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Estrogen-inducible uterine flavonoid binding sites: is it time to reconsider?

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Abstract

Epidemiological data support the beneficial effect of plant flavonoids on human health including anti-inflammatory and cancer preventing actions. The phytoestrogen flavonoids might interfere with estrogen action. The possible relations between the steroid- and the flavonoid-signalling in animal and plant cells have been addressed in numerous studies in the past decade. In search for possible sites of conjunction between these phenomena the post-receptor targets must not be disregarded.

The estrogen-inducible type II estrogen binding sites of rat uteri have first been reported 25 years ago by Clark and coworkers [Biochem. Biophys. Res. Commun. 81 (1978) 1]. These sites are known to bind catecholic flavonoids with considerable affinity. Behaviour of the tyrosinase-like enzymatic activity associated with these sites appeared reminiscent to the recently described dopachrome oxidase or tautomerase activity exhibited by the cytokine macrophage migration inhibitory factor (MIF) inasmuch as it also accepts a broad range of catecholic melanogenic precursors. Therefore we assessed, whether the known type II ligand flavonoids interfere with the MIF tautomerase. We report here, that luteolin and quercetin have a biphasic effect on the enol–keto conversion of phenylpyruvate mediated by MIF tautomerase. We also demonstrate the presence of MIF immunoreactivity by Western blotting in rat uterine nuclear extracts prepared according to the method that yields high type II binding activity. These data support the possible participation of MIF in type II estrogen binding phenomena.

Keywords: Phytoestrogen; Flavonoid–steroid link; Type II binding; MIF; Tautomerase

1. Introduction

Analogies between flavonoid signalling of Leguminoseae with nitrogen-fixing soil bacteria, and the steroid hormone signalling of vertebrates have been discussed lately considering either evolutionary aspects [1] or the action of environmental endocrine disrupting chemicals [2]. A recent paper reports on disruption of ecdysteroid signalling in arthropods by some flavonoids [3] found to act as 'phytoestrogens' in mammals [4] as well. It may seem that certain representatives of flavonoids have emerged in a role of being versatile biologically active agents designed and produced by plants to intervene in the diverse steroid hormone signalling repertoire of several animal species positioned at quite a distance on the evolutionary tree.

Discussions on the novel aspects of the flavonoid–steroid link, however, must not divert attention from issues that have longer history, yet still remain unsettled. Among those one may find a flavonoid binding site, usually referred as the type II estrogen binding site. This estrogen-inducible low affinity estrogen binder has first been reported by Clark and coworkers [5] a quarter of a century ago in rat uteri and its ability to bind flavonoids has become known a decade later [6]. The purification of this binding site has been reported recently [7]. Nevertheless, the identity of this flavonoid binding site has remained elusive though histone H4 reportedly binds type II ligands [8].

Though the isoflavonoid and lignan groups of phytoestrogens are more recent additions most of the flavonoids had certainly been produced by plants well before hormonal steroid receptors of higher order animals evolved. Therefore animals and human beings have been continually exposed to these compounds especially via the food consumed [9], and, most notably, it seems to exert a beneficial effect on human health. Remarkably, the ancestor of all other steroid receptors was most likely an ancient estrogen receptor [10]. Certain estrogenic and/or anti-estrogenic effects of

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the flavonoids may still be mediated at post-receptor steps of the hormone signalling path [11]. The exact nature and sites of these downstream events remain to be elucidated.

Type II binding site needs to be considered for certain flavonoids' action concerning parallel endocrine disrupting phenomena in vertebrates and arthropods. This binding site reportedly co-purifies with a tyrosinase-like enzymatic activity from rat uterine nuclear fraction [12]. The relationship of the type II binding site with the tyrosinase-like activity has been disputed later [13], although it might have helped to explain the perplexing spontaneous affinity labeling detected with its highest affinity flavonoid ligand, luteolin [14]. The exact mechanism of the affinity labeling by luteolin has not since been taken under scrutiny despite aiding the recent purification attempts of these sites [7,8].

Plants are known to produce a variety of tyrosinase inhibitors including those flavonoids [15] denoted as endocrine disruptors in animals. Ecdysteroids regulate molting that intensely relies on the activity of the phenoloxidase enzyme, the arthropod tyrosinase. Despite the known estrogen-dependent changes in the tyrosinase activity of the skin causing altered pigmentation in mammals (e.g. chloasma gravidarum of human subjects) the integument is not considered a typical estrogen target tissue there. Mammalian tyrosinase, however, is not abundant in the principal estrogen target sites, the reproductive organs.

An alternate function of phenoloxidase is in arthropods' immune defense against infections [16]. Similarly, a peculiar immune mediator cytokine of vertebrates, the macrophage migration inhibitory factor (MIF), has been described to catalyse an enzymatic step of the tyrosinase pathway, tautomerisation of dopachrome [17], although it prefers the 'D-' isomer, nevertheless, fairly accepts L-dopachrome methyl ester. This cytokine is known to be present in several tissues and it is also produced by the reproductive organs [18]. Affinity labelling of the tyrosinase-like enzymatic activity from rat uterus by [^{125}I] estradiol yielded a ~36 kDa band [19], that closely matches both, the molecular weight of trimeric MIF [20], and the molecular weight of the type II estrogen/luteolin binding sites [21]. In rat uterine nuclear extract, featuring type II sites, a tautomerase acivity resembling to MIF has been detected [22]. Therefore, it was of interest to assess the effect of estrogenic flavonoids known as good type II ligands on the enzymatic activity of MIF.

2. Experimental

Chemicals were of the highest available purity from Sigma unless stated otherwise. To perform the animal experiments the permission from the regional ethical committee on biomedical research had been obtained. The MIF-catalysed tautomeric enol–keto conversion of phenylpyruvate, was monitored as the velocity of decrease in absorbance at 288 nm on a Pharmacia-Biotech Ultrospec 3000 UV-Vis photometer according to Taylor et al. [23] with minor modifications. The 0.5 ml reaction mixture contained the following reagents in reaction buffer (50 mM sodium phosphate pH 6.5): the enzymes, recombinant human MIF from R&D Systems and bovine kidney tautomerase from Sigma [16], were dissolved in reaction buffer (at 0.1 and 1.8 mg/ml concentrations, respectively) and applied in 1 μl aliquots. Phenylpyruvic acid (the enolic form) diluted in 98% ethanol was also applied in 1 μl buffer to yield final substrate concentration of 100 μM. Luteolin (from Carl Roth GmbH) was diluted and applied likewise to yield the final concentrations desired (2 nM–100 μM). Spontaneous tautomerisation was accounted for in the results presented. Luteolin or quercetin at the concentrations applied here had no effect on the spontaneous tautomerisation of the substrate.

Five weeks old female CFY rats implanted with 100 μg estradiol-17β in s.c. beeswax pellets under short ether

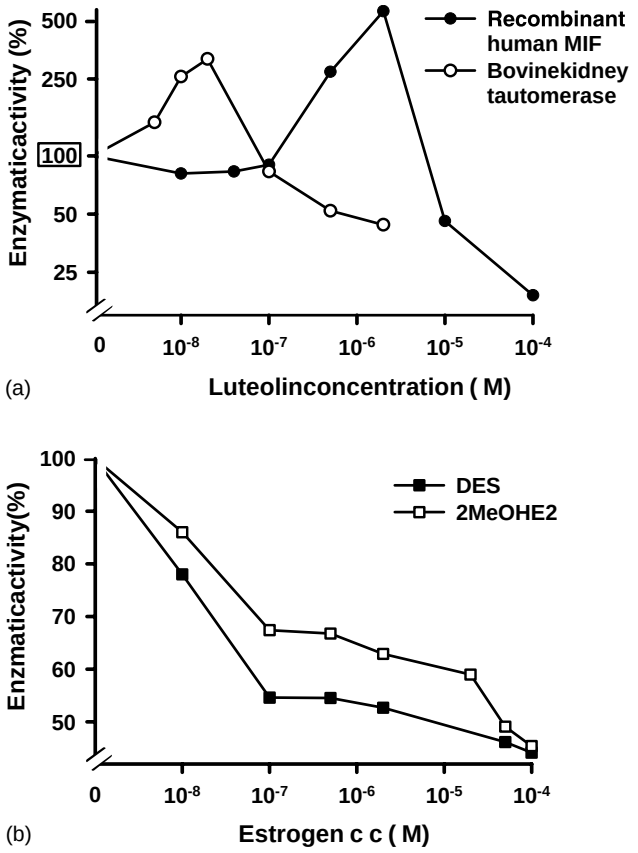

Fig. 1. (a) Enol–keto conversion of 100 μM phenylpyruvate catalysed by recombinant human MIF (closed symbols) and by bovine kidney tautomerase (open symbols). The 100% activity of the enzymes as assessed at 288 nm by the initial rate of dE/min decrease were 1.31 and 1.35, respectively. Note that the unusual logarithmic scale of the vertical axis was chosen for a better visual perception of both, the stimulatory and the inhibitory effect of luteolin. (b) Effect of estrogen compounds on the enol–keto conversion of 100 μM phenylpyruvate catalysed by bovine kidney tautomerase: diethylstilbestrol (DES: closed symbols); 2-methoxy-estradiol (2MeOHE2: open symbols). For further technical details see Section 2.

anaesthesia were sacrificed 71–73 h following the implantation. Uteri were removed and the uterine nuclear extract was prepared by the procedure that yields high type II binding activity [18]. The extracted proteins were separated on 10% polyacrylamide gels according to the method of Laemmli [24] with 0.1% SDS using bromophenol-blue as tracking dye. Gels were loaded with 25 μg protein per lane. The prestained molecular weight markers from Sigma were as follows: ovalbumin (45 kDa), carbonic anhydrase (29 kDa), alpha lactalbumin (14.2 kDa) and aprotinin from bovine milk (6.5 kDa). The gel was run in Bio-Rad Mini Protean II chambers then equilibrated for 3 × 20 min with the transfer buffer (50 mM sodium phosphate; 0.05% SDS; 20% methanol, pH 6.8). The separated proteins were transferred overnight onto nitrocellulose membrane (Hoefer) at 150 mA, 4 °C. The membrane was blocked for 2 h at room temperature with 3% BSA in NEHTG buffer (50 mM HEPES; 5 mM EDTA; 150 mM NaCl; 0.05% Triton-X100; 0.25% gelatin, pH 7.4), further placed for 3 h in a solution of 4 M urea; 150 mM NaCl; 20 mM EDTA pH 7.0, then washed in NEHTG buffer for 3 × 5 min. The membrane was incubated overnight at 4 °C with rabbit anti-human MIF antibody from R&D Systems (1:2000) in NEHTG buffer containing 1% BSA, then, after three washes, with the secondary antibody (peroxidase-tagged goat anti rabbit IgG from DAKO 1:2000) for 4 h at room temperature. After four washes the Western blot image was developed with Amersham's enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions.

3. Results and discussion

Luteolin at rather low concentrations (5–20 nM) stimulates the enzymatic activity of bovine kidney tautomerase (MIF) but the enzyme was inhibited at higher concentrations (Fig. 1a). Recombinant human MIF exhibited similar behaviour but at higher concentrations of luteolin. With quercetin the stimulation was obtained at slightly higher concentrations for both enzymes (not shown), also followed by inhibition when concentrations of this flavonoid were further increased. The synthetic estrogen diethylstilbestrol (DES) and the estrogen metabolite 2-methoxy-estradiol (2MeOHE2) showed partial inhibition, but no stimulation of the tautomerase activity in the concentration range applied (Fig. 1b).

Uterine nuclear extract prepared according to the method that yields high type II binding activity also features MIF immunoreactivity when processed on SDS–polyacrylamide gel electrophoresis followed by Western blotting (Fig. 2).

Both, luteolin and quercetin are known as type II binding site ligands. The relatively high sensitivity of MIF's enzymatic activity toward low concentrations of these flavonoids make this cytokine a possible site where flavonoids interfere with estrogen's action. These data support participation of MIF's enzymatic activity in the type II luteolin binding phenomena. Hence adopting Thierry Calandra's term on MIF [25] it could be renamed a Most Interesting Flavonoid Binding Site (MIFBS) i.e. 'macrophage migration inhibitory factor'-related flavonoid binding site.

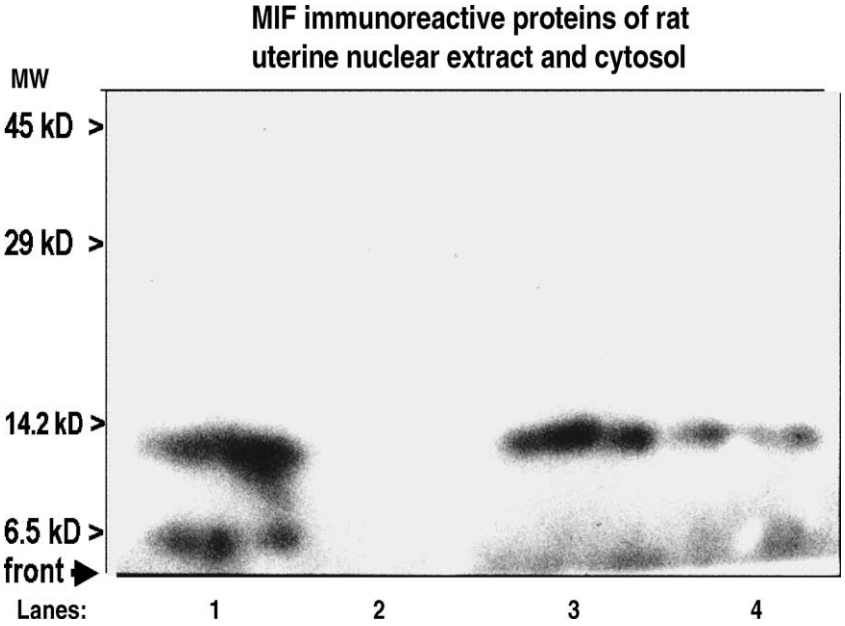

Fig. 2. SDS–polyacrylamide gel electrophoresis and Western blot of bovine kidney tautomerase (lane 1), rat uterine cytosol (lane 4), and nuclear extract exhibiting high nuclear type II estrogen binding (lane 3). Bovine serum albumin (lane 2) was included as negative control. The proteins were separated on 10% polyacrylamide gel, blotted onto nitrocellulose membrane, then reacted with polyclonal rabbit anti-human MIF IgG (1:2000). The secondary antibody was peroxidase-tagged goat anti rabbit IgG (1:2000). The image was developed with an enhanced chemiluminescence kit (ECL). The molecular weight markers shown are: ovalbumin (45 kDa), carbonic anhydrase (29 kDa), alpha lactalbumin (14.2 kDa), and aprotinin from bovine milk (6.5 kDa). For further technical details see Section 2.

The luteolin concentration stimulatory for bovine kidney tautomerase is lower than for the recombinant human MIF. The difference might be due to the presence of yet unknown 'sensitising' (co)factors in the extract prepared from the kidney tissue. On the other hand, the recombinant protein (or a portion of it) may be in a 'crumbled' state (i.e. not properly folded), or its subunits perhaps not properly assembled under the conditions applied here.

A functional homologue of MIF, D-dopachrome tautomerase, shares structural features with this cytokine and linkage of their genes have been reported [26], therefore it might be expected to exhibit type II-like estrogen binding characteristics, nevertheless, it needs to be assessed by future studies. On the other hand, the dopamine binding site of Y79 retinoblastoma cells [27], that has similar binding characteristics, but distinct ligand specificity in comparison with the type II estrogen binding, may, in fact, represent authentic L-dopachrome tautomerase since its expression has recently been described in this cell line [28].

It is a striking parallel that progesterone reportedly is a strong antagonist to the pro-inflammatory action of estrogen in the uterus [29], and also prevents induction of uterine nuclear type II sites by estrogen [30]. Our data may also shed light on a possible mechanism of certain flavonoids' anti-inflammatory action [31]. If the pro-inflammatory function of MIF relies on its enzymatic activity those flavonoids (like luteolin or quercetin) that interfere with it may alleviate the inflammatory process. Under certain conditions luteolin might also completely block the enzymatic function behaving as a suicide substrate/ligand as it could be conferred from its spontaneous crosslinking with the type II binding site. Certain known subtrates of MIF reportedly also crosslink with the enzyme [32]. The most potent inhibitor of MIF's tautomerase reported so far is a chromene structure [33] with close similarity to catecholic flavonoids like luteolin or quercetin. All hypotheses concerning the functional role of MIF's enzymatic activity, however, remain highly speculative until the 'true natural' substrate for MIF is found.

The implication of type II binding sites in estrogen-dependent tumor promotion and its antagonism by the type II site ligand flavonoids [6] has prompted several studies scrutinising the underlying mechanisms. The involvement of MIF in tumor growth [34] assessed in view of the so called type II binding phenomena might help to delineate anti-proliferative action of certain flavonoids on estrogen-dependent cancers [35,36].

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