Caffeic Acid - Potential Antioxidant in Cultured Human Retinal Pigment Epithelial Cells

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Abstract. Oxidative stress causes biological changes responsible for carcinogenesis and aging in human cells. The retinal pigmented epithelium is continuously exposed to oxidative stress. Therefore reactive oxygen species (ROS) and products of lipid peroxidation accumulate in RPE. Neutralization of ROS occurs in retina by the action of antioxidant defence systems. In the present study, the protective effect of caffeic acid (3,4-dihydroxy cinnamic acid), a dietary phenolic compound, has been examined in normal and in oxidative stress conditions (500 µM peroxide oxygen) in cultures human epithelial pigment retinal cells (Nowak, M. et al.). The cell viability, the antioxidant enzymes activity (CAT, GPx, SOD) and the level of intracellular reactive oxygen species (ROS) were determined.

Exposure to 100 µM caffeic acid for 24 h induced cellular changes indicating the protective effect of caffeic acid in RPE cells. Caffeic acid did not show any cytotoxic effect at concentrations lower than 200 µM in culture medium. Treatment of RPE cells with caffeic acid causes an increase of catalase, glutathione peroxidase and superoxide dismutase activity, especially in cells treated with hydrogen peroxide. Caffeic acid causes a decrease of ROS level in cells treated with hydrogen peroxide. This study proved that caffeic acid or food that contain high levels of this phenolic acid may have beneficial effects in prevention of retinal diseases associated with oxidative stress by improving antioxidant defence systems.

INTRODUCTION

Phenolic acids can be divided in two main groups: benzoic acid and cinnamic acid derivatives. The presence of the group -CH=CH-COOH in hydroxycinamic acids is the key of a more effective antioxidant activity, than the group -COOH of hydroxybenzoic acids (White, P. J. and Xing, Y., Shahidi, F., 1997). Hydroxycinnamic acid compounds are widely distributed in the plant kingdom. They have been described as chain-breaking antioxidants, probably acting through a radical-scavenging mechanism, which is related to their hydrogen-donating capacity and to their ability to stabilize the resulting phenoxyl radical (Siquet, C. et al., 2006). These compounds usually exist as esters of organic acids or glycosides. According to the results of Cuvelier et al. (Cuvelier, M. et al., 1992), esterification of caffeic acid by a sugar moiety decreases its antioxidant activity.

Caffeic acid (C9H8O4) (Fig.1) is a natural organic compound which belongs to the class of hydroxycinnamic acids. It is found in almost all plants and is an intermediate in lignin biosynthesis.

![Fig 1. Chemical structure of caffeic acid](image-url)
Caffeic acid has been isolated from *Ilex paraguariensis* (15 mg/100 g), *Melissa officinalis* (39.3 mg/100 g), *Baccharis genistelloides* (8 mg/100 g) and *Achyrocline satureioides* (4 mg/100 g) (Marques, V. and Farah, A., 2009). Caffeic acid is also present in beverages like wine, tea, coffee and apple juice (Marques, V. and Farah, A., 2009). Studies on rats and humans proved that caffeic acid is efficiently absorbed in small intestine (Azuma, K. et al., 2000); (Olthof, M. R. et al., 2001). Coffee was shown to increase plasma antioxidant capacity in humans (Natella, F. et al., 2002) and increases the incorporation of conjugated forms of caffeic acid into LDL particles improving the oxidation-resistance of LDL (Natella, F. et al., 2007). Caffeic acid was reported to have a wide variety of pharmacological activities including antioxidants (Nardini, M. et al., 1999), immunomodulatory, antiviral, anticarcinogenic and anti-inflammatory effect (Fesen, M. R. et al., 1994). Caffeic acid and its derivatives such as chlorogenic acid and caffeic acid phenyl ester are the major compounds in plants and act as substrate for polyphenols oxidases or peroxidases (Bassil, D. et al., 2005).

It was found that some phenolic acids tested on the line T47D tumor breast, have inhibitory effects on cell growth, with an order of: caffeic acid > ferulic acid = protocatechuic acid > sinapic acid = syringic acid (Kampa, M. et al., 2004). Caffeic acid was also found to show potent hepatoprotective activity by protecting against injuries induced by CCl₄ and alcohol (Janbaz, K. H. et al., 2004).

Our previous results showed that exposure of RPE to hydrogen peroxide (500 µM) affects antioxidant defence system. Since reactive oxygen species (ROS) induce cellular damages, antioxidants might be helpful to minimize oxidative stress-induced pathologies, like age related macular degeneration. The present study examines the antioxidant effect of caffeic acid in untreated or hydrogen peroxide treated RPE cells.

**MATERIAL AND METHODS**

**Cell culture and culture conditions**

Human adult retinal epithelial cells D407 were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum at 37°C, 5% CO₂, and 95% relative humidity. Upon reaching confluence, cells were trypsinized and seeded into the appropriate tissue culture vessels. The cells were seeded in 25 cm³ flask at a concentration of 6 x 10⁵. At 90% confluence, growth medium was removed and replaced with medium containing 100 µM caffeic acid (Sigma, USA). After 24 hours of caffeic acid treatment, the medium was removed and the cells were exposed to 500 µM H₂O₂ at 37 °C for 1 hour in serum free DMEM medium.

**Cell viability assay**

RPE cells were seeded (10,000 cells per well) in 96-well plates and after the cells attached, they were incubated for 24 h with different concentration of caffeic acid (10 – 250 µM). The number of viable cells for each concentration was determined with the thiazolyl blue tetrazolium bromide (MTT) cell proliferation reagent. This method uses the property of viable cells to reduce MTT to a coloured formazan, product which is detected by reading the absorbance at 570 nm with a 96-well plate reader. Cell viability was expressed as a percentage of control (cells incubated in normal medium only).
Antioxidant enzyme activity assay

Protein concentrations for each sample were determined using the bicinchoninic acid assay (Sigma). Activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were measured using commercial kits (Cayman Chem).

Measurement of reactive oxygen species

The determination of intracellular reactive oxygen species is based on the oxidation of 2’,7’-dichlorodihydrofluorescein (DCHF) by intracellular oxygen radicals, forming the fluorescent compound 2’,7’-dichlorofluorescein (DCF). The fluorescence was measured by a BioTek Synergy microplate reader. Cells were incubated with dichlorofluorescein diacetate (DCFDA) (stock solution 20 mM) using dilution 1:1000 in PBS. Fluorescence was monitored for 4h at 37 ºC at excitation 485/10 nm and emission 528/20 nm.

Statistical analysis

Statistical analysis were done using GraphPad Prism version 5.00. The points or bars represent the mean ± SEM, calculated from three experimental values.

RESULTS AND DISCUSSIONS

Viability of pigmented epithelial cells from human retina (D407) treated with acid caffeic

Human cells were grown to confluence and exposed to different concentrations of caffeic acid (10-250 µM) for 24 h. Cell viability was stimulated after treatment with caffeic acid at concentrations lower than 250 µM. A decrease of cellular viability of D407 cells was observed after exposure to concentrations higher than 200 µM caffeic acid. Concentration 100 µM is not cytotoxic to RPE cells Fig.2.

Evaluation of antioxidant enzymes in RPE cells treated with caffeic acid

Tests were performed in standard cultivation conditions and in induced oxidative stress. The experimental induction of oxidative stress was done by adding 500 µM hydrogen peroxide in the culture medium, and cell exposure for 1 hour. We previously demonstrated that this dose is non-lethal, but induce a decrease of cell viability and an alteration of antioxidant status in RPE cells.

The activity of key antioxidant enzymes - glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) - were monitored after 24 hours of cell treatment with 100 µM caffeic acid and 1 hour treatment with 500 µM hydrogen peroxide.

Treatment with hydrogen peroxide alone causes a decrease of all antioxidant enzyme activity compared with control cells, but more significant for GPx and SOD. A small increase of CAT (Fig.3) and SOD (Fig.5) was observed in cells treated with caffeic acid and no hydrogen peroxide.

In cells treated with both caffeic acid and hydrogen peroxide catalase activity recorded increased activity compared to cells treated only with hydrogen peroxide.
GPx activity in RPE is enhanced after treatment with hydrogen peroxide and caffeic acid, comparing with the control treated with hydrogen peroxide alone (Fig.4). Caffeic acid causes also an increase in SOD activity in RPE cells treated both with caffeic acid and hydrogen peroxide (Fig.5).

**Effect of caffeic acid on the generation of intracellular reactive oxygen species**

RPE cells treatment with hydrogen peroxide induced an increased generation of intracellular ROS, demonstrated by an intensification of DCF fluorescence. Data recorded after 4 hours of DFF-DA incubation showed that cells pretreated with caffeic acid and treated with hydrogen peroxide present a significantly decreased DCF fluorescence, compared with cells receiving only H\textsubscript{2}O\textsubscript{2} (Fig. 6). Similar results were found for other phenolic compounds – flavonoids – in RPE cells exposed to \textit{t}-butyl hydroperoxide (Hanneken, A. et al., 2006).

**CONCLUSIONS**

During this work we investigated antioxidant properties of caffeic acid in human retinal pigment epithelial cells.

Caffeic acid did not show any cytotoxic effect at concentrations lower than 200 µM in culture medium. Treatment of RPE cells with caffeic acid causes an increase of catalase, glutathione peroxidase and superoxide dismutase activity, especially in cells treated with hydrogen peroxide. Caffeic acid causes a decrease of ROS level in cells treated with hydrogen peroxide.

It is believed that oxidative stress in the RPE is one of the major causes of retinal diseases such as age related macular degeneration disease. RPE is continuously subjected to the aggressive attack of ROS, resulting from the phagocytosis and other metabolic processes. This study demonstrates that caffeic may protect cultured RPE cells against the aggression of ROS by intensifying the activity of antioxidant enzymes or by neutralizing directly the free radicals. Studies are required in humans to demonstrate the \textit{in vivo} preventive effect of caffeic acid in retinal degenerative diseases.

![Fig. 2. MTT test of proliferation (viability%) for epithelial cells from human retina (D407) treated with acid caffeic](image1)

![Fig. 3. Catalase activity in RPE cells after treatment with 100 µM caffeic acid](image2)
Fig. 4. Glutathione peroxidase activity in RPE cells after treatment with 100 µM caffeic acid

Fig. 5. Superoxide dismutase activity in RPE cells after treatment with 100 µM caffeic acid

Fig. 6. Determining the level of intracellular reactive oxygen species in pigmented epithelial cells of human retina

C = control; Cox = control+ H₂O₂; Ca = caffeic acid; Caox = caffeic acid + H₂O₂

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REFERENCES


