ELSEVIER

Steroids 67 (2002) 637–645

Steroids

Intravenous injection of human sex steroid hormone-binding globulin in mouse decreases blood clearance rate and testicular accumulation of orally administered [2-^{125}I]iodobisphenol A

Nadia Bendridi[a,b], Elisabeth Mappus[b], Catherine Grenot[b], Hervé Lejeune[a,c], Claude Yves Cuilleron[b], Michel Pugeat[a,b,*]

[a]*Hospices Civils de Lyon, Laboratoire de la Fédération d'Endocrinologie, Hôpital de l'Antiquaille, Lyon, France*
[b]*INSERM U 329, Lyon, France*
[c]*INSERM U 418, Hôpital Debrousse, Lyon, France*

Received 31 July 2001; received in revised form 14 December 2001; accepted 19 December 2001

Abstract

Bisphenol A, an environmental compound with estrogenic activity, has been shown to bind human sex steroid hormone-binding globulin (hSHBG), the main plasma transport protein which regulates the metabolism of androgens and estrogens and limits their access to target organs. The present study was conducted to determine whether physiologically relevant concentrations of hSHBG can influence the blood clearance rate of bisphenol A and its accumulation in the testes. A radioactive [2-^{125}I]iodobisphenol tracer was synthesized with an association constant (Ka) for binding to hSHBG of $0.14 \pm 0.01 \times 10^6$ M^{-1} at 37°C, a value much lower than for [2-^{125}I]iodoestradiol, which was also synthesized. We used i.v. injection of immunopurified hSHBG in adult male mice to maintain hSHBG levels within the physiologically possible range for humans (27–267 nM) before gavage administration of [2-^{125}I]iodobisphenol or [2-^{125}I]iodoestradiol, for measuring the blood clearance rate of radioactive signal in blood samples taken during the following 120 min. Testicular accumulation of radioactivity was measured 24 h and 48 h after gavage of [2-^{125}]iodobisphenol A. In mice receiving immunopurified hSHBG or vehicle, the time-dependent blood clearance of radioactivity exhibited a bi-exponential decrease which indicated α-diffusion and β-elimination phases for both radioactive ligands. The presence of circulating hSHBG significantly and dose-dependently lowered the clearance rate of radioactivity. However, much higher circulating levels of hSHBG were required to retard the blood clearance of [2-^{125}I]iodobisphenol A as compared to those required for [2-^{125}I]iodoestradiol, in keeping with the important difference in their respective Ka value for binding to SHBG. In addition, mice treated with hSHBG exhibited significantly ($P = 0.036$) reduced testicular accumulation of radioactivity 24 h and 48 h after ingestion of [2-^{125}I]iodobisphenol A. Provided that the binding properties of bisphenol A for hSHBG are not substantially different from those measured for [2-^{125}I]iodobisphenol A, these findings suggest that, although hSHBG binds 2-mono-iodobisphenol A with a relatively low binding affinity, high enough concentrations of circulating hSHBG (range concentrations between 85 and 267 nM) are potentially able to exert a protective effect against exposure to bisphenol A.

Keywords: Endocrine disruptor; Bisphenol A; Iodinated bisphenol A derivative; Human SHBG; Steroid; Clearance

1. Introduction

Although the question is still debated, several studies have claimed that sperm counts in human males have decreased by an average of 50% since 1940 [1,2]. In addition, increased frequencies of cryptorchidism and hypospadia, as well as of cancer of testis [3] have been reported. Exposure to xenoestrogens has been postulated as playing a role in the decline of testicular function and increase in genital tract malformations, but experimental support is lacking.

Bisphenol A (4,4′-isopropylidenediphenol) is a monomer widely employed in the manufacture of polycarbonate, epoxy, and polyester–styrene resins. Trace levels of bisphenol A leach from the linings of food cans and dental resins [4,5]. High doses were reported to cause reproductive toxicity and affect cellular development in rats and mice [6,7]. Recently, low levels of bisphenol A administered orally (2 ng/g) to mice (CF-1) during pregnancy have been found to

* Corresponding author. Tel.: +33-4-72-38-50-70; fax: +33-4-72-25-65-86.
E-mail address: michel.pugeat@chu-lyon.fr (M. Pugeat).

0039-128X/02/$ – see front matter
PII: S0039-128X(02)00014-4

reduce daily sperm production in male offspring and to reduce the size of the accessory reproductive organs [8]. Estrogenic activity of bisphenol A has been demonstrated in water-sterilized polycarbonate flasks [9]. In addition, bisphenol A has been shown to bind estrogen receptors [10], to stimulate cell proliferation, and also to induce the synthesis of progesterone receptor in estrogen-dependent MCF-7 breast cancer cell lines [9,11,12]. Since these initial findings, estrogenic activity has been reported for many natural environmental or synthetic nonsteroidal substances. Some of these xenoestrogens are structurally similar to diethylstilbestrol and can be active as estrogen agonists or antagonists [13]. More specifically, the binding of some chemical estrogens to estrogen receptor-α has been shown to be involved in the activation of the uterine insulin-like growth factor 1 signaling pathway [14]. In vivo experiments have shown that bisphenol A-treated Fischer 344 rats have stimulated proliferation of pituitary lactotroph cells with increased expression and secretion of prolactin, these effects being species-specific and not observed in Sprague Dawley rats [15].

Danzo [16] reported that bisphenol A binds human sex hormone-binding globulin (hSHBG) with a much lower binding affinity than its natural ligands, testosterone and 17β-estradiol. These findings were confirmed by our laboratory [17]. It has been postulated that SHBG-binding might be a limiting step for intracellular access and biological activity of several xenoestrogens that specifically bind SHBG [16,18]. However, most in vivo studies reporting the effects of xenoestrogen exposure were performed in rodents which have low, if any, circulating levels of SHBG during adult life, although they do have intra-testicular androgen-binding protein (ABP) [19,20]. Therefore, the question is whether the main function of hSHBG, which is to regulate the bioavailability of steroids to their target tissues [21,22], might be relevant for low binding affinity ligands such as bisphenol A in a natural endogenous steroid environment. To elucidate this question, radioinert and radioactive iodinated derivatives of bisphenol A (2-mono-iodobisphenol A and 2,2′-di-iodobisphenol A) and estradiol (2-iodoestradiol and 2,4-di-iodoestradiol) were prepared and employed to determine the influence of iodination on the binding affinity for SHBG. In a second step, [2-^{125}]iodobisphenol A and [2-^{125}I]iodoestradiol were orally administered to adult mice which were i.v. injected with immunopurified hSHBG or vehicle, in order to study the effects of hSHBG-binding on the time-dependent clearance rate of total blood radioactivity and testicular accumulation.

2. Experimental

2.1. Reagents

[1,2,6,7-^{3}H]Testosterone (3.44 TBq/mmol) (Amersham France SA, Les Ulis, France), testosterone (17β-hydroxy-4-androsten-3-one) and estradiol (1,3,5(10)-estratriene-3,17β-diol) (Sigma Chemical Co, Saint-Louis, MO, USA) were dissolved in ethanol prior to appropriate dilutions. Bisphenol A (4,4′-isopropylidenediphenol) (purity $>$ 99%) was supplied by Fluka (France), Concanavalin A-Sepharose (ConA-Sepharose) from Pharmacia Fine Chemicals AB (Uppsala, Sweden), Cytoscint® TM scintillation fluid from ICN Pharmaceuticals (France), NaI from Riedel-De Häen (Hanover, Germany) and Chloramine T and sodium metabisulfite from Aldrich-Chemie (Steinheim, Germany). ^{125}NaI was purchased from ICN pharmaceutical (100 mCi/ml). Radioactivity was counted in a γ counter (1900 model or Cobra II auto-γ, Packard Instrument Co., Downers Grove, IL, USA). NMR spectra were recorded at the Centre Commun de RMN (ESCPE-Université Claude Bernard, Villeurbanne, France). ^{1}H NMR spectra of iodinated estradiol and bisphenol A derivatives were recorded at 300 MHz on a Brüker (Wissenbourg, France) spectrometer equipped with an Aspect 300 computer. Chemical shifts were measured relative to tetramethylsilane Si(CH). Samples were prepared by dissolving 10 mg of steroid in 0.75 ml of CD_3OD (99.8%), purchased from CEA (Saclay, France). The ^{1}H chemical shifts were estimated to be accurate to $\pm$ 0.01 ppm, and coupling constants to $\pm$ 0.5 Hz.

2.2. Preparation of iodinated estradiol and bisphenol A derivatives, 2-iodoestradiol and 2,4-di-iodoestradiol

A solution of estradiol (544 mg, 2.13 mmol) in 1,4-dioxane (10 ml) was mixed with a solution of NaI (150 mg, 2 mmol), in 2 ml of 50 mM sodium phosphate buffer pH 7.6 (PBS), before adding a solution of Chloramine T (540 mg, 2.37 mmol) in the same buffer (5 ml). The reaction was allowed to proceed at 20°C for 5 min with constant mixing, before the addition of a solution of sodium metabisulfite (1.9 g, 9.99 mmol) in the same buffer (5 ml). Distilled water (20 ml) was added and the mixture was extracted twice with ethyl acetate (50 ml). The pooled extracts were evaporated to dryness and the residue dissolved in a small volume of methanol/water (7:3 v/v) mixture. The reaction products were separated by preparative high-performance liquid chromatography (HPLC) on a 250 $\times$ 10 mm μBondapak C18 reverse phase column (Waters RCM - 10 μM - 125 Å) using methanol/water (7:3 v/v) mixture as eluent (elution rate 8 ml/min with continuously monitored effluent at 280 nm). Separate peaks were collected and evaporated to dryness. The reaction products were also separated by thin-layer chromatography (TLC) on silica plates, using toluene/ethyl acetate (3:1 v/v) as the developing mixture (Rf of 2-iodoestradiol: 0.44; Rf of 2,4-di-iodoestradiol: 0.60). The corresponding [2-^{125}I]iodoestradiol was prepared with [^{125}I]Na using the same procedure and separated by TLC using radioinert 2-iodoestradiol as reference compound. ^{1}H NMR 2-iodoestradiol: ^{1}H NMR (CD_3OD) δ0.77 (3H, s, CH_3-18), 3.65 (H, m, H-17), 6.53 (H, s, H-4), 7.50 (H, s,

H-1). ^{1}H NMR 2,4-di-iodoestradiol: ^{1}H NMR (CD_3OD) δ0.87 (3H, s, CH_8), 3.65 (H, m, H-17), 7.60 (1H, s, H-1).

2-*Mono-iodobisphenol A and 2,2′-di-iodobisphenol A* were prepared according to the above method. The crude reaction products were dissolved in a minimum volume of methanol and separated using TLC on silica gel with a chloroform/ether (4:1 v/v) mixture (Rf of 2-mono-iodobisphenol A: 0.54; Rf of 2,2′-di-iodobisphenol A: 0.65). The corresponding ^{125}I-labeled derivatives were prepared with [^{125}I]Na and analyzed by TLC using radioinert 2-mono-iodobisphenol A and 2,2′-di-iodobisphenol A as reference compounds ([2-^{125}I]iodobisphenol A (a.s.: 0.013×10^{13} dpm/mmol)). ^{1}H NMR bisphenol A: ^{1}H NMR (CD_3OD) δ1.57 (6H, s, CH), 7.01 (4H, d: J = 8.8 Hz, arom. H-meta), 6.66 (4H, d: J = 8.8 Hz, arom. H-ortho). ^{1}H NMR 2-mono-iodobisphenol A: ^{1}H NMR (CD_3OD) δ1.56 (6H, s, CH), 6.68 (2H, d: J = 8.7 Hz, H-2′-6′), 6.70 (1H, d: J = 8.4 Hz, H-6), 7.00 (1H, dd: J = 8.6 and 2.3 Hz, H-5), 7.01 (2H, d: J = 8.8 Hz, H-3′-5′), 7.47 (1H, d: J = 2.3 Hz, H-3). ^{1}H NMR 2,2′-di-iodobisphenol A: ^{1}H NMR (CD_3OD) δ.55 (6H, s, CH), 6.72 (2H, d: J = 8.5 Hz, H-6), 6.99 (2H, dd: J = 8.6 and 2.3 Hz, H-5), 7.49 (2H, d: J = 2.4 Hz, H-3).

2.3. Purification and measurement of hSHBG

Purification of hSHBG was performed by immunoaffinity chromatography of a pool of human serum using an immobilized anti-hSHBG antibody, as previously described [23]. The serum concentration of hSHBG was measured using an immunoradiometric assay for hSHBG (^{125}I-labeled SHBG-Coatria, BioMérieux, Marcy l'Etoile, France).

2.4. Binding assays

2.4.1. Scatchard plot analysis

The respective association constants (Ka) of 2-mono-iodobisphenol A and 2,2′-di-iodobisphenol A for binding to immunopurified hSHBG were calculated by Scatchard plot analysis using the corresponding radioiodinated tracers [24] and a solid phase binding assay [25].

2.5. Relative binding assays of iodinated bisphenol A and estradiol derivatives

The competition curves were obtained from binding assays, performed on immunopurified hSHBG incubated with [^{3}H]testosterone, in the presence of increasing concentrations of each studied ligand (bisphenol A, iodinated bisphenol A derivatives, 17β-estradiol, iodinated estradiol derivatives and testosterone), as previously described [25,26]. Briefly, 500 μl of 50% Con A-Sepharose in Tris-HCl buffer were added to an immunopurified hSHBG solution (100 nM in 100 μl of Tris-HCl buffer). After 30 min of incubation at 20°C, 2 ml of Tris-HCl was added. The pellets obtained after centrifugation for 5 min at 3000 × *g* were washed three times with Tris-HCl buffer. Pellets were then added to a mixture of [^{3}H]testosterone (50 000 cpm in 100 μl of Tris-HCl buffer), and various final concentrations of the radioinert ligand: 0–500 μM for bisphenol A, 2-mono-iodobisphenol A, 2,2-di-iodobisphenol A, and 0–0.03 μM for 17β-estradiol, 2-iodoestradiol, 2,4-di-iodoestradiol or testosterone, and incubated in a total volume of 2.3 ml of Tris-HCl buffer, for 90 min at 37°C under shaking. After centrifugation for 5 min at 3000 × *g*, the pellets were counted for radioactivity in CytoScint® scintillation fluid in a beta counter (Packard Instrument Co.). Specific binding, in the presence of competitor, was expressed as a percentage of the maximum binding that was calculated by subtracting non-specific binding (estimated in the presence of 100-fold excess of testosterone) from total binding. Relative binding affinities (RBA) of ligands to hSHBG were calculated as follows:

$$\text{RBA} = [\text{IC}_{50}, \text{Testosterone}/\text{IC}_{50}, \text{Competitor}] \times 100.$$

The IC_{50} value is the concentration of radioinert competitor required to reduce [^{3}H]testosterone specific binding by 50%. The RBA value for testosterone was arbitrarily set at 100%.

2.6. Influence of iodinated bisphenol A derivatives on testosterone binding to immunopurified hSHBG

Immunopurified hSHBG (100 nM in 100 μl of Tris-HCl pH 7.6 buffer) was incubated with ConA-Sepharose (500 μl of 50% diluted gel) for 30 min at 20°C, and the solid phase was washed three times with 2 ml of Tris-HCl and centrifuged for 5 min at 3000 × *g*. The pellets were incubated with a mixture of [^{3}H]testosterone (50 000 cpm in 100 μl of Tris-HCl buffer), increasing radioinert testosterone for final concentrations ranging from 0 to 30 nM, and fixed concentrations of bisphenol A (20 μM), 2-mono-iodobisphenol A (20 and 25 μM) or 2,2′-di-iodobisphenol A (10 and 25 μM) in a total volume of 2.5 ml of Tris-HCl buffer. After incubation for 90 min at 37°C and centrifugation, the radioactivity of pellets was counted. Changes in association constant and/or in number of hSHBG binding sites for testosterone were studied by comparing Scatchard plots established in the presence or in the absence of bisphenol A or iodinated bisphenol A derivatives.

2.7. Animals, treatments, and sample collection

Adult male mice (Balb/C), 60–70 days old, with weights ranging from 17 to 24 g (purchased from Elevage Scientifique des Dombes, Châtillon sur Chalaronne, France), were housed individually at 20°C with alternating darkness and artificial light cycles, with food and water available ad libitum. In a first set of experiments, one group of male adult Balb/C mice ($n = 6$) was given a single oral bolus of 500 000 cpm of [2-^{125}I]iodobisphenol A and a second group ($n = 6$) was given a single oral bolus of 500 000 cpm of

[2-^{125}I]iodoestradiol, in 0.1 ml of 5% glucose vehicle. Blood samples were taken (200–300 μl) by perocular sinus puncture at 5, 10, 15, 20, 25, 30, 40, 60, and 80 min after gavage, placed in preweighted tubes containing heparin (Sanofi-Winthrop, l5 I.U.), and counted for total radioactivity without any prior purification. Thus, the radioactivity measured in the samples may correspond either to metabolites or unmetabolised iodinated bisphenol A derivatives. In a second set of experiments, two groups of adult male Balb/C mice were treated by tail-vein injection of purified hSHBG (9 μM) in 0.1 ml of PBS. One hour later, a blood sample was taken to measure serum hSHBG concentration, before oral gavage administration of [2-^{125}I]iodobisphenol A ($n = 7$) or [2-^{125}I]iodoestradiol ($n = 2$). In a further set of experiments, one group ($n = 18$, $n = 3$ per time tested, 6 times tested) of male adult Balb/C mice was injected with purified hSHBG or with vehicle ($n = 18$, $n = 3$ per time tested, 6 times tested) before receiving an oral bolus of 500 000 cpm of [2-^{125}I]iodobisphenol A. The animals were sacrificed by cardiac puncture for blood sampling at 1.5, 3, 5, 15, 24 and 48 h after gavage, and the tissue distribution and accumulation patterns of radioactivity were determined by immediate sampling of the main organs for weighing and counting.

2.8. Statistics

Data are expressed as the mean ± S.D. Multifactorial analysis of variance (ANOVA) was performed for: (i) analysis of hSHBG binding affinity of iodinated bisphenol A and estradiol derivatives; (ii) analysis of the relative binding affinities (RBA) of ligands to hSHBG; (iii) assessment of the effect of fixed concentrations of bisphenol A and iodinated bisphenol A derivatives on testosterone-binding to hSHBG. Multifactorial analysis of variance (ANOVA) was performed to assess the effects of hSHBG on the time-dependent disappearance of [2-^{125}I]iodoestradiol. The correlation between the time-dependent disappearance of [2-^{125}I]iodobisphenol A and SHBG blood concentrations was established using the least square method, which could be applied in this case, owing to the higher number of experiments conducted for [2-^{125}I]iodobisphenol A as compared with [2-^{125}I]iodoestradiol. These two relationships were studied at 40 min, 60 min and 80 min after oral administration. In both cases, comparisons yielding more than 95% statistical confidence ($P < 0.05$) were considered significant.

3. Results

3.1. Scatchard plot analysis of iodinated bisphenol A derivatives

Scatchard plot analysis, using either [2-^{125}I]iodobisphenol A or [2,2′-^{125}I]iodobisphenol A, showed a single population of hSHBG binding sites with Ka values of 0.14 ± 0.01 × 10^6 M^{-1} or 0.62 × 10^6 ± 0.07 M^{-1} at 37°C, respectively. The differences between these two affinity constants was significant ($P < 0.0005$, $n = 4$).

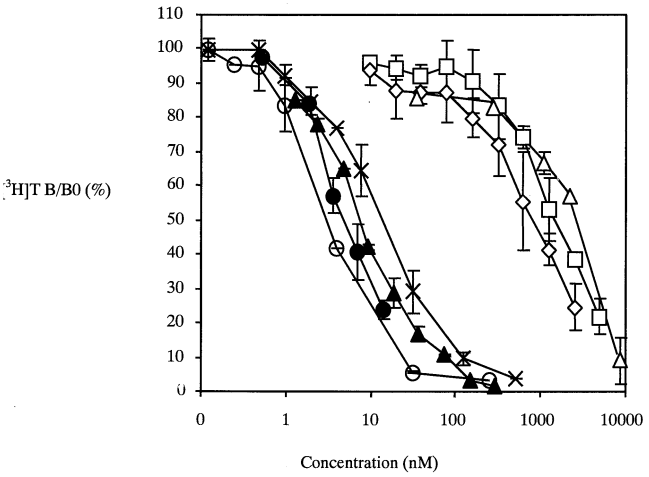

Fig. 1. Competition curves (B/B0 × 100) of tritiated [^{3}H]testosterone from human SHBG by increasing concentrations of radioinert testosterone (●), estradiol (▲), 2-iodoestradiol (○), 2,4-di-iodoestradiol (×), bisphenol A (Δ), 2-mono-iodobisphenol A (□), 2,2′-di-iodobisphenol A (◇). Purified human SHBG (100 nM in Tris-HCl buffer) was incubated with [^{3}H]testosterone (50 000 cpm) in the presence of estradiol, iodinated estradiol, bisphenol A and iodinated bisphenol A derivatives or unlabeled testosterone for 1.5 h at 37°C. Bound/free separation was achieved with Con-A Sepharose. The data are the average ± S.D. of 2–4 independent experiments.

3.2. Competitive binding studies to hSHBG

The relative binding affinities (RBAs) of bisphenol A, 2-mono-iodobisphenol A, and 2,2′-di-iodobisphenol A for human SHBG were determined from their ability to compete with [^{3}H]testosterone (Fig. 1). RBA was also measured for estradiol, 2-iodoestradiol and 2,4-di-iodoestradiol. All of the ligands tested were able to compete significantly ($P < 0.001$) with tritiated testosterone for binding to human SHBG. Bisphenol A and the two iodinated bisphenol A derivatives showed low binding affinities. The RBA values (Table 1) were 0.20% ± 0.01 for bisphenol A, 0.32% ± 0.10 for 2-mono-iodobisphenol, 0.47% ± 0.12 for 2,2′-di-iodobisphenol A, 175.5% ± 27.6 for 2-iodoestradiol, 23.8% ± 3.1 for 2,4-di-iodoestradiol and 58.9% ± 1.6 for estra-

Table 1
Relative binding affinities for hSHBG

Ligands	RBA (%)
Testosterone	100
Estradiol	58.9 ± 1.6*
2-Iodoestradiol	175.5 ± 27.6*
2,4-Di-iodoestradiol	23.90 ± 3.06*
Bisphenol A	0.20 ± 0.01*
2-Mono-iodobisphenol A	0.325 ± 0.100*
2,2′-Di-iodobisphenol A	0.48 ± 0.12*

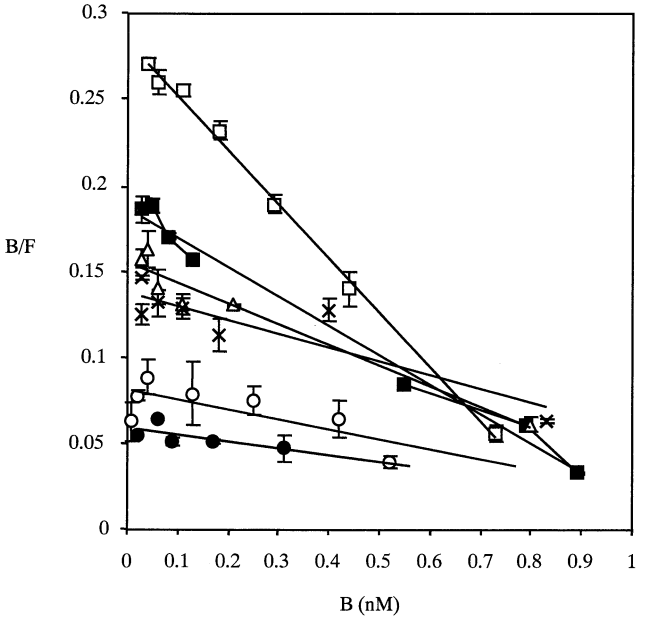

Fig. 2. Scatchard plot analysis of testosterone binding in the presence or in the absence of constant concentrations of radioinert bisphenol A or iodinated bisphenol A derivatives. Purified hSHBG (100 nM in Tris-HCl buffer pH 7.6) bound to Con-A was incubated for 1.5 h at 37°C, with [^{3}H]testosterone in 100 μl of Tris-HCl buffer pH 7.6, with increasing amounts (0–30 nM) of unlabeled testosterone (□) in the absence or in the presence of bisphenol A 20 μM (■), 2-mono-iodobisphenol A 20 μM (**Δ**) or 25 μM (×), and 2,2′-di-iodobisphenol A 10 μM (***o***) or 25 μM (●) in 100 μl of the same buffer. Separation of bound and free was achieved with Con-A Sepharose. The data are on average ± S.D. of 3 experiments.

diol. The affinity ranking for the various tested ligands on the basis of statistical differences in RBA value was 2-iodoestradiol > testosterone > estradiol > 2,4-di-iodoestradiol > 2,2′-di-iodobisphenol A > 2-mono-iodobisphenol A > bisphenol A.

3.3. Influence of iodinated bisphenol A derivatives on testosterone binding to purified hSHBG

Scatchard plots for testosterone binding to purified hSHBG in the presence or the absence of iodinated bisphenol A derivatives are shown in Fig. 2. The presence of bisphenol A (20 μM), or 2-mono-iodobisphenol A (20 and 25 μM) or 2,2′-di-iodobisphenol A (10 and 25 μM) significantly decreased ($P < 0.0001$) the apparent Ka for testosterone binding to hSHBG, but with an essentially unchanged number of binding sites (Table 2). This inhibitory effect was more pronounced for 2,2′-di-iodobisphenol A than for 2-mono-iodobisphenol A, a result in agreement with the differences between their respective binding affinities for hSHBG. Even the lowest concentration of 2,2′-di-iodobisphenol A (10 μM) exerted a stronger inhibition than the highest concentration of 2-mono-iodobisphenol A (25 μM). However, the differences in inhibiting effects between the two tested concentrations of each of these two compounds did not reach statistical significance ($P > 0.1$).

Table 2
Relative constant affinity for testosterone binding to hSHBG

Ligands	Ka of testosterone (37°C) ($X10^9$) (M^{-1})
Testosterone	0.395 ± 0.048
Testosterone + 20 μM of bisphenol A	0.185 ± 0.005*
Testosterone + 20 μM of 2-mono-iodobisphenol A	0.107 ± 0.016*
Testosterone + 25 μM of 2-mono-iodobisphenol A	0.093 ± 0.023*
Testosterone + 10 μM of 2,2′-di-iodobisphenol A	0.073 ± 0.026*
Testosterone + 25 μM of 2,2′-di-iodobisphenol A	0.056 ± 0.016*

Conversely, the differences in the inhibitory effect observed with the three ligands, bisphenol A, 2-mono-iodobisphenol A, and 2,2′-di-iodobisphenol A, were statistically significant, as established for the comparison of the effects of a 20 μM concentration of 2-mono-iodobisphenol A or bisphenol A ($P < 0.01$) or of a 25 μM concentration of 2,2′-di-iodobisphenol A and 2-mono-iodobisphenol A ($P < 0.01$). Taken together, these results indicate that iodinated bisphenol A derivatives and testosterone compete for the same hSHBG steroid binding site.

3.4. Effects of hSHBG on blood clearance of [2-^{125}I]iodinated bisphenol A and estradiol derivatives

Typical time-dependent clearance curves for radioactivity in the blood after oral administration of [2-^{125}I]iodinated bisphenol A or [2-^{125}I]iodinated estradiol derivatives are shown in Fig. 3 and Fig. 4. Radioactive material was detectable in the blood shortly after oral administration. The

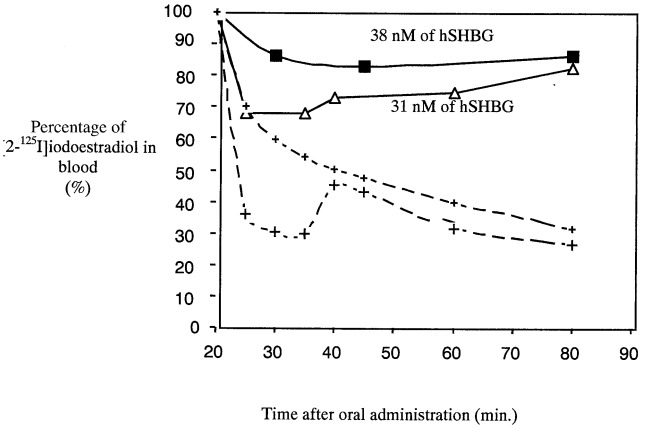

Fig. 3. Time-dependent blood clearance of orally ingested [2-^{125}I]iodoestradiol in the absence of blood hSHBG (− − −) (the broken lines represent the highest and the lowest values of radioactivity measured in the absence of circulating hSHBG) or in the presence of 31 nM (**Δ**) and 38 nM (■) of blood hSHBG concentrations. The maximum blood concentration of [2-^{125}I]iodoestradiol at $t = 20$ min is taken as 100%.

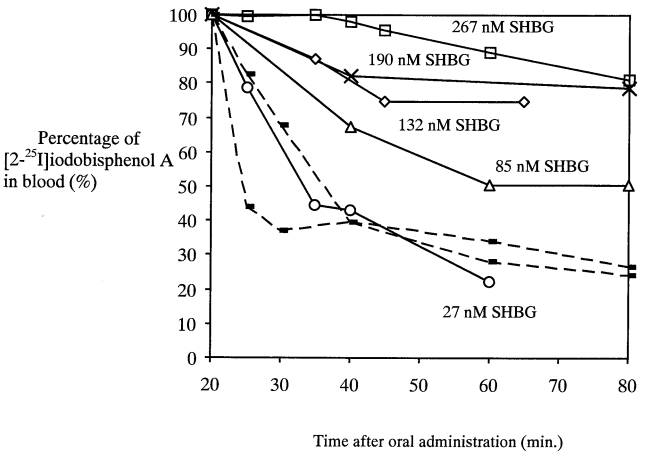

Fig. 4. Time-dependent blood clearance of orally ingested [2-^{125}I]iodobisphenol A in the absence of blood hSHBG (− − −) (the broken lines represent the highest and the lowest values of radioactivity measured in the absence of circulating hSHBG), or in the presence of 27 nM (***o***), 85 nM (**Δ**), 132 nM (◇), 190 nM (×) and 267 nM (□) of blood hSHBG concentrations. The maximal blood concentration of [2-^{125}I]iodobisphenol A at t = 20 min is taken as 100%.

time-dependent decrease exhibited a three-phase profile corresponding to: (i) resorption (data not shown), with a radioactive peak appearing in less than 20 min, (ii) α-diffusion from the vascular compartment during the next 15 min, (iii) β-elimination (reflecting metabolic degradation, mostly in liver, kidney and intestinal compartments) during the following 35 min, respectively. The presence of hSHBG at concentrations ranging from 31 to 27 nM significantly reduced the time interval for α-diffusion and β-elimination phases of total radioactivity in blood sampling after oral administration of [2-^{125}I]iodoestradiol, as compared to control animals receiving no hSHBG (Fig. 3). However, the presence of 20 or 27 nM concentrations of hSHBG did not cause any significant decrease in the α-diffusion or β-elimination phases of [2-^{125}I]iodobisphenol A derivatives. In the latter case, much higher hSHBG concentrations, ranging from 85 to 267 nM, were required to achieve a significant increase in [2-^{125}I]iodobisphenol A blood concentration (Fig. 4). A multifactorial analysis of variance taking into account the concentrations of hSHBG for the two radiolabeled derivatives showed that the increase in blood radioactive material (at 60 min or 80 min after gavage) due to the presence of hSHBG was significant (P = 0.002) for both iodinated derivative ligands. Regression coefficients for total radioactivity in blood sampling at 40, 60 and 80 min after the oral administration of [2-^{125}I]iodobisphenol, in the presence of various concentrations of hSHBG, were obtained using the linear least square method: (i) r = 0.972, P = 0.0002, n = 7 at 40 min; (ii) r = 0.946, P = 0.0177, n = 5 at 60 min; (iii) r = 0.919, P = 0.0095, n = 6 at 80 min. These results indicate that the highest hSHBG blood concentrations corresponded to the lowest [2-^{125}I]iodobisphenol A blood clearance rates.

3.5. Effects of hSHBG on the distribution and organ accumulation of orally ingested [2-^{125}I]iodobisphenol A in mice

[2-^{125}I]Iodobisphenol A orally ingested by mice was readily adsorbed from the gastrointestinal tract and distributed to organs. About 6% of the radioactivity was accumulated in the liver during the first 3 h, but accumulation reached 41% within 48 h. After 15 h, 31% of the radioactivity was eliminated in the feces, which suggested enterohepatic circulation of the radioactive material. In addition, only 3% of the radioactivity was detected in the urine after 5 h. The kinetics of accumulation of [2-^{125}I]iodobisphenol A in the testes and epididymes in the presence or the absence of hSHBG were studied in three different experiments. In the absence of injected hSHBG, a time-dependent accumulation of [2-^{125}I]iodobisphenol A in the testes and epididymes was observed up to 24 h. At this point in time, the percentages of the total orally administered radioactivity recovered in the testes and in epididymes were 0.25% ± 0.028 and 0.29% ± 0.35, respectively (Table 3). In the presence of i.v. hSHBG, although hSHBG concentration decreased time-dependently (158 nM ± 32 at 3 h, 109 nM ± 2 at 15 h, 65 nM ± 6 at 24 h and 55 nM ± 21 at 48 h), the accumulation of [2-^{125}I]iodobisphenol A in testes and epididymes, up to 24 h, was less than 0.06% in both cases. A multifactorial analysis of variance taking into account the differences in hSHBG concentration confirmed that the presence of hSHBG significantly decreased (P = 0.036) the testicular accumulation of radioactive material after admin-

Table 3
Percentage of ingested radioactivity measured in testes and epididymes

Time after ingestion of [2-^{125}I]iodobisphenol A (h)	Testes		Epididymes	
	Absence of hSHBG	Presence of hSHBG	Absence of hSHBG	Presence of hSHBG*
1.5	0.020 ± 0.010	0.020 ± 0.010	0.050 ± 0.014	0.063 ± 0.042*
3	0.017 ± 0.012	0.007 ± 0.006*	0.030 ± 0.000	0.040 ± 0.030*
5	0.010 ± 0.014	0.010 ± 0.000*	0.015 ± 0.021	0.043 ± 0.029*
15	0.280 ± 0.014	0.017 ± 0.015*	0.330 ± 0.000	0.040 ± 0.010*
24	0.250 ± 0.028	0.053 ± 0.040*	0.290 ± 0.350	0.057 ± 0.060*
48	0.370 ± 0.335	0.190 ± 0.130*	0.285 ± 0.370	0.237 ± 0.159*

istration of an oral dose of radiolabeled [2-^{125}I]iodobisphenol A as compared to control mice, untreated with hSHBG.

4. Discussion

Human SHBG binds estradiol with twice as low a binding affinity as testosterone [27]. By this property, SHBG influences the androgen-to-estrogen balance, at least in vitro [28]. In screening the biochemical activities of xenoestrogens, several groups have reported that bisphenol A, as well as some phytoestrogens, can interact specifically with human SHBG but with low binding affinity [16,17,29]. This raises the general question of the effects of hSHBG on metabolism, blood clearance and organ accumulation of low affinity SHBG-ligands. The aim of the present study was to use radioactive iodinated bisphenol A derivative and a mouse model with various concentrations of hSHBG obtained by i.v. tail injection of purified hSHBG with full binding activity.

Because 2-iodoestradiol exhibited a much higher affinity than estradiol, as expected from a previous study by Fernlund and Gershagen [30], it was important to investigate whether iodinated derivatives of bisphenol A had increased hSHBG binding affinity. In fact, [2,2′-^{125}I]iodobisphenol A, a derivative of bisphenol A symmetrically iodinated on both phenolic rings, exhibited a slightly higher affinity for hSHBG than [2-^{125}I]iodobisphenol A. This suggested a limited effect of iodination, when a phenolic ring remains unsubstituted as in [2-^{125}I]iodobisphenol A. Moreover, measured RBAs for bisphenol A, 2-mono-iodobisphenol A, and 2,2′-di-iodobisphenol A, by using a tritiated testosterone tracer, indicated stepwise but only slight increased affinity for hSHBG. Therefore, the small increase in binding affinity, resulting from 2-iodination of bisphenol A, is unlikely to lead to overestimating the hSHBG binding effect on clearance and testicular accumulation of the 2-mono-iodobisphenol A tracer. Since mice do not secrete hepatic SHBG, i.v. tail injection of immunopurified hSHBG that conserved full binding activity [31] was performed to achieve serum hSHBG concentrations that were between 27 and 267 nM - i.e. within the physiological range for normal humans [25]. Although measured in a single experiment, the half-life of hSHBG was found to be close to 20 h in mouse, in agreement with the half-life value of hSHBG that we had previously reported in rabbit [32]. This result indicates that hSHBG concentration decreased by at least 50% during our 24–48 h experimental protocols, minimizing its potential activity as compared to the normal human situation of constant liver production of hSHBG that maintains a relatively stable hSHBG concentration [27].

Because of the small sample size, only total radioactivity could be measured in blood samples. We found that, in mice serum samples with hSHBG concentrations of 12 or 25 nM, treatment with Con A-Sepharose, a lectin that has been used to adsorb serum glycoproteins including hSHBG [25], precipitated 22 or 36% of total radioactivity. Although not extensive, these results suggested that a significant proportion of radioactive [2-^{125}I]iodobisphenol A (and/or the metabolites) may circulate in the blood in a partly hSHBG-bound form. They also suggested that, even for low hSHBG concentrations, hSHBG binding sites were not saturated by mouse-endogenous sex steroid hormones [33], a finding in agreement with the model of physiological plasma steroid-transport developed by Dunn et al. [25] in humans.

The measurement of blood-clearance after a bolus of [2-^{125}I]iodobisphenol A in mice suggests that radioactive material follows a classic pathway of resorption, with appearance in the blood in the 20 min following oral administration, diffusion from the vascular compartment, and β-elimination, found mostly in liver, renal and intestinal compartments. The rapid first-pass elimination by the liver and the evidence for enterohepatic recirculation of radioactive material are in agreement with the toxicokinetic properties recently reported for bisphenol A in DA/Han rats [34].

The presence of circulating levels of hSHBG in mice was found to induce a significant increase in the blood retention time of [2-^{125}I]iodobisphenol A (Fig. 4) whereas a much stronger effect was observed for [2-^{125}I]iodoestradiol, as expected from the higher hSHBG binding affinity for 2-iodoestradiol than for 2-mono-iodobisphenol A. However, the 24 h testicular accumulation of 2-mono-iodobisphenol A, that was 0.25% in mice with no circulating hSHBG, was no longer detectable in the presence of high circulating concentrations of hSHBG. These results suggested that the low hSHBG binding affinity for 2-mono-iodobisphenol A could be overcome by high hSHBG concentrations so as to significantly enhance blood retention time and prevent testicular accumulation. One can speculate that, during prenatal and prepubertal exposure to some environmental xenoestrogens [35], SHBG levels, which are much higher than during adult life [36,37], may have the capacity to limit the biologic effects of xenoestrogens that specifically bind hSHBG. This protective effect of hSHBG was not anticipated from the low hSHBG binding affinity for bisphenol A. It appears to be strong enough to counterbalance a potential ‘shuttle’ mechanism of testicular accumulation of bisphenol A. Indeed, high testicular concentration of testosterone, one of the best natural ligands for hSHBG, could have displaced low-affinity hSHBG-bound molecules, facilitating their testicular accumulation. This mechanism may even be further amplified by the presence of intra-testicular ABP/SHBG [20]. The use of a transgenic mouse model that expresses human hSHBG transgenes [38] and maintains SHBG concentrations in the physiological range for humans would certainly be a powerful tool for assessing the influence of hSHBG on bisphenol A metabolism, cellular disposal, and testicular toxicity. It might also highlight the importance of the first liver by-pass for drugs or xenobiotics interaction with hSHBG during oral exposure.

In conclusion, the present data suggest that the presence of circulating hSHBG in mice facilitates sequestration of

bisphenol A (and/or metabolites) in blood, despite a low binding affinity for hSHBG. The data suggest that SHBG-binding could exert a preventive effect on testicular bisphenol A accumulation. However, potential extrapolation from animal models to humans may be hazardous because of the great variability of endogenous sex steroid hormones [39] and of liver metabolism [40]. Even so, however controversially, it has been claimed that the deleterious effects of exposure to bisphenol A could occur at extremely low doses, at least in some mice strains [41]. Therefore, in a human context, a potential protective effect of hSHBG could become particularly relevant in low bisphenol A exposure conditions - especially when hSHBG levels are very high, as during the prepubertal period.

Acknowledgments

The authors are much indebted to Geoffrey L. Hammond (London, Ontario, Canada) for numerous suggestions about all aspects of this work, Henri Déchaud for his kind help in binding assays, and Christine Baret for excellent technical assistance.

This work was supported by grant N°EN98D08 from the French 'Ministère de l'Environnement et de l'Aménagement du Territoire.'

References

[1] Carlsen A, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during the past 50 years. Br Med J 1992;5:609–13.
[2] Sharpe RM. Declining sperm counts in men: is there an endocrine cause? J Endocrinol 1993;36:357–60.
[3] Forman D, Moeller H. Testicular cancer. Cancer Surv 1994;9/20: 323–41.
[4] Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 1995;3:608–12.
[5] Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrel A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 1996;4(3):298–305.
[6] Reel JR, George JD, Lawton AD, Myers CB, Lamb JC. Bisphenol A: reproduction and fertility assessment in CD-1 mice when administered in feed. NTP/NIEHS Report 1985.
[7] Morrisey RE, George JD, Price CJ, Tyl RW, Marr MC, Kimmel CA. The developmental toxicity of bisphenol A in rats and mice. Fundament Appl Toxicol 1987:571–82.
[8] Vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nag SC, Parmigiani S, Welshons WV. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production and behavior. Toxicol Ind Health 1998;14:239–60.
[9] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol A, an estrogenic substance, is released from polycarbonate flasks during autoclaving. Endocrinology 1993;32:2279–86.
[10] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor. Endocrinology 1998;39:4252–63.
[11] Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 1995;3:113–22.
[12] Villalobos M, Olea N, Brotons JA, Olea-Serrano F, Ruiz de Almodovar JM, Pedraza V. The E-screen assay: a comparison of different MCF7 cell stocks. Environ Health Perspect 1995;3:844–50.
[13] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;1:378–84.
[14] Klotz DM, Hewitt SC, Korach KS, Diaugustine RP. Activation of a uterine insulin-like growth factor I signalling pathway by clinical, and environmental estrogens: requirement of estrogen receptor-α. Endocrinology 2000;41:3430–9.
[15] Steinmetz, R, Brown NG, Allen DL, Bigsby RM, Ben-Jonathan N. The environmental estrogen bisphenol A stimulates prolactin *in vitro* and *in vivo*. Endocrinology 1997;38:1780–6.
[16] Danzo BJ. Environmental xenobiotics may disrupt endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding protein. Environ Health Perspect 1997;5:294–301.
[17] Déchaud H, Ravard C, Claustrat F, Brac de la Perrière A, Pugeat M. Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). Steroids 1999;64:328–34.
[18] Hodgert Jury HH, Zacharewski TR, Hammond GL. Interactions between human plasma sex hormone-binding globulin, and xenobiotic ligands. J Steroid Biochem Mol Biol 2000;75:167–76.
[19] Gunsalus GL, Musto NA, Bardin CW. Immunoassay of androgen binding protein in blood: a new approach for the study of the seminiferous tubule. Science 1978;65–6.
[20] Joseph D. Structure, function and regulation of androgen-binding protein/sex hormone-binding globulin. Vitamin Horm 1994;9:180–97.
[21] Hammond GL. Molecular properties of corticosteroid-binding globulin and the sex-steroid binding proteins. Endocrine Rev 1990;1:65–79.
[22] Rosner W. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocrine Rev 1990;1: 80–91.
[23] Grenot C, de Montard A, Blachere T, Rolland de Ravel M, Mappus E, Cuilleron CY. Characterization of Met-139 as the photolabeled amino acid residue in the steroid-binding site of sex hormone-binding globulin using Δ-6 derivatives of either testosterone or estradiol as unsubstituted photoaffinity labeling reagents. Biochemistry 1992;1: 7609–21.
[24] Scatchard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci 1949;1:660–72.
[25] Dunn JF, Nisula B, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 1981;3:58–68.
[26] Pugeat M, Dunn JF, Nisula BC. Transport of steroid hormones: interaction of 70 drugs with testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 1981;3:69–75.
[27] Rosner W. Plasma steroid-binding proteins. Endocrinol Metab Clin North Am 1991;20:697–720.
[28] Burke CW, Anderson DC. Sex hormone-binding-globulin is an oestrogen amplifier. Nature 1972;240:38–40.
[29] Kloas W, Schrag B, Ehnes C, Segner H. Binding of xenobiotics to hepatic estrogen receptor, and plasma sex steroid-binding protein in the teleost fish, the common carp (Cyprinus carpis). General and Comparative Endocrinol 2000;119:287–99.

[30] Fernlund P, Gershagen S. 2-Iodoestradiol binds with high affinity to human sex hormone-binding globulin SHBG. J Steroid Biochem 1990;36:75–81.
[31] Grenot C, de Montard A, Blachere T, Mappus E, Cuilleron CY. Identification of a photolabeled site of the plasma binding protein for testosterone and estradiol (SBP) using tritiated 17 β-hydroxy-4,6-androstadien-3-one. C R Acad Sci III 1988;307:391–6.
[32] Cousin P, Déchaud H, Grenot C, Lejeune H, Pugeat M. Human variant sex hormone-binding globulin (SHBG) with an additional carbohydrate chain has a reduced clearance rate in rabbit. J Clin Endocrinol Metab 1998;83:235–40.
[33] Jean-Faucher C, Berger M, de Turckheim M, Veyssiere G, Jean C. Permanent changes in the functional development of accessory sex organs and in fertility in male mice after neonatal exposure to cyproterone acetate. J Endocrinol 1985;104:113–20.
[34] Upmeier A, Degen GH, Diel P, Michna H, Bolt HM. Toxicokinetics of bisphenol A in female DA/Han rats after a single i.v. and oral administration. Arch Toxicol 2000;4(8):431–6.
[35] Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM. Comparative effects of neonatal exposure of male rats to potent, and weak (environmental) estrogens on spermatogenesis at puberty, and the relationship to adult testis size, and fertility: evidence for stimulatory effects of low estrogen levels. Endocrinology 2000;141:3898–907.
[36] Apter D, Bolton NJ, Hammond GL, Vihko R. Serum sex hormone-binding globulin during puberty in girls and in different types of adolescent menstrual cycles. Acta Endocrinol 1984;107:413–9.
[37] Lee IR, Lawder LE, Townend DC, Wetherall JD, Hahnel R. Plasma sex hormone-binding globulin concentration and binding capacity in children before and during puberty. Acta Endocrinol 1985;109:276–80.
[38] Jänne M, Deol HK, Power SG, Yee S, Hammond GL. Human sex hormone-binding globulin gene expression in transgenic mice. Mol Endocrinol 1998;12:23–136.
[39] Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Exposure to bisphenol A advances puberty. Science 1999; 1:763–4.
[40] Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PC, Nephew KP, Bigsby RM. Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Perspect 2000;8:243–7.
[41] Kaiser J. Panel cautiously confirms low-dose effects. Science 2000; 90:695–6.