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

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Nigericin decreases intracellular pH (pHi) and stimulates prostanoid (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). cPLA2 was immuno-detected 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.

PROSTACYCLIN AND PROSTAGLANDIN E2 (PGE2); CEREBRAL 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 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).

In this paper, we investigated whether changes in [Ca2+] 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 Dickinson (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; bromoelanol lactone (HELSS) was from Biomol. Fura 2-AM and Fluronic F-127 were purchased from Molecular Probes (Eugene, OR). Phenylarsine 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).
were plated onto 12-well cell culture plates (10^5 cells/well) from isolated cerebral microvessels (60–300 µm) by collagenase-dispase treatment (1 mg/ml for 2 h at 37°C). Digested cells were separated by Percoll density gradient and plated onto 12-well Costar plates coated with Matrigel or onto Matrigel-coated glass coverslips (9 × 35 mm) within a Leighton tissue culture tube (16 × 93 mm). Endothelial cells were cultured in DMEM with 20% fetal bovine serum (FBS), 30 µg/ml endothelial cell growth supplement (ECGS), 1 U/ml heparin, and an antibiotic-antimycotic mixture in a 5% CO2-air incubator at 37°C for 5–6 days to reach confluence. Primary cultures consisted of >95% endothelial cells, identified by cellular morphology and fluorescence staining with antibodies to von Willebrand factor.

Stock cultures of Swiss 3T3 fibroblasts from the American Type Culture Collection were maintained in DMEM supplemented with 10% FBS (24). For experimental purposes, cells were plated onto 12-well cell culture plates (10^6 cells/well) and grown in DMEM with 20% FBS in a 5% CO2-air incubator for 5–6 days to reach confluence. All experiments were performed on confluent quiescent cells. To achieve quiescence, cells were exposed to a serum-depleted medium (0% FBS; 0% ECGS) for 15–20 h before the experiment. Measurement of [Ca^{2+}], for the measurement of [Ca^{2+}], endothelial cells grown on Matrigel-coated coverslips were loaded with the fluorescent Ca^{2+}-sensitive dye fura 2-AM (5 µg/ml) in the presence of Pluronic F-127 (0.01%) in Ca^{2+}-free Krebs buffer (in mM: 5.0 KCl, 0.6 MgSO4, 1.8 CaCl2, 120 NaCl, 6 glucose, 10 HEPES; pH 7.4) for 15 min at 37°C (14). Coverslips were washed twice with the buffer, placed into an LS-50 spectrofluorometer (Perkin-Elmer; 2-ml cuvette), and superfused with Ca^{2+}-free Krebs buffer at 37°C (perfusion rate, 0.8 ml/min). The fluorescence intensity of fura 2 was measured with the excitation wavelength pair of 340/380 nm and an emission wavelength of 510 nm (14). To determine the effects of nigericin on [Ca^{2+}], cells were superfused with nigericin (10^{-4} M) in Ca^{2+}-free Krebs buffer for 10 min. For calibration purposes, ionomycin (5 × 10^{-6} M) was applied at the end of each experiment; this was followed by the Ca^{2+}-free Krebs containing 4 mM EGTA.

To determine the effects of nigericin on Ca^{2+} entry, cells were loaded with fura 2-AM (5 µg/ml) in the presence of Pluronic F-127 (0.01%) in Ca^{2+}-free Krebs buffer (in mM: 5.0 KCl, 0.6 MgSO4, 1.8 CaCl2, 120 NaCl, 6 glucose, 10 HEPES; pH 7.4) for 15 min at 37°C. Coverslips with cells were placed in the spectrofluorometer cuvette (2 ml) and superfused with Ca^{2+}-free Krebs buffer for 5 min at 37°C (perfusion rate, 0.8 ml/min). After a constant fluorescence ratio was observed, cells were superfused with Ca^{2+}-free Krebs buffer for 3 min and then by Ca^{2+}-free Krebs buffer for 3 min. This cycle was repeated without and with nigericin (10^{-5} M). For calibration purposes, ionomycin (5 × 10^{-6} M) was applied at the end of each experiment; this was followed by Ca^{2+}-free Krebs containing 4 mM EGTA.

PG production. Cells were rinsed with PBS and incubated for 10–30 min in 1 ml of artificial cerebrospinal fluid (aCSF), an incubation medium similar to cortical CSF (in mM: 3.0 KCl, 1.5 MgCl2, 2.5 CaCl2, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO3 equilibrated with 5% CO2 and 21% O2 to pH 7.4–7.5; PCO2, 32–36 mmHg; PO2, 100–120 mmHg). To achieve the hypercapnic condition, aCSF was preequilibrated with 14% CO2 and 21% O2 (pH, PCO2, and PO2 were in the range of 7.00–7.10, 70–80 mmHg, and 100–120 mmHg, respectively) (25). Nigericin or ionomycin was added directly to the incubation medium. To determine whether CaPLA2 contributes to the basal or nigericin-induced PG synthesis, cells were pretreated for 15 min with 5–10 µM HELSS (13). To evaluate the effects of protein phosphorylation on PG production, we preincubated 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 mM 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 our 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.

In preliminary experiments, we determined that the vehicles for the inhibitors (DMSO and ethanol) at the concentrations used did not affect PG production and did not interfere with PG determinations.

COX activity. To evaluate COX activity in intact cells, we determined PG production (prostacyclin and PGE2) 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 nigericin (10^{-6}–10^{-4} M). The incubation medium was aspirated and stored at −20°C for PG determination.

PG assays. Concentrations of 6-ketoprostaglandin F1α, (6-keto-PGF1α, the stable hydrolysis product of prostacyclin) and PGE2 in the cell incubation medium were determined by RIA calibrated with previously published data (24). The PG concentration was normalized to cell protein.

PLA2 activity. PLA2 activity was detected as a release of arachidonic acid from intact cells (20). Cells grown on 12-well culture plates were loaded 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 nigericin 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 cPLA2 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 Laemml 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; Jackson 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 phosphorimager 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 prostacycin 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+.
nigericin does not increase \([Ca^{2+}]_i\) (Fig. 3A). After removal of nigericin from the incubation medium, the cells responded to ionomycin \((10^{-5} \text{ M})\) by increasing \([Ca^{2+}]_i\) (Fig. 3A). We have demonstrated earlier that intracellular acidification significantly increases the dissociation constant \(K_d\) of \(Ca^{2+}\) binding to fura 2 (under our experimental conditions, \(K_d = 713 - 89 \times \text{pH}\)) \((12)\), which may account for the apparent decrease in the original \([Ca^{2+}]_i\) tracing on nigericin application. Using \(pH_i\) values for control and nigericintreated cerebral microvascular endothelial cells \((12)\) as well as \(pH\)-corrected \(K_d\) values for fura 2 \((12)\), we determined \([Ca^{2+}]_i\) values based on the ratio of fluorescence at 340 nm to that at 380 nm \((340/380 \text{ ratio})\) in three separate experiments as 50 ± 3 and 48 ± 2 nM in the absence and presence of nigericin, respectively. Therefore, our data demonstrate that nigericin does not increase \([Ca^{2+}]_i\) in endothelial cells from cerebral microvessels of newborn pigs. To investigate whether nigericin affects \(Ca^{2+}\) entry, endothelial cells were loaded with fura 2 in a \(Ca^{2+}\)-free Krebs solution and then transferred to a \(Ca^{2+}\)-Krebs solution with or without nigericin \((10^{-5} \text{ M})\). Control cells responded to extracellular \(Ca^{2+}\) by rapidly increasing \([Ca^{2+}]_i\), whereas the subsequent removal of \(Ca^{2+}\) from the incubation medium resulted in a rapid decrease in \([Ca^{2+}]_i\); this cycle could be repeatedly reproduced without desensitization (Fig. 3B). When nigericin \((10^{-5} \text{ M})\) was added to the incubation medium, \(Ca^{2+}\) entry was apparently inhibited; the removal of nigericin did not restore the ability of endothelial cells to respond to extracellular \(Ca^{2+}\) within at least 15–20 min (Fig. 3B). \([Ca^{2+}]_i\) values (calculated as described above) were determined as 52 ± 3 and 54 ± 2 nM in the absence and presence of nigericin, respectively. The ability of endothelial cells to respond to ionomycin \((5 \times 10^{-6} \text{ M})\) by increasing \([Ca^{2+}]_i\) remained intact (Fig. 3B). These data indicate that nigericin does not increase \(Ca^{2+}\) entry or the \([Ca^{2+}]_i\) level in cerebral microvascular endothelial cells.

**Role of CaIPLA2 in the production of endothelial PG.** Because the endothelial effects of nigericin are not associated with the increase in \([Ca^{2+}]_i\), we investigated whether CaIPLA2 contributes to nigericin-stimulated PG production. We used HELSS, 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 \(\mu\text{M HELSS}\) for 15 min. HELSS did not alter basal or nigericin-stimulated PG production by endothelial cells (Table 1). Similarly, 10 \(\mu\text{M HELSS}\) did not affect endothelial PG production (data not shown). As a negative control for inhibitor selectivity, we determined the effects of HELSS on PG production stimulated by \(Ca^{2+}\) ionophore A-23187. A-23187 \((10^{-7} \text{ M})\) added directly into the incubation medium increased PG synthesis 2.5–3-fold in both control and HELSS-pretreated cells in 15 min (Table 1), thus confirming that \(Ca^{2+}\)-dependent PG production is not affected by HELSS. These results may indicate that...
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) and 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 PG biosynthesis by cerebral microvascular endothelial cells.

Table 1. Effects of HELSS on prostanoid production by cerebral microvascular endothelial cells

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<tr>
<th>Experimental Conditions</th>
<th>6-Keto-PGF₁α Production, pg/mg protein</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Basal</td>
<td>1.8 ± 0.3</td>
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<td>(n = 29)</td>
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<td>Nigericin (5 µM) stimulation</td>
<td>3.1 ± 0.4*</td>
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<td>(n = 29)</td>
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<tr>
<td>A-23187 (10⁻⁷ M) stimulation</td>
<td>4.5 ± 0.2*</td>
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Values are means ± SE. HELSS, bromoenol lactone; 6-keto-PGF₁α, 6-ketoprostaglandin F₁α. *P < 0.05 compared to basal values.
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 PLA_{2} activity.** The effects of nigericin on PLA_{2} 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 PLA_{2} activity (Fig. 8B). In control cells, nigericin (10^{-5} M) slightly increased PLA_{2} activity (Fig. 8B). In cells pretreated with sodium orthovanadate or PMA (but not PAO), the effects of nigericin on PLA_{2} activity were amplified (Fig. 8B). It is known that protein phosphorylation greatly potentiates the activation of cPLA_{2} by ionomycin (19). In our experiments,
ionomycin (10^{-6} M) stimulated PLA_2 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% CO_2) rapidly decrease pH_i 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% CO_2) extracellular pH = 7.1) on the production of prostacyclin and PGE_2 (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 PGE_2 were greatly amplified in endothelial cells pretreated with sodium orthovanadate, PAO, and PMA (Fig. 9). These data indicate that the decrease in pH_i 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⁺ and H⁺ 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 isofrom, 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 (10⁻⁶–10⁻⁴ 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-
ous findings), but nigericin did not cause any additional changes. Swiss 3T3 fibroblasts do not functionally express COX-2 and do not respond to tyrosine phosphorylation by COX activation (24). However, nigericin stimulated PG production in Swiss 3T3 cells even more effectively than in cerebral endothelial cells. Altogether, our data demonstrate that COX is not affected by nigericin.

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 Ca₁-independent PLA₂ (sPLA₂) and Ca²⁺-dependent PLA₂ (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 alkalization 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 [Ca²⁺]i, whereas we have found no changes in absolute [Ca²⁺], (calculated using pH-i-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 CaPLA₂. It has been shown that CaPLA₂ contributes to PG production in a cell-specific and a signal-specific manner (1, 3, 13, 18). HELSS, a CaPLA₂ 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 CaPLA₂ 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. PKC 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 (cPLA₂γ) (31) to nigericin-induced phospholipase activation should be considered.
Our data indicate that the significant part of cPLA2 (about 30% of the total amount) is associated with the detergent-insoluble cytoskeleton. The association of cPLA2 with the endothelial cytoskeleton is regulated by selected protein tyrosine phosphorylation pathways. The amount of detergent-insoluble cPLA2 is increased up to 60% when protein phosphorylation is upregulated with PAO, but not with sodium orthovanadate or PMA. The association of cPLA2 with the cytoskeleton is subject to a dynamic regulation. It appears that nigericin caused rapid dissociation of cPLA2 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, cPLA2 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 cellular 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 PLA2 and PG synthesis.

Other agents that rapidly decrease pH in cerebral microvascular endothelial cells in a manner similar to that of nigericin, such as sodium propionate and increased CO2, also stimulate PG production in a protein phosphorylation-dependent manner. The effects of sodium propionate and 14% CO2 on PG production in cerebral endothelial cells were greatly amplified in cells primed with PTP inhibitors or PMA. These data indicate that decreased pH 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 CO2 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. cPLA2, a potential target enzyme in the mechanism of stimulation of PG production by decreased pH, is immunodetected in cell lysates. The significant part of immunodetectable cPLA2 is tightly associated with the endothelial cytoskeleton. Intracellular acidification may cause a rearrangement of the endothelial cytoskeleton that results in the dissociation of cPLA2 from the cytoskeleton and translocation of the enzyme to other compartments. Phosphorylation-mediated activation of cPLA2 by nigericin does not appear to involve MAP kinase (ERK1 and ERK2).

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