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Inhibition of astroglia-induced endothelial differentiation by inorganic lead: A role for protein kinase C

(lead toxicity/angiogenesis/blood–brain barrier/neurotoxicity)

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ABSTRACT **Microvascular endothelial function in developing brain is particularly sensitive to lead toxicity, and it has been hypothesized that this results from the modulation of protein kinase C (PKC) by lead. We examined the effects of inorganic lead on an *in vitro* model of central nervous system endothelial differentiation in which astroglial cells induce central nervous system endothelial cells to form capillary-like structures. Capillary-like structure formation within C6 astroglial–endothelial cocultures was inhibited by lead acetate with 50% maximal inhibition at 0.5 μM total lead. Inhibition was independent of effects on cell viability or growth. Under conditions that inhibited capillary-like structure formation, we found that lead increased membrane-associated PKC in both C6 astroglial and endothelial cells. Prolonged exposure of C6 cells to 5 μM lead for up to 16 h resulted in a time-dependent increase in membranous PKC as determined by immunoblot analysis. Membranous PKC increased after 5-h exposures to as little as 50 nM lead and was maximal at ≈1 μM. Phorbol esters were used to determine whether PKC modulation was causally related to the inhibition of endothelial differentiation by lead. Phorbol 12-myristate 13-acetate (10 nM) inhibited capillary-like structure formation by 65 ± 5%, whereas 4α-phorbol 12,13-didecanoate was without effect. These findings suggest that inorganic lead induces cerebral microvessel dysfunction by interfering with PKC modulation in microvascular endothelial or perivascular astroglial cells.**

Lead toxicity has recently been identified as the most important environmental health hazard affecting children in the United States (1). It is now generally accepted that blood lead levels as low as 0.5 μM are potentially neurotoxic in children (1). Thus, up to 60% of children in certain older American cities may be at risk for lead toxicity.

Experimental evidence points to multiple sites, including neurons, astroglia, and the microvasculature, at which lead may act in the developing brain (2). The brain microvasculature of the suckling rat preferentially accumulates lead relative to brain parenchyma (3) and is particularly sensitive to its toxic effects (4, 5). Acute neonatal exposure to lethal lead concentrations results in cerebellar hemorrhage, cerebral edema, and increased blood–brain barrier permeability (6, 7). Lower systemic levels also damage the blood–brain barrier, as shown by increased permeability to serum albumin, without causing significant hemorrhage or cerebral edema (8). Susceptibility to these vascular effects in the suckling rat coincides with the period of active microvessel growth and blood–brain barrier development (5, 9). Conversely, susceptibility diminishes markedly as vessel growth slows and the blood–brain barrier matures (3, 5). Thus, it appears that lead interferes with the development and differentiation of brain microvessels.

It has been proposed that lead neurotoxicity results from interference with normal calcium-mediated cellular processes (10). Evidence in support of this hypothesis includes effects of lead on calcium-regulated neurotransmitter release, intracellular calcium levels, calcium channels, and calcium-binding regulatory proteins. Lead can substitute for calcium in the activation of protein kinase C (PKC) (11) and exposure of isolated neonatal rat brain microvessels to lead results in translocation of PKC from cytosolic to membrane-associated pools (12). Since PKC regulates many transcriptional and posttranslational events linked to cell proliferation and differentiation (13), altered PKC metabolism might explain some of the neurovascular effects of lead toxicity.

In this report, we establish that inorganic lead inhibits astroglia-induced microvessel formation *in vitro*, an experimental model of central nervous system microvessel development (14, 15). We also show that under similar conditions lead increases membrane-associated PKC pools in astroglial and microvascular endothelial cells. A causal relationship between PKC activation and the inhibition of astroglia-induced microvessel formation *in vitro* is suggested by the response to phorbol esters, well-characterized PKC activators (16). Our findings suggest that cerebral microvascular dysfunction of lead toxicity results from altered astroglial–endothelial interactions secondary to aberrant PKC modulation.

METHODS

Homologous Cell Cultures. Bovine retinal microvessel endothelial (BRE) cells were isolated by the method of Bowman *et al.* (17) as modified by Laterra *et al.* (14). Endothelial cells were cultured as described (14) and shown to be essentially 100% endothelial by labeling with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein (DiI-acyl-LDL) (18). Cells at passage 3–12 were used in all experiments.

C6 cells (19) were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) bovine calf serum and gentamicin sulfate (50 μg/ml) in humidified 5% CO_2/95% air at 37°C.

Proliferation Studies. BRE and C6 cells were grown in the presence of lead acetate at the indicated concentrations. Cells

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Abbreviations: PKC, protein kinase C; DiI-acyl-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein; PMA, phorbol 12-myristate 13-acetate; BRE, bovine retinal endothelial.

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were plated at subconfluency in 24-well plates. For each of 5 days beginning with the day after plating, triplicate wells were treated with trypsin under conditions that quantitatively removed all cells, and cells were then counted with a Coulter counter. Doubling times were calculated during exponential cell growth.

Cocultures. C6–BRE cell cocultures were established by a modification of the method of Laterra *et al.* (14). C6 cells were suspended in complete medium, with or without the indicated concentration of lead acetate, and plated at 7500–15,000 cells per chamber into 8-chamber fibronectin-coated Lab-Tek slides. After incubation for 24 h, the C6 medium was removed and replaced with 40,000–80,000 BRE cells suspended in 0.25 ml of stock endothelial cell medium (14) containing an equivalent concentration of lead acetate and fresh ascorbic acid (50 μg/ml). Control slides lacked either the astroglial or endothelial cell inoculum. After an additional 72 h at 37°C, cocultures were rinsed incubated with DiI-acyl-LDL, fixed, and mounted as described (14). To determine the effects of phorbol esters, a subset of cocultures received 10 μl of stock phorbol 12-myristate 13-acetate (PMA) or 4α-phorbol 12,13-didecanoate [250 mM in phosphate-buffered saline (PBS)] 48 h after plating. Capillary-like structure formation was quantitated by computer-assisted image analysis with use of the Microcomputer Imaging Device (MCID) software package of Imaging Research (Brock University, St. Catherines, ON, Canada) (14, 15).

PKC Assays. Confluent cell monolayers were exposed to lead acetate or phorbol ester in complete medium at 37°C for the indicated times. Controls were incubated in medium alone. The monolayers were then rinsed with ice-cold PBS, and the cells were scraped off and centrifuged at 200 × *g* for 5 min. Cell pellets were resuspended in 350 μl of 20 mM Tris·HCl (pH 7.5) containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, 2 μM leupeptin, trypsin inhibitor (10 μg/ml), and aprotinin (100 μg/ml) (Tris buffer), disrupted by 40 strokes with a Dounce tissue grinder, and centrifuged for 60 min at 100,000 × *g* at 4°C. Supernatant fluids were saved as cytosolic fractions. The pellets were sonicated on ice in Tris buffer containing 0.5% Triton X-100 and centrifuged at 20,000 × *g* for 5 min, and supernatant fractions were saved as membrane fractions. Protein was measured by the method of Bradford (20). Membrane and cytosolic fractions were solubilized in SDS/PAGE sample buffer containing mercaptoethanol, heated to 100°C for 2 min, subjected to polyacrylamide gel electrophoresis in a 7.5% polyacrylamide gel (21), and blotted onto ECL nitrocellulose membranes (Amersham; ref. 22). Blots were serially incubated with 2% (wt/vol) bovine serum albumin, mouse monoclonal antibody against the rat brain cytosol PKC α (1 μg/ml, UBI, Lake Placid, NY) (23) for 16 h, and horseradish peroxidase-conjugated anti-mouse IgG (1:5000 dilution, Amersham) in 50 mM NaCl/0.05% Tween-20/2 mM Tris·HCl, pH 7.5. Specific binding was detected by the ECL detection system (Amersham). The white light absorbance of the 80-kDa immunoreactive bands was quantitated by computer-assisted densitometry with the Microcomputer Imaging Device (MCID) software package of Imaging Research.

RESULTS

Astroglial Induction of Capillary-Like Structures. When cultured alone on fibronectin-coated substrata, BRE cells formed confluent monolayers with a typical cobblestone morphology. DiI-acyl-LDL was used to identify endothelial cells within C6–endothelial cocultures since C6 cells do not incorporate this marker efficiently (14, 18). In contrast to the cobblestone monolayers generated by BRE cells when cultured alone, BRE cells organized into capillary-like structures within 3 days of addition to C6 astroglial cultures (Fig. 1). These 10- to 20-μm diameter structures that consist of numerous linearly associated endothelial cells have been shown to resemble capillaries in size, associated extracellular matrix deposition, and presence of central lumens (14, 15).

Effects of Lead on Cell Growth and Differentiation. To determine whether inorganic lead altered capillary-like structure formation independent of cell proliferative effects or frank cell toxicity, we first examined the effects of lead on the growth and morphology of BRE cells and C6 cells. Doubling times during exponential cell growth are presented in Table 1. Lead added to final concentrations of 0.1–50 μM had no effect on BRE doubling times. C6 growth was not influenced by lead concentrations of <10 μM, but lead concentrations of 10 and 50 μM increased C6 cell doubling times by 31% and 115%, respectively. Neither BRE nor C6 cell morphologies were altered after exposure for 3–4 days to any of the lead concentrations as determined by phase-contrast microscopy.

C6–BRE cocultures were established and maintained for 72 h in 0.1–5 μM lead acetate and the extent of endothelial differentiation into capillary-like structures was examined. Lead concentrations of 0.5, 1, and 5 μM inhibited capillary-like structure formation by 29% ($P < 0.05$), 56% ($P < 0.01$), and 70% ($P < 0.01$), respectively (Fig. 2). In contrast to these higher concentrations of lead, 0.1 μM occasionally appeared to enhance this morphogenic response. This however was not a consistent finding and experiments using lower lead concentrations revealed no significant effects below 0.5 μM.

Effects of Lead on Cellular PKC. Earlier studies assessing enzyme activity had demonstrated that lead substitutes for

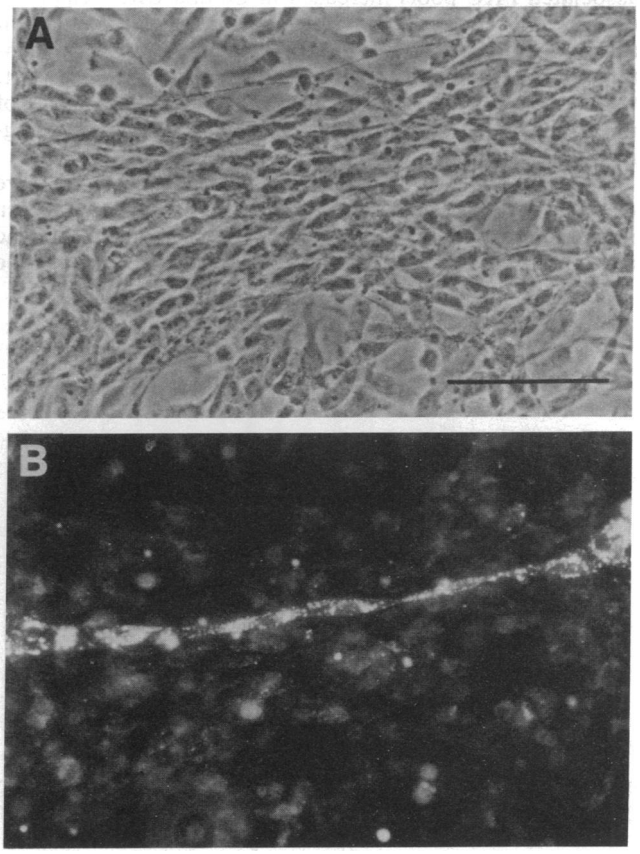

FIG. 1. Astroglia-induced endothelial differentiation into capillary-like structures. Endothelial cells were cocultured with C6 cells for 72 h and then labeled with the fluorescent endothelial marker DiI-acyl-LDL. (*A*) Phase-contrast photomicrograph of C6–endothelial coculture. (*B*) Fluorescent photomicrograph of the field in A showing a capillary-like structure consisting of linearly aligned overlapping endothelial cells. (Bar = 100 μm.)

Table 1. Effects of lead on C6 and BRE cell growth

Lead, μM	Doubling time, h	
	C6	BRE
0	13	14
0.1	13	13
0.5	12	14
1	12	14
10	17	15
50	28	15

Cells were plated sparsely in medium containing 0–50 μM lead acetate and at 24-h intervals cell numbers in triplicate samples were quantified. Doubling times were calculated during logarithmic growth.

calcium in the activation of PKC (11) and stimulates PKC translocation from cytosol to plasma membrane in isolated immature microvessels from rat brain (12). Therefore, we examined the effects of lead on the cytosolic and membrane-associated PKC pools in cultured C6 or BRE cells under conditions that also inhibited C6-induced microvessel morphogenesis. Cultures of either C6 or BRE cells were exposed to lead acetate for various periods of time and their cytosolic and membrane fractions were analyzed for PKC by Western blot analysis with a monoclonal antibody to the PKC α isozyme (23). Cells exposed to PMA were used as positive controls. Both cytosolic and membranous fractions derived from C6 and BRE cells contained an 80-kDa immunoreactive protein consistent with PKC (Fig. 3). The membrane-associated PKC pools increased in C6 and BRE cells after exposure to either 50 nM PMA or 5 μM lead acetate. In addition, immunoblots from BRE cell fractions also contained immunoreactive bands of lower molecular weight, within a 58- to 70-kDa range (Fig. 3*B*). These bands are of uncertain significance and may represent proteolytic fragments as described (24).

The time and concentration dependence of lead exposure on membranous and cytosolic PKC pools was examined in more detail. Prolonged incubation of cells in 5 μM lead acetate resulted in a gradual increase in the membrane-bound PKC pool (Fig. 4). In C6 cells, this pool increased to 187% of control after 3 h of lead exposure and to 250% of control after 16 h of lead exposure; cytosolic PKC concurrently decreased under these conditions. In contrast to this relatively slow response to lead, 50 nM PMA induced a rapid increase in membranous PKC concurrent with a decrease in the cytosolic PKC pool (Fig. 3). As shown in Fig. 5*B*, exposing C6 cells to total lead concentrations as low as 0.03 μM for 5 h resulted in detectable increases in membrane-associated PKC pools. A maximal response occurred at lead concentrations of 1–10 μM (Fig. 5*A*).

The Effects of Phorbol Esters on Capillary-Like Structure Formation. We found that, under similar conditions of exposure, lead acetate inhibited C6-induced endothelial differentiation into capillary-like structures and increased membrane-associated PKC pools in cultured BRE and C6 cells. To determine whether these effects of lead were causally related, we examined the influence of phorbol esters, well-characterized PKC activators, on C6-induced capillary-like structure formation. C6–BRE cocultures were treated for 24 h with either PMA or 4α-phorbol 12,13-didecanoate, a phorbol ester without PKC-activating activity (16). PMA (10 nM) inhibited C6-induced capillary-like structure formation by 70% ($P < 0.01$), whereas treatment of cocultures with 10 nM 4α-phorbol 12,13-didecanoate under identical conditions had no effect.

DISCUSSION

Microvessel development is a complex process involving endothelial migration, proliferation, cord formation, lumen formation, basement membrane deposition, and tissue-specific endothelial differentiation. In both brain and retina, these endothelial events occur under the influence of perivascular astrocytes (25, 26) and result in the formation of blood–brain and blood–retinal barriers. These barriers consist of anatomic and biochemical endothelial properties that regulate the passage of water-soluble compounds across central nervous system capillaries (27). Barrier expression depends upon intimate astroglial–endothelial interactions

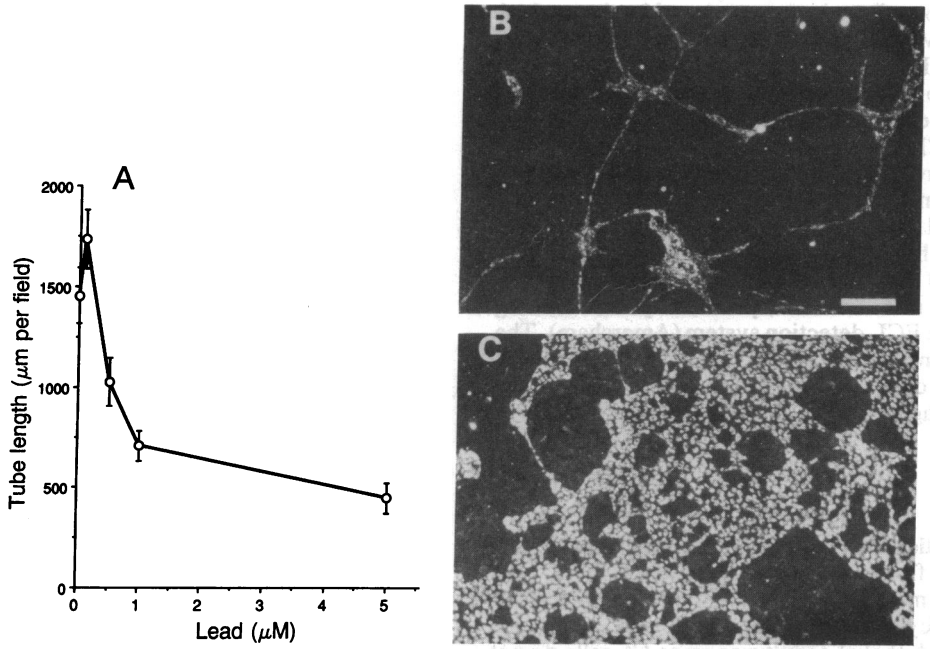

FIG. 2. Effect of lead on capillary-like tube formation. Endothelial cells were cocultured with C6 cells in the presence of lead acetate (0–5 μM) for 72 h and capillary-like structure formation was quantitated. (*A*) Data are from 12 representative fields (mean ± SEM). Fluorescent photomicrographs show control cocultures (*B*) and cocultures containing 5 μM lead acetate (*C*). (Bar = 250 μm.)

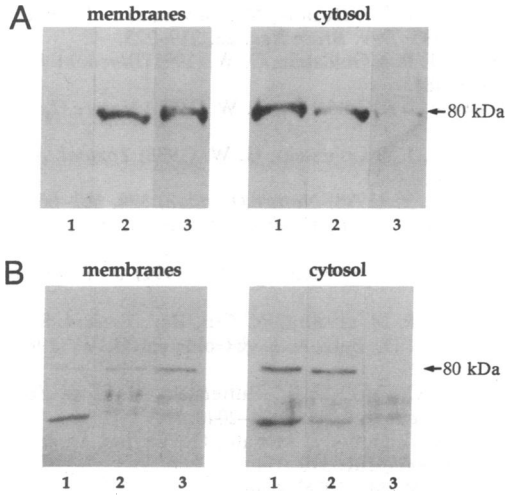

FIG. 3. Effect of lead on PKC in C6 and endothelial cells. Confluent cultures were exposed to medium alone (lanes 1), medium containing 5 μM lead acetate for 5 h (lanes 2), or medium containing 50 nM PMA for 30 min as a positive control (lanes 3). Cells were then fractionated and cytosolic and membrane fractions were analyzed for PKC on an immunoblot. (*A*) C6 cells. (*B*) BRE cells.

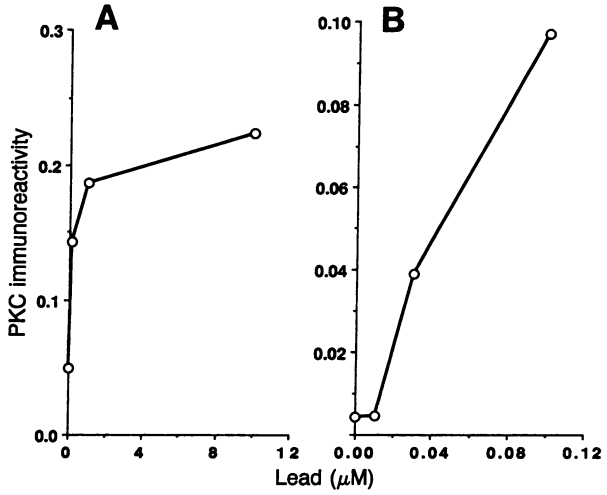

FIG. 5. Concentration dependence of the effect of lead on PKC in cultured C6 cells. C6 cells were exposed to lead acetate (0–10 μM) for 5 h after which cell membrane fractions were isolated and analyzed on an immunoblot for PKC, and the 80-kDa immunoreactive product was quantitated and expressed as OD. *A* and *B* represent data from separate experiments.

(28). The blood–brain barrier of immature brain is particularly sensitive to the toxic effects of lead (4–7).

We examined the effects of inorganic lead on an *in vitro* model of brain microvessel morphogenesis (14) to explore potential mechanisms by which lead damages microvascular function in immature brain. Lead acetate inhibited astroglial-induced endothelial differentiation into capillary-like tubes in a concentration-dependent fashion, with 50% maximal inhibition occurring at a total lead concentration of $\approx$0.5 μM (Fig. 2). Inhibition of morphogenesis was shown to be independent of conventional cell toxicity since lead concentrations below 10 μM had no effect on either endothelial or C6 cell proliferation (Table 1). The presence of *in vitro* "functional toxicity" in the absence of signs of conventional cell toxicity or death is consistent with an effect of lead on mechanisms of cell–cell signaling and with pathological demonstrations of altered capillary permeability but otherwise normal vascular histology in animals with lead toxicity (4).

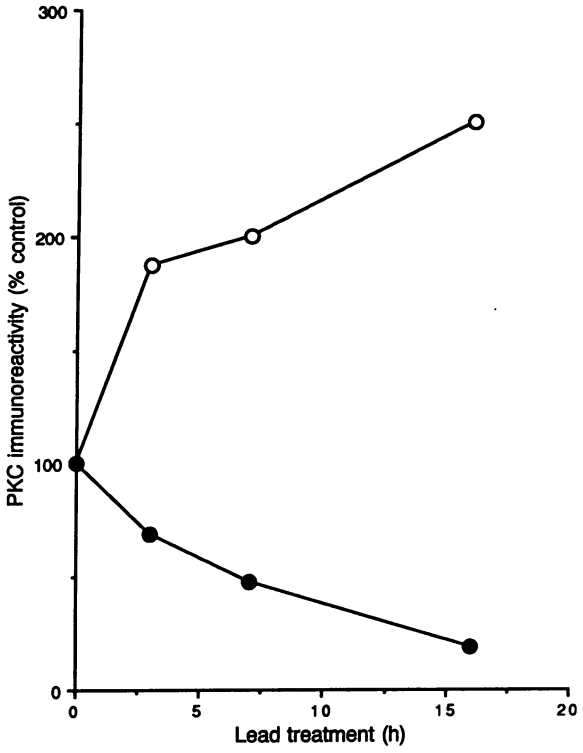

FIG. 4. Time dependence of the lead effect on PKC in cultured C6 cells. C6 cells were exposed to 5 μM lead acetate for up to 16 h and then cytosolic (●) and membranous (○) fractions were analyzed for PKC on an immunoblot. The optical density of the 80-kDa immunoreactive PKC was quantitated and plotted as percent of PKC in untreated controls.

It is well established that lead interferes with a number of diverse calcium-sensitive processes (10). Of these, PKC activation is particularly relevant to the regulation of microvascular cells (13, 16). PKC is normally translocated from cytosol to membrane when activated by diacylglycerol or calcium, the cytoplasmic concentrations of which are regulated by environmental signals via second messenger intermediaries (13). Lead might alter PKC function by interacting with calcium binding (10, 11) or cysteine-rich zinc-finger-like diacylglycerol-binding sites (29, 30). Indeed, lead induces PKC translocation to membrane in isolated neonatal rat brain microvessels *in vitro* (12) and substitutes for calcium in phospholipid-dependent PKC activation (11). In this report, we show that lead concentrations that inhibited astroglial-induced *in vitro* capillary-like structure formation increased membrane-associated PKC pools in both microvessel endothelial and astroglial cells (Figs. 3–5). Interestingly, both effects saturated at $\approx$1 μM (Figs. 2 and 5). This total lead concentration is undoubtably much higher than the free lead concentration due to the chelating characteristics of cell culture medium. Measurements of free lead concentrations in cell culture medium with a lead-sensitive electrode by Audesirk *et al.* (31) suggest that free lead levels in our experiments were in the nanomolar range. Thus, the current findings are consistent with our previous studies on lead-induced PKC translocation and enzyme activity in isolated brain microvessels (11, 12).

PMA inhibited astroglial-induced microvessel morphogenesis at concentrations consistent with a PKC-dependent mechanism (data not shown). That 4α-phorbol 12,13-didecanoate, a PMA analogue that does not activate protein kinase C, was without effect also supports a requirement for PKC activation. Likewise, we have recently found that the structurally distinct PKC activator 1-oleoyl-2-acetylglycerol

also inhibited capillary-like tube formation (unpublished observation). Inhibition by these well-characterized pharmacologic PKC agonists supports a causal relationship between lead-induced PKC translocation and the inhibition of capillary-like structure formation.

Aberrant PKC modulation has been implicated in the pathophysiology of tumor promotion (16) and secondary complications of diabetes mellitus (32). Our findings extend this mechanism of tissue dysfunction to the microvasculopathy associated with lead neurotoxicity. We show that lead alters PKC pools in a manner consistent with enzyme activation in endothelial cells and astroglia and further link this biochemical event to the disruption of functional astroglial–endothelial interactions. Since lead was found to alter PKC modulation in both endothelial and astroglial cells, its inhibitory effect on astroglial-induced endothelial differentiation may result from a primary influence on either astroglial inducer or endothelial effector cells (33). This is consistent with the ability of PKC agonists to activate endothelial cells (34, 35) and dedifferentiate astroglial cells (36, 37). Our findings suggest that lead damages brain microvessels by altering PKC modulation that results in endothelial dedifferentiation and blood–brain barrier dysfunction.

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