BIOCHEMICAL STUDIES ON A NATURAL ANTIOXIDANT ISOLATED FROM ROSEMARY: DERMOCOSMETIC IMPLICATIONS FOR ITS APPLICATION IN HUMAN ANTIAGING SKIN CARE

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Received: July 23, 1998

Key words: Skin aging, oxidative stress, antioxidant defense, antioxidant biotechnology.

Synopsis

It is generally accepted that lipid peroxides play an important role in the pathogenesis of free radical-induced cellular injury and that antioxidants such as glutathione, ascorbic acid or alpha-tocopherol are vital in cellular defense against endogenous or exogenous oxidants. The purpose of this work was to investigate the effectiveness of a natural extract derived from rosemary to control free radical-induced lipid peroxidation and tissue damage of skin. In the present study we provide evidence that an alcoholic extract of rosemary leaves is endowed with a strong antioxidant activity and is capable of inhibiting free radical-mediated reactions, as evaluated by both in vitro and in vivo biochemical systems. The present study is a preclinical perspective on the interface between the biochemical properties of a natural extract isolated from rosemary leaves, a better understanding of the endogenous antioxidant potential of skin and the real validity of a natural antioxidant biotechnology in the antiaging management of skin.

Riassunto

È generalmente riconosciuto che i perossidi svolgono un ruolo importante nella patogenesi del danno da radicali liberi e che gli antiossidanti come il glutatnone ridotto, l'ascorbato o la vitamina E sono essenziali nelle difese cellulari contro ossidanti endogeni o esogeni. Lo scopo del presente lavoro è stato quello di esaminare l'efficacia di un composto naturale, derivato da Rosmarinus officinalis L., nella protezione della cute dal danno da radicali liberi. Nel presente studio dimostriamo che un estratto alcolico derivante da foglie di rosmarino possiede una notevole attività antiossidante, essendo capace di limitare significativamente reazioni da radicali liberi, come osservato da test effettuati sia in vitro che in vivo. Lo studio offre una prospettiva preclinica all'interfaccia tra proprietà di un estratto naturale isolato dalle foglie di rosmarino, potenziale antiossidante della pelle ed efficacia di una biotecnologia antiossidante naturale nel trattamento antiaging della cute.
INTRODUCTION

In assessing dietary modulation of disease processes focus shifted over the years from the recognition in the late 1800s that diseases could be prevented by replacement of missing components, to concerns in the 1960s regarding toxic food constituents. Recently the pendulum has swung back to recognizing that much of the impact of diet on disease can be attributed to the presence or absence of dietary protective agents.

Among the dietary “chemoprotective” agents, compounds endowed with antioxidant properties, such as carotenoids, ascorbate, and tocoherol have been intensively investigated (1-5).

It has been now extensively demonstrated that oxygen derived active species cause damage to DNA, structural proteins, carbohydrates, enzymes and, especially, the lipid components of membranes (6), and there is growing evidence that free radical-induced perturbation of oxidant/antioxidant balance in cell is a primary pathogenic event in a number of human diseases such as Alzheimer’s disease, ataxia telangiectasias, atherosclerosis, Parkinson’s disease, cataracts, aging and carcinogenesis (7-10).

Previous studies have also indicated that spontaneous ultraweak light emission from biological systems, due to the decomposition of long-lived intermediates produced by oxidative processes at the cellular level, and attributed to the relaxation of electronically excited states generated from biochemical pathways involving the interaction of free radicals and enzymatic reactions, is a reflection of in vivo oxidative stress.

Consequently, the development of an in vivo oxidative stress condition may lead to enhanced chemiluminescence, as found in experimental pathological situations such as chronic ethanol intoxications, ischemic and post-ischemic brain damage or barbital treatment (11).

Skin, highly differentiated and certainly complex organizational structure, is particularly vulnerable to free radical damage, because of its contact with oxygen and with other environmental stimuli. It also have been demonstrated that UV light irra-
MATERIAL AND METHODS

Reagents

Linoleic acid (18:2), Fiske-Subbarow reagent, ammonium molybdate, thiobarbituric acid, hydrogen peroxide, cytochrome c (horse-heart type VI), nitro-blue tetrazolium, hypoxanthine, superoxide dismutase (bovine) were obtained from Sigma (St. Louis, USA). Xanthine oxidase was from Boehringer. All other chemicals were analytical grade and were obtained from Merck (Germany).

Isolation of fractions from rosmarinus officinalis leaves

Rosmarinus officinalis L. was obtained from the local growers in the region of Siracusa. The leaves were harvested and homogenized with 50% methanol at room temperature, and the residue was reextracted with 50% methanol. The extract was washed with n-hexane and after adding water to make the concentration of 10% methanol the combined extract was chromatographed over MCI-gel CHP 20P using a stepwise gradient elution of water and methanol. The 30% MeOH fraction was applied to a Sephadex LH-20 column and eluted with 50% MeOH to obtain the fraction F which was used in the course of this study.

Thin Layer Chromatography (TLC)

Milligram quantities of compounds present in fraction F were prepared by TLC on Silica Gel (Merck 60 F254). Resolution was accomplished with chloroform-MeOH-H2O (7:3:0.5). After TLC, the band, visualized under UV light (254 nm) or by spraying 10% H2SO4 reagent followed by heating, was scraped and eluted with 50% methanol.

Evaluation of antioxidant activity

A) The antioxidative activity of fraction F was evaluated by a ferric thiocyanate method, according to (24). Briefly, different amounts of samples dissolved in 120 µl of ethanol were added to a reaction mixture in a screwcap vial. Each reaction mixture consisted of 2.88 ml of 2.51% linoleic acid in ethanol and 9 ml of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40°C in the dark. At intervals during incubation, a 0.1 ml aliquot of the mixture was diluted with 9.7 ml of 75% ethanol, which was followed by adding 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% HCl to the reaction mixture the absorbance at 500 nm was measured.

B) Antioxidant activity of fraction F purified by TLC was also evaluated for their quenching activity on the superoxide radical generated in vitro by the hypoxanthine-xanthine oxidase system. The reaction was carried out essentially as described by Halliwell et al. (25). The reaction mixture in a total volume of 3 ml contained 0.1 ml of 30 mM EDTA, 10 µl of 30 mM hypoxanthine, 100 µl of 3 mM cytochrome c or 3 mM Nitro-blue tetrazolium (NBT). The solution was brought to volume with 50 mM potassium phosphate buffer pH 7.4. The reaction was started by adding 0.2 ml of xanthine oxidase freshly diluted in phosphate buffer pH 7.4 to 1 unit/ml. Rates of cytochrome c or NBT reduction were measured at 560 nm or 550 nm respectively at 25°C.

The antioxidant activity of the fraction isolated from the hydrophilic fraction of rosemary was measured on the basis of the percent inhibition in the reduction of cytochrome c or NBT. The concentration of cytochrome c in solution was calculated by measuring the absorbance at 550 nm before and after addition of excess sodium dithionite, using a molar reduced minus oxidized extinction coefficient of 1.85 x 104 at 550 nm. Xanthine oxidase activity was monitored in the absence of cytochrome c or NBT by measuring the absorbance at 290 nm due to ureate production.

This assay was included in order to ascertain that the compounds tested did not interfere with xanthine oxidase activity.
Sampling of skin surface lipids from healthy volunteers

The study was limited to 30 adult male volunteers. The age of the subjects ranged from 18-52 years old. The mean age in the group was 33 ± 11. The subjects were requested to avoid the use of hair lotions or other oil containing ointments during the duration of the experimental period. The experimental procedure was designed to evaluate the effectiveness of the hydrophilic fraction isolated from rosemary in preventing lipid peroxidation of skin surface lipids, as compared to vitamin E.

The study was carried out in the Experimental Dermatology Clinic at the University Hospital. The 30 volunteers were divided randomly into two groups (A, and B) of 15 individuals. A sample of skin surface lipids was obtained from the forehead of each individual, as control.

Then group A was asked to apply for a week vitamin E dissolved in ethanol 20%, whereas the group B was asked to use fraction F dissolved in 20% ethanol, for the same period. The experimental groups were asked to wash the forehead thoroughly with neutral soap before treatments, which were repeated each day at the same hour (10:00 p.m.). The morning following, respectively, the first, third and the last treatment, samples of skin lipids, obtained by the swabbing technique as described below, were taken. The surface lipids were obtained by swabbing an area of the forehead (3 cm x 3 cm) with a cotton swab. The procedure was standardized such that the area was swabbed three times horizontally and three times vertically for each individual.

Extraction of lipids

The cotton swabs were extracted for their lipid content with 3 ml of chloroform:methanol mixture (1:2.5). The extraction procedure was allowed to continue at room temperature for 2 hours in the presence of heneicosanoic acid (10 µg) as internal standard. The cotton was removed and re-extracted with fresh chloroform:methanol solution (1 ml). The extraction solutions were combined and 1% NaCl in 0.01 M HCl was added to the mixture and centrifuged. The chloroform layer from both extractions was pooled and washed with 3 ml methanol:water (1:1). The solution was centrifuged and after phase separation, the chloroform layer was recovered and evaporated to dryness under a stream of nitrogen gas in the dark. The dried lipids were dissolved in 3 ml chloroform-methanol solution (2:1) and the solution was stored at -20°C in the dark until analysis of lipid content and lipid peroxidation studies.

Analysis of lipid content

The content of amount of lipid extracted was assessed by the determination of phosphate, as described elsewhere (16).

Peroxidative stress of skin surface lipids and analysis of chemiluminescence

The sensitivity of skin surface lipids extracted from individuals in the three experimental groups was evaluated by measuring the susceptibility of the lipids to oxidative stress in the presence of t-butyl hydroperoxide (3 mM).

Oxidative damage to lipids generally leads to the formation of long-lived intermediates, attributed to the relaxation of electronically excited states generated from free radicals reactions, and hence enhances chemiluminescence, as a reflection of in vivo oxidative stress, which has been investigated extensively as a marker for lipid peroxidation processes either in vitro or in vivo.

Measurement of chemiluminescence was accomplished with a Turner TD 20/20 luminometer, according to (26) and results were expressed as per cent of peroxidation.

Statistical Analysis

Results are the means ± S.E.M. of four to six independent experiments. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan’s new multiple-range test. Dif-
ferences were considered significant at $P<0.05$.

**RESULTS**

Figures 1-2 show the antioxidant activity obtained with the aqueous extract of *Rosmarinus officinalis* L. The results are reported as percent inhibition of cytochrome c (Fig. 1) or NBT reduction (Fig. 2) in the presence of $\mu$g amounts of the fraction examined. Alpha-tocopherol was used for comparison of the antioxidant activity. As can be seen, significant inhibition of the rate of reduction of cytochrome c at 550 nm was observed in the presence of fraction F. Similar results were obtained when testing for reduction of NBT.

In both cases a dose-dependent inhibition was found relative to control values. However, this result was somewhat lower than the antioxidant effect found.
with corresponding amounts of vitamin E. Antioxidant activity of this fraction was investigated using the model of peroxidation of linoleic acid (18:2), as revealed by the thiocyanate method. As shown in Figure 3, the effects of different concentrations of compound F on the time course of peroxidation of 18:2 was monitored. Peroxidation of linoleic acid led to a time-dependent oxidative cleavage of this fatty acid, which was oxidized progressively during six days of incubation at 40°C in the dark. Addition of increasing amounts from 10 to 100 µg of rosemary extract delayed considerably and in a dose-dependent manner the oxidative breakdown of linoleic acid.

The susceptibility of lipids to peroxidative stress was evaluated using lipid samples collected in the study performed with human volunteers. As can be seen from Figure 4, lipids collected from...
the forehead of healthy volunteers, were subjected to in vitro oxidative stress, performed by incubation of lipid extract with 1 mM hydrogen peroxide and the chemiluminescence measured during the time course. Alternatively, lipid extract was studied with the thiocyanate method during six days of incubation as described above, and the results showed in Figure 5.

As can be seen from both figures, comparable protective effects were observed either in the presence of rosemary extract or vitamin E towards lipid exposed to oxidative damage.

In Figure 6, we report the interesting finding of a protection afforded by topical application of the hydrophilic extract from rosemary on skin surface lipids, against oxidative challenge of the skin. In fact, lipids extracted at different times, after topical treatment with the rosemary extract exhibited a signi-
significant higher resistance towards lipoperoxidative chain reactions.
In the same figure are reported the results of application of vitamin E, which showed to be of comparable extent respect to the rosemary extract. This demonstrates that treatment with an hydrophilic fraction derived from rosemary extract significantly inhibited peroxidative damages to skin lipids.

**DISCUSSION**

It is increasingly evident that free radicals play a key role in determining the general appearance of the skin. Skin, highly differentiated and certainly complex organizational structure, is particularly vulnerable to free radical damage, because of its contact with oxygen and with other environmental stimuli. The primary target are the unsaturated lipids in the stratum corneum, the outermost layer of the skin. Most of these lipids, consist of ceramides with unsaturated fatty acyl side chains and cholesterol which also has unsaturated double bonds (27).

The stratum corneum also contains squalene, a hydrocarbon which is readily peroxidized upon irradiation with U.V. light in the presence of oxygen. It also have been demonstrated that UV light irradiation leads to a considerable decrease in the concentration of antioxidant naturally present in the skin (15). As a result, a large increase in lipid peroxidation occurs. This sensitive marker of free radical attack well correlates with irreversible damage of cellular lipid, protein and nucleic acid constituents. Moreover, lipid peroxides resulting from lipoperoxidative processes, are themselves irritating to skin, as demonstrated in human studies.

The prevention of oxidative reactions in the skin lipids, through exogenous supplementation of antioxidants could have cosmetic benefits, in that may represent an efficient tool to mitigate the consequences of skin photoaging.

In the present study we employed peroxidative systems to mime pathogenic conditions supposed to be involved in skin aging processes, in order to evaluate the effectiveness of a natural antioxidant, isolated from rosemary leaves in inhibiting both in vitro and in vivo free radical-mediated reactions. In this study we provide evidence that the compound isolated from a hydrophilic extract of rosemary leaves is endowed with strong antioxidant activity, as revealed by the in vitro tests of inhibition of reduction of cytochrome c or nitro-blu tetrazolium, as well as by studying the susceptibility of linoleic acid (18:2) to peroxidative breakdown. The present compound revealed a strong antioxidant activity, of level comparable to vitamin E. This activity was observed also in vivo either by exposing skin surface lipids to oxidative stress, performed in absence and in presence of added antioxidants, or after topical application of this compound to the skin of healthy human volunteers, whose surface lipids taken and analysed for their resistance to oxidative stress.

In this last case, a lower susceptibility to oxidative stress was found, and this was even higher than the effect of vitamin E, as revealed by chemiluminescence analysis. Our data clearly demonstrate that the fraction F is endowed with antioxidant activity and therefore it may afford an efficient pharmacological tool to control lipoperoxidative changes of the skin, pointing out the importance of a natural antioxidant biotechnology in the anti-aging treatment of skin.
REFERENCES


