Supplemental material to this article can be found at:
http://jpet.aspetjournals.org/content/suppl/2017/09/01/jpet.117.241646.DC1

1521-0103/363/2/126–135$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2017 by The American Society for Pharmacology and Experimental Therapeutics
https://doi.org/10.1124/jpet.117.241646
J Pharmacol Exp Ther 363:126–135, November 2017

The Journal of PHARMACOLOGY And Experimental Therapeutics

Upregulation of Epac-1 in Hepatic Stellate Cells by Prostaglandin E_2 in Liver Fibrosis Is Associated with Reduced Fibrogenesis[S]

Marlies Schippers, Leonie Beljaars, Eduard Post, Sophie Lotersztajn, Catharina Reker-Smit, Bing Han, Pablo Munoz-Llancao, Martina Schmidt, and Klaas Poelstra

Groningen Research Institute for Pharmacy, Department of Pharmacokinetics, Toxicology and Targeting (M.S., L.B., E.P., C.R.-S., K.P.), and Groningen Research Institute for Pharmacy, Department of Molecular Pharmacology (B.H., P.M.-L., M.S.), University of Groningen, Groningen, The Netherlands; and INSERM U1149, Center for Research on Inflammation, Université Paris Diderot, Paris, France (S.L.)

Received April 28, 2017; accepted August 28, 2017

ABSTRACT

Exchange protein activated by cAMP (Epac-1) is an important signaling mechanism for cAMP-mediated effects, yet factors that change Epac-1 levels are unknown. Such factors are relevant because it has been postulated that Epac-1 directly affects fibrogenesis. Prostaglandin E_2 (PGE_2) is a well-known cAMP activator, and we therefore studied the effects of this cyclo-oxygenase product on Epac-1 expression and on fibrogenesis within the liver. Liver fibrosis was induced by 8 weeks carbon tetrachloride (CCL_4) administration to mice. In the last 2 weeks, mice received vehicle, PGE_2, the cyclo-oxygenase-2 inhibitor niflumic acid (NFA), or PGE_2 coupled to cell-specific carriers to hepatocytes, Kupffer cells, or hepatic stellate cells (HSC). Results showed antifibrotic effects of PGE_2 and profibrotic effects of NFA in CCL_4 mice. Western blot analysis revealed reduced Epac-1 protein expression in fibrotic livers of mice and humans compared with healthy livers. PGE_2 administration to fibrotic mice completely restored intrahepatic Epac-1 levels and also led to reduced Rho kinase activity, a downstream target of Epac-1. Cell-specific delivery of PGE_2 to either hepatocytes, Kupffer cells, or HSC identified the latter cell as the key player in the observed effects on Epac-1 and Rho kinase. No significant alterations in protein kinase A expressions were found. In primary isolated HSC, PGE_2 elicited Rap1 translocation reflecting Epac-1 activation, and Epac-1 agonists attenuated platelet-derived growth factor–induced proliferation and migration of these cells. These studies demonstrate that PGE_2 enhances Epac-1 activity in HSC, which is associated with significant changes in (myo)fibroblast activities in vitro and in vivo. Therefore, Epac-1 is a potential target for antifibrotic drugs.

Introduction

Liver fibrosis is caused by an imbalanced remodeling process due to chronic inflammation associated with excessive scar tissue formation (Seki and Schwabe, 2015). Hepatic stellate cells (HSC), key players in this process, transform from quiescent cells into proliferative, fibrogenic, and contractile myofibroblast-like cells in response to growth factors such as transforming growth factor (TGF)β and platelet-derived growth factor (PDGF)-BB (Seki and Schwabe, 2015). The differentiation and activation of HSC are tightly controlled by the activity of cAMP (Mallat et al., 1998). Increased intracellular cAMP levels inhibit fibroblast migration and proliferation and block the phenotype switch into myofibroblasts, leading to less scar tissue formation (Swaney et al., 2005).

Protein kinase A (PKA) is a well-known second-messenger system that mediates the effects of cAMP. More recently, exchange protein activated by cAMP (Epac) was identified as an important signaling mechanism for cAMP-mediated effects (Insel et al., 2012; Schmidt et al., 2013; Lezoualc'h et al., 2016). Epac is involved in the regulation of key cellular processes, such as calcium handling, neural signaling, inflammation, proliferation, and migration by promoting the exchange of GTP and GDP in the GTPase cycle (Schmidt et al., 2013; Yang et al., 2016; Yu et al., 2016). Two isoforms of Epac have been identified: Epac-1, found in heart, vasculature, brain, kidney, and lungs, and Epac-2, mostly found in brains and adrenal glands. Of interest, Epac-1 is reported to be involved in fibrogenesis. In several reports, significant lower Epac-1 expressions were observed during activation of myofibroblasts (Yokoyama et al., 2008; Insel et al., 2012; Schmidt et al., 2013).

This work was supported by Dutch Technology Foundation STW [VICI Grant GFA.6797], and grants from NanoNext.NL [Program 10.03] and The Netherlands Institute for Regenerative Medicine.
https://doi.org/10.1124/jpet.117.241646.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: ALT, alanine transferase; CCL_4, carbon tetrachloride; CE3F4, 5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2*H*)-quinolinecarboxaldehyde; COX, cyclo-oxygenase; Epac, exchange protein activated by cAMP; HSA, human serum albumin; HSC, hepatic stellate cell; LH, lactosylated HSA; MH, mannosylated HSA; NFA, niflumic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; PH, pPB coupled to HSA; PKA, protein kinase A; PLH, PGE_2-lactosylated HSA; PMH, PGE_2-mannosylated HSA; PMLC, phosphorylated myosin light chain; pPB, peptide derived from PDGF B chain; PPH, PGE_2 coupled to pPB-HSA; pSMAD, phosphorylated SMAD; rHSC, rat hepatic stellate cells; SMA, smooth muscle actin; TGF, transforming growth factor.

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

However, to date, nothing is known about mechanisms or factors that can enhance Epac-1 expression and the consequences of this enhancement for fibrogenic processes in vivo (Insel et al., 2012).

The cyclo-oxygenase (COX) product prostaglandin E_2 (PGE_2) affects cAMP levels directly in most cell types. In fibroblasts, PGE_2 exerts antifibrotic activities through cAMP activation by binding to EP2 or EP4 receptors (Ruwart et al., 1989; Mallat et al., 1998; Weinberg et al., 2009). We therefore investigated the effects of PGE_2 and the COX-inhibitor niflumic acid (NFA) on Epac-1 expression levels in the carbon tetrachloride (CCL_4)-induced mouse model for liver fibrosis. In addition, we used a cell-specific delivery approach to deliver PGE_2 to several resident hepatic cells to identify its most important target cell in vivo. Our studies show a decreased Epac-1 protein expression in fibrotic livers relative to normal livers. These Epac-1 levels were restored by PGE_2 delivery to HSC, but not by its delivery to hepatocytes or Kupffer cells. In HSC this was associated with reduced fibrogenic activity both in vitro and in vivo. Our studies indicate a significant role for Epac-1 during liver fibrosis and delineate a pathway to enhance these Epac-1 levels.

Materials and Methods

Synthesis of Drug Carriers and PGE_2 Constructs

PDGFβ-receptor-recognizing peptides [peptide derived from PDGF B chain (pPB); prepared by Ansynth Service BV, Roosendaal, The Netherlands], mannose (p-aminophenyl-a-D-mannopyranoside; Sigma-Aldrich, St. Louis, MO), and lactose (Merck, Darmstadt, Germany) were covalently coupled to human serum albumin (HSA) to yield, respectively, pPB coupled to HSA (PH), lactosylated HSA (LH), or mannosylated HSA (MH), as previously described (Beljaars et al., 1998, 2003).

PGE_2 (Cayman Chemicals, Ann Arbor, MI) was coupled to PH, LH, and MH, yielding PGE_2 coupled to pPB-HSA (PPH), PGE_2-lactosylated HSA (PLH), and PGE_2-mannosylated HSA (PMH). First, the carboxylic acid group of PGE_2 was activated by N,N-dicyclohexylcarbodiimide and N-hydroxysuccinimide. This PGE_2/N,N-dicyclohexylcarbodiimide/hydroxysuccinimide solution was reacted overnight at room temperature with PH, LH, or MH (molar ratio HSA:PGE_2 = 1:60). All products were subsequently dialyzed for at least 24 hours against phosphate-buffered saline (PBS) using a 10-kDa dialysis cassette (Slide-A-Lyzer 10K; Pierce Biotechnology, Rockford, IL) and further purified using chromatographic techniques. Conjugates were lyophilized and stored at −20°C. The amount of PGE_2 coupled to each carrier was assessed by mass spectrometry.

Cell Experiments

Primary rat HSC were isolated from livers of male Wistar rats (>400 g; Harlan, Zeist, The Netherlands), as described (Beljaars et al., 2003), and cultured on plastic for 10 days to obtain an activated cell type.

HepG2, RAW264.7, NIH/3T3 fibroblasts (all derived from American Type Culture Collection, Manassas, VA), and LX2 cells were used as model cells for, respectively, hepatocytes, macrophages, and HSC. Data are presented as the mean of three experiments, each performed in duplicate.

Concentrations used (unless otherwise stated) were 5 μm PGE_2, 50 μM Epac-1 agonist (8-pCPT-2′-O-Me-cAMP; Biolog, Bremen, Germany), 500 μM PKA agonist (N^6-benzoyladenosine-3′,5′-cyclic monophosphate sodium salt; Biolog), 50 ng/ml PDGF-BB (PeproTech, Rocky Hill, NJ), and 5 ng/ml TGFβ (Roche Diagnostics, Mannheim, Germany).

Proliferation Assay. Primary rat HSC (2×10^5 cells/well) were seeded in 12-well plates and grown for 2 days. After 24-hour starvation, PGE_2, Epac-1, or PKA agonist was added along with PDGF-BB. After 18 hours, ^{3}H-thymidin (0.25 μCi/ml) was added for 6 hours. Cells were washed, fixed with 5% trichloroacetic acid, and lysed with 1 M NaOH, and radioactivity was counted.

Migration Assay. Primary rat HSC (6×10^4 cells/well) were cultured in Transwell chambers (8 μm pore size; Costar, New York, NY) with PGE_2 or agonists. PDGF-BB was added to the lower chamber. After 24 hours, membranes were fixed and stained with hematoxylin. Cells on both sides of the membrane were counted in at least five fields/membrane (magnification, 40×). Migration was calculated as percentage of cells in the lower chamber relative to total cell number.

In Vitro Effects of PGE Conjugates. HepG2 cells (150,000 cells/well) were incubated with PGE_2 and equimolar amounts of PLH for 60 minutes. Cells were processed and used for Western blot analysis using vasodilator-stimulated phosphoprotein-1 antibody. RAW264.7 cells were incubated with 100 ng/ml lipopolysaccharide plus 10 μM PGE_2, equimolar amounts of PMH, or vehicle. After 24 hours, medium was harvested and total nitric oxide production was assessed (Melgert et al., 2001). LX2 cells were grown to confluency in 12-well plates. A standard scratch was made, after which cells were incubated for 24 hours with 10 ng/ml PDGF-BB plus 5 μM PGE_2, equimolar amounts of PPH, or vehicle. The scratch size was measured using imaging techniques at t = 0 and t = 24 hours, yielding the percentage wound healing.

Epac-1 and PKA Expression. The 3T3 fibroblasts (100,000 cells/well) were incubated with PGE_2 (0.1, 1, 5, and 10 μM) for 6, 24, and 48 hours. Cells were homogenized and processed for Western blot and polymerase chain reaction (PCR) analysis.

Rap1 Expression. The 3T3 cells (25,000 cells/well), seeded in glass labteks, were incubated with 10 μM PGE_2 and 20 μM Epac-1 antagonist 5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2*H*)-quinolinecarboxaldehyde (CE3F4) (Courilleau et al., 2012) for 60 minutes. After fixation in acetone/methanol (1:1), Rap1 was stained (overnight, 4°C) using the primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany), and subsequently visualized using Alexa488-goat anti-rabbit IgG (ThermoFisher Scientific; Landsmeer, The Netherlands) and 4′,6′-diamidino-2-phenylindole.

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen (Groningen, The Netherlands).

CCL_4 Model. Male BALB/c mice (20–22 g; Harlan) received CCL_4 for 8 weeks, according to standard protocols (Beljaars et al., 2003). At weeks 7 and 8, mice also received (i.v., 3 times/week) 0.5 mg/kg PGE_2, 5 mg/kg NFA, vehicle (PBS), PPH, PH, PMH, MH, PLH, or LH (n = 6–9/group). All animals received the same PGE_2 dose either free or bound to the albumin drug carrier. Mice were sacrificed at week 8. Two animals from each group received i.v. a PGE_2-loaded carrier 15 minutes prior to sacrifice to check whether the PGE_2-carrier conjugate still accumulated in the designated target cell. The liver enzymes alanine transaminase (ALT) and aspartate transaminase, reflecting liver damage, were measured in plasma according to routine methods at the clinical chemistry laboratory of the University Medical Center Groningen (Groningen, The Netherlands).

Human Tissue

Human liver tissue samples were obtained from the Department of Surgery and Liver Transplantation (University Medical Center Groningen). All patients signed an informed consent for the use of residual tissue for research purposes. Experimental protocols were approved by the Medical Ethical Committee, and anonymized tissue samples were used according to Dutch guidelines (http://www.federa.org/gedragscodes-codes-conduct-en). Normal human liver tissue was obtained from donor livers discarded for transplantation for technical reasons. Cirrhotic human liver tissue was obtained from patients undergoing liver transplantation, and indications for transplantation were primary sclerosing cholangitis, primary biliary cirrhosis, congenital cirrhosis, alcohol-induced liver disease, acute liver failure, and Wilson's cirrhosis. All human liver material was anonymized, and available patient characteristics are listed in Table 1.

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

Tissue Analysis

Immunohistochemistry. Cells and cryostat sections were stained according to standard methods. Primary antibodies used in this study were as follows: mouse anti–α-smooth muscle actin (SMA) and anti-desmin from Sigma-Aldrich; goat anti-collagen I from Southern Biotechnology Associates (Birmingham, AL); antiphosphorylated myosin light chain (PMLC)2 (Ser19) from Cell Signaling Technology (Danvers, MA); rat anti-CD68 (AbD Serotec, Dusseldorf, Germany); rabbit anti-CYP2E1 (Millipore, Darmstadt, Germany); and goat anti-HSA (MPbiomedicals, Eschwege, Germany). Species-specific horseradish peroxidase and fluorescein isothiocyanate/tetramethylrhodamine isothiocyanate–conjugated secondary antibodies were from Dako (Glostrup, Denmark). Stainings were quantified by analyzing complete sections from three different liver lobes of each animal at magnification 10 × 10 using Cell D image-analyzing software (Olympus, Hamburg, Germany).

Western blot analysis was performed according to standard methods with 100 μg protein loaded on a 12% gel and using the primary antibodies, as follows: goat anti–phosphorylated SMAD (pSMAD) 2/3 and goat anti–Epac-1 (Santa Cruz Biotechnology); goat anti-PKA [Cα] (BD Transduction Laboratories, Vianen, The Netherlands); and mouse anti–α-SMA, goat anti-collagen I, and β-actin (Sigma-Aldrich). Signals were quantified with Genetools (Syngene, Cambridge, UK) using ECL reagent (Perkin-Elmer, Boston, MA). Of each animal, samples from three different liver lobes were analyzed and corrected for the housekeeping gene β-actin.

Quantitative Real-Time PCR. Total RNA was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using cDNA synthesis kit (Promega, Leiden, The Netherlands). Reverse transcription PCR was performed using SYBR Green PCR Master Mix (ThermoFisher Scientific) or SensiMix SYBR kit (Bioline, London, UK), according to standard methods, with β-actin as housekeeping gene. Primers (Sigma Genosys, Haverhill, UK) used were as follows: α-SMA (forward: 5′-ACTACTGCCGAGCGTGAGAT-3′, reverse: 5′-CCAATGAAAGATGGCTGGAA-3′), collagen 1a1 (forward: 5′-TGACTGGAAGAGCGGAGAGT-3′, reverse: 5′-ATCCATCGGTCATGCTCTCT-3′), Epac-1 (forward: 5′-CAGTGCTGCTCTGGCCGGGA-3′, reverse: 5′-GTTCCTGCAGGCTGGGGCTC-3′), human EPAC-1 (forward: 5′-CATGTGAAACACGACTGGGC-3′, reverse: 5′-GAGGTCCAGCTCTTCATCCG-3′), PKA (catalytic subunit) (forward: 5′-GGTTCAGTGAGCCCCACGCC-3′, reverse: 5′-GGGGGTCCCACACAAGGTCCA), and β-actin (forward: 5′-GGCATCCTGACCCTGAAGTA-3′, reverse: 5′-GGGGTGTTGAAGGTCTCAAA-3′).

PGE_2 Enzyme-Linked Immunosorbent Assay. Liver samples were homogenized, and PGE_2 amounts were assessed using the monoclonal PGE_2 enzyme immunoassay kit (Cayman Chemicals), according to manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± S.E.M. Statistical analyses were performed using the Mann–Whitney test and considered significant at $P < 0.05$.

TABLE 1
Patient characteristics of the human livers used in this study

	Normal Livers	Cirrhotic Livers
	N = 7	N = 6
Age (min–max) years	41 (10–57)	49 (35–66)
Gender	n = 4: F	n = 3: F
	n = 2: M	n = 1: M
	n = 1: not known	n = 2: not known

F, female; M, male; max, maximum; min, minimum.

Results

PGE_2 and NFA Affect Liver Fibrosis in CCL_4-Treated Mice. Mice received CCL_4 for 8 weeks to induce liver fibrosis. During the last 2 weeks of CCL_4 administration, the mice were treated with PGE_2 (n = 9), the COX-2 inhibitor NFA (n = 6), or vehicle (PBS; n = 9). Normal mice, receiving only PBS, served as control (n = 9). Plasma levels for ALT and aspartate transaminase indicated significant liver damage in all CCL_4-treated mice (see Table 2). No major differences in damage were seen between fibrotic animals receiving different compounds in the final 2 weeks, although a reduced average in plasma ALT level was seen in NFA-treated animals ($P < 0.05$). Markers reflecting HSC activation (α-SMA) and matrix deposition (collagen I) were examined both at the mRNA (Supplemental Fig. 1) and protein level (Fig. 1). Immunohistochemistry and reverse transcription PCR analysis showed that PGE_2-treated mice displayed significantly less intrahepatic α-SMA and collagen I expression levels compared with CCL_4 mice receiving no treatment (Fig. 1, A–D). In contrast, NFA induced a significant increase in α-SMA and collagen I protein levels in fibrotic mice compared with CCL_4 mice receiving no treatment. pSMAD2/3 expression levels, indicating TGFβ signaling, were strongly enhanced in CCL_4-receiving mice treated with PBS (Fig. 1E). Treatment with PGE_2 significantly reduced these levels, whereas NFA administration enhanced pSMAD2/3 levels in fibrotic animals.

Measurement of intrahepatic PGE_2 levels revealed that CCL_4 mice displayed increased intrahepatic PGE_2 levels relative to healthy animals ($P < 0.01$, Fig. 1F). PGE_2 treatment significantly reduced these levels compared with untreated fibrotic mice ($P < 0.01$). NFA treatment completely abolished intrahepatic PGE_2 production in fibrotic animals.

PGE_2 Enhances Epac-1 Expression and Attenuates Rho Activity In Vivo. Previous in vitro studies showed that PGE_2 activates cAMP in myofibroblasts (Huang et al., 2008). We now examined intrahepatic expression levels of both cAMP mediators PKA and Epac-1 in CCL_4-treated mice. In fibrotic livers, a significant reduction in Epac-1 protein expression levels was observed relative to healthy mice livers (Fig. 2A), in agreement with previous reports (Yokoyama et al., 2008; Insel et al., 2012; Schmidt et al., 2013). PGE_2 treatment completely restored Epac-1 levels in fibrotic livers. In contrast, NFA treatment further reduced Epac-1 levels in fibrotic animals. Western blot analysis did not reveal significant changes in PKA expression levels in CCL_4-treated animals as compared with normal mice, and expression levels for PKA were also not affected by PGE_2 or NFA treatment.

We also examined human livers for Epac-1 expression. Western blots revealed that Epac-1 expression levels were significantly reduced also in human fibrotic livers, irrespective of etiology, compared with control livers (Fig. 2C).

Rho kinase is reported to be a downstream target of Epac-1 (Roscioni et al., 2011; Schmidt et al., 2013). Rho kinase in turn stimulates fibroblast-to-myofibroblast transdifferentation, migration, and contraction of these cells via phosphorylation of the myosin light chain (i.e., formation of PMLC) (Mack et al., 2001). This PMLC stimulates myosin II ATPase activity and contraction of actin fibers in HSC, leading to portal hypertension, one of the hallmarks of fibrosis (Klein et al., 2012). Recently, reports showed that Epac-1 inhibits phosphorylation of myosin light chain. In fibrotic mice, clear

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

TABLE 2
Plasma ALT and aspartate aminotransferase levels (avg. ± S.E.M.) in each experimental group at the time of sacrifice

	AST (U/L)	ALT (U/L)
Healthy	33 ± 9	13 ± 3
CCL_4	572 ± 129 (**relative to healthy)	182 ± 37 (***relative to healthy)
CCL_4 + PGE_2	326 ± 117 (ns relative to CCL_4)	152 ± 34 (ns relative to CCL_4)
CCL_4 + NFA	704 ± 118 (ns relative to CCL_4)	62 ± 12 (*relative to CCL_4)

AST, aminotransferase; ns, nonsignificant.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

staining for PMLC in the fibrotic bands was visible, whereas little or no expression was seen in healthy animals (Fig. 2D). PMLC staining colocalized with desmin staining, the marker for HSC. Fibrotic animals treated with PGE$_2$ displayed no PMLC staining in fibrotic bands or in any desmin-positive cell at all.

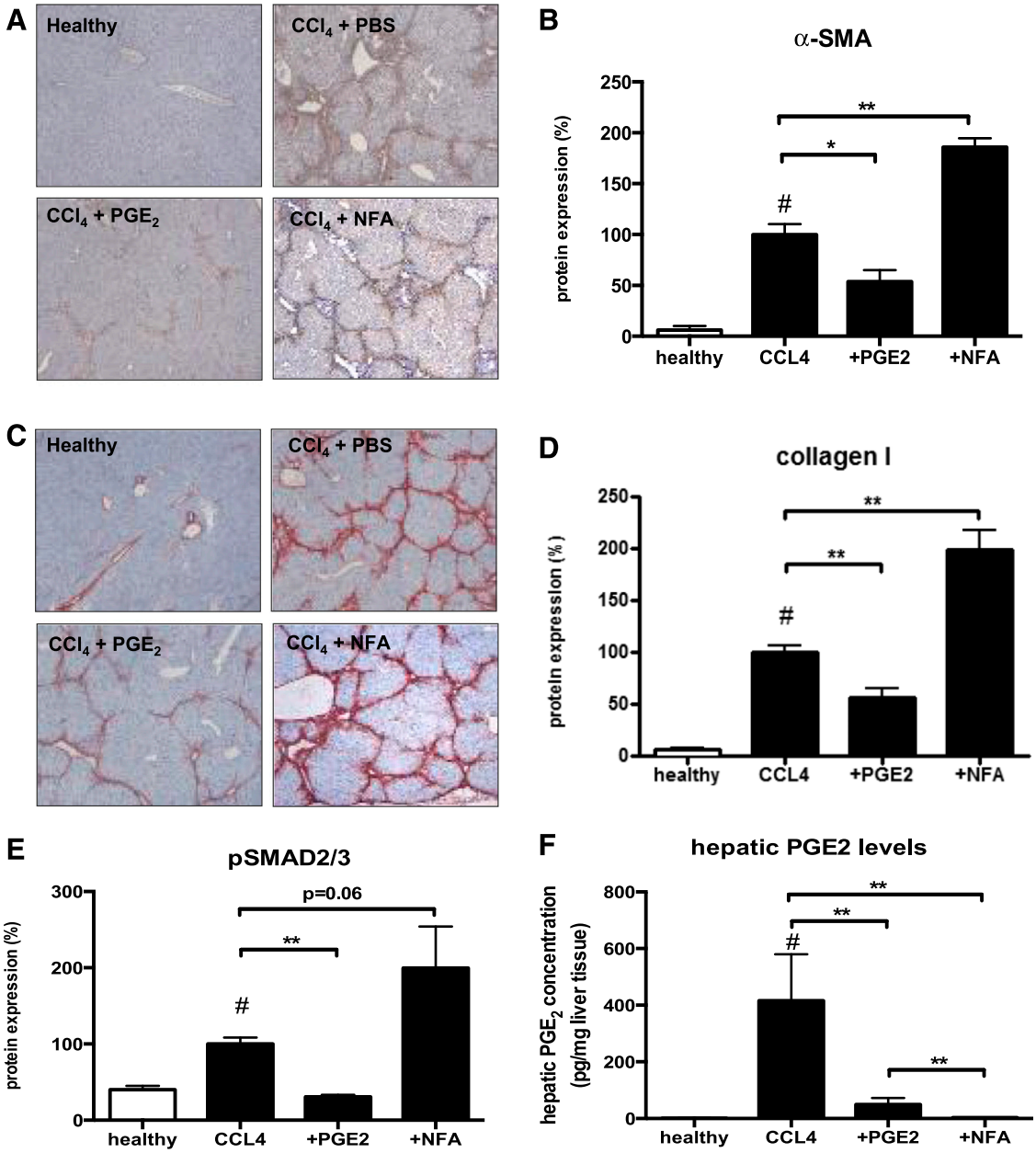

Fig. 1. Effect of PGE$_2$ and NFA (COX inhibitor) on fibrosis-related parameters in mice with CCL_4-induced liver fibrosis. Representative pictures of (A) α-SMA and (C) collagen type I staining in healthy and CCL_4 mice receiving treatment with PBS, PGE$_2$, or NFA (original magnification, 40×). Quantitative analysis of (B) α-SMA– and (D) collagen-stained liver sections. (E) Expression of pSMAD2/3, as analyzed by Western blot. (F) Analysis of hepatic PGE$_2$ levels measured by enzyme-linked immunosorbent assay in healthy mice and fibrotic mice treated with PBS, PGE$_2$, or NFA (*$P < 0.05$, **$P < 0.01$ compared with CCL_4; $^{\#}P < 0.05$ compared with healthy; n = 6–9 per group).

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

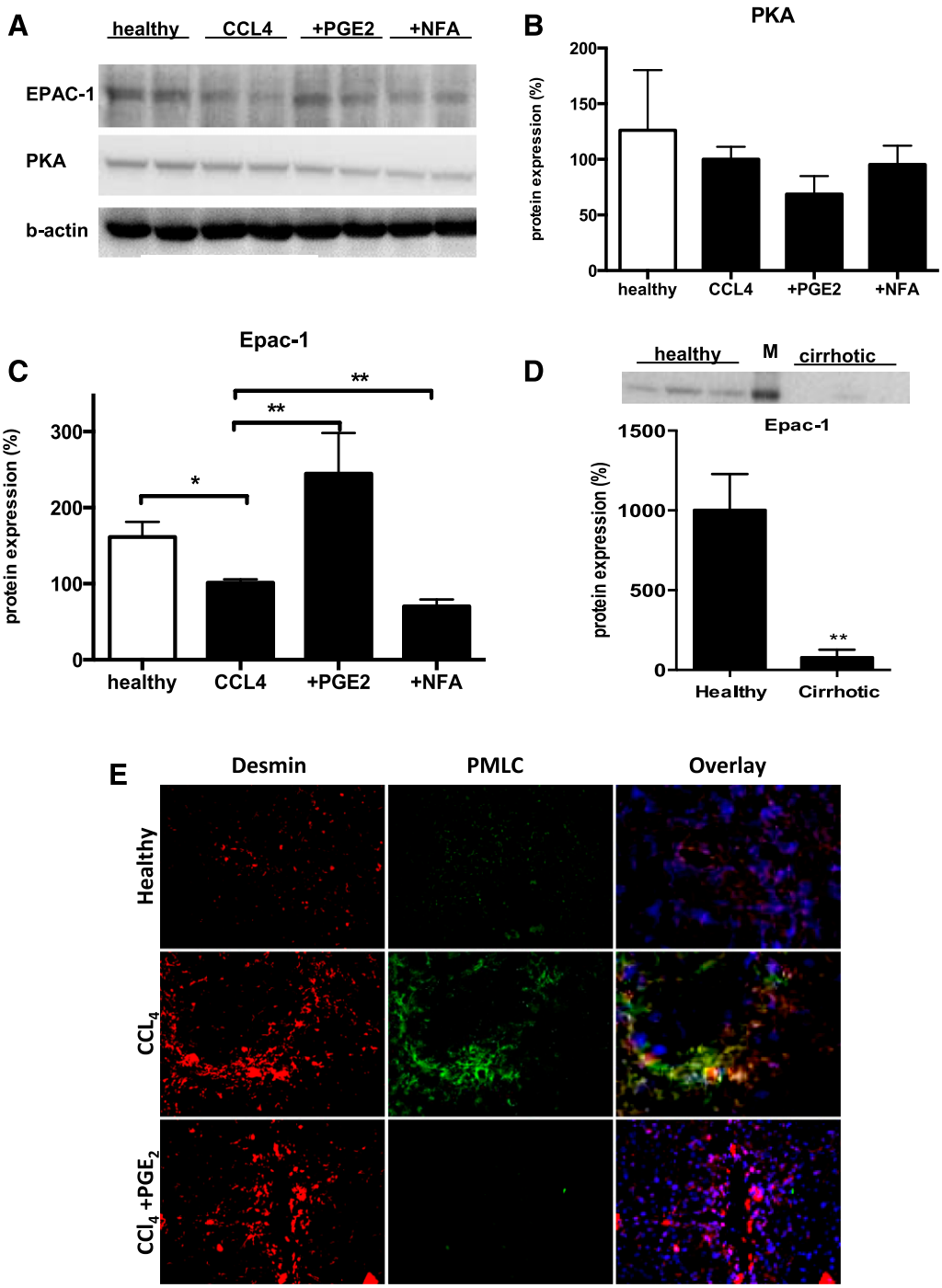

Fig. 2. Expression of EPAC-1, PKA, and PMLC in fibrotic livers. Effects of PGE_2 and NFA on intrahepatic expression of EPAC-1 (A and C) and PKA (A and B) in fibrotic mice, as determined by Western blot analysis. (D) Epac-1 expression in healthy and cirrhotic human livers. (E) Rho kinase activity, as reflected by PMLC staining, and desmin staining, as marker for HSC, in fibrotic livers. Note the colocalization of desmin and PMLC and the absence pf PMLC staining after PGE_2 treatment (original magnification, 100×). $^*P < 0.05$; $^{**}P < 0.01$.

Collectively, our data show that PGE_2 attenuated α-SMA and collagen I expression in fibrotic mice, which is associated with an increase in Epac-1 levels and inhibition of its downstream target Rho kinase compared with untreated fibrotic mice. In contrast, NFA increased HSC activation and collagen deposition and further reduced Epac-1 expression in fibrotic animals.

Cell-Specific Delivery of PGE_2 to Resident Hepatic Cells. Epac-1 proteins are expressed in many resident and circulating cells. We therefore used a drug-targeting approach to assess which cell type is involved in the observed effects on intrahepatic Epac-1 levels and fibrogenesis. We therefore coupled PGE_2 to cell-specific carriers directed at either HSC, Kupffer cells, or hepatocytes (Fig. 3A). Lactosylated human serum albumin (LH), which binds to the asialoglycoprotein receptor, is a well-known carrier to hepatocytes (Beljaars et al., 1998). PDGFβ receptors and mannose receptors are highly expressed on respectively activated HSC (Seki and Schwabe, 2015) and macrophages (Beljaars et al., 1998;

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

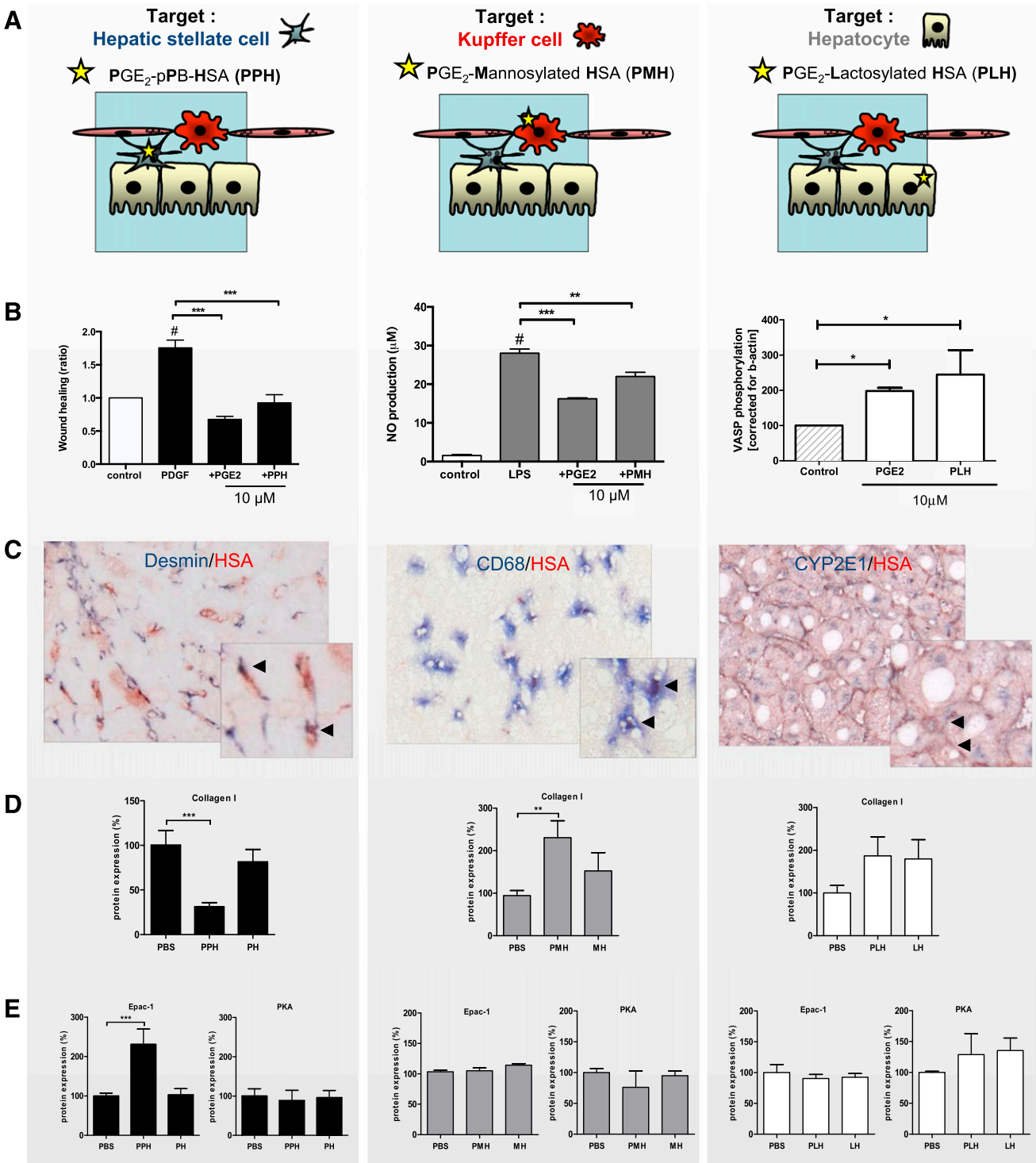

Fig. 3. Cell-specific effects of PGE$_2$ targeted to HSC (PPH), to Kupffer cells (PMH), or to hepatocytes (PLH) on fibrogenic markers and on Epac-1 and PKA expressions in fibrotic livers in mice. (A) Schematically outlines the different constructs and target cells. (B) Pharmacological effects of PGE$_2$ coupled to pPB-HSA (PPH), to mannosylated HSA (PMH), or to lactosylated HSA (PLH) in cell cultures. A wound-healing assay in cultures of fibroblasts was used to test the activity of PPH. The biologic activity of PMH was assessed by lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophages, and the phosphorylation of vasodilator-stimulated phosphoprotein in HEPG2 cells was used to assess the activity of PLH. (C) Double staining for the PGE$_2$ conjugate (with an antibody directed against HSA) and the cellular marker desmin (marker for HSC), CD68 (marker for KC), or CYP2E1 (hepatpocyte marker). Arrowheads show double staining for desmin and PPH, double staining of CD68 and PMH, and double staining for CYP2E1 and PLH in livers of fibrotic mice, 30 minutes after injection of the compounds (original magnification, 20 × 10, inserts 40 × 10). (D) Protein

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

Melgert et al., 2001), which both are abundantly present in fibrotic livers. These receptors are suitable targets for respectively HSC and KC delivery. Therefore, we coupled PDGFβ receptor-recognizing peptides to HSA (pPB-HSA) to obtain a HSC-selective carrier and attached mannose to HSA (MH) to reach macrophages. The cell specificity of all these carriers has been described in vivo in earlier reports (Beljaars et al., 1998, 2003; Melgert et al., 2001). PGE_2 was subsequently coupled to each carrier. Cell specificity of carriers was retained after attachment of PGE_2: PGE_2 attached to pPB-HSA (=PPH) was found in Desmin-positive cells, reflecting HSC, 15 minutes after its administration to fibrotic mice. Mannosylated HSA (=PMH) was found in CD68-positive KC cells and lactosylated HSA (=PLH) costained with the hepatocyte marker (CYP2E1) (Fig. 3C). All three PGE_2 conjugates, i.e., PPH, PMH, and PLH, also were pharmacologically active compounds as tested in cell cultures: PPH significantly attenuated PDGF-induced activities of fibroblasts in vitro, PMH significantly attenuated the lipopolysaccharide-induced nitrogen oxide species production of RAW264.7 macrophages, and PLH induced vasodilator-stimulated phosphoprotein phosphorylation in HEPG2 cells, all similar to free PGE_2 (Fig. 3B).

Fibrotic mice treated for 2 weeks with the HSC-selective PGE_2 conjugate PPH displayed a significant reduction in collagen I expression compared with untreated fibrotic mice (Fig. 3D). Mice receiving the carrier alone (i.e., PH) displayed no such reduction. Treatment of fibrotic mice with the KC-selective PMH conjugate or the carrier MH alone led to the opposite; upregulation of collagen I compared with the vehicle group was found. Treatment with the hepatocyte-selective PLH conjugate or the carrier alone (LH) did not induce significant alterations in collagen I deposition.

Subsequently, we studied Epac-1 and PKA expression levels in mice treated with PGE_2 conjugates or their respective control compounds. Only in livers of PPH-treated mice did we find significant upregulation of Epac-1 expression compared with PBS-treated fibrotic mice (Fig. 3E). All other treatments did not induce any alteration in Epac-1 expression. PKA expression was not different from untreated fibrotic mice in any of the groups (Fig. 3E). The increase in Epac-1 expression in mice treated with the HSC-selective PGE_2 conjugate was, similar to PGE_2 alone (see also Fig. 2E), associated with a significant reduction in PMLC expression in HSC (desmin-positive cells) in fibrotic livers (Fig. 4). Other treatments (i.e., PLH and PMH) and controls did not induce a change in hepatic PMLC staining relative to untreated fibrotic mice (data not shown). This indicates that HSCs are the key players of PGE_2-mediated effects on Epac-1 levels and fibrogenic processes within fibrotic livers.

Collectively, these drug-targeting studies show that HSC, rather than Kupffer cells, hepatocytes, or circulating immune cells, are the effector cells of PGE_2-induced changes in hepatic Epac-1 levels. These data also confirm the association between Epac-1 and Rho kinase activity in HSC in vivo.

PGE_2 Induces Rap1 Translocation In Vitro. To test whether PGE_2 is able to directly activate Epac-1 in fibroblasts, we added PGE_2 to 3T3 cells and examined Epac-1 expression at the mRNA and protein level as well as Epac-1 activation. No change in Epac-1 levels at mRNA nor protein levels was found (data not shown). However, a change in Rap1 staining was noted (Fig. 5). Epac-1 is known to induce translocation of Rap1 from the cytosol to plasma membranes (Yang et al., 2016). Figure 5 shows Rap1 staining evolving from a general weak cytoplasmatic staining within fibroblasts to a more concentrated intense staining along membranes after addition of PGE_2, indicating relocalization and concentration of Rap1. In turn, the PGE_2-induced relocalization was inhibited by the Epac-1 inhibitor CE3F4 (Courilleau et al., 2012). This indicates the involvement of Epac-1 in this PGE_2-mediated effect.

Effects of Epac-1 and PKA Agonists in Primary HSC. Our in vivo data implicate that fibrogenic mediators reduce Epac-1 expression, whereas Epac-1 enhancement leads to inhibition of HSC activities. We tested this in vitro using primary HSC, isolated from rats. In these primary cells, PDGF-BB and TGFβ significantly reduced Epac-1 mRNA expression levels compared with unstimulated cells, whereas no changes in PKA mRNA levels were seen (Fig. 6A). Subsequently, the PKA agonist N^6-benzoyladenosine-3′,5′-cyclic monophosphate sodium salt and the Epac-1 agonist 8-pCPT-2′-O-Me-cAMP (Roscioni et al., 2009, 2011) were used to study the effects of the Epac-1 and PKA signaling pathway on PDGF-induced migration and proliferation in recombinant HSC (rHSC). Both agonists attenuated PDGF-induced migration of rat hepatic stellate cells (rHSC) (Fig. 6B). The Epac-1 agonist also inhibited PDGF-induced proliferation in rHSC, whereas the PKA agonist had no effect (Fig. 6B).

Discussion

This study demonstrates the involvement of the cAMP effector Epac-1 in the regulation of hepatic fibrogenesis in vitro and in vivo. Epac proteins, alone or in concert with PKA, are closely involved in the regulation of pivotal processes, including cell survival, proliferation and differentiation, immune responses, and signaling cascades (Yokoyama et al., 2008; Roscioni et al., 2011; Lezoualc'h et al., 2016). Despite these activities, little is known about Epac during liver fibrosis in vivo. The relevance of Epac-1 during fibrogenesis has been inferred from studies showing a reduced Epac-1 expression after fibroblast activation in several organs (Roscioni et al., 2011; Insel et al., 2012; Schmidt et al., 2013; Lezoualc'h et al., 2016). It was therefore suggested that upregulation of Epac-1 might attenuate fibrosis (Insel et al., 2012; Schmidt et al., 2013). However, methods to achieve such an upregulation were unknown (Insel et al., 2012). Our in vivo experiments in fibrotic mice now show that Epac-1 can be upregulated in HSC by PGE_2. This is associated with reduced fibrogenesis in vivo.

PGE_2 was found to exert significant antifibrotic effects in mice with liver fibrosis, as reflected in this study by reduced collagen I, α-SMA, and pSMAD2/3 levels. The COX-2 inhibitor NFA induced the opposite effects on these parameters. This profibrotic effect cannot be explained by increased damage induced by NFA (see Table 2). Also, others found a profibrotic effect of COX-2 inhibitors (Gilroy et al., 1999), but the mechanism behind this always has been unclear. Our studies suggest a role for Epac-1 in this cascade of cyclo-oxygenase–induced effects.

levels for collagen type I in livers of fibrotic mice treated with PPH (n = 9/group), PMH (n = 6/group), or PLH (n = 6/group) measured by Western blot analysis. (E) Hepatic protein levels for cAMP effectors Epac-1 and PKA in mice after treatment with the different PGE_2 constructs. Vehicle (PBS) or drug carriers without PGE_2 (i.e., PH, MH, or LH) served as controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, #$P < 0.05$ as compared to control.

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

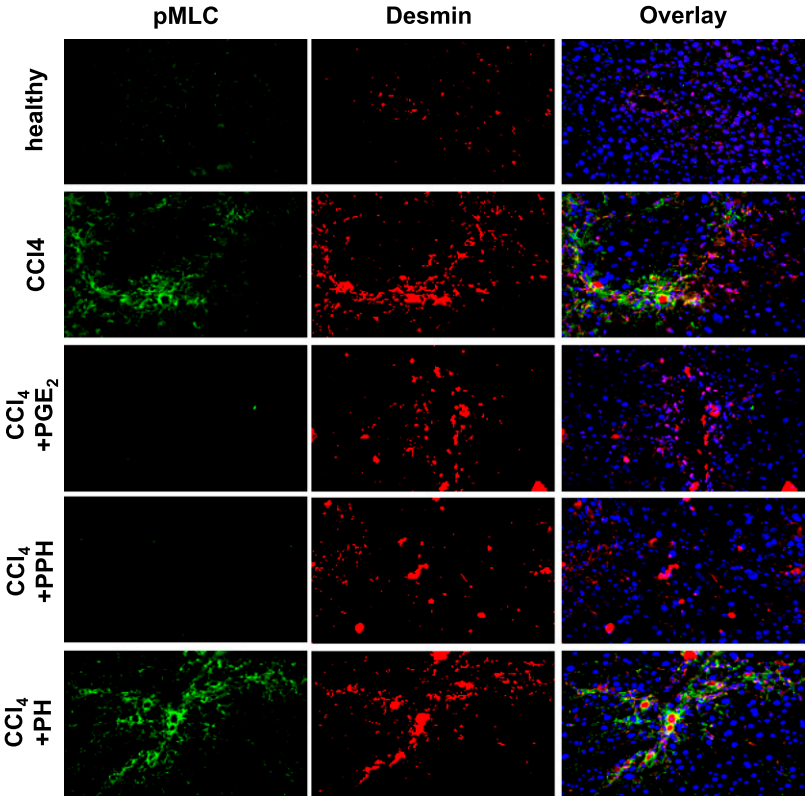

Fig. 4. Effects of PGE$_2$ targeted to HSC (PPH), free PGE$_2$, or carrier (PH) on intrahepatic pMLC expression (reflecting Rho kinase activity) in fibrotic livers in vivo. Representative pictures of immunofluorescent staining for PMLC (=Rho kinase marker: green) and desmin (HSC marker: red). Strong staining for PMLC can be seen in desmin-positive septae, but this staining is absent in fibrotic animals treated with PPH and PGE$_2$.

PGE$_2$ signals through intracellular cAMP inducing PKA or Epac-1 activation. Whereas PKA levels did not change in our studies, Epac-1 dropped significantly during hepatic fibrogenesis in mice and after HSC activation in vitro, confirming earlier reports (Insel et al., 2012). A limited survey in human tissue samples indicates that this reduction also occurs in patients with different liver diseases. PGE$_2$ and NFA displayed opposite effects in fibrotic livers: a strong upregulation of Epac-1 was seen after PGE$_2$ treatment, and a downregulation after NFA treatment. NFA treatment was shown to abolish intrahepatic PGE$_2$ production, confirming effective inhibition. These observations suggest that the antifibrotic effects of PGE$_2$ are, at least partly, mediated by Epac-1.

Our data seemingly reveal a contradiction. We showed that PGE$_2$ administration enhanced Epac-1 expression in fibrotic livers, yet liver fibrosis is associated with high hepatic PGE$_2$ levels (Fig. 1F) and reduced Epac-1 expression (Fig. 2C). Moreover, PGE$_2$ treatment attenuated intrahepatic PGE$_2$ production, yet enhanced Epac-1 levels. It is clear that total intrahepatic PGE$_2$ content does not correlate with Epac-1 or fibrosis. Of note, the plasma half-life of the administered PGE$_2$ (5 minutes) is too short to be detectable at the time of sacrifice (24 hours after injection). PGE$_2$ and NFA are both anti-inflammatory compounds and consequently can both attenuate PGE$_2$ production by inflammatory cells (Gilroy et al., 1999). At the same time, they have opposite effects on fibrogenesis, HSC activation, and Epac-1 levels (see Figs. 1 and 2A). Cell-specific delivery of PGE$_2$ to HSC did enhance intrahepatic Epac-1 levels and attenuated fibrogenesis and HSC activation. Therefore, it can be deduced that PGE$_2$ affects fibrosis via an effect on Epac-1 levels specifically within HSC, irrespective of the total PGE$_2$ content within livers.

One of the downstream targets of Epac-1 is Rho kinase (Yu et al., 2016), which was found to be downregulated by Epac-1 (Roscioni et al., 2011; Rajagopal et al., 2013; Schmidt et al., 2013). Rho kinase stimulates the transdifferentiation of HSC into collagen-producing myofibroblast-like cells via phosphorylation of its downstream substrate, myosin light chain (Mack et al., 2001). This prompted us to examine PMLC levels in fibrotic livers. Indeed, PMLC was abundantly expressed in desmin-positive cells in fibrotic septa in livers of untreated mice. In contrast, in fibrotic mice treated with PGE$_2$, PMLC

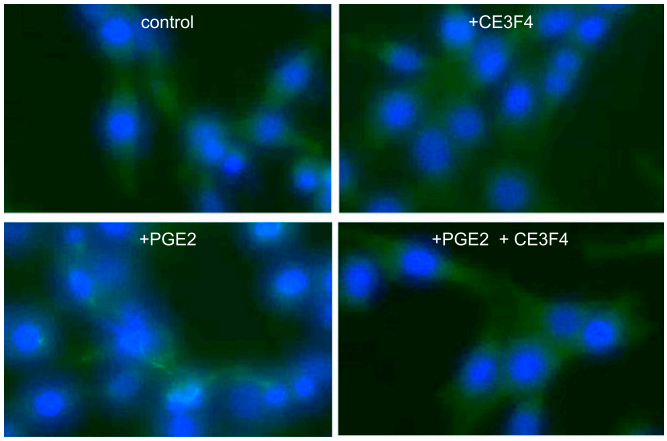

Fig. 5. Effects of PGE$_2$ on intracellular localization of Rap1 in NIH/3T3 cells. Pictures show fluorescent staining for Rap1, which is a downstream effector of Epac-1. Note the more intense staining along the plasma membranes in PGE$_2$-treated cultures, which is reversed by addition of the Epac-1 inhibitor CE3F4 (original magnification, 40 × 10).

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

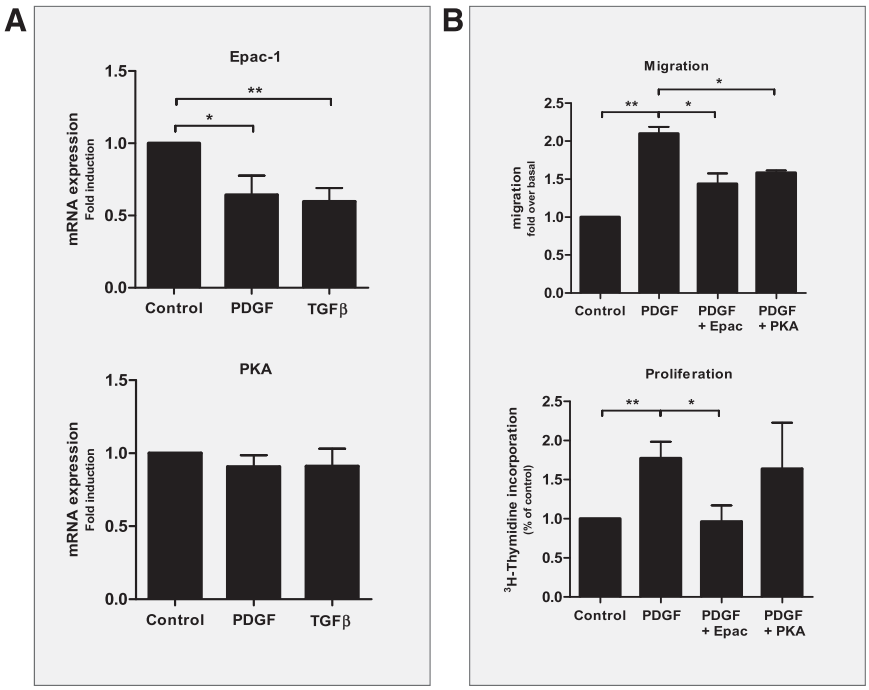

Fig. 6. In vitro effects of PDGF-BB and TGFβ on Epac-1 and PKA expression (A) and effects of Epac-1 and PKA agonists on PDGF-induced biologic responses in fibroblasts (B). (A) Quantitative real-time PCR analysis of Epac-1 and PKA mRNA expression levels after PDGF- or TGFβ-induced activation of primary HSC, normalized to β-actin expression. (B) The effects of Epac-1 agonist 8-pCPT-2′-O-Me-cAMP and the PKA agonist 6-Bnz-cAMP on PGDF-induced migration and proliferation in primary HSC. Data are presented as the mean (±S.E.M.) of four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

formation was completely absent, indicating a lower Rho kinase activity at these sites. Rho kinase has profound effects on portal pressure via actin-filament contraction in HSC (Klein et al., 2012), and portal hypertension is a key factor in clinical complications during cirrhosis, so the modulation of Rho kinase activity by PGE_2 in HSC is quite relevant.

PGE_2 is pleiotropic molecule affecting many cell types, including (circulating) inflammatory cells, smooth muscle cells, hepatocytes, and macrophages (Hui et al., 2004; Haag et al., 2008). To investigate which cell type was responsible for the observed alterations, we used different albumin-based cell-selective carriers. Cell selectivity of the applied carriers has been extensively demonstrated in previous studies (Beljaars et al., 1998, 2003). Cell specificity was verified for all PGE_2 constructs: all constructs were taken up by the designated target cells, as illustrated by double staining for albumin and specific cell markers. Also, pharmacological activity of constructs was verified in cell lines expressing the designated target receptor (i.e., the PDGFβ receptor, mannose receptor, or the asialoglycoprotein receptor), and we started therapies in a late stage of disease, when target receptor expression is high. In vivo, only HSC-selective PGE_2 delivery inhibited the CCL_4-induced scar tissue formation, normalized Epac-1 expression levels, and abolished PMLC protein expression, thereby excluding a role for KC, hepatocytes, and circulating or infiltrating inflammatory cells. We did not test the effect of PGE_2 on sinusoidal endothelial cells yet. Studies with PGE_2 coupled to endothelial-specific carriers need to be performed to exclude a role for this cell type. Our approach, however, led to insight in a very complex situation, in which inflammation and fibrosis interact and PGE_2 exerts many effects in multiple cell types. Collectively, our cell-selective approach shows that PGE_2 increases Epac-1 within HSC, which is associated with local inhibition of Rho kinase activity and reduced fibrogenesis in CCL_4-treated mice.

In vitro studies confirmed Epac-1–mediated effects by PGE_2 on fibroblasts: Rap1 translocation to the plasma membrane, reported to be induced by Epac-1 (Yang et al., 2016), was seen after addition of PGE_2 to fibroblast, and this was inhibitable by the Epac-1 inhibitor CE3F4 (Courilleau et al., 2012). Although PGE_2 is known for its role in inflammation and fibrosis (Mallat et al., 1998; Haag et al., 2008; Huang et al., 2008; Weinberg et al., 2009), literature on the effects of PGE_2 on liver fibrosis in vivo is scarce. One study (Ruwart et al., 1989) demonstrated reduced collagen deposition in cirrhotic rats treated with PGE_2, although the mechanism of action behind this was unknown. This lack of clarity is most likely due to the pleiotropic effects of PGE_2 and its poor pharmacokinetic profile; it is rapidly cleared by the liver and kidneys, or inactivated by plasma proteins. In fact, it is surprising that, despite its short half-life, significant effects of PGE_2 were found on liver fibrosis. Similar to PGE_2, the effect of COX activity on fibrogenesis in vivo is unclear. COX inhibitors are reported to be either profibrotic (Gilroy et al., 1999) or antifibrotic (Chavez et al., 2010), which may indicate a dual effect on fibrogenesis; their anti-inflammatory effects may lead to antifibrogenic effects, whereas effects on fibrogenic cells, for instance via inhibition of PGE_2 production, may stimulate fibrogenesis. This remains to be elucidated, but the present study provides clues for such a dual effect of PGE_2 and COX inhibitors.

In summary, our study shows the involvement of the cAMP effector Epac-1 in the regulation of liver fibrosis. Fibrogenesis is associated with a reduced Epac-1 expression in vivo. Restoration of Epac-1 levels by PGE_2 is associated with attenuated fibrogenic activity. In contrast, the COX inhibitor NFA attenuates hepatic Epac-1 expression, which is paralleled by an increased HSC activation and fibrogenesis. In HSC, but not in hepatocytes or KC, PGE_2 restores Epac-1 expression levels and strongly affects Rho activity in these cells. Our studies reveal a pathway to modulate Epac-1 levels and thereby fibrogenesis, although our studies also show that

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022

cell specificity is important when interfering with Epac-1 levels. Detailed insight into this pathway is essential to identify drug targets and understand the effects of COX inhibitors in chronic liver diseases.

Acknowledgments

CE3F4 was a gift of Dr. Frank Lezloalc'h (INSERM, UMR-1048, France Université de Toulouse, France). LX2 cells were kindly provided by S. L. Friedman (Mount Sinai Hospital, New York, NY).

Authorship Contributions

Participated in research design: Schippers, Beljaars, Lotersztajn, Schmidt, Poelstra.

Conducted experiments: Schippers, Post, Reker-Smit, Han, Munoz-Llancao.

Performed data analysis: Schippers, Beljaars, Poelstra.

Wrote or contributed to the writing of the manuscript: Schippers, Beljaars, Schmidt, Poelstra.

References

Beljaars L, Poelstra K, Molema G, and Meijer DK (1998) Targeting of sugar- and charge-modified albumins to fibrotic rat livers: the accessibility of hepatic cells after chronic bile duct ligation. *J Hepatol* **29**:579–588.

Beljaars L, Weert B, Geerts A, Meijer DK, and Poelstra K (2003) The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. *Biochem Pharmacol* **66**:1307–1317.

Chávez E, Segovia J, Shibayama M, Tsutsumi V, Vergara P, Castro-Sánchez L, Salazar EP, Moreno MG, and Muriel P (2010) Antifibrotic and fibrolytic properties of celecoxib in liver damage induced by carbon tetrachloride in the rat. *Liver Int* **30**: 969–978.

Courilleau D, Bisserier M, Jullian JC, Lucas A, Bouyssou P, Fischmeister R, Blondeau JP, and Lezoualc'h F (2012) Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. *J Biol Chem* **287**:44192–44202.

Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clark MJ, and Willoughby DA (1999) Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med* **5**:698–701.

Haag S, Warnken M, Juergens UR, and Racké K (2008) Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol* **378**:617–630.

Huang SK, Wettlaufer SH, Chung J, and Peters-Golden M (2008) Prostaglandin E2 inhibits specific lung fibroblast functions via selective actions of PKA and Epac-1. *Am J Respir Cell Mol Biol* **39**:482–489.

Hui AY, Dannenberg AJ, Sung JJ, Subbaramaiah K, Du B, Olinga P, and Friedman SL (2004) Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells. *J Hepatol* **41**:251–258.

Insel PA, Murray F, Yokoyama U, Romano S, Yun H, Brown L, Snead A, Lu D, and Aroonsakool N (2012) cAMP and Epac in the regulation of tissue fibrosis. *Br J Pharmacol* **166**:447–456.

Klein S, Van Beuge MM, Granzow M, Beljaars L, Schierwagen R, Kilic S, Heidari I, Huss S, Sauerbruch T, Poelstra K, et al. (2012) HSC-specific inhibition of rho-kinase reduces portal pressure in cirrhotic rats without major systemic effects. *J Hepatol* **57**: 1220–1227.

Lezoualc'h F, Fazal L, Laudette M, and Conte C (2016) Cyclic AMP sensor EPAC proteins and their role in cardiovascular function and disease. *Circ Res* **118**:881–897.

Mack CP, Somlyo AV, Hautmann M, Somlyo AP, and Owens GK (2001) Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem* **276**:341–347.

Mallat A, Gallois C, Tao J, Habib A, Maclouf J, Mavier P, Préaux AM, and Lotersztajn S (1998) Platelet-derived growth factor-BB and thrombin generate positive and negative signals for human hepatic stellate cell proliferation: role of a prostaglandin/cyclic AMP pathway and cross-talk with endothelin receptors. *J Biol Chem* **273**:27300–27305.

Melgert BN, Olinga P, Van Der Laan JM, Weert B, Cho J, Schuppan D, Groothuis GM, Meijer DK, and Poelstra K (2001) Targeting dexamethasone to Kupffer cells: effects on liver inflammation and fibrosis in rats. *Hepatology* **34**:719–728.

Rajagopal S, Kumar DP, Mahavadi S, Bhattacharya S, Zhou R, Corvera CU, Bunnett NW, Grider JR, and Murthy KS (2013) Activation of G protein-coupled bile acid receptor, TGR5, induces smooth muscle relaxation via both Epac- and PKA-mediated inhibition of RhoA/Rho kinase pathway. *Am J Physiol Gastrointest Liver Physiol* **304**:G527–G535.

Roscioni SS, Kistemaker LE, Menzen MH, Elzinga CR, Gosens R, Halayko AJ, Meurs H, and Schmidt M (2009) PKA and epac cooperate to augment bradykinin-induced interleukin-8 release from human airway smooth muscle cells. *Respir Res* **10**:88.

Roscioni SS, Maarsingh H, Elzinga CR, Schuur J, Menzen M, Halayko AJ, Meurs H, and Schmidt M (2011) Epac as a novel effector of airway smooth muscle relaxation. *J Cell Mol Med* **15**:1551–1563.

Ruwart MJ, Wilkinson KF, Rush BD, Vidmar TJ, Peters KM, Henley KS, Appelman HD, Kim KY, Schuppan D, and Hahn EG (1989) The integrated value of serum procollagen III peptide over time predicts hepatic hydroxyproline content and stainable collagen in a model of dietary cirrhosis in the rat. *Hepatology* **10**:801–806.

Schmidt M, Dekker FJ, and Maarsingh H (2013) Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. *Pharmacol Rev* **65**:670–709.

Seki E and Schwabe RF (2015) Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology* **61**:1066–1079.

Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, and Insel PA (2005) Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proc Natl Acad Sci USA* **102**: 437–442.

Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, and Weinreb M (2009) Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and Epac. *J Cell Biochem* **108**:207–215.

Yang Y, Yang F, Wu X, Lv X, and Li J (2016) EPAC activation inhibits acetaldehyde-induced activation and proliferation of hepatic stellate cell via Rap1. *Can J Physiol Pharmacol* **94**:498–507.

Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, and Insel PA (2008) The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci USA* **105**:6386–6391.

Yu JL, Deng R, Chung SK, and Chan GC (2016) Epac activation regulates human mesenchymal stem cells migration and adhesion. *Stem Cells* **34**:948–959.

Address correspondence to: Dr. Klaas Poelstra, Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: k.poelstra@rug.nl

Downloaded from jpet.aspetjournals.org at ASPET Journals on February 24, 2022