The anti-inflammatory pharmacology of Pycnogenol® in humans involves COX-2 and 5-LOX mRNA expression in leukocytes

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1. Introduction

Inflammation is per se an important response of mammalians aiming to counter a wide spectrum of potentially harmful stimuli and recover a homeostatic condition compatible with normal cellular activities and functions. Similarly to any kind of physiological response, inflammation requires a delicate and tight feedback regulation in order to avoid the consequences of a continuous activation which can eventually lead to chronic inflammatory condition, associated with tissue injury and dysfunction. Many aspects of inflammation are mediated or modulated by the mediators arising from the cyclooxygenase (COX) and lipoxygenase (LOX) cascade [1]. The cascade initiates with the release of arachidonic acid (AA) by phospholipase A2 (PLA2) action; while the final products are prostaglandins (PGs), thromboxanes (TXB) and leukotrienes (LTs) that are the members of a group of biologically active oxygenated fatty acids, named eicosanoids. PGs are potent vasodilators which account for the increased blood flow in inflamed areas. On the other hand, TXB acts as a potent vasoconstrictor and a stimulator of platelet aggregation [2]. LTs (LTB4 and cysteinyl-LT (cys-LT) play a multifaceted role within the inflammatory process. They are important modulator of pain in inflammation and immunomodulatory agents. LTB4 is a chemotaxtractant for leukocytes and may initiate the monocytes recruitment. Cys-LT increase vascular permeability, dilate blood vessels, contract non-vascular smooth muscle, and cause pain [3]. LTs have been recognized to play an important role in different chronic inflammatory disease such as asthma, allergic rhinitis, colitis, dermatitis rheumatoid arthritis and septic peritonitis [3,4].

Plant phenolic compounds have attracted growing interest as owing advantageous health effects. Pycnogenol® (PYC) is a patented standardised extract from the bark of the French maritime pine, Pinus pinaster (Horphag Research, Ltd, Geneva, Switzerland), mainly composed of procyanidins and flavonoid monomers and phenolic or cinnamic acids and their glycosides as minor constituents. About 65–75% of Pycnogenol components are procyanidins made of catechin and epicatechin subunits of varying chain lengths [5,6]. In addition to antioxidant activities resulting from hydroxyl groups of the polyphenol components. Pycnogenol has also been shown to exert a variety of biological activities [7,8] and in particular it has been reported to exert anti-inflammatory effects. Pycnogenol metabolites contained in plasma of human volunteers after ingestion have been shown to inhibit COX-1 and COX-2 enzyme activity, in vitro [9]. Moreover, two studies have suggested Pycnogenol as a valuable candidate tool in the management of asthma. A double-blind placebo controlled crossover study performed on 22 subjects suffering of...
asthma, showed that 4 weeks Pycnogenol supplementation induced a significant recovery of symptoms. The improvement of airway function paralleled the reduction of LTs levels in the blood [10]. More recently, a randomized, placebo controlled, double-blind study showed that urinary cyst-LTs were significantly reduced after PYC supplementation [11]. However, the molecular basis of Pycnogenol activity on LTs biosynthesis is still scarcely known limiting the utilization of this nutritional supplement as a possible dietary tool in the management of mild chronic inflammatory diseases such as asthma. Therefore, the purpose of this study was to investigate the molecular basis of the anti-inflammatory effect of Pycnogenol supplementation in activated human polymorphonuclear leukocytes (PMNL).

2. Materials and methods

2.1. Experimental model

6 healthy volunteers (35–50 years) participated to this study which was approved by the Ethical Committee of the National Institute for Food and Nutrition Research. The investigation conforms with the principles outlined in the Declaration of Helsinki. All subjects underwent a flavonoid free diet for 12 h and then referred to the Institute for blood withdrawal to obtain PMNL and plasma in basal condition. All subjects were supplemented for 5 days with 150 mg tablet per day of Pycnogenol® [9,12]. The supplementation was under conditions of the experimental protocol was decided in order to have a combination steady state and acute effect and on the basis of previous studies indicating that after 5 days a steady state level of circulating constituents is reached and that polyphenol absorption peaks at about 60 min from ingestion [13]. On each day of blood isolation, PMNL were isolated and activated. PMNL (1×10⁷/ml) were resuspended in RPMI 1640 w/o phenol red plus 10% autologous plasma and primed with 1 µg/ml lipopolysaccharides (LPS) for 30 min at 37 °C. At the end of LPS priming, PMNL were centrifuged, resuspended in RPMI 1640 w/o phenol red plus 10% autologous plasma and stimulated by adding 0.1 µM formyl-methionyl-leucyl-phenylalanine (fMLP), an agonist mimicking the structure of bacterial wall peptides. Priming of human granulocytes with LPS enhances the biosynthesis of leukotrienes in response to soluble agonist like fMLP, due to a transient increase in the potential for both arachidonic acid (AA) mobilization via cPLA2 (cytoplasmatic PLA2) and activation of 5-LOX [14]. Cells were incubated in the presence of fMLP for 15 s, 1.5 and 5 min. At the end of the incubations, the medium was collected and stored at −80 °C for LT, PG and TXB measurement by ELISA. Cells were pelleted and treated for RNA or protein isolation. The results obtained with PMNL isolated after Pycnogenol® supplementation were compared with those obtained with PMNL isolated in fasting condition in each subject.

2.2. PMNL isolation

PMNL were isolated from blood as previously described [15]. Briefly, venous blood was obtained from healthy volunteers using heparin as anticoagulant. Blood was centrifuged (250 × g for 20 min) and plasma separated for further uses. Erythrocytes were removed by dextran sedimentation. The leukocyte rich supernatant was centrifuged and the remaining erythrocytes were removed by hypotonic lysis. Cells were layered over Ficoll-Paque and centrifuged to separate mononuclear cells from PMNL. The PMNL rich pellet was recovered and washed in PBS/EDTA. PMNL preparation was checked by trypan blue dye exclusion analysis for cell viability and purity. Neutrophils are the most representative cell population of PMNL (>95%) as assessed by May-Grunwald Giemsa staining.

2.3. Real time PCR

RNA was isolated using RNeasy Mini kit (QIAGEN) according to the manufacturer protocol. The quantification of gene expression was determined by real-time quantitative PCR with the ABI PRISM® 7900 HT Instrument (Applied Biosystem, Foster City CA, USA) using the Sybr green JumpStart™ Taq Ready Mix kit (SIGMA). The oligonucleotide used for PCR studies are listed in Table 1. Data were collected and processed with SDS2.2 software and given as threshold cycle (Ct), C values for each target and reference genes were obtained and their difference was calculated (ΔCt). Primer efficiencies for the test genes and β-actin (reference gene) were similar. Transforming Ct values to absolute value considering the efficiency of the primers of target genes (2−ΔCt) was the last step in the quantification of gene expression. The results are expressed as arbitrary units, normalized for β-actin level.

Table 1

<table>
<thead>
<tr>
<th>Primer design for quantitative PCR analysis.</th>
<th>Primers</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>Primers</td>
</tr>
<tr>
<td>5-LOX</td>
<td>FW:5′TGCTGAGGCAACAGAAGA</td>
</tr>
<tr>
<td>FLAP</td>
<td>FW:5′GCCAGGTCGACAAGATGC</td>
</tr>
<tr>
<td>COX-2</td>
<td>FW:5′CTCTGCTGACAGACTTTC</td>
</tr>
<tr>
<td>COX-1</td>
<td>FW:5′CTGCTGACAGACTTTC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>FW:5′AGAAGAATTCCTGCTGCGG</td>
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5-LOX, 5-lipoxygenase; FLAP, five lipoxygenase activating protein; COX-2, cyclooxygenase-2; COX-1, cyclooxygenase-1.

Fig. 1. Effect of fMLP agonist stimulation on 5-LOX and FLAP gene expression in PMNL isolated before (A) and after (B) Pycnogenol® supplementation. Before fMLP activation cells were primed with LPS for 30 min at 37 °C. Data are expressed as fold of changes with respect to control. Control is gene expression before any treatments. *P<0.05 compared to PMNL isolated before Pycnogenol® supplementation.
2.4. LTBI PGE2 and TXB assay

LTBI, PGE2 and TXB levels in the supernatant of PMNL challenged with inflammatory stimuli was measured using EIA kit (CAYMAN chemical).

2.5. PLA2 activity

Cells, pelleted after all treatments, were homogenized in buffer containing 50 mM HEPES pH 7.4, EDTA 1 mM and a protease inhibitor mix. PLA2 activity was assessed by using arachidonoyl thio-PC (CAYMAN chemicals) as a synthetic substrate. Free thiols released by PLA2 after hydrolysis of the arachidonoyl thioester bond in the sn-2 position was detected by DTNB (Elmann’s reagent) at 415 nm.

2.6. Statistical analysis

Data are expressed as mean±SEM. Statistical analysis of the data was performed by using paired t-test. Data are normalized to the results obtained with the untreated cells isolated before and after Pycnogenol® supplementation. A P<0.05 was considered significant.

3. Results

3.1. Kinetic of 5-LOX and FLAP mRNA expression after LPS-fMLP stimulation

In PMNL collected in fasting conditions before Pycnogenol® supplementation, LPS priming (30 min at 37 °C) determines an increase of 5-LOX expression (about 2 times of untreated control cells). After cell priming, fMLP stimulation induced a further increase of 5-LOX mRNA transcription at 3 min of incubation (Fig. 1, panel A). The increase of 5-LOX mRNA expression is associated with a decrease of FLAP mRNA levels at all the experimental time points. Interestingly, following 5 days of Pycnogenol® supplementation an inhibition of the LPS-fMLP induced up-regulation of 5-LOX was found (Fig. 1, panel B) and this coincided with a lack of down-regulation of FLAP gene expression.

3.2. Kinetic of LTBI generation after LPS-fMLP stimulation

The release of LTBI from PMNL obtained from donors before Pycnogenol® administration began to increase 15 s after fMLP stimulation (about 40% of increase compared to untreated control cells). The highest level of LTBI production was measured 5 min after fMLP stimulation. As expected from the inhibitory effect of Pycnog

3.4. Kinetic of PMNL COX-2 transcription activation subsequent to LPS-fMLP stimulation

COX-2 expression is typically inducible and its mRNA has a relatively short half life compared to COX-1. After, PMNL priming with LPS, fMLP stimulation induced a 3-fold increase of COX-2 mRNA expression after 3 min of incubation in baseline PMNL. Pycnogenol® supplementation was associated with a significant down-regulation of LPS-fMLP induced COX-2 gene expression at any time points (Fig. 4).
biosynthesis and the release of AA, following cell activation, depends on PLA2 activation. In PMNL isolated in fasting condition, before Pycnogenol® administration, PMNL priming with LPS and the following stimulation with fMLP was associated with an increased PLA2 activity (Fig. 7). Pycnogenol® supplementation for 5 days inhibits the increased PLA2 activity induced by LPS priming and fMLP treatment in isolated PMNL.

4. Discussion

Human neutrophils play an important role in inflammation; they are attracted to the inflammation loci and release inflammatory mediators, such as lysosomal enzymes, AA derivatives, cytokines, metabolites, and toxic oxygen derivatives. Many aspects of inflammation are mediated or modulated by the upstream mediators arising from the COX and LOX cascade. The generation of free AA, is the rate limiting step in leukotriene, thromboxane and prostaglandin synthesis; such as mast cells, eosinophils or endothelial cells to form LTC4, the first product of the cys-LTs [21]. The evidence for the presence of LTs in disease development was often obtained from the detections of these mediators in inflammatory exudates and further supported by the efficacy of LT antagonists for abolishing or reducing the inflammatory condition [22]. Pycnogenol® has been demonstrated to possess a variety of anti-inflammatory effects. In asthmatic patients, Pycnogenol® was found to significantly lower leukotriene levels [10,11]. The lowered leukotriene levels in asthmatic patients may, however, have been secondary effects. To date there is no data available suggesting a pharmacologic effect of Pycnogenol® for inhibition of the AA pathway on a molecular level. Our experiments show that LPS-fMLP stimulation of PMNL, obtained from healthy fasting volunteers before Pycnogenol® administration, up-regulate 5-LOX and down-regulate FLAP mRNA transcription. These observations are in agreement with previous reports of increased levels of FLAP mRNA generally overlapping a decrease of 5-LOX and vice versa [23]. 5-LOX and FLAP co-regulated expression determine the increase of LTB4 production. Pycnogenol® supplementation suspended the

3.5. Kinetic of PMNL TXB2 generation after LPS-fMLP treatment

The increase of COX-1 and COX-2 gene expression in PMNL collected before Pycnogenol® administration to the donors was associated with the increase of TXB2 synthesis and release (+40% of untreated cells) into the culture supernatant (Fig. 5). Pycnogenol® supplementation lead to the inhibition of TXB2 release during the first 2 min after fMLP stimulation. However, 5 min after stimulation the amount of TXB2 in the supernatant was higher than that found in LPS-fMLP treated PMNL, isolated before Pycnogenol® supplementation.

3.6. Kinetic of PMNL PGE2 generation after LPS-fMLP treatment

As shown in Fig. 6, PGE2 production increases after LPS-fMLP stimulation in PMNL collected before Pycnogenol® supplementation. In fact, PGE2 levels were 50% higher than in untreated control cells, at 5 min after stimulation. Moreover, PGE2 production doubled compared to baseline values (207%) 30 min after fMLP stimulation (data not shown). Pycnogenol® supplementation was associated with a 3-fold increase of PGE2 release with a peak of production 2 min after fMLP stimulation. The decrease of PGE2 release after 5 min could be the result of a dynamic balance between inside and outside of the cells [17].

3.7. Kinetic of PMNL PLA2 after LPS-fMLP treatment

The availability of AA, the substrate of 5-LOX and COX enzymes, is the rate limiting step in leukotriene, thromboxane and prostaglandin biosynthesis and the release of AA, following cell activation, depends on PLA2 activation. In PMNL isolated in fasting condition, before Pycnogenol® administration, PMNL priming with LPS and the following stimulation with fMLP was associated with an increased PLA2 activity (Fig. 7). Pycnogenol® supplementation for 5 days inhibits the increased PLA2 activity induced by LPS priming and fMLP treatment in isolated PMNL.

Fig. 5. Effect of fMLP agonist stimulation on TXB2 release in PMNL isolated before and after Pycnogenol® supplementation. Before fMLP activation cells were primed with LPS for 30 min at 37 °C. Data are expressed as % compared with control. Control is TXB2 released in the supernatant of PMNL before any treatments. Pre-PYC are PMNL isolated before Pycnogenol® supplementation, post-PYC are PMNL isolated after Pycnogenol® supplementation. ★P<0.05 compared to pre-PYC PMNL.

Fig. 6. Effect of fMLP agonist stimulation on PGE2 release in PMNL isolated before and after Pycnogenol® supplementation. Before fMLP activation cells were primed with LPS for 30 min at 37 °C. Data are expressed as % compared with control. Control is PGE2 released in the supernatant of PMNL before any treatments. Pre-PYC are PMNL isolated before Pycnogenol® supplementation, post-PYC are PMNL isolated after Pycnogenol® supplementation. ★P<0.05 compared to pre-PYC PMNL.

Fig. 7. Effect of fMLP agonist stimulation on PLA2 enzyme activity in PMNL isolated before and after Pycnogenol® supplementation. Before fMLP activation cells were primed with LPS for 30 min at 37 °C. Data are expressed as % compared with control. Control is PLA2 activity before any treatments. Pre-PYC are PMNL isolated before Pycnogenol® supplementation, post-PYC are PMNL isolated after Pycnogenol® supplementation. ★P<0.05 compared to pre-PYC PMNL.
interdependency between 5-LOX and FLAP mRNA expression. Therefore, Pycnogenol® supplementation appears to be associated with a different regulation of 5-LOX and FLAP expression in turn leading to a decreased LTβ4 production as opposed to stimulated PMNL collected before Pycnogenol® supplementation.

COX-1 enzyme is ubiquitously expressed and is responsible for the production of PGs involved in the maintenance of homeostatic functions. In contrast, COX-2 is usually induced in response to inflammatory stimuli [24]. Nevertheless, it has been described that COX-1 can be regulated during development or by specific hormones and growth factors. COX-2 is constitutively expressed in specific tissues such as the brain, reproductive organs, kidney and thymus [25]. Moreover, available data also suggest that COX-1 may be induced at the site of inflammation [26].

Our results show that LPS priming and FMLP treatment induce mRNA expression of both COX-1 and COX-2 in PMNL collected before Pycnogenol® administration to blood donors. The highest levels of COX-1 were induced 15 s after FMLP stimulation, while the highest COX-2 expression was observed 3 min after FMLP stimulation. Pycnogenol® supplementation was associated with a down-regulation of COX-2 transcription and with a significantly higher expression of COX-1 compared to pre-PIC-stimulated PMNL.

Moreover, our results indicate that the increase of PGE2 and TXB2 release, in PMNL collected after Pycnogenol® oral administration is the result of the increase of COX-1 mRNA levels. In fact Pycnogenol® supplementation is associated with a lack of induction of COX-2, mRNA and baseline protein concentration is obviously absent.

A functional coupling between brain cPLA2 and COX-2 was suggested by 50–60% decrease of COX-2 mRNA and protein levels in cPLA2 knockout mice [27]. Our data show a decrease of PLA2 activity in stimulated PMNL obtained after Pycnogenol® supplementation, NF-κB transcription factor has been reported to act as transcriptional regulator of both PLA2 and COX-2 but also of 5-LOX expression [28–30]. We can, therefore, hypothesize that Pycnogenol® supplementation is associated to a decrease of NF-κB-mediated response triggered by the inflammatory stimuli, correlated to the observed lack of induction of 5-LOX and COX-2 expression and possibly to the decrease of PLA2 activity. These findings are associated with a compensatory up-regulation of COX-1 expression [31]. This hypothesis is supported by data indicating a Pycnogenol® dependent inhibition of NF-κB in endothelial cells and macrophages, in inflammatory condition [12,32].

Our results indicate that Pycnogenol® supplementation in humans is able to modulate the AA cascade, blocking COX-2 and 5-LOX pathways and leading to a shift of the metabolic pathways in favour of COX-1 activity. Future studies should elucidate the molecular effects of Pycnogenol® on the translation of COX and LOX enzymes in myeloid cell lines in response to pro-inflammatory stimuli. This would help to better understand the kinetics of prostaglandin and leukotriene synthesis involved in the anti-inflammatory activity of Pycnogenol®.

Acknowledgment

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References