Functional contribution of P2Y₁ receptors to the control of coronary blood flow

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Bender SB, Berwick ZC, Laughlin MH, Tune JD. Functional contribution of P2Y₁ receptors to the control of coronary blood flow. J Appl Physiol 111: 1744–1750, 2011. First published September 22, 2011; doi:10.1152/japplphysiol.00946.2011.—Activation of ADP-sensitive P2Y₁ receptors has been proposed as an integral step in the putative “nucleotide axis” regulating coronary blood flow. However, the specific mechanism(s) and overall contribution of P2Y₁ receptors to the control of coronary blood flow have not been clearly defined. Using vertically integrative studies in isolated coronary arterioles and open-chest anesthetized dogs, we examined the hypothesis that P2Y₁ receptors induce coronary vasodilation via an endothelium-dependent mechanism and contribute to coronary pressure-flow autoregulation and ischemic coronary vasodilation. Immunohistochemistry revealed P2Y₁ receptor expression in coronary arteriolar endothelial and smooth muscle cells. The ADP analog 2-methylthio-ADP induced arteriolar dilation in vitro and in vivo that was abolished by the selective P2Y₁ antagonist MRS-2179 and the nitric oxide synthase inhibitor N' nitro-l-arginine methyl ester. MRS-2179 did not alter baseline coronary flow in vivo but significantly attenuated coronary vasodilation to ATP in vitro and in vivo and the nonhydrolyzable ATP analog ATPS in vitro. Coronary blood flow responses to alterations in coronary perfusion pressure (40–100 mmHg) or to a brief 15-s ischemia were unaffected by MRS-2179. Our data support the hypothesis that P2Y₁ receptors modulate coronary vascular resistance via activation of P2Y₁ receptors in vivo but do not provide evidence for a prominent role of P2Y₁ receptors in vivo.

METHODS

All protocols were approved by an Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, Revised 1996). Male mongrel dogs (20–30 kg) were sedated with morphine (3 mg/kg sc) and anesthetized with α-chloralose (100 mg/kg iv). After completion of experimental protocols, hearts were fibrillated and excised as recommended by the American Veterinary Medical Association guidelines on euthanasia (June 2007). Surgical preparation. The methods for these procedures are described in detail elsewhere (4, 13). Briefly, after induction of anesthesia, intubation, and ventilation, the left femoral artery was catheterized to supply blood to an extracorporeal perfusion system used to maintain cardiac output during the period of coronary vascular isolation.

The P2Y₁ receptor is a member of the G protein-coupled family of P2Y receptors, for which ATP and ADP are endogenous ligands (41, 48). In general, the P2Y₁ receptor demonstrates greater sensitivity to ADP and differs from the P2Y₂ receptor, which is readily activated by triphosphates such as ATP and UTP (41, 48). In the vasculature, both receptors are expressed in endothelial and vascular smooth muscle cells, and their activation induces vasodilation in normal whole vessel preparations (18, 41). However, the relative contribution of endothelium-dependent vs. -independent pathways to ADP-mediated vasodilation varies considerably across circulations (5, 7, 24, 26). In particular, Brayden (5) reported that removal of the endothelium eliminates ADP-mediated vasodilation in middle cerebral arteries but only attenuates this response ~40% in skeletal muscle arterioles and ~10% in mesenteric resistance arteries. Previous work in the coronary circulation yielded conflicting results regarding the mechanism(s) of ADP-induced dilation, with some studies supporting primary endothelial dependence (31, 36) and others suggesting little to no involvement of the endothelium (17, 21, 22, 30, 42). These equivocal findings could be related to the use of nonselective and low-potency agonists such as the ATP analog 2-methylthio-ATP, which also activates P2X receptors (17, 33, 35, 37, 46) and subsequent confounding endothelium-dependent responses (25, 26, 51, 52). Thus the specific mechanisms and relative contribution of P2Y₁ receptors to the control of coronary blood flow have not been clearly defined.

The purpose of this investigation was to test the hypothesis that P2Y₁ receptors induce coronary vasodilation via an endothelium-dependent mechanism and contribute to physiological coronary responses to changes in perfusion pressure (autoregulation) and/or cardiac ischemia (reactive hyperemia). This hypothesis was examined by vertically integrated experiments in isolated, pressurized coronary arterioles in vitro and open-chest anesthetized dogs in vivo. Expression and localization of P2Y₁ receptors were also assessed by Western blotting and immunohistochemistry.
perfuse the left anterior descending (LAD) coronary artery. The right femoral vein was also catheterized for injection of supplemental anesthetic, heparin, and sodium bicarbonate as necessary based on periodic arterial blood gas determinations. After a left lateral thoracotomy and heparin administration (500 U/kg), a proximal portion of the LAD was isolated and cannulated with a stainless steel cannula attached to the extracorporeal perfusion system. Coronary perfusion pressure was maintained at 100 mmHg throughout experimental protocols, unless otherwise noted, by a servo-controlled roller pump. Blood flow in the coronary perfusion line was measured by an in-line flow transducer (Transonic, Ithaca, NY). Data were collected using Iox acquisition software (Emka Technologies, Falls Church, VA). Hemodynamic parameters were allowed to stabilize for ~30 min before initiation of experimental protocols.

In vivo experimental protocols. ATP (1–10 μg·kg⁻¹·min⁻¹) and the selective P2Y1 receptor agonist 2-methyl-ADP trisodium salt (2-MeS-ADP) were infused at a constant rate into the LAD perfusion circuit before and during administration of the selective P2Y1 receptor antagonist MRS-2179 (0.5 mg/min ic). Infusion of MRS-2179 was initiated 5 min prior to agonist infusions. The infusion rate of 2-MeS-ADP was adjusted for each animal based on basal coronary flow and hematocrit to achieve a calculated plasma concentration of 10 μM. In separate animals, coronary flow responses to 2-MeS-ADP were examined after administration of the nitric oxide (NO) synthase (NOS) inhibitor N³-nitro-L-arginine-methyl ester (L-NAME, 150 μg/min ic). Coronary reactive hyperemia was assessed by a 15-s occlusion of the LAD coronary artery before and after administration of MRS-2179 (0.5 mg/min ic). Coronary pressure-flow autoregulation was examined via stepwise reductions in coronary perfusion pressure between 100 and 40 mmHg before and during administration of MRS-2179 (0.5 mg/min ic). All drugs were prepared in 0.9% saline. LAD coronary artery perfusion territory was estimated as previously described by Feigl et al. (20).

Isolated arterioles. For in vitro studies, separate dogs were euthanized with pentobarbital sodium, and the heart was excised. An apical myocardial section was immersion-fixed in 10% buffered formalin for ~24 h and processed through standard paraffin embedding. Myocardial sections (5 μm thick) were cut with an automated microtome, floated onto positively charged slides, and deparaffinized. Slides were then steamed in citrate buffer at pH 6.0 (target retrieval solution S1699, Dako) for 30 min and incubated for 30 min. The slides were washed sequentially with Triton X-100 and water after each step in the staining protocol. Sections were incubated with avidin-biotin two-step blocking solution (Vector SP-2001) to inhibit background staining and in 3% hydrogen peroxide to inhibit endogenous peroxidase. Nonserum protein block (catalog no. X909, Dako) was applied for 1 h with secondary antibody conjugated to horseradish peroxidase, and protein expression was detected by enhanced chemiluminescence on a Kodak Image Station.

Immunohistochemistry. Cellular localization of P2Y1 receptors was determined via immunohistochemical staining of coronary arterioles in dog myocardial sections, similar to a previous study (27). An apical section of myocardium was immersion-fixed in 10% buffered formalin for 24 h and processed through standard paraffin embedding. Myocardial sections (5 μm thick) were cut with an automated microtome, floated onto positively charged slides, and deparaffinized. Slides were then steamed in citrate buffer at pH 6.0 (target retrieval solution S1699, Dako) for 30 min and then cooled for 30 min. The slides were washed sequentially with Triton X-100 and water after each step in the staining protocol. Sections were incubated with avidin-biotin two-step blocking solution (Vector SP-2001) to inhibit background staining and in 3% hydrogen peroxide to inhibit endogenous peroxidase. Nonserum protein block (catalog no. X909, Dako) was applied to inhibit nonspecific protein binding, and slides were incubated in primary rabbit polyclonal anti-P2Y1 receptor antibody (catalog no. APR-009, Alomone Labs) alone or after prior incubation of the antibody with the synthetic control peptide, according to the manufacturer’s instructions. The primary antibody was prepared in PBS containing 1% bovine serum albumin. Membranes were then incubated for 1 h with secondary antibody conjugated to horseradish peroxidase, and protein expression was detected by enhanced chemiluminescence on a Kodak Image Station.

In vitro arteriolar diameter results were normalized to maximum diameter, and presented as percent maximal dilation, calculated as follows: \(\frac{D_d - D_b}{D_{max} - D_b} \times 100\), where \(D_d\) is diameter after a drug intervention, \(D_b\) is baseline preconstricted diameter, and \(D_{max}\) is maximum passive diameter. EC₅₀ values (pD₂ = −log EC₅₀) were determined for individual concentration-response curves of each agonist, and reactive hyperemic volumes were calculated as the area under the curve using Prism software (GraphPad, San Diego, CA). Hyperemic volumes were normalized to the amount of coronary flow debt incurred during coronary occlusion (i.e., repayment-to-debt ratio). The duration of hyperemia was evaluated at the point when coronary flow returned to within 5% of baseline. Statistical analysis was performed using a t-test or one-
RESULTS

Coronary P2Y1 protein expression. Western blots of canine LCX coronary artery revealed three bands for the P2Y1 receptor (~42, 168, and 210 kDa; Fig. 1A), similar to findings with the same primary antibody in human and porcine vascular tissue (42, 49). P2Y1 receptor oligomerization has been suggested to account for these multiple bands, such that the band at 168 kDa represents a P2Y1 tetramer (49, 54). Immunohistochemistry revealed abundant localization of P2Y1 receptors to endothelial and vascular smooth muscle cells in canine coronary arterioles (Fig. 1B). Prior incubation of the primary antibody with the synthetic control peptide eliminated all three immunoblot bands and positive staining of arterioles, confirming P2Y1 specificity (not shown). Omission of the primary antibody also eliminated positive staining of arterioles (not shown).

Mechanism of P2Y1-mediated coronary vasodilation. Coronary arterioles with a passive internal diameter of 129 ± 7 μm were used in this study. Twenty-six arterioles from 17 dogs were used (i.e., multiple arterioles were isolated from several dogs, each for a different agonist). ET-1-induced tone averaged 45 ± 3%. All purinergic agonists induced dose-dependent coronary arteriolar dilation with order of potency as follows: ATP > ATPγS > 2-MeS-ADP; pD2 values were 5.2 ± 0.3, 5.1 ± 0.2, and 4.2 ± 0.1 for ATP, ATPγS, and 2-MeS-ADP, respectively (P < 0.05 for 2-MeS-ADP vs. other agonists). Activation of P2Y1 receptors with 2-MeS-ADP induced maximal dilation of 59 ± 6% at 100 μM, and this dilation was abolished by the selective P2Y1 antagonist MRS-2179 (10 μM; Fig. 2A). Treatment of arterioles with MRS-2179 tended to reduce basal ET-1-induced tone (~18 ± 9%, P = 0.055 vs. baseline). Administration of the NOS inhibitor l-NAME (300 μM) abrogated coronary dilation to 2-MeS-ADP to an extent similar to MRS-2179 (Fig. 2A). Inhibition of P2Y1 receptors with MRS-2179 also significantly attenuated dilation to ATP (1 μM–100 μM), reducing maximal dilation from 82 ± 5% to 45 ± 8% (Fig. 2B). To address whether activation of P2Y1 receptors occurs directly by ATP or via its breakdown products, additional vasoreactivity studies were conducted with the nonhydrolyzable ATP analog ATPγS. MRS-2179 shifted the ATPγS concentration-response curve to the right but did not affect the maximal dilatory response (74 ± 6% vs. 80 ± 3%; Fig. 2C), indicating modest direct P2Y1 activation by ATP.

In vivo P2Y1-mediated coronary vasodilation. Twelve dogs were dedicated for in vivo blood flow studies (i.e., no arterioles isolated for in vitro work). Infusion of the selective P2Y1 agonist 2-MeS-ADP (plasma concentration = 10 μM) into the LAD coronary artery increased coronary flow by 1.3 ± 0.2 ml·min⁻¹·g⁻¹ (Fig. 3A). In agreement with our findings in isolated arterioles, this increase in coronary flow was markedly attenuated by inhibition of P2Y1 receptors with MRS-2179 (0.5 mg/min ic) or NO production with l-NAME (150 μg/min ic; Fig. 3A). Intracoronary infusion of MRS-2179 or l-NAME...
did not alter baseline coronary flow (transiently or persistently), mean arterial pressure, or heart rate (Table 1). Infusion of MRS-2179 also had no effect on cardiac work, indicated by no change in rate-pressure product (systolic blood pressure × heart rate), before and after infusion (9.219 ± 974 and 10.328 ± 1.139, respectively, P = 0.11). Inhibition of P2Y₁ receptors with MRS-2179 also reduced coronary vasodilation to ATP (1–10 μg·kg⁻¹·min⁻¹ ic) in vivo by ~20% (Fig. 3B).

Contribution of P2Y₁ receptors to pressure-flow autoregulation and coronary reactive hyperemia. Pressure-flow autoregulation was assessed via servo-control of coronary perfusion pressure over a range of 100 to 40 mmHg. Under untreated-control conditions, coronary blood flow averaged 0.48 ± 0.04 ml·min⁻¹·g⁻¹ at a perfusion pressure of 100 mmHg and 0.34 ± 0.03 ml·min⁻¹·g⁻¹ at a perfusion pressure of 60 mmHg. Inhibition of P2Y₁ receptors with MRS-2179 did not significantly affect flow transiently at each pressure step or the relationship between coronary blood flow and coronary perfusion pressure, i.e., autoregulatory capability (Fig. 4A). Coronary zero flow pressure was also unchanged by MRS-2179 (26 ± 1 vs. 28 ± 3 mmHg).

The role of P2Y₁ receptors in coronary reactive hyperemia was assessed by the flow response following a 15-s coronary artery occlusion before and during infusion of MRS-2179 (Fig. 4B). Inhibition of P2Y₁ receptors did not significantly alter coronary vasodilation in response to this brief episode of cardiac ischemia, as MRS-2179 did not affect the peak hyperemic response (2.6 ± 0.2 vs. 2.5 ± 0.2 ml·min⁻¹·g⁻¹), the duration of hyperemia (52 ± 4 vs. 52 ± 3 s), the volume of repayment (0.7 ± 0.1 vs. 0.8 ± 0.1 ml/g), or the repayment of coronary flow debt (636 ± 33% vs. 737 ± 74%).

Table 1. Effect of antagonists on baseline hemodynamic variables in anesthetized open-chest dogs

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<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>CBF, ml/min</th>
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<tr>
<td>Baseline</td>
<td>102 ± 5</td>
<td>70 ± 5</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>+MRS-2179</td>
<td>104 ± 4</td>
<td>89 ± 11</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>+l-NAME</td>
<td>101 ± 9</td>
<td>82 ± 7</td>
<td>26 ± 5</td>
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Values are means ± SE. MAP, mean arterial blood pressure; HR, heart rate; CBF, coronary blood flow; l-NAME, N³-nitro-l-arginine methyl ester.

DISCUSSION

A nucleotide axis involving the activation of P2 purinergic receptors by ATP and its degradation product ADP has been proposed as a mechanism for the moment-to-moment regulation of coronary blood flow (8, 23). However, a role for endogenous purinergic compounds, notably ATP and ADP, in the control of coronary blood flow has remained an area of substantive debate. Activation of endothelial P2Y₁ receptors by ADP may therefore represent an integral step in this cascade. Vasodilation to ADP has been linked to the release of NO, prostacyclin, and endothelium-derived hyperpolarizing factor (5, 18, 41). In this context, the present study was designed to examine the mechanisms underlying P2Y₁ receptor-mediated dilation in the coronary circulation, as well as the functional contribution of these receptors to coronary pressure-flow autoregulation and reactive hyperemia. The major findings of this study are as follows: 1) P2Y₁ receptors are expressed in coronary microvascular endothelial and vascular smooth muscle cells; 2) ADP-induced coronary arteriolar dilation is predominantly mediated via a P2Y₁ and NO-dependent mechanism; and 3) P2Y₁ receptors contribute only partially (~20%) to ATP-induced coronary dilation. Despite these effects, inhibition of P2Y₁ receptors did not affect coronary blood flow under baseline-resting conditions, in response to changes in coronary perfusion pressure (autoregulation), or in response to a brief coronary artery occlusion (reactive hyperemia). Taken together, these data indicate that although P2Y₁ receptors represent a critical component of purinergic coronary vasodilation, they do not play an essential role in physiological coronary responses to alterations in perfusion pressure or cardiac ischemia.

Mechanisms of P2Y₁-mediated coronary vasodilation. Vascular P2Y₁ receptors demonstrate high affinity for ADP relative to P2Y₂ receptors, which more readily bind triphosphates such as ATP and UTP (41). Previous studies demonstrated that the mechanism(s) of ADP-induced dilation varies substantially across vascular beds (5, 7, 24, 34). Some, but not all, previous work in the coronary circulation demonstrated that ADP dilation is largely endothelium-dependent, as it is markedly attenuated by endothelial denudation (17, 21, 22, 30, 31, 36, 40, 42). However, the specific endothelial pathways involved remain
unclear. Our results extend these findings by demonstrating that coronary arteriolar dilation to the ADP analog 2-MeS-ADP is P2Y₁ receptor- and NO-dependent, as it is similarly blocked by MRS-2179 and l-NAME in vivo and in vitro. These findings are consistent with the coronary vasodilator effect of dinucleotide polyphosphates such as Ap5A and Ap6A (46) and indicate that coronary endothelial P2Y₁ receptors signal primarily through NO, with potentially minor roles for prostacyclin or endothelium-derived hyperpolarizing factor. ADP and P2Y₁ signaling to NOS has been linked to rapid (<1-min) endothelial NOS activation in cultured endothelial cells through phosphorylation of stimulatory serine residues (Ser¹⁷⁹ and Ser⁶₃⁵) and concomitant dephosphorylation of the inhibitory Ser¹¹⁶ site (10, 28). This effect is consistent with the time course of ADP-induced vasodilation in the present study and that reported for NO release after P2Y₁ stimulation in the mesenteric and placental endothelium (6, 7). The endothelial dependence of ADP-mediated coronary vasodilation is also consistent with other vascular beds such as the cerebral circulation (5, 55) but differs from others, i.e., mesenteric and skeletal muscle (5). Our data are intriguing in this regard, as we found strong P2Y₁ immunoreactivity in coronary microvascular smooth muscle, despite little to no vasomotor effect of 2-MeS-ADP in the presence of the NOS inhibitor l-NAME. To our knowledge, this is the first study to demonstrate the specific endothelial pathway involved in ADP-mediated coronary vasodilation in vivo.

The putative role of P2Y₁ receptors in the regulation of coronary blood flow in vivo would likely involve the breakdown of endogenous ATP to ADP (19, 23). Therefore, we conducted additional experiments to examine the contribution of P2Y₁ receptors to ATP-mediated coronary vasodilation. ATP has long been recognized as a potent coronary vasodilator through direct effects on vascular cells and indirect effects via breakdown products such as ADP (18, 30, 41). Regarding the P2Y₁ receptor, our data support both of these mechanisms, as P2Y₁ inhibition abolished ADP-mediated coronary vasodilation while modestly attenuating ATP-mediated vasodilation in vitro and in vivo. This finding is consistent with other reports (22, 23) and indicates robust P2Y₁ activation by ADP, with a more modest role for P2Y₁-mediated NO production in ATP-induced coronary vasodilation (29). Our in vitro data indicate that coronary P2Y₁ receptors are also directly activated by ATP, as dilation to the nonhydrolyzable ATP analog ATP₇S was attenuated by inhibition of P2Y₁ receptors with MRS-2179. Maximal dilation to ATP₇S was not altered by P2Y₁ blockade, however, indicating that the direct effects of ATP involve other dilator receptors/systems. We propose that this direct effect is related to the activation of P2Y₂ receptors, as Gorman et al. (23) recently reported that the highly selective P2Y₂ agonist MRS-2768 induces coronary vasodilation in the in situ dog heart.

P2Y₁ receptor involvement in coronary blood flow control. It has been proposed that the local liberation of ATP from various sources (i.e., red blood cells, endothelial cells, and myocytes) is sensitive to myocardial metabolism and, thus, may contribute to the matching of coronary flow to myocardial metabolism (12, 19, 23). This hypothesis is supported by previous reports that coronary venous plasma ATP levels increase in proportion to cardiac work during exercise (19) and that inhibition of purinergic vasodilation (combined blockade with adenosine and P2Y₁ receptor antagonists and l-NAME) decreases the balance between coronary blood flow and myocardial metabolism at rest and during exercise in dogs (23). These findings implicate ATP and its breakdown products in the negative-feedback control of the coronary circulation (8, 23, 53). However, the present data importantly indicate that P2Y₁ receptors do not significantly contribute to the regulation of basal coronary tone, as MRS-2179 did not significantly reduce resting coronary flow. These data are consistent with previous in vivo studies in dogs and swine (23, 39). Since P2Y₁ receptor activation stimulates NO production and numerous earlier studies demonstrated little to no effect of NOS inhibition on baseline coronary flow, the lack of effect of P2Y₁ inhibition on baseline coronary flow is not surprising (1, 3, 14, 43, 44).

Coronary blood flow responses to changes in perfusion pressure and following brief coronary artery occlusion have previously been reported to be modulated, at least in part, by NO (9, 11, 13, 43, 50). The production of NO upon endogenous stimulation of coronary P2Y₁ receptors may therefore contribute to coronary pressure-flow autoregulation and/or reactive hyperemia. Our data are the first to examine the contribution of P2Y₁ receptors to coronary pressure-flow autoregulation and demonstrate that blockade of these receptors does...
not modulate the autoregulation of coronary flow in response to changes in perfusion pressure. Additionally, inhibition of P2Y1 receptors with MRS-2179 had little to no effect on the coronary reactive hyperemic response. This finding differs from a previous study by Olivecrona et al. (39), who reported a 25–50% decrease in the coronary hyperemic response to 10 min of ischemia following an intracoronary bolus of MRS-2179 in swine. Besides potential species differences, these discrepant findings may be the result of the significant difference in the duration of ischemia: 15 s in the present study and 10 min in the study of Olivecrona et al. Longer durations (5–20 min) of ischemia have been associated with ultrastructural changes in the myocardium, which are absent following acute (<5 min) ischemia (32). Taken together, these data suggest that P2Y1 involvement in coronary ischemic dilation may be dependent on the accumulation of endogenous P2Y1 agonists over time, i.e., modulation of vasodilation in response to prolonged (rather than acute) bouts of myocardial ischemia. This contention is supported by studies that reported increased release of ATP from red blood cells and cardiac myocytes in response to reductions in blood PO2 and tissue PO2 after minutes of ischemia, as opposed to seconds of ischemia (15, 16, 47). The potential time course of P2Y1 activation following cardiac ischemia warrants further examination. In summary, the present data demonstrate that endogenous activation of P2Y1 receptors is not required for the acute regulation of coronary flow. Thus the contribution of NO to coronary autoregulation and reactive hyperemia does not require activation of these receptors (9, 13, 50). Additional studies are needed, however, to address whether the negligible effect of P2Y1 receptor inhibition could be related to the activation of compensatory pathways or other purinergic receptor subtypes such as endothelial P2Y2 or P2Y6, which could mask the effect of P2Y1 receptor inhibition on coronary blood flow regulation. The fact that P2Y1 blockade inhibits ~20% of in vivo coronary dilation to ATP suggests significant redundancy in the nucleotide axis. Alternatively, recent evidence also suggests a potential role for endothelial P2X receptors in the regulation of vascular tone; however, expression of these receptors in the coronary vasculature has not been determined (25, 26, 51, 52). It is also possible that the production of ADP and ATP increased to ATP suggests significant redundancy in the nucleotide axis hypothesis: ATP release and metabolism by coronary artery stenosis. Further work is necessary to delineate the potential involvement of other purinergic receptors. However, such studies are hampered by a lack of potent and selective receptor agonists and antagonists.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.B.B., M.H.L., and J.D.T. are responsible for conception and design of the research; S.B.B., Z.C.B., and J.D.T. performed the experiments; S.B.B., Z.C.B., and J.D.T. analyzed the data; S.B.B., M.H.L., and J.D.T. interpreted the results of the experiments; S.B.B. prepared the figures; S.B.B. drafted the manuscript; S.B.B., Z.C.B., M.H.L., and J.D.T. edited and revised the manuscript; S.B.B., Z.C.B., M.H.L., and J.D.T. approved the final version of the manuscript.

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