

# Dipeptidyl Peptidase-4 Inhibitor Anagliptin Prevents Intracranial Aneurysm Growth by Suppressing Macrophage Infiltration and Activation

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**Background**—Chronic inflammation plays a key role in the pathogenesis of intracranial aneurysms (IAs). DPP-4 (dipeptidyl peptidase-4) inhibitors have anti-inflammatory effects, including suppressing macrophage infiltration, in various inflammatory models. We examined whether a DPP-4 inhibitor, anagliptin, could suppress the growth of IAs in a rodent aneurysm model.

**Methods and Results**—IAs were surgically induced in 7-week-old male Sprague Dawley rats, followed by oral administration of 300 mg/kg anagliptin. We measured the morphologic parameters of aneurysms over time and their local inflammatory responses. To investigate the molecular mechanisms, we used lipopolysaccharide-treated RAW264.7 macrophages. In the anagliptin-treated group, aneurysms were significantly smaller 2 to 4 weeks after IA induction. Anagliptin inhibited the accumulation of macrophages in IAs, reduced the expression of MCP-1 (monocyte chemoattractant protein 1), and suppressed the phosphorylation of p65. In lipopolysaccharide-stimulated RAW264.7 cells, anagliptin treatment significantly reduced the production of tumor necrosis factor  $\alpha$ , MCP-1, and IL-6 (interleukin 6) independent of GLP-1 (glucagon-like peptide 1), the key mediator in the antidiabetic effects of DPP-4 inhibitors. Notably, anagliptin activated ERK5 (extracellular signal-regulated kinase 5), which mediates the anti-inflammatory effects of statins, in RAW264.7 macrophages. Preadministration with an ERK5 inhibitor blocked the inhibitory effect of anagliptin on MCP-1 and IL-6 expression. Accordingly, the ERK5 inhibitor also counteracted the suppression of p65 phosphorylation in vitro.

**Conclusions**—A DPP-4 inhibitor, anagliptin, prevents the growth of IAs via its anti-inflammatory effects on macrophages. (*J Am Heart Assoc.* 2017;6:e004777. DOI: 10.1161/JAHA.116.004777.)

**Key Words:** dipeptidyl peptidase-4 inhibitor • extracellular signal-regulated kinase 5 • glucagon-like peptide-1 • intracranial aneurysm • macrophage

The worldwide prevalence of intracranial aneurysms (IAs) is estimated as 3.2%.<sup>1</sup> The mortality rate from subarachnoid hemorrhage, caused mainly by ruptured IAs, is estimated to be between 8% and 67%,<sup>2</sup> with 40% of survivors experiencing severe permanent damage<sup>2</sup> including loss of consciousness, hemiparesis, aphasia, and neuropsychological disorders. Caring for these disabled patients also places a

burden on health care. Consequently, preventing the growth and rupture of IAs is one of the most important challenges for neurosurgeons and health care. Although surgical clipping and coiling have become established therapies for IAs, effective noninvasive medical therapies that reduce the risk of aneurysm growth do not exist. Recent studies have revealed that chronic inflammatory responses underlie IA growth.<sup>3,4</sup> In

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## Clinical Perspective

### What Is New?

- Oral administration of a dipeptidyl peptidase-4 inhibitor, anagliptin, inhibits macrophage infiltration and activation, resulting in the suppression of intracranial aneurysm growth in rats.
- Anagliptin mediates its anti-inflammatory effects, at least in part, through a glucagon-like peptide 1 receptor-independent pathway. Anagliptin activates extracellular signal-regulated kinase 5, which is known to play key roles in the anti-inflammatory function of statins in vascular cells, and may inhibit the proinflammatory nuclear factor- $\kappa$ B pathway in macrophages.

### What Are the Clinical Implications?

- Although surgical clipping and coiling are established therapies for intracranial aneurysms, effective medical therapies that prevent aneurysm growth do not exist.
- The dipeptidyl peptidase-4 inhibitor anagliptin could be a candidate for the medical therapy for intracranial aneurysms.
- Activation of extracellular signal-regulated kinase 5 and subsequent inhibition of the nuclear factor- $\kappa$ B pathway may provide a promising direction for the development of new therapeutic targets for intracranial aneurysms.

fact, several anti-inflammatory agents inhibit aneurysms in rodent models,<sup>5,6</sup> yet no clinical trial has conclusively demonstrated the effectiveness of these reagents in humans.

DPP-4 (dipeptidyl peptidase-4) inhibitors are widely used antidiabetic drugs that affect the metabolism of incretins, such as GIP (gastric inhibitory polypeptide) and GLP-1 (glucagon-like peptide 1), to enhance glucose-induced insulin secretion. Notably, the expression of DPP-4 increases during inflammation, suggesting that, in addition to its function in blood glucose homeostasis, DPP-4 participates in the pathogenesis of inflammatory diseases. Accordingly, the DPP-4 inhibitor anagliptin prevents the progression of atherosclerosis<sup>7</sup> and abdominal aortic aneurysms<sup>8</sup> by mitigating macrophage activation and accumulation. During the growth of IAs, macrophage infiltration disrupts the extracellular matrix through the secretion of macrophage-derived matrix metalloproteinases including MMP-2 and MMP-9.<sup>9</sup> In this study, we focused on the anti-inflammatory effects of a DPP-4 inhibitor and examined whether anagliptin prevents the growth of aneurysms in an experimentally induced IA model.

## Methods

### Operating Procedure to Induce IAs in a Rat Model

All animal experiments were conducted following the guidelines for Japan's Act on Welfare and Management of Animals. The study

protocol was approved by the institutional animal care and use committees and the ethics committee of Kyoto University. Male Sprague Dawley rats aged 7 weeks were used in all experiments. IAs were induced using a previously described method.<sup>10</sup> Briefly, the left renal artery and the left common carotid artery were ligated under general anesthesia induced with an intraperitoneal injection of 50 mg/kg pentobarbital. Animals were fed with chow containing 8% sodium chloride and 0.12% 3-aminopropionitrile, with or without 300 mg/kg anagliptin. Rats were maintained with free access to chow, and their casual blood glucose was measured with Glutest Every (Sanwa Kagaku Kenkyusho, Aichi, Japan) at 2 weeks after IA induction. A BP-98A (Softron, Tokyo, Japan) blood pressure meter was used without anesthesia. We trained rats on a different day before measurement of blood pressure on the day of euthanasia. Rats were euthanized under general anesthesia 2 or 4 weeks after the operation. In total, 106 rats (control group, n=6; vehicle group, n=50; anagliptin group, n=50) were used in the present study.

### Morphologic Evaluation of IAs

Bifurcations of the right olfactory artery and the anterior cerebral artery were stripped and frozen in OCT compound. Samples were sliced 5- $\mu$ m thick with a Cryostat CM1860 (Leica, Wetzlar, Germany) and stained with Elastica van Gieson. Four parameters of the IAs were measured: aneurysm size, (maximum height plus maximum width of the lumen) divided by 2; size of the internal elastic lamina disruption, (end-to-end dimension of internal elastic lamina plus length of perpendicular line from tip of the aneurysm) divided by 2; wall thickness ratio, minimum width of aneurysmal wall divided by average thickness of normal arterial wall; and lumen area of aneurysms, calculated by the BZ-7000 (Keyence, Osaka, Japan).

### Immunohistochemistry

After blocking with 2% bovine serum albumin, samples were incubated with primary antibodies overnight, followed by incubation with fluorescence-labeled secondary antibodies (Alexa Fluor 488 and 594) for 2 hours. Slides were covered with Prolong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) and viewed under a fluorescence confocal microscope (FV1000; Olympus, Tokyo, Japan). The primary antibodies used in immunohistochemistry were anti-Iba1 (anti-ionized calcium binding adaptor molecule 1), anti-phosphorylated p65, and anti-MCP-1 (anti-monocyte chemoattractant protein 1).

### Quantitative Real-Time Polymerase Chain Reaction

Whole Willis arterial rings were stripped, and total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany),

followed by the conversion to single strand cDNA with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative real-time polymerase chain reaction was performed using the SYBR Green PCR Master Kit and Applied Biosystems' 7300 Real-Time PCR System. The primers used in this study were as follows: *β-actin*, forward 3'-cctgagcgcgaagtactctgtgt-5', reverse 5'-gctgatccacatctgctggaa-3'; *Vcam-1* (vascular cell adhesion molecule 1), forward 3'-gcgaaggaaactggagaagaca-5', reverse 5'-acacattaggaccgtgcagtt-3'; *Icam-1* (intercellular adhesion molecule 1), forward 3'-cgggagatgaatgtacctaca-5', reverse 5'-tgcacgtccctggtgatact-3'; *E-selectin*, forward 3'-ccattcggcctctcagcta-5', reverse: 5'-tgcagctcacagagccattc-3'; *MCP-1* (encoded by *Ccl2*), forward 3'-cctccaccactatgcaggtctc-5', reverse 5'-gcacgtggatgctacaggc-3'; and *Cd68*, forward 3'-actgggctcttgaaactacac-5', reverse 5'-cc ttggtttgttcgggttca-3'.

### Western Blotting

RAW264.7 cells, a murine macrophage-like cell line, were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, L-glutamine, and 1% penicillin, streptomycin, and amphotericin B. Cells were pretreated with or without 10 nmol/L BIX 02189 (an ERK5 [extracellular signal-regulated kinase 5] inhibitor) for 90 minutes, followed by treatment with or without 100 μmol/L anagliptin for 10 minutes. Finally, cells were stimulated with or without 10 ng/mL lipopolysaccharide. The cell lysate was used for Western blotting. The antibodies used were as follows: anti-β actin, anti-p65, anti-phospho-p65, anti-ERK1/2, anti-phospho-ERK1/2, anti-ERK5, and anti-phospho-ERK5.

### Quantitative Assay for Inflammatory Cytokines

Exendin fragment 9–39, an inhibitor of GLP-1 receptor, or BIX 02189 was added to RAW264.7 cells before a 10-minute anagliptin treatment. Cells were then stimulated with 10 ng/mL lipopolysaccharide for 24 hours, and the supernatant was analyzed using the BD Cytometric Bead Array Mouse Inflammation Kit (BD Bioscience, San Diego, CA) and FACSCalibur (BD Bioscience).

### Measurement of DPP-4 Activity

The DPP activity fluorometric assay kit (BioVision Inc., Milpitas, California, USA) was used according to the manufacturer's instruction. Briefly, a quenched fluorescent group, 7-amino-4-methylcoumarin (excitation/emission: 360/460 nm) is released when DPP-4 cleaves a substrate, and this fluorescence was measured with SpectraMax M2 (Molecular Devices, Sunnyvale, CA).

### Measurement of Lysyl Oxidase Activity

A lysyl oxidase activity assay kit (Fluorometric; Abcam, Cambridge, UK) was used, according to the manufacturer's instruction. Signals were read by a SpectraMax M2 at excitation/emission of 540/590 nm.

### Statistical Analysis

Results are presented as mean±SEM. Data were analyzed between 2 groups using the Student *t* test or the Wilcoxon rank sum test. The Bonferroni adjustment method was used for multiple comparisons after the Kruskal–Wallis test. *P*<0.05 was considered statistically significant.

## Results

### Administration of Anagliptin Prevented the Growth of Experimental IAs

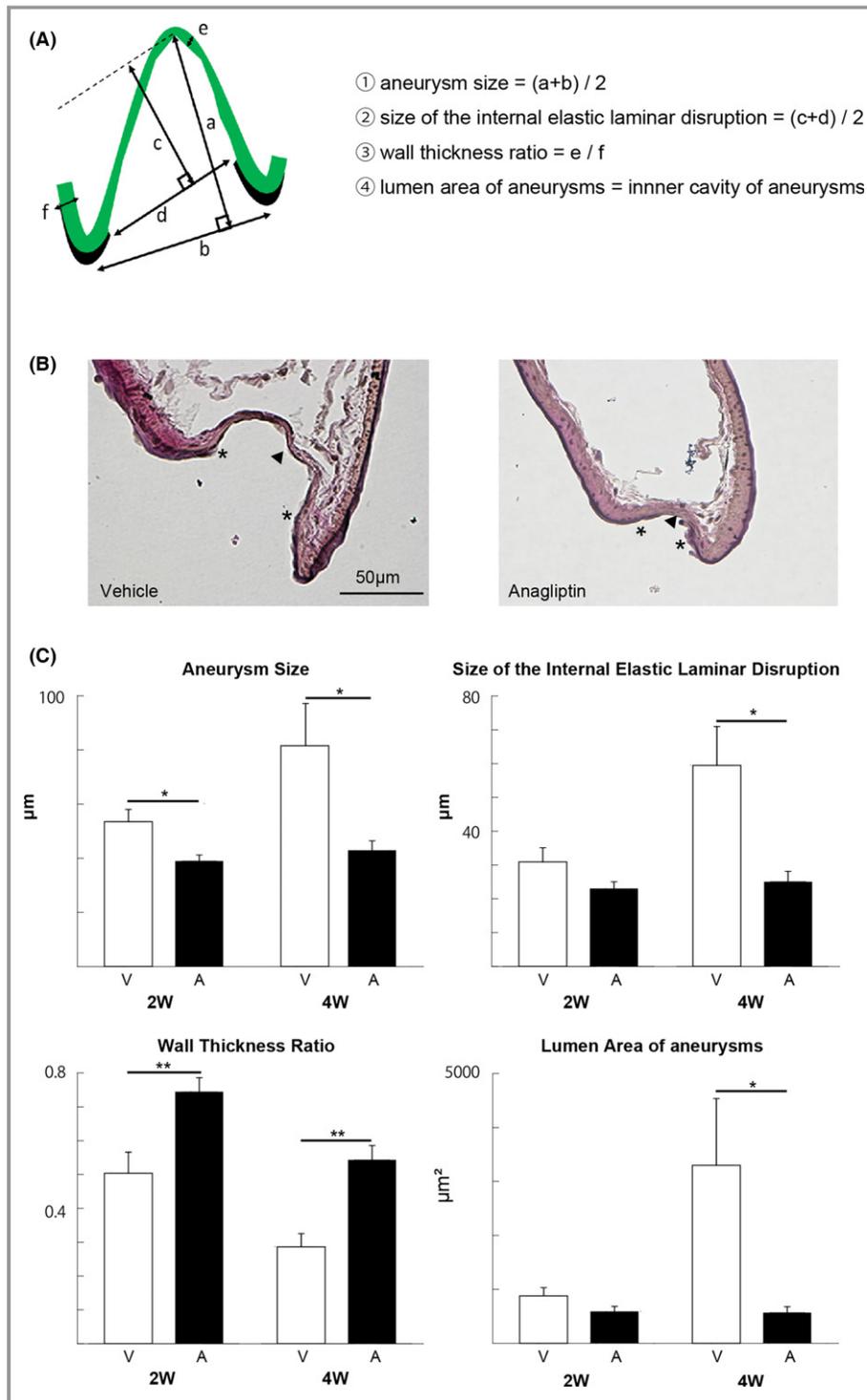
Oral administration of anagliptin did not affect body weight (data not shown) and did not influence systolic or diastolic blood pressure and heart rate (Table, Figure S1). Although DPP-4 activity in serum was inhibited in the anagliptin-treated group (*P*<0.01, Table), there was no significant difference in the casual blood glucose level between groups (Table).

The quantitative morphologic evaluation found that treatment with anagliptin suppressed the growth of IAs in rats (Figure 1). The anagliptin-treated group had a significant decrease in aneurysm size compared with the vehicle group at both 2 and 4 weeks after surgical IA induction (2 weeks: 53.6±4.5 versus 39.0±2.2 μm, *P*<0.05; 4 weeks: 81.6±15.6 versus 42.9±3.5 μm, *P*<0.05). In addition, administration of anagliptin protected the wall thickness ratio (2 weeks: 0.50±0.06 versus 0.74±0.04 μm, *P*<0.01; 4 weeks: 0.29±0.04 versus 0.54±0.04 μm, *P*<0.01). The size of the internal elastic lamina disruption and the lumen area of the aneurysms were also smaller at 4 weeks after IA induction in the anagliptin-treated group (size of the internal

**Table.** Background Parameters

	Vehicle (n=8)	Anagliptin (n=7)	<i>P</i> Value
Systolic blood pressure, mm Hg	144.2±7.0	140.2±8.7	0.72
Diastolic blood pressure, mm Hg	65.7±5.6	68.4±5.1	0.73
Heart rate, bpm	350.6±7.5	356.4±11.3	0.69
Blood glucose, mg/dL	99.6±3.1	93.0±9.9	0.51
DPP-4 activity, μU/mL	625.4±80.5	81.5±7.9	<0.01

Results are presented as mean±SEM. DPP-4 indicates dipeptidyl peptidase-4.



**Figure 1.** Administration of anagliptin prevents the growth of intracranial aneurysms (IAs). A, Scheme of the morphological assessment of IAs. The green heavy line marks the aneurysmal wall and the black line represents the internal elastic lamina. The 4 parameters were defined as shown. B, Examples of IAs in the 2 groups. The aneurysm at 4 weeks (4W) after the operation in the vehicle group (left) and in the anagliptin group (right). Arrowheads indicate the luminal side of IAs. Asterisks show the disruption of the internal elastic lamina. Scale bar=50  $\mu\text{m}$ . C, Morphological assessments were performed with 4 parameters. Data were analyzed using the Student *t* test. Results are presented as mean  $\pm$  SEM (n=5-8 each). \**P*<0.05, \*\**P*<0.01. A indicates anagliptin group; 2W, 2 weeks; V, vehicle group.

elastic lamina disruption:  $59.5 \pm 11.5$  versus  $25.1 \pm 3.0 \mu\text{m}^2$ ,  $P < 0.05$ ; lumen area of aneurysms:  $3.30 \times 10^3 \pm 1.24 \times 10^3$  versus  $5.67 \times 10^2 \pm 1.11 \times 10^2 \mu\text{m}^2$ ,  $P < 0.05$ ).

In the present study, we fed rats on a 3-aminopropionitrile-containing diet, which regulates extracellular matrix organization, to facilitate IA formation in this rodent model, as reported previously.<sup>3,9,11,12</sup> Importantly, anagliptin treatment did not affect lysyl oxidase activity in the blood (Figure S2), meaning that the effect of anagliptin on IA growth may not directly associate with the reagent 3-aminopropionitrile, an inhibitor of the catalytic activity of lysyl oxidase.

### Treatment With Anagliptin Suppressed Macrophage Infiltration and Activation in IAs

Macrophage infiltration and activation play key roles in prolonged inflammation and are associated with the growth of IAs.<sup>9</sup> As shown in Figure 2A, Iba1-positive macrophages had less infiltration into the IAs of the anagliptin-treated group than those in the vehicle group (Figure 2A). Accordingly, *Cd68* gene expression in cerebral arteries was significantly decreased in rats treated with anagliptin (Figure 2C). Because MCP-1 is crucial for macrophage infiltration and IA development,<sup>9,11</sup> we examined MCP-1 expression and found that anagliptin treatment impaired the expression of MCP-1 protein in IAs (Figure 2B) and had a tendency to suppress its RNA expression in cerebral arteries (4 weeks,  $P = 0.095$ ; Figure 2C).

Next, we examined whether anagliptin treatment suppresses the activation of infiltrated macrophages. Given that the amount of phosphorylated p65 was decreased in the macrophages that accumulated in the IAs of anagliptin-treated rats (Figure 2B), anagliptin may inhibit macrophage activation, at least partially, by inactivating the NF- $\kappa$ B (nuclear factor- $\kappa$ B) pathway, leading to the suppression of aneurysm growth.

Increased expression of *Vcam-1*, *Icam-1*, and *E-selectin* are markers of vascular endothelial cell activation and dysfunction; however, anagliptin treatment did not influence their expression in cerebral arteries (Figure 2C, Figure S3); therefore, we focused on macrophages as the main targets of anagliptin in the prevention of IA growth.

### Anagliptin Treatment Attenuated the Inflammatory Activation of Murine Macrophage-Like Cells

The anti-inflammatory effect of anagliptin was verified using lipopolysaccharide-stimulated RAW264.7 murine macrophage-like cells. Anagliptin pretreatment attenuated the elevated production of proinflammatory cytokines and

chemokines caused by lipopolysaccharide, including TNF- $\alpha$  (tumor necrosis factor  $\alpha$ ), IL-6 (interleukin 6), and MCP-1 (Figure 3A). Pretreated cells had impaired phosphorylation of p65 (Figure 3B) and ERK1/2 (Figure 3C), indicating that anagliptin suppressed the inflammatory activation of macrophages by inhibiting the NF- $\kappa$ B and ERK1/2 pathways.

The antihyperglycemic action of DPP-4 inhibitors, including anagliptin, depends on GLP-1 and GLP-1 receptor.<sup>13</sup> Consequently, we investigated whether a GLP-1-dependent pathway is involved in the anti-inflammatory action of anagliptin. Exendin fragment 9–39 is an inverse agonist of the GLP-1 receptor and inhibits insulin secretion by GLP-1 in pancreatic  $\beta$  cells. As shown in Figure 3D, pretreatment with exendin fragment 9–39 did not block the inhibitory effect of anagliptin on TNF- $\alpha$  production in lipopolysaccharide-stimulated RAW264.7 cells. Together, these results indicate that the anti-inflammatory effects of anagliptin in macrophages is at least partly dependent on the NF- $\kappa$ B and MAPK pathways and independent of a GLP-1 receptor signaling pathway.

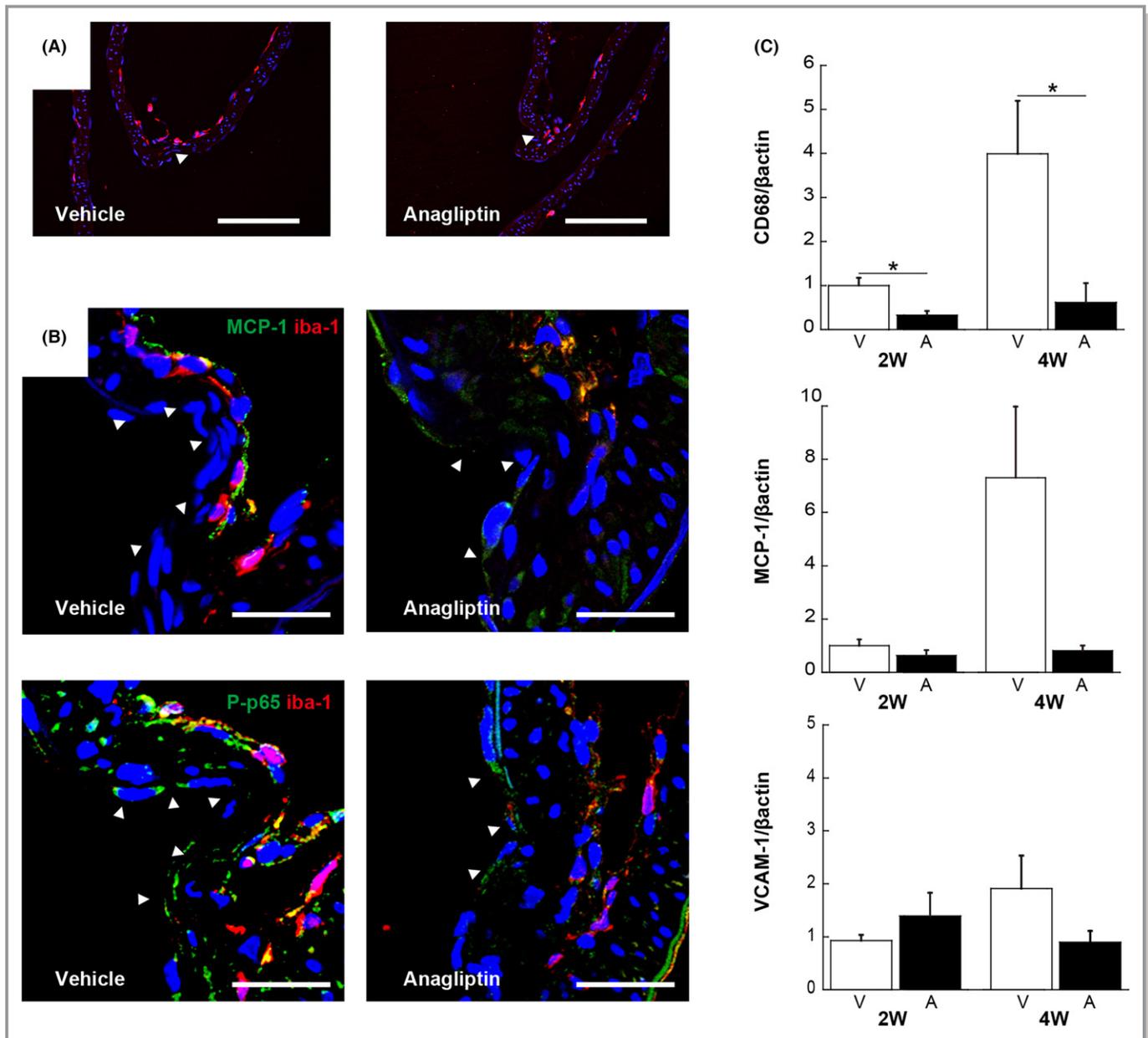
### ERK5 Activation Mediates the Anti-Inflammatory Effect of Anagliptin in Macrophages

Statins, commonly used lipid-lowering drugs, prevent the growth of experimental IAs by inhibiting local immune responses.<sup>5</sup> ERK5 activation plays key roles in the anti-inflammatory effect of statins in endothelial cells,<sup>14</sup> and in macrophages, statins increase ERK5 kinase activity, resulting in increased capacity to phagocytose apoptotic cells and inhibited atherosclerotic plaque formation.<sup>15</sup> Notably, anagliptin robustly activated ERK5 in RAW264.7 cells regardless of lipopolysaccharide treatment (Figure 4A). In addition, pretreatment with a selective ERK5 inhibitor, BIX02189, impaired the inhibitory effect of anagliptin on MCP-1 expression ( $P < 0.01$ ) in lipopolysaccharide-stimulated RAW264.7 cells (Figure 4B). Suppression of IL-6 expression by anagliptin also tended to be blocked ( $P = 0.06$ ) by BIX02189 (Figure 4B).

To verify the involvement of ERK5 in anagliptin-associated inhibition of intracellular proinflammatory signaling, immunoblotting was performed using lipopolysaccharide-stimulated RAW264.7 cells. As shown in Figure 4C, the attenuation of p65 phosphorylation by anagliptin was blocked by pretreatment with an ERK5 inhibitor, indicating that anagliptin suppresses the NF- $\kappa$ B signaling pathway via ERK5 activation in macrophages.

### Discussion

In this study, we demonstrated that oral administration of a DPP-4 inhibitor, anagliptin, inhibits macrophage infiltration and activation, resulting in the suppression of IA growth in

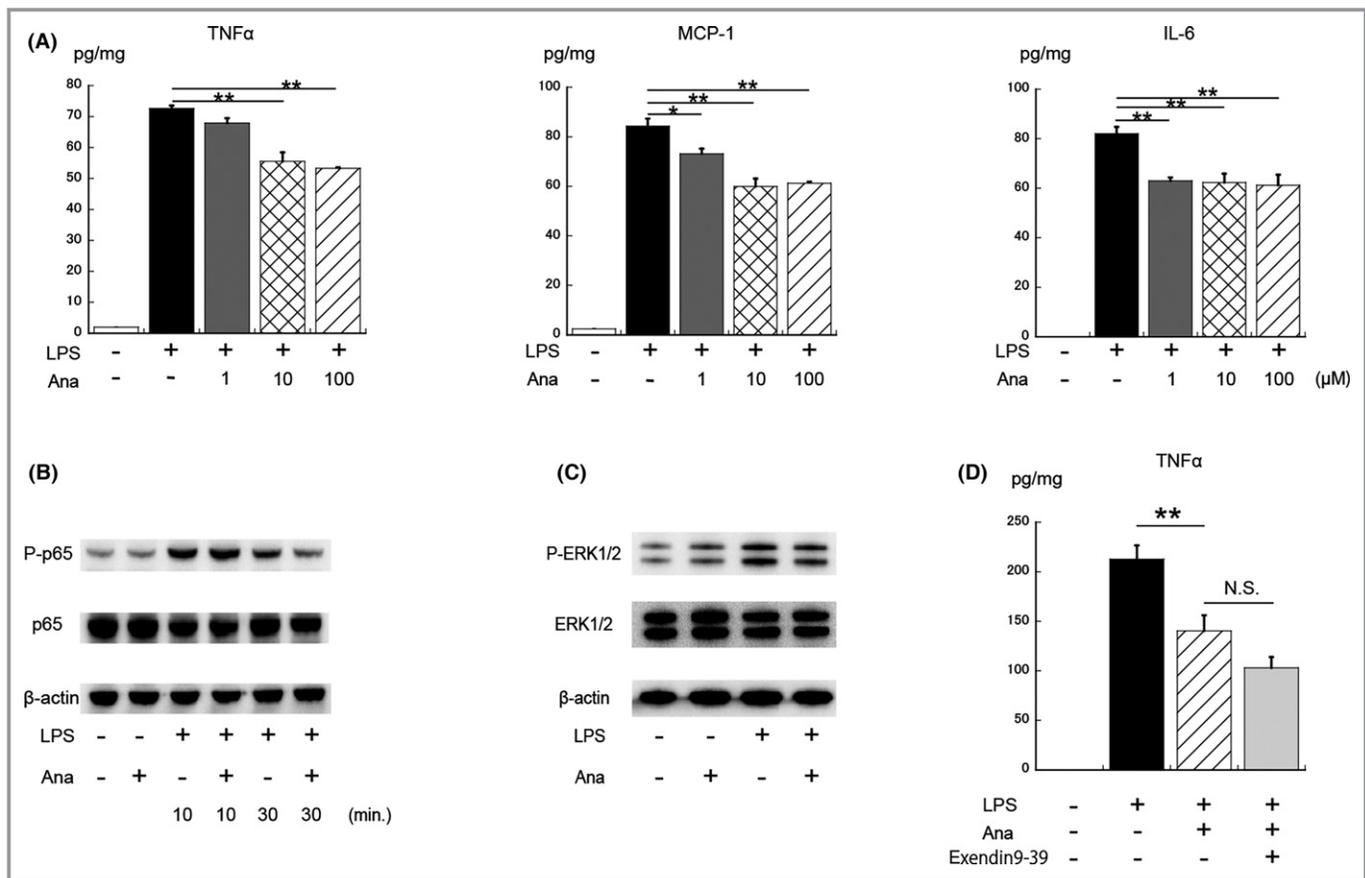


**Figure 2.** Treatment with anagliptin suppresses macrophage infiltration and activation in intracranial aneurysms (IAs). A, Macrophages were stained with anti-Iba1 (anti-ionized calcium binding adaptor molecule 1) antibody in the vehicle (left) and anagliptin groups (right). Arrowheads indicate the luminal side of the IAs. Scale bar=50  $\mu$ m. B, In the upper panels, IAs were double-stained using anti-MCP-1 (monocyte chemoattractant protein-1) (green) and anti-Iba1 (red) in the 2 groups. In the lower panels, IAs were stained using anti-phosphorylated p65 (green) and anti-Iba1 (red). Arrowheads indicate the luminal side of the IAs. Scale bar=20  $\mu$ m. C, The gene expression of *Cd68*, *MCP-1/Ccl2*, and *Vcam-1* (vascular cell adhesion molecule 1) in cerebral arteries was evaluated using real-time polymerase chain reaction. Data were analyzed using the Wilcoxon rank sum test. Results are presented as mean  $\pm$  SEM (n=5 each). \* $P$ <0.05. A indicates anagliptin group; 4W, 4 weeks; 2W, 2 weeks; V, vehicle group.

rats. The quantitative morphologic evaluation found that anagliptin suppressed IA growth, and its inhibitory effects were more prominent in late-stage IA growth, as was the case in statin treatment.<sup>5</sup> In addition, we found that anagliptin treatment attenuated the inflammatory activation of macrophages, at least in part, through a GLP-1 receptor-independent pathway. Anagliptin activated ERK5 in macrophages,

which mediated its anti-inflammatory effects including inhibiting the proinflammatory NF- $\kappa$ B pathway.

Hemodynamic stress on the endothelial cells of the cerebral arteries triggers the formation and growth of IAs, causing prolonged and excess inflammation in the vessel wall.<sup>4</sup> Chronic inflammation involves the infiltration of monocytes/macrophages<sup>11</sup> and the subsequent release of



**Figure 3.** Anagliptin treatment inhibits the inflammatory activation of RAW264.7 macrophages. A, Murine macrophage cell line RAW264.7 cells were treated with or without anagliptin (1.0–100 μmol/L) for 10 minutes, then incubated with 10 ng/mL lipopolysaccharide (LPS) for 24 hours. The levels of proinflammatory cytokines in cell culture supernatants were measured. Results are presented as mean±SEM (n=3 each). \* $P<0.05$ , \*\* $P<0.01$ . B, RAW264.7 cells were treated with or without 100 μmol/L anagliptin, followed by LPS stimulation for 10 to 30 minutes. Cells were lysed and phosphorylation of p65 was evaluated by Western blotting. A representative figure of 3 consecutive experiments is shown. C, Anagliptin was administered, then RAW264.7 cells were incubated with LPS for 10 minutes. The levels of P-ERK1/2 (phosphorylated extracellular signal-regulated kinase 1/2) were examined by Western blotting. A representative figure of 3 consecutive experiments is shown. D, Before the pretreatment of anagliptin, 50 nmol/L extendin fragment 9–39 was administered for 10 minutes. Cells were stimulated with LPS and TNF-α (tumor necrosis factor-α) protein levels in cell culture supernatants were measured. Results are presented as mean±SEM (n=4 each). \* $P<0.05$ , \*\* $P<0.01$ . Ana indicates anagliptin; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; N.S., not significant.

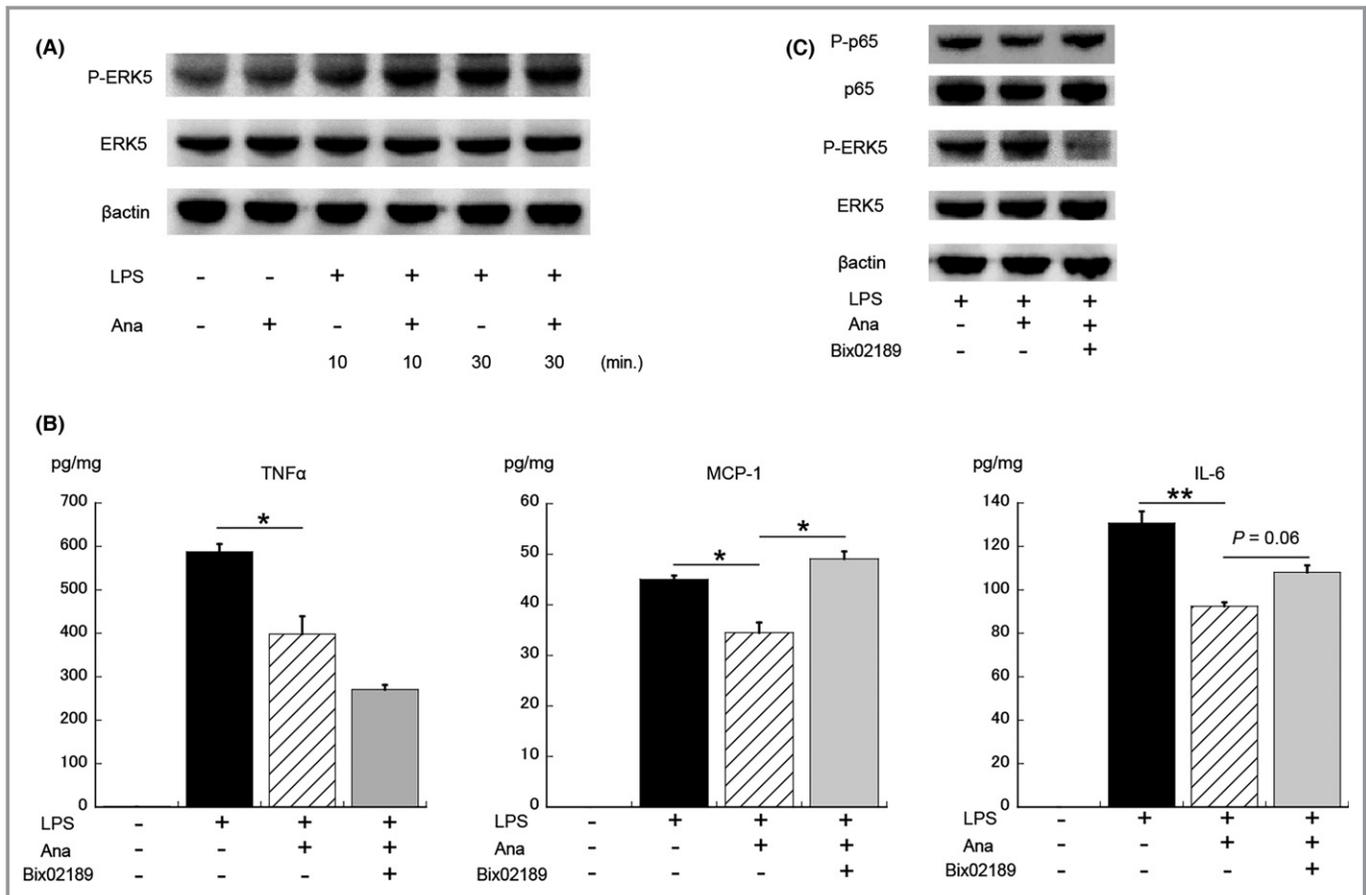
proinflammatory cytokines, chemokines,<sup>11</sup> and matrix-degrading proteinases, such as matrix metalloproteinases,<sup>9</sup> that induce cell death and the destruction of the extracellular matrix, causing the thinning of the aneurysmal wall. Inhibiting NF-κB and ETS-1 (E26 transformation-specific sequence 1) with chimeric decoy oligodeoxynucleotides decreases expression of MCP-1 and macrophage infiltration in rat IA models,<sup>12</sup> indicating that inflammatory processes play a crucial role in the formation and growth of IAs.

DPP-4 inhibitors are widely used antidiabetic drugs; however, the anti-inflammatory effects against cardiovascular diseases, including myocardial infarction,<sup>16</sup> atherosclerosis,<sup>7,17</sup> and abdominal aortic aneurysms,<sup>8</sup> have been reported in animal models. Anagliptin, a DPP-4 inhibitor, also has

anti-inflammatory effects, and according to phase 3 clinical trials, anagliptin treatment improves serum lipid profiles.<sup>18</sup>

DPP-4 is expressed in T lymphocytes, macrophages, dendritic cells, adipocytes, and endothelial cells.<sup>19</sup> DPP-4 consists of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain.<sup>20</sup> The extracellular domain contains binding sites for its ligands such as fibronectin and adenosine deaminase. The catalytic region in the C-terminal extracellular domain is responsible for the dipeptidase activity on substrate peptides including incretins, neuropeptides, chemokines, and cytokines.<sup>20</sup>

DPP-4 inhibitors, such as anagliptin, inhibit the extracellular catalytic domain of DPP-4,<sup>21</sup> preventing the degradation of substrates including stromal cell-derived factor 1<sup>22</sup> and



**Figure 4.** ERK5 (extracellular signal-regulated kinase 5) activation mediates the anti-inflammatory effect of anagliptin in macrophages. A, RAW264.7 cells were pretreated with or without 100  $\mu$ mol/L of anagliptin for 10 minutes, followed by lipopolysaccharide (LPS; 10 ng/mL) stimulation for 10 to 30 minutes. Phosphorylation of ERK5 (P-ERK5) was evaluated by Western blot analysis. A representative figure of 3 consecutive experiments is shown. B, After incubation with or without 10 nmol/L BIX02189 (selective ERK5 inhibitor) for 90 minutes, cells were treated with anagliptin, followed by incubation with LPS for 24 hours. LPS-induced expression of proinflammatory cytokines in the supernatant was measured. Results are presented as mean $\pm$ SEM (n=3 each). \* $P$ <0.05, \*\* $P$ <0.01. C, RAW264.7 cells were pretreated with or without BIX02189, then treated with anagliptin. Cells were stimulated with LPS for 30 minutes, and the levels of phosphorylated forms of p65 and ERK5 were examined by Western blotting. A representative figure of 3 consecutive experiments is shown. Ana indicates anagliptin; BIX, BIX02189.

platelet-activating factor.<sup>23</sup> Soluble DPP-4, shed from the cell surface, lacks the cytoplasmic and transmembrane domains but has preserved catalytic capacity. Treatment with soluble DPP-4 augments intracellular inflammatory signaling pathways in macrophages and induces vascular smooth muscle cell proliferation.<sup>24</sup> This may explain the pleiotropic effects of DPP-4 inhibitors.

GLP-1 is a major substrate for DPP-4 and is related to its inhibitors' antidiabetic effects; however, the anti-inflammatory mechanisms of DPP-4 inhibitors involve pathways other than GLP-1 and GLP-1 receptor-dependent signaling. For instance, administering antagonists of GLP-1 and GIP incompletely blocks the antiatherosclerotic effects of vildagliptin in diabetic, apolipoprotein E-deficient mice.<sup>25</sup> Anagliptin suppresses lipopolysaccharide-induced production of proinflammatory cytokines in human and mouse macrophage-like cells by inhibiting the NF- $\kappa$ B and MAPK pathways.<sup>7,26</sup> In this study,

we demonstrated that the anti-inflammatory action of anagliptin in macrophages was at least partly independent of GLP-1 receptor signaling.

We found that anagliptin activated ERK5. ERK5 is a member of the MAPK family and has both kinase and transcription activity. ERK5 is activated by various stimuli, such as colony-stimulating factor 1,<sup>27</sup> growth factors,<sup>28</sup> reactive oxygen species, and lipopolysaccharide,<sup>29</sup> and its activation regulates various cellular processes, including differentiation, cell survival,<sup>30</sup> and proliferation.<sup>27</sup> These in turn contribute to the pathogenesis of various diseases including atherosclerosis and cancer. Regarding its roles in vessel walls, ERK5 activation inhibits inflammatory responses in vascular endothelial cells.<sup>31</sup>

Several lines of evidence indicate that statins have anti-inflammatory functions, and statin treatment ameliorates aneurysm growth in a IA model.<sup>5</sup> Statins robustly activate

ERK5 in endothelial cells, preventing endothelial dysfunction.<sup>14</sup> In macrophages, ERK5 activation by statins enhances efferocytosis and plays a key role in atherosclerotic plaque formation in hyperlipidemic mice.<sup>15</sup> Our study revealed that, similar to statins, anagliptin activates ERK5 in macrophages and inhibits inflammatory activation of RAW264.7 macrophage-like cells.

Krüppel-like transcription factors KLF2 and KLF4 are downstream targets for statin-mediated ERK5 activation<sup>14</sup>; however, we detected no significant increase in KLF2 or KLF4 expression in RAW264.7 cells treated with anagliptin (data not shown), suggesting that the downstream effects of anagliptin-mediated ERK5 activation in macrophages may be different from those of statins. Previous reports have indicated that ERK5 activation reduces cellular inflammatory responses by inhibiting the NF- $\kappa$ B pathway.<sup>14,32</sup> In contrast, in the setting of leukemic T lymphocytes<sup>33</sup> or acute inflammation induced by Toll-like receptor ligands or ischemia-reperfusion injury,<sup>34</sup> ERK5 activation enhances NF- $\kappa$ B signaling. In this study, we showed that anagliptin treatment impaired the activation of the NF- $\kappa$ B pathway in macrophages in an ERK5-dependent manner.

A previous study demonstrated that ERK5 silencing does not alter ERK1/2 activation in macrophages. Likewise, pharmacological inhibition of ERK1/2 does not impair ERK5 activation.<sup>27</sup> In our study using lipopolysaccharide-stimulated RAW264.7 macrophages, pretreatment with an ERK5 inhibitor blocked the anagliptin-mediated reduction of MCP-1 expression but not TNF- $\alpha$  expression, meaning that the production of MCP-1 and TNF- $\alpha$  are differentially regulated in cells.

This study is associated with several limitations. First, because we administered only anagliptin, we could not exclude the possibility that the inhibition against IA growth is one of the off-target effects of anagliptin but is not common in DPP-4 inhibitors. However, a number of previous reports have described that in *in vivo* disease models, other DPP-4 inhibitors including sitagliptin<sup>35</sup> and alogliptin<sup>8</sup> improved the inflammatory phenotypes via inhibition of macrophage infiltration. This suggested that an anti-inflammatory function may be a class effect of this type of drug, and it seems reasonable to assume that DPP-4 inhibitors in general could prevent, at least partially, IA growth through the anti-inflammatory functions. Second, regarding the roles of the GLP-1 or ERK5 pathway in anagliptin-mediated anti-inflammation, we performed *in vitro* experiments using only murine macrophage-like cells. Further *in vivo* studies will provide evidence for the direct involvement of GLP-1–GLP-1 receptor signaling or ERK5 activation in IA growth.

In summary, we demonstrated that a DPP-4 inhibitor, anagliptin, prevents the growth of IAs via its anti-inflammatory effects on macrophages. Activation of ERK5 and subsequent inhibition of the NF- $\kappa$ B pathway may be key for the anti-

inflammatory mechanisms of anagliptin and a promising direction for the development of new therapeutic targets for the treatment of IAs.

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## Disclosures

None.

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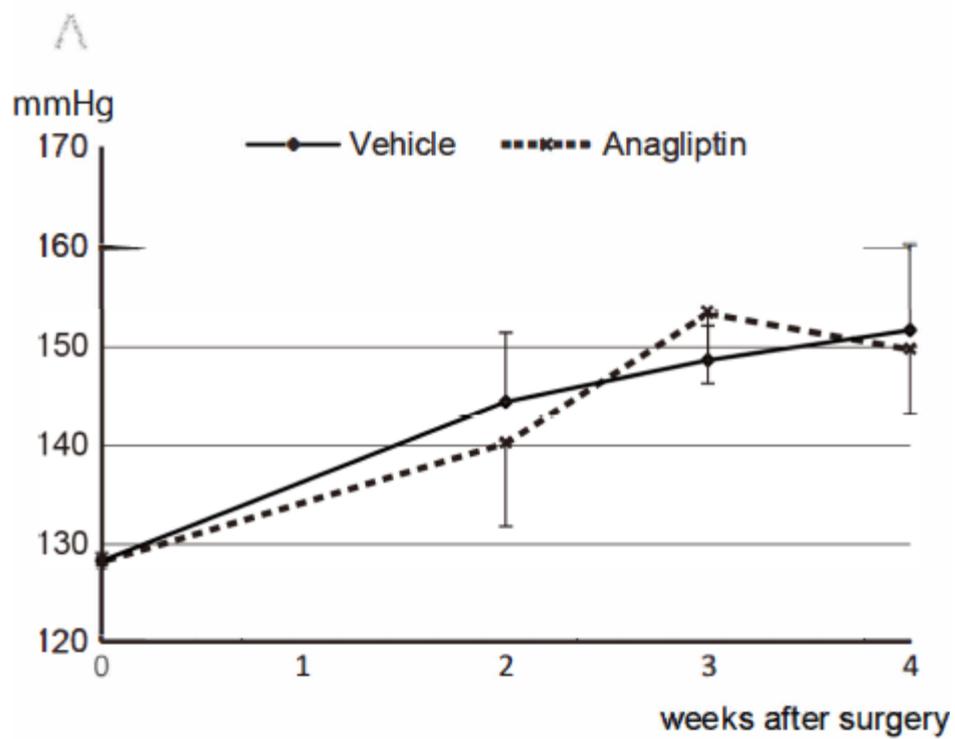
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# **Supplemental Material**

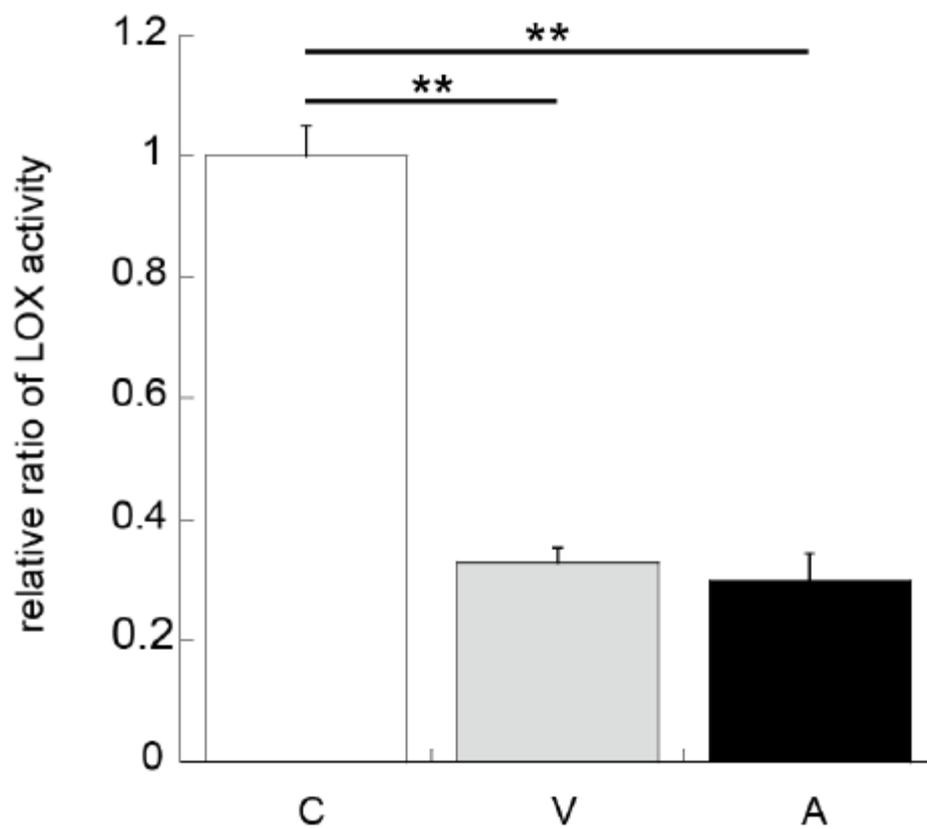
**Figure S1.** Blood pressure without anesthesia was measured by tail cuff at each time point.

Data were analyzed using the Student's t-test. Results are presented as mean  $\pm$  SEM (n = 6–8 each).



**Figure S2.** Lysyl oxidase (LOX) activity was measured in serum samples of rats in the two groups. Relative ratio of LOX activity of the vehicle and the anagliptin groups to that of the control group were statistically compared. Results are presented as mean  $\pm$  SEM (n = 6–7 each).

\*\*  $P < 0.01$ . C; control, V; vehicle group, A; anagliptin group



**Figure S3.** Anagliptin treatment did not suppress inflammation in endothelial cells of IAs. The gene expression of *intercellular adhesion molecule 1 (Icam-1)* and *E-selectin* in cerebral arteries was evaluated using real-time PCR. Results are presented as mean  $\pm$  SEM (n = 6–8 each). \*  $P < 0.05$ , \*\*  $P < 0.01$ . V; vehicle group, A; anagliptin group

