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Endothelial Cell-Specific Deletion of $P2Y_2$ Receptor Promotes Plaque Stability in Atherosclerosis-Susceptible ApoE-Null Mice

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Abstract

Objective—Nucleotide $P2Y_2$ receptor ($P2Y_2R$) contributes to vascular inflammation by increasing vascular cell adhesion molecule (VCAM)-1 expression in endothelial cells (EC), and global $P2Y_2R$ deficiency prevents fatty streak formation in $ApoE^{-/-}$ mice. Since $P2Y_2R$ is ubiquitously expressed in vascular cells, we investigated the contribution of endothelial $P2Y_2R$ in the pathogenesis of atherosclerosis.

Approach and Results—EC-specific $P2Y_2R$-deficient mice were generated by breeding VEcadherin5-Cre mice with the $P2Y_2R$ "floxed" mice. Endothelial $P2Y_2R$ deficiency reduced eNOS activity and significantly altered ATP-and UTP-induced vasorelaxation without affecting vasodilatory responses to acetylcholine. Telemetric blood pressure and echocardiography measurements indicated that EC-specific $P2Y_2R$-deficient mice did not develop hypertension. We investigated the role of endothelial $P2Y_2R$ in the development of atherosclerotic lesions by crossing the EC-specific $P2Y_2R$ knockout mice onto an $ApoE^{-/-}$ background and evaluated lesion development after feeding a standard chow diet for 25 weeks. Histopathological examination demonstrated reduced atherosclerotic lesions in the aortic sinus and entire aorta, decreased macrophage infiltration and increased smooth muscle cell and collagen content leading to the formation of a subendothelial fibrous cap in EC-specific $P2Y_2R$-deficient $ApoE^{-/-}$ mice. Expression and proteolytic activity of matrix metalloproteinase (MMP)-2 was significantly reduced in atherosclerotic lesions from EC-specific $P2Y_2R$-deficient $ApoE^{-/-}$ mice. Furthermore, EC-specific $P2Y_2R$ deficiency inhibited NO production leading to significant increase in SMC migration out of aortic explants.

Conclusions—EC-specific $P2Y_2R$-deficiency reduces atherosclerotic burden and promotes plaque stability in $ApoE^{-/-}$ mice through impaired macrophage infiltration acting together with reduced MMP-2 activity and increased SMC migration.

Graphical Abstract

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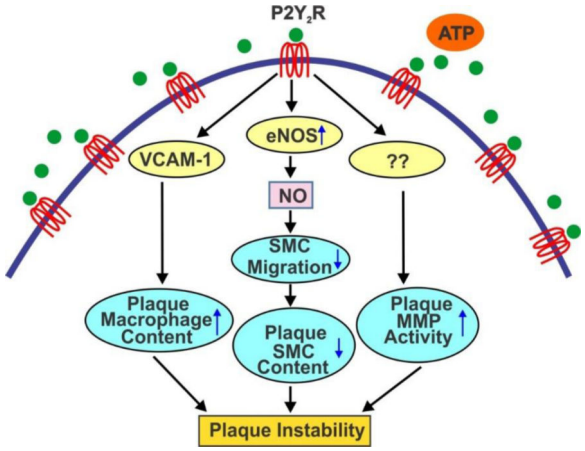
Disclosures: None



Keywords

Adenosine 5'-trisphosphate; Atherosclerosis; Endothelial cell; Receptor; Smooth muscle cell

Introduction

Endothelial dysfunction and monocyte trans-endothelial migration have been recognized as integral components in the initiation and progression of atherosclerosis (1). $P2Y_2$ receptor ($P2Y_2R$) activation *in vivo* enhances neointimal hyperplasia in response to vascular injury (2).We recently reported that $P2Y_2R$ deficiency prevents fatty streak lesion formation in $ApoE^{-/-}$ mice (3). During atherosclerosis, inflammation-induced endothelial barrier dysfunction promotes lipoprotein and leukocyte transendothelial flux in the vascular wall (4). Extracellular nucleotides acting on $P2Y_2R$ regulate the expression of VCAM-1 (5), one of the earliest molecular events in the pathogenesis of atherosclerosis (6). These data suggest a prominent role for endothelial $P2Y_2R$ in the formation of atherosclerotic lesions. However, given that this nucleotide receptor is ubiquitously expressed in vascular and blood-derived cells (7-10), the relative contribution of endothelial $P2Y_2R$ to the pathogenesis of atherosclerosis is unclear. We hypothesized that endothelial cell $P2Y_2R$ contributes to atherosclerosis by conferring an endothelial inflammatory phenotype and promoting monocyte infiltration. In the present study, we have generated EC-specific $P2Y_2R$-deficient mice on an $ApoE^{-/-}$ background, and characterized the functional contribution of endothelial $P2Y_2R$ to the development of atherosclerosis.

We determined that in $ApoE^{-/-}$ mice, loss of endothelial cell $P2Y_2R$ inhibited the progression of atherosclerosis and increased SMC and collagen content in atherosclerotic plaques resulting in the formation of a subendothelial fibrous cap. Mechanistically, we showed that endothelial $P2Y_2R$ deficiency decreased the proteolytic activity of matrix metalloproteinase MMP-2 and inhibited NO production leading to significant increase in SMC migration. Our findings suggest that targeted inhibition of this nucleotide receptor may increase plaque stability.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Generation of mice with endothelial-specific deletion of $P2Y_2$ receptor

A conditional "floxed" allele of the murine $P2Y_2R$ genomic locus was generated by flanking the coding region of the $P2Y_2R$ gene with loxP sites (Fig. 1A). The $P2Y_2R^{fl/fl}$ mice were bred to cadherin 5-Cre (Cre) transgenic mice to generate $_{EC}P2Y_2R$-KO ($P2Y_2R^{fl/fl}$ $Cre^{-/+}$) and control $P2Y_2R^{fl/fl}$ mice. All mice are on a C57BL/6 background. Immunostaining of the mouse aorta with antibodies against $P2Y_2R$ detected the presence of $P2Y_2R$ in both EC and SMC in control mice (Fig. 1A). As expected, $P2Y_2R$ was expressed in SMC but not in EC in the aorta of $_{EC}P2Y_2R$-KO mice (Fig. 1B) whereas, no $P2Y_2R$-positive staining was detectable in the aorta of total body $P2Y_2R$ knockout mice (Fig. 1B). Western blot analysis of lysates from primary EC cultures further confirmed endothelial-specific knockout of the $P2Y_2R$ (Fig. 1C). In addition, endothelial deletion of $P2Y_2R$ did not result in $P2Y_2R$ deletion from peripheral blood mononuclear cells (Fig. 1C). The $_{EC}P2Y_2R$-KO mice and controls were born at the expected Mendelian ratios, and exhibited no obvious developmental defects. These data indicated that the $P2Y_2R$ is efficiently and specifically deleted in the endothelial compartment of $_{EC}P2Y_2R$-KO mice.

Endothelial $P2Y_2$ receptor mediates nucleotide-induced vasodilation

As extracellular nucleotides evoke endothelium-dependent relaxation in the mouse aorta (11) we used the $_{EC}P2Y_2R$-KO mice to clarify the role of the $P2Y_2R$ in this process. Aortic ring constrictions in response to KCl or phenylephrine (PE) were similar between control and $_{EC}P2Y_2R$-KO mice (Fig. 2A). Acetylcholine-induced dilations of PE preconstricted aortic arch rings were also not significantly different between the two groups (Fig. 3A). However, the vasodilatory responses evoked by ATPγS were significantly smaller in the $_{EC}P2Y_2R$-KO mice compared to controls (56.5 ± 3.8%, n=8 in controls vs 19.2 ± 10.0%, n=5 in $_{EC}P2Y_2R$-KO; Fig. 2B). UTPγS-induced dilations were also significantly impaired in the $_{EC}P2Y_2R$-KO aortic rings (80.4 ±8.4%, n=7 in controls vs. 43.6 ± 13.7%, n=5 in $_{EC}P2Y_2R$-KO; Fig. 2B). Aortic rings in control and $_{EC}P2Y_2R$-KO mice showed complete relaxation in response to the $P2Y_6$ receptor agonist UDP (56.5 ± 7.7%, n=9 in controls vs 66.4 ± 11.5%, n=6 in $_{EC}P2Y_2R$-KO; Fig. 2C). Surprisingly, the $P2Y_1R$ agonist ADP, even at the highest concentration of 10 μM, dilated neither control nor $_{EC}P2Y_2R$-KO aortic rings (Fig. 2C). These data together with the normal vasodilatory responses to Ach demonstrate that although the $P2Y_2$-deficiency mice have reduced eNOS activity, this is not sufficient to result in systemic arterial hypertension.

Endothelial $P2Y_2$ receptor deletion attenuates eNOS activity *in vivo* but does not result in hypertension

Decreased endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production are critical contributors to endothelial dysfunction (12). Interestingly, extracellular nucleotides have also been implicated in activation of eNOS, leading to NO

generation and vasodilation (13). To explore the mechanisms underlying the reduction of endothelium-dependent relaxation in response to nucleotides, we therefore examined the effect of $_{EC}P2Y_2R$ deletion on eNOS expression and activity. Western blot analysis demonstrated a significant (**$p<0.01$) decrease in eNOS protein levels in the $_{EC}P2Y_2R$KO mouse aorta compared to control mice (Fig, 2D). The decreased eNOS protein expression correlated with a decrease in plasma nitrate levels in $_{EC}P2Y_2R$-KO mice (Fig. 2E, (**$p<0.01$). A tamoxifen-inducible endothelium-specific $P2Y_2$-deficient mouse (Tie2-CreER^{T2} $P2Y_2R^{fl/fl}$) has been previously reported to develop systemic hypertension (14). Blood pressure measurements were thus recorded in our $_{EC}P2Y_2R$-KO mice to determine if they also develop hypertension. Radio-telemetry monitoring of blood pressure and heart rate showed no difference between $_{EC}P2Y_2R$-KO and control mice (Table 1). Likewise, acute blood pressure measurements showed no difference between control and $_{EC}P2Y_2R$-KO mice (Table 2). Furthermore, transthoracic echocardiography revealed no significant difference in right ventricle diameter or thickness in 5 month-old $_{EC}P2Y_2R$-KO mice relative to control mice (Table 3), indicating that these mice did not develop pulmonary hypertension. Likewise, left ventricle thickness and diameter were similar in mice of both genotypes. Additional parameters of LV function (% fractional shortening, mitral and aortic velocities) are also similar between groups (data not shown).The blood pressure data coupled with the lack of hypertrophy in both ventricles indicates a lack of systemic hypertension in $_{EC}P2Y_2R$-KO mice. Together, these data demonstrate that endothelial $P2Y_2R$-deficiency strongly reduces eNOS activity *in vivo* resulting in reduced NO formation which was not accompanied by arterial hypertension.

Loss of endothelial $P2Y_2$ receptor reduces atherosclerotic lesions in ApoE$^{-/-}$ mice

Endothelium-dependent vasodilation is involved in atherosclerotic lesion formation (1). To examine the contribution of endothelial cell $P2Y_2R$ to atherosclerosis, we generated atherosclerosis-susceptible mice with endothelial-specific deletion of $P2Y_2R$. To do this, we crossed both the $P2Y_2R^{fl/fl}$ mice and the Cad5Cre mice onto an ApoE$^{-/-}$ background to generate $P2Y_2R^{fl/fl}$ ApoE$^{-/-}$ Cad5Cre$^{+/-}$ (referred to as $_{EC}P2Y_2R$/ApoE$^{-/-}$) experimental mice and $P2Y_2R^{fl/fl}$ ApoE$^{-/-}$ Cad5Cre$^{-/-}$ controls (referred as to control/ApoE$^{-/-}$). The mice were maintained on a standard chow diet for 25 weeks and analyzed for lipoprotein and atherosclerotic lesion characteristics. The two groups of mice did not differ significantly in body weight, total plasma cholesterol, or fasting lipoprotein profile (Table 3). En face aortic preparations stained with oil-red-O indicate a significant (*$p<0.05$) reduction in total plaque area in $_{EC}P2Y_2R$/ApoE$^{-/-}$ mice as compared to control/ApoE$^{-/-}$ (Fig. 3A). Cross sections of aortic sinus specimens stained with Mason's trichrome showed a 40.6% decrease in the mean lesion area in the aortic root of $_{EC}P2Y_2R$/ApoE$^{-/-}$ mice compared to control/ApoE$^{-/-}$ mice (0.320 ±0.019 μm^2 , n=5 vs. 0.193 ± 0.009 μm^2, n=6 respectively; (**$p<0.01$); Fig. 3A). These results demonstrate that endothelial deletion of $P2Y_2R$ reduces atherosclerotic plaque burden.

$P2Y_2$ receptor deficiency attenuates inflammation and endothelial barrier dysfunction

We next determined the effect of endothelial cell $P2Y_2R$ deficiency on the cellular composition of atherosclerotic lesions. Immuno-staining with the anti-macrophage antibody Mac-3 revealed that macrophage plaque content in the aortic root lesions of $_{EC}P2Y_2R$/

ApoE$^{-/-}$ mice was reduced by more than 66% as compared with control mice (***p<0.001) Fig. 3B-C). To determine the mechanisms underlying the reduced macrophage plaque content in $_{EC}$P2Y$_2$R/ApoE$^{-/-}$ mice, we examined the effect of endothelial P2Y$_2$R deletion on the transmigration of peripheral blood monocytes across endothelial monolayers. As shown in Figure 3D, monocyte chemoattractant protein (MCP)-1 increased monocyte transmigration across endothelial monolayers from control P2Y$_2$R $^{fl/fl}$ mice whereas loss of endothelial P2Y$_2$R ($_{EC}$P2Y$_2$R-KO mice) significantly impaired monocyte migration. Consistent with reduced plaque macrophage content, the percentage of lesion area positive for VCAM-1 staining was significantly reduced in the aortic root lesions of $_{EC}$P2Y$_2$R ApoE$^{-/-}$ mice(***p<0.001, Fig. 3B-C). These results suggest that absence of endothelial P2Y$_2$R attenuates endothelial barrier dysfunction under pathological conditions. To examine the effect of endothelial P2Y$_2$R deletion on vascular integrity *in vivo*, mice were injected with Evans blue dye, and vascular permeability within the aorta was assessed by measuring leakage of the dye. As shown in Figure 3E, there was no significant (***p<0.001) difference in the amount of dye recovered between control and $_{EC}$P2Y$_2$R-KO mice on a wild type background. In contrast, on an ApoE$^{-/-}$background, loss of endothelial P2Y$_2$R significantly reduced the amount of dye deposited in the aorta compared to control/ApoE$^{-/-}$ mice (Fig. 3E, (***p<0.001). These results indicate that absence of endothelial P2Y$_2$R attenuates endothelial barrier dysfunction under pathological conditions.

Endothelial P2Y$_2$ receptor deficiency promotes fibrous cap formation

The decrease in macrophage content observed in $_{EC}$P2Y$_2$R ApoE$^{-/-}$ mice suggested that there may be an overall increase in SMC or collagen content of the lesions. Total SMC content in $_{EC}$P2Y$_2$R ApoE$^{-/-}$ lesions measured by the SMC marker α-SM-actin represented 18% of the total lesion area and was 2.2 fold higher that that seen in control/ApoE$^{-/-}$ mice (***p<0.001, Fig. 4B-C). Cap-like structures were observed in $_{EC}$P2Y$_2$R/ApoE$^{-/-}$ atherosclerotic lesions but not in control/ApoE$^{-/-}$ lesions (Fig. 4A). The increase in collagen content observed in $_{EC}$P2Y$_2$R/ApoE$^{-/-}$ mouse plaques correlated with a dramatic increase in the cross-section area stained with Masson's Trichrome (53.4%± 3.6% as compared to 23.6 ± 2.0% in controls; ***p<0.001; Fig. 4A-C). Our results strongly suggest that deletion of endothelial P2Y$_2$R shifts plaque phenotype from an inflammatory status to a more stable plaque characterized by an abundance of SMC and fibrous connective tissue.

Endothelial deletion of P2Y$_2$R decreases MMP-2 activity in atherosclerotic lesions

MMPs are thought to enhance migration and proliferation of SMC during early stages of the atherosclerotic process while in advanced plaques, inflammation-derived proteolytic activity may weaken the plaque. As atherosclerotic lesions in $_{EC}$P2Y$_2$R/ApoE$^{-/-}$ mice exhibited increased SMC and collagen content compared to lesions in ApoE$^{-/-}$ mice, we examined whether endothelial P2Y$_2$R deficiency alter MMP proteolytic activity in atherosclerotic lesions of 25 week old mice. Gelatin zymography analysis of protein extracts from the aortic arch revealed a significant (*p < 0.05) decrease in MMP-2 activity in $_{EC}$P2Y$_2$R/ApoE$^{-/-}$ mice compared to control/ApoE$^{-/-}$ (Fig. 5A). This reduction in MMP-2 activity was paralleled by a decrease in MMP-2 mRNA expression as demonstrated by qPCR (***p < 0.001; Fig. 5B). Pro-MMP-9 expression in the lesions was similar between control/ApoE$^{-/-}$ and $_{EC}$P2Y$_2$R/ApoE mice. However, MMP-9 proteolytic activity was not detected in the

lesions. Protein and mRNA levels of tissue inhibitor of metalloproteinase (TIMP)-2 were significantly (**$p < 0.01$) higher in the aortic arch of $_{EC}P2Y_2R/ApoE^{-/-}$ mice whereas TIMP-1 expression levels were similar between the two genotypes (Fig. 5B-C). These data indicate that endothelial $P2Y_2R$ deficiency inhibits MMP-2 activity in the atherosclerotic lesions in $ApoE^{-/-}$ mice.

Endothelial deletion of $P2Y_2R$ increases SMC migration ex vivo

To explore the mechanisms underlying the increased accumulation of SMC in $_{EC}P2Y_2R$ $ApoE^{-/-}$ lesions, we examined the effect endothelial $P2Y_2R$ deletion on migration of SMC out of aortic explants. NO derived from endothelial cells has been shown to inhibit SMC migration and proliferation (15-16). We, therefore, examined if SMC migration is altered in $_{EC}P2Y_2R$-KO mice that exhibit reduced eNOS levels. We first established that aortic explants from $_{EC}P2Y_2R$-KO mice released significantly less NO in the culture medium than explanted aortas from control mice (Fig. 6A, **$p < 0.01$, n=7). About 90% of aortic explants from $_{EC}P2Y_2R$-KO mice exhibit migrating SMC at day 10 compared to only 65% in control/ $ApoE^{-/-}$ (**$p < 0.01$), Fig. 6B). At day 7, an average of 15± 4.3 ($p<0.05$, n=7) migrated cells per explant ($p<0.05$, n=7) was observed in $_{EC}P2Y_2R/ApoE^{-/-}$ mice compared to 6± 2.2 migrated cells per explant (n=15) in control mice. Immunofluorescence staining indicated that the migrated cells expressed the SMC marker desmin and were negative for the endothelial cell marker CD31 (not shown). Notably, addition of the nitric oxide synthase inhibitor L-NAME (10 mmol/L) to the culture media equally enhanced SMC migration out of the explants from the control mice such that there was no difference in the migration of $P2Y_2R$ and control cells (Fig. 6C). These data indicate that loss of endothelial $P2Y_2R$ enhances SMC migration through decreased NO release thereby contributing to SMC accumulation in $_{EC}P2Y_2R$-KO lesions.

Discussion

To examine the contribution of endothelial $P2Y_2$ receptor ($P2Y_2R$) to the pathogenesis of atherosclerosis, we generated mice harboring an EC-specific $P2Y_2R$ deletion mice on an $ApoE^{-/-}$ background. We demonstrated that on the $ApoE^{-/-}$ background EC-specific $P2Y_2R$ deficiency attenuates endothelial barrier dysfunction, decreases both inflammation and the burden of atherosclerosis. Most notably, EC-specific $P2Y_2R$ deficiency increases SMC and collagen plaque content leading to the formation of sub-endothelial fibrous cap. Mechanistically, endothelial $P2Y_2R$-deficiency decreases VCAM-1 expression resulting in decreased recruitment of macrophages into the atherosclerotic plaques. Conversely, endothelial $P2Y_2R$ deficiency also decreases eNOS activity resulting in reduced NO production and increased SMC migration. Futhermore, we demonstrated that EC-specific $P2Y_2R$ deficiency reduced MMP-2 proteolytic activity in atherosclerotic lesions in $ApoE^{-/-}$ mice. Thus, our data suggest that therapies directed against EC $P2Y_2R$ might decrease plaque burden and increase plaque stability.

Extracellular nucleotides are maintained at very low levels due to rapid hydrolysis by ectonucleotidases (17). Under physiological conditions ATP released from the endothelium may exert a potent regulation on vascular tone (18). Aortic rings from $P2Y_2$ receptor

knockout mice ($P2Y_2R^{-/-}$) exhibit impaired vasorelaxation in response to nucleotides (19). In these mice, it was reported that ATP-induced dilation is mediated by both $P2Y_1$ and $P2Y_2$ receptors, while UTP-dependent vasodilation is attributed mostly to the $P2Y_6R$. As these $P2Y_2R^{-/-}$ mice lack the $P2Y_2R$ in both endothelial and smooth muscle cells, in the present study we sought to clarify the role of endothelial $P2Y_2R$ in endothelium-dependent relaxation. Using mice with EC-specific deletion of $P2Y_2R$, we demonstrated that the vasodilatory effects of ATP and UTP are mainly mediated by endothelial $P2Y_2R$. In addition, we established that $P2Y_1R$ is not involved in ATP-induced relaxation since ADP even at high concentrations could not elicit relaxation of aortic rings in either control or $_{EC}P2Y_2R$-KO mice. Furthermore, our results showed that Ach-induced dilation of PE pre-constricted aortic rings were similar between control and $_{EC}P2Y_2R$-KO mice, indicating that loss of endothelial $P2Y_2R$ does not result in generalized endothelial dysfunction. Although nucleotide-mediated vasomotor responses may vary among different vascular beds (20), our findings reveal that endothelial $P2Y_2R$ is the main receptor that mediates ATP -and UTP-dependent vasodilation in the aorta.

Impaired endothelium-dependent vasodilation is associated with the development of essential hypertension in humans and mice (20-21). $P2Y_2R^{-/-}$ mice were found to develop salt-resistant hypertension (22), and $P2Y_2R$ activation decreases blood pressure in mice (23). Furthermore, a tamoxifen-induced endothelium-specific $P2Y_2$-deficient mouse (Tie2-$CreER^{T2}$ $P2Y_2R^{fl/fl}$) was reported to develop spontaneous hypertension (14). Interestingly, our $_{EC}P2Y_2R$-KO did not develop hypertension as assessed by either acute or chronic measurements of blood pressure and the lack of hypertrophy in either ventricle. We demonstrated that endothelial deletion of $P2Y_2R$ using cadherin 5-Cre transgenic mice did not result in $P2Y_2R$ deletion from peripheral blood mononuclear cells. As Tie2-Cre–mediated deletion of $P2Y_2R$ may also lead to excision from $P2Y_2R$ bone marrow derived cells, together these data suggest that it may be the loss of $P2Y_2R$ from bone marrow derived cells that accounts for the development of hypertension in $P2Y_2R^{-/-}$ mice. Heterologous bone marrow transplants into global $P2Y_2R^{-/-}$ mice will be required to resolve this issue.

Although our data suggest that loss of endothelial $P2Y_2R$ does not result in systemic hypertension, functional impairment of the vascular endothelium is found in most forms of cardiovascular diseases including atherosclerosis (1). Endothelial dysfunction is associated with increased leukocyte transmigration and increasd permeability of endothelium (1, 24). Significant amounts of extracellular ATP may locally accumulate in the vasculature during traumatic and pathological events (7) and $P2Y_2R$ activation has been shown to induce a transient increase in venular permeability to albumin (25), suggesting that the $P2Y_2R$ may play a role in regulating vascular permeability. Accordingly, we found that EC-specific $P2Y_2R$ deletion attenuated endothelial barrier dysfunction that occurs in $ApoE^{-/-}$ mice as evidenced by decreased Evans blue dye accumulation observed in $_{EC}P2Y_2R/ApoE^{-/-}$ mice compared to control/$ApoE^{-/-}$ mice. In addition, monocyte transmigration across endothelial monolayers isolated from $_{EC}P2Y_2R$-KO mice was significantly decreased compared to endothelial cells isolated from control mice. These data strongly imply that the decreased monocyte content of atherosclerotic lesions in $_{EC}P2Y_2R/ApoE^{-/-}$ mice is likely to be due at least in part to impaired monocyte transmigration. We have also shown that loss of $P2Y_2R$ attenuates VCAM-1 expression in endothelial cells (3) suggesting that decreased binding of

the monocytes likely also contributes to the decreased monocytes seen in atherosclerotic plaques of $_{EC}P2Y_2R/ApoE^{-/-}$ mice. Significantly less VCAM-1-positive areas were also observed in atherosclerotic lesions in $_{EC}P2Y_2R/ApoE^{-/-}$ mice. We recently reported that global $P2Y_2R$ deficiency prevents fatty streak lesion formation in $ApoE^{-/-}$ mice (3). Altogether, these data indicate a major effect of endothelial $P2Y_2R$ deletion may be to reduce vascular inflammation.

The formation of fibrous caps in atherosclerotic lesions of $_{EC}P2Y_2R/ApoE^{-/-}$ mice was a major finding of the present study. SMC accumulation could be the result of both increased matrix metalloproteinase activity and migration of vascular SMC. However, gelatin zymography showed significantly lower activity of MMP-2 in the lesions of 25 week-old $_{EC}P2Y_2R/ApoE^{-/-}$ mice and no MMP-9 activity was detected at this time point. The decrease in MMP-2 activity was paralleled by a significant increase in TIMP-2 protein and mRNA levels in the plaques. Although these data do not rule out a possible transient increase in MMP activity at an earlier stage of plaque development, they suggest that in mature plaques decreased MMP2 activity in $_{EC}P2Y_2R/ApoE^{-/-}$ mice could have a beneficial effect by increasing plaque stability. Despite the decreased MMP activity, we observed a significant increase in SMC migration of aortic segments from $_{EC}P2Y_2R/ApoE^{-/-}$ mice (Figure 6B) suggesting that the increased SMC content of the fibrous plaque may be due to increased SMC migration. We postulate that this occurs as a consequence of decreased endothelial NO. In support of this, studies have shown that transfection of eNOS gene into SMC can effectively inhibit their migration in response to chemotactic agents (26). Futhermore, the difference in SMC migration between explants derived from the control and $_{EC}P2Y_2R/ApoE^{-/-}$ mice was abrogated in the presence of a specific eNOS inhibitor (L-NAME, Figure 6C). Together these findings suggest that EC-specific deletion of $P2Y_2R$ may be contributing to plaque stability by not only increasing its SMC content but also by downregulating MMP-2 activity.

In conclusion, this study shows that decreased VCAM-1 expression leading to impaired macrophage infiltration acting together with MMP-2 activity and increased SMC migration resulting from diminished eNOS activity could explain the beneficial effects on plaque stabilization observed in $_{EC}P2Y_2R$ $ApoE^{-/-}$ mice. Thus, targeted inhibition of $P2Y_2R$-mediated signals in endothelial cells may be a viable approach to decrease plaque burden and improve plaque stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations

ATP	Adenosine 5-triphosphate
ADP	Adenosine diphosphate
ApoE$^{-/-}$	Applipoprotein E null
EC	Endothelial cells
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
PBMC	Peripheral blood mononuclear cells
MMP	Matrix metalloproteinases
SMC	Smooth muscle cells
SMA	Smooth muscle actin
TIMP	Tissue inhibitor of matrix metalloproteinase

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Highlights

- Endothelial cell-specific deletion of $P2Y_2$ receptor reduces eNOS activity and reveals a major role for $P2Y_2$ receptor in nucleotide-mediated vasodilation.
- Endothelial cell-specific deletion of $P2Y_2$ receptor attenuates vascular permeability in ApoE-null mice.
- Endothelial cell-specific deletion of $P2Y_2$ receptor decreases metalloproteinase activity and smooth muscle migration in atherosclerotic lesions.
- Endothelial cell-specific deletion of $P2Y_2$ receptor promotes fibrous cap formation in ApoE-null mice.

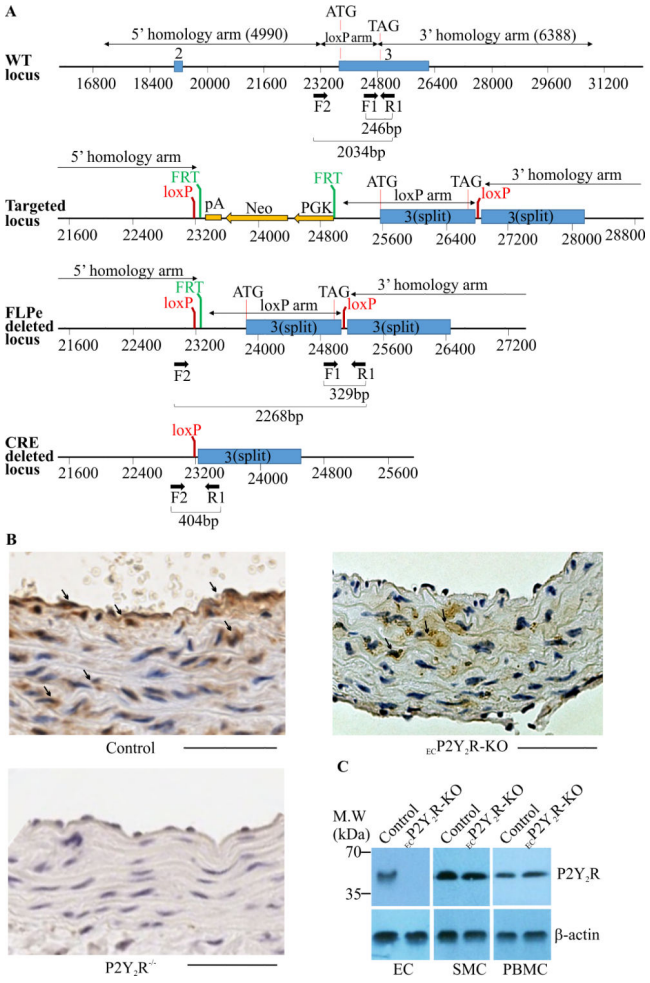

Figure 1.

Generation of $_{EC}P2Y_2R$-KO mice. **A.** The wild type mouse $P2Y_2R$ locus is indicated in the upper panel. The positions of the 5', 3' and loxP homology arms used to generating the targeting vector are indicated. Exons 2 and 3 of the gene are indicate by blue boxes. The positions of primers used for genotyping are also indicated. Schematics of the targeted locus, the FLPe deleted locus, in which the neo cassette was removed and the final cre deleted or knockout locus are also shown. Cre-mediated deletion results in excision of the entire coding region of the $P2Y_2R$ gene. **B,** Immunostaining of $P2Y_2R$ in cross sections of aortic vessels in control, $_{EC}P2Y_2R$-KO and $P2Y_2R^{-/-}$ (total body $P2Y_2R$ knock out) mice. $P2Y_2R$ is expressed in both EC and SMC in aorta of control mice whereas in $_{EC}P2Y_2R$-KO mice expression was only detected in SMC. $P2Y_2R$ staining was absent from EC and SMC in the aorta of $P2Y_2R^{-/-}$mice. Scale bar represents 50μm. **C,** Western blot analysis of $P2Y_2R$ expression in primary cultured EC, SMC and PBMC from control and $_{EC}P2Y_2R$-KO mice. Data shown are representative of experiments performed from 4 mice for each genotype.

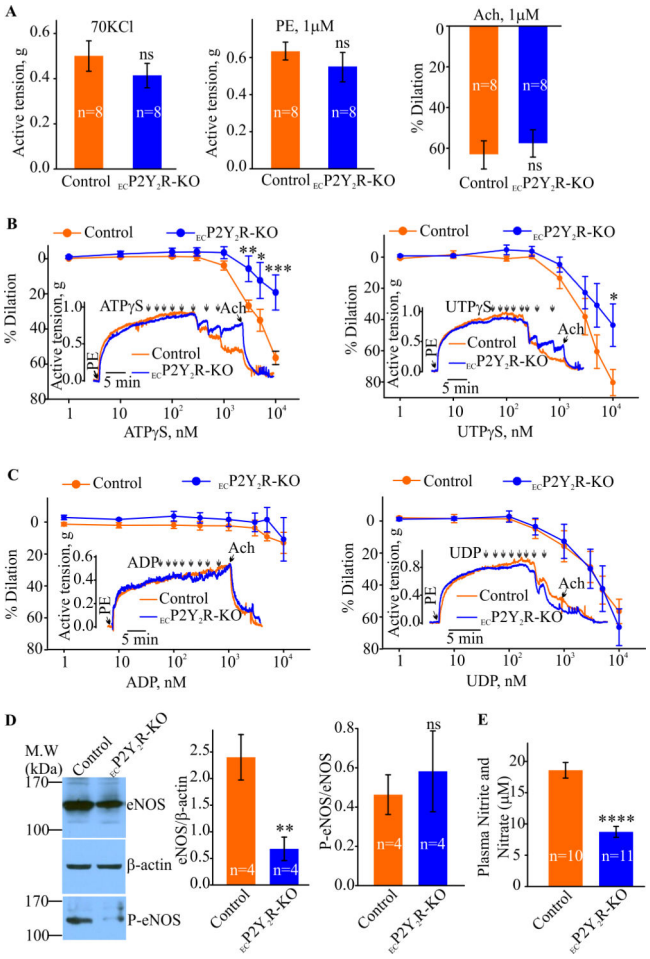

Figure 2.

Endothelial deletion of $P2Y_2R$ results in significant decrease in nucleotide- induced vasodilation and eNOS activity. ATPγS- or UTPγS-induced dilations are significantly decreased in the aortic rings from $_{EC}P2Y_2R$-KO mice. Phenylephrine (PE)- and 70 mM KCl-induced contractions and Ach-induced dilations in PE pre-constricted aortic rings from control and $_{EC}P2Y_2R$- KO mice are shown in **A**. Sample traces and concentration-response curves for ATPγS, UTPγS and ADP, UDP induced effects in PE pre-constricted aortic rings are shown in **B** and **C**, respectively. *p<0.05, **p<0.01 and ***p<0.001. **D,** Western blot analysis of total eNOS protein and phosphorylated eNOS (serine 1176) in aortic arch lysates from control and $_{EC}P2Y_2R$-KO. Densitometry analysis are shown in graphs (n= 7, **p < 0.01). **E,** Plasma nitrate and nitrite levels in control (n=10) and $_{EC}P2Y_2R$-KO mice (n= 11). Data are mean ± SEM; ****p<0,0001 by 2-tailed Student's t test.

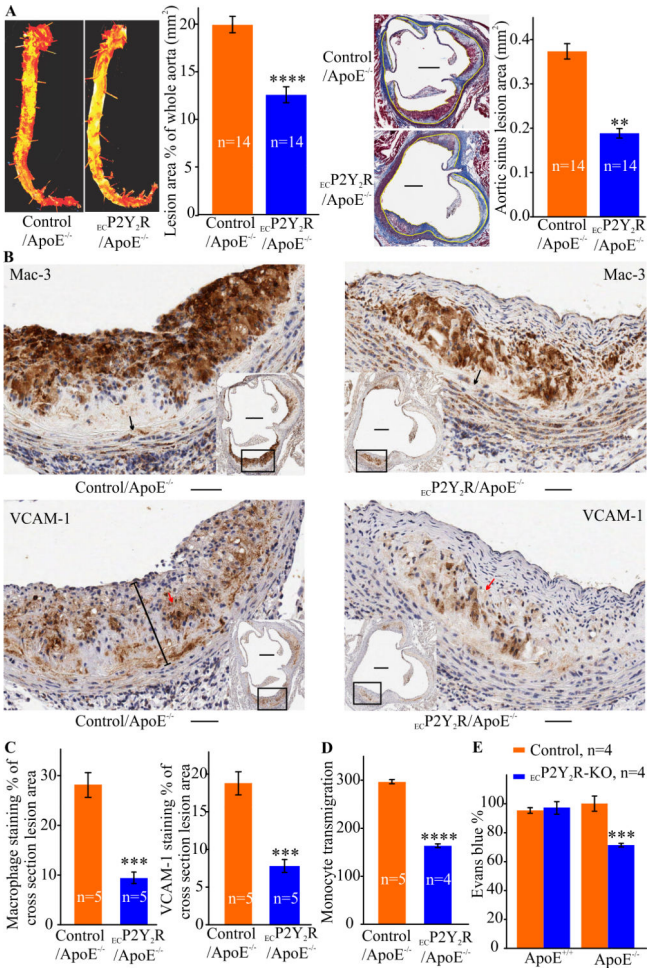

Figure 3.

Endothelial cell-specific deletion of $P2Y_2R$ reduces atherosclerosis, inflammation and endothelial barrier dysfunction in $ApoE^{-/-}$ mice. **A**, Analysis of atherosclerotic lesions in $_{EC}P2Y_2R$ $ApoE^{-/-}$ and control $ApoE^{-/-}$ mice fed standard chow diet for 25 weeks. Left panel shows representative photomicrographs of Oil red O-stained aortas collected between the subclavian and iliac branches and quantitative computer- assisted image analysis of lipid deposition in the entire aorta. Data represent the percentage surface area of the aorta occupied by atherosclerotic lesions in control (n=14) and $_{EC}P2Y_2R/ApoE^{-/-}$ mice (n=14). ****p<0.0001. Right panel shows representative images of cross sections of aortic sinus from $_{EC}P2Y_2R/ApoE^{-/-}$ and control/$ApoE^{-/-}$ mice stained with Masson's trichrome. Scale bar represents 250 μm. The adjacent graphs shows the mean ±SEM lesion area measured in 5 sections for each of 14 animals. The lesion area in the aortic sinus was determined by computer-assisted image analysis. The yellow lines delineate the lesions. **p < 0.01. **B**, Representative images of immunohistological staining of atherosclerotic lesions in the aortic sinus stained with the anti-macrophage Mac-3 antibody or VCAM-1 as indicated. Scale bar represents 50 μm and the scale bar in inset represents 250 μm. Morphometric analysis of the stained areas are indicated in panel **C**. Data are the mean ±SEM of the percentage staining of the total plaque area in 5 consecutive sections in 5 mice for each genotype. ***p<0.001. **D**,

Monocyte transmigration across endothelial cell monolayers from control and $_{EC}P2Y_2R$-KO mice. EC were grown on transwell membrane with monocytes from wild-type added to the top chamber and MCP-1 to the bottom. Transmigrated monocytes were counted and normalized to the non-treated condition (without MCP-1). ****$p < 0.0001$. **E**, Mice were injected with Evans blue dye and leakage of the dye into the aortas was quantified after extracting the dye and measuring absorbance at 620 nm. Values were normalized to the dry weight of the aorta. Data represent mean ±SEM; ($n = 5$). ***$p < 0.001$.

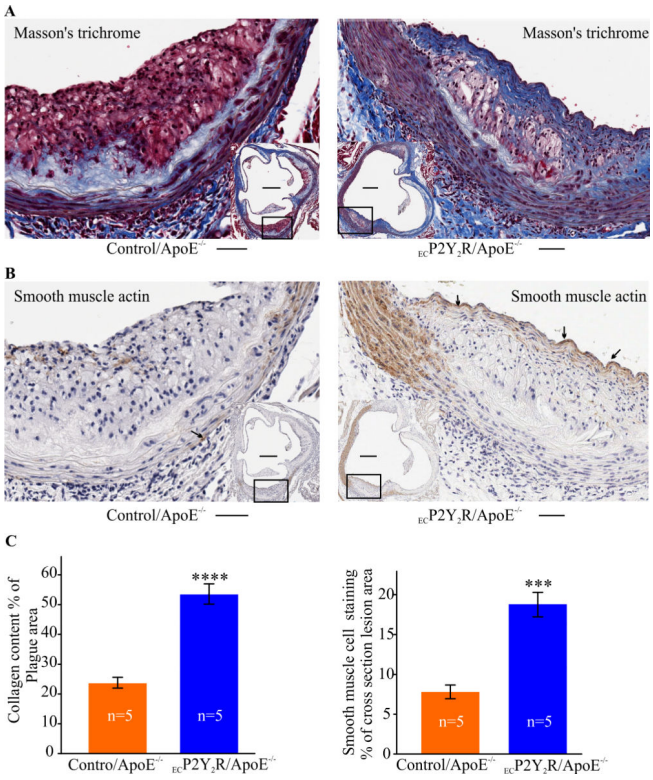

Figure 4.

Endothelial cell-specific deletion of $P2Y_2R$ promotes fibrous cap formation. **A**, Masson's trichrome showing collagen fibers (blue) and immunohistochemical staining for αSM-actin **(B)** in atherosclerotic lesions of $ApoE^{-/-}$ and $_{EC}P2Y_2R/ApoE^{-/-}$ mice. The areas occupied by α-SM actin staining or collagen were expressed as percentage of total cross-section lesion area. Scale bar represents 50 μm and the scale bar in inset represents 250 μm. (**C**). A continuous fibrous cap can be observed in the lesions of $_{EC}P2Y_2R/ApoE^{-/-}$ mice but was notably absent in lesions from $ApoE^{-/-}$ mice. Data represent mean ±SEM; ***p<0.001, ****p<0.0001. (n=5 mice for each genotype).

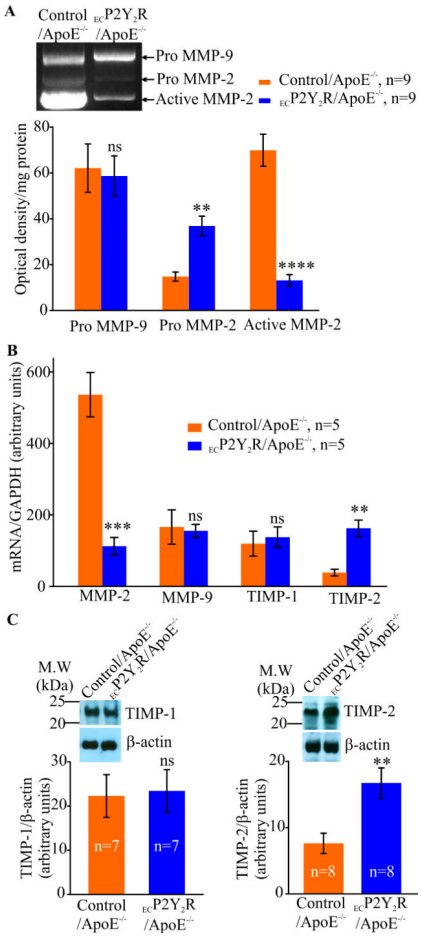

Figure 5.

Endothelial deletion of $P2Y_2R$ increases MMP-2 activity in atherosclerotic lesions. **A,** Reduced gelatinolytic activity in 25 week-old $^{EC}P2Y_2R/ApoE^{-/-}$ mice. Representative zymogram demonstrating a significantly reduced MMP-2 activity (****p<0.0001) in the $^{EC}P2Y_2R/ApoE^{-/-}$ aortic arch compared to control/$ApoE^{-/-}$. Data represent mean ±SEM; (n=9 mice per group); ****p <0.0001. There was no significant differences in pro-MMP-9 activity between control/$ApoE^{-/-}$ and $_{EC}P2Y_2R/ApoE^{-/-}$ mice. This decrease in MMP-2 activity was correlated with decreased MMP-2 mRNA levels (**p<0.01, n=5) as shown by qPCR (**B**).**C,** Western blot analysis shows a significant increase in TIMP-2 protein levels in $_{EC}P2Y_2R/ApoE^{-/-}$ mice compared to control/$ApoE^{-/-}$ (n=5, **p< 0.01) whereas TIMP-1 protein levels were unchanged.

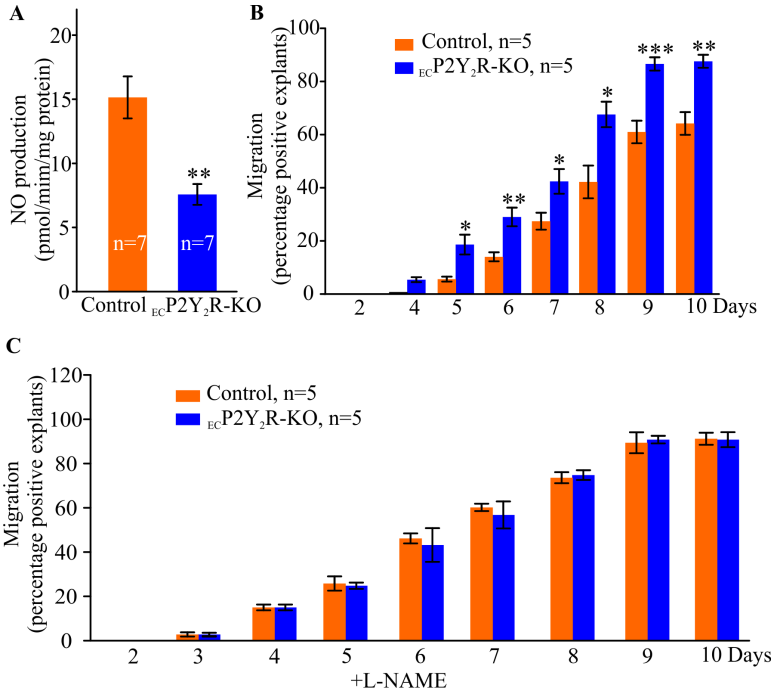

Figure 6.

Endothelial deletion of $P2Y_2R$ decreases NO production but increases SMC migration from aortic explants. **A**, NO release from the aortic explants from control and $_{EC}P2Y_2R$-KO mice (n=7 for each genotype) was measured by determining the production of nitrite in the cultured medium after 4 days. Data represent mean ±SEM, $^{**}p < 0.01$. **B**, Cell migration was expressed as percent of explants exhibiting at least one SMC outside the explant. SMC migration was significantly increased in explants from $_{EC}P2Y_2R/ApoE^{-/-}$ mice as compared to control/$ApoE^{-/-}$ mice (n=10 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$ by 2-tailed Student's *t* test. **C**, SMC migration out of aortic explants in presence of the eNOS inhibitor L-NAME (10 mmol/L) was similar in control and $_{EC}P2Y_2R$-KO mice.

Table 1

Radiotelemetry blood pressure measurements

	Control (n=5)	$_{EC}$P2Y$_2$R-KO (n=5)
Baseline SPB (mmHg)		
Night time	144.92±12.51	143.06±11.50
Daytime	93.28±13.06	88.40±13.53
Heart rate (beats/min)		
Night time	579.04±74.70	585.10±56.67
Daytime	402.68±57.38	374.03±47.14

Table 2

Acute blood pressure measurements

	Control (n=12)	$_{EC}$P2Y$_2$R-KO (n=12)
Baseline SPB (mmHg)	82.84±4.05	82.43±4.50
Heart rate (beat/min)	524.01±33.94	520.11±95.13

Table 3

Echocardiography data in conscious mice

Mice	Weight (g)	RVW (mm)	RVD (mm)	LVD (mm)	LVW (mm)
Control	30.25±2.63	0.36±0.09	1.84±0.21	4.07±0.23	0.64±0.09
ECP2Y2R-KO	30.58±3.09	0.36±0.09	1.72±0.23	4.15±0.45	0.63±0.08

P<0.05 versus RVW, Right ventricle wall thickness; RVD, Right ventricle chamber diameter; LVD, Left ventricle chamber diameter; LVW, Left ventricle posterior wall thickness.

Table 4

Body weight and plasma lipid profile of 25-week old $_{EC}P2Y_2R/ApoE^{-/-}$ and control/$ApoE^{-/-}$

	Control/$ApoE^{-/-}$ (n=12)	**$_{EC}P2Y_2R/ApoE^{-/-}$ (n=12)**
Body weight (g)	34.4± 4	33.7± 5
Total cholesterol (mg/dl)	489± 28	481± 22
Triglycerides (mg/dl)	68± 7	67± 6