Cellular and Molecular Life Sciences (2019) 76:341–354
https://doi.org/10.1007/s00018-018-2937-2

REVIEW

The role of $P2Y_{12}$ receptor in ischemic stroke of atherosclerotic origin

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Received: 30 March 2018 / Revised: 11 September 2018 / Accepted: 5 October 2018 / Published online: 9 October 2018

Abstract

Atherosclerosis is a chronic and progressive disease of the arterial walls and a leading cause of non-cardioembolic ischemic stroke. $P2Y_{12}$ is a well-recognized receptor that is expressed on platelets and is a target of thienopyridine-type antiplatelet drugs. In the last few decades, $P2Y_{12}$ receptor inhibitors, such as clopidogrel, have been applied for the secondary prevention of non-cardioembolic ischemic stroke. Recent clinical studies have suggested that these $P2Y_{12}$ receptor inhibitors may be more effective than other antiplatelet drugs in patients with ischemic stroke/transient ischemic attack of atherosclerotic origin. Moreover, animal studies have also shown that the $P2Y_{12}$ receptor may participate in atherogenesis by promoting the proliferation and migration of vascular smooth muscle cells (VSMCs) and endothelial dysfunction, and affecting inflammatory cell activities in addition to amplifying and maintaining ADP-induced platelet activation and platelet aggregation. $P2Y_{12}$ receptor inhibitors may also exert neuroprotective effects after ischemic stroke. Thus, $P2Y_{12}$ receptor inhibitors may be a better choice for secondary prevention in patients with atherosclerotic ischemic stroke subtypes because of their triple functions (i.e., their anti-atherosclerotic, anti-platelet aggregation, and neuroprotective activities), and the $P2Y_{12}$ receptor may also serve as a noval therapeutic target for atherosclerosis. In this review, we summarize the current knowledge on the $P2Y_{12}$ receptor and its key roles in atherosclerosis and ischemic stroke of atherosclerotic origin.

Keywords $P2Y_{12}$ · Atherosclerosis · Ischemic stroke · Platelet · Smooth muscle cell

Introduction

In the past several decades, stroke has become a global burden because of its long course and high morbidity and mortality. Antiplatelet treatment is recommended for secondary prevention of non-cardioembolic ischemic stroke. However, unlike cardiovascular disease, which is caused by coronary atherosclerosis, the etiology of ischemic stroke is very complicated and affects its prognosis, outcomes, and management. According to the TOAST classification system, non-cardioembolic ischemic stroke includes four subtypes: large-artery atherosclerosis, small-artery occlusion, stroke of other determined etiology, and stroke of undetermined etiology. Few antiplatelet drug trials have been performed according to ischemic stroke subtype [1]. Therefore, it is uncertain whether there is significant heterogeneity in the effects of antiplatelet therapy on secondary prevention among the different non-cardioembolic ischemic stroke subtypes. A recent prespecified exploratory subgroup analysis of the SOCRATES clinical trial demonstrated the superiority of ticagrelor, a $P2Y_{12}$ antagonist, over aspirin in stroke/TIA patients with ipsilateral atherosclerotic stenosis but not in patients without ipsilateral atherosclerotic stenosis or those with potential causal small-vessel disease [2]. Atherosclerosis, a chronic disease of the medium and large arteries, is one of the main causes of stroke. Its pathological process is complicated and includes damage to vessel endothelial cells (ECs), the adhesion and activation of platelets, the recruitment of monocytes, the proliferation and migration of smooth muscle cells (SMCs), and the release of inflammatory mediators. Other clinical and basic studies have also indicated that the $P2Y_{12}$ receptor plays a role

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in atherosclerosis. Thus, $P2Y_{12}$ receptor inhibitors may be a better choice than other antiplatelet drugs in patients with atherosclerotic ischemic stroke subtypes because of their potential anti-atherogenic effect besides the antiplatelet effects. In this review, we summarize what is currently known about the $P2Y_{12}$ receptor and the key role it plays in atherosclerosis and atherosclerotic ischemic stroke.

Introduction to the $P2Y_{12}$ receptor

Discovery of the $P2Y_{12}$ receptor

In 1978, Bennett et al. reported that a polypeptide involved in adenosine diphosphate (ADP)-induced platelet aggregation may represent an ADP receptor. This effect was inhibited by 5′-*p*-fluorosulfonyl-benzoyladenosine (FBSA), an adenine nucleotide analog that inhibits platelet aggregation. The receptor was then found to be sensitive to thienopyridines and to couple to inhibitory trimeric GTP-binding regulatory protein (G_i), which was called the $P2T_{AC}$ receptor [3]. Finally, this receptor was identified in humans and rats and designated $P2Y_{12}$ by Hollopeter et al. [4], and it was shortly thereafter identified in humans [5, 6] and mice [7] by other groups. Patients with congenital $P2Y_{12}$ receptor deficiency were first described in 1992 [8] and 1995. $P2Y_{12}$ receptor deficiency is an autosomal recessive disorder characterized by a severe selective impairment of platelet reactivity to ADP and low concentrations of collagen. In Hollopeter's study, evidence was provided indicating that affected patients have a defect in the *p2y*$_{12}$-gene [4].

Structure of the $P2Y_{12}$ receptor

The *P2Y*$_{12}$ gene resides on chromosome 3q24-25 [4, 9]. The $P2Y_{12}$ receptor contains 342 amino acids and is a member of the P2Y purinergic receptor family, which is composed of two main groups: ligand-gated intrinsic ion channel P2X receptor subtypes ($P2X_1$–$P2X_7$) [10, 11] and G-protein-coupled P2Y-receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) [4, 12, 13]. Similar to other classes of G-protein-coupled receptors (GPCRs), the crystal structure of the $P2Y_{12}$ receptor contains seven α-transmembrane helices that interact with each other to stabilize overlapping regions. In all of these transmembrane domains, helix III is located in the center of the structure. In the $P2Y_{12}$ receptor, helix V is straight and long, which is a distinct difference between this receptor and other GPCRs [14]. In addition, four extracellular cysteines located at positions 17, 97, 175, and 270 form two disulfide bonds that link the amino terminus (C17) to helix VII (C270) and helix III (C97) to the extracellular loop (EL2, C175) [14–16] (Fig. 1). $P2Y_{12}$ receptors localize on the surfaces of mammalian cells and freshly isolated platelets as monomers, dimers, and oligomers, each of which may be associated with different physiological characteristics and functions [16, 17]. ADP is the endogenous agonist of $P2Y_{12}$ receptors [18], and adenosine triphosphate (ATP) and its triphosphate analog act as its antagonists [19–21]. The $P2Y_{12}$ receptor triggers different intracellular signaling cascades in different cells, resulting in different functions.

Distribution of the $P2Y_{12}$ receptor

In the past, the $P2Y_{12}$ receptor was thought to be expressed on platelets and in subregions of the brain including the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, and thalamus [4]. In recent years, $P2Y_{12}$ receptors have also been found on M2 microglia cells [22, 23], dendritic cells [24], oligodendrocytes [25], oligodendrocyte precursor cells [26], astrocytes [27], endothelial cells [28–30], vascular smooth muscle cells (VSMCs) [31, 32], osteoclasts [33], macrophages [34, 35], and subpopulations of leukocytes [36].

Blocking the P2Y12 receptor ameliorates atherosclerosis and atherosclerotic ischemic stroke

Genetic abnormalities and $P2Y_{12}$ receptor inhibitors induce structural and functional deficiencies, respectively, in this receptor. Here, we summarize the anti-atherosclerotic effects of $P2Y_{12}$ receptor blockade.

$P2Y_{12}$ antagonists may be more effective than aspirin in patients with ischemic stroke/TIA of atherosclerotic Origin

Two main types of $P2Y_{12}$ receptor inhibitors are used in a clinical setting: prodrug thienopyridines such as ticlopidine, clopidogrel, and prasugrel, and direct-acting antagonists such as ticagrelor and cangrelor [37–39]. In recent years, among these drugs, clopidogrel and ticagrelor have been the two most widely used clinically as antiplatelet agents, along with aspirin, for secondary prevention in patients who have experienced non-cardioembolic ischemic stroke. However, among trials of antiplatelet therapies intended for secondary prevention, few analyses have characterized patients according to ischemic stroke subtypes. In the recent SOCRATES Study, ticagrelor was found to produce effects that were no better than those of aspirin in preventing stroke, myocardial infarction (MI) or death at 90 days in patients with non-cardioembolic, non-severe ischemic stroke (IS), or high-risk transient ischemic attack (TIA) [40]. Surprisingly, a prespecified exploratory subgroup analysis of the SOCRATES data

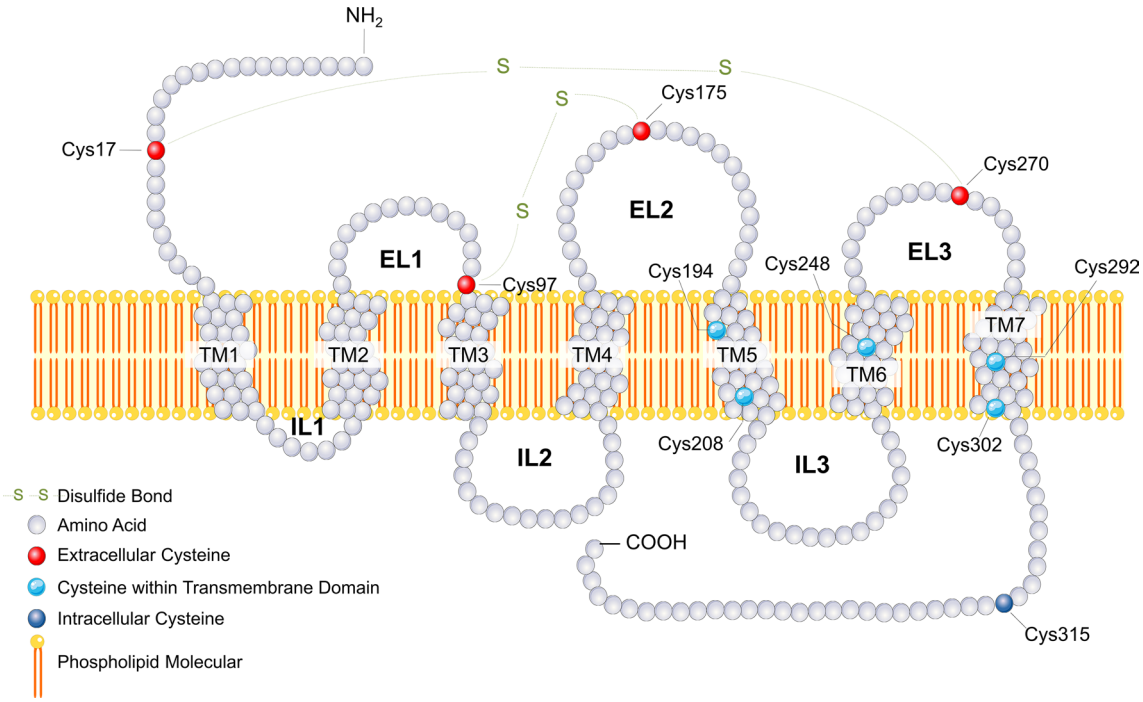

Fig. 1 Predicted secondary structure of the $P2Y_{12}$ receptor. The $P2Y_{12}$ receptor is a member of GPCRs, with seven α-transmembrane helices, three extracellular loops (EL1–EL3) and three intracellular loops (IL1–IL3), and consists of 342 amino acids in total. There are four extracellular cysteines at positions 17, 97, 175 and 270, five cysteine within transmembrane domain at positions 194, 208, 248, 292, and 302, and one intracellular cysteine at position 315. In addition, there are two disulfide bonds bridging Cys97 with Cys 175, and Cys17 with Cys 270, respectively. *Cys* cysteine; *EL* extracellular loop; *IL* intracellular loop; *TM* transmembrane domain (Adapted from Ref. [16])

showed that ticagrelor was superior to aspirin as a secondary preventive agent in patients with ipsilateral large-artery atherosclerotic stenosis, but not in patients without ipsilateral large-artery atherosclerotic stenosis or in patients with potentially causal small-vessel disease [2]. The subgroup analysis focused on the presence or absence of symptomatic disease with a potential causal relationship with the index stroke or TIA, and provided evidence that a treatment effect was modified by atherosclerotic stenosis.

Coincidentally, some previous studies have produced similar results (Table 1). As early as 1996, the CAPRIE study suggested that clopidogrel exerts a better effect than that was achieved by aspirin in decreasing the occurrence of stroke, MI or vascular death in patients with atherosclerotic vascular disease consisting of recent IS or MI or symptomatic peripheral arterial disease [41]. Specifically, in the ischemic stroke subgroup, the risk of a reduced primary outcomes was relatively higher (7.3%) for clopidogrel treatment than for aspirin. The later CAPRIE-subgroup analysis showed that the benefit of clopidogrel was amplified in patients with documented prior symptomatic atherosclerotic disease [42]. In the CHARISMA-subgroup study, patients with prior ischemic events who were at high risk of atherothrombotic events received more benefit from a dual therapy consisting of clopidogrel and aspirin than from aspirin therapy alone. It is a pity that the atherosclerotic disease origin was not assessed in patients with ischemic stroke or TIA as this may have decreased the power of the test [43]. The results of the CAPRIE and CHARISMA studies indicated that clopidogrel may benefit atherosclerotic patients when used for secondary prevention of cardiocerebrovascular events and that the additive effect of clopidogrel may derive from the fact that it likely exerts an anti-atherosclerotic effect in addition to its antiplatelet effect.

However, in the COMPRESS study, there was no significant difference between combination medication consisting of clopidogrel and aspirin and aspirin alone for preventing the recurrence of IS in patients with acute IS of the large-artery atherosclerosis type according to the TOAST classification [44]. The primary outcome of the COMPRESS study was new ischemic lesion on MRI within 30 days. First, this primary outcome is a surrogate marker, the detection of which was approximately 15-fold greater than the recurrent clinical ischemic stroke incidence. Second, the follow-up duration was 30 days, which might be slightly short to detect the anti-atherogenic effect of drug treatments for new ischemic lesions and particularly for symptomatic clinical events. The follow-up durations of the SOCRATES,

Table 1 Overview of studies relating $P2Y_{12}$ antagonists in ischemic stroke/TIA patients of atherosclerotic origin

RCT (year)	Patients	Treatments (*n*)	Time[a]	Follow-up	Outcome	RR/HR (95% CI), *p*	RR of BE (95% CI)
SOCRATES subgroup (2017) [2]	Non-severe AIS or high-risk TIA associated with IAS	1. Tica 90 mg bid. (1542) 2. ASA 100 mg qd. (1539)	24 h	90 d	Death, MI, or Stroke	0.68 (0.53–0.88), 0.003	NS
	Non-severe AIS or high-risk TIA without IAS	1. Tica 90 mg bid. (5047) 2. ASA 100 mg qd. (5071)	24 h	90 d	Death, MI, or Stroke	0.97(0.84–1.13), 0.72	NS
CAPRIE (1996) [41]	Symptomatic atherosclerotic vascular disease[b]	1. Clop 75 mg qd. (9599) 2. ASA 325 mg qd. (9586)	NA	1-3 y	Stroke, MI, or vascular death	8.7% (0.3–16.5)[c], 0.043	NS
CAPRIE-subgroup (2004) [42]	CAPRIE patients with prior IS/MI	1. Clop 75 mg qd. (2249) 2. ASA 325 mg qd. (2247)	NA	1-3 y	Stroke, MI, or vascular death	14.9% (0.3–27.3)[c], 0.045	NA
CHARISMA subgroup (2007) [43]	CHARISMA patients[d] with prior IS, MI or symptomatic PAD	1. Clop 75 mg qd. + ASA 75–162 mg qd. (4735) 2. ASA 75–162 mg qd. (4743)	NA	27.6 m[e]	Stroke, MI, or cardiovascular death	0.86 (0.76–0.96), 0.008	Moderate bleeding: 1.60 (1.16-2.20) Others: NS
COMPRESS (2016) [44]	AIS of LAA	1. Clop 75 mg qd. + ASA 100 mg qd. (167) 2. ASA 100 mg qd. (167)	48 h	30 d	New IS on MRI	1.02 (0.77–1.35), 0.91	1.59 (0.91-2.68)
CHANCE-subgroup (2015) [45]	Minor IS or high-risk TIA with ICAS	1. 21 d-ASA 100 mg qd. + Clop 75 mg qd. (300) 2. ASA 100 mg qd. (308)	24 h	90 d	Stroke	0.79 (0.47–1.32), –	2.83 (0.57-14.1)
	Minor IS or high-risk TIA without ICAS	1. 21 d-ASA 100 mg qd. + Clop 75 mg qd. (231) 2. ASA 100 mg qd. (250)	24 h	90 d	Stroke	1.12 (0.56–2.25), –	1.02 (0.35-2.97)

AIS acute ischemic stroke; *ASA* aspirin; *BE* bleeding events; *CI* confidence interval; *Clop* clopidogrel; *d* day; *h* hour; *HR* hazard ratio; *IAS* ipsilateral atherosclerotic stenosis; *ICAS* intracranial arterial stenosis; *IS* ischemic stroke; *LAA* large-artery atherosclerosis; *m* month; *MI* myocardial infarction; *MRI* magnetic resonance Imaging; *NA* not available. *NS* no significance; *PAD* peripheral arterial disease; *RCT* randomized controlled trial; *RR* relative risk; *SVD* small-vessel disease; *TIA* transient ischemic attack; *Tica* ticagrelor; *y* year

[a]Time from onset of symptoms to randomization

[b]Manifested as recent ischemic stroke, recent myocardial infarction, or symptomatic peripheral arterial disease

[c]Relative risk reduction of clopidogrel group comparing to aspirin group

[d]Patients with multiple atherothrombotic risk factors, coronary disease, cerebrovascular disease, or symptomatic peripheral arterial disease

[e]The median follow-up duration, patients were followed up and evaluated at 1, 3, 6 months and every 6 months thereafter until the end of the trial

CAPRIE, and CHARISMA studies were all more than 90 days. Therefore, the findings need to be confirmed by more randomized clinical trials that use an adequate clinical end point as a primary end point and follow-up for a longer duration.

An analogous result was shown in the CHANCE-subgroup study in which the addition of clopidogrel to aspirin

did not significantly alter the risk of recurrent stroke in patients with minor stroke or high-risk TIA, regardless of the presence of intracranial arterial stenosis [45]. Clopidogrel requires conversion to an active metabolite by hepatic cytochrome p450 (CYP) isoenzymes to target the P2Y12 receptor, and polymorphisms of the *CYP2C19* gene have been identified as strong predictors of clopidogrel non-responsiveness. The CHANCE study focused on Asian populations, and the percentage of carriers of *CYP2C19* loss-of-function alleles is higher in Asians (52.5%) than in western populations (27.9%) [46, 47], which may have played an important role in reducing the efficacy of clopidogrel and narrowing the differences observed between groups [48].

In conclusion, $P2Y_{12}$ receptor inhibitors may exert an anti-atherosclerotic effect in addition to its antiplatelet effect in non-cardioembolic ischemic stroke of presumed atherosclerotic disease origin. However, most of these studies were subgroup analyses or even post hoc subgroup analyses, and these designs dramatically diminish the reliability of their results. Additional multicenter, randomized, and controlled trials are needed to confirm these results.

Effects of $P2Y_{12}$ receptor blockade in animal atherosclerotic models

Recently, pharmacologic and genetic studies have implicated the $P2Y_{12}$ receptor in atherosclerosis in different animal models of atherosclerosis (Table 2).

$p2y_{12}$-gene deletion improves atherosclerosis lesion development in mice

The effect of $P2Y_{12}$ on the progression of atherosclerosis was tested in $ApoE^{-/-}$ mice. These mice develop atherosclerosis after being provided a high-fat diet. Li et al. reported that when the $p2y_{12}$-gene was deleted in $ApoE^{-/-}$ mice, the size of the lesion area was reduced to different degrees in aortic arches (~60%), abdominal aortas (~60%), and the aortic root after 20 weeks on a high-fat diet [49]. Following this study, West et al. reported similar results in the aortic arch, aortic sinus and brachiocephalic artery in $p2y_{12}$/*ApoE* double-knockout mice that were fed a high-fat diet for 4 weeks [50]. However, there were no significant decreases in lesion area in the carotid artery and descending aortae when $p2y_{12}$ was knocked out, potentially due to differences in hemorheology. Compared with the aortic sinus and aortic arch, the carotid artery and descending aortae are exposed to lower levels of shear stress and faster blood flow velocities, which provide natural protection against atherosclerotic plaques. Furthermore, $p2y_{12}$/*ApoE* double-knockout mice had a higher plaque fibrous content and exhibited preserved fiber integrity [49, 50], which may have increased the stability of atherosclerotic plaques by reducing the risk of rupture [51].

Some other studies have used the $ldlr^{-/-}$ mouse model. Similar to $ApoE^{-/-}$ mice, $ldlr^{-/-}$ mice with $p2y_{12}$ deficiency had smaller plaque areas in the aortic sinus and aortic root than were found in $ldlr^{-/-}$ mice in addition to fewer macrophages and neutrophils within the plaques after they were fed a high-fat diet for 12 weeks; both of these effects indicate that atherosclerosis development was improved [52].

A transplanted-arteriosclerosis model was also established in $p2y_{12}$ knockout mice and wild-type mice into which a carotid artery with atherosclerotic plaques was transplanted. The results showed that there was less luminal occlusion, a lower intima–media ratio, and fewer host-derived smooth muscle-like cells in the carotid allografts transplanted into the $p2y_{12}$ knockout mice than in those transplanted into the wild-type mice [53, 54].

$P2Y_{12}$ receptor inhibitors diminish the size of plaques in animal atherosclerosis models

$P2Y_{12}$ receptor inhibitors play a role similar to that of $p2y_{12}$ deficiency in $ApoE^{-/-}$ mice fed a high-cholesterol or normal diet. Quantitative analyses have shown that clopidogrel inhibits atherogenesis in the aortic sinus, aortic arch, carotid artery, brachiocephalic artery, and whole aortae [55–58]. The effect of $P2Y_{12}$ antagonists on stabilizing plaques has also been explored in the past several years. Clopidogrel was found to decrease the area of low mass density, which is composed of lipids and macrophages, and to increase the area of high mass density, which is composed of SMCs and collagen [55, 57], thus leading to more stable plaques.

Delaying treatment with clopidogrel after atherogenesis has been reported to inhibit the infiltration of macrophages and $CD4^{+}T$ cells into the plaques [58]. Delayed treatment with ticagrelor was also investigated and was found to reduce the sizes of necrotic areas and to thicken the fibrous caps of plaques [59]. However, neither treatment reversed lesion remodeling [58], [59]. In agreement with this finding, Li et al. reported that clopidogrel induced significant decreases in the intimal thickness, the intimal area, and the intima–media ratio by reducing both the area and the thickness to lower than was achieved by treatment with aspirin or atorvastatin. In addition, clopidogrel additively decreased the media thickness and area to levels lower than were observed in the placebo group in rabbit vascular-injury models [60].

As shown in Table 2, the majority of animal experiments have verified the anti-atherogenic effect of $P2Y_{12}$ receptor inhibitors, with the exception of West's Study. In West's study, both clopidogrel and ticagrelor were administered for only 4 weeks, which may be too short to affect plaque formation and development in a high-fat/cholesterol-fed

Table 2 Animal experiments about effects of blocking $P2Y_{12}$ receptors on atherosclerosis lesions

References	Model[a]	Treatment	Control	Time[b]	Results (treatment group vs. control group)
[49]	*ApoE*$^{-/-}$ + HFD	*p2y*$_{12}^{-/-}$	WT	20 w	Reduced lesion area in AR, AA and thoracic-abdominal aorta, and Mon./Mø. infiltration of the lesion in AR; increased fibrous contents at plaque sites in AR
[50]	*ApoE*$^{-/-}$ + HFD	*p2y*$_{12}^{-/-}$	WT	4 w	Reduced lesion area in AS, AA and BA; NS in lesion area in TA or DA
		200 mg/kg/d tica	Mannitol	4 w	NS in lesion area in AS, AA, TA, DA or whole aortae
		20 mg/kg/d clop	Mannitol	4 w	NS in lesion area in AS, AA, TA, DA or whole aortae
[52]	*Ldlr*$^{-/-}$ + HFD	*p2y*$_{12}^{-/-}$	WT	12 w	Reduced lesion area in AS and AR
[53]	CAT[c]	*p2y*$_{12}^{-/-}$	WT	1/2/4/8 w	Reduced IMR of carotid allografts at 2/4/8 w, and reduced narrowing of carotid allografts at 4/8w after transplantation
[54]	CAT[d]	*p2y*$_{12}^{-/-}$	WT	8 w	Reduced luminal occlusion and smooth muscle-like cells in carotid allografts
[55]	*ApoE*$^{-/-}$ + ND	(1 mg/2 mg)/d clop	Placebo	10 w	Reduced lesion area in AS
[56]	*ApoE*$^{-/-}$ + HFD	20 mg/kg/d clop	Placebo	12 w	Reduced lesion area in AA or whole aortae
[57]	*ApoE*$^{-/-}$ + HCD	0.1% clop	Placebo	10 w	Reduced lesion volumes in BA; decreased lipid and Mø content and increased collagen content in BA; NS in lesion volume in AS
[58]	*ApoE*$^{-/-}$ + HCD	1 mg/kg/d clop	Placebo	6 m	Reduced lesion area and luminal occlusion in TA and AA; reduced CD4 +, CD8 + and Mø filtration in AA
	ApoE$^{-/-}$ + 60d-HCD	1 mg/kg/d clop	Placebo	4 m	Reduced CD4 +, CD8 + and Mø filtration in AA; NS in lesion area and luminal occlusion in TA
[59]	20 w-old *ApoE*$^{-/-}$ + ND	100 mg/kg/d tica	Placebo	25 w	Reduced area of necrotic core and increased fibrous caps thickness in AS. NS in lesion area in AS
[60]	Balloon injury + 6 w-HCD[e]	4 mg/kg/d clop	Placebo	6 w	Reduced intimal area and thickness, medial area and thickness, and intima–media area and thickness ratio in the injured iliac-femoral artery
		4 mg/kg/d clop	12 mg/kg/d-ASA or 2.5 mg/kg/d Atorv	6 w	Reduced intimal area and thickness and intima–media area and thickness ratio in injured iliac-femoral artery; NS in medial area and thickness in the injured iliac-femoral artery

A acetylsalicylic acid; *AA* aortic arch; *AR* aortic root; *AS* aortic sinus; *ASA* aspirin; *Atorv* atorvastatin; *BA* brachiocephalic artery; *CAT* carotid artery transplantation; *Clop* clopidogrel; *d* day; *DA* descending aortae; *HCD* high-cholesterol diet; *HFD* high-fat diet; *IMR* intima–media ratio; *m* month; *min* minute; *Mon.* monocyte; *Mø.* macrophage; *ND* normal diet; *NS* no significant difference; *s* second; *TA* thoracic aorta; *Tica* ticagrelor; *w* week; *WT* wild type

[a]The animals used for model are mice unless otherwise stated

[b]Time of treatment duration

[c]Carotid artery transplantation from 129X1 mice

[d]Carotid artery transplantation from C3H/He (H-2k) mice

[e]Modeling based on New Zealand White rabbits

ApoE$^{-/-}$ mouse model. The authors found that there was no significant difference in the sizes of the lesion areas among the clopidogrel, ticagrelor and control groups.

In summary, experimental evidence from animal models, especially *p2y*$_{12}$-gene knockout animal models, generally supports the idea that inducing a blockade against the $P2Y_{12}$ receptor via either gene deficiency or an antagonist exerts inhibitory effects on atherosclerosis initiation and progression. The effects may at least partially explain the additive effect of $P2Y_{12}$ receptor inhibitors observed in patients with non-cardioembolic ischemic stroke of

presumed atherosclerotic disease origin. However, these conclusions remain under investigation in humans.

Mechanisms of P2Y12 receptor involvement in atherosclerosis progression

As previously mentioned, inducing a blockage against $P2Y_{12}$ receptors ameliorates atherosclerosis. $P2Y_{12}$ receptors are expressed on the surface of different types of cells, and each of these cell types can potentially contribute to the progression of atherosclerosis [61]. Some of the relevant mechanisms have been confirmed, while others remain unclear (Fig. 2).

The role of $P2Y_{12}$ receptors expressed on platelets in atherosclerosis

Activated platelets, represent an important resource of inflammatory mediators, and play a critical role in atherogenesis. The $P2Y_{12}$ receptor is expressed on platelets, in which its expression is upregulated by nicotine and high glucose levels [30, 62] to mediate the activation of different downstream effectors. To date, three main signaling pathways have been shown to act downstream of $P2Y_{12}$ in platelets (Fig. 3).

The $G_{\alpha i2}$–AC–cAMP–PKA pathway

Once ADP binds to a $P2Y_{12}$ receptor, the coupled G_{i2} protein dissociates into $G_{\alpha i2}$ and $G_{\beta\gamma}$. The dissociated $G_{\alpha i2}$ inhibits the activation of adenylyl cyclase (AC), the production of cyclic adenosine monophosphate (cAMP), and the phosphorylation of protein kinase A (PKA), which causes a change in the level of vasodilator-stimulated phosphoprotein (VASP), an upstream effector of platelet shape [63, 64] and the release of α-granules [49]. However, the relationship between VASP and α-granules is currently uncertain. As a result of granule release, plasma levels of pro-inflammatory factors, such as platelet-derived growth factor (PDGF) and platelet factor 4 (PF4) increase, and the exposure levels of P-selectin and CD40 ligand (CD40L) are upregulated [65]. These factors trigger inflammatory cascades, and may promote the development of atherosclerosis.

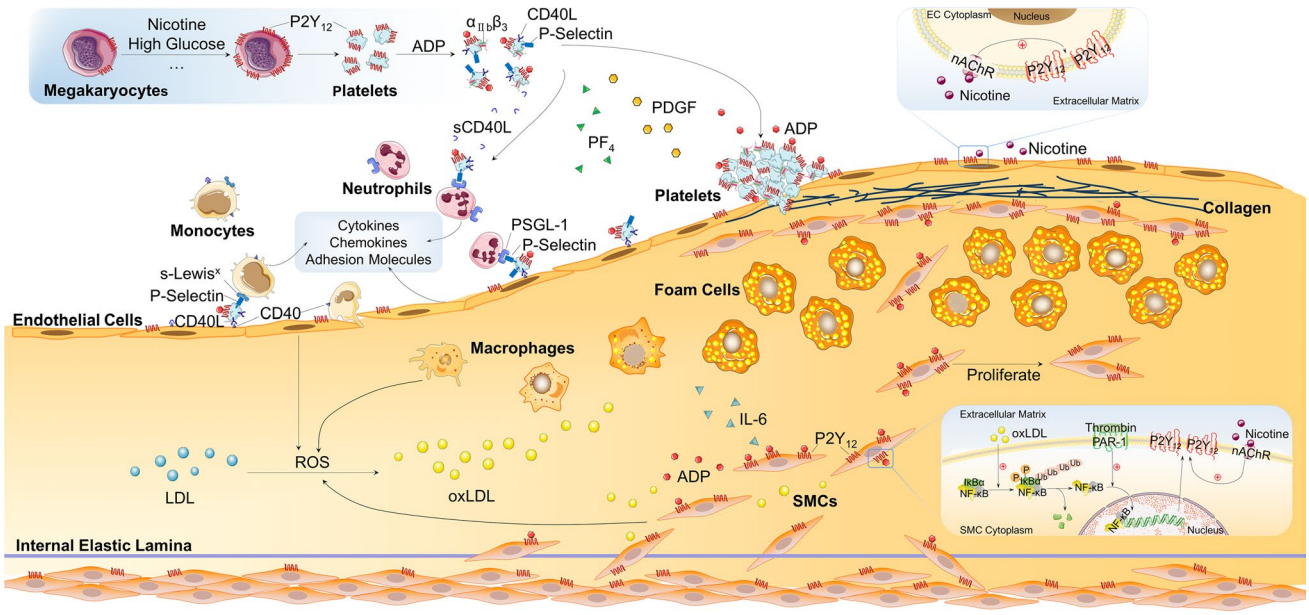

Fig. 2 Mechanisms of $P2Y_{12}$ receptor involvement in pathological process of atherosclerosis. The $P2Y_{12}$ receptor on platelet, upregulated under nicotine or high glucose condition, activates platelets, leading to platelet aggregation to damaged ECs and increased expression and release of inflammatory molecules, which attracting recruitment and infiltration of inflammatory cells, such as monocytes and neutrophils. The platelets, inflammatory cells and damaged ECs interact with each other and induce inflammatory cascades. The SMC $P2Y_{12}$ receptor plays a key role in the proliferation, IL-6 secretion and migration of SMCs into the intima and plaque. The expression of $P2Y_{12}$ receptor on SMC can be upregulated by oxLDL or thrombin via NF-κB pathway. Levels of $P2Y_{12}$ receptor on SMCs and ECs can be increased by nicotine via nAChR. *EC* endothelial cell; *IL-6* interleukin-6; *LDL* low density lipoprotein; *nAChR* nicotinic acetylcholine receptor; *NF-κB* nucleus factor-κB; *oxLDL* oxidized low density lipoprotein; *PAR-1* protease activated receptors-1; *PDGF* platelet-derived growth factor; *PF4* platelet factor 4; *PSGL-1* P-selectin glycoprotein ligand-1; *SMC* smooth muscle cell (Adapted from Ref. [61])

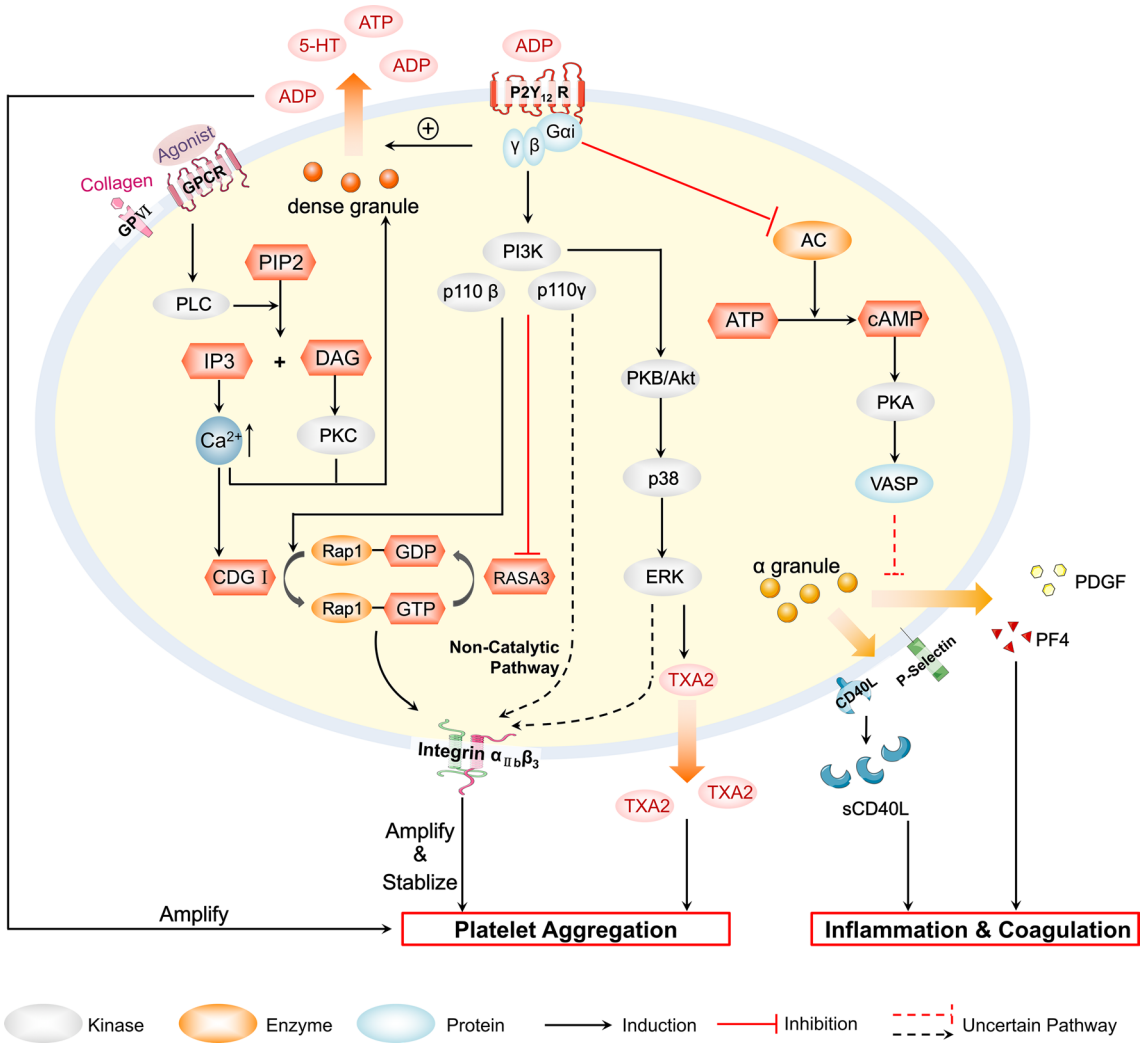

Fig. 3 Mechanisms of platelet $P2Y_{12}$ receptor involvement in progression of atherosclerosis. ADP binds to the P2Y12-receptor and activates the PI3K via coupled Gβγ protein. PI3K isoform p110β and (or) p110γ and regulates activation of PKB/Akt and RAP1 as well as inhibition of RASA3, which balances the circle of Rap1-GDP and Rap1-GTP with CDGI. The phosphorylated Akt mediates an Akt-p38-ERK pathway and TXA2 generation. Both Akt and RAP1 pathways stimulate the inside-out activation of integrin $\alpha_{II}b\beta3$, which allows for amplification and stabilization of platelet aggregation. The $P2Y_{12}$-promoted release of dense granule amplifies the platelet aggregation. On the other hand, the activated Gαi protein mediates the inhibition of AC and decrease of cAMP and PKA levels, resulting in α-granules release containing PDGF and PF4, and the exposure of P-selectin and CD40L, which leads to inflammation and coagulation. *AC* adenylyl cyclase; *ADP* adenosine diphosphate; *ATP* adenosine triphosphate; *cAMP* cyclic adenosine monophosphate; *CDGI* Ca2+ and diacylglycerol regulated guanine nucleotide exchange factor I; *RASA* Ras activator; *CD40L* CD40 ligand; *ERK* extracellular signal-regulated kinase; *GTP* guanosine triphosphate; *PDGF* platelet-derived growth factor; *PF4* platelet factor 4; *PI3K* phosphoinositide 3-kinase; *PKA* protein kinase A; *PKB* protein kinase B; *PKC* protein kinase C; *PLC* phospholipase C; *RAP* Ras-related protein; *TXA2* thromboxaneA2; *VASP* vasodilator-stimulated phosphoprotein

The $G_{\beta\gamma}$-PI3K–Rap1 pathway

Rap1, a member of the small GTPase family, plays a key role in platelet aggregation. After ADP binds to the $P2Y_{12}$ receptor, the dissociated $G_{\beta\gamma}$ activates phosphoinositide 3-kinase (PI3K) [66]. Then, RAP1 is activated via GTP-loading from the inactive GDP-bound form and the subsequent inside-out activation of integrin $\alpha_{IIb}\beta_3$ is synergized by CalDAG-GEFI (CDGI) [67, 68]. Integrin $\alpha_{IIb}\beta_3$ is an important signaling molecule known to contribute to the amplification and stabilization of platelet aggregation [69–71]. Deficiency in one of two PI3K isoforms (p110β or p110γ) inhibits ADP-induced RAP-1b and platelet aggregation to some degree, but does not completely block either [72, 73]. However, Jackson's team proposed an opposite view, whereby p110γ may mediate non-catalytic signaling to activate $\alpha_{IIb}\beta_3$ [74].

In addition, the $P2Y_{12}$ receptor and PI3K also play key roles in downregulating the activity of RASA3, one of the

most highly expressed GTPase activating proteins (GAPs) to hydrolyze GTP in platelets. They thereby exert a synergistic effect that results in the upregulation of PI3K-Rap1 and CDGI-Rap1 activity to sustain the integrin activation [75, 76]. As a result, CDGI induces the rapid but reversible activation of $\alpha_{IIb}\beta_3$ and platelet aggregation, while PKC and $P2Y_{12}$–PI3K complementarily induce the delayed but sustained activation of $\alpha_{IIb}\beta_3$ and platelet aggregation.

The $G_{\beta\gamma}$-PI3K–PKB/Akt–ERK pathway

PI3K also induces the sequential phosphorylation of protein kinase B(PKB)/Akt and activation of mitogen-activated protein kinase (MAPK) p38 and extracellular regulated protein kinases (ERK). Briefly, the $P2Y_{12}$-receptor mediates the PI3K–PKB/Akt–p38-ERK pathway, leading to the generation of thromboxane A2 and platelet aggregation [77, 78]. In addition, because it shares this signaling pathway in common with integrin activation, the p38-ERK pathway may also act as a stimulator of the inside-out activation of $\alpha_{IIb}\beta_3$ [79].

In summary, the activation of $P2Y_{12}$ receptors located on platelets mainly results in three effects. First, the activated platelets release a collection of cytokines, adhesion molecules and chemokines to attract leukocytes such as neutrophils, lymphocytes and monocytes, to the area around the damaged endothelium. The leukocytes release more inflammatory molecules, adhere to the endothelium and migrate through the endothelial layer, where they become involved in atherogenesis. Second, membrane proteins and receptors, such as glycoprotein (GP) IIb/IIIa, are activated to recruit many more platelets, resulting in the induction of platelet aggregation, which plays a key role in plaque formation after endothelial damage and thrombogenesis after plaque rupture. Third, the ADP-induced activation promotes the release of the dense granules induced by other agonists [80–82] and then activates the recruitment of more platelets and initiates a positive feedback mechanism that contributes to an inflammatory storm and amplifies and stabilizes platelet aggregation.

The role of $P2Y_{12}$ receptors expressed on VSMCs in atherosclerosis

Fewer studies have explored the functions of the $P2Y_{12}$ receptor in VSMCs. The first report, by Wihlborg et al. in 2004, showed that $P2Y_{12}$ receptors are expressed on human internal mammary artery SMCs [32]. Sane et al. then reconfirmed that $P2Y_{12}$ is expressed on human aortic and saphenous vein SMCs and that its expression is enhanced by nicotine via nicotinic acetylcholine receptors (nAChR) [30] and by thrombin via the NF-κB pathway [83]. Subsequently, the $P2Y_{12}$ receptor was also found to be expressed in human carotid atherosclerotic plaques, especially at plaque ruptures, and that it colocalizes with SMCs [83]. The level of $P2Y_{12}$ expression observed in culprit coronary plaques, in which it mostly colocalizes with ECs and SMCs but not macrophages, was higher in acute MI patients than in stable angina pectoris patients [84], implicating the $P2Y_{12}$ receptor in plaque destabilization. It has been reported that in mice, the level of expression of the $P2Y_{12}$ receptor on VSMCs is only approximately 7% of that on platelets, and that its expression on VSMCs may have less effect than its expression on platelets with regard to the size of plaques during atherogenesis [49]. However, Storey's team performed an interesting experiment, in which they obtained platelet $p2y_{12}$-deficient and vessel wall $p2y_{12}$-deficient $ApoE^{-/-}$ mice from $ApoE^{-/-}$ and $ApoE^{-/-}p2y_{12}^{-/-}$ male mice undergoing bone marrow transplantation. After the mice were fed a high-fat diet for 4 weeks, the authors surprisingly found that in the vessel wall $p2y_{12}$-deficient mice compared with $ApoE^{-/-}$ controls, plaque sizes were significantly lower in the aortic sinus, aortic arch, and brachiocephalic artery, while in the platelet $p2y_{12}$-deficient mice, there were no significant differences, indicating that the expression of $P2Y_{12}$ receptors in vessel walls, but not platelets, promotes early atherogenesis [50].

As was observed in platelets, $P2Y_{12}$ receptors reduced the cAMP levels via Gi proteins in human VSMCs [32, 83], but the influence of this function remains unclear. In vitro, the $P2Y_{12}$ receptors present on SMCs were also found to enhance the expression of IL-6 [83], thereby leading to an increase in the production of chemokines, an increase in intercellular adhesion molecule-1 (ICAM-1) levels via the endothelial cell response, and the subsequent recruitment and transmigration of leukocytes [85, 86]. Moreover, in vitro, $P2Y_{12}$ receptors enhanced the proliferation of human SMCs [83], which is an important step in the development of atherosclerosis. Furthermore, our group found that in a high-fat-diet-fed $ApoE^{-/-}$ mouse model, the number of $P2Y_{12}$-positive SMCs in plaques was linearly correlated with the plaque area in the aortic arch. In an $ApoE^{-/-}$ mouse model, in the clopidogrel-treated group compared with a placebo group, the abundance of SMCs was lower in the lesions of mice fed a western diet for 12 weeks, potentially because SMC migration was suppressed by the $P2Y_{12}$ receptor blockade-mediated inhibition of PKA and cofilin dephosphorylation [56]. In addition, the $P2Y_{12}$ receptors on VSMCs mediate the 2-MesADP-induced contraction of human blood vessels, especially small veins [32], an effect that may be associated with vasospasm and could further contribute to atherosclerosis. However, it is a pity that these effects were not inhibited by clopidogrel in humans [32].

Thus, the $P2Y_{12}$ receptor-mediated proliferation and migration of VSMCs play important roles during atherogenesis. It has been reported that up to 50% of the

macrophage-like cells in plaques are derived from SMCs [87] and VSMC phenotypic switching are also critical in both atherogenesis and advanced plaques. However, to indentify whether the $P2Y_{12}$ receptors expressed on VSMCs involve in phenotypic switching and SMC-derived foam cells formation in atherosclerotic lesions and further effect plaque vulnerability, more evidence need to be provided.

The role of $P2Y_{12}$ receptors expressed on inflammatory cells in atherosclerosis

Although there is almost no $P2Y_{12}$ receptor expression in white blood cells in mice [49], $P2Y_{12}$ receptors could affect inflammatory cell activities, such as rolling and recruitment, via the cytokines, adhesion molecules and chemokines that are released from the abovementioned cells to promote atherosclerotic processes.

Reports have shown that in $ApoE^{-/-}$ mice, genetically knocking out the $p2y_{12}$ gene decreases monocyte/macrophage infiltration within vascular walls [49]. A recent study reported that prasugrel inhibited the platelet-induced release of interferon-γ (IFN-γ) and the differentiation of $CD4^+$ T cells into pro-inflammatory $CD4^+$ phenotypes; that report investigated the role of $P2Y_{12}$ receptor inhibitors in indirect immune responses to platelet activation [88]. Clopidogrel induced a similar effect in addition to reducing the levels of P-selectin, E-selectin, PDGF-β, ICAM-1, vascular adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) and decreasing the numbers of $CD4^+$ and $CD8^+$ T cells in the vascular walls of animal models [58, 60]. Its effects on cellular infiltration may represent an outcome of the $P2Y_{12}$ blockade-mediated down-regulation of cytokines, adhesion molecules and chemokines [89], as no report to date has shown that $P2Y_{12}$ receptors exert a direct effect on monocytes, macrophages, or T cells.

Surprisingly, clopidogrel was found to upregulate $CD4^+CD25^+$ regulatory T cells (Tregs) in the spleen in addition to splenic but not bone marrow-derived endothelial progenitor cells in a mouse model, and these increases may have contributed to decreases in the volumes and increases in the stability of plaques [55]. Clopidogrel inhibited the proliferation of splenocytes in vitro but had no influence on the oxLDL-mediated immunoproliferative response of splenocytes [55]. The effects of clopidogrel on the spleen suggest that $P2Y_{12}$ receptors play a role in systemic inflammation, for which inflammatory factor levels in the blood are much more commonly used indicators. For example, $P2Y_{12}$ antagonists decrease serum levels of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), IL-6 and IL-8, chemokine (C–C motif) ligand (CCL)-2, and growth colony-stimulating factor (G-CSF) and platelet–leukocyte aggregation in humans [83, 90–93]; the levels of ICAM-1, VACM-1, MCP-1, PF_4, PDGF-β and platelet aggregations in experimental animal models [49, 58, 60]; and P-selectin and soluble CD40L levels in both humans and experimental animal models [58, 83]. All of these inflammatory mediators interact with each other and give rise to a cascade of inflammatory events, including inflammatory cell recruitment and infiltration and the release of reactive oxygen species and matrix degrading enzymes, which are partially responsible for the pathological processes that lead to atherosclerosis.

The role of $P2Y_{12}$ receptors expressed on ECs in atherosclerosis

ECs, as important components of the vessel wall, act as a natural barrier and maintain vascular integrity. The $P2Y_{12}$ receptor is likely to be expressed on ECs in culprit coronary plaques in patients with acute MI, indicating a potential association between EC-$P2Y_{12}$ and atherosclerosis [84]. Similar to platelets and SMCs, the expression of the $P2Y_{12}$ receptor is upregulated by nicotine on ECs via nAChR [30]. A recent study suggested that ADP-$P2Y_{12}$ signaling mediated JNK activation, induced increases in the levels of ICAM-1, VACM-1, and MCP-1, and impaired endothelium-dependent vascular vasodilation, all of which form the basis for atherogenesis and the recruitment of inflammatory cells, including monocytes [94]. The ameliorated inflammation and endothelial dysfunction induced by ticagrelor may partially contribute to its protective effect against the formation or rupture of plaques.

The neuroprotective effects of $P2Y_{12}$ receptor inhibitors in animal model of ischemic stroke

In addition to its antiplatelet and anti-atherogenic effects, some animal studies have also suggested that $P2Y_{12}$ receptor inhibitors may have neuroprotective effects. The $P2Y_{12}$ receptor is expressed on resting microglia but not activated microglia, and it at least partly mediates microglia activation and migration towards damage sites following brain injury [23, 95]. However, ticagrelor reduced infarct volumes and ameliorated the neurological deficits induced by cerebral focal ischemia in a rat permanent middle cerebral artery occlusion (MCAO) model, potentially by inhibiting the activation and chemotaxis of microglia [23]. Yamauchi et al. reported that ticagrelor exerted a similar protective effect, including improved cerebral blood flow, to the ischemia reperfusion injury area in a mouse transient MCAO model by modulating endothelial nitric oxide synthase (eNOS) and ERK1/2 signaling in ECs during the early phase after reperfusion [96]. This neuroprotective effect was also confirmed in prasugrel and non-human primate models. The significantly increased MCA patency, reduced infarct volume and

reduced neurological deficits observed in these models support the notion that these agents may act by inhibiting $P2Y_{12}$ to limit the development of ischemic stroke [97, 98]. These findings indicate that neurological function scores may also be considered an end point in clinical trials.

Conclusion

$P2Y_{12}$ receptor inhibitors are considered antiplatelet agents and are widely used in clinical settings, especially to benefit patients with acute coronary syndromes or non-cardioembolic ischemic stroke. However, a growing amount of both clinical and experimental evidences suggests that in addition to their well-known antiplatelet effects, they also have antiatherosclerotic effects that could be achieved through targeting platelets or VSMCs or other cells, such as inflammatory cells and ECs, and neuroprotective effects. In this review, we summarized the roles of the $P2Y_{12}$ receptor and its inhibitors in atherosclerotic ischemic stroke and atherosclerosis and considered the mechanisms potentially underlying these effects, thereby providing a fundamental basis for the multitarget effects of $P2Y_{12}$ receptors.

In future studies, some points should be taken into consideration, such as the limitation of data for ischemic stroke of atherosclerotic origin, the choice of target study population (i.e., gene polymorphisms associated with drug metabolism), the size of the study population, the length of follow-up and the precise definition of the end point. More evidence is needed to increase our understanding of the role of the $P2Y_{12}$ receptor in atherosclerotic ischemic stroke and atherosclerosis.

Acknowledgements This work was supported by National Natural Science Foundation of China (No. 81571139 to LM, No. 81571119 to BH) and National Key Research and Development Program of China (No. 2018YFC1312200).

Compliance with ethical standards

Ethical approval These experiments comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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