Published in final edited form as:

J Vasc Res. 2016 ; 53(3-4): 163–171. doi:10.1159/000449059.

Direct Evidence for $P2Y_2$ Receptor Involvement in Vascular Response to Injury

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Abstract

Objectives—Extracellular nucleotide release at the site of arterial injury mediates proliferation and migration of vascular smooth muscle cells (SMC). Our aim was to investigate the role of the $P2Y_2$ nucleotide receptor ($P2Y_2R$) in neointimal hyperplasia.

Approach and Results—Vascular injury was induced by implantation of a polyethylene cuff around the femoral artery in wild-type and $P2Y_2$ receptor-deficient mice ($P2Y_2R^{-/-}$). Electron microscopy was used to analyze monocyte and lymphocyte influx to the intima 36 hours post-injury. Compared to wild-type (WT) littermates, $P2Y_2R^{-/-}$ mice exhibited a 3-fold decreased number of mononuclear leukocytes invading the intima ($p<0.05$). Concomitantly, migration of smooth muscle cells was decreased by more than 60% ($p<0.05$) a resulting in a sharp inhibition of intimal thickening formation in $P2Y_2R^{-/-}$ mice (n=15) 14 days after cuff placement. *In vitro*, loss of $P2Y_2$ receptor significantly impaired monocyte migration in response to nucleotide agonists. Furthermore, transgenic rats over-expressing the $P2Y_2R$ developed accelerated intimal lesions resulting in more than 95% luminal stenosis ($P<0.05$, n=10).

Conclusions—Loss-and gain-of-function approaches established a direct evidence for $P2Y_2$ receptor involvement in neointimal hyperplasia. Specific anti-$P2Y_2$ receptor therapies may be used against restenosis and bypass graft failure.

Introduction

Neointimal hyperplasia is a key feature of early atherosclerosis and restenosis after angioplasty.[1-2] Migration and proliferation of vascular smooth muscle cells (SMC) caused by injury are the underlying causes of intimal hyperplasia.[3] In experimental intimal hyperplasia, an influx of leukocytes precedes the migration of SMC to the intima.[4] Studies have shown that blockade of mononuclear leukocyte influx but not that of polymorphonuclear leukocytes prevent intimal accumulation of SMC in the collar model of injury[4-5], thus excluding neutrophils as initiators of the migration of SMC into the intima.

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Conflict of interest: None

Mononuclear cells have been shown to induce cytokine expression, vascular SMC proliferation, and arterial occlusion after endothelial injury.[6] Increases in tumor necrosis factor (TNF)-α levels precede migration of SMC into the intima in the balloon-injured rat aorta.[7] A more direct evidence for TNF-α involvement in intimal hyperplasia has been also established.[8]

Similar to cytokines, alterations in extracellular nucleotide concentrations in response to vascular stress conditions have been documented. Extracellular nucleotides are released in the vascular wall from perivascular nerves, activated platelets, and mechanically stretched cells.[9-12] Extracellular nucleotides induce both proliferation and migration of vascular SMC.[13-19] Mice deficient in CD39, the ectoenzyme which is mainly responsible for nucleotides degradation in vascular SMC, exhibit reduced intimal thickening in response to vascular injury.[20] Up-regulation and activation of the $P2Y_2$ receptor subtype has been shown to stimulate neointimal growth in the collared rabbit carotid artery.[21] In this model, local UTP delivery induced intimal accumulation of monocytes. In addition, nucleotide binding to $P2Y_2$ receptor stimulates the expression of vascular cell adhesion molecule-1, which suggests a role for UTP in early of leukocyte infiltration during vascular injury. However, the contribution of extracellular nucleotides and their receptors to intimal hyperplasia is still unclear.

Our aim was to establish a cause and effect relationship with respect to $P2Y_2$ receptor activation and neointimal hyperplasia. We used loss-and gain-of-function approaches to show that $P2Y_2$ receptor regulates early mononuclear lymphocyte influx to the intima in the cuff model of femoral artery injury. We demonstrated that mice lacking $P2Y_2$ receptor develop less neointimal area following arterial injury, whereas over-expression of $P2Y_2$ receptor led to the formation of accelerated intimal thickening in rats. These data unequivocally provide direct evidence for $P2Y_2$ receptor involvement in neointimal hyperplasia setting the stage for anti-$P2Y_2$ receptor therapies against graft failure and post-angioplasty restenosis.

Methods

Animals

Eight week-old wild-type and transgenic rats (Sprague-Dawley; Harlan, Indianapolis, IN) were used in this study. Transgenic rats over-expressing the $P2Y_2$ receptor were produced as previously described using a lentiviral vector system and bred for 10 generations to ensure stability of the transgene.[22] Wild-type and $P2Y_2R^{-/-}$ mice (C57BL/6) were purchased from Jackson laboratory and used at 8 weeks of age. Animal protocols were approved by the University of Missouri Animal Care and Use Committee.

Femoral artery cuff placement

Animals were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by intraperitoneal injection. The right femoral artery of the mouse was isolated from the surrounding tissues. A polyethylene cuff made of PE-50 tubing (inner diameter, 0.56 mm; outer diameter, 0.965 mm; Becton Dickinson, Mountain View, CA) was cut longitudinally to

open the tube, loosely placed around the artery and closed with suture. The cuff is larger than the vessel, therefore blood flow was not obstructed. After the experimental period, the mice were killed by an overdose of the anesthesia. The cuffed artery was removed and micro-dissected from the cuff.

Tissue harvesting and histologic staining

After sacrifice, femoral arteries were fixed in situ by constant pressure fixation at 100 mmHg with 10% formalin using a 22-gauge butterfly catheter placed in the left ventricle of the heart. Cuffed and sham-operated femoral arteries were then embedded in paraffin. Cross-sections (5μM thin) were continuously cut from one edge to the other edge of the cuffed and the control femoral artery and mounted in order on five series of slides. Cross sections were then stained with elastica van Gieson, hematoxylin and eosin or used for immunohistochemistry.

Morphometry

Hematoxylin and eosin–stained cross sections were used for morphometric analysis. For this purpose, serial cross-sections (5 μm thick) were obtained every 75 μm throughout the entire length of the cuffed mouse femoral artery. To quantify intimal thickening, 5 equally spaced cross-sections in each animal were photographed, and the images were digitized. The areas of the neointima, the media, and the adventitia were measured using an image analyzing software (NIH image) by an observer blinded as to the animal genotype or the treatment. Neointima was defined as the area between the vessel lumen and the internal elastic lamina. Media was defined as the area between the internal and external elastic lamina. Adventitia was defined as the area outside the external elastic lamina.

Incorporation and detection of 5-bromo-2′-deoxyuridine (BrdU)

Mice were injected intraperitoneally with BrdU (25mg/kg; Sigma) 3, 2, and 1 day before euthanasia. Immunohistochemistry was performed using anti-BrdU antibody following the manufacturer's instructions (Zymed Laboratories). The number of BrdU-positive nuclei were counted in 5 cross-sections and expressed as a percentage of the total number of nuclei.

Electron microscopy

Femoral arteries were removed from the cuff at the end of the experimental period and processed for transmission electron microscopy as previously described. [5] Ultrathin sections were collected on 75-mesh copper grids, stained with lead citrate, and examined with a Zeiss EM 10. Intimal cells (monocytes, lymphocytes, neutrophils, basophils, and eosinophils and smooth muscle cells were identified on the basis of their ultrastructural features as previously reported.[5] The number of intimal cells beneath the endothelium were determined for each cell type and expressed in relation to the number of endothelial cells overlying the lesion as previously described.[5]

Monocyte migration assay

Monocytes were is isolated from mouse blood using the EasySep™ mouse monocyte enrichment kit (StemCell Technologies) according to the manufacturer's instructions. Cell

migration assay was performed in a 48-well chemotaxis chamber (Neuro Probe Inc, Bethesda, MD) using polyvinylpyrrolidone-free polycarbonate filters with 5-μm diameter pores. The lower chamber contained RPMI 1640 medium, in the presence or absence (negative control) of stimulus, and the upper chamber contained the cell suspension (2×10^6 cells/ml). The chemoattractant peptide *N*-formyl-methionylleucyl-phenylalanine (fMLP) served as positive control. The chamber was incubated at for 1 hour at 37 °C in air containing 5% CO_2. At the end of the experiment, cells that have migrated were stained with hematoxylin. The number of migrated cells was determined after subtracting non-specific migration (negative control).

Statistical analyses

Data are presented as mean ± SEM. Statistical analysis was performed with Graphpad Prism using either two-tailed unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by post hoc comparison. Differences with P values <0.05 were considered significant.

Results

$P2Y_2$ receptor-deficiency reduces neointimal hyperplasia

To establish a cause-effect link between $P2Y_2R$ and intimal hyperplasia, we subjected wild-type (n=15) and $P2Y_2R^{-/-}$ mice (n=15) to cuff-induced injury of the femoral artery. We examined intimal lesion formation 14 days after injury, the time corresponding to a peak SMC accumulation of SMC. Figure 1A shows representative cross sections of the cuffed femoral arteries. Neointimal hyperplasia was uniformly distributed across the arterial lumen. $P2Y_2R^{-/-}$ mice developed 7-fold less neointimal area than corresponding WT littermates (Figure 1B). Sham-operated arteries did not exhibit neointimal thickening (Figure 1B). Comparison of intima-to-media ratios confirmed the reduction in neointimal size observed in $P2Y_2R^{-/-}$ mice (Figure 1C). The mean percent luminal stenosis was significantly higher in WT compared to $P2Y_2R^{-/-}$ mice (Figure 1D). Cell proliferation as assessed by BrdU incorporation into SMC 4 days after cuff placement was significantly suppressed in $P2Y_2R^{-/-}$ mice (Figure 1E). Intimal thickening in both groups were mainly composed of SM actin-positive cells (Figure 1F).

$P2Y_2$ receptor deletion inhibits mononuclear lymphocyte influx to the intima

Mononuclear leukocytes (monocytes and lymphocytes) not neutrophils mediate the subsequent migration of medial SMC to the intima following arterial injury.[4-5] We next asked if deletion of the $P2Y_2$ receptor affects the influx of mononuclear leukocytes to artery following arterial injury. Electron microscopy has been used to distinguish granulocytes, (i.e., neutrophils, basophils, and eosinophils) from monocytes and lymphocytes by their specific granules.[5] Because this method cannot always sharply distinguish between monocytes and lymphocytes, we therefore grouped lymphocytes and monocytes as mononuclear leukocytes as previously done.[5] In WT mice, a significant number of mononuclear lymphocytes invaded the nascent intimal thickening 36h following cuff implantation (Figure 2A). Notably, mononuclear lymphocytes influx after cuff placement

was significantly suppressed in $P2Y_2R^{-/-}$ mice (Figure 2A). These data clearly show that absence of $P2Y_2R$ blocks the influx of monocytes and lymphocytes during arterial injury.

Loss of $P2Y_2$ receptor *inhibits smooth muscle cell migration*

As previously reported in the cuff model of injury, SMC migration is the dominant factor contributing to early accumulation of SMC in the nascent intima.[4] We and others have shown that extracellular nucleotides acting through $P2Y_2R$ regulate SMC migration.[18-19, 23] Therefore, we asked if deletion of $P2Y_2R$ influences the migration of medial SMC to the intima. We identified SMC on the basis of their ultrastructural features using electron microscopy, and determined their number by quantitative analysis of ultrathin sections. SMC started to migrate from the underlying media into the intimal compartment 36h following cuff implantation. At this time point, the number of SMC that invaded the intima in $P2Y_2R^{-/-}$ mice was strikingly lower as compared to wild-type littermates (Figure 2B). In contrast, no SMC was detectable in the intima in sham-operated arteries from either genotype (Figure 2B). These data demonstrate that loss of $P2Y_2R$ inhibits early migration of SMC to the intima.

Extracellular nucleotides acting through $P2Y_2$ receptor promote monocyte migration

To elucidate the mechanism by which $P2Y_2R$ regulates mononuclear leukocyte influx, we tested whether $P2Y_2$ receptor agonists promote monocyte migration. Using a chemotaxis assay, we found that UTPγS, a slowly hydrolysable analog of the $P2Y_2R$ promotes migration of monocyte from wild-type mice in a dose-dependent manner (Figure 3). In contrast, $P2Y_2$ receptor deficiency significantly impaired monocyte migration in response to UTPγS (Figure). ATP, another potent agonist of $P2Y_2R$ also induced the migration of monocytes from WT but not from $P2Y_2R^{-/-}$ mice (data not shown), confirming the involvement of $P2Y_2R$ in monocyte migration. As expected, monocyte migration in response to fMLP was similar between wild- type and $P2Y_2R^{-/-}$ mice (Figure 3).

Over-expression of $P2Y_2R$ accelerates neointimal hyperplasia

We generated the first transgenic rat over-expressing a purinergic receptor.[22] We next used this gain of function approach to test if over-expression of $P2Y_2R$ promotes neointimal hyperplasia. Wild- type and $P2Y_2R$ transgenic rats were subjected to femoral artery injury and intimal lesions were assessed at day 14. WT animals developed a modest intimal thickening (Figure 4A-B) consisting of α-SMC actin-positive cells (not shown). Rats over-expressing the $P2Y_2R$ transgene exhibited a dramatic increase in neointimal area compared to WT littermates (Figure 4A-B), whereas sham-operated arteries did not exhibit intimal thickening (Figure 4B). There was no significant difference in the medial area between wild-type and transgenic rats. As expected, the intima/media ratio was greater in the transgenic rats compared to wild-type controls (Figure 4C). The mean percent luminal stenosis in the transgenic rats was over 95% ($p<0.001$; Figure 4D), resulting in near blockade of the arterial lumen. These results further confirm that the $P2Y_2R$ is involved in neointimal hyperplasia.

Discussion

Several lines of evidence support a role for extracellular nucleotides in the response to vascular injury. We first reported that $P2Y_2R$ is over-expressed in intimal lesions of the rat carotid artery.[23] In addition, local delivery of nucleotides via a mini-osmotic pump to the collared rabbit carotid artery accelerates the development of intimal hyperplasia.[21] Furthermore, mice deficient in CD39 (the ectoenzyme which is mainly responsible for nucleotides degradation) exhibit reduced intimal thickening in response to vascular injury[20], thus confirming the crucial role of extracellular nucleotides in the development of intimal hyperplasia. However, given that purinergic receptors are ubiquitously expressed in vascular cells, it was necessary to determine if the $P2Y_2R$ is directly involved in the vascular response to injury. In this study, we used loss-and gain-of-function approaches to directly assess the contribution of $P2Y_2R$ to neointimal hyperplasia. We demonstrated that $P2Y_2R$ deficiency decreased neointimal area induced by placement of a cuff around the mouse femoral artery. Conversely, over-expression of $P2Y_2R$ in rats accelerated neointimal hyperplasia resulting in near complete arterial luminal stenosis.

The $P2Y_2R$ is expressed in virtually all blood-derived cells.[12] It was reported that blockade of mononuclear leukocyte influx but not that of neutrophils decreased intimal accumulation of SMC in the femoral artery cuff model of vascular injury[4-5], thus excluding neutrophils as initiators of the migration of SMC into the intima. Activation of $P2Y_2R$ stimulates monocyte recruitment in the collared rabbit carotid artery.[21] Mechanistically, we found that absence of $P2Y_2R$ significantly impaired mononuclear lymphocyte influx to the intima. In addition, monocyte lacking $P2Y_2R$ failed to migrate in response to nucleotide agonists, demonstrating the importance of monocyte $P2Y_2R$ in the early response to vascular injury. Stimulation of P2 receptors is coupled to the release of the pro-inflammatory cytokines interleukins[24-27] that are of obvious relevance to the development of neotimal hyperplasia. We showed that $P2Y_2R$ activation regulates the secretion of lymphotoxin-α (member of the TNF ligand family) in murine monocytes.[28] Interestingly, $ApoE^{-/-}$ mice deficient in $P2Y_2R$ exhibit decreased production of lymphotoxin-α and delayed onset of atherosclerosis. Furthermore, administration of lymphotoxin-α neutralizing antibody also prevents lesion formation in $ApoE^{-/-}$ mice.[29] These data suggest expressed in monocyte $P2Y_2R$ contributes to neointimal hyperplasia at least by modulating the secretion of cytokines that can directly influence SMC proliferation.

Ectoenzymes that metabolize ATP play important roles in regulating the response to endothelial dysfunction. In many cell types, including endothelial cells, nucleoside triphosphate diphosphohydrolase-1/CD39 and ecto-5'-nucleotidase/CD73 dephosphorylate ATP and ADP to AMP. AMP is then converted to adenosine by CD73 or other enzymes. The importance of this extracellular purinergic flux in controlling inflammation is apparent in mice genetically engineered to lack CD39.[30-31] In these animals, activation of inflammatory cells such as platelets, macrophages, and neutrophils, and their adhesion to the vascular endothelium, are enhanced. Mice deficient in CD39, exhibit reduced intimal thickening in response to vascular injury.[20] The $P2Y_2R$ is distinguished among G protein-coupled receptors in its ability to interact directly with growth factor receptors to transactivate their signal transduction pathways. For example, we have shown the importance of $P2Y_2R$

interaction with vascular endothelial cell growth factor receptor (VEGFR-2) in the regulation of VCAM-1 expression.[32] Since VCAM-1 plays a crucial role in both atherosclerosis and neointimal hyperplasia, targeted control of endothelial $P2Y_2R$ could prevent intimal hyperplasia that accompanies these processes.

Extracellular nucleotides acting via G protein-coupled $P2Y_2$receptors serve as potent stimulators for migration SMC *in vitro*.[18-19] Here, we demonstrated that $P2Y_2R$ deficiency prevent the early migration of SMC to the intima in response to cuff-induced injury. ATP stimulates release of matrix metalloproteinase-2 (MMP-2) from human aortic SMC[33], and MMP-2-induced matrix degradation facilitates migration and proliferation of SMC.[34] Interestingly, both ATP and UTP are potent chemotactic agents that stimulate VSMC migration via $P2Y_2R$.[18-19] We have shown that $P2Y_2$ receptor interacts with the actin-binding protein filamin A to mediate SMC migration.[35] Altogether, these mechanisms may explain our findings that perivascular infusion of UTP into the collared rabbit carotid arteries enhanced neointimal development[21] and $P2Y_2R$ expression is associated with coronary in-stent restenosis.[36] Abundant sources for extracellular ATP in the vessel and the trophic effects on vascular SMC suggest that SMC $P2Y_2R$ might contribute to intimal hyperplasia by stimulating proliferation of vascular SMC. In support of this idea, a significant number of BrdU-positive SMC were detected in the cuffed artery of wild-type mice. In contrast, the percentage of proliferating cells was strikingly lower in $P2Y_2R^{-/-}$ mice. Subsequently, we found that wild-type mice exhibited 7-fold more neointimal area compared to $P2Y_2R^{-/-}$ mice. Furthermore, over-expression of the $P2Y_2$ receptor drastically increased intimal hyperplasia causing a near complete blockade of the arterial lumen only 14 days after injury. Thus, we conclude that $P2Y_2R$ contributes to neointimal hyperplasia by stimulating both migration proliferation of vascular SMC. In summary, we demonstrated that $P2Y_2$ receptor directly regulates the influx of mononuclear lymphocytes that are essential for subsequent migration and accumulation of SMC into the neointima. Therefore, this nucleotide receptor might be a therapeutic target for the prevention of graft failure and post-angioplasty restenosis.

Acknowledgment

This work was supported by a grant from the National Institute of Health (1R01HL112883) to Cheikh I. Seye

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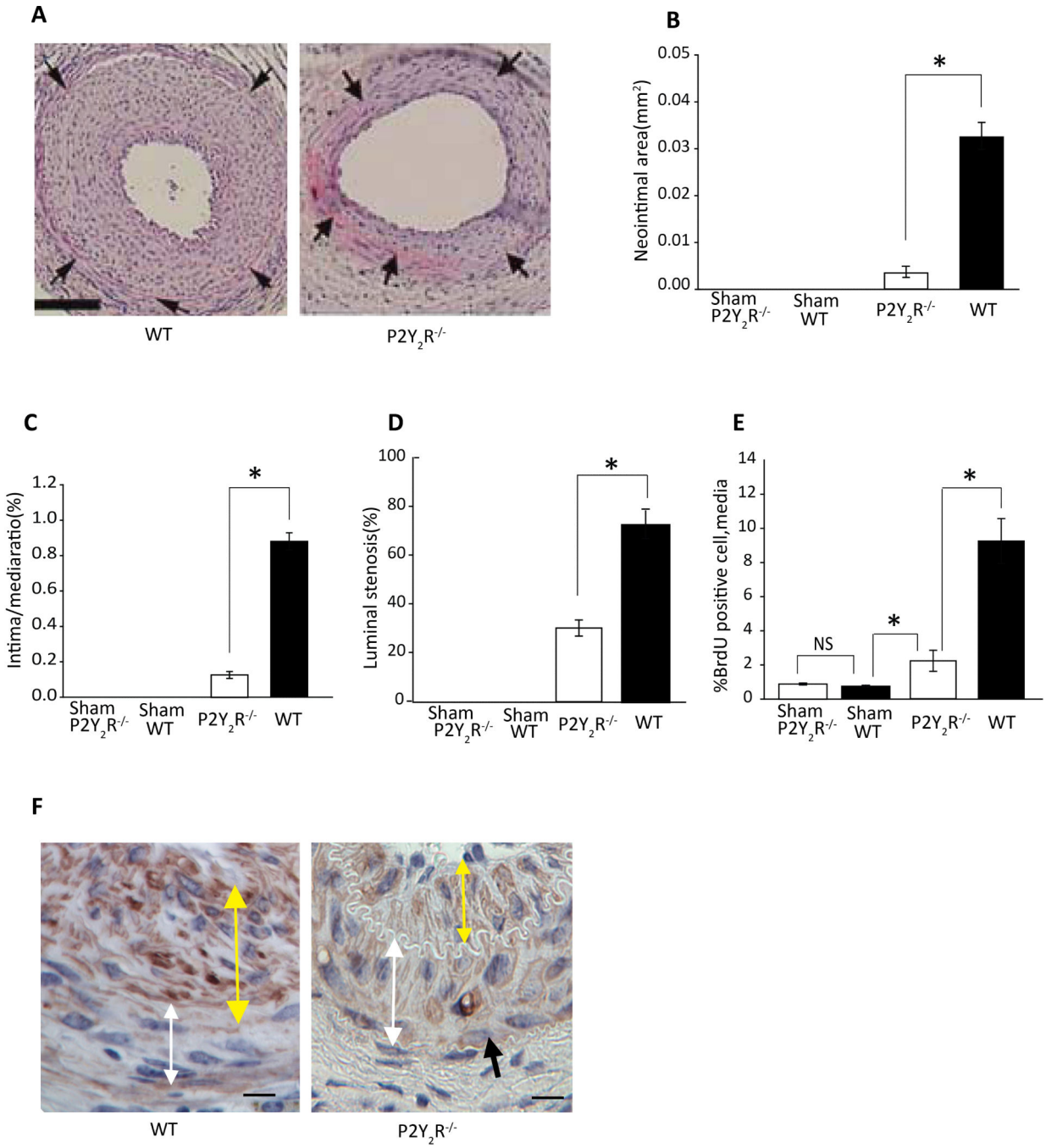

Figure 1.

Morphometric analysis of cuffed femoral arteries in wild-type (WT) and $P2Y_2$ receptor-deficient ($P2Y_2R^{-/-}$) mice. A, representative images of hematoxylin-eosin staining of femoral arteries 14 days after cuff placement. Black arrows indicates internal elastic laminae, white and yellow arrows delineate the media and the intimal thickening, respectively. Scale bar represents 25μm. B, Cross-sectional intimal surface area of femoral arteries in WT and $P2Y_2R^{-/-}$ mice. The intima to media ratio was measured in panel C and luminal stenosis (D) was calculated as the percentage of the area inside the internal elastic

lamina occupied by the intimal area (100 × IA/IELA). E, smooth muscle cell proliferation (% of BrdU-positive cells). Data are mean ± SEM. *P<0.05, one-way ANOVA with Tukey's multiple comparison post analysis. F, Immunohistochemical staining of smooth muscle α - actin in injured femoral arteries from representative WT and $P2Y_2R^{-/-}$ mice 14 days after cuff-induced injury. White and yellow arrows delineate the media and the intimal thickening, respectively. Scale bar represents 25μm.

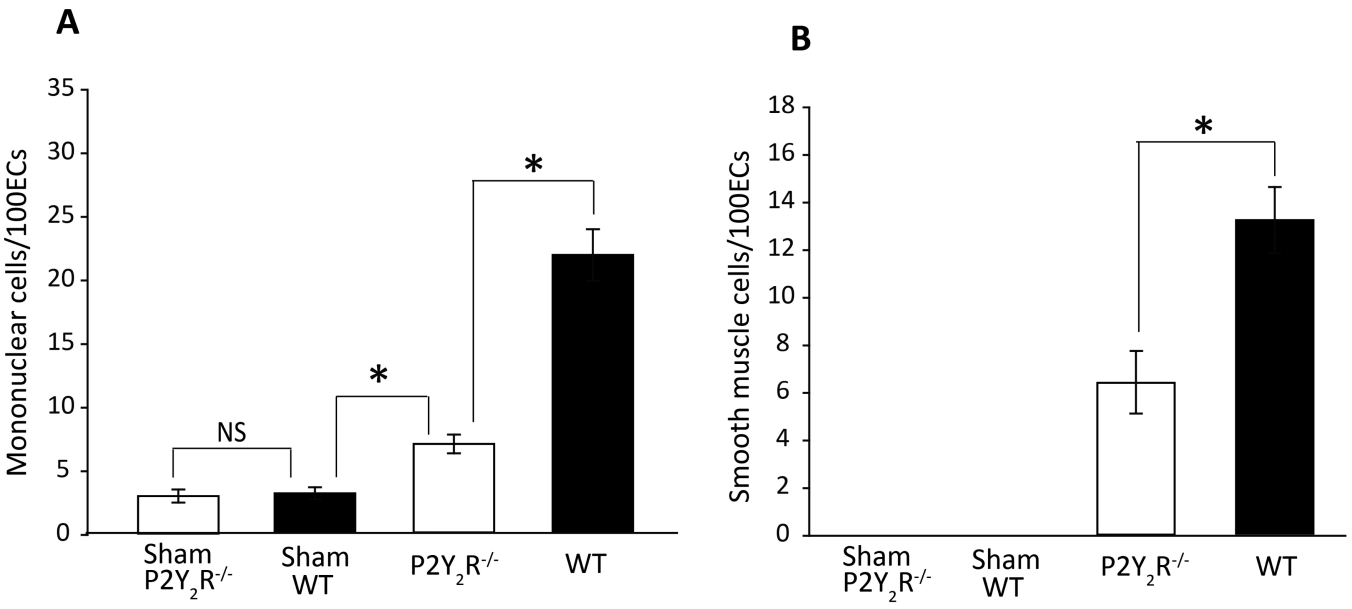

Figure 2.

Intimal accumulation of mononuclear leukocytes and smooth muscle cells. Bar graph showing accumulation of mononuclear leukocytes (right) and smooth muscle cells (left) within the intimal lesion of WT and $P2Y_2R^{-/-}$ mice. For quantitative analysis, ultrathin cross sections through the midregion of the cuff-bearing artery segment were selected from a minimum of four different planes separated by at least 0.1 mm as previously described.[4] The number of intimal cells beneath the endothelium were determined for each cell type and expressed in relation in relation to 100 endothelial cells. Data are mean ± SEM. *P<0.05, one-way ANOVA with Tukey's multiple comparison post analysis.

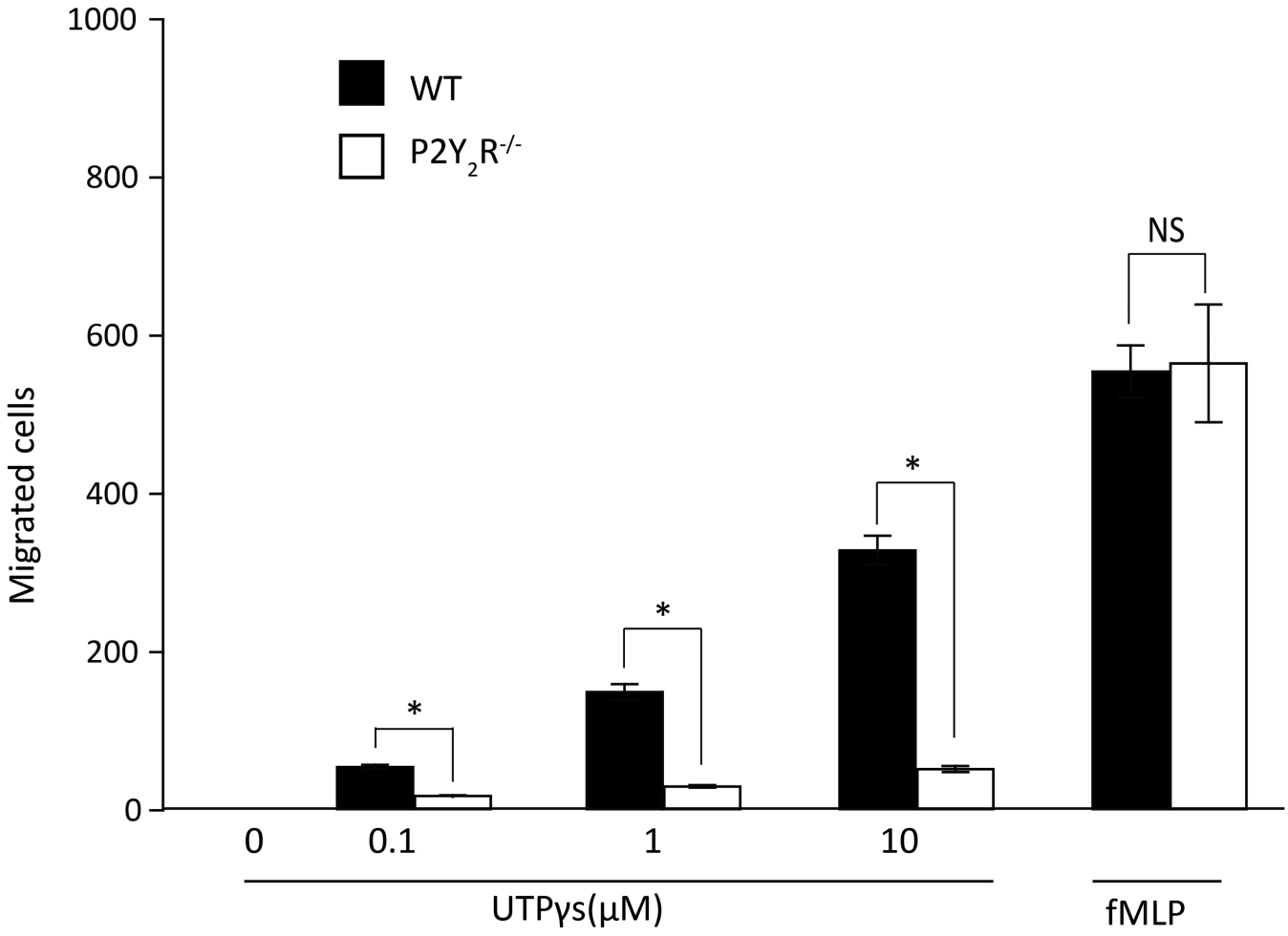

Figure 3.

Monocyte migration in response to nucleotide stimulation. Migration assays were performed in modified Boyden chambers. Increasing concentrations of nucleotides were added to the lower well as indicated. The chemoattractant peptide fMLP was used as a positive control. Cell migration assay was performed in a 48-well chemotaxis chamber, and cells that have migrated were stained with hematoxylin. The number of migrated cells was determined after subtracting non-specific migration (negative control). Five independent experiments were performed. Data are mean ± SEM. *P<0.05, one-way ANOVA with Tukey's multiple comparison post analysis.

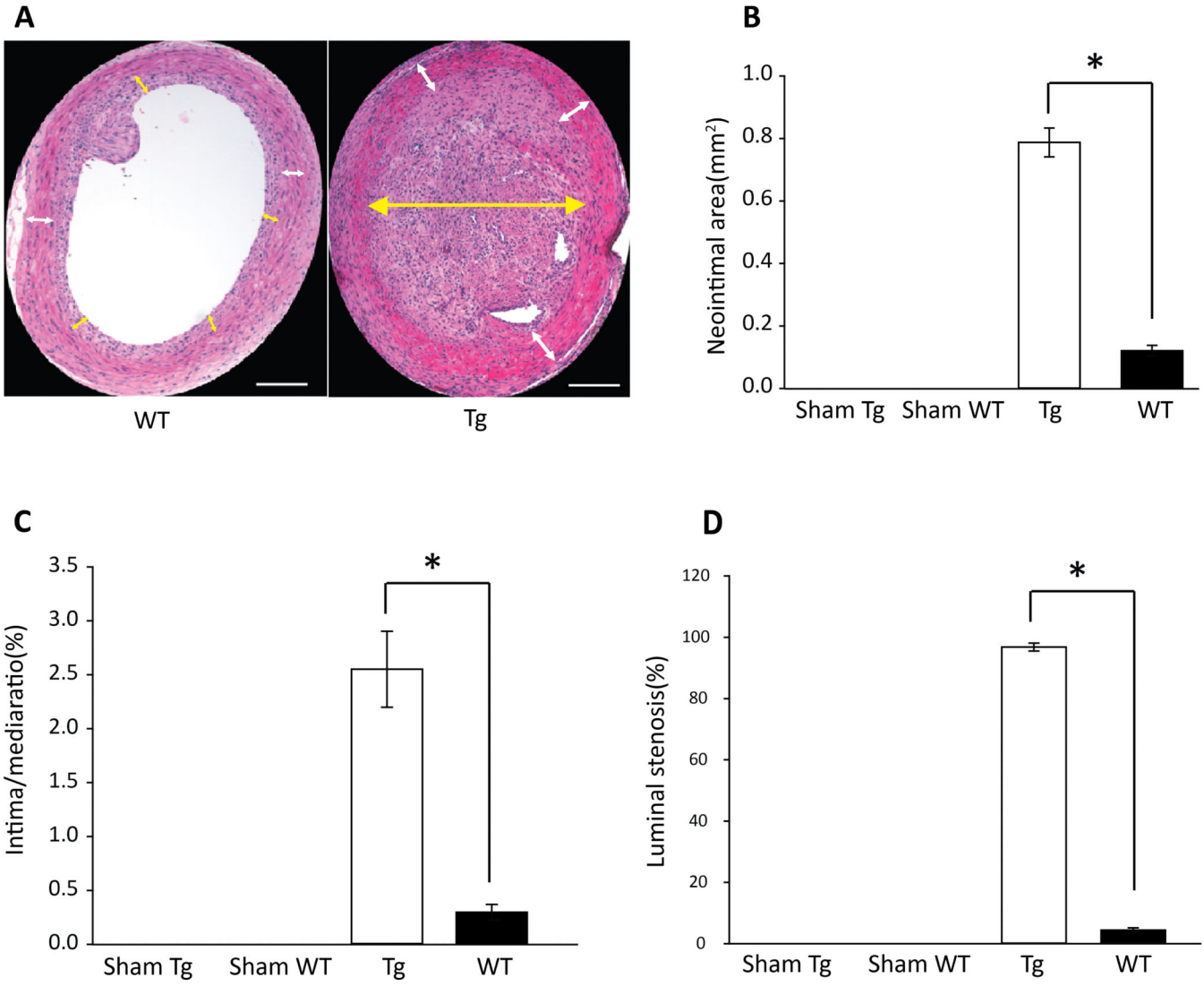

Figure 4.

Morphometric analysis of cuffed femoral arteries in wild-type and transgenic rats over-expressing the $P2Y_2$ receptor. A, representative images of hematoxylin eosin-stained cross section of injured femoral artery of WT and $P2Y_2R$ over-expressing rats 14 days after cuff placement. White and yellow arrows delineate the media and the intimal thickening, respectively. Scale bar represents 25μm. B, Cross-sectional intimal surface area of femoral arteries in WT and $P2Y_2R$ over-expressing rats. Quantitative computer-assisted image analysis of lesions was performed at day 14. Five μm-thick serial cross-sections were obtained every 100 μm throughout the entire length of the cuffed mouse femoral artery. To quantify neointimal area, 5 equally spaced cross-sections in each animal (n=10) were photographed, and the images were digitized. Intima/media ratio (C) and percentage of luminal stenosis (D) were calculated as described in Figure 1. Values are mean ± SEM. *P<0.05, one-way ANOVA with Tukey's multiple comparison post analysis.

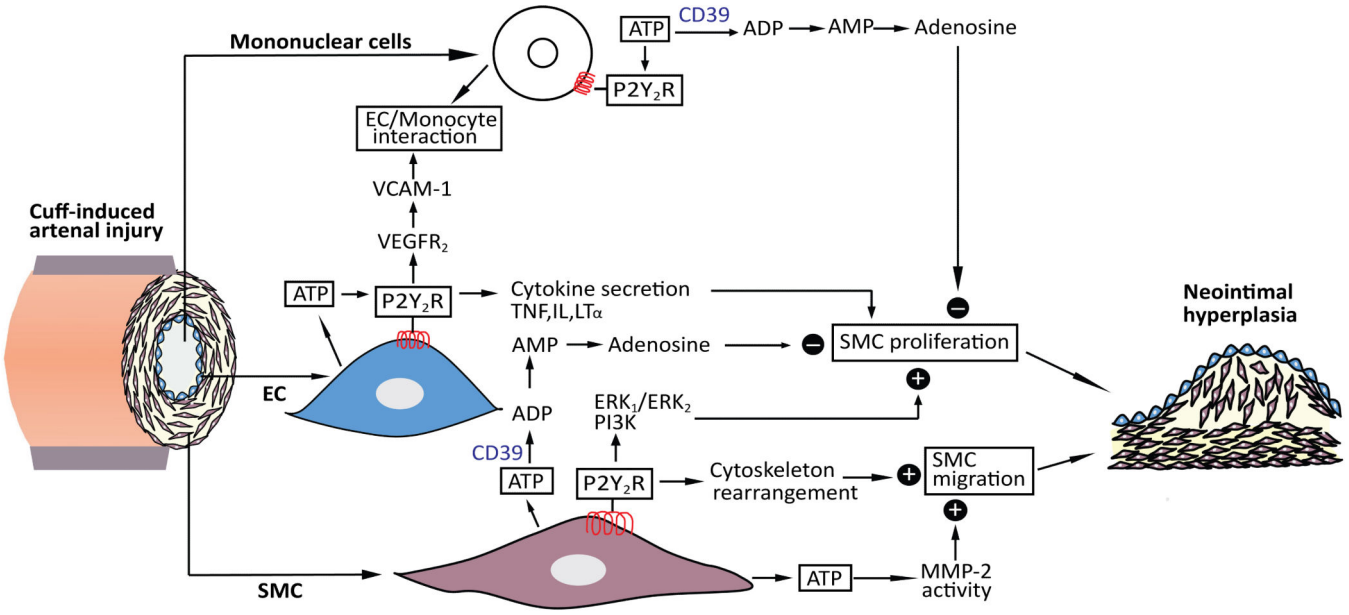

Figure 5.

Schematic depicting the role of $P2Y_2$ receptor in vascular response to injury. Mononuclear cells: ATP release during cuff-induced vascular injury activates $P2Y_2R$ in monocytic cells leading to cytokine secretion which in turn stimulates SMC migration and proliferation. Adenosine generated through degradation of ATP initiated by the ectoenzyme CD39 acts as inhibitor of SMC proliferation. Endothelial cells (EC): in endothelial cells, ATP acting through $P2Y_2R$ stimulates VCAM-1 expression via phosphorylation of VEGFR2 receptor. VCAM-1 which enables interaction between EC and monocytes plays a pivotal role in neointimal hyperplasia. Smooth muscle cells: $P2Y_2R$ activation in SMC mediates proliferation through ERK1/ERK2 and Phophosinositide 3-kinase (PI3-K) mechanisms. ATP acting on $P2Y_2R$ causes cytoskeleton rearrangement and matrix metalloproteinase (MMP-2) activation leading to SMC migration.