A novel adipocytokine, omentin, inhibits platelet-derived growth factor-BB-induced vascular smooth muscle cell migration through antioxidative mechanism

Kyosuke Kazama, Muneyoshi Okada, and Hideyuki Yamawaki
Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Towada, Aomori, Japan

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Kazama K, Okada M, Yamawaki H. A novel adipocytokine, omentin, inhibits platelet-derived growth factor-BB-induced vascular smooth muscle cell migration through antioxidative mechanism. Am J Physiol Heart Circ Physiol 306: H1714–H1719, 2014. First published April 11, 2014; doi:10.1152/ajpheart.00048.2014.—Omentin is a novel adipocytokine expressed in visceral adipose tissue. Secretion and blood concentration of omentin decrease in the obese subjects. We previously demonstrated that omentin is anti-inflammatory in vascular smooth muscle cells (SMCs). While vascular remodeling via migration of SMCs is also important for hypertension development, it remains to be clarified whether omentin affects this process. Here we examined whether omentin controls SMC migration. Omentin (300 ng/ml, 2 h) significantly inhibited platelet-derived growth factor (PDGF)-BB (10 ng/ml, 6 h)-induced migration of rat mesenteric arterial SMCs, as determined by Boyden chamber assay. Omentin (300 ng/ml, 2 h) significantly inhibited PDGF-BB (10 ng/ml, 30 min)-induced phosphorylation of p38 and heat shock protein (HSP) 27. Omentin (300 ng/ml, 2 h) significantly inhibited PDGF-BB (10 ng/ml, 30 min)-induced NADPH oxidase (NOX) activation as determined by lucigenin assay. Omentin (300 ng/ml, 24 h) significantly inhibited fetal bovine serum (5%, 4 days)-induced SMC outgrowth from rat isolated mesenteric artery. In vivo, omentin significantly inhibited carotid intimal hyperplasia in mouse ligation model. In summary, we for the first time demonstrate that omentin prevents PDGF-BB-induced SMC migration by preventing NOX/ROS/HSP27 pathways, which might be at least partly responsible for the preventive effects on neointimal hyperplasia. Our data suggest that omentin may be protective against hypertension development by inhibiting vascular structural remodeling.

adipokine; vascular smooth muscle; migration; oxidative stress; cell signaling

Obesity with an accumulation of visceral fat is one of the major risk factors for various health problems including hypertension. Adipose tissue is recently recognized as an endocrine organ that can secrete various cytokines termed adipocytokine. In the adipocytes enlarged by obesity, imbalanced production and secretion of adipocytokine may occur. It is now known that adipocytokine can regulate obesity-related hypertension by directly acting on blood vessel system (21).

Omentin is a relatively recently identified adipocytokine originally discovered in omental fat (11). Secretion and plasma concentration of omentin decrease in the obese subjects (1). A decrease in circulating omentin level is related to the occurrence of type 2 diabetes and metabolic syndrome (19). It was also demonstrated that a decrease in circulating omentin level independently correlated with an increased arterial stiffness and carotid plaque in type 2 diabetes (23). These epidemiologic data collectively suggest that omentin is related to the pathogenesis of obesity-induced cardiovascular diseases including hypertension. However, the underlying mechanisms for this remain to be fully determined. We and others previously demonstrated that omentin is anti-inflammatory in cultured vascular endothelial and smooth muscle cells (SMCs) (6, 22, 25), whereas the specific receptor for omentin has not yet been identified. It was also shown that omentin induced vasodilation in rat isolated blood vessels via stimulating endothelial nitric oxide (NO) production (23). Vascular structural remodeling via SMC migration is one of the critical processes for chronic hypertension development. Reactive oxygen species (ROS) production by NADPH oxidase (NOX)-1 plays an important role for neointimal formation via SMC migration (9, 12, 20). Furthermore, p38/heat shock protein (HSP) 27 pathway plays a role in SMC migration (9). We then hypothesized that omentin can prevent vascular SMC migration by inhibiting NOX/ROS/p38/HSP27 pathways, which may lead to the preventive effects on vascular structural remodeling. To test the hypothesis, we performed in vitro, ex vivo, and in vivo experiments.

Materials and Methods

Approval of animal study. Animal care and treatment were conducted in conformity with institutional guidelines of The Kitasato University and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Animal research was approved by the ethical committee of the School of Veterinary Medicine, The Kitasato University.

Materials. Reagent sources were as follows: human omentin (BioVender, Candler, NC), murine platelet-derived growth factor (PDGF)-BB (PeproTech, Rocky Hill, NJ), DMEM and NADPH (Wako, Osaka, Japan), lucigenin (Alexis, San Diego, CA), and gp91ds-tat (Anaspec, Fremont, CA). Antibody sources were as follows: phospho-p38 (Thr180/Tyr182); phospho-Akt (Ser473); phospho-ERK (Thr202/Tyr204); phospho-PDGFB receptor (Tyr751); total PDGF-B receptor (Cell Signaling, Beverly, MA); total p38, total Akt, total ERK (Santa Cruz Biotech, Santa Cruz, CA); HSP27 (Enzo Life Science, Plymouth Meeting, PA); and α-smooth muscle actin (DAKO, Glostrup, Denmark).

Isolation and culture of vascular SMCs. Isolation and culture of vascular SMCs were performed as previously described (6). Male Wistar rats (7–10 wk old, 0.2–0.4 kg) were anesthetized with urethane (1.5 g/kg ip) and euthanized by exsanguination. The main branch of superior mesenteric artery was harvested, and endothelium was removed. SMCs were obtained by an explant method and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). We confirmed the positive staining of the cells to α-smooth muscle actin antibody by immunofluorescence. Passage 10 to 25 SMCs at 80 to 90% confluence were growth arrested in serum-free DMEM for 24 h before stimulation. We confirmed that
there are minimal variations in signal responses between the passages of SMCs (6).

**Boyden chamber assay.** Boyden chamber assay was performed in Transwell chambers (Costar, Cambridge, MA) as previously described (18). The polycarbonate membranes with 8-μm pores were coated with 2% gelatin. Serum-free DMEM (600 μl) was added in the lower chamber. The upper chamber was added with 5 × 10⁵ cells in 100 μl DMEM/well. PDGF-BB (10 ng/ml, 6 h) was added to the lower chamber in the absence or presence of omentin (50–300 ng/ml, pretreatment for 2 h). Control was without any treatment. The membranes were fixed with methanol and stained with Giemsa (Nacalai Tesque, Kyoto, Japan). The number of migrated cells through the membranes was randomly counted in ×100 fields under a light microscope (CKX31, Olympus, Tokyo, Japan) and averaged.

**Western blot analysis.** After SMCs were treated without (control) or with PDGF-BB (10 ng/ml, 30 min) in the absence or presence of omentin (300 ng/ml, pretreatment for 2 h), total cell lysates were harvested by homogenizing SMCs with Triton X-100-based lysis buffer. Protein concentration was determined using a bicinchoninic acid method (Pierce, Rockfold, IL). Equal amounts of proteins (10–20 μg) were separated by SDS-PAGE (7.5–10%) and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI). After being blocked with 3% bovine serum albumin (phosphorylation antibody) or 0.5% skim milk (nonphosphorylation antibody), membranes were incubated with primary antibodies (1:500 dilution) at 4°C overnight, and the membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological Industries, Kibbutz Beit-Haemek, Israel). Equal loading of protein was confirmed by measuring total protein or α-smooth muscle actin expression. The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

**Lucigenin assay.** After SMCs were treated without (control) or with PDGF-BB (10 ng/ml, 30 min) in the absence or presence of omentin (300 ng/ml, pretreatment for 2 h), total cell lysates were harvested. NOX activity was determined by lucigenin assay as previously described (6). The reaction was carried out in total volume of 200-μl assay buffer containing 5 μM lucigenin, 1 mM NADPH, and 30 μg of cell lysates and was measured by a TriStar LB941 lumino meter (Berthold, Bad, Wildbad, Germany). Chemiluminescence was continuously measured for 30 min. Chemiluminescence of relative light units per second was obtained every 10 s, and the area under the curve was compared.

**Rat-isolated mesenteric arterial ring assay.** The main branch of superior mesenteric artery was harvested from male Wistar rats (7–10 wk old, 0.2–0.4 kg), and endothelium was removed as described above. We used collagen gel (Nitta-zelatin, Osaka, Japan) for stabilizing the mesenteric artery. After mesenteric arterial rings were stabilized for 30 min in serum-free DMEM, they were treated without (control) or with FBS (5%, 4 days) in the absence or presence of omentin (300 ng/ml, pretreatment for 24 h), and SMC outgrowth was observed in a light microscope (CKX-31).

**Mouse carotid ligation model.** To induce neointimal hyperplasia, we used carotid ligation model (18). Male BALB/c mice (10 wk old, ~30 g) were anesthetized with pentobarbital sodium (1.5 g/kg ip). Left carotid artery was ligated (Ligation), and right carotid was used as sham-operated control (Sham). After ligation, the mice were treated

Fig. 1. Effects of omentin on smooth muscle cell (SMC) migration. A: representative photomicrographs of SMCs treated without (control, Cont) or with platelet-derived growth factor (PDGF)-BB (10 ng/ml, 6 h) in the absence or presence of omentin (50–300 ng/ml, pretreatment for 2 h) were shown (n = 4). B: migration was determined by Boyden chamber assay and shown as fold increase relative to PDGF. **P < 0.01 vs. Cont; ##P < 0.01 vs. PDGF. Scale bar = 100 μm; n represents the number of independent sets of experiments.
with omentin (5 μg/mouse; + omentin) or saline (50 μl/mouse) for 4 wk. We used a biodegradable gelatin hydrogel (Medgel, 5 mg/mouse; MedGEL, Tokyo, Japan) (7) for chronic local administration of omentin by implanting it on the carotid bifurcation position. Because medgel was slowly resolved by collagenase in vivo, we can administrate omentin gradually for a long period of time. A previous report confirmed that the drug was sustained releasing from medgel for 14 days after implantation in vivo (7). In the in vitro preliminary experiment, we found that omentin in the medgel was released into water at 0.15 μg/24 h. Based on the result, we applied 5 μg omentin/mouse in medgel.

Histology. The carotid arteries were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. The 4 μm-thick sections were stained with hematoxylin and eosin. The images were obtained using a light microscope (BX-51). Intima-to-media ratio was calculated by using ImageJ software (National Institutes of Health, Bethesda, MD).

**RESULTS**

Effects of omentin on PDGF-BB-induced SMC migration. We first examined whether omentin pretreatment (50–300 ng/ml, 2 h) affects PDGF-BB-induced SMC migration by using Boyden chamber assay. Omentin inhibited PDGF-BB (10 ng/ml, 6 h)-induced SMC migration in a dose-dependent manner (Fig. 1A). Omentin at 300 ng/ml produced the maximal inhibitory effects (0.08 ± 0.02-fold relative to PDGF; n = 4, P < 0.01, Fig. 1B).

Statistical analysis. Date are shown as means ± SE. Statistical evaluations were performed by one-way analysis of variance followed by Bonferroni’s test (Figs. 1–3) or Student’s t-test (Fig. 4). Values of P < 0.05 were considered statistically significant.

Fig. 2. Effects of omentin on PDGF-BB-induced phosphorylation (p) of p38 and heat shock protein (HSP) 27 in SMCs. After SMCs were treated without (control) or with PDGF-BB (10 ng/ml, 30 min) in the absence or presence of omentin (300 ng/ml, 2 h; A–E) or gp91ds-tat (1 μM, 2 h; G), total cell lysates were harvested. Phosphorylation of p38 (n = 19–23 or 10; A or G), HSP27 (n = 14–17; B), Akt (n = 4; C), ERK (n = 4; D), and PDGF-β receptor (n = 6; E) was determined by Western blot analysis and shown as fold increase relative to PDGF. Equal protein loading was confirmed using total or α-actin antibody. F: effects of omentin on PDGF-BB-induced NADPH oxidase (NOX) activation. After SMCs were treated without (control) or with PDGF-BB (10 ng/ml, 30 min) in the absence or presence of omentin (300 ng/ml, pretreatment for 2 h), total cell lysates were harvested. NOX activity was determined by a lucigenin assay (n = 6). NOX activity was shown as fold increase relative to PDGF. **P < 0.01 vs. Cont; #P < 0.05 and ###P < 0.01 vs. PDGF; n represents the number of independent sets of experiments.

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Effects of omentin on PDGF-BB-induced signals related to SMC migration. Since p38 and HSP27 are known to mediate SMC migration (4), we next examined the effects of omentin on PDGF-BB-induced phosphorylation of p38 and HSP27. Omentin (300 ng/ml, 2 h) significantly inhibited PDGF-BB (10 ng/ml, 30 min)-induced phosphorylation of p38 (omentin: 0.80 ± 0.05-fold relative to PDGF, n = 19–23, P < 0.01, Fig. 2A) and HSP27 (omentin: 0.79 ± 0.05-fold relative to PDGF, n = 14–17, P < 0.01, Fig. 2B). We confirmed that omentin (300 ng/ml, 2 h) had no effects on PDGF-BB (10 ng/ml, 30 min)-induced phosphorylation of Akt, ERK, and PDGF-β receptor (Fig. 2C–E, n = 4–6), suggesting the specificity of omentin effect to the p38/HSP27 signal.

Effects of omentin on PDGF-BB-induced superoxide generation. Since O$_2^-$ generation through the activation of NOX is a major contributor for SMC migration (9, 12, 20), we further examined the effects of omentin on PDGF-BB-induced NOX activation by lucigenin assay. Omentin (300 ng/ml, 2 h) significantly inhibited PDGF-BB (10 ng/ml, 30 min)-induced NOX activation in SMCs (omentin: 0.89 ± 0.04-fold relative to PDGF, n = 6, P < 0.05, Fig. 2F). We also confirmed that omentin (300 ng/ml, 2 h) inhibited PDGF-BB (10 ng/ml, 30 min)-induced O$_2^-$ generation in SMCs as determined by O$_2^-$-sensitive dye dihydroethidium staining (n = 6). We further confirmed that gp91ds-tat, a specific inhibitor of NOX, can inhibit the PDGF-BB-induced activation of p38 (Fig. 2G), suggesting the causality that NOX exists upstream of p38.

Effects of omentin on FBS-induced SMC outgrowth from rat-isolated mesenteric artery. As an ex vivo study, we next examined the effects of omentin on FBS-induced SMC outgrowth from isolated mesenteric artery. Omentin (300 ng/ml, 24 h) significantly inhibited FBS (5%, 4 days)-induced SMC outgrowth (omentin: 0.23 ± 0.15-fold relative to FBS, n = 5, P < 0.01, Fig. 3A and B).

Effects of chronic omentin treatment on carotid intimal hyperplasia in mouse ligation model. Finally, we examined the effects of chronic omentin treatment on carotid intimal hyperplasia in mouse ligation model. Omentin significantly inhibited the increased carotid intima-to-media ratio (ligation, 1.84 ± 0.53 vs. ligation + omentin, 0.19 ± 0.13, n = 5 to 6, P < 0.01, Fig. 4, A and B).

DISCUSSION

In this study, we investigated the effects of omentin on vascular structural remodeling with a focus on SMC migration and for the first time demonstrate that omentin prevents PDGF-BB-induced SMC migration via inhibiting NOX/O$_2^-$/p38/HSP27 pathways, which is at least partly responsible for the omentin inhibition of carotid neointimal hyperplasia. Since
SMC inflammation is also important for vascular neointimal formation (13) and we previously found that omentin is anti-inflammatory in SMCs (6), it might be possible that omentin prevents neointimal formation by the anti-inflammatory mechanism.

Blood omentin concentration decreased in the obese subjects (310 ng/ml) compared with that in the lean subjects (370 ng/ml) (1). It was also reported that blood omentin concentration decreased in the obese diabetic woman (119 ng/ml) compared with that in the nondiabetic obese woman (207 ng/ml) (17). In addition, it was reported that blood omentin concentration decreased in the men with metabolic syndrome (130 ng/ml) compared with the men without metabolic syndrome (186 ng/ml) (19). From these reports, it is considerable that the concentration of omentin used in this study is within the (patho)physiological range.

In this study, we showed that omentin inhibited PDGF-BB-induced NOX activation. Furthermore, we have previously shown that omentin inhibited tumor necrosis factor-α-induced SMC inflammatory responses via inhibiting NOX activation (6). These data indicate that omentin may affect the common pathways that are downstream of the receptors. The data that omentin had no effects on the phosphorylation of the PDGF-β receptor (Fig. 2E) may support the hypothesis. There is a report demonstrating that PDGF stimulated NOX activity via activation of p47^phox^, a component of NOX through the activation of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) pathways (14). Thus it might be possible that omentin inhibits PDGF-stimulated NOX activation through the inhibition of PKC/PI3K/ p47^phox^ pathways. There are several NOX isoforms expressed in vascular SMCs including NOX-1, NOX-2, and NOX-4 (3, 8, 15). There are reports showing that NOX-1 plays an important role for neointimal formation via SMC migration (9, 12, 20). We also showed that gp91ds-tat, a NOX inhibitor, can inhibit PDGF-BB-induced activation of p38. It is then suggested that omentin prevents vascular SMC migration by inhibiting NOX-1-derived O$_2^-$ generation and subsequent activation of p38/HSP27, which may lead to the preventive effects on neointimal hyperplasia.

There is no report so far identifying the omentin receptor. There are several reports regarding the receptors for adiponectin, a representative of “good adipocytokine.” In addition to the classical G protein-coupled receptors (AdipoR1 in skeletal muscle and AdipoR2 in liver), a previous report demonstrated that T-cadherin serves as a signaling receptor for adiponectin and mediates an inhibitory effect for atherosclerosis progression (16). We suppose that some unknown intracellular signal transducers may mediate the effect of omentin.

Previous in vitro studies demonstrated that omentin is anti-inflammatory in both vascular SMCs (6) and endothelial cells (22, 25). In addition, our ex vivo study demonstrated that omentin stimulates NO production and induces vasodilation in rat aorta (23). Furthermore, our current report demonstrated that acute intravenous administration of omentin inhibits agonists-induced increases of blood pressure in rats (5). To further advance these findings, we for the first time demonstrate that omentin inhibits SMC migration and vascular structural remodeling in this study. Recently, not only our group but also others explored cardioprotective effects of omentin. For example, a decrease in circulating omentin level was associated with
left ventricular diastolic dysfunction in type 2 diabetes. In addition, omentin pretreatment prevented the induction of contractile dysfunction and insulin resistance in rat cardiomyocytes (2). It was also demonstrated that omentin promotes revascularization in ischemic muscle of mice through inducing endothelial cell differentiation and survival via activation of Akt/endothelial NO synthase pathway (10). Considering the endothelial cell differentiation and survival via activation of revascularization in ischemic muscle of mice through inducing omentin promotes contractile dysfunction and insulin resistance in rat cardiomyocytes (2). It was also demonstrated that omentin promotes revascularization in ischemic muscle of mice through inducing endothelial cell differentiation and survival via activation of Akt/endothelial NO synthase pathway (10).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

K.K., M.O., and H.Y. approved final version of manuscript; H.Y. edited and revised results of experiments; K.K. prepared figures; K.K. drafted manuscript; K.K., M.O., and H.Y. analyzed data; K.K., M.O., and H.Y. interpreted DISCLOSURES

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