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# Clopidogrel, independent of vascular $P2Y_{12}$ receptor, improves the arterial function in small mesenteric arteries from Ang II-hypertensive rats

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## Abstract

The $P2Y_{12}$ receptor antagonist clopidogrel blocks platelet aggregation, improves systemic endothelial nitric oxide bioavailability, and has anti-inflammatory effects. Since $P2Y_{12}$ receptors have been identified in the vasculature, we hypothesized that clopidogrel ameliorates angiotensin II (Ang II) -induced vascular functional changes by blockade of $P2Y_{12}$ receptors in the vasculature. Male Sprague Dawley rats were infused with Ang II (60 $ng.min^{-1}$) or vehicle for 14 days. The animals were treated with clopidogrel (10$mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle. Vascular reactivity was evaluated in second-order mesenteric arteries. Clopidogrel treatment did not change systolic blood pressure [(mmHg) control-vehicle, 117±7.1 vs. control- Clopidogrel, 125±4.2; AngII-vehicle, 197±10.7 vs. AngII-Clopidogrel, 198±5.2], but it normalized increased phenylephrine-induced vascular contractions [(%KCl) vehicle-treated, 182.2±18 vs. Clopidogrel, 133±14%), as well as impaired vasodilation to acetylcholine [(%) vehicle-treated, 71.7±2.2 vs. Clopidogrel, 85.3±2.8) in Ang II-treated animals. Vascular expression of $P2Y_{12}$ receptor was determined by western blot. Pharmacological characterization of vascular $P2Y_{12}$ was performed with the $P2Y_{12}$ agonist 2-MeS-ADP. Although 2-MeSADP induced endothelium-dependent relaxation [(Emax %) = 71%±12), as well as contractile vascular responses (Emax %=83±12) these actions are not mediated by $P2Y_{12}$ receptor activation. 2-MeS-ADP produced similar vascular responses in control and Ang II rats. These results indicate potential effects of Clopidogrel, such as improvement of hypertension-related vascular functional changes that are not associated with direct actions of clopidogrel in the vasculature, supporting the concept that activated platelets contribute to endothelial dysfunction, possibly via impaired NO bioavailability.

### Keywords

Clopidogrel; P2Y receptors; endothelium; adenosine-5'-diphosphate (ADP); angiotensin II

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## INTRODUCTION

Adenosine-5'-diphosphate (ADP) has been established for a long time as an aggregating agent [1, 2]. Activation of platelets by ADP leads to rapid $Ca^{2+}$ entry, mobilization of intracellular $Ca^{2+}$ stores and activates platelet $P2Y_1$ and $P2Y_{12}$ purinoceptors to inhibit adenylyl cyclase. Extracellular ADP, via those receptors, elicit these physiological responses [3].

There are 2 principal classes of nucleotide receptors: P2X receptors, which are ligand-gated ion channels; and P2Y receptors, which belong to the G-protein coupled receptor family [4, 5]. Extracellular nucleotides, such as ADP, activate P2Y purinoreceptors located both in vascular smooth muscle and endothelial cells, generating vasoconstriction and endothelium-dependent vasodilatation, respectively [6-8].

$P2Y_{12}$ receptors were identified in human blood vessels, where they induce contraction [9]. Controversially, $P2Y_{12}$ receptors do not play a role in vascular function in aorta from rats[7]. Although no data regarding the function of $P2Y_{12}$ receptors in resistance vasculature are available.

The first clinical application of P2 receptor antagonism involved the use of thienopyridines, such as the $P2Y_{12}$ ADP-receptor antagonist Clopidogrel, a platelet aggregation inhibitor [8]. Besides the important role of these drugs in preventing platelet aggregation, recent studies identified direct effects of thienopyridines on endothelial function. Clopidogrel treatment improves endothelial nitric oxide (NO) bioavailability and decreases proinflammatory and prothrombotic-related events in humans and in experimental animals [10-14].

Complications during coronary heart disease, ischemic stroke, and peripheral vascular disease, are related to thrombosis rather than hemorrhage. Some complications related to elevated blood pressure, heart failure, and atrial fibrillation are also associated with thromboembolism. Thus it seems plausible that antithrombotic therapy may be particularly useful in preventing thrombosis-related vascular complications of elevated blood pressure [15]. However, whether these beneficial effects of clopidogrel are all related to prevention of platelet aggregation or if there is a direct effect in the vasculature is still unknown.

We hypothesized that treatment with clopidogrel ameliorates hypertension-associated vascular dysfunction, in part by blocking vascular $P2Y_{12}$ receptors. To test our hypothesis, we assessed the effects of clopidogrel treatment on vascular reactivity in control and angiotensin II (Ang II)-treated rats. Pharmacological and molecular approaches were used to establish the presence and functionality of vascular $P2Y_{12}$ receptors in mesenteric resistance arteries from these animals.

## METHODS

### Animals and blood pressure measurement

Eight week-old male Sprague-Dawley rats (230–250g; Harlan Laboratories, Indianapolis, IN), maintained on a 12:12-h light-dark cycle with rat chow and water *ad libitum*, were used in these studies. All procedures were conducted in accordance with the *Guide for the Care*

*and Use of Laboratory Animals* of the National Institutes of Health and were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Rats were anesthetized with a mixture of ketamine (80mg.kg$^{-1}$) and xylazine (10mg.kg$^{-1}$) and osmotic mini pumps (0.5µl per hour - 14 days - model 2002, Alzet Co., Cupertino, CA) were implanted subcutaneously. Animals were divided into two groups: a control group infused with saline only, and the other infused with Ang II (60 ng.min$^{-1}$) for a period of 14 days. Both groups were simultaneously treated either with clopidogrel (Plavix® - 10mg.kg$^{-1}$.day$^{-1}$) or vehicle (peanut butter, 1g) for 14 days. At day 0 (before experimental procedure) and day 14, systolic blood pressure (SBP) was measured by tail cuff plethysmography in conscious rats. Weight gain was also evaluated by measurement of the body weight at days 0 and 14. The efficacy of treatment with clopidogrel was evaluated by determination of bleeding time, as previously described [16]. Briefly, after 14 days of treatment with clopidogrel or vehicle, rats were placed in individual restrainers and the tip of the tail (3mm) was cut and blood drops were collected on filter paper. The duration of bleeding was recorded.

## Vascular functional studies

After euthanasia, the mesentery was rapidly excised and placed in an 4°C cold physiological salt solution (PSS), containing (mM): NaCl, 130; $NaHCO_3$, 14.9; KCl, 4.7; $KH_2PO_4$, 1.18; $MgSO_4{\cdot}7H_2O$ 1.18; $CaCl_2{\cdot}2H_2O$, 1.56, EDTA, 0.026, glucose 5.5. Second-order branches of mesenteric artery (≈ 2 mm in length with internal diameter ≈ 150 to 2 µm) were carefully dissected and mounted as ring preparations on two stainless steel wires. The second-order mesenteric arteries were mounted in an isometric Mulvany-Halpern small-vessel myograph (40 µm diameter; model 610 *DMT*-USA, Marietta, GA) and recorded by a PowerLab 8/SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO). One wire was attached to a force transducer and the other to a micrometer. Both dissection and mounting of the vessels were carried out in cold (4°C) PSS. The second-order mesenteric arteries were adjusted to maintain a passive force of 3mN. Arteries were equilibrated for 45 min in PSS at 37°C, and continuously bubbled with 5% $CO_2$ and 95% $O_2$. Arterial integrity was assessed first by stimulation of vessels with 120 mM KCl and, after washing and a new stabilization time, by contracting the segments with phenylephrine (PE; 10µM) followed by relaxation with acetylcholine (ACh; 10µM). Endothelium-dependent relaxation was assessed by measuring the dilatory response to ACh (1nM to 10µM) in PE-contracted vessels (3µM). ACh responses were also evaluated after a 30-minute incubation with vehicle or with the NO synthase inhibitor N$^{\omega}$-nitro-*L*-arginine methyl ester (*L*-NAME 100µM) plus indomethacin (10µM), an inhibitor of prostanoid synthesis. A concentration-response curve to PE (1nM to 100µM) was performed to evaluate vascular contractility. The response to 2-MeS-ADP [2-(Methylthio) adenosine 5'-trihydrogen diphosphate trisodium; 0.1 to 100µM] was evaluated in arteries on basal tonus and after PE-induced (3µM) contraction. To avoid the possibility of tachyphylatic responses, concentration-response curves to 2-MeS-ADP were performed by testing only one concentration of 2-MeS-ADP in each vascular preparation. Therefore, various vascular preparations from one animal were stimulated with only one concentration of 2-MeS-ADP in this study (0.01 to 100µM), to construct the

concentration-response curve. Responses to 2-MeS-ADP (100μM) were also evaluated in arteries after incubation with *L*-NAME (100μM) plus indomethacin (10μM), both on basal tonus and after PE-induced (3μM) contraction. In addition, 2-MeS-ADP-induced responses (both contraction and relaxation) were determined in the presence of selective antagonists for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors: MRS-2179, MRS-2395 and MRS-2211, respectively.

### Western blot for detection of vascular $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors

Proteins (40 μg) extracted from small mesenteric arteries were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were incubated with antibodies overnight at 4°C. Antibodies were as follows: $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$ (1:200; Alomone Labs) and β-actin (1:1000; Sigma). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results are normalized to β -actin protein and expressed as arbitrary units.

### Data Analysis

The results are shown as mean ± SEM where “n” represents the number of rats used in the experiments. Contractions were recorded as changes in the displacement (mN) from baseline and normalized by KCl contraction and are represented as percentage of KCl-induced contraction. Relaxation is expressed as percent change from the PE contracted levels. Concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA, U.S.A.). Values of $P<0.05$ were considered a statistically significant difference. Statistical analysis was performed using two-way analysis of variance plus Newman-Keuls post hoc analysis to compare the concentration-responses curves between all the groups. The other analyses were performed with one-way analysis of variance plus Newman-Keuls post hoc analysis.

### Chemicals

Acetylcholine chloride, angiotensin II, indomethacin, $N^{\omega}$-nitro-*L*-arginine methyl ester (L-NAME), 2-(Methylthio) adenosine 5’-trihydrogen diphosphate trisodium (2-MeS-ADP), MRS-2395 [2,2-Dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester], sodium nitriprusside (SNP) and phenylephrine hydrochloride, were purchased from Sigma Aldrich (St. Louis, MO). MRS-2179 tetrasodium salt [2′-Deoxy-N6-methyladenosine 3′,5′-bisphosphate tetrasodium salt] and MRS-2211 2 disodium salt {[(2-Chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde} were purchased from Tocris (Ellsville, MO).

## RESULTS

### Physiologic Parameters and bleeding time

Systolic blood pressure (SBP) and body weight were similar in all groups of animals at the beginning of the study. Ang II significantly increased SBP, whereas clopidogrel treatment did not change SBP either in control or Ang II-hypertensive rats (Table 1). The bleeding

time of rats treated with clopidogrel was significantly prolonged in both normotensive and hypertensive groups, showing efficacy of treatment (Table 1). Ang II-treated animals displayed a tendency to reduced weight gain and clopidogrel treatment had no effect on body weight gain (Table 1).

### Vascular reactivity studies

Mesenteric resistance arteries (Figure 1A) from Ang II-treated animals displayed impaired relaxation to ACh (71.8±2.5% vs. 86.1±2.5%, control), which was significantly improved in vessels from Ang-II infused rats treated with clopidogrel (85.4±2.5%). Clopidogrel treatment had no effects on ACh responses in vessels from control animals (85.5±2% vs. 86.1±2.5%, vehicle). Incubation of mesenteric resistance arteries with L-NAME plus indomethacin abolished the differences in ACh-induced relaxation between the groups (Figure 1B). SNP-induced relaxation was similar amongst mesenteric arteries from all groups (data not shown).

Mesenteric resistance arteries from Ang II-hypertensive rats showed increased contractile responses to PE (182.2±18%) when compared to that in arteries from control animals (132.2±8% - Figure 1C). Treatment with clopidogrel normalized PE-induced contraction in arteries from Ang II hypertensive rats (133 ±14%) but did not change the response in arteries from control animals (136±8%). In addition, incubation of mesenteric resistance with L-NAME plus indomethacin abolished the differences in ACh-induced relaxation between the groups (Figure 1D)

This first set of results indicates that treatment with clopidogrel prevents dysfunction associated with Ang-II hypertension. In addition, the results also suggest that effects of clopidogrel are possibly associated with increased NO bioavailability and prostanoid synthesis.

### 2-MeS-ADP-induced vascular responses

Pharmacological characterization of vascular $P2Y_{12}$ receptors was performed with 2-MeS-ADP. 2-MeS-ADP is the most potent $P2Y_{12}$ receptor agonist available.

**Relaxant Action—**Treatment of mesenteric resistance arteries on passive basal tonus with 2-MeS-ADP did not produce changes in force levels. After contraction with PE (3μM), a significant relaxation to 2-MeS-ADP (10μM) was observed (Figure 2A). Concentration-response curves to 2-MeS-ADP (0.01 to 100μM) showed that maximum relaxation in mesenteric arteries (71%±12) is obtained with 100μM 2-MeS-ADP (Figure 2B).

Vascular responses to 2-MeS-ADP, after constriction with PE (3μM), were determined in all experimental groups and no statistical differences were observed. Figure 2C illustrates relaxant responses to 2-MeS-ADP (100μM) in mesenteric arteries from the four experimental groups.

Although the adenosine diphosphate analogue 2-MeS-ADP is the most potent $P2Y_{12}$ receptor agonist available, this drug can also activate $P2Y_1$ and $P2Y_{13}$ receptors [22]. Therefore, after determining the effects of the 2-MeS-ADP in mesenteric resistance arteries,

we performed a functional characterization of the receptor subtype(s) that mediate 2-MeS-ADP responses in rat second-order mesenteric arteries, by using MRS-2179, a $P2Y_1$ antagonist; MRS-2395, a $P2Y_{12}$ antagonist; and MRS-2211, a $P2Y_{13}$ antagonist. The effects of 2-MeS-ADP were tested after incubation of the vessels with different concentration of each antagonist (0.01, 0.1 and 1.0 μM), for 30 minutes. The concentrations chosen for the experiments were 1μM (MRS-2211) and 0.1 μM (MRS-2179 and MRS-2395), since no further inhibitory effects were observed with greater concentrations of the $P2Y_1$ or $P2Y_{12}$ antagonists (0.01, 0.1 and 1.0 μM).

In second-order mesenteric arteries from control normotensive rats, blockade of $P2Y_1$ receptors, with MRS-2179 (0.1μM), and $P2Y_{13}$, with MRS-2211 (1μM), produced approximately a 50% and 40 % inhibition of 2-MeS-ADP-induced relaxation, respectively (Figure 2D). In contrast, blockade of $P2Y_{12}$ receptors, with MRS-2395 (0.1μM), did not interfere with 2-MeS-ADP-induced relaxation. The simultaneous incubation with both $P2Y_1$ and $P2Y_{12}$ or $P2Y_{13}$ and $P2Y_{12}$ receptors antagonists did not produce greater inhibition of 2-MeS-ADP-induced relaxation (Figure 2D). However, $P2Y_1$ and $P2Y_{13}$ receptors antagonists resulted in almost 75% inhibition of 2-MeS-ADP-induced relaxation. The same profile of results was found in mesenteric arteries from Ang II-induced hypertensive rats, except that concomitant blockade of $P2Y_1$ and $P2Y_{13}$ receptors antagonists did not produce greater inhibition of 2-MeS-ADP-induced relaxation in hypertensive rats (Figure 2D).

**Contractile Action—**Similar to what was observed in non-treated mesenteric arteries; 2-MeS-ADP did not induce changes in level of force in endothelium-intact mesenteric resistance arteries treated with L-NAME (100μM plus indomethacin (10 μM). However, in the presence of L-NAME plus indomethacin, it further increased tension development when tested in PE (3μM)-contracted vessels (Figure 3A). Concentration-response curves to 2-MeS-ADP (0.01 to 100μM), after pre-incubation with L-NAME (100μM) plus indomethacin (10 μM) were performed in PE-constricted mesenteric arteries The maximum contractile response in mesenteric arteries (83%±12) from control rats was obtained with 100μM 2-MeS-ADP (Figure 3B).

Vascular responses to 2-MeS-ADP, in vessels incubated with L-NAME (100μM), plus indomethacin (10 μM), after constriction with PE (3μM) were determined in all experimental groups. Figure 3C illustrates that contractile responses to 2-MeS-ADP (100μM) were not different among the groups.

The blockade of $P2Y_1$ receptors with MRS-2179 or $P2Y_{13}$ receptors with MRS-2211 partially inhibited 2-MeS-ADP-induced contraction in second-order mesenteric arteries, whereas blockade of $P2Y_{12}$ receptors with MRS-2395 produced no significant effect in the response to 2-MeS-ADP. The concomitant blockade of $P2Y_1$ and $P2Y_{12}$ or $P2Y_{13}$ and $P2Y_{12}$ did not produce further inhibition of 2-MeS-ADP-induced contraction (Figure 3D) whereas simultaneous incubation of $P2Y_1$ and $P2Y_{13}$ resulted in reduction of 2-MeS-ADP-induced contraction. The same profile of results was found in mesenteric arteries from Ang II-induced hypertensive rats, but again, simultaneous blockade of $P2Y_1$ and $P2Y_{13}$ receptors antagonists did not produce greater inhibition of 2-MeS-ADP-induced contraction (Figure 2D).

These data from functional experiments suggest that in mesenteric arteries, $P2Y_1$ and possibly $P2Y_{13}$ are involved in both relaxant and contractile responses induced by 2-MeS-ADP.

### Protein expression of vascular $P2Y_{1/12/13}$ receptors

Protein expression of $P2Y_{12}$ receptor was determined in mesenteric arteries from control and Ang II-hypertensive animals. Expression of $P2Y_1$ and $P2Y_{13}$ receptors was also determined because the functional data suggest that actions of the most selective $P2Y_{12}$ agonist available are mediated by $P2Y_1$ and $P2Y_{13}$.

Protein expression of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors was observed in mesenteric arteries. No differences in $P2Y_1$ and $P2Y_{12}$ protein expression were observed between mesenteric arteries from control and Ang II-hypertensive animals (Figure 4A). Interestingly, expression levels of $P2Y_{13}$ receptors were very low in mesenteric arteries from Ang II-hypertensive animals (Figure 4B).

## DISCUSSION

Clopidogrel, an anti-platelet drug, is prescribed to prevent coronary artery disease and thrombotic events. In patients with elevated blood pressure, anti-platelet therapy is recommended for secondary prevention because the magnitude of the absolute benefit in vascular alterations is many times greater [15]. More recently, clopidogrel treatment has been shown to improve endothelial NO bioavailability and ameliorate proinflammatory and prothrombotic-related events [10-12]. Additionally, $P2Y_{12}$ receptor polymorphisms are more frequently present in patients with vascular disease than in healthy people [17].

Due to these additional actions of clopidogrel on endothelial function, and considering that very few studies have addressed the effects of clopidogrel on vascular reactivity, we hypothesized that clopidogrel, by direct actions on the vasculature, is able to attenuate hypertension-related vascular functional changes. The Ang II-induced hypertension model was chosen due to well-characterized changes in vascular reactivity and due to the vasoconstrictor, pro-growth and pro-inflammatory actions of Ang II [18].

PE-induced contraction and ACh-induced relaxation were impaired in mesenteric resistance arteries from rats infused with Ang II. Clopidogrel treatment completely prevented the effects of Ang II. Since the differences in the ACh-induced relaxation were abolished among the groups after inhibition of NO and prostaglandin production, Ang II-induced impaired vascular reactivity is associated with altered endothelial function. These results also indicate that the improvement generated by clopidogrel is partially endothelium-dependent. Additionally, It is well established that Ang II decreases NO bioavailability [19] and that the preservation of NO bioavailability is essential for endothelium function [20].

Heitzer and colleagues [10] showed that ADP receptor blockade by clopidogrel improves endothelium-dependent vasodilation to acetylcholine and vascular bioavailability of NO in the human forearm of patients with symptomatic coronary artery disease. In addition, clopidogrel reduced inflammatory and oxidative parameters [10]. Our findings support the

concept that activated platelets contribute to endothelial dysfunction and impaired NO bioavailability. However, considering that $P2Y_{12}$ receptors have been described in vascular tissue, it is possible that some of the beneficial effects of clopidogrel are due to blockade of vascular $P2Y_{12}$ receptors, and may not reflect only its antiplatelet effects.

Because the presence of $P2Y_{12}$ receptors in the vasculature is the central point in our hypothesis, both pharmacological and molecular approaches were used to characterize the presence of vascular purinergic receptors, more specifically expression of vascular $P2Y_{12}$ receptors. The effects of the most potent analogue of ADP, 2-MeS-ADP, which is known as a partial agonist for $P2Y_{12}$ receptors, was tested in arteries from normotensive and hypertensive rats treated with the $P2Y_{12}$ receptor antagonist, Clopidogrel.

One of the findings of this manuscript is that 2-MeS-ADP display a dual vascular response in rat small mesenteric arteries contracted with PE, but has no effects on basal tonus. 2-MesADP was able to promote endothelium-dependent vasodilation and also induced smooth muscle contraction. In arteries treated with inhibitors of the NO synthase and cyclooxygenase enzymes, not only the relaxant responses induced by 2-MeS-ADP were abrogated, but also contractions in response to this agonist were enhanced. These data suggest that purinergic receptors activated by 2-MeS-ADP are present both in vascular smooth muscle and endothelial cells.

Protein for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors was expressed in mesenteric arteries. No changes in $P2Y_1$ or $P2Y_{12}$ were detected in mesenteric arteries from Ang II-infused rats when compared with the control animals. $P2Y_{13}$ receptor expression was decreased in mesenteric arteries from Ang II hypertensive rats. Considering that similar functional responses to 2-MeS-ADP were observed in arteries from control and Ang II-treated rats, it is possible that activation of $P2Y_1$ compensates for the decreased expression of $P2Y_{13}$.

The functional characterization of receptors mediating the vascular responses to 2-MeS-ADP suggested the involvement of $P2Y_1$ and $P2Y_{13}$, but not $P2Y_{12}$, receptors both in vascular smooth muscle and endothelial cells. As mentioned, responses of mesenteric resistance arteries to 2-MeS-ADP were not different between the control and hypertensive groups. Furthermore, our functional studies show that in small mesenteric arteries, responses mediated by $P2Y_1$ and $P2Y_{13}$ receptors are not altered in vessels from Ang II hypertensive rats.

Our results are in accordance with a previous report showing that 2-MeS-ADP induces endothelium-dependent, NO-mediated relaxation of rat aortic rings, an effect that was resistant to clopidogrel treatment [9]. On the other hand, Wihlborg et al [8] reported contractile responses to 2-MeS-ADP in human internal mammary artery segments, which were blocked by the selective $P2Y_{12}$ antagonist AR-C67085. They also demonstrated that $P2Y_{12}$ receptor mRNA was highly expressed (expression of $P2Y_{12}$ was higher than $P2Y_1$ and $P2Y_{13}$ receptors) in human vascular smooth muscle cells. Interestingly, 2-MeS-ADP-induced contraction was not reduced in vascular segments from patients using clopidogrel [8], which may indicate that 2-MeS-ADP responses are mediated by other subtypes of P2Y receptors.

As shown here, clopidogrel treatment increased bleeding time, showing the efficacy of the treatment. No pharmacological target in the vasculature was found for Clopidogrel. Considering that 1) clopidogrel restores impaired vascular reactivity; 2) low expression of $P2Y_{12}$ receptors were detected in mesenteric resistance arteries; and 3) there were no changes in 2-MeS-ADP-induced contractile and relaxant responses in arteries from control and Ang II-hypertensive rats, it seems that the beneficial actions of clopidogrel are not due to direct effects of clopidogrel in the vasculature, but rather due to its anti-platelet actions.Alternativelly, one may speculate that clopidogrel may exert its beneficial actions by blocking other subtypes of P2Y receptors, in addition to $P2Y_{12}$ receptors, both in the platelets and vasculature.

Although a large number of studies have reported that Clopidogrel inhibits platelets activation, further studies addressing platelets interaction with the vasculature in angiotenisn II hypertension are necessary. Accordingly, experiments measuring the expression of platelet surface receptors (e.g. CD62P (P-selectin) and CD42b (a component of von Willebrand factor receptor). Antigens, by flow cytometry), and platelet function both in basal condition and after stimulation with ADP or 2-MeS-ADP and collagen, will be helpful to better understand Clopidogrel benefits.

In summary, our results indicate that potentially beneficial effects of Clopidogrel, such as improvement of hypertension–related vascular functional changes, are not due to direct effects in the vasculature, and support the concept that activated platelets contribute to endothelial dysfunction, possibly via impaired NO bioavailability and prostanoid synthesis.

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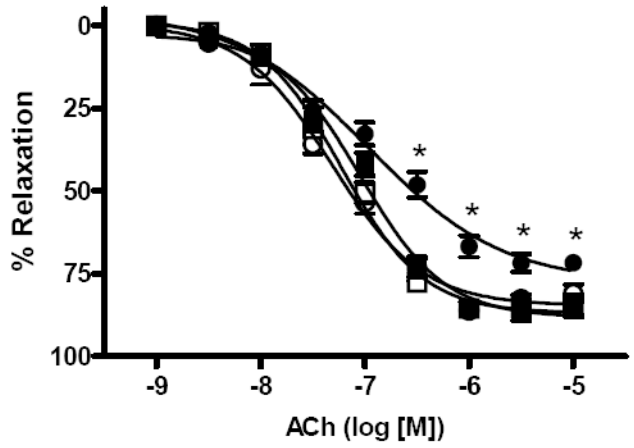
## ABBREVIATIONS

| | |
|---|---|
| **2-Mes-ADP** | 2-(Methylthio) adenosine 5’-trihydrogen diphosphate trisodium |
| **ACh** | acetylcholine |
| **ADP** | adenosine-5’-diphosphate |
| **Ang II** | angiotensin II |
| **Emax** | maximum effect elicited by the agonist |
| **eNOS** | endothelial nitric oxide synthase |

| | |
|---|---|
| **KCl** | potassium chloride |
| **L-NAME** | Nω-nitro-L-arginine methyl ester |
| **NO** | nitric oxide |
| **P2X receptors** | ligand-gated ion channels of the P2 nucleotide receptors family |
| **P2Y receptors** | G-protein coupled receptors of the P2 nucleotide receptors family |
| **PE** | phenylephrine |
| **PSS** | physiological salt solution |
| **ROS** | reactive oxygen species |
| **SNP** | sodium nitroprusside |
| **NOx** | nitrite/nitrate |

A

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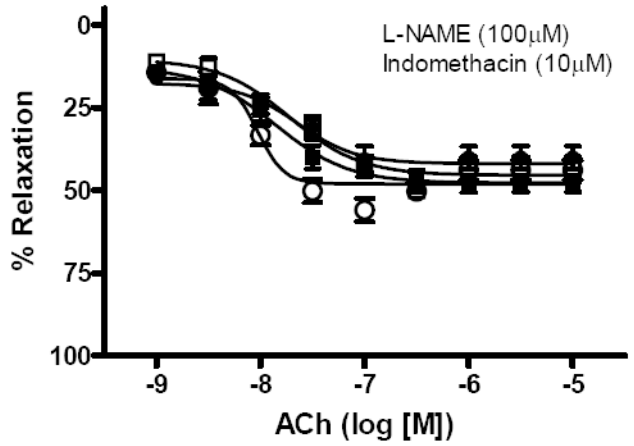


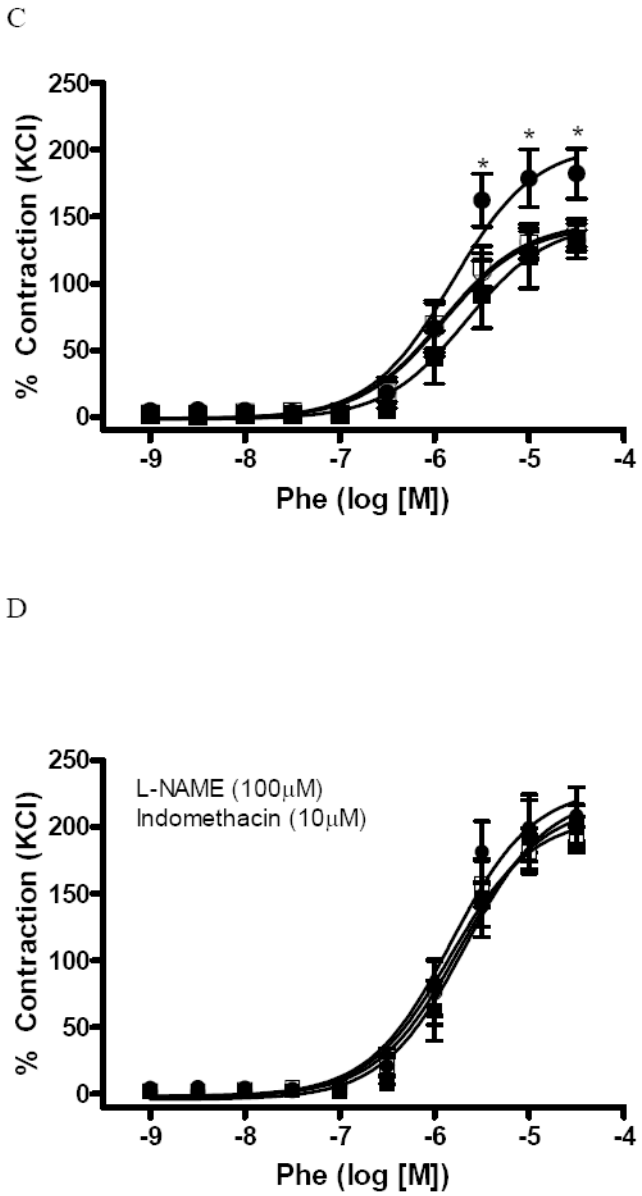
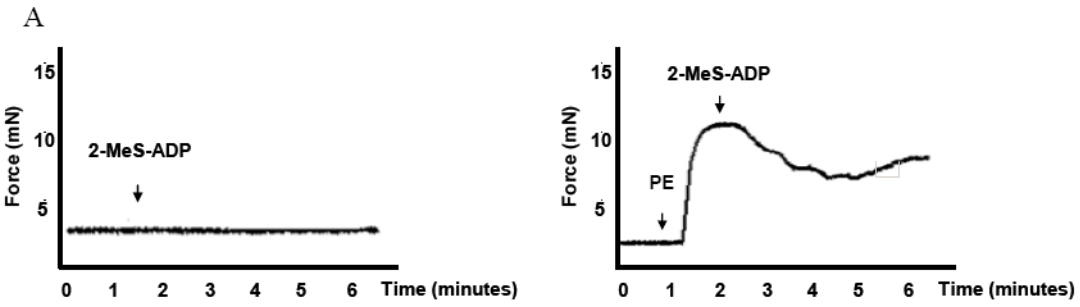
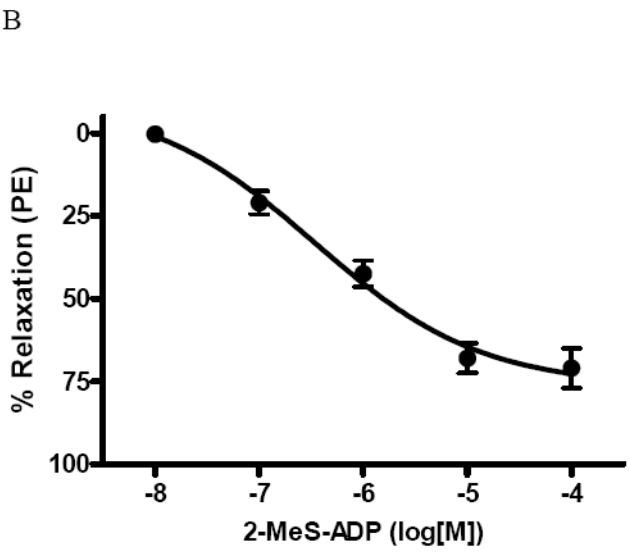



**Figure 1. Clopidogrel improves ACh-induced relaxation and prevents increased responses to PE in arteries from Ang II-treated rats**

Concentration-response curves to ACh in second-order mesenteric arteries in the absence (A) or in the presence (B) of L-NAME (100μM) plus indomethacin (10μM). Concentration-response curves to PE in the absence (C) or in the presence (D) of L-NAME (100μM) plus indomethacin (10μM). in arteries from (○) control + vehicle, (□) control + Clopidogrel, (●) Ang II + vehicle and (■) Ang II + clopidogrel rats. Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100% (% of relaxation). Experimental values for PE-induced contraction are represented as % of KCl-induced response. Results are presented as mean ± SEM of n=6 in each experimental group. *, $P<0.05$ compared with other groups.



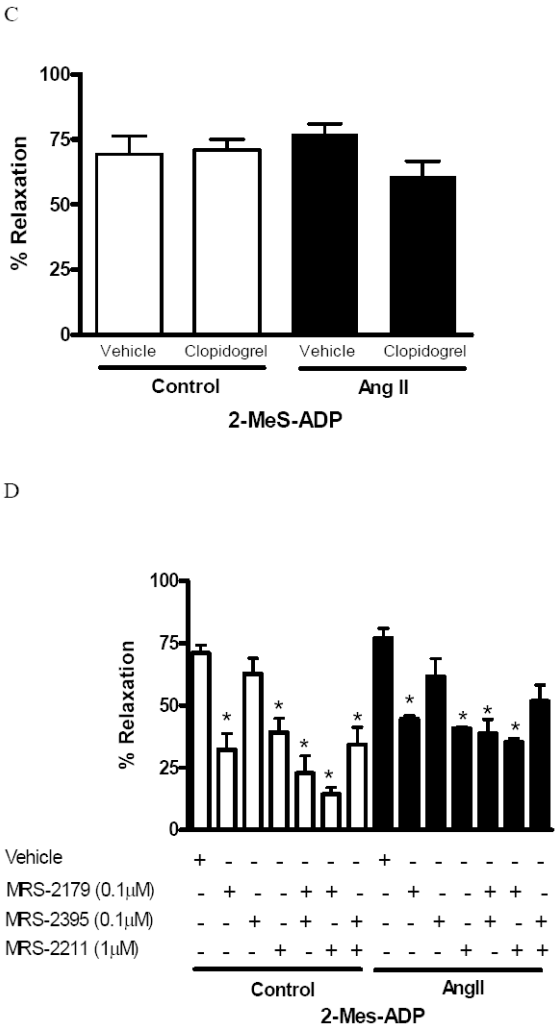
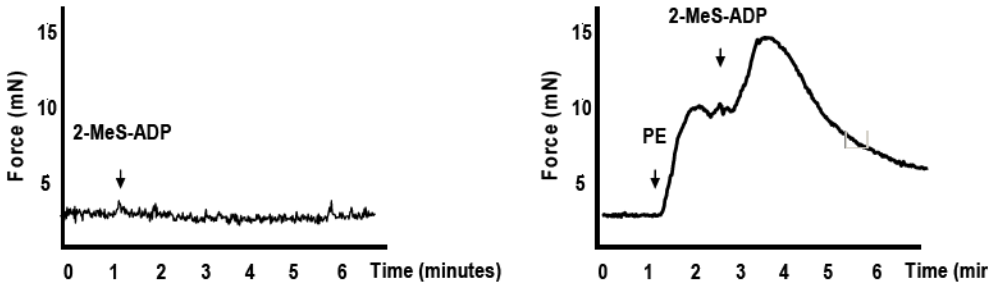



**Figure 2. Effects of 2-MeS-ADP in endothelium-intact second-order mesenteric arteries from control and Ang II-hypertensive rats**

**(A)**, Representative tracings showing that, upon stimulation with 2-MeS-ADP (10μm), mesenteric arteries in basal tonus display no changes in force levels, whereas PE-contracted (3 μM) arteries exhibit a relaxant response. **(B)** Concentration-response curve to 2-MeS-ADP (0.1 to 100 μM) in PE-contracted (3 μM) arteries from control rats. **(C)** 2-MeS-ADP (100 μM) induced similar relaxation in mesenteric arteries from control and Ang II-treated rats treated with vehicle or Clopidogrel. **(D)** Relaxant responses to 2-MeS-ADP (100 μM) were determined in arteries incubated with MRS-2179 (0.1 μM), a $P2Y_1$ receptor antagonist; MRS-2395 (0.1 μM), a $P2Y_{12}$ receptor antagonist; MRS-2211 (1μM), a $P2Y_{13}$ receptor antagonist or with the combination of these antagonist. Then, they were stimulated with PE (3 μM) and, after contractile responses were obtained, 100 μM 2-MeS-ADP was added to the bath. Experimental values of the relaxation induced by 2-MeS-ADP were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Results are presented as mean ± SEM of n=5 in each experimental group. *, $P<0.05$ vs. vehicle in their respective group.

A

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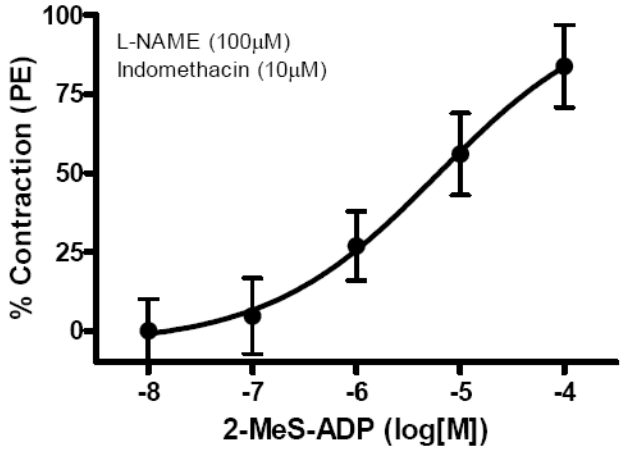

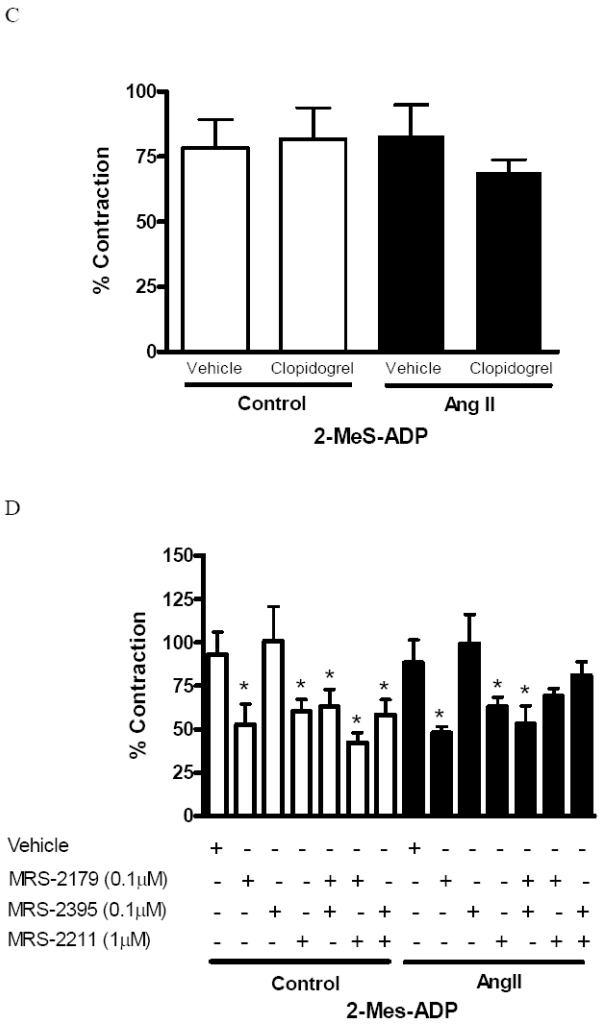


**Figure 3. Effects of 2-MeS-ADP in endothelium-intact second-order mesenteric arteries, incubated with L-NAME plus indomethacin, from control and Ang II-hypertensive rats**

**(A)** Representative tracings showing that, upon stimulation with 2-MeS-ADP (10μm), mesenteric arteries, incubated with L-NAME (100μM) plus indomethacin (10μM), display no vascular responses, whereas 2-MeS-ADP (100μm) induces contraction in PE (3 μM)-stimulated vessels. **(B)** Concentration-response curve to 2-MeS-ADP (0.1 to 100 μM) in PE-contracted (3 μM) arteries incubated with L-NAME plus indomethacin. **(C)** 2-MeS-ADP (100 μM) induced similar contraction in mesenteric arteries from control and Ang II-treated rats treated with vehicle or Clopidogrel, after incubation with L-NAME plus indomethacin. **(D)** Contractile-responses to 2-MeS-ADP (100 μM) were determined in arteries incubated with MRS-2179 (0.1 μM), a $P2Y_1$ receptor antagonist; MRS-2395 (0.1 μM), a $P2Y_{12}$ receptor antagonist; MRS-2211 (1μM), a $P2Y_{13}$ receptor antagonist or with the combination of these antagonist. Then, they were stimulated with PE (3 μM) and, after contractile responses were obtained, 100 μM 2-MeS-ADP was added to the bath. Experimental values for 2-MeS-ADP-induced contraction are represented as percentage of PE-induced contraction. Results are presented as mean ± SEM of n=5 in each experimental group. *, $P<0.05$ vs. respective control group.

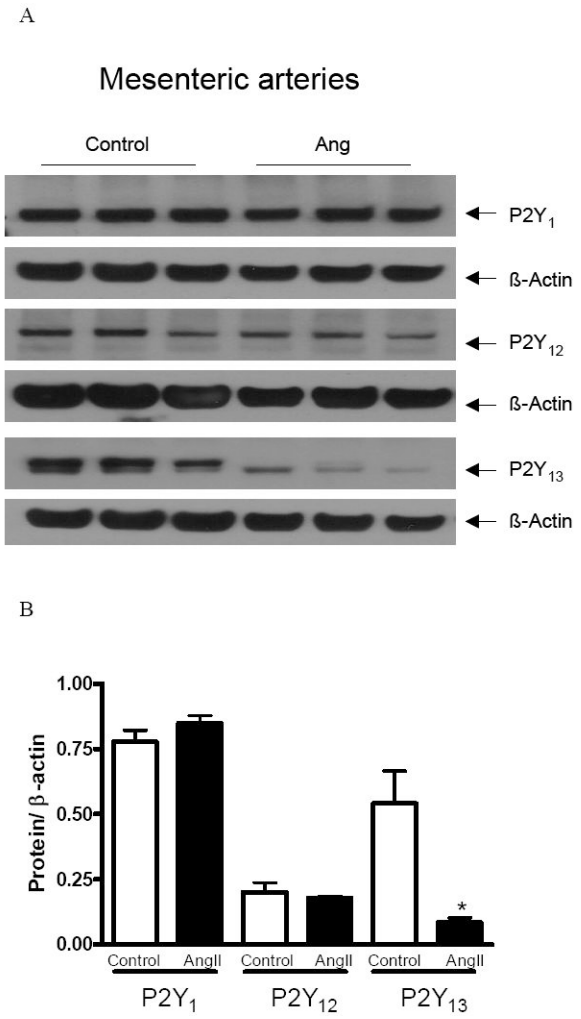


**Figure 4. Protein expression of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors in arteries from control and Ang II-hypertensive rats**

(A), representative images of P2Y receptors expression in rat mesenteric arteries from control and Ang II-treated rats. (B) Bar graphs showing the relative vascular expression of P2Y receptors after normalization to β-actin expression. Results are presented as mean ± SEM of n= 3-4 blots in each experimental group. *, P<0.05 vs. respective control group.

## Table 1

Effects of clopidogrel treatment on systolic blood pressure, bleeding time and weight gain in control and Ang II-treated rats.

| | Control + Vehicle | Control + Clopidogrel | Ang II + Vehicle | Ang II + Clopidogrel |
|---|---|---|---|---|
| SBP (mmHg) | 117±7.1 | 125±4.2 | 197 ±10.7 * | 198 ±5.2 * |
| Bleeding time (s) | 424±31 | > 1200 † | 470±42 | >1200† |
| Weight gain (g) | 32±8 | 34±9 | 21±5 | 26±5 |

*P<0.05 vs. respective control;

†P<0.05 vs. vehicle-treated. n=16 in each group.