British Journal of Pharmacology (2009), 156, 835–845

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Prostaglandin E_2 induces contraction of liver myofibroblasts by activating EP_3 and FP prostanoid receptors

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Background and purpose: Increased portal pressure in liver injury results from hypercontraction of perivascular non-parenchymal cells including liver myofibroblasts (MFs). Prostaglandin E_2 (PGE_2) is the major eicosanoid which is released around the venous system during liver injury, but little is known about their contractile effect on MFs.
Experimental approach: Contraction of primary rat liver MFs was measured by a collagen gel contraction assay. Expression of E prostanoid (EP) receptor subtypes was assessed by reverse transcription-polymerase chain reaction. Fura-2 fluorescence was used to determine intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Phosphorylation of protein kinase C (PKC) was detected by Western blot analysis.
Key results: Liver MFs expressed mRNAs for all four EP receptors. PGE_2 induced contraction in a dose- and time-dependent manner, and slightly increased $[Ca^{2+}]_i$ only at high concentrations (10 $\mu mol \cdot L^{-1}$). An agonist selective for EP_3 receptors, ONO-AE-248, dose-dependently induced MF contraction but did not increase $[Ca^{2+}]_i$. Pretreatment with rottlerin (a specific novel PKC inhibitor) and Ro 31-8425 (a general PKC inhibitor) significantly reduced 1 $\mu mol \cdot L^{-1}$ PGE_2- or ONO-AE-248-induced contractions. Furthermore, 1 $\mu mol \cdot L^{-1}$ PGE_2 stimulated phosphorylation of PKC isoforms PKCδ and PKCε. The F prostanoid (FP) receptor antagonist AL8810 abolished the $[Ca^{2+}]_i$ elevation and the rapid contraction induced by 10 $\mu mol \cdot L^{-1}$ PGE_2.
Conclusions and implications: Lower concentrations up to 1 $\mu mol \cdot L^{-1}$ of PGE_2 induce liver MF contraction via a $[Ca^{2+}]_i$-independent PKC-mediated pathway through the EP_3 receptor, while higher concentrations have an additional pathway leading to Ca^{2+}-dependent contraction through activating the FP receptor.
British Journal of Pharmacology (2009) **156**, 835–845; doi:10.1111/j.1476-5381.2008.00051.x; published online 23 February 2009

Keywords: liver myofibroblast; prostaglandin E_2; EP_3 receptor; FP receptor; protein kinase C

Abbreviations: ECM, extracellular matrix; GPCR, G-protein-coupled receptor; HSC, hepatic stellate cell; MF, myofibroblast; mPGES, microsomal prostaglandin E synthase; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

Introduction

In cirrhosis, portal hypertension is caused by an increase in vascular resistance and splanchnic blood flow, which are caused primarily by structural changes such as fibrotic scar tissue and by elevated intrahepatic vascular tone (Laleman *et al.*, 2005). Several *in vitro* and *in vivo* studies have highlighted the role of non-parenchymal cells including liver myofibroblasts (MFs) and hepatic stellate cells (HSCs). MFs are located around the central vein and the portal area, while HSCs are in the space of Disse (Knittel *et al.*, 1999a). In response to liver injury, these cells undergo activation, and produce increased quantities of extracellular matrix (ECM) protein (Friedman, 2000). Besides a fibrogenic response, they also acquire enhanced contractile properties upon activation, which is characterized by greater expression of α-smooth muscle actin (Knittel *et al.*, 1999b; Desmouliere *et al.*, 2003). It is possible that contraction of these non-parenchymal cells, in response to secreted vasoconstrictors, results in an increase in the sinusoidal pressure gradient and resistance, and consequently, in the progress of portal hypertension (Shah *et al.*, 1998; Reynaert *et al.*, 2002). Therefore, MFs and HSCs are now considered to be therapeutic targets to decrease portal hypertension in advanced chronic liver disease. Although most studies have focused on HSC contractility and suggested that various stimulants cause contraction both in Ca^{2+}-dependent

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Received 28 April 2008; revised 23 September 2008; accepted 6 October 2008

and -independent manner (Kawada *et al*., 1993; Pinzani *et al*., 1996; Melton *et al*., 2006; Laleman *et al*., 2007), few studies have yet addressed MF contractility.

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin biosynthesis from arachidonic acid and exists as two isoforms. COX-1 is constitutively expressed in most tissues and fulfills housekeeping functions. On the other hand, COX-2 is the inducible isoform that accounts for the increased production of PGs in response to pro-inflammatory and mitogenic stimuli (Hu, 2003). Prostaglandin E_2 (PGE_2) exerts both pro-inflammatory and anti-proliferative effects via G-protein-coupled receptors (GPCRs) (Narumiya *et al*., 1999; Sugimoto and Narumiya, 2007). There are four GPCRs responding to PGE_2 designated as EP_1, EP_2, EP_3 and EP_4. The E prostanoid (EP) receptors exhibit differences in signal transduction depending upon coupled G protein subtypes. EP_1 receptors couples to G_q and increases intracellular Ca^{2+}. EP_2 and EP_4 receptors couples to G_s and stimulate adenylate cyclase with a subsequent increase in intracellular cyclic adenosine monophosphate (cAMP). Binding of PGE_2 to the EP_3 receptor, on the other hand, activates multiple signaling pathways, including Ca^{2+}, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) signaling pathways (Breyer *et al*., 2001). In the liver, non-parenchymal cells produce mainly PGE_2 and also other PGs, and release them around the vessels during liver injury (Enomoto *et al*., 2000; Neyrinck *et al*., 2004).

Previous *in vivo* studies reported that COX-2 is highly expressed in cirrhotic liver, mainly in the sinusoidal area and area around the vessels, indicating that COX-2 and the resulting product PGE_2 may modulate the function of non-parenchymal cells during liver injury (Cheng *et al*., 2002; Mohammed *et al*., 2004). However, the role of PGE_2 in liver MF bioactivity still remains unknown.

In this study, we isolated rat liver MFs and assessed the changes in their contractility and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by PGE_2 treatment. Low doses of PGE_2 caused MF contraction via Ca^{2+}-independent PKC activation through the EP_3 receptor. In addition to this mechanism, high doses of PGE_2 activated the F prostanoid (FP) receptor and subsequently induced Ca^{2+}-dependent contraction.

Methods

Cell isolation and culture

All animal procedures were performed in accordance with the guidelines of the University of Tokyo. Rat liver MFs were isolated from male Sprague-Dawley rats (300–500 g) as described previously (Kojima *et al*., 2007). In brief, the liver was perfused with collagenase (Sigma, USA) and protease (Merck, Germany), following removal of hepatocytes and cell debris by low-speed centrifugation. A cell fraction containing MFs was obtained by density gradient centrifugation using 14.6% Nycodenz (Sigma, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, USA), and passaged two to three times before use. Cells between passage 3 and 8 were used for experiments. We confirmed that isolated cells from rat liver express α-smooth muscle actin, fibulin-2 and interleukin-6 at the mRNA level prior to the experiments (data not shown). These have been suggested as specific markers for MFs (Knittel *et al*., 1999b; Ramadori and Saile, 2002; Tateaki *et al*., 2004).

Collagen gel contraction assay

Contraction of liver MFs was examined as previously described (Reynaert *et al*., 2001; Maruyama *et al*., 2008). In brief, collagen gels were prepared by mixing 70% type I collagen from porcine tendon (Nitta Gelatin, Japan), 20% 5 × DMEM and 10% 0.05 N NaOH under ice-cold conditions (final collagen concentration, 2.1 $mg \cdot mL^{-1}$). The solution was added to each well of 12 well plates and incubated at 37°C for 1 h. Cells were plated on top of the collagen gels at a concentration of 1×10^5 cells per well. After incubation overnight to allow cell attachment, serum-free conditions were introduced for 48 h. Gels were then detached from the plates using a microspatula. The surface area of the gels was quantified using ImageJ (National Institutes of Health, USA). Relative contraction of the gels was expressed as a percentage according to the following formula: [(gel surface area of buffer – gel surface area of test substance)/(gel surface area of buffer – gel surface area of 5% FBS stimulation for 24 h)] × 100%.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured using fura-2 AM as previously described (Kojima *et al*., 2007). In brief, liver MFs on glass coverslips in HEPES-buffered solution were loaded with 3 $\mu mol \cdot L^{-1}$ fura-2 AM (Dojindo Laboratories, Japan) for 40 min in a dark room at 37°C and placed in a bath on the stage of an inverted microscope (TE-300, Nikon, Japan) equipped with a 40-fold objective lens. Using a fluorescence imaging system (Hamamatsu Photonics, Japan), images of 510 nm fluorescence were captured every 3 s using 340 nm and 380 nm wavelength light, and the fluorescence of an image at 340 nm (F340) was divided by the fluorescence at 380 nm (F380) giving a ratio (F340/F380). $[Ca^{2+}]_i$ was calculated using the following formula described previously (Takahashi *et al*., 1999): $[Ca^{2+}]_i = K_d \times (S_{f2}/S_{b2}) \times (R - R_{min})/(R_{max} - R)$, where K_d is the effective dissociation constant of fura-2 and has a value of 224 $nmol \cdot L^{-1}$, R is the fluorescence ratio, R_{min} and R_{max} are the ratios in absence and presence of Ca^{2+}, respectively, and S_{f2} and S_{b2} are the emissions at 380 nm in the absence and presence of Ca^{2+} respectively. Calibration was accomplished after permeabilization of the cells with 3 $\mu mol \cdot L^{-1}$ ionomycin and measurement of fluorescence at both wavelengths under Ca^{2+}-free (in 0.5 $mmol \cdot L^{-1}$ EGTA) or Ca^{2+}-saturated (in 1.5 $mmol \cdot L^{-1}$ $CaCl_2$) conditions to obtain R_{min}, R_{max}, S_{f2} and S_{b2}. The area under the Δratio per time curve (area under curve, AUC) was calculated to assess the response.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver MFs, and the concentration of total RNA was adjusted to 1 $\mu g \cdot \mu L^{-1}$ with RNase-free distilled water. RT-PCR was performed as previously described (Kojima *et al*., 2007). After denaturation at 95°C for 10 min,

Table 1 RT-PCR primer pairs

Target gene	*5′ sequence*	*3′ sequence*	*Product (bp)*
GAPDH	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	308
EP_1	ATGGTCTTCTTTGGCCTGTG	GTTCTCTCGGAAACGTCGAG	387
EP_2	GAACGCTACCTCGCCATCGG	CGAAGGTGATGGTCATAATGGC	421
EP_3	CTTTGCCTCCGCCTTCGCC	CTTAGCAGCAGATAAACCCAGG	363
EP_4	CATCTTACTCATCGCCACCTCTC	GTTAGGTCTGGCAGGTATAGGAGG	393
FP	GACTCTTAGCTCTCGGCATCTC	CGTAGCAGAATGTAGACCCAGG	298
COX-1	CCGGATTGGTGGGGGTAG	AGGGGCAGGTCTTGGTGTTG	434
COX-2	CTGTATCCCGCCCTGCTGGTG	ACTTGCGTTGATGGTGGCTGTCTT	282
PGE synthase-1	GTTTGGTGATGGAGAACAGC	GTAGACGAAACCAAGGAAGAGG	257
PGE synthase-2	CCAGTACAAGACATGTCCCTTC	GTACACGTTGGGAGAGATGAGA	439

COX, cyclooxygenase; EP, E prostanoid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE, prostaglandin E; RT-PCR, reverse transcription-polymerase chain reaction.

36 cycles of amplification at 94°C for 40 s, at 60°C for 1 min and at 72°C for 1 min were performed using a thermal cycler (Takara Bio, Japan). The PCR products were electrophoresed onto a 2% agarose gel containing ethidium bromide at 0.2 $\mu g \cdot mL^{-1}$. The detectable fluorescence bands were visualized using an ultraviolet transilluminator. The forward and the reverse primers for EP_1 through EP_4 receptors, FP receptor, COX-1, COX-2, microsomal prostaglandin E synthase-1 (mPGES-1), mPGES-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed as shown in Table 1.

Western blot analysis

To detect phosphorylated PKCδ and PKCε, Western blots were carried out in accordance with the method described previously (Vary *et al.*, 2005), with modifications noted below. Liver MFs were transferred to ice-cold homogenization buffer [50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 $mmol \cdot L^{-1}$ NaCl, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ Na_3VO_4, 1 $mmol \cdot L^{-1}$ NaF, 1 $mg \cdot mL^{-1}$ Pefabloc SC (protease inhibitor) and 1 $mg \cdot mL^{-1}$ Complete (protease inhibitor cocktail)] followed by centrifugation at 20 000× *g* for 30 min at 4°C, and the supernatant was collected for analysis. The blots on polyvinylidene difluoride (PVDF) membranes were probed with anti-phospho-PKCδ^{Thr505} and phospho-PKCε^{Ser729} antibodies (1:500 each). For secondary reaction, anti-mouse IgG (Alexa Fluor 680) or anti-rabbit IgG (IRDye800) were used (1:10 000 each). Bands were detected and quantified with the Odyssey system (LI-COR Biosciences, USA). To correct for loading variations, the result was expressed as a ratio of phospho/total PKC with the control ratio set at 1.0.

Cell morphology and viability

For Giemsa staining, MFs were fixed in 4% paraformaldehyde for 5 min, incubated in Giemsa solution (2% in 10 $mmol \cdot L^{-1}$ phosphate buffer, pH 7.4) for 1 h, washed in distilled water, dehydrated and covered with glass. For actin staining, cells were fixed in 4% paraformaldehyde and incubated with 0.01% Triton and 10% normal goat serum (Chemicon International, USA) for 30 min at room temperature. Cells were probed with anti-smooth muscle actin antibody (1:100) followed by anti-mouse IgG (Alexa Fluor 568, 1:100). Cells were finally incubated with DAPI (1 $\mu g \cdot mL^{-1}$) for 5 min. For Trypan blue staining, cells were trypsinized, resuspended in 0.3% Trypan blue solution and counted using a haemocytometer. The cells with and without blue dye staining were recorded as dead and alive respectively.

Materials

Drug and molecular target nomenclature conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008). The chemicals obtained were: PGE_2 and AL8810 (Cayman Chemical, USA), ATP disodium salt, ionomycin calcium salt and DAPI (Sigma, USA), Ro 31-8425 (Calbiochem, Germany), phorbol 12,13-dibutyrate (PDBu) and rottlerin (Biomol International, USA), Pefabloc SC and Complete (Roche Applied Science, USA), anti-PKCδ and anti-PKCε antibodies (BD Biosciences, USA), anti-phospho-PKCδ^{Thr505} antibody (Cell Signaling Technology, USA), anti-phospho-PKCε^{Ser729} antibody (Upstate Biotechnology, USA), anti-rabbit IgG conjugated to Alexa Fluor 680 and anti-mouse IgG conjugated to Alexa Fluor 568 (Molecular Probes, USA), anti-mouse IgG conjugated to IRDye800 (Rockland, USA) and anti-smooth muscle actin antibody (DAKO, Denmark). ONO-DI-004 (EP_1 receptor agonist), ONO-AE1-259-01 (EP_2 receptor agonist), ONO-AE-248 (EP_3 receptor agonist) and ONO-AE1-329 (EP_4 receptor agonist) were gifts kindly provided by Ono Pharmaceutical Company Ltd. (Osaka, Japan).

Statistical analysis

The results of the experiments are expressed as mean ± SEM. Statistical evaluation of the data was performed by paired or unpaired Student's *t*-test for comparison between two groups, and by one-way analysis of variance followed by Dunnett's test for comparisons between more than two groups. Statistical significance was established at *P*-values lower than 0.05.

Results

EP receptor subtypes

We performed RT-PCR in liver MFs to investigate the expression of the four subtypes of EP receptor. As shown in Figure 1A, MFs expressed mRNAs for all four EP receptor subtypes: EP_1, EP_2, EP_3 and EP_4. They also expressed COX-1, COX-2, mPGES-1

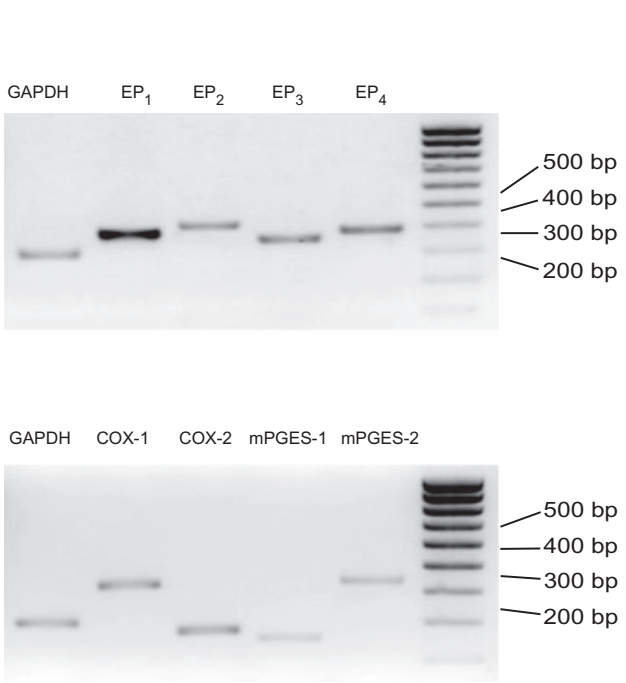

Figure 1 Expression of the four subtypes of E prostanoid (EP) receptor EP_1 through EP_4 (A) and cyclooxygenase-1 (COX-1), COX-2, microsomal prostaglandin E synthase-1 (mPGES-1) and mPGES-2 (B) in liver myofibroblasts (MFs). Figures show representative results from RT-PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

and mPGES-2, which are the essential enzymes in PGE_2 biosynthesis from arachidonic acid (Figure 1B).

Effect of PGE_2 on liver MF contraction

We next performed the collagen gel contraction assay in order to investigate the contractile effect of PGE_2. Hydrated collagen gels provide a potent model for examining *in vitro* the reciprocal mechanical interactions that occur between cells and the ECM (Tomasek *et al.*, 2002). Liver MFs cultured on collagen gels were stimulated with PGE_2 after cell attachment and serum starvation. As shown in Figure 2A, PGE_2 induced cell contraction in a dose-dependent manner (0.1–10 $\mu mol \cdot L^{-1}$, $n = 5$). Contractile effects of PGE_2 were observed at each time point during the experiment. PGE_2 (1 $\mu mol \cdot L^{-1}$) induced cell contraction of 44.8 $\pm$ 3.0% at 8 h. Data are shown as the percentage of maximal contraction induced by 5% FBS.

Effect of EP receptor-selective agonists on cell contraction

Liver MFs cultured on collagen gels were stimulated with a selective agonist of each EP receptor to examine which subtype is responsible for the contractile effect of PGE_2. The EP_3 receptor agonist ONO-AE-248 (1 $\mu mol \cdot L^{-1}$) produced contraction of 31.7 $\pm$ 1.7% at 8 h after stimulation ($P < 0.01$), whereas neither ONO-DI-004 (EP_1 receptor agonist), ONO-AE1-259-01 (EP_2 receptor agonist), nor ONO-AE1-329 (EP_4 receptor agonist) induced contraction. The EP_4 receptor agonist ONO-AE1-329 showed a slight suppressive effect at 1 h (Figure 2B, $P < 0.05$, $n = 6$). As shown in Figure 2C, the contractile effects of ONO-AE-248 were dose-dependent (0.1–10 $\mu mol \cdot L^{-1}$, $n = 7$). These results suggest that PGE_2 induces contraction in MFs via the EP_3 subtype of the PGE receptor.

$[Ca^{2+}]_i$ response to PGE_2 and a selective agonist of the EP_3 receptor

To explore the downstream pathways of EP_3 receptor, we examined the involvement of intracellular Ca^{2+} in liver MFs treated with PGE_2 or an EP_3 selective agonist. PGE_2 (1 $\mu mol \cdot L^{-1}$) and ONO-AE-248 (1–10 $\mu mol \cdot L^{-1}$) did not increase $[Ca^{2+}]_i$, while PGE_2 induced a slight but apparent increase in $[Ca^{2+}]_i$ at a concentration of 10 $\mu mol \cdot L^{-1}$ (Figure 3A). We previously reported that ATP induces sufficient $[Ca^{2+}]_i$ increase in MFs (Kojima *et al.*, 2007). Compared with $[Ca^{2+}]_i$ increase by 10 $\mu mol \cdot L^{-1}$ ATP, the amount of change in AUC induced by 10 $\mu mol \cdot L^{-1}$ PGE_2 was only 23% (AUC, 0–2 min). Because of their similarity of chemical structure, prostanoids are known to react with different prostanoid receptors, especially at higher concentration. We thus examined the effect of antagonism of the FP receptor on $[Ca^{2+}]_i$ elevation induced by10 $\mu mol \cdot L^{-1}$ PGE_2. As shown in Figure 3B, the FP receptor antagonist AL8810 (3 $\mu mol \cdot L^{-1}$) completely inhibited this $[Ca^{2+}]_i$ increase ($P < 0.01$, $n = 4$).

Effect of PKC inhibitors on cell contraction

We next examined the effect of PKC inhibitors on cell contraction induced by PGE_2 or the EP_3 receptor agonist. Liver MFs on collagen gels were pretreated with compounds that differentially inhibit different PKC isozymes. Ro 31-8425 is a broad range PKC inhibitor for both Ca^{2+}-dependent conventional PKC (cPKC) and Ca^{2+}-independent novel PKC (nPKC), showing threefold to fourfold selectivity for cPKCs versus PKCε. Rottlerin specifically inhibits nPKC isozyme PKCδ. In the present study, cells were pretreated with these inhibitors for 30 min prior to the treatment with 1 $\mu mol \cdot L^{-1}$ of PGE_2 or ONO-AE-248. As shown in Figure 4A,B, Ro 31-8425 (30–300 $nmol \cdot L^{-1}$) significantly inhibited PGE_2- and ONO-AE-248-induced MF contractions ($P < 0.05$, $n = 5$). Rottlerin (0.3–3 $\mu mol \cdot L^{-1}$) also inhibited cell contraction in a dose-dependent manner (Figure 4C,D, $P < 0.01$, $n = 5$).

Effect of PKC inhibitors on cell morphology and viability

We confirmed whether both PKC inhibitors influence MF morphology and viability under the present conditions. As shown in Figure 5A, we could not observe any changes in MF cell shape and expression of α-smooth muscle actin after 8 h treatment with Ro 31-8425 (300 $nmol \cdot L^{-1}$) or rottlerin (3 $\mu mol \cdot L^{-1}$). We also examined cell viability using Trypan blue staining. MFs were stimulated for 8 h with Ro 31-8425 (300 $nmol \cdot L^{-1}$) or rottlerin (3 $\mu mol \cdot L^{-1}$) and the percentage of viable cells were not different from that under unstimulated condition (Figure 5B, $n = 5$).

PGE_2 promotes PKCδ and PKCε phosphorylation

To directly examine whether PGE_2 activates nPKC, we next evaluated the phosphorylation of nPKC isozymes, PKCδ (Thr505, Figure 6A) and PKCε (Ser729, Figure 6B). While 5 min stimulation with 1 $\mu mol \cdot L^{-1}$ PGE_2 did not phosphorylate both PKC isozymes, 15 min stimulation significantly increased phosphorylation of both $PKC\delta^{Thr505}$ ($P < 0.01$, $n = 4$)

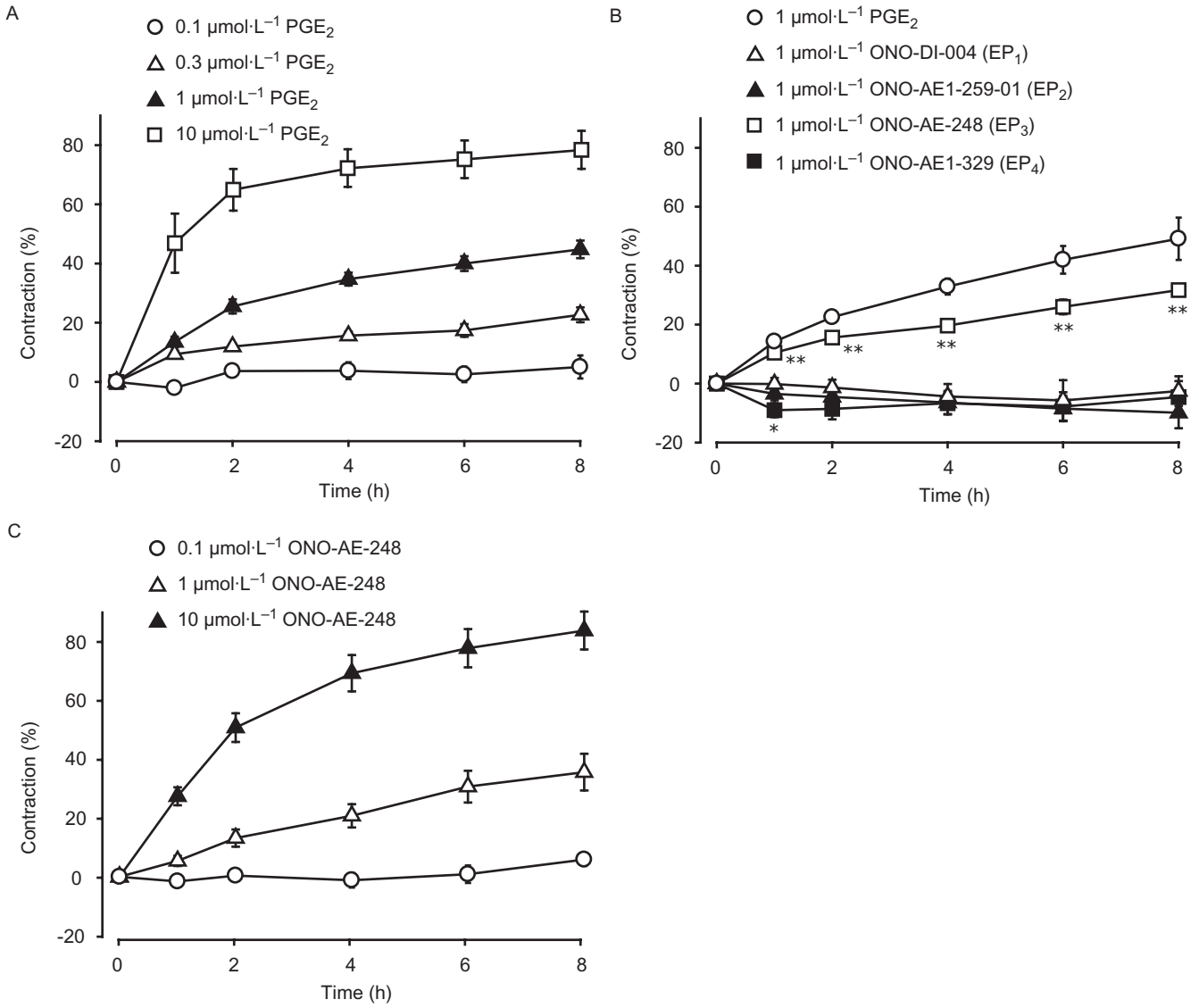

Figure 2 Prostaglandin E_2 (PGE_2) induced collagen gel contraction in liver myofibroblasts. (A) Collagen gels were treated in DMEM containing a range of concentrations of PGE_2: 0.1, 0.3, 1 and 10 μmol·L^{-1}. (B) Effect of E prostanoid receptor subtype-selective agonists on collagen gel contraction: EP_1 (ONO-DI-004), EP_2 (ONO-AE1-259-01), EP_3 (ONO-AE-248) and EP_4 (ONO-AE1-329). All the agonists were used at a concentration of 1 μmol·L^{-1}. (C) Effect of EP_3 receptor agonist ONO-AE-248 over a range of concentrations: 0.1, 1 and 10 μmol·L^{-1}. Gels were photographed to quantify the surface area. Relative contraction was calculated as shown in Methods. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with unstimulated condition. DMEM, Dulbecco's modified Eagle's medium.

and PKCε^{Ser729} ($P < 0.05$, $n = 4$). For both PKC isozymes, we confirmed that a known PKC activator PDBu (1 μmol·L^{-1}, 5 min) increased significantly their phosphorylation (Figure 6A,B, far right lane). We next examined whether PKC inhibitors can block these phosphorylations. 30 min pretreatment with Ro 31-8425 (300 nmol·L^{-1}) and rottlerin (3 μmol·L^{-1}) influenced neither PKCδ (Figure 6C) nor PKCε (Figure 6D) phosphorylation induced by PGE_2 (1 μmol·L^{-1}, 15 min).

FP receptor antagonist partially blocked 10 μmol·L^{-1} PGE_2-induced cell contraction

We examined the effect of the FP receptor antagonist AL8810 on cell contraction induced by PGE_2. As shown in Figure 7A, 30 min pretreatment with AL8810 (3 μmol·L^{-1}) partially blocked the contraction induced by 10 μmol·L^{-1} PGE_2. ONO-AE-248 (10 μmol·L^{-1}) induced only 59.7% contraction as compared with PGE_2 at 1 h, while the difference was abolished by AL8810 treatment ($P < 0.05$, $n = 4$). In contrast, AL8810 did not affect cell contraction induced by ONO-AE-248 or 1 μmol·L^{-1} PGE_2 ($n = 4$, data not shown). When assessed by RT-PCR, MFs expressed FP receptor as well as EP receptors (Figure 7B).

Discussion and conclusions

In the present study, we examined the effect of PGE_2 on isolated liver MF contractility. We demonstrated that PGE_2

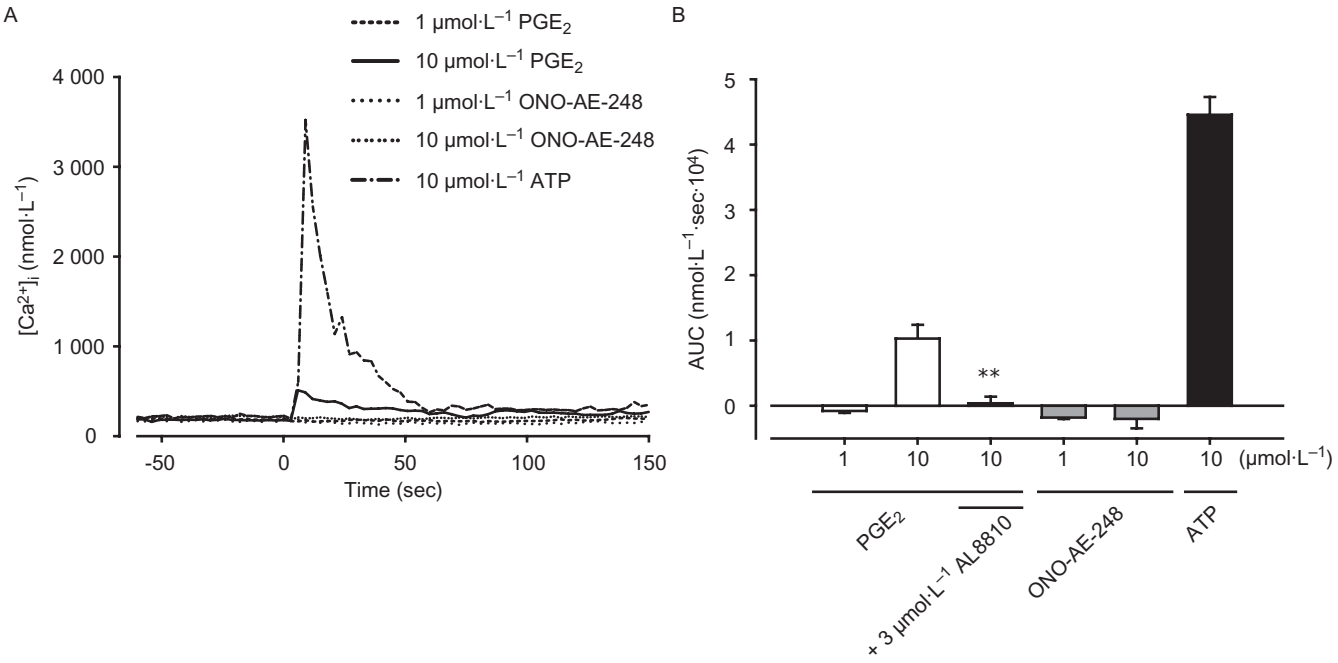

Figure 3 Effect of prostaglandin E_2 (PGE_2) and ONO-AE-248 on $[Ca^{2+}]_i$. (A) Liver myofibroblasts cultured on glass coverslips were stimulated with PGE_2 and ONO-AE-248 (1 or 10 μmol·L^{-1}), or ATP (10 μmol·L^{-1}). Changes in $[Ca^{2+}]_i$ were measured using Ca^{2+} fluorescent dye fura-2. (B) The area under $[Ca^{2+}]_i$-time curve after stimulation (AUC: 0–2 min). Data are presented as mean ± SEM of 52–110 cells from four separate experiments. $^{**}P < 0.01$ compared with 10 μmol·L^{-1} PGE_2-treated cells. AUC, area under curve.

up to 1 μmol·L^{-1} induced cell contraction via the EP_3-PKC pathway which was not accompanied by a detectable $[Ca^{2+}]_i$ increase and that at higher concentrations, PGE_2 activated the FP receptor resulting in a $[Ca^{2+}]_i$ increase and additional cell contraction.

Recent studies have clearly shown that MFs possess a different origin from HSCs (Knittel *et al.*, 1999a; Kinnman *et al.*, 2003). Although both cells exhibit a similar phenotype regarding contractility and ECM secretion, some reports have provided evidence that these cells differentially contribute to liver cirrhosis. Proliferation of MFs observed in a cirrhotic liver suggests a pivotal role of this cell type, while HSCs also undergo activation during liver injury but fail to proliferate *in vivo* and also *in vitro* (Ramadori and Saile, 2002; Saile *et al.*, 2002). In this study, we sought to investigate the contractility of MFs which still remained largely unknown.

Many reports have shown that non-parenchymal cells in sinusoidal spaces were positively stained with COX-2 in various models of liver disease (Yamamoto *et al.*, 2003; Planaguma *et al.*, 2005). These results allow us to hypothesize that liver MFs are one of the major sources of PGE_2 upon liver injury. Indeed, MFs expressed COX-2 as well as mPGES-1 and mPGES-2, indicating that secreted PGE_2 may control the function of adjacent non-parenchymal cells in an autocrine and/or paracrine manner. Previous reports show that liver injury or inflammation increases the local PGE_2 concentration in sinusoids up to several hundred or thousand nanomolar (Neuschafer-Rube *et al.*, 1993; Devaux *et al.*, 2001). These reports support that PGs in our study are within a physiologically relevant concentration in the liver. In the present study, MFs expressed a noticeable level of COX-2 expression even under unstimulated conditions (Figure 1B). We previously reported that scaffold materials influence MF bioactivity (Kojima *et al.*, 2007). Therefore incubation on plastic dishes may have provided some stimulation of COX-2 expression in MFs. Further investigation is needed to clarify this point.

Prostaglandin E_2 is thought to regulate important liver functions such as glucose homeostasis, delivery of blood substances to hepatocytes and lipid oxidation (Bradford *et al.*, 1999; Enomoto *et al.*, 2000; Pestel *et al.*, 2002). In this study, we focused on its contractile effects on liver MFs and demonstrated that exogenous administration of PGE_2 promoted cell contraction. PGE_2 is known to produce a broad range of biological effects through its binding to specific receptors namely EP_1, EP_2, EP_3 and EP_4. In liver MFs, all four EP receptor subtypes were observed at mRNA level. We then applied four EP subtype-selective agonists to see their contractile effects individually and found that only ONO-AE-248, a selective agonist for EP_3, receptors, induced contraction. Our results are consistent with the previous reports showing that EP_3 receptors mediate PGE_2-induced contraction in human pulmonary artery (Qian *et al.*, 1994). Another group also suggested that both EP_1 and EP_3 receptors are involved in constriction of adult porcine large cerebral arteries (Jadhav *et al.*, 2004). However, the selective agonist for EP_1 receptors did not induce contraction in rat liver MFs, even at a concentration of 10 μmol·L^{-1} (data not shown).

Some other studies clarified that PGE_2 exhibits vasodilator effects through EP_2 receptor in human dermal fibroblasts, or through EP_4 receptor in mice aorta and human middle cerebral arteries, which couple efficiently to G_s or G_i (Davis

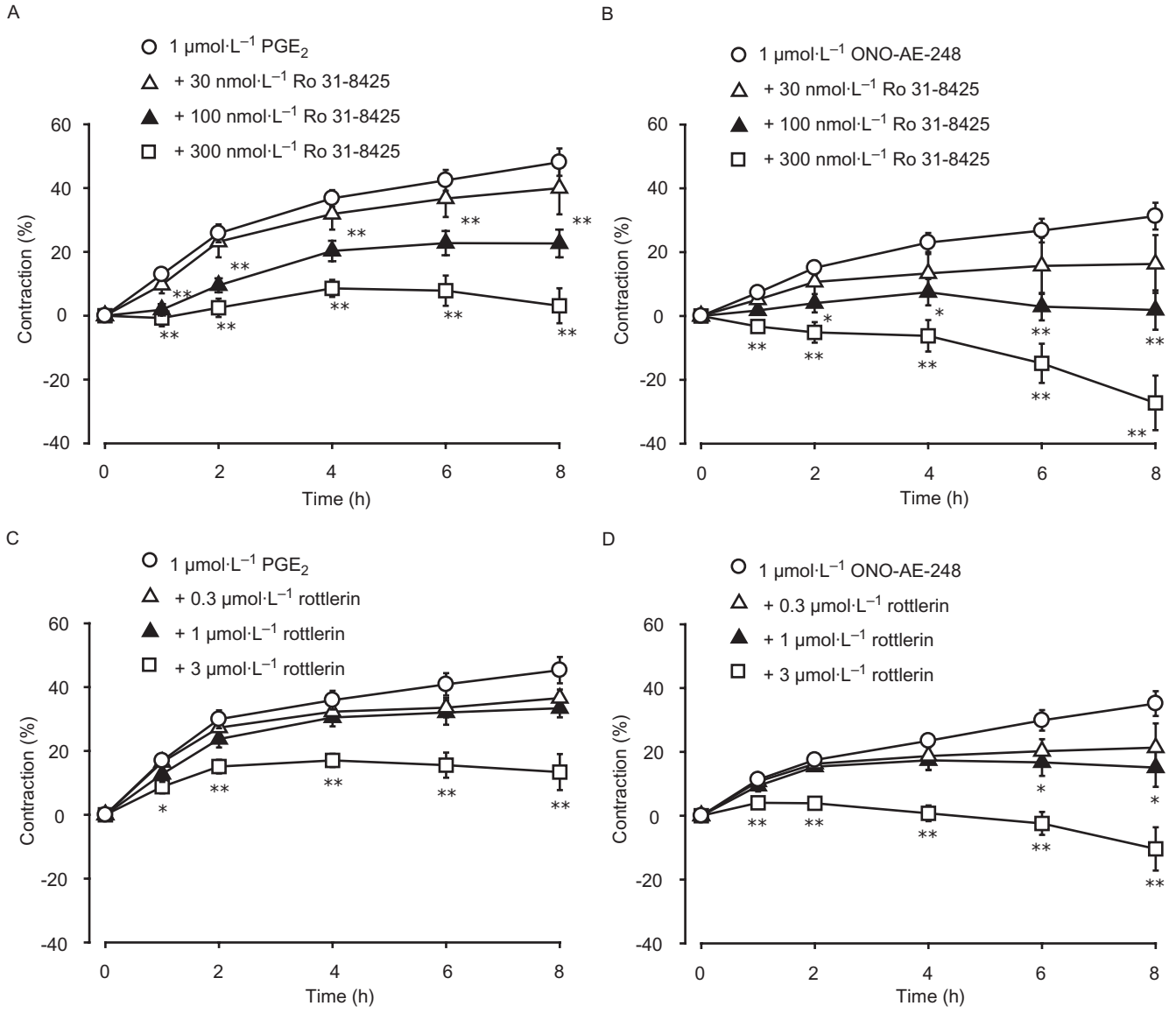

Figure 4 Ro 31-8425 and rottlerin inhibited liver myofibroblast (MF) contractions induced by PGE_2- or ONO-AE-248. Cells were stimulated with 1 μmol·L^{-1} of PGE_2 (A, C) or ONO-AE-248 (B, D) after pretreatment for 30 min with either protein kinase C (PKC) inhibitor: Ro 31-8425 (general PKC inhibitor: 30, 100 and 300 nmol·L^{-1}) (A, B) or rottlerin (specific nPKC inhibitor: 0.3, 1 and 3 μmol·L^{-1}) (C, D). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with 1 μmol·L^{-1} PGE_2- or ONO-AE-248-treated cells.

et al., 2004; Sandulache *et al.*, 2006). Consistent with these observations, we showed that the EP_4 selective agonist ONO-AE1-329 slightly inhibited rat liver MF contraction at an early period of PGE_2 treatment. This dilation may be mediated through intracellular cAMP generation resulting from EP_4 receptor-coupled G_s activation. In this study, we could not observe any contractile effect after EP_1 and EP_2 receptor stimulation. These receptors may be involved in other pathophysiological roles in MFs, such as cell proliferation, migration, or ECM secretion.

Although a previous study indicated that EP_3 receptor stimulation caused smooth muscle contraction through Ca^{2+}-signaling pathways in guinea-pig aorta (Jones *et al.*, 1998), our study demonstrated that PGE_2 or a EP_3 selective agonist caused MF contraction without changing $[Ca^{2+}]_i$ at a concentration of 1 μmol·L^{-1}. We therefore sought to reveal the Ca^{2+}-independent pathway in MF contraction induced by 1 μmol·L^{-1} of PGE_2 or ONO-AE-248.

In vascular smooth muscle, a Ca^{2+}-independent pathway, known as Ca^{2+} sensitization, is proposed to be an important component of the constrictor response to many receptor agonists. Several key intracellular protein kinases including PKC have been thought to be involved in pathways leading to Ca^{2+}-sensitization (Somlyo and Somlyo, 2003). PKC can be subdivided into three classes based on primary structure and biological properties: conventional PKC isozymes (cPKC), novel PKC isozymes (nPKC) and atypical PKC isozymes (aPKC) (Ward *et al.*, 2004). Our results showing no $[Ca^{2+}]_i$ increase in the presence of 1 μmol·L^{-1} of PGE_2 or ONO-AE-248 rule out the involvement of cPKC, which

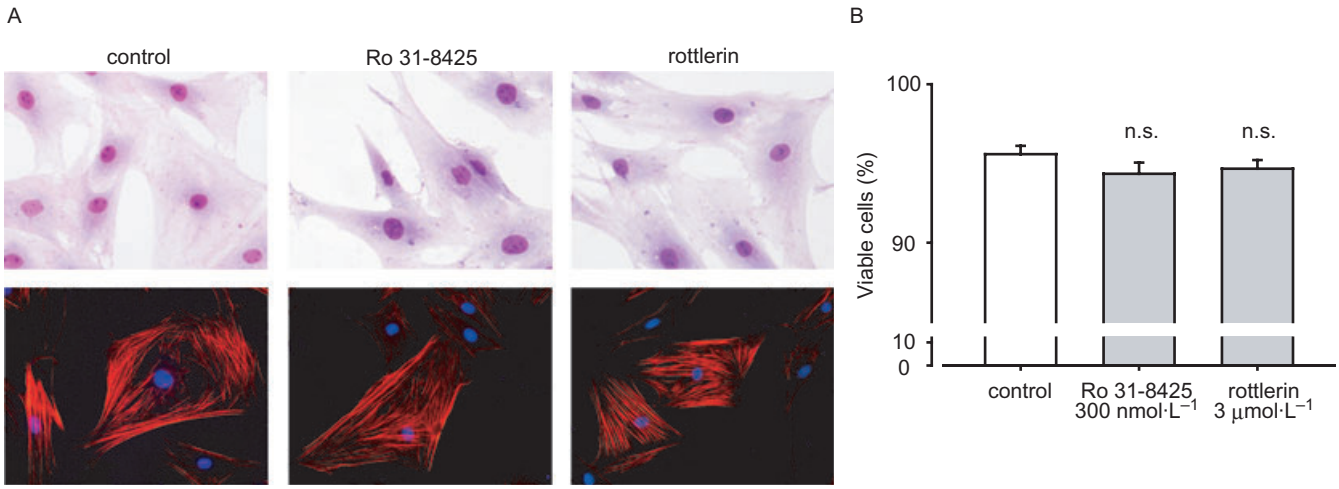

Figure 5 Ro 31-8425 and rottlerin did not influence myofibroblast (MF) morphology and viability. MFs were treated with Ro 31-8425 (300 nmol·L^{-1}) or rottlerin (3 μmol·L^{-1}) for 8 h. (A) MF were fixed in paraformaldehyde and stained in Giemsa solution (upper row) or probed with anti-smooth muscle actin antibody (lower row). (B) Cells after 8 h stimulation were resuspended in 0.3% Trypan blue solution, and viable and dead cells were counted. Data are presented as mean ± SEM. No significant difference was observed between control and 8 h stimulated conditions.

requires $[Ca^{2+}]_i$ increase on activation. In our study, we revealed that rottlerin (a specific nPKC inhibitor) as well as Ro 31-8425 (a general PKC inhibitor) strongly inhibited 1 μmol·L^{-1} PGE_2- or ONO-AE-248-induced contraction. The general PKC inhibitor Ro 31-8425 is known to inhibit both cPKCs- and PKCε-activity while rottlerin inhibits PKCδ more potently than other nPKC isozymes. The effects of these inhibitors were specific to the contractile response against PGE_2, because the administration of each PKC inhibitor alone did not induce toxicity in terms of cell morphology and viability. We also observed that PKCδ and PKCε undergo rapid phosphorylation in response to MF stimulation with PGE_2. These data show the possible involvement of PKCδ and PKCε in the primary component of EP_3 selective agonist action.

Ro 31-8425 and rottlerin are known to bind to the ATP binding site at the catalytic domain of PKC (Muid *et al.*, 1991; Gschwendt *et al.*, 1994). Although it is well-known that cPKCs are phosphorylated by a Ro 31-8425- and rottlerin-insensitive kinase, PDK-1 (phosphoinositide-dependent kinase-1), the detailed relationship of nPKC phosphorylation and the inhibitory effect of PKC inhibitors is still unclear (Way *et al.*, 2000; Steinberg, 2004). As shown in Figure 6C,D, pretreatment with Ro 31-8425 and rottlerin did not influence nPKC phosphorylations. Our results suggest that nPKCs may be phosphorylated by a kinase insensitive to Ro 31-8425 and rottlerin in liver MFs. Further investigation should be made to reveal the detailed interactions between PKC inhibition and the inhibition of contractile activity by these agents.

As well as PKC signaling pathways, it has been suggested that the other pathways also involve in Ca^{2+} sensitization. Recently, the small GTPase, Rho, and Rho-kinase has been investigated as an important regulator of HSC proliferation, migration and contraction (Tangkijvanich *et al.*, 2001; Ramm *et al.*, 2003; Laleman *et al.*, 2007) but the involvement of Rho and other related factors in MF contraction still remains to be tested.

A higher concentration of PGE_2 (10 μmol·L^{-1}) caused rapid and greater MF contraction compared with that with the low concentration (1 μmol·L^{-1}). A notable finding in the $[Ca^{2+}]_i$ measurement is that this PGE_2-induced contraction at 10 μmol·L^{-1} is accompanied by a small increase of $[Ca^{2+}]_i$, suggesting that different signal pathways are involved. PGE_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are structurally identical, except at the C-9 position in the cyclopentane ring where PGE_2 has a keto substituent and $PGF_{2\alpha}$ has a hydroxyl. Also, PGE_2 binds to the FP receptor in CHO cells and HEK-293 cells (Kiriyama *et al.*, 1997; Fujino *et al.*, 2004). Therefore, it is possible that the $[Ca^{2+}]_i$ increase by 10 μmol·L^{-1} PGE_2 in rat liver MFs was due to FP receptor activation, whose downstream pathway has been classically characterized by Ca^{2+} signaling (Breyer *et al.*, 2001). We demonstrated here that the selective antagonist for FP receptor, AL8810, significantly inhibited $[Ca^{2+}]_i$ increase by 10 μmol·L^{-1} PGE_2. AL8810 also inhibited the rapid contraction by 10 μmol·L^{-1} PGE_2, which indicates the involvement of the FP receptor in PGE_2-induced MF contraction, especially at higher concentrations.

In summary, we showed that low concentrations of PGE_2 induced contraction in liver MFs via EP receptor subtype EP_3. This effect appears to involve an enhancement of the Ca^{2+} sensitization through a Ca^{2+}-independent, novel PKCδ and/or PKCε-mediated pathway. We also demonstrated that a high concentration of PGE_2 activated the $PGF_{2\alpha}$ receptor FP and increased $[Ca^{2+}]_i$, leading to stronger MF contraction than through the EP_3-PKC pathway. A better understanding of the intracellular signal transduction mechanisms leading to MF contraction might lead to the identification of novel potential targets for the treatment of portal hypertension and cirrhosis. The present results may provide new insights into the development of a

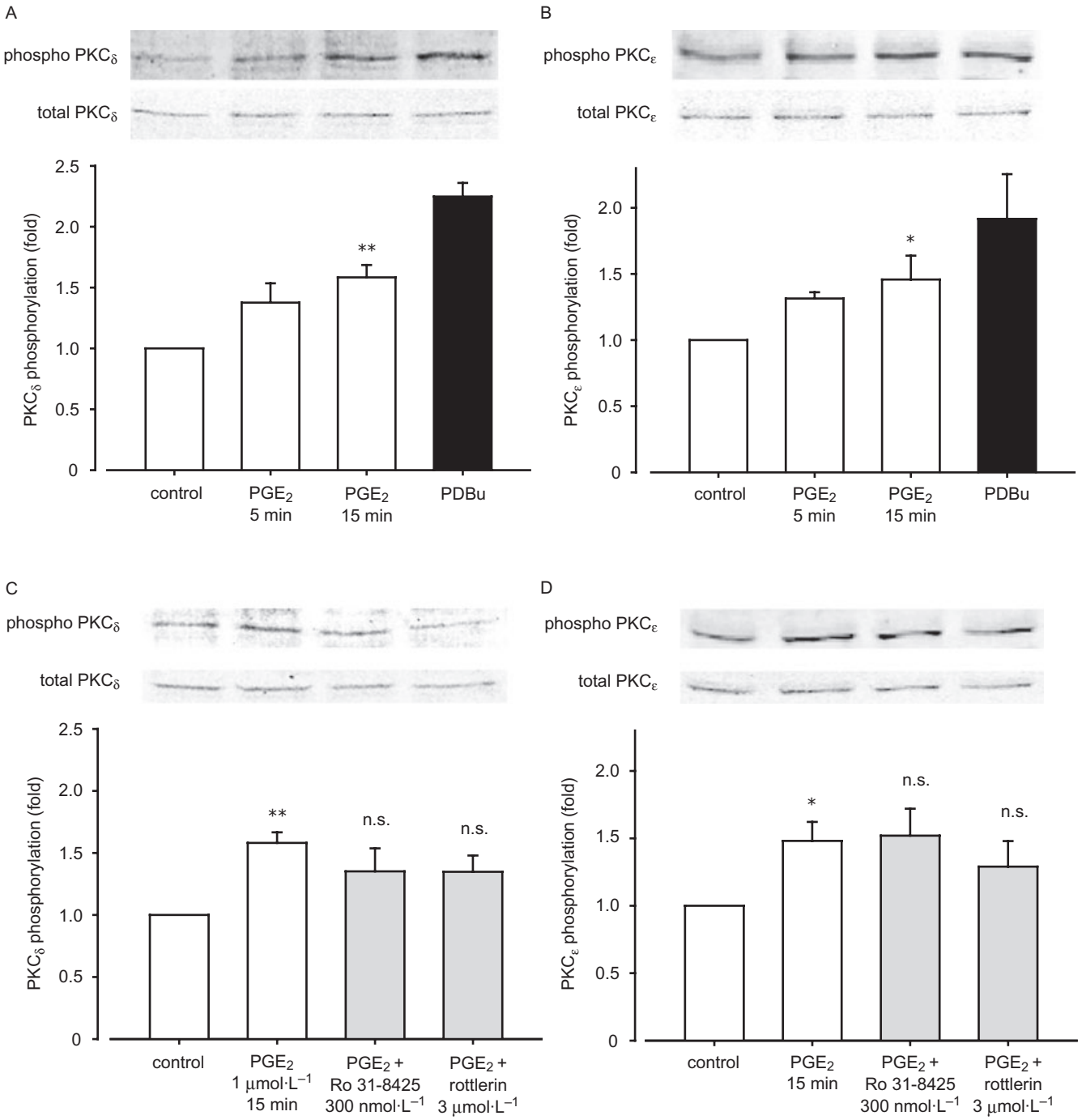

Figure 6 Effect of prostaglandin E_2 (PGE_2) on protein kinase C (PKC) phosphorylations. Cell homogenates were analysed by Western blotting after stimulation with: (A, B) 1 μmol·L^{-1} PGE_2 (5 or 15 min) or 1 μmol·L^{-1} PDBu (5 min), (C, D) 30 min pretreatment with Ro 31-8425 or rottlerin followed by 1 μmol·L^{-1} PGE_2 (15 min). Anti-phospho-PKCδ^{Thr505} antibody (A, C) or anti-phospho-PKCε^{Ser729} (B, D) antibody was used. The phosphorylation was measured by densitometry and normalized as described in Methods. Representative pictures are also shown. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ compared with basal control.

pharmacological therapeutic strategy targeting PGE_2 and its receptor subtypes.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology (Japan) and the Japan Society for the Promotion of Science.

Conflicts of interest

None.

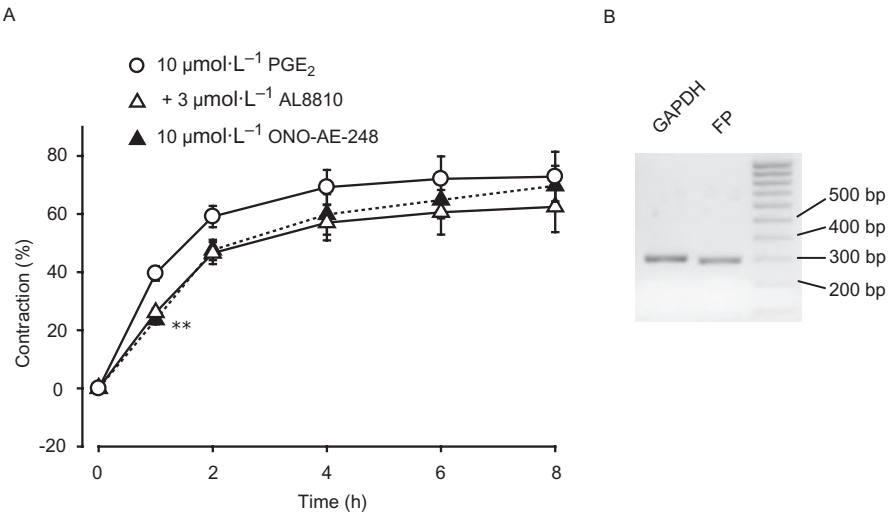

Figure 7 The F prostanoid (FP) receptor selective antagonist AL8810 partially inhibited 10 μmol·L^{-1} prostaglandin E_2 (PGE_2)-induced liver myofibroblast (MF) contraction. (A) Cells were stimulated with 10 μmol·L^{-1} PGE_2 after pretreatment for 30 min with AL8810 at a concentration of 3 μmol·L^{-1}. Data are presented as mean ± SEM. $^{**}P < 0.01$ compared with 10 μmol·L^{-1} PGE_2-treated cells. (B) Representative results from RT-PCR showing the expression of the FP receptor. RT-PCR, reverse transcription-polymerase chain reaction.

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