Figure Legends

Figure I. Effects of temocapril on EC apoptosis after H_2O_2 treatment in rat carotid artery. Representative *en face* specimens stained with Hoechst 33342 are shown. Nuclear morphology of intact EC (round), smooth muscle cells (spindle shaped) and apoptotic EC (arrow heads), as determined by the presence of chromatin condensation, nuclear fragmentation or apoptotic bodies, can be observed. Scale bars indicate 10 μm.

Figure II. Effects of temocapril on neointimal formation after H_2O_2 treatment in rat carotid artery. Temocapril (10 mg/kg/day; n=9) or its vehicle (n=9) was administered orally for 3 days before H_2O_2 treatment. Histological analyses were performed 2 weeks after H_2O_2 treatment. Representative cross-sections with Elastica van Gieson staining (left panels) and morphometric analyses (right panels) are shown. Scale bars indicate 100 μm. *$P<0.05$ vs. vehicle. Values are expressed as mean ± SEM.

Figure III. Effects of PD123319 (A) and L-NAME (B) on H_2O_2-induced EC apoptosis in culture. PD123319 (10 μmol/L), L-NAME (10 μmol/L), temocapril (100 μmol/L) or their vehicle was added to the culture medium 24 hr before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 hr after H_2O_2 treatment (0.2 mmol/L) by means of DNA fragmentation. NS, not significant. Values are expressed as mean ± SEM (n=3). Similar results were obtained in three independent experiments.

Figure IV. Effects of temocapril and olmesartan on 2',7'-dichlorofluorescein (DCF) formation in cultured EC. Temocapril (100 μmol/L), olmesartan (10 μmol/L) or their vehicle was added to the culture medium 24 hr before H_2O_2 treatment until assay. Intracellular DCF intensity were measured using the Metamorph software 3 hr after H_2O_2 treatment. Values are expressed as mean ± SEM (n=3). Similar results were obtained in three independent experiments.

Figure I.

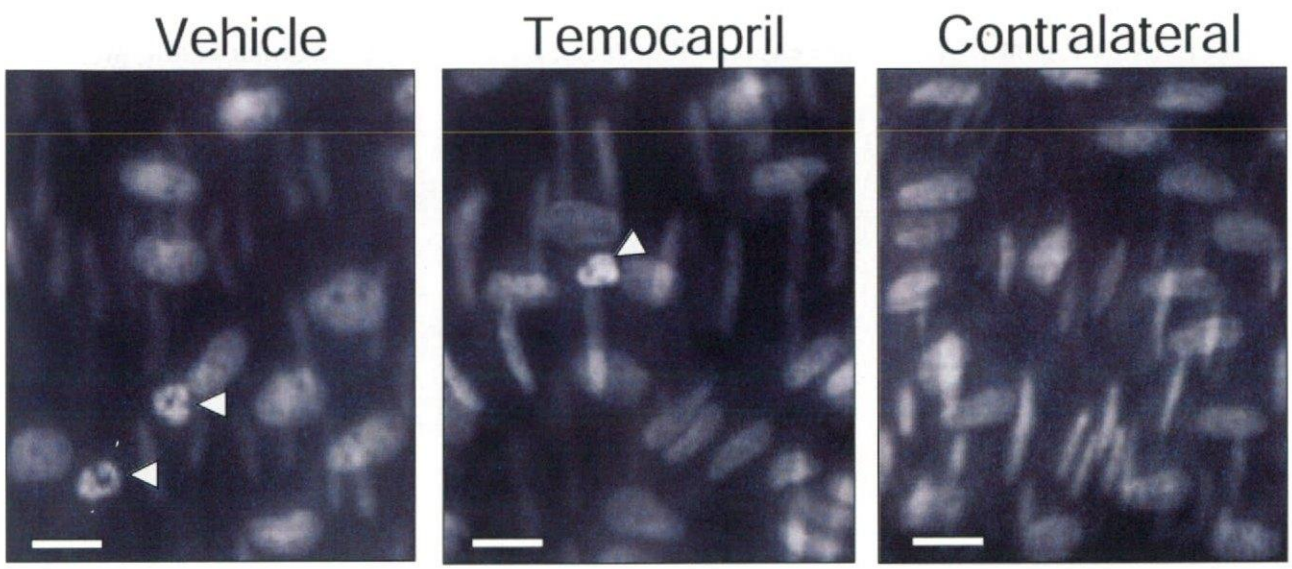


Figure II.

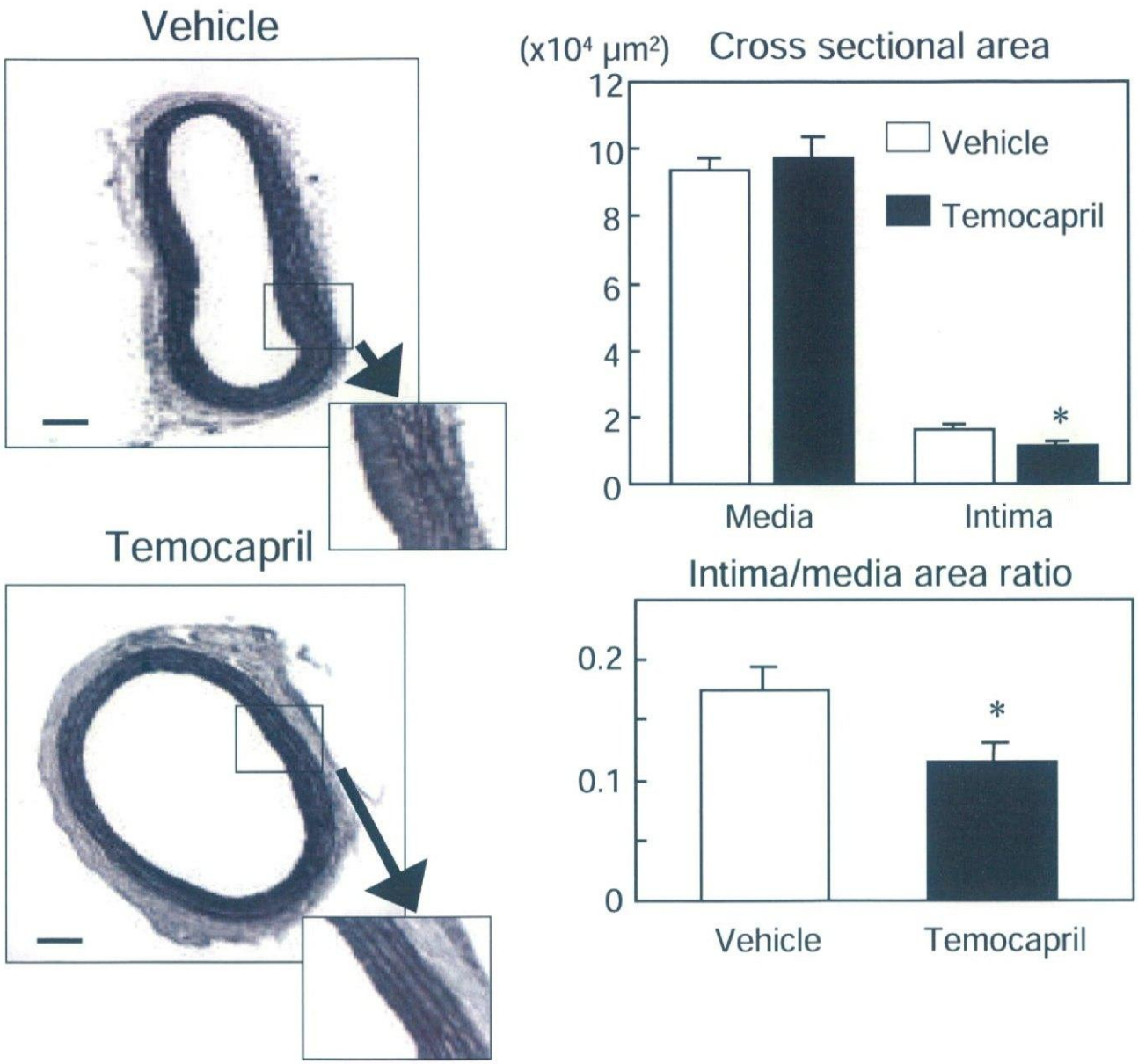
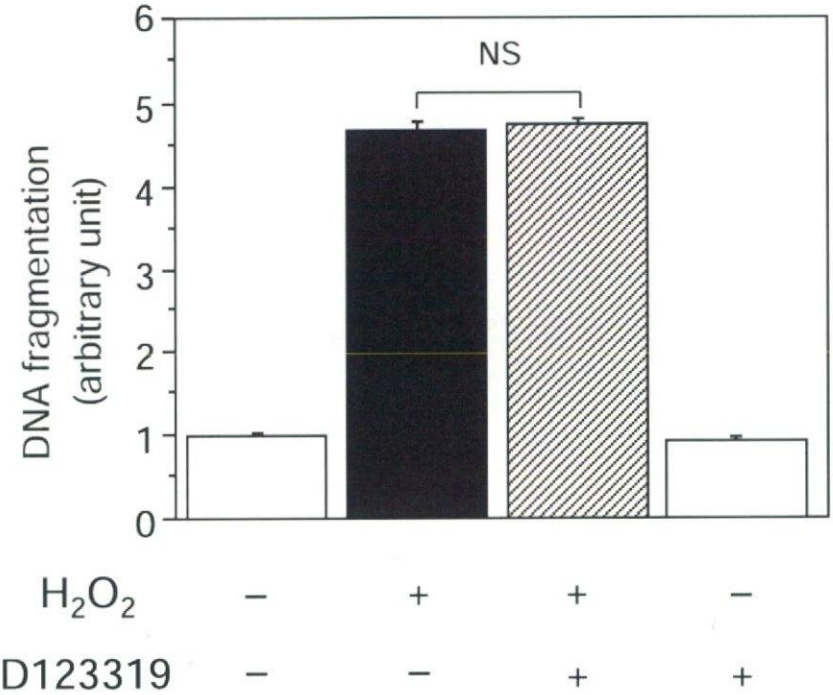


Figure III.

A.



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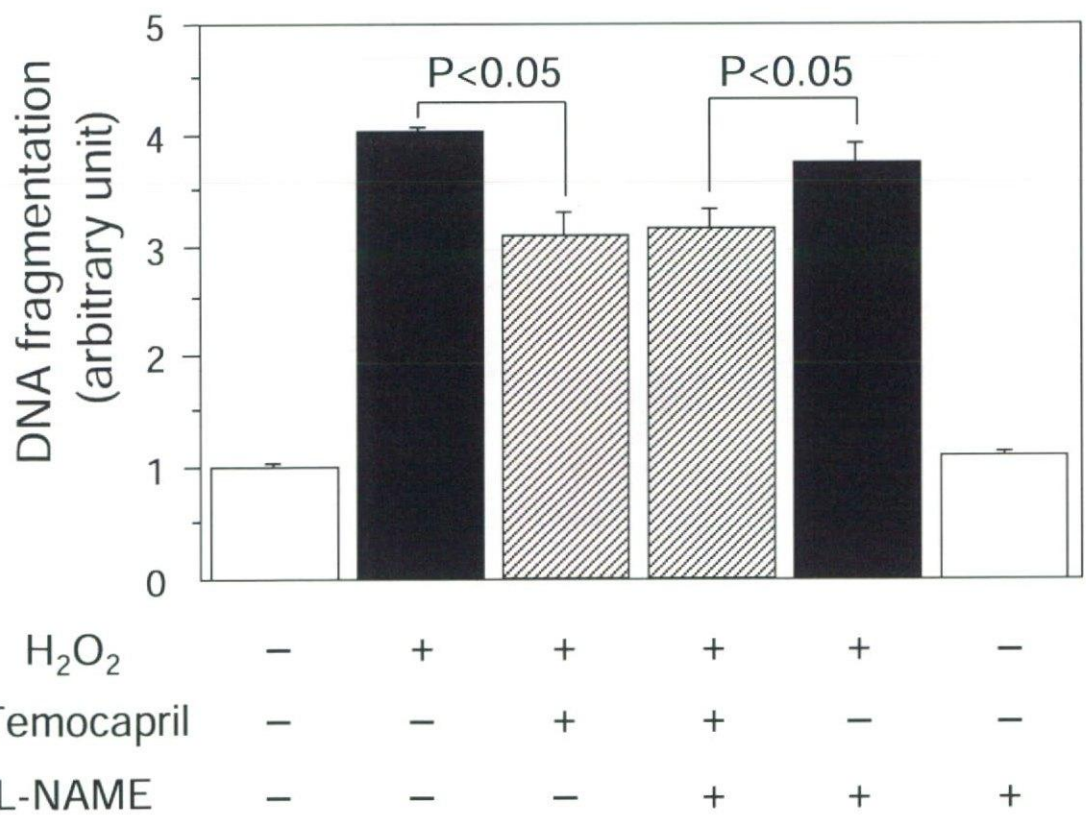
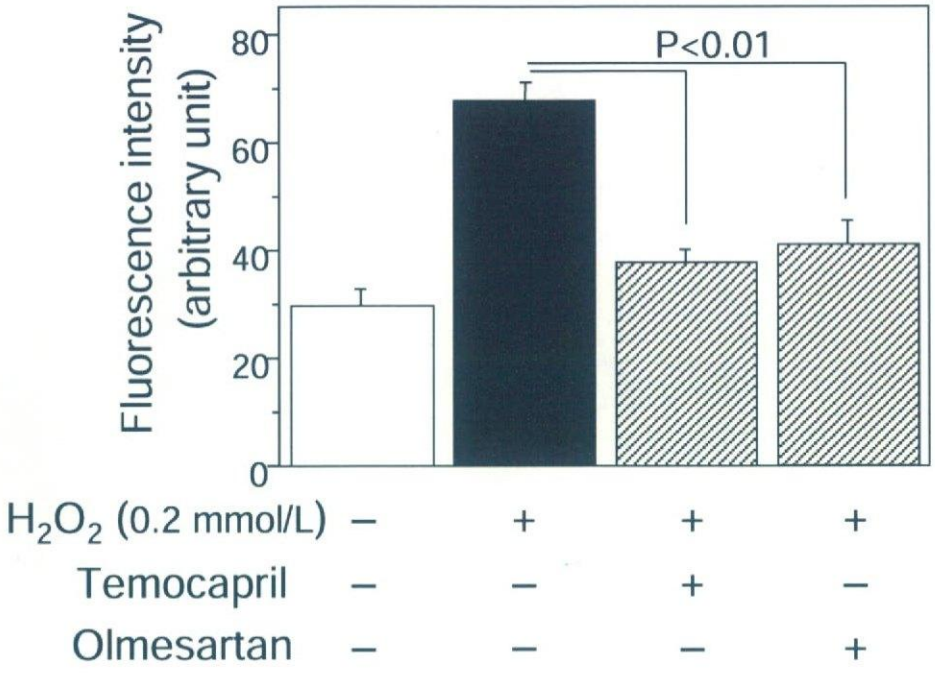


Figure IV.



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5α-Bile alcohols function as farnesoid X receptor antagonists ☆

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Abstract

The farnesoid X receptor (FXR) is a bile acid/alcohol-activated nuclear receptor that regulates lipid homeostasis. Unlike other steroid receptors, FXR binds bile acids in an orientation that allows the steroid nucleus A ring to face helix 12 in the receptor, a crucial domain for coactivator-recruitment. Because most naturally occurring bile acids and alcohols contain a *cis*-oriented A ring, which is distinct from that of other steroids and cholesterol metabolites, we investigated the role of this 5β-configuration in FXR activation. The results showed that the 5β-(A/B *cis*) bile alcohols 5β-cyprinol and bufol are potent FXR agonists, whereas their 5α-(A/B *trans*) counterparts antagonize FXR transactivation and target gene expression. Both isomers bound to FXR, but their ability to induce coactivator-recruitment and thereby induce transactivation differed. These findings suggest a critical role for the A-ring orientation of bile salts in agonist/antagonist function.

Keywords: Nuclear receptor; Farnesoid X receptor; Bile acid; Bile alcohol; Agonist; Antagonist; Coactivator

The farnesoid X receptor (FXR; NR1H4) is a nuclear receptor that is activated by bile acids [1,2] and bile alcohols [3,4], and it plays an essential role in bile acid/cholesterol homeostasis [5,6]. FXR belongs to the steroid hormone receptor superfamily, however, crystal structure studies have suggested that bile acids bind FXR with their steroid backbone flipped head to tail, the reverse orientation of all other steroid hormones, when they bind to their cognate receptors. Steroid hormones, such as testosterone, glucocorticoids, and estrogen, are oriented with their D rings facing helix 12 of their respective receptors [7–9], whereas the A ring of bile acids faces helix 12 of FXR [10,11].

Helix 12, the most C-terminal helix in the ligand binding domain of nuclear receptors, plays a crucial role in ligand-dependent receptor activation. Binding of an agonist to a receptor leads to a conformational change that allows the receptor to interact with a coactivator, which mediates ligand-dependent transcription of the receptor [12]. In this activated state, helix 12, the activation function 2 (AF2), functions as a molecular switch and forms one side of the coactivator binding pocket [13]. Structural analysis studies have demonstrated that agonist and antagonist bind at the same site within the core of the ligand-binding domain, but induce different conformations [8,10,11,14]. Agonists have been shown to stabilize the agonist conformation of helix 12 via direct or indirect interactions, and partial agonists or antagonists have been shown to destabilize it.

Unlike those of other steroids and cholesterol metabolites, the A rings of most naturally occurring bile acids are *cis*-oriented (5β-configuration). Because structural studies have shown that the A ring of bile acids is in contact with several amino acid residues on helices 11 and 12

☆ *Abbreviations:* FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α-hydroxylase; SHP, the small heterodimer partner; BSEP, bile salt export pump; PXR, pregnane X receptor.

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[10,11], it seems likely that the 5β-(A/B *cis*) ring juncture of bile acids plays a critical role in stabilizing the agonist-bound conformation of helix 12.

Bile alcohols are produced as intermediates in the bile acid synthetic pathway in mammals and as end-products of cholesterol catabolism in most evolutionarily primitive vertebrates [15]. We have shown that bile alcohols possess FXR-ligand properties similar to those of the corresponding bile acids [3]. Although the majority of naturally occurring bile alcohols are 5β-bile alcohols, few species of fishes and frogs produce 5α-bile alcohols containing a *trans*-oriented A ring. The bile alcohol 5α-cyprinol was originally isolated from the bile of *Cyprinus carpi* [16], the Asiatic carp, and 5α-bufol was isolated from the bile of lungfish [17] and frogs [18] (Fig. 1). Since our preliminary experiments showed that their 5β-counterparts, 5β-cyprinol and 5β-bufol, are potent agonists of human FXR, in this study we investigated whether these 5α-bile alcohols possess the ability to bind to FXR and recruit a coactivator. The results showed that both of these 5α-bile alcohols are capable of binding to FXR but are unable to induce coactivator-association, and as a result antagonize FXR activation.

FXR activation has been shown to repress the expression of cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme in the bile acid biosynthetic pathway, by inducing an orphan nuclear receptor, the small heterodimer partner (SHP) [19,20]. FXR also up-regulates expression of the bile salt export pump (BSEP) [21], which represents the major canalicular bile salt export pump of the liver. We also investigated whether the 5α-bile alcohols modulate FXR-target gene expression.

Materials and methods

Bile alcohols and chemicals. Cholic acid and chenodeoxycholic acid were commercial products. 5α- and 5β-Cholestane-3α,7α,12α,26,27-pentols (5α-cyprinol and 5β-cyprinol) were isolated from the bile of carp [16,18]. 5α- and 5β-Cholestane-3α,7α,12α,25,26-pentols (5α-bufol and 5β-bufol) were isolated from the bile of frogs and toads, respectively [18,22]. GW4064 is synthesized according to the published procedures [23].

Transient transfections and reporter gene assays. HepG2 cells were maintained in DMEM containing 10% FCS and 100 μg/ml kanamycin, and they were seeded in 24-well plates 24 h prior to transfection. Cells were transfected with 85 ng pFXRE-tk-Luc [3], 25 ng each of the pcDNA3.1 expression vectors for human FXR (NR1H4) and RXRα, and either 65 ng of *Renilla* luciferase vector (phRL-TK) or pSV-β-galactosidase vector (Promega) with Effectene (Qiagen). Three hours after transfection, cells were exposed for 24 h to bile acids or bile alcohols in the medium containing 0.5% delipidated FBS. Cells were lysed and luciferase activity was determined. Firefly luciferase activity was normalized to *Renilla* luciferase or β-galactosidase activity for each well.

Coactivator-association assay using fluorescence polarization. The assay was performed essentially according to the published procedure [4]. TAMRA-labeled peptide (100 nM, with amino acid sequence ILRKLLQE) was incubated for 1 h with 1.5 μM of purified GST-fused human FXR ligand binding domain (residues 244–472) and ligands in 100 μl of buffer (10 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT at pH 7.9) in a black polypropylene 96-well plate on a shaker. Ligand-dependent recruitment of the coactivator peptide was measured as increases in fluorescence polarization with a Mithras LB-940 multilabel reader (Berthold).

mRNA analysis by real-time quantitative RT-PCRs. Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism

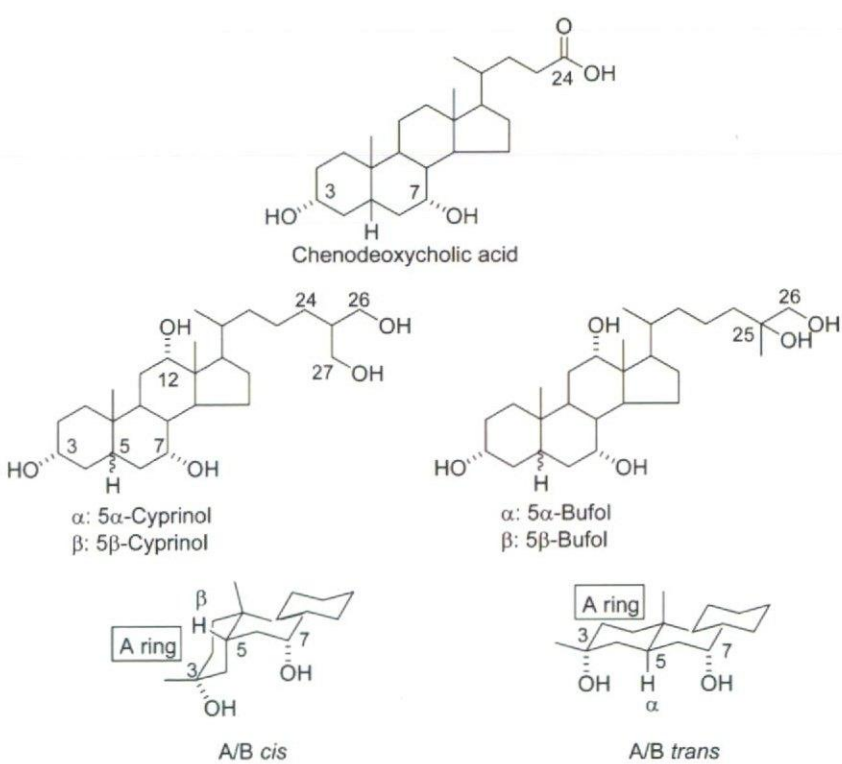

Fig. 1. Structure of bile alcohols and chenodeoxycholic acid (CDCA). 5α- and 5β-Cyprinol, 5α- and 5β-cholestane-3α,7α,12α,26,27-pentols; 5α- and 5β-bufol, 5α- and 5β-cholestane-3α,7α,12α,25,26-pentols; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.

7700 sequence detection system (Applied Biosystems). Human hepatoma-derived HepG2 cells were exposed to bile acids or bile alcohols in DMEM containing 0.5% delipidated FBS for 20 h. Total RNA extracted with the RNeasy Mini Kit (Qiagen) was treated with DNAase according to the manufacturer's instructions (Qiagen). The relative expression levels of mRNA were determined using the TaqMan one-step RT-PCR Master Mix Reagent Kit. The primer/probe sequences for human BESP, CYP7A1, and SHP have been reported previously [4,24].

Other methods. Cell viability was checked by leakage of lactate dehydrogenase into the medium. Statistical significance was determined by ANOVA followed by the Student Newman–Keuls method.

Results

The ability of bile alcohols to activate human FXR was assessed by means of a transient transfection assay. HepG2 cells were cotransfected with a FXRE-driven luciferase reporter plasmid and expression plasmids for FXR and RXRα. Exposure of the cells to 5β-cyprinol or 5β-bufol, bile alcohols containing two hydroxyl groups in their side chain (Fig. 1), led to the induction of luciferase activity at levels comparable to that of the most potent physiological FXR ligand, CDCA (Fig. 2A), whereas their 5α-counterparts, 5α-cyprinol and 5α-bufol, had little effect. However, these 5α-bile alcohols inhibited the transactivation elicited by either 50 μM CDCA (Fig. 2B) or 1 μM GW4064, a synthetic FXR agonist structurally unrelated to bile acids [23] (Fig. 2C).

In an in vitro coactivator-recruitment assay, 5β-cyprinol and 5β-bufol induced a dose-dependent interaction of SRC-1 peptide with FXR, but not with LXRα (Fig. 3A). By contrast, 5α-cyprinol induced a very weak interaction, accounting for 20% of 5β-cyprinol-induced interaction (Fig. 3B). 5α-Bufol induced no interaction at all. When assayed with 1 μM GW4064, these 5α-bile alcohols reduced (by 80%) the GW4064-elicited interaction (Fig. 3C). These findings show that 5α-cyprinol and 5α-bufol act as FXR antagonists, whereas their 5β-counterparts, 5β-cyprinol and 5β-bufol, are FXR agonists.

We used real-time quantitative RT-PCR to investigate the effect of these bile alcohols on the expression of FXR-target genes in HepG2 cells. 5β-Cyprinol and 5β-bufol increased the BSEP mRNA level, whereas 5α-cyprinol and 5α-bufol had little effect (Fig. 4A). When combined with 50 μM CDCA, these 5α-bile alcohols decreased CDCA-elicited induction of BSEP mRNA in a dose-dependent manner (Fig. 4B).

5β-Cyprinol and 5β-bufol increased the SHP mRNA level and markedly reduced the CYP7A1 mRNA level (Fig. 4A). The SHP mRNA elevation by 5α-bufol and 5α-cyprinol was small or insignificant. However, unexpectedly, these 5α-bile alcohols markedly repressed CYP7A1 expression (by 90% and 80%, respectively, at 50 μM). By contrast, 90% reduction in the CYP7A1 expression by CDCA was accompanied by a 5.2-fold elevation of the SHP level. When combined with CDCA, these 5α-bile alcohols further enhanced CDCA-elicited repression of CYP7A1, although SHP expression was unchanged or decreased instead (Fig. 4B).

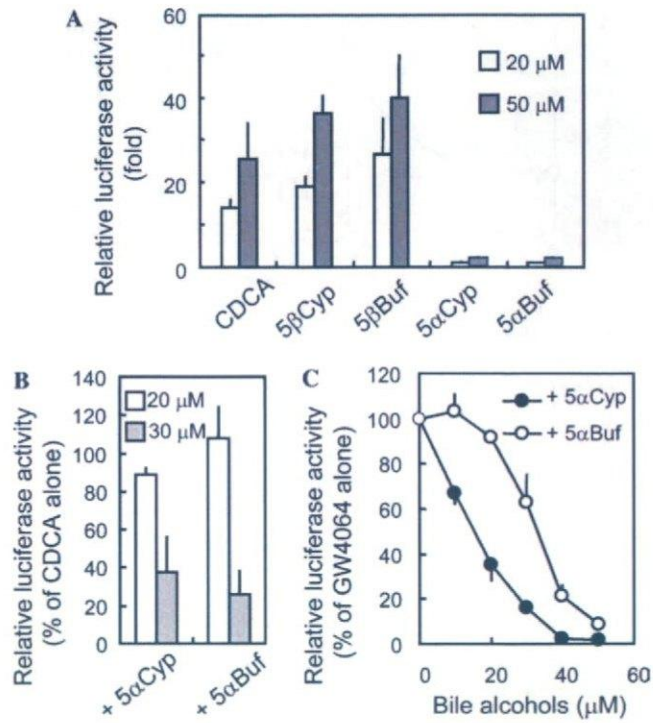

Fig. 2. 5β-Bile alcohols activate FXR, but 5α-bile alcohols function as antagonists in the cellular transactivation assay. (A) HepG2 cells were transfected with expression plasmids for human FXR and RXRα, and the $FXRE_{PLTP}$x4-tk-luc reporter plasmid together with a *Renilla* luciferase plasmid as a control. Cells were exposed to vehicle alone or to 20–50 μM of the bile alcohols indicated. Luciferase activity in the cell extract was normalized to *Renilla* luciferase activity and expressed as fold induction relative to vehicle-exposed cells. The values are means ± SD of three experiments. (B,C) Cells were transfected as in (A), except that β-Gal was used as an internal control, and exposed to 50 μM CDCA (B) or 1 μM GW4064 (C) in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. Exposure of cells to CDCA or GW4064 alone caused a 70- or 115-fold induction, respectively, relative to vehicle-exposed cells. The values are the means ± SD of three experiments.

Discussion

Bile acids and bile alcohols are produced as the terminal catabolites of cholesterol, and as amphipathic steroids they also play important roles in intestinal lipid absorption. Their unusual 5β-A/B *cis* ring juncture provides a structure that allows them to function as excellent detergents [5]. In addition, by activating FXR as physiological ligands, bile acids and alcohols directly modulate expression of genes involved in the biosynthesis/catabolism, excretion, and absorption of bile acids and cholesterol [6]. In the present study, we investigated the role of the A/B ring juncture configuration of bile salts in ligand-activation of FXR.

The results of both cell-based and in vitro assays showed that 5β-cyprinol and 5β-bufol, but not their 5α-counterparts, 5α-cyprinol and 5α-bufol, function as potent FXR agonists (Figs. 2A and 3A), indicating that the *cis*-orientation of the A ring is essential for FXR activation. It was noteworthy that, when assayed with CDCA, the 5α-alcohols potently inhibited agonist-elicited FXR transactivation

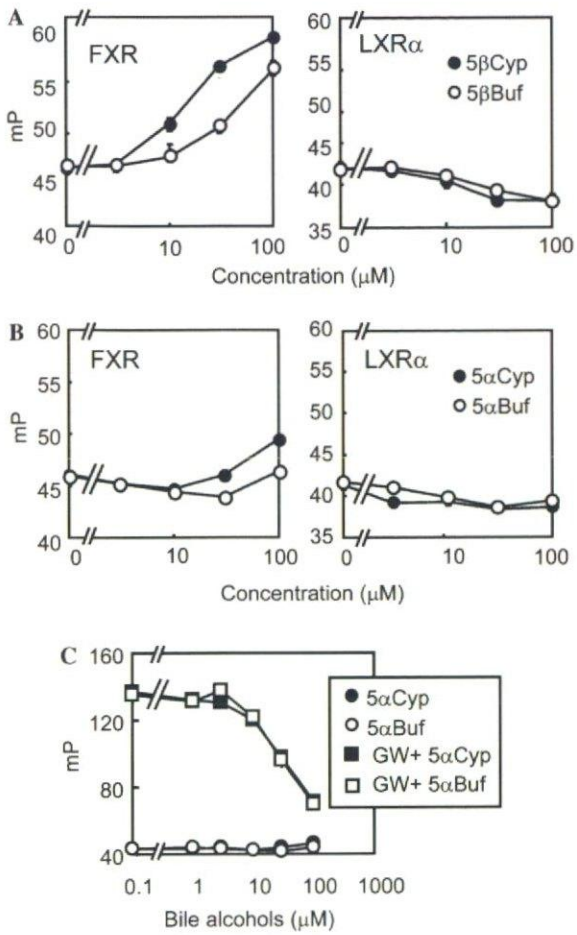

Fig. 3. 5β-Bile alcohols promote association between FXR and SRC-1 peptide in vitro, whereas 5α-bile alcohols function as antagonists, as determined by fluorescence polarization assay. (A,B) A fluorescence-tagged SRC-1 peptide (0.1 μM) was incubated with 1.5 μM GST-FXR or GST-LXRα in the presence of various concentrations of 5β-cyprinol (5β Cyp), 5β-bufol (5β Buf), 5α-cyprinol (5α Cyp), or 5α-bufol (5α Buf) indicated. Ligand-induced SRC-1 peptide association with the receptor was monitored by increases in millipolarization fluorescence units (mP). (C) Changes in fluorescence polarization caused by 3 μM GW4064 were measured in the presence of the concentrations of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated. The values are means ± SD of three experiments. Some error bars are not visible within symbols.

(Fig. 2B). These two 5α-bile alcohols might have inhibited CDCA transport into the cells, and 5α-cyprinol has actually been shown to inhibit taurocholate uptake by *asbt*, the ileal conjugated bile acid transporter [25]. However, their inhibition of GW4064-elicited FXR transactivation and coactivator-association (Figs. 2C and 3C) indicated that they both competitively inhibit agonist-induced FXR activation. The results of the in vitro experiment (Fig. 3) also indicated that these bile alcohols directly activate FXR as ligands without being metabolized. These findings clearly show that 5α-cyprinol and 5α-bufol function as FXR antagonists.

The ability of 5β-cyprinol and 5β-bufol, and inability of their 5α-counterparts to promote coactivator-association to the receptor (Fig. 3) indicate that the A/B *cis* ring juncture (5β) is required for this process. However, the inhibition of GW4064-induced coactivator-association by the 5α-bile alcohols indicates that they are capable of binding to the receptor. Crystal structure studies have demonstrated interaction between the *cis*-oriented A ring of 5β-bile acids and residues on helix 12, corroborating the association between coactivator peptide and the receptor [10,11]. It is conceivable that the *trans*-oriented A ring in the 5α-bile alcohols destabilizes this agonist conformation of helix 12, thereby preventing coactivator-association.

Although 5α-cyprinol and 5α-bufol efficiently inhibited agonist-induced FXR transactivation and FXR-target gene expression, they had no effect in the absence of agonists. In a FXRE-dependent transactivation assay using HepG2 cells, luciferase activity of no-ligand control was very low, suggesting that the level of endogenous FXR ligands is negligible. Indeed, the bile acids produced in hepatic cells in vitro do not accumulate within the cells but are rapidly released to the medium [26], whereas in vivo the liver is constantly supplied with bile acids via the enterohepatic circulation. It is possible that the 5α-bile alcohols may inhibit FXR activation in the liver.

5α-Cyprinol and its sulfate are toxic and sometimes cause renal and hepatic failure after ingestion of goldfish or carp gallbladders [27,28]. A study has shown that 5α-cyprinol inhibits taurocholate uptake [25], but the mechanism of its toxicity is not well understood. By down-regulating BSEP and up-regulating CYP7A1 antagonizing FXR may lead to an increase in the intracellular level of toxic bile acids. In this study, we found that 5α-cyprinol and 5α-bufol antagonize CDCA-induced BSEP mRNA expression in HepG2 cells at concentrations that do not affect cell viability. Because BSEP plays the major role in bile acid excretion by the liver into the bile [29], and genetic defects in BSEP have been shown to cause progressive familial intrahepatic cholestasis [30], reduced BSEP expression may be involved in the mechanism of the toxicity.

The enhancement of CDCA-induced CYP7A1 mRNA repression by 5α-cyprinol and 5α-bufol was an unexpected finding (Fig. 4B). FXR antagonists should inhibit CDCA-elicited SHP induction and thereby diminish CYP7A1 repression. Exposure of cells to these 5α-bile alcohols alone also led to repression of CYP7A1 mRNA (Fig. 4A), although the SHP mRNA elevation was small or insignificant, suggesting a FXR/SHP-independent mechanism. It is noteworthy that ursodeoxycholic acid has been shown to repress CYP7A1 expression despite its negligible ability to activate FXR [31]. Studies have shown that CYP7A1 can be repressed by bile acids via redundant pathways, including repression through activation of the xenobiotic receptor pregnane X receptor (PXR) or activation of c-Jun N-terminal kinase mediated by TNFα or FXR-inducing FGF19 production [5,32–34]. A recent study has shown that 5α-cyprinol activates mouse PXR, but not human PXR [35], suggesting that the activation of a PXR-mediated pathway is unlikely in our experiments on HepG2 cells.

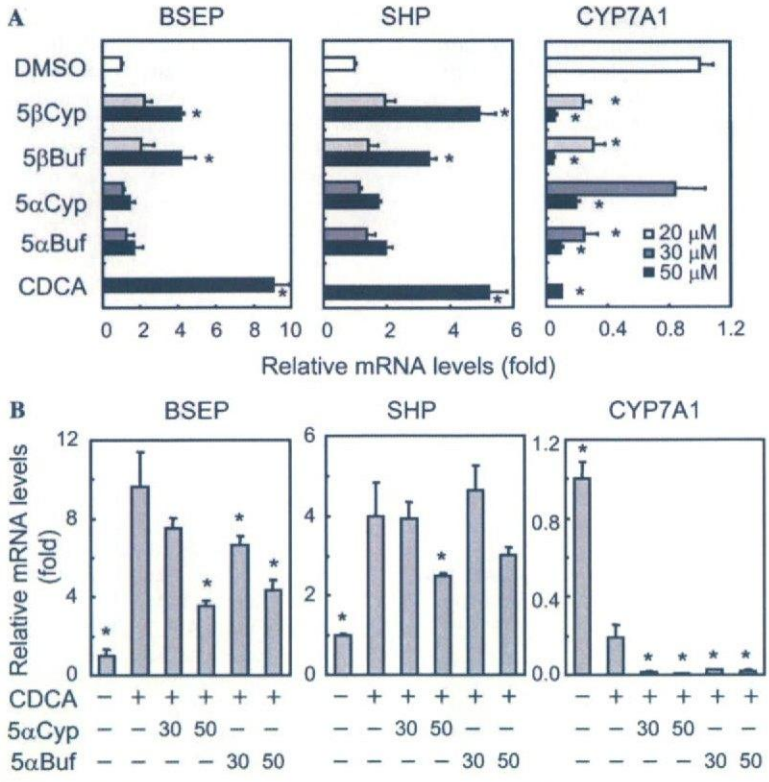

Fig. 4. Regulation of gene expression by various bile alcohols. HepG2 cells were treated for 20 h with vehicle (DMSO) alone, 50 μM CDCA, or the concentrations (20–50 μM) of bile alcohols indicated (A), or with 50 μM CDCA in the presence of the concentrations (μM) of 5α-cyprinol (5α Cyp) or 5α-bufol (5α Buf) indicated (B). Total RNA was isolated from the cells, and the levels of BSEP, SHP, and CYP7A1 mRNA were measured by real-time quantitative RT-PCR. Data were normalized to 18S rRNA levels. The values represent the means ± SD relative to vehicle-exposed cells (taken as 1) from three experiments. Statistically significant differences from respective controls (A) or the cells exposed to CDCA alone (B) are indicated by an asterisk ($*P < 0.01$).

A naturally produced FXR antagonist that lowers cholesterol has been identified [36], and an FXR agonist has been shown to prevent cholesterol gallstone formation [37]. Our findings may provide insights that will be useful for drug development.

Acknowledgments

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Possible Involvement of Increase in Spinal Fibronectin Following Peripheral Nerve Injury in Upregulation of Microglial $P2X_4$, a Key Molecule for Mechanical Allodynia

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KEY WORDS
ATP; purinergic; glia; extracellular matrix; pain

ABSTRACT
We have recently demonstrated that the $P2X_4$ receptor, an ATP-gated cation channel, in spinal microglia is a key molecule that mediates the mechanical allodynia induced by peripheral nerve injury. Although microglial $P2X_4$ receptor expression is increased after peripheral nerve injury, the molecular mechanism(s) underlying its upregulation remains largely unknown. Fibronectin is a member of the extracellular matrix molecules and is actively produced in response to injury and diseases in the CNS. Here, we describe the influence of fibronectin on $P2X_4$ receptor expression in microglia and the upregulation of fibronectin after peripheral nerve injury. Microglia that were cultured on fibronectin-coated dishes showed a marked increase in $P2X_4$ receptor expression, both at the mRNA and protein levels, as compared to those cultured on control dishes. Fibronectin also enhanced the microglial Ca^{2+} responses mediated by $P2X_4$ receptors. Moreover, Western blot examination of the spinal cord from a rat with spinal nerve injury indicated that fibronectin was upregulated on the ipsilateral side. Interestingly, intrathecal injection of ATP-stimulated microglia to the rat lumber spinal cord revealed that microglia cultured on fibronectin-coated dishes was more effective in the induction of allodynia than microglia cultured on control dishes. Taken together, our results suggest that spinal fibronectin is elevated after the peripheral nerve injury and it may be involved in the upregulation of the $P2X_4$ receptor in microglia, which leads to the induction of neuropathic pain.

INTRODUCTION

Extracellular nucleotides act as signaling molecules in numerous tissues. Two groups of purinoceptors with distinct signal transduction mechanisms are known to exist. P2X purinoceptors are ligand-gated ion (cation) channels, whereas P2Y purinoceptors are members of the superfamily of G protein-coupled receptors. The P2X family consists of seven different subunits that can form homo- or hetro-oligomeric assemblies, and each subunit has two transmembrane regions with intracellular N- and C-termini. The $P2X_4$ receptor has a broad expression pattern in the periphery, and it predominates in the CNS (Le et al., 1998; Soto et al., 1996). With regard to the physiological and pathological importance of $P2X_4$ in the CNS, we have recently showed that $P2X_4$ receptors in the spinal cord are upregulated after peripheral nerve injury, which is responsible for the induction of mechanical allodynia in rats (Tsuda et al., 2003). Interestingly, the $P2X_4$ receptor is upregulated in microglia but not in neurons in the spinal cord. Allodynia is a form of neuropathic pain that is caused by normally innocuous stimuli, such as touch, and although the symptom has been recognized for over a century, its cellular mechanisms are largely unknown. Microglial $P2X_4$ receptors in the spinal cord could be a key molecule that induces the mysterious neuropathic pain, allodynia.

Microglia are brain-specific macrophages, and their activation is a general response to pathological processes in the CNS. They are in a quiescent state in the normal brain, but become rapidly activated upon brain injury, inflammation, or diseases, transforming from ramified microglia into an amoeboid macrophage-like phenotype. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated (Milner and Campbell, 2002, 2003). Fibronectin is one of the extracellular matrix (ECM) molecules, and it is a large, multi-domain glycoprotein existing both as a cell surface protein and in plasma. Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion (Adams and Watt, 1993; Hynes, 1992; Raghow, 1994). The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the normal CNS. Some ECM molecules including fibronectin, however, are upregulated following adult CNS injury (Jones, 1996). These

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data suggest that fibronectin is a key molecule involved in the overexpression of $P2X_4$ in microglia after nerve injury.

In the present study, we demonstrate that: (1) culturing primary microglia on fibronectin induces the upregulation of functional $P2X_4$ receptors on the cell surface in vitro, (2) increased expression of fibronectin was observed in the ipsilateral side of the spinal cord taken from allodynia rats, and (3) intrathecal administration of ATP-stimulated microglia that had been treated with fibronectin enhanced the allodynic response in rats in vivo. All these findings suggest that the increase in spinal fibronectin after a spinal nerve injury is a critical event in the upregulation of microglial $P2X_4$ receptors, which would be important for the onset of mechanical allodynia.

MATERIALS AND METHODS

Isolation of Microglia

The primary cultures of rat microglia were derived from the forebrains of neonatal Wistar rats (Nakajima et al., 1992). In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and with DNase, and then centrifuged to remove dead cells. The pellet was resuspended in DMEM, filtrated, and cultured in medium with 10% fetal bovine serum for 12–23 days. Microglia were isolated on day 10 and day 15 by gently shaking the flasks for 2 min.

Quantitative RT-PCR

Microglia were plated on tissue culture dishes that had been coated with fibronectin (Sigma, Missouri, USA) at 10 μg/ml or non-treated, and kept at 37°C for 3 h. Then, the cells were washed with warm DMEM twice and the total RNA was extracted using the RNeasy mini kit (QIAGEN Japan, Tokyo, Japan). Real time RT-PCR was performed using the TaqMan One-Step RT-PCR Master Mix Kit (Applied Biosystems, CA), $P2X_4$ primers, and TaqMan GAPDH Control Reagents (Applied Biosystems). The forward and reverse primer pairs for $P2X_4$ were:

F: 5′-TGGCGGACTATGTGATTCCA-3′

R: 5′-GGTTCACGGTGACGATCATG-3′

The PCR reaction was carried out by One Step RT-PCR in a total volume of 25 μl using the ABI PRISM 7700 Sequence Detection system (Applied Biosystems). All values were normalized with the GAPDH expression.

Western Blotting

Microglia were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% SDS, 5 mM EDTA, protease inhibitors cocktail) and mixed with Laemmli sample buffer. For the rat spinal cord homogenates, the L5 corresponding spinal cord was collected from control or allodynia rats of 1-, 3-, 7-day post operation and the area of dorsal horn was excised. Then the tissue was homogenized in homogenization buffer (PBS, 1% NP-40, 1% Triton X-100, 5mM EDTA, protease inhibitors cocktail) for 20 s on ice, centrifuged thoroughly to remove cell debris, and mixed with Laemmli sample buffer. All samples were subject to BCA assay to adjust the loading protein amount. Cell lysates or tissue homogenates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, CA). The membrane was blocked with TBS-Tween 0.05%, 1% BSA, 0.02% NaN_3, and probed with primary antibodies: anti-$P2X_4$ (Alomone, Jerusalem, Israel, 1:200 dilution), anti-β-actin (Sigma, 1:1000 dilution), anti-ERK2 (Santa Cruz, CA, 1:200 dilution), or anti-fibronectin (Dako, Glostrup, Denmark, 1:100 dilution). The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (Amersham Biosciences, NJ, 1:1000 dilution) and visualized with the ECL system (Amersham Biosciences). Bands were quantified using NIH Image J 1.33u software.

Intracellular Calcium Concentration ($[Ca^{2+}]i$) Measurement

Microglia were cultured for 24 h at 37°C on an appropriately coated Flexiperm cover glass. Then the culture medium was replaced with balanced salt solution (BSS at pH 7.4: 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, 10 mM D-glucose). Cells were loaded with fura-2 by incubating them with 5 μM fura-2-acetoxymethylester in BSS for 1 h at room temperature. Changes in $[Ca^{2+}]i$ were assessed by ratiometric images (F340/F380) of fura-2 fluorescence, which were detected with Aquacosmos/HiSca (Hamamatsu Photonics, Hamamatsu, Japan). For TNP-ATP(100 μM), PPADS (10 μM), or 0 Ca^{2+} (removal of extracellular Ca^{2+}) experiments, cells were treated with these antagonists or the 0 Ca^{2+} solution 2 min before and during ATP-applications.

Chung Model

All experiments were performed using 8-week-old male Wistar rats. All surgeries were performed under inhalation anesthesia using Forene in 100% O_2, induced at 5% and maintained at 2%. The spinal nerve on the left side was exposed at a proximal location under an aseptic condition. Then the 5^{th} lumber spinal nerve was tightly ligated with a silk suture (5-0) and its peripheral side was completely transected. The muscle and the skin were sutured closed, and the animal was allowed to recover before the behavioral testing. To evaluate allodynia, von Frey filaments were applied to the plantar surface of the hindpaw, and the withdrawal from mechanical stimulus was monitored as previously reported (Tsuda et al., 2003).

Intrathecal Catheterization and Injections of Microglia

For intrathecal microglia administration, intrathecal catheterization was performed on Wistar rats (12 weeks, male) (Tsuda et al., 2003). Briefly, with the rat under inha-

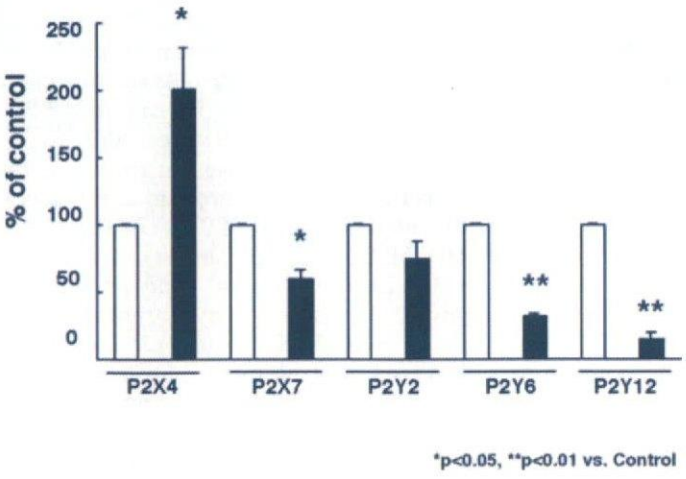

Fig. 1. The effect of fibronectin on the mRNA expression of microglial $P2X_4$. Fibronectin increased the expression of $P2X_4$ in microglia at the mRNA level. Microglia were cultured on fibronectin for 3 h at 37°C, and the expression of $P2X_4$ was assessed by quantitative RT-PCR. $P2X_4$ was markedly upregulated by fibronectin, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ purinoreceptors were significantly decreased. Data are mean ± SE of 3 separate experiments. Asterisks show significant difference from control (*$P < 0.05$, ** $P < 0.01$ vs. control, Student's t-test).

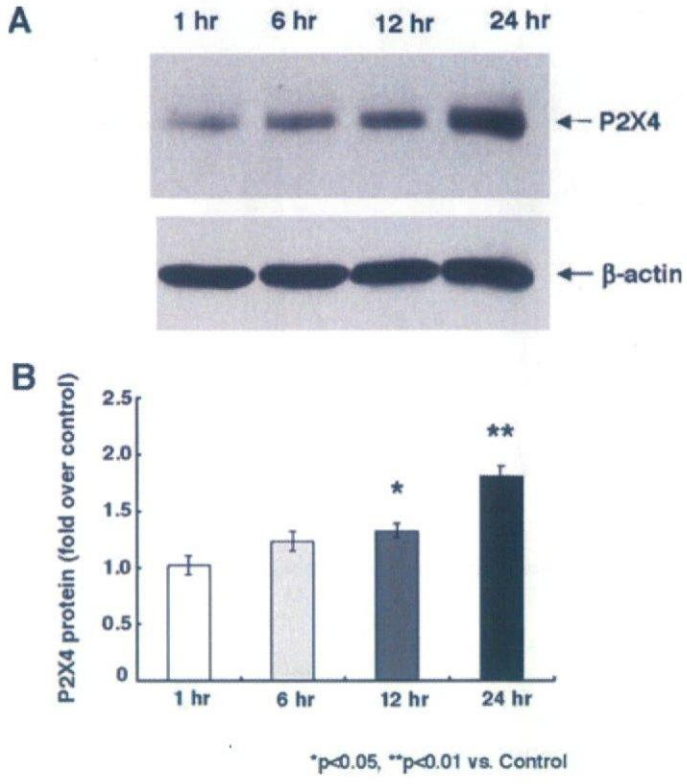

Fig. 2. Time-course study of the microglial $P2X_4$ upregulation. Fibronectin increased the expression of $P2X_4$ in microglia at the protein level. Microglia were cultured on fibronectin for 1, 6, 12, and 24 h at 37°C, and the protein expression of $P2X_4$ receptors was analyzed by Western blotting. The protein expression of $P2X_4$ receptors began to increase after 12 h of incubation and increased strongly after 24 h of incubation. The intensity of the bands was quantified with a computing densitometer using NIH Image J 1.33u image analysis software. Asterisks show significant difference from control (*$P < 0.05$, ** $P < 0.01$ vs. control, Student's t-test).

lation anesthesia, an incision was made in the atlanto-occipital membrane and the catheter was inserted caudally to the lumber enlargement (close to L4-L5 segments) of the spinal cord. Verification of the catheter placement was made by the observation of hind limb paralysis after intrathecal injection of lidocaine (2%, 5 μl) 3 days after catheterization. Animals that failed the verification for the catheter placement were not included in the data analyses. Microglia were cultured on uncoated- or fibronectin-coated dishes for 24 h at 37°C, washed twice with PBS, and harvested. After adjusting their concentrations, cells were stimulated with ATP at 0, 0.5, and 5 μM, incubated for 1 h at 37°C, and subsequently microinjected. Animals were subject to the behavioral testing 5 h after the injection.

Statistical Analysis

The von Frey test results were analyzed by the Mann-Whitney U-test and values with $P < 0.05$ were considered statistically significant. For the other data, the Student's t test was performed, and values with $P < 0.05$ (or $P < 0.01$ where appropriate) were considered statistically significant as compared to controls.

RESULTS

Fibronectin Increased the Expression of $P2X_4$ Receptors in Microglia at both the mRNA and Protein Levels

Microglia were plated onto fibronectin or control plastic, and their P2 receptor expression was studied by quantitative RT-PCR (Fig. 1). To normalize the results, we used the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and, therefore, the P2 receptor gene expression was given as the ratio P2X(Y)/GAPDH. As shown in the figure, incubation of microglia on fibronectin at 10 μg/ml for 3 h resulted in the marked upregulation of $P2X_4$ gene expression, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ were all rather diminished (Fig. 1), suggesting that the $P2X_4$ receptor is unique among the purinoceptors on microglia.

To confirm this effect of fibronectin on the $P2X_4$ receptor at the protein level, we examined its expression by Western blotting using anti-$P2X_4$ antibody (Fig. 2). As seen in Fig. 2, microglial $P2X_4$ appeared as a single band at ~75 kDa, and since its predicted molecular weight from its protein sequence is 43 kDa, the molecule seems to be heavily glycosilated (Soto et al., 1996). We previously reported that fibronectin induces profound microglial proliferation through β1 integrin (Nasu-Tada et al., 2005), and thus the protein amount loaded on the gel was carefully adjusted. In addition, β-actin was used as the endogenous control to normalize the Western blot data. Each band was quantified using computing software, and the basal value of β-actin was subtracted from the $P2X_4$ results. The increase in $P2X_4$ expression became evident after 12 h of fibronectin stimulation (Fig. 2) (1.3-fold as compared to 1 h incubation, $P < 0.05$), and the increase continued until it reached an

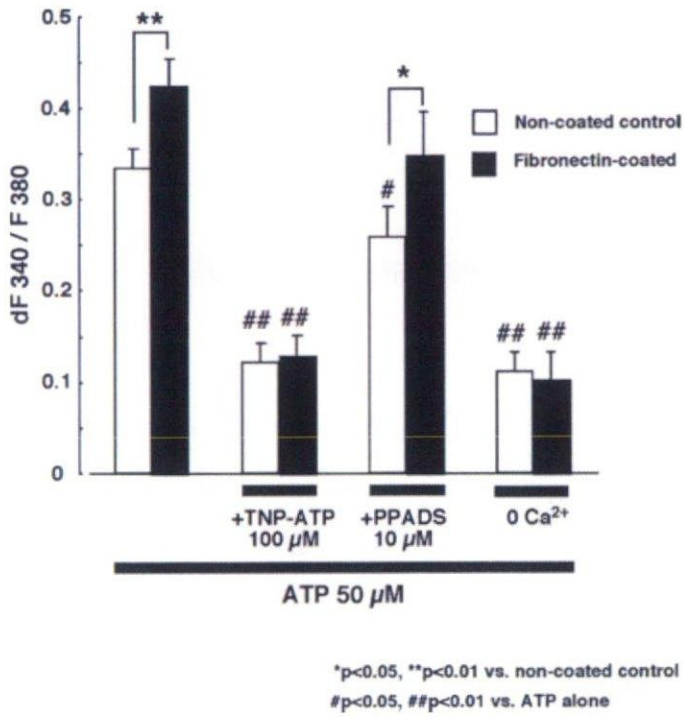

Fig. 3. Enhancement by fibronectin of the function of $P2X_4$ receptors in microglia. The function of microglial $P2X_4$ was assessed by fura-2 based $[Ca^{2+}]i$ imaging (ratio of F340/F380). Microglia cultured on fibronectin showed an increase in the Ca^{2+} response to stimulation with ATP 50 μM. Microglia were cultured for 24 h on fibronectin or on a control, and pretreated with TNP-ATP (100 μM) or PPADS (10 μM) for 2 min where required. Flexiperm cover glass (i.e., non-coated) and the ATP (50 μM)-evoked increase in $[Ca^{2+}]i$ was monitored. 0 Ca^{2+} indicates removal of Ca^{2+} from the extracellular medium. Asterisks and #s show significant difference from non-coated control and ATP alone, respectively (*$P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. ATP alone, Student's t-test).

approximately 2-fold increase after 24 h incubation ($P < 0.01$).

Microglia Cultured on Fibronectin Showed an Increase in $[Ca^{2+}]i$ in Response to ATP Stimulation

To confirm that fibronectin upregulates functional $P2X_4$ receptors on microglia, the ATP-evoked increases in $[Ca^{2+}]i$ were subsequently studied. Microglia were cultured for 24 h on fibronectin or on uncoated Flexiperm cover glass (control), and the changes in $[Ca^{2+}]i$ in response to ATP (50 μM) were detected by the conventional fura-2 method., i.e., the ratiometric images of fura-2 fluorescence. The nucleotide receptors that are known to be expressed in microglia include $P2X_4$, $P2X_7$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$ (Inoue, 2002; Sasaki et al., 2003; Tsuda et al., 2003), and possibly $P2Y_{13}$ due to its abundant mRNA in the brain and the immune system (Zhang et al., 2002). $P2Y_{12}$ and $P2Y_{13}$ receptors are Gi-coupled P2 receptors, and the activation of these receptors, in general, does not cause an elevation in $[Ca^{2+}]i$ but decreases the intracellular cAMP.

In the absence of extracellular Ca^{2+} (Fig. 3, 0 Ca^{2+}), neither the control nor microglia cultured with fibronectin showed much response to ATP 50 μM stimulation, suggesting that Gq/11-phospholipase C coupled P2Y receptors, which are dependent on intracellular Ca^{2+} storage, were not relevant to this case. In contrast, in the presence of extracellular Ca^{2+}, microglia on fibronectin showed a significant increase ($P < 0.01$) in the Ca^{2+} response to ATP 50 μM (Fig. 3, ATP alone), indicating that the expression of the ion-channel type purinoceptors, i.e., P2X receptors, is augmented by fibronectin and that these are likely to be $P2X_4$ receptors, since the $P2X_7$ receptor is activated at a relatively high concentration of ATP (i.e., concentrations greater than 100 μM) (Ralevic and Burnstock, 1998). Pretreatment of cells with TNP-ATP (an antagonist of $P2X_{1-4}$ receptors) dramatically reduced the $[Ca^{2+}]i$ response in microglia on both control and fibronectin-coated dishes, indicating that basal response to ATP at 50 μM as well as its augmented response on fibronectin substrate mostly result from microglial $P2X_4$ receptor. On the other hand, pretreatment with PPDAS (an antagonist of $P2X_{1,2,3,5,7}$) did not fundamentally affect but only slightly reduced the $[Ca^{2+}]i$ response in both populations. PPADS is known to inhibit $P2Y_{1-2}$ receptors as well and, therefore, the result indicates that microglial $P2Y_2$ receptor also constitutes the $[Ca^{2+}]i$ response to ATP stimulation. In conclusion, fibronectin upregulated the functional $P2X_4$ receptors on the microglial surface, and this lead to an enhancement of the increase in $[Ca^{2+}]i$ evoked by ATP 50 μM via $P2X_4$ receptors.

Fibronectin Was Upregulated in the Allodynia Rat Spinal Cord

As described earlier, the importance of the microglial $P2X_4$ receptor in the induction of mechanical allodynia after nerve injury has recently become evident (Tsuda et al., 2003). We sought to determine the profile of fibronectin expression in the spinal cord of nerve-injured rats, where the microglial $P2X_4$ receptor expression is increased. L5 spinal cord segments were harvested from rats of control, 1-, 3- and 7-day post nerve injury, and the expression of fibronectin was assessed by Western blotting. There has been little evidence for fibronectin in the normal CNS other than in the basement membrane of endothelial, pial, and ependymal cells, and our result showed that Naive and Day 1 rats exhibited slight signs of fibronectin (Fig. 4). However, spinal fibronectin became evident on the ipsilateral side at 3 and 7 days following the nerve injury (Fig. 4, Day 3 ipsi, Day 7 ipsi). The contralateral side remained unchanged throughout the experiment.

Upregulation of Microglial $P2X_4$ Receptors Lowered the Threshold of Pain Responses Caused by Intrathecal Transfer of the Cells

In the study by Tsuda et al. (2003), the intrathecal transfer of ATP-treated microglia induced mechanical allodynia

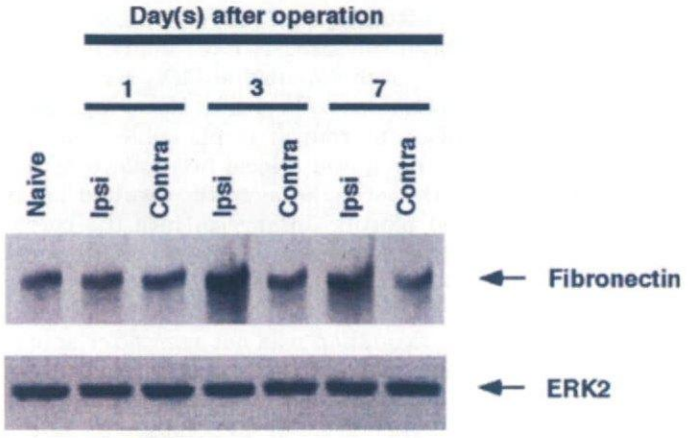

Fig. 4. Time-course study of fibronectin expression in the allodynia rat spinal cord. The rat spinal nerve on the left side was exposed, tightly ligated with a silk suture, and its peripheral side was completely transected. On Days 1, 3, and 7 post-operation, L5 spinal cords from control and allodynia rats were collected and the tissues were subjected to homogenization and Western blotting. Anti-fibronectin (Dako, 1:100 dilution) antibody and HRP-conjugated anti-rabbit IgG (Amersham) antibody were used for the detection. The data represent 3 independent experiments.

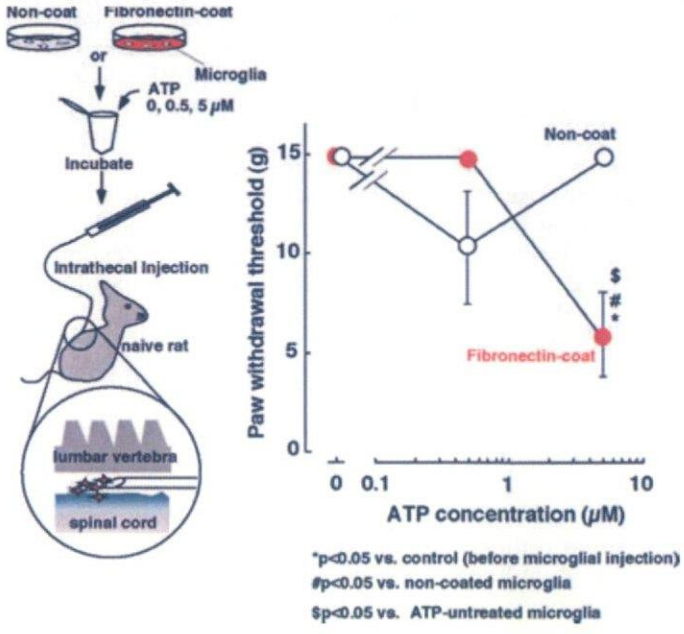

Fig. 5. Changes in nociceptive response after intrathecal transfer of microglia with elevated expression of P2X$_4$ receptors. Microglia were cultured either on fibronectin (red circle) or on control plastic (white circle) for 24 h at 37°C and both groups were subsequently stimulated with ATP at 0 (control), 0.5, and 5 μM for 1 h at 37°C. Without intrathecal microinjection of microglia, no rats showed pain behavior. Then the cells were intrathecally transferred to the lumbosacral spinal cord of a normal rat. Five h after the microinjection, nociceptive responses were evaluated by measuring the 50% paw withdrawal threshold to mechanical stimuli. Six rats were used in each group for this study. The Mann-Whitney U-test was performed and statistical significance was set at $P < 0.05$ [$*P < 0.05$ vs. control (before microglial injection); $\#P < 0.05$ vs. non-coated microglia; $\$P < 0.05$ vs. ATP-untreated microglial injection]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in normal rats, and microglial P2X$_4$ receptors were mainly responsible for this effect. Therefore, we hypothesized that microglia with more P2X$_4$ receptors expressed on the surface are capable of causing severer mechanical allodynia. To examine this hypothesis, microglia were cultured either on fibronectin or on control plastic for 24 h, stimulated with 0.5 or 5 μM of ATP for 1 h or left untreated as the control, then intrathecally transferred to normal rats, and their pain behavior was monitored 5 h after the microinjection using von Frey hairs to calculate the 50% paw withdrawal threshold (Fig. 5). Without intrathecal injection of microglia, no rat showed any pain behavior (data not shown). As seen in Fig. 5, no pain response was observed at ATP 0 (control) or 0.5 μM. An interesting difference, however, was seen at ATP 5 μM, where a significant decrease in the 50% withdrawal threshold was observed with fibronectin-treated microglia as compared with the non-treated microglia. Additionally, it was clearly demonstrated that intrathecal transfer of microglia that were treated with ATP at 50 μM, in the absence of fibronectin, was capable of inducing allodynia in the recipient rat (Tsuda et al., 2003). Collectively, these results suggest that upregulation of P2X$_4$ receptors by fibronectin lowered the threshold for the response to mechanical allodynia.

DISCUSSION

In the present study, we demonstrated that: (1) the treatment of microglia with fibronectin enhanced the expression of functional P2X$_4$ receptors, (2) the spinal fibronectin was upregulated after the peripheral nerve injury, and (3) the fibronectin treatment of microglia lowered the concentration of ATP that was necessary to cause mechanical allodynia by intrathecal transfer. Because the upregulation of P2X$_4$ receptors in spinal microglia is a critical event in the induction of mechanical allodynia after peripheral nerve injury (Tsuda et al., 2003, 2005), our present results may partially clarify the mechanism of mechanical allodynia.

The Correlation Between Fibronectin and Microglial P2X$_4$ Receptor

The P2X$_4$ receptor displays a broad tissue distribution, especially in the CNS (Soto et al., 1996). Although the precise physiological roles of P2X$_4$ receptors remain unknown, it has been reported that the upregulation of P2X$_4$ receptors is linked to several pathological conditions, such as nerve injury (Tsuda et al., 2003), ischemia (Cavaliere et al., 2003), and muscular dystrophy (Yeung et al., 2004). In the present study, we showed that fibronectin increased the microglial P2X$_4$ expression at the mRNA level by more than 2-fold. The elevation was also confirmed at the protein level, and these upregulated P2X$_4$ receptors were shown to be functional by the increase in $[Ca^{2+}]i$ mediated by P2X$_4$ receptors.

These findings are of interest because some ECM molecules, including fibronectin, are known to be upregulated following adult CNS injury (Jones, 1996). Fibro-

nectin is involved in neurite growth during development (Matthiessen et al., 1989) and plays an important role in the spinal cord development in humans (Krolo et al., 1998). The expression of fibronectin is regionally and developmentally regulated in the brain, and its presence is relatively minor in the normal CNS (Jones, 1996). However, once injuries occur, its expression is dramatically increased (Hoke and Silver, 1996; Pasinetti et al., 1993). Fibronectin also exists at high concentrations in the blood plasma, and a breakdown of the blood-brain barrier (BBB) should result in an increase in its local concentration in the CNS. Thus, it is highly plausible that the increased fibronectin may somehow upregulate $P2X_4$ receptors in microglia.

The signaling pathway(s) by which fibronectin promotes $P2X_4$ upregulation is currently under investigation. Microglia possess functional β1 integrin, which is one of the receptors for fibronectin, and they undergo firm adhesion, activation (Milner and Campbell, 2002, 2003), and proliferation (Nasu-Tada et al., 2005) through this molecule, presumably by regulating intracellular signaling cascades. Thus, fibronectin-to-integrin-mediated signals may be critical for the $P2X_4$ receptor upregulation. It is interesting that, among various P2 receptors expressed in microglia, i.e., $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors, only the $P2X_4$ receptor is upregulated by fibronectin (Fig. 1). This result suggests that the $P2X_4$ receptor gene may undergo unique transcriptional regulation by the linkage of fibronectin to integrin.

The Correlation Between Spinal Fibronectin and Mechanical Allodynia in Rats

We showed that spinal fibronectin was upregulated after the nerve injury. Several lines of evidence indicate that fibronectin is directly upregulated at the site of injuries in the PNS (Lefcort et al., 1992; Martini, 1994; Vogelezang et al., 1999) and CNS (Hoke and Silver, 1996; Pasinetti et al., 1993); but to our knowledge, there has been no report demonstrating that fibronectin is upregulated at a distal region, i.e., in the CNS, after peripheral nerve injury. The clinical signs of allodynia induced in rat in the Chung model become evident by 3-day post operation and the phenotype reached the maximum by 1-week post operation. The concomitant upregulation of the microglial $P2X_4$ receptors, which is observed only on the ipsilateral side of the spinal cord, follows the same time-course profile (Tsuda et al., 2003). In this study, the expression of the spinal fibronectin was strongly augmented, but again only on the ipsilateral side, during the course of mechanical allodynia, and the expression pattern was also similar to the time-course of the above. Therefore, our results strongly suggest that the upregulation of the ipsilateral fibronectin correlates both with the induction of allodynia and with the upregulation of the microglial $P2X_4$ receptor. Since it is usually observed that mechanical allodynia spans several weeks, it is still necessary for us to investigate the relationship between the upregulation of fibronectin and chronic allodynia. Our data so far postulate that fibronectin may be involved in the onset of the disease, most likely by initiating the upregulation of the microglial $P2X_4$ receptor.

Since fibronectin is known to be present at a high concentration in the blood plasma, it is plausible that the peripheral nerve injury caused a local breakdown of the central BBB, and that the breakdown resulted in a transfer of the blood plasma fibronectin into the corresponding part of the CNS. In a cortical cold-injury model, fibronectin was found to leak from blood vessels (Nag et al., 2001; Nourhaghighi et al., 2003); but in another report, such exudation was not seen after spinal cord injury (Farooque et al., 1992). Interestingly, the plasma fibronectin expression is known to be elevated after tissue injury (Thompson et al., 1992). Recently, plasma fibronectin was reported to support neuronal survival and reduce bran injury following transient focal cerebral ischemia, but it was not essential for skin-wound healing and hemostasis (Sakai et al., 2001). Alternatively, fibronectin may be synthesized by neuronal and glial cells in the CNS. Fibronectin is a vital molecule in neural development and regeneration (Venstrom and Reichardt, 1993), and astrocytes are known to synthesize and release fibronectin (Jiang et al., 1994; Matthiessen et al., 1989; Price and Hynes, 1985). A recent report by Tom et al. (2004) revealed that astroglial-associated fibronectin plays a key role in axonal regeneration in the white matter and, indeed, astrocytes produce and release fibronectin in response to ATP stimulation (unpublished observation). However, so far we have not identified the source of the upregulated fibronectin, and the mechanism by which fibronectin is upregulated after peripheral nerve injury remains to be clarified.

The effect of fibronectin was further highlighted by the experiment involving intrathecal transfer. In our previous study (Tsuda et al., 2003), microglia that were treated with 50 μM ATP could induce mechanical allodynia when they were intrathecally transferred into a normal rat. Microglia that were cultured on fibronectin and treated with 5 μM ATP were capable of inducing mechanical allodynia. In contrast, control microglia were not able to induce mechanical allodynia at that ATP concentration. The result suggests that fibronectin lowered the threshold of pain sensation. Fifty μM of ATP was adequate to cause mechanical allodynia by intrathecal transfer in both groups, suggesting that the effect of microglia in causing allodynia in response to ATP stimulation is saturated at an ATP concentration of 50 μM. Although microglia have other P2 receptors, i.e., $P2X_7$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors, only $P2X_4$ receptors are involved in the induction of mechanical allodynia (Tsuda et al., 2003). Interestingly, fibronectin upregulated only $P2X_4$ receptors but downregulated other P2 receptors on microglia at the mRNA level (Fig. 1). Thus, the involvement of other microglial P2 receptors in pain sensation, the threshold of which was lowered by fibronectin, would be negligible. Altogether, the results suggest that microglia with upregulated $P2X_4$ receptors by fibronectin treatment were able to transduce signals that lead to allodynia at a lower concentration of ATP.

In summary, we demonstrated that fibronectin induces the upregulation of $P2X_4$ receptors on microglia in vitro, and that fibronectin is increased in the spinal cord in vivo when mechanical allodynia is induced after peripheral nerve injury. When microglia are intrathecally administered into normal rats, they induce mechanical allodynia only if pre-stimulated with ATP (Tsuda et al., 2003). Our ex vivo experiments showed that fibronectin lowers the ATP concentration that is necessary for microglia to induce this pain behavior. Although both the signaling pathway by which fibronectin promotes $P2X_4$ upregulation and the source of increased fibronectin are currently under investigation, all these findings suggest that the upregulation of spinal fibronectin may be involved in the onset mechanism of mechanical allodynia after nerve injury.

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Direct excitation of deep dorsal horn neurones in the rat spinal cord by the activation of postsynaptic P2X receptors

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ATP mediates somatosensory transmission in the spinal cord through the activation of P2X receptors. Nonetheless, the functional significance of postsynaptic P2X receptors in spinal deep dorsal horn neurones is still not yet well understood. Using the whole-cell patch-clamp technique, we investigated whether the activation of postsynaptic P2X receptors can modulate the synaptic transmission in lamina V neurones of postnatal day (P) 9–12 spinal cord slices. At a holding potential of −70 mV, ATPγS (100 μM), a nonhydrolysable ATP analogue, generated an inward current, which was resistant to tetrodotoxin (1 μM) in 61% of the lamina V neurones. The ATPγS-induced inward current was accompanied by a significant increase in the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) in the majority of lamina V neurones. The ATPγS-induced inward current was not reproduced by P2Y receptor agonists, UTP (100 μM), UDP (100 μM), and 2-methylthio ADP (100 μM), and it was also not affected by the addition of guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) into the pipette solution, thus suggesting that ionotropic P2X receptors were activated by ATPγS instead of metabotropic P2Y receptors. On the other hand, α,β-methylene ATP (100 μM) did not change any membrane current, but instead increased the mEPSC frequency in the majority of lamina V neurones. The ATPγS-induced inward current was suppressed by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (10 μM), but not by trinitrophenyl-ATP (TNP-ATP) (1 μM). Furthermore, we found that ATPγS (100 μM) produced a clear inward current which was observed in all lamina V neurones over P16 spinal cord slices, in contrast to P9–12. These results indicate that distinct subtypes of P2X receptors were functionally expressed at the post- and presynaptic sites in lamina V neurones, both of which may contribute to the hyperexcitability of lamina V in a different manner. In addition, the data relating to the developmental increase in the functional P2X receptors suggest that purinergic signalling may thus be more common in somatosensory transmission with maturation.

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Extracellular ATP plays a crucial role in nociceptive transmission in the central and peripheral nervous systems (Burnstock & Wood, 1996; Hamilton & McMahon, 2000; Chizh & Illes, 2001; Kennedy *et al.* 2003; Liu & Salter, 2005). ATP receptors are divided into two classes, ionotropic P2X receptors (Khakh, 2001; North, 2002) and G-protein-coupled metabotropic P2Y receptors (von Kügelgen & Wetter, 2000). To date, seven P2X receptor subunits ($P2X_1$ to $P2X_7$) have been cloned (North & Surprenant, 2000). Each P2X subunit has two transmembrane domains with a cysteine-rich extracellular loop, which contains an ATP-binding site. A functional P2X receptor is composed of three or more P2X subunits, forming a pore structure that is permeable to cations including Ca^{2+}. Assembled from seven P2X subunits, at least 11 subtypes of functional P2X receptors can thus be formed in heterologous expression systems. These 11 P2X receptors are homomeric $P2X_1$ to $P2X_7$ receptors, heteromeric $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$ and $P2X_{4/6}$ (Khakh *et al.* 2001; North, 2002).

The spinal dorsal horn (DH) is the first site in the central nervous system where somatosensory information is

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