選択的 $P2Y_1$ 受容体アゴニストである ADPβS 及び 2MeSADP(各 1 μM)は ATP と同じく H_2O_2 誘導性細胞死からアストロサイトを強く保護した (図 5A)。更に、$P2Y_1$ 受容体選択的アンタゴニストである MRS2179 によって ATP の保護作用は濃度依存的に抑制される事が明らかとなった (図 5B)。この事から ATP の保護作用は主に $P2Y_1$ 受容体を介した経路で発揮されている事が明らかとなった。

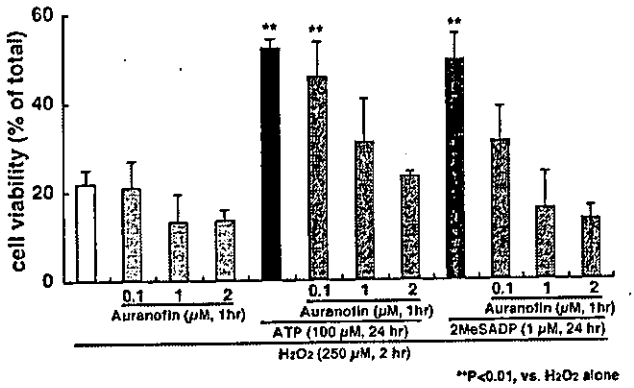

図 6. ATP/2MeSADP 誘導性保護作用に対する thioredoxin reductase 選択的阻害剤の効果

Thioredoxinreductase 特異的阻害剤 auranofin(0.1-2μM)は ATP(100μM)及び 2MeSADP(1 μM)による保護作用を濃度依存的に抑制した。Auranofin 単独ではアストロサイトの cellviability には顕著な影響を与えず、H_2O_2 誘導性細胞死に対しても影響を与えなかった。ATP/2MeSADP は H_2O_2 処置前 24 時間前に細胞に処置し、auranofin は H_2O_2 処置前 1 時間前に処置した。

ATP によって発現誘導されたこれらの遺伝子が、実際に H_2O_2 に対する保護作用に関係するかどうかを確認した。ATP (100 μM)及び 2MeSADP (1 μM)処置(24 hr)によって誘導される H_2O_2 に対する保護作用は、TrxR 選択的阻害剤である auranofin によって濃度依存的(0.1-2 μM)に抑制された(図 6)。

D　考察

BBB を形成する血管内皮細胞、周皮細胞、アストロサイトのうち、外側の 2 種の細胞、周皮細胞とアストロサイトが ATP を介してコミュニケーションをとっていることが明らかとなった。アストロサイト及び周皮細胞は、血管の収縮・弛緩等、機械刺激に頻繁に曝されているが、これら機械刺激は両細胞からの ATP 放出を誘発する。また、細胞内には約 5 mM 以上と計算されている濃度の ATP が存在しており、障害時、例えば BBB が破綻した等の細胞傷害時には、漏出し、周皮細胞及びアストロサイトはこれら細胞外 ATP に曝されることになる。従って、血管を取り巻く細胞は、生理的にも、病体生理時にも ATP に曝される機会が多いと考えられる。特に、BBB 破綻時には、血液成分中の高濃度の ATP も漏出してくる可能性が高い。この様な細胞外 ATP の情報を、アストロサイトは P2Y1 受容体を介し受容し、また周皮細胞は主に P2X 受容体を介して受容し (結果未発表)、様々な応答を呈する。今回、このうちアストロサイトは、細胞外 ATPP2Y1 受容体を介し、種々の酸化ストレスに抵抗性を示す分子種の発現を亢進させることが明らかとなった。また、実際 ATP 曝露により、アストロサイトは酸化ストレスに抵抗性を示すようになり、この抵抗性の獲得は thioredoxin reductase 阻害剤により消失した。

アストロサイトは血管壁を取り巻く BBB の最外層に位置し、周皮細胞と積極的にコミュニケーションをとり、BBB の機能維持に役立っている。このアストロサイトの機能を支える、ATP/P2Y1 受容体の動態、さらにこの刺激により惹起される酸化ストレス軽減因子の動態は、BBB 機能に強く影響するものと考えられる。今後、*in vitro* 及び *in vivo* BBB モデルを用いた検討により、ATP、P2Y1 受容体、さらにこれら刺激により誘発される遺伝子群の動態と BBB 機能の関連性を明らかとすることが重要である。

E　結論

BBB 機能維持に重要な役割を果たす、周皮細胞とアストロサイトが、ATP を介してコミュニケーションを取っていることが明らかとなった。このうち、アストロサイトは ATP/P2Y1 受容体を介するシグナルにより、酸化ストレスに対する抵抗性を獲得していた。ATP は BBB 障害時に放出されると考えられており、アストロサイトは ATP に曝される機会が多い。ATP/P2Y1 受容体を介するアストロサイトの応答性が実際の BBB 機能とどのようにリンクしているか、また、この P2Y1 受容体及び、この受容体シグナルにより誘導される酸化ストレスを軽減遺伝子群が、BBB 機能にどのように影響売るかを明らかにするこ必要がある。

F　健康危険情報

特筆事項無し

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G　知的財産権の出願・登録状況

1.特許取得

2.実用新案登録

無し

3.その他

無し

研究成果の刊行に関する一覧表

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分担研究報告書

「血液脳関門破綻に基づく医薬品副作用の予測系の確立」に関する研究
(In vitro BBB 病態モデル作成及び各種薬物の脳内移行性評価)

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研究要旨

薬物の脳移行は血液脳関門(BBB)により制限されている。しかし、脳への難移行性薬物である免疫抑制薬等が中枢性有害作用を発現することから、これら薬物の BBB 機能に与える影響を明らかにする必要がある。そこで、血液脳関門再構成系 in vitro BBB 常態モデルを作製し、免疫抑制薬 cyclosporin A (CsA)の BBB 透過機序解明に着手した。CsA は、脳血管内皮細胞由来 adrenomedullin (AM)産生阻害に基づく cAMP/protein kinase A (PKA)経路の低下およびペリサイト由来 transforming growth factor-β (TGF-β)の産生阻害により BBB 機能を破綻させることが判った。

A．研究目的

医薬品による中枢性有害作用は重篤なものが多い。例えば免疫抑制薬や NSAIDs などによる脳炎、脳症、振戦、けいれんなどである。これら薬剤は、BBB により脳内移行が制限されている。しかし、中枢性有害作用が発現することから医薬品の BBB 透過が懸念される。また、病態時の BBB 機能変化は明らかになっておらず、各医薬品によるその機能への影響も不明である。そこで、常態時および病態時の in vitro BBB モデルを作製し、医薬品による BBB 機能に対する影響を検討することを企てた。本成績と中枢性有害作用との関連性を明らかにし医薬品の安全性確立を目指す。

B．研究方法

血液脳関門再構成系 in vitro BBB 常態モデル：マウス脳毛細血管内皮細胞株（MBEC4）単独培養系(monolayer)、MBEC4 と脳ペリサイト(rat、human pericyte)の共培養系(pericyte coculture)を作製した。

BBB 透過性評価：上記モデルを使用し、血管側に sodium fluorescein (Na-F)を入れ、脳実質側から回収し透過係数を算出し、MBEC4 透過性の指標とした。

P-gp 排出機能評価：MBEC4 培養プレートもしくは上記モデルを使用し、rhodamine 123 を加えて、一時間後に NaOH で可溶化し、MBEC4 内 rhodamine 123 (R123)蓄積量を測定し、P-gp 機能の指標とした。

（倫理面への配慮）

本研究施設に設置されている動物実験等倫理委員会の承認の元、実験を行った。

C．研究結果

BBB 構成細胞である脳毛細血管内皮細胞、アストロサイト、ペリサイトの共培養系において、経内皮電気抵抗の上昇および tight junction 構成蛋白質(occludin, claudin-5, ZO-1)の発現量の増加を確認した。Monolayer において AM は MBEC4 透過性および R123 蓄積量を減少させた。このとき cAMP 産生量は増加した。これら作用は AM アンタゴニストおよび PKA 阻害剤(H89)により抑制された。Pericyte coculture では monolayer と比較し、MBEC4 透過性および R123 蓄積量は減少した。この減少は TGF-β1 抗体および TGF-β1 受容体阻害剤処理により monolayer と同程度まで上昇した。一方、CsA は MBEC4 透過性および R123 蓄積量を増大した。この増大は pericyte coculture において著明であった。このとき MBEC4 産生 AM、cAMP およびペリサイト産生 TGF-β は減少した。また H89 と CsA の併用によ

りMBEC4透過性は上昇した。

D．考察

BBB は、脳毛細血管内皮細胞、ペリサイト及びアストロサイトの３種類の細胞が互いに協調することで機能を維持する。この維持機構に、脳毛細血管内皮細胞産生 AM およびペリサイト産生 TGF-βが促進的に関与していることが判った。また、CsA は脳血管内皮細胞由来 AM 産生の低下に伴う cAMP/PKA 経路の阻害および直接的な cAMP/PKA 経路の阻害によって BBB 機能を低下させ CsA の脳内への侵入を許す。脳内に侵入した CsA はペリサイトの TGF-β産生を阻害し、さらに BBB 機能を破綻させると考える。

我々は今回 in vitro BBB 常態モデルを作製し、これを用いて CsA の脳移行機序の一部を明らかにした。今後、病態下における CsA の脳移行機序について in vitro BBB 病態モデルを作製し検討していく必要がある。

E．結論

今回作製した血液脳関門再構成系 in vitro BBB 常態モデルは in vivo に概挿しうるモデルであり、医薬品の脳移行性および BBB 機能への影響を簡便に評価でき、中枢性有害作用の予測に有用である。

F．健康危機情報

該当なし

G．研究発表

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H．知的財産権の出願・登録状況

（予定も含む。）

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

研究成果の刊行に関する一覧表

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厚生労働科学研究費補助金（医薬品医療機器等レギュラトリーサイエンス総合研究事業）
分担研究報告書

「血液脳関門破綻に基づく医薬品副作用の予測系の確立」に関する研究
血液脳関門を介した異物排泄メカニズムにおける薬物間相互作用の予測システムの開発

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研究要旨　血液脳関門の異物排泄過程における薬物間相互作用予測システムの構築を目指して研究を行った。候補遺伝子同定のために、マウス in situ 脳灌流法を用いて野生型マウスと遺伝子欠損マウスで、脳移行性を比較解析した。BCRP 阻害剤 GF120918 処理により、DHEAS と mitoxantrone の脳内移行性は増加した。しかし、BCRP 欠損マウスと野生型マウスで脳内移行性に差は見られないことから、これらの薬物における BCRP を介した排出輸送の寄与率は小さいことが示唆された。一方で、Mrp4 欠損マウスでは、DHEAS の脳内移行性は増加しており、Mrp4 が血液脳関門の関門機構として重要なトランスポーターであることが示唆された。

A.研究目的

血液脳関門の関門機能の破綻には、tight junction の障害による生理的な破綻と併用薬との薬物間相互作用による異物排泄メカニズムの機能低下による薬物動態学的な破綻の２つの可能性を考慮することが必要である。後者については、キニジンとロペラミドとの薬物間相互作用は血液脳関門を介した異物排泄メカニズムを介したものであることが示唆されている。このような薬物間相互作用を予測・評価するシステムを開発することは、安全な薬剤療法を達成するために必須である。

本研究では、特に後者による関門機能の破綻に焦点をあて、異物排泄に働くトランスポーターを明らかにし、薬物間相互作用の可能性について検討、ならびにその評価系を確立することを目的とした。

B.研究方法

マウス *in situ* 脳灌流法を用いて、脳移行性を測定した。脳毛細血管内皮細胞の管腔側に発現していることが明らかにされている２つの ABC トランスポーター、BCRP と MRP4 に焦点をあてた。BCRP 阻害剤である GF120918 (10mg/kg、iv)による効果を検討した。BCRP-、MRP4 欠損マウスを用いて、野生型マウスについて、脳移行性の比較を行った。

C.研究結果

GF120918 処理群では、dehydroepiandrosterone sulfate (DHEAS)、ならびに抗癌剤 mitoxantrone の脳内移行性(CL_{uptake})は増加した。しかし、BCRP 欠損マウスにおいても、この GF120918 処理による効果は観察された。更に、BCRP 欠損マウスと野生型マウスで、脳内移行性を比較した場合に、BCRP 欠損による脳内移行性の増加は観察されなかった。mitoxantrone については、P-glycoprotein 欠損マウスにおいて、脳内濃度が増加していることから、GF120918 の効果は一部 P-gp の阻害であることが示唆されている。

Mrp4 欠損マウスを用いて同様の実験をおこなったところ、Mrp4 欠損マウスでは、DHEAS の脳内移行性が野生型マウスに比べて 1.6 倍に増加した。しかし、GF120918 の効果は、依然として Mrp4 欠損マウスにおいても観察された。

D.考察

DHEAS と mitoxantrone は BCRP の基質になるものの、BCRP 欠損マウスでは脳内移行性に差がみられないことから、少なくともこの 2 化合物については、血液脳関門において BCRP による排出の寄与率は小さいことが示唆された。mitoxantrone については、一部 P-gp により説明されることが明らかになった。Mrp4 を欠損した場合では、DHEAS の脳内移行性が増加していることから、Mrp4 が血液脳関門において異物排泄に関与していることが示唆された。Mrp4 がどのような薬物について脳内

からの排出に関与するのか、更なる解析が必要である。

E.結論

少なくとも、DHEAS と mitoxantrone については、血液脳関門において BCRP が主要な排出トランスポーターではないことが示唆された。一方で、DHEAS についてはMrp4 によるくみ出しが示唆された。

GF120918 による脳内移行性の増加については、BCRP・MRP4 以外のトランスポオーターの阻害によることが示唆された。

BCRP と MRP4 欠損マウスとの比較を行い、更に関門における排出トランスポーターとしての重要性を明らかにすることが必要である。

F.健康危険情報

なし

G.研究発表

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H.知的財産権の出願・登録状況

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GLIA 39:89–95 (2004)

Activation of p38 Mitogen-Activated Protein Kinase in Spinal Hyperactive Microglia Contributes to Pain Hypersensitivity Following Peripheral Nerve Injury

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KEY WORDS mitogen-activated protein kinases; dorsal horn; tactile allodynia; neuropathic pain

ABSTRACT Neuropathic pain is an expression of pathological operation of the nervous system, which commonly results from nerve injury and is characterized by pain hypersensitivity to innocuous stimuli, a phenomenon known as tactile allodynia. The mechanisms by which nerve injury creates tactile allodynia have remained largely unknown. We report that the development of tactile allodynia following nerve injury requires activation of p38 mitogen-activated protein kinase (p38MAPK), a member of the MAPK family, in spinal microglia. We found that immunofluorescence and protein levels of the dually phosphorylated active form of p38MAPK (phospho-p38MAPK) were increased in the dorsal horn ipsilateral to spinal nerve injury. Interestingly, the phospho-p38MAPK immunofluorescence in the dorsal horn was found exclusively in microglia, but not in neurons or astrocytes. The level of phospho-p38MAPK immunofluorescence in individual microglial cells was much higher in the hyperactive phenotype in the ipsilateral dorsal horn than the resting one in the contralateral side. Intrathecal administration of the p38MAPK inhibitor, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), suppresses development of the nerve injury-induced tactile allodynia. Taken together, our results demonstrate that nerve injury-induced pain hypersensitivity depends on activation of the p38MAPK signaling pathway in hyperactive microglia in the dorsal horn following peripheral nerve injury.

INTRODUCTION

Nerve injury arising from disease or physical trauma produces long-lasting abnormal hypersensitivity to innocuous stimuli, a phenomenon known as tactile allodynia (Woolf and Mannion, 1999; Scholz and Woolf, 2002). Tactile allodynia is a hallmark, and the most troublesome, of neuropathic pain syndrome in humans. It is nearly always resistant to known treatments such as nonsteroidal antiinflammatory agents (NSAIDs) or even narcotics (Woolf and Mannion, 1999; Scholz and Woolf, 2002). The mechanisms by which nerve injury develops tactile allodynia have remained largely unknown. It is thus essential to identify the molecular changes that lead to tactile allodynia in an effort to both understand its mechanisms and develop new therapies.

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Several lines of evidence have proposed that induction of tactile allodynia is attributed to central hyperactive states resulting from multiple plastic alterations in dorsal horn neurons as well as glia following nerve injury (Woolf and Mannion, 1999; Woolf and Salter, 2000; Watkins et al., 2001; Scholz and Woolf, 2002). Recent models of nerve injury-induced plasticity in the dorsal horn postulate that induction of the spinal plasticity requires activation of intracellular signaling events including protein kinases for transcriptional and posttranscriptional modifications of various proteins such as cell surface receptors (Woolf and Salter, 2000). It is thus expected that protein kinases, including protein kinase Cγ (Malmberg et al., 1997), in the dorsal horn must regulate nerve injury-induced tactile allodynia; however, their remains poorly understood.

We report that development of tactile allodynia following nerve injury depends on p38 mitogen-activated protein kinase (p38MAPK), one of four subgroups of the MAPK family (Ono and Han, 2000); activation of p38MAPK is found in hyperactive microglia, but not in neurons or astrocytes in the dorsal horn after nerve injury. Thus, the present study suggests that p38MAPK in spinal microglia is an essential intracellular protein kinase that regulates pain hypersensitivity following peripheral nerve injury. Preliminary results of this study have been reported in abstract form (Tsuda et al., 2002a,b).

MATERIALS AND METHODS

Animals

Male Wistar rats were used in this study: rats weighing 200–230 g for the biochemical and immunohistochemical experiments, and rats weighing 270–290 g for the behavioral experiments testing the effect of intrathecal treatment with a p38MAPK inhibitor. We have confirmed that p38MAPK phosphorylation in the dorsal horn following nerve injury is also observed in both weight ranges of rats (data not illustrated). Rats were housed at a temperature of 22 ± 1°C with a 12-h light/dark cycle (light on 8:30 to 20:30) and were fed food and water ad libitum. All the animals used in the present study have been treated in accordance with the guidelines of National Institute of Health Sciences.

Neuropathic Pain Model

We used the spinal nerve injury model (Kim and Chung, 1992) with some modifications. A unilateral L5 spinal nerve of rats was tightly ligated and cut just distal to the ligature under isoflurane (2%) anesthesia. To assess the tactile allodynia, the calibrated von Frey filaments (0.4–15.1 g; Stoelting, Wood Dale, IL) were applied to the plantar surface of the hindpaw from below the mesh floor. The 50% paw withdrawal threshold was determined by the up-down method (Dixon, 1980; Chaplan et al., 1994).

Immunohistochemistry

The rats were deeply anesthetized by pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 150 ml of phosphate-buffered saline (PBS; composition in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.5, NaH_2PO_4 8.1; pH 7.4), followed by 300 ml of ice-cold 4% paraformaldehyde. The L5 segment of the lumbar spinal cord was removed, postfixed in the same fixative, and placed in 30% sucrose solution for 24 h at 4°C. Transverse L5 spinal cord sections (30 μm) were incubated in a blocking solution (3% normal goat serum [NGS]) and then incubated for 48 h at 4°C in the primary antibody, anti-phospho-p38MAPK (1:200; Cell Signaling, Beverly, MA). Markers of microglia, OX42 (anti-OX42, 1:100, Chemicon, Temecula, CA); astrocytes, glial fibrillary acidic protein (GFAP; anti-GFAP, 1:500; Boehringer-Mannheim, Indianapolis, IN); and neurons, NeuN (anti-NeuN, 1:200; Chemicon) were used to identify the type of phospho-p38MAPK-positive cells. Following incubation, tissue sections were washed and incubated for 3 h at room temperature in the secondary antibody solution (anti-rabbit IgG-conjugated Alexa Fluor™ 488 or anti-mouse IgG-conjugated Alexa Fluor 546, 1:1,000; Molecular Probes, Eugene, OR). The spinal cord sections were analyzed using a MicroRadiance Confocal Imaging System (Bio-Rad, Hercules, CA) and an Olympus IX70 microscope (Olympus Optical, Tokyo, Japan) equipped for epifluorescence. For quantitative assessment of the immunofluorescence staining of cells, we randomly selected dorsal horn fields displayed at high magnification. Microglia, as identified by OX42 immunofluorescence, were outlined; the immunofluorescence intensity of the phospho-p38MAPK was determined as the average pixel intensity within each cell. Background fluorescence intensity was determined and was subtracted from the value obtained for microglia.

Western Blotting

The rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.). The lumbar and sacral spinal cord was quickly removed and placed on a dish with ice-cold PBS. We identified the cord from L4 to L6 by the entry area of the dorsal roots and the shape of the cord under a microscope and cut at the boundary between L3 and L4 and between L6 and S1. The spinal cord segments L4-L6 ipsilateral to the nerve injury were homogenized in ice-cold PBS containing a mixture of phosphatase inhibitors (Sigma-RBI, St. Louis, MO) and protease inhibitors (Calbiochem, San Diego, CA). The homogenates were incubated with DNase and were sonicated. The resulting homogenate (20 μg) was subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred electrophoretically to nitrocellulose membranes. After blocking, the membranes were incubated with anti-phospho-p38MAPK antibody (1:1,000; Cell Signal-

ing) or anti-p38MAPK antibody (1:1,000; Cell Signaling) and then were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The blots were detected with a chemiluminescence method (LumiGLO; Cell Signaling) and exposed to autoradiography films (Hyperfilm-ECL; Amersham, Arlington Heights, IL).

Spinal Administration of p38MAPK Inhibitor

Under isoflurane (2%) anesthesia, rats were implanted with catheters for intrathecal injection according to the method described previously (Yaksh et al., 1980). A polyethylene tube (PE-10; 7.5 cm) was inserted through the atlanto-occipital membrane and to the lumbar enlargement (close to L4-L5 segments) and externalized through the skin. Rats were injected intrathecally with 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; 30 nmol/10 μl) (Sigma-RBI) or vehicle [2% dimethylsulfoxide (DMSO)/10 μl] using a 25-μl Hamilton syringe with 28-gauge needle. Intrathecal injection of SB203580 or vehicle was started immediately after nerve injury (day 0) and given once a day for 14 days. SB203580 was dissolved in 100% DMSO and diluted by PBS (final concentration of DMSO: 2%). Behavioral testing was done 12–14 h after the injection of SB203580.

Statistical Analysis

Statistical analyses of the results were evaluated using the Student's *t*-test, the Student's paired *t*-test or the Mann-Whitney U-test.

RESULTS

p38MAPK Is Activated Exclusively in Hyperactive Microglia in the Dorsal Horn Following Peripheral Nerve Injury

Animals with L5 spinal nerve injury displayed tactile allodynia; the withdrawal threshold of the hindpaw, ipsilateral to nerve injury, to mechanical stimulation decreased progressively from 15.1 ± 0.1 g before the injury (day 0) to 3.0 ± 0.5 g at day 7 and to 2.1 ± 0.4 g ($n = 7$) at day 14 ($P < 0.001$, significantly different from the threshold on day 0) (Fig. 1A). In contrast, paw withdrawal threshold of the contralateral hindpaw was not changed significantly by nerve injury (Fig. 1A). To examine whether p38MAPK is activated in the dorsal horn of the spinal cord in rats that have developed tactile allodynia, we carried out immunofluorescence analysis with an antibody targeting the dually phosphorylated p38MAPK (phospho-p38MAPK), because p38MAPK members have a Thr-Gly-Tyr dual phosphorylation motif, requiring phosphorylation for its activation (Ono and Han, 2000). In L5 dorsal spinal cord

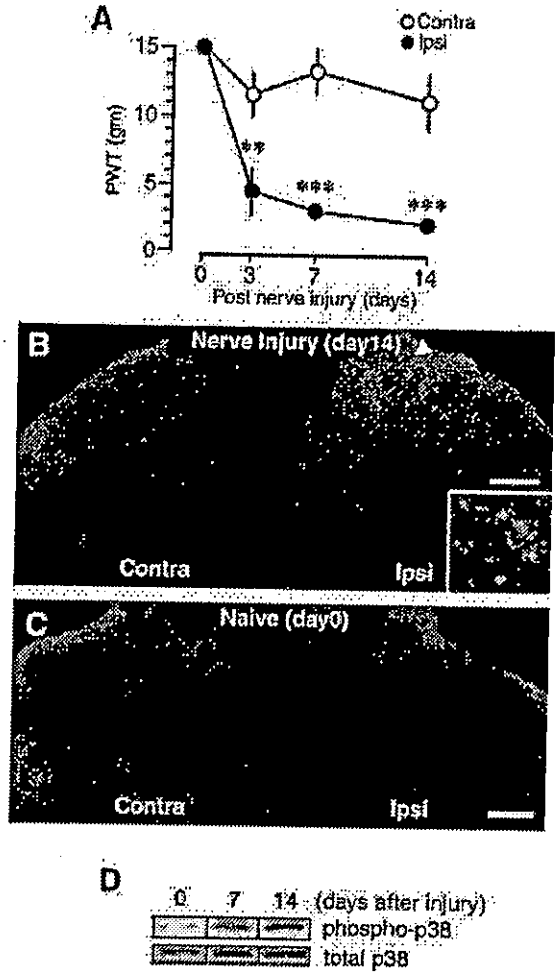

Fig. 1. p38 mitogen-activated protein kinase (p38MAPK) is dramatically activated in the spinal dorsal horn following L5 spinal nerve injury. **A:** The withdrawal threshold of tactile stimulation to the ipsilateral and contralateral hindpaw (PWT) was examined before nerve injury, 3, 7, and 14 days after nerve injury. Each data point represents the mean ±SEM of paw withdrawal threshold (in grams) (**$P < 0.01$, ***$P < 0.001$ by Student's paired *t*-test, compared with the threshold on day 0, $n = 7$). **B,C:** Immunoreactivity of phospho-p38MAPK (green) detected by an antibody for dual-phosphorylated p38MAPK in L5 dorsal spinal cord 14 days after nerve injury (B) and in that of naive rat (C) was visualized by immunofluorescence analysis using confocal microscopy. Highly magnified picture of the area (arrowhead) in B, shown in inset of B. **D:** Total protein from the spinal cord ipsilateral to the nerve injury on days 0 (naive), 7, and 14 was subjected to Western blot analysis. The proteins of phospho-p38MAPK and total p38MAPK were detected by an antibody for dual-phosphorylated and nonphosphorylated p38MAPK, respectively. Scale bars = 200 μm in B,C.

sections, at 14 days after nerve injury, we observed strong and punctate phospho-p38MAPK immunofluorescence on the ipsilateral side (Fig. 1B). The punctate labeling observed at low magnification was due to immunofluorescence of individual small cells, as shown under highly magnification (Fig. 1B, inset). In contrast, phospho-p38MAPK immunofluorescence was weaker and much less extensive in the dorsal horn contralat-

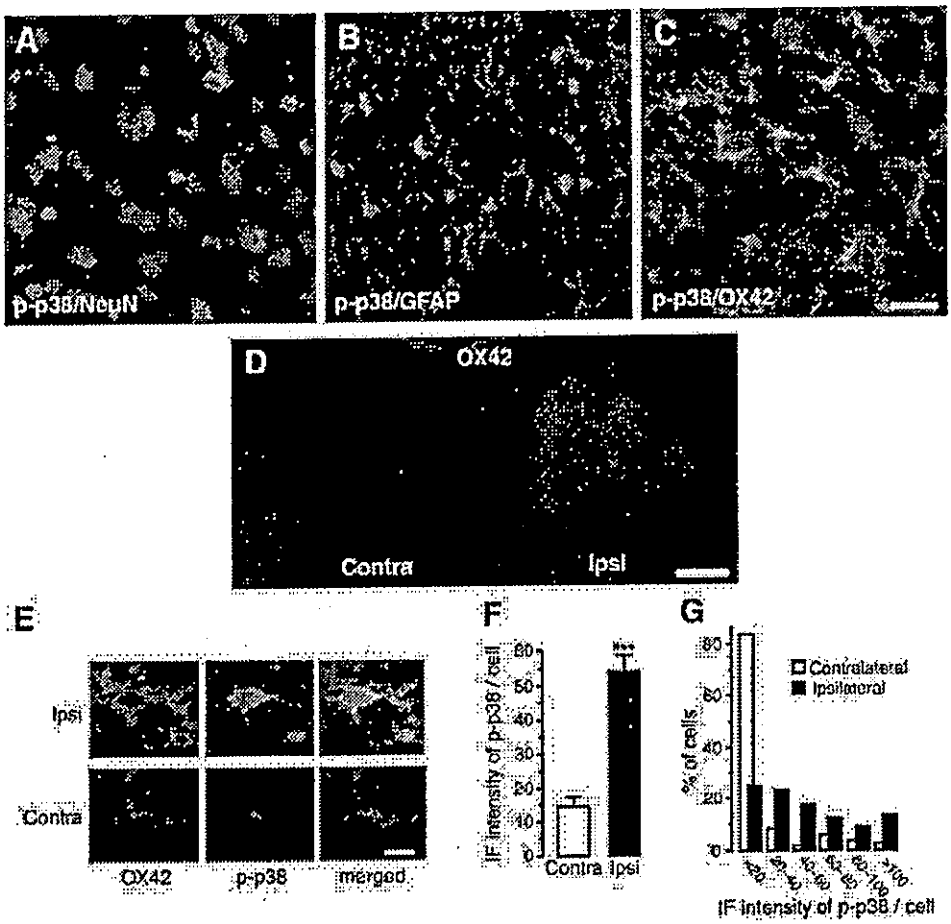

Fig. 2. p38 mitogen-activated protein kinase (p38MAPK) is activated in individual hyperactive microglia, but not in neurons or astrocytes in the dorsal horn following L5 spinal nerve injury. Immunostaining was carried out in L5 dorsal spinal cord sections at 14 days after nerve injury, using confocal microscopy. A–C: Double immunofluorescent labels of phospho-p38MAPK (green) with NeuN (A, red), a marker of neurons, glial fibrillary acidic protein (GFAP) (B, red), a marker of astrocytes, and OX42 (C, red), a marker of microglia, were analyzed. D: The change of the level of OX42 immunofluorescence (red) following nerve injury was examined in transverse section of L5 dorsal horn at 14 days after nerve injury. E: The different activation of p38MAPK in ipsilateral (Ipsi, top three panels) and contralateral (Contra, bottom three panels) microglia was examined. OX42, phospho-p38MAPK (p-p38) and merged immunofluorescences are shown in red, green, and yellow, respectively. F: Immunofluorescence intensity of phospho-p38MAPK in individual microglia was determined as the average pixel density in the ipsilateral (Ipsi; n = 83 OX42-positive cells) and contralateral (Contra; n = 74 OX42-positive cells) dorsal horn. Each data point represents the mean ±SEM of immunofluorescence (IF) intensity of phospho-p38MAPK (p-p38) per cell (***$P < 0.001$ by the Mann-Whitney U-test, compared with the value of contralateral dorsal horn). G: Histogram of the percentage of dorsal horn microglia displaying ranges of IF intensity values of phospho-p38MAPK in individual microglia. Ipsi, ipsilateral; Contra, contralateral. Scale bars = 25 μm in C; 200 μm in D; 10 μm in E.

eral to the nerve injury (Fig. 1B) or in that of naive rats (Fig. 1C). We further examined the level of phosphorylated p38MAPK protein in homogenates from the spinal cords of naive and nerve-injured rats by Western blot analysis; we found that the band intensity of phospho-p38MAPK protein in the ipsilateral spinal cord increased dramatically 7 and 14 days after nerve injury compared with that in naive rat (day 0) (Fig. 1D). The bilateral difference in phospho-p38MAPK levels parallels the emergence of the tactile allodynia (Fig. 1A). These results indicate that the p38MAPK is activated in the dorsal horn ipsilateral to the nerve injury, which may correlate with the nerve injury-induced tactile allodynia.

To identify the type of cell in which p38MAPK was phosphorylated after nerve injury, we carried out double immunolabeling for phospho-p38MAPK and for cell type-specific markers: for neurons, NeuN; for astrocytes, GFAP; or for microglia, OX42 (Honore et al., 2000). We found that cells showing phospho-p38MAPK immunofluorescence were not double-labeled for NeuN (0%, calculated in 110 cells, representative shown in Fig. 2A) or GFAP (0%, calculated in 132 cells, representative shown in Fig. 2B). Rather, almost all phospho-p38MAPK-positive cells (99%, calculated in 187 cells) were double-labeled with OX42 (representative shown in Fig. 2C), indicating that activation of p38MAPK in the dorsal horn is highly restricted to

microglia, but not found in neurons or astrocytes. OX42 recognizes the complement receptor type 3 (CR3), expression of which is greatly increased in hyperactive versus resting microglia (Aldskogius and Kozlova, 1998). We found that OX42 labeling was greater in the dorsal horn ipsilateral to the nerve injury (Fig. 2D), whereas OX42 labeling in the dorsal horn was low bilaterally in sham-operated animals (not illustrated). OX42-positive cells were more numerous (Fig. 2D) and displayed hypertrophic morphology (Fig. 2E) in the dorsal horn on the side of the nerve injury as compared with the contralateral side (Fig. 2D,E). These results indicate that nerve injury induced a switch from the resting to the hyperactive phenotype in the population of microglia in the dorsal horn. The cells labeled intensely with OX42 showed high levels of phospho-p38MAPK immunofluorescence (Fig. 2E, top panels). In contrast, resting microglia that showed a low level of OX42 had no or weak phospho-p38MAPK immunofluorescence (Fig. 2E, bottom panels). The mean level of intensity of phospho-p38MAPK immunofluorescence per OX42-positive cell was on average 3.7-fold higher in the ipsilateral (n = 83 cells) as compared with the contralateral dorsal horn (n = 74 cells) ($P < 0.001$; Fig. 2F). The distribution of phospho-p38MAPK immunofluorescence intensities per OX42-positive cell was skewed to the right (Fig. 2G). We conclude that, in the dorsal horn following nerve injury, hyperactive microglia are the cell type in which p38MAPK is activated and that the level of p38MAPK phosphorylation is dramatically increased in individual microglia. As shown in Figure 2E, subcellular distribution between phospho-p38MAPK and OX42 immunofluorescence is different, but our confocal microscopic Z-series analyses demonstrated that phospho-p38MAPK signals were found in the inside of OX42 signals which is known to localize on the cell surface (data not illustrated).

p38MAPK Activation in the Spinal Cord Is Required for Development of Tactile Allodynia Following Peripheral Nerve Injury

We examined whether intrathecal treatment with a potent inhibitor of p38MAPK, SB203580, through a catheter whose tip was positioned near the L4-L5 dorsal horn alters the development of tactile allodynia following nerve injury. Catheterized rats were treated with vehicle (2% DMSO/10 μl, n = 7) or SB203580 (30 nmol/10 μl, n = 9) once a day for 14 days, beginning on the day of the nerve injury. Intrathecal vehicle-treated rats displayed a marked decrease in paw withdrawal threshold following nerve injury ($P < 0.01$, significantly different from the threshold on day 0) (Fig. 3). In contrast, intrathecal SB203580-treated rats showed only a slight decrease in paw withdrawal threshold; paw withdrawal thresholds was not significantly decreased except for day 3 ($P < 0.01$, significantly different from the threshold on day 0). Paw withdrawal thresholds on day 7 and 14 were significantly greater in animals treated with SB203580 (n = 9) as compared with that in animals treated with vehicle (n = 7) (day 7: $P < 0.01$, day 14: $P < 0.001$, significantly different from the threshold of vehicle-treated group on days 7 and 14, respectively) (Fig. 3). These results suggest that intrathecal treatment with an inhibitor for p38MAPK in the spinal cord, the distribution of which is highly restricted in microglia, suppresses the development of tactile allodynia following spinal nerve injury.

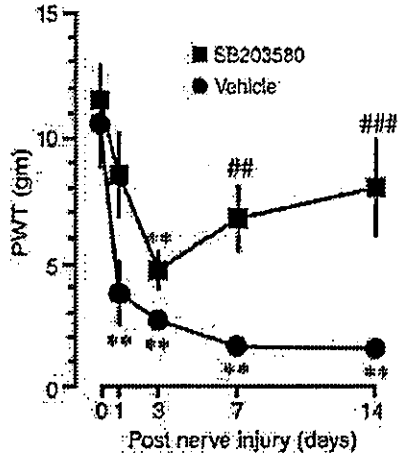

Fig. 3. Intrathecal administration of a potent inhibitor for p38 mitogen-activated protein kinase (p38MAPK), SB203580, suppresses development of tactile allodynia caused by L5 spinal nerve injury. Rats were injected intrathecally with SB203580 (30 nmol/10 μl, n = 9) or vehicle (2% dimethylsulfoxide [DMSO]/10 μl, n = 7) once a day for 14 days. The withdrawal threshold of tactile stimulation to the ipsilateral hindpaw (PWT) was examined on days 0 (before nerve injury), 1, 3, 7, and 14 at 12–14 h after intrathecal injection. Each data point represents the mean ±SEM of paw withdrawal threshold (in grams). $^{**}P < 0.01$ by the Student's paired *t*-test, compared with threshold on day 0; $\#\#P < 0.01$; $\#\#\#P < 0.001$, by the Mann-Whitney U-test, compared with the threshold of vehicle-treated group.

DISCUSSION

Our principal conclusion from the present findings is that following spinal nerve injury p38MAPK is activated in individual hyperactive microglia in the dorsal horn, leading to the development of nerve injury-induced pain hypersensitivity tactile allodynia, a major functional consequence of peripheral nerve injury. p38MAPK is the first intracellular signaling event, activation of which occurs exclusively in microglia, that regulates pain hypersensitivity caused by nerve injury. We showed a marked increase in immunofluorescence and protein levels of dual-phosphorylated p38MAPK in the dorsal horn after spinal nerve injury. These results are supported by previous findings that phosphorylation of p38MAPK is increased in response to the damage of the sciatic nerve or dorsal root (Murashov et al., 2001; Nomura et al., 2001; Kim et al., 2002) that projects to the dorsal spinal cord. p38MAPK has been

reported to be expressed in a number of cell types in the CNS in vivo (Lee et al., 2000; Maruyama et al., 2000). We found that in the neuropathic pain state, p38MAPK activation in the dorsal horn was not observed in neurons or astrocytes but, rather, occurred exclusively in microglia. As shown previously (Aldskogius and Kozlova, 1998), the number of microglia increased in the dorsal horn on the side of the nerve injury following nerve injury. We have recently demonstrated that the number of microglia is on average 2.2-fold greater in the ipsilateral side of the dorsal horn than in the contralateral side (Tsuda et al., 2003). We also show that marked phosphorylation of p38MAPK is observed in individual microglia in the ipsilateral dorsal horn (3.7-fold, as compared with the contralateral side), particularly in hyperactive microglia that dramatically expressed complement receptor type 3 recognized by OX42, and displayed hypertrophic morphology. The increase is more striking than the increase in the number of microglia in the ipsilateral side of the dorsal horn. Therefore, in the dorsal horn, following nerve injury, hyperactive microglia are the cell type in which p38MAPK is activated; in addition to an increase in the number of cells, the increased p38MAPK phosphorylation in individual hyperactive microglia would be one of the major components of p38MAPK activation in the dorsal horn following nerve injury. Moreover, we show that intrathecal treatment with SB203580, which binds to the ATP pocket in p38MAPK, and consequently inhibits its enzymatic activity (Tong et al., 1997), led to a statistically significant suppression of tactile allodynia on days 7 and 14 after nerve injury when the increase in p38MAPK phosphorylation was found. It appears that the suppression of tactile allodynia by SB203580 might be related to its inhibitory effect on the increased p38MAPK activity in the dorsal horn after nerve injury, based on the observations that intrathecal administration of SB203580 has no effect on basal pain responses in naive rats, which have a very low level of p38MAPK phosphorylation (Watkins et al., 1997; Murashov et al., 2001; Nomura et al., 2001; Ji et al., 2002; present data). Taking these results together, we conclude that development of tactile allodynia following nerve injury depends on activation of the p38MAPK signaling pathway in hyperactive microglia in the dorsal horn, although we cannot exclude the possible involvement of p38MAPK in DRG neurons (Kim et al., 2002).

p38MAPK activation in microglia is quite different from that of other MAPKs, extracellular signal-regulated kinases (ERK) and c-jun N-terminal or stress-activated protein kinases (JNK/SPAK), activation of which is found in dorsal horn astrocytes, but not in microglia, after peripheral nerve injury (Ma and Quirion, 2002). Thus, microglial regulation of tactile allodynia may require activation of p38MAPK, but not other MAPKs. Several extracellular substances have been reported to trigger p38MAPK activation in microglia in vitro, thereby regulating microglial functions (Koistinaho and Koistinaho, 2002). Tikka et al. (2001) have shown that glutamate-evoked proliferation of microglia and interleukin-1β (IL-1β) and nitric oxide release from microglia in the spinal cord primary culture depend on its p38MAPK activation. We have recently demonstrated that extracellular ATP activates p38MAPK in cultured microglia, thereby releasing tumor necrosis factor-α (TNF-α) and IL-6 (Hide et al., 2000; Shigemoto-Mogami et al., 2001). These cytokines are increased in the spinal cord following spinal nerve injury (Sweitzer et al., 2001; Winkelstein et al., 2001) and are involved in induction of nerve injury-induced tactile allodynia (Ramer et al., 1998; Sommer et al., 1998; Sweitzer et al., 2001). Therefore, elucidating p38MAPK activity-dependent microglial outputs, including the production of these cytokines in the dorsal horn in vivo, would help in understanding the mechanisms underlying the induction of pain hypersensitivity following nerve injury.

Nerve injury and peripheral inflammation that produce neuropathic and inflammatory pain states, respectively, have been known to induce distinct sets of neurochemical changes in the dorsal horn (Honore et al., 2000). In contrast to the present findings with peripheral nerve injury, Ji et al. (2002) have shown that p38MAPK activation does not occur in the dorsal horn under a sustained inflammation by intraplantar injection of complete Freund's adjuvant, which produces prolonged hypersensitivity to pain. Thus, p38MAPK activation in dorsal horn microglia would be a unique intracellular change following nerve injury, contributing to the development of nerve injury-induced pain hypersensitivity. This approach may provide a new therapeutic strategy specially targeting neuropathic pain. Importantly, in naive animals, p38MAPK activation is very weak in the dorsal horn (Fig. 1), and basal pain sensitivity is not affected by spinal administration of p38MAPK inhibitors (Watkins et al., 1997; Ji et al., 2002). This suggests a therapeutic benefit of interfering with p38MAPK activation in the treatment of neuropathic pain, without affecting normal pain sensitivity.

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