Disruption of cAMP and Prostaglandin E2 Transport by Multidrug Resistance Protein 4 Deficiency Alters cAMP-Mediated Signaling and Nociceptive Response

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ABSTRACT

Multidrug resistance protein 4 (MRP4; ABCC4) is a member of the MRP/ATP-binding cassette family serving as a transmembrane transporter involved in energy-dependent efflux of anticancer/antiviral nucleotide agents and of physiological substrates, including cyclic nucleotides and prostaglandins (PGs). Phenotypic consequences of mrp4 deficiency were investigated using mrp4-knockout mice and derived immortalized mouse embryonic fibroblast (MEF) cells. Mrp4 deficiency caused decreased extracellular and increased intracellular levels of cAMP in MEF cells under normal and forskolin-stimulated conditions. Mrp4 deficiency and RNA interference-mediated mrp4 knockdown led to a pronounced reduction in extracellular PGE2 but with no accumulation of intracellular PGE2 in MEF cells. This result was consistent with attenuated cAMP-dependent protein kinase activity and reduced cyclooxygenase-2 (Cox-2) expression in mrp4-deficient MEF cells, suggesting that PG synthesis is restrained along with a lack of PG transport caused by mrp4 deficiency. Mice lacking mrp4 exhibited no outward phenotypes but had a decrease in plasma PGE metabolites and an increase in inflammatory pain threshold compared with wild-type mice. Collectively, these findings imply that mrp4 mediates the efflux of PGE2 and concomitantly modulates cAMP mediated signaling for balanced PG synthesis in MEF cells. Abrogation of mrp4 affects the regulation of peripheral PG levels and consequently alters inflammatory nociceptive responses in vivo.

MRP4 (ABCC4) is a member of the multidrug resistance proteins (MRPs) belonging to the C group of the ATP-binding cassette (ABC) protein superfamily. To date, nine total MRP members, MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11), and MRP9 (ABCC12), have been identified. MRP4 functions as an energy-dependent, transmembrane efflux transporter closely related to three other MRP family members, MRPs 5, 8, and 9, with respect to structure and functional characteristics (Kruh and Belinsky, 2003; Deeley et al., 2006). Based upon predicted membrane topology, MRPs 4, 5, 8, and 9 consist of a cytoplasmic segment (L0) and a P-glycoprotein-like core structure but lack the NH2-terminal domain (TMD0) that is present in all of the other MRPs (Kruh and Belinsky, 2003; Deeley et al., 2006). MRPs 1, 2, 3, and 4 function physiologically as organic anion transporters mediating the efflux of glutathione, glucuronate, and sulfate conjugates whereas MRP5 does not (Kruh and Belinsky, 2003; Deeley et al., 2006).

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Both MRPs 4 and 5 are capable of transporting cAMP and cGMP (Jedlitschky et al., 2000; Chen et al., 2001), the cyclic nucleotides that play critical roles in intracellular signaling. This unique transport capacity also led to the findings that MRPs 4 and 5 mediate the efflux of purine nucleotide analogues derived from the anticancer 6-thiopurines and of the antiviral purine nucleotide analog 9-(2-phosphonylmethoxy-

ABBREVIATIONS: MRP/mrp, human/murine multidrug resistance protein; MEF, mouse embryonic fibroblast; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PG, prostaglandin; PKA, cAMP-dependent protein kinase; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; Cox, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; kb, kilobase pair(s); bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; EIA, enzyme immunoassay; PGEM, PGE2 metabolite; shRNA, short hairpin RNA; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; ANOVA, analysis of variance; siRNA, short interfering RNA.
mice. Through behavioral analyses of gene knockout of mrp4 in the efflux transport of PGs and the cAMP-mediated mechanism for the antinociceptive effects of nonsteroidal (Burian and Geisslinger, 2005). Therefore, inhibition of Cox-2 effects of inflammatory mediators at the site of inflammation involved in hyperalgesia through enhancing nociceptive effects. Cox-1 is constitutively expressed in most tissues, whereas Cox-2 is catalyzing the synthesis of PGs from arachidonic acid. Cox-1 is rapidly induced in response to mitogens, growth factors, and inflammatory cytokines (Smith et al., 2000). The Cox-2 gene is characterized physiological molecules with pivotal roles in cellular communication and signaling. cAMP functions as an intracellular second messenger that mediates a broad range of cellular responses by activation of cAMP-dependent protein kinase (PKA) (Mayr and Montminy, 2001). The activation of PKA involves the binding of cAMP to regulatory subunits and the subsequent dissociation and nuclear translocation of catalytic subunits. Nuclear-localized catalytic subunits mediate the phosphorylation of the cAMP-response element (CRE)-binding protein (CREB) at Ser133, which promotes the transcription of a variety of target genes (Mayr and Montminy, 2001).

Two isoforms of cyclooxygenases (Cox) are required for catalyzing the synthesis of PGs from arachidonic acid. Cox-1 is constitutively expressed in most tissues, whereas Cox-2 is rapidly induced in response to mitogens, growth factors, and inflammatory cytokines (Smith et al., 2000). The Cox-2 gene contains a consensus CRE motif, TGACGTCA, in its promoter sequences and is one of many genes transcriptionally targeted by the cAMP-dependent signaling pathway (Mayr and Montminy, 2001). The induction of Cox-2 is responsible for an increase in the production of PGE₂, a primary PG involved in hyperalgesia through enhancing nociceptive effects of inflammatory mediators at the site of inflammation (Burian and Geisslinger, 2005). Therefore, inhibition of Cox-mediated PG synthesis is broadly accepted as the primary mechanism for the antinociceptive effects of nonsteroidal anti-inflammatory drugs (NSAIDs).

In the present study, the functional importance of mrp4, the murine ortholog of MRp4, in the transport of cAMP and PGE₂ was investigated through establishment of mice with a homozygous disruption of the mrp4 gene. The involvement of mrp4 in the efflux transport of PGs and the cAMP-mediated signaling pathway was also explored with immortalized embryonic fibroblast cell lines established from mrp4-deficient mice. Through behavioral analyses of gene knockout animals, a phenotypic consequence of the mrp4 deficiency involving altered inflammatory nociceptive response was also elucidated. The findings demonstrate that mrp4 contributes to the transport of PGs and the regulation of the cAMP-mediated signaling pathway leading to PG synthesis.

Materials and Methods

Generation and Breeding of Mrp4-Null (Mrp⁴⁻) Mice. The embryonic stem cell line RR212 (strain 129P2/Ola Hsd) containing a heterozygous gene trap disruption of the abec4 gene was obtained from BayGenomics/Multiple Myeloma Research Consortium (MMRC) (University of California Davis, Davis, CA). Chimeras were generated from this cell line by standard blastocyst microinjection and embryo transfer by the Animal Genomics Services at the Yale University School of Medicine (New Haven, CT). High percentage male chimeras were mated with C57BL/6 female mice (Charles River, Wilmington, MA), and agouti F1 offspring were genotyped using PCR analyses for the presence of the neomycin sequence (see Genotyping). Positive, mrp4²⁻/⁻ F1 mice were subsequently mated to yield P2 animals with homozygous disruption of the mrp4 gene (mrp4⁻/⁻).

Genotyping. Tail DNA was purified using the Puregene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instructions. Initial PCR neomycin cassette-based genotyping of the F1 heterozygotes was conducted according to the protocol of BayGenomics/MMRC using the neomycin sequence forward primer 5'-CTGGGAGGAGGCCTTTC-3' and the reverse primer 5'-AGGTGAGATCACGAGGATC-3'. Subsequent genotyping was directed at unique wild-type and knockout alleles by Southern blot and/or PCR analyses. NCBI-BLAST genomic DNA homology searching placed the 5′-rapid amplification of cDNA ends mrp4 sequence of the RR212 ES clone within the Mus musculus clone RP24-212C1 (AC122778). AC122778 contains EcoRV restriction sites at positions 21258 and 38577, yielding a 17.5-kb wild-type band on Southern blotting. Gene trap insertion of the disrupting vector sequence (pGTL1fx; BayGenomics) between the wild-type EcoRV sites of AC122778 introduced a third EcoRV site yielding a diagnostic 11.5-kb knockout allele. Hybrid-N² membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) blots of EcoRV digested genomic DNA (15 µg) were probed with a 3²P-labeled 514-bp PCR generated probe, located 5′ to the disrupted intronic sequence of AC122778. The probe was prepared by amplifying the sequence of AC122778 corresponding to position 21752 through 22266 with the forward/reverse primer pair 5′-TAGGTTGAGATGGTGAGATGGTGCAG-3′/5′-CCTTAGATGTTGGCTGTTG-3′. Subsequently, all genotyping was performed by PCR using a cocktail of three primers: 5′-TCCATGAGACATGAGGATGACTGTC-3′ (wild-type forward), 5′-GGACCACTAGTAACTGCAGTCAAT (wild-type reverse), and 5′-ATGAGATGAGTTGGGATGCTACTGTC-3′ (knockout reverse) under the conditions of one cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 63°C for 1 min, 72°C for 1 min, and finally one cycle of 72°C for 7 min. The PCR reaction yielded a 582-bp wild-type product and a 220-bp knockout product. Sequence analysis of the knockout PCR product localized the insertion of the disrupting vector sequence to the intronic sequence of AC122778 immediately 3′ to nucleotide 29580, with the most 5′ portion of the disrupting vector, pGTL1fx, being nucleotide 1196 of the Es2 sequence.

Generation and Immortalization of Primary Embryonic Fibroblasts. Mrp4²⁻/⁻ and mrp4⁻/⁻ primary embryonic fibroblasts were generated by pairing heterozygous mrp4⁻/⁻ parents, followed by harvesting embryos on days 14.5 to 15.5 and genotyping. The pregnant dam was euthanized by carbon dioxide asphyxiation and the uterus was removed. The 12 embryos were pooled for each of the experiment and were frozen at passage 2. The procedure for transfection and immortalization of primary embryonic fibroblast cells was described previously (Lin et al., 2002) with some modification. In brief, primary embryonic fibroblast cells were cotransfected with pC2Pa and pCMV-Bsd plasmids. At 48 h after transfection, 4 µg/ml of Blasticidin S (Invitrogen, Carlsbad, CA) was added for 10 days to select resistant colonies. The total population of resistant colonies was pooled for each of the mrp4⁻/⁻ and mrp4⁻/⁻ fibroblasts, which were continuously cultured in the presence of Blasticidin S for another month and expanded into immortalized cell lines.
RT-PCR Analysis. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Primer sequences of wild-type mrp4 (AY415588) for RT-PCR were forward 5'-TGTGCCTCTAAATCTGTTG-3' and reverse 5'-GCAATT-CTCAGTGGTCCG-3'. For the knockout mrp4/-/geo (β-galactosidase-neomycin fusion) RT-PCR reaction, the same forward primer for wild-type mrp4 and a reverse primer 5'-TCCAGTCCAGGTGT-AA-3', corresponding to the sequence of pT21txf vector from position 1645 through 1664, were used. Primer sequences of murine mrp1, mrp5, and β-actin and RT-PCR conditions used were described previously (Lin et al., 2002).

Cytotoxicity Assay. The sensitivity of immortalized fibroblasts to PMEA (Moravek, Brea, CA; Gilead, Foster City, CA) was determined by the MTS cytotoxicity assay as described previously (Lin et al., 2002).

cAMP-dependent Protein Kinase Assay. Cell pellets were resuspended in ice-cold PKA extraction buffer (25 mM Tris-Cl, pH 7.4, 2 mM EDTA, 0.5 mM PMSF, and proteinase inhibitor cocktail; Roche, Indianapolis, IN), sonicated, and centrifuged to clear the lysates. Aliquots of lysates were used to measure protein concentrations by the Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. PKA activity was determined using C-18 Amprep minicolumns (GE Healthcare). Duplicate sets of samples were also saved for determination of cellular PGE2 by the MTS cytotoxicity assay (as described previously (Lin et al., 2002)). PKA activity was determined by the MTS cytotoxicity assay as described previously (Lin et al., 2007). Cells were plated in six-well plates, and stored at 4°C for 24 h. Thereafter, 10 μl of phosphate buffer and 7.5 μl of EIA buffer (Cayman Chemical) were added to the plasma, which was processed for measurement of PGEM using the Prostaglandin E Metabolite EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Measurement of Plasma PGE Metabolite. Mice were euthanized by CO2 asphyxiation, and at least 500 μl of blood were immediately obtained and transferred into a tube containing EDTA (Microtainer; BD Biosciences, Franklin Lakes, NJ). The tubes were centrifuged and 25 μl of supernatant containing plasma was transferred to a tube in which 7.5 μl of carbonate buffer (Cayman Chemical, Ann Arbor, MI) was added. The plasma was then incubated at 37°C for 24 h. After incubation, 10 μl of phosphate buffer and 7.5 μl of EIA buffer (Cayman Chemical) were added to the plasma, which was processed for measurement of PGEM using the Prostaglandin E Metabolite EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Western Blot Analysis. The procedures were as described previously (Lin et al., 2002). Anti-mrp4 (M4I-10) antibody was obtained from Abcam (Cambridge, MA). Anti-mrp5 (C-17), Cox-1 (H-62) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was supplied by Sigma-Aldrich (St. Louis, MO).

Generation of shRNA and Transient Transfections. Mouse mrp4 shRNAs were derived from the 19-base sequence of mouse mrp4 cDNAs CCACAGGGCAGATGGTTA and CCTCGGCTTAACAA-TATT (666–668 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336). The control shRNA was a nontargeting sequence (TATT (666–684 and 4041–4059, respectively, GenBank accession number NM_001033336).

Determination of Inflammatory Pain Thresholds. Behavioral analyses were performed to assess mechanical and thermal nociceptive thresholds in a model of inflammatory pain. Mrp4+/− mice and control mrp4+/− were exposed to 50 μl of a 6% capsaicin solution in 50% DMSO by intradermal injection into the glabrous surface of the hind paw using 3% halothane anesthesia. After acclimation to the testing area (30 min), mechanical sensory thresholds were determined by paw withdrawal from the application of a series of von Frey filaments (Stoelting, Wood Dale, IL) to the glabrous surface of the paw. After application of calibrated von Frey filaments (0.4–26 g, verified by calibration against a force transducer) with enough force to cause buckling of the filament, a modification of the “up-down” method of Dixon (1980) was used to determine the value at which paw withdrawal occurred 50% of the time (Chaplan et al., 1994), interpreted to be the mechanical nociceptive threshold.

After acclimation to the test chamber, thermal hyperalgesia was assessed by measuring the latency of paw withdrawal in response to a radiant heat source (Dirig et al., 1997). Mice were placed in Plexiglas boxes on an elevated glass plate under which a radiant heat source (4.7 amps) was applied to the glass plate of the paw through the glass plate. The heat source was turned off automatically by a photocell upon limb-lift, allowing the measurement of paw
Results

Homologous Disruption of Mrp4 in Mice and MEFs. The mrp4 gene was disrupted by a GeneTrap vector through an insertional mutation in the intron sequence between exons 18 and 19. As a result, the normal transcript processing of mrp4 mRNA was interrupted by the exon of the β-geo gene from the inserted GeneTrap vector (Fig. 1A). Disruption of both alleles of the mrp4 gene was confirmed by Southern blotting (Fig. 1B) and PCR analysis (Fig. 1C) of tail genomic DNA. Mice containing homologous disruption of the mrp4 gene bred normally and did not display an outward phenotype appreciably different from their wild-type counterparts (data not shown).

Immortalization of mrp4+/+ and mrp4−/− primary embryonic fibroblasts derived from these mice was carried out by the previously described method of stable transfection with the simian virus 40 large T antigen (Lin et al., 2002). Abrogation of mrp4 expression at the mRNA level by the GeneTrap vector was confirmed by RT-PCR analyses for these immortalized cell lines. Using primers corresponding to the sequences in exons 16 and 20 of mrp4, a predicted 356-bp RT-PCR product was amplified in mrp4+/+ MEF cells but not in mrp4−/− MEF cells (Fig. 1D). Because the disruption by the GeneTrap vector resulted in β-geo sequences joined to the exon sequences of mrp4 after exon 18 (Fig. 1A), a predicted 290-bp, mrp4-β-geo fusion PCR product was amplified only in mrp4+/− MEF cells when the same forward primer of wild-type mrp4 and a reverse primer of the β-geo gene in the vector were used. RT-PCR products for mrp1, mrp5, and β-actin mRNA were amplified at comparable levels among mrp4−/− and mrp4+/− MEF cells. Furthermore, a complete elimination of mrp4 protein was also verified in mrp4−/− MEF cells by Western blot analysis (Fig. 1E). The level of mrp5 protein in mrp4−/− MEF cells remained similar to that of mrp4+/− MEF cells.

Augmentation of PMEA Cytotoxicity and Intracellular cAMP Levels in Mrp4-Deficient MEF Cells. MRP4 reportedly serves as a high-affinity transporter for the nucleotide analog PMEA, and overexpression of MRP4 has been shown to confer resistance to the cytotoxicity of PMEA (Schuetz et al., 1999; Lee et al., 2000). To determine the effects of mrp4 abrogation on the sensitivity of cells to PMEA, the cytotoxicity of this agent to MEF cells after 72 h of exposure was determined by the MTS cytotoxicity assay. Mrp4−/− MEF cells exhibited a marked increase (10-fold) in sensitivity to PMEA compared with mrp4+/+ MEF cells (Fig. 2A). In contrast, both MEF cell lines exhibited similar sensitivity to vinblastine, a cytotoxic agent that is not transported by Mrp4 (Fig. 2B). We have also found that treatment with MK-571, an inhibitor of Mrp4 (Chen et al., 2002), partially sensitized mrp4−/− MEF cells to PMEA (data not shown). These results are consistent with a recent report of independently developed mrp4-deficient mice (Belinsky et al., 2007) and indicate that the loss of Mrp4 augments the cytotoxicity of PMEA as a result of a decrease in efflux of this agent in mrp4−/− MEF cells.

Using models of overexpressing cell lines and/or membrane vesicles, MRP4 has been demonstrated to mediate the efflux of cAMP (Chen et al., 2001; Wielinga et al., 2003). To corroborate this phenomenon with cells deficient in Mrp4, we measured the levels of extracellular and intracellular cAMP in MEF cells stimulated with forskolin (FSK), an activator of adenylate cyclase that induces a rapid elevation of the intracellular cAMP level (Seamon et al., 1981). Exposure to FSK for 1 h caused a significant increase in extracellular and intracellular cAMP levels in both mrp4+/+ and mrp4−/− MEF cells (Fig. 2C). The FSK-induced increase in the level of extracellular cAMP in mrp4+/+ MEF cells was significantly greater than that occurring in mrp4−/− MEF cells. The lower basal and stimulated levels of extracellular cAMP in mrp4−/− MEF cells may be produced by the efflux activity of Mrp5 (Jedlitschky et al., 2000). In contrast, exposure to FSK produced a significantly
greater increase in the level of intracellular cAMP in mrp4<sup>−/−</sup> MEF cells than that occurring in mrp4<sup>+/−</sup> MEF cells. The basal level of intracellular cAMP in mrp4<sup>−/−</sup> MEF cells was significantly higher than that in mrp4<sup>+/−</sup> MEF cells. These findings suggest that the disruption of mrp4 reduces the efflux of cAMP and therefore leads to an elevated level of intracellular cAMP under normal and stimulated conditions.

**Effects of Mrp4 Deficiency on PKA Activity and Cox-2 Expression.** The activity of PKA is modulated by changes in the intracellular level of cAMP. Therefore, we determined whether mrp4 deficiency had effects on basal and stimulated PKA activity in MEF cells. To further augment cAMP levels and PKA activity, 3-isobutyl-1-methylxanthine (IBMX), a broad-spectrum phosphodiesterase inhibitor that blocks the hydrolysis of cAMP (Chasin and Harris, 1976), was also used in combination with FSK. Mrp4<sup>−/−</sup> MEF cells exhibited a significant decrease in basal PKA activity (∼50%) relative to mrp4<sup>+/−</sup> MEF cells (Fig. 3A). Treatment with IBMX alone for 1 h had minimal effect on basal PKA activity but augmented FSK-stimulated PKA activity in both mrp4<sup>−/−</sup> and mrp4<sup>+/−</sup> MEF cells. Exposure to FSK or FSK in combination with IBMX for 1 h seemed to cause an increase in PKA activity of greater magnitude in mrp4<sup>−/−</sup> MEF cells compared with that occurring in mrp4<sup>+/−</sup> MEF cells. These findings indicate that PKA activity responds to a rapid change in the intracellular level of cAMP stimulated by FSK treatment. In addition, the basal and overall PKA activities are attenuated in mrp4-deficient cells, possibly as a result of a prolonged accumulation of intracellular cAMP.
treatment with the cAMP-modulating agents described in Fig. 3A for 6 h were also quantified. Treatment of \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) MEF cells with FSK or FSK in combination with IBMX produced an increase in the levels of Cox-2 protein in a manner analogous to that of PKA activity (Fig. 3B). These findings suggest that the expression of Cox-2 is under the control of PKA activity and is also attenuated as a result of \( m{r}p4 \) deficiency in MEF cells.

To test whether the attenuation of the PKA activity and Cox-2 expression was caused by a constant increase in basal level of intracellular cAMP in \( m{r}p4 \)-deficient cells, the level of Cox-2 protein after prolonged exposure of MEF cells to IBMX was determined by Western blot analysis (Fig. 3C). Treatment of \( m{r}p4^{+/+} \) MEF cells with IBMX at all tested concentrations for 24 h had no effect on the level of Cox-2. In contrast, the same treatment produced a further decrease in Cox-2 levels in \( m{r}p4^{-/-} \) MEF cells, presumably as a result of a lack of or a slowdown in the removal of accumulated intracellular cAMP by \( m{r}p4 \). This finding confirms that a persistent increase in intracellular cAMP is accountable for the attenuation of PKA activity and Cox-2 expression in \( m{r}p4 \)-deficient cells.

Contribution of Cox-2 and Mrp4 to the Efflux of PGE2 in MEF Cells. MRPs have been shown to mediate the efflux of PGE2 from cells, in an ATP-dependent manner (Reid et al., 2003; Sauna et al., 2004; Rius et al., 2005). To characterize the kinetics of \( m{r}p4 \)-mediated prostaglandin transport and the effects of \( m{r}p4 \) deficiency, the time course of changes in extracellular and intracellular levels of PGE2 in MEF cells was determined, PGE2 levels being measured at 3, 8, and 24 h after cell plating. Extracellular PGE2 rapidly reached the highest level at 3 h followed by a gradual decline between 8 and 24 h (Fig. 4A), with \( m{r}p4^{+/+} \) MEF cells exhibiting a much greater increase in the level of extracellular PGE2 than \( m{r}p4^{-/-} \) cells over the time course of measurement. At 24 h, \( m{r}p4^{-/-} \) MEF cells showed a moderate decline (39% of the highest level reached) in the level of extracellular PGE2, whereas \( m{r}p4^{-/-} \) MEF cells exhibited a greater reduction (62% of the highest level reached) in extracellular PGE2. The kinetics of intracellular PGE2 levels were similar to that of extracellular PGE2 in \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) MEF cells (Fig. 4B). However, both \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) MEF cells exhibited a substantial reduction in the intracellular PGE2 (~65% of the highest levels reached) at 24 h. To illustrate the contribution of \( m{r}p4 \) to the efflux of PGE2, the ratios of extracellular PGE2 to intracellular PGE2 levels were calculated to correct for the differences in the intracellular PGE2 level between \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) cells (Fig. 4C). The ratio for \( m{r}p4^{-/-} \) MEF cells was higher than that of \( m{r}p4^{-/-} \) cells and continued to rise with time, whereas the lower ratio for \( m{r}p4^{-/-} \) cells reached a plateau at 3 h. At 24 h, the ratio for \( m{r}p4^{+/+} \) MEF cells was significantly higher than that of \( m{r}p4^{-/-} \) cells, suggesting that \( m{r}p4 \) contributes to the considerable level of extracellular PGE2 in \( m{r}p4^{-/-} \) MEF cells. The expression of Cox-2 protein in \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) MEF cells was also determined simultaneously with the PGE2 measurements. \( m{r}p4^{+/+} \) MEF cells exhibited a greater induction of Cox-2 protein than \( m{r}p4^{-/-} \) cells at each time point over the course of the measurements (Fig. 4D). The kinetics of Cox-2 induction corresponded closely to that of intracellular PGE2 levels and both \( m{r}p4^{+/+} \) and \( m{r}p4^{-/-} \) MEF cells exhibited a substantial decline in Cox-2 protein levels at 24 h.

The contribution of cAMP-mediated signaling and Cox-2 to extracellular PGE2 was also examined. Treatment of \( m{r}p4^{+/+} \) MEF cells with H-89, a specific PKA inhibitor (Chijiwa et al., 1990), led to a partial decrease (53%) in the level of extracellular PGE2 (Fig. 4E). Furthermore, treatment of \( m{r}p4^{-/-} \) MEF cells with the Cox-2 preferential inhibitor 6-methoxy-2-naphthyl acetic acid (Meade et al., 1993) caused a marked reduction (88%) in the level of extracellular PGE2. Both inhibitors produced a significant reduction in the level of extracellular PGE2 produced by \( m{r}p4^{-/-} \) cells but no significant effects on \( m{r}p4^{+/+} \) MEF cells (one-way ANOVA). Taken together, these findings suggest that the level of extracellular PGE2 is attributable to the combination of \( m{r}p4 \)-mediated efflux PGE2 and cAMP signaling-modulated Cox-2 expression in MEF cells.
Effects of Mrp4 Knockdown by siRNA on the Extracellular and Intracellular Levels of PGE₂ in MEF cells.

To provide further evidence that mrp4 was required for the efflux of PGE₂ in MEF cells, transient knockdown of mrp4 using RNA interference was carried out in mrp4⁻/⁻ MEF cells. Transfection of mrp4⁻/⁻ MEF cells with either of two different mrp4-siRNAs (1 and 2) caused partial but considerable knockdown of mrp4 concurrently with a relatively moderate reduction in Cox-2 (Fig. 5A). This concomitant reduction in mrp4, albeit to a lesser extent, was consistent with that occurring in mrp4⁻/⁻ MEF cells (Fig. 3B). Whether mrp4 knockdown in mrp4⁻/⁻ MEF cells had effects on the transport of PGE₂ was investigated by measuring extracellular and intracellular levels of PGE₂. Transfection of mrp4⁻/⁻ MEF cells with either of the mrp4-siRNAs resulted in a marked decrease (>70%) in the extracellular levels of PGE₂ (Fig. 5B). In contrast, the intracellular levels of PGE₂ were minimally affected by the knockdowns of mrp4 (Fig. 5C). These results corroborate the findings with mrp4 deficient MEF cells, indicating that mrp4 is responsible for the efflux of PGE₂ in MEF cells.

Plasma Levels of PGE Metabolite and Inflammatory Nociceptive Threshold in Mrp4-Deficient Mice. Because a marked reduction in the extracellular levels of PGE₂ occurred in mrp4⁻/⁻ MEF cells relative to mrp4⁺/⁺ MEF cells, we assayed whether the genetic deficiency of mrp4 in mice affected the plasma levels of PGE₁ and PGE₂. Because of the rapid and extensive degradation in vivo, PGEM, the PGE₁ and PGE₂ metabolite present in the plasma, was measured to estimate the level of PGE₂ secreted into the extracellular fluids and the circulation. Plasma samples were collected from six to seven mice of each genotype. Consistent with the finding with MEF cells, mrp4⁻/⁻ mice exhibited an approximate 50% decrease in the levels of PGEM in the plasma relative to wild-type animals (Fig. 6A).

Because PGE₁ and PGE₂ in the periphery enhance nociceptive pathways to produce hyperalgesia or pain hypersensitivity (Burian and Geisslinger, 2005), we measured whether mrp4 deficiency resulted in an altered responsiveness to pain in mice after a local inflammatory challenge. To accomplish this, wild-type and mrp4⁻/⁻ mice were assessed for thermal and mechanical nociceptive thresholds after unilateral intradermal injection of capsaicin, a local irritant producing an inflammatory response. The ipsilateral hindpaws were injected with capsaicin, whereas contralateral hindpaws not injected with capsaicin were used as controls. Both wild-type and mrp4⁻/⁻ mice had a similar latency of paw withdrawal, or baseline threshold, in thermal and me-
mechanical responses without administration of capsaicin (Fig. 6, B and C). It is noteworthy that mrp4−/− mice exhibited a prolonged latency of withdrawal from a thermal source (Fig. 6B) and an increase in tolerance to weight application (Fig. 6C) in ipsilateral hindpaws compared with wild-type animals. These findings suggest that a deficiency in mrp4 causes an increase in the threshold of pain responsiveness possibly attributable to a reduction in peripheral PGE1 and PGE2 levels.

Discussion

The discovery of MRP4 as a transporter of cyclic nucleotides and prostaglandins has important implications in the homeostasis of these signaling molecules inside cells. The efflux properties of MRP4 for cAMP and cGMP provide an alternative or complementary mechanism to the phosphodiesterases as modulators of the intracellular concentration of these second messengers (Chen et al., 2001; Adachi et al., 2002). It has been proposed that MRP4 functions as an overload mechanism for exceedingly high levels of cAMP and is less likely to regulate intracellular cAMP levels because of its relatively low affinity for cyclic nucleotides (Adachi et al., 2002; Borst et al., 2004). However, our results with MEF cell lines deficient in mrp4 demonstrate that a decrease in intracellular cAMP occurs concurrently with an increase in the intracellular level of this cyclic nucleotide under conditions of normal growth and upon stimulation of cAMP synthesis. These findings provide evidence that MRP4/mrp4 can play a role in the regulation of intracellular cAMP levels.

An increase in the level of intracellular cAMP as a result of a deficiency in mrp4 has a significant impact on cAMP-mediated signaling events. Our results demonstrate that abrogation of mrp4 leads to attenuated PKA activity and down-regulated Cox-2 expression in MEF cells. We also show that prolonged treatment with the phosphodiesterase inhibitor IBMX produces a further down-regulation of Cox-2 in mrp4−/− MEF cells. The down-regulation does not occur in wild-type MEF cells exposed to IBMX because mrp4 remains available to bring down any increase in the level of intracellular cAMP. It has also been reported that long-term exposure of cells to a cAMP agonist causes a loss of the C-subunit of PKA and attenuates CREB transcriptional activity as a result of dephosphorylation of CREB at Ser133 (Mayr and Montminy, 2001). Thus, the attenuation of cAMP-mediated signaling in mrp4-deficient cells represents the cellular adaptation to the persistent elevation in intracellular cAMP or acts as a negative feedback mechanism to prevent excessive stimulation of the expression of target genes such as Cox-2.

Our gene knockout and knockdown studies support previously reported findings with overexpressed MRP4 that mrp4 mediates the efflux of PGE2 (Reid et al., 2003; Sauna et al., 2004; Rius et al., 2005). Thus, mrp4 deficiency leads to a pronounced decrease in the levels of extracellular PGE2. Noticeably, mrp4−/− cells still exhibit the kinetics of extracellular PGE2 levels essentially paralleling that of extracellular PGE2 in wild-type MEF cells during the early time points of the measurements. This small rise in extracellular PGE2 levels in mrp4−/− cells, which coincides with the induction of Cox-2 protein after cell plating, may result from outward passive diffusion (Schuster, 2002) or the action of unidentified PGE2 efflux transporters. However, mrp2, which has also been identified to mediate active efflux of PGE2 (de Waart et al., 2006), does not play a role in this case because its expression is not detectable in MEF cells (Lin et al., 2002). A decline in extracellular PGE2 levels in both mrp4−/− and mrp4−/− cells at 24 h is attributable to the combined effects of the prostaglandin transporter-mediated influx (Schuster, 2002) and a decrease in Cox-2 expression.

The data on intracellular levels of PGE2, however, contradicted the assumption that an increase in intracellular PGE2 would occur in the absence of the mrp4 transporter. In fact, the level of intracellular PGE2 in mrp4-deficient cells is low, at approximately half that in mrp4−/− cells. We postulate that this phenomenon is attributable to an increase in the intracellular degradation of PGE2 (Schuster, 2002) and/or a decrease in the de novo synthesis of PGE2 as a result of attenuated Cox-2 expression in mrp4-deficient cells. Similar results were also obtained using the approach of RNA interference-mediated knockdown of mrp4 in wild-type cells. Mrp4 knockdown produces a relatively smaller reduction in basal Cox-2 levels than that occurring in mrp4−/− cells because the depletion of mrp4 by this approach is only partial. For the same reason, the intracellular PGE2 levels may be less affected compared with those occurring in mrp4−/− cells. Collectively, the findings suggest that mrp4 functions as a major efflux transporter for PGE2 and a mechanism for regulating PGE2 synthesis in MEF cells. The reduction in Cox-2 expression occurs secondarily to the absence of mrp4, presumably serving to prevent the build-up of intracellular PGE2 and/or excessive PGE2 synthesis. The interplay between MRP4 and prostaglandin-synthesizing enzymes has been suggested by the recent report of Rius et al. (2005) demonstrating that MRP4 and Cox-2 are coexpressed and colocalized in epithelia cells of the human seminal vesicles, a source of prostaglandins, in the urogenital tract. This finding substantiates our assertion that mrp4 is indispensable for cells actively producing and secreting prostaglandins.

Consistent with the consequences of an mrp4 deficiency in vitro, mrp4−/− mice display an approximate 50% decrease in the plasma level of the PGE metabolite. Mrp2 may be responsible for the remaining 50% plasma level of PGE metabolite produced by mrp4 deficient mice. With respect to tissue distribution, MRp4 is reportedly expressed in the apical membrane of the proximal tubule of the kidney at a high level (van Aubel et al., 2002; Maher et al., 2005). Thus, the notion was that mrp4−/− mice should manifest an increase in the plasma PGE metabolite level because of impaired renal excretion. This contradiction to our finding may be reconciled by the assumption that the renal clearance of PGE metabolite still occurs possibly due to compensation by mrp2 in the kidney. In addition, the overall PGE synthesis and/or secretion by tissues of mrp4−/− mice is reduced. It has been broadly recognized that NSAIDs exert analgesic effects by inhibiting prostaglandin synthesis and preventing the lowering of the nociceptive threshold at both peripheral and central sites (Dionne et al., 2001; Burian and Geisslinger, 2005). Moreover, recent reports demonstrate that mrp4 is inhibited by indomethacin and several other NSAIDs at physiologically relevant concentrations (Reid et al., 2003; El-Sheikh et al., 2007). Therefore, the reduction in plasma PGE metabolite levels in mrp4−/− mice is likely to cause the phenotype of an
increase in nociceptive thresholds in response to mechanical and thermal stimuli. Our findings provide the first evidence that mrp4-mediated prostaglandin transport has physiologic significance in the regulation of inflammatory nociceptive responses in vivo. However, we cannot rule out a possible contribution of altered ion channel activity by PKA in nociceptive neurons (Fitzgerald et al., 1999) to the attenuation of pain responses in mrp4−/− mice. CAMP and cAMP agonists have been implicated in promoting PGE2-induced primary afferent hyperalgesia (Taiwo et al., 1989). Because mrp4 deficiency causes the attenuation of PKA activity and Cox-2 expression in vitro, the involvement of the cAMP-mediated signaling pathway in modulating inflammatory nociceptive responses in vivo will be worthy of further investigation.

References


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