Phosphorylation-dependent stimulation of prostanooid synthesis by nigericin in cerebral endothelial cells

HELENA PARFENOVA, JOHN HAFFNER, AND CHARLES W. LEFFLER

Laboratory for Research in Neonatal Physiology, Department of Physiology, University of Tennessee, Memphis, Tennessee 38163

Parfenova, Helena, John Haffner, and Charles W. Leffler. Phosphorylation-dependent stimulation of prostanooid synthesis by nigericin in cerebral endothelial cells. Am. J. Physiol. 277 (Cell Physiol. 46): C728–C738, 1999.—Nigericin decreases intracellular pH (pHi) and stimulates prostanooid (PG) synthesis in endothelial cells from cerebral microvessels of newborn pigs. Nigericin-induced PG production was abolished by protein tyrosine kinase (PTK) inhibitors and amplified by phorbol 12-myristate 13-acetate (PMA) or protein tyrosine phosphatase (PTP) inhibitors. Nigericin-induced PG production in PMA-primed cells was potentiated by PTP inhibitors and abrogated by PTK inhibitors. Phospholipase A2 (PLA2) activity was stimulated by nigericin in a phosphorylation-dependent manner. Nigericin’s effects on PG production and PLA2 activity were reproduced by ionomycin, which activates cytosolic PLA2 (cPLA2). PLA2 was immunodetected in endothelial cell lysates. We found no evidence that nigericin’s effects are mediated via mitogen-activated protein (MAP) kinase (extracellularly regulated kinase 1 (ERK1) and ERK2) activation: although nigericin stimulated detergent-soluble MAP kinase, its effects were not amplified by PMA or PTP inhibitors. Phosphorylation-dependent stimulation of PG synthesis was also observed when pHi was decreased by sodium propionate or a high level of CO2. Altogether, our data indicate that nigericin and decreased pHi stimulate PG synthesis by a protein phosphorylation-dependent mechanism involving cross talk between pathways mediated by PTK and PTP and by protein kinase C; cPLA2 appears to be a key enzyme affected by nigericin and decreased pHi.

Hypercapnia is an important physiological stimulus that regulates cerebral blood flow. In newborn pigs, the vasodilation response of pial arterioles to hypercapnia is accompanied by an increased production of major vasodilation response of pial arterioles to hypercapnia that regulates cerebral blood flow. In newborn pigs, the production of prostacyclin and PGE2 (15). We have demonstrated that the effects of hypercapnia on endothelial PG production can be reproduced by compounds that rapidly decrease intracellular pH (pHi; nigericin and sodium propionate), whereas decreasing extracellular pH does not affect PG production (14). Therefore, the rapid decrease in pH, caused by hypercapnia is essential for stimulation of endothelial PG production (14). Nigericin, a K+ and H+ ionophore, rapidly decreases pH in cerebral microvascular endothelial cells [pH 7.2 and 6.9 in absence and presence, respectively, of nigericin (5 μM)] and stimulates PG production 1.5- to 2-fold (14). Therefore, nigericin appears to be a useful tool for investigating mechanisms by which endothelial PG production is stimulated on a rapid decrease in pH1.

Phospholipase A2 (PLA2) and cyclooxygenase (COX) are rate-limiting enzymes in PG synthesis. Rapid changes in PG production might be due to a rapid alteration in the activity of either enzyme. PLA2, which releases arachidonic acid from the sn-2 position of membrane phospholipids, is represented by several distinct types (10, 19). Ca2+-dependent types of PLA2 [the 14-kDa secreted enzyme (sPLA2) and the 85-kDa cytosolic enzyme (cPLA2)] are regulated by changes in the intracellular Ca2+ level ([Ca2+]i) in response to different stimuli (16, 19, 22). In addition, the activity of cPLA2 is rapidly regulated by protein phosphorylation (16, 19). A Ca2+-independent PLA2 (CalPLA2; also called iPLA2) that has been recently identified in many cell types also contributes to PG production in a variety of cell types (1, 3, 13, 18). We have recently demonstrated that COX-2, a major functional constitutive isofrom in cerebral microvascular endothelial cells from newborn pigs, is posttranslationally activated by tyrosine phosphorylation (24). Therefore, endothelial PG production could be rapidly regulated at the level of either PLA2 (Ca2+; phosphorylation) or COX-2 (tyrosine phosphorylation) or both.

In this paper, we investigated whether changes in [Ca2+]i and/or protein phosphorylation contribute to the stimulation of PG production in response to the nigericin-induced decrease in pHi in cultured endothelial cells from cerebral microvessels of newborn pigs.

MATERIALS AND METHODS

Protocols involving animals were approved by the Animal Care and Use Committee at the University of Tennessee-Memphis. All procedures were done by aseptic techniques.

Materials. Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD) and Sigma (St. Louis, MO). Matrigel (growth factor reduced) was purchased from Becton Dickenson (Bedford, MA). Nigericin, ionomycin (Ca2+ salt), A-23187, genistein, sodium orthovanadate, phorbol 12-myristate 13-acetate (PMA), and myelin basic protein were obtained from Sigma; bromoeno lactone (HELSS) was from Biomol. Fura 2-AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Phosphatase oxide (PAO) and tyrphostin 47 were from Biomol. Protein G-Sepharose was from Pharmacia Biotech (Piscataway, NJ). Arachidonic acid was from Cayman Chemical (Ann Arbor, MI). [5,6,8,9,11,
were plated onto 12-well cell culture plates (10^5 cells/well) and cultured in DMEM with 20% fetal bovine serum (FBS) (24). For experimental purposes, cells were depleted of serum by incubating the cells for 15 min with 5–10 µM HEPLSS (13). To evaluate the effects of protein phosphorylation on PG production, we preincubated the cells for 30 min with protein tyrosine kinase (PTK) inhibitors (300 µM genistein and tyrphostin) (24), protein tyrosine phosphatase (PTP) inhibitors (1 mM sodium orthovanadate and 10 µM PAO) (24), or the protein kinase C (PKC) activator PMA (1 µM); all inhibitors were also included in the incubation medium. To determine the effects of protein tyrosine phosphorylation on PMA-induced PG synthesis, cells were preincubated first with PMA for 15 min and then with inhibitors of protein kinase and protein phosphatase for an additional 20 min; all inhibitors were also included in the incubation medium. The active doses of the inhibitors as well as the treatment conditions for endothelial cells were determined based on previously published data (24). After a 15-min incubation at 37°C, the medium was aspirated and stored at −20°C for PG determination. For protein determination, cells were extracted with 0.1 N HCl; protein was detected with the Micro BCA assay (Pierce Chemical, Rockford, IL). The viability of the cells (indicated by the protein mass of the cells attached to the wells at the end of the experiment) was not altered by any of the treatments.

Measurement of [Ca^{2+}]. To evaluate COX activity in intact cells, we determined PG production (prostacyclin and PGE_2) from the exogenous substrate, arachidonic acid (24). Cells were washed twice with PBS and incubated with 10 µM arachidonic acid in 1 ml of aCSF for 10 min at 37°C without or with nigerin (10^-6–10^-4 M). The incubation medium was aspirated and stored at −20°C for PG determination.

PG assays. Concentrations of 6-ketoprostaglandin F_1α, the stable hydrolysis product of prostacyclin (PGH_2) and PGE_2 in the cell incubation medium were determined by RIA (Pierce Chemical, Rockford, IL) and previously published data (13). The PG concentration was normalized to cell protein.

PLA_2 activity. PLA_2 activity was detected as a release of arachidonic acid from intact cells (20). Cells grown on 12-well culture plates were placed on ice overnight with [3H]arachidonic acid (0.5 µCi/ml) in serum-free DMEM. To prevent further conversion of arachidonic acid, cells were treated with 10^{-4} M indomethacin for 30 min immediately before the experiment. When indicated, cells were pretreated with 1 mM sodium orthovanadate for 30 min or with 0.5–5 µM PMA for 10 min; these compounds were also included in the incubation medium. Control and pretreated cells were washed three times to remove unincorporated tracer and then incubated in serum-free DMEM without or with nigerin or ionomycin (concentrations as indicated in legends for Figs. 4 and 5) for 30 min. The medium was aspirated, cleared by centrifugation, and used for detection of released [3H]arachidonic acid. The cell monolayer was lysed with 1% Triton X-100 for 30 min and used to estimate total incorporation of [3H]arachidonic acid into cells. Incorporated and released [3H]arachidonic acid was detected in samples by liquid scintillation. In control unstimulated cells, 3–4% of the total incorporated arachidonic acid was released under basal conditions.

Detection of cPLA_2 and mitogen-activated protein kinase proteins. Quiescent cells were extracted by agitation on ice for 30 min with the extraction buffer [1% Nonidet P-40 (NP-40),
0.5% sodium deoxycholate, 0.1% SDS in PBS] containing protease and phosphatase inhibitors (200 µM leupeptin, 40 µg/ml aprotinin, 200 µM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 1 mM sodium-EDTA, 1 mM sodium fluoride, 100 µg/ml trypsin inhibitor). Detergent-soluble proteins were collected by aspiration, mixed with two volumes of 3X concentrated Laemmli sample buffer (0.12 M Tris·HCl, pH 6.8, 8% SDS, 4.5% dithiothreitol, 30% glycerol, 0.02% bromphenol blue), and boiled for 10 min. Detergent-insoluble proteins were scraped and solubilized with the Laemmli sample buffer (10 min, 100°C). The amount of protein in the samples was quantified by dot-blot staining with amido black as described previously (24). Detergent-soluble and detergent-insoluble proteins (20–50 µg protein/lane) were separated by SDS-7.5% PAGE and transferred to nitrocellulose membranes. Membranes were probed with 1) a monoclonal antibody to the amino-terminal domain of cPLA2 of human origin (Santa Cruz Biotechnology; dilution 1:2,000) and then a peroxidase-conjugated donkey anti-mouse IgG (dilution 1:10,000; J. ackson Immunoresearch, West Grove, PA) and 2) a polyclonal antibody to the carboxy-terminal domain of ERK1 (p44) or ERK2 (p42) of rat origin (Santa Cruz Biotechnology; dilution 1:5,000) and then a peroxidase-conjugated donkey anti-rabbit IgG (dilution 1:10,000; Jackson Immunoresearch). Detection of MAP kinase activity. Detergent-soluble and detergent-insoluble fractions of endothelial cells were collected with the extraction buffer with the protease and phosphatase inhibitors described above. Detergent-soluble proteins were collected by aspiration. Detergent-insoluble proteins were partially solubilized by sonication of extracted cell debris in the extraction buffer and clarified by centrifugation. The amount of protein in the samples was quantified by dot-blot staining with amido black as described previously (24). Mitogen-activated protein (MAP) kinase activity was detected as described previously (30) with modifications. Briefly, MAP kinase was immunoprecipitated from detergent-soluble and detergent-insoluble fractions (200–300 µg protein; 500 µl) by ERK1 and ERK2 antibodies (dilution 1:100; Santa Cruz Biotechnology) and 25 µl of protein G-Sepharose for 2 h on ice. Immunoprecipitated ERK1 and ERK2 were pelleted by centrifugation. To determine MAP kinase activity, Sepharose-conjugated proteins were incubated with 10 µg of myelin basic protein, 10 µM cold ATP, and 2.5 µCi of [γ-32P]ATP (10 Ci/mmol; Amersham) in 50 µl of the kinase buffer (in mM: 30 Tris·HCl, 20 MgCl₂, 2 MnCl₂; pH 8.0). The reaction was terminated by Laemmli sample buffer (10 min, 100°C). Samples were resolved by electrophoresis in 14% gels. MAP kinase activity, expressed as 32P incorporation into myelin basic protein, was quantitated by phosphorimagery analysis.

Statistical analysis. Data are presented as means ± SE of absolute values or percentages of control. Statistical significance was assessed with Student’s t-test. A level of P < 0.05 was considered significant.

RESULTS

Effect of nigericin on PG production and COX activity. Nigericin (10⁻⁶–10⁻⁵ M), added directly into the incubation medium, rapidly (in 10–15 min) increased PG production (both prostacyclin and PGE₂) by endothelial cells two- to threefold (Fig. 1A). However, nigericin (10⁻⁶–10⁻⁵ M) did not alter the COX activity measured as PG production from exogenous arachidonic acid (Fig. 1B). These data indicate that COX is not affected by nigericin.

**Effect of extracellular Ca²⁺ removal on PG production.** Stimulation of PG production by nigericin was observed in the presence of the Ca²⁺ (1.5 mM) normally included in the incubation medium (aCSF). We investigated whether removal of Ca²⁺ from the incubation medium affects basal or nigericin-stimulated PG production. In the absence of extracellular Ca²⁺ (nominally Ca²⁺-free media), the production of 6-keto-PGF₁α and PGE₂ was decreased by 40–50% (Fig. 2). However, nigericin (5 and 10 µM) stimulated the production of prostacyclin in a Ca²⁺-free medium (1.5 ± 0.3- and 2.2 ± 0.4-fold, respectively) to the same extent as in a Ca²⁺-containing medium (1.7 ± 0.2- and 2.1 ± 0.2-fold, respectively) (Fig. 2). Similarly, the production of PGE₂ was stimulated by nigericin in a Ca²⁺-free medium (2.4 ± 0.4- and 2.9 ± 0.3-fold in the presence of 5 and 10 µM nigericin, respectively) to the same extent as in a Ca²⁺-containing medium (2.4 ± 0.4- and 2.5 ± 0.3-fold, respectively) (Fig. 2). Therefore, stimulation of PG production by nigericin does not require extracellular Ca²⁺.

**Effect of nigericin on [Ca²⁺]i in endothelial cells.** When endothelial cells were loaded with fura 2-AM in a Ca²⁺-free solution, nigericin (10⁻⁵ M) caused a rapid decrease in the original [Ca²⁺]i. The tracking was main-
Role of CaIPLA₂ in the production of endothelial PG. Because the endothelial effects of nigericin are not associated with the increase in \([\text{Ca}^{2+}]\), we investigated whether CaIPLA₂ contributes to nigericin-stimulated PG production. We used HEISSL, an inhibitor of CaIPLA₂ that has been shown to selectively inhibit the enzyme at concentrations of 1–5 \(\mu\text{M}\) (2, 10, 18). Cells were preincubated with 5 \(\mu\text{M}\) HEISSL for 15 min. HEISSL did not alter basal or nigericin-stimulated PG production by endothelial cells (Table 1). Similarly, 10 \(\mu\text{M}\) HEISSL did not affect endothelial PG production (data not shown). As a negative control for inhibitor selectivity, we determined the effects of HEISSL on PG production stimulated by \(\text{Ca}^{2+}\) ionophore A-23187. A-23187 (10⁻⁷ \(\text{M}\)) added directly into the incubation medium increased PG synthesis 2.5- to 3-fold in both control and HEISSL-pretreated cells in 15 min (Table 1), thus confirming that \(\text{Ca}^{2+}\)-dependent PG production is not affected by HEISSL. These results may indicate that...

---

**Fig. 2. Effects of extracellular \(\text{Ca}^{2+}\) removal on prostacyclin (as 6-keto-\(\text{PGF}_{1\alpha}\)) and PGE₂ production by cerebral microvascular endothelial cells.** Confluent quiescent cells were incubated for 15 min with nigericin in artificial cerebrospinal fluid (aCSF) with (+\(\text{Ca}^{2+}\)) or without (−\(\text{Ca}^{2+}\)) 1.5 \(\mu\text{M}\) Ca²⁺. Each point represents mean ± SE of 3 experiments. *P < 0.05 compared with control in each group.

---

**Fig. 3. Original representative tracing illustrating effect of nigericin (10⁻⁵ \(\text{M}\)) on intracellular concentration of free \(\text{Ca}^{2+}\) (as ratio of fluorescence at 340 nm to that at 380 nm (340/380 ratio)) in cerebral microvascular endothelial cells (confluent quiescent cells were loaded with fura 2-AM (5 \(\mu\text{g/ml}\)) in presence of 0.01% Pluronic F-127) in \(\text{Ca}^{2+}\)-Krebs solution for 15 min at 37°C (A) and \(\text{Ca}^{2+}\) entry (as 340/380 ratio) into cerebral microvascular endothelial cells (B). Confluent quiescent cells were loaded with fura 2-AM (5 \(\mu\text{g/ml}\)) in presence of 0.01% Pluronic F-127 in \(\text{Ca}^{2+}\)-free Krebs solution for 15 min at 37°C.
Table 1. Effects of HELSS on prostanoid production by cerebral microvascular endothelial cells

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>6-Keto-PGF₁α Production, pg/mg protein</th>
<th>5 µM HELSS preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.8 ± 0.3 (n = 29)</td>
<td>1.8 ± 0.2 (n = 29)</td>
</tr>
<tr>
<td>Nigericin (5 µM)</td>
<td>3.1 ± 0.4* (n = 29)</td>
<td>3.3 ± 0.3* (n = 29)</td>
</tr>
<tr>
<td>A-23187 (10⁻⁷ M)</td>
<td>4.5 ± 0.3* (n = 24)</td>
<td>4.5 ± 0.4* (n = 24)</td>
</tr>
</tbody>
</table>

Values are means ± SE. HELSS, bromoenol lactone; 6-keto-PGF₁α, 6-ketoprostaglandin F₁α. *P < 0.05 compared to basal values.

Cal PLA₂ is not involved in basal and nigericin-induced PG production by cerebral microvascular endothelial cells.

Effect of increased protein phosphorylation on nigericin- and ionomycin-induced PG synthesis. We investigated the effects of protein tyrosine phosphorylation on the responses to nigericin. Nigericin-stimulated PG production was inhibited by genistein (300 µM), a PTK inhibitor (Fig. 4). Similarly, genistein inhibited PG production was inhibited by genistein (300 µM), a PTK inhibitor (Fig. 4). We further investigated the effects of PTK-PTP- and PKC-dependent phosphorylation on PG production in cerebral microvascular endothelial cells and in Swiss 3T3 fibroblasts in response to nigericin and ionomycin. As we demonstrated previously, PTP inhibitors rapidly stimulate COX activity in cerebral microvascular endothelial cells from newborn piglets, but not in Swiss 3T3 fibroblasts (26). As a result, the basal level of PG production by endothelial cells (but not 3T3 cells) pretreated for 30 min with sodium orthovanadate (1 mM) or PAO (10 µM) is elevated (Fig. 5, A and B). In endothelial cells pretreated with PTP inhibitors, nigericin (10⁻⁵ M) caused a 7- to 12-fold increase in PG production compared with a 1.5- to 2-fold increase in control untreated cells (Fig. 5A). The greatly amplified response of PG synthesis to nigericin was also observed in Swiss 3T3 fibroblasts treated with PTP inhibitors (Fig. 5B). The PKC activator PMA (0.5–5 µM) increased basal PG production in endothelial cells (3- to 4-fold) (Fig. 5A). The PKC activator PMA (0.5–5 µM) increased basal PG production in endothelial cells (3- to 4-fold) (Fig. 5A). The PKC activator PMA (0.5–5 µM) increased basal PG production in endothelial cells (3- to 4-fold) (Fig. 5A), but not in Swiss 3T3 fibroblasts (Fig. 5B). However, both cell types pretreated with PMA (1 µM) demonstrated an amplified response to nigericin (13- to 15-fold increase above the control basal level) (Fig. 5, A and B).

The “priming” effects of protein phosphorylation on the induction of PG synthesis by nigericin in both endothelial and 3T3 cells were unaffected on removal of Ca²⁺ from the incubation medium (data not shown) and, therefore, did not require the presence of extracellular Ca²⁺. However, we found a striking similarity between the effects of nigericin and ionomycin in cells primed with protein phosphorylation. In control untreated endothelial and Swiss 3T3 cells, ionomycin (10⁻⁶ M) increased PG production 2- to 4-fold, whereas in cells pretreated with either sodium orthovanadate, PAO, or PMA ionomycin caused a 5- to 20-fold increase in PG production (Fig. 5, A and B).

We investigated interactions between tyrosine phosphorylation and PKC-dependent phosphorylation in nigericin-induced PG production (Fig. 6). The effect of
nigericin in PMA-primed cells was further potentiated in cells pretreated with sodium orthovanadate or PAO and completely abolished in cells pretreated with PTK inhibitors (genistein and tyrphostin; 150 µM) (Fig. 6). H-7, a serine/threonine kinase inhibitor (150 µM), only partially (by 50%) inhibited the effect of nigericin in PMA-primed cells (Fig. 6). Overall, these data indicate that protein tyrosine phosphorylation and PKC-dependent serine/threonine phosphorylation by closely interrelated mechanisms greatly amplify the ability of endothelial and Swiss 3T3 cells to respond to nigericin by increasing PG synthesis.

Effect of nigericin on COX activity. We investigated the effects of nigericin on COX activity in cerebral microvascular endothelial cells primed with protein phosphorylation (Fig. 7). In cells pretreated with PTP inhibitors, COX activity was increased (Fig. 7), confirming our previous data (26). In cells treated with PMA (5 µM), the COX activity was unaltered (Fig. 7). Nigericin (10⁻⁵ M) did not alter COX activity in cells treated with either modulator of protein phosphorylation (Fig. 7). Increasing the nigericin concentration up to 10⁻⁴ M also had no effect on the COX activity (data not shown). Therefore, nigericin does not alter the COX activity in cerebral microvascular endothelial cells primed with protein phosphorylation.

Effect of nigericin and ionomycin on PLA₂ activity. The effects of nigericin on PLA₂ activity were determined in endothelial cells (control or primed with protein phosphorylation) (Fig. 8B). Short pretreatment (20–30 min) of endothelial cells with sodium orthovanadate (1 mM) and PAO (10 µM), but not with PMA (1 µM), increased basal PLA₂ activity (Fig. 8B). In control cells, nigericin (10⁻⁵ M) slightly increased PLA₂ activity (Fig. 8B). In cells pretreated with sodium orthovanadate or PMA (but not PAO), the effects of nigericin on PLA₂ activity were amplified (Fig. 8B). It is known that protein phosphorylation greatly potentiates the activation of cPLA₂ by ionomycin (19). In our experiments,
Ionomycin (10⁻⁶ M) stimulated PLA₂ activity. Responses to ionomycin were greatly potentiated in cells pretreated with sodium orthovanadate or PMA, but not with PAO (Fig. 8B). The close similarity between the phosphorylation-dependent effects of ionomycin and nigericin on PLA₂ activity may indicate that cPLA₂ is affected by nigericin.

cPLA₂ was immunodetected in endothelial cell extracts (Fig. 8A). Although most of the cPLA₂ could be solubilized in detergent-containing extraction buffer, a significant part of it (~30%) remained tightly bound to the detergent-insoluble cytoskeleton. The association of cPLA₂ with the endothelial cytoskeleton was increased in cells pretreated with PAO, but not with sodium orthovanadate or PMA (Fig. 8A). These data indicate the presence of two distinct pools of cPLA₂ in endothelial cells.

Effects of propionate and hypercapnia on PG synthesis in cells primed with protein phosphorylation. Propionate (80 mM) and hypercapnia (14% CO₂) rapidly decrease pH₁ in endothelial cells to the same extent as 5 μM nigericin (pH 6.9–7.0) (12). We compared the effects of nigericin (5 μM), propionate (80 μM), and hypercapnia (14% CO₂, extracellular pH = 7.1) on the production of prostacyclin and PGE₂ (Fig. 9) in endothelial cells with an increased level of protein phosphorylation. In control untreated cells, all agents stimulated PG production 1.5- to 2-fold. Similar to the effects of nigericin, the effects of propionate and hypercapnia on the production of prostacyclin and PGE₂ were greatly amplified in endothelial cells pretreated with sodium orthovanadate, PAO, and PMA (Fig. 9). These data indicate that the decrease in pH₁ is a major factor that potentiates the effects of nigericin in cells primed with protein phosphorylation.

MAP kinase in cerebral endothelial cells. MAP kinase, as ERK1 (44 kDa) and ERK2 (42 kDa) proteins (4), and MAP kinase activity were detected in cell lysates (Fig. 10, A and B). About 80% of the total MAP kinase protein (Fig. 10B) and MAP kinase activity (data not shown) is soluble in the detergent-containing buffer for immunoprecipitation (radioimmunoprecipitation assay buffer), and 20% of the MAP kinase remains insoluble. Endothelial MAP kinase is rapidly (in 30 min) activated in cells with upregulated protein phosphorylation (Fig. 10A). Our data demonstrate a distinct
functional diversity between detergent-soluble and detergent-insoluble MAP kinases with respect to protein phosphorylation. Sodium orthovanadate greatly increases MAP kinase activity in the detergent-soluble fraction (3- to 4-fold), with much less effect (1.5- to 2-fold stimulation) on detergent-insoluble MAP kinase. In contrast, PAO stimulates exclusively a detergent-insoluble MAP kinase (2- to 2.5-fold increase), with no effect on detergent-soluble MAP kinase. PAO also caused translocation of MAP kinase from the detergent-soluble to the detergent-insoluble endothelial fraction: soluble MAP kinase protein decreased to 40%, whereas detergent-insoluble MAP kinase protein increased to 60% (Fig. 10B). No protein redistribution in the cells treated with sodium orthovanadate and PMA was observed (Fig. 10B). PMA stimulated MAP kinase activity in both fractions evenly (Fig. 10A). Tyrphostin did not alter the PMA-induced stimulation of MAP kinase (data not shown), indicating no interaction between protein tyrosine- and PKC-mediated phosphorylation in MAP kinase activation.

We found that nigericin has a dual effect on MAP kinase activity in control cells: it stimulates detergent-soluble MAP kinase and inhibits detergent-insoluble MAP kinase (Fig. 11). In cells with upregulated protein phosphorylation (both tyrosine and serine/threonine), we observed a reversal of the nigericin-induced activation of detergent-soluble MAP kinase, along with a potentiation of the nigericin-induced inhibition of detergent-insoluble MAP kinase (Fig. 11). Therefore, it appears that nigericin facilitates the translocation of MAP kinase from the detergent-insoluble fraction to the detergent-soluble fraction in both control cells and cells with elevated protein tyrosine phosphorylation.

DISCUSSION

In newborn pigs, as well as in newborn human babies, endothelially derived PGs are major factors that control cerebral vascular tone (17). Therefore, mechanisms that rapidly regulate PG synthesis are of special physiological importance in the newborn cerebral circulation. Using primary cultures of endothelial cells from cerebral microvessels of newborn pigs, we investigated mechanisms of increased endothelial PG production in response to hypercapnia (high level of CO₂). Our major finding is that the decreased pHᵢ caused by nigericin, propionate, and hypercapnia stimulates PG production in endothelial cells by targeting PLA₂ via a protein phosphorylation-dependent mechanism. Protein tyrosine phosphorylation and PKC-mediated phosphorylation greatly potentiate the ability of intracellular acidification to stimulate PG production and PLA₂ activity, whereas inhibition of PTK abrogates the stimulation. The effects of PTK-, PTP-, and PKC-mediated phosphorylation are additive, and tyrosine phosphorylation may be essential for maximum effect. cPLA₂ appears to be a key enzyme affected by nigericin and decreased pHᵢ.

Primary cultures of endothelial cells from the cerebral cortices of newborn pigs respond to high CO₂ by an immediate increase in PG synthesis (14, 25). Although hypercapnia decreases both extracellular pH and pHᵢ, the decrease in pHᵢ (from 7.2 under normocapnic conditions to 7.0–6.9 on exposure to 14% CO₂) is a leading factor in increasing endothelial PG synthesis (14). Similar to high CO₂, pharmacological compounds that rapidly decrease pHᵢ (nigericin and sodium propionate) also rapidly stimulate endothelial PG synthesis (14). In contrast, agents that selectively alter extracellular pH do not affect PG production (14). Nigericin, a K⁺ ionophore, is a powerful tool for selectively decreasing pHᵢ in a variety of cultured cells. In cerebral microvascular endothelial cells from newborn pigs, nigericin (5 μM) decreases pHᵢ to 7.0–6.9 and rapidly stimulates PG production (14). We used nigericin as a tool to investigate the mechanisms by which the rapid decrease in pHᵢ stimulates PG production.

COX and PLA₂ are key rate-limiting enzymes in PG production. Because reactivity to hypercapnia includes a variety of PGs (15), we assume that the key enzyme(s) in PG synthesis rather than individual prostaglandin synthases is involved in the response to intracellular acidification.

In cerebral microvascular endothelial cells from newborn piglets, COX-2, a major constitutively functioning COX isozyme, is posttranslationally activated by tyrosine phosphorylation (24). Therefore, rapid responses to nigericin could be associated with COX activation. However, nigericin in a wide range of concentrations (10⁻⁶ to 10⁻⁴ M) did not alter COX activity in endothelial cells. When cells were pretreated with PTP inhibitors to upregulate protein tyrosine phosphorylation, basal COX activity was stimulated (consistent with our previ-
PLA₂ cleaves arachidonic acid by hydrolyzing membrane phospholipids at the sn-2 position, thus largely contributing to PG synthesis (6, 7, 10, 19, 20). Several types of PLA₂ have been described, including Cal PLA₂ and Ca²⁺-dependent PLA₂ (sPLA₂ and cPLA₂) (3, 7, 10, 18–20). First, we investigated whether the effects of nigericin on PG production are Ca²⁺ dependent. The effects of nigericin do not require extracellular Ca²⁺ removal of Ca²⁺ from the incubation medium did not prevent activation of PG production. However, similarity between the effects of nigericin and ionomycin on endothelial PG production indicated a possibility that nigericin may elevate [Ca²⁺]. Indeed, in some cell types (pancreatic acinar cells, gastric parietal cells, platelets, and neutrophils) intracellular acidification by nigericin triggered Ca²⁺ mobilization from intracellular stores independently of extracellular Ca²⁺ (12, 22, 30). In contrast, in endothelial cells (8), fibroblasts (9), and vascular smooth muscle cells (2, 28), intracellular alkalinization elevates cytosolic Ca²⁺ via release from intracellular stores. As we have previously reported, in endothelial cells from cerebral microvessels of newborn pigs, intracellular acidification by high CO₂ decreased an original [Ca²⁺] tracing; when the tracing was corrected for the pH₁ effect on Kd for fura 2, no changes in [Ca²⁺] were found (14). Our present data demonstrate that the application of nigericin to cerebral endothelial cells also decreased the 340/380 ratio independently of extracellular Ca²⁺; however, we have found no changes in absolute [Ca²⁺], (calculated using pH₁-corrected Kd values for fura 2) in nigericin-stimulated cells. Altogether, these data indicate that the effects of intracellular acidification on endothelial PG production are not mediated by Ca²⁺.

We addressed the possibility that nigericin stimulated PG production by targeting Cal PLA₂. It has been shown that Cal PLA₂ contributes to PG production in a cell-specific and a signal-specific manner (1, 3, 13, 18). HESL, a Cal PLA₂ selective inhibitor, at concentrations of 5 µM that maximally inhibit the enzyme in a variety of cell types (1, 3, 13), did not alter basal or nigericin-stimulated PG production in cerebral endothelial cells. These data do not confirm that Cal PLA₂ is involved in the mechanism by which nigericin stimulated endothelial PG production.

The effects of nigericin on PG production are modulated by protein phosphorylation. Genistein abrogated the stimulation of PG production, indicating that the basal level of protein tyrosine phosphorylation is essential for the effects of nigericin. In concert with these data, the effects of nigericin on PG production were greatly amplified in cells with increased protein phosphorylation. Priming cells with either a PTP inhibitor (sodium orthovanadate or PAO) or a PKC activator (PMA) greatly increased the responses to nigericin. Amplification of responses to nigericin in cells primed with protein phosphorylation is not limited to cerebral microvascular endothelial cells but is also observed in other cell types, such as Swiss 3T3 fibroblasts. The detection of PLA₂ activity unveiled a similar pattern: nigericin rapidly stimulated enzyme activity in cells with upregulated protein phosphorylation.

We investigated the relationship between PKC and protein tyrosine phosphorylation in priming endothelial cells for a nigericin response. Nigericin’s stimulation of PG production in PMA-primed cells was greatly potentiated by PTP inhibitors (vanadate and PAO) and completely abolished by PTK inhibitors (tyrphostin and genistein). These data indicate a close interaction between a PTK(s) or PTP(s) and a PKC-dependent pathway(s) involved in the mechanism of nigericin’s stimulation of PG production. PTK is possibly downstream of PKC in the mechanism of nigericin-stimulated PLA₂ activity.

cPLA₂ is the only well-characterized PLA₂ that is posttranslationally activated by protein phosphorylation via diverse cell-specific signaling pathways that may involve PKC, MAP kinase, and PTK (4, 6, 10, 11, 16, 19, 21, 23, 26, 32). A characteristic feature of the response is that phosphorylation of cPLA₂ is not sufficient for full activation of the enzyme; the enzyme also requires an additional stimulus that is necessary for a translocation of cPLA₂ to the cell membrane to have full access to substrate phospholipids (6, 19, 23, 27). It has been shown that ionomycin serves as such an additional signal, facilitating the Ca²⁺-dependent translocation of cPLA₂ to the membrane via an as yet unknown mechanism (19). To elucidate whether nigericin could also promote full activation of cPLA₂, we compared the effects of nigericin and ionomycin on PG production in cells primed with protein phosphorylation. Our data demonstrate that in cerebral microvascular endothelial cells stimulation of PG production by ionomycin was blocked by PTK inhibition and amplified by PTK- and PKC-mediated phosphorylation. We also found a striking similarity between the effects of ionomycin and nigericin in Swiss 3T3 fibroblasts primed with PTP inhibitors or PMA. Therefore, nigericin, as well as ionomycin, may serve as an additional signal that promotes full activation of cPLA₂. These data demonstrate that the effects of ionomycin on PG production and cPLA₂ activity in endothelial cells and Swiss 3T3 fibroblasts primed with protein phosphorylation could be fully reproduced by nigericin. Because nigericin does not increase [Ca²⁺], our finding may suggest that the increase in Ca²⁺ influx caused by ionomycin could be irrelevant to the ability of ionomycin to activate cPLA₂. Indeed, an increase in [Ca²⁺] was not required for cPLA₂ activation in PMA-primed macrophages and neutrophils (26). The contribution of a novel, Ca²⁺-independent cPLA₂ homologue (cPLA₂-γ) (31) to nigericin-induced phospholipase activation should be considered.
Our data indicate that the significant part of cPLA$_2$ (about 30% of the total amount) is associated with the detergent-insoluble cytoskeleton. The association of cPLA$_2$ with the endothelial cytoskeleton is regulated by selected protein tyrosine phosphorylation pathways. The amount of detergent-insoluble cPLA$_2$ is increased up to 60% when protein phosphorylation is upregulated with PAO, but not with sodium orthovanadate or PMA. The association of cPLA$_2$ with the cytoskeleton is subject to a dynamic regulation. It appears that nigericin caused rapid dissociation of cPLA$_2$ from the cytoskeleton and translocation of the enzyme to other cellular compartments.

What is the mechanism by which protein phosphorylation is involved in nigericin’s stimulation of phospholipase activity? In some, but not all, cell types, cPLA$_2$ is phosphorylated and activated by MAP kinase (4, 5, 11, 21, 23). MAP kinase activity itself is regulated by both serine/threonine and tyrosine phosphorylation and, therefore, could serve as an integrating effector in cell signaling (5). We investigated the possibility that MAP kinase is involved in the mechanism of the endothelial response to nigericin. We detected two structurally and functionally distinct pools of ERK1 and ERK2 kinase (detergent soluble and detergent insoluble) in cerebral microvascular endothelial cells. Under basal conditions, ~80% of the total ERK1 and ERK2 kinase protein and activity can be solubilized by detergents (1% NP-40, 0.5% sodium deoxycholate, and 1% SDS in PBS), whereas ~20% of MAP kinase remains detergent insoluble. According to our preliminary data, the detergent-insoluble fraction also contains a variety of cytoskeletal proteins (actin, tubulin, and paxillin). We found that both soluble and cytoskeletal fractions of ERK1 and ERK2 kinase are activated by protein phosphorylation. PMA stimulates ERK1 and ERK2 kinase activity in both fractions two- to threefold. However, PTP inhibitors exhibit strong selectivity to subcellular fractions of MAP kinase. Sodium orthovanadate stimulates soluble MAP kinase to a greater extent than detergent-insoluble cytoskeletal MAP kinase (3.5- and 1.5-fold, respectively), whereas PAO activates exclusively cytoskeletal ERK1 and ERK2 kinase. PAO also induces translocation of MAP kinase to the cytoskeleton, thus increasing the amount of the detergent-insoluble fraction up to 60% of the total MAP kinase protein. Although endothelial MAP kinase activity is regulated by both serine and threonine phosphorylation, we did not observe cross talk between tyrosine kinase- and PKC-mediated pathways. MAP kinase activation by PMA was not altered by inhibitors of PTK or PTP (data not shown). Therefore, it is unlikely that MAP kinase provides an interaction between tyrosine kinase- and PKC-mediated pathways in the stimulation of endothelial PG synthesis.

Nigericin increased ERK1 and ERK2 activity in the detergent-soluble fraction and decreased their activity in the detergent-insoluble cytoskeleton, thus indicating the translocation of MAP kinase from the cytoskeleton to other compartments. A dissociation of MAP kinase from the cytoskeleton was also observed in nigericin-stimulated cells pretreated with sodium orthovanadate and PAO, but not with PMA. Therefore, it appears that nigericin may cause cytoskeletal rearrangements that result in the dissociation of MAP kinase and, probably, other proteins from the cytoskeleton. However, no potentiation of the effects of nigericin on MAP kinase activity has been observed in cells primed with protein phosphorylation. Therefore, MAP kinases ERK1 and ERK2 are not involved in the mechanism of nigericin-induced activation of PLA$_2$ and PG synthesis.

Other agents that rapidly decrease pH$_i$ in cerebral microvascular endothelial cells in a manner similar to that of nigericin, such as sodium propionate and increased CO$_2$, also stimulate PG production in a protein phosphorylation-dependent manner. The effects of sodium propionate and 14% CO$_2$ on PG production in cerebral endothelial cells were greatly amplified in cells primed with PTP inhibitors or PMA. These data indicate that decreased pH$_i$ is a leading factor in the ability of nigericin to stimulate PG production via a protein phosphorylation-dependent mechanism.

Taken together, our data demonstrate that the intracellular acidification pH caused by nigericin and high CO$_2$ levels stimulates PG production in cerebral microvascular endothelial cells via a protein phosphorylation-dependent mechanism that may involve cross talk between PTK(s) or PTP(s) and PKC. cPLA$_2$, a potential target enzyme in the mechanism of stimulation of PG production by decreased pH$_i$, is immunodetected in cell lysates. The significant part of immunodetectable cPLA$_2$ is tightly associated with the endothelial cytoskeleton. Intracellular acidification may cause a rearrangement of the endothelial cytoskeleton that results in the dissociation of cPLA$_2$ from the cytoskeleton and translocation of the enzyme to other compartments. Phosphorylation-mediated activation of cPLA$_2$ by nigericin does not appear to involve MAP kinase (ERK1 and ERK2).

We thank A. Fedinec and M. Jackson for their technical assistance, D. Morse for the illustrations, and J. Emerson-Cobb for editorial assistance.

This research was supported, in part, by National Heart, Lung, and Blood Institute Grants HL-42851 and HL-34059. P. Parfenova was supported by a grant-in-aid from the Southeast Affiliate of the American Heart Association.

Address for reprint requests and other correspondence: H. Parfenova, Dept. of Physiology, Univ. of Tennessee-Memphis, 894 Union Ave., Memphis, TN 38163.

Received 24 February 1999; accepted in final form 10 June 1999.

REFERENCES


