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Identification of biologically active δ-lactone eicosanoids as paraoxonase substrates

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ABSTRACT

The mammalian paraoxonases (PONs 1, 2 and 3) are a family of esterases that are highly conserved within and between species. They exhibit antioxidant and anti-inflammatory activities. However, their physiological function(s) and native substrates are uncertain. Previous structure-activity relationship studies demonstrate that PONs have a high specificity for lipophilic lactones, suggesting that such compounds may be representative of native substrates. This report describes the ability of PONs to hydrolyze two bioactive δ-lactones derived from arachidonic acid, 5,6-dihydroxy-eicosatrienoic acid lactone (5,6-DHTL) and cyclo-epoxycyclopentenone (cyclo-EC). Both lactones were very efficiently hydrolyzed by purified PON3. PON1 efficiently hydrolyzed 5,6-DHTL, but with a specific activity about 15-fold lower than PON3. 5,6-DHTL was a poor substrate for PON2. Cyclo-EC was a poor substrate for PON1 and not hydrolyzed by PON2. Studies with the PON inhibitor EDTA and a serine esterase inhibitor indicated that the PONs are the main contributors to hydrolysis of the lactones in human and mouse liver homogenates. Studies with homogenates from PON3 knockout mouse livers indicated that >80% of the 5,6-DHTL and cyclo-EC lactonase activities were attributed to PON3. The findings provide further insight into the structural requirements for PONs substrates and support the hypothesis that PONs, particularly PON1 and PON3, evolved to hydrolyze and regulate a class of lactone lipid mediators derived from polyunsaturated fatty acids.

1. Introduction

Mammalian paraoxonases (PONs) are a unique family of calcium-dependent esterases consisting of PONs 1, 2 and 3 [1]. All have antioxidative and anti-inflammatory properties; however, their physiological functions are uncertain [1,2]. The family is highly conserved within and between species [1,3]. The redundancy in structure within the family and their high level of conservation suggest native physiological substrates exist for which they evolved to hydrolyze and regulate. However, such substrates remain to be clearly identified.

PONs hydrolyze a range of esters including organophosphates, arylesters and lactones (internal cyclic esters) and exhibit overlapping, but also distinct, substrate specificities [2]. However, most PON substrates identified to date are not naturally occurring compounds and/or are inefficiently hydrolyzed and likely represent promiscuous activities of the enzymes. Native substrates for an enzyme would be expected to occur naturally *in vivo*, have biological activity and be efficiently metabolized by the enzyme. Comprehensive structure-activity relationship studies with PON1 suggested that its native activity is that of a lactonase [4]. Further structure-activity studies demonstrated that all three PONs share a high specificity towards lipophilic five and six membered ring

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; cyclo-EC, cyclo-epoxycyclopentenone; 5,6-DHTL, 5,6-dihydroxy-eicosatrienoic acid lactone; EC, epoxycyclopentenone; 5,6-EET, 5,6-epoxyeicosatrienoic acid; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phophocholine; GFP, green fluorescent protein; HBSS, Hanks Balance Salt Solution; HEK, human embryonic kidney 293; 5-HL, 5-hydroxy-eicosatetraenoic acid, 1,5-lactone; PECPC, 1-palmitoyl-2-(5,6-epoxyisoprostane A2)-sn-glycero-3-phosphocholine; PON, paraoxonase; PUFA, polyunsaturated fatty acid.

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lactones, i.e. γ- and δ-lactones [2,5]. Among such substrates, a γ- and δ-lactone derivative of docosahexaenoic acid and arachidonic acid, respectively, were exceptionally efficiently metabolized by the PONs. Enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids (PUFA) generates enormously diverse classes of lipid mediators including the classic eicosanoid families and continually emerging isoprostanes and prostanoid-like compounds [6]. Thus, the high specificity of PONs for lactone derivatives of docosahexaenoic acid and arachidonic acid suggest that lactone derivatives of oxidized PUFAs may be a subclass of lipid signaling molecules that are native endogenous PON substrates.

In this report we extend previous structure-activity relationship studies and characterize the ability of PON1, PON2 and PON3 to hydrolyze two δ-lactones derived from arachidonic acid, cyclo-epoxycyclopentenone (cyclo-EC) and 5,6-dihydroxy-eicosatrienoic acid lactone (5,6-DHTL; Fig. 1). Epoxycyclopentenone (EC) is the free fatty acid epoxyisoprostane component of 1-palmitoyl-2-(5,6-epoxyisoprostane A2)-sn-glycero-3-phophocholine (PECPC) that can be released by the action of phospholipase A_2 [7]. PECPC is a bioactive oxidized phospholipid generated by oxidation of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phophocholine (PAPC), typically under conditions of oxidative stress. Both PECPC and EC exhibit anti-inflammatory activity [8]. However, EC is more potent than PECPC, suggesting that EC is the bioactive component of PECPC. Because of the proximity of the carboxyl group to the 5,6-epoxide in EC, it can undergo a spontaneous intermolecular reaction (i.e., lactonization) to the δ-lactone, cyclo-EC, at physiological pH (Fig. 1) [9]. Both EC and cyclo-EC have potent anti-inflammatory activities *in vitro* and *in vivo*, with the potency of cyclo-EC exceeding that of EC [8].

5,6-Epoxyeicosatrienoic acid (5,6-EET) is a cytochrome P450 metabolite of arachidonic acid that is an agonist of transient receptor potential channels with pleiotropic biological actions. In an analogous manner to EC, 5,6-EET can spontaneously lactonize to 5,6-DHTL [10] (Fig. 1). Recently, PON1 was shown to hydrolyze 5,6-DHTL to its corresponding 5,6-dihydroxytrienoic acid [10]. In this same study 5,6-DHTL was detected in mouse kidney extracts, with higher levels in the extracts from PON1 knockout mice, compared to wild type mice. These findings suggest that 5,6-EET is converted *in vivo* to 5,6-DHTL and PONs may be important regulators of the lactone's activity or disposition.

This report demonstrates that cyclo-EC and 5,6-DHTL are substrates for the PON family, with PON3 being particularly efficient and PON2 exhibiting little to no activity with the lactones. Also, we provide evidence that PONs are the main enzymes contributing to hydrolysis of the lactones in human and mouse liver homogenates. The findings provide greater insight into the structural requirements for the PONs' substrates and support the hypothesis that PONs, particularly PON1 and PON3, have specialized to metabolize and regulate a subclass of lactone lipid mediators.

2. Materials and methods

2.1. Materials

(±)-5,6-DHTL and (±)-5-hydroxy-eicosatetraenoic acid, 1,5-lactone (5-HL) were from Cayman Chemical (Ann Arbor, MI). Cyclo-EC and cyclo-EC lactam were synthesized and purified as described [9,11]. 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was from Sigma-Aldrich (St. Louis, MO). Human embryonic kidney 293 (HEK) cells were from American Type Culture Collection (Manassas, VA). Stable HEK cells expressing recombinant human PON2-enhanced green fluorescent protein (GFP) and PON3-GFP constructs were prepared as described [12] and provided by Dr. Sven Horke (Johannes Gutenberg-Universität Mainz, Mainz, Germany). Pooled EDTA and PMSF free human liver S9 fractions were from BioIVT (Westbury, NY). Recombinant human PONs were expressed in *Trichopulsia ni* High Five insect cells and purified as described [2]. PON3 knockout mice were generated as described [13]. All other reagents were of analytical or cell culture grade from commercial sources.

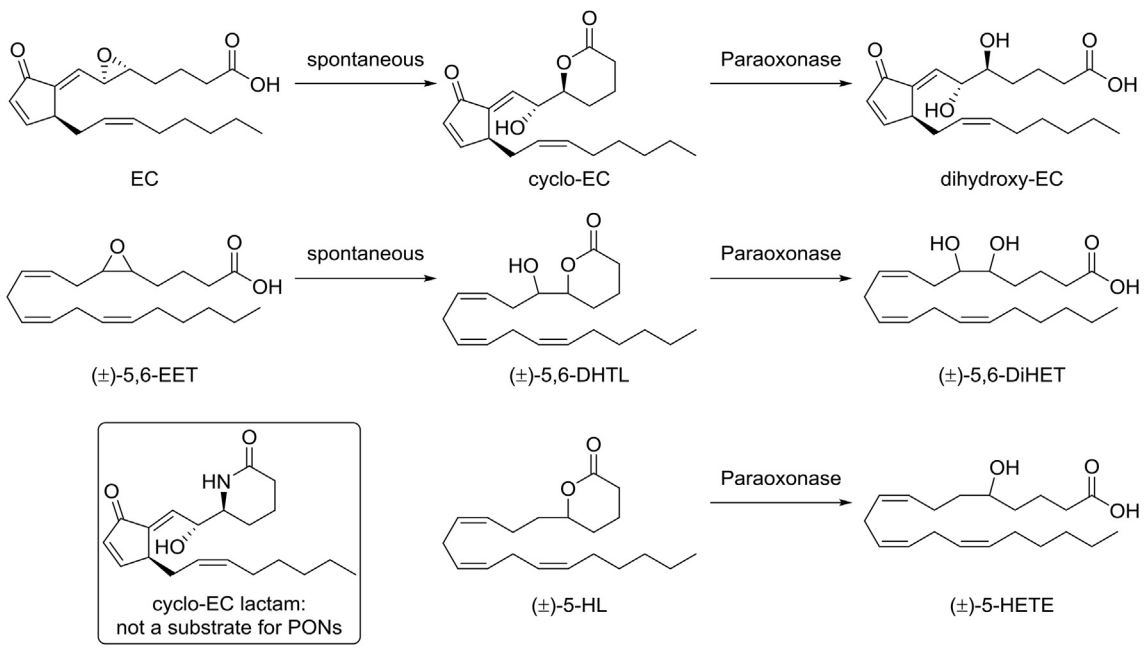

Fig. 1. Structures of lactones evaluated in this study. EC is the 5,6-epoxyisoprostane component of the oxidized phospholipid 1-palmitoyl-2-(5,6-epoxyisoprostane A2)-sn-glycero-3-phosphocholine released by hydrolysis at the sn2 ester linkage. Cyclo-EC lactam is the lactam analog of cyclo-EC that is not a PON substrate. (±)-5,6-EET is an epoxyeicosatrienoic acid formed by oxidation of arachidonic acid by cytochrome P450s. The 5,6-epoxides can spontaneously react to the corresponding δ-lactones. (±)-5 HL is the PON substrate previously characterized [2]. The δ-lactones are hydrolyzed by PONs to their corresponding hydroxy acids.

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