Ovarian cancer G protein-coupled receptor 1-dependent and -independent vascular actions to acidic pH in human aortic smooth muscle cells

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Atherosclerosis is a chronic inflammation disease characterized by acidic micromilieu; vascular action; lysophosphatidic acid receptor

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Atherosclerosis is a chronic inflammation disease characterized by acidic micromilieu, which are partly attributed to increased lactate production by the anaerobic glycolysis of inflammatory cells (25) and the accumulation of numerous bioactive lipid mediators, such as lysophosphatidic acid (LPA) and prostaglandins (PGs) in addition to cytokines and plasmogen activator inhibitor (PAI)-1 expression, in the atherosclerotic lesion (10, 40, 44, 51). Vascular smooth muscle cells (SMCs) play a pivotal role in the progression of atherosclerosis (40). Its proliferation is regulated by MAPK phosphatase (MKP)-1 (2, 24). Acidification induces a variety of responses in the vascular system. Thus, acidification causes vasodilatation (16, 17, 19, 49, 56, 58) and inhibition of proliferation and migration of vascular SMCs (15, 39); however, the molecular mechanisms underlying these responses have not been fully elucidated (55). As for the lipid mediators (LPA and PGs), their roles in atherogenesis seem to be different. LPA is found in the lipid-rich core of atherosclerotic plaques (44) and is known as a proatherogenic factor. LPA is an activator of platelets and an effective phenotypic modulator of vascular SMCs, thereby promoting the development of intimal hyperplasia (45). LPA acts mainly through five G protein-coupled receptors (GPCRs), LPA1–LPA5 receptors, to stimulate a wide variety of intracellular signaling pathways (7). On the other hand, PGI2, the production of which is mediated through cyclooxygenase (COX), plays an antiatherogenic role in the vascular system (6, 13). PAI-1 levels are altered in vascular disorders, including atherosclerosis, and modulate SMC responses, leading to vascular remodeling (10). MKP-1 expression is induced by various stimuli and regulates vascular function (2, 4, 21, 24, 26, 27, 54).

We and others (18, 29, 31, 33, 38, 50, 57) have recently found that ovarian cancer GPCR-1 (OGR1) family receptors, including OGR1, GPR4, G2A, and TDAG8, which have previously been proposed as receptors for lysolipids, sense extracellular protons, resulting in the stimulation of intracellular signaling pathways. In human aortic SMCs (AoSMCs), we (52) found that extracellular acidification caused PGI2 production and cAMP accumulation via the OGR1/phospholipase C/COX pathway. These vascular actions occurred within 30 min of stimulation. However, changes in cellular activities under chronic acidic micromilieu, other than short-term PGI2 production and cAMP accumulation, remain unknown.

As described above, LPA is thought to be atherogenic. However, a recent animal study (36) using LPA receptor knockout mice showed that LPA receptors are not always atherogenic and, in some cases, appear to work as antiatherogenic receptors. These results suggest that LPA actions may be modulated by cellular circumstances. In the present study, we examined the long-term effects of extracellular acidification on the functions of AoSMCs, especially focusing on the role of OGR1. We found that extracellular acidification induced COX-2 protein and mRNA expression, PGI2 production, MKP-1 mRNA expression, PAI-1 mRNA expression, and inhibition of proliferation in AoSMCs. Our results suggested that OGR1 mediates COX-2 induction, subsequent PGI2 production, and MKP-1 expression, but not PAI-1 expres-
sion and inhibition of proliferation, in these cells. We also found that LPA synergistically induced COX-2 expression, PGI2 production, and MKP-1 expression under acidic pH through cross-talk between LPA receptors and OGR1.

MATERIALS AND METHODS

Materials. Normal human AoSMCs were purchased from Kurabo (Osaka, Japan), NS-398 and 1-oleoyl-sn-glycero-3-phosphate (LPA) were from Cayman Chemical (Ann Arbor, MI), PDGF-BB was from...
Peprotech, [3H]thymidine (20 μCi/ml) was from American Radiolabeled Chemicals (St. Louis, MO), fatty acid-free BSA was from Calbiochem-Novabiochem (San Diego, CA), anti-β-actin antibody was from Cell Signaling Technology (Danvers, MA), and pertussis toxin was from List Biological Laboratories (Campbell, CA). 3-[4-(4-[1-(2-chlorophenyl)-ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl]benzylsulfonylpropanoic acid (Ki-16425) (34) was generously provided by Dr. H. Ohta (Kyowa-Kirin, Tokyo, Japan), and YM-254890 (47) was generously provided by Dr. M. Taniguchi (Astellas, Tsukuba, Japan). Bera-
prost sodium was kindly provided by Dr. M. Yajima (Kaken Pharma-
ceutical, Tokyo, Japan). Anti-COX-2-antibody was prepared against a synthetic COOH-terminal peptide (ASSSRSGLDDINPT) of COX-2 (53). Specificity of the anti-COX-2 antibody was confirmed by Western blot analysis using detergent lysates of normal human dermal fibroblasts. The anti-COX-2 antibody reacted with COX-2, which was detected as bands at ~72 kDa (30, 37, 53), but did not react with COX-1. The sources of all other reagents were the same as those previously described (9, 52).

Cell culture. AoSMCs were cultured (~1.3 × 10^6 cells/cm²) using HuMedia from Kurabo (Osaka, Japan). Cells from passages 7 to 10 were used for all experiments. Twenty-four hours before the experiments, the medium was changed to fresh DMEM without serum containing 0.1% (wt/vol) BSA (fraction V). For Western blot analysis and mRNA expression experiments, cells were plated on 6-cm dishes. For the PGI₂ assay, cells were plated on 12 multiplates. The pH of the DMEM was adjusted by titration with HCl or NaOH to mimic metabolic acidosis. We added 25 mM HEPES, 27 mM NaHCO₃, and 0.1% BSA to DMEM (D-5523) from Sigma-Aldrich (St. Louis, MO). This DMEM maintained the pH more stably (HEPES-buffered DMEM). Cells were incubated for the indicated times under the indicated pH values in HEPES-buffered DMEM at pH 7.4. All data in this report are referenced to pH 7.4.
PGL₂ measurements. PGL₂ levels were determined using an enzyme immunoassay kit according to the manufacturer’s instructions (Cayman Chemical). Cells were cultured in 12 multiplates. After 24 h of serum starvation before the experiments, cells were then incubated for the indicated times at the indicated pH values in HEPES-buffered DMEM in the presence or absence of 100 nM NS-398. The control vehicle of NS-398 was DMSO. The supernatant (0.3 ml) was immediately collected after the incubation and transferred to microtubes (1.5 ml) on ice. The amounts of PGL₂ released in the supernatant were estimated as the levels of its stable metabolite (6-keto-PGF₁α).

cAMP measurements. AoSMCs were washed once and preincubated for 20 min with or without 1 μM indomethacin at 37°C in HEPES-buffered medium. HEPES-buffered medium was composed of 20 mM HEPES (pH 7.6), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 5 mM glucose, and 0.1% (wt/vol) BSA (fraction V). Cells were then incubated for 30 min under the indicated pH values in the presence of 0.5 mM IBMX with or without 1 μM indomethacin in a final volume of 0.6 ml. Where indicated, appropriate test agents (10 μM LPA, 10 ng/ml PDGF, or 100 nM beraprost sodium) were supplemented with the incubation medium. All data in this report are referenced to pH at room temperature. The reaction was terminated by the addition of 100 μl of 1 N HCl. cAMP in the acid extract was measured using a cAMP radioimmunooassay kit (Yamasa, Choshi, Japan).

TaQMan PCR analysis. Quantitative RT-PCR was performed using real-time TaqMan technology as previously described (59). TaqMan probes were obtained from Applied Biosystems (Foster City, CA). The identification number of each specific probe is Hs01573469 for COX-2, Hs00203431 for G2A, Hs00268858 for OGR1, Hs00269247 for TDA8, Hs00720999 for GPR4, Hs00173500 for LPA₁, Hs00173704 for LPA₂, Hs00173857 for LPA₃, Hs00271072 for LPA₄, Hs01051307 for LPA₅, Hs06610257 for MKP-1, Hs01126606 for PAI-1, and Hs99999905 for GAPDH, respectively. The expression level of the target mRNA was normalized to the expression of GAPDH mRNA.

Transfection of small interfering RNA. Nonsilencing RNA or small interfering (si)RNA targeted against OGR1 or LPA₁ (60 pmol) was transfected into cells (~1 × 10⁶ cells) using RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Cells were cultured for 3 days after siRNA transfection, and total RNA was then purified using an RNaseasy kit according to the manufacturer’s instruc-
tions (Qiagen). OGR1 siRNA, LPA₁ siRNA, and nonsilencing RNA were obtained from Dharmaco (Lafayette, CO). The identification number is M-005591 for OGR1, L-003656 for LPA₁, and D-001206 for nonsilencing RNA.

Western blot analysis. Cells were cultured in 6-cm dishes for the indicated times at the indicated pH values in HEPES-buffered DMEM. After stimulation, incubation was terminated by two washes with ice-cold PBS and the addition of 0.5 ml lysis buffer composed of 50 mM HEPES (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 100 mM NaF, 0.2 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Cells were then harvested from the dishes with a rubber policeman. The recovered lysate was incubated for 30 min on ice and centrifuged at 14,000 g for 20 min. The supernatant was subjected to 10% SDS-PAGE, and proteins in the gel were transferred to a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) by electroblot. Membranes were blocked with 5% dry milk for 2 h and incubated with primary antibodies (1:500 for COX-2 and 1:2,000 for β-actin) for 2 h. Membranes were then incubated with a second antibody conjugated with alkaline phosphatase for 1 h and visualized using the nitroblue teterazolium/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt system (42).

Cell growth. To examine DNA synthesis, [3H]thymidine incorpo-
ratiom was measured. AoSMCs were seeded on 12-well plates at 1 × 10⁶ cells in 1 ml. Twenty-four hours before experiments, the medium was changed to DMEM containing 0.1% BSA at pH 7.4. Cells were then stimulated for 18 h in HEPES-buffered DMEM containing 0.1% BSA at the indicated pH values in the presence of 10 ng/ml PDGF. After 18 h, cells were supplemented with [3H]thymidine (1 μCi/ml) and cultured for a further 6 h. The radioactivity in the trichloroacetic acid-insoluble fraction was measured as previously described (48). To examine cell proliferation, a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphe-
nyleltrazolium bromide (MTT) assay was used. AoSMCs were seeded on 12-well plates at 1 × 10⁶ cells in 1 ml. Twenty-four hours before experiments, the medium was changed to DMEM containing 0.1% BSA at pH 7.4. Cells were then stimulated for the indicated times in

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Fig. 1. Effect of extracellular acidification on PGI₂ production and cyclooxygenase (COX)-2 expression in human aortic smooth muscle cells (AoSMCs). A: AoSMCs were incubated at pH 7.6 or 6.3 in HEPES-buffered DMEM containing 0.1% BSA. The production of 6-keto-PGF₁α, a stable metabolite of PGI₂, in the medium at the indicated times was measured. B: AoSMCs were preincubated with 100 nM NS-398 or DMSO (Me₂SO; as a vehicle) for 10 min and then incubated for 24 h at pH 7.6 or 6.3 in the presence or absence of the inhibitor to measure the 6-keto-PGF₁α production. Data are means ± SE from 3 experiments. C: COX-2 protein expression was measured by Western blot analysis. AoSMCs were incubated for the indicated times at pH 7.6 or 6.3 in DMEM as described above. Each lane was loaded with an equal amount of cell extract (16 μg), which was confirmed by β-actin expression. A representative result is shown. The other two experiments gave similar results. D: COX-2 mRNA expression was assessed by real-time TaqMan PCR. AoSMCs were incubated at the indicated times at pH 7.6 or 6.3 in DMEM. Results are expressed as relative ratios to GAPDH mRNA expression. Data are means ± SE from at least 3 experiments. E: AoSMCs were incubated for 8 h at the indicated pH values, and COX-2 protein expression was measured by Western blot analysis. A representative result is shown. The other two experiments gave similar results. *The effect of acidic pH was significant (P < 0.05).
HEPES-buffered DMEM containing 2% FBS at pH 7.6 or 6.3 in the presence of 10 μM LPA and 10 ng/ml PDGF. After the appropriate time, the medium was changed to DMEM containing 0.1% BSA at pH 7.4, and cells were then supplemented with 0.1 ml MTT (5 mg/ml, Dojindo, Kumamoto, Japan) in each well and cultured for a further 4 h. The reaction was stopped by the addition of isopropanol with 0.04 N HCl (1 ml/well). MTT values were measured by absorbance with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Data presentation. All experiments were performed in duplicate or triplicate. Results of multiple observations are presented as means ± SE from more than two different batches of cells unless otherwise stated. Statistical significance was assessed by ANOVA; values were considered significant at P < 0.05.

RESULTS

Human AoSMCs sense extracellular pH, resulting in the expression of COX-2 and production of PGI2. We first examined whether long-term acidification of the extracellular medium modulates PGI2 production in AoSMCs. We measured PGI2 because PGD2 and PGE2 exerted only a small effect on cAMP accumulation compared with PGI2 in AoSMCs (52). To observe the time dependency of PGI2 production, cells were stimulated at pH 7.6 or 6.3 for the indicated times (Fig. 1A). PGI2 production was clearly detected at 0.5 h, as we have previously reported (52). Production was continuously increased up to 24 h after acidification. In contrast, production was not as significant at pH 7.6 as it was at pH 6.3 during this period. PGI2 production at 24 h after acidification was attenuated by NS-398, a COX-2-specific inhibitor, indicating that PGI2 was produced through COX-2 (Fig. 1B). As expected, the acidification of the extracellular medium stimulated COX-2 protein expression (Fig. 1C). The expression was increased from 4 h to 8 h and then decreased, but it was still detected even 24 h after acidification. The expression was not increased at pH 7.6 during this period. Figure 1D shows that acidification also stimulated COX-2 mRNA expression. mRNA expression was detected from 1 h after acidification, peaked at 2 h, sustained relative high expression up to 8 h, and decreased at 24 h after acidification. mRNA expression was low and did not increase during this incubation period at pH 7.6. Thus, mRNA expression preceded protein expression, suggesting that acidification of the extracellular medium stimulates COX-2 expression at a transcriptional level in AoSMCs. To observe the pH dependency of COX-2 protein expression, we stimulated the cells at the indicated pH values in medium for 8 h. As shown in Fig. 1E, a significant
increase of COX-2 protein expression was detected at a pH lower than 6.8.

**OGR1 is involved in proton-induced COX-2 expression and PGI2 production.** To examine the possibility of whether OGR1 family receptors are involved in the induction of COX-2 and production of PGI2 in AoSMCs, we first measured the mRNA expression of OGR1, GPR4, TDA9, and G2A, which have been reported as proton-sensing GPCRs. The expression of OGR1 mRNA was clearly detected (Fig. 2A). To prove the involvement of OGR1 in the proton-induced actions, we performed siRNA experiments. As shown in Fig. 2, B–D, siRNA targeted against OGR1 markedly inhibited proton-induced PGI2 production (Fig. 2B), COX-2 protein expression (Fig. 2C), and mRNA expression (Fig. 2D). On the other hand, the IL-1α-induced actions (Fig. 2, B–D) were hardly attenuated by the same siRNA treatment. This result indicates that OGR1 siRNA specifically reduced the proton-induced actions. Thus, OGR1 may sense extracellular acidification and thereby stimulate PGI2 production through COX-2 expression in AoSMCs.

**G protein coupling to proton-induced COX-2 expression and PGI2 production.** We next examined which heterotrimeric G proteins are coupled to proton-induced COX-2 expression and PGI2 production. As expected, YM-254890, a specific Gq/11 protein inhibitor (47), markedly attenuated proton-induced PGI2 production (Fig. 3A), COX-2 protein expression (Fig. 3B), and mRNA expression (Fig. 3C). Pertussis toxin, which inhibits Gi protein activity, also slightly, but not significantly, reduced PGI2 production (Fig. 3A) and COX-2 expression (Fig. 3, B and C). Thus, Gq/11 protein seemed to be engaged in the proton-induced actions.

**LPA enhancement of proton-induced COX-2 expression and PGI2 production through LPA1 receptors.** LPA is found in the lipid-rich core of atherosclerotic plaques (44). We next examined the possibility that LPA may influence the proton-induced actions because the LPA action on atherosclerosis seems to depend on the surrounding environment (36). As shown in Fig. 4A, exogenously applied LPA enhanced PGI2 production at pH 6.3 but hardly affected production at pH 7.6. PGI2 production at pH 6.3 was attenuated by Ki-16425, a specific LPA1 and LPA3 receptor antagonist (34), suggesting that PGI2 production is stimulated through LPA receptors. Proton-induced COX-2 mRNA expression was also significantly enhanced by the addition of LPA at acidic pH but not at pH 7.6, suggesting that the enhancement is at a transcriptional level (Fig. 4B). The result indicates that LPA permissively activates proton-induced COX-2 expression. As shown in Fig. 4C, the LPA-induced enhancement of COX-2 protein expression was clearly detected from 10 nM LPA at pH 6.3. On the other hand, the enhancement was hardly detected at pH 7.6 even in the presence of 10 μM LPA. As shown in Fig. 4D, this LPA enhancement of COX-2 expression was clearly detected at a pH lower than 6.8. The enhancement of acidic pH-induced COX-2 expression and PGI2 production by LPA was accompanied by the enhancement of cAMP accumulation, which was sensitive to indomethacin (Fig. 4E). Consistent with the results of the involvement of OGR1 in the acidic pH-induced actions, the synergistic stimulation of PGI2 production by LPA was also attenuated by the downregulation of OGR1 expression by OGR1 siRNA (Fig. 4F).

To clarify the subtype of LPA receptors responsible for the enhancement of the proton-induced actions, we first examined the mRNA expression of LPA1–LPA5 receptors, which have been reported as the major LPA receptor subtypes. The expression of LPA1 was clearly detected in AoSMCs (Fig. 5A). To prove the involvement of LPA1 in the proton-induced actions, we performed siRNA experiments. siRNA targeted against LPA1 specifically attenuated LPA1 mRNA expression but not OGR1 mRNA expression (Fig. 5A), indicating that LPA1 siRNA specifically inhibits its expression. Under this condition, siRNA treatment markedly inhibited proton-induced PGI2 production in the presence of 10 μM LPA (Fig. 5B). LPA-enhanced COX-2 expression was also markedly inhibited by LPA1 siRNA (Fig. 5C).

![Figure 3](http://apjheart.physiology.org/)

**Fig. 3. Involvement of Gq/11 and Gq proteins in proton-induced COX-2 expression and PGI2 production.** A–C: AoSMCs were pretreated with or without 50 ng/ml pertussis toxin (PTX) for 16 h. Cells were then treated with 1 μM YM-254890 (YM) or Me2SO (as a vehicle) for 10 min and further incubated at the indicated pH values for 24 h to measure PGI2 production (A), 8 h to measure COX-2 expression (B), and 2 h to measure COX-2 mRNA expression (C). Data are means ± SE from at least 3 experiments (A and C) or shown as a representative result (B). The other two experiments gave similar results (B). *The effect of acidic pH was significant (P < 0.05).
Taken together with the results of the inhibition by Ki-16425 (Fig. 4A), these results indicate that at least LPA₁ is involved in the enhancement of proton-induced OGR1-mediated COX-2 expression and PGI₂ production in AoSMCs.

OGR1 is unlikely involved in acidic pH-induced inhibition of proliferation in AoSMCs. Acidic pH (15), PGI₂ (13), and cAMP (22) have been shown to exert inhibitory actions on the proliferation of vascular SMCs. cAMP accumulation by acidic
pH and LPA (Fig. 4E) was parallel with PGI2 production under similar conditions (Fig. 5B), suggesting that cAMP accumulation is mediated through the OGR1/COX-2/PGI2 pathway. Supporting this, cAMP accumulation was attenuated by indomethacin (Fig. 4E). As shown in the Supplemental Material (Supplemental Fig. 1A), beraprost, a stable synthetic analog of PGI2, inhibited PDGF-induced DNA synthesis in AoSMCs.1

We therefore speculated that OGR1-mediated PGI2/cAMP pro-

1Supplemental Material for this article is available online at the American Journal of Physiology-Heart and Circulatory Physiology website.
duction might be involved in the inhibitory DNA synthesis. As shown in Fig. 6A, PDGF-induced DNA synthesis was markedly attenuated by acidic pH; however, the acidic pH-induced action was not affected by the downregulation of OGR1 expression. This is not due to the lower incorporation of \([3H]\)thymidine into the cells under acidic pH because total incorporation was not affected by extracellular pH, i.e., 6.4 ± 0.23 × 10^5 disintegrations/min at pH 7.6 and 5.8 ± 0.21 × 10^5 disintegrations/min at pH 6.3 (number of the observations: 4). In agreement with the results of DNA synthesis, OGR1 siRNA and the Gq/11 protein inhibitor failed to ameliorate the PDGF-induced cell growth as measured by MTT formazan formation under the acidic pH condition even in the presence of LPA (Fig. 6B). Moreover, indomethacin hardly affected the inhibitory DNA synthesis by acidic pH regardless of the presence of LPA (Supplemental Fig. 1B). These results suggest that OGR1 plays a minor, if any, role in the acidic pH-induced inhibition of DNA synthesis and proliferation in AoSMCs.

**Effects of low pH and LPA on MKP-1 and PAI-1 expression in AoSMCs.** We finally examined whether acidic pH influences atherogenesis-related vascular responses other than COX-2 expression, PGI2 production, and proliferation. Similar to COX-2 induction, acidic pH stimulated mRNA expression of both MKP-1 (Fig. 7A) and PAI-1 (Fig. 7B); however, in this case, LPA alone also significantly stimulated their expression. Thus, both acidic pH and LPA caused similar stimulatory effects on their expression. However, whereas MKP-1 expression was synergistically stimulated by acidic pH and LPA (Fig. 7A), acidic pH attenuated rather than enhanced LPA-induced stimulatory PAI-1 expression to the level attained by acidic pH alone (Fig. 7B). Moreover, the OGR1 dependency of the acidic pH-induced actions was totally different between their expressions. Treatment with OGR1 siRNA attenuated acidic pH-induced MKP-1 expression regardless of the presence of LPA (Fig. 7A). On the other hand, both stimulatory and inhibitory PAI-1 expression in response to acidic pH were hardly affected by the downregulation of OGR1 expression (Fig. 7B), as was

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**Fig. 6.** OGR1 is unlikely to be involved in inhibition of proliferation of AoSMCs by acidic pH. A: AoSMCs were transfected with NS RNA or OGR1 siRNA. DNA synthesis was evaluated for PDGF (10 ng/ml) under the indicated pH values as described in MATERIALS AND METHODS. DNA synthesis is expressed as a percentage of the value at pH 7.6. The value at pH 7.6 was 6.1 ± 0.51 × 10^4 disintegrations/min for NS RNA and 4.1 ± 0.58 × 10^4 disintegrations/min for OGR1 siRNA. Data are means ± SE from at least 3 experiments. B: AoSMCs were transfected with NS RNA or OGR1 siRNA. Cells were then pretreated with 1 μM YM or MeSO (as a vehicle) for 10 min and further incubated at the indicated pH values for the indicated times. Cell proliferation was then evaluated for PDGF (10 ng/ml) in the presence of LPA (10 μM) by a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in MATERIALS AND METHODS. Data are expressed as differences in the absorbance at 570 nm from that at 630 nm. Data are means ± SE from at least 3 experiments.

**Fig. 7.** Effect of OGR1 siRNA on proton-induced MAPK phosphatase (MKP)-1 and plasminogen activator inhibitor (PAI)-1 expression. AoSMCs were transfected with NS RNA or OGR1 siRNA. Cells were then incubated at the indicated pH values for 3 h in the presence or absence of 10 μM LPA to measure MKP-1 mRNA expression (A) and PAI-1 mRNA expression (B). Expression of each mRNA was assessed by real-time TaqMan PCR. Results are expressed as relative ratios to GAPDH mRNA expression. Each RT-PCR assay was performed at least three times. Data are expressed as means ± SE. *The effect of OGR1 siRNA was significantly different from NS RNA (P < 0.05); #expression was significantly different from that of NS RNA at pH 7.6 (P < 0.05).
the case in acidic pH-induced inhibition of proliferation (Fig. 6). These results suggest that acidic pH-induced MKP-1 expression, but not PAI-1 expression, is regulated by OGR1.

We further characterized OGR1-dependent MKP-1 expression (Supplemental Fig. 2). The stimulatory acidic pH effect on MKP-1 expression was significantly inhibited by YM-254890, a Gq/11 protein inhibitor, but not by indomethacin regardless of the presence of LPA. Thus, whereas acidic pH-induced expression of both MKP-1 and COX-2 are mediated by the OGR1/Gq/11 protein pathway, MKP-1 expression seems to be regulated independently of COX-2. On the other hand, LPA-induced stimulatory MKP-1 expression was remarkably attenuated by the LPA receptor antagonist Ki-16425 irrespective of the difference in extracellular pH (Supplemental Fig. 2), as was case for LPA enhancement of PGI2 production (Fig. 4A). Interestingly, the LPA effect at pH 7.6 was also significantly inhibited by the Gq/11 protein inhibitor, suggesting that the LPA receptors involved in the MPK-1 expression are in part coupled to Gq/11 protein.

DISCUSSION

In the present study, we confirmed previous results (52) showing that extracellular acidification stimulated cAMP accumulation through OGR1-mediated PGI2 production in AoSMCs during a short-term incubation of 30 min. We also found that extracellular acidification stimulated COX-2 protein and mRNA expression and subsequent PGI2 and cAMP production through OGR1/Gq/11 protein in AoSMCs. Thus, extracellular acidification-induced PGI2 production was composed of two phases: i.e., a short-phase production without COX-2 expression (~2 h) and a late-phase production with COX-2 expression (4–8 h; Fig. 1C). OGR1 appears to be involved in both phases of PGI2 production. Moreover, we showed that extracellular acidification caused the inhibition of proliferation of SMCs and stimulation of mRNA expressions of MKP-1 and PAI-1, although all the responses were not OGR1 dependent. Thus, whereas acidic pH-induced MKP-1 expression is dependent on OGR1, PAI-1 expression and inhibition of proliferation seem to be independent of OGR1. In addition to the acidification effects, we showed new roles for LPA in the LPA1 receptor in cross-talk with OGR1. These results support the idea that extracellular acidification, through OGR1 family receptors and/or unidentified mechanisms, and LPA, through the LPA1 receptor, at vascular inflammatory lesions may play critical roles in the regulation of some long-term actions involved in the initiation and progression of inflammatory diseases such as atherosclerosis.

The synthesis and activity of PGs are critical for the initiation and development of inflammation. COX catalyzes the conversion of arachidonic acid into PGG2, leading to other PGs after the action of a variety of PG synthases. COX existed in two isoforms: the constitutively expressing COX-1 isoform, which is mainly responsible for homeostatic prostaglandins, and an inducible COX-2 isoform, which is localized in inflammatory cells and tissues and upregulated during the inflammatory response (12, 46, 60). The expression of both COX isoforms is detectable in atherosclerotic lesions (1, 43). However, the impact of COX inhibition on lesion progression is controversial. COX-2-selective inhibitors might have protective effects within the cardiovascular system or even reduce atherogenesis by virtue of their anti-inflammatory effects (5).

On the other hand, there is an increased risk of atherothrombosis in individuals taking COX-2-selective inhibitors (20). Studies in mice have also shown conflicting data indicating that COX-2 inhibition accelerates (41), does not alter (35), or retards (3) atherogenesis. These differences among the studies may be partly due to the formed PGs, which vary with cell type and have divergent effects on disease progression and, perhaps, plaque stability. Among the PGs, PGI2 plays an important antiatherogenic role. Disruption of PGI2 signaling by genetic deletion of the receptor is associated with increased atherosclerosis and restenosis after injury in animal models (6). The cardioprotective effects of estrogen in premenopausal women are due in part to the induction of PGI2 (11). PGI2 potently inhibits platelet aggregation (14) and vascular SMC proliferation (13).

MKP-1 dephosphorylates and inactivates MAPKs such as ERK and p38 MAPK. MKP-1 inhibits vascular SMC growth (24), endothelial cell apoptosis (4), and contraction of brain vessels (54) and promotes endothelial cell migration (21). Thus, MKP-1 is thought to be an antiatherogenic enzyme. On the other hand, the role of PAI-1 in atherogenesis is controversial. PAI-1 inhibits plasminogen-mediated fibrinolysis and has been reported to promote the stimulation of vascular remodeling as a proatherogenic factor. On the other hand, PAI-1 is also shown to inhibit vascular proliferation through binding to vitronectin and blocking its interaction with urokinase-type plasminogen activator receptor as an antiatherogenic factor (10). Acidic pH inhibited LPA-induced PAI-1 expression (Fig. 7B). With respect to cell growth, the finding that acidic pH stimulated MKP-1 and PAI-1 expression is consistent with the observation of inhibition of DNA synthesis and proliferation under acidic pH (Fig. 6).

Proliferation of vascular SMCs is a hallmark of the progression of atherosclerosis. It is well known that acidification inhibits the proliferation of such cells (15, 55); however, the molecular mechanism of the inhibition is obscure. cAMP causes inhibition of the proliferation of vascular SMCs (22). PGI2 also induces inhibition of proliferation of SMCs (13) and suppression of the progression of atherosclerosis (6). We, therefore, assumed that PGI2 production and the associated cAMP accumulation through the proton/OGR1 pathway could be involved in acidic pH-induced inhibition of proliferation of AoSMCs. However, our present results suggested that the OGR1/COX-2/PGI2 pathway does not play a critical role in acidic pH-induced inhibition of proliferation of AoSMCs. However, our present results suggested that the OGR1/COX-2/PGI2 pathway does not play a critical role in acidic pH-induced inhibition of DNA synthesis and proliferation, as evidenced by the findings of lack of effects of knockdown of OGR1 expression by OGR1 siRNA (Fig. 6, A and B), inhibition of Gq/11 protein by YM-254890 (Fig. 6B), and inhibition of COX-2 by indomethacin (Supplemental Fig. 1B). Intracellular acidification caused by extracellular acidification might induce the stronger inhibitory effect than the cAMP-induced effect on the proliferation of AoSMCs (55), although the precise mechanism of the inhibition remains unknown. As a result, the OGR1-dependent inhibitory action might be masked by the OGR1-independent strong inhibitory action. Thus, acidification effects are complex in vascular systems, as the process of atherosclerosis is complex. However, the present study strongly suggests that OGR1-dependent stimulation of the COX-2/PGI2/cAMP pathway and MKP-1 expression and OGR1-independent regulation of PAI-1 expression and SMC proliferation may play critical roles at acidic micromilieu under acidic pH. 

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OG1-DEPENDENT AND -INDEPENDENT RESPONSES

The results suggest that the enhancement of PGI2 production be differentiated into OGR1-dependent and -independent models is required. Molecular mechanism and role of LPA in atherosclerosis in vivo might be mediated by the OGR1-independent pathway. We also suggest that the LPA stimulates PGI2 production and cAMP accumulation. The synergistic enhancement of MKP-1 but not PAI-I was observed. LPA concentrations in human plasma are ~0.08–5 μM (45), which are high enough to induce the enhancement of COX-2 expression (Fig. 4C). Further investigation of the molecular mechanism and role of LPA in atherosclerosis in vivo models is required.

In conclusion, acidic pH-induced vascular actions of AOprostacyclin and cAMP accumulation. The synergistic enhancement induced by acidic pH and LPA was suppressed by either OGR1 siRNA (Fig. 4F) or LPA1 siRNA (Fig. 5B). These results suggest that the enhancement of PGI2 production was due to cross-talk between OGR1 and the LPA1 receptor rather than changes in the biological activities of LPA and/or its receptors under the acidic micromilieu, although the molecular mechanism underlying the enhancement is currently unknown. LPA concentrations in human plasma are ~0.08–5 μM (45), which are high enough to induce the enhancement of COX-2 expression (Fig. 4C). Further investigation of the molecular mechanism and role of LPA in atherosclerosis in vivo models is required.

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DISCLOSURES

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

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