Paraoxonase-1 Deficiency does not Influence Clopidogrel Antiplatelet Function in Mice

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Abstract

Background: Clopidogrel is an antiplatelet agent used in the treatment of vascular diseases. It requires in vivo bio activation linked to the cytochrome P450. Several studies reported that paraoxonase-1 (PON1) was a crucial enzyme in clopidogrel activation, and that patients carrying a variant of the PON192 gene polymorphism have a high risk of thrombosis. However, these reports were not confirmed by subsequent results. The present study was aimed at investigating whether PON1 deficiency affects the biological action of clopidogrel in mice.

Methods: PON1-deficient mice (n = 50) and wild type animals (n = 50) received different treatments for 3 days: a) clopidogrel, b) aspirin, c) cilostazol, d) clopidogrel + aspirin, and e) clopidogrel + aspirin + cilostazol. Blood was collected for the Platelet Function Analysis (PFA-100).

Results: The different anticoagulant treatments resulted in higher aggregation times in all the mice, compared to the internal PFA control; demonstrating the anti-platelet effect of these compounds. We did not observe any significant alterations on the PFA assay in PON1-deficient mice, relative to wild type animals.

Conclusion: PON1 deficiency does not influence the antiplatelet action of clopidogrel in mice, and supports the proposition that this enzyme is not involved in clopidogrel bio activation.

Keywords: Antiplatelet agents; Clopidogrel; Paraoxonase-1; Vascular diseases

Abbreviations

ADP: Adenosine Diphosphate; CYP: Cytochrome; PFA: Platelet Function Analysis; PON1: Paraoxonase-1

Introduction

Clopidogrel is an oral, thienopyridide-class, antiplatelet agent used in the treatment of vascular diseases, including peripheral vascular disease [1]. This compound is a pro-drug that requires enzymatic biotransformation into the active thiol metabolite to facilitate its action of inhibiting platelet adenosine diphosphate (ADP) P2Y12 receptor [2]. Early studies described in vivo bio activation of clopidogrel as a two-step process closely linked to the cytochrome P450 (CYP) 2C19 enzyme [3]. However, Bouman et al. [4] reported that paraoxonase-1 (PON1) was a crucial enzyme in clopidogrel metabolic activation, especially in the second-step. Their article described the first step as an oxidation of clopidogrel to 2-oxo-clopidogrel catalyzed by cytochromes, and the second step as a hydrolytic cleavage (catalyzed by PON1) of the γ-thiobutyrolactone ring of 2-oxo-clopidogrel to the pharmacologically-active thiol metabolite. They also highlighted that individuals carrying the QQ isoform of PON1 gene polymorphism have a higher risk of stent thrombosis. Tselepis et al. [5] showed an inverse association between PON1 activity and platelet activation following clopidogrel administration, and suggested that PON-1 is an important determinant of clopidogrel antiplatelet efficacy in these patients. Dansette et al. [6] reported that PON1 catalyzes the formation of a minor thiol metabolite, while the biosynthesis of the major clopidogrel metabolite is CYP P450 dependent. These reports generated considerable controversy, and methodological concerns regarding the Bouman et al. article have been published [7]. Subsequent studies failed to show any influence of serum PON1 activity or genetic polymorphisms on clopidogrel bio activation [8-12]. Ancrizen et al. [12] reported that CYP2C19, CYP2B6 and CYP3A were the most important determinants in the bio activation of clopidogrel in vitro, and a retrospective study by Ohmori et al. [10] did not find any significant association between PON1 polymorphism and clopidogrel response in patients who had a myocardial infarction. The present study, conducted in mice, was aimed at investigating whether PON1 deficiency affects the biological action of clopidogrel.

Materials and Methods

The study adhered to rules for the protection of animals in research, and was approved by the Committee for Animal Experimentation of the Universitat Rovira i Virgili. Genetically modified PON1-deficient mice (n = 50) and wild type animals (n = 50) received an atherogenic diet for 24 weeks. The last 3 days before their sacrifice, they were subdivided into 5 groups to receive different treatments: Group I: clopidogrel [22 mg/kg/day]; Group II: aspirin [60 mg/kg/day]; Group III: cilostazol [50 mg/kg/day]; Group IV: clopidogrel + aspirin; Group V: clopidogrel + aspirin + cilostazol. All treatments were administered by oral gavage. These drugs, alone or in combination, are currently used for the treatment of coagulation disorders in patients with PAD [13,14]. Cilostazol is a phosphodiesterase inhibitor with antiplatelet properties which plays an important role in thrombosis prevention [14]. PON1-deficient animals of the C57BL/6J genetic background

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were the progeny of mice provided by the Division of Cardiology of the University of California in Los Angeles [15]. Wild-type animals were from the C57BL/6 strain (Charles River Labs., Wilmington, MA, USA). Mice were bled by cardiac puncture and all the blood obtained (about 2 mL) was collected into citrate-containing tubes for the Platelet Function Analysis (PFA-100). This is an in vitro method that replicates the platelet aggregation process. Using a membrane coated with collagen which promotes platelet adhesion, the membranes are covered with either epinephrine or ADP to promote platelet activation. The PFA is a functional assay, and samples are interpreted as having an inhibited coagulation when the measured coagulation times are higher than those of the internal controls (control collagen/epinephrine: 113-137 seconds; control collagen/ADP: 87-105 seconds). The high volume of sample required to perform this technique precluded other analytical determinations being performed in serum.

To ensure that genetically modified mice were really PON1-deficient, we measured PON1 lactonase activity in excised liver homogenates, since the liver is a key organ for clopidogrel activation, and this was the reason for PON1 deficiency within the platelet adhesion and aggregation pathways. One of the most common reasons for PFA prolongation of dysfunction within the platelet adhesion and aggregation pathways. One of the most common reasons for PFA prolongation is the administration of platelet anti-aggregants [17-19]. Collagen, epinephrine and ADP, under in vivo physiological conditions, promote substances facilitating adhesion and aggregation of platelets. This assay has been studied and validated in rats and mice, and closure in control animals are approximately 100 seconds [20]. Our results show that PON1 deficiency does not produce any significant alteration in the anticoagulant function of the tested drugs. These results support, and extend, recent findings in humans. Sibbing et al. [21] analyzed the ADP-induced platelet aggregation in relation to PON1<sub>192</sub> and CYP2C19 gene polymorphisms in patients following a coronary stent insertion. The results showed that the PON1<sub>192</sub> polymorphism did not influence platelet response to clopidogrel nor the risk of thrombosis in these patients. The CYP2C19 polymorphism, however, had an impact on the antiplatelet effect of clopidogrel and on thrombosis risk. Further, the results of a systematic review and meta-analysis from Reny JL et al. [22] did not support PON1<sub>192</sub> genotype as a major determinant of the biological response to clopidogrel, nor as a risk-factor for major cardiovascular ischemic events in clopidogrel-treated patients.

Results and Discussion

The different treatments produced higher aggregation times in all the mice, compared to the internal PFA control and, as such, demonstrating the anti-platelet effect of these compounds against collagen-induced platelet aggregation. We did not observe any significant alteration in the PFA assay in PON1-deficient mice, relative to wild type animals (Table 1). In addition, hepatic PON1 activity was measurable only in wild type mice, but not in PON1-deficient animals.

PFA has been developed as a standard test for the detection of dysfunction within the platelet adhesion and aggregation pathways. One of the most common reasons for PFA prolongation is the administration of platelet anti-aggregants [17-19]. Collagen, epinephrine and ADP, under in vivo physiological conditions, promote substances facilitating adhesion and aggregation of platelets. This assay has been studied and validated in rats and mice, and closure in control animals are approximately 100 seconds [20]. Our results show that PON1 deficiency does not produce any significant alteration in the anticoagulant function of the tested drugs. These results support, and extend, recent findings in humans. Sibbing et al. [21] analyzed the ADP-induced platelet aggregation in relation to PON1<sub>192</sub> and CYP2C19 gene polymorphisms in patients following a coronary stent insertion. The results showed that the PON1<sub>192</sub> polymorphism did not influence platelet response to clopidogrel nor the risk of thrombosis in these patients. The CYP2C19 polymorphism, however, had an impact on the antiplatelet effect of clopidogrel and on thrombosis risk. Further, the results of a systematic review and meta-analysis from Reny JL et al. [22] did not support PON1<sub>192</sub> genotype as a major determinant of the biological response to clopidogrel, nor as a risk-factor for major cardiovascular ischemic events in clopidogrel-treated patients.

Conclusion

We conclude that PON1 deficiency does not influence the antiplatelet action of clopidogrel in mice, thus supporting the proposition that this enzyme is not involved in its bio activation.

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Conflicts of Interest

The authors report no conflicts of interest.

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Table 1: Plasma PFA assay and hepatic PON1 activity in the groups of experimental mice.

| Group | Parameters | Wild type | PON1 deficient |
|-------|------------|-----------|----------------|
| I: Clopidogrel (n = 10) | Collagen/EP (seconds) | >300 | >300 |
| II: Aspirin (n = 10) | Collagen/EP (seconds) | >300 | >300 |
| III: Cilostazol (n = 10) | Collagen/EP (seconds) | >300 | >300 |
| IV: Clopidogrel + Aspirin (n = 10) | Collagen/EP (seconds) | >300 | >300 |
| V: Clopidogrel + Cilostazol (n = 10) | Collagen/EP (seconds) | >300 | >300 |

< DL: below detection limit, Values are expressed as means with standard deviations in parentheses.
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