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Rapid Induction of mRNA for Prostaglandin H Synthase in Ovine Meningeal Fibroblasts

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We examined effects of interleukin 1 α (IL1 α) and phorbol 12, 13 dibutyrate (PDB), an activator of protein kinase C, on mRNA for Prostaglandin H synthase (PGHS) and prostanoid production in cultured ovine meningeal fibroblasts. Immuno- and morphologically-identified fibroblasts were derived from cerebral cortex and white matter from fetal lambs (approximately 120 days gestation) and grown to confluence on glass coverslips in 12 well plates. Levels of prostaglandin $F_{2\alpha}$ and the stable hydrolysis product of prostacyclin (i.e., 6-keto-$PGF_{1\alpha}$) were determined using enzyme immunoassay. Relative amounts of mRNA were determined by in situ hybridization using ovine cDNA for PGHS1. IL1 α (10 ng/ml) increased mRNA levels over baseline by 62±19% ($p<0.05$) at 60 min., 37±12% (NS) at 120 min., and 36±18% (NS) at 240 min (n=12). Levels of 6-keto-$PGF_{1\alpha}$ were 148±18 pg/ml during baseline, 246±41 pg/ml at 60 min., 248±40 pg/ml at 120 min., and 259±62 pg/ml at 240 min (all $p<0.05$) (n=12). $PGF_{2\alpha}$ was increased although it wasn't statistically significant. However, IL1 α decreased PGE_2 level significantly (all $p<0.05$). PDB (10^{-6}M) increased mRNA levels over baseline by 25±6% after 30 min., 40±6% after 60 min., and 20±8% after 90 min. (n=9) (all $p<0.05$). Levels of 6-keto-$PGF_{1\alpha}$ were 200±43 pg/ml during baseline, 202±43 pg/ml after 30 min. (NS), 268±58 pg/ml after 60 min. ($p<0.05$), and 296±60 pg/ml after 90 min. ($p<0.05$) (n=9). Levels of $PGF_{2\alpha}$ were 178±26 pg/ml during baseline, 300±30 pg/ml after 30 min., 299±35 pg/ml after 60 min., and 355±32 pg/ml after 90 min (all $p<0.05$) (n=6). Actinomycin-D (1 mg/ml) prevented increases in mRNA, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$ at 60 min. for both IL1 α and PDB. We conclude that cerebral fibroblasts are avid producers of prostanoids, and that enhanced production of PGHS is responsible for augmented $PGF_{2\alpha}$ and prostacyclin production in the presence of an activator of protein kinase C and for decreased PGE_2 and increased prostacyclin production in the presence of IL1 α.

Key Words: Prostaglandins, Cyclooxygenase, Cell culture, Prostacyclin, Interleukin, Protein kinase C, Phorbol ester

INTRODUCTION

Meningeal fibroblasts are in immediate contact with cerebrospinal fluid (CSF) and probably are important contributors of prostaglandins to this fluid. Prostaglandins in CSF and brain extracellular fluid in general are important because they participate in regulation of cerebral hemodynamics (Busija & Leffler, 1987; Busija et al, 1992; Shibata et al, 1991; Ellis et al, 1979) and probably also are involved in regulation of neuronal (Hedqvist, 1977; Hillier & Templeton, 1982; Kimura et al, 1985) and glial functions (Into et al, 1992). Further, prostaglandins from fibroblasts might sensitize sensory nerve endings of trigeminal origin which invest the meninges covering the brain surface and blood vessels (Liu-Chen et al, 1983). However, virtually nothing is known about the regulation of prostaglandin pro-

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duction by cerebral fibroblasts except that these cells are avid producers of prostacyclin (Murphy et al, 1985). Fibroblasts derived from other tissues such as skin have been shown to increase prostaglandin production in response to interleukin 1 α (IL1 α) (Raz et al, 1989; Raz et al, 1988). Interleukin 1 α production and accumulation in brain and CSF are associated with trauma and inflammation (Woodroofe et al, 1991; Morganti-Kossmann et al, 1992). In the case of IL1 α, augmented prostaglandin production in peripheral fibroblasts is due primarily to increased synthesis of prostaglandin H synthase (PGHS; commonly referred to as cyclooxygenase) (Raz et al, 1988). However, it is unclear whether regulation of prostaglandin production in cerebral fibroblasts is similar to fibroblasts in other tissues and organs. The purpose of this study was to examine regulation of prostanoids production in cerebral fibroblasts in response to application of IL1 α. Specifically, we examined the ability of IL1 α to alter prostanoids production and messenger RNA (mRNA) expression for PGHS in cultured cerebral fibroblasts. In addition, we also examined prostanoids and PGHS mRNA responses in fibroblasts exposed to phorbol 12, 13 dibutyrate, another agent known to increase brain production of prostaglandins (Kimura et al, 1985).

METHODS

Primary cultures

Primary cultures of fibroblasts were prepared from cerebral hemispheres taken from fetal lambs via modification of standard methods (McCarthy & de Villis, 1980). The animals were sacrificed and pieces of cortex and underlying white matter were aseptically removed. Using microforceps, relatively large pieces of dura and visible vessels were pulled away and discarded. The cortical pieces were cut into smaller pieces, trypsinized (0.25%), dispersed through the tip of pipets with decreasing bore size, and passed through a 130 μm nitex screen. The filtered suspension was centrifuged at 100 g for 10 min. The pellet was resuspended into 20~30 ml of Earle's basal media (supplemented with glucose, glutamine, 10% fetal calf serum, penicillin/streptomycin, and fungizone) (BME-C) and filtered through a 35 μm screen. The cell suspension then was added to 75 cm^3 flasks containing BME-C. Within 10~14 days, the cells had grown to confluence. At this time, the cells were mixed fibroblasts and astroglia. When confluent, the cells were subcultured onto glass cover slips or into wells of 12-well (22 mm) plates at 1×10^5 cells/well in BME-C without fungizone. By subculturing the cells relatively lightly onto uncovered glass coverslips, we were able to favor fibroblasts over astroglia. The cells were confluent and ready for study after 4~14 days. Other potential cells types, such as astroglia, were not substantially present at the time of the experiments. Virtually all of the cells studied were fibronectin positive and glial fibrillary acidic protein-negative.

IL1 α experiments

The cells in each well were exposed to either media alone or 10 ng/ml recombinant human IL1 α for 60, 120, and 240 minutes. In addition, cells were exposed to actinomycin D (1 mg/ml) alone or actinomycin D together with IL1 α for 60 min. At the end of the experiments the media was collected and frozen and the cells fixed for determination of mRNA levels.

PDB experiments

The cells in each well were exposed to media or 10^{-6}M PDB for 30, 60, and 90 min. Also, cells were exposed to actinomycin D (1 mg/ml) plus PDB for 60 min. At the end of the experiments the media was collected and frozen and the cells fixed for determination of mRNA levels.

Measurement of prostanoids

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 6-keto-$PGF_{1\alpha}$ levels in media were determined using enzyme immunoassay kits (Oxford Biomedical Res. Inc., Oxford, MI) which utilize specific antibodies. 6-keto-$PGF_{1\alpha}$ was determined as the stable hydrolysis product of prostacyclin.

Determination of mRNA levels

We used a method similar to that of Strulovici et al (Strulovici et al, 1989). After the experimental protocols were completed, the cell-bearing coverslips were fixed with methanol and hybridized in the original 12-well culture plate. After a brief rinse with

RNase-free PBS, cells were fixed with ice cold methanol for 20 minutes. After fixation, coverslips were rinsed again with PBS and equilibrated in 5X SSC (sodium chloride, sodium citrate, citric acid) until prehybridization. Cells were prehybridized at 49°C for approximately 1 hour in 0.5 ml/well standard hybridization buffer without cDNA probe (50% formamide, 5X Denhardt's sol, 0.1% SDS, 5X SSC and 100 ug/ml salmon sperm DNA). After prehybridization, prehybridization solution was replaced with hybridization buffer containing 1×10^6 cpm/ml of ^{32}P-labeled PGHS cDNA probe. The ovine cDNA probe was obtained from DeWitt (DeWitt, 1991) and purified before use by trough elution, and labeled with ^{32}P-dCTP by random prime labeling (Amersham, Burckinghamshire, England). Cells were hybridized in a water bath overnight at 49°C. After hybridization, cells and coverslips were washed twice at room temperature with 2X SSC containing 0.1% SDS. Coverslips were checked after the second 2X SSC wash for background radiation levels with a Geiger counter. If necessary to decrease background radiation, coverslips were washed once or occasionally twice more with 0.5X SSC/0.1% SDS for 10 min. each wash.

Coverslips were then briefly rinsed twice with distilled water, dehydrated by dipping in absolute ethanol, and dried on filter paper before aligning on plastic wrap for image analysis of β-radiation emission using a Betascope 603 Blot Analyzer (Betagen Corp. Waltham, MA). Hybridized coverslips were routinely analyzed for 2 hours and data expressed as count per minute (cpm).

Statistical analysis

Values are reported as means ± sem. Values (controls versus IL1 α; controls versus PDB; or controls versus IL1 α or PDB plus actinomycin D were compared using repeated measures analysis of variance followed by pair-wise comparisons using the Student-Newman-Kuels test. $P<0.05$ level was used in all statistical tests. Percent change values were calculated as the difference between control and treatment values divided by control values times 100. For the statistical analysis utilizing mRNA values, it was necessary to multiply counts by 10 for coverslips from two plates done separately in order to compensate for differences in nuclide half-life and differences in washing of coverslips following hybridization. This statistical adjustment did not alter the proportional changes in counts due to treatments and paired t-tests using the Bonferroni correction to adjust overall significance level to the $p<0.05$ level resulted in identical results using percent change data as with repeated measures analysis of variance on the raw count data.

RESULTS

Effects on hybridization

IL1 α increased hybridization at 60 min. (Fig. 1). However, although there was a tendency for hybridization to remain high at 120 and 240 min., these levels were not significantly different from baseline. Coapplication of actinomycin-D prevented increases in hybridization, maintaining 10% below control hybridization. PDB increased hybridization at 30, 60, and 90 min., with the peak response occurring at 60 min. (Fig. 1). Coapplication of actinomycin-D prevented increases in hybridization from occurring, maintaining 30% below control hybridization.

Effects on prostanoids levels

IL1 α increased levels of 6-keto-$PGF_{1\alpha}$ over baseline at 60, 120, and 240 min. (Fig. 2) Baseline values were 148 ± 18 pg/ml. $PGF_{2\alpha}$ was increased although it isn't statistically significant. However, IL1 α decresed PGE_2 level significantly (all $p<0.05$). Coapplication with actinomycin-D prevented an in-

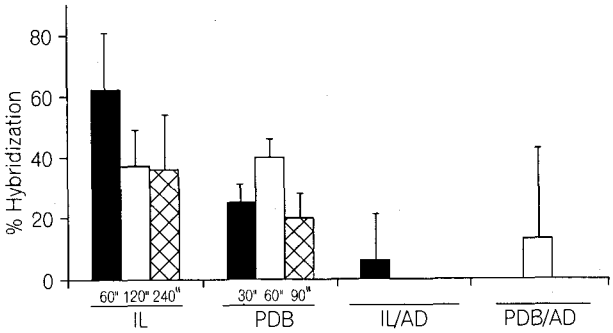

Fig. 1. Percent change in hybridization of mRNA using sheep cDNA for PGHS1 in meningeal fibroblast exposed to 10^{-6}M PDB or 10 ng/ml IL1 α. Baseline values were measured before application of IL1 α and PDB. Values are means ± standard error. *$p<0.05$, compared to baseline. n=4. AD, actinomycin D.

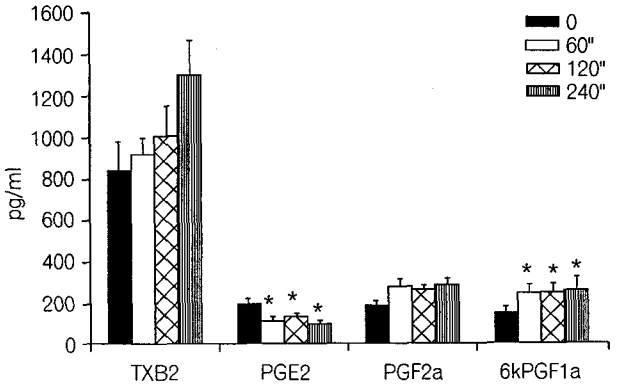

Fig. 2. Prostanoids levels in meningeal fibroblast exposed to 10 ng/ml IL1 α. Values are means $\pm$ standard error. Baseline values are control prostanoids levels of fibroblast which was not exposed to IL1 α. *$p<0.05$, compared to baseline. n=5.

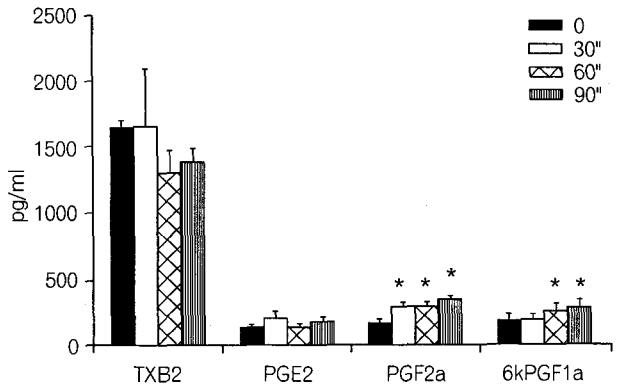

Fig. 3. Prostanoids levels in meningeal fibroblast exposed to 10^{-6}M PDB. Values are means $\pm$ standard error. Baseline values are control prostanoids levels of fibroblast which was not exposed to PDB. *$p<0.05$, compared to baseline. n=4.

crease in prostacyclin and $PGF_{2\alpha}$ production. For actinomycin-D treated fibroblasts, the 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ levels were 152 ± 19 pg/ml and 230 ± 15 pg/ml. For fibroblasts treated with both actinomycin-D and IL1 α, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ levels were 162 ± 33 pg/ml and 267 ± 62 pg/ml.

PDB did not increase prostacyclin production at 30 min., but increased levels at 60 and 90 min. (Fig. 3). Baseline values were 200 ± 43 pg/ml. Levels of $PGF_{2\alpha}$ were increased at 30, 60, 90 min. (all $p<0.05$). Coapplication with actinomycin-D prevented an increase in prostacyclin and $PGF_{2\alpha}$ production. For fibroblasts treated with both actinomycin-D and PDB, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ levels were 166 ± 37 pg/ml and 302 ± 12 pg/ml, which was not different from fibroblasts treated with actinomycin-D alone (see previous paragraph for values).

DISCUSSION

The major finding of the current study is that there is rapid induction of the PGHS system in meningeal fibroblasts in response to IL1 α and PDB. Thus, both IL1 α and PDB are able to increase levels of mRNA and prostanoids production within 1 hour. In particular for PDB, in which determinations were made at several early times, increases in mRNA level preceded increases in prostanoids production by at least 30 minutes, thereby allowing adequate time for translation of mRNA and production of PGHS. Further, inhibition of transcription by actinomycin-D prevented both increases in mRNA and prostacyclin production in response to both IL1 α and PDB. These results indicate that meningeal fibroblasts are avid producers of prostaglandins, that the cellular levels of PGHS can be quickly increased, and that some stimuli increase prostanoids production by increasing cellular PGHS levels.

Since prostaglandins are important regulatory substances in blood circulation, general prostaglandin synthesis by cells is tightly controlled at two levels--availability of free arachidonic acid and amount of PGHS. Arachidonic acid availability usually is tightly controlled by enzymes such as phospholipase A_2, and also in astrocytes by phospholipase C and diacylglycerol lipase (Xie et al, 1992; Smith et al, 1991). Activation of PLA_2 results in rapid release of arachidonic acid and allows almost immediate increases in cellular synthesis of prostaglandins. We have shown recently in piglet astroglia (Thore et al, 1994) and others have shown in rat astroglia that activation of protein kinase C (PKC) results in a fast increase in prostaglandin production via activation of PLA_2 (Hartung & Toyka, 1987; Jeremy et al, 1987). However, those experiments were of relatively short duration (40 minutes) and thus did not examine possible effects on gene transcription. Activation of PKC has been shown to increase PGHS levels in noncerebral fibroblasts and other cells (Raz et al, 1989; Korn et al, 1989; Wu et al, 1988). In endothelial cells, both interleukin and phorbol esters increase mRNA and protein levels for PGHS, and result in increased synthesis of prostaglandins (Wu et

al, 1988; Frasier-Scott et al, 1988). The time needed for this effect is surprisingly short, so that increased PGHS levels are detectable after 2 hours and peak at 4~6 hours (Frasier-Scott et al, 1988; Zyglewska et al, 1992; Wu et al, 1991; Snoek & Levine, 1983; Maier et al, 1990; Fagan & Goldberg, 1986; Wong et al, 1989; Frasier-Scott et al, 1988). Although two forms of PGHS are now known to exist, we focussed on PGHS1 because this form is constitutively expressed in the meningeal fibroblasts. In addition, the close correspondence between magnitude of the increase in mRNA for PGHS1 and the increase in prostanoids production indicates that we can account for virtually all of the effects of IL1 α and PDB with this form.

Although fibroblasts are usually considered to be dormant structural components of tissues, cytokines and platelet-derived growth factor released at the site of injury or inflammation can induce these cells to divide and produce secondary factors such as prostaglandins and growth factors which may promote inflammation and/or wound repair (DeWitt, 1991). Further, enhanced prostaglandin production by fibroblasts following injury or during inflammation can lead to sensitization of sensory fibers and increased pain perception. Cerebral blood vessels and meningeal tissues are heavily innervated by sensory fibers originating from the trigeminal ganglia, and irritation of these tissues or distention of the blood vessels is associated with pain (Liu-Chen et al, 1983). Migraine and subarachnoid hemorrhage are two examples of intense pain associated with activation of this pathway. Maintained or enhanced synthesis and release of prostaglandins by fibroblasts could lead to greater pain sensation.

Prostaglandins released into CSF by fibroblasts might also participate in blood flow regulation by brain. Cerebral resistance vessels are very sensitive to prostaglandins in CSF (Busija & Leffler, 1987; Busija et al, 1992; Shibata et al, 1991; Ellis et al, 1979) which surrounds pial arterioles on three sides. It has generally been assumed that the major contributors of prostaglandins to CSF have been from glia, neurons, and vascular cells. However, the potential contribution of fibroblasts to CSF prostaglandins has not been previously considered. Fibroblasts associated with the brain surface, with the dura mater, and blood vessels are in immediate contact with CSF. Thus, probably during normal conditions, and especially following injury and during inflammation, fibroblasts are important contributors of prostaglandins to CSF. A recent study has provided indirect, quantitative evidence concerning the contribution of prostaglandins by fibroblasts to CSF. In that study, administration of aspirin completely blocked prostacyclin production by cerebral microvessels, while only partially attenuating prostacyclin levels (measured as 6-keto-$PGF_{1\alpha}$) in CSF (Copeland et al, 1992). Since cerebral fibroblasts produce prostacyclin in many species (Murphy, 1985), it seems likely that fibroblasts are the remaining source of prostacyclin to CSF. In addition to influencing cerebral vessels, extracellular fluid prostaglandins might also influence glial and/or neuronal function (Hillier & Templeton, 1982; Kimura et al, 1985; Into, 1992; Mikami & Miyasaka, 1979; Martin et al, 1987). For example, prostaglandins are involved in various neuronal functions such as control of neurotransmitter release. Under pathological conditions, metabolism of arachidonic acid by PGHS is the primary source of oxygen free radicals produced in brain (Pourcyrous, 1990). Fluctuations of PGHS levels in cerebral tissues would result in various levels of free radicals being produced.

The ways in which PDB and IL1 α are able to enhance PGHS1 transcriptional activity are unclear. It is possible that PDB, by activation PKC, could phosphorylate a nuclear activator or reduce repressor activity. It seems that IL1 α also operates through increased PKC activity.

In summary, exposure of meningeal fibroblasts to IL1 α and PDB results in rapid increases in mRNA levels for PGHS1 which precede or accompany augmented production of prostacyclin. Thus, it appears that these stimuli result in increased prostacyclin production via increased synthesis of PGHS.

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