Involvement of P2Y<sub>12</sub> receptor in vascular smooth muscle inflammatory changes via MCP-1 upregulation and monocyte adhesion

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on this putative effect (28). For further elucidation of the atherosclerotic disease mechanism, which takes into account platelets, it is important to know whether ADP-dependent P2 receptors whose functions have been well demonstrated in platelets are also involved in the regulation of important inflammatory mediators such as MCP-1 or not. We investigated the hypothesis that ADP, via vascular smooth muscle P2Y12 receptor, may induce vascular wall inflammatory changes by upregulating MCP-1 and promoting monocyte adhesion.

MATERIALS AND METHODS

Reagents. ADP was purchased from Sigma. R-138727, the active metabolite of prasugrel, a P2Y12 inhibitor, was a kind gift from Daiichi Sankyo (Tokyo, Japan). Suramin, a nonselective antagonist of P2 receptors, was from Wako Pure Chemical Industries (Osaka, Japan). The reactive oxygen species (ROS) inhibitors N-acetylcysteine (NAC), diphenyleneiodonium (DPI), and Tempol were from Sigma-Aldrich (St. Louis, MO). SC-202525 and RS504393, antagonists of MCP-1 receptor CCR2, were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-ERK 1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), and total JNK were from Cell Signaling Technology (Danvers, MA). Antibody against total ERK 1/2 was from New England Biolabs (Ipswich, MA). Antibody against MCP-1 was from LifeSpan BioSciences (Seattle, WA).

Cell culture. VSMCs were obtained from the aorta of 6-wk-old male Wistar rats following the explant method previously described (25). Animals were handled in accordance with the protocols submitted to and approved by the University of Tokyo Committee on Institutional Animal Care and Use. Cells were cultured and propagated in DMEM containing 10% FBS. Cells passaged three to eight times were used for the experiments. For serum deprivation, cells were incubated in DMEM containing 0.2% FBS for 48 h. The human monocytic cell line THP-1 cells, obtained from ATCC (Manassas, VA), were used for assays of monocyte binding to VSMCs. THP-1 cells were cultured and propagated in RPMI 1640 medium containing 10% FBS. In all the experiments using receptor or signal inhibitors, incubation with the inhibitors was started 30 min before the start of ADP stimulation and was continued until the end of the stimulation unless otherwise stated.

siRNAs. Two different siRNAs (Stealth RNAiPre-Designed siRNAs) predesigned by the manufacturer and targeted against rat P2Y12 receptor were purchased from Invitrogen (Carlsbad, CA). Sequences of the two siRNAs (siRNA-P2Y12-1 and siRNA-P2Y12-2) were as follows: siRNA-P2Y 12-1: 5'-CCUUGAACGCCUGCCUUGAUC-CAUU-3' and 5'-AAUGGAUCAAGGCAGGCUUCAAGG-3'; and siRNA-P2Y12-2: 5'-CACGAACAGCUUGGCGAUGAGGAUU-3'.
and 5′-AAUCCUACGCGCAUGCUAGUG-3′. The same manufacturer’s recommended negative control siRNA (Stealth RNA siRNA Negative Control Hi GC) was also used. For transfection with siRNAs only, transfection reagent (sc-29528) from Santa Cruz Biotechnology was used following the manufacturer’s instructions.

**Quantitative real-time PCR.** Total RNA from VSMCs was extracted using TRIZOL reagent (Invitrogen). One microgram of total RNA per sample was subjected to reverse transcription using the Omniscript RT kit (Qiagen, Hilden, Germany). Expression of MCP-1 or GAPDH was examined by SYBR green-based real-time PCR. Primers used were as follows: MCP-1 sense: 5′-CTCAGCCAGATGCAGGAATGC-3′; MCP-1 antisense: 5′-TCTCCAGCGACTCATGGG-3′; and GAPDH sense: 5′-GTATGACTCTACCCACGGCAAGT-3′, GAPDH antisense: 5′-TTCCCCTTGTAGCACAGCAGCTT-3′. Real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA).

**ELISA.** MCP-1 concentrations in culture medium were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Before the assays were started,

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**Fig. 2.** Involvement of nonspecific P2 receptor(s) and of P2Y12, specifically, in ADP-induced MCP-1 upregulation in VSMCs. Effects of suramin (A), a nonselective inhibitor of P2 receptors, of R-138727 (B), a P2Y12 receptor inhibitor, and of 2 different P2Y12 siRNAs (P2Y12-1 and P2Y12-2; C) compared with negative control siRNA (NC) on MCP-1 mRNA upregulation as examined by real-time PCR (n = 3). VSMCs were stimulated with 10⁻⁵ mol/l ADP for 4 h. D: effects of R-138727 on ADP-induced MCP-1 protein upregulation as examined by ELISA (n = 3). VSMCs were stimulated with 10⁻⁵ mol/l ADP for 16 h. E: effects of P2Y12 siRNAs on ADP-induced MCP-1 protein upregulation as examined by ELISA (n = 3). ★P < 0.05 vs. ADP (+); ■P < 0.05 vs. untreated ADP (+).
VSMCs were washed twice with phosphate-buffered saline and medium was replaced with phenol red-free culture medium. Medium was collected after the incubation period and centrifuged for 1 min. The supernatants were stored at −80°C until the assay.

**Western blot analysis.** VSMC lysates for Western blot analysis were prepared with Nonidet P-40 cell lysis buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40] containing 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 2 µg/ml apro tin. Cell lysates were subjected to SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody for 1 h at room temperature, immunoreactive proteins were visualized by chemiluminescence.

**Plasmids and luciferase assay.** The 5'-flanking region of rat MCP-1 from base −3,555 to base +74 was amplified by PCR using rat genomic DNA as the template and cloned into the KpnI-XhoI site of pGL3-basic plasmid (Promega, Fitchburg, WI). Primers used for the PCR were as follows: sense: 5'-GGGGTACCGTTTGAGTGTTCCAGAGG-3', and antisense: 5'-CCGCTCGAGTCTCATGATAGTGTTGAGGAAGA-3'.

With this wild-type MCP-1 promoter region as the template, plasmids containing a mutated MCP-1 promoter were generated using QuickChange mutagenesis kit (Promega). Primers used for the mutation of two NF-κB binding sites (∆NF-κB) at base −2287 to base −2278 (site A) and base −2261 to base −2252 (site B) were as follows: site A sense: 5'-CTTCTCTTGGAAGGCTCTGGGAACCTCG-3', site B sense: 5'-CATTCCTGAG-GATTCCTTCCAGAGC3', and antisense: 5'-CTTCTTGAG-3'. A plasmid in which all of the above NF-κB sites and the AP-1 site were mutated (NF-κB/ AP-1) was also generated. All the sequences were confirmed by DNA sequencing.

DNA plasmid transfections were performed with TransIT reagent (Takara Bio, Otsu, Japan) following the manufacturer’s instructions. Fifty nanograms of reporter plasmid together with 50 ng of pRL-SV40 (Promega), a reference renilla luciferase reporter plasmid employed to normalize for transfection efficiency, were used for the transfection in each 0.32-cm2 well. Cotransfections with DNA plasmid and siRNA were performed with jetPrime reagent (Polyplus Transfection, Illkirch, France). Luciferase assay was performed using the Dual Glo Reporter Assay System (Promega) according to the manufacturer’s instructions. The activity of promoters was expressed as the ratio of firefly luciferase activity to renilla luciferase activity.

**Fig. 3.** JNK and ERK 1/2 are involved in ADP-induced MCP-1 upregulation. ADP-induced JNK, but not ERK, activation is mediated by P2Y12. A: MCP-1 mRNA upregulation, as examined by real-time PCR, was significantly diminished by an ERK 1/2 inhibitor U0126 at 10−6 mol/l and by a JNK inhibitor SP600125 at 10−5 mol/l (n = 3). VSMCs were stimulated with 10−5 ADP for 4 h. B: ADP-induced MCP-1 protein upregulation was significantly inhibited by U0126 and SP600125 by ELISA (n = 3). P < 0.05 vs. ADP (−); #P < 0.05 vs. untreated ADP (+). C: ADP-induced time-dependent JNK phosphorylation. Representative image of 2 independent experiments is shown. D: ADP-induced and JNK activation was significantly inhibited by R-138727 at 10−5 mol/l (n = 3). VSMCs were stimulated with 10−5 mol/l ADP for 15 min. JNK phosphorylation level was examined by Western blot analysis. Representative band images of 3 independent experiments are shown. ★P < 0.05 vs. ADP (−); †P < 0.05 vs. untreated ADP (+). E: ADP-induced JNK activation was significantly inhibited by P2Y12 siRNAs (n = 3). VSMCs were stimulated with 10−5 mol/l ADP for 15 min. F: lack of effects of ADP and P2Y12 siRNAs on JNK protein expression levels. Representative image of 2 independent experiments is shown.
Assays of monocytes binding to VSMCs. For the determination of VSMC monocyte binding activity, human monocyte cell line THP-1 cells labeled with calcein, a fluorescent dye, were used. Molecule function generalizability despite cell species difference can be expected to some extent and has been shown in previous similar study (31). THP-1 cells were labeled with 2.5 μg/ml calcein (Dojindo Laboratories, Kumamoto, Japan) in foil-covered tubes for 30 min at 37°C and washed twice with PBS to remove dye that was not incorporated. Then, the THP-1 cells (5 × 10⁶ cells per well in 6-well plates) were placed evenly over VSMCs that had been stimulated, or not, with ADP. After the THP-1 cells and the VSMCs were incubated together for 30 min, all the cells were washed gently twice with PBS to remove unbound THP-1 cells. Then, cell lysate was extracted from all the remaining cells using Nonidet P-40 cell lysis buffer. Fluorescence intensities were measured using a plate reader with excitation/emission wavelengths of 495/515 nm, respectively.

Statistics. Values are shown as means ± SE of data. For comparisons between groups, Student’s t-test or ANOVA followed by the Student-Newman-Keuls test was used where appropriate. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

ADP-induced MCP-1 mRNA and protein upregulation in VSMCs. The effects of ADP stimulation on VSMC MCP-1 mRNA expression were examined by real-time PCR. ADP at 10⁻⁵ mol/l upregulated MCP-1 mRNA expression in a time-dependent manner, reaching a maximal 3.6 ± 0.3-fold increase compared with control 4 h after the start of stimulation (Fig. 1A). This upregulation was dose dependent. At 4 h, upregulation started to be detected significantly at ∼10⁻⁵ mol/l and continued to increase dose dependently up to 2 mol/l at 4 h, where a 6.3 ± 0.5-fold increase was observed (Fig. 1B). ADP apparently was not cytotoxic even at this highest dose examined.

To examine MCP-1 protein levels, ELISA was performed using culture medium of VSMCs stimulated, or not, with ADP. ELISA showed that ADP-induced MCP-1 protein upregulation compared with unstimulated control was significant after 2–16 h but was not significant at 24 h or later due to basal time-dependent increase of MCP-1 expression in unstimulated VSMCs (Fig. 1C). To confirm the ELISA results, we performed Western blotting using cell lysate protein. We detected ADP-induced MCP-1 bands at ∼25 kDa, which were the same size as the positive control band induced by VSMC stimulation with TNF-α (Fig. 1D). By Western blotting, ADP-induced MCP-1 upregulation compared with control was observed from 2 to 8 h after the start of stimulation (Fig. 1D) but was not observed at 24 h, which was roughly the same result as ELISA.

Involvement of P2Y12 in ADP-induced MCP-1 upregulation. To examine whether ADP-induced MCP-1 upregulation was mediated by P2 receptors, VSMCs were incubated with suramin, a nonspecific P2 receptor family antagonist. ADP-induced MCP-1 mRNA upregulation was significantly inhibited by suramin (Fig. 2A). As an inhibitor of P2Y12, we used R-138727, the active metabolite of prasugrel, which has been shown to effectively antagonize the receptor and potently inhibit ADP-induced platelet aggregation (18). R-138727 at 10⁻⁵ mol/l or two different siRNAs directed against P2Y12 significantly inhibited MCP-1 mRNA upregulation (Fig. 2, B and C). ELISA showed that ADP-induced MCP-1 upregulation was attenuated by R-138727 and siRNAs (Fig. 2, D and E).

Involvement of JNK pathway downstream of P2Y12 receptor. It has been shown that in the modulation of MCP-1 expression transcriptional activation by factors such as NF-κB and AP-1 is important and there exist multiple signaling pathways involved in the process, which include MAPKs such as ERK 1/2 or JNK (6, 9).

For the exploration of the signal transduction mechanisms in ADP-induced MCP-1 upregulation, specific inhibitors of multiple representative pathways were employed. Among them, incubation with U0126, an ERK 1/2 pathway inhibitor, and with SP600125, a JNK inhibitor, significantly inhibited the MCP-1 mRNA increase induced by ADP (Fig. 3A). U0126 and SP600125 also inhibited ADP-induced MCP-1 protein increase significantly by ELISA (Fig. 3B). Accordingly, we examined the effects of ADP on activities of ERK 1/2 and JNK by Western blot analysis using phospho-specific antibodies. Among the several time points preliminarily examined, ADP enhanced the phosphorylation of both pathways significantly and almost maximally at 15 min after the start of stimulation.
Fig. 5. Consensus NF-κB and AP-1 binding sites are involved in ADP-induced MCP-1 promoter activation. P2Y<sub>12</sub> receptor-mediated MCP-1 promoter activation involves NF-κB. A: ADP-induced wild-type MCP-1 promoter activation was inhibited by R-138727 as examined by luciferase assay (n = 4). Firefly luciferase activity of the pGL3-basic plasmid containing a 3.6-kb rat wild-type MCP-1 promoter fragment was normalized by renilla luciferase activity of the cotransfected pRL-SV40 plasmid. Transfected VSMCs were stimulated with 10<sup>-5</sup> mol/l ADP for 3 h. B: ADP-induced wild-type MCP-1 promoter activation was inhibited by P2Y<sub>12</sub> siRNAs (n = 4). *P < 0.05 vs. ADP (-); #P < 0.05 vs. untreated ADP (+). C, Site-directed mutagenesis of the 2 consensus NF-κB sites (ΔNF-κB), of the AP-1 site (ΔAP-1) or of all these sites (ΔNF-κB and AP-1) in the MCP-1 promoter diminished the ADP-induced activation compared with the wild-type promoter (WT) as examined by luciferase assay (n = 4). Transfected VSMCs were stimulated with 10<sup>-5</sup> mol/l ADP for 3 h. *P < 0.05. D: ADP-induced NF-κB activation as examined with pNF-κB-luc vector was inhibited by R-138727 and suramin (n = 4). Transfected VSMCs were preincubated with 10<sup>-5</sup> mol/l R-138727 or with 10<sup>-4</sup> mol/l suramin before a 3-h stimulation with 10<sup>-5</sup> mol/l ADP. E: ADP-induced NF-κB activation as examined with pNF-κB-luc vector was inhibited by P2Y<sub>12</sub> siRNAs. *P < 0.05 vs. ADP (-); #P < 0.05 vs. untreated ADP (+).
However, the examination of the effects of P2Y12 inhibition at this time point revealed that P2Y12 was consistently involved only in JNK phosphorylation. In our time-course experiment, JNK phosphorylation could be observed by a 5- to 15-min ADP stimulation and could not be seen at 30 min or later (Fig. 3C). R-138727 and P2Y12 siRNAs significantly inhibited JNK phosphorylation at 15 min (Fig. 3, D and E). We also examined the effects of P2Y12 siRNAs on JNK expression at several time points in a longer time course up to 8 h. There were no effects on JNK expression within this time course (Fig. 3F).

Involvement of ROS. Since ROS are known to be involved in the JNK pathway (15), we used NAC, DPI, and Tempol to examine whether ROS are involved in ADP-induced MCP-1 upregulation and JNK pathway or not. These agents significantly inhibited ADP-induced MCP-1 mRNA and protein upregulation (Fig. 4, A and B), as well as JNK phosphorylation (Fig. 4C).

MCP-1 promoter activation via P2Y12 depends on NF-κB. To determine whether MCP-1 promoter activity is involved in ADP-induced MCP-1 upregulation, luciferase assay was performed using a reporter plasmid containing the MCP-1 promoter region. ADP significantly activated the promoter, which was inhibited by R-138727 and P2Y12 siRNAs (Fig. 5, A and B). For the elucidation of the cis-elements involved in the ADP-induced MCP-1 transcription, reporter plasmids in which consensus NF-κB and AP-1 sites in the MCP-1 promoter region were mutated were utilized. Deletion of both two NF-κB sites at bases −2,287 to −2,278 and at bases −2,261 to −2,252 (∆NF-κB), or deletion of AP-1 site at bases −54 to −44 (∆AP-1) significantly inhibited the ADP-induced promoter activation (Fig. 5C). Deletion of all these sites (∆NF-κB and AP-1) further inhibited the activation (Fig. 5C). ADP stimulation also increased the signal of pNF-κB-luc reporter plasmid, which contains multiple consensus NF-κB sites, demonstrating the NF-κB signal transduction pathway activation by ADP (Fig. 5D). This NF-κB signal activation was inhibited by R-138727 and suramin (Fig. 5D), as well as by P2Y12 siRNAs (Fig. 5E). On the other hand, ADP-induced AP-1 signal activation as shown by pAP1-luc reporter plasmid was inhibited by suramin but not by R-138727 (data not shown).

P2Y12 contributes to monocyte binding to VSMC. It has been shown that MCP-1 promotes binding of monocytes to VSMCs (23). We next tried to determine if P2Y12 affects monocyte binding to VSMCs. VSMCs seeded in six-well plates were stimulated with ADP for 8 h, and then human monocyte cell line THP-1 cells, which had been labeled with calcine, were evenly placed over them and incubated for 30 min. After the cells were washed twice with PBS to remove unbound THP-1 cells, the lysates were extracted from all the remaining cells. Fluorescence intensity was measured using a plate reader. Fluorescence intensity was significantly (1.7 ± 0.2-fold) increased when VSMCs had been stimulated with ADP compared with unstimulated controls (Fig. 6A). P2Y12 siRNAs significantly diminished the ADP-induced binding (Fig. 6A). To investigate MCP-1 involvement in this binding, two antagonists of MCP-1 receptor CCR2 were added before incubation of VSMCs and THP-1 cells. Both these antagonists, SC-202525 and RS504393, significantly inhibited ADP-induced binding (Fig. 6B).

DISCUSSION

This study offers several novel findings (Fig. 7). First, we have demonstrated that extracellular ADP upregulates the expression of a major chemokine, MCP-1, in VSMCs. Second, this upregulation is mediated by P2Y12 receptor. Third, JNK pathway is implicated in this upregulation in ROS-dependent manners. NF-κB-dependent MCP-1 promoter activation is also
involved. Lastly, P2Y12 via MCP-1 mediates ADP-induced monocyte binding to VSMCs.

Platelets are known to be mediators not only of clot formation and coagulation but also of inflammation (29). It has been demonstrated that already in the early phases of atherogenesis, platelets, like white blood cell types such as macrophages and neutrophils, attach to the activated endothelium, via adhesion molecules including E-selectin (13). Platelets activated in the process of adhesion can in turn activate endothelial cells, thus promoting the vicious cycle of local inflammation. Moreover, platelets also interact with leukocytes and enhance their recruitment to the vascular wall by expressing adhesive molecules such as PECAM-1 and by releasing inflammatory mediators and cytokines (22). It might be expected that extracellular nucleotides, including ADP, and inflammatory cytokines released by activated platelets could contribute to the development of this process. Further elucidation of the direct effects of these molecules on vascular wall cells, including those mediated by P2 receptors, is necessary because these can become targets for pharmacological intervention.

In the vascular cells, there have been several reports on the expression and functions of ADP-sensitive P2 receptors such as P2Y12 and P2Y1. In cultured rat VSMCs, it has been shown that P2Y1 is expressed at levels that can evoke Ca2+ signals and that P2Y12 is also expressed at relatively abundant levels (16). It has been reported that ADP induces VSMC contraction via P2Y12 (30) and promotes proliferation (7). P2Y12 is reported to be upregulated when VSMCs undergo changes from the contractile to the synthetic phenotype (12). It has been shown that P2Y1 deleterion decreases macrophage infiltration and VCAM-1 expression in aortas of apolipoprotein E (ApoE)-deficient mice, ameliorating atherosclerotic lesions (19). A recent study has demonstrated that thrombin induces P2Y12 expression and contributes to interleukin-6 upregulation and mitogenesis in VSMCs (24). However, the effects of P2Y12 on the expression of other atherogenic cytokines or the intracellular mechanisms involved remain largely unknown. Our present study may presumably be the first to demonstrate the effects on expression of MCP-1, a major chemokine, and the signal transduction mechanisms involved.

In contrast to the above potentially atherogenic effects, it has been demonstrated that ADP-sensitive P2Y1 is involved in nitric oxide release from the endothelium and in vascular relaxation (8). Several nucleotides activate endothelial nitric oxide synthase in endothelial cells via P2 receptors, including P2Y1 (26). Recently, P2Y1 has been shown to promote endothelial cell migration by activating MAPK pathways, such as ERK 1/2, JNK, and P38 (27). Thus the total pathophysiological significance of vascular ADP-sensitive receptors in terms of atherogenesis has not been clarified yet, necessitating further elucidation.

In the signal transduction mechanism of VSMCs leading to MCP-1 upregulation, implication of ROS-sensitive pathways including MAPKs and transcription factors, such as NF-κB or AP-1, has been demonstrated (6, 9). In the present study, ADP-induced intracellular signal activation and MCP-1 upregulation were sensitive to inhibition by the ROS inhibitors NAC, DPI, and Tempol. As for activation of MAPKs via P2Y1 receptor, as mentioned, a recent report showed that ADP promotes migration of endothelial cells by activating ERK 1/2, JNK, and P38 (27). In our study, JNK was involved in P2Y12-mediated MCP-1 upregulation by ADP. ERK was activated by ADP, but this was apparently not mediated by P2Y12. Involvement of other ADP-sensitive P2 receptors such as P2Y1 in ERK activation is a possible explanation, but further investigation is required for confirmation.

There are previous reports showing that NF-κB and AP-1 are implicated in P2 receptor functions, including those via P2X7 or P2Y6, mostly in immune or related cell types (1). As for P2Y12, previous reports are scarce showing its involvement in these transcription factors. Our data indicate that ADP activates NF-κB and AP-1 in VSMCs. We have further demonstrated that P2Y12 specifically is at least implicated in the activation of NF-κB. For the clarification of the specific P2 receptors involved in ADP-induced proinflammatory effects in vascular cells, further studies are required.

Taken together, our data indicate that in VSMC P2Y12 mediates ADP-induced MCP-1 expression via the ROS-sensitive JNK pathway and transcription factor NF-κB. P2Y12 contributes to ADP-induced monocyte binding to VSMC. These findings provide additional insight into the roles played by P2Y12, which is critically involved in both platelet activation and vascular wall cell inflammation and thus represents an important therapeutic target in atherosclerotic diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.S. and Y.H. conception and design of research; H.S., D.N., M.T., A.K., M.M., and D.F. performed experiments; H.S. and Y.H. analyzed data; H.S. and Y.H. interpreted results of experiments; H.S. prepared figures; H.S. drafted manuscript; H.S., T.N., R.N., and Y.H. edited and revised manuscript; H.S., D.N., M.T., A.K., M.M., D.F., T.I., T.N., R.N., and Y.H. approved final version of manuscript.

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