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Original paper

Effects of chlorogenic acid on the liver cell metabolism under high glucose conditions

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Abstract

This work aimed to investigate the potential of chlorogenic acid (CGA) to modulate the response of human hepatocytes under hyperglycemic condition.

Markers of oxidative stress such as reduced glutathione content, malondialdehyde levels, advanced oxidation protein products, and nitric oxide production, as well as protein expression of Nrf2 transcription factor were measured in HepG2 cells cultured in normoglycemic (5.55 mM) and high-glucose medium (25 and 35 mM) pre- and post-treated with different concentrations of CGA (5, 10, 50 μM).

Our results brought evidence of the protective effect of CGA on hepatic cells under hyperglycemic conditions that act through modulation of the intracellular reduced glutathione content and Nrf2 protein expression. The efficiency of CGA was more pronounced in the pre-treatment experiment. The findings of the present investigation demonstrated the protective role of CGA on hepatocytes by attenuating some markers of hyperglycemia-mediated oxidative stress.

Keywords : *chlorogenic acid, HepG2 cells, hyperglycemia, Nrf2, oxidative stress*

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Introduction

Hyperglycemia is a hallmark sign of type 1 and type 2 diabetes. Especially type 2 diabetes has reached serious epidemic proportions and the number of people affected by it is in a continuous alarming growing (W. FAN & al. [1]). Despite all efforts and investments made in diabetes research, there is still no cure for diabetics. Most treatments help patients to manage the symptoms to a certain extent, but they still face multiple long-term health complications including damage of blood vessels, kidney disease, problems with eyes, gums, feet infections and nerve damage (NATIONAL KIDNEY FOUNDATION [2]). In addition, researchers found a link between diabetes and liver which is the primary organ involved in glucose metabolism, through a complex and bi-directional relationship. First, the type 2 diabetes could develop in persons with non-alcoholic fatty liver disease and second, the liver injury could result from the potentially detrimental effects of type 2 diabetes (P. LORIA & al. [3]). Hyperglycemia was found to dysregulate the glucose, lipids, and bile acids metabolism and homeostasis in liver (A. DEY & K. CHANDRASEKARAN [4]). *In vitro* and *in vivo* studies showed that hyperglycemia induced structural and functional changes in liver cells through several mechanisms.

Mitochondrial dysfunction induced by hyperglycemia is one of the main mechanisms responsible for hepatic injury. Hyperglycemia induces overproduction of reactive oxygen species (ROS) as a result of an increased input of reducing equivalents into the mitochondrial electron transport chain which is the major cause of the diabetes clinical complications (A. DEY & K. SWAMINATHAN [5]). In liver, mitochondrial dysfunction mediated by hyperglycemia results as a consequence of a reduced mitochondrial oxidative phosphorylation and oxygen consumption, increased protein carbonyl formation, nitrosative stress, and lipid peroxidation as well as changes in the ultrastructure of mitochondria such as: fragmentation, and hypertrophy with an increased number of abnormal cristae (A. DEY & K. SWAMINATHAN [5]).

Previous studies on cell culture models showed that mimicking of the hyperglycemic condition in liver cells caused increased ROS generation, structural and functional mitochondrial alterations, changes in ATP and glucose transporters level and dysregulation of glucose metabolism (A. DEY & K. CHANDRASEKARAN [4]). C.M. PALMEIRA & al., [6] studied the effect of high glucose concentrations on HepG2 cells and noticed a progressive increase in ROS generation followed by induction of oxidative stress, protein oxidations, decrease in mitochondrial DNA content and inhibition of mitochondrial function. Also, using clone 9 rat liver cell line treated with high glucose doses, T. YU & al. [7] observed rapid fragmentation of mitochondria and an increase of ROS production. Furthermore, in hepatic stellate cells, high glucose concentration stimulated ROS production by activating NADPH oxidase through protein kinase C and induced MAPK phosphorylation and Type I collagen production (R. SUGIMOTO & al. [8]).

Other deleterious effects of hyperglycemia in the liver include activation of stress signaling pathways, attenuation of insulin signaling, elevation of pro-inflammatory cytokine levels and impairment of several cellular processes such as proteasome activity and expression of molecular chaperones (A. DEY & K. CHANDRASEKARAN [4]).

Many studies showed that flavonoids and other polyphenols play a protective role in hepatic alterations due to their high antioxidant capacity (N.G. SHEHAB & al. [9]), (M. SUCIU & al. [10]), (S. LI & al. [11]), (H. BAZOOL FARHOOD & al. [12]). Among them, caffeoylquinic acid (chlorogenic acid, CGA), was found to have great potential as it possesses antioxidant, antibacterial and anti-inflammatory activities (A.A.P. ALMEIDA & al. [13]), (M.D. dos SANTOS & al. [14]). CGA is mainly found in green coffee but is also produced by many herbs (tea plants), fruits (e.g., apples, pears, berries), and vegetables (e.g., eggplant, tomatoes, carrots, sweet potatoes) (N. LIANG & D.D. KITTS, [15]). The caffeoylquinic acid is the most abundant and studied member of the so-termed “chlorogenic acids (CGAs)” family, a class of esters formed from hydroxycinnamic acids (caffeoyl-, feruloyl-, dicaffeoyl-and coumaroyl quinic acids) with quinic acid.

Several studies revealed that CGA is involved in the controlled release of glucose into the bloodstream and may, therefore, help in delaying the development

of diabetes. A series of other health benefits have been associated with the consumption of CGA in the last few years, such as: reduction of the relative risk of cardiovascular disease, diabetes type 2, hepatic steatosis, obesity, cancer, and Alzheimer's disease and support of the brain, skin and digestive system (J. LINDSAY & al. [16]; E. SALAZAR-MARTINEZ & al. [17]; T. RANHEIM & B. HALVORSEN [18]).

In this context, the aim of our study was to investigate the potential of CGA to modulate the in vitro oxidative stress in human hepatocytes cells under hyperglycemic (HG) conditions.

Materials and Methods

1. Cell culture and treatment

The HepG2 cell line derived from liver tissue of a patient with hepatocellular carcinoma was purchased from the American Type Culture Collection (HB-8065, Manassas, VA, USA) and cultured in normoglycemic (NG, 5.55 mM glucose) Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotic-antimycotic solution and 10% fetal bovine serum. The cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Besides their ability to easily grow in both, low- and high-glucose media, HepG2 cells are a well-established model and an important tool for the study of hyperglycemia *in vitro* (A. DEY & K. CHANDRASEKARAN [4]). The cells were seeded at a density of 5×10^4 cells/mL in culture flasks or plates depending on the experimental procedure. A 20 mM stock solution of chlorogenic acid (CGA, 3-(3,4-dihydroxycinnamoyl)quinic acid) (C3878, Sigma) was prepared in phosphate-buffered saline (PBS) solution and sterilized by filtration before the cell treatment. Two experimental set-ups were designed for this study: (A) Pre-treatment with CGA - the cells were incubated with CGA (5, 10 and 50 µM) for 24 h and then the medium was removed and replaced by a medium supplemented with glucose (final concentrations in the medium 25 mM and 35 mM) and incubated for another 24 h and (B) Post-treatment with CGA – the cells were first incubated in hyperglycemic (HG) medium (25 mM and 35 mM glucose) and after 24 h, different concentrations of CGA (5, 10 and 50 µM) were added for 24 h in fresh NG medium. Cells cultured in NG medium and without chlorogenic acid were used as controls.

2. Preparation of cell lysate

After treatment, the HepG2 cells were collected from culture flasks, washed with PBS and centrifuged at 1500 rpm for 5 min. Cells pellets were re-suspended in 0.5 mL of PBS and then sonicated on ice three times, for 30 s each. The total extract was centrifuged at 3000 rpm for 10 min at 4 °C. Aliquots of the supernatant were used for further determinations.

3. Protein concentration

The protein concentration (mg/mL) was measured according to Bradford method (M.M. BRADFORD [19]) using bovine serum albumin (BSA) as a standard.

4. Glutathione assay

The reduced glutathione (GSH) content in HepG2 cells was measured by the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to a yellow product, 5-thio2-nitrobenzoic acid (TNB) spectrophotometrically detected at 405 nm. First, the cellular lysate was deproteinized with 5% sulfosalicylic acid and after centrifugation (10000 rpm, 10 min, 4°C), a volume of 10 µL supernatant was mixed with 150 µL of 40 µg/mL DTNB solution. GSH contents in samples were obtained using a standard curve prepared using pure GSH in the range between 0.0312 and 2 nmoles. The results were calculated as nmoles/mg protein.

5. Lipid peroxidation assay

Lipid peroxidation was evaluated by measuring the malondialdehyde (MDA) levels in samples. Briefly, the cell lysate was incubated with 0.025 M thiobarbituric acid (TBA) for 65 min at 37 °C and the MDA-TBA adducts formed were fluorimetrically recorded using a 520 nm/ 549 nm (excitation/emission) filter (A. DINISCHIOTU & al. [20]). A calibration curve in the range 0.05–5 µM 1,1,3,3-tetramethoxypropane was used to calculate the sample MDA concentration. The results were expressed as nmoles of MDA/mg protein.

6. Advanced oxidation protein products assay

Advanced oxidation protein products (AOPP) levels in the exposed HepG2 cells were estimated as

we described before (M. RADU BALAS & al. [21]). Briefly, 200 µL of cell lysate and 10 µL of 1.16 M potassium iodide were mixed in a 96-well plate and incubated on a shaker for 5 min at room temperature. Then, a volume of 20 µL of glacial acetic acid was added in each well. The optical density was recorded at 340 nm after 1 min. A stock solution of 100 µM Chloramine T was used as standard. The results were calculated as µmoles of AOPP/mg protein.

7. Nitric oxide determination

NO concentration was indirectly assessed by quantitating the nitrite in the sample (Griess reaction) (D.L. GRANGER & al. [22]). The nitrite treated with a sulfanilamide (S) forms an unstable diazonium salt which turns into a colored compound in the presence of naphthyl ethylene diamine (NED). Medium (80 µL) of exposed HepG2 cells collected after treatment was pipetted into a 96-well plate and mixed with 80 µL of NED:S solution (1:1 ratio). The optical density was read at 540 nm. The NO concentrations were expressed in µM.

8. Western blot analysis

HepG2 cell lysates were run on sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gels, using the Mini-Protean 3 system (Bio-Rad Laboratories, Hercules, CA, USA) for detection of nuclear factor erythroid 2-related factor 2 (Nrf2) protein. The protein bands were electro-blotted on PVDF membranes, using the Mini Trans-Blot system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were maintained for 30 min in a blocking solution, washed for 5 min in MiliQ water and incubated for one hour with anti-Nrf2 primary antibody (sc-722, Santa Cruz, Biotechnology, Dallas, TX). After rinsing three times with wash solution, the membrane was developed with Western Breeze Chromogenic Immunodetection Kit (Thermo Fisher Scientific) using 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate for alkaline phosphatase. Beta-actin protein (42 kDa) was used as an internal standard. The blot images were acquired and quantified using the Chemidoc (Bio-Rad) equipment and ImageLab (both from Bio-Rad) analysis software. The values for each band were normalized to β-actin and expressed as % from control.

9. Statistical analysis

Data were expressed in terms of mean ± standard deviation (SD) of triplicate measurements from three independent experiments. Student's t-test was used to analyze the differences between the samples untreated and treated with CGA in hyperglycemic conditions. The p values <0.05 were considered significant.

Results

The response of HepG2 cells to the CGA treatment was evaluated in two different experimental set-ups: first, the liver cells exposed to a high glucose environment were treated for 24 h with CGA (post-treatment) and secondly, the CGA treatment was applied with 24 h before the addition of the high glucose culture medium (pre-treatment). In this study, the focus was targeted on the evaluation of oxidative stress markers in order to highlight the role of CGA on human hepatocytes subjected to HG conditions.

As shown in Figure 1A, the HG media decreased GSH content in HepG2 cells in a concentration-dependent manner compared to NG one.

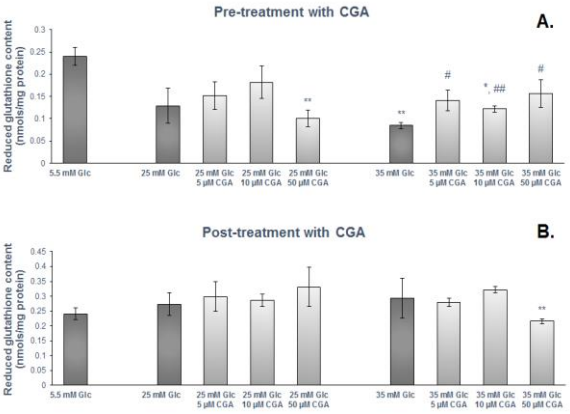

Figure 1. *Effects of glucose and CGA on intracellular GSH content* (A) pre-treatment of HepG2 cells with different concentrations of CGA (5, 10 and 50 µM) for 24 h before glucose supplementation to a final concentration of 25 mM and respectively 35 mM and (B) post-treatment of HepG2 cells for 24 h with different concentrations of CGA after supplementation of culture medium with glucose. Values of GSH are represented as means of three biological replicates ± SD. All samples were compared to NG control (*) and CGA-treated samples were compared with their correspondent HG control (#). Values were significant when p < 0.5 (* or #) and p < 0.01(** or ##).

The 24 h exposure of HepG2 cells to CGA prior

to glucose supplementation of culture medium to 25 mM, induced no significant changes of the intracellular GSH level except the maximum dose of CGA (50 µM). In this condition, the GSH content decreased by 1.8 folds ($p < 0.01$**) compared with the one found in NG cells. The exposure of liver cells to 35 mM glucose in the culture media after CGA pre-treatment led to an important increase of GSH content compared to HG cells a trend of restoration up to that of cells grown in NG medium being noticed. On the other hand, the GSH content remained unchanged after the post-treatment of HG cells with CGA (Figure 1B). Only, the addition of 50 µM CGA to HepG2 cells pre-exposed to 35 mM glucose, induced a significant decrease by 1.1 folds ($p < 0.01$**) compared to NG cells.

In the pre-treatment experiment, the MDA level decreased by 1.53 folds in the cells grown in 35 mM glucose medium compared to those cultivated in NG and 25 mM media.

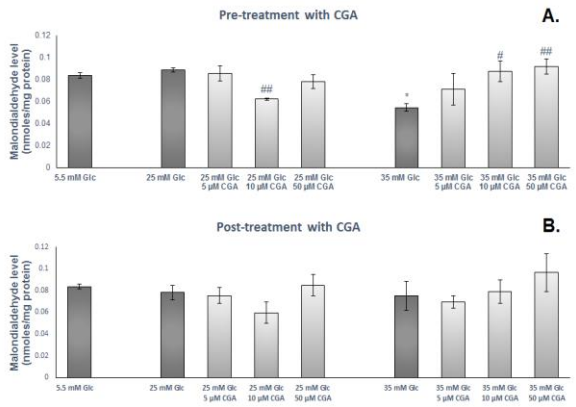

Figure 2. *Effects of glucose and CGA on MDA level.* (A) pre-treatment of HepG2 cells with different concentrations of CGA (5, 10 and 50 µM) for 24 h before glucose supplementation to a final concentration of 25 mM and respectively 35 mM and (B) post-treatment of HepG2 cells for 24 h with different concentrations of CGA after supplementation of culture medium with glucose. Values of MDA are represented as means of three biological replicates ± SD. All samples were compared to NG control (*) and CGA-treated samples were compared with their correspondent HG control (#). Values were significant when $p < 0.5$ (* or #) and $p < 0.01$(** or ##).

Also in HepG2 cells grown in 25 mM glucose medium pre-exposed to CGA (Figure 2A) was insignificantly changed, whereas in those cultivated in 35 mM glucose medium it increased significantly by 1.6 ($p < 0.05$#) and 1.7 folds ($p < 0.01$##) only in the presence of 10 and respectively 50 µM CGA, compared to the correspondent free-CGA control.

Interestingly, a considerable decrease by 1.42 folds ($p < 0.01$##) was observed after pre-treatment with 10 µM CGA and incubation of cells in 25 mM glucose medium. The post-treatment with CGA induced no significant alteration of MDA level (Figure 2B).

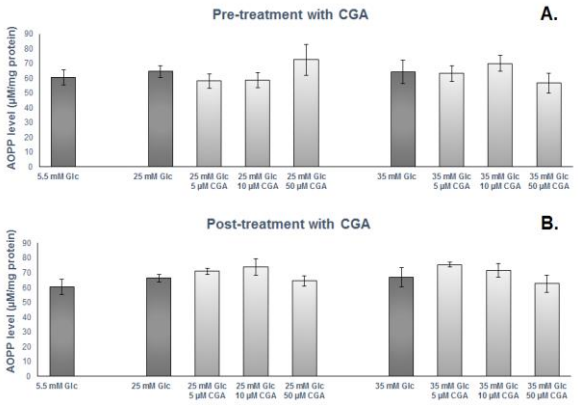

Figure 3. *Effects of glucose and CGA on AOPP level.* (A) pre-treatment of HepG2 cells with different concentrations of CGA (5, 10 and 50 µM) for 24 h before glucose supplementation to a final concentration of 25 mM and respectively 35 mM and (B) post-treatment of HepG2 cells for 24 h with different concentrations of CGA after supplementation of culture medium with glucose. Values of AOPP are represented as means of three biological replicates ± SD.

Figure 3 presents the levels of AOPP in the HepG2 cells after exposure to the two experimental set-ups. After evaluation of this parameter, the obtained results indicated no changes compared to NG control in any of the conditions.

Figure 4 shows the level of NO production in the culture medium after the pre- and post-treatment with CGA. In the first situation, we observed a slight increase of NO production by 1.11 folds ($p < 0.05$#) in the medium of cells treated with 50 µM CGA and 25 mM glucose compared with the one found in HG correspondent control cells. In the cell medium containing 35 mM glucose, the NO level was unchanged compared to NG control except in the medium of cells treated with 50 µM CGA where the NO level increased by 1.24 folds ($p < 0.01$##) vs. HG correspondent control.

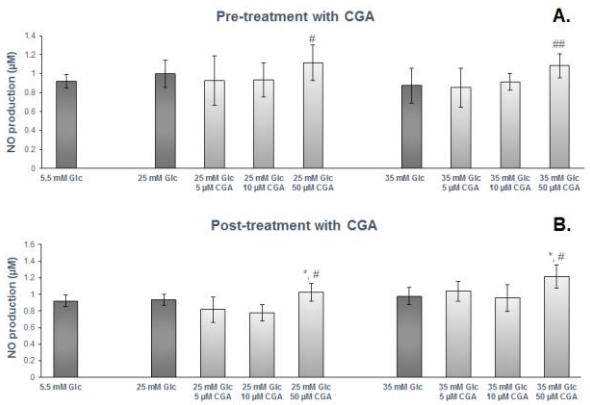

Figure 4. *Effects of glucose and CGA on NO production.* (A) pre-treatment of HepG2 cells with different concentrations of CGA (5, 10 and 50 µM) for 24 h before glucose supplementation to a final concentration of 25 mM and respectively 35 mM and (B) post-treatment of HepG2 cells for 24 h with different concentrations of CGA after supplementation of culture medium with glucose. Values of NO are represented as means of three biological replicates ± SD. All samples were compared to NG control (*) and CGA-treated samples were compared with their correspondent HG control (#). Values were significant when $p < 0.5$ (* or #) and $p < 0.01$(** or ##).

The addition of CGA after incubation of cells in HG medium resulted in a different response on NO production (Figure 4B). Cells grown in HG medium, either with 25 or 35 mM glucose, produced no change of NO level compared with NG cells. The same situation was also registered in cells treated with 5 and 10 µM CGA. Higher doses of 50 µM CGA, caused a significant elevation of NO level in medium with 25 and 35 mM glucose by 1.1 fold ($p < 0.05$#) and respectively by 1.24 fold ($p < 0.001$###) compared with their HG correspondent controls.

The expression of Nrf2 protein, a key regulator of the cellular antioxidant response was determined by western blot analysis.

As shown in Figures 5A and 5B, the expression variation of Nrf2 depended on the HG conditions: 25 mM respectively 35 mM in the culture media. The pre-treatment with CGA up-regulated the Nrf2 expression irrespective of the dose used in the case of cells grown in 25 mM glucose media. On the other hand, in HepG2 cells cultivated in 35 mM glucose media, the treatment with 5 µM CGA a slight increase of Nrf2 expression was noticed, followed by a significant decrease after exposure to 10 and 50 µM CGA compared to 35 mM glucose and NG conditions.

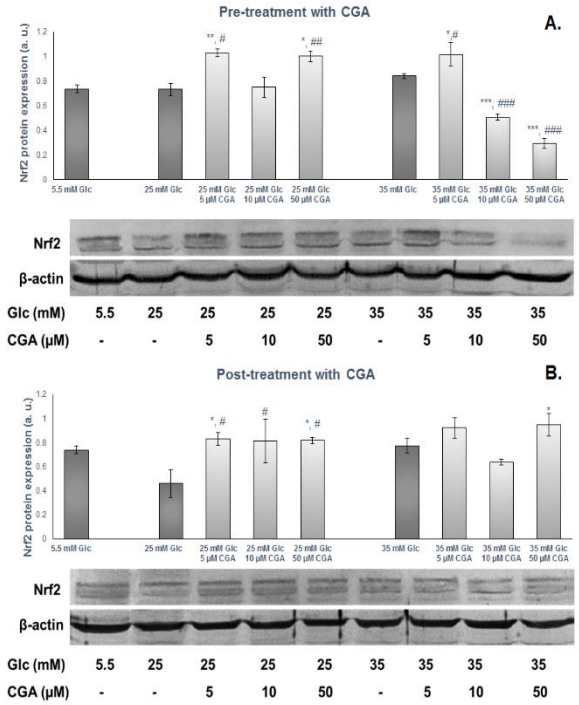

Figure 5. *Effects of glucose and CGA on Nrf2 protein expression.* (A) pre-treatment of HepG2 cells with different concentrations of CGA (5, 10 and 50 µM) for 24 h before glucose supplementation to a final concentration of 25 mM and respectively 35 mM and (B) post-treatment of HepG2 cells for 24 h with different concentrations of CGA after supplementation of culture medium with glucose. The blot images (below graphs) were quantified and the values for each band of Nrf2 protein were expressed in arbitrary units (a.u.) and represented as means of three biological replicates ± SD. All values were normalized to β-actin. All samples were compared to NG control (*) and CGA-treated samples were compared with their correspondent HG control (#). Values were significant when $p < 0.5$ (* or #), $p < 0.01$(** or ##) and $p < 0.001$(*** or ###).

The post-treatment with all three doses of CGA up-regulated this expression in 25 mM glucose conditions as well as the dose of 50 µM CGA in 35 mM glucose medium compared to NG control.

Discussions

This study aimed to reveal the role of 3-(3,4-

dihydroxycinnamoyl)quinic acid (CGA isomer) on hepatocytes response to high glucose concentration in order to gain more comprehensive understanding regarding the mechanisms that may suppress the effects of hyperglycemia in liver cells. To accomplish that, we compared the pre- and post-treatment with CGA on HG HepG2 cells. Our previous work (H. BAZOOL FARHOOD & al. [12]) proved that both the pre- and post-treatment with CGA reduces the HG-increased cell metabolic activity and ROS production close to normal, in human hepatocytes. Thus, the antioxidant capacity of CGA was appreciated through the evaluation of intracellular GSH concentration. The GSH is the most important non-enzymatic antioxidant and is synthesized in the framework of the γ-glutamyl cycle in the liver. This organ is the primary site for total body GSH turn-over and accounts for over 90% of GSH inflow into the systemic circulation. Considering these, liver cells dysfunction might affect endogenous production and utilization of GSH, and possibly further lead to dysregulation of the GSH-dependent antioxidant system (S.B. CHENG & al. [23]). The antioxidant activity of GSH is provided by the thiol group (−SH) of the cysteine which is involved in reduction and conjugation reactions. These reactions provide the means for removal of hydroperoxides, quenching of free radicals and detoxification of many xenobiotic compounds (H.J. FORMAN & al. [24]).

Our results showed that GSH level decreased in cells exposed to 35 mM glucose concentration. The pre-treatment with CGA (at any dose) resulted in the restoration of GSH content close to that of NG cells. This effect was not observed when cells were post-treated with CGA. Moreover, a decrease of GSH concentration was noticed when HG cells were post-treated with the highest concentration of CGA (50 µM) tested. These opposite results could indicate that CGA could promote both antioxidant and pro-oxidant effects in HG HepG2 cells depending by dose and moment of exposure. Relative to our previous findings, these results indicate that GSH concentration in HG HepG2 cells is not influenced significantly by ROS formation in post-treatment, probably due to an increased biosynthesis stimulated by Nrf2 up-regulation.

The CGAs exert antioxidant activity by donating hydrogen atoms to reduce free radicals such as superoxide anions (O2´), hydroxyl radicals (¨OH), 2,21-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and peroxylnitrite (ONOO´) and by inhibiting oxidation of lipids and DNA damage (N. LIANG & D.D. KITTS [15]). Antioxidant effect of CGA was demonstrated in several *in vitro* studies. It was shown that three CGA isomers (3-caffeoylquinic acid, 4-caffeoylquinic acid, and 5-caffeoylquinic acid) had the ability to suppress H_2O_2 generation and protect PC12 cells against apoptosis by inhibiting the mitochondrial membrane depolarization caused by oxidative stress (J.B. PARK, [25]). Also, it was demonstrated that 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid had a protective effect against ROS generation induced by t-butyl hydroperoxide in HepG2 cells (G. BAEZA & al. [26]). However, the pro-oxidant activity of CGA was also described and occur in the presence of oxygen and transition metals, such as Cu and Fe, producing ROS capable of damaging macromolecules, such as DNA and lipids (Y. SAKIHAMA & al. [27]).

Lipid peroxidation is a chain reaction easily initiated *in vitro* after ROS formation by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA). This process leads to the accumulation of lipid peroxidation products such as hydroperoxides and malondialdehyde (MDA), characteristic components of the first and respectively the second stages of lipid oxidation reactions. Lipid peroxidation can also cause damage to proteins and DNA.

According to our data, the HG conditions did not induce changes on the MDA level except in HepG2 cells exposed to 35 mM glucose in the pre-treatment set-up. Contrary to our expectation, in this case, it was observed a reduction in the formation of MDA products in HG control cells but an increase dependent on dose in those pre-treated with CGA. An inhibition of MDA under hyperglycemic condition was not reported until now.

MDA products can be generated through the decomposition of arachidonic acid and larger PUFAs, by either enzymatic or non-enzymatic processes. Once formed MDA can be enzymatically metabolized which could be a possible explanation for the reduction of MDA level observed in HepG2 cells. Glucose-6-phosphate isomerase or phosphoglucose isomerase

(PGI) (EC 5.3.1.9) plays a central role in glucose metabolism, increasing the amount of free glucose that can diffuse out of the cellular membrane. On the other hand, this enzyme is responsible for the biotransformation of cytoplasmic MDA to methylglyoxal (MG) and further to D-lactate by enzymes of the glyoxalase system by using GSH as a cofactor (Z.S. AGADJANYAN & al. [28]).

The HG conditions, as well as CGA treatment, did not determine advanced oxidation of proteins.

Under physiological or low lipid peroxidation rates (subtoxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or signaling pathways activation that upregulates antioxidant proteins resulting in an adaptive stress response. A commonly used redox biomarker for quantitating response of cells to oxidative stress is the transcription factor Nrf2. That is a member of the basic leucine zipper NF-E2 family and plays an essential role in the antioxidant response element-mediated expression of phase II detoxifying enzymes, including glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD) (N. LIANG & D.D. KITTS, [15]) as well as enzymes implicated in GSH synthesis. The Nrf2 antioxidant response has been shown to protect against a wide range of diseases including diabetes, cardiovascular disease, inflammation, cancer, aging etc. Previously, it was demonstrated that the Nrf2 pathway protects mice podocytes exposed to hyperglycemia partly through regulation of sirtuin 1 (Sirt1) activity (Q. ZHANG & al. [29]). Another study reported that the expression of Nrf2 is enhanced by ROS formation and inhibited by antioxidants in hepatic cells (C. MA-ON & al. [30]).

Addition of CGA resulted in the elevation of Nrf2 protein expression both in the pre and post-treatment set-ups in most of the sample. However, a pronounced decrease was also noticed in cells cultured in 35 mM glucose and pre-treated with 10 and 50 µM CGA and respectively post-treated with 10 µM CGA. The reduction of Nrf2 protein expression could be explained by its translocation into the nucleus where it activates an antioxidant response. Recently, H. ZHANG & al. [31] demonstrated that fisetin, a dietary flavonoid with antioxidant activity upregulates the protein level of Nrf2 and downregulates the protein level of Kelch-like ECH-associated protein 1 (Keap1) in HepG2 cells (H. ZHANG et al., [31]). When exposed to toxic substances, thus leading to oxidative stress, Nrf2 dissociates from Keap1 and enters into the nucleus to protect from ubiquitination degradation and activates an antioxidant response.

Nrf2 could also play a pivotal role in inflammation. Several studies have demonstrated that Nrf2 contributes to the anti-inflammatory process by regulating gene expression through the antioxidant response element (ARE) inhibiting the progression of inflammation (S.M.U. AHMED & al. [32]). Furthermore, it was shown that Nrf2–Keap1 signaling pathway could be activated by nitric oxide (NO) and participates in cell responses to oxidative and nitrosative stresses caused by excessive NO exposure (C.Q. Li & al. [33]). NO is a physiological signaling molecule that plays an important role in many metabolic processes but at high concentrations has toxic effects acting as a pro-inflammatory mediator.

Exposure of HepG2 cells to high glucose medium resulted in no elevation of NO production in both set-up experiments. Moreover, the addition of CGA caused no significant change on NO levels compared to HG correspondent controls except for the highest CGA dose (50 µM). At this concentration of CGA, the NO level significantly increased in any of the conditions (no more than 32%) suggesting rather a pro-oxidant effect of this compound and possible an inflammatory response of HepG2 cells.

According to previous literature, elevated levels of glucose may enhance NO production through increased expression of inducible and endothelial nitric oxide synthases (iNOS and eNOS) gene and protein levels (R. ADELA & al. [34]). In hepatocytes, production of NO is mediated by iNOS. Under hyperglycemic conditions, iNOS produces large amounts of NO though is still not clear whether higher NO levels are beneficial or detrimental to the cell metabolism. Experimental evidence revealed that NO could be either a primary mediator of liver cell injury or a potent protective mechanism against injurious stimuli depending upon the NO concentration (Y. IWAKIRI & M.Y. KIM, [35]). According to our study, the potential of CGA to influence NO production is dose-dependent. While the low doses of CGA registered no significant impact on NO level, at a higher

dose of 50 µM CGA, a clear elevation of NO production was observed. Epidemiological studies reveal that a diet rich in polyphenols contributes to the counteraction of oxidative stress and enhancement of NO generation with many beneficial effects on human health. Flavonoids may affect NO production by different mechanisms such as NO radical scavenging, modulating of iNOS enzyme activity and gene expression as well as protein-binding properties but also by decreasing its breakdown, promoting higher levels overall. A study conducted by Q. GUO & al., [36], showed that stimulation of NO production could vary depending on polyphenol type in murine macrophages. Thus, they found that NO radical formation remained largely unaffected by the monomeric flavonoids catechin and epicatechin, whereas the addition of 50 µg/ml taxifolin to the medium significantly decreased NO production in RAW 264.7 cells by about 60% and increased NO radical formation up to 80% in the presence of the dimeric procyanidins B 1, B2, and B3.

Overall, our data brought evidence of the protective effect of CGA on hepatic cells under hyperglycemic conditions by modulating the expression of molecules implicated in cell response to oxidative stress including the reduced glutathione and Nrf 2 protein.

Conclusions

The findings of the present investigation demonstrated the protective effects of CGA on human hepatocytes by attenuating markers of hyperglycemia-mediated oxidative stress. The efficiency of CGA was evident particularly when it was used in the pre-treatment experiment. Our data revealed that CGA is an important bioactive compound which is involved in the counteracting the damaging effects induced by hyperglycemia in HepG2 cells.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

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