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Characterisation of bioactive compounds in infant formulas using immobilised recombinant estrogen receptor-α affinity columns

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ABSTRACT

In this study, the use of recombinant estrogen receptor alpha (ERα)-based affinity columns was reported, for the isolation and the identification of estrogenic substances present in complex matrices, focusing on bioactive compounds present in foodstuff. The capability of affinity columns to trap high, but also low-affinity radio-labelled ligands (17β-estradiol, genistein and bisphenol A) was demonstrated. Three pooled samples of infant formulas (milk-based, hypoallergenic and soy-based formulas for infants aged 0–4 months) from a EU market basket were prepared by the CASCADE Network of Excellence. After determining the estrogenic activity of these food samples, human recombinant ERα ligand binding domain (LBD) based affinity columns combined with suitable analytical methods (high resolution LC–MS/MS) were used to identify the bioactive compounds present in the soy-based formula extract, namely phytoestrogens (genistein and daidzein) involved in the agonistic activity measured. Incubations of genistein with liver microsomes were carried out and the extracts analysed following the same protocol, demonstrating that hERα affinity columns can also be used for trapping active metabolites. This approach combining bioluminescent cell lines with this useful tool based on hERα-LBD affinity columns thus allowed the purification and the concentration of both known and unknown estrogenic ligands prior to investigation of their structure using LC–MS.

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1. Introduction

The increasing awareness that diet can play a significant role in the onset of diseases that afflict Western populations has led to better recognition that there are many classes of bioactive nonnutrients. Bioactive food components and contaminants need to be characterised both regarding their structure and biological activity. The complexity of food matrices is however a major problem when trying to achieve this goal. Food components and contaminants able to activate nuclear receptors are of major concern. These compounds, which are potential endocrine disruptors (EDs), can interfere with endocrine regulations in the human body. During the two recent decades several studies have shown that EDs can be involved in the onset of diseases such as cancers (Maffini et al., 2006; Caserta et al., 2008), allergies, diabetes (Van Den Hazel et al., 2006) and various disorders of the reproductive system (Skakkebaek et al., 2001; Ivell and Hartung, 2003).

At the cellular level, one of the most common pathways for endocrine disruption is the binding of exogenous compounds to estrogen receptors (ERs). Two ER isoforms, ERα and ERβ, are found with a different tissue subtype distribution in the human body (Kuiper et al., 1997; Pearce and Jordan, 2004). The two receptors have a similar affinity for their natural ligand, 17β-estradiol (E_2),

Abbreviations: BPA, bisphenol A; BSA, bovine serum albumin; DCC, dextran-coated chacoal; DMEM, Dulbecco's modified eagle medium; E_2, 17β-estradiol; EB, eluting buffer; EDs, endocrine disruptors; ERs, estrogen receptors; ERβ, estrogen receptor-β; ESI, electrospray ionisation; ESI-MS/MS, electrospray-mass spectrometry/mass spectrometry; EU, European union; FCS, fetal calf serum; HAf, Hypoallergenic formulas; hERα, human estrogen receptor-α; HPLC, high performance liquid chromatography; K_d, dissociation constant; LBD, ligand binding domain; LC, liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; Mf, milk formulas; MS, mass spectrometry; NR, nuclear receptor; Ni-NTA, nickel-nitrilotriacetic acid; OD, optical density; R_T, retention time; TIC, total ion current; UV, ultraviolet; WB, washing buffer.

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but differences have also been observed for other ligands (Kuiper et al., 1998). Food contaminants which are able to interfere with ERs may be molecules of natural origin, pharmaceuticals, or xenobiotics used for industrial purposes, in agriculture, or in the manufacture of consumer's goods (Kuiper et al., 1997). Natural and pharmaceutical estrogens have high-affinity (dissociation constant $K_d < 1$ nM) for ERs, whereas most xenoestrogens have lower affinity constants ($K_d > 10$ nM). Some of these xenoestrogens are well-known molecules produced in large volumes. Bisphenol A (BPA), a plasticizer that has been used, among others, in the manufacture of food and beverage containers, including baby bottles and nipples, can be released into food and was shown to bind to both ER subtypes (Kuiper et al., 1998; Kurosawa et al., 2002). In contrast, natural components such as phytoestrogens, which are widely found in soy-based products, are complete agonists for both ERs, but their binding activity was shown to be more potent with respect to ERβ than ERα (Casanova et al., 1999).

Many methods have been developed so far with the aim to elucidate both the structure and the biological activity of the EDs present in environmental or food extracts. One possible strategy is to combine high performance liquid chromatography fractionation with *in vitro* estrogenicity bioassays and mass spectrometric analysis (Cargouet et al., 2004; Noppe et al., 2007). However, the scope of such strategy is limited by the fact that only molecules whose structure is previously known can be monitored (Delmonte and Rader, 2006). We have begun to investigate the suitability of a new tool allowing characterisation of contaminants from complex mixtures. This tool is based on nuclear receptor (NR) ligand binding domain (LBD) immobilised on agarose columns and on the capability of NR to bind EDs ligands. This approach has already been tested with standard estrogenic compounds and environmental samples on recombinant ERα-LBD affinity columns (Pillon et al., 2005). We hypothesized that such columns may be successfully adapted for the investigation of the structure of biologically active components and contaminants in food extracts.

The objective of this study is to develop a tool for characterising substances that mediate estrogenic activity in complex matrices, that is, to isolate ER activators based on their direct binding to recombinant ERα-LBD. In a first step, the binding capacity of the recombinant ERα immobilised on agarose columns was evaluated using pure radio-labelled compounds. The binding affinity of both natural estrogen (17β-estradiol) and low-affinity ligands (bisphenol A and genistein) was tested. The combination of these columns with analytical methods such as HPLC coupled with mass spectrometry (ESI-MS/MS, Orbitrap-MS/MS) was further investigated with the aim to achieve the structural identification of the estrogenic substances found in the tested food matrices. The method was applied to samples obtained by pooling "starting" infant formulas from several EU countries, within the frame of a large program initiated by CASCADE (Chemicals as contaminants in the food chain) Network of Excellence, an FP6 EU funded consortium, targeting health risks in food. This particular typology of products is consumed by infants who are not exclusively breastfed in the first period of life (0–4 months); moreover, it is the unique food consumed by infants who are not breastfed at all. The prevalence of breastfeeding varies very much throughout the world (Zetterstrom, 1999) according to the WHO Global Data Bank on Breastfeeding (WHO, 2000), which covers 94 countries and 65% of the world's infant population of ages <12 months, only 35% of them are exclusively breastfed between 0 and 4 months of age. Concerns about the possible adverse effect of phytoestrogens present in soy-based infant formulas on the development of infants have been raised but the prevalence of soy-based infant formulas feeding is not available for most EU countries (COT Report, 2003). In 2002 in UK, soy-based formulas was fed to >2% of infants aged 10–14 weeks (Department of Health, 2002) and in 1992, it was estimated that in Ireland 5% of infants aged between 3 and 9 months were being fed either a soy-based infant formula or a hydrolysate formula (COT Report, 2003). In this study, the estrogenic activity of the different infant formula extracts was first evaluated using MELN and HELN reporter cell lines. Further, we explored the capability of ERα affinity columns to retain bioactive compounds, as well as their possible biotransformation products, present in these complex matrices.

2. Materials and methods

2.1. Chemicals

Genistein (CAS number: 446-72-0), daidzein (CAS number: 486-66-8), biochanin A (CAS number: 491-80-5), apigenin (CAS number: 520-36-5) and 17β-estradiol (CAS number: 50-28-2) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). [^{14}C]-E_2 (specific activity, 1.9 GBq/mmol) and [^{14}C]-Genistein (592 MBq/mmol) were purchased from NEN Life Sciences Products (Paris, France) and Isotopchim (Ganagobie-Peyruis, France), respectively. [^{3}H]-bisphenol A (specific activity, 185 GBq/mmol) was purchased from Moravek Biochemicals (CA, USA). The radiopurity of each radio-labelled compound was above 99.9%, as controlled by radio-HPLC. Standards of genistein metabolites obtained from rat liver microsome incubations were available from INRA UMR1089.

All solvents (analytical grade) were purchased from Scharlau Chemie SA (Barcelona, Spain). Ultrapure water from Milli-Q system (Millipore, Saint Quentin en Yvelines, France) was used for *in vitro* procedures and for the preparation of HPLC mobile phases. All solvents used for food extraction were picograde quality.

Materials for cell culture were obtained from Invitrogen (Cergy-Pontoise, France). Luciferin was purchased from Promega (Charbonnieres, France).

2.2. Determination of estrogenic activity of infant formula extracts

2.2.1. Infant formula samples

Infant formulas from the "EU basket". In order to choose the infant formula products, an EU infant formula basket was designed based on the market share data of 2007 for infant formulas were purchased from the Company "Food for Thought" (www.fft.com). The first Holding Companies that altogether constitute over 80% of the EU market of infant formulas were identified and the websites of the companies were searched or the companies were contacted in order to find the names of all products for each of the three following categories of infant formulas: milk-based, soy-based and hypoallergenic. The products were purchased in different EU countries. The pooled sample of milk-based infant formulas includes 11 different products; the pooled sample of soy-based infant formulas (Sf) includes six different products; the pooled sample of hypoallergenic infant formulas includes nine different products. The weight of each product in the pooled samples was proportional to its market share.

2.2.1.1. Sample homogenisation. A total of three pooled samples of "starting" infant formulas of respectively milk-based (Mf), soy-based (Sf) and hypoallergenic (HAf) formulas were prepared. The weighing procedure was performed in a purified glovebox under nitrogen atmosphere and the pooled infant formulas were further homogenized in a rotoshaker (Heidolph, Kelkheim, Germany) for 8 h. The samples were kept at room temperature and stored in brown glass jars protected from direct light. In order to prevent any contamination, each further opening or closure of the sample collector was performed under nitrogen atmosphere.

2.2.1.2. Sample extraction. Extraction of 20 g of infant formula sample was carried on, using Accelerated Solvent Extractor (ASE 200) device (Dionex, Sunnyvatem CA, USA). The procedure was performed using mixture of *n*-hexane:acetone (75:25, v/v) at 120 °C and at a pressure of 12 MPa. Two static cycles of 10 min were applied for a complete extraction. Then, estrogenic activity of food extracts was evaluated using reporter cell lines.

2.2.2. Cell culture conditions

For strain cultures, cells were grown in phenol red containing Dulbecco's modified Eagle medium (DMEM F12), 1 g/l glucose, supplemented with 5% fetal calf serum (FCS), and 1% antibiotic (penicillin/streptomycin) in a 5% CO_2 humidified atmosphere at 37 °C. Given the known estrogenicity of phenol red and FCS, *in vitro* experiments were achieved in DMEM F12 supplemented with 5% dextran-coated charcoal (DCC)-treated FCS (test culture medium).

2.2.2.1. Reporter cell lines. The stably transfected luciferase reporter MELN cell line was obtained as described (Balaguer et al., 2001). Briefly, ERα-positive breast cancer MCF-7 cells were transfected with the estrogen-responsive gene ERE-βGlob-Luc-SV-Neo (Balaguer et al., 1999). Selection of resistant clones by geneticin was performed at 1 mg/ml. MELN cells were cultured in DMEM F12 with phenol red, supplemented with 10% FCS and 1 mg/ml G418 in a 5% CO_2 humidified atmosphere at

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