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Assessment of the Antioxidant Potential Effect of Caffeic Acid in Retinal Pigmented Epithelial Cells

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Abstract. The aim of this study was to demonstrate that caffeic acid, an antioxidant with phenolic structure, can improve the antioxidant status of retinal pigment epithelium (RPE), when oxidative stress is induced by high glucose concentrations in culture medium. We evaluate the high glucose and caffeic acid cytotoxicity and their action on retinal pigmented epithelial cell viability, intracellular reactive oxygen species generation, superoxid dismutase and gluthatione reductase activity. Caffeic acid did not show any cytotoxic effect on RPE cells up to 250 μM in culture medium and protect them against induced oxidation. Caffeic acid protects RPE cells by quenching the intracellular ROS generation and by enhancing the activity of glutathione peroxidase and superoxide dismutase. Caffeic acid treatment of cultured RPE cells can contribute to antioxidant defence in oxidative stress conditions induced by hyperglycaemia.

Keywords: caffeic acid, antioxidant, pigmented epithelial cells

INTRODUCTION

Diabetic retinopathy is the most common diabetic eye disease and the leading cause of blindness in adults. Inefficient control of blood glucose levels is one of the complications of diabetic retinopathy. So, in the development of retinopathy, hyperglycemia is the initiating event [1], high concentrations of glucose being a powerful stress for diabetic retina, in which evolve an overproduction of ROS. The retinal pigment epithelium is a monolayer of cuboidal cells located between photoreceptors of the neurosensory retina and the choroidal capillary bed. As part of the outer blood retinal barrier, retinal pigment epithelium facilitates selective molecular transport between choroidal blood supply and the outer retina. Being a part the retinal blood barrier the retinal pigment epithelium (RPE) has been reported to be affected in diabetes. Oxidative stress in RPE results after a progressive cellular damage caused by reactive oxygen species, contributing to protein misfolding and functional abnormalities of the RPE cells during cellular senescence and age-related macular degeneration pathology [5]. Retinal pigmented epithelium is also the site of advanced glycosylation end product formation [6]. RPE cells are normally well protected against oxidative damage by their antioxidant mechanisms such as catalase, superoxide dismutase, glutathione peroxidase, which act by scavenging the superoxide and hydroxyl anions to prevent ROS-induced damage [7]. We aimed to determine whether the addition of an antioxidant into a high-glucose culture medium might counteract the oxidatively induced damages of RPE cells.

It is well known that caffeic acid, a dietary phenolic compound, acts as antioxidants [8] and have a wide variety of pharmacological activities including immunomodulatory, antiviral, anticarcinogenic and anti-inflammatory effect [9]. This dietary phenolic compound, caffeic acid, is found in a wide variety of plant derived materials such as vegetables, fruits, olive, sunflower, tea and especially red wine (5 mg caffeic acid/L) [10]. In this study we demonstrated that caffeic acid protects RPE cells from glucose-induced oxidative stress,

knowing that glucose is the major energy source for retinal metabolism [11]. Subcytotoxic levels of caffeic acid and glucose were administrated to RPE cells to evaluate their influence on D407 cell viability.

MATERIALS AND METHODS

2.1. Chemicals

GIBCO® Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, California, USA). Fetal bovine serum, penicillin, streptomycin, amphotericin B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), caffeic acid standard were purchase from Sigma (St. Louis, USA). Methanol, acetic acid, sodium piruvate, hydrogen peroxide and all the others chemicals used were of analytical grade and supplied from Merck (Darmstadt, Germany).

2.2. Cell culture and treatment

Human adult retinal pigment epithelial cells D407 were maintained in Dulbecco's Modified Eagle Medium with high glucose (25mM), supplemented with 10% fetal bovine serum, 1 mM sodium piruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B, at 37◦C, 5% CO_2, and 95% relative humidity. The cells were seeded in 25 cm^3 flask at a concentration of 6 x 10^5. After reaching 90% confluence, growth medium was removed and replaced with medium containing caffeic acid. Caffeic acid was solubilized in water until the final concentration of 100 µM in medium.

2.3. Exposure of cells to high glucose treatments

After 24 hours from caffeic acid treatment, the culture media was removed and, after washing with PBS, the cells were exposed to 25, 40, 50, 70, 100 µM glucose for 1h in DMEM medium. Thereafter, the cells were washed twice with cold PBS, and then specificaly lysed for each enzyme determination.

2. 4. MTT assay

D407 cells were plated (10^4 cells/ well) in 96-well plates and after the cells attached, they were incubated for 24h with caffeic acid and for 1h with different concentration of glucose. The number of viable cells at each time point was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation reagent. Briefly, the cells were washed with PBS and 200 µl MTT solution in HBSS buffer were added to each well. After 2 h of incubation the MTT reagent was removed and the formazan particles were solubilized with 200 µl DMSO. The absorbance was read at 550 nm, respectively at 630 nm (for background) with a microplate plate reader HT BioTek Synergy (BioTek Instruments, USA). Cell viability was expressed as a percentage of control (cells incubated in normal medium only).

2. 5. Glutathione peroxidase assay

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, using reduced glutathione in order to protect the cell from oxidative damage. Cayman Chemical (USA) GPx Assay kit measures GPx activity indirectly by a coupled reaction with gluthatione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is the rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

The cells collected in PBS were homogenized in 100 µl cold buffer (50mM Tris-HCl, pH 7.5, 5 mM EDTA and 1mM DTT) and centrifuged at 1500 g for five minutes at 4 ºC. A volume of 20 µl of sample was treated with 100 µl of assay buffer, 50 µl of co-substrate mixture (NADPH, glutathione and gluthatione reductase). The reaction is initiated by adding 20 µl of cumene hydroperoxide quickly. The plate is shaked few seconds and the absorbance is read at 340 nm. GPx activity was calculated, using the NADPH extinction coefficient of 0.00373 μM^{-1} and the rate of ΔA_{340}/ min. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25 ºC.

2. 6. Superoxide dismutase assay

Superoxide dismutase (SOD) assay kit (Cayman Chemical Company, Michigan, USA) which utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. This method measures the activity of all three SOD types.

The cells were homogenized in 200 µl cold 20mM HEPES buffer, pH 7.2, containing 1mM EDTA. The cells were centrifuged in 1500 g for five minutes at 4 ºC and the supernatant was used for enzyme assay. A volume of 200 µl buffer solution containing tetrazolium salt and hypoxanthine was mix with 10 µl of sample. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all wells. The plate was incubated on a shaker for 20 minutes at room temperature. The absorbance was monitored at 460 nm using a microplate reader. A standard curve made with bovine erythrocyte SOD was used for determination of enzyme activity. One unit of enzyme is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

2. 7. Intracellular reactive oxyspecies assay

The determination of intracellular reactive oxygen species (ROS) is based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCHF) by intracellular peroxides, forming the fluorescent compound 2',7'-dichlorofluorescein (DCF), which is measured by a BioTek fluorescence plate reader. Cells were cultured in 96 well black plates and incubated with dichlorofluorescein diacetat (DCFDA). Fluorescence was monitored for 4h at 37 ºC at excitation 485/10 nm and emission 528/20nm.

2.8. Statistical analysis was done using one-way analysis of variance ANOVA, Bonferroni Multiple Comparison Test of Graph Pad Prism version 5.00. Significant differences are designated by $p<0.05$ and notation ***extremely significant, **very significant, *significant. The points or bars represent the mean ± SEM, calculated from three experimental values.

RESULTS AND DISCUSSIONS

Diabetic retinopathy affects virtually all subjects who suffer from type I diabetes by at least 20 years and 80% of those with type II diabetes for the same period. Most of the effects of high glucose concentration are actually related to increased metabolism. Thus, there is an increase in glycolysis, in pyruvate production, and in oxidative phosphorylation. Oxidative phosphorylation is one of the physiological processes that generate reactive oxygen species. D407 RPE cells are usually cultivated in medium containing 25 mM glucose, which represents a high glucose concentration. In the first part of this study we evaluated the influence of different higher glucose concentration in culture medium, mimicking hyperglycaemia that occurs in diabetes, on the viability and antioxidant status of RPE cells.

3. 1. Viability of human retinal pigmented epithelial cells (D407) treated with caffeic acid

Human retinal pigmented epithelial cells were cultivated until 80% confluence and then were exposed to different concentrations of caffeic acid (0-250µM) for 24 h. Cellular viability was stimulated by caffeic acid treatment with concentrations lower than 250 µM. A decrease of cellular viability occurred after caffeic acid treatment with concentrations higher than 200 µM. In conclusion the concentration of 100 µM caffeic acid is not cytotoxic for RPE cells (Fig.1).

3.2. Viability of human retinal pigmented epithelial cells (D407) treated with different concentrations of glucose

D407 retinal human cells were exposed to glucose concentrations between 25-100 mM for 24 h. A semnificative decrease of viable cells number was recorded after exposure to 100 mM glucose concentration (Fig. 2). As can be observed in Fig. 2 and Fig. 3, the increase of glucose concentration had positive effects on cells viability up to 70 mM but determined a decrease of viability at 100 mM glucose.

3.3. Evaluation of glutathione peroxidase activity

Cells treatment with 50 mM glucose induced a small but not significant increase of GPx activity. Treatment with caffeic acid for 24 h resulted in a very significant increase of GPx activity in both control and high glucose condition. It is known that glutathione peroxidase acts at lower level of peroxides. Abu-Amsha et al. 1996 reported that GPX was up-regulated in high glucose condition [10]. GPX-1 is an important antioxidant enzyme that scavenge hydrogen peroxide in mammalian cells [16]. The up-regulation of GPX-1 observed in RPE cells under high glucose condition further implicates a reactive and protective mechanism against hyperglycemia-induced oxidative stress [17]. Also the administration of caffeic acid to streptozocin-induced diabetic rats elevated the activity of glutathione peroxidase in the cardiac tissue, but reduced the activities of superoxide dismutase [18]. Prasad et al., 2009 reported that the treatment with caffeic acid increases the activities of antioxidant enzymes such as SOD and GPx in UVB-irradiated lymphocytes [9].

3.4. Evaluation of superoxide dismutase activity

SOD catalyzes the conversion of superoxide radicals ($O2^{\cdot -}$) to hydrogen peroxide (H_2O_2), which is the first step in metabolic defense against cellular oxidative stress [13]. High glucose concentration induced a small but not statistically significant decrease of SOD activity, results in the accumulation of $O2^{\cdot -}$ in RPE cells. The positive influence of caffeic acid on SOD activity was more evident in control cells that in high glucose treated cells (Fig. 6). The influence of higher glucose concentrations on antioxidant enzymes were also investigated by Yokoyama et al. They reported that high concentrations of glucose in culture medium (33mM) increased expression level of glutathione peroxidase, but decreased the SOD activity which also shifted the isoelectric point toward acidic region. The authors concluded that the RPE cells respond to acute pathologically high glucose levels by elevating yhe expression of anti-oxidant and proteolytic enzymes [12].

3.5. Determination of intracellular reactive oxygen species

RPE cells treated with 25 mM glucose exhibit lower level of fluorescence, indicating a low level of reactive oxigen species formed in D407 cells. Cells treated with higher glucose concentrations (50 mM) exhibit increased fluorescence comparing to control cells (Fig. 4). Very high concentration of glucose (70 and 100 %) increased significantly the ROS generation, as can be seen from the time-course increase of fluorescence. Yokoyama et al. also reported that in RPE cells treated with 33 mM glucose reactive oxygen species increased

[12]. Glucose at 50 mM was chosen for all experiments, based on the fact that it has positive effect on the cell viability but significantly increase the ROS generation compared with control. There is direct evidence that the hyperglycaemia accelerates the rate of oxidative phosphorylation, which leads to ROS, especially $O_2^{\cdot -}$ production [13].

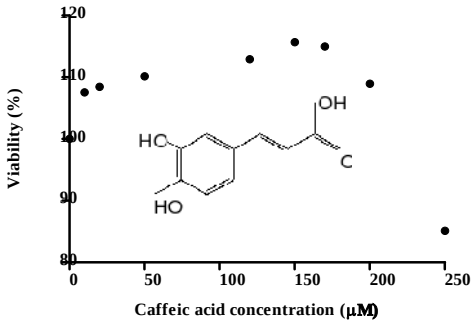

Fig. 1. MTT proliferation test (viability %) of human retinal epithelial cells (D407) treated with caffeic acid

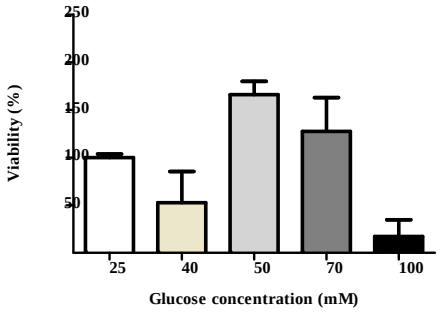

Fig. 2. MTT proliferation test (viability %) of human retinal epithelial cells (D407) treated with different glucose concentrations

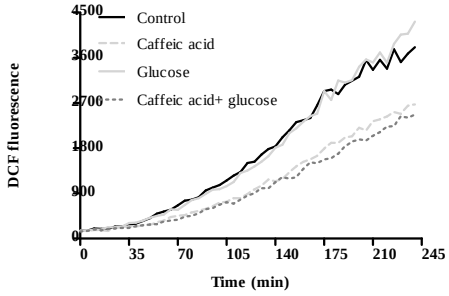

Fig. 3. Determination of total reactive oxygen species content in human retinal epithelial cells after treatment with caffeic acid (100 μM) and/or glucose (50 mM)

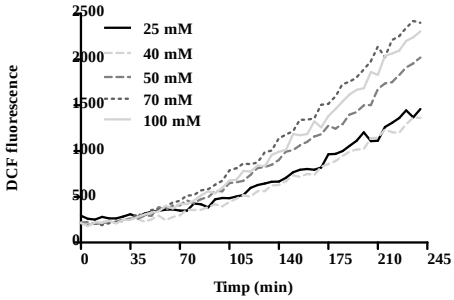

Fig. 4. Determination of total reactive oxygen species content in human retina epithelial cells after treatment with different glucose concentrations

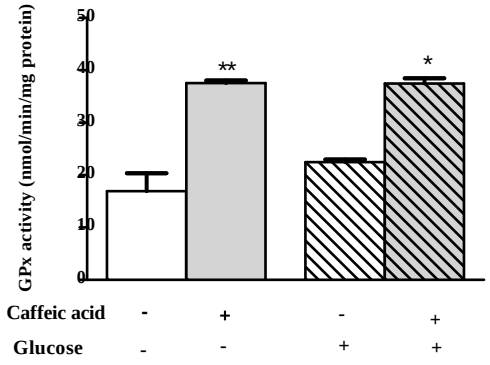

Fig. 5. GPx activity (nmol/min/mg protein) in D407 retina cells treated with caffeic acid and/or glucose (50 mM)
Statistic: one-way ANOVA analysis of variance, Tukey test, $p<0.05$, *- significant, **- very significant.

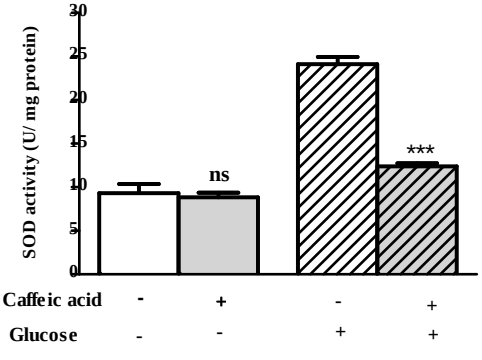

Fig. 6. SOD activity (nmol/min/mg protein) in D407 retina cells treated with caffeic acid and/or glucose (50 mM)
Statistic: one-way ANOVA analysis of variance, Bonferroni test, $p<0.05$, ns - non significant, ***- extremely significant.

CONCLUSIONS

We examined the effect of high concentration of glucose, in the presence or in the absence of caffeic acid on the viability and oxidative status of cultured retinal pigment epithelial cells. Caffeic acid did not show any cytotoxic effect on RPE cells up to 250 μM in culture medium and protect them against induced oxidation. Caffeic acid protects RPE cells by quenching the intracellular ROS generation and by enhancing the activity of glutathione peroxidise and superoxide dismutase. Caffeic acid treatment of cultured RPE cells can contribute to antioxidant defence in oxidative stress conditions induced by hyperglycaemia.

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