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Research article

ANTIOXIDANT EFFECT OF A FERMENTED POWDER OF LADY JOY BEAN IN PRIMARY RAT HEPATOCYTES

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Abstract: The role and beneficial effects of plant and food extracts against various diseases induced by oxidative stress have received much attention in recent years. Legumes are rich in bioactive compounds, and some studies suggest a correlation between their consumption and a reduced incidence of diseases. Primary cultures of rat hepatocytes were used to investigate whether and how an extract obtained from a fermented powder of bean named Lady Joy (*Phaseolus vulgaris* L.) is able to regulate antioxidant and detoxifying enzymes through the NRF2 pathway, inhibit NF-kB activation, and reduce H_2O_2-induced endoplasmic reticulum (ER) stress. All of the antioxidant and detoxifying enzymes studied were significantly up-regulated by Lady Joy treatment. Western blot showed that Nrf2 was activated by Lady Joy treatment. Also, cells treated with this fermented bean were partially protected against NF-kB activation resulting from H_2O_2 stress. As a link between oxidative stress and

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Abbreviations used: ALA – α-lipoic acid; ARE – antioxidant responsive element; BiP – immunoglobulin binding protein; CHOP – C/EBP homologous protein; ER – endoplasmic reticulum; GADD153 – growth arrest and DNA-damage-inducible protein; GRP – glucose-regulated protein, 78 kDa; GSH – glutathione; GSSG – oxidized glutathione; GST – glutathione-S-transferase; H_2O_2 – hydrogen peroxide; HO-1 – heme oxygenase-1; IkBα –nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Keap1 – Kelch-like ECH-associated protein 1; NF-kB – nuclear factor-kappa B; NQO1 – NAD(P)H:quinone oxidoreductase; Nrf2 – nuclear factor E2-related protein; ORAC – oxygen radical absorbance capacity; PARP-1 – poly [ADP-ribose] polymerase 1; ROS – reactive oxygen species

ER dysfunction is hypothesized, we verified whether Lady Joy was able to protect cells from H_2O_2-induced ER stress, by studying the response of the proteins CHOP, BiP and caspase 12. The results of this study show that Lady Joy can induce the Nrf2 pathway, inhibit NF-kB, and protect ER from stress induced by H_2O_2.

Keywords: Fermented bean, Antioxidants, Anti-inflammatory, Oxidative stress, Hepatocytes, NRF2, NF-kB, Antioxidant enzymes

INTRODUCTION

Oxidative stress, defined as a loss of balance between the cellular concentration of reactive oxygen species and the antioxidant capacity of the cell itself, is implicated in the onset of various diseases [1]. Some natural substances interrupt the chain reactions that lead to the formation of additional radicals, thus preventing the propagation of cell damage, while others play the role of *scavengers* of reactive oxygen species [2]. In recent years, several plants have been studied because of their antioxidant properties that could be useful in the prevention of oxidative stress reactions, such as those mediated by the formation of free radical species. Legumes are an important group of plant food, consisting in edible seeds belonging to the *Leguminosae* or *Fabaceae* family. The proteins contained in legumes are deficient in sulfur amino acids and tryptophan, though other essential amino acids, such as lysine and threonine, are abundant [3]. The lipids present in this food group are generally polyunsaturated fatty acids, such as linoleic acid, linolenic acid and lipoic acid. Many epidemiological and clinical studies have shown a correlation between the consumption of beans and a decrease in the incidence of various diseases, such as cancer and cardiovascular disease [4]. Beans also contain phytohemagglutinin, which is toxic if not thermally denatured, and an alpha-amylase inhibitor, which is active against salivary and pancreatic human alpha-amylase [5]. To cope with this problem, a bean variety, named Lady Joy, has been developed and shown to be devoid of lectin activity (6; patent no. EP1732397 A2). Recent studies have consistently shown that some phytochemicals are able to regulate antioxidant and phase II enzymes through the activation of a nuclear factor E2-related protein (Nrf2) [7]. In its inactive state, Nrf2 is found in a protein complex with Keap1. Various agents, including ROS and weak electrophiles, can phosphorylate or alkylate one or more cysteine residues on Keap1, which releases Nrf2 from the complex. Nrf2 then binds to antioxidant responsive element (ARE) sites in the promoter regions of antioxidant and phase II genes, thereby inducing transcription of those genes [8]. In recent years, many authors have theorized the existence of a cross-talk between the Nrf2/ARE and the nuclear factor-kappa B (NF-kB) systems in response to inflammation [9]. In the absence of stimuli, NF-kB is associated with an inhibitor protein, IkBα. Upon stimulation, IkBα is rapidly phosphorylated on serine residues, which targets the

inhibitor to ubiquitination and degradation by proteasomes. Nrf2 -/- cells are sensitive to a variety of ER stress inducing agents, and Nrf2 over-expression enhances survival during the unfolded protein response [9]. Perturbations in ER homeostasis, due to certain stress stimuli such as nutrient deprivation, oxidative stress or ER Ca^{2+} depletion, lead to the accumulation of unfolded or misfolded proteins within the lumen of the ER, a condition known as ER stress [10-11].
In the present study, we investigated the effects of a fermented powder of the bean Lady Joy, on the antioxidant and drug-metabolizing enzymes in primary rat hepatocytes. In addition, the effect of Lady Joy on the NF-kB pathway was examined. Finally, to investigate the possible link between oxidative stress and endoplasmic reticulum dysfunction, we tested the ability of Lady Joy to reduce H_2O_2-induced ER stress.

MATERIALS AND METHODS

Chemicals

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-Nrf2 (sc-13032), anti-heme oxygenase-1 (sc-10789), anti-NF-kB (sc-7178), anti-β-actin (sc-130657), anti-PARP-1 (sc-25780), anti-GADD153 (sc-793), anti-caspase-12 (sc-5627), anti-GRP78 (sc-13968) and goat anti-rabbit (1:2000 or 1:5000) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Collagen (type I) was prepared by the method of Beken et al. [12].

Preparation of Lady Joy

The cultivar Lady Joy (*Phaseolus vulgaris*) is a common bean genetically devoid of the toxic constituent phytohemagglutinin [6]. The lysate of Lady Joy was prepared by Agrisan SRL (Larciano, PT, Italia). The starting cultures for fermentation typically consist of a mix of lacto-bacillus and natural yeast strains. Lady Joy lysate and rough powder (flour of bean) were extracted with distilled water, sonicated and centrifuged. The supernatants were collected for cell treatments.

Primary rat hepatocyte isolation, cell culture and treatments

Hepatocytes were isolated from 200-300 g Wistar male rats with free access to drinking water and food and on a 12 h light/dark cycle. The research using animals was approved by the Italian Ministry of Health in compliance with European Community law no. 116/92. The approved protocol number is 10/09. Rats were anesthetized with 40 mg/kg ketamine, and the liver was perfused as described previously [12-13]. The cells were dispersed in Williams' E medium containing 39 ng/ml dexamethasone, 0.5 U/ml insulin, 0.007 µg/ml glucagon, 5 µg/ml penicillin and streptomycin, 5 µg/ml ampicillin and kanamycin and 10% fetal bovine serum. The cells were plated on a collagen pre-coated dish. After 5 h, the medium was replaced with serum-free Williams E medium. After 24 h, the second layer of type I collagen was added to create a collagen-gel sandwich culture [12]. Cells were divided into four different groups: in the first group (control), the cells

were treated with medium only; in the second one with Lady Joy 0.7 mg/ml (LJ); in the third group with H_2O_2 200 μM; and in the last group, the H_2O_2 200 μM was added after 1 h pre-treatment with Lady Joy 0.7 mg/ml (LJ + H_2O_2). We used H_2O_2 200 μM based on our previous work [14]. To study endoplasmic stress, we used tunicamycin 10 μg/ml as a positive control for H_2O_2. We used for Lady Joy different concentrations from 0.1 to 2.8 mg/ml, and 0.7 mg/ml was the best concentration in terms of maximum protective effect and without toxicity. At 0.7 mg/ml concentrations, Lady Joy produced no adverse cytotoxic effects over the exposure periods compared with the vehicle-only controls, as measured by lactate dehydrogenase assay.

Characterization of phytocompounds

Total phenolics content was determined according to the method described by Singleton [15]. Total flavonoid content was measured using the aluminum chloride method [16]. Total flavonols content was determined by the method described by Romani et al. [17]. Total monomeric anthocyanins were determined as described by Lee et al. [18]. The antioxidant capacity of lysate and flour of bean was determined using the oxygen radical absorbance capacity (ORAC) assay, according to Ninfali et al. [19]. Peroxyl radicals were generated by 2,2-azobis (2-amidinopropane) dihydrochloride, and fluorescein was used as a probe. In each experiment, Trolox was used as a standard.

Preparation of subcellular fractions and enzymatic activities

After 24 h treatment, a collagenase solution was added for 30 minutes; then recovered cells were centrifuged (400 xg) at 4°C. The cell pellet was sonicated and used for the mitochondrial [20] and microsomal preparation [21]. The protein content was determined by the method of Lowry et al. [22], using bovine serum albumin as a standard. NAD(P)H:quinone oxidoreductase (NQO1) activity was measured by the method of Bensen et al. [23]. Glutathione-S-transferase (GST) activity was quantified as previously described by Habig et al. [24]. The GSSG reductase activity was measured by monitoring the consumption of NADPH for 1 minute at 340 nm, while the catalase activity was monitored as described by Cao and Li [25]. Heme oxygenase-1 (HO-1) activity was determined by the method of Naughton et al. [26]. Lactate dehydrogenase activity was assayed as previously described [27]. Reduced GSH was measured using the method previously described by Hissin and Hilf [28].

Purification of total RNA, cDNA synthesis and real-time PCR

Total cellular RNA was extracted from primary rat hepatocytes 4 h after treatment, using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using NanoDrop (Celbio, Mi, Italy). Genomic DNA elimination and reverse transcription of total RNA were performed using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time amplification reactions were performed using Rotor-Gene 3000 (Corbett) by adding 2 μl of 10-fold diluted cDNA and 400 nM of each primer to a real-time PCR supermix (SsoFast EvaGreen

Supermix, Bio-Rad, Hercules, CA, USA). All the primer pairs were designed with the Beacon Designer 5.0 software and synthesized by Sigma (St. Louis, MO, USA) (Table 1). Amplification reaction efficiency of each sample was confirmed to be similar and higher than 1.6. The relative expression levels were calculated with respect to the expression of the controls, the mean of which was assigned a value of 1.

Table 1. Primer pairs, annealing temperature (T) and product size for real-time PCR experiments.

Gene	Forward (5'-3')	Reverse (5'-3')	Annealing T (°C)	Product size (bp)
NQO1	GGTGGAAGAAGCGTCTGGAG	TGGTTGTCGGCTGGAATGG	58	187
HO-1	TCTGGAATGGAAGGAGATGCC	GAACAGCCGCCTCTACCG	56	130
GCL (c)	GCACATCTACCACGCAGTCAAGG	ACATCGCCGCCATTCAGTAAC	58	129
GCL (m)	GCGAGGCAGCGGTCTTCC	CCCAGTTGAGCAGGTTCCC	58	160
β-actin	TGGGACGATATGGAGAAGATTTGG	GGACAACACAGCCTGGATGG	60	189

Preparation of nuclear fractions

Nuclear and cytosolic extracts were prepared by previously established methods [29]. Cells were harvested in PBS and centrifuged at 800 g for 3 min at 4°C. The pellet was resuspended in 200 µl of cold hypotonic buffer, consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 µM dithiothreitol and a complete protease inhibitor cocktail (Sigma antiprotease cocktail P8340). After addition of NP40 to a final concentration of 0.3%, the cells were vortexed and centrifuged at 4°C. The resulting nuclear pellet was resuspended in cold nuclear extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 µM dithiothreitol, 25% glycerol and protease inhibitors) and incubated on ice for 30 min. The nuclear extract was finally centrifuged and the supernatant containing nuclei proteins was aliquoted and stored at -30°C.

Immunoblot analysis

Nuclear and microsomal proteins were separated according to Laemmli [30] on SDS-10% (v/v) polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes following the method of Towbin et al. [31]. Antibodies used were anti-Nrf2 (1:10000), anti-heme oxygenase-1 (1:1000), anti-β-actin (1:1000), anti-PARP-1 (1:1000), anti-NF-kB (1:1000), anti-GADD153 (1:1000), anti-caspase-12 (1:1500), anti-GRP78 (1:1500) and goat anti-rabbit (1:2000 or 1:5000). Immunoreactive proteins were visualized with a chemiluminescence reaction kit (EuroClone, Mi, Italy) and bands obtained from five independent rat experiments were electronically scanned and quantified by Image J software.

Statistical analysis

Data are expressed as mean ± S.D. from five independent experiments, and statistical significance was evaluated by Student's t-test or one-way ANOVA followed by Bonferroni post-test. Data were considered statistically significant when $p < 0.05$.

RESULTS

Characterization of phytocompounds

In these experiments, we chose to study total polyphenols, flavonoids, and flavonols. We compared the ORAC values in the lysate and in the flour of the bean. The total polyphenol content in the lysate was significantly higher than in the flour (3.8 ± 0.24 and 2.348 ± 0.05 mg GAE/g dw respectively, $p < 0.001$). Flavonoid levels in the lysate and the flour were found to be statistically significantly different (2.1 ± 0.1 and 1.6 ± 0.1 mg CE/g dw respectively, $p < 0.01$). Flavonol concentration was reduced after fermentation (0.4 ± 0.1 and 1.6 ± 0.05 mg QE/g dw respectively, $p < 0.001$). The antioxidant activity in the bean lysate and flour, evaluated with ORAC assay, was 1233 ± 23 and 730 ± 30 μmol TE/100 g sample, dw, respectively ($p < 0.001$). Since the bean lysate showed higher polyphenol and flavonoid content and a stronger total antioxidant capacity than the bean flour, we decided to treat the rat hepatocytes only with the Lady Joy lysate.

Effect of Lady Joy on phase II enzymes and Nrf2

We investigated the effect of Lady Joy on both cytosol and mitochondrial reservoirs of GSH individually. Cytosol GSH levels rose significantly above the control value (109.2 ± 1.2 nmol/mg prot) in both LJ + H_2O_2 (163.5 ± 1.4 nmol/mg prot) and LJ-only (141.8 ± 1.1 nmol/mg prot) treated cells. The same pattern was observed for mitochondrial GSH levels, though to a different extent; mitochondrial GSH levels in the control was 64.1 ± 1.6 nmol/mg prot) and increased significantly in both LJ + H_2O_2 (160.5 ± 2.4 nmol/mg prot) and LJ-only (131.4 ± 2.1 nmol/mg prot). Furthermore, both Lady Joy and Lady Joy + H_2O_2 were able to enhance NQO1 activity by about 0.5- and 0.2-fold, respectively (Fig. 1A). HO-1 (Fig. 1B) activity was increased above the control levels by Lady Joy (by about 0.7 times the control value) and Lady Joy + H_2O_2 (by about 0.3 times the control value) treatment. GST activity followed the same pattern (Fig. 1C), being increased by both Lady Joy (by about 0.4 times the control value) and Lady Joy + H_2O_2 (by about 0.3 times the control value), but not by the 24 h treatment with H_2O_2 alone. We observed the same pattern for catalase activity (Fig. 1D). Indeed, catalase activity increased following Lady Joy treatment by about 0.4 fold and by about 0.3 fold following Lady Joy + H_2O_2 treatment, but remained unchanged after 24 h treatment with H_2O_2.

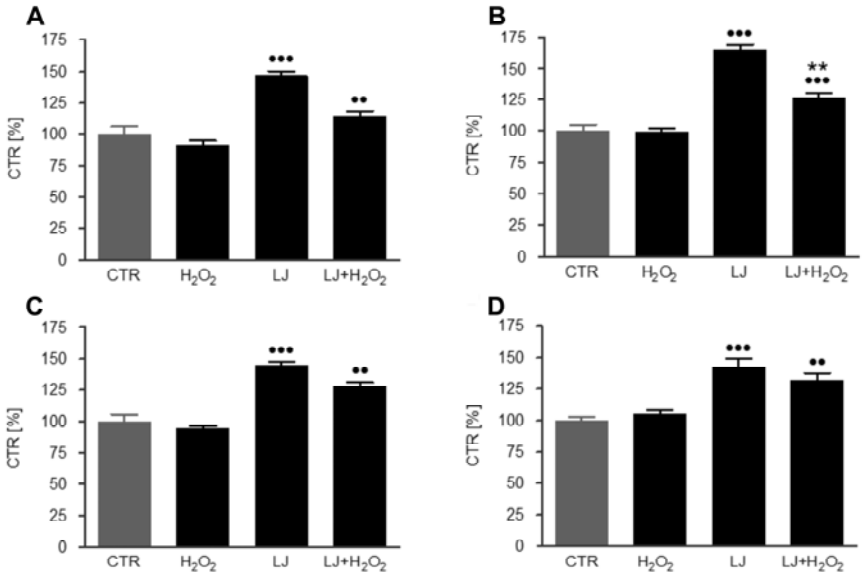

Fig. 1. Antioxidant enzyme activity: A – NAD(P)H:quinone oxidoreductase; B – heme oxygenase-1; C – glutathione-S-transferase; D – catalase. Results are expressed as percentages of control values. **Significantly different from controls, $p < 0.01$. ***$p < 0.001$. ••Significantly different from H_2O_2, $p < 0.01$. •••$p < 0.001$.

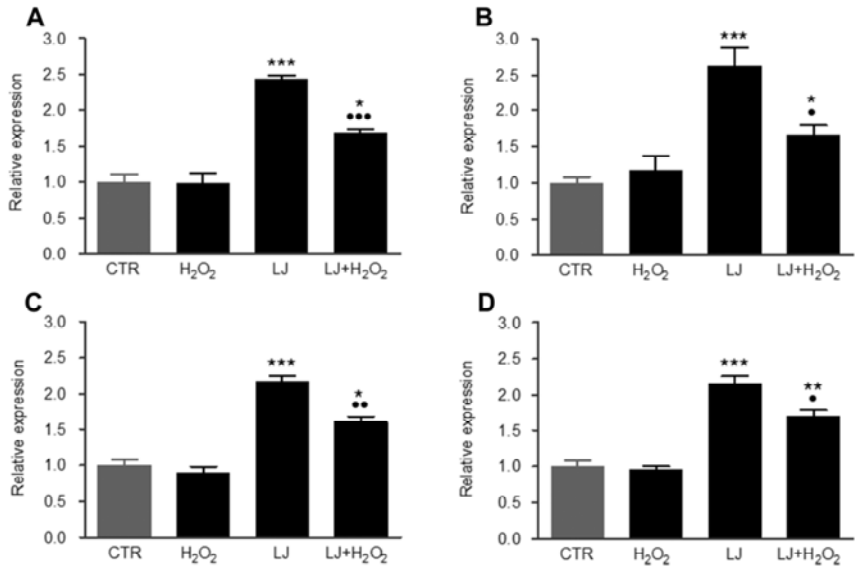

Fig. 2. Real-time PCR analysis: A – NAD(P)H:quinone oxidoreductase; B – heme oxygenase-1; C – GCL(c); D - GCL(m). Results are normalized against β-actin housekeeping genes and referred to the control mean, which was assigned the value 1. *Significantly different from controls, $p < 0.05$. **$p < 0.01$. ***$p < 0.001$. •Significantly different from Lady Joy, $p < 0.05$. ••$p < 0.01$. •••$p < 0.001$.

The NQO1 and HO-1 enzymes and GSH were chosen for additional analysis at the transcriptional level. Since GSH is a heterodimer, comprising a catalytic subunit (GCL(c)) and a regulatory subunit (GCL(m)), we verified the effect of Lady Joy in the presence and absence of H_2O_2, on both subunits at the transcriptional level. Cells were treated with 0.7 mg/ml Lady Joy and 200 μM H_2O_2 for 4 h. As shown in Figs. 2A-B-C-D, treatments with Lady Joy and Lady Joy + H_2O_2 increased the expression of all the genes examined in a similar manner. The effect of Lady Joy on heme oxygenase-1 was also assessed at the protein level by western blot (Fig. 3). In microsomes from hepatocytes treated with Lady Joy, the anti-rat HO-1 antibodies revealed a significant increase in intensity of an immunoreactive protein band of about 1.7 fold with respect to the control. As expected, 24 h of treatment with 200 μM H_2O_2 had no effect.

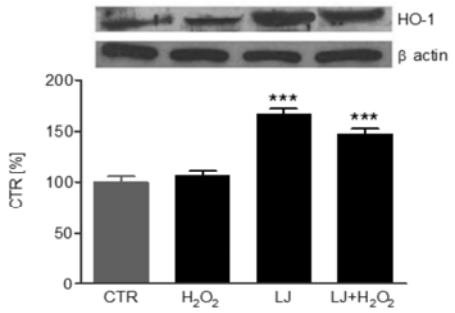

Fig. 3. Western blot analysis of heme oxygenase-1 protein in microsomes. Densitometric analysis of the western blot data are shown in the histograms below. The results have been normalized to β-actin levels and are expressed as percentages of control. ***Significantly different from controls, $p < 0.001$.

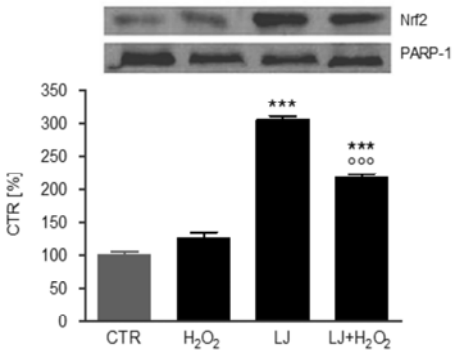

Fig. 4. Western blotting analysis of Nrf2 in nuclear extracts of control cells (CTR) and cells treated with Lady Joy or Lady Joy + H_2O_2. Densitometric analysis of the western blot data are shown in the histograms below. The results have been normalized to PARP-1 levels and are expressed as percentages of control. ***Significantly different from controls, $p < 0.001$. •••Significantly different from Lady Joy, $p < 0.001$.

Pre-treatment (1 h) with Lady Joy before H_2O_2 raised HO-1 protein levels above the control value, but to a lesser extent than in cells treated with Lady Joy only. To verify whether Nrf2 was activated in primary rat hepatocytes treated for 1 h with Lady Joy, we analyzed the nuclear fraction using western blot (Fig. 4). In the Lady Joy treated cells, the intensity of the protein band increased to about 300% of the control value, whereas in the Lady Joy + H_2O_2 treated cells, the band intensity was a bit lower – about 200% of the control value.

Effect of Lady Joy on H_2O_2-induced lactate dehydrogenase

Lactate dehydrogenase is a stable cytosolic enzyme that is released when the cell is lysed or when there is any injury to the cell membrane. Results were calculated as percentages, of 100% activity of total lysed cells (Fig. 5). A significant increase in lactate dehydrogenase (LDH) activity (15% of whole cell activity) was observed in the medium when cells were exposed to H_2O_2 alone. Interestingly, when cells were pretreated for 1 h with the Lady Joy (LJ) extract before H_2O_2 treatment, a reduction in H_2O_2-induced cell injury was observed as assessed by a decreased LDH release (5%).

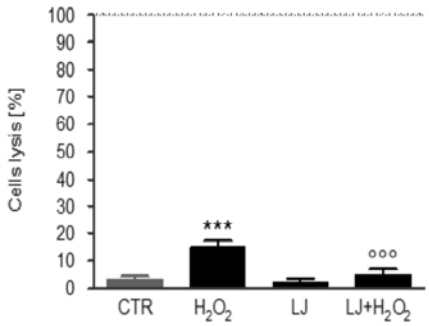

Fig. 5. Lactate dehydrogenase. Results are expressed as percentages, of 100% activity of total lysed cells. ***Significantly different from controls, $p < 0.001$. °°°Significantly different from H_2O_2, $p < 0.001$.

Effect of Lady Joy on NF-kB and endoplasmic reticulum (ER) stress

It is known that the oxidative stress that occurs in the presence of extracellular hydrogen peroxide can trigger NF-kB nuclear translocation [32]. Therefore, we analyzed the presence of NF-kB in the nuclear fraction of control and treated hepatocytes using western blot (Fig. 6). As expected, in nuclei of H_2O_2-treated hepatocytes, the anti-NF-kB immune reactive protein used in this assay produced a strong signal (350% of the control value). In the nuclei of control cells and in those treated with Lady Joy, the protein band was very weak. An important result was found in the nuclear extracts of hepatocytes treated with Lady Joy + H_2O_2. Indeed, the intensity of the protein band was reduced compared to cells treated with H_2O_2 only, to about 160% of the control value.

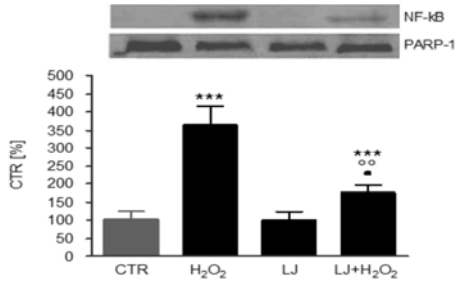

Fig. 6. Western blotting analysis of NF-kB in nuclear extracts. Densitometric analyses of the western blot data are shown in the histograms below. The results have been normalized to PARP-1 levels and are expressed as percentages of control. ***Significantly different from controls, $p < 0.001$. °°Significantly different from H_2O_2, $p < 0.01$. •Significantly different from Lady Joy, $p < 0.05$.

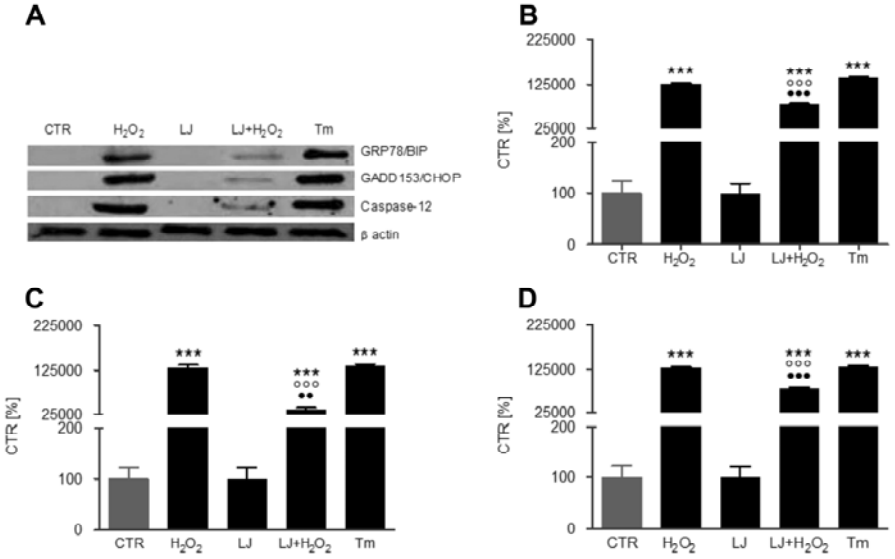

Fig. 7. Western blotting analysis: A – BIP, CHOP and caspase 12. Densitometric analysis of the western blot data are shown in the histograms below: B – BIP; C – CHOP; D – caspase 12. The results have been normalized to β-actin levels and are expressed as percentages of control. ***Significantly different from controls, $p < 0.001$. °°°Significantly different from H_2O_2, $p < 0.001$. •••Significantly different from Lady Joy, $p < 0.001$; ••$p < 0.01$.

Since recent studies have postulated a link between oxidative stress and endoplasmic reticulum dysfunction [33], we verified whether Lady Joy was able to protect cells from H_2O_2-induced ER stress. Fig. 7A shows western blots of three important proteins (BIP, CHOP, caspase 12), which are up-regulated during ER stress. Indeed, the blots show that tunicamycin treatment induced

a very strong increase of BIP, CHOP and caspase 12 levels. This result is also presented in the form of histograms in Figs 7B, C and D. Lady Joy treatment, on the other hand, did not cause any change in the immunoreactive bands of these proteins with respect to their control levels. Pretreatment with Lady Joy before H_2O_2 markedly reduced the intensity of all the three protein bands analyzed, indicating a clear protective effect of the bean lysate on ER stress induced by H_2O_2.

DISCUSSION

In the present study, we took advantage of the Lady Joy variety, which is devoid of phytohemagglutinin, to investigate whether a fermented powder of this bean was able to regulate phase 2 enzymes by causing the Nrf2 protein to translocate into the nucleus, which happens when this factor is activated by a trigger. We also explored the possibility that Lady Joy could prevent NF-kB activation following cell exposure to H_2O_2. Preliminary tests established that a 200 μM solution of H_2O_2, used to induce ROS, was unable to trigger Nrf2 or, consequently, to induce expression of its target genes (data not shown). Our results show that NQO1 and HO-1 (targets of Nrf2 activation) do not respond to H_2O_2, whereas they are induced by Lady Joy treatment. Many phytochemicals can induce detoxifying enzymes; curcumin, for example, has been observed to protect primary rat hepatocytes against ethanol-induced oxidative stress, via dose- and time-dependent induction of Nrf2-regulated enzymes [34]. A recent paper of ours showed that Lisosan G can regulate phase 2 enzymes in primary rat hepatocytes, through the activation of the Nrf2 pathway and by reducing the translocation of NF-kB to the nucleus [13]. Immunoblot of nuclei showed that Nrf2 was activated by Lady Joy treatment and that NF-kB, which usually is activated by H_2O_2 stress, was less induced following treatment with the bean lysate. Here, we investigated the ability of Lady Joy to restrict H_2O_2-induced NF-kB activation. As expected, Lady Joy pretreatment reduced the amount of NF-kB protein found in the nuclei compared with hepatocytes treated only with H_2O_2. The experiments on H_2O_2-induced ER stress indicate that this fermented bean powder provides partial cytoprotection. The work of Tanaka et al. [35] is one of the few studies available discussing the ability of natural compounds to help the ER cope with this stress; it showed that tunicamycin-induced cytotoxicity in Neuro2a cells is significantly suppressed by pretreatment with genipin (found in gardenia fruit extract). As reported in the results section, Lady Joy is rich in polyphenolic and flavonoid compounds, and unpublished results have shown that this lysate also contains α-lipoic acid (ALA) at a concentration of 186 mg/kg. In human leukemia HL-60 cells and neuroblastoma SH-SY5Y cells, ALA was shown to upregulate NQO1 gene transcription [36-37]. Ogborne et al. [38] have shown that in human monocytic cells, ALA induces HO-1 expression via Nrf2. It is known that ALA is an inhibitor of NF-kB [39]. Many of the major phytochemicals such as flavonoids and terpenoids, driven by NF-kB signaling, possess significant therapeutic properties including anti-

inflammatory effects [40]. Gallotannin, a polyphenolic hydrolyzable tannin, has anti-inflammatory and anti-cancer properties, and this may be mediated in part by the suppression of NF-kB activation [41]. The high content of vitamins, proteins, fiber, and also phytochemicals, in beans is responsible for some of the health effects associated with the consumption of this food, such as antioxidant capacity and decrease in the incidence of cancer and cardiovascular disease [4]. In the present study we evaluated some beneficial effects of Lady Joy, a variety of bean which not only shows the properties mentioned above but also a special feature, as it comes from a cultivar genetically selected as devoid of toxic phytohemagglutinin. Basically it was possible to uncover some of the molecular mechanisms associated with the treatment with Lady Joy: up-regulation of the NRF2 pathway and inhibition of NF-kB, as well as protection of the endoplasmic reticulum against oxidative stress induced by H_2O_2. In conclusion, a diet supplemented with Lady Joy may exert positive effects on human health.

REFERENCES

1. Valko, M., Leibfritz, D., Moncol, J., Cronin M.T.D., Mazur, M. and Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. **Int. J. Biochem. Cell Biol.** 39 (2007) 44-84. DOI: 10.1016/j.biocel.2006.07.001.
2. Rice-Evans, C.A., Miller, N.J., Bolwell, P.J., Bramley, P.M. and Pridham, J.B. The relative antioxidant activities of plant derived polyphenolic flavonoids. **Free Radic. Res.** 22 (1995) 375-383.
3. Chagas, E.P. and Santoro, L.G. Globulin and albumin proteins in dehulled seeds of three Phaseolus vulgaris cultivars. **Plant Foods Hum. Nutr.** 51 (1997) 17-26.
4. Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. and Etherton, T.D. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. **Am. J. Med.** 113 (2002) 71S-88S. DOI: 10.1016/S0002-9343(01)00995-0.
5. Lioi, L., Galasso, I., Lanave, C., Daminati, M.G., Bollini, R. and Sparvoli, F. Evolutionary analysis of the APA genes in the Phaseolus genus: wild and cultivated bean species as sources of lectin-related resistance factors? **Theor. Appl. Genet.** 115 (2007) 959-970. DOI: 10.1007/s00122-007-0622-1.
6. Confalonieri, M., Bollini, R., Berardo, N., Vitale, A. and Allavena, A. Influence of phytohemagglutinin on agronomic performance of bean (*Phaseolus vulgaris*. L.). **Plant Breeding** 109 (1992) 329-334.
7. Wu, X., Zhou, Q.H. and Xu, K. Are isothiocyanates potential anti-cancer drugs? **Acta Pharmacol. Sin.** 30 (2009) 501-512. DOI: 10.1038/aps.2009.50.
8. Buckley, B.J., Marshall, Z.M. and Whorton, A.R. Nitric oxide stimulates Nrf2 nuclear translocation in vascular endothelium. **Biochem. Biophys. Res. Commmun.** 307 (2003) 973-979. DOI: 10.1016/S0006-291-X(03)01308-1.

9. Saw, C.L., Qing, W. and Ah-Ng, T.K. Anti-cancer and potential chemopreventive actions of ginseng by activating Nrf2 (NFE2L2) anti-oxidative stress/anti-inflammatory pathways. **Chin. Med.** 5 (2010) 37. DOI: 10.1186/1749-8546-5-37.
10. Cullinan, S.B. and Diehl, J.A. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following ER stress. **J. Biol. Chem.** 279 (2004) 20076-20087. DOI: 10.1074/jbc.M314219200.
11. Schroder, M. and Kaufman, R.J. ER stress and the unfolded protein response. **Mutat. Res.** 569 (2005) 29-63.
12. Beken, S., Vanhaecke, T., De Smet, K., Pauwels, M., Vercruysse, A. and Rogiers, V. Collagen-gel cultures of rat hepatocytes: collagen-gel sandwich and immobilization cultures. **Method Mol. Biol.** 107 (1998) 303-309. DOI: 10.1385/0-89603-519-0:303.
13. De Smet, K., Beken, S., Vanhaecke, T., Pauwels, M., Vercruysse, A. and Rogiers, V. Isolation of rat hepatocytes. **Methods Mol. Biol.** 107 (1998) 295-302. DOI: 10.1385/0-89603-519-0:295.
14. La Marca, M., Beffy, P., Pugliese, A. and Longo, V. A fermented powder of wheat is an inducer of the antioxidant/detoxifying system in primary rat hepatocytes. **PLoS One** 8 (2013) 1-9. DOI: 10.1371/journal.pone.0083538.
15. Singleton, V.L., Orthofer, R. and Lamuela-Raventos, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. **Meth. Enzymol.** 299 (1999) 152-178.
16. Kim, D., Chun, O., Kim, Y., Moon, H. and Lee, C. Quantification of phenolics and their antioxidant capacity in fresh plums. **J. Agric. Food Chem.** 51 (2003) 6509-6515.
17. Romani, A., Mancini, P., Tatti, S. and Vincieri, F. Polyphenols and polysaccharides in Tuscan grapes and wines. **Ital. J. Food Sci.** 8 (1996) 13-24.
18. Lee, J., Durst, R.W. and Wrolstad, R.E. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. **J. AOAC Int.** 88 (2005) 1269-78.
19. Ninfali, P., Mea, G., Giorgini, S., Rocchi, M. and Bacchiocca, M. Antioxidant capacity of vegetables, spices and dressings relevant to nutrition. **Br. J. Nutr.** 93 (2005) 257-266. DOI: 10.1079/BJN20041327.
20. Robin, M.A., Sauvage, I., Grandperret, T., Descatoire, V., Pessayre, D. and Fromenty, B. Ethanol increases mitochondrial cytochrome P450 2E1 in mouse liver and rat hepatocytes. **FEBS Lett.** 579 (2005) 6895–6902. DOI: 10.1016/j.febslet.2005.11.029.
21. Jagow, R., Kampffmeyer, H. and Kinese, M. The Preparation of microsomes. **Naunyn-Schmiedebergs Arch. Pharmak.** 251 (1965) 73-87.
22. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent. **J. Biol. Chem.** 193 (1951) 265-276.

23. Bensen, A.M., Hunkeler, M.S. and Talalay, P. Increase of NAD(P)H: quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. **Proc. Nat. Acad. Sci. USA** 77 (1980) 5216-5220.
24. Habig, W.H., Pabst, M.J. and Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. **J. Biol. Chem.** 249 (1974) 7130-7139.
25. Cao, Z. and Li, Y. Chemical induction of cellular antioxidants affords marked protection against oxidative injury in vascular smooth muscle cells. **Biochem. Biophys. Res. Co.** 292 (2002) 50-57. DOI: 10.1006/bbrc.2002.6614.
26. Naughton, P., Foresti, R., Bains, S.K., Hoque, M., Green, C.Y. and Motterlini, R. Induction of heme oxygenase 1 by nitrosative stress. **J. Biol. Chem.** 277 (2002) 40666-40674. DOI: 10.1074/jbc.M203863200.
27. Deker, T. and Lohmann-Matthes, M. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. **J. Immunol. Methods** 15 (1988) 61-69.
28. Hissin, P.J. and Hilf, R. A fluorimetric method for determination of oxidized and reduced glutathione in tissues. **Anal. Biochem.** 74 (1976) 214–226. DOI: 10.1016/0003-2697(76)90326-2.
29. Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C.J., Foresti, R., Alam, J. and Motterlini, R. Curcumin activates the heme oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. **Biochem. J.** 371 (2003) 887–895. DOI:10.1042/BJ20021619.
30. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227 (1970) 680-685.
31. Towbin, H.T., Staehelin, P. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. **Proc. Natl. Acad. Sci. USA** 76 (1979) 4350-4354.
32. Schreck, R. and Baeuerle, P.A. A role for oxygen radicals as second messengers. **Trends Cell Biol.** 1 (1991):39–42.
33. Srinivasan, K. and Sharma, S.S. 3-Bromo-7-nitroindazole attenuates brain ischemic injury in diabetic stroke via inhibition of endoplasmic reticulum stress pathway involving CHOP. **Life Sci.** 90 (2012) 154-160. DOI: 10.1016/j.lfs.2011.10.017.
34. Bao, W., Li, K., Rong, S., Yao, P., Hao, L., Ying, C., Zhang, X., Nussler, A. and Liu, L. Curcumin alleviates ethanol-induced hepatocytes oxidative damage involving heme oxygenase-1 induction. **J. Ethnopharm.** 128 (2010) 549–553. DOI: 10.1016/j.jep.2010.01.029.
35. Tanaka, M., Yamazaki, M. and Chiba, K. Neuroprotective action of genipin on tunicamycin-induced cytotoxicity in Neuro2a cells. **Biol. Pharm. Bull.** 32 (2009)1220-1223.
36. Jia, Z., Hallur, S., Zhu, H., Li, Y. and Misra, H. Potent upregulation of glutathione and NAD(P)H:quinone oxidoreductase 1 by alpha-lipoic acid in human neuroblastoma SH-SY5Y cells: protection against neurotoxicant-

elicited cytotoxicity. **Neurochem. Res.** 33 (2008) 790–800. DOI 10.1007/s11064-007-9496-5.

37. Elangovan, S. and Hsieh, T.C. Control of cellular redox status and upregulation of quinone reductase NQO1 via Nrf2 activation by alpha-lipoic acid in human leukemia HL-60 cells. **Int. J. Oncol.** 33 (2008) 833–838. DOI: 10.3892/ijo_00000071.
38. Ogborne, R.M., Rushworth, S.A. and O'Connell, M.A. α-Lipoic acid–induced heme oxygenase-1 expression is mediated by nuclear factor erythroid 2-related factor 2 and P38 mitogen-activated protein kinase in human monocytic cells. **Arterioscler. Thromb. Vasc. Biol.** 25 (2005) 2100–2105. DOI: 10.1161/01.ATV.0000183745.37161.6e.
39. Packer, L., Witt, E.H. and Tritschler, H.J. Alpha-lipoic acid as a biological antioxidant. **Free Radic. Biol. Med.** 19 (1995) 227–250. DOI: 10.1016/0891-5849(95)00017-R.
40. Salminen, A., Kauppinen, A. and Kaarniranta, K. Phytochemicals suppress nuclear factor-kB signaling: impact on health span and the aging process. **Curr. Opin. Clin. Nutr. Metab. Care** 15 (2012) 23-28. DOI: 10.1097/MCO.0b013e32834d3ae7.
41. Al-Halabi, R., Bou Chedid, M., Abou Merhi, R., El-Hajj, H., Zahr, H., Schneider-Stock, R., Bazarbachi, A. and Gali-Muhtasib, H. Gallotannin inhibits NF-kB signaling and growth of human colon cancer xenografts. **Cancer Biol. Ther.** 12 (2011) 59-68. DOI: 10.4161/cbt.12.1.15715.