Interaction Between Retinoids and Eicosanoids: Their Relevance to Cancer Chemoprevention

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Abstract: Carcinogenesis involves a growing accumulation of genetic and epigenetic aberrations, leading to the deregulation of cellular homeostasis, followed by neoplastic progression. Although nutritional lipids play a critical role the specific transcriptional mechanisms involved in this process are not completely understood. In this review, we examine the biological effects of dietary essential fatty acids (n-3 and n-6 EFAs) and vitamin A, and the common pathways related to cancer chemoprevention. Eicosanoids (EFAs derivates) and retinoids (vitamin A derivates) are major mediators that act on their corresponding RXR-heterodimerized receptors (PPAR and RAR) and modify the carcinogenetic signalling pathways. Several effects of these mediators, mainly at DNA level, depend on specific molecular properties of the receptor isoforms and their differential affinities for their ligands, whose availability can be intentionally managed through diet. Nevertheless, the previous grade of differentiation in normal development or in cancer cells is an important modulatory factor of the cellular responses, especially when differentiating agents are evaluated. The potential of dietary EFAs and retinoids in chemoprevention and chemotherapy, through their actions on the cellular proliferation and differentiation processes, with particular reference to human breast cancer is discussed herein.

Keywords: Cancer chemoprevention, eicosanoid, fatty acid, PPAR, retinoid, RXR.

1. INTRODUCTION

Carcinogenesis is a multistage process, which involves a gradual accumulation of genetic and epigenetic aberrations that ultimately results in the deregulation of cellular homeostasis [1]. Thus, the initiation and progression of the neoplastic process depend on individual susceptibility, which is determined by hereditable characters and their interactions with different environmental factors [2]. Furthermore, nutrition plays a critical role in cancer development and treatment [3], with there being increasing evidence that some dietary agents, such as phytochemicals (with antioxidant and anti-inflammatory properties), essential unsaturated fatty acids, among others, exhibit unique anti-tumour effects that could delay the onset of cancer and thus, serve as chemopreventive agents. Moreover, as many of these agents have selective tumoricidal actions on certain varieties of cancer cells, they may be called chemotherapeutic ones [4], with experimental and clinical-epidemiological data supporting the use of these compounds in the prevention and control of different epithelial malignancies (colon, lung, prostate, breast, among others). However, specific mechanisms of several of these nutrients/biodrugs, mainly at the transcriptional level, are still not fully understood [5]. In the present work, the interaction of fatty acids and vitamin A-like compounds are analyzed in order to describe common pathways and their potential biomedical implications.

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2. DIETARY LIPIDS: ESSENTIAL n-3 AND n-6 POLYUNSATURATED FATTY ACIDS (PUFA)

2.1. Sources and Metabolism of Essential PUFA

Essential PUFA are classified in different families according to the position of the first double bond from the last carbon (mainly in the n-3 and n-6 families), of which precursors can not be synthesized de novo in the body. Dietary sources of the 18-carbon n-3 fatty acid (Linolenic acid: LNA) are soybean, canola, wheat germ, and walnut oils. Linoleic acid (LA), an 18-carbon n-6 fatty acid, is found in sunflower, corn, soybean, and cottonseed oils. Concerning respective derivates, 20- and 22-carbon n-3 PUFA sources are mainly fish oils, whereas beef products are the main source of the long chain n-6 fatty acid, arachidonic acid (AA, C20:4n-6), by far the most abundant one in western dietary habits. The 18-carbon PUFA derived from plants can be converted by the same enzyme pools into cellular cytoplasmatic desaturated and elongated PUFA. Thus, LA is converted to AA, while LNA is converted to EPA (eicosapentaenoic acid), leading to chemical modifications and biological activation [6].

The biologically-active lipids derived from the 20-carbon PUFA are eicosanoids (prostaglandins, prostacyclins and thromboxanes), which arise from enzymatic cyclooxygenation (COX) of the linear AA. This cascade first produces the release of AA from membrane phospholipids by phospholipase A, followed by its subsequent metabolism to prostanoids, with AA being the main precursor for this. These include stable prostaglandins (PG), e.g. PGE_2, $PGF_{2\alpha}$, PGD_2, PGJ_2, as well as labile prostanoids, such as PG endoperoxides (PGG_2, PGH_2), thromboxane A_2 (TXA_2) and

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prostacyclin (PGI_2). In addition, PGD_2 and PGE_2 can be transformed into PGJ_2 and PGA_2, respectively, through either non-enzymatic rearrangement or by dehydration. Even though COX enzymes have an elevated affinity for AA (a substrate widely present in foods, such as red meats), when the dietary availability of other 20-C precursors (i.e. EPA) is increased, these enzymes can also be metabolized. Subsequently, the relative levels of dietary precursors affect the levels of the PG formed, which may be deleterious for the body if the level of non-EFAs are abnormally high in the diet [7].

Other groups of bioactive lipid molecules derive from enzymatic lipoxygenation (LOX) of 20-C unsaturated fatty acids, including leukotrienes (LT) and lipoxins [8]. Furthermore, the cytochrome P450 epoxygenase pathway can generate other compounds, such as the 11, 12-epoxyeicosatrienoic acid, shown in Fig. (**1**). Other eicosanoids result from the lipid oxidative metabolism, such as the endocannabinoids (i.e. anandamide, arachidonoylethanol-amide, 2-arachidonoylglycerol), which are endogenous AA metabolites produced in the brain and other tissues that bind and activate the cannabinoid receptors (CB1 and CB2) [9]. These have also been implicated in a wide array of physiological and pathological processes, including cancer, obesity and diabetes [10].

2.2. Effects of Dietary PUFA on Cell Physiology and their Implications in Disease

Dietary essential PUFAs are important precursors for the signaling molecules that control many facets in cell physiology. When quantitatively-altered lipid intake occurs, metabolic perturbations may favour many chronic degenerative diseases (diabetes, obesity, vessel dysfunctions, etc.), and cancer development may also take place [11,12]. On the other hand, some PUFAs belonging to the n-3 family have chemopreventive properties against prostate cancer [13]. Indeed, it is known that the incidence of many cancers, which differs widely among geographical areas [14], is related to the relative contribution of various fatty acids to the total dietary intake [15,16]. Moreover, it is possible to

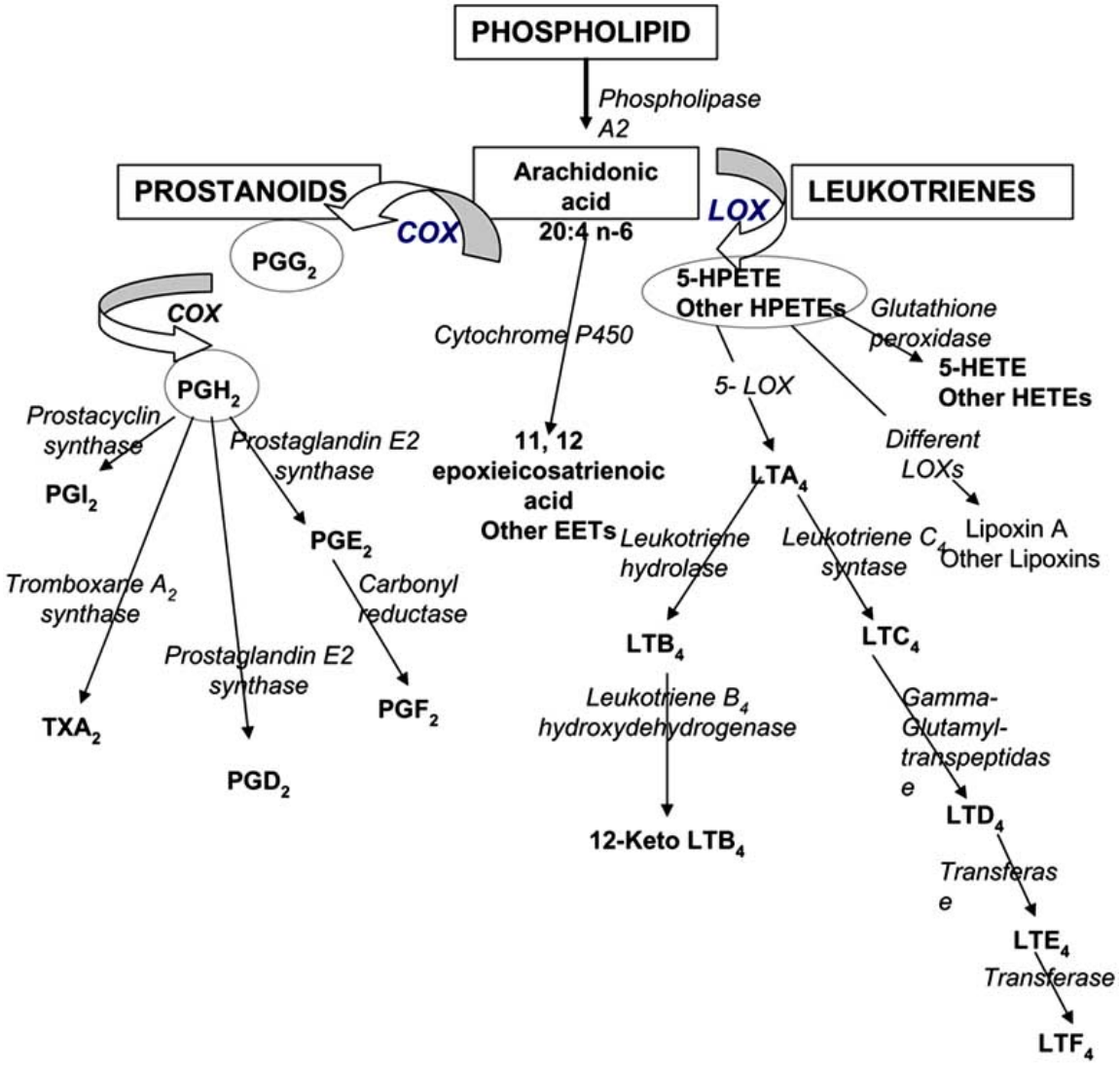

Fig. (1). Eicosanoid biosynthesis.

modify experimentally the plasma membrane phospholipid composition, where pivotal copies of many enzymes are found, by changing the up-stream of the PUFA precursor through lipid intake (i.e. amounts of AA or EPA in foods). Since AA is a precursor of the series 2 of PG and TX, and also of the series 4 of LT, whereas EPA is a precursor of the series 3 and 5, different availabilities of these fatty acids lead to changes in the quantities of their derivates. Thus, the influence of dietary habits is relevant, as fatty acids with 20 carbons play a structural role in all biomembranes. Also, they are substrates for endocannabinoid and eicosanoid formation, with the latter being oxygenated derivates with a wide range of hormonal, pro-inflammatory and regulatory functions [17].

Eicosanoids participate in carcinogenesis, as they are able to regulate cellular proliferation and differentiation. Nevertheless, they can also act in a bimodal way, as happens for other PUFAs derivates, with it being demonstrated that the blocking of the derivate 5-HETE (hydroxyeicosatetraenoic acid) by the LOX antagonist action consistently induces apoptosis and regulates growth-related signal pathways [18]. On the other hand, the modulation of PPAR (peroxisome proliferating-activating receptors), mainly through the activation of PPARγ, should also be taken into account since binding occurs with metabolites formed by alternative 20-carbon PUFA, such as the eicosanoids derived from AA or EPA [18]. COX-derived eicosanoids, such as PGE_2, induce down-regulation of glycoprotein E-cadherin, a major cell adhesion molecule widely used as a differentiation marker for epithelia-derived cancers [19], and playing an important role in the development and progression of a wide range of carcinomas [20,21]. It is well known that *in vitro* and clinical studies to evaluate malignant cells from skin and other cancer lines suggest that decreased E-cadherin is associated with neoplastic progression [22].

2.3. PUFA-Related Signalling

Dietary essential fatty acids and their eicosanoid derivates are natural ligands of PPAR, which in turn have been implicated in cancer regulation (see Fig. **2**). These nuclear receptors, first described as being activated by peroxisome proliferators [23], are represented by three isoforms (PPARγ, PPARα and PPARδ/β) encoded by different genes. When these bind to their corresponding ligands, they trigger the regulation of important cellular functions, including cell proliferation and differentiation, as well as stress responses. Thus, PPAR are ligand-regulated transcription factors, which control gene expression by binding to specific response elements (PPRE) comprising promoters [24]. The use of synthetic PPAR ligands has allowed the unveiling of many of their potential effects on pathological states, including atherosclerosis, hypertension, inflammation, infertility, demyelination, and cancer [25].

PPARγ deserves special attention, since its activation results in beneficial anti-cancer effects in many cell lines triggered by the release of eicosanoids and fatty acids. Among these, PGJ_2 is a strong endogenous ligand [26]. Also, LA metabolic products, such as 9-HODE (hydroxy-

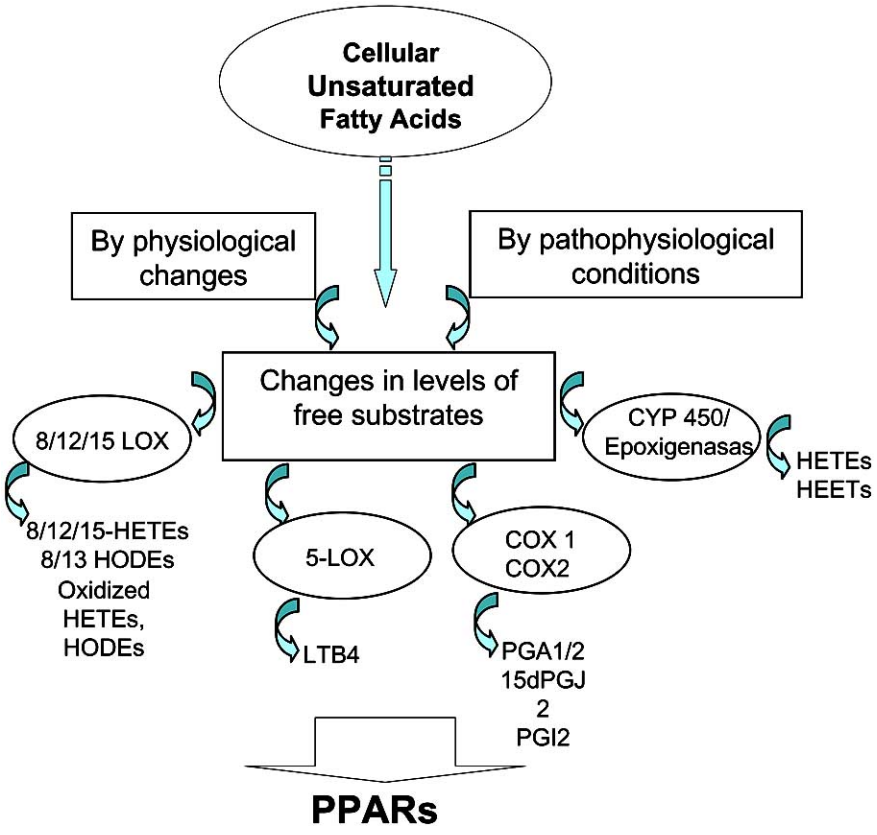

Fig. (2). Transcriptional activity of dietary lipid derivates.

octadecadienoic acid), 13-HODE, and 13-OXO (oxooctadecadienoic acid), are other well-known PPARγ ligands [27]. Regarding PPARγ's chemopreventive properties, n-3 fatty acids induce up-regulation of syndecan 1, a glycosaminoglican leading to arrest of the prostate cancer progression [28]. Other examples of anti-tumour PPARγ ligands are LA and CLA (conjugated linoleic acid) in colorectal carcinoma, where continuous treatment induces quiescence up to 5-7 weeks after exposition. This effect has also been seen with Ku-7 and DU-145 cells belonging to bladder and prostate cancer cells lines, respectively [29]. Indeed, CLA is a variety of LA with strong anti-cancer activity [30].

3. DIETARY VITAMIN A DERIVATES AND ANALOGUES: RETINOIDS AND REXINOIDS

3.1. Sources and Metabolism of Retinoids and Rexinoids

Retinoids and rexinoids are chemically related to vitamin A (retinol), an organic compound required as an essential nutrient for humans, even at minimal amounts [31]. Accordingly, naturally-occurring and synthetic derivates are named retinoids, a term coined by Sporn in 1976 [32]. Most recently, the synthetic retinoids have been alternatively called rexinoids, given their high affinity for RXR (retinoid X receptors) [33]. Natural retinoids are mostly represented by retinyl esters, which release retinol by hydrolysis.

In animal organisms, retinal, also known as retinaldehyde, or other closely related compounds, such as 3-hydroxy-retinal, are molecules found in the photoreceptor cells of the retina. These serve as the chromophore of various visual pigments (rhodopsins) [34]. The best described active retinoid metabolites to date are 11-cis-retinal and retinoic acid (RA) isomers (*all-trans* and 9-*cis*-retinoic acid). In plants, edible coloured tissues (leaves, fruits, roots) contain dietary pro-vitamin A compounds (carotenoids), which can be converted to vitamin A in animal tissues by oxidative cleavage [35]. The conversion of ß-carotene occurs by two different carotenoidoxygenases, named CMO1 and CMO2, belonging to a family of structurally related non-heme iron oxygenases, with CMO1 being the key enzyme for the conversion of ß-carotene to vitamin A in mammals [36,37]. CMO1 converts ß-carotene to retinaldehyde by a centric oxidative cleavage at the C15´-C15´ double bond for all varieties of biologically active retinoids, including 11-cis-retinal and RA [38-40], whereas CMO2 catalyzes an excentric oxidative cleavage of carotenoids (carotene and lycopene) at the C9' and C10' double bonds [39] (Fig. **3**).

The retinol-binding protein (RBP) is the seric transport protein of retinol in the circulation from the liver to its target tissues. The existence of a cell-surface receptor on the target cells, which mediates the uptake of retinol from RBP, has been known since 1975. Recently, this receptor was identified as a trans-membrane protein named STRA6, with its expression being induced by RA in certain cancer cell lines. This molecule was found to be highly specific for RBP, and was identified in those tissues known to require retinol for their functions [40].

3.2. Physiological and Pathological Roles of Dietary Retinoids

Retinol seems to regulate the growth, development, and epithelial maintenance in vertebrates by conversion to the active form, RA [41]. This is an important signaling molecule, which influences developmental processes and cell differentiation by binding nuclear receptors, causing transcriptional regulation of its target genes [42,43]. Regarding this, the RA mechanism of action is similar to that of steroid and thyroid hormones, involving induction of the expression of specific genes, and thus placing retinoids in the category of hormones, which regulate cytodifferentiation, apoptosis, tissue growth, and embryonic development [44]. Other major physiological processes regulated by RA include vision, reproduction, bone formation, and haematopoiesis.

Since retinoids control cell growth, differentiation and apoptosis, they have become potential chemopreventive and chemotherapeutic agents. Furthermore, they have been shown to suppress carcinogenesis in various organs (e.g. head-neck, skin, bladder, lung, prostate and breast cancers) in animal models [45]. For example, the *all-trans* RA (ATRA) causes fast terminal differentiation of acute promyelocytic leukemia cells *in vitro* and *in vivo* (humans), although unfortunately it is less active in other malignancies. However, clinically retinoids showed promising effects for reversing other pre-malignant human epithelial lesions, and in preventing head and neck, lung, liver and breast tumours [46]. ATRA is also able to regulate the tumour necrosis factor-induced pathway, among others [47,48]. Moreover, retinoids play a central role in the control of tumour progression, inducing stromal proliferation by regulating the expression of several key molecules, such as matrix metalloproteinases [49], transforming growth factor-β, and also cell cycle-regulating proteins [50], for example, cyclin dependent kinase I, p16, and p21 [51].

3.3. Retinoid-Related Signalling

The retinoid pleiotropic regulating functions on cell physiology depend on their interaction with two types of nuclear receptors: retinoic acid receptors (RAR) and RXR [52,53], which belong to a superfamily of steroid and thyroid hormone receptors [54]. Each receptor (RAR and RXR) has three subtypes (α, β and γ). Also, each subtype can possess some isoforms which differ physiologically [55]. For example, RARβ has four isoforms with distinct retinoid affinities and different biological functions. The loss of $RAR\beta_2$ is associated with tumorigenesis and retinoid resistance, whereas its induction prevents this carcinogenetic condition. On the other hand, the expression of $RAR\beta_4$ is increased in various types of cancers. In fact, its over-expression in transgenic rodents causes hyperplasias and neoplasias in various tissues, whereas its induction increases proliferation of certain tumour cells which do not express $RAR\beta_2$ [56].

RA possesses a dual role in carcinogenesis, hence precluding a modulating, homeostatic capability. When it

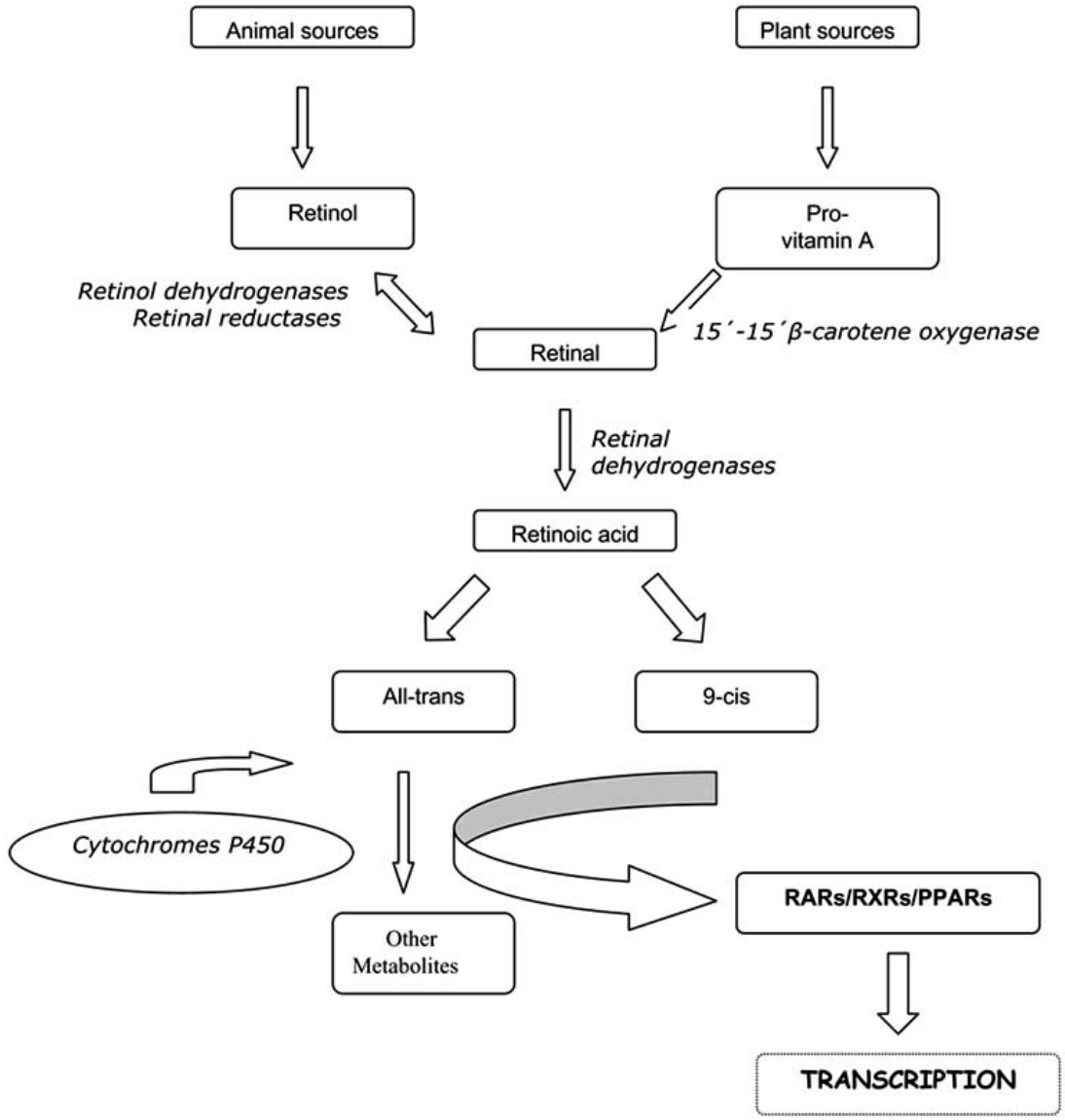

Fig. (3). Metabolism and transcriptional activity of dietary retinoids.

binds to RAR, neoplastic growth is inhibited, whereas if it activates the nuclear receptor PPARβ/δ, stimulation of cell growth and inhibition of apoptosis are the results. The choice of the target receptor seems to be determined at the cytosol environment by the differential transport systems required for nuclear location. Before RAR recognition, the cellular RA-binding protein II (CRABP II) operates as the carrier, while the fatty acid-binding protein 5 is necessary for PPARβ/δ ligation [57]. There are two CRABPs (I and II), both belonging to the highly-conserved family of small cytosolic lipid binding proteins. CRABP I retains RA in the cytoplasm and prevents its nuclear uptake, hence working to modulate the balance system, with CRABP II being over-expressed in a wide variety of cancers, such as neuro-blastoma and Wilms' tumour [58]. In the nucleus, RAR binds retinoids, ATRA and the closely-related isomer *9-cis*-RA. RXR binds *9-cis* and *all-trans* isomers of RA and rexinoids [59-61].

Both *in vitro* and *in vivo* studies have revealed that several nuclear receptors need to constitute a heterodimer with RXR in order to exert transcriptional functions. The first identified heterodimeric partners were the receptors for thyroid hormones, retinoids, and vitamin D. The PPAR, liver X receptors, farnesoid X receptor, pregnane X receptor, and constitutively activated receptors are also included in this group [60]. All three RXR subtypes are common hetero-dimerization partners for members of the so-called subfamily 1 nuclear receptors. They exert their actions as ligand-dependent transcriptional regulators by binding to the specific DNA-response elements found in the promoter region of target genes, whose interaction with RXR increases with their DNA-binding efficiency [61]. ATRA, for example, activates RAR/RXR heterodimers and exerts its biological actions by binding to retinoic acid response elements (RARE) [62]. The CRABP II combined with RA acts as a co-activator for RAR/RXR, interacting specifically with the receptor complex RAR/RXR, which is bound to the

RARE of particular genes in order to greatly activate their expression [63]. In addition, retinoids can either activate or repress gene expression through RAR/RXR heterodimers interacting with other transcription factors, such as AP-1, estrogen receptor α, and nuclear factor NF-κB [64].

4. PATHWAY INTERACTIONS: CO-MODULATION OF PPAR/RXR AND RAR/RXR SIGNALLING BY DIETARY PUFA DERIVATES EICOSANOIDS AND RETINOIDS

The transcriptional activity of eicosanoids is mediated by ligation with PPAR, which act in their corresponding PPRE. The retinoid transcriptional activity is carried out by RA (the active form), which binds to RAR in order to interact with their corresponding RARE. Nevertheless, RA also possesses other complementary actions. It binds to RXR, which in turns activates either PPAR or RAR by heterodimerization. Moreover, RA can bind directly to PPAR. Consequently, RA is able to modulate gene expression directly by ligation with PPAR or RAR, and indirectly by binding with RXR (complexes PPAR/RXR and RAR/RXR). Concomitantly, eicosanoid-related regulation can be modified by RA-mediated co-modulation. As mentioned above, the availability of these lipid mediators can be changed in a quailtative-quantitative manner by dietary PUFAs supply.

Interestingly, there is a regulatory link between carotenoids/pro-vitamin A and fatty acid metabolism, with it being reported that the CMO1 gene can be transcriptionally regulated by the action of PPAR/RXR, either in mice or in humans [65]. Furthermore, our work on two varieties of human breast cancer cells adds further support to the significant interrelation existing between PUFA (n-3: EPA; n-6: GLA -γ-linolenic acid-) and ATRA administered in the culture media, with the differential responses (Quiroga *et al*., unpublished data). Thus, as shown in Table **1**, we can observe that for the lines ZR-75-1 and MCF-7 (which exhibit dissimilar grades of differentiation [66]), COX activity (marker: release levels of 12-HHT) predominates over LOX activity (marker: release levels of 12-HETE). ATRA increases eicosanoid formation (COX and LOX pathways) in ZR-75-1 (undifferentiated line with low basal levels of eicosanoids), but it strongly reduces this synthesis in MCF-7 (the more differentiated line exhibiting the upper basal levels of eicosanoids). When ATRA is co-administered with GLA, the retinoic effects on LOX activity are suppressed. On the other hand, co-treatment with EPA decreases COX activity in both lines, independently of the ATRA effect. The ZR-75-1 cells treated with ATRA+GLA produce higher 12-HETE than cells treated with ATRA+EPA, whereas the inverse is true for MCF-7 cells.

Our results agree with previous ones showing that the combination of certain dietary PUFA and retinoids consistently inhibited the progression of mammary carcinogenesis in both cell cultures and animal studies, prompting future research to clarify these findings [67]. In another work, the combination of RXR and PPARγ synthetic agonists (bexarotene and rosiglitazone, respectively) in the treatment of colon cancer produced a greater efficacy in growth inhibition than either single agent. Also, co-treatment cooperatively decreases COX-2 expression and PGE_2 synthesis, increasing the desirable expression of the differentiation markers [68].

5. CONCLUSIONS

In the framework of the available data, the outcome of a nutritional intervention with two types of molecules (derivates of fatty acids and vitamin A) may implicate interactions of major molecular pathways, involving the regulation of critical common points in cell proliferation, differentiation and death. Thus, the chemopreventive and anti-cancer activities of these compounds depend on synergic effects at the transcriptional level. Nevertheless, the previous grade of differentiation of the tumour tissue should be considered, since it is a pre-condition for cellular responses, especially when cytoprotective and cytodifferentiating agents are evaluated. Overall, the neoplastic phenotype will be determined by the existing interplay between the different pathways. Regarding this, the sum of the effects will depend on their DNA binding sites and on the specific molecular properties of the receptor isoforms and their differential

Table 1. Eicosanoid Formation in Breast Cancer Cell Lines (BCCL) Co-Treated with ATRA and EFAs*

BCCL	Treatment	LOX Activity (12-HETE)	COX Activity (12-HHT)
MCF-7	C	15.98 ± 7.86 (c)	206.22 ± 12.31 (b)
	ATRA	2.02 ± 0.45 (a-b)	4.23 ± 0.15 (a)
	ATRA-GLA	14.48 ± 1.23 (b-c)	2.16 ± 0.81 (a)
	ATRA-EPA	0.00 ± 0.00 (a)	0.00 ± 0.00 (a)
ZR-75-1	C	5.52 ± 1.02 (a-b)	69.86 ± 12.63 (b-c)
	ATRA	14.75 ± 1.16 (c)	183.13 ± 26.76 (c)
	ATRA-GLA	3.72 ± 0.85 (a)	39.94 ± 5.78 (a-b)
	ATRA-EPA	10.40 ± 3.96 (b-c)	24.41 ± 0.57 (a)

*C: control; ATRA: *all-trans*-retinoic acid; GLA: γ-linoleic acid; EPA: eicosapentaenoic acid.

*Different letters in round brackets indicate statistical differences (ANOVA followed by LSD Fisher test; $p<0.05$).

affinities for dietary ligands, whose availability in foods and meals may be intentionally-managed.

Taken together, the evidence suggests that EFAs and retinoids, as natural dietary compounds, possess an important biomedical potential, with chemoprevention and chemotherapy being the main aims, given their regulating activities on cellular proliferation and differentiation. Also, the nutrigenomic study of different receptors for dietary ligands will promote future nutritional, clinical and epidemiological approaches.

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Anti-breast cancer activity of curcumin on the human oxidation-resistant cells ZR-75-1 with γ-glutamyltranspeptidase inhibition†

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Since curcumin, a polyphenol extracted from the rhizomes of *Curcuma longa* L. (*Zingiberaceae*), has been proposed for breast cancer chemoprevention, the aim of the present work was to determine if it had anti-tumour effects on mammary cells which are resistant to oxidative damage. ZR-75-1 cells were treated with curcumin and copper(II) sulphate in order to evaluate cell death and γ-glutamyltranspeptidase (GGTP) activity. Curcumin was cytotoxic in a dose-dependent manner (loss of viability with lactate-dehydrogenase release) with apoptotic effects on ZR-75-1 cells. Also, curcumin displayed an antioxidant effect only on the copper-oxidized cells. The GGTP activity was decreased in a dose-dependent manner by curcumin, with the changes in this parameter accounting for neoplastic inhibition (direct relation between the enzyme activity and cellular viability). Summing up, our results suggest that curcumin induced apoptosis in ZR-75-1 with an antioxidant activity performed on those treated with copper(II) sulphate, which should be explored more thoroughly with the involvement of the GGTP enzyme activity as biomarker of their malignancy.

Key words: Apoptosis, biomarker, breast cancer, curcumin, copper, γ-glutamyltranspeptidase, oxidative stress

INTRODUCTION

Breast cancer is one of the most frequent causes of female death worldwide, with its pharmacological treatment being a medical challenge due to its malignancy (1). Regarding this, curcuminoids have been proposed as potential anti-tumour agents for refractory cases (2,3). Thus, the effects of curcumin, a polyphenolic antioxidant extracted from the rhizomes of *Curcuma longa* L. (*Zingiberaceae*), need to be evaluated in human aggressive cancer cells, such as the line ZR-75-1.

With regard to preventive strategies, the use of dietary antioxidants has been proposed to counteract the damages induced by oxidative stress (4). Several studies have been directed towards the evaluation of biomedical properties of different plant biomolecules which might be nutraceutical (5). Concerning the potential of these substances, one of the most pursued is the anti-tumour activity acting at different carcinogenetic stages (6). From many phytochemicals, the curcumin was proposed as a chemopreventive and chemotherapeutic agent in breast cancer (7). Related to this, curcumin could inhibit cancer initiation, promotion, progression and dissemination in animal models (8). However, given its antioxidant activity, the effects

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may differ depending on the cellular redox state. Consequently, in vitro probes should be used in cells challenged with an oxidant agent, such as copper(II) sulphate. Also the xenobiotics (i.e. curcumin and cupric salts), which form part of human diet, could modulate different cellular parameters such as the membrane γ-glutamyltranspeptidase (GGTP), which is involved in cellular antioxidant defence (9). Therefore, GGTP modulation could be considered in oncological interventions, as it may have an active role in cellular proliferation and malignant behaviour (10).

The aim of the present work was to determine the curcumin anti-tumour effect on ZR-75-1 cells, which can resist usual oxidative therapy, by assessing cell death and GGTP activity.

MATERIALS AND METHODS

Chemicals

Curcumin (CAS nº 458-37-7, $[HOC_6H_3(OCH_3)CH=CHCO]_2CH_2$, MW=368.38 g/mol) and copper(II) sulphate (CAS nº 7758-98-7, $CuSO_4$, MW=159.61g/mole) were obtained from Sigma-Aldrich Inc. (USA). The kits for in vitro enzymatic determinations (γ-glutamyltranspeptidase -GGTP-, lactate-dehydrogenase -LDH-) were purchased from Wiener Lab (Argentina). Staining chemicals (Hoechst 33342, methylthiazolyldiphenyl-tetrazolium bromide -MTT-, *N,N,N',N'*-tetramethyl-p-phenylendiamine1,4,dihydrochloride -TMPD-), culture reagents and other substances were obtained from Sigma-Aldrich Inc. (USA).

Cell culture and treatments

The human breast cancer cell line ZR-75-1 (American Type Culture Collection) was cultured in RPMI-1640 completed with 10% foetal bovine serum (FBS), 100 U/mL penicillin G and 40 μg/mL gentamycin sulphate, incubated at 37ºC in a 5% CO2 atmosphere. After 24 h post-seeding in 96-well plates (30,000 cells/well), cells were incubated for 24 h in medium containing curcumin (0,5,10,20 and 40 μM, dissolved in dimethylsulphoxide (DMSO, at a final concentration below 0.05% in the media) with cupric sulphate (0,2.5 and 10 μM, dissolved in water). The dose range of curcumin used for experiments were chosen in accordance to other researchers in other to modulate molecular targets of malignant development (11), while the copper concentrations were those required for in vitro protein oxidation (12).

Cellular viability assessment

After treatment and discarding the media, viable cells were cultured with 60 μL MTT (0.25% in culture media without phenol red) for 4 h. After washing with PBS, the stained cells were solubilized with 100 μL Triton X-100 (10%, 20 min). Results were recorded using a Bio-Rad 680 microplate reader and the relative absorbance was calculated (percentage with respect to Control) at 540 nm (13).

LDH activity measurement

Technical requirements were first established to avoid interferences in the study of this enzyme for the experimental conditions of this work. The released LDH by death cells was measured following the LDH-P UV AA kit manufacturer's instructions, and results (IU/L converted to percentages) were recorded at 340 nm (14).

Cytological characterization

Cells were first stained with 1μg/mL of Hoechst 33342 (15 min at 37ºC in darkness). After washing three times with PBS, the vital/death phenotype was determined using a fluorescence microscope (Axiovert 100, Zeiss) under UV light (365/380 nm). Images were analysed using the Axio-Vision software (Zeiss) (15).

GGTP activity measurement

After the medium was discarded, the active enzyme was released from the cellular membranes using 20 μL of Triton X-100 (10%, 20 min). Then, GGT was measured following the γ-G-test kinetic AA kit manufacturer's instructions, adapted to determinations in cultured cells (10). Proteins were measured in 10 μL of the samples by the Bradford method (16), in order to report results as mIU/mg of protein (specific activity).

Free radical detection

After the treatments, plates were washed three times with PBS, and cells were lysed with 15 μL of sodium dodecylsulphate (1%, 20 min). 5 μL of the samples were separated for protein determination by the Lowry method (17). Samples (10 μL) were mixed with 50 μL of 16 mM TMPD, incubated for 30 min and measured at 540 nm. Given the high susceptibility of TMPD to oxidizing agents (4), it was prepared in DMSO in order

to stabilize solutions and improve the technical efficiency. The presence of reactive oxygen species (ROS) was calculated by a calibration curve, with results being reported as μM of H2O2/mg of proteins.

Statistical analysis

Data were expressed as means ± standard error (SE) from four separate experiments performed in triplicate. ANOVA models were used to evaluate differences among the treatments. For the comparison of means, Tukey tests were used ($p<0.01$). Associations between different cellular responses were established by the Pearson coefficient. The statistical analyses probes were performed using the InfoStat 2008e.1 software.

RESULTS

Cellular death

The percentage of viable cells respect to controls was significantly decreased by curcumin in a dose-dependent manner ($p<0.0001$), with non-copper(II) sulphate-related effects present on this variable (Table). In order to confirm cell toxicity, the LDH activity in the culture media was assayed. A strong inverse correlation between this variable and the decreasing cellular viability (Pearson coefficient=-0.70) was found. In a dose-dependent manner, curcumin increased the LDH release from injured cells ($p<0.0001$). Although this effect was more notable in cells treated with 10 μM copper (II) sulphate, this salt did not modify the enzyme release (Table). Morphologically, cultures with decreased cellular viability exhibited several apoptotic figures (Figure).

Specific GGTP activity and its correlation with cellular viability

In order to establish the value of this parameter as a breast cancer biomarker, it was necessary to decide an appropriate experimental cell density. Regarding this, a number of 30,000 cells per well was chosen after performing experiments on a wide range of seeded cells (10,000-70,000 cells/well). A direct correlation was found between the specific GGTP activity and the cellular viability assessed by the MTT assay (Pearson coefficient=0.68). Concerning this, curcumin was able to diminish the enzymatic activity dose-dependently, which was enhanced by incorporation of copper (II) sulphate ($p<0.0001$); (Table).

Free radical level

The cellular oxidative level was significantly increased by copper (II) sulphate at both concentrations (2.5 and 10 μM). Curcumin behaved as an antioxidant agent under copper-related oxidative stress ($p<0.0001$), with effects being clearer in cells treated with 10 μM of copper (II) sulphate, and absent in copper-unexposed cells (Table).

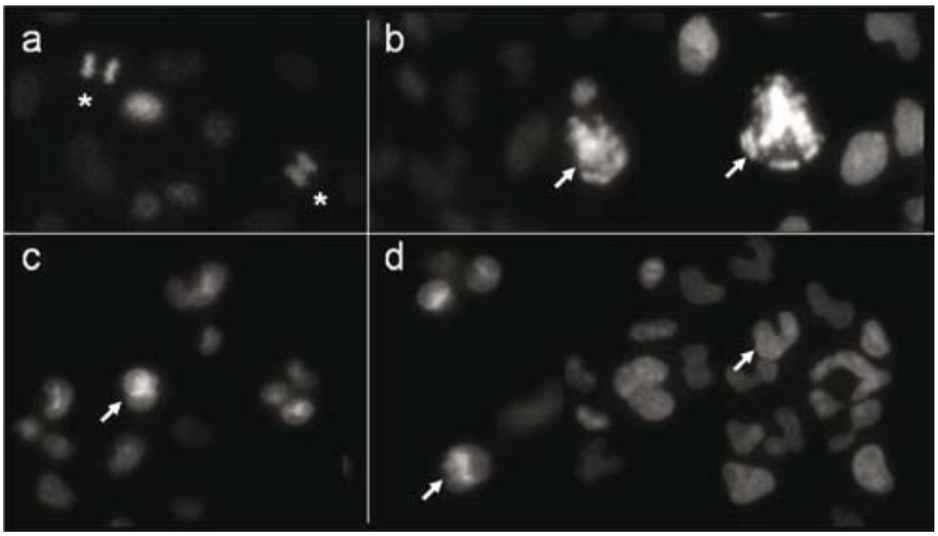

Figure. Cytofluorescence (Hoechst 33342 staining, 400x): ZR-75-1 cells were incubated for 24 h with curcumin at different concentrations (0,5, 10, 20 and 40 μM). The images were representative of those from four separate experiments, with mitotic figures being found in non-treated cells (a), whereas curcumin-treated cells showed several apoptotic figures (b-d).

Table. In vitro parameters in ZR-75-1 cells treated with curcumin and copper

Cellular viability (% respect to controls):				
		Copper(II) sulphate (μM)		
		0	2.5	10
Curcumin (μM)	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	5	102.94 ± 2.94	110.00 ± 3.33	103.33 ± 3.33
	10	85.29 ± 5.88	76.67 ± 3.33*	73.33 ± 6.67*
	20	38.24 ± 2.94*	40.00 ± 3.33*	40.00 ± 3.33*
	40	20,59 ± 0.68*	26.67 ± 3.33*	20.00 ± 3.33*
Lactate-dehydrogenase release (% respect to controls):				
		Copper(II) sulphate (μM)		
		0	2.5	10
Curcumin (μM)	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	5	108.29 ± 15.94	85.87 ± 5.40	215.18 ± 34.76
	10	152.76 ± 24.48	124.12 ± 24.45	182.50 ± 27.69
	20	242.55 ± 48.96*	167.20 ± 21.91	547.62 ± 56.08*
	40	216.63 ± 22,11*	153.65 ± 23.48	508.57 ± 82.85*
Specific γ-glutamyltranspeptidase activity (mIU/mg of protein):				
		Copper(II) sulphate (μM)		
		0	2.5	10
Curcumin (μM)	0	6.87 ± 0.81	6.27 ± 0.62	3.82 ± 0.58*
	5	3.21 ± 0.34*	3.44 ± 0.33*	1.62 ± 0.17*
	10	1.63 ± 0.37*	1.51 ± 0.59*	0.40 ± 0.15*
	20	0.21 ± 0.05*	0.29 ± 0.16*	0.06 ± 0.04*
	40	1.00 ± 0.26*	0.34 ± 0.06*	0.46 ± 0.14*
H2O2 formation (μM/mg of protein):				
		Copper(II) sulphate (μM)		
		0	2.5	10
Curcumin (μM)	0	69.42 ± 5.92	206.14 ± 18.70*	204.38 ± 16.84*
	5	53.73 ± 1.14	60.23 ± 3.63	116.31 ± 15.27
	10	69.72 ± 9,06	70.73 ± 8.47	88.36 ± 15.07
	20	36.69 ± 2.45	79.43 ± 10.37	32.37 ± 13.08
	40	101.01 ± 9.12	115.33± 12.69	48.36 ± 4.74

Data were expressed as means ± SE of four separate experiments (*p<0.01).

DISCUSSION

Dietary polyphenols, such as curcumin, could be considered for cancer chemoprevention, which can be primary (preventing illness appearance) and/or secondary (preventing illness progression), and for therapeutic schemes (chemotherapy and chemoadjutancy) (18). In the present study, curcumin was found to be cytotoxic for ZR-75-1 cells in a dose-dependent manner, despite the fact that it retained its antioxidant activity on copper-oxidized cells. These results indicate that cell death was induced by mechanisms different from oxidative damage (19), with the role of thiol-reactive metal ions requiring further studies due to the existence of contradictory data (20). Nonetheless it was previously established that curcumin induced apoptosis on the human breast cancer line MCF-7 (21), the ZR-75-1 cell line was used because it represents a clear example of cells which can tolerate traditional anticancer oxidative stress-based treatments. Moreover, the viability of this cell line was not compromised by copper-induced peroxide formation. This finding show that these cells resist different kinds of oxidizing agents, such as arsenic and other xenobiotics reported by Soria et al. (10).

Concerning curcumin molecular targets (22), an incipient theory sustains that neoplastic cells have deregulated pathways triggered during cancer initiation and promotion, which are essential for tumour development, such as the GGTP activity. As a result, their inhibition by antioxidants may lead to cellular death (23,24). Also, curcumin can activate the steroid/xenobiotic receptor, which is antiproliferative in breast cancer cells, including ZR-75-1 (25). Regarding this, given the indirect relation between cellular viability and LDH release, we conclude that curcumin was cytotoxic for ZR-75-1 cells leading them to acquire apoptotic features.

Related to cancer development and pathologically-activated antioxidant defences, the GGTP activity has been proposed as a tumour biomarker due to its cytoprotective and pro-proliferating activities (26). Moreover, it was shown to be a sensitive variable with respect to culture conditions (i.e. cellular density), with a strong direct relation being found between the enzyme activity and ZR-75-1 viability, thus supporting its biological role in malignant behaviour. Furthermore, curcumin decreased the GGTP activity (firstly described finding) and compromised the tumour viability. In this regard, the impairment of this antioxidant enzyme may lead to cell death by down-regulation of enzyme-related pro-tumour pathways (27,28). In this regard, it is important to keep in mind that this parameter depends on the cyto-differentiation grade for several dietary compounds, showing a direct relation with the viability of undifferentiated cells (i.e. ZR-75-1), whereas other more differentiated types (i.e. MCF-7) exhibit an inverse relation (10; Quiroga et al., unpublished data), with further studies involving a wide range of tumour cell types being encouraged.

CONCLUSIONS

Summing up, the dietary antioxidant curcumin reduced the viability of ZR-75-1 cells, with GGTP being an appropriate biomarker for evaluating the cancer cytotoxic response. In consequence, there is evidence to encourage the use of curcumin as an effective phytochemical in combating tumour growth under conditions of oxidative stress (such in case of the traditional breast chemotherapy) involving an apoptosis-like cell death.

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Differential effects of quercetin and silymarin on arsenite-induced cytotoxicity in two human breast adenocarcinoma cell lines

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Abstract

Arsenic has been proposed as a chemotherapeutic agent for leukemia and other solid tumors. However, its environmental exposure has been linked epidemiologically with an elevated carcinoma risk (i.e. skin, bladder and lung), with cellular oxidative stress being implicated in both induced-arsenic toxicity and carcinogenicity. Consequently, antioxidants may differentially interfere in these effects. The human mammary adenocarcinoma lines MCF-7 and ZR-75-1 were treated in vitro with 200 μM $NaAsO_2$ (As), 5 μM silymarin (S) and/or 50 μM quercetin (Q). The following biomembrane parameters were assessed: sialic acid (SA) in gangliosides, γ-glutamyltranspeptidase activity (GGT), conjugated dienes and free radical activity, in order to evaluate the arsenite–flavonoid interactions. The time-dependent arsenite toxicity was not prevented by flavonoids in ZR-75-1 cells, whereas quercetin protected MCF-7 cells for 8 h. With regard to GGT, only quercetin protected ZR-75-1 cells against stress. In MCF-7 cells, the arsenite-induced GGT activity was not counteracted by either quercetin or silymarin. S, Q, As and As + S treatments reduced the SA content only in the MCF-7 membrane, while As + Q treatment increased it in both lines. The membrane resistance to lipid oxidation in these cells enclosed the up-regulation of GGT activity and sialylglycolipid content. Taking these results together, quercetin interfered with arsenite toxicity, whereas silymarin was not able. Thus, the potential role of flavonoids as co-adjutants may differ widely in therapeutic protocols.

Keywords: Arsenite; Breast cancer cells; γ-glutamyltranspeptidase; MCF-7; Oxidative stress; Quercetin; Sialic acid; Silymarin; ZR-75-1

Introduction

Arsenic has been proposed as a chemotherapeutic agent for human leukemia and other solid tumors (Ling et al., 2002). On the other hand, its chronic exposure has been linked epidemiologically with an elevated risk of urinary, lung, skin, colon, and liver carcinomas, in areas with high levels of arsenic in the drinking water (Paoloni et al., 2005). Oxidative stress is being increasingly recognized as a possible mechanism implicated in both induced-arsenic apoptosis and carcinogenicity (Shi et al., 2004; Valko et al., 2006). Consequently, the potential of several antioxidants to counteract arsenic injury has been tested in vitro and in vivo (Wei et al., 2005; Bongiovanni et al., 2007). Silymarin and quercetin are polyphenolic antioxidant flavonoids, which possess cytoprotective and anticarcinogenic effects, and are widely found in vegetable sources (Volate et al., 2005). These compounds are usually presented as equivalent (Khanna et al., 2007). However, this is not always the case. Regarding this, quercetin is a more powerful antioxidant than silymarin against the dose-dependent cytotoxicity of hydrogen peroxide (Svobodova et al., 2006). Also, the former is a non-competitive inhibitor of arylamine *N*-acetyltransferase 1 and 2 enzymes in the metabolic activation of aromatic and heterocyclic amines, whereas silymarin is not

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(Kukongviriyapan et al., 2006). Due to their similarities and differences, they could play distinct roles in antineoplastic chemotherapy (Kanadaswami et al., 2005). Therefore, it is important to acquire further knowledge about the interactions among arsenite and antioxidant flavonoids in cancer and normal cells.

Cellular membrane integrity plays a critical role in cell functionality and viability, which is relevant in carcinogenesis and oncological pharmacology (Hossain et al., 2000; Pasqualini et al., 2003). In this regard, the lipid sialylation and the ectoenzyme γ-glutamyltranspeptidase activity (GGT, CD-224, EC 2.3.2.2) were studied as markers of the cell membrane status. Gangliosides (sialylglicolipids) have previously been found to be involved in cellular surface-related regulation and in biomembrane resistance to oxidation (Proia, 2003; Sergent et al., 2005). Furthermore, since some cancer cells present aberrant glycosilation, with the sialic acid content (SA) being a useful tumor marker (Narayanan, 1994). Finally, GGT protects cells from oxidative stress with constitutive high enzyme levels, being associated with cancer development and chemoresistance (Hanigan et al., 1999). In conclusion, the aim of this work was to study the arsenite–flavonoid interactions and their effect on some properties of membrane components, and also on the viability of human mammary cancer cells.

Materials and methods

Chemicals

Quercetin ($C_{15}H_{10}O_7$ 2 H_2O, MW=338.27 g/mol) was obtained from E. Merck (Germany). Sodium arsenite ($NaAsO_2$) was purchased from Anedra SA (Argentina, www.anedra.com.ar). Silymarin ($C_{25}H_{22}O_{10}$ 2 H_2O, MW=482.4 g/mol), *N*,*N*,*N*',*N*'-tetramethyl-p-phenylendiamine1,4 dihydrochloride ($C_{10}H_{16}$ N_2 2HCl, TMPD, MW=237.2), culture reagents and other chemicals were obtained from the Sigma-Aldrich Co. (USA). The γ-*G*-test kinetic AA kit™ for in vitro diagnosis was from Wiener Laboratories (Argentina, www.wiener-lab.com.ar).

Cell culture

MCF-7 and ZR-75-1 cells (American Type Culture Collection), obtained from mesothelial carcinomatosis exudates of two Caucasian 60–70 year-old women with human mammary ductal adenocarcinoma (Engel and Young, 1978), were cultured in Dulbecco's modified Eagle's medium completed with 10% fetal bovine serum (FBS), 100 IU/mL penicillin G and 40 μg/mL gentamycin sulphate, incubated at 37 °C in a 5% CO_2 atmosphere.

Treatments

After 48 h post-seeding (40,000 cells/cm^2), cells were incubated under all of the following conditions (acute treatment): 200 μM $NaAsO_2$ (As), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM NaAsO2 plus 5 μM silymarin (As + S), 200 μM NaAsO2 plus 50 μM quercetin (As + Q), and controls having no treatment (C). Flavonoid concentrations were used in agreement with the oral bioavailability obtained in clinical assays at high doses, (Williamson and Manach, 2005). Treatments were continued for 0–8 h in order to evaluate cellular viability, while other variables were studied after a 2 h exposure. Additionally, GGT activity was also measured after treatment and allowed to recover for 2 h in free treatment medium. Consequently, cells could be obtained both with (RC) or without recovery (NRC).

Crystal violet staining (cellular viability)

After 48 h attachment in 96-well plates (10,000 cells/well), viable cells were stained with 0.5% crystal violet in 50% methanol for 15 min. After washing with 50% methanol three times, the stained cells were solubilized with 20% methanol in a sodium citrate solution (0.1 M, pH 5.4). Results, consistent with cellular density, were recorded by a BioRad 680 microplate reader and presented for relative absorbance (percentage calculated with respect to C) at 570 nm.

Membrane obtention

After enzymatic harvesting with porcine trypsin, cells were homogenized at 20,000 rpm for 30 seconds in 1 mL of 10 mM HEPES buffer (pH 7.4, containing 2 μg/mL leupeptin and 1 mM EDTA). They were centrifuged at 100,000 g for 1 h at 4 °C to recover the pellet (P), which was then resuspended in 200 μL of 10 mM HEPES buffer. The protein content was determined according to the Lowry method, and each P suspension (150 μL) was mixed with 750 μL chloroform/methanol (2:1 v/v, Fölch extraction). The mixture was centrifuged at 1,000 *g* for 10 min, and then the upper layer was used for sialic acid determination. The lower layer was washed twice with chloroform/methanol/water (3:48:47 v/v/v) and dried under a pure nitrogen flow at room temperature. Lipids were resolubilized in 200 μL ethanol (eP samples).

GGT activity measurement

The modified Szasz method was used following the γ-*G*-test kinetic AA kit manufacturer's instructions (Szasz, 1969). Samples were mixed with 1% Triton X-100 and 100 mM Tris-HCl substrate buffer pH 8.5 (containing 2.9 mM L-γ-glutamyl-3-carboxi-4-nitroanilide and 100 mM GLY–GLY) in the proportion 1:1:9 (v/v/v). Then, the absorbance at 405 nm was recorded. Results were expressed in mIU/mg of proteins (1 IU=1 μmole of product/min at pH 8.5 and 25 °C). After sample solubilization with Triton X-100, the enzyme activity was recorded for 24 h, using different sample concentrations (protein amount) and the substrate buffer alone as blank. Concerning this, activity was recorded for 10 min in samples containing 1.5 mg/mL of protein.

SA content measurement (membrane sialylation)

After Fölch extraction, the SA content (nmole/mg of protein) was measured in the upper phase at 580 nm according to Miettinen and Takki-Luukkainen (1959).

Membrane oxidation assessment

The conjugated dienes (CD) were measured as lipid oxidation markers under low-temperature conditions in eP samples at 234 nm against ethanol (Recknagel and Glende, 1984). Results (DO/mg of protein) were expressed as relative absorbance compared to controls.

Free radical activity was detected by the oxidized TMPD radical measurement (Cornelli et al., 2001). Briefly, each eP sample was mixed with 2 mM TMPD in ethanol (1:1 v/v), and kept in an oxygen-free environment at room temperature for 30 min. Results (DO/mg of protein) were recorded at 560 nm and expressed as relative absorbance compared to controls.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) from four separate experiments performed in triplicate, unless otherwise noted. ANOVA models were used to evaluate differences of cellular viability and membrane parameters (GGT and SA) among treatments (C, S, Q, As, As + S, As + Q). For the comparison of means, Tukey tests were used, considering a significance level of $\alpha = 0.05$. The paired *t*-student test was used to compare GGT activity in the NRC and RC (p-values < 0.05). The association between these variables was determined using the correlation coefficient (CC), while the time-related cellular viability was assessed by single linear regression. Analytical probes were performed using the InfoStat 2007e.1 software.

Results

Cellular viability

The studied human breast lines exhibited different susceptibilities to the treatments (Table 1), with the following treatments decreasing cellular viability significantly in a time-dependent way: As, As + S and As + Q on ZR-75-1 cells, and As and As + S on MCF-7 cells.

GGT activity

The specific activity of GGT, depicted in Fig. 1, had quantifiable basal values in both breast cancer lines, although MCF-7 cells exhibited significant lower activity than ZR-75-1 cells ($p < 0.05$) under the different experimental conditions. It was done with different comparisons in order to analyze the enzyme data:

- *GGT activity before recovery*. MCF-7 cells did not response to the 2-hour treatments. On the other hand, the enzymatic activity was increased in ZR-75-1 cells by exposure to flavonoids with respect to control, although quercetin had the strongest effect ($p < 0.05$), while As, As + S and As + Q had non-significant lower activities than control ($p < 0.09$).
- *GGT activity after recovery*. MCF-7 cells treated with S, As, As + S and As + Q increased the GGT activity with respect to control ($p < 0.05$). A decreased activity was seen with the Q treatment (4.4 fold lower; $p < 0.02$) when NRC and RC results were compared, whereas it was increased after As exposure (2.1 fold higher; $p < 0.05$). ZR-75-1 cells treated with As and As + S had significant lower activities than control ($p < 0.05$). Additionally, an increased GGT activity was found in C, S, As, As + S and As + Q treatments when NRC and RC results were compared: 3.6, 2.4, 3.8, 5.1 and 5.7 folds, respectively ($p < 0.05$).

Membrane sialylation

Despite the fact that ZR-75-1 and MCF-7 cells had similar basal levels of SA in their membranes (288.8 ± 36 and 232.2 ± 36 pmoles of SA/mg of proteins, respectively), they responded by different extents, being the SA content in MCF-7 lower than in ZR-75-1 under the five treatments ($p < 0.05$): 3.4 fold (S), 18.9 fold (Q), 8.8 fold (As), 7.4 fold (As + S) and 4.1 fold (As + Q). In regard to As, S, Q and As + S treatments, they decreased SA amounts with respect to control only in MCF-7 cells, while As + Q treatment increased the membrane SA content independently of the tumor line ($p < 0.05$) (Fig. 2).

Table 1
Cellular viability of the ZR-75-1 and MCF-7 lines (LR, linear regression)

Cell line	Treatment	Time (hours)				Time LR (slope, R^2)
		2	4	6	8	
ZR-75-1	S	77.14±7.81	108.54±28.6	65.44±22.38	83.26±21.63	−2.26, 0.17
	Q	93.8±14.7	71.52±11.52	79.64±0.92	83.75±23.19	−2.33, 0.43
	As	72.15±6.96	80.43±21	35.29±14.46↓	31.9±10.35↓	−8.65, 0.86
	As+S	58.0±14.0↓	52.96±0.44↓	49.54±4.63↓	33.44±12.27↓	−7.08, 0.81
	As+Q	102.24±10.65	80.19±2.43	70.45±15.82	43.29±0.22↓	−7.26, 0.9
F-values (5,18)		8.78	5.12	9.43	12.65	
MCF-7	S	111.47±7.0	82.99±5.61	102.15±9.7	93.64±10.61	−1.1, 0.11
	Q	133.81±27.18	110.87±33.39	130.23±25.84	115.04±13.19	0.29, 0.01
	As	93.36±10.15	82.61±5.9	54.69±12.53↓	46±5.87↓	−7.33, 0.95
	As+S	92.51±16.55	89.61±15.02	58.79±13.0↓	53.59±7.31↓	−6.33, 0.89
	As+Q	113.22±10.45	106.84±14.32	92.5±0.8	95.92±7.46	−1.44, 0.3
F-values (5,18)		3	1.66	12.34	10.22	

Cells were incubated under the following conditions: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data are expressed as% of controls (mean ± SD). ↓Significant decrease compared with the corresponding untreated controls at each time ($p < 0.05$).

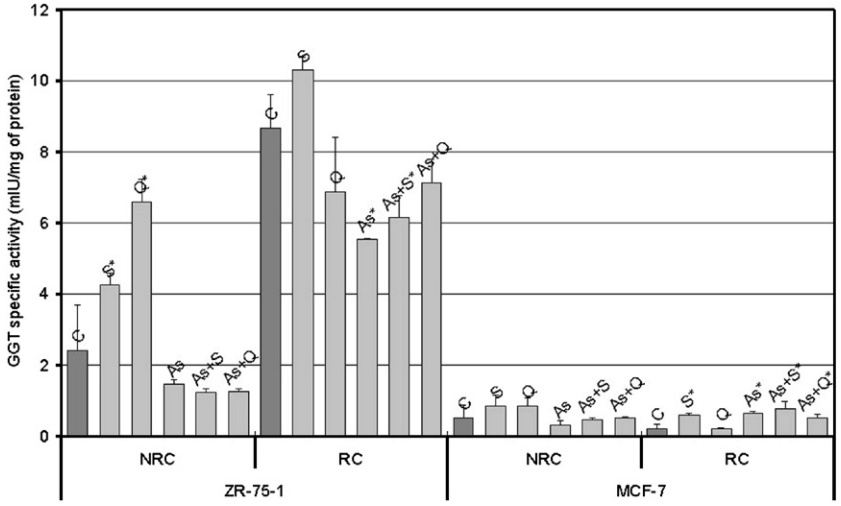

Fig. 1. GGT specific activity in ZR-75-1 and MCF-7 cells. Cells were incubated under the following conditions: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data (mean ± SD) were obtained from non-recovered cells (NRC: 2 hour treatment) and recovered cells (RC: 2 hour treatment followed by 2 hour recovery). * Statistical differences compared with the corresponding control (C, $p<0.05$): $F(5,18)=57.59$ (ZR-75-1 NRC), $F(5,18)=18.32$ (ZR-75-1 RC), $F(5,18)=1.94$ (MCF-7 NRC), and $F(5,18)=14.21$ (MCF-7 RC).

Membrane oxidation and its relation with other parameters

In ZR-75-1 cells, the CD levels were similar: 100±0 (C), 112±9.8 (S), 126±16.7 (Q), 121±26.1 (As), 113±23.6 (As + S), and 95±26 (As + Q). S, Q and As + Q treatments decreased TMPD measures <5% of control ($p<0.03$), while As + S reduced them by 50%. Subsequently, the TMPD oxidation was not strongly related to CD formation (CC<0.5). In MCF-7 cells, the CD levels were also similar: 100±0 (C), 102±5.6 (S), 96±8.4 (Q), 95±11.4 (As), 99±15.9 (As + S), and 85±20 (As + Q). In contrast to ZR-75-1 cells, the TMPD oxidation was reduced by the treatments to some extent and this was closely related to CD formation (CC>0.5). In both breast lines, the CD elevation enclosed upper GGT activity (CC>0.5). Although this enzyme and the SA content had a non-linear relationship, both were linked with decreased TMPD data (CC<−0.5). Moreover, increased SA content was associated with lower CD formation (CC<−0.5).

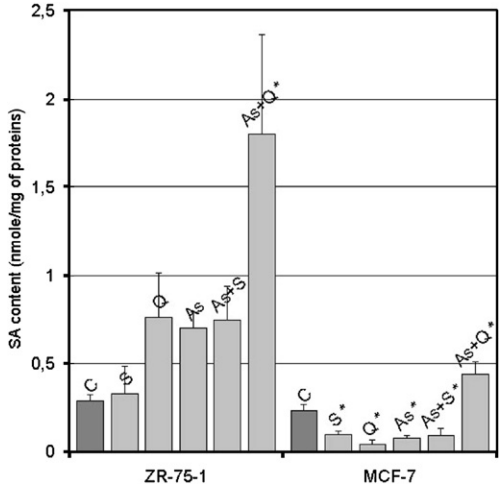

Fig. 2. Sialic acid (SA) content in ZR-75-1 and MCF-7 cells. Cells were incubated under the following conditions: none (C), 5 μM silymarin (S), 50 μM quercetin (Q), 200 μM sodium arsenite (As), As + S and As + Q. Data are mean ± SD. * Statistical differences compared with the corresponding control (C, $p<0.05$): $F(5,18)=15.72$ (ZR-75-1), and $F(5,18)=32.37$ (MCF-7).

Discussion

The malignant behavior of breast cancer cells is closely linked to their membrane properties. In the present work, the different lines were treated following a previous protocol (Bongiovanni et al., 2007). Accordingly, CHO-K1 cells needed a recovery time in order to show quantifiable amounts of Hsp70 and GGT, indicating a low or absent constitutive presence. The arsenite-induced stress produced increases in GGT (1.73 fold), CD (1.85 fold) and Hsp70 (3.79 fold), which were counteracted by flavonoids.

Since ZR-75-1 and MCF-7 cells, together with other lines positive for estrogen receptors (ER+), share substantial global similarities in the phenotypic characteristics and in the structures of their respective transcriptomes with human ER + breast tumors, these lines are good experimental models in which to identify events that are likely to be important in these cancers (Zhu et al., 2006). Nonetheless, in the presence of breast cancer heterogeneity, some biomedical relevant differences can be found. Indeed, ZR-75-1 cells are selectively insensitive to the antiproliferative actions of 2-methoxyestradiol due to their high levels of 17-β-HO-steroid dehydrogenase II, which rapidly inactivate the steroid (Liu et al., 2005). Furthermore, ZR-75-1 and MCF-7 lines reflect dissimilar grades of differentiation, as the latter retains several characteristics of differentiated epithelium (Engel and Young, 1978). Here, these differences were supported by the fact that the ZR-75-1 cells showed an elevated constitutive GGT activity, an extended SA induction and a weaker response to the trophic effect of a phytoestrogen (i.e. quercetin, Oh and Chung, 2004; Ise et al., 2005) in the crystal violet staining. For this assay, the time-dependent arsenite-

induced toxicity on ZR-75-1 cells was not prevented by either flavonoid, while quercetin protected the MCF-7 cells for 8 h.

With regard to the GGT, which is constitutively present in several tumors (Hanigan et al., 1999), both breast lines exhibited detectable basal levels. Since the enzymatic activity of GGT in ZR-75-1 was increased in RC with respect to NRC including controls, the change of culture medium resulted in a self-sufficient stress condition. This phenomenon was not seen in quercetin treated cells, which had a significant rise after the first 2 h of treatment (2.73 fold), but without showing any variation after the medium change. Since GGT expression is regulated by the cytosolic glutathione pool and steroids (Rasmussen et al., 2005; Chinta et al., 2006), we speculated that the increased quercetin-induced activity might generate enough intracellular glutathione to sustain the enzymatic activity level reached during the recovery time. Conversely, although silymarin increased GGT activity 1.76 fold, it did not protect cells from the medium change-related stress. In arsenite-treated cells, only quercetin protected cells from the arsenite-induced inhibition of GGT, requiring at least 2 h of recovery. In MCF-7 cells, the enzyme was less activated by stressors (medium change or treatments) with a low basal GGT activity (respect to ZR-75-1 cells). Arsenite increased it after the recovery time, and this was not diminished by either quercetin or silymarin.

In the upper phase of the Fölch partition from extra-neural sources, the sialic acid (SA) content represents essentially monosialylated gangliosides from the plasma membrane surface (Hammache et al., 1999). Related to this, ZR-75-1 cells had a greater SA value than MCF-7 cells, with As + Q treatment increasing it for both lines, whereas As, S, Q and As + S treatments reduced the SA content only in the MCF-7 line. Although SA content was not related to cell viability in this work, its pharmacological regulation could be important, since ganglioside molecular interactions are important in cancer growth due to their role in the immune response and in the metastatic process (Hakomori, 2002; Varki and Varki, 2007).

The GGT activity and the SA content were chosen as membrane markers with reference to our previous results, since they were susceptible to prolonged arsenite exposure in the CHO-K1 cells, showing increased GGT activity and decreased SA content (Bongiovanni, 2006; Bongiovanni et al., 2007). This line is usually used as a non-tumoral referent and its responses are compared with human cancer cells (Ling et al., 2002). Moreover, these cells are positive for estrogen receptors and have a similar SA content to the breast cells studied (Nethrapalli et al., 2005; Bongiovanni, 2006). Since the inadequate sialylation and the sustained activation of GGT are tumoral factors (Narayanan, 1994; Hanigan et al., 1999), they should be taken in account in the carcinogenetic compound prospecting.

Concerning lipo-oxidation, distinct lipid sensitivities were found between ZR-75-1 and MCF-7 cells (different TMPD-CD correlation coefficients). The regulation of the GGT activity and the sialylglycolipid content may modulate the membrane resistance to oxidation, leading to a decrease in free radical activity and CD formation. Since the GGT kinetics and redox status can be modulated by changes in membrane lipid composition (Medina Basso et al., 2006), the quercetin and silymarin effects on arsenite-treated cells may also respond to the differential flavonoid affinity for the lipid bilayer. Moreover, the weak relation between cellular viability and oxidative responses involves non-unidirectional mechanisms converging in both arsenite and antioxidant actions (Piga et al., 2007).

Conclusion

Taken together, our results show that there were differences between breast adenocarcinoma lines, which must be taken into account when extrapolations are made. Regarding the flavonoids studied, which protect non-tumoral cells from arsenite-induced stress (Bongiovanni et al., 2007), silymarin should be considered in clinical investigations as an adjuvant agent in chemotherapy. However, the use of quercetin should be carefully evaluated in women under oncological management, since this flavonoid increased the GGT and SA levels, which have been considered tumor markers. Furthermore, quercetin could interfere with the arsenite toxicity in therapeutic approaches.

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85- EFECTO DE CURCUMINA EN CÈLULAS DE MAMA HUMANA ZR-75-1

[Effect of curcumine on cultured ZR-75-1 cancer cells]

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RESUMEN La curcumina, diferuloilmetano, 1,7-bis-(4-hidroxi-3-metoxi fenil)-hepta-1,6-dieno-3,5-diona, es un compuesto polifenólico, que se extrae de los rizomas de la *Curcuma Ionga* a los que le da el color amarillo característico y ha sido utilizado como aditivo alimenticio. Posee actividad antiinflamatoria, antioxidativa, pro-apoptótica, antitumoral y anticancerosa. La gama glutamiltranspeptidasa (GGTP) es una enzima de membrana que metaboliza el glutation, molécula con elevada capacidad antioxidativa. Es marcadora de estrés celular y de algunos cánceres. Valoramos el efecto de la curcumina, en células ZR-75-1 con o sin la adición de Sulfato de Cobre, un agente oxidativo, sobre la proliferación, la actividad de la enzima GGTP, y la visualización de la apoptosis. La proliferación celular disminuye significativamente con el tratamiento con curcumina (20 y 40 μM) pero no es afectada significativamente con la adición de Sulfato de Cobre (2.5 y 10 μM). La curcumina (5 y 10 μM) disminuye la actividad específica de la enzima GGTP presentando un comportamiento bifásico con un punto de inflexión a los 20 μM de curcumina e incrementando a la concentración de 40 μM. La adición de 2.5 μM de Sulfato de Cobre no modificó el efecto de la curcumina sin embargo 10 μM de Sulfato de Cobre provocó un efecto aditivo en la disminución de la actividad de la enzima GGTP. Se observaron figuras apoptóticas ante el tratamiento con curcumina con y sin el agregado de Sulfato de Cobre. La curcumina indujo apoptosis y afectó la proliferación de células ZR-75-1 y en forma bi-fásica la enzima GGTP lo cual fue potenciado con Sulfato de Cobre (10 μM).

PALABRAS CLAVES curcumina, quimioprevención, carcinogénesis.

ABSTRACT The curcumine, diferuloylmetane, 1,7-bis-(4-hidroxy-3-methoxy fenil)-hepta-1,6-diene-3,5-dione, is a polyphenolic compound that is extracted of the rhizomes of the *Curcuma Ionga* to those that it gives the characteristic yellow color and that it has been used as food additive. It has antiinflammatory, antioxidative, pro-apoptotic, antitumoral and anticancer activity. The gamma glutamyltranspeptidase (GGTP) is a membrane enzyme that metabolizes the glutathion, molecule which has a high antioxidative capacity. It is a marker of cellular stress and of some cancers. We added curcumine (0, 5, 10, 20, 40 μM) on cultured ZR-75-1 cancer cells with or without Copper Sulfate (2.5-10 μM) during 24hs and measured the cellular proliferation, the activity of the enzyme GGTP and visualized the induction of apoptosis. The cellular proliferation diminishes significantly with the curcumine treatment (20 and 40 μM) but it is not affected significantly with the addition of Copper Sulfate (2.5 and 10 μM). Curcumine (5 and 10 μM) decreased the GGTP activity with a biphasic behavior with an inflexion point at 20 μM of curcumine and increasing at 40 μM. The adittion of 2.5 μM Copper Sulfate did not modified the curcumine effect. However, 10 μM of Copper Sulfate, induced an additive effect on the diminished activity of the GGTP. It is observed apoptotic figures before the treatment with curcumine with and without the adittion of Sulfate of Copper. Curcumine induced apoptosis and affected the proliferation of ZR-75-1 cells and GGTP activity in bi-phasic form and it had an additive effect with Copper Sulfate (10 μM).

KEYWORDS curcumine, chemoprevention, carcinogenesis

INTRODUCCIÓN

La curcumina, 1,7-bis-(4-hidroxi-3-metoxi fenil)-hepta-1,6-dieno-3,5-diona, es un compuesto polifenólico, que se extrae de los rizomas de la *Curcuma Ionga*. Esta asociada a la prevención de lipoperoxidación y disminución del estrés oxidativo por su acción antioxidante (Rajagopalan & Kallikat, 2004). Inhibe la actividad del factor de transcripción NF-KB induciendo la apoptosis y por ende, inhibiendo el desarrollo tumoral (Braiteh & Kurzrock, 2005). La curcumina (fitoquímico, antioxidante) y el cobre (en forma de sal, oxidante) son agentes que se ingieren con la dieta y pueden afectar la enzima gama glutamiltranspeptidasa (GGTP) vinculada al metabolismo celular del glutatión, un importante reductor celular (Rukkumani, 2004) y es activada por estrés oxidativo (Kugelman et al, 1994). Su actividad ha sido referida como un factor de reconstitución de defensas celulares oxidantes / antioxidantes afectando el proceso de carcinogénesis por la activación del factor de transcripción NF-kappaB (Cremonezzi et al, 2001) Los objetivos son valorar el efecto de la curcumina y/o Sulfato de Cobre sobre la actividad de la enzima gama glutamiltranspeptidasa (GGTP), la proliferación celular y la apoptosis.

MATERIALES Y MÉTODOS

Los reactivos se obtuvieron de Wienner y Sigma Chemical Co. La línea celular ZR-75-1, de la American Type Cell Collection. Las células cultivadas en medio DMEM completo, fueron tratadas durante 24 horas, con curcumina (0, 5, 10, 20 y 40 μM) en ausencia / presencia de Sulfato de Cobre (2.5 y 10 μM). Se determinó la proliferación celular con el colorante MTT según Li y col (2005), las proteínas con la técnica de Bradford (1976) y la actividad de GGTP con el ensayo comercial de Wienner conteniendo Triton X-100. Se utilizó la coloración con HOECHST (1μg/ml) para visualizar apoptosis.

Figura 1. Proliferación de células humanas ZR-75-1 expuestas a curcumina y/o Sulfato de Cobre.

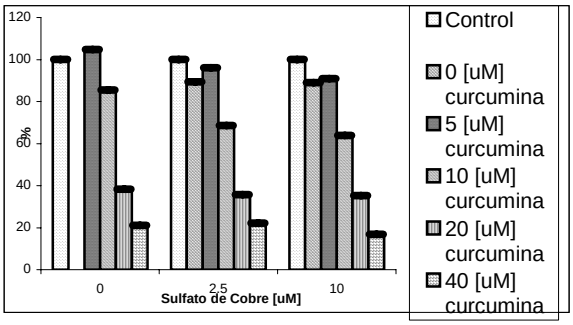


Figura 2. Efecto de curcumina sobre las células humanas ZR-75-1 sin/con Sulfato de Cobre.

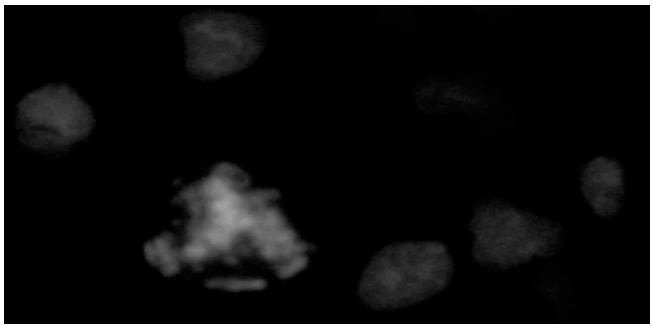
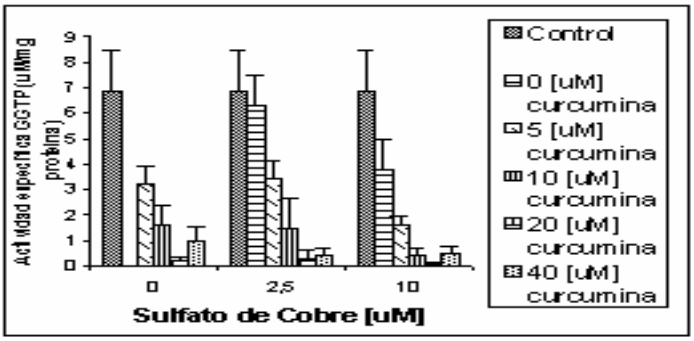


Figura-3. Apoptosis en células tratadas con curcumina sin/con sulfato de Cobre.



RESULTADOS

El control no contiene curcumina ni Sulfato de Cobre. En cada barra se ha indicado la desvia-ción estándar de la media. La proliferación celu-lar disminuye significativamente con el trata-miento con curcumina (20 y 40 μM) pero no es afectada por Sulfato de Cobre (2.5 y 10μM) (Figura 1). La curcumina (5 y 10 μM) disminuye la actividad de la enzima GGTP presentando un comportamiento bifásico con un punto de inflexión a los 20 μM de curcumina e incrementa a 40 μM. La adición de Sulfato de Cobre (2.5 μM) no modificó el efecto de la curcumina sin embargo 10 μM provocó un efecto aditivo en la disminución de la actividad de la enzima GGTP (Figura 2). Se observaron figuras apoptóticas ante el tratamiento con curcumina con y sin el agregado de Sulfato de Cobre (Figura 3).

DISCUSIÓN Y CONCLUSIONES

La inhibición en la proliferación y la apoptosis de células ZR-75-1 por la curcumina fue coincidente con Radhakrishna Pillai et al (2004) con células de cáncer de pulmón. En tanto, la disminución de la actividad de GGTP con el incremento de curcumina (5 a 20 μM) y su recuperación a los 40 μM y la potenciación de de la disminución de GGTP frente a 10μM de Sulfato de Cobre, un agente oxidante, no fue observado por otros investigadores. Asì, Farombi et al (2007) encontraron que la GGTP , en testículo de rata, incrementó frente a la curcumina y un agente oxidante y Rukkumani et al (2004) encontraron que el alcohol incrementó la actividad enzimática y no potenció el efecto de la curcumina. Encontramos que la curcumina indujo apoptosis e inhibición de crecimiento de células ZR-75-1 y no presentó un efecto protector frente un oxidante como el Sulfato de Cobre.

AGRADECIMIENTOS Este trabajo ha sido subvencionado por la SECYT-UNC.

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Universidad Nacional de Córdoba
Facultad de Ciencias Médicas
Secretaría de Ciencia y Tecnología

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Ha participado como Autor del Trabajo:

Modulación de parámetros de malignidad tumoral en células cancerosas de mama ZR-75-1 por ácido retinoico y ácidos grasos insaturados.

En las 7mas Jornadas de Investigación Científica de la Facultad de Ciencias Médicas, Universidad Nacional de Córdoba

Se le expide el presente

Certificado

Dado en Córdoba a los 3 Días del Mes de Noviembre del 2006

7 JIC

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Secretario de Ciencia y Tecnología
Facultad de Ciencias Médicas - UNC

Prof. Dr. José María Willington
Decano
Facultad de Ciencias Médicas - UNC

258 P

ESTUDIO DEL POTENCIAL FARMACOLOGICO DEL ACIDO NORDIHIDROGUIARETICO DE *LARREA DIVARICATA* (JARILLA; CORDOBA, ARGENTINA) EN DOS MODELOS CELULARES

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El género *Larrea (L.)* posee especies nativas en Córdoba (Argentina) de valor farmacológico por su contenido de ácido nordihidroguaiarético (NDGA), inhibidor de la vía enzimática lipoxigenasa (LOX). Esta vía y la ciclooxigenasa (COX), participan en la producción de eicosanoides implicados en procesos fisiológicos (función renal) y patológicos (cáncer). Nos propusimos evaluar comparativamente, la actividad in vitro del NDGA presente en *L. divaricata (L. d)* en dos modelos celulares con diferentes extractos de la planta. Se incubaron células VERO (riñón primate; $2x10^6$ cél.) y ZR-75-1 (adenocarcinoma mamario humano; $2.5x10^6$ cél.) con extractos orgánicos de *L. d* o con NDGA purificado (vehículo: DMSO). La producción de eicosanoides 12-HETE (marcador LOX) y 12-HHT (marcador COX) se midió por HPLC. La viabilidad celular (VC) se valoró por tinción vital (MTT/RN). Análisis: ANOVA + test de Tukey ($p<0,05$). Las concentraciones de extracto con VC 80% en VERO fueron (µg/ml): 132,2 (hexano); 21,1 (cloroformo); 143,4 (metanol) y 17-20 (NDGA). El NDGA inhibió la formación de 12-HETE (22,1±11 ng) con respecto a control (397,4±41,5 ng), sin afectar al 12-HHT (Control: 23,2±9,5 ng; NDGA: 22,6±10,4 ng). Empleando esas concentraciones, se trataron las células ZR-75-1 (n = 3), donde sólo NDGA disminuyó la VC por debajo del 75% respecto al control ($p<0,0001$), inhibiendo totalmente el 12-HETE pero sin afectar al 12-HHT (controles: 4,9±0,8 ng y 71,6±2,8 ng, respectivamente). Estos resultados se correlacionaron con el contenido de NDGA presente en cada tratamiento (en mayor grado con el purificado, menor con los extractos crudos y nulo en los controles). Conclusión: En dos modelos in vitro: uno no tumoral (VERO) y otro tumoral (ZR-75-1), se estableció que NDGA (*L. d.* nativa) posee potencial antitumoral sin ser nefrotóxico, en asociación con la inhibición especifica de la vía LOX.

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BIOCELL

225.
CURCUMIN CYTOTOXIC EFFECT ON THE HUMAN BREAST CANCER CELLS ZR-75-1

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Introduction: The curcumin (dipheruloylmethane), a food additive, is a polyphenolic compound obtained from *Curcuma longa* rhizomes, which is used as an antitumour agent. The tumour development depends on the misbalance between cellular proliferation and death. Objective: To assess the cytotoxic effect of curcumin. Methodology: It was evaluated the curcumin effect (0, 5, 10, 20, 40 µM) with out cupric sulphate (2.5, 10 µM) on: viability (MTT assay), LDH (lactate dehydrogenase) release and oxidant level (TMPD assay) of ZR-75-1 cell. Data were analysed by ANOVA followed by Tukey test ($p<0.05$). Results: The cellular viability was decreased by curcumin, without significant cupric effect. Also, it was inversely correlated with LDH release (Pearson Coeff.=-0.7). The cell cupric-induced oxidation was diminished significantly by curcumin. Discussion: Although curcumin acted as antioxidant against cupric sulphate, this plant polyphenol exerted a cytotoxic effect in dose-dependent way on the studied cell line with increasing cell death-associated LDH release.

226.
CHARACTERIZATION OF THE PROTEIN PROFILE OF STRAIN RC-98 OF BOVINE RESPIRATORY SYNCYTIAL VIRUS BY SDS-PAGE

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Bovine Respiratory Syncytial Virus (BRSV) is an enveloped virus. Its genome is RNA and encodes for eleven proteins that migrate in SDS-PAGE with variations in their molecular weights (MW) according to the sub-group the strain belongs to. The objective was to identify the protein profile of strain RC-98. The virus was purified in discontinuous saccharose gradients from which electroforetic runs were carried on. The gels were prepared according to the system of discontinuous buffer and gels in 12.5 and 15% polyacrilamide. The electrophoretic patterns obtained were characteristic of BRSV. In the 12.5% gel the following transmembrane proteins were identified: G-84kD, F0-77kD, and its cleaved polypeptides F1-46kD and F2-23kD. The ribonucleoproteic complex N-43kD, P-34kD and L-200kD were also observed. Matrix proteins M-29kD, M2 -22kD and M2 -11kD were visualized in the 15% gel. In addition another band of 18kD was identified that could be one of the nonstructural proteins NS1 or NS2, or the corresponding glycoprotein SH, which can appear glycosilated, or not with a MW that varies from 7.5 to 21kD. The main surface glycoproteins, such as G and F of the strain RC-98 were identified with neutralizing antibodies. On the basis of the pattern of MW of polypeptides obtained by protein F cleaving we suggest that the strain in study belongs to the intermediate sub-group (AB) of VSRB.

227.
IMMUNOCYTOCHEMICAL CHARACTERIZATION OF THE CYTOPATHIC EFFECT INDUCED BY THE BOVINE RESPIRATORY SYNCYTIAL VIRUS

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Bovine Respiratory Syncytial Virus (BRSV) produces a cytopathic effect (CPE) characterized by intracytoplasmic inclusion bodies and syncytial cells which relates to its name. The aim of this work was to characterize the CPE of the BRSV strain RC-98 by immunocytochemistry in cultures of Hep-2 cells. Monolayers of Hep-2 cells were grown over coverslips, then infected with the BRSV strain RC-98. When the CPE affected extensively about 50% of the monolayers, the cells was fixed and immunocytochemistry was performed, using a polyclonal antibody anti-BRSV (VMRD) and commercial kit (Vectastain Elite ABC Vector). Viral specific signals were observed in the cytoplasm and nucleus of a high number of cells, and consisted in protein clusters of multiple irregular forms surrounded by a clear haul, corresponding to intracytoplasmic and intranuclear inclusion bodies. Another finding observed was the presence of syncytial cells which were very evident and showed specific signal in the cytoplasm of polykaryocytes. Intranuclear inclusion is very intriguing and appears to disagree with investigations on the replicative cycle of BRSV indicating that the events leading into viral replication occur in the cytoplasm, without involving the nucleus. Intranuclear inclusions can be a particular characteristic induced by the strain RC-98, nevertheless intranuclear inclusions have been also described for other viruses of the same family but not for the BRSV.

228.
REPRODUCTIVE CYCLE OF THE EUROPEAN HARE AND ITS RELATION WITH THE DATE OF COMMERCIAL HUNTING

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The harvest of the *European hare* in the Pampean region of Argentina, is historically done during the months of May, June and July, which coincides with the date of hunting and the beginning of the reproductive period. The aim of this work was to analyze the reproductive cycle of the *European hare* through their gestational state and relate it to the present date of commercial hunting. In a slaughterhouse plant of Rio Cuarto dedicated to the processing of hares for export, a study of the total number of hares was carried out during ten consecutive days (from the 15 to the 25 July, 2004). This observation was done by Veterinary. The total of examined hares 7315, 38% of the total (2780) were male; 61.7% adult females (4513) and 0.3% corresponded to young hare (22) that were not discriminated by sex. Of the total of adult females, 75% were pregnant (3385) with embryos in different periods of gestation. The percentage of pregnancy found in the month of July coincides with the findings of other authors, and with the great number of females during the period of study. The present date stipulated for the season of commercial hunting of the European hare in the Province of Cordoba would be affecting the reproductive cycle, it is proposed to begin a month before the habitual period (April) and to finish no further the last days of the month of June.

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221.

D-ALANYL-D-ALANINE CARBOXIPEPTIDASE IS ASSOCIATED TO SALT TOLERANCE IN *Ochrobactrum sp.* 11a

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Salinity is a severe stress condition in agriculture, which results in a decreased productivity of crop plants and has a detrimental effect on soil-microorganisms.Recently, some effective salt-tolerant-PGPR belonging to *Ochrobactrum* genus were isolated. These strains are able to promote the maize growth. Since salt tolerance in bacteria is a complex phenotype, in which many regulatory mechanisms are involved it is necessary to characterize in detail genes conferring a salt tolerant phenotype in these PGPR. Random transposon Tn5-B21 mutagenesis was used to generate salt sensitive mutants of *Ochrobactrum* sp. 11a. The obtained transconjugants were tested for growth in minimal medium supplemented with NaCl 300 mM. Chromosomal and plasmid DNA isolation, Southern blotting and the construction of recombinant plasmids were carried out as described by Sambrook *et al*, 1989. Mutants which showed a salt sensitive phenotype were selected for further genotypic characterization. DNA sequence analysis of the locus tagged in the mutant called 1a showed significant similarity with the *dacB* gene of *Brucella suis* 1330 encoding an D-alanyl-D-alanine carboxipeptidase. DacB is a periplasmic protein involved in cell wall biosynthesis. It can be assumed that the adaptation to high salt concentrations involves modifications of the external cell layers. These alterations in the cell wall probably establish an enhanced diffusion barrier to reduce the influx of inorganic ions into the periplasm.

222.

ETHANOL INDUCED CHANGES ON MIGRATORY PARAMETERS OF *IN VITRO* NEURAL PROGENITORS OF THE OLFACTORY INTERNEURONS

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In the mammal forebrain, subventricular zone contain progenitors of the olfactory interneurons, this cells migrate during fetal and postnatal period to the olfactory bulb through the rostral migratory stream (RMS). It has been shown that ethanol induces changes in the pattern of migration of some neural cells. The aim of the present work was to evaluate ethanol's effects upon morphometric and dynamic parameters of *in vitro* RMS neuroblasts. RMS explants from rat fetuses at gestacional day 20 were used. Coverslips carrying cultures were mounted in a Sykes-Moore chamber and perfused with ethanol 150mM, 38mM or culture medium (Control). Cells parameters were determined after 2,5 hs of time-lapse videorecording. We found that neuroblasts exposed to 150mM ethanol doses, exhibited a decrease in velocity and a reduction of distances traveled, as well as significant changes regarding cell shape when compared with Control cultures. 38mM ethanol dose failed to exert significant effect. The results indicated that ethanol has a deleterious effect upon migrating bulbar interneurons that could affect the development of olfactory bulb during fetal period. Taking into account that precursor cells for the olfactory interneurons migrate during early development and throughout postnatal life, present results suggest that the critical period for ethanol-induced damage may to involve pre- and post-natal stages in the main olfactory bulb. Therefore, odor encoding and discrimination may be affected by ethanol throughout lifespan.

223.

ETHER STRESS: STUDIES IN DIABETIC MURINE MODELS

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Stress could be defined as the adaptive response of the body to stimuli interfering with internal homeostasis. Although ether anesthesia is widely used in experimental procedures, ether itself might activate the hypothalamus- hypophysis-adrenal axis and the sympathetic adrenal axis.
The effect of ether stress in genetically diabetic rats, eSS and eSMT, and α eumetabolic controls, was studied. Glycaemia was assessed pre (G0, mg/dl) and 120 min after glucose overload (G120) in 6 and 12 month-old rats. Data were analyzed by ANOVA followed by multiple comparison tests, and expressed as mean ± standard error mean for each group.
In 6 month old-animals, higher G0 y G120 values were found after ether treatment both in eSS and in eSMT (eSS: G0=98±26, n=120 vs 130±19, n=10 $p<0.001$; G120=194±51, n=120 vs 233±46, n=10, $p<0.05$; eSMT: G0=143±27, n=45 vs 170±17, n=9, $p<0.01$; G120=224±35, n=45 vs 317±78, n=9, $p<0.001$). The eumetabolic line α, showed no changes in G0 (69±22, n=27 vs 88±11, n=8, $p>0.05$) and enhanced values in G120 (106±20,n=27 vs 171±14, n=8, $p<0.001$). Twelve month-old eSMT and α rats followed the same tendency.
In basal conditions, only eumetabolic α rats managed to maintain glucose levels post ether treatment. Moreover, ether stress resulted in changes in the glycaemic homeostatic control after glucose overload both in diabetic and non-diabetic lines.

224.

RETINOIC ACID AND FATTY ACIDS: EFFECTS ON HUMAN BREAST CANCER CELLS

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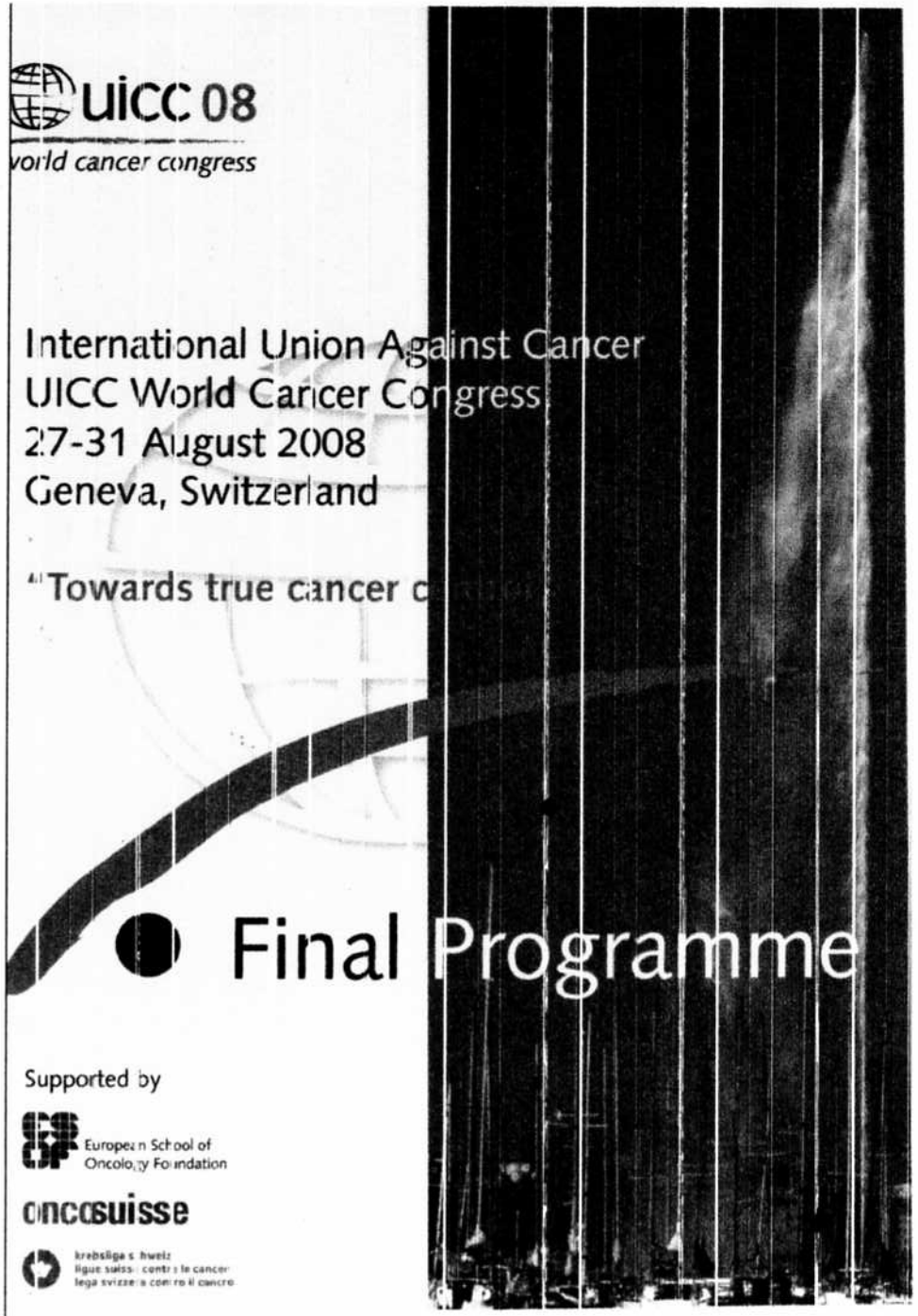
Introduction: The unsaturated fatty acids (UFA) and the all-trans retinoic acid (RA) could play a modulating role on the carcinogenic process, but their combined use has not been completely studied. Objective: To modulate tumor parameters in ZR-75-1 cells by RA and/or UFA (families: n-3, n-6 y n-9). Methodology: The cells were treated with RA 1 μM and UFA 50 μM: eicosapentaenoic (EPA, n-3), γ-linolenic (GLA, n-6), oleic (OA, n-9) or eicosatrienoic (ETA, n-9). It was assessed cellular proliferation (CP; MTT assay), eicosanoid production (HPLC), lipid profile (GC) and E-cadherin expression (Immunocytochemistry). Statistics: ANOVA – LSD Fisher test ($p<0.05$). Results: The membrane lipid profile showed the incorporation of the corresponding UFA. The CP was diminished by RA (-34.5%) and the UFA inclusion enhanced this antiproliferative effect. There was a 60% correlation between the 12-HETE formation (12-LOX pathway) and the CP. The E-cadherin expression was increased mainly by the n-9 serie. Discussion: The antitumoral effect observed with RA+UFA (decreasing CP) and the increasing differentiation (E-cadherin expression) were associated differentially to the specific UFA used into a synergic interaction.



[PUB-185] DIFFERENTIAL EFFECTS OF RETINOIC ACID PLUS N-9 UNSATURATED FATTY ACIDS ON TWO HUMAN BREAST CANCER LINES

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Background: All-trans retinoic acid (RA) and unsaturated fatty acids (UFAs) exhibit different effects on tumor parameters such as proliferation and differentiation. Although both varieties of nutrients *per se* may play a modulating role on the carcinogenic process (De Antueno et al., 1997; Hansen et al., 2000), the combined use of n-9 UFAs plus RA has not been fully studied. **Objectives:** The purpose of this work was to modulate certain tumoral parameters related to cancer differentiation on two mammary cancer cell lines (ZR-75-1 and MCF-7) by RA, both with and without n-9 UFAs. **Methods:** At 90% confluence, cells were incubated with RA 1 µM or RA plus n-9 UFAs 50 µM: oleic acid (OA) or eicosatrienoic acid (n-9, ETA), an unusual UFA related to EFA (Essential Fatty Acids) deficiency and cancer (Eynard et al. 1998). The lipid profile (GLC), γ-glutamyltranspeptidase activity (GGTP, Szasz method), E-cadherin and actin expression (immunocytochemistry) were assessed. Statistics: ANOVA + LSD Fisher test ($p<0.05$). **Results:** After confirming the membrane lipid profile enrichment with the corresponding added UFA, the outcomes were presented in table. In MCF-7, ETA diminished E-cadherin expression respect to OA, whereas actin was increased. In ZR-75-1, ETA increased the three protein parameters compared with OA.

Effect of RA and RA plus some n-9 UFAs on three tumoral proteins parameters

Cell Lines	Agents	Actin	E-cadherin	GGTP
MCF-7	RA	162.4**	64.5*	21.6*
	RA+OA	170.6**	107.0**	16.0*
	RA+ETA	245.2**	86.0*	25.3*
ZR-75-1	RA	111.6	140.7**	87.3
	RA+OA	168.5**	119.2**	59.6*
	RA+ETA	265.7**	171.6**	103.3

All values were expressed as % respect to control: *decrease, **increase ($p<0.05$).

Conclusions: This model allowed tumor parameters to be compared in two RA-treated cell lines with distinct differentiation grades (Soria et al. 2007), modulated by two n-9 UFAs with different saturation status (OA: precursor; ETA: derived abnormal metabolite). Varying degrees of effects were found for the UFAs studied, with ETA boosting the RA-induced actin expression in both lines. All treatments increased the E-cadherin expression in ZR-75-1, but only OA achieved this in MCF-7. Moreover, OA decreased GGTP activity in ZR-75-1, whereas the other three treatments had this inhibitory effect in MCF-7. Summing up, regardless the cell line, only OA showed a potential beneficial effect in the enhancement of E-cadherin and actin expressions with GGTP down-regulation.

References: De Antueno R. et al. Br J Cancer 1997;75:1812-1818. Hansen LA et al. Carcinogenesis 2000;21:1271-1279. Soria EA et al. Life Sci 2007;81:1397-1402. Eynard et al. Prostagl Leuk Essential Fatty Acids 1998;59:371-377.

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6 hs. Asimismo, las células tratadas con DHMC experimentan una disminución del potencial de membrana mitocondrial a las 14 hs (Citometría con $DiOC_6$). Estos resultados demuestran que el efecto pro-apoptótico de DHMC en la línea celular U937 está mediada por la vía intrínseca de apoptosis.

287. (68) MODULACIÓN MUSCARÍNICA DE LA PROLIFERACIÓN EN CÉLULAS SCA-9. PARTICIPACIÓN DE LAS ÓXIDO NÍTRICO SINTASAS, ARGINASAS Y CICLOOXIGENAS

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Se ha descrito la importancia de las enzimas óxido nítrico sintasa (NOS), arginasa (A) y ciclooxigenasa (COX) en la biología tumoral. Decidimos estudiar la participación de las NOS, A y COX así como su regulación recíproca, en el efecto del agonista muscarínico carbacol (Carb) sobre la proliferación de células tumorales de glándula submaxilar murina, SCA-9. La proliferación se determinó por MTT; la actividad de NOS por el reactivo de Griess; la actividad de A por un método colorimétrico que detecta urea y la actividad de COX por RIA de PGE_2. El tratamiento con Carb aumentó la proliferación con una concentración efectiva máxima de 10^{-9}M (31±2% vs. basal p<0,001). Dicho efecto se revirtió en todos los casos por la preincUBAción con el antagonista musacarínico atropina (AT) (10^{-5}M) o con los inhibidores enzimáticos: de COX, indometacina (INDO) (10^{-4}M), de NOS, L-NMMA (10^{-4}M) o de A, NOHA (10^{-4}M) (n=8 p<0,001 vs Carb). También observamos que el Carb estimuló la actividad de NOS, siendo la concentración efectiva máxima 10^{-9}M (72±26% vs. basal p<0,001). Este efecto fue revertido por la preincUBAción con L-NMMA e INDO, e incrementado por la preincUBAción con NOHA (n=5 p<0,001 vs. Carb). El Carb estimuló la actividad de A a una concentración de 10^{-9}M (171±16% vs basal p<0,001) y este efecto se incrementó en presencia de L-NMMA lo que confirma que NOS y A comparten el mismo sustrato. Además la preincUBAción con INDO potenció el efecto del Carb sobre la A revelando una modulación negativa de los productos de COX (n=6 p<0,001 vs Carb). Al estudiar la actividad de COX, observamos que el Carb produjo un efecto estimulante a la concentración de 10^{-9}M (85±6% vs basal p<0,001) que fue revertido al preincUBAr las células con NOHA, e incrementado al preincUBArlas con L-NMMA (n=6 p<0,001 vs. Carb). Concluimos que la estimulación muscarínica promueve la proliferación de células SCA-9 mediante un mecanismo complejo de "cross talk" entre las enzimas NOS, A y COX.

288. (405) REDUCCIÓN POR LANTANA GRISEBACHII DE LA LINFOTOXICIDAD INDUCIDA POR ARSÉNICO

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La respuesta inmune puede ser interferida por diferentes condiciones inmunosupresoras, tal como la exposición tóxica a arsénico induciendo estrés oxidativo en linfocitos. Dado que antioxidantes dietarios podrían ser tener actividad fitorremediadora, el objetivo de este trabajo fue identificar ex vivo un extracto vegetal bioactivo frente As. Se evaluaron extractos (hexano, agua TA, agua 95°C; hw) de M. pentlandiana (MP) y L. grisebachii (LG) in cultivos linfocitarios (provenientes de bazo de rata Wistar) tras determinar el contenido de polifenoles de cada uno, estudiándose sus efectos en la viabilidad celular y la formación de oxidantes celulares (ANOVA, p<0,05). La extracción con hw permitió obtener un extracto con 20% de contenido polifenólico a partir de los tejidos aéreos de ambas plantas, superando a los otros solventes (p<0,05). Tras descartar los extractos hexánicos por ser tóxicos (p<0,0001), se determinó que LG(hw) fue el extracto con mayor capacidad antioxidante que MP(hw), reduciendo la formación de nitritos y radicales en linfocitos T y B provenientes de animales control y crónicamente expuestas a arsénico (p<0,05). Se encontró que la concentración de arsénico tenía relación directa con el nivel de oxidantes en la serie T (p<0,05; R^2=0,33), mientras que B era menos sensible a dicho efecto (p=0,08; R^2=0,19). La reducción de la viabilidad linfocitaria (% respecto a control) por 1 µM de arsenito sódico fue prevenida por LG(hw) de manera dosis-dependiente (p<0,0001): 10,5±0,7, 30,2±1,5, 19,9±1,4, 60,6±1,0 (linfocitos T); 61,3±4,3, 36,8±1,8, 79,5±5,6, 67,5±1,1 (linfocitos B), 0, 1, 10 y 100 µg/mL de extracto, respectivamente. En conjunto, los resultados indican que el extracto acuoso de *L. grisebachii* es antioxidante y linfoprotector frente al efecto deletéreo del arsénico, pudiendo estar esto asociado a su alto contenido polifenólico.

289. (598) EL RANELATO DE ESTRONCIO REVIERTE LOS EFECTOS DELETÉREOS CAUSADOS POR LOS AGE SOBRE OSTEOBLASTOS EN CULTIVO. ROL DE LOS CANALES DE CALCIO.

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En pacientes con Diabetes mellitus tipo 2 la acumulación de productos de glicación avanzada (AGE) está implicado en el desarrollo de complicaciones crónicas tales como las óseas. Previamente hemos demostrado que los AGE inhiben el crecimiento y la diferenciación de los osteoblastos. Recientemente se ha demostrado que el ranelato de estroncio (RaSr) actúa como un agente anabólico y antirresortivo sobre el hueso. Sin embargo no se ha reportado hasta ahora el efecto de este fármaco sobre complicaciones esqueléticas causadas por la diabetes. En el presente trabajo hemos evaluado si el RaSr es capaz de revertir los efectos deletéreos de la albúmina (ASB) modificada por AGE sobre osteoblastos en cultivo MC3T3E1 de ratón, así como el rol del calcio en su mecanismo de acción. Encontramos que los AGE causan inhibición de la proliferación osteoblástica (efecto máximo a 200 ug/ml de AGE: 63± 6% vs. ASB control, p<0,01). Por otro lado, el RaSr 0,1mM estimula la proliferación celular (121±6% vs. ASB, p<0,05), mientras que su co-incUBAción con AGE revierte completamente la inhibición de la proliferación causada por AGE (152±3% vs. AGE, p<0,01). Cuando evaluamos la diferenciación celular, encontramos que RaSr 0,1mM produce un incremento en la actividad de fosfatasa alcalina (FAL; 125±7% vs. ASB, p<0,01) y en la producción de colágeno tipo 1 (Col1, 118±3% vs. ASB, p<0,05); mientras que 100 ug/ml de AGE produce una disminución significativa en ambos parámetros (FAL: 65±5% vs. ASB, p<0,001; Col1: 66±7% vs. ASB, p<0,001). El co-tratamiento de RaSr con AGE revierte los efectos deletéreos de los AGE (FAL: 213±14% vs. AGE, p<0,01; Col1: 126±3% vs. AGE, p<0,05). Por otro lado la co-incUBAción con nifedipina bloquea los efectos del RaSr, pero no los de los AGE. Estos resultados muestran que el RaSr actúa como un agente anabólico sobre osteoblastos en cultivo, y que es capaz de revertir efectos deletéreos inducidos por AGE, por mecanismos que implican la activación de canales de calcio.

290. (199) LA CANTIDAD DIARIA DE VITAMINA D2 Y D3 RECOMENDADA ES SUFICIENTE PARA ALCANZAR LOS NIVELES DESEABLES DE 25OHD?

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Existen controversias acerca de si la vitamina D2 (D2) es tan efectiva como la vitamina D3 (D3) respecto de los niveles de 25hidroxivitamina D (25OHD) al administrarlas en dosis equivalentes. Objetivo: determinar en un modelo de insuficiencia de vitamina D (vitD) y osteopenia establecida si la administración diaria de D2 y D3 en dos dosis diferentes (100 y 200IU/kg) son igualmente efectivas para aumentar los niveles de 25OHD. Se ovariectomizaron (OVX) 64 ratas Wistar (200±50g) las que durante 15 días