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Research Paper

Endothelium-independent vasorelaxation by ticlopidine and clopidogrel in rat caudal artery

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Abstract

Objectives Thienopyridines are prodrugs currently used as anti-aggregating agents. The aim of this study was to determine if these compounds might have vascular activity independent of hepatic bioactivation.

Methods The direct activity of thienopyridines was studied in rat caudal arterial rings and aortic smooth muscle cells in culture.

Key findings Both compounds (0.01 μM–100 μM) showed a concentration-dependent vasorelaxation in arterial tissues precontracted with phenylephrine, 5-hydroxytryptamine and KCl. The relaxation induced by 100 μM ticlopidine and clopidogrel was greater than 80%. The relaxation by ticlopidine was compared with the activity of acetylcholine. These two agents showed similar potency, although ticlopidine was slightly more active. Pretreatment with the nitric oxide synthase inhibitor L-NAME inhibited the relaxation by acetylcholine but not that by ticlopidine. To further study vasorelaxation by ticlopidine, other pharmacological inhibitors including propranolol, nifedipine and suramin were used. These compounds lacked inhibitory effects on the vasorelaxation by ticlopidine. In vascular smooth muscle cells, 1 μM ticlopidine induced a decrease in cell proliferation, while incubation with both ticlopidine and ADP or 2-methioADP led to an additive effect.

Conclusions The data suggest that ticlopidine and clopidogrel cause relaxation of arterial tissues and influence vascular smooth muscle cell proliferation directly without hepatic biotransformation. Furthermore, the arterial relaxation induced *in vitro* by thienopyridines is endothelium independent, and β-adrenergic and P2 receptors are not involved.

Keywords β-adrenergic receptors; clopidogrel; P2 receptors; ticlopidine; vasorelaxation

Introduction

Antiplatelet drugs are widely used for the prevention of atherosclerotic and thrombotic events related to ischaemia.[1] These include compounds such as acetylsalicylic acid, glycoprotein IIb/IIIa receptor antagonists, and thienopyridines such as ticlopidine and clopidogrel, which act as prodrugs blocking platelet ADP receptors.[2–4] Ticlopidine is used in the prevention of thrombosis during and after coronary stent placement and has been found to be at least equivalent to aspirin in the prevention of events in patients with cerebrovascular disease. It is known that treatment with thienopyridines reduces the binding of ADP to low affinity binding sites on platelets, named $P2Y_{12}$ receptors, inducing anti-aggregating activity.[5–8] Thienopyridines do not affect ADP-induced platelet aggregation *in vitro* and hepatic metabolism is necessary for the effect to occur *in vivo*.[9–12] It has been shown that the inhibition of aggregation in rats treated either intravenously or orally with clopidogrel is abolished after functional hepatectomy by a porto-jugular shunt.[13] Furthermore, in isolated blood-perfused rat livers, clopidogrel inhibited ADP-induced platelet aggregation, supporting the fact that the activity of clopidogrel is dependent on hepatic metabolism.[8] Thus, ticlopidine and clopidogrel are antiplatelet agents known as ADP receptor antagonists *in vivo* but without activity *in vitro*. Few studies have considered the thienopyridines for any activity other than their anti-aggregating activity and generally used *in vivo* models.[14–16] It has been shown that clopidogrel reduces inflammation and increases endothelium-derived nitric oxide synthase protein in rabbit ischaemic coronary artery, suggesting a protective role of thienopyridine.[14] Also, clopidogrel and ticlopidine (10 mg/kg i.v., 30 min before the

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animals were killed) were found to produce dose-dependent vasomodulatory action in arterial preparations isolated from rabbit, rat or dog.[17,18]

In contrast with the majority of the literature, Weber *et al*. showed direct inhibition by clopidogrel on ADP-induced aggregation in washed platelets.[19] Moreover, Jakubowski *et al*. found an immediate and direct endothelial action in isolated guinea-pig heart, independent from antiplatelet activity.[20] Thus, there are a few suggestions in the literature of possible activity of thienopyridines other than their known anti-aggregating activity *in vivo*; even direct vascular effects may be involved in their cardiovascular action. In this study, we hypothesized that the thienopyridines may have in-vitro activity on vascular tissues, in the absence of hepatic metabolism. We investigated the direct action of ticlopidine and clopidogrel in isolated resistance vessels using rat caudal arterial rings and vascular smooth muscle cells (VSMCs).

Materials and Methods

In-vitro pharmacology

This study conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). The protocols were approved by the Research Ethics Committee of the University of Padua.

Adult male Wistar rats (6 months old, 300–400 g) were anaesthetised by inhalation of methoxyflurane and then killed. The caudal artery was removed and rinsed with Krebs solution. The proximal 2 cm of the caudal artery was cleaned of adhering tissue and the arterial rings (3 mm long) were placed in a tissue bath under a resting tension of 19.6 mN. Extreme care was taken not to damage the endothelium. In some experiments, the endothelial cell layer was removed by rubbing the luminal side of the vessel with an L-shaped stainless steel wire. The tissue bath was filled with Krebs-Ringer solution at 37°C, with the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2{\cdot}2H_2O$ 2.5, $MgSO_4{\cdot}7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-Glucose 11.1, bubbled with 95% O_2 and 5% CO_2 ($pO_2 = 345 \pm 8$ mmHg), pH = 7.2. Tissues were allowed to equilibrate for 1 h before viability was assessed with standard start procedures. The isometric tension was recorded by means of a high-sensitivity transducer connected to a chart recorder (type DYO and Unirecord System, model 7050; Ugo Basile, Comerio, Italy).

In experiments carried out on depolarised tissue, the Krebs-Ringer solution was modified to counterbalance the increased concentration of KCl (80 mM) by an equal reduction of NaCl.

Clopidogrel and ticlopidine were dissolved and diluted in saline. In intravenous bolus studies, 10 mg/kg of each compound was administered to groups of rats ($n = 6$), the caudal segments being obtained after 30 min of treatment. Control rats ($n = 5$) received saline and were kept under the same conditions as drug-treated animals.

Cell culture

Primary VSMCs were derived from the thoracic aorta of adult male Wistar rats as described previously.[21] First, the aorta was isolated and the adventitia was stripped off. Then, the aorta was minced and cultured in DMEM containing 20% fetal bovine serum until VSMCs were isolated. Cells were used at or below 10 passages and were grown in DMEM containing 10% fetal bovine serum 1% Pen/Strep. All reagents were from Cambrex, Lonza (New York, NY, USA). The smooth muscle phenotype of cell lines was verified by positive immunofluorescence for smooth muscle α-actin.

Cell counting and MTT assay

The cell proliferation assay was performed using cell counting and MTT assay.[22] In brief, 5000 cells/well were seeded into flat-bottomed, 96-well plates. After 24 h, the media was removed and replaced with serum-free media for 24 h to achieve synchronous growth arrest. At 48 h after the experimental stimulation, MTT was added (final concentration 0.5 µg/ml) for 4 h, followed by the addition of 150 µl dimethylsulfoxide to dissolve the formed formazan crystals. The absorbance was recorded at 490 nm with a microtiter plate reader (Bio-Rad, Hercules, CA, USA) after 90 min incubation at 37°C. Results are reported as relative optical densities from at least three independent experiments. Cell counting analysis by trypan blue staining was used to confirm the MTT analysis. VSMCs were resuspended in 0.05% trypsin and 0.02% EDTA, and cell numbers were counted with a hemocytometer.

Drugs

Propranolol was dissolved in dimethylsulfoxide and the other drugs were dissolved in saline. Compounds were obtained from Sigma-RBI (St. Louis, MO, USA) except for clopidogrel which was a kind gift of Dr Jean-Marc Herbert (Cardiovascular Thrombosis research Dept, Sanofi-Synthelabo Toulouse Cedex, France). All reagents were of analytical grade and the purity of all compounds was >98%.

Statistical analysis

All values presented are means ± SEM of at least six experiments. Changes in vascular tension are expressed as a percentage of the contraction induced by the agonist taken as 100%. All concentrations expressed indicate the final concentration in the tissue bath. E_{max} is the maximum response evoked by the agonist, while EC50 is half the maximum effective concentration.

Sigmoid curve fitting was performed by the GraphPadPrism program (GraphPad Software, San Diego, CA, USA). Based on the principal equation for a sigmoidal curve, the program makes iterative computations to derive a best fit based upon the actual experimental values. Simple pair-wise comparisons were done by Student's *t*-test. Multiple comparisons of the effects of treatments against control and inhibitors against treatments were performed by two-way analysis of variance followed by Dunnett's test. $P \leq 0.05$ was considered to be significant.

Results

In-vivo and in-vitro effects of ticlopidine and clopidogrel

Phenylephrine and 5-hydroxytryptamine (5-HT) induce vasoconstriction in vessels by activating α_1-adrenergic and $5\text{-}HT_{2A}$ receptors, respectively. We studied the concentration–

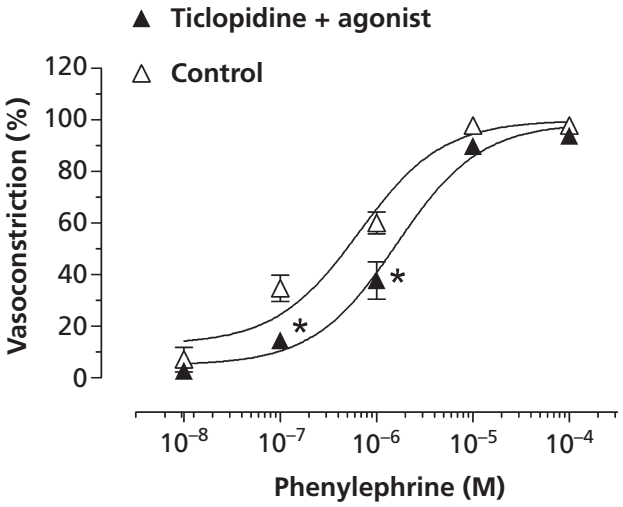


Figure 1 Pretreatment with ticlopidine. In-vitro effect of pretreatment with 1 μM ticlopidine on concentration–vasoconstriction curve induced by phenylephrine in rat caudal arteries. Values are mean ± SEM of six experiments. SEMs not indicated fall within respective symbols. *$P < 0.05$, significantly different compared with control.

response curves obtained by phenylephrine or 5-HT *in vivo* in caudal arterial rings from rats treated or untreated (controls) with ticlopidine or clopidogrel (10 mg/kg i.v.). The ticlopidine-treated group showed a slight increase in the contractile response by phenylephrine compared with the controls, while the clopidogrel-treated group showed a weak inhibition of the vasoconstriction induced by 5-HT (data not shown). Thus, the thienopyridines did not significantly change the vasoconstriction caused by phenylephrine or 5-HT in arterial tissues *in vivo*.

In-vitro pretreatment with 1 μM ticlopidine (30 min) caused a slight inhibition of the vasoconstriction–concentration curve obtained with phenylephrine (Figure 1), whereas it did not alter the 5-HT curve (data not shown). Under the same experimental conditions, 1 μM clopidogrel (30 min) did not change the constriction by phenylephrine or 5-HT (data not shown).

Thienopyridines (0.01 μM–100 μM) did not change the resting tension of rat arterial rings, although at 10 and 100 μM, ticlopidine slightly decreased the basal tone of tissues ($P > 0.05$). However, ticlopidine and clopidogrel caused an evident relaxation of arteries precontracted with 0.5 μM phenylephrine, 0.5 μM 5-HT or 80 mM KCl (Figure 2). The unexpected vasorelaxation was concentration dependent and was promptly reversible after washout. The potency of ticlopidine was greater in tissues precontracted with 5-HT or KCl than in tissues precontracted with phenylephrine ($ticlopidine_{5\text{-}HT} = ticlopidine_{KCl} > ticlopidine_{phenylephrine}$). Clopidogrel showed similar behaviour ($clopidogrel_{5\text{-}HT} > clopidogrel_{KCl} > clopidogrel_{phenylephrine}$). In KCl-pretreated tissues, the pD_2 (–log EC50) was 5.5 ± 0.2 and 5.1 ± 0.1 for ticlopidine and clopidogrel, respectively.

In-vitro vasorelaxation by ticlopidine

To investigate the vasomodulation of thienopyridines we compared the action induced by ticlopidine with acetylcholine, a physiological endothelium-dependent vasorelaxant, in KCl-pretreated tissues (Figure 3). Unexpectedly, ticlopidine showed the same potency as acetylcholine. Moreover, the maximal vasorelaxant effect obtained with 100 μM ticlopidine was greater than with the muscarinic agonist: the E_{max} values were 89.8 ± 0.9 and 58.6 ± 2.3, respectively ($P < 0.001$). To evaluate the role of endothelium-derived nitric oxide in the relaxation caused by ticlopidine and acetylcholine, the concentration–response curves were obtained in the presence of the nitric oxide synthase inhibitor L-N^G-nitro-arginine methyl ester (L-NAME, 100 μM, 30 min).[23] As expected, this inhibitor reduced relaxation by the muscarinic agonist almost completely but did not change vasorelaxation by ticlopidine (Figure 3). We also performed experiments with caudal arteries deprived of endothelium and found that the vasorelaxation induced by acetylcholine was completely inhibited, whereas dilatation by ticlopidine was unaffected (data not shown).

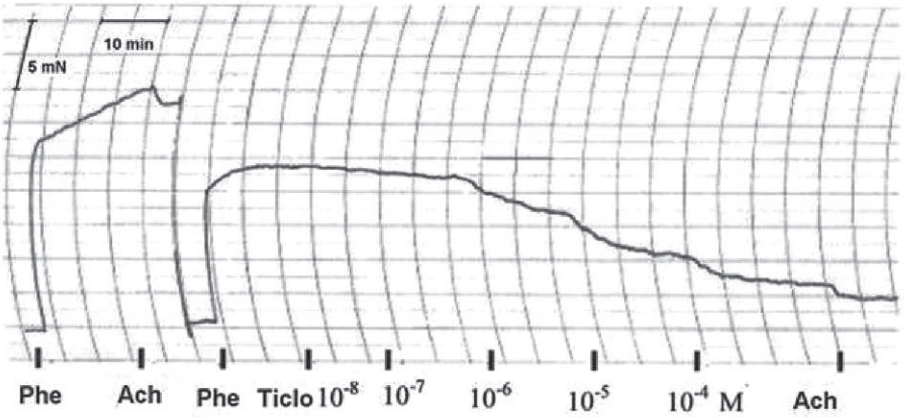
Propranolol, nifedipine and suramin were used to determine the mechanism of vascular activity of ticlopidine *in vitro*. The β-antagonist propranolol[24] (0.5 μM incubated for 30 min) did not influence vasodilatation induced by ticlopidine (data not shown), whereas 0.01 μM nifedipine, an L-type voltage-gated calcium channel inhibitor,[25] significantly increased the vascular effect induced by ticlopidine: the pD_2 was 5.2 ± 0.2 and 6.1 ± 0.3 for ticlopidine alone and ticlopidine in the presence of nifedipine, respectively. Suramin (300 μM), a well known P2X and P2Y receptor antagonist,[26,27] was tested to determine if ticlopidine acted through interaction at P2 receptors. In the presence of suramin, the vasorelaxation caused by ticlopidine was slightly increased (Figure 4). To define the role of $P2Y_{12}$ receptors in arterial tissues, we used 2-methioADP, a stable analogue of ADP with a high affinity for P2Y1 and $P2Y_{12}$ receptors.[28,29] 2-MethioADP (10 μM) induced a vasoconstriction of 22.2 ± 5.1%, which was completely inhibited by pretreatment with 300 μM suramin (data not shown).

In-vitro effects of ticlopidine on VSMC proliferation

To further investigate the direct action of the thienopyridines we carried out a set of experiments in VSMCs derived from rat aorta and measured cell vitality using the MTT test. In VSMCs, 1 μM and 10 μM ticlopidine decreased cell proliferation to 76.8 ± 1.6% and 86.4 ± 2.3%, respectively, whereas at the higher concentration of 100 μM, ticlopidine increased cell growth (124.8 ± 2.1%). In the presence of 50 μM ADP, a known physiological activator of VSMC proliferation by P2 receptors, or 0.1 μM 2-methioADP, a synthetic activator of P2 receptors, ticlopidine did not decrease cellular proliferation but added to the effects of the P2 receptor agonist (Figure 5).

Discussion

The results suggest that ticlopidine and clopidogrel can directly induce vasorelaxation of rat caudal arteries. The effects observed were quantitatively similar to the vasorelaxation induced by acetylcholine. Further, ticlopidine directly influences VSMC proliferation. All these effects were induced by thienopyridines without in-vivo metabolism.



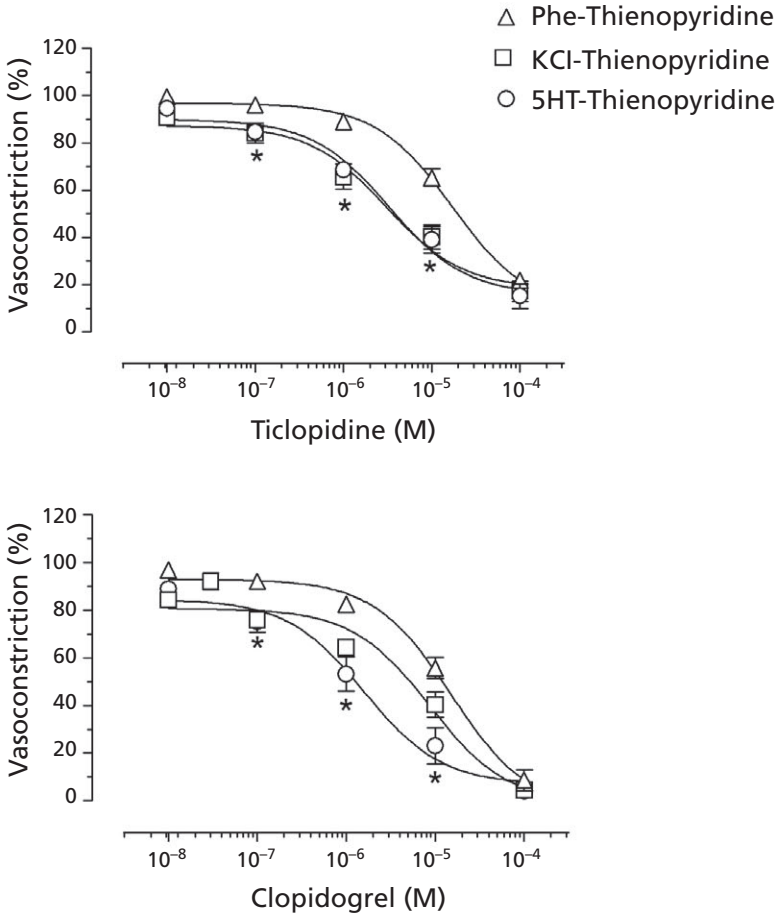


Figure 2 Ticlopidine on precontracted tissues. Example of an original tracing showing the vasorelaxation induced by cumulative concentrations of ticlopidine (Ticlo), in rat caudal artery precontracted by 0.5 μM phenylephrine (Phe) *in vitro*. The presence of endothelium was evaluated by acetylcholine (Ach), and concentration–effect curves obtained with ticlopidine and clopidogrel in rat tissues precontracted by 0.5 μM Phe, 80 mM KCl and 0.5 μM 5-hydroxytryptamine (5-HT). Values are mean ± SEM of six to ten experiments. SEMs not indicated fall within respective symbols. $^*P < 0.05$, significantly different compared with KCl-thienopyridine.

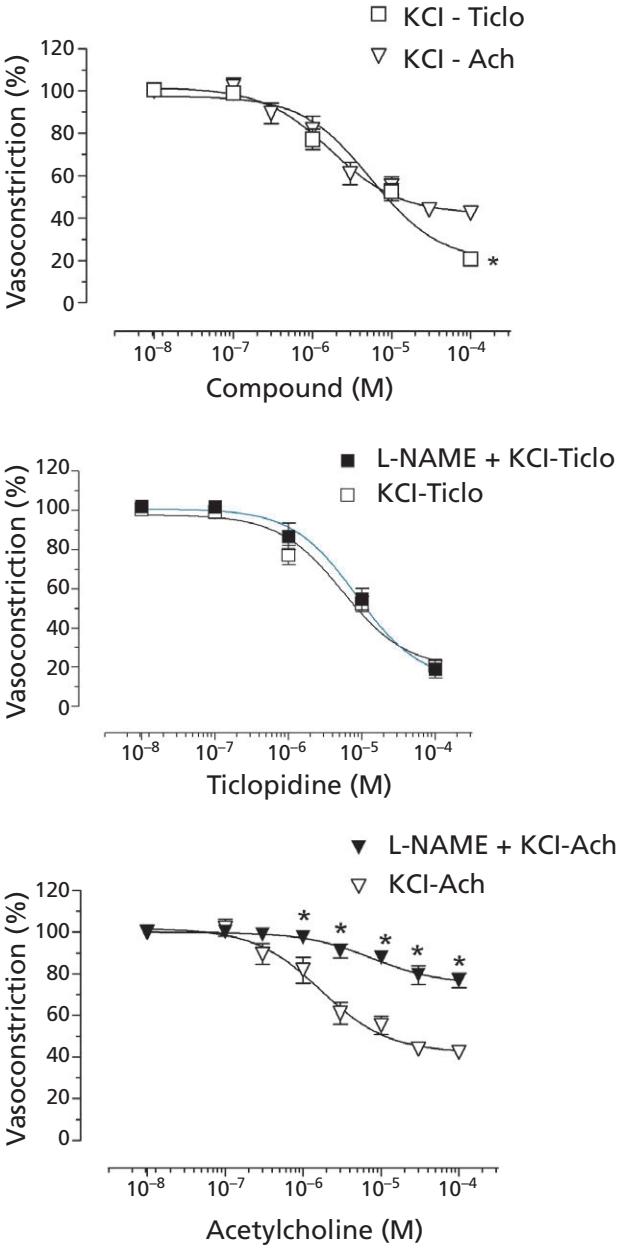


Figure 3 Role of endothelium. Concentration–effect curves obtained by ticlopidine (Ticlo) and acetylcholine (Ach) in KCl-precontracted caudal arteries alone or in the presence of 100 μM L-NAME. Values are mean ± SEM of six to eight experiments. SEMs not indicated fall within respective symbols. $*P < 0.05$, significantly different compared with acetylcholine alone.

These observations are surprising because it is known that thienopyridines act after hepatic metabolism through cytochrome P450 2C19, cytochrome P450 3A4 and cytochrome P450 3A5.[12,30,31] However, the results of this study show that the thienopyridines may directly induce vasorelaxation and influence VSMC proliferation without hepatic biotransformation and so we suggest that the thienopyridines are not only prodrugs but could have vascular activity independent of their metabolic activation.

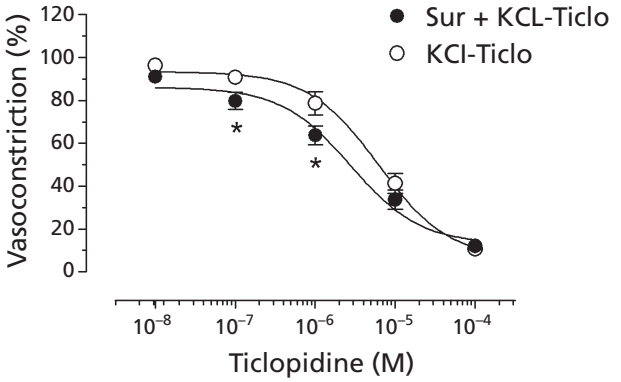


Figure 4 Role of P2 receptors. Concentration–effect curves obtained by ticlopidine (Ticlo) alone or in the presence 300 μM suramin (Sur) in KCl-precontracted caudal arteries. Values are mean ± SEM of six to eight experiments. SEMs not indicated fall within respective symbols. $*P < 0.05$, significantly different compared with ticlopidine alone.

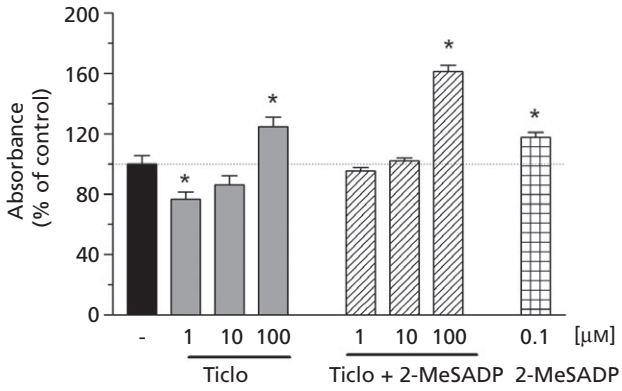


Figure 5 Ticlopidine on cell proliferation. Effect of ticlopidine (Ticlo) alone and in the presence of 0.1 μM 2-methioADP on proliferation of vascular smooth muscle cells derived from rat aorta. Values are mean ± SEM from at least three independent experiments in quadruplicate. $*P < 0.05$, significantly different compared with controls (black column).

In this study, pretreatment *in vivo* with ticlopidine or clopidogrel caused only modest changes on vasoconstriction induced *in vitro* by phenylephrine and 5-HT in rat arterial tissues. In contrast, another study showed that pretreatment with clopidogrel or ticlopidine (30 min before the animals were killed) was able to decrease vasoconstriction by 5-HT, endothelin-1, and serum and platelet-rich plasma/arachidonic acid mixtures in rat and rabbit aortic rings.[17] The reasons for this difference are not clear but may be related to the different blood vessels used in the experiments.

Regarding the mechanism implicated in the vasorelaxation induced by thienopyridines *in vitro*, the results showed that the effect is not related to endothelium-derived nitric oxide, because pre-incubation with the nitric oxide synthase inhibitor L-NAME did not decrease vasorelaxation caused by ticlopidine, as was the case for acetylcholine. Further, in endothelium-deprived arterial tissues the vasorelaxation induced by ticlopidine was unchanged. β_2-Adrenergic receptors and P2 receptors seem not to be involved, since

propranolol and suramin did not inhibit the dilation induced by the thienopyridine. The $P2Y_{12}$ receptor is expressed in platelets, where it plays an important role in blood aggregation, but is also present in human blood vessels, where it contributes to vasoconstriction. This receptor is activated by adenine diphosphate derivatives, with 2-methioADP being much more powerful than ADP. Further, $P2Y_{12}$ receptors were detected *in vitro* in rat and human vascular smooth muscle cells.[32] In the present study, 2-methioADP induced vasoconstriction and increased VSMC proliferation, highlighting the presence of $P2Y_{12}$ receptors both in rat caudal artery and VSMCs. These data show that vasorelaxation induced by thienopyridines is not related to $P2Y_{12}$ receptors, and this is consistent with suramin completely inhibiting the vasoconstriction by 2-methioADP, leaving the relaxant effect of ticlopidine almost unchanged. Moreover, in the literature it is reported that patients treated with clopidogrel did not show any inhibition of $P2Y_{12}$ receptor-mediated vasoconstriction,[32] and even high oral doses of clopidogrel had no inhibitory effect on vasoconstriction caused by receptor $P2Y_{12}$.[33]

The incubation of arterial tissues with the L-type voltage-gated calcium-channel inhibitor nifedipine[25,34] strongly increased the vasorelaxation observed with ticlopidine, suggesting additive pathways between the two drugs. Thus, the vasorelaxant effects of thienopyridines might be related to cellular calcium levels. This observation could have therapeutic interest because there may be conditions in which patients, for example with instable angina pectoris, receive co-medication of ticlopidine and nifedipine, and there may be a synergistic vasorelaxation in these patients. Further clinical evaluations are needed to confirm this.

Few studies have suggested the possibility that thienopyridines may induce direct effects, regardless of their metabolic activation. Jakubowski *et al*. reported an immediate and direct endothelial action of thienopyridines in the isolated guinea-pig heart, independent of antiplatelet activity.[20] Also, it has been suggested that ticlopidine enhances the interleukin 1β-stimulated nitric oxide release in cultured rat smooth muscle cells via cAMP- and protein kinase A-dependent mechanisms.[35,36] In cultured endothelium, thienopyridines also directly stimulated the release of PGI_2.[37] Recently, it has been shown that ticlopidine may reduce the atherosclerotic process *in vitro* by blocking monocyte adhesion and migration through reducing monocyte chemoattractants protein-1 (MCP-1), interleukin 8, and vascular cell adhesion molecule 1 levels in cytokine-stimulated endothelial cells.[38] Whether this experimental observation has therapeutic implications is difficult to assess. The results suggest that it is important to consider the effects of thienopyridines not only on platelets but also on vascular resistance. Other experiments *in vitro* and *in vivo* are necessary to better clarify the mechanisms of action of these compounds and their therapeutic application.

Conclusions

To our knowledge, this study provides the first evidence for in-vitro vasorelaxation by thienopyridines. The data shows that thienopyridines may induce direct effects on vascular tissues and in smooth muscle cell cultures without hepatic biotransformation. Ticlopidine and clopidogrel showed a concentration-dependent vasorelaxation, the mechanism of action of which is independent of endothelium-derived nitric oxide, β-adrenergic receptors and P2 receptors, but may be related to the homeostasis of calcium ions.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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