Intracellular-specific colocalization of prostaglandin E₂ synthases and cyclooxygenases in the brain

Alejandro Vazquez-Tello,¹ Li Fan,¹ Xin Hou,¹ Jean-Sébastien Joyal,¹ Joseph A. Mancini,² Christiane Quiniou,¹ Ronald I. Clyman,¹ Fernand Gobeil, Jr.,¹ Daya R. Varma,³ and Sylvain Chemtob¹

1 Departments of Pediatrics and Ophthalmology and Pharmacology, Research Center of Hôpital Sainte-Justine, Montréal H3T 1C5; 2 Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Kirkland H9R 4P8; 3 Department of Pharmacology and Therapeutics, McGill University, Montréal H3G 1Y6; and 4 Department of Pharmacology, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

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To date, several glutathione (GSH)-dependent PGESs have been purified from different tissues, and their genes have been cloned and characterized to some extent. MGST1-L1, a member of the superfamily of membrane-associated proteins in eicosanoid and GSH metabolism (MAPEG) was identified as microsomal PGES (mPGES-1) (12, 24). mPGES-1/MGST1-L1 mRNA is expressed widely among tissues and can be stimulated both in vivo and in vitro by proinflammatory cytokines, growth factors, and bacterial lipopolysaccharide (12, 20, 24). More recently, another membrane-associated PGES, mPGES-2, was purified from the bovine heart, and its cDNA was cloned (31, 37). In contrast to mPGES-1, mPGES-2 enzyme activity is preferentially stimulated by DTT and responds, albeit to a lesser extent, to GSH and 2-mercaptoethanol: mPGES-2 mRNA is expressed in many tissues including the brain (24). Moreover, PGES activities have also been detected in cytosolic fractions. For instance, several glutathione-S-transferase (GST) isoforms of the μ-class such as GSTM2–2 and GSTM3–3 convert PGH₂ into PGD₂, PGE₂, and PGF₂α (3, 25, 34). In addition, a GSH-dependent cytosolic PGES (cPGES) enzyme was purified from the rat brain and found to be identical to the ubiquitous p23, a previously characterized chaperone-like protein that is involved in the stabilization of steroid hormone receptor multiprotein (14, 32).

Because PGE₂ exerts actions on the plasma membrane and separately on intracellular compartments via its receptors (4, 10, 21), and because its precursor PGH₂ is extremely short lived, a coordinated interaction between COX and PGES is essential to produce PGE₂ for actions on its vicinal receptors. Currently, a hypothetical model proposed that in microsomes mPGES-1 is preferentially coupled with COX-2 for PGE₂ biosynthesis, whereas COX-1 activity is coordinately associated with the specific cytosolic cPGES (24, 32). This rather simplified model was based on in vitro conditions of cultured HEK-293 cells overexpressing a combination of COX and PGES enzymes. Nevertheless, mice with disrupted mPGES-1 gene are fertile and develop normally (33), which questions an exclusive COX-2/mPGES-1 and COX-1/cPGES interaction.

In the mammalian brain, prostanoid levels are normally low (15). At birth, the concentration of PGE₂ in both cerebrospinal fluid and brain plasma of the perinate reach high levels, decreasing rapidly thereafter (15, 27). In the cerebral cortex...
and hippocampus, COX-1 along with COX-2 is expressed constitutively in discrete populations of neurons as well as in the brain vasculature, particularly during development (16, 17, 26, 27). For instance, in the rat cortex, there is a marked increase in COX-2 expression at 3–4 wk of age (juvenile), which coincides with the late phase of dendritic formation and the maturity of synaptic patterns (16, 17); postsynaptic membrane excitability is significantly reduced by the COX-2-specific inhibitor NS-398, and this effect is reversed by exogenous PGE2 but not by PGF2α or PGD2 (6) despite the fact that PGD2 is the most abundant prostaglandin in the rat brain (1). Correspondingly, an important expression profile of PGE2 receptors homologously regulated by PGE2 in synaptosomes of the perinatal pig has been reported (19). Another major function attributed to PGE2 during perinatal development of the male rat brain applies to its role in the organization of neuronal structures necessary to fashion male sexual behavior (2). Altogether, the expression profile of PGE2 biosynthetic enzymes appears to vary with age. Accordingly, to sustain PGE2 biosynthesis during brain development, the expression profile of PGES enzymes during ontogeny would be anticipated to correspond to that of COX-2 for appropriate coordination; however, this compartmentalization and functional coordination of COX and PGES enzymes in the brain tissue, particularly during development, have yet to be shown.

We therefore hypothesized that in brain tissue, PGE2 biosynthesis is regulated by a coordination of COX-1 or COX-2 with either mPGES-1 or cPGES. Because it is speculated that colocalization of COX with PGES enzymes could vary as a function of age, we analyzed these parameters in different age groups of species that exhibit distinct prostaglandin abundance.

To obtain a more complete profiling of the intracellular cocompartmentalization of COX-1/2 with cPGES and mPGES-1, localization of these proteins was studied in the brain of two distinct animal species, each at different age groups. Fetal (~60% gestation) and adult (6–8 mo old) rats, were obtained from a breeder (St-Hélène, Québec, Canada). Newborn (<15 h after birth) and juvenile pigs (12 wk) were acquired from Fermes Ménard (L’Ange-Gardien, Québec, Canada). Adult male and pregnant Wistar Rats (3–4 mo old) were obtained from Charles River Canada; juvenile (4 wk old) rats were raised in our animal care facility. Animals were anesthetized with halothane (~2.5–3.5%) and killed with pentobarbital sodium (120 mg/kg) in accordance with regulations of the Canadian Council on Animal Care Committee and with approval of the Sainte-Justine Hospital Animal Care Committee. The brains were removed and placed immediately in ice-cold PBS buffer (pH 7.4). Brain tissues were either used immediately or frozen in liquid N2 and stored at ~−80°C.

Preparation of microsomes from pig brain microvasculature. Brain microvessels (~30 μm) were purified from adult rats and from pigs of different age groups (newborn, juvenile, and adult) as described previously (27). These microvessels are largely composed of endothelium.

Cell culture. To further ascertain that COX-c/mPGES localization is more complex than previously described (24, 32) and possibly different than that seen in the brain, cultured cell lines were also studied. Accordingly, expression of these proteins in microsomes and nuclei was determined in cultured primary cerebrovascular endothelial cells (31) as well as in various cell types, specifically, HEK-293, A549, HTC4, COS-1, and COS-7 cells (American Type Culture Collection; Manassas, VA). Cultures were performed as previously described in detail (4, 21).

Presentation of microsomal membrane fractions. Microsomes were purified from porcine and rat brain and derived cerebral vasculature as described elsewhere (28). All solutions, samples, and glassware were kept on ice, and all centrifugations were done at 4°C. Briefly, the tissues (0.1–0.5 g) were disrupted with a polytron (Omni 2000) for 1 min at medium speed in 10 vol of homogenization buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM PMSF, and a cocktail of protease inhibitors from Roche and 10 mM each of EDTA and EGTA when indicated]. Debris, nuclei, and organelles were removed by two successive centrifugations (2,000 and 5,000 g, 10 min), and the microsomes were sedimented (175,000 g, 1 h). These fractions were highly enriched in the plasma membrane marker 5′-nucleotidase (210 ± 25 U/mg protein) (4) and in the endoplasmic reticulum marker glucose-6-phosphatase (22.5 ± 4.3 mmol PO4 released/mg protein) (4). For analysis of cPGES association with membranes, microsomes were incubated either in 0.1 M sodium carbonate (pH 11.0), 0.1 M Tris-base (pH 11.1; h on ice), or buffer containing 8 M urea (1 h at 4°C) or 50 mM DTT (2 h at 4°C or 22°C) and then washed twice by ultracentrifugation (175,000 g, 1 h) followed by resuspension. The protein concentration was quantified with the Bio-Rad Protein Assay reagent. For cross-linking experiments, washed microsomes were adjusted to 1 or 5 mg/ml protein and incubated with 0.5–5.0 mM of the cross-linker reagent EGD or at room temperature or at 0°C according to the manufacturer’s instructions.

Isolation of cell nuclei from pig brains. Brain cell nuclei were purified through discontinuous isosmotic buffered Percoll gradients following a modified procedure described elsewhere (35). Pig brain tissues (10 of all age groups was mixed with a scalpel and gently disrupted using a glass potter and loose and tight pestles, sequentially, in 5 vol of isosmotic buffer [25 mM Tris·HCl (pH 7.4), 0.3 M sucrose, 20 mM KCl, 25 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.1% Biotech); Bio-Rad Protein Assay reagent (Bio-Rad Laboratories); PolyFeC reagent (Qiagen); DMEM (Life Technologies); ethylene glycol-bis(succinimidylsuccinate) (EGS; Pierce); and Western Lightening chemiluminescence reagent (Perkin-Elmer). All other chemicals were purchased from Sigma.

Materials. The purified cPGES protein, monoclonal JJ3 antibody, and p23 cDNA were gifts from Dr. David O. Toft (Mayo Clinic; Rochester, MN). The following items were purchased: anti-COX-2 and anti-mPGES-1 polyclonal antibodies (Cayman); anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Oncogene Research Products); anti-von Willebrand factor monoclonal antibody (DAKO, Diagnostics Canada; Ontario, Canada); anti-FLAG M2 monoclonal antibody (Stratagene); biotin-conjugated F(ab’)2 fragments of goat anti-mouse and anti-rabbit IgG and streptavidin conjugated to Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probes); deoxyoligonucleotides (Alpha DNA); plasmids pCMV-Taq2 (Stratagene) and pcDNA3.1 (Invitrogen); platinum Ptx DNA polymerase (Invitrogen); M-MLV reverse transcriptase (Stratagene); RNA guard RNase inhibitor, arachidonic acid, Percoll, and PGH2 (Amersham Pharmacia

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2-mercaptoethanol, and 0.5 mM PMSF] containing 20% glycerol. The nuclei were sedimented (1,500 g, 10 min) and resuspended in isoosmotic buffer containing 0.1% Nonidet P-40 (instead of Triton X-100), a milder nonionic detergent that does not destroy the nuclear envelope (21, 35). After filtration through 70-μm nylon mesh, the above centrifugations and resuspensions were repeated twice. The pellet was suspended in 10–14 ml buffer and gently ground with a glass potter and tight fitting pestle (20–30 strokes). Samples were stained with Trypan blue and viewed under a light microscope to verify satisfactory cell disruption. The samples were made to 20% Percoll (40 ml), and 7–10 ml were loaded over discontinuous isoosmotic three-layer Percoll gradients (12 ml/layer; 65%, 50%, and 35% Percoll) and centrifuged (7,000 g, 40 min). Nuclei were collected at the 35/50% Percoll interface, washed, resuspended in 100–300 l, and costained with 4',6-diamino-2-phenylindole (DAPI) and Trypan blue, and the intactness and purity of the preparation verified under epifluorescence microscopy. Nuclei contained on average 5.3 ± 1.5 U/mg protein of 5'-nucleotidase activity and 13.4 ± 3.7 mmol PO_4 released/mg protein of glucose-6-phosphatase, which is <3% and >55%, respectively, of the total activities in microsomal fractions; the endoplasmic reticulum is contiguous with the outer nuclear membrane. In the case of cultured cells, nuclei were purified by the NP-40 lysis method as described previously (21).

**SDS-PAGE and Western immunoblot.** Protein samples (10–40 μg, depending on the experiment) were resolved by SDS-PAGE on 10% or 15% gels along with precise amounts (1–30 ng) of purified human cPGES or mPGES-1 proteins. Equal loading was ensured by staining parallel gels with Coomassie blue. After electrotransfer, polyvinylidene difluoride membranes were probed with primary antibodies to COX-2 (polyclonal), COX-1 (polyclonal), mPGES-1 (polyclonal), cPLA2 (polyclonal), and cPGES (monoclonal). Secondary antibodies were added (1:2,500 dilution) and revealed with Western Lightning chemiluminescence reagent (Perkin-Elmer) and exposed to X-ray film (Kodak). The integrated signal intensities of samples and control

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**Fig. 1.** The cytosolic prostaglandin E synthases (cPGES) isoform is also associated to membranes. **A:** membrane-associated cPGES (cPGES-m) immunoblot in microsomal membranes (30 μg protein) from porcine newborn brain treated with 10 mM each of EDTA and EGTA, 0.1 M Tris (pH 11.0), 0.1 M sodium carbonate (pH 11.0), and 8 M urea buffer alone and 50 mM DTT at 4 or 22°C; the last 3 lanes contained cytosolic protein (15 μg, 175,000-g supernatant) from fetal (F), newborn (NB), and adult (A) pig brains, respectively. **B:** quantitative densitometry of cPGES-m immunoblots (n = 3) with microsomes from porcine newborn brain treated with Na_2 CO_3 (pH 11), 8 M urea, and 50 mM DTT, expressed as the percentage of untreated microsomes. *P < 0.05 compared with control values. C: cPLA2 immunoblot from porcine newborn brain microsomes purified in the presence or absence of 10 mM EGTA. D: cPGES immunoblot with porcine newborn brain microsomes cross-linked (5 mg protein/ml, 30 min at 22°C) with ethylene glycol-bis(succinimidylsuccinate) (EGS). In the last lane, microsomes (1 mg protein/ml) were preincubated (2 h at 4°C) with 50 mM DTT and cross-linked with EGS (30 min at 22°C). The first lane was loaded with 10 ng of purified cPGES. **E.i:** cPGES immunoblot of microsomes from transfected COS-1 cells with green fluorescent protein (GFP)-tagged wild-type (WT), C58K mutated, and COOH-terminal 35-amino acid-deleted (CA35) cPGES. U, untransfected cells. **E.ii:** GFP fluorescence of COS-1 cells transfected with GFP-tagged cPGES constructs used for the immunoblot shown in **E.i.** Note the comparable transfection (fluorescence) for all constructs.
proteins run in the same gel were determined by scanning densitometry using Image-Pro Plus version 4.5 software (Media Cybernetics).

**Immunohistochemistry.** Adult male Wistar rats (n = 2) brains were perfused with PBS buffer and processed as described previously (9). Neurons in the 10-μm-thick sections were distinguished from glial cells using classic Nissl staining (hematoxylin-eosin and cresyl violet) or Neurotrace Fluorescent Nissl Stain (Molecular Probes). The sections (>10 selected sections of 100 sections/brain, including preliminary attempts to optimize the conditions) were incubated overnight at 4°C with the primary antibodies to COX-2 (1:400 dilution), mPGES-1 (1:200 dilution), cPGES (1:250 dilution), GFAP (monoclonal, 1:300 dilution), or von Willebrand factor (monoclonal, 1:50). After being rinsed several times with PBS, the sections were incubated for 2 h at room temperature with biotin-conjugated F(ab′)2 fragments of goat antimouse and anti-rabbit IgG (each at 1:300 dilution), rinsed, and then further incubated with streptavidin conjugated to Alexa Fluor 594 or mouse and anti-rabbit IgG (each at 1:300 dilution), rinsed, and then further incubated with streptavidin conjugated to Alexa Fluor 594 or Alexa Fluor 488 (1:1,000 dilution, 1 h at room temperature) (Molecular Probes). Nuclei were counterstained with 50 nM DAPI. Omission of primary antibodies was used as negative controls. Immunopositive cells were quantified on selected microscope field areas of cortex and hippocampus (C1–C4 sections) by optical and/or epifluorescence microscopy (Nikon Eclipse E600) using AxioVision 2.05 software (Zeiss); these regions were selected because of the abundance of COX-2 expression, allowing us to investigate its possible previously undescribed colocalization with cPGES-m in line with our hypothesis.

**PGES enzyme assays.** The purified nuclei and microsomal preparations from the porcine brain (fetal and newborn) were used to assess the conversion of PGH2 to PGE2 by radioimmunoassay as described elsewhere (32). Briefly, the equivalent of 10 μg of membrane protein were preincubated at 25°C for 5 min in 100 μl of 0.1 M potassium-phosphate buffer (pH 7.0) with or without 1 mM GSH or DTT. Reactions were started with the addition of 10 μM PGH2, stopped after 30 s by adding 1 vol of 15 mM stannous chloride-0.1% HCl, and frozen immediately on dry ice. Conversion of arachidonic acid to PGE2 with brain microsomes was also determined (27). PGE2 was quantified by radioimmunoassay (Amersham) as described previously (27).

**Plasmid constructs, cell transfection, and confocal microscopy.** Human cPGES [wild type, mutant cPGES (C58K)], and the 35-amino acid COOH-terminal deletion] and full-length mPGES-2 cDNAs were amplified by PCR with primers generating EcoRI sites, Platinum Pfx DNA polymerase (Invitrogen), and plasmids containing the p23 and NotI fragment of goat anti-mouse and anti-rabbit IgG (each at 1:300 dilution), rinsed, and then further incubated with streptavidin conjugated to Alexa Fluor 594 or Alexa Fluor 488 (1:1,000 dilution, 1 h at room temperature) (Molecular Probes). Nuclei were counterstained with 50 nM DAPI. Omission of primary antibodies was used as negative controls. Immunopositive cells were quantified on selected microscope field areas of cortex and hippocampus (C1–C4 sections) by optical and/or epifluorescence microscopy (Nikon Eclipse E600) using AxioVision 2.05 software (Zeiss); these regions were selected because of the abundance of COX-2 expression, allowing us to investigate its possible previously undescribed colocalization with cPGES-m in line with our hypothesis.

**Statistical analysis.** Data from scanning densitometry and enzyme assays are presented as means ± SE, with each treatment performed in triplicate. Values were analyzed by one-way ANOVA, and comparison among means was determined by the Tukey-Kramer method. Statistical significance was set at P < 0.05.

**RESULTS**

cPGES is associated with membranes. Although cPGES is considered to be exclusively cytosoluble (13, 32), our immunoblots with porcine and rat brain microsomal membranes and soluble fractions consistently detected the presence of cPGES (Fig. 1A); cPGES was of course also present in cytosolic fractions (Fig. 1A). The binding of cPGES to the membranes was not disrupted with EDTA-EGTA washes (Fig. 1, A and B), indicating that calcium is not involved in the cPGES-membrane interaction, whereas similar washes diminished the presence of cPLA2 (Fig. 1C). A harsh alkaline treatment [0.1 M Tris (pH 11) or 0.1 M sodium carbonate (pH 11)], which effectively removes peripheral proteins from membranes (22), only slightly altered the abundance of cPGES in membranes (Fig. 1, A and B). To determine whether cPGES is intrinsic or associated with the membranes by protein-protein interactions, the microsomal membranes were treated with 8 M urea or 50 mM DTT. The effects of urea were negligible (Fig. 1, A and B), whereas DTT strongly dissociated about 90% of cPGES (Fig. 1, A and B). This may infer the involvement of disulfide bridges in the cPGES-membrane interaction.

To examine the possibility that cPGES interacts with an intramembrane protein, the purified microsomes were cross-linked with EGS, followed by SDS-PAGE and Western

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Fig. 2. cPGES-m but not microsomal PGES (mPGES)-1 colocalizes with cyclooxygenase (COX)-2 and COX-1 in microsomes from porcine cerebrum. A: immunoblots with microsomes (40 μg membrane protein) purified from fetal, newborn, juvenile (J), and adult pig brains. The + control lane contained 1 ng of purified mPGES-1 protein (approximately the limit of detection by the anti-mPGES-1 antibody in our conditions). B and C: relative abundance of cPGES-m, COX-2, and COX-1 estimated by densitometry of immunoblots from pig (B) and rat (C) brain cerebrum microsomal membranes (no EDTA-EGTA). Results are expressed as the percentage of values from newborn samples set at 100%. *P < 0.05 compared with corresponding newborn values (n = 3).
immunoblot. Remarkably, a new immunoreactive band of about 220 kDa appeared with concomitant disappearance of 23-kDa cPGES with increasing concentrations of EGS (Fig. 1D). Pretreatment of membranes with DTT (50 mM) prevented the formation of this heavier immunoreactive band by EGS (Fig. 1D); this provides further evidence for the formation of a complex dependent on disulfide bonds.

The cysteine at position 58 and the carboxyl terminal 35 amino acids of cPGES have been suggested to contribute to the interaction of cPGES to proteins (39, 40). We examined their involvement in the association of cPGES to membranes by mutation (C58K) or deletion (CΔ35); proteins were GFP tagged for identification in transfected COS-1 cells. The C58K mutation and CΔ35 deletion markedly diminished or nearly abolished the cPGES association with membranes (Fig. 1E). Altogether, this membrane-associated isomform is designated as cPGES-m to distinguish it from the soluble protein.

Colocalization of cPGES-m with COX-1 and COX-2 in microsomes from porcine and rat brain tissue. The pattern of expression of PGE2 biosynthetic enzymes was analyzed by Western blot on cerebrum microsomes from pigs and rats of different developmental stages. mPGES-1 protein was undetectable in these samples (Fig. 2, A–C). The overall profile of expression of cPGES-m, COX-2, and COX-1 in the rat brain was generally similar to that in the pig brain with the exception that cPGES-m was most abundant in the fetal pig and COX-2 in juvenile rats (Fig. 2, B and C); modest developmental changes in cPGES-m and COX-1 were observed compared with the marked ones in COX-2.

mPGES-1, cPGES-m, and COX-2 in microsomes from cerebral microvasculature. We also examined the expression of mPGES-1, cPGES-m, COX-1, and COX-2 in microsomes from the microvasculature of the pig brain. Although cPGES-m, COX-2, and COX-1 were present at all developmental stages studied, mPGES-1 was again barely detectable in juvenile and adult microvascular microsomes (Fig. 3).

Presence of mPGES-1 and COX-2 in nuclear fractions from brain tissue and various cell lines. Because the cell nucleus may also be a source of prostaglandins (30), we examined the

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**Fig. 3.** A: immunoblots of mPGES-1, cPGES, COX-1, and COX-2 in microsomes from cerebral microvasculature isolated from newborn, juvenile, and adult pigs. The + control lane contained 15 and 10 ng of purified human mPGES-1 and cPGES proteins, respectively. B: relative abundance of cPGES-m, mPGES-1, COX-2, and COX-1 proteins in microsomes from pig newborn, juvenile, and adult brain microvessels, assessed by densitometry of immunoblots and expressed as the percentage of values in newborn. *P < 0.05 compared with corresponding newborn values (n = 3).

**Fig. 4.** mPGES-1 and COX-2 colocalize in purified nuclei from newborn pig brain. A: immunoblots of mPGES-1, cPGES, COX-1, and COX-2 in nuclei (40 μg protein) from fetal, newborn, and adult brain parenchyma. The + control lane contained 15 and 10 ng of purified mPGES-1 and cPGES proteins, respectively. B: immunoblots of nuclei and microsomes purified from cultured HEK-293 (293), porcine brain microvascular endothelial (EC), A549, and HTC4 cells. The + control lane contained 10 and 20 ng of purified mPGES-1 and cPGES proteins, respectively. C: glutathione-dependent conversion of PGH2 to PGE2 in nuclei and microsomes from fetal and newborn porcine brain parenchyma. *P < 0.05 compared with other values. D: conversion of arachidonic acid to PGE2 in microsomes of newborn brain parenchyma. *P < 0.05 compared with other values.
expression profile of COX-1/2 and c/mPGES in this compartment. In contrast to microsomes, nuclei from pig brain parenchyma contained mPGES-1, COX-2, and cPGES (except in the newborn; Fig. 4A); COX-1 was undetectable in nuclei.

The pattern of expression of these proteins in nuclear and microsomal compartments was also further explored in various cell types, namely, HEK-293, primary porcine brain microvascular endothelium, A549, and HTC4. In nuclear fractions, COX-2 (but not COX-1) was detected only in endothelial cells along with both mPGES-1 and cPGES (Fig. 4B). In microsomes, the four proteins were present in all cells with the exception of A549 cells, which contained only COX-1 (but not COX-2), which colocalized with both mPGES-1 and cPGES-m.

Activities of PGES in nuclei and microsomes from brain parenchyma. PGES activity (glutathione dependent as is the case for cPGES and mPGES-1) in nuclei of the newborn brain was greater than that in the fetus (Fig. 4C), consistent with more abundant mPGES-1 expression (Fig. 4A). The addition of DTT to unveil mPGES-2 activity negligibly augmented PGE2 formation, confirming a major role for mPGES-1 in nuclei from the newborn brain (where cPGES is absent). In brain microsomes, PGE2 formation was greater in the fetus than newborn. The latter was not affected by DTT, consistent with a major role for cPGES in microsomes of brain tissue devoid of mPGES-1 (see Fig. 2); however, the contribution of mPGES-2 in PGE2 formation in the postnatal brain cannot be excluded (31). To further verify that COX-2 can coordinate its enzyme activity with a PGES other than mPGES-1 (24), microsomes of newborn brain parenchyma were treated with MK886 or NS398, which are inhibitors of mPGES-1 and COX-2, respectively (7). MK886 negligibly affected conversion of arachidonic acid into PGE2, consistent with immunoblot data (Fig. 2), whereas NS398 diminished PGE2 formation by 75% (Fig. 4D) (cPGES-selective inhibitors are not yet available).

Intracellular localization of cPGES and mPGES-1 in transfected cells. The localization of cPGES and mPGES-1 was further studied in cells transfected with GFP-tagged cPGES or FLAG-tagged mPGES-1 plasmid constructs and visualized by confocal microscopy. cPGES was present throughout the cytoplasm and nucleus, whereas mPGES-1 was present throughout the cytoplasm and perinucleus (Fig. 5), as seen for COX-2 (30). mPGES-2 (tagged with GFP) localization was only found throughout the cytoplasm (but not at the nucleus).

Colocalization of COX-2 with cPGES and mPGES-1 in brain neurons. The colocalization of COX-2 with cPGES and mPGES-1 was analyzed by immunofluorescence microscopy in the cortex and hippocampus of adult rat brain sections. COX-2 immunostaining was detected in the perinuclear region and cytoplasm of cortical (Fig. 6B) and hippocampal (Fig. 6C) neurons coidentified with Nissl staining (Fig. 6D), consistent with immunoblots (Fig. 2). COX-2 was not seen in astroglial cells identified with an anti-GFAP antibody (data not shown), as previously documented in vivo (11). Likewise, mPGES-1 was mostly observed at the perinuclear region (Fig. 6E) of neurons (Fig. 6F). COX-2 could be found colocalized with cPGES (Fig. 6G) or mPGES-1 (Fig. 6H).

DISCUSSION

PGE2 is a major prostaglandin produced by many cells and is crucial for maintenance of tissue homeostasis. In the brain, this prostanoid is involved in numerous processes including synaptic plasticity and development, long-term potentiation, neurogenesis, and circulatory adaptation as well as being a key mediator of pyrogenesis (2, 5, 6, 29). A coordinated interaction of COX-1/2 with PGES is essential to generate PGE2, which in

Fig. 5. Intracellular localization of cPGES-GFP, FLAG-mPGES-1, and mPGES-2-GFP in transfected HEK-293 cells (A–C) or COS-1 cells (D–I). Nuclei were stained with propidium iodide (red; A and D) or 4′,6-diamino-2-phenylindole (DAPI; blue; G); C, F, and I are merged images. Scale bar = 10 μm.
turn mediates its pleiotropic cell surface and intracellular effects, especially as it pertains to complex brain functions (2, 6, 16, 17). Identification of the colocalization of COX/PGES underlies their interaction and may assist in understanding the regulation of these therapeutic targets.

Previously, in vitro experiments with HEK-293 cells co-transfected with different combinations of COX and PGES cDNAs have led to the suggestion of the model of COX-2/mPGES-1 and COX-1/soluble cPGES coordination (24, 32). However, in vivo corroboration of this model in brain tissues has never been substantiated. In fact, this rather limited view has been contradicted by observations in noncerebral cell lines, which revealed the presence of both COX-1 and COX-2 at the same intracellular location (23, 26, 30), consistent with observations in the present study (Fig. 4B). Moreover, mPGES-1-deficient mice were found to exhibit normally important functions mediated by PGE2, including renal development and fertility (33), suggesting that other PGES coordinate with COX-1/2 to fulfill the homeostatic requirements of PGE2. In the present study, our findings unveil a more complex interaction between COX-2 and PGESs, such that membrane-associated COX-2 can colocalize and coordinate its activity not only with mPGES-1 but also with cPGES-m, which was convincingly found present on membranes (Fig. 1); this more intricate interaction between COXs and PGESs was particularly appreciated by the distinct developmental profile of subcellular localization of these proteins (Figs. 2–4). Moreover, although subcellular localization of COX-2 in the cerebrum reveals its presence in microsomes and the perinuclear region (nuclei), mPGES-1 was only detected in the latter fraction of the same tissue, implying alternative cellular localization for this alleged microsomal protein.

A salient feature of our findings applies to cPGES localization. We disclosed novel evidence that cPGES is not exclusively a soluble protein, as previously thought, but is also associated with microsomal membranes from the cerebrum and cerebral microvasculature at all developmental stages as well as microsomes from various cell lines. This cPGES-m protein appears to anchor to the membranes by involving disulfide links. These disulfide bridges may be intermolecular, which is similar to the case of the metalloendopeptidase heterodimer meprin for which the α-subunit localizes to the plasma membrane with the membrane-integrated β-subunit (13). The disulfide bridges may also be intramolecular, particularly relevant because cPGES contains five cysteine residues; in this case, conformational changes would likely enhance protein-protein interactions. Consistent with this notion, deletion of the COOH-terminal 35 amino acids virtually abolished the membrane association of cPGES (Fig. 1E). The membrane localization of cPGES-m in the proximal vicinity of COX-1/2 would be biophysically coherent for the efficient conversion of the unstable arachidonic acid-derived PGH2 into PGE2 (Fig. 4, C and D). Hence, cPGES-m is a major PGES in microsomes and perinuclear regions.
from cerebral parenchyma and microvasculature, irrespective of developmental stages, and may coordinate with COX-2 and/or COX-1; this clearly is the case in the newborn brain, where COX-2 and to a lesser extent COX-1 contribute to PGE$_2$ formation (27). These findings further emphasize the point that COX-2 does not exclusively colocalize and coordinate with mPGES-1.

By combining cell fractionation, immunoblotting, immunohistochemistry, and enzyme assays, we provide evidence that PGE$_2$ biosynthetic enzymes (COX and PGES) display distinct association patterns depending on the tissue (brain parenchyma vs. vasculature), the developmental stage, and the subcellular compartment (perinuclear and microsomal). Specifically, in contrast to COX-2, mPGES-1 was virtually undetectable in the cytoplasm (Fig. 6, B, C, and F) and microsomes (Figs. 2 and 4D) based on immunoreactivity and enzyme assay data. Therefore, in brain microsomes, COX-2 seemed to coordinate its activity with cPGES rather than mPGES-2; in these assays, DTT, which is expected to enhance mPGES-2 enzyme activity (31, 37, 38), was hardly effective (in the newborn). On the other hand, mPGES-1 was concentrated at the nuclear/perinuclear region (Figs. 4A and 6F) with COX-2 (but not COX-1) (Figs. 4, A and B, and 6H); the presence of perinuclear COX-2 is consistent with previous observations (30), and the greater abundance of mPGES-1 and COX-2 in the newborn (Figs. 3 and 4A) results from their enhanced induction during parturition (18). In cerebral microvessels mostly composed of endothelium (27), mPGES-1 expression in microsomes was dependent on the developmental stage but was virtually absent in the juvenile and adult despite the presence of COX-2 (Fig. 3). Thus our findings in the brain do not support an exclusive colocalization and interaction between COX-2 and mPGES-1 or between COX-1 and cPGES as previously reported in vitro on HEK-293 cells (24, 32). This inference is further substantiated by studying various cell lines, in which colocalization of COX-2 with mPGES-1 pattern is again not exclusive; besides, in some cell lines, notably A549, COX-2 is absent such that COX-1 compartmentalizes with both mPGES-1 and cPGES-m (Fig. 4B).

Convincing evidence indicates that PGE$_2$ is important for the development of certain brain structures and functions as well as perinatal hemodynamic changes (2, 19). Correspondingly, developmental changes in COX-PGES expression enzymes (2, 16, 17) reflect those of PGE$_2$ levels (1) that are essential for the maturation of neuronal networks. Interestingly, the timing of such dendritic formation and synaptogenesis is coincidental with COX-2 expression (16, 17), which impacts on the regulation of vasomotor tone (19).

To conclude, PGE$_2$ biosynthesis in the brain seems to be mediated by the concerted actions of microsomal COX-1/COX-2/cPGES-m and by nuclear/perinuclear COX-2/mPGES-1/cPGES, the relative expressions of which are developmentally regulated. The former complex possibly would favor generation of PGE$_2$ to be released extracellularly and exert paracrine effects such as those involved in pyrogenesis, inflammation, and circulatory control, whereas the nuclear complex would supply PGE$_2$ to its vicinal recently described perinuclear receptors to regulate gene expression (4, 10).

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