# Conjugation and Deconjugation Reactions within the Fetoplacental Compartment in a Sheep Model: A Key Factor Determining Bisphenol A Fetal Exposure

Tanguy Corbel, Elisabeth Perdu, Véronique Gayrard, Sylvie Puel, Marlène Z. Lacroix, Catherine Viguié, Pierre-Louis Toutain, Daniel Zalko, and Nicole Picard-Hagen

Institut National de Recherche Agronomique, Unité Mixte de Recherche 1331, Toxalim, Research Centre in Food Toxicology, F-31027; and Université de Toulouse, Institut National Polytechnique de Toulouse, Ecole Nationale Vétérinaire de Toulouse, Ecole d'Ingénieur de Purpan, Université Paul Sabatier, F-31076, Toulouse, France

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# ABSTRACT

The widespread human exposure to bisphenol A (BPA), an endocrine disruptor targeting developmental processes, underlines the need to better understand the mechanisms of fetal exposure. Animal studies have shown that at a late stage of pregnancy BPA is efficiently conjugated by the fetoplacental unit, mainly into BPAglucuronide (BPA-G), which remains trapped within the fetoplacental unit. Fetal exposure to BPA-G might in turn contribute to in situ exposure to bioactive BPA, following its deconjugation into parent BPA at the level of fetal sensitive tissues. The objectives of our study were 1) to characterize the BPA glucurono- and sulfoconjugation capabilities of the ovine fetal liver at different developmental stages, 2) to compare hepatic conjugation activities in human and sheep, and 3) to evaluate the extent of BPA

# Introduction

Bisphenol A (BPA) is a xenoestrogen widely used as a monomer in the manufacture of epoxy resins and polycarbonate plastics, which contribute to the almost ubiquitous human exposure to BPA across a life span (Vandenberg et al., 2010). Much of the concern regarding BPA safety has arisen from reported adverse effects of BPA, such as the impairment of reproductive development, as well as effects on energy metabolism and cognitive development even at low doses when exposure occurs during the perinatal period (Vandenberg et al., 2013).

In adult humans, BPA is almost completely eliminated in urine (Volkel et al., 2002) after an extensive hepatic first-pass conjugation into BPA-glucuronide (BPA-G) by UDP-glucuronosyltransferases (UGT) and

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Part of this work was presented as follows: Corbel T, Perdu E, Lacroix MZ, Puel S, Gayrard V, Viguié C, Toutain PL, Zalko D, and Picard-Hagen N (2012) The free/ conjugated bisphenol A ratio in fetoplacental compartment: a key parameter determining bisphenol A prenatal exposure. *Connaissances récentes sur les effets des perturbateurs endocriniens sur l'environnement et la santé;* 2012 Dec 10–11; Paris, France. Ministère de l'Écologie et du Développement Durable et de l'Énergie/ National Endocrine Disrupter Research Programme (PNRPE), Paris, France. dx.doi.org/10.1124/dmd.114.061291. conjugation and deconjugation processes in placenta and fetal gonads. At an early stage of pregnancy, and despite functional sulfoconjugation activity, ovine fetuses expressed low hepatic BPA conjugation capabilities, suggesting that this stage of development represents a critical window in terms of BPA exposure. Conversely, the late ovine fetus expressed an efficient detoxification system that metabolized BPA into BPA-G. Hepatic glucuronidation activities were quantitatively similar in adult sheep and humans. In placenta, BPA conjugation and BPA-G deconjugation activities were relatively balanced, whereas BPA-G hydrolysis was systematically higher than BPA conjugation in gonads. The possible reactivation of BPA-G into BPA could contribute to an increased exposure of fetal sensitive tissues to bioactive BPA in situ.

to a lesser extent into BPA-sulfate (BPA-S) by sulfotranferases (SULT). These reactions are usually considered as mechanisms of detoxification because these metabolites are not estrogenic (Nakagawa and Tayama, 2000; Matthews et al., 2001). In humans, in the fetoplacental compartment, highly variable BPA and BPA-G levels have been reported in cord blood, placenta, and amniotic fluid (Vandenberg et al., 2010; Gerona et al., 2013). However, these biomonitoring data remained limited and were therefore unlikely to reflect fetal exposure throughout pregnancy. Thus, many uncertainties remain regarding the actual fetal internal exposure to BPA in humans during this critical period of pregnancy, which consequently prevents a sound evaluation of human health risks, based on the effects observed in animal studies and on the determination of BPA fetal internal exposure in animal models.

Previous BPA toxicokinetic investigations have shown the preponderance of the glucuroconjugated form of BPA in the plasma of fetal monkeys (Patterson et al., 2013) and sheep (Corbel et al., 2013; Viguie et al., 2013) after maternal exposure to BPA. It has been established from both the pregnant ewe (Corbel et al., 2013) and the isolated perfused human placenta (Corbel et al., 2014) models that placental permeability toward BPA-G is very limited. Such observations suggest that toward the end of gestation, the BPA-G present in fetal blood may originate from fetal phase II metabolism rather than from a maternal supply.

**ABBREVIATIONS:** BPA, bisphenol A; BPA-G, bisphenol A-glucuronide; BPA-S, bisphenol A-sulfate; CL, clearance; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; LOQ, limit of quantification; MS, mass spectrometry; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SULT, sulfotransferase; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

It is noteworthy that the results obtained in near-term fetuses are not necessarily representative of BPA fetal exposure at earlier stages of pregnancy, including during the most critical period of development, which are characterized by a weak expression of drug-metabolizing systems (Domoradzki et al., 2004). Moreover, conjugated metabolites can be regenerated back to the corresponding parent compounds by  $\beta$ -glucuronidases and sulfatases, which are expressed in many tissues (Warren and French, 1965; Sperker et al., 1997). Accordingly, it has been shown that 4.4% of the BPA-G dose passed through the placental membrane is converted back to parent BPA in the rat fetal compartment (Nishikawa et al., 2010). Thus, high levels of BPA metabolites in the fetal unit raise the question of the deleterious consequences of in situ reactivation of BPA metabolites through deconjugation at the level of fetal tissues. Together, these data highlight the urgent need for models relevant to the human situation for investigating of the dynamics of the balance between BPA conjugation and back deconjugation throughout the whole gestation period.

In this context, the present study 1) characterizes in vitro fetal hepatic BPA conjugation activities at different pregnancy stages in sheep, 2) compares in vitro hepatic conjugation activities between adult humans and sheep to ascertain the validity of the sheep model, and 3) evaluates ex vivo the BPA conjugation and back deconjugation in the placenta and fetal gonads, which are target organs susceptible to estrogenic interferences, at different developmental stages.

## Materials and Methods

#### Animals and Tissue Collection

All animal procedures were performed in accordance with accepted standards of humane animal care under agreement number 31-1155545 from the French Ministry of Agriculture and were approved by the Midi-Pyrénées ethics committee (ref: MP/01/15/03/12).

Three nonpregnant and 12 pregnant Lacaune ewes (54–57 days of gestation, corresponding to early-stage fetus, n = 8; and 120–135 days of gestation corresponding to near-term fetus, n = 4) were used. Ewes were sacrificed by electrical stunning immediately followed by exsanguination. Fetuses were rapidly extracted from the uterus (six males and eight females, and three males and three females, for the stages of 54–57 days and 120–135 days of pregnancy, respectively).

Fetal gonads and placental cotyledons were immediately collected and chilled in saline solution at 4°C and were incubated within 1 hour after collection. Ovaries were also collected from the three nonpregnant ewes and from three adult ewes at 120–135 days of pregnancy.

For early-stage fetuses, fetal livers were collected, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}$ C. The livers from six near-term fetuses and from three nonpregnant ewes were immediately perfused for 5 minutes after their collection using a saline solution at 4°C. A fragment of about 10 g of the right lobe was frozen in liquid nitrogen and stored at  $-80^{\circ}$ C.

## Characterization of BPA Conjugation/Deconjugation Activities in Hepatic Subcellular Fractions

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

**Preparation of Hepatic Subcellular Fractions.** Because of the small size of the organs (around 2g; Bazer et al., 2012), the livers from six male and six female early-stage fetuses were pooled as follows: three pools of two males and three pools of two females. Livers from the six near-term fetuses (three males, three females) and from the three nonpregnant ewes were processed individually. Hepatic subcellular fractions were prepared by differential centrifugation to obtain microsomal and cytosolic fractions as previously described elsewhere (Zalko et al., 2006) within 1 month after collection. All fractions were then stored in sodium phosphate buffer (0.1 M), pH 7.4, containing 20% glycerol at  $-80^{\circ}$ C until the assays.

Human microsomal fractions were obtained from pools of 10 female donors and purchased from TEBU (Le Perray-en-Yvelines, France), BD Gentest (Erembodegem, Belgium) and Life Technologies (Saint-Aubin, France). Cytosolic fractions were purchased from TEBU (pools of 10 female donors), Life Technologies and Sigma-Aldrich (pools of 10 mixed gender donors).

In Vitro Incubations. The protein content of the subcellular fractions was determined with bovine serum albumin as a standard (Bradford, 1976). The linearity of the UGT reaction with time was investigated using incubation times of 10, 20, and 30 minutes, with a liver microsomal protein concentration of 1 mg/ml; the linearity of the SULT reaction with time was investigated using incubation times of 10, 20, 30, and 60 minutes, with a liver cytosolic protein concentration of 0.4 mg/ml. All assays were optimized with respect to substrate concentration range. All preincubations and incubations were performed in glass vials containing 0.5 M Tris-Maleate buffer (pH 7.4) and 10 mM MgCl<sub>2</sub> at 37°C under shaking. BPA (purity  $\geq$ 99%) and BPA-G and BPA-S (purity  $\geq$ 95%) stock solutions were dissolved in ethanol and were diluted extemporaneously in the incubation buffer. The final concentration of ethanol in the incubation media was 0.5% for BPA, 0.8% for BPA-G, and 0.6% for BPA-S.

**Glucuronidation Assays.** In vitro glucuronidation of BPA was evaluated by incubating 0.5 mg of hepatic microsomal proteins for 20 minutes with BPA at different concentrations (2.5, 5, 10, 50, 100, and 200  $\mu$ M), in a volume of 0.5 ml buffer containing 1 mM UDP-glucuronic acid (UDPGA). The reaction was stopped by adding 1 ml of ice-cold acetonitrile and the samples were stored at  $-20^{\circ}$ C until assayed. Blank samples without UDPGA or without microsomal proteins were used as controls.

Sulfoconjugation Assays. In vitro sulfoconjugation activity was evaluated using cytosolic fractions. For each assay, 0.2 mg of cytosolic protein was preincubated for 30 minutes with 5 mM ATP and 2 mM Na<sub>2</sub>SO<sub>4</sub> in a volume of 0.5 ml of incubation buffer. The assay conditions for the generation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in vitro by preincubation of cytosolic proteins at 37°C for 30 minutes with inorganic sulfate (2 mM) and ATP (5 mM) in presence of Mg<sup>2+</sup> (10 mM) were adapted from the method optimized by Nemoto et al. (1978), Wong et al. (1991), and Wong and Wong (1996). In a preliminary experiment, we showed that the rate of BPA-S formation was similar in presence of a PAPS generation system or of 240 µM PAPS, indicating that this preincubation would generate a sufficient amount of PAPS to optimize BPA sulfoconjugation. BPA at different concentrations (2.5, 5, 10, 50, 100, 200, 500, and 1000  $\mu$ M) was then added to the incubation medium. The reaction was stopped after 20 minutes of incubation by adding 1 ml of ice-cold acetonitrile and the samples were stored at  $-20^{\circ}$ C until assayed. Blank samples without ATP and Na2SO4 or without cytosolic proteins were used as controls.

**Deconjugation Assays.** Hepatic deconjugation activity was evaluated on ovine microsomal liver fractions from one pool of early-stage fetuses, one nearterm fetus, and one adult ewe. We incubated 0.5 mg of microsomal protein for 20 minutes with BPA-G or BPA-S at three different concentrations (2.5, 50, and 100  $\mu$ M) without cofactors under the same experimental conditions as those of the conjugation assay. The reaction was stopped by adding 1 ml of ice-cold acetonitrile and the samples were stored at  $-20^{\circ}$ C until assayed.

Analytic Procedure. BPA and its metabolites concentrations were determined after extraction with acetonitrile using a validated ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) system, adapted for incubation media, from a method previously described elsewhere (Lacroix et al., 2011). The calibration curves ranged from 4.4 nM to 219  $\mu$ M, 24.8 nM to 248  $\mu$ M, and 32.5 nM to 325  $\mu$ M, for BPA, BPA-G, and BPA-S, respectively. The mean intraday and interday coefficients of variation for three concentrations of BPA, BPA-G, and BPA-S were lower than 15%, and the limits of quantification (LOQ) were validated at 4.4 nM, 24.8 nM, and 32.5 nM, respectively.

**Determination of Michaelis-Menten Parameters.** All data were computed using WinNonlin software (version 5.3; Pharsight Corporation, Mountain View, CA) and the Michaelis-Menten parameters  $V_{\text{max}}$  and  $K_{\text{m}}$  were determined by nonlinear regression analysis using the following model:

$$V = \frac{V_{\max} \times [S]}{K_m + [S]} \tag{1}$$

where  $V_{\text{max}}$  was the maximum reaction velocity expressed in pmol per mg of microsomal or cytosolic protein and per min,  $K_m$  was the Michaelis constant which corresponds to the BPA micromolar concentration at half  $V_{\text{max}}$ , and [S] was the micromolar substrate concentration.

For the incubations of hepatic subcellular pool fractions of early-stage fetuses, several concentrations of BPA-G were below the LOQ. Consequently, the sparse data option of WinNonlin was used for each sex group, allowing a computation of the different standard errors (S.E.) associated with the estimated parameters.

**Hepatic Intrinsic Clearance.** From the Michaelis-Menten parameters determined both for the glucurono- and sulfoconjugation reactions, the hepatic intrinsic clearance (CL<sub>int</sub>) was calculated with the following equation, expressed in ml/min per g of liver:

$$CL_{\rm int} = \frac{V_{\rm max}}{K_m} \times A \tag{2}$$

where *A* was the amount of protein (microsomal or cytosolic for glucuronidation and sulfoconjugation, respectively) per gram of liver. In sheep, the amount of protein per gram of liver was determined by the Bradford method (Bradford, 1976). For human subcellular fractions, the values of the amounts of microsomal protein and cytosolic protein per gram of liver were 45 mg/g (Riley et al., 2005) and 80.7 mg/g (Cubitt et al., 2011), respectively.

**Correction for Nonspecific Protein Binding.** Glucuronidation and sulfoconjugation BPA intrinsic clearances were corrected for nonspecific protein binding in the microsomal or cytosolic incubation medium ( $fu_{inc}$ ) using the following equation:

$$CL_{\text{int},u} = \frac{Cl_{\text{int}}}{fu_{inc}} \tag{3}$$

where  $CL_{int,u}$  was the unbound intrinsic clearance and  $fu_{inc}$  the percentage of BPA unbound from protein in the incubation.

The free fraction of BPA in the microsomal incubation medium ( $fu_{inc}$ ) was estimated with the following equation (Austin et al., 2002):

$$fu_{inc} = \frac{1}{C \times 10^{0.56 \times \log P/D - 1.41} + 1}$$
(4)

where *C* was the microsomal protein concentration in the incubation medium (mg/ml) and  $\log P/D$ , corresponding to  $\log Kow$  for a basic compound, was 3.4 for BPA (Austin et al., 2002; Mazur et al., 2010).

To verify the predicted  $fu_{inc}$  determined by eq. 4 in our experimental conditions,  $fu_{inc}$  was determined experimentally in duplicate by equilibrium dialysis over 20 minutes at 37°C, with a cellulose membrane with a molecular mass cutoff of 5 kDa, as described previously elsewhere (Corbel et al., 2013). The binding of BPA to microsomal proteins was determined at protein concentrations of 1 mg/ml for only one adult sheep and for three BPA concentrations (1, 10, and 100  $\mu$ M).

Because no equation was described to estimate the free fraction of BPA in the cytosolic incubation medium, the binding of BPA to cytosolic proteins was determined for hepatic cytosolic fractions at a protein concentration of 0.4 mg/ml from one pool of early-stage fetuses, one near-term fetus, one adult sheep, and one human pool and for two concentrations of BPA (10 and 200  $\mu$ M).

BPA concentrations in the incubation medium and dialyzed subcellular fraction sample compartments were measured by ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS), as described earlier. The free fraction of BPA ( $fu_{inc}$ ) was determined as the ratio of the BPA concentration measured in the incubation medium compartment to that in the dialyzed compartment containing the microsomal or the cytosolic fractions.

In Vitro–In Vivo Extrapolation for Hepatic Clearance. To understand better the species- and development stage-dependent variation in BPA hepatic pharmacokinetics, the unbound intrinsic clearance ( $CL_{int,u}$ ) was incorporated into the well-stirred model (Ito and Houston, 2004) to calculate the hepatic clearance ( $CL_{H}$ ) for ovine near-term fetuses and for adult sheep and humans, as shown in the following equation derived from Miners et al. (2006):

$$CL_{H} = \frac{Q_{H} \times fu \times (B \times CL_{\text{int},u})}{Q_{H} + fu \times (B \times CL_{\text{int},u})}$$
(5)

where  $Q_{\rm H}$  was the liver blood flow [75, 50, and 23 ml/kg per min in near-term fetuses (Rudolph, 1985), adult ewes (Boxenbaum, 1980), and humans (Arms and Travis, 1988), respectively], *fu* was the free fraction of BPA in blood [11%, 7%, and 5% in near-term fetuses, adult ewes (Corbel et al., 2013) and humans

(Csanady et al., 2002), respectively], and *B* was the ratio between the liver weight and the body weight [12 g/kg in sheep (Boxenbaum, 1980) and 26 g/kg in humans (Arms and Travis, 1988)].

#### **BPA Conjugation/Deconjugation Reactions in Target Tissues**

**Biochemical Synthesis of [<sup>3</sup>H]-BPA-Glucuronide and [<sup>3</sup>H]-BPA-Sulfate.** Tritiated metabolites of BPA, not available commercially, were synthesized from [<sup>3</sup>H]-BPA (radiopurity: >97%, specific activity: 185 GBq/mmol; Moravek Biochemicals, Brea, CA) as previously described by Bursztyka et al. (2008).

[<sup>3</sup>H]-BPA-glucuronide ([<sup>3</sup>H]-BPA-G) was produced by incubating 2 mg of guinea pig liver microsomal protein with [<sup>3</sup>H]-BPA (1133 kBq) fortified with 40 nmol BPA and 2 mM UDPGA in 1 ml 0.5 M Tris buffer (pH 7.4) containing 10 mM MgCl<sub>2</sub> and 0.1% Triton X-100 for 2 hours at 37°C. After the addition of 3 volumes of methanol and a 10-minute centrifugation at 8000g, the supernatant was collected, and [<sup>3</sup>H]-BPA-G synthesis was checked using the radio-high-pressure liquid chromatography (HPLC) system described later. [<sup>3</sup>H]-BPA-G was collected using the same HPLC system coupled to a Gilson model 201/202 fraction collector. [<sup>3</sup>H]-BPA-G was purified using a 0.5 g Chromabond C18 glass cartridge (Macherey Nagel, Hoerdt, France) as described previously elsewhere (Cabaton et al., 2008). The [<sup>3</sup>H]-BPA-G structure, with a specific activity of 28.3 GBq/mmol, was confirmed by mass spectrometry.

[<sup>3</sup>H]-BPA-sulfate ([<sup>3</sup>H]-BPA-S) was produced by incubating 2 mg of guinea pig liver cytosolic protein with 40 nmol [<sup>3</sup>H]-BPA (1133 kBq) and 2 mM PAPS in a final volume of 1 ml 0.5 M Tris buffer (pH 7.4) containing 10 mM MgCl<sub>2</sub> for 2 hours at 37°C. After protein precipitation using 3 volumes of methanol and centrifugation, the supernatant was evaporated. [<sup>3</sup>H]-BPA-S production was checked by radio-HPLC. [<sup>3</sup>H]-BPA-S was collected and purified using a 0.5 g Chromabond C18 glass cartridge (Macherey Nagel) as described elsewhere (Cabaton et al., 2008). The [<sup>3</sup>H]-BPA-S structure, with a specific activity of 28.3 GBq/mmol, was confirmed by mass spectrometry.

Ex Vivo Incubations of [<sup>3</sup>H]-BPA, [<sup>3</sup>H]-BPA-G, and [<sup>3</sup>H]-BPA-S with Target Organs from Adult Sheep and Fetuses. Approximately 5 mg of fetal ovaries or fetal testes at an early stage of pregnancy (54–57 days, n = 3) and approximately 50 mg of fetal testes and fetal ovaries from 120- to 135-day-old fetuses (n = 3), placental cotyledons, and maternal ovaries (n = 3) were incubated in 12-well plastic culture plates containing 100 pmol [<sup>3</sup>H]-BPA (2.83 kBq) in 1 ml Dulbecco's modified Eagle's medium (DMEM), or 100 pmol [<sup>3</sup>H]-BPA-G (2.83 kBq) in 1 ml sodium chloride 0.9%, or in 1 ml sodium acetate buffer 0.2 M, pH 4.5, or 100 pmol [<sup>3</sup>H]-BPA-S (2.83 kBq) in 1 ml sodium chloride 0.9% .

For the three molecules, control incubations were performed in the same conditions with 100 pmol [<sup>3</sup>H]-BPA, [<sup>3</sup>H]-BPA-G, or [<sup>3</sup>H]-BPA-S (2.83 kBq) without tissues. All incubations were maintained for 8 hours at 37°C under 90 rpm shaking and an  $O_2/CO_2$  95%/5% atmosphere. At the end of the incubations, media and tissues were collected separately in glass vials. Tissues were extracted in 1 ml of ethanol. All samples were kept at  $-20^{\circ}$ C until analysis.

**Analytic Procedure.** Radioactivity recovery and BPA and metabolite quantifications were performed with the analytic procedures previously described by Bursztyka et al. (2008). The radioactivity in each incubation medium and tissue was measured from aliquots (10  $\mu$ l) by liquid scintillation counting in a Packard Tricarb 4430 counter with Ultima Gold (Packard Instruments, Meriden, CT) as the scintillation cocktail. Aliquots of each incubation medium were analyzed directly by radio-HPLC. Ethanolic extracts from tissues were evaporated before radio-HPLC analysis.

The HPLC system used to analyze BPA metabolites consisted of a Spectra Physics system (P1000) coupled to Flo-One A500 detector, with Flo-scint II as scintillation cocktail (Packard Instruments) for online radioactivity detection. The column was a Zorbax C18 column ( $250 \times 4.6$  mm,  $5 \mu$ m) (Interchim, Montluçon, France) coupled to a Kromasil C18 guard column ( $18 \times 4.6$  mm,  $5 \mu$ m). The mobile phases consisted of ammonium acetate buffer (20 mM, pH 3.5) and acetonitrile 95:5 v/v and 10:90 v/v in A and B, respectively. The flow rate and temperature were set at 1 ml/min and  $35^{\circ}$ C. The gradient used was 0–4 minutes 100% A; 4–6 minutes of linear gradient from 100% A to A:B 85:15 v/v; 6–16 minutes A:B 85:15 v/v; 16–18 minutes linear gradient from 15% B to 25% B; 18–28 minutes A:B 75:25 v/v; 28–30 minutes linear gradient from 25% B to 30% B; 30–37 minutes A:B 70:30 v/v; 37–39 minutes linear gradient from 30% B to 70% B; 39–45 minutes A:B 30:70 v/v.

BPA and metabolites were identified by comparison of radio-HPLC retention times with those of authentic standards before and after enzymatic hydrolysis. Metabolites were quantified by integrating the area under the detected radioactive peaks.

#### Statistical Analysis

Data were expressed as mean  $\pm$  S.D., unless otherwise specified. Statistical analyses were performed using Systat 12.0 software (SPSS/IBM, Chicago, IL). For the early-stage fetuses (only for sulfoconjugation) and the 120- to 135-day-old fetuses, the formation rate of BPA metabolites by subcellular hepatic fractions was analyzed by a two-way analysis of variance (ANOVA) with substrate concentration, sex, and their interaction as the fixed-effect factors; individuals were nested within the sex group as a random-effect factor. As no gender effect was observed, male and female fetuses were grouped together. The effect of the developmental stage (early-stage versus near-term fetuses only for sulfoconjugation) and the effect of species (human versus ovine) on the liver metabolic parameters ( $V_{max}$ ,  $K_m$ , and intrinsic clearance) were evaluated by Student's *t* test or by Aspin-Welch test in the case of unequal variance. For each analysis, *P* values were corrected by the number of comparisons.

## Results

# Characterization of BPA Conjugation/Deconjugation Activities in Hepatic Microsomes

The mean ( $\pm$  S.D.) amounts of microsomal proteins were 5.15  $\pm$  1.24 and 6.80  $\pm$  1.95 mg/g in early-stage and near-term fetal livers, respectively, and were about 2-fold higher in adult livers (12.65  $\pm$  2.12 mg/g, *t* test *P* < 0.05). The mean ( $\pm$  S.D.) amounts of cytosolic proteins were 10.78  $\pm$  1.49, 12.45  $\pm$  4.37, and 16.28  $\pm$  1.65 mg/g in early-stage, near-term fetal livers and in adult livers, respectively. The average body weight of the near-term fetuses was 2.93  $\pm$  0.86 kg, and their liver weight was 89.2  $\pm$  23.8 g.

**Correction for Nonspecific Protein Binding.** The experimental BPA free fraction ( $fu_{inc}$ ) in the microsomal incubation medium under all conditions was similar to the predicted value (eq. 4) (mean ± S.D.:  $0.26 \pm 0.04$  versus 0.24). The experimental  $fu_{inc}$  in cytosol was close

to 1 (mean  $0.97\pm0.10$ ; range: 0.84–1.07) under all conditions. Thus, the nonspecific protein binding correction in the cytosol was disregarded to calculate CL<sub>int.u</sub>.

**Glucuronidation of BPA.** The formation of BPA-G by hepatic microsomal fractions of human, adult ewes, and early- and late-ovine fetuses, expressed as a function of BPA concentration, followed Michaelis-Menten kinetics (Fig. 1). BPA-G formation showed enzyme saturation for BPA concentrations around 10  $\mu$ M and 50  $\mu$ M, in ovine fetuses at early and late stages of pregnancy, respectively, and around 100  $\mu$ M in adult ewes and humans (Fig. 1). In near-term ovine fetuses, the kinetics of BPA-G formation did not differ according to gender (ANOVA, N.S.).

Table 1 presents the mean  $\pm$  S.D. values for the Michaelis-Menten parameters ( $V_{\text{max}}$  and  $K_{\text{m}}$ ) and the hepatic unbound intrinsic clearance (CL<sub>int,u</sub>) of BPA glucuronidation in humans, in ovine adults and in early- and late-stage ovine fetuses. The mean  $V_{\text{max}}$  of glucuronidation increased throughout the development: it was 512-fold lower in earlystage fetuses and 13-fold lower in near-term fetuses (P = 0.012) than in adult sheep. The mean  $V_{\text{max}}$  of BPA glucuronidation was significantly higher for incubations performed with adult ovine hepatic microsomal fractions, compared with human hepatic microsomes (6086  $\pm$  862 versus 2770  $\pm$  250 pmol/min per mg, P = 0.006).

The Michaelis-Menten constant ( $K_m$ ) of BPA glucuronidation increased with the developmental stage in sheep: it was about 20-fold and 10-fold lower (P = 0.03) for early (4.06  $\mu$ M) and late (9.59  $\mu$ M) stages of pregnancy, respectively, compared with adults (82.0  $\mu$ M). In adults, the  $K_m$  for glucuronidation was not significantly different between ewes and humans (59.6  $\mu$ M, N.S.).

In sheep, the hepatic unbound intrinsic clearance of BPA glucuronidation was about 65-fold and 2-fold lower for early (0.060 ml/min per g of liver) and late (1.73 ml/min per g of liver, P = 0.04) stages of pregnancy, respectively, compared with adults (3.93 ml/min per g of liver). The human unbound intrinsic clearance (9.00 ml·min per g of liver) was in the same range as observed for adult sheep, although it tended to be higher (P = 0.06).



Fig. 1. Kinetics of bisphenol A glucuronidation by liver microsomes. BPA-G formation rate (pmol/mg microsomal protein per min) as a function of BPA concentrations by liver microsomes from ovine fetus at early and late stages of pregnancy and from adult ewes and humans, incubated for 20 minutes. Symbols indicate mean ( $\pm$  S.D.) values of three determinations, and lines indicate fitted values of BPA-G formation.

## Fetoplacental BPA Conjugation/Deconjugation Reactions

#### TABLE 1

## Bisphenol A glucuronidation by hepatic microsomes

Michaelis-Menten apparent kinetic constants ( $V_{max}$  and  $K_m$ ) for BPA glucuronidation by hepatic microsomal fractions of early-stage (54- to 57-day-old) and near-term (120- to 135-day-old) ovine fetuses and of adult sheep and humans after incubation with BPA for 20 minutes. Hepatic glucuronidation unbound intrinsic clearance ( $CL_{int,u}$ ) and extrapolated hepatic clearance ( $CL_H$ ) (mean  $\pm$  S.D.).

| Subjects                 | $V_{ m max}$                       | $K_{ m m}$       | CL <sub>int,u</sub>   | $CL_{H}$        |
|--------------------------|------------------------------------|------------------|-----------------------|-----------------|
|                          | pmol/mg microsomal protein per min | $\mu M$          | ml/min per g of liver | ml/kg per min   |
| Ovine fetuses            |                                    |                  |                       |                 |
| Early stage <sup>a</sup> |                                    |                  |                       |                 |
| Female $(n = 3)^b$       | 15.3 (S.E. 2.26)                   | 4.71 (S.E. 2.95) | 0.075                 | NA              |
| Male $(n = 3)^b$         | 8.50 (S.E. 1.70)                   | 3.41 (S.E. 2.93) | 0.044                 | NA              |
| Near-term                |                                    |                  |                       |                 |
| Female $(n = 3)$         | $519 \pm 258$                      | $10.8 \pm 5.94$  | $1.53 \pm 1.16$       | $4.76 \pm 3.68$ |
| Male $(n = 3)$           | $446 \pm 316$                      | $8.38 \pm 7.05$  | $1.93 \pm 1.21$       | $6.40 \pm 4.28$ |
| Adult                    |                                    |                  |                       |                 |
| Ewes $(n = 3)$           | $6086 \pm 862$                     | $82.0 \pm 18.1$  | $3.93 \pm 0.85$       | $3.14 \pm 0.43$ |
| Humans $(n = 3)$         | $2770 \pm 250$                     | $59.6 \pm 15.6$  | $9.00 \pm 2.47$       | $7.67 \pm 1.35$ |
|                          |                                    |                  |                       |                 |

NA, not applicable.

<sup>a</sup> At 54-57 days of pregnancy, several concentrations of BPA-G were below the LOQ (25 nM), so the data were computed together for

each sex group to estimate Michaelis-Menten parameters and the associated standard errors (S.E.).

Three pools of two fetuses.

**Sulfoconjugation of BPA.** The formation of BPA-S by hepatic cytosolic fractions of humans adult ewes and early- and late-ovine fetuses, expressed as a function of BPA concentration, followed Michaelis-Menten kinetics (Fig. 2). BPA-S formation showed enzyme saturation for a BPA concentration around 50  $\mu$ M for ovine fetuses, regardless of the stage of pregnancy, and for 100 and 500  $\mu$ M, for humans and adult ewes, respectively (the latter concentration is not shown in Fig. 2). In ovine, hepatic sulfoconjugation plots clearly suggest similar sulfoconjugation activities, whatever the stage of pregnancy. By contrast, the sulfoconjugation rate calculated for the 3 human cytosolic fraction pools showed considerable variation, and was lower than observed for adult sheep. For the early- and late-stage ovine fetuses, the kinetics of BPA-S formation did not differ according to the sex (ANOVA, N.S.).

Table 2 gives the mean ( $\pm$  S.D.) values of Michaelis-Menten parameters ( $V_{\text{max}}$  and  $K_{\text{m}}$ ) for BPA-S formation and for the hepatic

unbound intrinsic clearance of BPA sulfoconjugation. The overall mean  $V_{\text{max}}$  of hepatic sulfoconjugation for early-stage and late-stage fetuses, respectively, was similar (78.3 ± 1.32 versus 104 ± 16.2 pmol/min per mg of cytosolic proteins, N.S.) but was lower than that calculated for ewes (655 ± 309 pmol/min per mg of cytosolic proteins). Compared with adult sheep, the mean human sulfoconjugation  $V_{\text{max}}$  (7.03 ± 4.37 pmol/min per mg of cytosolic proteins) was very low.

The overall mean  $K_{\rm m}$  for sulfoconjugation was similar between earlyand late-stage fetuses (15.67 ± 8.11 versus 7.51 ± 1.48  $\mu$ M, N.S.) and was 37- and 77-fold lower than in the adult sheep (575 ± 267  $\mu$ M), respectively. In the adult, the  $K_m$  for sulfoconjugation was 14-fold lower when using human cytosols (40.2 ± 6.16  $\mu$ M) than when using adult sheep cytosols.

In sheep, the hepatic unbound intrinsic clearance of BPA sulfoconjugation was 3-fold lower for early-stage fetuses than it was for near-term



Fig. 2. Kinetics of bisphenol A sulfoconjugation by liver cytosols. BPA-S formation rate (pmol/mg cytosolic protein per min) as a function of BPA concentrations using liver cytosol from ovine fetuses at early and late stages of pregnancy and from adult ewes and humans, incubated for 20 minutes. Symbols indicate mean ( $\pm$  S.D.) values of three determinations, and lines indicate fitted values of BPA-S formation.

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#### TABLE 2

#### Bisphenol A sulfoconjugation by hepatic cytosols

Michaelis-Menten apparent kinetic constants ( $V_{max}$  and  $K_m$ ) for BPA sulfoconjugation by hepatic cytosolic fractions of 54- to 57-dayold (early-stage) and near-term ovine fetuses and of adult sheep and humans. Hepatic sulfoconjugation unbound intrinsic clearance ( $CL_{int,u}$ ) and extrapolated hepatic clearance ( $CL_H$ ) (mean values  $\pm$  S.D.)

| Subjects           | $V_{ m max}$                    | K <sub>m</sub>  | CL <sub>int,u</sub>   | $CL_{H}$          |
|--------------------|---------------------------------|-----------------|-----------------------|-------------------|
|                    | pmol/mg cytolic protein per min | $\mu M$         | ml/min per g of liver | ml/kg per min     |
| Ovine fetuses      |                                 |                 |                       |                   |
| Early stage        |                                 |                 |                       |                   |
| Female $(n = 3)^a$ | $79.2 \pm 5.74$                 | $12.3 \pm 0.84$ | $0.279 \pm 0.020$     | NA                |
| Male $(n = 3)^a$   | $77.3 \pm 18.3$                 | $19.0 \pm 11.4$ | $0.203 \pm 0.076$     | NA                |
| Near term          |                                 |                 |                       |                   |
| Female $(n = 3)$   | $116 \pm 27$                    | $7.96 \pm 0.44$ | $0.660 \pm 0.370$     | $2.18 \pm 1.36$   |
| Male $(n = 3)$     | $93.0 \pm 24.0$                 | $7.05 \pm 2.16$ | $0.716 \pm 0.070$     | $2.50 \pm 0.64$   |
| Adults             |                                 |                 |                       |                   |
| Ewes $(n = 3)$     | $655 \pm 309$                   | $575 \pm 267$   | $0.081 \pm 0.034$     | $0.070 \pm 0.034$ |
| Humans $(n = 3)$   | $7.03 \pm 4.37$                 | $40.2 \pm 6.16$ | $0.015 \pm 0.012$     | $0.020\pm0.015$   |
|                    |                                 |                 |                       |                   |

NA, not applicable.

<sup>a</sup> Three pools of two fetuses

fetuses (0.241  $\pm$  0.065 versus 0.688  $\pm$  0.240 ml/min per g of liver, P = 0.015). The adult ewes hepatic unbound intrinsic clearance of BPA sulfoconjugation was 8-fold lower than that of fetuses at a late stage of pregnancy (P = 0.007) (Table 2). The hepatic unbound intrinsic clearance of sulfoconjugation was in the same range in humans and in ewes (0.015  $\pm$  0.012 versus 0.081  $\pm$  0.034 ml/min per g of liver, N.S.).

In sheep, the ratio between the unbound intrinsic clearance of BPA glucuronidation and BPA sulfoconjugation increased with the stage of pregnancy from 0.24 (early stage) to 2.5 (late stage), and reached 48 in adult ewes. In adult humans, BPA hepatic glucuronidation was highly predominant, with a ratio of about 600.

Prediction of In Vivo Hepatic Clearance from the In Vitro Data on BPA-G and BPA-S Formation by Hepatic Subcellular Fractions. The glucuronidation hepatic clearance  $CL_H$  (Table 1) was of the same order of magnitude in ovine near-term fetuses and in adult sheep (5.58 ± 3.68 versus 3.14 ± 0.43 ml/kg per min, N.S.). However, it was 2-fold higher in humans compared with adult sheep (7.67 ± 1.35 versus 3.14 ± 0.43 ml/kg per min; P = 0.01).

The sulfoconjugation hepatic clearance (Table 2) was very low in adult sheep and humans (0.070  $\pm$  0.034 and 0.020  $\pm$  0.015 ml/kg per min) and accounted for only 2.2% and 0.26% of the overall conjugation hepatic clearance, respectively. The near-term fetal sulfoconjugation hepatic clearance was 33-fold higher than that in adult sheep (2.34  $\pm$  0.97 ml/kg per min; P = 0.001) and was as high as 30% of the conjugation hepatic clearance.

**Deconjugation of BPA Metabolites.** The deconjugation assays (glucuronidase and sulfatase activities) performed with ovine hepatic microsomal fractions, under the same conditions of incubation as the conjugation assays but without cofactors, showed a very low percentage of hydrolysis of BPA-G and BPA-S into BPA: below 1.5% and 2.5% for the sulfatase and the glucuronidase activities, respectively, whatever the concentration of the substrate and the stage of development.

Because glucuronidation of BPA and hydrolysis of BPA-G were measured under the same incubation conditions, resulting in the maximal glucuronidation and hydrolysis rates, the fraction of BPA-G formed in presence of 100  $\mu$ M of BPA (with UDPGA) and the fraction of BPA-G (100  $\mu$ M) hydrolyzed were compared. In early-stage fetuses, the percentage of BPA-G hydrolyzed (0.72%) was very low, even if it was 3-fold higher than the rate of BPA-G formed (0.24%). In ovine near-term fetuses and in adult sheep, the rate of hydrolysis of BPA-G was low, even negligible, compared with the percentage of glucuronidation (2.38% versus 12.7% in near-term fetuses and 1.15% versus 72.8% in adult sheep).

## **BPA Conjugation/Deconjugation Reactions in Target Tissues**

Overall radioactivity recovery for incubation media and tissues (fetal gonads, adult ovaries, and placenta), after 8 hours of incubation with 100 pmol [<sup>3</sup>H]-BPA, [<sup>3</sup>H]-BPA-G, or [<sup>3</sup>H]-BPA-S (2.83 kBq) was 105  $\pm$  15%. The fraction of the radioactivity recovered from incubation media was 65%  $\pm$  18% for [<sup>3</sup>H]-BPA (DMEM), 57%  $\pm$  23% for [<sup>3</sup>H]-BPA-G (sodium acetate buffer), 80%  $\pm$  15% for [<sup>3</sup>H]-BPA-G (saline solution) and 74%  $\pm$  16% for [<sup>3</sup>H]-BPA-S (saline solution). The respective percentages of radioactivity recovered for the same assays, from tissue extracts, were 57%  $\pm$  12%, 45%  $\pm$  21%, 20%  $\pm$  13%, and 21%  $\pm$  8%.

Representative radio-HPLC profiles for these assays are displayed in Fig. 3 for 100 pmol [<sup>3</sup>H]-BPA incubated in 1 ml of DMEM, and 100 pmol [<sup>3</sup>H]-BPA-G incubated in saline solution without tissue (control) or incubated with placenta obtained at 54–57 days of pregnancy. Blank incubations of radiolabeled substrates without tissue did not reveal any degradation of BPA or of BPA-G. Under our HPLC conditions, the retention times of [<sup>3</sup>H]-BPA, [<sup>3</sup>H]-BPA-G, and [<sup>3</sup>H]-BPA-S were 43.8, 29.0, and 42.5 minutes, respectively. After incubation of BPA with placenta, the only metabolite produced was BPA-S. Incubation of BPA-G with placenta tissue in saline solution showed a hydrolysis of BPA-G into BPA. Integrating the area under the radioactive peaks permitted quantification of BPA and metabolites in pmoles and comparison of BPA conjugation with BPA-G hydrolysis.

Figure 4 shows for each tissue (fetal and adult gonads, placenta) the amount of  $[^{3}H]$ -BPA that was conjugated and the amount of  $[^{3}H]$ -BPA-G that was hydrolyzed after 8 hours of incubation of 100 pmol of radiolabeled BPA (in DMEM) or 100 pmol of radiolabeled BPA-G (in saline solution). In placenta (Fig. 4A), at early as well as at late stages of pregnancy, BPA conjugation (range: 4.89–29.64 pmol/8 h and 6.67–47.7 pmol/8 h, respectively) was highly variable and was mainly associated with the production of BPA-S.

In incubations performed with adult ovaries, the BPA conjugation rates (median and range) were 21.8 pmol/8 h (11.6–35.3) for ovaries obtained from pregnant ewes and 18.2 pmol/8 h (1.08–24.9) for ovaries of nonpregnant ewes (Fig. 4B).

In fetal gonads, the conjugation of BPA was either very low or undetectable, whether obtained from early- or late-stage pregnancy fetuses, and ranged from 0 to 2.29 pmol/8 h (median: 0 pmol/8 h) in fetal ovaries (Fig. 4C) and from 0 to 8.48 pmol/8 h (median: 4.15 pmol/8 h) in fetal testes (Fig. 4D).

In optimal conditions for glucuronidase activity (incubation in sodium acetate buffer, pH 4.5), the overall mean hydrolysis percentage



Fig. 3. Representative radiochromatograms showing BPA conjugation and BPA-G deconjugation by placenta tissue. Representative radiochromatographic profiles obtained after incubation of 100 pmol [<sup>3</sup>H]-BPA for 8 hours in DMEM medium without tissue (A) or with early-stage pregnancy placenta (B) and from incubation of 100 pmol [<sup>3</sup>H]-BPA-G for 8 hours in saline solution without tissue (C) or with early-stage pregnancy placenta (D).

of radiolabeled BPA-G was high,  $91\% \pm 15\%$ , whatever the tissue and the stage of development, indicating the presence and functionality of this enzyme in our ex vivo conditions.

In saline solution, for fetal gonads, BPA-G hydrolysis rates were relatively high both for early- and late-stage pregnancy [median (range): 6.99 pmol/8 h (4.13–15.9) for fetal ovaries (Fig. 4C) and 27.5 pmol/8 h (16.8–59.3) for fetal testes (Fig. 4D)]. For adult ovaries (Fig. 4B), BPA-G hydrolysis rates were similar between pregnant and nonpregnant ewes [median (range): 49.5 pmol/8 h (49.1–58.4) versus 43.1 pmol/8 h (26.0–54.8)]. In our experimental conditions, for fetal gonads (at all stages of pregnancy) and adult ovaries, the BPA-G hydrolysis rate was systematically higher than the BPA conjugation rate. For placenta, the BPA-G hydrolysis rate was lower at an early stage than at a late stage of pregnancy [median (range): 3.42 pmol/8 h (3.13–10.1) and 24.5 pmol/8 h (18.3–28.6), respectively] (Fig. 4A).

[<sup>3</sup>H]-BPA-S hydrolysis rates (data not shown) were relatively low (median: 1.44 pmol/8 h) and ranged from 0 to 5.20 pmol/8 h for the fetal gonads (all stages of pregnancy) and adult ovaries. For placenta, BPA-S hydrolysis rates ranged from 0 to 7.58 pmol/8 h and from 3.92 to 14.0 pmol/8 h at early and late stages of pregnancy, respectively, and were highly variable, precluding any comparison between conjugation and BPA-S deconjugation activities throughout gestation.

## Discussion

Pregnancy is a critical window of exposure during which developmental processes are extremely sensitive to estrogenic interferences. BPA is a xenoestrogen of major concern for fetal development. The degree of human fetal exposure to BPA and its metabolites is still unclear. Addressing the metabolism of the fetoplacental unit in animal models is a key issue for the understanding of fetal exposure to bioactive (parent) BPA. In the present study, we used human and ovine subcellular liver fractions as well as sheep target tissues to evaluate the ontogeny of metabolic activities involved in BPA conjugation/deconjugation reactions. Hepatic BPA Conjugation/Deconjugation Activities. Our study showed that the ovine fetus at the first third of pregnancy, expressed limited activities of BPA glucuronidation, with an intrinsic clearance about 30-fold lower than in the last third of pregnancy. By contrast, the capability of the liver to glucurono-conjugate BPA in near-term lambs was nearly as high as in adults. These results are consistent with the development of the expression of isoforms of UGT in the ovine near-term fetus and newborn (Dvorchik et al., 1986; Pretheeban et al., 2011). The two parameters  $V_{max}$  and  $K_m$  increased throughout pregnancy and in adulthood, indicating both an increase in UGT enzyme expression and a decrease in their affinity, depending on the UGT isoforms. However, as the in vitro BPA glucuronidation was evaluated under native incubation conditions (without activation), we cannot rule out the hypothesis that the UDPGA cofactor transport could be a rate-limiting step for BPA glucuronidation in ovine fetuses.

In human liver microsomes, BPA is mainly glucuronidated by the UGT2B15 and 2B7 isoforms (Hanioka et al., 2008; Mazur et al., 2010). The expression of these two isoforms is detectable in human fetal livers during the second trimester of pregnancy, and has been stated to account for 18% (Divakaran et al., 2014) or 10%–20% of the values calculated in adults.

We showed that for BPA the glucuronidation unbound intrinsic clearance of hepatic microsomal fractions in humans was close to that calculated for sheep. This suggests that sheep, unlike immature rats (Matsumoto et al., 2002), represent a valuable model to evaluate metabolic processes toward BPA.

The estimated value of hepatic glucuronidation clearance for adult humans (7.7 ml/kg per min) was consistent with the values determined in in vitro studies using liver microsomal fractions [13.7 ml/kg per min (Elsby et al., 2001); 15.5 ml/kg per min (Mazur et al., 2010)] or cryopreserved hepatocytes [4.4 ml/kg per min (Kurebayashi et al., 2010); 6 ml/kg per min (Kuester and Sipes, 2007)].

We found no gender-related differences for the  $V_{\text{max}}$  or  $K_{\text{m}}$  values, nor for the unbound intrinsic glucuronidation clearance of BPA in



Fig. 4. BPA conjugation/deconjugation reactions in placenta and in gonads. Conjugation rate of  $[^{3}H]$ -BPA and hydrolysis rate of  $[^{3}H]$ -BPA-G in fetal and adult ovine tissues after incubation for 8 hours in DMEM and saline solution, respectively. BPA conjugation and BPA-G hydrolysis activities are represented individually for three fetuses at an early stage of pregnancy (solid symbols) and three fetuses at a late stage of pregnancy (open symbols): (A) in placenta, (B) in fetal ovaries, (C) in fetal testes, and (D) in adult ovaries of three pregnant (solid symbols) and three nonpregnant ewes (open symbols).

ovine fetuses. This suggests that UGT's activities toward BPA are similar in male and female fetal livers at the examined stages of pregnancy. This result is in agreement with data obtained with human liver microsomes (Elsby et al., 2001; Mazur et al., 2010) and hepatocytes (Kuester and Sipes, 2007), as well as with the BPA pharmacokinetic data previously reported for sheep (Corbel et al., 2013). By contrast, a gender difference in the hepatic glucuronidation of BPA was observed in vitro in adult rats (Mazur et al., 2010).

We also assessed the contribution of the hepatic sulfoconjugation pathway using liver cytosols. The ability of ovine fetal livers to conjugate BPA through sulfoconjugation was found to be high, even at an early stage of pregnancy. In fact, it was about 3- to 8-fold higher in fetuses than in adults. This precocious expression of phenol liver sulfoconjugation activities has already been reported in midgestation human fetuses, with levels slightly below or equal to the activities calculated in adults (Pacifici, 2005; Stanley et al., 2005; Hines, 2008).

The respective contribution of these two conjugative pathways to the hepatic clearance of BPA was then evaluated. In early-stage fetuses, the unbound intrinsic sulfoconjugation clearance of BPA was 4-fold higher than that of glucuronidation. These data are in accordance with the exposure to BPA-S observed in human umbilical cord blood at midgestation (Gerona et al., 2013). Conversely, BPA's sulfoconjugation hepatic clearance was very low in adult sheep and humans, accounting for only 2.2% and 0.26% of the total conjugation hepatic clearance. This result is consistent with the low fraction of BPA-S (1.4% of total BPA) observed at a steady state in ewes after BPA intravenous infusion (Corbel et al., 2013). In adult human hepatocytes incubated with BPA, it was previously reported that the BPA-S fraction was rather high (9% of the total metabolites) but remained low compared with BPA-G (Kurebayashi et al., 2010). In the near-term ovine fetus, the contribution of hepatic BPA sulfoconjugation was more pronounced in vitro (30%) than previously reported in vivo, at a steady state in the fetal lamb after intravenous infusion of BPA (4%; Corbel et al., 2013). This difference could be due to the limitation of in vitro assays (Miners et al., 2006).

Inasmuch as conjugation plays an important role in the detoxification of BPA, it is necessary to examine the possible contribution of the liver to the deconjugation of BPA metabolites, which could in turn modulate the elimination of BPA. Our results indicate that  $\beta$ -glucuronidase activity had only a minor influence on the liver BPA glucuronidation activity of ovine near-term fetuses and adult sheep. By contrast, in early-stage fetuses, as the glucuronidation activity was very low, the balance between glucuronidation and deconjugation was in favor of hydrolysis, reinforcing the limited capacity of detoxification by BPA glucuronidation pathway in the fetus at an early-stage of pregnancy.

**BPA Conjugation/Deconjugation Reactions in Target Tissues.** The placenta is considered to play a protective role during gestation through the metabolism and clearance of xenobiotics. In our study, the placenta showed wide interindividual variability regarding BPA conjugation and hydrolysis activities. These data are in good accordance with the highly variable ratio (from 0.32 to 6.6) of conjugated over unconjugated BPA measured in human placenta (Zhang et al., 2011). Similar high interindividual variations in UGT and  $\beta$ -glucuronidase expression and activities have previously been reported for human midterm and late-term placenta (Collier et al., 2002a; Collier et al., 2002b). This variability remained unexplained, but was partly attributed to a variability in the fraction of trophoblast contained in the placental samples. Indeed, UGT enzymes are localized in the trophoblast layer.

Under our conditions, BPA-S was the main metabolite produced by ovine placental tissues, and the sulfoconjugation activity was already observed at an early stage of pregnancy. This result is consistent with the fact that the SULT1A family is expressed in human placenta (Stanley et al., 2001), and this isoform is known to provide a relevant contribution to the sulfoconjugation of BPA (Nishiyama et al., 2002). Furthermore, the BPA-G and BPA-S hydrolysis activities were low at an early stage of pregnancy, when hepatic sulfoconjugation was predominant. These results suggest that the deconjugation of BPA metabolites does not occur to a significant extent and that the placenta may play a metabolic detoxification role with regard to BPA in the early stages of pregnancy, when the fetus has limited metabolic capacities. However, at a late stage of pregnancy, BPA-G and BPA-S hydrolysis activities were demonstrated to be active in the placenta, suggesting that the placenta would not only be involved in the detoxification of BPA but may also contribute to the reactivation of BPA metabolites.

The amount of BPA-conjugates hydrolysis in relation to their formation was also investigated ex vivo using ovine gonads, which are potential targets for BPA's endocrine effects. The conjugation/deconjugation balance was clearly in favor of BPA-G deconjugation in ovine fetal gonads (ovaries and testes) with an activity of BPA-G hydrolysis 10-fold higher than the activity of BPA conjugation. As the total BPA levels in adult rodent gonads exceeded those in plasma (Yoo et al., 2000; Zalko et al., 2003; Kim et al., 2004), the capacity of gonads to hydrolyze BPA-G could lead to a reactivation of BPA directly at the level of target tissues. The close proximity of both the enzymes responsible for the formation (UGT) as well as the hydrolysis (*β*-glucuronidase) of BPA-G could result in a cycle of glucuronidation and deglucuronidation. Such futile cycling would greatly influence the apparent glucuronidation rate of BPA and could thereby modify BPA disposition at the level of tissues, leading to prolonged effects of BPA in all tissues in which such a cycle would be present, including the gonads. This process may explain the sustained levels of unconjugated BPA in rat testis 8 hours after an oral administration of BPA, even though plasma concentrations had already decreased (Miyakoda et al., 2000).

In conclusion, our data suggest that the dynamics of the balance between the conjugation of BPA and the deconjugation of BPA-G throughout development in hepatic, placental, and gonadal tissues could play a role in determining the level of exposure of target tissues to BPA, providing key information for predicting human fetal BPA exposure. At an early stage of pregnancy, despite a significant sulfoconjugation activity, ovine fetuses were shown to express poor detoxification systems toward BPA, supporting previous assumptions that early development is a critical window of exposure for this endocrine disruptor. By contrast, near-term ovine fetuses expressed detoxification systems able to efficiently metabolize BPA into BPA-G, and, to a lesser extent, into BPA-S. However, we previously demonstrated that both metabolites remain trapped in the fetoplacental compartment, leading to a high fetal exposure to the BPA conjugates. Furthermore, our data show that the  $\beta$ -glucuronidase activity in the fetal gonads could lead to a reactivation of BPA-G to BPA, thereby modifying locally the exposure to active BPA. The critical importance of this conjugation-deconjugation cycle must be further investigated, to determine whether the fetal overexposure to BPA conjugates, assumed to be inactive in terms of estrogenicity, has to be taken into account in risk assessment for human health related to fetal BPA exposure.

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# Authorship Contributions

Participated in research design: Corbel, Perdu, Gayrard, Lacroix, Viguié, Toutain, Zalko, Picard-Hagen.

Conducted experiments: Corbel, Perdu, Gayrard, Picard-Hagen, Puel. Contributed new reagents or analytic tools: Perdu, Puel, Lacroix. Performed data analysis: Corbel, Perdu, Gayrard, Toutain, Picard-Hagen.

Wrote or contributed to the writing of the manuscript: Corbel, Perdu, Gayrard, Lacroix, Viguié, Toutain, Zalko, Picard-Hagen.

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Address correspondence to: Nicole Picard-Hagen, Ecole Nationale Vétérinaire de Toulouse, 23, Chemin des Capelles, 31076 Toulouse CEDEX 3, France. E-mail: n.hagen-picard@envt.fr