Free Radical Biology and Medicine 53 (2012) 305–313

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Facilitated cellular uptake and suppression of inducible nitric oxide synthase by a metabolite of maritime pine bark extract (Pycnogenol)

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ARTICLE INFO

Article history:
Received 9 November 2011
Received in revised form
1 April 2012
Accepted 13 April 2012
Available online 23 April 2012

Keywords:
RAW 264.7 macrophages
Human monocytes
EA.hy 926
Inducible nitric oxide synthase
Nitric oxide
Phloretin
Free radicals

ABSTRACT

Many natural products exhibit anti-inflammatory activity by suppressing excessive nitric oxide (NO) production by inducible NO synthase (iNOS). The maritime pine bark extract Pycnogenol has been formerly shown to decrease nitrite generation, taken as an index for NO, but so far it was not clear which constituent of the complex flavonoid mixture mediated this effect. The purpose of this study was to elucidate whether the in vivo generated Pycnogenol metabolite M1 (δ-(3,4-dihydroxyphenyl)-γ-valerolactone) displayed any activity in the context of induction of iNOS expression and excessive NO production. For the first time we show that M1 inhibited nitrite production (IC_{50} 1.3 μg/ml, 95% CI 0.96–1.70) and iNOS expression (IC_{50} 3.8 μg/ml, 95% CI 0.99–14.35) in a concentration-dependent fashion. This exemplifies bioactivation by metabolism because the M1 precursor molecule catechin is only weakly active. However, these effects required application of M1 in the low-micromolar range, which was not consistent with concentrations previously detected in human plasma samples after ingestion of maritime pine bark extract. Thus, we investigated a possible accumulation of M1 in cells and indeed observed high-capacity binding of this flavonoid metabolite to macrophages, monocytes, and endothelial cells. This binding was distinctly decreased in the presence of the influx inhibitor phloretin, suggesting the contribution of a facilitated M1 transport into cells. In fact, intracellular accumulation of M1 could explain why in vivo bioactivity can be observed with nanomolar plasma concentrations that typically fail to exhibit measurable activity in vitro.

Introduction

Many plant secondary metabolites exhibit some degree of biological activity in humans [1]. Various classes of phytochemical compounds exhibit anti-inflammatory effects by targeting key mediators of the complex inflammatory pathways [2,3]. Uncontrolled inflammatory processes often play a pivotal role in chronic or degenerative diseases such as arthritis, vascular disorders, or neurodegeneration. In the inflammatory network nitric oxide (NO) is an established effector mediating inflammatory cell damage. Whereas a low level of NO production is essential for maintaining homeostasis, excess generation of NO after de novo synthesis of inducible nitric oxide synthase (iNOS) is the hallmark of inflammatory disorders. Numerous plant extracts or extract constituents have been examined for their potential to inhibit NO production or suppression of iNOS expression. In this context, the most popular test system is the murine cell line of RAW 264.7 macrophages, which reliably express high levels of iNOS after activation with inflammatory agents [4]. Typically, the extent of nitrite production, taken as an index for NO, is determined in the cell culture supernatants. Indeed various phytochemicals decrease the nitrite generation in this test system. Regarding the concentrations for inhibiting nitrite production by 50% (IC_{50}) whole-plant extracts often require higher concentrations (≥ 50 μg/ml [5–7]) for the effect compared with defined single constituents. Among those defined single compounds many exhibit activity in the concentration range 10–30 μM, whereas only few have reported IC_{50} values below 10 μM [8,9].

Another natural product being widely used for inflammatory conditions is French maritime pine bark extract [10,11], which is monographed as a food supplement in the *United States Pharmacopoeia* [12]. A standardized bark extract that complies with this monograph is derived from of *Pinus pinaster* Ait. (Pycnogenol; Horphag Research Ltd., UK). Pycnogenol was formerly investigated for effects on nitrite production and iNOS expression and found to decrease nitrite generation in murine RAW 264.7 macrophages by 40% at a concentration of 100 μg/ml [13]. In a test system with renal tubular cells even lower concentrations of Pycnogenol (10 μg/ml) exhibited distinct effects on nitrite production and iNOS expression [14]. Recently it was reported that Pycnogenol attenuated induced iNOS gene expression and NO production in chondrocytes [15]. Maritime pine bark extract is

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.04.013

a complex mixture comprising 65%–75% procyanidins that consist of catechin and epicatechin subunits of varying chain lengths. Other constituents are flavonoids, phenolic or cinnamic acids, and their glycosides.

One important factor that needs consideration when the bioactivity of plant extracts is investigated is the bioavailability of their constituents [16]. Clearly, highly condensed procyanidins cannot be absorbed and thus cannot interact with target cells in vivo. We recently analyzed plasma samples of volunteers after oral intake of single and multiple doses of Pycnogenol and found catechin, ferulic acid, caffeic acid, and taxifolin in addition to 10 other yet unidentified compounds [17]. We also detected a pine bark extract metabolite in plasma samples, δ-(3,4-dihydroxyphenyl)-γ-valerolactone, or 5-[(3,4-dihydroxyphenyl)methyl]oxolan-2-one according to IUPAC nomenclature, which we named M1. This metabolite was also found in urine samples after ingestion of Pycnogenol [18]. Earlier we showed that M1 is a bioeffective compound displaying anti-inflammatory and antioxidant activity [19]. Notably, M1 is not a constituent of the extract, but it is generated in vivo from catechin units by gastrointestinal microbial activity [20–24] (Fig. 1). This metabolite, M1, has also been detected in human urine after ingestion of green tea [24].

The purpose of this study was to elucidate whether the Pycnogenol metabolite M1 displays any activity in the context of dysfunctional induction of iNOS expression and excessive NO production and might thus present an active principle contributing to the extract's anti-inflammatory effects. In this context we also sought to consider whether M1 concentrations detected in vivo would be consistent with M1 concentrations necessary to induce any effect in vitro. Therefore, we determined the disposition of M1 in the presence of cells that are present in the blood such as monocytes/macrophages and endothelial cells. Furthermore, we investigated the possibility of a facilitated uptake into cells by co-incubation of M1 with the influx inhibitor phloretin (Fig. 2). Phloretin, or dihydronaringenin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one), is the aglycon of phlorizin, which is a dietary constituent found in various fruit trees [25]. Phloretin inhibits various membrane transporters such as GLUT-1 glucose transporter [26], red blood cell urea [27], or monocarboxylate transporters [28].

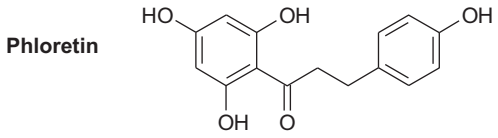

Fig. 2. Structural formula of phloretin (dihydronaringenin).

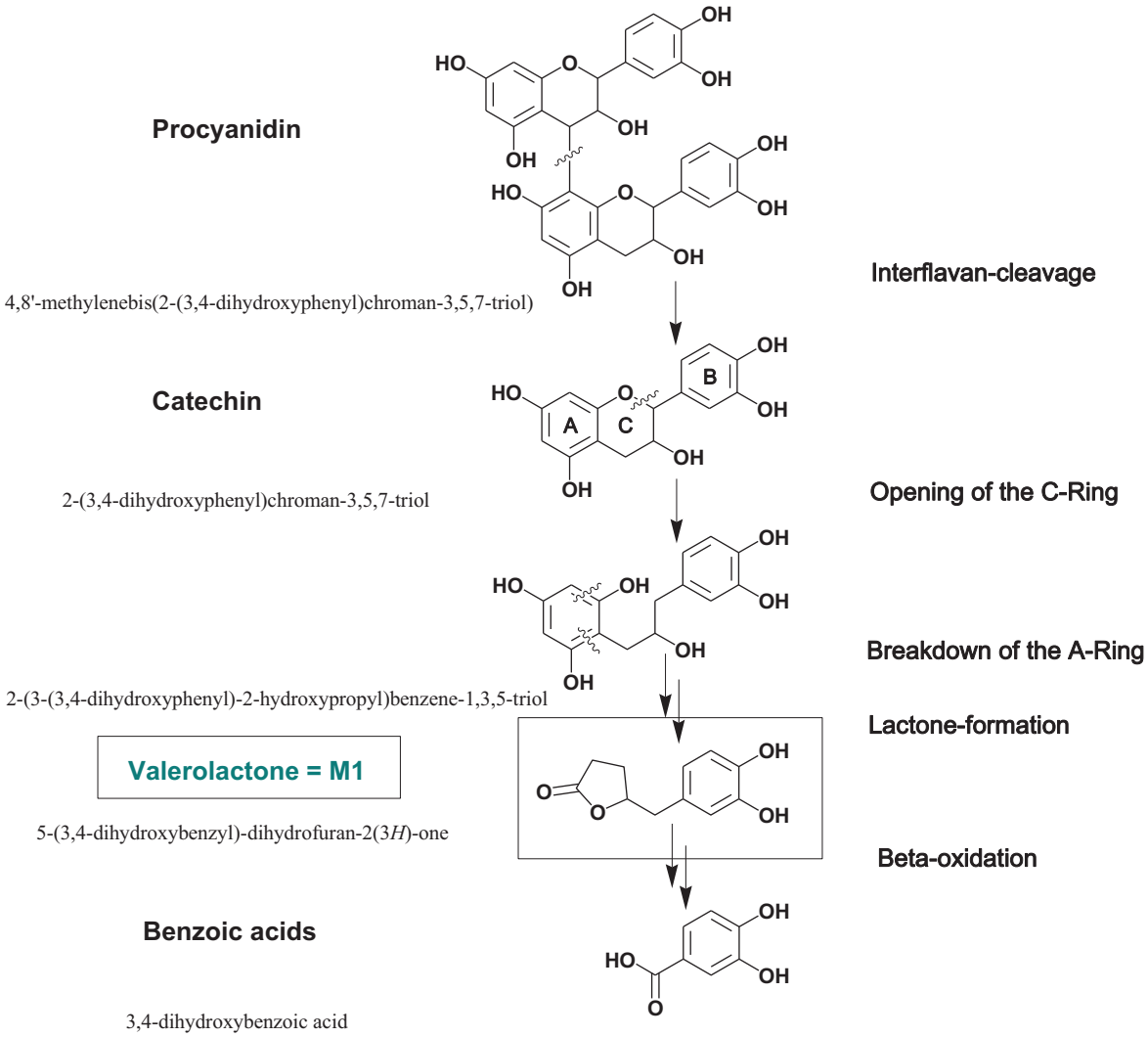

Fig. 1. Proposed pathway for formation of metabolite M1 from flavan-3-ol structures in vivo by metabolizing microbiota in the human colon (based on [23,24]).