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Journal of Cellular Biochemistry 114:2785–2794 (2013)

Journal of Cellular Biochemistry

Effect of Xanthohumol and 8-Prenylnaringenin on MCF-7 Breast Cancer Cells Oxidative Stress and Mitochondrial Complexes Expression

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ABSTRACT

Xanthohumol (XN) and 8-prenylnaringenin (8PN) are hop (*Humulus lupulus* L.) polyphenols studied for their chemopreventive effects on certain cancer types. The breast cancer line MCF-7 was treated with doses ranging from 0.001 to 20 μM of XN or 8PN in order to assess the effects on cell viability and oxidative stress. Hoechst 33342 was used to measure cell viability and reactive oxygen species (ROS) production was determined by 2′,7′-dichlorofluorescein diacetate. Catalase, superoxide dismutase, and glutathione reductase enzymatic activities were determined and protein expression of sirtuin1, sirtuin3, and oxidative phosphorylation system (OXPHOS) were done by Western blot. Treatments XN 0.01, 8PN 0.01, and 8PN 1 μM led to a decrease in ROS production along with an increase of OXPHOS and sirtuin expression; in contrast, XN 5 μM gave rise to an increase of ROS production accompanied by a decrease in OXPHOS and sirtuin expression. These results suggest that XN in low dose (0.01 μM) and 8PN at all assayed doses (0.001–20 μM) presumably improve mitochondrial function, whereas a high dose of XN (5 μM) worsens the functionality of this organelle. J. Cell. Biochem. 114: 2785–2794, 2013.

KEY WORDS: BREAST CANCER; MCF-7; 8-PRENYLNARINGENIN; XANTHOHUMOL; OXIDATIVE STRESS; OXIDATIVE PHOSPHORYLATION SYSTEM; MITOCHONDRIA; SIRTUINS

Xanthohumol (XN) is the main prenylated flavonid of the female inflorescence of the hop plant, *Humulus lupulus* L. (Cannabaceae). This plant also contains the estrogenic flavonid 8-prenylnaringenin (8PN), considered to be the most potent phytoestrogen isolated to date. Hops are used in beer elaboration process to add flavor and bitterness, thereby the main dietetic source of XN and 8PN is through beer consumption [Stevens and Page, 2004].

For over a decade, studies have been performed to evaluate the chemopreventive and chemotherapeutic potential of these and other hop compounds against cancer. In fact, XN is the hops compound which has been given more attention, owing to its ability to inhibit in vitro the states of initiation, promotion, and progression of carcinogenesis, hence XN seems to have a wide spectrum as chemopreventive agent [Stevens and Page, 2004; Gerhauser, 2005; Colgate et al., 2007]. Specifically, XN has been shown to inhibit growth and to induce apoptosis in breast cancer line MCF-7, among others [Lust et al., 2005; Vanhoecke et al., 2005; Zanoli and Zavatti, 2008]. One of the mechanisms through which XN inhibits cellular proliferation is by causing an oxidizing effect through inducing an increase in reactive oxygen species (ROS) in tumor cells [Yang et al., 2007; Strathmann et al., 2010; Festa et al., 2011].

ROS, mainly generated through the mitochondrial respiratory chain, are necessary for proper cell function by acting as intracellular messengers regulating proliferation and other biological processes [Festa et al., 2011]. Nevertheless, when produced in excess, ROS induce lipid peroxidation, protein carbonylation, and DNA damage leading to the activation of apoptotic pathways and cell death [Festa et al., 2011]. For this reason, enzymatic (glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase) and non- enzymatic detoxification systems are necessary for removing

Grant sponsor: Instituto de Salud Carlos III; Grant number: PI12/01827; Grant sponsor: FEDER-Unión Europea ("Una manera de hacer Europa"); Grant sponsor: Centro de Información Cerveza y Salud (CICS, Madrid, Spain).

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Manuscript Received: 19 March 2013; Manuscript Accepted: 26 June 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 8 July 2013

DOI 10.1002/jcb.24627 • © 2013 Wiley Periodicals, Inc.

ROS excess and to maintain a low level of oxidative stress. A common feature of cancer cells is that they have elevated levels of ROS and therefore they have a higher level of oxidative stress without an activation of the apoptotic pathways. Among the explanations given for the high level of ROS in cancer cells are either the existence of defects in the oxidative phosphorylation system (OXPHOS) or by a malfunction of antioxidant systems. Because of their cancer promoting effect, high levels of ROS are considered to be adverse factors; however, the oxidative damage caused by these radicals can be seen as an opportunity to exploit their cytotoxic potential by using exogenous agents that increase intracellular ROS levels and induce cell death [Trachootham et al., 2006]. Thus, compounds with oxidant activity may act selectively on those cells with an increased level of oxidative stress by inducing apoptosis.

Sirtuins are a class of proteins which have recently been given importance in the oxidative stress response. These proteins are NAD^+ dependent class III histone deacetylases and of which seven types have been described [Hallows et al., 2008; Park et al., 2011]. Through their deacetylase action, sirtuins are able to modify histones causing chromatin remodeling so that they can regulate gene expression [Shahbazian and Grunstein, 2007]. Sirtuins are involved in several physiological processes such as stress response, metabolism regulation, gene silencing, aging and carcinogenesis [Haigis and Guarente, 2006; Finkel et al., 2009]. Two sirtuin isoforms, sirtuin1 (Sirt1) and sirtuin3 (Sirt3), have been shown to play a central role in the regulation of mitochondrial maintenance and metabolism [Lombard et al., 2007]. In fact, studies have shown that Sirt3 acts as a tumor suppressor due to its ability to reduce mitochondrial ROS production [Kong et al., 2010; Bell et al., 2011; Yu et al., 2012]; however, the mechanism that explains how Sirt3 regulates ROS production and carcinogenesis has not been fully elucidated. Moreover, Sirt1 controls mitochondrial biogenesis induction and progression through PGC-1α regulation [Menzies and Hood, 2012]. Interestingly, phytoestrogens have been shown to modulate sirtuin levels and activity; for instance, genistein at 25 μM is capable of reducing the expression of Sirt1 [Kikuno et al., 2008] while resveratrol activates both Sirt 1 transcription and function [Tseng et al., 2011; Shakibaei et al., 2012] promoting mitochondrial biogenesis [Rasbach and Schnellmann, 2008]. These data suggest that phytoestrogens may exert effects on ROS production by modulating sirtuin levels and activity.

Taking this background information into account, the objective of this paper was to investigate the effect of XN and 8PN on the viability and oxidative stress levels of breast cancer cells. In tackling this aim, ROS production, antioxidant enzyme activities as well as Sirt1, Sirt3, and OXPHOS protein levels were determined in MCF-7 cells treated with different doses of these phytoestrogens.

MATERIALS AND METHODS

CHEMICALS

XN and 8PN were purchased from Sigma–Aldrich (St. Louis, MO, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

CELL CULTURES AND TREATMENTS

Human breast cancer cell line MCF-7 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin and streptomycin) in a 5% CO_2 humidified atmosphere at 37°C. To evaluate the effects of XN and 8PN, cells were shifted 24 h prior to treatment to a phenol red-free DMEM containing 10% charcoal-stripped FBS and 1% antibiotic (penicillin and streptomycin). XN and 8PN treatments were performed when cell cultures reached confluence by providing fresh medium supplemented with XN or 8PN for 48 h. Control cells were treated with 0.1% DMSO as a vehicle. For cell proliferation and ROS production assay, cells were plated in 96-well plates, whereas for Western blot and enzyme assays, cells were cultured in 100 mm culture dishes.

CELL VIABILITY ASSAY

Cells were plated at 8,000 cells per well in 96-well plates and shifted to phenol red-free medium 24 h prior to treatment as described previously. Cells were treated with XN or 8PN at different concentrations (0.001, 0.01, 0.1, 1, 5, 10, 15, and 20 μM) for 48 h. After treatment, cell culture medium was removed and DNA was stained with Hoechst 33342 (Sigma–Aldrich) at a concentration of 0.01 mg/ml. Plates were incubated for 5 min at 37°C and the fluorescence was measured using a microplate fluorescence reader FLx800 (BIO-TEK Winooski, Vermont, USA) set at 360 nm excitation and 460 nm emission wavelengths.

ROS PRODUCTION ASSAY

Cells were plated at 8,000 cells per well in 96-well plates and shifted to phenol red-free medium 24 h prior to treatment as described previously. Cells were treated with XN or 8PN at the same concentrations used in cell viability assay for 48 h. After treatment, cell culture medium was removed and cells were treated with PBS supplemented with glucose 20 mM and 2′, 7′-dichlorofluorescein diacetate (DCFDA) (Sigma–Aldrich) 10 μM. Plates were incubated for 15 min at 37°C and the assay was performed at the same temperature in a 96-well microplate fluorescence reader FLx800 (BIO-TEK Winooski) set at excitation and emission wavelengths of 485 and 528 nm, respectively. To normalize ROS production to cell number, cell culture medium was removed, and cells were stained with Hoechst 33342 (Sigma–Aldrich) as described below.

MEASUREMENT OF ANTIOXIDANT ACTIVITY

Cells were harvested by scraping them out with PBS buffer and then were centrifuged at 5,000 rpm for 5 min at 4°C to remove cell debris. The resultant cell pellet was resuspended in RNAse-free water and the lysates were kept on ice and the protein content was determined by a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). Catalase (CAT; EC 1.11.1.6) activity was measured according to an adaptation of the Johansson method [Johansson and Borg, 1988] based on the peroxidative function of the enzyme; superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by following the reduction of cytochrome c by measuring the absorbance at 550 nm on a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) at 37°C, as described previously [Quick et al., 2000].

Finally, glutathione reductase (GRd; 1.8.1.7) activity was measured monitoring the oxidation of NADPH at 340 nm, according to an adaptation of the Carlberg method [Carlberg and Mannervik, 1985].

WESTERN BLOT ANALYSIS

Cells were harvested by scraping them out with lysis buffer [Miro et al., 2011] and disrupted by sonication. Afterwards, protein content was determined with a bicinchoninic acid protein assay kit (Pierce). For Western blot analysis, 40 μg of protein from cell lysates were separated on a SDS–PAGE gel (15% for OXPHOS and 12% for the other proteins) and electrotransferred onto nitrocellulose membranes. After the transfer, membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20). Antisera against Sirt1 (H-300; Sc-15404) and α-tubulin (B-7; Sc-5286), the latter used as a housekeeping protein, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); while Sirt3 (#07-1596) was from Millipore (Billerica, MA, USA) and total OXPHOS (#MS601) was from MItoSciences (Eugene, OR, USA). Finally, protein bands were visualized by Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One Software (Bio-Rad).

MEASUREMENT OF CARBONYL CONTENT

The presence of carbonyl groups, a measure of protein oxidation, was determined by an immunological method using the OxySelect™ Protein Carbonyl Immunoblot kit (Cell Biolabs, San Diego, CA). For this purpose, 20 μg of protein from cell lysate were separated on a 12% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Protein carbonyls were detected by incubating the membrane with 2, 4-dinitrophenylhydrazine (DNPH) for 5 min. Unspecific biding sites on the membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20). After incubation with the DNP-antibody, bands were visualized using the Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One software (Bio-Rad Laboratories).

STATISTICAL ANALYSIS

All data are expressed as means ± SEM (standard error of the mean) with n = 6. Statistical analysis was carried out using the Statistical Program for the Social Sciences software (SPSS 18.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between treated and control cells were analyzed with the unpaired Student's *t*-test for proliferation and ROS production assays. One-way ANOVA analysis was used for data of Western blot and enzyme assays.

RESULTS

EFFECTS OF XN AND 8PN ON CELL VIABILITY

To evaluate the effect of XN and 8PN on MCF-7 breast cancer cell line viability, cells were treated with different concentrations of these compounds and cell number was assessed using Hoechst 33342 staining (Fig. 1). Treatments with XN in a concentration range of 0.001–20 μM for 48 h had a biphasic effect on cell proliferation in a

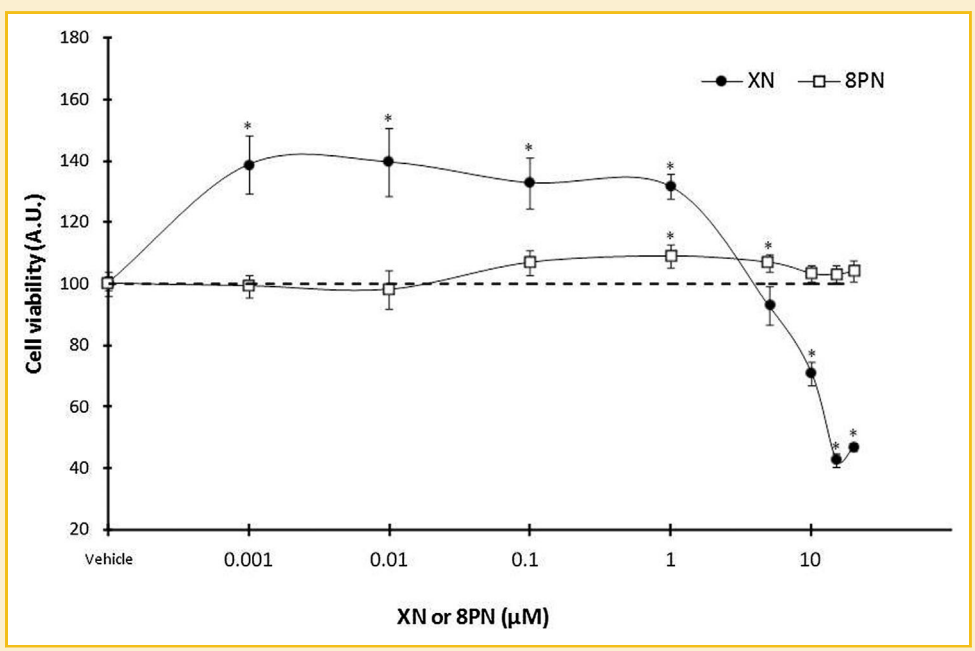

Fig. 1. Influence of XN and 8PN on MCF-7 cell line viability. Cells were plated in 96-well plates and treated with XN or 8PN (0.001, 0.01, 0.1, 1, 5, 10, 15, and 20 μM) for 48 h. Cell viability was assessed by DNA staining with Hoechst 33342 and represented as percentage with respect to vehicle-treated cells (0.1% DMSO, showed as dashed line at 100%). A.U.: arbitrary units. XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

dose-dependent manner; low concentrations of XN (0.001–1 μM) significantly increased cell proliferation (+39.62% at XN 0.01 μM with respect to vehicle-treated cells) whereas high concentrations (10–20 μM) caused a statistically significant decrease on cell proliferation (−57.32% at XN 15 μM compared to vehicle-treated cells). Otherwise, 8PN resulted in a slightly but statistically significant increase of cell number at 1 and 5 μM.

EFFECTS OF XN AND 8PN ON ROS PRODUCTION

To evaluate the antioxidant properties of XN and 8PN on MCF-7 cell line, ROS production was analyzed by the DCFDA assay (Fig. 2). Cells treated with low XN doses resulted in a statistically significant reduction of ROS production at 0.001 μM (−32.16% with respect to vehicle-treated cells) and 0.01 μM (−27.65% compared to vehicle-treated cells); otherwise, high XN doses ranging from 1 to 15 μM resulted in a marked increase of ROS production with a maximum peak at 5 μM (three times higher than vehicle-treated cells). Samples treated with 8PN showed a significant decrease of ROS production at all tested concentrations except at the lowest one (0.001 μM), reaching at 15 μM dose a 26.07% decrease with respect to vehicle-treated cells.

EFFECTS OF XN AND 8PN ON ANTIOXIDANT ENZYME ACTIVITY, PROTEIN OXIDATION LEVELS AND SIRT1, SIRT3, AND OXPHOS EXPRESSION LEVELS

To examine the effect that phytoestrogens XN and 8PN exert on antioxidant enzymes and different mitochondrial proteins, cells were treated with two different concentrations of each compound depending on the results obtained in cell viability and ROS production assays. A XN concentration was chosen at which this compound decreased ROS levels (0.01 μM) as well as a concentration at which there was a maximum ROS production (5 μM). Nevertheless, 8PN reduced ROS production at all tested doses so a low concentration (0.01 μM) and a high one (1 μM) were chosen to detect possible differing effects to dosage.

ANTIOXIDANT ENZYMES ACTIVITY

Figure 3 shows the activities of the antioxidant enzymes CAT, SOD, and GR. There was a statistically significant decrease on CAT activity with respect to vehicle-treated cells with XN 0.01 μM (−44.72%) and 5 μM (−31.83%) as well as with 8PN 0.01 μM (−41.24%) and 1 μM (−32.24%) (Fig. 3A). In the case of SOD activity (Fig. 3B), a statistically significant decrease on the activity with respect to vehicle-treated cells could also be observed with XN 0.01 μM as well as with 8PN 0.01 and 1 μM (in all there was a 50% decrease); treatment with XN 5 μM resulted in a non-statistically significant decrease of SOD activity with respect to vehicle-treated cells. Finally, GR activity (Fig. 3C) showed a statistically significant decrease with respect to vehicle-treated cells with XN 0.01 μM (−38.17%), 8PN 0.01 μM (−45.28%), and 1 μM (−44.70%); treatment with XN 5 μM resulted in an increase of GRd activity with respect to treatment XN 0.01 μM (+39.72%).

PROTEIN OXIDATIVE DAMAGE LEVELS

The presence of carbonyl groups, a marker of protein oxidation, was determined in order to evaluate the damage protein levels on treated cells (Fig. 4). Cells treated with XN 0.01 μM and 8PN showed a non-statistically significant downward trend in their carbonyl content with respect to vehicle-treated cells. In contrast, cells treated with XN 5 μM showed an upward trend in carbonyl content, with levels reaching statistically significant differences compared to XN 0.01 μM XN treated cells.

SIRT1 AND SIRT3 EXPRESSION LEVELS

Figure 5 shows the effects of XN and 8PN on Sirt1 (Fig. 5A) and Sirt3 (Fig. 5B) expression levels. Treatment with XN 0.01 μM induced a

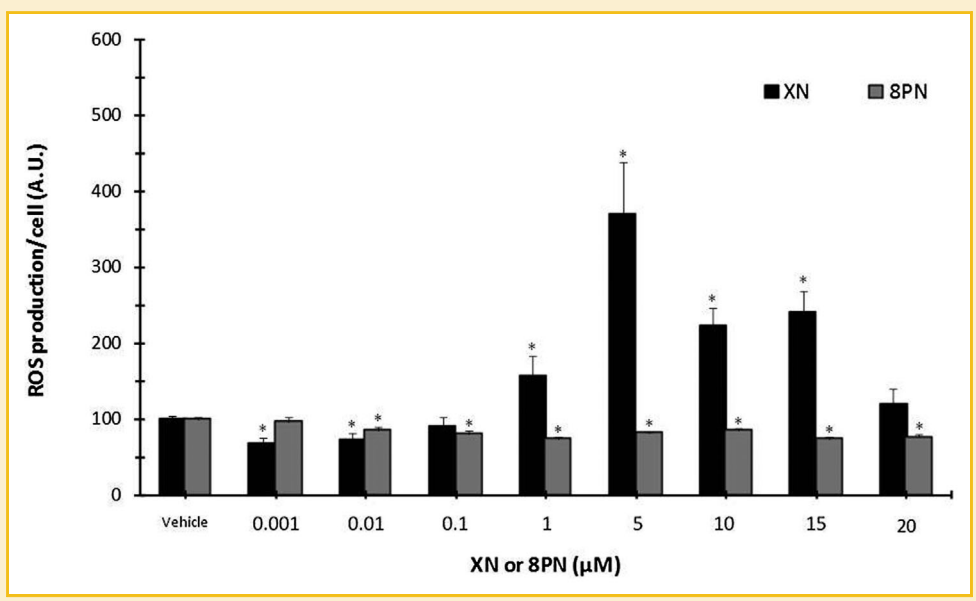

Fig. 2. Effect of XN and 8PN on MCF-7 cell line ROS production. Cells were plated in 96-well plates and treated with XN or 8PN (0.001, 0.01, 0.1, 1, 5, 10, 15, and 20 μM) for 48 h. ROS production was determined by DCFDA and represented as percentage with respect to vehicle-treated cells (0.1% DMSO, showed as 100%). A.U.: arbitrary units; XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

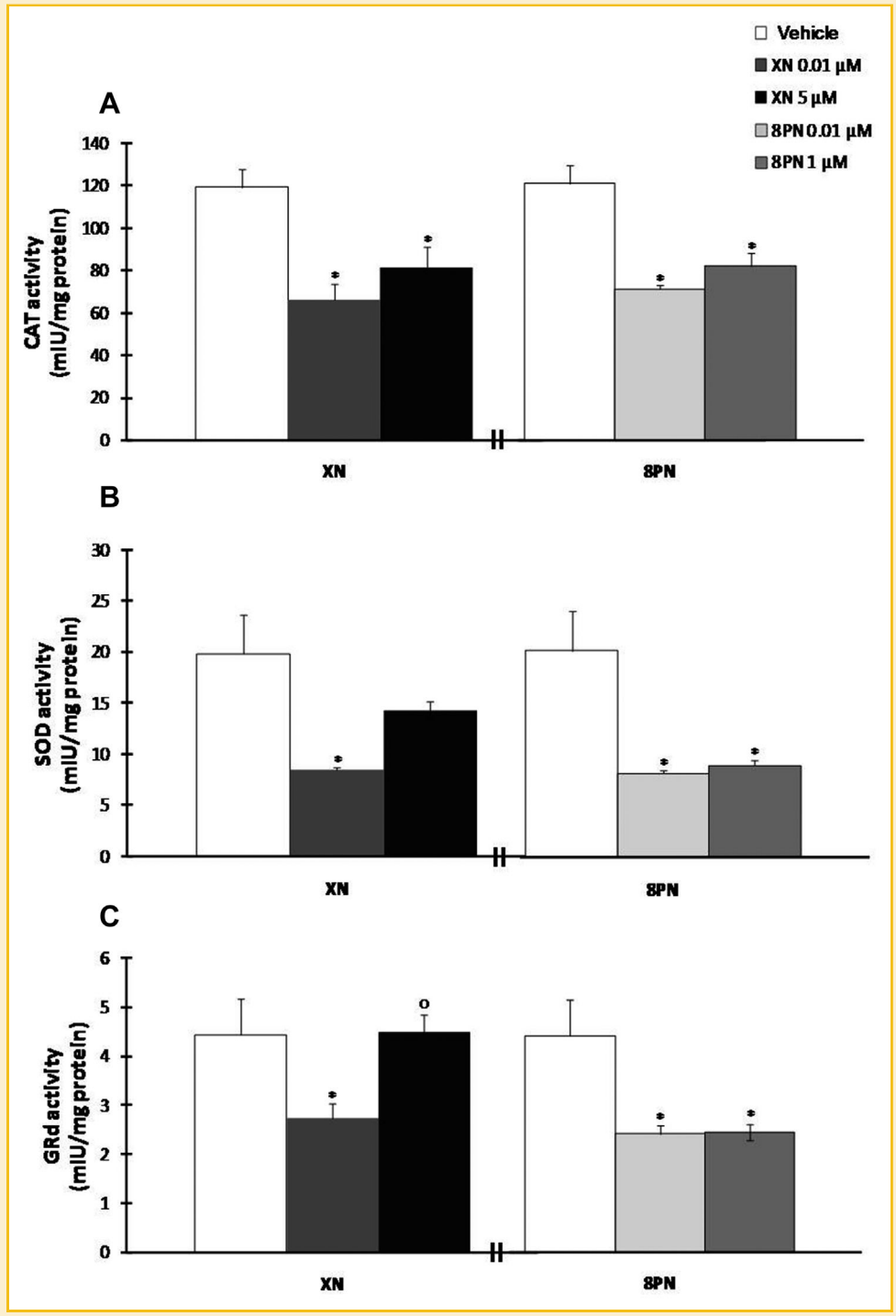

Fig. 3. Influence of XN and 8PN on MCF-7 cell line antioxidant enzymes activity CAT (A), SOD (B), and GRd (C). Cells were treated with XN (0.01 and 5 μM) or 8PN (0.01 and 1 μM) for 48 h. Spectrophotometric methods were used and data are presented as mIU/mg protein. Vehicle-treated cells were treated with 0.1% DMSO. CAT, SOD, and GRd: catalase, superoxide dismutase, and glutathione reductase; IU: international units; XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means ± SEM. *Statistically significant difference between treated and vehicle-treated cells (One-way ANOVA test; $P < 0.05$, n = 6). ° Statistically significant difference respect previous treatment (One-way ANOVA test; $P < 0.05$, n = 6).

sixfold significant increase in sirt1 expression; moreover, Sirt3 also experimented an increase in expression with this treatment but it was not statistically significant. Treatments with 8PN resulted in an increase of Sirt1 and Sirt3 expression with respect to vehicle-treated cells in a dose-dependent manner, although this increase was only significant in the case of Sirt1 with 8PN 1 μM (sixfold over vehicle-treated cells). In contrast, treatment with XN 5 μM resulted in a statistically significant decrease of Sirt1 with respect to treatment XN

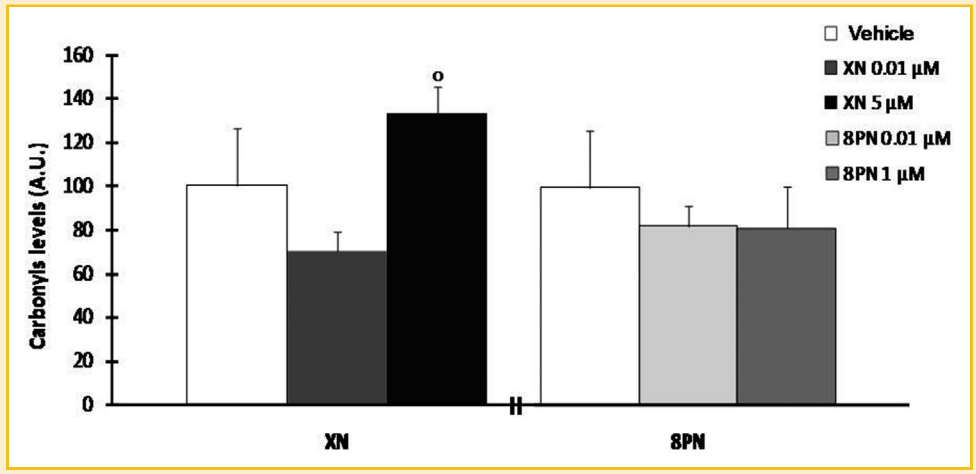

Fig. 4. Effect of XN 0.01 μM, XN 5 μM, 8PN 0.01 μM, and 8PN 1 μM on carbonylated protein levels after 48 h of treatment. Levels were determined by Western blot and represented as percentage of carbonyls with respect to vehicle-treated cells (0.1% DMSO, showed as 100%). A.U.: arbitrary units; XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means ± SEM. *Statistically significant difference between treated and vehicle-treated cells (One-way ANOVA test; $P < 0.05$, n = 6). ° Statistically significant difference respect previous treatment (One-way ANOVA test; $P < 0.05$, n = 6).

0.01 μM, equalizing vehicle-treated cells levels; XN 5 μM also decreased Sirt3 expression with respect to vehicle-treated cells but it was not statistically significant.

OXPHOS EXPRESSION LEVELS

OXPHOS expression levels (Fig. 6A and B) were determined as a measure of mitochondrial functionality after treatment with XN and 8PN at selected treatments. Treatment with XN 0.01 μM caused an expression increase of all mitochondrial complexes with respect to vehicle-treated cells, with this increase only significant in case of complex II (+43.37%) and V (+75.77%). Treatment with 8PN 0.01 and 1 μM also led to an upward trend in the expression of all mitochondrial complexes with respect to vehicle-treated cells but only complex II (+41.48% with 8PN 1 μM), III (+71.66% with 8PN 0.01 μM and +77.85% with 8PN 1 μM), and V (+71.23 with 8PN 1 μM) increments were statistically significant. Otherwise, treatment with XN 5 μM produced a downward trend on all mitochondrial complexes with respect to vehicle-treated cells except on complex III and V, where a slight increase was observed; and although these results were not statistically significant with respect to vehicle-treated cells they were with respect to treatment XN 0.01 μM on complexes I, II, and IV.

DISCUSSION

The aim of the present study was to determine the effects that phytoestrogens XN and 8PN, which are present in beer, exert on mitochondrial oxidative metabolism of the MCF-7 breast cancer cell line. In this study, we have observed that i) these compounds have effects on oxidative stress by means of changes in mitochondrial OXPHOS and sirtuins and ii) these effects are opposite in a dose-dependent manner (see summary Table I).

Low concentrations of XN (0.001–0.01 μM) and almost all tested doses of 8PN (0.001–20 μM) exerted an antioxidant function when causing significant decrease of ROS levels (−32% with XN 0.001 μM and −26% with 8PN 15 μM) along with an increase in cell viability (+40% with XN 0.01 μM and + 9% with 8PN 1 μM). This increase of cell viability caused by 8PN has already been observed in previous studies although these authors reported that this compound also exerts cytotoxic effects on MCF-7 cell line when used at concentrations of 1 μM or higher [Matsumura et al., 2005; Brunelli et al., 2009]. Otherwise, higher doses of XN (1–15 μM) resulted in a marked increase of ROS production (near threefold vs. vehicle-treated cells) along with a marked decrease of cell viability (−57% with XN 15 μM). These results are consistent with previous works in other cell lines treated with XN [Miranda et al., 1999; Lust et al., 2005; Pan et al., 2005; Vanhoecke et al., 2005; Delmulle et al., 2006; Monteiro et al., 2007; Yang et al., 2007; Ho et al., 2008; Monteiro et al., 2008; Szliszka et al., 2009; Deeb et al., 2010; Dorn et al., 2010; Strathmann et al., 2010; Drenzek et al., 2011; Festa et al., 2011; Zajc et al., 2012]. In fact, despite that many dietary polyphenols have been studied for their antioxidant activity, recent publications have shown that several polyphenols may also act as oxidants [Lee and Lee, 2006; Antosiewicz et al., 2008; Khan et al., 2008; Trachootham et al., 2009; Strathmann et al., 2010].

Protein carbonyl content, which is an end-marker of oxidative damage, showed a downward trend consistent with the mild decrease in ROS production induced by XN 0.01, 8PN 0.01, and 8PN 1 μM. The lack of statistical significance in the decrease of protein oxidation may be in relation to its end-marker nature, since antioxidant activities, protein turnover, and repairing systems may blunt the ROS effects. Moreover, the low antioxidant activity observed in these same treatments could be explained by the fact that they are not activated precisely because ROS levels are low, and therefore, the low oxidative stress observed in these treatments could not be due to detoxifying activity of antioxidant enzymes. These results could indicate that XN and 8PN exert a modulatory effect on ROS production although by a separate path to the regulation of the antioxidant enzyme activity.

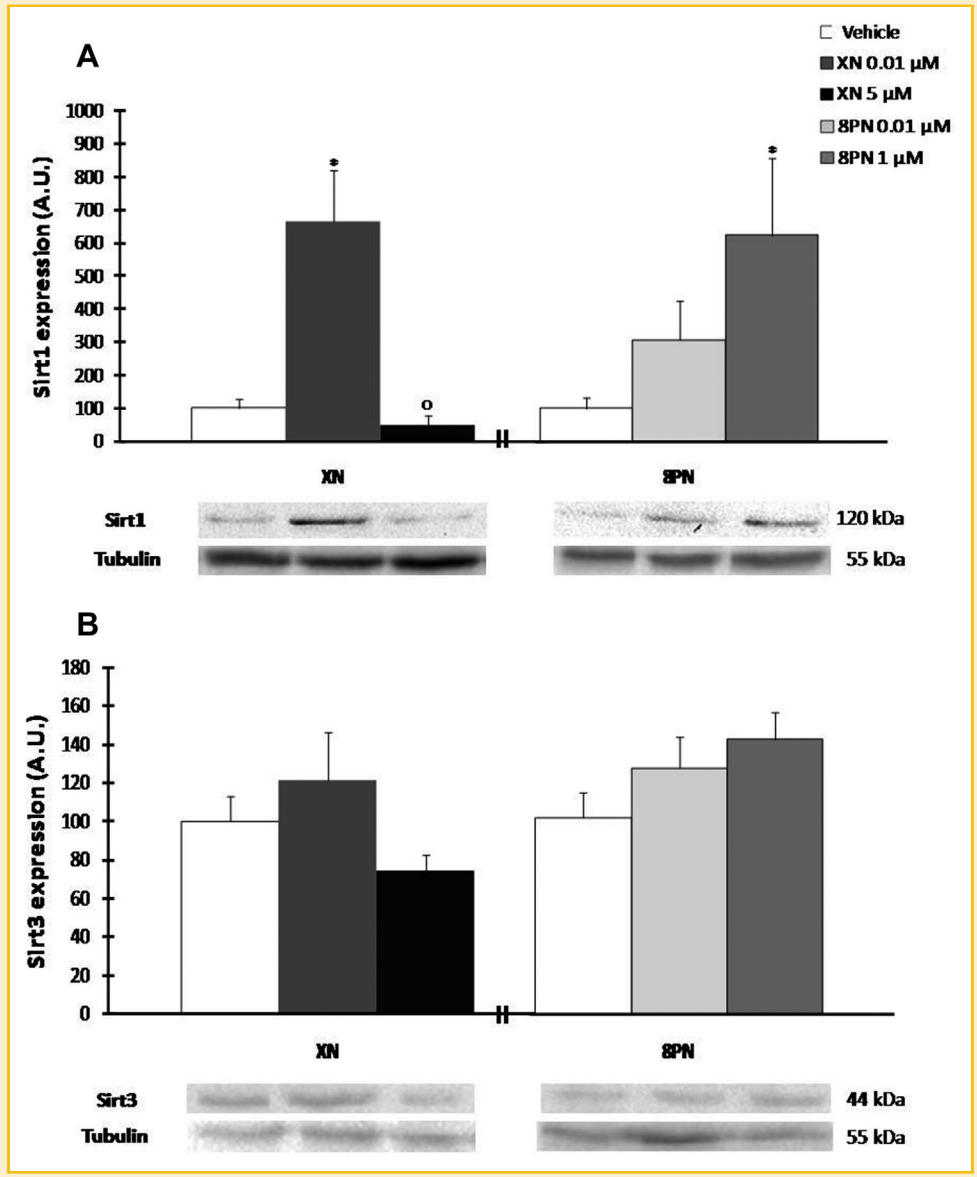

Fig. 5. Influence of XN 0.01 μM, XN 5 μM, 8PN 0.01 μM, and 8PN 1 μM on Sirt1 (A) and Sirt3 (B) expression levels after 48 h of treatment. Levels were determined by Western blot and represented as percentage of expression with respect to vehicle-treated cells (0.1% DMSO, showed as 100%). Representative bands of Western blot are shown. A.U.: arbitrary units; XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means ± SEM. *Statistically significant difference between treated and vehicle-treated cells (One-way ANOVA test; $P < 0.05$, n = 6). °Statistically significant difference respect previous treatment (One-way ANOVA test; $P < 0.05$, n = 6).

Furthermore, these treatments (XN 0.01, 8PN 0.01, and 8PN 1 μM) caused an increase of OXPHOS expression levels, as well as a marked increase of Sirt1. From these results it appears that the reduction of ROS production observed in these treatments might be due to an improvement in mitochondrial function.

In contrast, samples treated with XN 5 μM showed an increase of carbonylated protein content in accordance with their higher ROS levels. Besides, this treatment leads to a downward trend in CAT and SOD activities; this could be due to the fact that this treatment causes an increase of ROS production too high to be offset by antioxidant enzymes activity or it even could be that their activity is damaged by ROS. Another possibility to explain this decrease of the antioxidant activities with XN 5 μM could be that XN exerts a direct inhibitory effect of antioxidant enzymes activity. Furthermore, the increase in ROS production induced by XN 5 μM may be due to decreased OXPHOS expression observed with this treatment which would indicate why perhaps mitochondrial functioning would be altered. In fact, in an earlier work with mitochondrial subparticles, it was already noted that high concentrations of XN cause a decrease of the activity of certain OXPHOS complexes accompanied by an increase of ROS levels [Strathmann et al., 2010]. Furthermore, this treatment also caused a significant reduction of Sirt1 and less marked Sirt3 expression.

These results show that XN exerts a dual effect on ROS production and cell viability in a dose-dependent manner and acts by reducing ROS production at low concentrations while promoting ROS

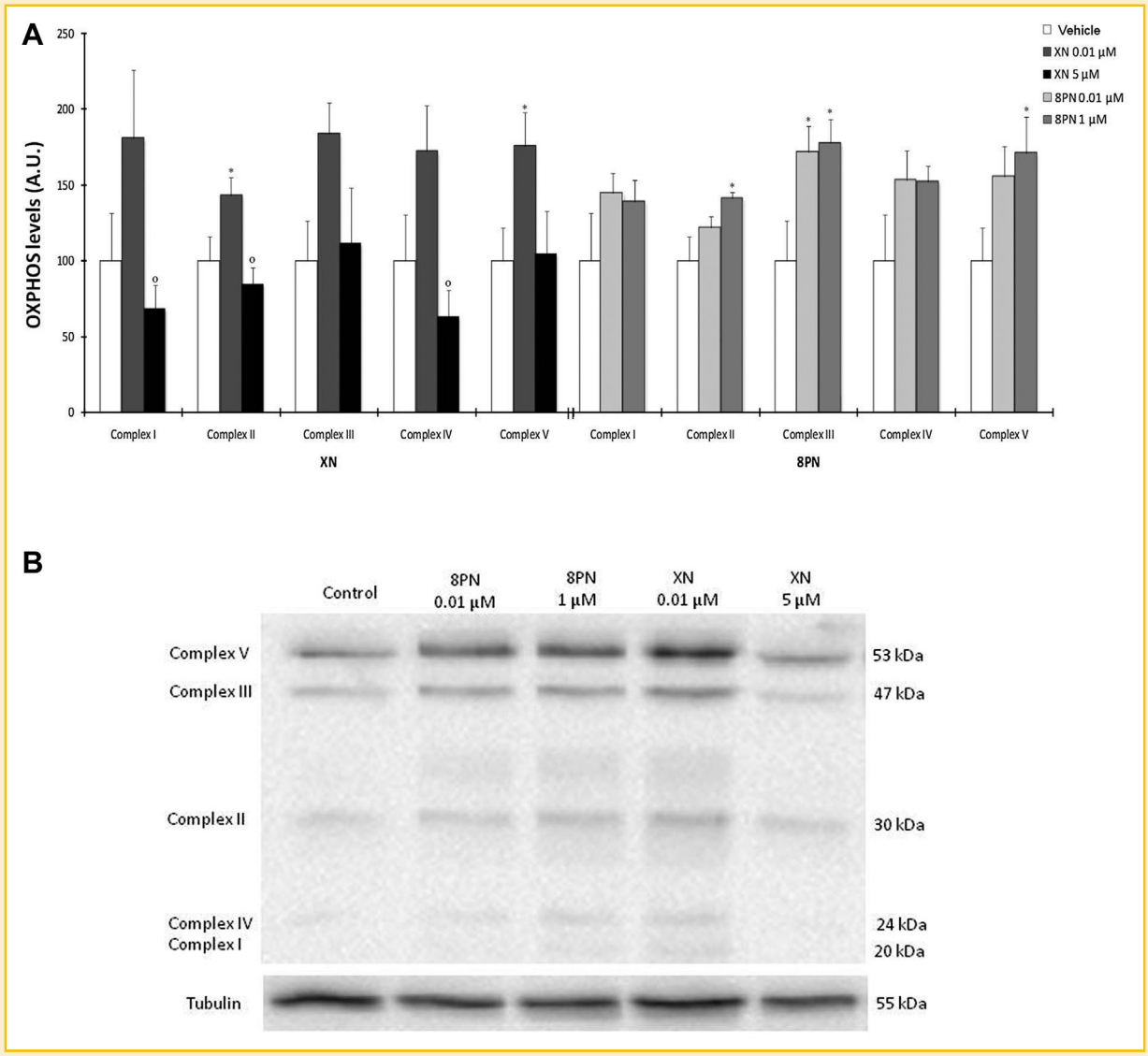

Fig. 6. A: Influence of XN 0.01 μM, XN 5 μM, 8PN 0.01 μM, and 8PN 1 μM on OXPHOS expression levels after 48 h of treatment. Levels were determined by Western blot and represented as percentage of expression with respect to vehicle-treated cells (0.1% DMSO, showed as 100%). A.U.: arbitrary units; XN and 8PN: xanthohumol and 8-prenylnaringenin. Data are means $\pm$ SEM. *Statistically significant difference between treated and vehicle-treated cells (One-way ANOVA test; $P < 0.05$, n = 6). ° Statistically significant difference respect previous treatment (One-way ANOVA test; $P < 0.05$, n = 6). B: Influence of XN 0.01 μM, XN 5 μM, 8PN 0.01 μM and 8PN 1 μM on OXPHOS expression levels after 48 h of treatment. Levels were determined by Western blot and representative bands are shown.

formation at high concentrations; this dual effect has already been observed previously for other phytoestrogens like genistein and resveratrol [Matsumura et al., 2005; Signorelli and Ghidoni, 2005]. In summary, from results one could postulate that XN at low concentrations and 8PN induce sirtuin expression and these proteins stimulate mitochondrial biogenesis which thereby decreases mitochondrial ROS production; otherwise, the oxidizing doses of XN might inhibit the expression of sirtuin proteins, which would adversely affect mitochondrial function which in consequence lead to an increase in ROS levels. Previous studies have found that phytoestogens may modulate cell sirtuin levels and activity; for instance, genistein at 50 μM is capable of reducing Sirt1 expression [Kikuno et al., 2008]. Sirtuins have been found to be involved in the modulation of mitochondrial function in the cell; as a matter of fact, Sirt1 controls mitochondrial biogenesis induction and progression [Menzies and Hood, 2012] and Sirt3 resides mainly in mitochondria and regulates oxidative stress through the deacetylation of substrates involved in both ROS production and detoxification, so that this protein could maintain mitochondrial redox homeostasis [Finley et al., 2011; Bause and Haigis, 2012; Giralt and Villarroya, 2012]. In the present study, OXPHOS expression levels showed a pattern of changes depending on the compound and dosage used for treatment. From these results, a possible regulation of ROS production by XN and 8PN could be proposed; these phytoestrogens may modulate mitochondrial function, and therefore ROS production, through Sirt1 and Sirt3, and thus sirtuins could mediate the action between

TABLE I. Statistically Significant Changes (Increase ↑ or Decrease ↓) with Respect to Vehicle-Treated Cells (Student's *t*-test and One-Way ANOVA Test; $P < 0.05$, n = 6)

	XN, 0.01 μM	XN, 5 μM	8PN, 0.01 μM	8PN, 1 μM
Cell growth	↑			↑
ROS levels	↓	↑	↓	↓
Antioxidant activity				
CAT	↓	↓	↓	↓
SOD	↓		↓	↓
GRd	↓		↓	↓
Carbonyl levels				
Sirt1	↑			↑
Sirt3				
OXPHOS				
Complex I				
Complex II	↑			↑
Complex III			↑	↑
Complex IV				
Complex V	↑			↑

MCF-7 cells were treated with XN or 8PN for 48 h. CAT, SOD, and GRd, catalase, superoxide dismutase, and glutathione reductase; OXPHOS, oxidative phosphorylation system; ROS, reactive oxygen species; Sirt1, sirtuin 1; Sirt3, sirtuin 3; XN and 8PN, xanthohumol and 8-prenylnaringenin.

these compounds and OXPHOS expression levels. These results suggest that XN at high concentrations share functional similarities with "mitocans," compounds that selectively affect and destabilize mitochondrial function until they kill the cells by a redox imbalance; in fact, several action targets of mitocans have been identified amongst which one is the OXPHOS complex [Strathmann et al., 2010]. Particularly, it has seen that XN precisely appears to act specifically against cells displaying altered redox balance [Jacob et al., 2011], so this compound may be a potential candidate as pro-oxidative drug in cancer treatment.

Beer is the most important dietary source of XN and 8PN. The USDA estimates that a daily average consumption of beer (225 ml) provides an intake of 0.14 mg of prenylflavonids (including XN, isoxanthohumol, and 8PN) [Stevens and Page, 2004]. On the other hand, other study found that serum of women receiving a dietary hop supplement (6.12 mg XN and 0.3 mg 8PN) during 5 days reached a 5 and 2 nM concentration of XN and 8PN, respectively. This study also detected these compounds in breast tissue, showing values in the range of pmols per g tissue [Bolca et al., 2010]. The doses observed by Bolca et al. [2010] in mammary gland of supplemented women were in the low range of the doses tested in our study and far from the high dose of XN (5 μM) eliciting oxidative effects. Considering the bioavailability data [Stevens and Page, 2004], it is likely that a dietary hop supplementation may provide low XN and 8PN doses ameliorating ROS production. On the other hand, the ROS promoting effect of high XN concentrations deserves special attention since it could be a promising chemotherapeutic agent in breast cancer therapy. Cancer cells have higher oxidative stress than normal cells but their ROS threshold for apoptotic induction is greater. Thus, high XN doses may prompt cancer cells to a higher oxidative stress able to trigger apoptosis. Future studies are needed to determine whether these oxidant effects of XN are specific and relevant enough in animal models to be considered a promising tool for breast cancer therapy.

ACKNOWLEDGMENTS

This work was supported by grants from Fondo de Investigaciones Sanitarias of Instituto de Salud Carlos III (PI12/01827) of the Spanish Government, cofinanced by FEDER-Unión Europea ("Una manera de hacer Europa"), and from Centro de Información Cerveza y Salud (CICS, Madrid, Spain).

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