Ticlopidine attenuates progression of atherosclerosis in apolipoprotein E and low density lipoprotein receptor double knockout mice

Jacek Jawien a, Gabor Csanyi a,1, Mariusz Gajda b, Lukasz Mateuszuk a, Magdalena Lomnicka a, Ryszard Korbut a, Stefan Chlopicki a,*

a Chair of Pharmacology, Jagiellonian University Medical College, Krakow, Poland
b Chair of Histology, Jagiellonian University Medical College, Krakow, Poland

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Abstract

Platelets are involved in the development of atherothrombosis. However, the anti-atherosclerotic effects of thienopiridines have not been, as yet, proven. We analyzed the effects of ticlopidine on atherogenesis in apolipoprotein E/low density lipoprotein receptor double knockout (apoE/LDLR−/−) mice. 2-month-old apoE/LDLR−/− mice fed a Western diet (21% fat, 0.15% cholesterol) were treated with ticlopidine (90 mg/kg/day) for a period of 4 months. In 6-month-old apoE/LDLR−/− mice treated with ticlopidine and in their non-treated counterparts we analyzed: cholesterol and triglyceride levels, the size of atherosclerotic plaques in aortic roots (oil red-O staining, cross-section method), and in the whole aorta (Sudan IV staining, en face method), the number of macrophages in atherosclerotic plaque (CD68 staining), as well as the endothelial function in the isolated thoracic aorta. Concentrations of total cholesterol and triglycerides in plasma were not altered by treatment with ticlopidine. However, the size of atherosclerotic plaques measured in aortic roots by the cross-section method and the number of macrophages estimated by anti-CD68 staining were significantly reduced by ticlopidine treatment. In contrast, the effect of ticlopidine on the area covered by plaques in the whole aorta (en face analysis) was not statistically significant. Importantly, acetylcholine-induced vasodilation in isolated aorta was improved in ticlopidine-treated apoE/LDLR−/− mice as compared to their non-treated counterparts. In conclusion, ticlopidine attenuates the progression of atherosclerosis and improves the endothelial function in apoE/LDLR−/− mice.

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1. Introduction

Atherosclerosis is the single largest cause of mortality in the world (Murray and Lopez, 1997; Ross, 1999; Lusis, 2000) and anti-platelet therapy with aspirin or/and thienopiridines (ticlopidine, clopidogrel) represents a mainstay of chronic treatment of this disease.

There is overwhelming evidence supporting excessive activation of platelets in patients with atherothrombosis (Ruggeri, 2002) and the role of platelet-dependent mechanisms in the development of vascular inflammation as well as atherosclerosis progression (Gawaz et al., 2005; Ramos et al., 1999; Huo et al., 2003). Indeed, activated platelets induce or accelerate endothelial inflammation — a crucial event in the progression of atherosclerosis (Sachais, 2001; Gawaz et al., 2005). Accordingly, platelets are again considered (Ross and Glomset, 1976) as an important player in the initiation and progression of atherosclerosis (Huo et al., 2003; Massberg et al., 2002; Gawaz et al., 2005).

In the light of the rapidly evolving knowledge on the key role of platelets in atherogenesis, it is quite surprising that experimental support for the anti-atherosclerotic action of anti-platelet drugs such as aspirin is limited, sometimes contradictory (Cayatte et al., 2000; Tous et al., 2004) or almost absent, as regards thienopiridines (Gu et al., 2005).
Indeed, the effects of aspirin on the progression of atherosclerosis has not been unequivocally demonstrated. Aspirin at a dose that selectively inhibits cyclooxygenase-1 (COX-1) in platelets was shown to blunt platelet activation, vascular inflammation and atherosclerosis progression in low density lipoprotein (LDL) receptor-knockout mice (Cyrus et al., 2002), but did not display a clear-cut anti-atherosclerotic effect in apolipoprotein E (apoE)-knockout mice (Cayatte et al., 2000; Tous et al., 2004).

Furthermore, it is still not proven whether thienopyridines afford protection against the progression of atherosclerosis in mouse models of this disease. Surprisingly, it was recently demonstrated that clopidogrel alone did not significantly inhibit atherosclerosis progression in the rabbit model of atherosclerosis (Gu et al., 2005). In our previous studies we have shown that thienopyridines, being antagonists of the P2Y<sub>12</sub> subtype of the ADP receptor in platelets (Solet et al., 2001; Storey, 2001; Gachet, 2001), exert pleiotropic action on endothelial cells as they have the ability to release prostacyclin (PGI<sub>2</sub>) and nitric oxide (NO) from endothelium in <i>vitro</i> as well as in <i>vivo</i> (Ziemianin et al., 1999; Dupin et al., 2002; Gryglewski et al., 1996, 2000; Jakubowski et al., 2005).

The aim of the present work was to examine the effects of ticlopidine on the progression of atherosclerosis in the unique mouse model of this disease: apoE/LDL receptor-double knockout mice (apoE/LDLR<sup>−/−</sup>) (Ishibashi et al., 1994; Witting et al., 1999). The size of atherosclerosis plaques, the abundance of macrophages in atherosclerotic plaques, as well as the endothelial function in aorta were analyzed in ticlopidine-treated apoE/LDLR<sup>−/−</sup> mice and compared to their non-treated counterparts.

2. Materials and methods

2.1. Animals and treatment

Female apoE/LDLR<sup>−/−</sup> mice of a C57BL/6J×129/SvJ background were obtained from Taconic (Ejby, Denmark). Mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22.5±0.5 °C, 50±5% humidity) and access to diet and water was provided <i>ad libitum</i>. At the age of 8 weeks mice were put on a Western diet (consisting of 21% fat by weight, 0.15% cholesterol by weight and no cholic acid; Sniff, Soest, Germany), for 4 months. 20 mice were randomly allocated to two groups: one receiving Western diet alone, the other receiving the same diet mixed with ticlopidine to yield a dose of 90 mg/kg/day (Sigma-Aldrich, St. Louis, MO, USA). This dose of ticlopidine was chosen on the basis of previous studies of the anti-platelet action of ticlopidine in mice (Suzuka et al., 1995; Fabra et al., 1987; Horisawa et al., 1999) and on the basis of a calculation of the equivalent dose in mice that would be relevant to the human dose of ticlopidine known to inhibit platelet aggregation (500 mg/kg, 7.14 mg/kg/day). The standard dose equivalent man/mouse (kg<sup>−1</sup>) equals 12.5 (Van Miert, 1989), thus the equivalent mouse dose was 7.14 mg/kg×12.5=90 mg/kg.

At the age of 6 months mice were injected with 1000 UI of fraxiparine (Sanofi-Synthelabo, Santea, France) i.p. and sacrificed under anesthesia (thiopental, 100 mg/kg) to quantify atherosclerosis plaques using cross-section and en face methods, to study the endothelial function in the isolated aorta as well as to analyze the total cholesterol and triglyceride levels in plasma. All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

2.2. Quantification of atherosclerosis in aortic roots (cross-section analysis)

In anesthetized mice, the thorax was longitudinally opened, the right atrium was incised and the heart was perfused by phosphate buffer saline (PBS, pH 7.4) through the apex of the left ventricle at a constant pressure of approximately 100 mm Hg. Next, the heart and the ascending aorta were dissected. The excised heart and ascending aorta were embedded in optimal cutting temperature (OCT) compound (CellPath, Oxford, UK) and snap-frozen. 10-µm-thick cryosections were cut from the aortic root using a standardized protocol (Nicoletti et al., 1998; Elhage et al., 2003). Serial sections were cut from the proximal 1 mm of the aortic root. Eight adjacent sections were collected at 100-µm intervals starting at a 100-µm distance from the appearance of the aortic valves. Sections were thaw-mounted on poly-L-lysine coated slides and air dried. After fixation in 4% paraformaldehyde (pH=7), sections were stained with Meyer’s hematoxylin and oil red-O (Sigma-Aldrich, St. Louis, MO, USA) and snap-frozen. Oil red-O-stained sections were examined under an Olympus BX50 (Olympus, Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using an Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024×768 pixels. The total area of the lesion was measured manually in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal a mean lesion area was calculated from eight sections, reflecting the cross-section area covered by atherosclerosis (Robertson et al., 2003).

2.3. Quantification of atherosclerosis in descending aorta (en face analysis)

The aorta from arch to bifurcation was dissected from surrounding tissues and fixed in 4% formaldehyde. Then it was opened longitudinally, pinned onto brown wax plates and stained with Sudan IV (Sigma-Aldrich, St. Louis, MO, USA) (Robertson et al., 2003). Oil red-O-stained sections were dissected from an Olympus BX50 microscope and used for quantitative evaluation. Images of the aorta were recorded using an Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024×768 pixels. The total area of the lesion was measured manually in each slide using LSM Image Browser software.

2.4. Quantification of inflammation in atherosclerotic plaques by immunohistochemistry

For indirect triple immunohistochemistry, acetone-fixed sections of ascending aorta were used. Sections were cut from the proximal 1 mm of the aortic root as for oil red-O staining. They were incubated overnight with primary antisera: Cy3-conjugated anti-smooth muscle α-actin (Sigma-Aldrich, St. Louis, MO, USA), rat anti-mouse CD68 (Serotec, Oxford, UK) and rabbit anti-CD3 (Calbiochem, Darmstadt, Germany). Then, goat anti-rat IgG biotinylated antibodies and goat anti-rabbit
2.2. Analysis of endothelial function in isolated mice aorta

After opening the thorax of anesthetized mice, the descending thoracic aorta was quickly removed and, after cleaning from surrounding tissue, it was cut into 3 rings, each approximately 3 mm in length. Vascular rings were then transferred to organ chambers filled with 5 ml of Krebs–Heinsleit solution (mM: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, glucose 10, pyruvic acid 2, HEPES 10 and EDTA 0.03) and maintained at 37 °C, pH 7.4 and gassed with 95% O2 and 5% CO2. Rings were mounted between 2 hooks attached to an isometric force transducer (Biegastab K30 type 351; Hugo Sachs March-Fr, Germany) that was connected for continuous recording of tension to a recorder (Graphtec WR3320, UK). Resting tension was increased by steps to reach a final value of 0.75 g, after which the rings were incubated for 30 min. After checking viability of the vessels with KCl, 30 mM, aortic rings were preconstricted with phenylephrine (10−6) and maintained at 37 °C, pH 7.4 and gassed with 95% O2 and 5% CO2. Rings were mounted between 2 hooks attached to an isometric force transducer (Biegastab K30 type 351; Hugo Sachs March-Fr, Germany) that was connected for continuous recording of tension to a recorder (Graphtec WR3320, UK). Resting tension was increased by steps to reach a final value of 0.75 g, after which the rings were incubated for 30 min. After checking viability of the vessels with KCl, 30 mM, aortic rings were preconstricted with phenylephrine (10−6) to obtain submaximal contraction (60–80% of KCl-induced maximum response). Then, cumulative concentrations of acetylcholine (10−9 to 10−6 M) were added to induce endothelium-dependent relaxation. The endothelium-independent vasorelaxation was evoked by S-nitroso-N-acetylpenicillamine (SNAP, 10−9 to 10−6 M). Basal NO production was measured on mildly preconstricted rings (20% of KCl-induced contraction) on the basis of the contraction induced by the nitric oxide synthase inhibitor — L-N(G)-nitroarginine methyl ester (L-NAME, 300 μM, 15 min). To test the contribution of endothelium-derived hyperpolarizing factor (EDHF) to acetylcholine-induced relaxation, it was repeated in phenylephrine preconstricted rings in the presence of L-NAME and indomethacin (1 μM, 30 min). The response attributed to EDHF was then analyzed in rings preconstricted with KCl (60 mM).

2.6. Analysis of total cholesterol and triglyceride concentration in plasma

Blood was collected from the right ventricle. Samples of plasma that were obtained by centrifugation at 1000 × g at 4 °C for 10 min. were immediately frozen and stored (−80 °C) until assayed. Total cholesterol and triglycerides were assayed using commercially available kits (Cormay, Lublin, Poland).

2.7. Statistical analysis

Results were expressed as mean±S.E.M. Differences between means were evaluated by ANOVA followed by post-hoc Scheffe’s test. For quantification of atherosclerosis, the nonparametric Mann–Whitney U test was used. p<0.05 was considered statistically significant.

3. Results

3.1. Effects of ticlopidine on atherosclerosis progression in apoE/LDLR−/− mice

In 6-month-old apoE/LDLR−/− mice treated with ticlopidine for 4 months (90 mg/kg/day), plasma levels of total cholesterol and triglycerides were similar to those in non-treated counterparts (total cholesterol: 28.9±1.1 mmol/l vs 26.8±1.3 mmol/l (n=5); triglycerides: 1.8±0.1 mmol/l vs 2.0±0.1 mmol/l (n=5) in the non-treated and the ticlopidine-treated group, respectively). However, in ticlopidine-treated apoE/LDLR−/− mice the area covered by atherosclerotic lesions in aortic roots was markedly diminished (oil red-O-stained lesions were 565 867±39 764 μm² vs 308 641±33 022 μm² in the non-treated and the ticlopidine-treated group, respectively, p<0.05, Figs. 1 and 2). In contrast to pronounced anti-atherosclerotic effect of ticlopidine in cross-section analysis, the effect of

![Fig. 1. Representative photomicrographs showing oil red-O-stained lesions in aortic roots (A, B) and Sudan IV-stained lesions in whole aorta (C, D) in apoE/LDLR−/− mice that were untreated (A, C) or treated with ticlopidine (90 mg/kg) (B, D).](image-url)
ticlopidine on the surface area covered by plaques in the whole aorta (en face analysis) was not evident (Sudan IV-stained area was 25.15±2.9% and 21.5±5.71% in the non-treated and the ticlopidine-treated group, respectively, $p=0.0617$; Figs. 1 and 2). Importantly, in aortic roots not only the size of atherosclerotic plaque but also the composition of the lesions was favorably changed by ticlopidine treatment. The area covered by immunopositive CD68 macrophages and the number of immunopositive CD3 T lymphocytes were reduced in ticlopidine-treated mice (Figs 2C, D and 3). Interestingly, in ticlopidine-treated mice the T lymphocytes were localized in peripheral parts of plaques, while in their non-treated counterparts they were distributed both in peripheral and in central regions of the plaques (Fig. 3). In addition, in ticlopidine-treated mice, the percentage of plaque area

![Fig. 2. Summarized data showing anti-atherosclerotic effects of ticlopidine (90 mg/kg) in aortic roots (A, B, C) and in whole aorta (D). A — the area of lesions in aortic roots measured by cross-section method and oil red-O staining ($\mu m^2 \times 10^5$), B — the percentage of plaque area occupied by CD68 positive cells, C — the number of CD3 positive cells per mm², D — the percentage of surface area of aorta occupied by Sudan IV staining as measured by en face method. Data represent mean±S.E.M. from $n=5–7$ mice in each group. *$p<0.05$.](image)

![Fig. 3. Triple immunohistochemical staining of aortic roots from non-treated (A–D) and ticlopidine-treated (E–H) apoE/LDLR$^{-/-}$ mice. The figure shows representative immunoreactivities for smooth muscle $\alpha$-actin (A, E), lymphocyte’s CD3 (B, F) and macrophage’s CD68 (C, G); as well as combined images showing color triple immunohistochemical staining for smooth muscle $\alpha$-actin, CD3 and CD68 (D, H). Notice abundant smooth muscle cells in the intima of ticlopidine-treated animals (A, E, arrows) and more abundant T lymphocyte in the intima (B, F, arrowheads) of aortic root of non-treated apoE/LDLR$^{-/-}$ mice. Numerous T lymphocytes in adventitia (B, F, arrows) as well as large infiltrations of macrophages (C, H, asterisks) can be observed both in non-treated and ticlopidine-treated apoE/LDLR$^{-/-}$ mice. L — lumen of the aorta.](image)
covered by continuous layers of smooth muscle cells was increased (8±2% vs 12±3% in the non-treated and the ticlopidine-treated group, respectively, \( p < 0.05 \)).

3.2. Effects of ticlopidine on endothelial function in thoracic aorta from apoE/LDLR–/– mice

Endothelium-dependent vasodilation induced by acetylcholine in phenylephrine-preconstricted aortic rings from 6-month-old apoE/LDLR–/– mice was impaired. (Fig. 4A). In 6-month-old apoE/LDLR–/– mice treated with ticlopidine, acetylcholine-induced vasodilation in aorta was markedly improved (Fig. 4A), while the magnitude of endothelium-independent vasodilation induced by SNAP remained unmodified (Fig. 4B). Basal NO production (as measured by the magnitude of the contraction induced by \( \text{L-NAME} \) (300 \( \mu \text{M} \)) in mildly phenylephrine-preconstricted aortic rings) was similar in ticlopidine-treated and non-treated apoE/LDLR–/– mice (Fig. 4C). Interestingly, in the presence of \( \text{L-NAME} \) acetylcholine-induced response was completely blocked in aortic rings from non-treated apoE/LDLR–/– mice, while in aortic rings from ticlopidine-treated apoE/LDLR mice the effect of \( \text{L-NAME} \) was less pronounced (Fig. 4D). The NO-independent component of the acetylcholine-induced response, that was up-regulated in ticlopidine-treated animals, was not modified by indomethacin (5 \( \mu \text{M} \)), but was absent when rings were preconstricted with KCl (60 mM) instead of phenylephrine.

4. Discussion

ApoE/LDLR–/– mice represent a unique and reliable model of atherogenesis that allows for the quantification of anti-atherosclerotic activity of pharmacological tools (Jawien et al., 2005, 2006; Olszanecki et al., 2005). Using this model, we found that chronic treatment of apoE/LDLR–/– mice with ticlopidine at a dose that affords anti-platelet activity (Suzuka et al., 1995) inhibited progression of atherosclerosis (in aortic roots), blunted inflammatory response in atherosclerotic plaque, and improved endothelial function without an effect on total cholesterol and triglyceride levels in the blood.

The negative results, as regards the ticlopidine effect on the area stained by Sudan-IV in the whole aorta (en face), is in line with the notion that en face analysis is a less sensitive method to quantify atherosclerosis (Nicoletti et al., 1998). It may well be that atherosclerotic plaques in the aortic arch, thoracic and abdominal aorta represent lesions at various stages of development, though there are invariably stained with Sudan IV. In contrast, in mouse models of atherosclerosis plaques appears firstly in the aortic root, which then is homogenously occupied by the advanced type of atherosclerotic plaques, so this localization is the best for consistent quantification of atherosclerosis (Nicoletti et al., 1998). It may well be that atherosclerotic plaques in the aortic arch, thoracic and abdominal aorta represent lesions at various stages of development, though there are invariably stained with Sudan IV. In contrast, in mouse models of atherosclerosis plaques appears firstly in the aortic root, which then is homogenously occupied by the advanced type of atherosclerotic plaques, so this localization is the best for consistent quantification of atherosclerosis (Nicoletti et al., 1998).
in en face analysis of the whole aorta does not preclude the major results shown here, supporting the anti-atherosclerotic action of ticlopidine in apoE/LDLR−/− mice. Indeed, ticlopidine treatment not only reduced the area of the plaque stained by oil red-O but also, as revealed by immunostaining, significantly down-regulated the number of macrophages and, to a lesser extent, the number of T lymphocytes in the plaques. It is well known that the intensity of vascular inflammatory response that is consistently associated with atherosclerosis depends on the abundance and activation of macrophages and T lymphocyte in the plaque (Elhage et al., 2003; Robertson et al., 2003). Thus, ticlopidine treatment limited vascular inflammatory response of atherosclerosis, as was previously found with other anti-platelet drugs (Ikonomidis et al., 1999; Cyrus et al., 2002; Woodward et al., 2004; Azar et al., 2006).

In the present work we also found that ticlopidine treatment improved acetylcholine-induced vasodilation. It is widely accepted that endothelial dysfunction is a key factor in the progression of atherosclerosis, thus it may well be that an improvement of the endothelial function by cardiovascular drugs contributes to their anti-atherosclerotic effects (Chlopicki and Gryglewski, 2005). Interestingly, we found that ticlopidine did not modify basal NO production, but up-regulated the NO-independent component of acetylcholine-induced vasodilation that could be attributed to EDHF. Indeed, in our experiments the cyclooxygenase inhibitor indomethacin did not affect the up-regulated NO-independent vasodilation in ticlopidine treated apoE/LDLR−/− mice, while this response was inhibited by elevated extracellular K+ concentration (60 mM). It is tempting to speculate that up-regulation of EDHF by ticlopidine plays a role in maintaining proper endothelial function and contributes to the anti-atherosclerotic effect of ticlopidine. Indeed, the function of EDHF is not limited to the regulation of vasomotor tone. EDHF displays potent anti-inflammatory action (Campbell, 2000; Selemidis and Cocks, 2002). We previously demonstrated that ticlopidine exerts pleiotropic action on endothelial cells that is not related to the anti-platelet activity of this drug (Gryglewski et al., 1996, 2000; Jakubowski et al., 2005). The endothelial action of thienopyridines, at least in the isolated guinea pig heart, was not mediated by endothelial purinergic receptors (Jakubowski et al., 2005). Further studies are needed to characterize in greater detail the vascular effects of ticlopidine, detected here as well as in our previous studies.

On the other hand, the improvement of endothelial function found in the present work may result from the anti-platelet action of ticlopidine. This notion seems to be consistent with the causal role of activated platelets in the vascular inflammatory response (Libby, 2000; Ruggeri, 2002) as well as with their role in the development of endothelial dysfunction (Gawaz et al., 2005). Indeed, inhibition of platelet activation and subsequent down-regulation of vascular inflammatory response was repeatedly shown to be associated with improved endothelial function (Husain et al., 1998; Kharbanda et al., 2002). It is, however, of note that ticlopidine most likely inhibits the activation of platelets and stimulates endothelial defensive pathways by distinct mechanisms. Further studies are required to understand the relative contribution of each of these mechanisms to the anti-atherosclerotic action of ticlopidine.

It may well be that clopidogrel is also endowed with anti-atherosclerotic activity. Interestingly, we demonstrated that clopidogrel enantiomer that is devoid of anti-platelet action possesses an identical PGI2- and NO-releasing capability as compared to enantiomer endowed with anti-platelet action (Gryglewski et al., 2000; Jakubowski et al., 2005). Recently it was shown that clopidogrel improved endothelial function in patients by platelet-dependent mechanisms (Heitzer et al., 2006). We believe that experimental studies on anti-atherosclerotic effects of clopidogrel enantiomers – alone and in combination – may shed new light on the complementary effects of thienopyridines on platelets and endothelium, and that this may in turn explain their good clinical efficacy in the fight against atherosclerosis.

Summing up, we demonstrate here, for the first time, that ticlopidine attenuates atherosclerosis development and improves endothelial function in the experimental mouse model of atherosclerosis. The question whether this effect is related to the anti-platelet effects of ticlopidine, to the endothelial action of ticlopidine, or to both, remains to be answered.

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