Inhibition of activated factor X by rivaroxaban attenuates neointima formation after wire-mediated vascular injury

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Abstract

Accumulating evidence suggests that activated factor X (FXa), a key coagulation factor, plays an important role in the development of vascular inflammation through activation of many cell types. Here, we investigated whether pharmacological blockade of FXa attenuates neointima formation after wire-mediated vascular injury. Transluminal femoral artery injury was induced in C57BL/6 mice by inserting a straight wire. Rivaroxaban (5 mg/kg/day), a direct FXa inhibitor, was administered from one week before surgery until killed. At four weeks after surgery, rivaroxaban significantly attenuated neointima formation in the injured arteries compared with control ($P<0.01$). Plasma lipid levels and blood pressure were similar between the rivaroxaban-treated group and non-treated group. Quantitative RT-PCR analyses demonstrated that rivaroxaban reduced the expression of inflammatory molecules (e.g., IL-1β and TNF-α) in injured arteries at seven days after surgery ($P<0.05$, respectively). In vitro experiments using mouse peritoneal macrophages demonstrated that FXa increased the expression of inflammatory molecules (e.g., IL-1β and TNF-α), which was blocked in the presence of rivaroxaban ($P<0.05$). Also, in vitro experiments using rat vascular smooth muscle cells (VSMC) demonstrated that FXa promoted both proliferation and migration of this cell type ($P<0.05$), which were blocked in the presence of rivaroxaban. Inhibition of FXa by rivaroxaban attenuates neointima formation after wire-mediated vascular injury through inhibition of inflammatory activation of macrophages and VSMC.

Keywords: macrophage, neointima formation, inflammation, activated factor X, rivaroxaban

1. Introduction

Angioplasty, especially stent implantation for coronary artery disease, is now established as a therapeutic strategy of great benefit (Stone, 2008a; b). However, restenosis remains the main limitation of percutaneous coronary intervention (Hoffmann et al., 1996; Lowe et al., 2002; Serruys et al., 2006). Excessive neointimal hyperplasia after vascular injury contributes to restenosis after angioplasty (Hoffmann and Mintz, 2000). Both monocyte/macrophage-dependent inflammation and vascular smooth muscle cell (VSMC) proliferation play causal roles in this disease process through multiple cellular and molecular mechanisms (Toutouzas et al., 2004).

Previous studies have demonstrated a link between the blood coagulation system and inflammatory diseases (Borissoff et al., 2011; Croce and Libby, 2007). A number of previous studies investigated the role of activated factor X (FXa) in vascular inflammation (Rothmeier and Ruf, 2012). Recently, we reported that a direct FXa inhibitor, rivaroxaban, attenuates atherosclerotic plaque progression and destabilization in apolipoprotein E-deficient mice through inhibition of pro-inflammatory activation of macrophages (Hara et al., 2015). Other studies also reported inflammatory activation of VSMC and endothelial cells by FXa in vitro (Busch et al., 2005; Kaiser et al., 2000). FXa-induced activation of protease activated receptor (PAR)-1 and PAR-2 is suggested to be an underlying mechanism. In fact, several studies have shown the contribution of PAR-1 and PAR-2 (Andrade-Gordon et al., 2001; Chieng-Yane et al., 2010; Takada et al., 1998; Tennant et al., 2008) to the development of vascular lesions and inflammation. However, the impact of rivaroxaban on neointima formation after mechanical vascular injury has not been evaluated.

In this study, we therefore examined the effects of rivaroxaban, the first oral

anticoagulant that directly inhibits FXa (Perzborn et al., 2011), on neointima hyperplasia and inflammatory features of injured arteries in mice, and also performed in vitro studies using macrophages and VSMC, key players in neointima formation, to reveal the mechanisms. Our results suggested that rivaroxaban attenuates inflammatory activation of macrophages and VSMC, leading to the suppression of neointima hyperplasia after mechanical vascular injury in mice.

2. Materials and Methods

2.1. Animals and treatments

C57BL/6J male mice purchased from Japan SLC, Inc. were used in this study. Mice received a normal chow diet supplemented with or without rivaroxaban (5 mg/kg body weight/day) from 7-weeks-old to kill. Rivaroxaban was supplied by Bayer Pharma AG. Non-treated mice served as the control. Femoral artery endovascular injury was induced in mice in both the rivaroxaban-treated and non-treated groups at the age of 8 weeks, as described previously (Sata et al., 2000). Mice were euthanized at 7 days or 4 weeks after surgery, and the femoral arteries were isolated for further analyses. The mice were housed in a room in which lighting was controlled (12 h on/12 h off), and room temperature was kept at 25 °C. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University.

2.2. Blood pressure and laboratory data

Blood pressure measurement was performed using a tail-cuff system (BP-98A, Softron) as described previously (Salim et al., 2016). Blood was collected from the left ventricle at the time of sacrifice, and plasma was isolated and stored at -80°C until required. Plasma lipid levels were

measured using commercially available kits (Wako Diagnostics). Rivaroxaban concentration was determined in lithium-heparin plasma by Shin Nippon Biomedical Laboratories, Ltd. (Japan).

2.3. Analyses of femoral arteries

For morphometric studies, the femoral arteries were harvested at 7 days or 4 weeks after surgery, and embedded in paraffin after fixation with 10% buffered formalin. Embedded femoral arteries were cut into 5-μm-thick sections for further analyses. Cross sections were stained with Elastica van Gieson staining to perform morphometric analysis. Digitalized images were analyzed with the FLOVEL Filing System (FLOVEL Company, Ltd., Tokyo, Japan). The lumen, internal elastic lamina, and external elastic lamina were defined. The areas of the intima (tissue between lumen and internal elastic lamina) and media (tissue between internal elastic lamina and external elastic lamina) were recorded. Neointima/media area ratio (I/M ratio) was calculated. Accumulation of smooth muscle cells or macrophages in neointima was examined by immunohistochemistry. Sections were incubated with alkaline phosphatase-conjugated anti-α-smooth muscle actin (α–SMA) antibody (Sigma) or anti-Mac3 (BD Pharmingen) antibody. Development was performed by Vector Red substrate (Vector) for α–SMA or the combination of HRP-conjugated secondary antibody and ImmPACT DAB substrate (Vector Laboratories) for Mac3. Sections were counterstained with hematoxylin. The percentage of Mac3-pisitive cells in neointima was calculated. For gene expression analysis, the femoral arteries were harvested at 7 days after surgery and snap-frozen in liquid nitrogen for gene expression analysis.

2.4. Cell culture

Murine resident peritoneal macrophages were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a 5% CO_2 humidified

atmosphere. To investigate the effects of FXa on macrophage activation, macrophages were stimulated with 10 nM FXa (Haematologic Technologies, Inc.) for 24 h after 24-h serum starvation. Additionally, 1 μM or 10 μM rivaroxaban was used to block the effects of FXa. To assess the effect of PAR-1 or PAR-2, the major receptor of FXa, on macrophage activation, cells were treated with 10 nM PAR-1 specific agonist peptide (AP-1; Sigma-Aldrich) or 10 nM PAR-2 specific agonist peptide (AP-2; Sigma-Aldrich) for 24 h after 24-h serum starvation.

2.5. Reverse transcription and real-time polymerase chain reaction

Extraction of total RNA from femoral arteries or cells, and reverse transcription were performed using an illustra RNAspin RNA Isolation Kit (GE Healthcare) and QuantiTect Reverse Transcription kit (Qiagen), respectively. Quantitative real-time PCR (qPCR) was performed using an Mx3000P (Agilent Technologies) and Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences of gene-specific primers are presented in Table 1. Data are expressed in arbitrary units normalized by β-actin.

2.6. Cell proliferation assay

Rat VSMC were isolated and cultured as described previously (Sreejayan and Yang, 2007). To evaluate the effects of FXa on migration of rat VSMC, a scratch wound assay was performed as reported previously. VSMC were seeded in 24-well plates and allowed to attach. After scraping off the cell monolayer in a straight line, cells were incubated with FXa (10 nM) with or without rivaroxaban (1 μM) for 24 h. Then, the distance between one side of the scratch and the other was measured.

A cell proliferation assay was performed using a commercially available kit (CellTiter 96, Promega) according to the manufacturer's instructions to evaluate the effect of FXa on the growth

of rat VSMC. Rat VSMC were seeded in 96-well plates at an initial density of 1×10^4 cells/well and allowed to attach for 24 h. After serum starvation for 24 h, cells were incubated with FXa (10 nM) with or without rivaroxaban (1 μM) for 24 h.

2.7. Statistical analysis

Numerical values are expressed as mean ± S.E.M. Comparison of parameters between two groups was performed using unpaired Student's t-test. Differences between multiple groups were analyzed by one-way analysis of variance, followed by the Scheffe's post hoc analysis. A value of $P < 0.05$ was considered significant.

3. Results

3.1. PAR-1 and PAR-2 increased in injured artery

We examined the expression of PARs in the femoral artery in wild-type mice. qPCR analysis showed that mechanical vascular injury significantly increased PAR-1 and tended to increase PAR-2 in injured arteries at 7 days after surgery compared with non-injured arteries (Fig. 1). There were no significant differences in the expression of PAR3 and PAR4 between injured and non-injured arteries.

3.2. Rivaroxaban did not change metabolic parameters

After 4 weeks of rivaroxaban administration, body weight gain, blood pressure, plasma glucose level, and plasma lipid levels did not differ between rivaroxaban-treated mice and non-treated mice (Table 2). After 4 weeks of treatment, plasma level of rivaroxaban was 28.1 ± 3.2 μg/l in the treated group.

3.3. Rivaroxaban attenuated neointima formation after vascular injury

Histological analysis using femoral arteries harvested at 4 weeks after surgery showed that rivaroxaban treatment significantly attenuated neointima hyperplasia compared with the non-treatment group (Fig. 2). Rivaroxaban significantly reduced neointima area (30,453 ± 1,982 vs. 19,582 ± 2,981 μm^2; P=0.01) and intima/media ratio (4.37 ± 0.49 vs. 2.60 ± 0.51; P=0.03) compared with control. The most of cell-type in the neointima at 4 weeks after surgery was smooth muscle cell. There was no difference in cross sectional area of the media between the groups (9,460 ± 1,723 vs. 10,215 ± 1,734 μm^2; P=0.76). Non-injured arteries did no show neointima in the rivaroxaban group and the control group. These results indicate that rivaroxaban attenuates neointima formation after vascular injury without alteration of metabolic parameters.

3.4. Rivaroxaban reduced expression of inflammatory molecules in injured artery

Previous studies demonstrated the contribution of pro-inflammatory activation of macrophages to the development of neointima formation at earlier time point of lesion development (Lavin et al., 2014; Schober and Weber, 2005). Therefore, we investigated the effect of rivaroxaban on vascular inflammation in injured arteries at 7 days after surgery. The result of immunohistochemistry demonstrated that rivaroxaban reduced macrophage accumulation in neointima (P=0.02) (Fig. 3A). qPCR analysis demonstrated that rivaroxaban reduced the expression of the macrophage marker F4/80 (P=0.006) compared with the control group. Rivaroxaban treatment also reduced the expression of inflammatory mediators, such as monocyte chemoattractant protein (MCP)-1 (P=0.009), interleukin (IL)-1β (P=0.01) and tumor necrosis factor (TNF)-α (P=0.03) (Fig. 3B). Rivaroxaban also decreased mRNA expression of transforming growth factor (TGF)-β1 (P=0.003), stromal cell-derived factor (SDF)-1 (P=0.0002) and granulocyte-macrophage colony stimulating factor (GM-CSF) (P=0.04) in the injured artery

(Fig. 3C).

3.5. FXa-PARs signal promoted pro-inflammatory activation of macrophages

To investigate the molecular mechanism by which inhibition of FXa signaling prevented vascular inflammation and neointima formation without improvement of metabolic parameters, we performed in vitro experiments using resident peritoneal macrophages obtained from wild-type mice. The results of qPCR analysis demonstrated that FXa, a major ligand of PAR-1 and PAR-2, significantly increased mRNA expression of inflammatory molecules (e.g., IL-1β, IL-6 and TNF-α), which was attenuated in the presence of rivaroxaban in a dose-dependent manner (Fig. 4A). Furthermore, a specific agonist peptide of PAR-1 or PAR-2 also increased the expression of inflammatory molecules in this cell type (Fig. 4B).

3.6. Rivaroxaban attenuated vascular smooth muscle cell proliferation and migration

To investigate the mechanism by which rivaroxaban attenuates lesion formation after mechanical vascular injury, we also investigated the effects of rivaroxaban on migration and proliferation of VSMC, which is one of the important mechanisms of neointima formation after vascular injury. The results of scratch-wound assay and MTS assay using rat VSMC demonstrated that FXa promoted both proliferation and migration of this cell type (Fig. 5A-C). Rivaroxaban attenuated FXa-induced proliferation and migration of VSMC.

4. Discussion

In this study, we demonstrated that rivaroxaban, a specific inhibitor of FXa, attenuated neointima formation after mechanical vascular injury in mice. We also demonstrated that rivaroxaban inhibited pro-inflammatory activation of macrophages, and proliferation and migration of VSMC,

which are important mechanisms of neointima hyperplasia. Accumulating evidence suggests that the FXa-PARs signaling contributes to the pathogenesis of inflammatory diseases (Borissoff et al., 2011; Croce and Libby, 2007), although little is known about the effect of rivaroxaban treatment on the development of neointima after mechanical vascular injury.

Previous studies reported the contribution of FXa-PARs signaling to the development of vascular lesions after vascular injury. Expression of PARs in the vasculature has been reported. In addition, several studies have reported that mechanical vascular injury increased the expression of PAR-1 and PAR-2 (Cheung et al., 1999; Damiano et al., 1999). The results of our present study are consistent with those studies. These results suggested that PAR-1 and PAR-2, and their ligand FXa may play a role in the pathogenesis of neointima formation after vascular injury. In a carotid ligation model, another commonly used vascular injury model, PAR-2 deletion significantly reduced neointima formation (Tennant et al., 2008). PAR-1, another FXa receptor, also participates in neointima formation after vascular injury. Previous studies reported that a selective blocking antibody or antagonists against PAR-1 reduced neointima formation in vascular injury models (Andrade-Gordon et al., 2001; Chieng-Yane et al., 2010; Takada et al., 1998). Furthermore, several studies demonstrated that pharmacological blockade of FXa suppressed neointima formation after vascular injury using animal models (Kaiser et al., 2000; Ragosta et al., 1994). The results of our present study demonstrating that rivaroxaban, the first direct oral anticoagulant that inhibits FXa, suppressed neointima hyperplasia correspond to the results of previous studies. These results implicate causal roles of FXa-PARs signaling in neointima hyperplasia after vascular injury, and suggested the potency of rivaroxaban, a clinical approved direct oral FXa inhibitor, for the inhibition of restenosis after angioplasty.

A large amount of evidence has demonstrated the involvement of various cellular and molecular pathways in neointima hyperplasia (Toutouzas et al., 2004). Angioplasty improves blood flow, but meanwhile causes endothelial denudation and impaired endothelial function, leading to activation of the coagulation system and/or endothelium-leukocyte interaction (Costa and Simon, 2005). Acceleration of neointima hyperplasia through activation of platelet and coagulation cascades has been already reported (Chandrasekar and Tanguay, 2000; Vicente et al., 2007). Therefore, the anti-coagulant effects of rivaroxaban might have an impact on suppression of neointima hyperplasia. FXa-PARs signaling plays a key role in the coagulation cascade, whereas accumulating evidence suggests that it also directly contributes to the pathophysiology of vascular inflammation and neointima hyperplasia. Studies using PAR-2-deficient mice showed reduced endothelium-leukocyte interaction after vascular injury (Tennant et al., 2008). Stimulation of vascular inflammation by PAR-2 signaling, especially in non-hematopoietic cells, in BERK mice, a model of sickle cell disease, was also reported (Sparkenbaugh et al., 2014). Pharmacological blockade of FXa also inhibited SMC proliferation in injured vessels (Kaiser et al., 2000). Furthermore, recent studies including our own revealed that FXa-PAR2 signaling promotes pro-inflammatory activation of monocyte/macrophages, key players in vascular inflammation (Hara et al., 2015). In this study, we demonstrated that injured arteries harvested from rivaroxaban-treated animals showed less macrophage accumulation and reduced expression of inflammatory molecules such as MCP-1. Rivaroxaban treatment reduced the expression of growth factors that stimulate proliferation and migration of SMC such as TGF-β1 (Ryan et al., 2003; Tsai et al., 2009) and SDF-1 (Schober et al., 2003; Zernecke et al., 2005). We further demonstrated that FXa promotes pro-inflammatory activation of macrophages and

proliferation and migration of SMC, which were blocked in the presence of rivaroxaban. The results of these in vivo and in vitro studies are consistent with those of previous studies and indicate inhibitory effects of rivaroxaban on the inflammatory activation of these cell types and neointima formation.

Recent clinical trials using rivaroxaban demonstrated that it might reduce cardiovascular events in patients with acute coronary syndrome compared with the control (Mega et al., 2009; Mega et al., 2012; Mega et al., 2013). Furthermore, recently, the result of the Cardiovascular Outcomes for People Using Anticoagulation Strategies (COMPASS) trial was reported (Eikelboom et al., 2017). In that study, rivaroxaban (2.5 mg twice daily) plus aspirin group had better cardiovascular outcomes compared with aspirin alone group. The anti-coagulation effects of rivaroxaban might play a role in these results, although, considering the results of our present study and previous studies, the suppression of neointima formation through the attenuation of vascular inflammation by rivaroxaban might also partially explain this result. Several direct oral anti-coagulants are now available (Furugohri et al., 2008; Perzborn et al., 2005; Pinto et al., 2007; Wienen et al., 2007). Further studies are needed to investigate the effects of these drugs on neointima hyperplasia and their underlying mechanisms.

In conclusion, rivaroxaban suppressed neointima hyperplasia in a mouse mechanical vascular injury model. Attenuation of vascular inflammation by inhibiting FXa-PARs signals might have at least partial roles in this result. Our results suggest that the blockade of FXa-PARs signals by rivaroxaban is a potential therapeutic strategy to attenuate neointima hyperplasia after mechanical vascular injury, which is a major limitation of angioplasty.

Competing interest

Dr. Sata received research funding from Bayer Yakuhin, Ltd. Other authors declare that they have no conflict of interest.

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References

Andrade-Gordon, P., Derian, C.K., Maryanoff, B.E., Zhang, H.C., Addo, M.F., Cheung, W., Damiano, B.P., D'Andrea, M.R., Darrow, A.L., de Garavilla, L., Eckardt, A.J., Giardino, E.C., Haertlein, B.J., McComsey, D.F., 2001. Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J. Pharmacol. Exp. Ther. 298, 34-42.

Borissoff, J.I., Spronk, H.M., ten Cate, H., 2011. The hemostatic system as a modulator of atherosclerosis. N. Engl. J. Med. 364, 1746-1760.

Busch, G., Seitz, I., Steppich, B., Hess, S., Eckl, R., Schomig, A., Ott, I., 2005. Coagulation factor Xa stimulates interleukin-8 release in endothelial cells and mononuclear leukocytes: implications in acute myocardial infarction. Arterioscler. Thromb. Vasc. Biol. 25, 461-466.

Chandrasekar, B., Tanguay, J.F., 2000. Platelets and restenosis. J Am Coll Cardiol 35, 555-562.

Cheung, W.M., D'Andrea, M.R., Andrade-Gordon, P., Damiano, B.P., 1999. Altered vascular injury responses in mice deficient in protease-activated receptor-1. Arterioscler. Thromb. Vasc. Biol. 19, 3014-3024.

Chieng-Yane, P., Bocquet, A., Letienne, R., Bourbon, T., Sablayrolles, S., Perez, M., Hatem, S.N., Lompre, A.M., Le Grand, B., David-Dufilho, M., 2010. Protease-activated receptor-1 antagonist F 16618 reduces arterial restenosis by down-regulation of tumor necrosis factor alpha and matrix metalloproteinase 7 expression, migration, and proliferation of vascular smooth muscle cells. J. Pharmacol. Exp. Ther. 336, 643-651.

Costa, M.A., Simon, D.I., 2005. Molecular basis of restenosis and drug-eluting stents. Circulation 111, 2257-2273.

Croce, K., Libby, P., 2007. Intertwining of thrombosis and inflammation in atherosclerosis. Curr. Opin. Hematol. 14, 55-61.

Damiano, B.P., D'Andrea, M.R., de Garavilla, L., Cheung, W.M., Andrade-Gordon, P., 1999. Increased expression of protease activated receptor-2 (PAR-2) in balloon-injured rat carotid artery. Thromb. Haemost. 81, 808-814.

Eikelboom, J.W., Connolly, S.J., Bosch, J., Dagenais, G.R., Hart, R.G., Shestakovska, O., Diaz, R., Alings, M., Lonn, E.M., Anand, S.S., Widimsky, P., Hori, M., Avezum, A., Piegas, L.S., Branch, K.R.H., Probstfield, J., Bhatt, D.L., Zhu, J., Liang, Y., Maggioni, A.P., Lopez-Jaramillo, P., O'Donnell, M., Kakkar, A., Fox, K.A.A., Parkhomenko, A.N., Ertl, G., Stork, S., Keltai, M., Ryden, L., Pogosova, N., Dans, A.L., Lanas, F., Commerford, P.J., Torp-Pedersen, C., Guzik, T.J., Verhamme, P.B., Vinereanu, D., Kim, J.H., Tonkin, A.M., Lewis, B.S., Felix, C., Yusoff, K., Steg, P.G., Metsarinne, K.P., Cook Bruns, N., Misselwitz, F., Chen, E., Leong, D., Yusuf, S., 2017. Rivaroxaban with or without Aspirin in Stable Cardiovascular Disease. N. Engl. J. Med. *in press*.

Furugohri, T., Isobe, K., Honda, Y., Kamisato-Matsumoto, C., Sugiyama, N., Nagahara, T., Morishima, Y., Shibano, T., 2008. DU-176b, a potent and orally active factor Xa inhibitor: in vitro and in vivo pharmacological profiles. J. Thromb. Haemost. 6, 1542-1549.

Hara, T., Fukuda, D., Tanaka, K., Higashikuni, Y., Hirata, Y., Nishimoto, S., Yagi, S., Yamada, H., Soeki, T., Wakatsuki, T., Shimabukuro, M., Sata, M., 2015. Rivaroxaban, a novel oral anticoagulant, attenuates atherosclerotic plaque progression and destabilization in ApoE-deficient mice. Atherosclerosis 242, 639-646.

Hoffmann, R., Mintz, G.S., 2000. Coronary in-stent restenosis - predictors, treatment and prevention. Eur. Heart. J. 21, 1739-1749.

Hoffmann, R., Mintz, G.S., Dussaillant, G.R., Popma, J.J., Pichard, A.D., Satler, L.F., Kent, K.M., Griffin, J., Leon, M.B., 1996. Patterns and mechanisms of in-stent restenosis. A serial intravascular ultrasound study. Circulation 94, 1247-1254.

Kaiser, B., Paintz, M., Scholz, O., Kunitada, S., Fareed, J., 2000. A synthetic inhibitor of factor Xa, DX-9065a, reduces proliferation of vascular smooth muscle cells in vivo in rats. Thromb. Res. 98, 175-185.

Lavin, B., Gomez, M., Pello, O.M., Castejon, B., Piedras, M.J., Saura, M., Zaragoza, C., 2014. Nitric oxide prevents aortic neointimal hyperplasia by controlling macrophage polarization. Arterioscler. Thromb. Vasc. Biol. 34, 1739-1746.

Lowe, H.C., Oesterle, S.N., Khachigian, L.M., 2002. Coronary in-stent restenosis: current status and future strategies. J. Am. Coll. Cardiol. 39, 183-193.

Mega, J.L., Braunwald, E., Mohanavelu, S., Burton, P., Poulter, R., Misselwitz, F., Hricak, V., Barnathan, E.S., Bordes, P., Witkowski, A., Markov, V., Oppenheimer, L., Gibson, C.M., 2009. Rivaroxaban versus placebo in patients with acute coronary syndromes (ATLAS ACS-TIMI 46): a randomised, double-blind, phase II trial. Lancet 374, 29-38.

Mega, J.L., Braunwald, E., Wiviott, S.D., Bassand, J.P., Bhatt, D.L., Bode, C., Burton, P., Cohen, M., Cook-Bruns, N., Fox, K.A., Goto, S., Murphy, S.A., Plotnikov, A.N., Schneider, D., Sun, X., Verheugt, F.W., Gibson, C.M., 2012. Rivaroxaban in patients with a recent acute coronary syndrome. N. Engl. J. Med. 366, 9-19.

Mega, J.L., Braunwald, E., Wiviott, S.D., Murphy, S.A., Plotnikov, A., Gotcheva, N., Ruda, M., Gibson, C.M., 2013. Comparison of the efficacy and safety of two rivaroxaban doses in acute coronary syndrome (from ATLAS ACS 2-TIMI 51). Am. J. Cardiol. 112, 472-478.

Perzborn, E., Roehrig, S., Straub, A., Kubitza, D., Misselwitz, F., 2011. The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor. Nat. Rev. Drug. Discov. 10, 61-75.

Perzborn, E., Strassburger, J., Wilmen, A., Pohlmann, J., Roehrig, S., Schlemmer, K.H., Straub, A., 2005. In vitro and in vivo studies of the novel antithrombotic agent BAY 59-7939--an oral, direct Factor Xa inhibitor. J. Thromb. Haemost. 3, 514-521.

Pinto, D.J., Orwat, M.J., Koch, S., Rossi, K.A., Alexander, R.S., Smallwood, A., Wong, P.C., Rendina, A.R., Luettgen, J.M., Knabb, R.M., He, K., Xin, B., Wexler, R.R., Lam, P.Y., 2007. Discovery of 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H -pyrazolo[3,4-c]pyridine-3-carboxamide (apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. J. Med. Chem. 50, 5339-5356.

Ragosta, M., Gimple, L.W., Gertz, S.D., Dunwiddie, C.T., Vlasuk, G.P., Haber, H.L., Powers, E.R., Roberts, W.C., Sarembock, I.J., 1994. Specific factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. Circulation 89, 1262-1271.

Rothmeier, A.S., Ruf, W., 2012. Protease-activated receptor 2 signaling in inflammation. Semin. Immunopathol. 34, 133-149.

Ryan, S.T., Koteliansky, V.E., Gotwals, P.J., Lindner, V., 2003. Transforming growth g/lSalim, H.M., Fukuda, D., Higashikuni, Y., Tanaka, K., Hirata, Y., Yagi, S., Soeki, T., Shimabukuro, M., Sata, M., 2016. Dipeptidyl peptidase-4 inhibitor, linagliptin, ameliorates endothelial dysfunction and atherogenesis in normoglycemic apolipoprotein-E deficient mice. Vascul. Pharmacol. 79, 16-23.

Sata, M., Maejima, Y., Adachi, F., Fukino, K., Saiura, A., Sugiura, S., Aoyagi, T., Imai, Y., Kurihara, H., Kimura, K., Omata, M., Makuuchi, M., Hirata, Y., Nagai, R., 2000. A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. J.

Mol. Cell. Cardiol. 32, 2097-2104.

Schober, A., Knarren, S., Lietz, M., Lin, E.A., Weber, C., 2003. Crucial role of stromal cell-derived factor-1alpha in neointima formation after vascular injury in apolipoprotein E-deficient mice. Circulation 108, 2491-2497.

Schober, A., Weber, C., 2005. Mechanisms of monocyte recruitment in vascular repair after injury. Antioxid. Redox. Signal. 7, 1249-1257.

Serruys, P.W., Kutryk, M.J., Ong, A.T., 2006. Coronary-artery stents. N. Engl. J. Med. 354, 483-495.

Sparkenbaugh, E.M., Chantrathammachart, P., Mickelson, J., van Ryn, J., Hebbel, R.P., Monroe, D.M., Mackman, N., Key, N.S., Pawlinski, R., 2014. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. Blood 123, 1747-1756.

Sreejayan, N., Yang, X., 2007. Isolation and functional studies of rat aortic smooth muscle cells. Methods. Mol. Med. 139, 283-292.

Stone, G.W., 2008a. Angioplasty strategies in ST-segment-elevation myocardial infarction: part I: primary percutaneous coronary intervention. Circulation 118, 538-551.

Stone, G.W., 2008b. Angioplasty strategies in ST-segment-elevation myocardial infarction: part II: intervention after fibrinolytic therapy, integrated treatment recommendations, and future directions. Circulation 118, 552-566.

Takada, M., Tanaka, H., Yamada, T., Ito, O., Kogushi, M., Yanagimachi, M., Kawamura, T., Musha, T., Yoshida, F., Ito, M., Kobayashi, H., Yoshitake, S., Saito, I., 1998. Antibody to thrombin receptor inhibits neointimal smooth muscle cell accumulation without causing inhibition of platelet aggregation or altering hemostatic parameters after angioplasty in rat. Circ. Res. 82, 980-987.

Tennant, G.M., Wadsworth, R.M., Kennedy, S., 2008. PAR-2 mediates increased inflammatory cell

adhesion and neointima formation following vascular injury in the mouse. Atherosclerosis 198, 57-64.

Toutouzas, K., Colombo, A., Stefanadis, C., 2004. Inflammation and restenosis after percutaneous coronary interventions. Eur. Heart. J. 25, 1679-1687.

Tsai, S., Hollenbeck, S.T., Ryer, E.J., Edlin, R., Yamanouchi, D., Kundi, R., Wang, C., Liu, B., Kent, K.C., 2009. TGF-beta through Smad3 signaling stimulates vascular smooth muscle cell proliferation and neointimal formation. Am. J. Physiol. Heart. Circ. Physiol. 297, H540-549.

Vicente, C.P., He, L., Tollefsen, D.M., 2007. Accelerated atherogenesis and neointima formation in heparin cofactor II deficient mice. Blood 110, 4261-4267.

Wienen, W., Stassen, J.M., Priepke, H., Ries, U.J., Hauel, N., 2007. In-vitro profile and ex-vivo anticoagulant activity of the direct thrombin inhibitor dabigatran and its orally active prodrug, dabigatran etexilate. Thromb. Haemost. 98, 155-162.

Zernecke, A., Schober, A., Bot, I., von Hundelshausen, P., Liehn, E.A., Mopps, B., Mericskay, M., Gierschik, P., Biessen, E.A., Weber, C., 2005. SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ. Res. 96, 784-791.

Figure legends

Fig. 1. Expression of PARs in injured artery.

Results of qPCR analysis using injured arteries in wild-type mice at 7 days after surgery demonstrated higher expression of PAR-1 and PAR-2 compared with non-injured arteries. Expression of PAR-3 and PAR-4 did not differ between injured and non-injured arteries. (n = 4-5). *P<0.05. All values are mean ± S.E.M.

Fig. 2. Effects of rivaroxaban on neointima formation.

Elastica van Gieson staining or immunostaining for α-SMA of injured arteries at 4 weeks after surgery. Rivaroxaban treatment reduced neointima area and intima/media ratio compared with control group. There was no difference in cross-sectional area of the media between the groups. The major cell population in the neointima at 4 weeks after surgery was smooth muscle cell. Non-injured arteries did no show neointima in the rivaroxaban group and the control group. Bar: 100 μm. (n = 10-12, per group). Ctrl; control, Riv; rivaroxaban, EVG; Elastica van Gieson, α-SMA; α-smooth muscle actin. Arrow heads indicate the internal elastic lamina. *P<0.05. All values are mean ± S.E.M.

Fig. 3. Effects of rivaroxaban on inflammation in injured artery.

(A) The results of immunohistochemistry against Mac3 on injured arteries at 7 days after surgery. Rivaroxaban reduced the infiltration of macrophages into neointima. (n = 4-5). Bar: 20 μm. **(B and C)** The results of qPCR analyses using injured arteries at 7 days after surgery. Rivaroxaban treatment decreased the expression of F4/80, a macrophage marker. Rivaroxaban also reduced

the expression of inflammatory molecules, such as MCP-1, IL-1β and TNF-α, in the injured arteries (B). Rivaroxaban also attenuated the expression of several growth factors, such as TGF-β1, SDF-1 and GM-CSF (C). (n = 4-5). Ctrl; control, Riv; rivaroxaban, L; lumen, M; media. *P<0.05 and **P<0.01. All values are mean ± S.E.M.

Fig. 4. Effects of FXa on macrophage activation.

(A) The results of qPCR analysis demonstrated that murine resident peritoneal macrophages treated with FXa showed increased expression of IL-1β, IL-6, and TNF-α compared with the control. The expression of these inflammatory molecules was attenuated in the presence of rivaroxaban in a dose-dependent manner. (All values are mean ± S.E.M. of 6 different experiments). **(B)** The results of qPCR analysis demonstrated that murine resident peritoneal macrophages treated with a specific agonist peptide of PAR-1 or a specific agonist peptide of PAR-2 showed increased expression of inflammatory molecules. (All values are mean ± S.E.M. of 4 different experiments). NT: no treatment, FXa: activated factor X, Riv: rivaroxaban, AP-1: specific agonist peptide of PAR-1, AP-2: specific agonist peptide of PAR-2. *P<0.05, **P<0.01, and ***P<0.0001.

Fig. 5. Effects of FXa on VSMC migration and proliferation.

(A and B) Representative figures of scratch-wound assay using rat VSMC at 24 h after treatment (A). FXa promoted the migration of this cell type, which was blocked in the presence of rivaroxaban (B). (n = 6 per group) **(C)** MTS proliferation assay using rat VSMC demonstrated that FXa promoted the proliferation of this cell type after 24 h of treatment. Rivaroxaban attenuated

FXa-induced proliferation. (n = 6 per group) NT: no treatment, FXa: activated factor X, Riv: rivaroxaban. *P<0.05, **P<0.01, and ***P<0.001. All values are mean ± S.E.M.

Table 1. Primer sequences.

	Forward	Reverse
PAR-1	5’-gtcttcccgcgtccctat-3’	5’-gggttcaccgtagcatctgt-3’
PAR-2	5’-ggaccgagaaccttgcac-3’	5’-gaacccctttcccagtgatt-3’
PAR-3	5’-ttctgccagtcactgtttgc-3’	5’-ggttggctttgctgagttgt-3’
PAR-4	5’-tgtagagagtaccaggggaagc-3’	5’-aggacttcggctccttgagt-3’
F4/80	5’-tgcatctagcaatggacagc-3’	5’-gccttctggatccatttgaa-3’
MCP-1	5’-ccactcacctgctgctactcat-3’	5’-tggtgatcctcttgtagctctcc-3’
IL-1β	5’-gcccatcctctgtgactcat-3’	5’-aggccacaggtattttgtcg-3’
TNF-α	5’-accctcacactcagatcatcttc-3’	5’-tggtggtttgctacgacgt-3’
TGF-β1	5’-ggagagccctggataccaac-3’	5’-cgcacacagcagttcttctc-3’
SDF-1	5’-gctctgcatcagtgacggta-3’	5’-taatttcgggtcaatgcaca-3’
GM-CSF	5’-atgcctgtcacgttgaatga-3’	5’-atatcttcaggcgggtctgc-3’
IL-6	5’-acaaccacggccttccctactt-3’	5’-cacgatttcccagagaacatgtg-3’
β-actin	5’-cctgagcgcaagtactctgtgt-3’	5’-gctgatccacatctgctggaa-3’

Table 2. Effects of rivaroxaban on metabolic parameters.

	Control (n=10)	Rivaroxaban (n=12)	P value
Body weight, g	24.8±0.4	25.2±0.4	0.40
Heart rate /min	706±14	677±13	0.63
Systolic BP, mmHg	111.8±2.2	108.2±2.0	0.15
Diastolic BP, mmHg	80.6±2.5	79.0±2.3	0.46
Plasma glucose, mg/dl	158.0±13.8	145.1±12.6	0.50
Triglyceride, mg/dl	62.4±6.7	66.2±6.1	0.68
Total cholesterol, mg/dl	89.3±2.6	89.8±2.4	0.90
HDL-C, mg/dl	49.7±2.2	48.8±2.0	0.77
LDL-C, mg/dl	27.1±2.2	27.7±2.0	0.85
Rivaroxaban, μg/l	N.D.	28.1±3.2	-

BP; blood pressure. HDL-C; high-density lipoprotein cholesterol. LDL-C; low-density lipoprotein cholesterol. N.D.: not detectable. All values are mean ± SEM.

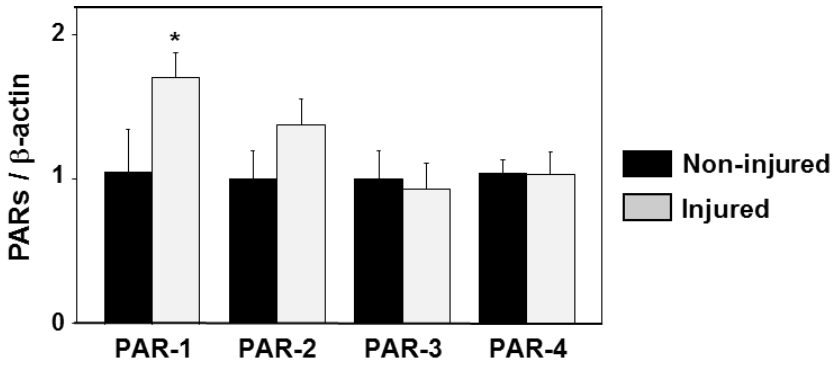


Fig. 1

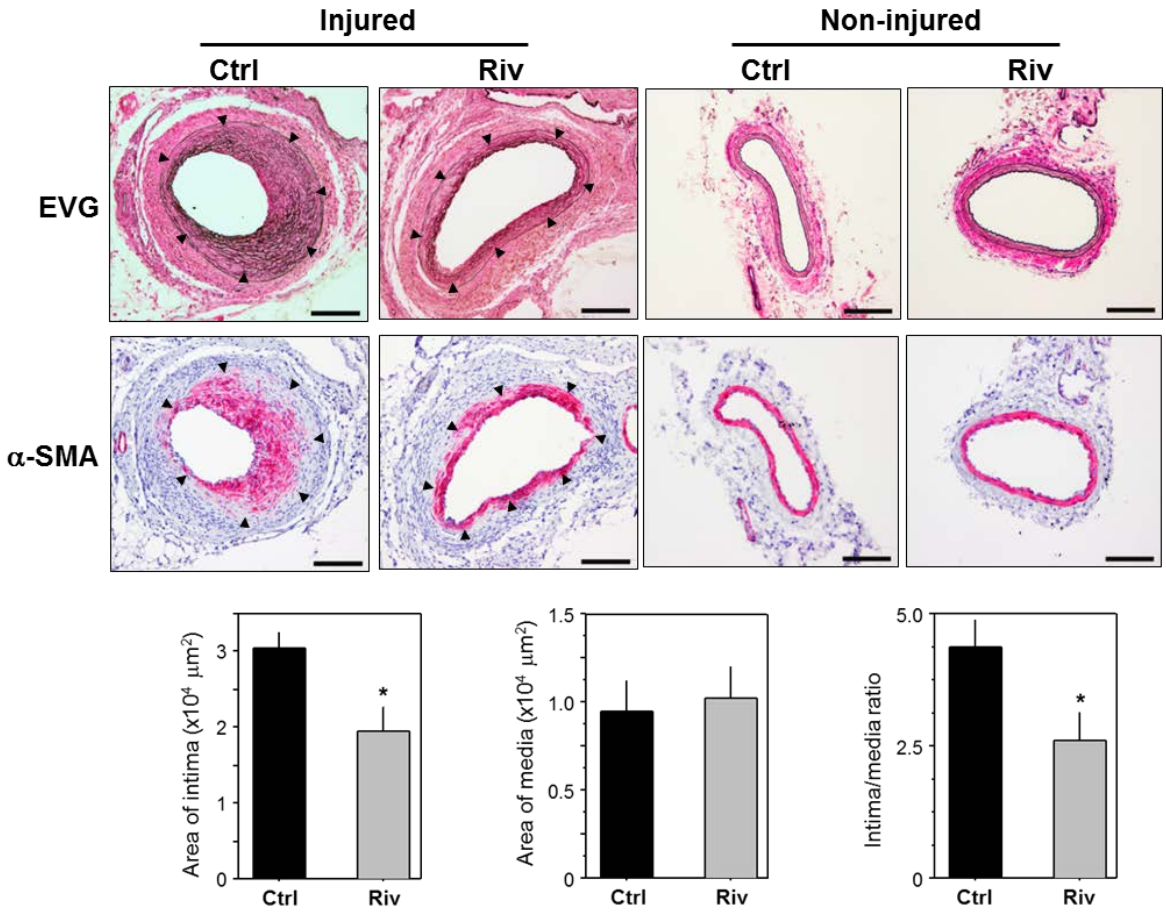
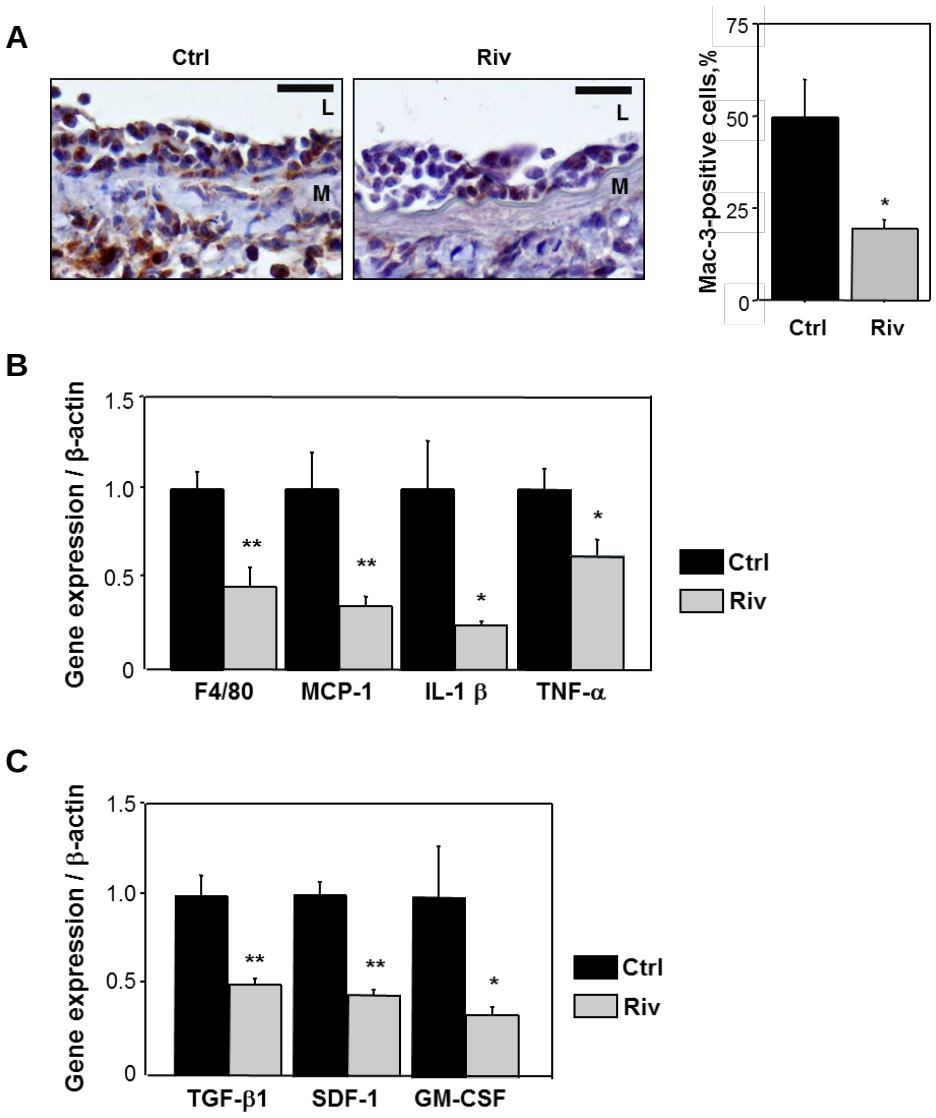

Injured
Non-injured
Ctrl
Riv
Ctrl
Riv
EVG
α-SMA
Area of intima (x10⁴ μm²)
Area of media (x10⁴ μm²)
Intima/media ratio
Ctrl
Riv
Ctrl
Riv
Ctrl
Riv
*
*

Fig. 2


A
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Riv
L
M
L
M
Mac-3-positive cells,%
75
50
25
0
*
Ctrl
Riv
B
Gene expression / β-actin
1.5
1.0
0.5
0
**
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*
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F4/80
MCP-1
IL-1 β
TNF-α
Ctrl
Riv
C
Gene expression / β-actin
1.5
1.0
0.5
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TGF-β1
SDF-1
GM-CSF
Ctrl
Riv

Fig. 3

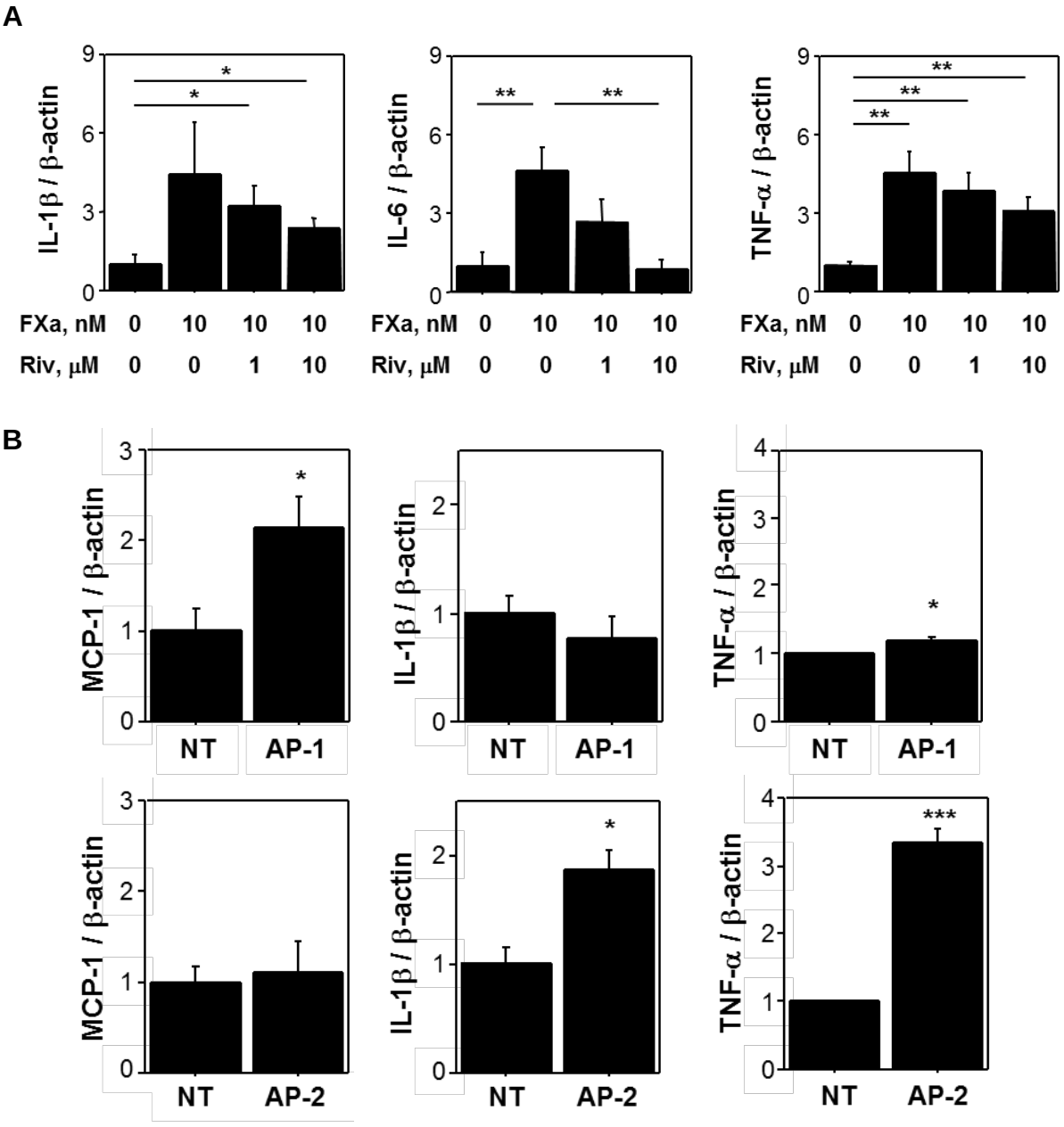
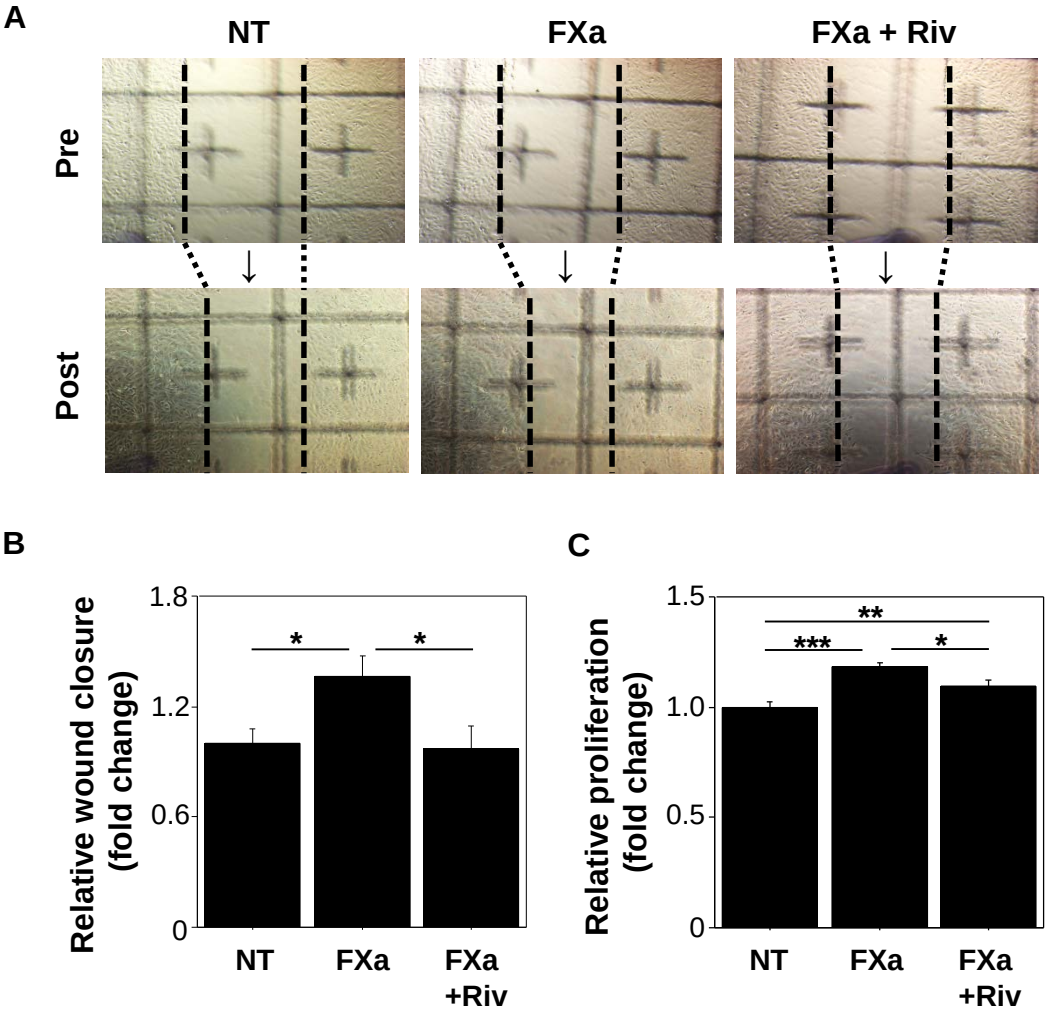

A
IL-1β / β-actin
IL-6 / β-actin
TNF-α / β-actin
FXa, nM 0 10 10 10
Riv, μM 0 0 1 10
B
MCP-1 / β-actin
IL-1β / β-actin
TNF-α / β-actin
NT
AP-1
AP-2

Fig. 4


A
NT
FXa
FXa + Riv
Pre
Post
B
Relative wound closure (fold change)
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1.2
0.6
0
*
*
NT
FXa
FXa +Riv
C
Relative proliferation (fold change)
1.5
1.0
0.5
0
**
***
*
NT
FXa
FXa +Riv

Fig. 5