J. Biochem. 2010;147(5):671–678 doi:10.1093/jb/mvp208

JB THE JOURNAL OF BIOCHEMISTRY

Lysophosphatidylinositol induces rapid phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2 in HEK293 cells expressing GPR55 and IM-9 lymphoblastoid cells

Received September 30, 2009; accepted December 17, 2009; published online January 4, 2010

Saori Oka, Shinji Kimura, Toshinobu Toshida, Ryo Ota, Atsushi Yamashita and Takayuki Sugiura*

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamihara, Kanagawa 229-0195, Japan

*Takayuki Sugiura, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamihara, Kanagawa 229-0195, Japan, Tel: +81 42 685 3746, Fax: +81 42 685 1345,
email: sugiurat@pharm.teikyo-u.ac.jp

Lysophosphatidylinositol (LPI) is an endogenous ligand for GPR55, a putative novel type of cannabinoid receptor. In this study, we first examined the effects of LPI on p38 mitogen-activated protein kinase in HEK293 cells expressing GPR55. LPI induced the rapid phosphorylation of p38 mitogen-activated protein kinase in GPR55-expressing cells. No apparent effect was observed in the vector-transfected cells. The exposure of GPR55-expressing cells to LPI also triggered the phosphorylation of activating transcription factor 2 downstream of the p38 mitogen-activated protein kinase. Treatment of the cells with Y-27632 [a Rho-associated kinase (ROCK) inhibitor] blocked the LPI-induced phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2, suggesting that the Rho-ROCK pathway is involved in these cellular responses. Notably, GPR55 was found to be abundantly expressed in lymphoid organs such as the spleen and thymus. We obtained evidence that rapid phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2 also takes place in IM-9 lymphoblastoid cells, which naturally express GPR55, after stimulation with LPI. These results suggest that GPR55 and its endogenous ligand LPI play essential roles in the homoeostatic responses to stress signals in several mammalian tissues and cells including certain types of immune cells.

Keywords: activating transcription factor 2/ cannabinoid/G protein-coupled receptor/ lysophosphatidylinositol/p38 mitogen-activated protein kinase.

Abbreviations: abn-CBD, abnormal cannabidiol; ATF-2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; MAPK, mitogen-activated protein kinase; Δ^9-THC, Δ^9-tetrahydrocannabinol.

Δ9-Tetrahydrocannabinol (Δ^9-THC), a major psychoactive constituent of marijuana, binds to specific receptor(s), i.e. the cannabinoid receptors, thereby eliciting a variety of pharmacological responses (*1*). To date, two types of cannabinoid receptors have been identified, the CB1 receptor and the CB2 receptor (*2*,*3*). Studies using the CB1/CB2 selective ligands and knockout mice have revealed that additional receptors for the cannabinoids exist (*4*–*6*). Recently, several groups proposed that orphan G protein-coupled receptor 55 (GPR55) (*7*) is such an additional cannabinoid receptor (*8*–*11*), yet it has a low sequence identity with the CB1 (13.5%) and CB2 (14.4%) receptors and lacks the CB1 and CB2 functional fingerprints (*12*, *13*).

To search for an endogenous ligand for GPR55, we generated HEK293 cells that stably expressed human GPR55 and found that lysophosphatidylinositol (LPI) induced rapid phosphorylation of extracellular signal-regulated kinase (ERK) and a transient increase in the intracellular free Ca^{2+} concentration (*14*). The effect of LPI was diminished by treatment of the cells with siRNA against GPR55. We also found that receptor desensitization occurred following exposure to LPI (*14*). These results strongly suggest that LPI is an endogenous ligand for GPR55.

Notably, GPR55 is widely expressed within the body, such as in the brain, spleen, tonsils, testes and ileum (*7*, *8*, *11*), suggesting that it plays a role in controlling a variety of physiological responses (*15*–*17*). However, the information available concerning the biological roles of GPR55 is limited. Furthermore, there are many inconsistencies regarding the effects of cannabinoid compounds, such as CP55940 and 2-arachidonoyglycerol, on GPR55 (*8*–*24*).

In this study, to assess the function of GPR55, we explored possible intracellular signalling molecules, downstreams of GPR55, using HEK293 cells expressing GPR55 as target cells and LPI and related molecules as receptor ligands. We found that LPI induced rapid phosphorylation of p38 mitogen-activated protein kinase (MAPK). We also found that LPI provoked the time-dependent phosphorylation of activating transcription factor 2 (ATF-2). Similar results were obtained for IM-9 lymphoblastoid cells, which naturally express GPR55.

Materials and Methods

Chemicals

Essentially fatty acid-free BSA, LPI sodium salt (derived from soybean), lysophosphatidylcholine (LPC) (derived from egg yolk) and

WIN55212-2 were purchased from Sigma (St Louis, MO, USA). CP55940 and abnormal cannabidiol (abn-CBD) were acquired from Tocris (Bristol, UK). O-1602 was purchased from the Cayman Chemical Co (Ann Arbor, MI, USA). Y-27632, PD98059, SB203580 and SP600215 were acquired from EMD Chemicals Inc. (Gibbstown, NJ, USA). Sucrose monolaurate (SM-1200), wortmannin and herbimycin A were obtained from Wako Pure Chem. Ind. (Osaka, Japan). The anti-phospho-p38 MAPK rabbit antibody, anti-p38 MAPK rabbit antibody, anti-phospho-ATF-2 rabbit antibody, anti-ATF-2 rabbit antibody and anti-rabbit IgG horseradish peroxidase-linked goat antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). The C3 toxin was acquired from List Biological Laboratories (Campbell, CA, USA).

Cloning

A DNA fragment containing the entire open reading frame of the human GPR55 gene (GenBank™ accession number NM_005683) was amplified from human spleen cDNA by PCR as described previously (*14*).

Cells

HEK293 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were then transfected with GPR55-pcDNA4/TO or an empty vector using Lipofectamine™ 2000 reagent. The stably transfected clones were selected in the presence of zeocin (100 μg/ml, Invitrogen). The human lymphoblastoid cell lines, Jurkat cells, Raji cells, Daudi cells and IM-9 cells were grown at 37°C in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Western blot analysis

The subconfluent HEK293 cells expressing GPR55 were incubated in 1 ml of DMEM containing 5 mM HEPES–NaOH (pH 7.4) and 0.1% BSA in the presence of LPI, various ligands or the vehicle (final concentration of DMSO: 0.02%, v/v) in 35-mm dishes at 37°C for the indicated periods of time. Following the incubation, the medium was aspirated and the cells were washed with ice-cold Tyrode's solution containing 5 mM HEPES–NaOH (pH 7.4). The IM-9 cells were incubated in 500 μl of RPMI1640 containing 5 mM HEPES–NaOH (pH 7.4) and 0.1% BSA in the presence or absence of LPI at 37°C for the indicated periods of time. Following the incubation, the cells were spun down and the medium was removed. Western blot analysis was carried out as previously described (*14*). The intensity of the bands was quantified using *ImageJ*, and the ratio of phospho-p38 MAPK to total p38 MAPK or the ratio of phospho-ATF-2 to total ATF-2 was calculated. The data were expressed as fold stimulation (compared to vehicle alone).

RT-PCR analysis

cDNA templates were synthesized from total RNA obtained from various human organs (Ambion). Real-time PCR was performed using SYBR® Premix Ex Taq™ (Takara Bio, Inc.). The following primers were used: GPR55: 5′-ACAGTTTGCAGTCCACATCC-3′ (sense), 5′-ACGCTTCCGTACATGCTGAC-3′ (antisense); GAPDH: 5′-ACAGTCCATGCCATCACTGC-3′ (sense), 5′-TCCA CCACCCTGTTGCTGTA-3′ (antisense). For conventional PCR analysis, total RNA was extracted from Jurkat cells, Raji cells, Daudi cells and IM-9 cells using ISOGEN (Nippon Gene Co., Ltd). The RNA was treated with RNase-free DNase I and reverse transcribed to cDNA. PCR was carried out using Ex Taq polymerase (Takara Bio, Inc.) under the following conditions: for amplification of the GPR55 gene, 30 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C; for amplification of the GAPDH gene, 25 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C.

RNA interference (RNAi) analysis

IM-9 cells were transfected with Duplex small interfering RNA (siRNA) for GPR55 using the Lipofectamine RNAiMAX reagent as previously described (*14*). Following transfection, the cells were incubated at 37°C for 48 h. A scrambled stealth RNAi (Invitrogen) was used as a negative control.

Statistical analysis

The data were analysed by analysis of variance (ANOVA) followed by Dunnett's test (Figs 2–4, 6A and B) or Tukey's test (Figs 5 and 6C). $P < 0.05$ was considered to be significant.

Results

Effects of LPI on p38 MAPK in GPR55-expressing HEK293 Cells

We first examined the effects of LPI on p38 MAPK in the vector-transfected cells and GPR55-expressing cells. As demonstrated in Fig. 1, LPI (1 μM) exerted no apparent effect on p38 MAPK in the vector-transfected cells. Overexpression of GPR55 modestly increased the basal phosphorylation level of p38 MAPK. Notably, treatment of the cells with LPI (1 μM) markedly enhanced the phosphorylation of p38 MAPK in the GPR55-expressing cells.

Figure 2 shows the time-dependent changes in the phosphorylation of p38 MAPK following stimulation with LPI (1 μM). LPI caused rapid phorphorylation of p38 MAPK in the GPR55-expressing cells, but not in the vector-transfected cells. The response was detectable 2.5 min after stimulation with LPI and persisted for at least 20 min.

LPI produced concentration-dependent increases in the phosphorylation of p38 MAPK in the GPR55-expressing cells (Fig. 3); the response was detectable from 30 nM and reached a plateau at 3 μM. The EC_{50} was 300 nM. On the other hand, LPI did not induce the phosphorylation of p38 MAPK at least upto 10 μM in the vector-transfected cells.

Effects of LPI, various cannabinoid receptor ligands and related molecules, on p38 MAPK in GPR55-expressing HEK293 cells

We next compared the ability of LPI and various cannabinoid receptor ligands as well as structurally related molecules to activate p38 MAPK. As demonstrated in Fig. 4A, no marked change was observed following the addition of various ligands to the vector-transfected cells. On the other hand, LPI (1 μM) evoked the phosphorylation of p38 MAPK in the GPR55-expressing

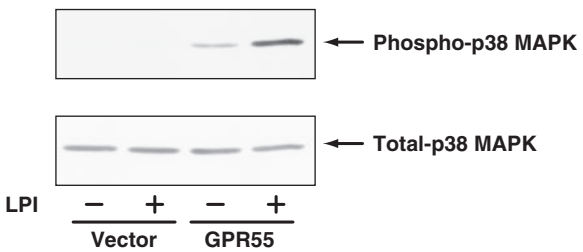

Fig. 1 Effects of LPI on p38 MAPK in GPR55-expressing HEK293 cells. Vector-transfected cells or GPR55-expressing cells were incubated in the presence or absence of LPI (1 μM) at 37°C for 10 min. A western blot analysis was performed as described in the 'Materials and Methods' section. The result is representative of four separate experiments with similar results.

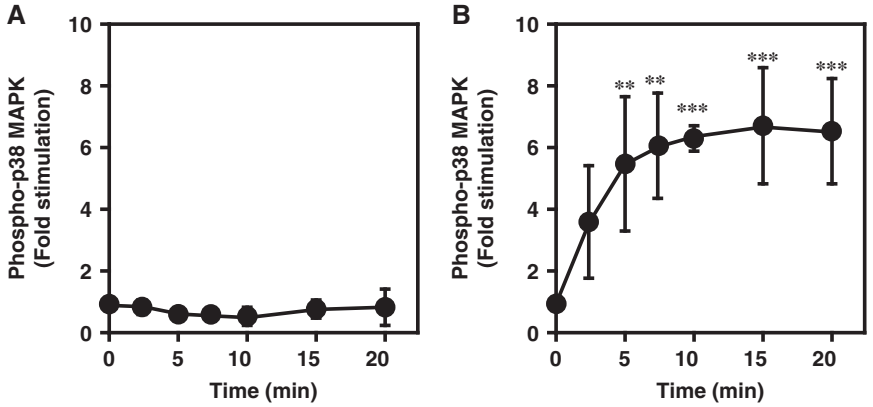

Fig. 2 Time-dependent changes in the phosphorylation of p38 MAPK in GPR55-expressing HEK293 cells. Vector-transfected cells (A) or GPR55-expressing cells (B) were incubated in the presence or absence of LPI (1μM) at 37°C for the indicated periods of time. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to time 0). The values are the means ±SD of four determinations. $^{**}P<0.01$; $^{***}P<0.001$.

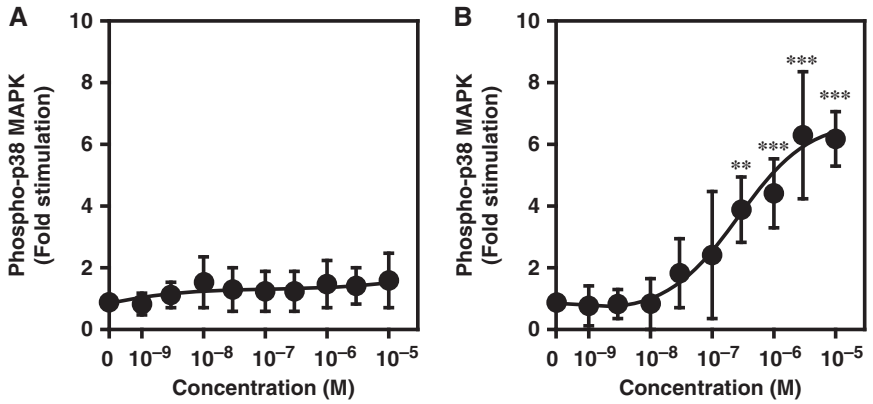

Fig. 3 Dose dependence of LPI-induced phosphorylation of p38 MAPK in GPR55-expressing HEK293 cells. Vector-transfected cells (A) or GPR55-transfected cells (B) were incubated in the presence or absence of various concentrations of LPI at 37°C for 10 min. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to vehicle alone). The values are the means ±SD of four determinations. $^{**}P<0.01$; $^{***}P<0.001$.

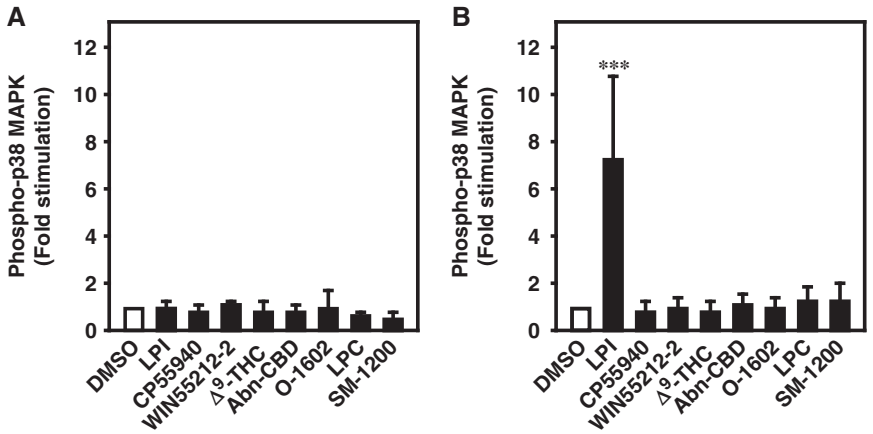

Fig. 4 Effects of LPI, various cannabinoid receptor ligands and related molecules on p38 MAPK in GPR55-expressing HEK293 cells. Vector-transfected cells (A) or GPR55-expressing cells (B) were incubated in the presence or absence of LPI, various cannabinoid receptor ligands and structurally related molecules (1μM each) at 37°C for 10 min. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to vehicle alone). The values are the means ±SD of four determinations. $^{***}P<0.001$.

cells (Fig. 4B). In contrast, various cannabinoid receptor ligands, such as CP55940, WIN55212-2, Δ^9-THC, abn-CBD and O-1602 (1 μM each), had no effect on the p38 MAPK level in the GPR55-expressing cells. LPC (1 μM) and the non-ionic detergent SM-1200 (1 μM) also had no effect on the phosphorylation of p38 MAPK in these cells.

Effects of several inhibitors on LPI-induced phosphorylation of p38 MAPK in GPR55-expressing HEK293 cells

To investigate the intracellular signalling pathways that are downstream of GPR55 and involved in the phosphorylation of p38 MAPK, we treated the GPR55-expressing cells with several inhibitors. As shown in Fig. 5A, treatment with Y-27632 [a Rho-associated kinase (ROCK) inhibitor; 20 μM] completely blocked the LPI (1 μM)-induced phosphorylation of p38 MAPK in the GPR55-expressing cells. On the other hand, PD98059 (a MEK inhibitor; 20 μM), wortmannin (a phosphatidylinositol 3-kinase inhibitor; 500 nM) and herbimycin A (a tyrosine kinase inhibitor; 20 μM) had no influence on the effect of LPI. The effect of the C3 toxin treatment is depicted in Fig. 5B. Treatment of the cells with the C3 toxin (20 μg/ml) markedly suppressed LPI-induced phosphorylation of p38 MAPK, indicating that RhoA is critically involved in the response.

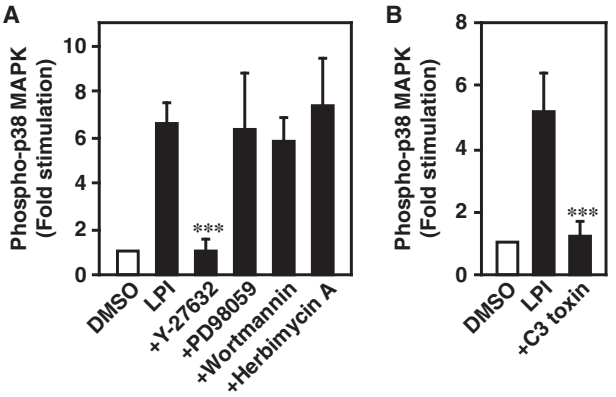

Fig. 5 Effects of various inhibitors on p38 MAPK in GPR55-expressing HEK293 cells. (A) GPR55-expressing cells were treated with the vehicle, Y-27632 (20 μM), wortmannin (500 nM), herbimycin A (20 μM) or PD98059 (20 μM) at 37°C for 1 h and then incubated in the presence of LPI (1 μM) at 37°C for 10 min. (B) GPR55-expressing cells were incubated in the presence or absence of C3 toxin (20 μg/ml) at 37°C for 24 h. The cells were then washed and challenged with LPI (1 μM) at 37°C for 10 min. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to vehicle alone). The values are the means ± SD of four determinations. ***$P < 0.001$.

Effect of LPI on the ATF-2 transcription factor in GPR55-expressing cells

The transcription factor ATF-2 is a downstream target of p38 MAPK. So, we tested the ability of LPI to activate ATF-2 in the GPR55-expressing cells. The activation of ATF-2 was evaluated by determining its phosphorylation. As demonstrated in Fig. 6B, the exposure of the cells to LPI (3 μM) markedly stimulated the phosphorylation of ATF-2. The response was detectable from 5 min after treatment with LPI and persisted for at least 120 min. No apparent effect of LPI on the phosphorylation of ATF-2 was observed in the vector-transfected cells (Fig. 6A).

We then examined the effects of several inhibitors on the LPI (3 μM)-induced phosphorylation of ATF-2 in the GPR55-expressing cells. As demonstrated in Fig. 6C, PD98059 (a MEK inhibitor; 20 μM) did not markedly influence the LPI-induced phosphorylation of ATF-2. SB203580 (a p38 MAPK α/β-isoforms inhibitor; 20 μM) also did not affect the phosphorylation of ATF-2. On the other hand, the LPI-induced phosphorylation of ATF-2 was markedly reduced by Y-27632 (a ROCK inhibitor; 20 μM), as in the case of the p38 MAPK activation shown in Fig. 5, suggesting that Rho-ROCK act as common upstream molecules for both p38 MAPK and ATF-2 activation.

Expression of GPR55 mRNA in various human organs and cell lines

In order to determine the effects of LPI on naturally GPR55-expressing cells, we investigated the expression of GPR55 in various human organs and cell lines. As illustrated in Fig. 7A, high levels of GPR55 expression were observed in the spleen, thymus, small intestine and testes. Substantial levels of GPR55 mRNA were also detected in the brain, lung and colon. On the other

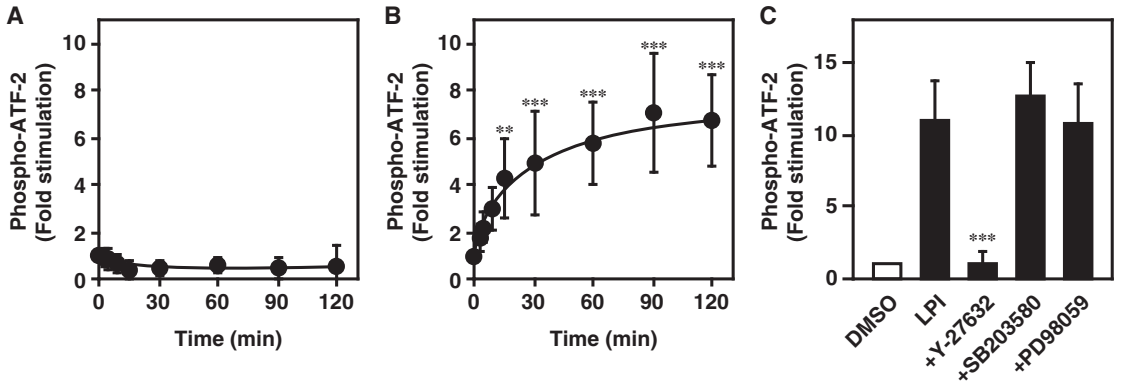

Fig. 6 Effects of LPI on the phosphorylation of ATF-2 in GPR55-expressing HEK293 cells. Vector-transfected cells (A) or GPR55-expressing cells (B) were incubated in the presence or absence of LPI (3 μM) at 37°C for the indicated periods of time. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to time 0). The values are the means ± SD of four determinations. **$P < 0.01$; ***$P < 0.001$. (C) GPR55-expressing cells were treated with the vehicle, Y-27632 (20 μM), SB203580 (20 μM) or PD98059 (20 μM) at 37°C for 1 h and then incubated in the presence of LPI (3 μM) at 37°C for 30 min. A western blot analysis was performed as described in the 'Materials and Methods' section. The data are expressed as the fold stimulation (compared to vehicle alone). The values are the means ± SD of four determinations. ***$P < 0.001$.

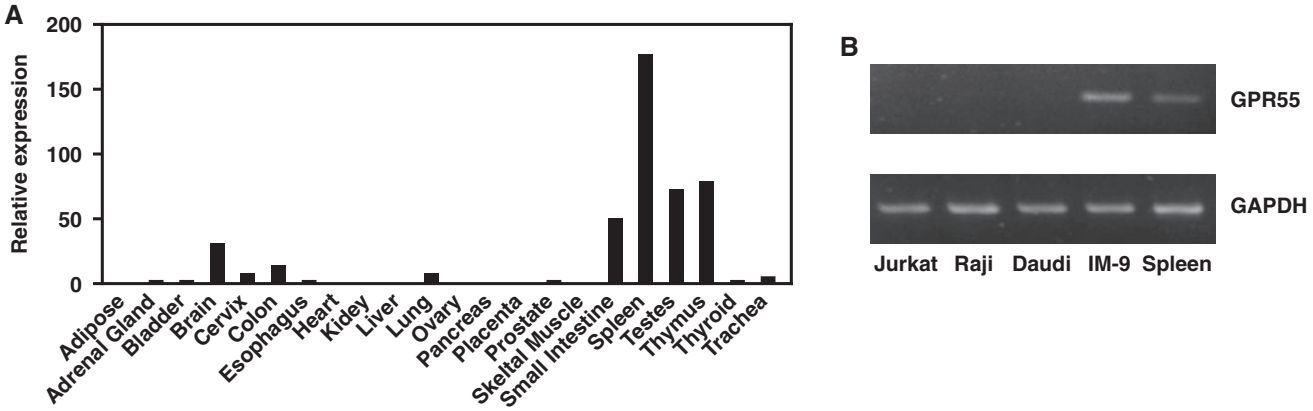

Fig. 7 Expression of GPR55 mRNA in various human organs and cell lines. (A) The expression of GPR55 mRNA in various human organs was determined by real-time PCR as described in the 'Materials and Methods' section. Results are expressed as arbitrary units and normalized against GAPDH mRNA expression. (B) The expression of GPR55 mRNA in several lymphoblastoid cell lines was determined by RT-PCR as described in the 'Materials and Methods' section.

hand, the expression levels of GPR55 mRNA in other organs, such as the liver, heart and kidney, were rather low.

We then explored the expression of GPR55 mRNA in several lymphoblastoid cell lines, because high levels of GPR55 mRNA expression were observed in the spleen and thymus. We found that GPR55 mRNA was abundantly expressed in IM-9 cells, while its expression in Jurkat cells, Raji cells and Daudi cells was negligible (Fig. 7B).

Effects of LPI on p38 MAPK and ATF-2 in IM-9 cells

The effects of LPI on p38 MAPK and ATF-2 in IM-9 cells were examined next. As demonstrated in Fig. 8A, LPI (1 μM) evoked the phosphorylation of p38 MAPK in IM-9 cells. LPI (3 μM) also induced the phosphorylation of ATF-2 (Fig. 8B). On the other hand, LPC (3 μM) did not influence the phosphorylation of p38 MAPK or ATF-2 in IM-9 cells (data not shown). Of note, treatment of the cells with the siRNA markedly reduced the LPI-induced phosphorylation of p38 MAPK (by 80%) and ATF-2 (by 60%), whereas treatment with a control siRNA did not exert any effect on the LPI-induced response (data not shown). Moreover, the LPI-induced phosphorylation of p38 MAPK or ATF-2 was not observed in Jurkat cells, Raji cells and Daudi cells (data not shown). These results suggest that the augmented phosphorylation of p38 MAPK and ATF-2 is a common cellular event in GPR55-expressing cells following stimulation with LPI.

Discussion

GPR55 is a rhodopsin-like G protein-coupled receptor that was identified in 1999 (*7*). Evidence is accumulating that GPR55 is expressed in various mammalian tissues and cells (*7*, *8*, *11*). Yet, the endogenous ligand for GPR55 was not determined for a long time. Recently, we started a search for the endogenous ligand for GPR55 and obtained evidence that LPI acts as an agonist towards GPR55. LPI induced the rapid phosphorylation of ERK and a Ca^{2+} transient in HEK 293 cells expressing GPR55 but not in vector-transfected cells (*14*, *20*). Several investigators also reported that LPI induces Ca^{2+} responses and activation of the nuclear factor of activated T cells (NFAT) in HEK293 cells expressing GPR55 (*21*), a Ca^{2+} transient in mouse dorsal root ganglion neurons (*18*) and the EA.hy926 human umbilical vein endothelial cell line (*19*), β-arrestin translocation in HEK293 cells expressing GPR55 (*22*) and enhanced phosphorylation of ERK in the BV-2 mouse microglial cell line (*25*). Nevertheless, not much information is currently available concerning LPI as a GPR55 ligand.

In this study, we examined in detail the effects of LPI on several other signalling molecules such as p38 MAPK. We found that LPI induced the phosphorylation of p38 MAPK in GPR55-expressing HEK293 cells (Figs 1–3). A similar result was obtained for IM-9 cells, which naturally express GPR55 (Fig. 8). The phosphorylation of p38 MAPK was blocked by a ROCK inhibitor and C3 toxin, suggesting that the effect of LPI on p38 MAPK was mediated by the Rho-ROCK pathway. On the other hand, MEK, phosphatidylinositol 3-kinase and tyrosine kinase seem not to be involved in the phosphorylation of p38 MAPK.

We also found that LPI caused the phosphorylation of the ATF-2 transcription factor, a member of the leucine zipper family of DNA binding proteins in GPR55-expressing HEK293 cells (Fig. 6B) and IM-9 cells (Fig. 8). ATF-2 is activated in response to signals that converge on the stress-activated protein kinases p38 MAPK and c-Jun N-terminal kinase (JNK) (*26*). It has also been reported that ATF-2 can be activated by ERK (*27*). As for JNK, it appears not to be involved, because LPI did not induce the activation of JNK, and the inhibition of this enzyme using a specific inhibitor (SP600125) did not influence the activation of either p38 MAPK or ATF-2 (data not shown). The blockage of the MEK/ERK pathway using PD98059 also did not affect the LPI-induced phosphorylation of ATF-2 (Fig. 6C), suggesting that the activation of ERK induced by LPI is not relevant to ATF-2 activation. Notably, SB203580, a p38 MAPK inhibitor, did not affect the LPI-induced

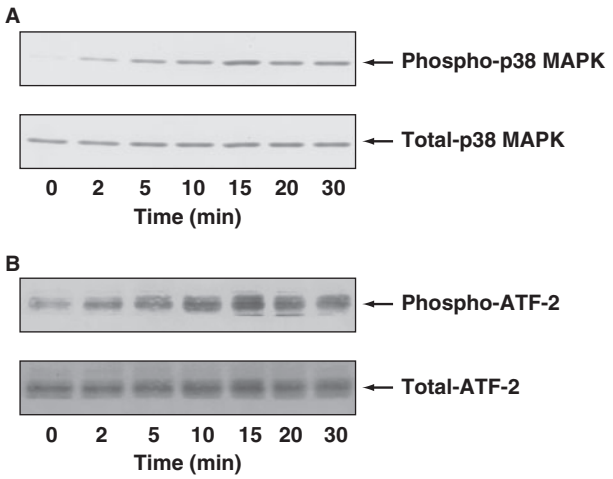

Fig. 8 Effects of LPI on the phosphorylation of p38 MAPK and ATF-2 in IM-9 cells. IM-9 cells were incubated in the presence or absence of LPI at 37°C for the indicated periods of time. A western blot analysis was performed as described in the 'Materials and Methods' section. (A) Effects of LPI (1 μM) on the phosphorylation of p38 MAPK. (B) Effects of LPI (3 μM) on the phosphorylation of ATF-2. The data are representative of three separate experiments with similar results.

phosphorylation of ATF-2 (Fig. 6C). This is not surprising, because SB203580 blocks the p38α- and β-isoforms, but not the γ- or δ-isoform (*28*), raising the possibility that LPI activated the p38 MAPK γ- or δ-isoform thereby inducing the phosphorylation of ATF-2. In relation to this, it should be noted that Marinissen *et al.* (*29*) reported that RhoA selectively activates p38γ, but not p38α. It is also worth noting that the LPI-induced phosphorylation of p38 MAPK in GPR55-expessing HEK293 cells was mediated by the Rho-ROCK pathway (Fig. 5). It is conceivable, therefore, that LPI stimulates p38 MAPK (presumably γ- or δ-isoform) primarily via the Rho-ROCK pathway, leading to the activation of ATF-2.

Thus, RhoA and ROCK are key molecules in the LPI-induced activation of p38 MAPK and ATF-2. This is consistent with the fact that GPR55 is thought to couple to the $G_{12/13}$ protein (*11*) and that the activation of the $G_{12/13}$ protein, in general, leads to an increase in RhoA guanine nucleotide exchange and ROCK activation (*30*, *31*). Several investigators have previously reported that the Rho-ROCK pathway is involved in GPR55-mediated cellular responses, *e.g.* the Ca^{2+} response and the activation of NFAT (*21*). We also found that the Rho-ROCK pathway participates in ERK activation (S. Oka *et al.*, unpublished results). It appears, therefore, that the $G_{12/13}$-RhoA-ROCK signalling pathway mediates various LPI- and GPR55-induced cellular events.

Meanwhile, GPR55 has been reported as a possible novel type of cannabinoid receptor (*8–11*, *18*). Several investigators have demonstrated that various cannabinoid receptor ligands stimulate the binding of [^{35}S]GTPγS to membranes prepared from GPR55-expressing HEK293 cells (*8–11*). Several cannabinoid receptor ligands have also been shown to induce Ca^{2+} responses in HEK293 cells expressing GPR55 (*18*, *19*, *21*). On the other hand, in our previous study, various cannabinoid receptor ligands (1 μM) failed to induce the phosphorylation of either ERK or a Ca^{2+} transient in HEK293 cells expressing GPR55 (*14*). Several cannabinoid receptor ligands also failed to induce the phosphorylation of p38 MAPK (Fig. 4). The reason for these discrepancies is not clear. The different experimental results, with respect to the effects of the cannabinoid receptor ligands, may be attributed, at least in part, to differences in the experimental conditions and protocols. In any case, the thorough elucidation of whether GPR55 acts as an effective and functional receptor for cannabinoids requires future investigation.

The physiological implications of the LPI-induced and GPR55-mediated phosphorylation of p38 MAPK have also not been fully elucidated. GPR55 has been shown to be expressed in certain regions of the brain and several peripheral tissues such as the spleen and tonsils (*7*, *8*, *11*). We also observed that GPR55 is highly expressed in the spleen and thymus (Fig. 7). Several investigators have reported that GPR55 may participate in a variety of physiological and pathophysiological processes, *e.g.* the regulation of cytokine production, inflammatory/neuropathic pain and the regulation of vascular functions (*15–24*, *32*). Considering that p38 MAPK is responsive to various cellular stresses including inflammatory cytokines, lipopolysaccharides, heat shock, osmotic shock and ultraviolet irradiation and is involved in cell differentiation and apoptosis (*33*, *34*), it seems very likely that GPR55 and its endogenous ligand, LPI, play essential roles in the self-defence mechanisms against stress stimuli in several biological systems such as the nervous system, immune system and vascular system. Several investigators have previously demonstrated that LPI exhibits biological activity towards certain types of mammalian cells (*35*), yet the details of the mechanisms behind these actions remain to be determined. Further detailed studies are thus needed to fully elucidate the physiological and pathophysiological roles of GPR55 and LPI in mammalian tissues.

In summary, we found that LPI induced the rapid phosphorylation of p38 MAPK in HEK293 cells expressing GPR55 and IM-9 cells. We also found that LPI provoked the phosphorylation of the transcription factor ATF-2. These results point to roles of GPR55 and LPI in the homeostatic responses to stress signals in several types of mammalian cells.

Funding
Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in Aid for Scientific Research (to S.O., partial); and a Japan Society for the Promotion of Science, Grant-in Aid for Scientific Research (to T.S.).

Conflict of interest
None declared.

References

1. Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M.,

Mackie, K., Martin, B.R., Mechoulam, R., and Pertwee, R.G. (2002) International Union of Pharmacology: XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* **54**, 161–202
2. Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., and Bonner, T.I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**, 561–564
3. Munro, S., Thomas, K.L., and Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**, 61–65
4. Di Marzo, V., De Petrocellis, L., Fezza, F., Ligresti, A., and Bisogno, T. (2002) Anandamide receptors. *Prostaglandins Leukot. Essent. Fatty Acids* **66**, 377–391
5. Di Marzo, V. and De Petrocellis, L. (2006) *Non-CB1, non-CB2 receptors for endocannabinoids in* Endocannabinoids: The Brain and Body's Marijuana and Beyond (Onaivi, E.S., Sugiura, T., and Di Marzo, V., eds.), pp. 151–174, Taylor & Francis, Boca Raton.
6. Begg, M., Pacher, P., Bátkai, S., Osei-Hyiaman, D., Offertáler, L., Mo, F.M., Liu, J., and Kunos, G. (2005) Evidence for novel cannabinoid receptors. *Pharmacol. Ther.* **106**, 133–145
7. Sawzdargo, M., Nguyen, T., Lee, D.K., Lynch, K.R., Cheng, R., Heng, H.H., George, S.R., and O'Dowd, B.F. (1999) Identification and cloning of three novel human G protein-coupled receptor genes GPR52, ψGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Res. Mol. Brain Res.* **64**, 193–198
8. Brown, A.J. and Wise, A. (2001) *Identification of Modulators of GPR55 Activity*. GlaxoSmithKline, Patent number, WO0186305
9. Drmota, T., Greasley, P., and Groblewski, T. (2004) *Screening Assays for Cannabinoid-ligand-type Modulators of GPR55*. Astrazeneca, Patent number, WO2004074844
10. Johns, D.G., Behm, D.J., Walker, D.J., Ao, Z., Shapland, E.M., Daniels, D.A., Riddick, M., Dowell, S., Staton, P.C., Green, P., Shabon, U., Bao, W., Aiyar, N., Yue, T.-L., Brown, A.J., Morrison, A.D., and Douglas, S.A. (2007) The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *Br. J. Pharmacol.* **152**, 825–831
11. Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N.-O., Leonova, J., Elebring, T., Nilsson, Drmota, T., and Greasley, P.J. (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br. J. Pharmacol.* **152**, 1092–1101
12. Petitet, F., Donlan, M., and Michel, A. (2006) GPR55 as a new cannabinoid receptor: still a long way to prove it. *Chem. Biol. Drug Des.* **67**, 252–253
13. Baker, D., Pryce, G., Davies, W.L., and Hiley, C.R. (2006) In silico patent searching reveals a new cannabinoid receptor. *Trends Pharmacol. Sci.* **27**, 1–4
14. Oka, S., Nakajima, K., Yamashita, A., Kishimoto, S., and Sugiura, T. (2007) Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochem. Biophys. Res. Commun.* **362**, 928–934
15. Hiley, C.R. and Kaup, S.S. (2007) GPR55 and the vascular receptors for cannabinoids. *Br. J. Pharmacol.* **152**, 559–561
16. Pertwee, R.G. (2007) GPR55: a new member of the cannabinoid receptor clan? *Br. J. Pharmacol.* **152**, 984–986
17. Brown, A.J. (2007) Novel cannabinoid receptors. *Br. J. Pharmacol.* **152**, 567–575
18. Lauckner, J.E., Jensen, J.B., Chen, H.-Y., Lu, H.-C., Hille, B., and Mackie, K. (2008) GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc. Natl Acad. Sci. USA* **105**, 2699–2704
19. Waldeck-Weiermair, M., Zoratti, C., Osibow, K., Balenga, N., Goessnitzer, E., Waldhoer, M., Malli, R., and Graier, W.F. (2008) Integrin clustering enables anandamide-induced Ca^{2+} signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered repression. *J. Cell Sci.* **121**, 1704–1717
20. Oka, S., Toshida, T., Maruyama, K., Nakajima, K., Yamashita, A., and Sugiura, T. (2009) 2-Arachidonoyl-sn-glycero-3-phosphoinositol: a possible natural ligand for GPR55. *J. Biochem.* **145**, 13–20
21. Henstridge, C.M., Balenga, N.A., Ford, L.A., Ross, R.A., Waldhoer, M., and Irving, A.J. (2009) The GPR55 ligand L-α-lysophosphatidylinositol promotes RhoA-dependent Ca^{2+} signaling and NFAT activation. *FASEB J.* **23**, 183–193
22. Yin, H., Chu, A., Li, W., Wang, B., Shelton, F., Otero, F., Nguyen, D.G., Caldwell, J.S., and Chen, Y.A. (2009) Lipid G protein-coupled receptor ligand identification using β-arrestin PathHunter assay. *J. Biol. Chem.* **284**, 12328–12338
23. Ross, R.A. (2009) The enigmatic pharmacology of GPR55. *Trends Pharmacol. Sci.* **30**, 56–63
24. Godlewski, G., Offertáler, L., Wagner, J.A., and Kunos, G. (2009) Receptors for acylethanolamides - GPR55 and GPR119. *Prostaglandins Other Lipid Mediat.* **89**, 105–111
25. Pietr, M., Kozela, E., Levy, R., Rimmerman, N., Lin, Y.H., Stella, N., Vogel, Z., and Juknat, A. (2009) Differential changes in GPR55 during microglial cell activation. *FEBS Lett.* **583**, 2071–2076
26. Gupta, S., Campbell, D., Dérijard, B., and Davis, R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389–393
27. Ouwens, D.M., de Ruiter, N.D., van der Zon, G.C., Carter, A.P., Schouten, J., van der Burgt, C., Kooistra, K., Bos, J.L., Maassen, J.A., and van Dam, H. (2002) Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO J.* **21**, 3782–3793
28. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
29. Marinissen, M.J., Chiariello, M., and Gutkind, J.S. (2001) Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 γ) MAP kinase pathway. *Genes Dev.* **15**, 535–553
30. Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G., and Sternweis, P.C. (1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science* **280**, 2109–2111
31. Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. *Science* **280**, 2112–2114
32. Staton, P.C., Hatcher, J.P., Walker, D.J., Morrison, A.D., Shapland, E.M., Hughes, J.P., Chong, E., Mander, P.K., Green, P.J., Billinton, A., Fulleylove, M., Lancaster, H.C., Smith, J.C.,

Bailey, L.T., Wise, A., Brown, A.J., Richardson, J.C., and Chessell, I.P. (2008) The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain* **139**, 225–236
33. Kumar, S., Boehm, J., and Lee, J.C. (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* **2**, 717–726
34. Ashwell, J.D. (2006) The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nat. Rev. Immunol.* **6**, 532–540
35. Corda, D., Iurisci, C., and Berrie, C.P. (2002) Biological activities and metabolism of the lysophosphoinositides and glycerophosphoinositols. *Biochim. Biophys. Acta* **1582**, 52–69