also observed following posthypoxic reoxygenation (95% room air-5% CO2; 2 h). Moreover, inhibition of PKC with chelerythrine chloride (10 μM) attenuated the hypoxia-induced increases in [14C]sucrose permeability. Similar to what was observed in RMECs, total PKC activity was also stimulated in cerebral microvessels isolated from rats exposed to hypoxia (6% O2–94% N2; 1 h) and posthypoxic reoxygenation (room air; 10 min). In contrast, hypoxia (6% O2–94% N2; 1 h) and posthypoxic reoxygenation (room air; 10 min) significantly increased the expression levels of only PKC-γ and PKC-δ in the in vivo hypoxia model. These data demonstrate that hypoxia-induced BBB paracellular permeability changes occur via a PKC-dependent mechanism, possibly by differentially regulating the protein expression of the 11 PKC isoforms.

Previously studies (36, 47) have demonstrated that H and H/R cause changes in paracellular permeability to [14C]sucrose in cerebral vascular endothelial cells. These permeability changes have been correlated with alterations in the expression and localization of several TJ proteins (36). More recently, nitric oxide (NO) and Ca2+ have been shown to partially mediate the effects of H and H/R on cellular permeability, though the mechanisms by which NO and Ca2+ may cause these permeability changes are still unclear (17, 35). However, it has recently been suggested that phosphorylation of the TJ proteins can affect the functionality of the TJ (4, 23, 33, 46). Several intracellular signaling molecules have been shown to regulate TJ protein phosphorylation, including tyrosine kinases, MAPK, and PKC (4, 33).

Recent studies (28, 30) have begun to investigate the mechanisms by which the different PKC isoforms (conventional, α, βI, βII, γ, novel, δ, ε, θ, μ, and atypical, λ) regulate the expression and function of the TJ proteins. These studies have shown that the activities of the various PKC isoforms have diverse effects on the function and the integrity of epithelial cell barriers. For example, activation of PKC-δ, PKC-λ, and PKC-δ is detrimental to the cytoskeletal structure and permeability of the intestinal epithelial barrier, whereas activation of PKC-ζ protects the integrity of this barrier (5, 6, 12).

Whether these effects of PKC activation also occur in endothelial cells at the BBB remains to be determined. There is increasing evidence that PKC alters BBB permeability by altering TJ function. Pertussis toxin increases permeability of the cerebral endothelial cells in vitro via a PKC-dependent pathway (19). Desai et al. (21) demonstrated that endothelial barrier dysfunction caused by interleukin-6 was mediated via PKC. Finally, studies (49) have also shown that PKC-α and PKC-β are both involved in regulating endothelial cell permeability after ischemia and inflammatory stimulation.

However, it remains to be determined whether the ischemic stressors H and H/R actually stimulate upregulation of PKC activity in endothelial cells of the BBB. Furthermore, it is unknown whether this PKC activation is involved in H- and H/R-induced BBB paracellular permeability changes. The goal
of this study was to determine whether H- and H/R-mediated changes in BBB permeability involve increased PKC activity and protein expression in BBB endothelial cells. This was investigated with both an in vitro tissue culture model and a global, nonocclusive in vivo H model. The data presented demonstrate that H and H/R regulate PKC activity in BBB endothelial cells and that PKC is involved in H-induced changes in BBB paracellular permeability. Additionally, this study demonstrates that the expression of the PKC isozymes is differentially regulated following both H and H/R in each of our BBB models.

MATERIALS AND METHODS

Chemicals and supplies. Plasma-derived horse serum (PDHS), MEM, Ham’s F-12 nutrient mixture, gentamicin, amphotericin B, heparin sodium, polymyxin B, and anti-rabbit peroxidase-conjugated secondary antibody were all purchased from Sigma (St. Louis, MO). Rabbit anti-PKCα, rabbit anti-PKCβI, rabbit anti-PKCβII, rabbit anti-PKCγ, rabbit anti-PKCδ, rabbit anti-PKCɛ, rabbit anti-PKCη, rabbit anti-PKCζ, and rabbit anti-PKCα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Complete Mini Protease Inhibitor and Complete Mini EDTA-free Protease Inhibitor were purchased from Roche Applied Science (St. Louis, MO). Nitrocellulose and 10% Tris-HCl Criterion gels were purchased from Bio-Rad Laboratories (Heracles, CA). [γ-32P]ATP and Western Lightning Chemiluminescence Reagent Plus were purchased from New England Nuclear/PerkinElmer Life Sciences (Boston, MA). The PKC Activity Assay kit was purchased from Calbiochem (La Jolla, CA). All other chemicals and supplies were purchased from Sigma.

Animals. Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Rats were housed individually in 12-h light-controlled facilities. Animals were fed ad libitum and had free access to water. All procedures were approved by the University of Arizona Institutional Animal Use and Care Committee.

RMEC isolation. Brains were removed from male and female Sprague-Dawley rats (250–300 g), and rat brain microvessel endothelial cells (RMECs) were isolated by using a modified method of Miller et al. (38). Briefly, microvessels were isolated from rat cortical gray matter using a sequence of enzymatic digestions and differential centrifugations, and cells were then suspended in MEM containing Ham’s F-12 nutrients (45%), PDHS (10%), gentamicin (50 μg/ml), amphotericin B, and heparin sodium. Cells were plated at 50,000 cells/cm² on collagen/fibronectin-coated dishes or 0.4 μm Transwell filters. Cells were fed 3 days after the initial plating and fed every 2 days afterward. The confluent cells were then used between days 11 and 14.

In vitro H treatment. Confluent RMEC monolayers were treated under hypoxic conditions using a temperature- and oxygen-controlled hypoxia workstation infused with 1% O2-99% N2 at 37°C for 24 hr. After the H treatment, a subset of RMECs was then incubated at 37°C for 2 hr in 95% room air-5% CO2 for the H/R treatment. For controls, RMECs were treated with room air for 24 hr. After treatment, the monolayers were then used to measure PKC activity, to measure [14C]sucrose permeability, or for Western blot analysis of PKC isozymes.

In vivo H treatment. As described previously (47), male or female Sprague-Dawley rats (200–250 g) were anesthetized with intraperitoneal injections (1 ml/kg) consisting of ketamine (78.3 mg/ml) and xylazine (3.1 mg/ml) and acepromazine (10 mg/ml) before treatment. Physiological body temperature was maintained using a heating pad. For H treatment, rats were placed in an oxygen-controlled hypoxia chamber at 6% O2-94% N2 for 1 hr. For H-treated rats, the brain was removed in the workstation chamber. For H/R treatment, rats were removed from the H chamber and placed in room air for 10 min. After H/R treatment, the brain was removed. As a control, rats were anesthetized and placed in room air for 1 hr. After treatment and removal of the brains, cerebral microvessels were isolated and analyzed for PKC activity and protein expression of PKC isozymes.

Rat cerebral microvessel isolation. Brain microvessels were isolated from rat cerebral gray matter for analysis of PKC activity and protein expression. Rats were anesthetized and treated as stated above. After removal of the brain, it was immersed in ice-cold buffer A containing (in mM) 103 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, and 15 HEPES (pH 7.4), and the meninges and choroid plexus were removed. The cortical mantles were weighed and then homogenized in a Teflon homogenizer in a fivefold volume of buffer B containing (in mM) 103 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 15 HEPES, 25 NaHCO3, 10 glucose, sodium pyruvate, and dextran (MW 64,000, 1 g/100 ml), pH 7.4 (Complete Mini Protease Inhibitor). After homogenization, an equal volume of 26% dextran was added, and the sample was centrifuged at 5,800 g at 4°C for 10 min. The supernatant was discarded, the pellet was resuspended in 10 ml of buffer B, and the suspension was then passed through a 100-μm mesh. The filtrate was centrifuged at 500 g for 5 min, and the pellet was resuspended in 1 ml of buffer B. The sample was centrifuged again at 14,000 rpm for 5 min at 4°C, and the pellet was resuspended in PKC extraction buffer containing (in mM) 25 Tris-HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, and 10 β-mercaptoethanol, and Triton X-100 (0.05%), leupeptin (1 μg/ml), and aprotinin (1 μg/ml). The sample containing PKC was then incubated with a PKC substrate and [γ-32P]ATP for 30 min at 37°C. The enzymatic reaction was terminated, and free avidin solution was added to the mixture. After a series of washes and centrifugations, avidin-bound biotinylated 32P-labeled peptide was removed. Samples were counted to determine the amount of radioactivity incorporated by the PKC. Specific activity [in pmol phosphate · min−1 · sample amount−1 (SA)] was calculated as follows:

\[
\text{PKC specific activity (pmol phosphate} \cdot \text{min}^{-1} \cdot \text{SA}^{-1}) = (75\text{[cpm(e + s)]} - \text{cpm(e)}) / \text{[cpm(r) · (t) · (SA)]}
\]

where SA is the amount of PKC sample in microliters, cpm(e + s) is the reference sample count in counts per minute, cpm(e) is the reaction sample count for the PKC sample, cpm(e) is the reaction sample count for the sample minus substrate, and t is the incubation period time.

[14C]sucrose permeability assay. The paracellular flux across the RMEC monolayer was determined as described previously (36). After treatment, the growth medium was replaced with assay buffer containing (in mM) 122 NaCl, 3 KCl, 1.4 CaCl2, 1.2 MgSO4, 25 NaHCO3, 10 HEPES, 10 glucose, and 0.5 K2HPO4 for 30 min at 37° C. Initially, 0.5 μCi of [14C]sucrose was added to the upper chamber (lumen). Immediately, 50 μl of solution were removed from the upper chamber to determine the initial concentration of [14C]sucrose. Samples (50 μl) were then removed from the lower chamber (abuminal) at times 0, 30, 60, and 120 min, and 50 μl of fresh assay buffer were added at each time. At the conclusion of the experiment, the amount of radioactivity in each sample was determined, and paracellular flux of [14C]sucrose across the membrane at 30, 60, and 120 min was calculated by using the following equation:

\[
\text{Flux} = V / (S \cdot A) \cdot C_i
\]

where V is the volume in the receiver chamber (1.5 ml), SA is the surface area of the monolayer (1 cm²), C is the concentration of
PKC MEDIATES HYPOXIA-INDUCED BBB PERMEABILITY CHANGES

H2014

[14C]sucrose in the donor chamber at time 0, and C, is the concentration of [14C]sucrose in the receiving chamber at either 30, 60, or 120 min. To determine the permeability coefficient, flux was plotted versus time, and the permeability coefficient (cm/min) was calculated by using linear regression analysis in Microsoft Excel.

Western blot analysis. SDS-PAGE and Western blot analysis were done as described previously (25). Briefly, proteins were separated on 10% Tris-HCl gels and transferred to nitrocellulose. The appropriate positive controls for each PKC isozyme were run alongside samples to ensure that the correct PKC isoform was being detected by each antibody. PKC-α, PKC-β, PKC-βII, PKC-γ, PKC-δ, PKC-ε, PKC-η, PKC-λ, PKC-μ, PKC-δ, and PKC-ζ were detected by chemiluminescence using rabbit anti-PKC-α (1:3,000), rabbit anti-PKC-β (1:1,000), rabbit anti-PKC-βII (1:15,000), rabbit anti-PKC-γ (1:10,000), rabbit anti-PKC-ζ (1:5,000), rabbit anti-PKC-δ (1:10,000), rabbit anti-PKC-η (1:1,000), rabbit anti-PKC-μ (1:1,000), rabbit anti-PKC-κ (1:15,000), rabbit anti-PKC-ζ (1:2,000), and rabbit anti-PKC-λ (1:1,000) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:16,000). After being exposed to films, all nitrocellulose membranes were washed and stained with India ink for normalization of protein loading. Films and membranes were scanned by using the Kodak Image Station, and semiquantifiable analysis of the protein was done by using the Kodak ID Imaging Software (Eastman Kodak, New Haven, CT).

Statistical analysis. All experiments were repeated at least three times, and all data are means ± SE. All statistical analysis was done by using Sigma Stat software. All activity and permeability data were analyzed using one-way ANOVA, followed by the Student-Newman-Keuls t-test. RMEC PKC activity data were also analyzed using a t-test to compare normoxia and H treatments. RMEC protein expression was analyzed using one-way ANOVA, followed by Bonferroni’s t-test. RCMV protein expression was analyzed using one-way ANOVA, followed by Fisher least significant difference test.

RESULTS

BBB endothelial cell PKC activity in vitro after H and H/R. The first series of experiments investigated the activation of PKC after H and H/R in an in vitro BBB model. In RMECs treated with H (1% O2-99% N2; 24 h), total PKC activity increased by 73% compared with that of normoxia (95% room air-5% CO2; 24 h)-treated controls (Fig. 1). After H/R (95% room air-5% CO2; 2 h), there was a reduction in total PKC activity in RMECs back toward normoxia levels (Fig. 1). This increase in PKC activity correlates with previous studies (1, 36, 47), demonstrating that exposure to H increases [14C]sucrose permeability.

PKC isoyme expression in RMECs after H and H/R. In the next set of experiments, we determined whether the expression of the PKC isoforms was altered during H and H/R in the in vitro BBB model. Based on previous studies (17) which demonstrated a role for Ca2+ in the H- and H/R-mediated changes on endothelial cell permeability, we first investigated the effects of H and H/R on the expression of the Ca2+-dependent PKCs (conventional) in RMECs. After treatment with H (1% O2-99% N2; 24 h), there was a significant increase in the expression of the PKC-βII and PKC-γ (Fig. 2A) in RMECs. The expression levels of both PKC-βII and PKC-γ remained elevated after 2 h of H/R (95% room air-5% CO2; Fig. 2A). There was no effect of H or H/R on the expression of PKC-α and PKC-β (Table 1) in RMECs.

Recent evidence (6) in the literature also suggests a role for the novel and atypical PKCs (Ca2+-independent) in modulating endothelial cell permeability. In RMECs treated with H (1% O2-99% N2; 24 h), there was a significant increase in the expression of the novel PKCs, PKC-η, and PKC-μ (Fig. 2B; Table 1), and the expression of these two PKC isoforms remained elevated following 2 h of reoxygenation. After H/R (95% room air-5% CO2; 2 h), there was a significant increase in the expression of PKC-ε (Fig. 2B; Table 1), which was not seen after hypoxic treatment. Both H and H/R had no significant effect on the expression of PKC-β or PKC-δ (Table 1) in RMECs. In addition, there were also changes in the protein expression of the atypical PKCs. After H (1% O2-99% N2; 24 h) in RMECs, an increase in the expression of PKC-λ protein was observed, and the protein levels of PKC-λ remained elevated during H/R (Fig. 2C; Table 1). Meanwhile, the protein expression of PKC-ζ was slightly reduced after H but then significantly increased above hypoxic levels after H/R (Fig. 2C).

Role of PKC in H- and H/R-induced paracellular permeability changes. Similar to previous studies (1, 36), there was an effect of oxygen concentration on paracellular permeability in BBB endothelial cells. Compared with normoxia treatment (room air; 24 h), H (1% O2-99% N2; 24 h) caused a significant increase (129%) in [14C]sucrose permeability in RMECs. This increase is reduced 42% by 2 h of posthypoxic reoxygenation (95% room air-5% CO2; Fig. 3). We next determined whether inhibition of PKC altered these responses. The data presented in Fig. 3 show that pretreatment with the PKC inhibitor chelerythrine chloride (10 μM; 15 min) before H (1% O2-99% N2; 24 h) significantly attenuates the H-induced increase in [14C]sucrose permeability in RMECs. Reoxygenation (95% room air-5% CO2; 2 h) following treatment with H (1% O2; 24 h) and chelerythrine chloride (C; 10 μM; 15 min) did reduce [14C]sucrose permeability similar to H/R alone (Fig. 3). However, the effects of PKC inhibition and H/R on [14C]sucrose permeability were not additive (Fig. 3). When compared with normoxic controls (95% room air-5% CO2; 24 h), chelerythrine chloride (10 μM; 15 min) had no effect on RMEC paracellular permeability to [14C]sucrose (Fig. 3).

H alters BBB endothelial cell PKC activity expression in vivo. We next wanted to determine whether PKC activity was also altered in our global, nonocclusive in vivo H model. Similar to what was found in the in vitro RMEC model, H stimulated PKC activity dramatically in isolated RCMV from...
PKC mediates hypoxia-induced BBB permeability changes

H2015

Fig. 2. Hypoxia and posthypoxic reoxygenation alter protein expression of conventional, novel, and atypical PKC isozymes in RMECs. Confluent monolayers of cultured RMECs were exposed to normoxia (N, room air, 24 h), hypoxia (H; 1% O2–99% N2; 24 h), or hypoxia followed by 2 h of posthypoxic reoxygenation (H/R, room air) and paracellular permeability was measured using Western blot analysis. Representative Western blot analysis showing protein expression was measured using bands of cultured RMECs were exposed to normoxia (N, room air, 24 h), hypoxia (H, 1% O2–99% N2; 24 h), or hypoxia followed by 2 h of posthypoxic reoxygenation (H/R; room air, 2 h). *P < 0.05 compared with normoxia; †P < 0.05 compared with hypoxia (n = 11–16).

Table 1. Effects of H and H/R on protein expression of conventional, novel, and atypical PKC isozymes in RMECs in vitro.

<table>
<thead>
<tr>
<th>Condition</th>
<th>PKCα (80 kDa)</th>
<th>PKCβ (80 kDa)</th>
<th>PKCβII (80 kDa)</th>
<th>PKCy (80 kDa)</th>
<th>PKCμ (115 kDa)</th>
<th>PKCe (95 kDa)</th>
<th>PKCη (80 kDa)</th>
<th>PKCδ (80 kDa)</th>
<th>PKCθ (80 kDa)</th>
<th>PKCζ (80 kDa)</th>
<th>PKCζ (80 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td><img src="image1" alt="Band" /></td>
<td><img src="image2" alt="Band" /></td>
<td><img src="image3" alt="Band" /></td>
<td><img src="image4" alt="Band" /></td>
<td><img src="image5" alt="Band" /></td>
<td><img src="image6" alt="Band" /></td>
<td><img src="image7" alt="Band" /></td>
<td><img src="image8" alt="Band" /></td>
<td><img src="image9" alt="Band" /></td>
<td><img src="image10" alt="Band" /></td>
<td><img src="image11" alt="Band" /></td>
</tr>
<tr>
<td>H</td>
<td><img src="image12" alt="Band" /></td>
<td><img src="image13" alt="Band" /></td>
<td><img src="image14" alt="Band" /></td>
<td><img src="image15" alt="Band" /></td>
<td><img src="image16" alt="Band" /></td>
<td><img src="image17" alt="Band" /></td>
<td><img src="image18" alt="Band" /></td>
<td><img src="image19" alt="Band" /></td>
<td><img src="image20" alt="Band" /></td>
<td><img src="image21" alt="Band" /></td>
<td><img src="image22" alt="Band" /></td>
</tr>
<tr>
<td>H/R</td>
<td><img src="image23" alt="Band" /></td>
<td><img src="image24" alt="Band" /></td>
<td><img src="image25" alt="Band" /></td>
<td><img src="image26" alt="Band" /></td>
<td><img src="image27" alt="Band" /></td>
<td><img src="image28" alt="Band" /></td>
<td><img src="image29" alt="Band" /></td>
<td><img src="image30" alt="Band" /></td>
<td><img src="image31" alt="Band" /></td>
<td><img src="image32" alt="Band" /></td>
<td><img src="image33" alt="Band" /></td>
</tr>
</tbody>
</table>

Data are means ± SE percentage control of normalized optical density of all PKC isozymes during normoxia (N; room air, 24 h), hypoxia (H; 1% O2–99% N2; 24 h), and posthypoxic reoxygenation (H/R; room air, 2 h). *P < 0.05 compared with normoxia; †P < 0.05 compared with hypoxia (n = 11–16).

for an increase in PKC-βIII expression after H and H/R in vivo. However, the increase was not statistically significant. As seen in RMECs, there was no affect of H or H/R on PKC-α and PKC-βI expression in the in vivo BBB model (Table 2). Additionally, the expression of the novel and atypical PKC isozymes was quite different in the in vivo model compared with the in vitro model. There was only an increase in PKC-θ after H/R in the in vivo model (Fig. 5B; Table 2). Furthermore, PKC-κ was unable to be detected in isolated RCMV (Table 2).

DISCUSSION

The data presented here demonstrate that PKC is involved in the paracellular permeability changes induced by H and H/R.
Stimulation of total PKC activity by H and H/R in BBB endothelial cells correlates with BBB paracellular permeability changes induced by H and H/R. Moreover, inhibition of PKC attenuates the H-induced increase in $[^{14}C]$sucrose permeability. The specific PKC isozymes involved in the H-induced paracellular permeability changes remain to be determined. However, results from the present study show that H and H/R regulate the expression of multiple isoforms of PKC, suggesting these isoforms may be mediating the effects of H and H/R on paracellular permeability.

One mechanism by which H and H/R may increase total PKC activity is enhanced PKC protein expression. There are 11 isoforms of PKC that are categorized based on their modes of activation. The conventional PKCs ($\alpha$, $\beta I$, $\beta II$, $\gamma$) require Ca$^{2+}$, whereas the novel ($\mu$, $\epsilon$, $\eta$, $\delta$, $\theta$) and the atypical ($\zeta$, $\lambda$) PKCs are Ca$^{2+}$-independent (28, 30). Although previous studies (32, 37) have shown the expression of at least 6 PKC isoforms ($\alpha$, $\beta$, $\delta$, $\epsilon$, $\eta$, $\zeta$) in endothelial cells, the data presented here demonstrate that all 11 isoforms of PKC are expressed in BBB endothelial cells in vitro (Table 1), and 10 PKC isoforms are expressed in vivo (Table 2). Neither of the previous studies investigated the expression of PKC-$\mu$, PKC-$\theta$, and PKC-$\lambda$. More of a surprise, however, is the fact that PKC-$\gamma$ is expressed in BBB endothelial cells because both of the previous studies (32, 37) were unable to detect this isozyme in endothelial cells. This could be due to the development of more specific antibodies for the various PKC isoforms, or it could be explained by differences in the endothelial cell models. For example, the first study used a cultured human umbilical vein endothelial cell line (HUVEC) and was unable to detect both PKC-$\delta$ and PKC-$\gamma$ while detecting four other PKC isoforms (37). The second study used an immortalized cell line, rat brain endothelial-4 (RBE4), isolated RMECs from 2-wk-old rats, aortic endothelial cells, and isolated cerebral capillaries, and they were able to detect six PKC isoforms but not PKC-$\gamma$ (32).

Because the expression of some of the PKC isoforms remains elevated even as PKC activity levels are decreasing and the fact that the expression of some PKC isoforms do not increase until PKC activity levels are decreasing suggest that other intracellular mechanisms besides increased protein expression may be involved in regulating PKC activity. Additionally, the large increases in PKC activity most likely cannot be explained by only increased protein expression. This suggests that PKC is being altered by other intracellular mechanisms. For example, it has been shown that activated PKC translocates from the cytoplasm to the membrane once it is activated (41). Furthermore, PKC itself is phosphorylated on activation (41). Although these studies focused on the changes in PKC isoform protein expression, it is important to note that there is most likely an increase in translocation and/or phosphorylation of the various PKC isoforms. Future studies will be required to determine the activation (translocation and/or
inhibited by the overexpression of PKC-ε to prevent the activation of the transcription factor NF-κB. Recently, it has been shown that the protective effect of EGF on BBB endothelial cell integrity during H and H/R. Also, our data, which show an increase in PKC-ε expression and localization after hypoxic stress. Because both studies (15) have indicated that PKC-δ, PKC-θ, and PKC-λ all play a role in mediating epithelial cell integrity. More specifically, these studies demonstrated that disruption of epithelial barrier integrity by oxidants involves upregulation of PKC-δ, which in turn modulates inducible NO synthase (iNOS) (6). Additionally, in vivo studies (15) have indicated that PKC-δ mediates injury caused by cerebral reperfusion. Although our results do not show an increase in PKC-δ expression, our results do not exclude the possibility that PKC-δ may play a role in altering BBB endothelial cell integrity during H and H/R. Also, our data, which show an increase in PKC-λ expression in RMECs, suggest that this may be one isozyme that is important in regulating endothelial barrier integrity.

Although our study along with the literature suggests a role for PKC in the detrimental effects of H on BBB function, we cannot exclude the possibility that the activation of PKC may improve BBB integrity as was found in epithelial cells. Recently, it has been shown that the protective effect of EGF on intestinal barrier integrity during exposure to oxidants is due to the activation of PKC-βII and PKC-ζ isozymes, which appears to prevent the activation of the transcription factor NF-κB and degradation of its endogenous inhibitor IκB by oxidants (7–10, 13). Another study (11) has shown that the upregulation of iNOS following stimulation by oxidants in epithelial cells is inhibited by the overexpression of PKC-ζ, and this leads to the protection of the epithelial barrier integrity. If PKC is important for protecting barrier integrity, it may explain why an increase in the expression of PKC-ζ, PKC-ε, and PKC-θ was not observed until H/R, a point when endothelial cell paracellular permeability is returning to basal levels. Although our study did not show an increase in total PKC activity after H/R, we cannot exclude the possibility that PKC-ζ, PKC-ε, and PKC-θ activity is upregulated after reoxygenation. Actually, it may be that H/R (in our model system) increases PKC-ζ, as well as PKC-ε and PKC-θ activity (and possibly activity) to reestablish the integrity of the BBB. There are several possible mechanisms by which H/R and PKC-ζ may be restoring BBB integrity. Previous studies (35) have shown that inhibition of iNOS attenuates H-induced increases in paracellular permeability and that iNOS expression increases with H and returns to normoxic levels after H/R. This suggests that H/R may be lowering iNOS protein levels by stimulating an increase in PKC-ζ expression. This reduction in iNOS could then be contributing to the effect of H/R on [14C]sucrose permeability. A second mechanism by which PKC-ζ may protect the BBB is by regulating NF-κB expression and activity, since previous studies (18, 48) have shown that these are altered at the BBB during H and H/R.

The one PKC isozyme that demonstrated a significant change in both our in vitro and in vivo models was PKC-γ. In addition, there were similar trends in the upregulation of PKC-βII both in vitro and in vivo. As stated earlier, cell permeability changes after ischemia and inflammatory stimulation are regulated by PKC-β (49). On the other hand, little is known about the function of PKC-γ in endothelial cells, because previous studies (32, 37) demonstrated a lack of expression of this isozyme. However, studies (17) have shown that H-mediated changes in endothelial cell permeability involve alterations in intracellular Ca²⁺. Additionally, studies (16) have implicated a role for Ca²⁺ in modulating occludin expression and localization after hypoxic stress. Because both PKC-βII and PKC-γ are categorized as conventional PKCs (28, 30), this suggests that PKC-βII and PKC-γ, in conjunction with changes in intracellular Ca²⁺, may be contributing to H-induced changes in paracellular permeability. Future studies are needed to investigate whether these two PKC isozymes are involved in the observed Ca²⁺-dependent alterations.

There are several possible mechanisms by which PKC may be altering BBB permeability. One possible mechanism is the direct regulation (phosphorylation) of the TJ proteins by PKC. PKC-ζ has been shown to colocalize with zona occludens (ZO-1) in Madin-Darby canine kidney (MDCK) and human casuarin colon adenocarcinoma-2 (Caco-2) cell lines (22). It has also been shown that occludin phosphorylation can alter its interactions with intracellular scaffolding proteins (33). This supports the hypothesis that PKC may directly regulate the structure and function of TJ proteins. Furthermore, studies (44, 45) have shown that the activation of PKC-α disrupts the TJ and causes disassembly of occludin at the TJ. Another hypothesis is that PKC may be indirectly altering the TJ proteins by activating a downstream intracellular signaling pathway that in turn directly affects the TJ proteins. Previous studies (17, 18) have demonstrated that changes in NO and intracellular Ca²⁺ contribute to increases in paracellular permeability induced by H and H/R. Increases in the expression of the Ca²⁺-dependent isozymes PKC-βII and PKC-γ along with PKC-ζ, which can regulate NOS (5), suggest that PKC may interact with this signaling molecule to contribute to BBB permeability alterations.

### Table 2. Effects of H and H/R on protein expression of conventional, novel, and atypical PKC isozymes in vivo

<table>
<thead>
<tr>
<th>PKC Isozyme</th>
<th>N</th>
<th>H</th>
<th>H/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>100±5.1</td>
<td>112.9±6.6</td>
<td>120.1±10.1</td>
</tr>
<tr>
<td>PKCβII</td>
<td>100±7.2</td>
<td>108.2±11.5</td>
<td>116.1±9.8</td>
</tr>
<tr>
<td>PKCδ</td>
<td>100±14.8</td>
<td>144.5±32.2</td>
<td>207.7±64.8</td>
</tr>
<tr>
<td>PKCγ</td>
<td>100±12.4</td>
<td>144.8±15.8*</td>
<td>146.1±13.9*</td>
</tr>
<tr>
<td>PKCε</td>
<td>100±4.7</td>
<td>95.8±7.7</td>
<td>99.9±9.4</td>
</tr>
<tr>
<td>PKCe</td>
<td>100±13.1</td>
<td>145.1±41.2</td>
<td>119.3±15.6</td>
</tr>
<tr>
<td>PKCg</td>
<td>100±4.8</td>
<td>108.3±10.8</td>
<td>96.6±6.9</td>
</tr>
<tr>
<td>PKCλ</td>
<td>100±5.9</td>
<td>110.8±5.7</td>
<td>98.1±7.1</td>
</tr>
<tr>
<td>PKCζ</td>
<td>100±14.8</td>
<td>109.6±13.3</td>
<td>154.4±18.5*</td>
</tr>
</tbody>
</table>

Data are means ± SE percentage control of normalized optical density of all the PKC isozymes during normoxia (N, room air, 1 h), hypoxia (H, 6% O₂-94% N₂; 1 h), and posthypoxic reoxygenation (H/R, room air, 10 min). ND, not detected. *P < 0.05 compared with normoxia (n = 10).
A final hypothesis to consider is that PKC may not alter the TJ proteins but affects other proteins located at the endothelial cell junction to regulate paracellular permeability. This idea is supported by a study (20) that showed that PKC mediates BBB function by altering ICAM and fluid phase endocytosis in tumor necrosis factor α-treated endothelial cells. It is also supported by a study (24) that demonstrated a minimal role for PKC in H-induced BBB permeability and TJ alterations induced by H and VEGF.

The unique aspect of the present study is our attempt to translate our results from the in vitro BBB model to our nonocclusive, global in vivo hypoxia model. It was interesting to note that in both models there was a significant increase in PKC activity after treatment with H and that this increase was reduced during H/R. Also of interest were the differences in the expression of the various PKC isozymes during the different treatment conditions in both models. In the in vitro model, the protein expression of 7 of 11 PKC isozymes was altered after either H and/or H/R. However, the protein expression of only 2 of the 11 PKC isozymes was significantly changed after H and/or H/R in the nonocclusive in vivo hypoxia model.

There are several possible explanations for these differences. It could be due to the fact that in the in vitro model is a monolayer of endothelial cells. This model is simplified and allows the investigator to study in great detail the response of only the endothelial cells to an insult. However, it is now understood that the endothelial cells, pericytes, glia, and even neurons, all contribute to maintaining the integrity of the BBB as a neurovascular unit (34, 39). This suggests that nonendothelial cells or factors released by these cells can also regulate PKC activity that in turn modulates BBB function. It also implies that endothelial cells in the in vitro model may be more sensitive to H and H/R due to the lack of pericytes, glia, and neurons, which constitute the intact neurovascular unit. This concept is supported by studies (2, 27, 42, 43) that show that endothelial cells co-cultured with glia and/or neurons respond differently to stimuli than do endothelial cells cultured alone. A second explanation for the differences between the in vitro and in vivo H models may be the level of H and duration of the hypoxic exposure. The Davis laboratory (35, 36, 47) fully characterized both models in previous studies, and the O2 levels and H duration were based on points where functional (paracellular permeability) changes in the BBB were observed. However, it may be that the in vitro and in vivo regulation of PKC activity and protein expression is different based on the levels of O2 and the duration of exposure.

In conclusion, this study demonstrates that the H-induced increases in PKC activity in BBB endothelial cells play a functional role in altering [14C]sucrose paracellular permeability. This study also shows for the first time that all 11 PKC isozymes are present in BBB endothelial cells. Furthermore, the results indicate that the expression of various isozymes of PKC is differentially regulated by H and H/R. These data suggest that different PKC isozymes may contribute to different cellular functions leading to alterations in endothelial cell integrity during physiological and pathophysiological states.

REFERENCES


GRANTS

This work was supported by a Postdoctoral National Research Service Award F32-NS-049894 (to M. A. Fleegal) and by RO1-NS-39592 (to T. P. Davis).

AJP-Heart Circ Physiol • VOL 289 • NOVEMBER 2005 • www.ajpheart.org


