Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by pomegranate polyphenols: A role for PPAR-γ pathway

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1. Introduction

Serum paraoxonase 1 (PON1) is an HDL-associated lipolactonase, which is synthesized and secreted by the liver [1]. It is a member of the paraoxonases family (PON1, PON2, PON3). PON1 has anti-oxidative properties, which are associated with the enzyme's capability to protect LDL [2], as well as HDL [3] from oxidation, to decrease macrophage oxidative status [4], to stimulate cholesterol efflux from macrophages [5], to decrease oxidative status in atherosclerotic lesions [6] and to attenuate atherosclerosis development.

A variety of non-genetic factors have been shown to influence serum PON1 levels and activity. PON1 undergoes inactivation under oxidative stress and its activity is preserved by dietary antioxidants [5]. Moderate daily consumption of alcohol [7], vitamin C and E [8], wine [9], or pomegranate juice [10], increased serum levels of PON1 in animals and in humans. Together with the enzyme secretion rate, enzymatic turnover and protein stability, the level of PON1 gene expression is a major determinant of PON1 status.

PO1 mRNA levels were shown to be modulated by inflammatory factors (interleukins 1 and 6, oxidized phospholipids, or tumor necrosis factor-α) [11], by hypolipidemic drugs [12], and also by polyphenols [13–15].

The mechanisms of action of polyphenols in the upregulation of PON1 remain largely unknown. Gouédard et al. showed that dietary polyphenols increase PON1 gene expression by an aryl hydrocarbon receptor-dependent mechanism [15]. However, PON1 was also shown to be regulated by Sp1 and protein kinase C (PKC) [16], by the mitogen-activated protein kinase (MAPK) signaling cascade [17], and also via the interaction of sterol regulatory element binding protein (SREBP-2) with Sp1 [12].

Pomegranate juice (PJ) contains poly-molecular ellagitannin compounds, such as punicalagin, which are potent antioxidant and anti-atherogenic agents [18,19]. Phenolic compounds [20] and pomegranate [21] were shown to activate the nuclear receptor paraoxonise proliferator-activated receptor γ (PPARγ). Because the analysis of the promoter sequence of PON1 gene indicates that it could possibly be regulated by nuclear receptors [22], and as we have recently shown that PON2, another member of the paraoxonases family, is indeed regulated by PJ polyphenols via a PPARγ pathway activation [23], we hypothesize that PJ polyphenols could regulate PON1 expression via PPARγ pathway activation.

In the present study we have investigated the in vitro effect of PJ and its major polyphenols: punicalagin, gallic acid (GA), and ellagic acid (EA), on the expression and activity of PON1 in hepatocytes.

2. Materials and methods

For detailed description of this section please see online supplement “Materials and Methods”.

2.1. Chemicals

Pomegranate juice and punicalagin were from PomWonderful CA, USA, and from Daneel Ferrara, University of Mississippi, USA.

2.2. Cells

The human hepatoma cell line HuH7 was cultivated in DMEM medium supplemented with 5% FCS, 100,000 U/L penicillin, 100 µg/mL pyruvate, and 100 mg/L streptomycin, at 37 °C in a humidified incubator (5% CO₂, 95% air).

2.3. Conditioned medium (CM) collection

HuH7 hepatocytes were treated with PJ, punicalagin, GA and EA at 37 °C for 24 h. Then, the medium was washed out and RPMI-1640 medium supplemented with 1 µg of protein/mL of HDL derived from serum of PON1 knockout mice was added. After five hours the medium was collected.

2.4. Cells-associated and secreted PON1 arylesterase activity

Cell-associated PON1 arylesterase activity was measured directly on the cells and secreted PON1 arylesterase activity was measured in the collected medium. Enzyme arylesterase activity was measured using Phenyl acetate as substrate. The absorbance was monitored at 0 and 1 min after substrates addition. Measurements were as follows: 1 U of arylesterase activity = 1 µmol of phenyl acetate hydrolyzed/min.

2.5. PON1 mRNA expression

Total RNA was extracted with Epicentre commercial kit (Tamar, Israel). cDNA was generated from 1 µg of total RNA using Thermo Scientific commercial kit (Tamar, Israel). Products of the RT were subjected to quantitative PCR using TaqMan gene expression assays.

2.6. PON1 protein determination by confocal microscopy analysis

Cells were grown on 1.5 mm cover-slide, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Permeabilized cells were stained directly with rabbit anti-human PON1 followed by donkey anti-rabbit antibody conjugated to cy3 and visualized in green. Propidium iodide visualized in red, was used for counterstaining the macropage nucleus. Negative controls consisted of slides treated with the second antibody alone.

2.7. PON1 promoter reporter studies

HuH7 cells were transfected with 1 µg DNA consisting of a 20:1 ratio of PON1 promoter construct and a renilla luciferase encoding control plasmid, respectively. Four hours after start, the transfection procedure was finished by medium replacement.

Specific polyphenols treatment occurred after finishing the transfection procedure. Then, firefly and Renilla luciferase activities were recorded using the dual-luciferase reporter assay system using a Clarity Luminescence Microplate Reader. Finally, firefly activity was normalized for Renilla activity to account for transfection efficiency and PON1 promoter activity was calculated as the ratio of firefly luciferase activity over Renilla luciferase activity.

2.8. PPARγ gene silencing by small interfering RNA (siRNA)

PPARγ was silenced using SmartPool siRNA and the DharmaFECT4 transfection reagent. Successful transfection and knockdown of PPARγ was verified by quantitative PCR. HuH7 hepatocytes were then subjected to treatment with PJ and its polyphenols and quantitative PCR was performed.

2.9. LDL and HDL oxidation

LDL or HDL (100 µg of protein/mL) in phosphate-buffered saline (PBS) was oxidized by CuSO₄ (5 µmol/L) for 90 min at 37 °C.

2.10. Statistical analyses

Each separate experiment was performed in triplicate, and each individual experiment was replicated at least 3 times (n=3). Statistical analyses used Student’s t test for comparing differences between the two groups, and one-way ANOVA followed by the Student-Newman–Keuls test was used for comparing differences between multiple groups.

3. Results

3.1. Pomegranate juice polyphenols upregulate PON1 expression and activity in HuH7 hepatocytes

To test whether PJ and its polyphenols affect PON1 expression in hepatocytes, HuH7 hepatocytes were incubated for 24 h at 37 °C with increasing concentrations of PJ, or with purified polyphenols that are present in abundance in PJ, i.e. punicalagin, GA and EA. Fig. 1A demonstrates that PJ, punicalagin, and GA significantly increased PON1 mRNA expression in hepatocytes by up to 4-, 6-, and 7-fold, respectively, compared to control untreated cells. In contrast, EA increased hepatocyte PON1 mRNA expression by only 50%, and only when the highest EA concentration was used (0.36 mmol GAE/L). Next, cells transfected with the PON1 reporter gene plasmid were exposed to 0.36 mmol GAE/L of PJ, punicalagin, GA, or EA, and luciferase activity was then measured. PJ, punicalagin, GA, or EA produced a significant stimulation of PON1 promoter activity, by 5.7-, 7.5-, 8.5- and 1.8-fold, respectively (Fig. 1B). Furthermore, PJ, punicalagin and GA significantly increased hepatic PON1 protein abundance, as determined by confocal laser scanning, by 6.5-, 13.8-, and 17.3-fold, respectively, whereas EA increased hepatic PON1 protein expression by only 70% (Fig. 1C and D). In accordance, PJ, punicalagin, GA and EA dose-dependently increased cell-associated PON1 arylesterase activity by up to 1.45-, 1.56-, 2.88-, and 1.40-fold, respectively (Fig. 2A). However, because PON1 is mostly secreted from the hepatocytes, we next determined PON1 arylesterase activity in medium collected from HuH7 hepatocytes, which were incubated for 24 h with medium (without FCS), in the absence (control), or presence of PJ or purified polyphenols. Since it was previously shown that PON1 secretion from cells can only be effective in the presence of an acceptor, i.e. HDL [1], we have added to the incubation medium HDL, which was separated from serum derived from PON1 knockout mice, in order to eliminate possible interference of HDL-associated PON1

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activity with the determination of cell-secreted PON1 activity. PJ, punicalagin and GA increased PON1 secretion from hepatocytes by 2.7-, 1.9- and 2.4-fold, respectively, compared to control untreated cells, whereas EA did not significantly affect PON1 secretion from hepatocytes.

3.2. Polyphenols induce the secretion of a biologically active PON1

Biological activity of PON1 includes the enzyme's capability to protect LDL, as well as HDL from oxidation. In order to determine whether PJ and its polyphenols induce the secretion of a biologically active PON1, conditioned medium (CM) collected from hepatocytes pretreated with GA (GA-CM), which exhibited the highest effect among all tested polyphenols, was examined for its capability to inhibit copper ion-induced oxidation of HDL, or of LDL. CM collected from untreated hepatocytes served as control (control-CM). Both control-CM and GA-CM inhibited copper ion-induced HDL (Fig. 3A) or LDL (Fig. 3B) oxidation in a dose-dependent manner. However, while control-CM inhibited lipoproteins oxidation just minimally, GA-CM substantially inhibited HDL or LDL oxidation, with an IC50 (the concentration needed to inhibit lipoprotein oxidation by 50%) of 22 or 6 µL/mL, respectively (Fig. 3A and B). In order to confirm that the CM-mediated inhibition of HDL or LDL oxidation could be specifically attributed to PON1 secreted from the cells, PON1 catalytic activity in the medium was inactivated by heating the medium at 56 °C for 90 min, or by pre-incubation of the CM with 2-hydroxyquinolin, a selective PON1 inhibitor. Copper ion-induced LDL oxidation was significantly inhibited by GA-CM, by 71%, and
Fig. 3. Pomegranate polyphenols induce secretion of biologically active PON1. (A) HDL and (B) LDL were oxidized by copper ions in presence of conditioned medium from untreated (control-CM) and from gallic acid treated cells (GA-CM). (C) LDL was oxidized by copper ions in presence of GA-CM that was heated or treated with 2-hydroxyquinoline. Results are expressed as mean ± SD (n = 3 separate experiments), *P < 0.01 vs. control-CM.

Fig. 4. Upregulation of hepatic PON1 involves PPARγ pathway activation. HuH7 cells were incubated for 24 h at 37°C with Rosiglitazone (50 μmol/L) or Wy14643 (100 μmol/L). (A) PON1 mRNA expression, (B) Cell-associated and (C) medium-secreted PON1 arylesterase activity. Results are expressed as mean ± SD (n = 3 separate experiments), *P < 0.01 vs. control untreated cells.

3.3. PJ and its polyphenols upregulate PON1 expression in hepatocytes via PPAR-γ pathway

PON1 gene promoter sequence indicates that it could possibly be regulated by nuclear receptors. Because we have recently shown that macrophage PON2, another member of the paraoxonases family, is regulated by PJ polyphenols via PPAR-α pathway activation, we questioned whether PJ polyphenols regulate PON1 expression also in hepatocytes via a similar PPAR-γ pathway activation.

First we incubated HuH7 hepatocytes at 37°C for 24 h with rosiglitazone, which is a PPAR-γ activator, or with Wy14643, which is a PPAR-α activator. PON1 mRNA expression (Fig. 4A), cell-associated PON1 arylesterase activity (Fig. 4B) and medium-secreted PON1 arylesterase activity (Fig. 4C) were all increased by 1.7-, 1.5- and by 2.2-fold, respectively, in cells incubated with rosiglitazone, whereas no significant effect was observed when the cells were treated with Wy14643, suggesting that indeed PON1 expression in hepatocytes, similarly to PON2 expression in macrophages, is modulated by PPAR-γ, and not by PPAR-α. Next, we analyzed whether PJ polyphenols increase PON1 expression in hepatocytes via PPAR-γ pathway. Because EA exhibited only a minimal effect on PON1 expression in hepatocytes, we explored the mechanism involved in the stimulation of PON1 expression by PJ, punicalagin and GA only. GW9662, an irreversible PPAR-γ antagonist, reduced the upregulation of PON1 mRNA expression.
mediated by PJ, punicalagin or GA by 54%, 44%, or 42%, respectively (Fig. 5A). Alternatively, the cells were incubated with PJ, punicalagin or GA in the presence of TPA, which was previously shown to cause nuclear export and downregulation of PPAR-γ via MEK activation [24]. TPA indeed reduced PJ polyphenols-mediated PON1 mRNA upregulation by 55%, 55%, or 42%, respectively (Fig. 5B). To further substantiate the above findings, we downregulated the PPAR-γ expression using siRNA duplexes that were introduced into the cells by transfection. Control cells were transfected with the same amount of nonspecific siRNA duplexes. The downregulation of PPAR-γ 24 h after cell transfection was verified by RT-PCR (Fig. 5C). When expression of PPAR-γ was downregulated, addition of PJ or GA did not stimulate cellular PON1 mRNA expression (Fig. 5D).

Taken together, the above results suggest a role for nuclear transcriptional PPAR-γ in the stimulation of hepatocyte PON1 expression by PJ polyphenols.

3.4. PJ polyphenols induce PON1 gene expression in HuH7 hepatocytes via cAMP-PKA signaling cascade

Upstream mediators of PPAR-γ activation include protein kinase A (PKA) activators [25], which act directly on cyclic AMP (cAMP) production [26]. In order to elucidate the initial signaling mechanisms involved in the induction of PON1 gene expression elicited by PJ polyphenols in hepatocytes, we first determined whether PKA activators can upregulate cellular PON1 mRNA expression. The HuH7 hepatocytes were incubated for 24 h at 37°C with increasing doses of dibutyryl cAMP (db-cAMP), which is a cAMP analogue. Fig. 6A shows that db-cAMP increases PON1 mRNA expression in a dose-dependent manner, by up to 2.57-fold, suggesting that PKA activators are stimulators of hepatocyte PON1 mRNA expression. Next, we tested whether PJ polyphenols-mediated activation of PON1 mRNA transcription also involves the PKA pathway. For this purpose we used the H89 compound, a specific inhibitor of PKA. Fig. 6B shows that H89 was able to repress the stimulation of PON1 mRNA transcription by PJ, punicalagin or GA by 45%, 37% or 61%, respectively. These results suggest that PJ and its polyphenols punicalagin and GA increase PON1 gene transcription activity via the PPAR-γ-PKA-cAMP signaling cascade.

4. Discussion

The present study demonstrates that PJ and its polyphenols punicalagin, GA and EA upregulate PON1 expression and PON1 release from hepatocytes, via the intracellular signaling cascade PPARγ-PKA-cAMP. Previous reports demonstrated similar PPARγ activator property for pomegranate flower extract, which contains both GA and EA [27], and GA has been shown to be responsible for the PPARγ activator property of the pomegranate flower extract. These effects were demonstrated in vitro, as well as in vivo in zucker diabetic fatty rats after administration of methanol extract from pomegranate flower [21]. Similarly, our present findings show that GA exhibited the most remarkable effect on PON1 upregulation, whereas EA had the lowest effect, although both compounds exhibit anti-oxidative properties. A possible explanation for this
effect is that the PJ polyphenols stimulate hepatic PON1 expression independent of their anti-oxidative capacity. This is sustained by previous findings showing that molecules having a chemical structure compatible with a putative antioxidant, can affect cellular functions at different levels beyond their anti-oxidative activity [28].

Activation of PPAR \textgamma{} by phytochemicals is not new and PON1 hepatic expression was previously shown to be stimulated by polyphenols other than those present in pomegranate, such as quercetin [14,15] or resveratrol [13]. However, our present study expanded these findings to an additional class of polyphenols that are prominent in pomegranate juice, the elagitannins. Furthermore, in the current study we have elucidated a multi-step pathway of the proximal signaling by which PJ polyphenols can regulate PON1 gene transcription in hepatocytes. Our data show that PJ polyphenols-mediated stimulation of PON1 expression in hepatocytes involves cAMP-PKA-PPAR signaling cascade. Consequently, PPAR \textgamma{} acts to upregulate PON1 gene transcriptional activity, and PON1 release from the hepatocytes to the medium. We have shown that activation of this signaling cascade significantly increases PON1 mRNA expression. On the contrary, inhibition of any of the cellular factors involved in the above signaling cascade inhibits PJ polyphenols upregulation by PJ or by its polyphenols.

PJ polyphenols-mediated upregulation of PON1 resulted in an increased secretion of a biochemically active PON1 from the hepatocytes, as shown by inhibition of lipoproteins oxidation by the secreted PON1. Blocking PON1 catalytic activity by its heating or by a specific inhibitor, diminished the potency of the medium to protect the lipoproteins from oxidation. We thus conclude that the anti-atherogenic characteristics of PJ or its polyphenols are modulated, at least in part, via an upregulation of PON1 expression in hepatocytes, and its subsequent release in the medium. These effects are consistent with the beneficial cardiovascular protective effects of PJ.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.08.051.


