Procyanidins extracted from pine bark protect α-tocopherol in ECV 304 endothelial cells challenged by activated RAW 264.7 macrophages: role of nitric oxide and peroxynitrite

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Abstract A co-culture system was used to study the effect of reactive nitrogen species (RNS) generated by RAW 264.7 macrophages grown on filters and activated by lipopolysaccharide and interferon-γ, on the α-tocopherol levels in ECV 302 endothelial cells. The results indicate that: RNS generated by activated macrophages or by direct administration of peroxynitrite lead to a significant loss of α-tocopherol in endothelial cells; pre-incubation with procyanidin extracted from pine bark (Pycnogenol) protects α-tocopherol of endothelial cells and enhances by about 15% basal endogenous levels of α-tocopherol. These results demonstrate flavonoids participate in the cellular antioxidant network and suggest that Pycnogenol may play an important role in the protection of endothelium from oxidative stress induced by reactive nitrogen species.

Key words: Reactive nitrogen species; Endothelial cell; Activated macrophage; α-Tocopherol; Procyanidin; Pycnogenol

1. Introduction

It is widely accepted that endothelial cell dysfunction is associated with an alteration in the cell redox status [1]. Vitamin E and in particular α-tocopherol is an essential component of the cellular antioxidant network which has been reported to exert a protective activity against cardiovascular diseases [2]. This effect is due either to its chain-breaking antioxidant activity in the lipid component of the cell, or to a specific regulatory function toward key enzymes involved in cell growth and differentiation [3].

When α-tocopherol levels are challenged by reactive oxygen species (ROS) and reactive nitrogen species (RNS), e.g. nitric oxide (NO) or peroxynitrite ($ONOO^-$), produced during pro-oxidative conditions such as in an inflammatory response, the whole cellular antioxidant network participates in maintaining the redox homeostasis compatible with cell functions [4].

Many plant phenolics and in particular some flavonoids possessing a strong antioxidant activity may also participate in antioxidant cell defenses either by efficiently scavenging both ROS and RNS, or by recycling ascorbate [5], and therefore may exert an α-tocopherol 'sparing effect' [6].

Different plant extracts have been used in the traditional medicine as remedies for circulatory diseases. Pycnogenol is the proprietary extract from the bark of *Pinus marittima* composed from phenolic molecules, broadly divided into monomers (catechin, epicatechin and taxifolin) and mainly condensed flavonoids classified as procyanidins/pro-anthocyanidins (oligomeric procyanidins). Pycnogenol also contains phenolic acids (such as caffeic, ferulic and *p*-hydroxybenzoic acids) as minor constituents and glycosylation products, i.e. glucopyranose derivatives of either flavanols or phenolic acids as lesser constituents [7]. Pycnogenol has been reported to enhance the antioxidant capacity of cultured endothelial cells by specifically increasing enzyme expression [8] and to protect them from organic peroxide-induced oxidative stress [8,9]. Further it has been demonstrated that Pycnogenol is a powerful ROS and RNS scavenger and it is able to modulate NO metabolism in activated macrophages [10].

Macrophages are the principal inflammatory cell type found in the atheromatous plaque microenvironment [11]. They interact with endothelial cells, smooth muscle cells and lymphocytes through a wide spectrum of different sensor and signal transducers between the endothelium and circulating blood cells. Activated macrophages produce massive amounts of ROS and RNS, generating a pro-oxidant environment which in turn further triggers cell responses through activation of the NF-κB transcription factor inducing a circular mechanism finally leading to endothelial cell dysfunction [12].

In this study we have utilized a novel co-culture system comprised of activated macrophages and endothelial cells separated by a filter to study, after macrophage activation the effect of a long term, sustained production of NO, at physiological concentrations, on α-tocopherol levels in the endothelial cells. The protective effect on α-tocopherol level by the standardized procyanidins-containing extract from the pine bark (Pycnogenol) in endothelial cells challenged either by activated macrophages or by direct addition of $ONOO^-$ was also investigated.

2. Materials and methods

2.1. Cell culture and treatments

ECV 304 and RAW 264.7 (obtained from the American Type Culture Collection, Rockville, MD, USA) were maintained at 37°C in 5% CO_2, and were grown and passaged according to standard protocols.

ECV 304 cells (human umbilical cord endothelial cell), are a spontaneously transformed immortal endothelial cell line established from an apparently normal human umbilical cord. Cells were grown up to 90–100% confluence in 6-well tissues culture plates (Falcon, Franklin Lakes, NJ, USA), in 199 medium supplemented with 10% fetal calf serum (FCS) (UCSF cell culture facilities) and streptomycin-penicillin solution. α-Tocopherol concentration of the medium was adjusted to 20 μM by adding a concentrated D-α-tocopherol solution dissolved in ethanol. The final ethanol concentration in the medium was less than 0.1%.

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RAW 264.7 cells, a murine line of monocyte-macrophages, were seeded on 0.4 μm pore size cell culture inserts (Falcon) and cultured in D-MEM containing 10% FCS and antibiotics. RAW 264.7 were activated with lipopolysaccharide (LPS) and γ-interferon (IFN-γ) (Genzyme, Cambridge, MA, USA) at 25 ng/ml and 10 U/ml respectively. After 1 h macrophages were transferred on the top of ECV wells in direct contact with 199 medium, and then co-cultured for 24 h at 37°C.

Pycnogenol (Horphag Research Ltd., Guernsey) was dissolved in dimethyl sulfoxide (DMSO) and added to ECV 304 culture medium at the indicated concentrations. Cells were incubated in the presence of Pycnogenol for at least 16 h and the treatment was not removed unless indicated. The final DMSO concentration in the medium was less than 0.1%. Control cells (no Pycnogenol added) were treated with identical concentrations of DMSO.

ECV 304 cells were treated with 250 μM $ONOO^-$ (Alexis, San Diego, CA, USA) in PBS for 2 h at 37°C after removing the medium. The excess of H_2O_2 was removed by passing through a manganese dioxide column. Actual $ONOO^-$ concentration was assessed by measuring the absorbance at 302 nm in 1 N NaOH. As negative controls, treatments with $ONOO^-$ were performed in parallel with a decomposed form of $ONOO^-$ prepared from the same stock as the active form and containing the same concentrations of nitrite and salts as negative controls.

All other chemicals were purchased from Sigma (St. Louis, MO, USA). HPLC reagents were from Fisher (Fair Lawn, NJ, USA).

2.2. Assessment of nitric oxide production and α-tocopherol measurements

NO concentration in both ECV 304 and RAW 264.7 media was assessed at the end of the co-culture, after the filters containing RAW 264.7 cells were removed, as NO_2^- and total nitroso compound (NOx) i.e. NO_3^- plus NO_2^-, using the Griess reagent as described previously [13].

α-Tocopherol and cholesterol in ECV 304 cells were simultaneously measured by HPLC coupled with EC and UV detectors after hexane extraction of the cell lysate as described [14] and α-tocopherol concentrations were normalized to cholesterol. Cholesterol levels were observed to be not affected by either Pycnogenol or macrophages treatment (data not shown).

2.3. Statistics and data presentation

Data in histograms are presented as a mean ± S.D. of at least three separate experiments performed in triplicate. Difference between treatments were analyzed by ANOVA and the minimum level of significance was defined at $P < 0.05$.

3. Results

3.1. NO generation by activated macrophages

LPS and IFN-γ induce the expression of the inducible form of nitric oxide synthase (iNOS). A significant amount of NO is produced over a long term. NO spontaneously oxidizes generating both nitrite (NO_2^-) and nitrate (NO_3^-). Moreover, when macrophages are stimulated the enzymatic machinery producing $O_2^{-\bullet}$ is also activated, eventually resulting in the formation of $ONOO^-$, which finally decomposes into NO_3^-. Therefore, the measurement of NO_2^- and total nitro compounds (NOx), i.e. NO_3^- plus NO_2^- in the medium following cell activation, is an index of both NO generation and NO reaction with superoxide.

NO_2^- and NO_3^- are accumulated in the medium after RAW 264.7 macrophages stimulation with LPS and IFN-γ (see Fig. 1). Non-activated RAW 264.7 cells produce only low levels of NO_2^- and NO_3^- but 24 h after treatment of cells with LPS and IFN-γ, the levels in the culture medium increased dramatically from 2.5 ± 0.3 to 56.4 ± 1.2 and from 7.3 ± 0.9 to 138.2 ± 3.4 μM respectively. Pycnogenol alone had no effect on the basal production of both NO_2^- and NO_3^-.

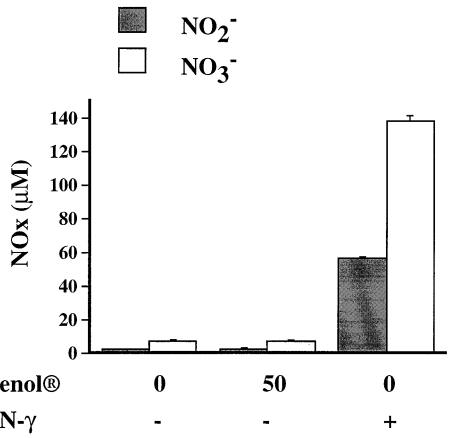

Fig. 1. NO_2^- and NO_3^- production by RAW 267.4 monocyte-macrophages cells after LPS and IFN-γ treatment. Bars represent the mean ± S.D. of at least three different experiments performed in triplicate.

3.2. Effect of the co-culture of activated RAW 264.7 macrophage and ECV 304 endothelial cells on the α-tocopherol levels in ECV 304 cells

The simultaneous production of ROS and RNS by activated macrophages induces a pro-oxidant environment which challenges the antioxidant system of ECV 304 endothelial cells. After 24 h of exposure to activated RAW 267.4 macrophages, α-tocopherol level of control ECV 304 cells (no pretreatment with Pycnogenol) was significantly lower in comparison to cells which were co-cultured with non-activated macrophages (Fig. 2). Co-culture with non-activated RAW 264.7 did not affect the α-tocopherol levels in ECV 304 endothelial cells (data not shown).

The pre-incubation of ECV 304 endothelial cells with Pycnogenol was associated with a significant protection from α-tocopherol loss. Treatment with 10 μg/ml Pycnogenol was associated with significantly higher levels of α-tocopherol,

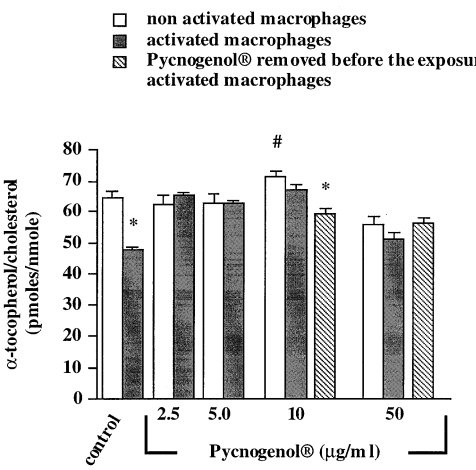

Fig. 2. α-Tocopherol levels and effect of 24 h preincubation with Pycnogenol in ECV 304 endothelial cells after the exposure to RAW 264.7 macrophages activated by LPS and IFN-γ. Bars represent the mean ± S.D. of at least three different experiments performed in triplicate. *Significant from 'non-activated RAW' within the same pre-treatment. Significant from control cells.

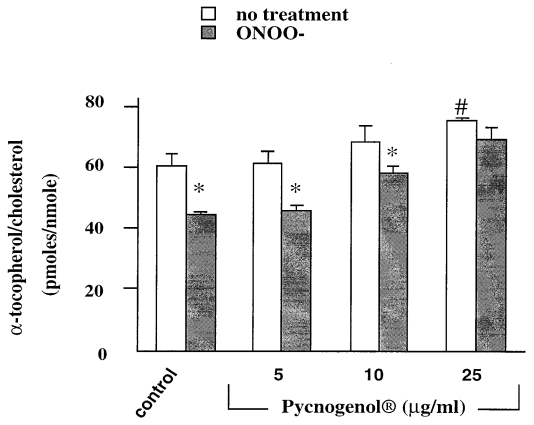

Fig. 3. α-Tocopherol levels in ECV 304 endothelial cells after 2 h exposure to 250 μM $ONOO^-$ and effect of 24 h preincubation with Pycnogenol. Bars represent the mean ± S.D. of at least three different experiments performed in triplicate. *Significant from 'no $ONOO^-$ treatment' within the same pre-treatment. Significant from control cells.

even in the absence of the exposure to RNS and ROS, indicating that procyanidins from pine bark affected cellular redox status in the basal condition. However, higher concentrations of Pycnogenol in the medium (50 μg/ml) were associated both with lower levels of α-tocopherol and with a less evident protection.

When Pycnogenol was removed from the medium before the exposure to macrophages, a lesser protective effect was observed, suggesting that some interaction between oxidizing species and flavonoids occurred in the medium. At the highest concentration tested, the removal of Pycnogenol from the medium, was not associated with any significant difference in α-tocopherol levels in EVC 302 endothelial cells after the exposure to activated RAW 264.7 macrophages.

3.3. Protective activity of Pycnogenol on $ONOO^-$ induced α-tocopherol loss in ECV endothelial cells

Due to the simultaneous production of NO and superoxide ($O_2^{-\bullet}$) in the course of macrophage activation, the generation of $ONOO^-$ is likely to occur. $ONOO^-$ is a more reactive pro-oxidant species than NO and $O_2^{-\bullet}$ radicals, and it has been proposed as a causative factor in cell injury. In order to assess if Pycnogenol could protect α-tocopherol from the direct exposure to $ONOO^-$, ECV 304 endothelial cells were incubated for 2 h in the presence of 250 μM $ONOO^-$.

Following incubation with $ONOO^-$, α-tocopherol levels of ECV 304 cells were significantly affected(Fig. 3). A loss of about 30% of the baseline α-tocopherol value was observed in control cells. As in the previously described experiments with activated macrophages, pre-incubation with 10 μg/ml Pycnogenol was associated both with higher baseline α-tocopherol levels and with protection of α-tocopherol after treatment with 250 μM $ONOO^-$. At 25 μg/ml Pycnogenol in the pre-incubation medium, α-tocopherol loss following $ONOO^-$ treatment was not significant. It is important that $ONOO^-$ was administered to ECV 304 cells in PBS, after washing away the culture medium, therefore eliminating the possibility of a direct interaction between $ONOO^-$ and Pycnogenol outside the cellular environment.

4. Discussion

In this study a novel co-culture system was utilized to demonstrate that macrophage activation, such as occurs during inflammatory processes, challenges endothelial cell antioxidant status, as indicated by a significant decrease of α-tocopherol levels. This model was characterized by a long-term, sustained generation of high levels of RNS, thus mimicking the occurrence of a mild 'physiological' oxidative stress. The combination of two non-homo-specific cell lines in the experimental design (RAW 264.7, a murine derived cell line, with ECV 304 endothelial cells of human origin) was chosen due to the need of avoiding the activation of endothelial cells through the species-specific cytokine utilized for macrophage activation. No effect was observed either on α-tocopherol levels or other functional cell parameters when ECV 304 cells were exposed to the medium utilized for RAW 264.7 culture after activation with LPS and IFN-γ.

We have previously demonstrated that Pycnogenol affects NO metabolism in the same monocyte-macrophage cell line utilized here for the co-culture. In the present study RAW 264.7 macrophages were in contact with Pycnogenol only after activation with LPS and IFN-γ, and in this condition no effect on NO_2^- or NO_3^- production was observed [10].

This study demonstrates that the generation of relatively high amounts of RNS by activated macrophages induces oxidative stress sufficient to alter the cellular antioxidant levels. These results help to clarify the complex role of NO in the endothelium, where it can act either as antioxidant [15] or as pro-oxidant as suggested by the results reported herein and previously [16,17], depending on the relative concentrations between NO and ROS such as $O_2^{-\bullet}$. Alterations in endothelial antioxidant levels eventually affect the cellular functions which, at least in part, are regulated by cellular redox status such as the expression of NF-κB. This in turn plays a key role in endothelial dysfunction and in atherogenesis [12].

The results also indicate that $ONOO^-$ is able to significantly deplete α-tocopherol levels in endothelial cells. These findings are in agreement with other reports [18,19] confirming the importance of α-tocopherol in RNS mediated oxidative stress.

Another important finding in this investigation is the demonstration that flavonoids, and in particular the procyanidin blend extracted from the bark of *Pinus marittima*, appear to have an important effect on the cellular antioxidant network both in the basal condition, as indicated by higher α-tocopherol levels in ECV 304 cells pre-incubated with Pycnogenol, and in pro-oxidant conditions induced by RNS, such as those due to the exposure to activated macrophages or to the direct administration of $ONOO^-$. Phenolics, and in particular some flavonoids, owing to their intermediate redox potential and physico-chemical characteristics, can possibly act at the interface between ascorbate and tocopherols. Ascorbic acid has been shown to directly regenerate α-tocopherol from α-tocopherol radical in lipoproteins and membranes, and is thus an integral part of the redox antioxidant-recycling network [20]. Pycnogenol has been shown to prolong ascorbyl radical lifetime [5] and can therefore contribute to prevent α-tocopherol loss, possibly by the enhancing ascorbate protective effect, which is likely to be one of the primary mechanism of tocopheryl radical reduction. However, it was observed that the highest concentration of Pycnogenol tested (50 μg/ml) were

associated with both slightly lower α-tocopherol levels in basal condition and to a lesser protection of ECV 304 endothelial cells challenged by activated macrophages, suggesting that high concentrations of phenolics may possibly have a pro-oxidant activity in these experimental conditions.

The delicate interaction between endothelial cells and macrophages is of interest. These cells interact together with lymphocytes and smooth muscle cells in determining the maintenance of blood vessel homeostasis [21]. In particular, endothelial cells participate in several aspects of vascular physiology and physio-pathology, contributing to the control and regulation of vessel tone, coagulation state, cell growth, proliferation and death, and leukocyte trafficking [22]. Occlusive vascular diseases, such as atherosclerosis, are in fact characterized by an abnormal proliferation of smooth muscle cells, inflammatory cells and extracellular matrix protein. Endothelial cells dysfunction leads to vasoconstriction, thrombus formation and inflammatory cells infiltration into a plaque [22].

α-Tocopherol plays a key role in the maintenance of endothelial functions and polyphenols may significantly contribute in protecting this antioxidant during pro-oxidative events [4,23]. Flavonoids and in general polyphenols are attracting increasing interest as nutritional compounds recognized to positively affect human health and also for their potential pharmacological activity. In recent years several different epidemiological reports have indicated that consumption of foods rich in polyphenols is associated with lower incidence of degenerative diseases [24] and experimental data are accumulating regarding phenolic compounds as natural phytochemical antioxidants important for human health [23]. The ascorbate recycling activity displayed by various flavonoids [5] confers to some plant extracts an important role in the cellular antioxidant network and confirms the early reports by Szent-Gyorgyi [25] who was the first investigator to propose phenols as important bio-active molecules.

Hence, this study provides a background for the better understanding of the complex activity of Pycnogenol and other related flavonoids, within the cellular antioxidant network, and about RNS as possible causative factors of endothelial dysfunction and cardiovascular disease.

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