Anti-inflammatory and anti-atherogenic effects of cathechin, caffeic acid and trans-resveratrol in apolipoprotein E deficient mice

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Abstract

A strong negative correlation between polyphenols consumption and coronary heart disease has been extensively documented. These results prompted investigations on the mechanisms responsible for polyphenols effects in cardiovascular disease.

The aim of this work was to investigate in apoE KO mice the effect of P183/1 (a mixture of cathechin, caffeic acid and resveratrol) on atherosclerosis and gene expression patterns in the vascular wall.

ApoE KO mice were fed a diet supplemented with P183/1, 40 and 160 mg/kg body weight/day for 8 weeks. The supplementation with the high dose of P183/1 significantly reduced the presence of atherosclerotic plaque by 40 and 36% in the aortic sinus and in the ascending aorta, respectively. This reduction was associated with a reduced expression of markers for macrophages, lymphocytes (both Th1 and Th2) and of MCP-1, MIP-1\textsubscript{α}, MIP-1\textsubscript{β}, CCR1, CCR2 and ET1 in the vascular wall. In conclusion, P183/1 supplementation significantly decreases atherosclerosis in ApoE KO mice by affecting inflammatory cells recruitment and expression of pro-inflammatory chemokines in the vascular wall.

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Keywords: Polyphenols; Chemokines; Inflammation

1. Introduction

A strong negative correlation between polyphenols consumption and coronary heart disease or ischaemic stroke mortality has been extensively documented [1,2]. These results prompted investigations on the effects of plant polyphenols in animal models and human beings. Polyphenolic compounds from red wine and green tea are able to prevent the progression of atherosclerosis in animal models [3–6], suggesting that the protective effect of dietary polyphenols against vascular risk might be attributed, at least in part, to their ability to retard the progression of early atherosclerotic lesions to advanced plaques which are prone to rupture with superimposed thrombosis. A major difficulty with this approach is the extreme complexity of the polyphenolic content of food and beverages. There are several hundred plant phenolic compounds including non-flavonoids (phenolic acids, stilbenes like trans-resveratrol) and flavonoids, i.e., molecules possessing two phenols joined by a pyran carbon ring structure (flavanols, flavonols and anthocyanins). Previous studies have been limited to the analysis of the effects of the consumption of tea extract [6], green and black tea [4], red wine, dealcoholized wine and wine polyphenols [5,7], but the anti-atherosclerotic effect of pure polyphenols has been demonstrated only for quercitin and catechin [3,8]. As the reduction of atherosclerosis lesion by polyphenol intake is not necessarily related to the modifications in the oxidative stress biomarkers [7] and lipid parameters it is possible that other mechanisms, such as

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anti-inflammatory or vasodilatory actions could be involved [1].

We have recently demonstrated that in humans, the antioxidant effect of wine is closely dependent upon its polyphenolic content [9]; moreover, a significant correlation was observed between polyphenol plasma levels and inhibition of oxidative stress [9].

The aim of this work was to investigate in apolipoprotein E knock out (apoE KO) mice the effect of a mix of pure polyphenols (catechin, caffeic acid and resveratrol) named P183/1 on the development of atherosclerosis and on the gene expression pattern in the vascular wall, focusing on the possible anti-inflammatory actions.

2. Materials and methods

2.1. Animals and treatment

Apolipoprotein E KO mice were purchased from Jackson (6-weeks old). After 2 weeks of housing the animals were divided in three groups (12 mice each group): the control group, the group receiving 40 mg/kg of body weight/day (low dosage, LD) of P183/1 and the group receiving 160 mg/kg of body weight/day (high dosage, HD) of P183/1. P183/1 is constituted by resveratrol 0.27%, caffeic acid 1.37% and catechin 8.35%. These dosages were chosen on the basis of previous observations indicating that the antioxidant effect of wine is related to its polyphenolic content [9]. The drug was administered for 8 weeks in a single pellet of western type diet (21% fat, 0.15% cholesterol and 19.5% casein, Piccioni Italy), then animals were fed ad libitum with chow diet. All experimental procedures were in accordance with the institutional guidelines for animal research. The mice were sacrificed with an injection of Avertin 2.5% (Aldrich Chemical Co. USA). The heart and the arterial tree were perfused with saline solution under physiological pressure. Then the aortas and the hearts were isolated, placed in a storing solution (RNA laterICE™, Ambion, Germany) at −20°C or paraffin embedded. For RNA isolation the samples were homogenized in a dismembrator (B. Braun, Melsungen AG, Germany) then processed as described [10].

2.2. Plasma lipid analysis

Blood samples were collected in EDTA tubes immediately before death by retro-orbital bleeding and plasma was separated by low-speed centrifugation at 4°C. The measurement of plasma lipids was performed by standard enzymatic techniques (ABX for Cobas Mira Plus, Montpellier, France); HDL-C was determined after precipitation of apoB-containing lipoproteins.

2.3. Quantification of atherosclerosis

Cross sequential sections 5 μm thick were prepared. For the aortic sinus and the ascending aorta the slides were stained with hematoxilin–eosin, for each tract of the aorta 15 sections were analyzed. Images of the aortas were captured with a Nikon digital camera and the atherosclerotic lesions were quantified by computer image analysis, using OPTIMAS 6.2 software.

Data are expressed as μm² and are calculated as the differences between the area of the media + intima subtracted of the media area.

2.4. Real-time quantitative polymerase chain reaction

Total RNA was extracted and reverse transcribed as described [11,12]. Three microlitre of cDNA were amplified by real-time quantitative PCR with 1× Syber green universal PCR mastermix (BioRad, Italy). The specificity of the Syber green fluorescence was tested as described in ref. [11]. The primers used are described in Table 1. Each sample was analyzed in duplicate using the IQ™-Cycler (BioRad). The PCR amplification was related to a standard curve ranging from 10⁻¹¹ to 10⁻¹⁴ mol/L and data were normalized for the housekeeping gene ribosomal protein L13a (RLP13a) (NM_009438).

2.5. Statistics

Data were analyzed using SPSS 12.0 for Windows (SPSS, Chicago, IL). Statistical analysis was performed with Mann–Whitney test, setting the significance level at \( p < 0.05 \).

3. Results

3.1. Body weight and plasma lipids

At 8 weeks of age, apo E⁻/⁻ mice were assigned randomly to three groups, receiving either the diet alone or with the polyphenols mixture added at low dose or high dose for 8 weeks. No significant differences were observed in the weight gain between the three groups, only a slight increase was observed in the group treated with the low dose of P183/1 (Table 2). Also plasma levels of total cholesterol, HDL and triglycerides were not different between the three groups (Table 2).

3.2. Effect of polyphenols on the extent of atherosclerosis

Morphometric analysis showed that the lesion area in the aortic sinus was reduced by 40% in animals treated with the high dose of P183/1 compared with control animals, while no difference was observed with the low dose of P183/1 (Table 2 and Fig. 1). When the presence of atherosclerosis was assessed in the ascending aorta a 36% reduction of the lesion size was observed in the mice treated with the high dose of P18371 compared to control mice (Fig. 2).
3.3. Cellular composition of the lesion (Fig. 3)

To evaluate the effects of P183/1 on vascular wall composition, a comprehensive gene expression analysis of specific markers of the cells present during the development of atherosclerosis was performed. As reported by the mRNA level for CD68, the number of monocyte/macrophages within the vascular wall of the aortic arch decreased significantly in the Apo E−/− mice treated with P183/1 compared to control mice (−49.4%). Similar results were obtained for the expression of the T-helper lymphocytes (Th) marker CD4 (−66.4%). Analysis of the chemokine receptor CCR4 expression, a Th lymphocyte type 2 marker, revealed a decreased recruitment into the vascular wall in P183/1 treated mice compared to control mice. Similarly, the amount of Th1 cells, as detected with the TIM3 probe (T-cell immunoglobulin domain, mucin domain) decreased in P183/1 treated mice. Also the expression of α-actin, a marker of smooth muscle cells, resulted decreased in P183/1 treated mice.

3.4. Gene expression in the vascular wall

As P183/1 treatment resulted in a decreased presence of monocyte/macrophages and CD4+ lymphocytes in the arterial wall, we investigated the expression of pro-inflammatory and anti-inflammatory mediators in the aorta. P183/1 treatment (high dose) decreased MCP-1, MIP-1α, MIP 1β, IL6 and IL10 expression (Fig. 4). We further investigated the expression of the chemokine receptors, CCR1, CCR2, CCR3, CCR5 and CXCR3. CCR1 and CCR2 expression was significantly decreased upon P183/1 treatment, a slight effect was observed on CCR3 expression while no effect was observed on CCR5 and CXCR3 expression (Fig. 4).

When we evaluated the effects of P183/1 on the expression of genes affecting vascular functions, the treatment with the high dose resulted in a significant decrease of endothelin-1 (ET-1) expression, a slight effect was observed also on inducible and endothelial nitric oxide while no effects were observed on tissue factor, transforming growth factor β1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Fw</th>
<th>Primer Rev</th>
</tr>
</thead>
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<tr>
<td>RLP-13a</td>
<td>5′CGCCAGAAGCAGGAGAA3′</td>
<td>3′GGAGACTGCTTGACCTTGAAGTT5′</td>
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<tr>
<td>α-Actina</td>
<td>5′GGGCTTGGTCCATCCATTA3′</td>
<td>3′AGGACTTGGTGGACCTTACAATC5′</td>
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<td>CD68</td>
<td>5′AGCTCCCTTGGGCGGAAG3′</td>
<td>3′AGGTGCAAGCTGAGCAGATT5′</td>
</tr>
<tr>
<td>CD4</td>
<td>5′GTGACCTGGGAGAAGGAAAG3′</td>
<td>3′GGCTGTCACCCGGACTGAA5′</td>
</tr>
<tr>
<td>TIM3</td>
<td>5′GGACACTGGTCAACAAATGGGA3′</td>
<td>3′CAGGCAGCTGAAAGCAGATG5′</td>
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<td>MCP-1 (CCL2)</td>
<td>5′TCTCCAGCGCCCTCACTTCT3′</td>
<td>3′CAGGCCCAGAAGCATGAC5′</td>
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<tr>
<td>MIP1-α (CCL3)</td>
<td>5′CAAGTCTTCTTCAGGCCATAG3′</td>
<td>3′TCCGCTGTAGGAGAAGCA5′</td>
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<tr>
<td>MIP1-β (CCL4)</td>
<td>5′TGCTCTGGTTGGCCCTTCT3′</td>
<td>3′CGGGAATGGGGGGGTCAGAG5′</td>
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<td>CCR1</td>
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<tr>
<td>CCR2</td>
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<td>3′GTGGCCCTTCCCACAGC5′</td>
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<td>3′TCCACCACCTGGAACAG5′</td>
</tr>
<tr>
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<td>ET-1</td>
<td>5′CCATGGCTGCTGGGATCT3′</td>
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<td>TF</td>
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<td>3′CCGGACCTCATGAGCAGTG5′</td>
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<td>5′TGCAATGAGCGCTGGTAA3′</td>
<td>3′GACGGTTAGTTGGGCGAGATTG5′</td>
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<td>TGF-β2</td>
<td>5′CCACCTCCCCCTGGAA3′</td>
<td>3′AGACATCAAAGGCGAGATT5′</td>
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</table>

Table 1
Forward and reverse primers used for real-time PCR experiments

Table 2
Effects of 8-week supplementation of P183/1 (low dosage, LD or high dosage, HD) on atherosclerotic lesions in hypercholesterolemic Apo E−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 12)</th>
<th>P183/1 LD (n = 12)</th>
<th>P183/1 HD (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at death (g)</td>
<td>27.2 ± 0.6</td>
<td>28.7 ± 1.5</td>
<td>28.2 ± 1.2</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>468 ± 108</td>
<td>489 ± 87</td>
<td>498 ± 97</td>
</tr>
<tr>
<td>HDL-Chol (mg/dL)</td>
<td>34 ± 6</td>
<td>24 ± 7</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>97 ± 15</td>
<td>109 ± 30</td>
<td>98 ± 16</td>
</tr>
<tr>
<td>Aortic sinus lesion (mm²)</td>
<td>0.51 ± 0.17</td>
<td>0.56 ± 0.21</td>
<td>0.30 ± 0.06 *</td>
</tr>
<tr>
<td>Ascending aorta lesion (mm²)</td>
<td>0.13 ± 0.08</td>
<td>0.17 ± 0.11</td>
<td>0.08 ± 0.05 *</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control group.
Fig. 1. Effect of oral P183/1 supplementation on the extent of atherosclerosis in Apo E−/− mice in the aortic sinus. Representative sections of aortic sinus from control mice (A) or mice supplemented with P183/1 low dose (B) or high dose (C) are shown. Arrows indicate the atherosclerotic lesions, bar 300 μm.

(TGF-β1), TGF-β2 and vascular endothelial growth factor (VEGF) expression (Fig. 5).

4. Discussion

This study demonstrate that P183/1 (a mixture of polyphenols, cathechin, caffeic acid and resveratrol) inhibits atherosclerosis progression in Apo E−/− mice. This effect is associated with a decrease recruitment of monocyte-macrophages and T lymphocyte in the vascular wall. As a consequence of this, the expression of pro-inflammatory cytokines and chemokines as well as the expression of their receptors is decreased in the vascular wall of animals treated with the P183/1 compound.
Previous studies analyzed the effect of the consumption of tea extract [6], green and black tea [4], red wine, deacholized wine and wine polyphenols [5,7] on atherosclerosis hypothesizing a possible role of their polyphenols components in this effect but lacking any explanation on the molecular mechanisms involved. The novelty of our approach is that we investigated for the first time the direct effect of the diet supplementation with a mixture of three pure polyphenols on vascular wall thickening and inflammation.

Hayek et al. [3] showed that the anti-oxidant effect of quercitin or catechin on LDL is associated with a reduced atherosclerosis progression, however a recent analysis on published human studies suggested that polyphenols transiently affect some markers of oxidative stress but the correlation with cardiovascular disease remain to be addressed [1]. Similarly the hypolipidemic effects of polyphenols are controversial [1] and in our experiment a reduction of atherosclerosis was observed in the absence of plasma lipid changes by P183/1. These observations prompted the investigators to study vascular protective mechanisms of polyphenols independent of their antioxidant effects [2] such as a potential reduction of the inflammatory response observed during atherogenesis [13,14].

In vitro resveratrol inhibits ICAM-1 and VCAM-1 expression induced by TNFα in endothelial cells [15] by interacting with the NF-kB pathway [16]. Furthermore, resveratrol, catechin and quercitin decrease monocyte adhesion to endothelial
cells in vitro [15,17]. To investigate whether these effects occur also in vivo, we analyzed the expression of specific markers for monocyte/macrophage and CD4+ lymphocyte in the vascular wall. The treatment with P183/1 significantly decreased the expression of monocyte/macrophage and CD4+ lymphocyte, for the latter this was true for the Th1 and Th2 sub-population, suggesting a more general reduced leucocytes recruitment by P183/1 in the vascular wall. We next analyzed whether this reduced cell recruitment resulted in a reduced inflammatory response in the vascular wall. In Apo E KO mice the expression pattern of inflammatory mediators increases during atherogenesis correlating with the presence of inflammatory cells in the atherosclerotic lesion [18]. After a while, depending on the weeks of diet, the inflammatory response reaches the steady state although the plaque size further increases [18]. Treatment with P183/1 significantly decreased the expression of MCP-1, MIP1α and MIP1β and of the chemokine receptors CCR1 and CCR2. The interaction of this chemokine network plays a key role in the pathogenesis of vascular disease [19], and is considered as an important therapeutic target to limit atherosclerosis progression [19]. In addition to chemokines also cytokines such as IL-6 and IL-10 are modulated by polyphenols treatment although to a less extent compared to chemokines. IL-6 and IL-10 posses pro-inflammatory and anti-inflammatory effects, respectively [18] and their decrease probably reflects a decrease in lymphocytes Th1 and Th2 in the vascular wall, suggesting a global inhibitory effect of polyphenols on lymphocytes recruitment in vivo. A more pronounced effect on pro-inflammatory mediators could result in a better outcome in the vascular wall of P183/1 animals.

Of note the protective effects of P183/1 were observed only in animals administered with the high dose of the drug. This dosage corresponded to 13.36 mg/kg of catechin, 2.2 mg/kg of caffeic acid and 0.44 mg/kg of resveratrol that are all below the values used in previous experiments with single polyphenols [1,8], suggesting a global protective effect of these polyphenol mixture.

Plant polyphenols exert also vasorelaxant, anti-angiogenic and anti-proliferative effects on cells of the vascular wall such as the endothelium or smooth muscle cells [2]. Chyu et al. [8] demonstrated that in vitro epigallocatechin gallate decrease vascular smooth muscle cell proliferation in vitro, here we confirm some of these findings showing in vivo a reduced presence of smooth muscle cell in the aortas of mice fed with the diet containing P183/1, however this difference did not reached a statistical significance due to an high variability in the same mice group.

Endothelial protection by polyphenols has been extensively documented [2]. Plant polyphenols vasorelaxation is mediated by the NO-cyclic GMP pathway [20] and induction of eNOS expression [21]. Nevertheless the latter effect was observe with a large volume of red wine [21]. In our experiments, however we did not observe any difference in eNOS expression in the vascular wall upon P183/1 treatment, thus suggesting that the increased nitric oxide bioavailabil-

ity observed with polyphenols [22] could depend from other mechanisms other than eNOS transcription [23] and that other effects could be responsible for vasorelaxation [2]. Indeed polyphenols inhibit the synthesis of endothelin-1, a potent vasoconstrictor, in human and bovine endothelial cells [24,25]. P183/1 treatment decreased endothelin-1 expression in the vascular wall, suggesting that this could be one of the mechanisms by which polyphenols may restore the balance between endothelium derived vasoconstrictor and vasodilating factors. Anti-angiogenic effect of polyphenols has also been reported [2] and depends mainly on the prevention of VEGF expression [26]. In our experiments, we observed only a slight reduction of VEGF expression or of the expression of TGF-β [27]. It is possible that as increased VEGF expression is required only in advanced plaque to support neo-vascularization [28] in our experiment we investigated the early phases of atherogenesis, where control of the VEGF pathway may not be required as yet.

The current evidence suggests that the protection against cardiovascular disease associated with polyphenols results from a variety of effects produced by different mechanisms. We show here that in atherosclerosis prone mice supplementation with catechin, caffeic acid and resveratrol significantly decrease atherosclerosis progression in the aortic arch, by affecting inflammatory cells recruitment in the vascular wall and subsequent expression of pro-inflammatory chemokines and cytokines and by decreasing endothelin-1 expression thus potentially affecting endothelial function.

Acknowledgment

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References


