Inhibition of Iodothyronine 5'-Deiodinase by Iopanoic Acid Does Not Block Nuclear T₃ Accumulation During Rat Fetal Development

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ABSTRACT. We studied the effect of iopanoic acid (IOP), an iodinated contrast medium, on iodothyronine 5'-deiodinase (5'D) and nuclear T₃ content (nT₃) in fetal tissues. In 18- and 20 day-old fetuses from control dams, nT₃ was higher in interscapular brown adipose tissue (IBAT, 69 ± 5 and 281 ± 8 fmol/mg of DNA) than in brain (16 ± 2 and 42 ± 3 fmol/mg of DNA) or liver (5.6 ± 1 and 27 ± 2 fmol/mg of DNA). IOP administration (10 mg, twice daily) to pregnant rats on days 18 and 19 postconception significantly blocked 5'D activity in fetal IBAT and brain at day 20. Liver 5'D was not affected. The rise in nT₃ was not modified by IOP treatment in IBAT, but it was enhanced in brain and liver of IOP-treated fetuses on day 20. In contrast, in adult rats, IOP treatment reduced IBAT nT₃. Prolongation of IOP treatment until day 21 decreased fetal body weight on day 22 and inhibited IBAT 5'D. No change was produced in mitochondrial oxidative capacity, the subunit II of cytochrome oxidase, or uncoupling protein mRNA expression in IBAT from IOP-treated fetuses. Thus, the finding that IOP does not decrease the nT₃ of fetal IBAT explains the lack of effect of IOP on uncoupling protein expression in fetuses, in contrast with the known decrease in adults. Present results also show that IOP increases nT₃ in brain and liver, indicating a general incapacity of IOP to decrease nT₃ in fetal tissues. It is concluded that the effects of IOP during fetal life differ from those in adults. (*Pediatr Res* 35: 91-95, 1994)

Abbreviations

IBAT, interscapular brown adipose tissue
T₃, 3,5,3'-triiodothyronine
T₄, thyroxine
rT₃, 3,3',5'-triiodothyronine
IOP, iopanoic acid
5'D-I, iodothyronine 5'-deiodinase type I
5'D-II, iodothyronine 5'-deiodinase type II
UCP, uncoupling protein
COII, subunit II of cytochrome oxidase
PTU, propylthiouracil
BAT, brown adipose tissue
PMSF, phenylmethanesulfonyl fluoride

A large proportion of the T₃ produced in humans and in rats is generated by 5'-deiodination (5'D) of T₄ in peripheral tissues (1). Two types of enzymatic system catalyzing 5'D have been described. Type I and type II 5'-deiodinases (5'D-I and 5'D-II) differ in catalytic residues, reaction kinetics, substrate and inhibitor specificities, tissue distribution and ontogenic profiles (2-9). 5'D-I is most abundant in liver and kidney, but is detectable in other tissues (4, 5). 5'D-II is present mainly in the brain, pituitary and BAT (5, 6). Interestingly, tissues expressing 5'D-I and 5'D-II differ in their dependence on T₃ or T₄ in plasma for intracellular T₃. IOP, a cholecystographic contrast agent, is an inhibitor of 5'D (10), and has been used to demonstrate the importance of local T₄-to-T₃ conversion in tissues expressing 5'D-II, such as pituitary (11), brain (12) and BAT (13).

BAT is the main site for nonshivering adaptative thermogenesis, and it has a high mitochondrial oxidative capacity and a unique mitochondrial UCP that causes dissipation of the energy of metabolic oxidation as heat (14). BAT must be fully differentiated at birth, as its thermogenic activity is especially important during the neonatal period (15). The onset of UCP gene transcription, which may be considered as the key point of brown fat differentiation *in vivo*, begins between days 18 and 19 of fetal life in the rat, and is followed by an abrupt increase in UCP mRNA levels (16, 17). BAT 5'D-II shows peak values on day 20 of fetal life (18), in parallel to the maturation of nuclear T₃ receptor expression and T₃ receptor occupancy, for which maximum values are also reached in IBAT at day 20 (17). In adult brown fat, UCP expression is highly dependent on local T₃ generation by 5'D-II because its blockade by IOP impairs UCP expression in response to cold stress (19). However, IOP administration to pregnant rats does not affect the rise in UCP expression in fetal IBAT (16). These discrepancies prompted us to reevaluate the effects of IOP administration to pregnant mothers on the thyroidal status of fetal tissues. In the present report, we determine the effect of IOP treatment from day 18 of gestation (onset of active thyroid function in the fetus) on the rise of 5'D activity and nuclear T₃ content in IBAT, brain, and liver between days 18 and 20 of fetal life. The effects of IOP treatment on the functionality of IBAT from fetuses at term have also been studied.

MATERIALS AND METHODS

Animals. Wistar rats from our breeding colony initially weighing 180-200 g were used. They were fed with a stock diet (A03 type, Panlab, Spain) and kept in a controlled environment (12-h light/dark cycles, 21°C, 85% humidity). They were maintained and treated in accordance with international recommendations for the care and use of laboratory animals. Female rats were mated and the day of pregnancy was determined by the presence of spermatozoa in vaginal smears. The administration of IOP

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was started at day 18 of gestation. Caesarean sections were performed at 18 (only untreated animals), 20 and 22 days of gestation. Fetuses were killed by decapitation, and IBAT, brain, and liver were removed. Tissue samples were immediately frozen in liquid nitrogen with the exception of brown fat from several 22-day-old fetuses, which was directly homogenized in 0.25 M sucrose for mitochondria isolation (20).

IOP treatment. IOP (Telepaque) was supplied by Sterwin Española. IOP solution (250 mg/12.5 mL) was prepared daily. IOP was dissolved in 0.05 N NaOH and pH was lowered to 7.5–7.8 by addition of 0.2 N HCl. Pregnant rats were injected subcutaneously with 10 mg of IOP every 12 h, from day 18 of gestation to 12 h before they were killed on the morning of day 20 or 22 of gestation. Control animals received the vehicle solution with identical timing. IOP effectiveness in decreasing IBAT nuclear T_3 was assessed by IOP administration to adult male rats (220–250 g body weight) following the same dose and time schedule as in pregnant dams during two days.

Iodothyronine 5'-deiodinase assays. Outer ring 5'-deiodinating activity was assayed by quantifying the $^{125}I^-$ liberated from rT_3 as described previously (9). Briefly, liver 5'-D-I was assayed at a final concentration of 0.5 μM rT_3 and 1 mM DTT (Calbiochem), whereas IBAT 5'-D-II and brain 5'-D activities were determined in the presence of 2 nM rT_3 and 20 mM DTT. Brain 5'-D-II was measured by the addition of 1 mM PTU (12). Incubations were performed at 37°C for 5 min (liver) or 60 min (brown adipose tissue and brain). Separation of iodine from iodothyronines was carried out by ion-exchange chromatography on Dowex 50W-X2 column (Bio-Rad) equilibrated with 10% acetic acid. The enzyme levels in liver are expressed as pmol $I^- \cdot h^{-1} \cdot mg$ protein $^{-1}$ whereas in BAT and brain they are expressed as fmol $I^- \cdot h^{-1} \cdot mg$ protein $^{-1}$.

Determination of nuclear T_3 content. Nuclei were isolated as described previously (17). Briefly, frozen tissues were homogenized in 10 vol/g of tissue buffer A (0.32 M sucrose, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, and 10 mM Tris, pH 7.5). The homogenates were filtered through four layers of nylon mesh and centrifuged at $4000 \times g$ for 10 min. The crude nuclear pellets were resuspended in 0.5 mL of buffer A, mixed with 4.5 mL of buffer B (2 M sucrose, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 10 mM Tris, pH 7.5), and centrifuged at $100,000 \times g$ for 60 min. The nuclear pellets were washed and resuspended in buffer C (0.32 M sucrose, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 10 mM Tris, pH 7.5) at 2 g of starting tissue per milliliter. Recovery of DNA in the final nuclear pellets ranged from 50–60 percent. Nuclear T_3 was extracted essentially as described by Hartong *et al.* (21). Aliquots of isolated nuclei resuspended in buffer C were extracted three times with 4 volumes of 99% ethanol. Parallel studies using nuclei prelabeled with $^{125}IT_3$ *in vivo* indicated that this procedure resulted in extraction of more than 85% of the nuclear labeled T_3 .

The combined ethanolic extracts were then dried by rotatory evaporation (Speed-Vac), and the residues were resuspended in the assay buffer. T_3 radioimmunoassay was performed as described elsewhere (17, 22) using antibodies from Henning (Berlin) and free acid L- T_3 (Sigma) as reference. Resuspended samples were assayed in triplicate. Two further aliquots were used to determine the individual nonspecific binding occurring in the absence of specific antibody. Correction of total binding for nonspecific binding was performed for each individual sample. Displacements with different dilutions of the samples were essentially parallel to those obtained for the standard curve in buffer. The recovery of T_3 added to resuspended samples averaged $96.6 \pm 8.3\%$. Parallel incubations with six increasing concentrations of IOP ranging from 0.5 to 5 μg of IOP per assay tube did not modify [^{125}I] T_3 binding, indicating that IOP does not cross-react in the T_3 radioimmunoassay. Estimated IOP concentration in sample tubes does not exceed 0.3 μg IOP, even assuming that IOP is homogeneously distributed between fetal and maternal

compartments, and 100% recovery of IOP in the ethanolic extract of the nuclear fraction.

RNA isolation and Northern analysis. Total RNA was prepared from frozen brown adipose tissue by homogenization in 4 M guanidine thiocyanate (23). RNA samples (10 μg per lane), containing 0.2 $\mu g/mL$ ethidium bromide for easy monitoring of the RNA (24), were subjected to electrophoresis on 1.5% agarose, 2 M formaldehyde gels. After fixation to nylon filters (Hybond N, Amersham), RNA was hybridized to the pUCP36 insert, containing full-length cDNA for the rat UCP (25), or to the 0.5-kb pIL-7 insert corresponding to part of the c-DNA for COII (26). The cDNA probes were labeled using [α - ^{32}P]dCTP (Amersham) by the random oligonucleotide-priming method. Filters were washed under stringent conditions (30 mM NaCl, 3 mM sodium citrate, 0.1% SDS, 65°C, 30 min). For quantification, autoradiographs were scanned using a densitometer (LKB).

Synthesis of labeled tracers. High specific radioactivity [$5'$ - ^{125}I] rT_3 and [$5'$ - ^{125}I] T_3 were obtained by radioiodination with $^{125}I^-$ (IMS 30; Amersham) using 3,3'-diiodothyronine and 3,5-diiodothyronine (Henning) as substrate, respectively (27).

Other determinations. DNA was determined by bisbenzimidazole (Hoechst 33258, Sigma) fluorescence (28). Protein was determined by a modification of the Lowry method in tissue homogenates and mitochondria pellets (29). Cytochrome oxidase activity was determined in tissue homogenates and mitochondria preparations (30). The recovery of cytochrome oxidase in each sample was used to determine the mitochondrial protein content of brown adipose tissue. Statistical comparisons were performed by unpaired or paired *t* test and analysis of variance.

RESULTS

IBAT and liver 5'-D activities increased significantly from day 18 to day 20 of fetal life (Fig. 1, A and C, respectively). Brain 5'-D total activity (type I + type II) did not change during this period, whereas 5'-D-II (PTU-insensitive) increased significantly (Fig. 1B). Nuclear T_3 content was also higher in IBAT, brain, and liver from 20-day-old fetuses than from those at day 18 (Fig. 1, D, E, F). Nuclear T_3 concentrations were higher in IBAT than in brain (4–6-fold) or liver (10-fold) during this period. The dose-response curve obtained with different dilutions of resuspended extracts prepared from IBAT of 20-day-old fetuses was parallel to the T_3 standard curve (Fig. 2). This result rules out the possibility that the high nuclear T_3 content determined in fetal IBAT was artifactual.

IOP administration to pregnant rats during days 18 and 19 postconception did not modify litter size (controls: 11.8 ± 0.5 fetuses/dam, IOP-treated: 11.6 ± 0.6 fetuses/dam) or body weight at day 20 (controls: 3.27 ± 0.12 g, IOP-treated: 3.42 ± 0.20 g). IOP treatment produced a significant blockage of 5'-D activity in IBAT and brain; in contrast, liver 5'-D was not modified. T_3 content was similar in IBAT and slightly increased in brain and liver nuclei of IOP-treated fetuses when compared with control fetuses at day 20 ($p < 0.05$) (Fig. 1, D, E, and F, respectively). However, when administered to adult rats, IOP produced a significant reduction in IBAT nuclear T_3 content and plasma T_3 concentration (Table 1).

Considering the significance of nuclear T_3 content in fetal IBAT when compared with other fetal tissues at day 20 and the fact that BAT continues its differentiation during late fetal life, the question arose as to whether longer IOP treatment might affect BAT functionality in fetuses at term. In the second experiment, IOP was injected to pregnant dams from day 18 to 21 of gestation (*i.e.* 4 days instead of 2 days). This treatment produced a significant decrease in the body weight of 22-day old fetuses (Table 2). The litter size was not modified (data not shown). IOP inhibition of IBAT 5'-D activity in fetuses at term was as effective as at day 20. The nuclear T_3 content in this experimental series was not determined because DNA recovery in the final nuclear pellets from both treated and untreated fetuses was too low for

Fig. 1. 5'D and nuclear T₃ content in IBAT, brain, and liver from fetuses obtained from control and IOP-treated dams. *Hatched bars* correspond to 20-d-old fetuses obtained from pregnant rats injected twice daily with IOP (10 mg in 500 μ L of alkaline isotonic saline, subcutaneously) on d 18 and 19 of gestation. *Open bars* correspond to 18- and 20-d-old fetuses obtained from untreated and vehicle-treated dams, respectively. 5'D activity (*left panels*) was determined in standard conditions for each tissue and is expressed as picomoles (liver) or femtomoles (BAT and brain) of I⁻ released · h⁻¹ · mg protein⁻¹. Brain PTU-insensitive activity (5'D-II) was measured by the addition of 1 mM PTU (*filled bars*). Nuclear T₃ content (*right panels*) was measured by T₃ RIA after ethanol extraction from isolated nuclei and is expressed as femtomoles of T₃ · mg of DNA⁻¹. Data are means $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ vs 18-d-old fetuses. ## $p < 0.01$, ### $p < 0.001$ 20 + IOP vs control group of the same age (by analysis of variance).

accurate T₃ determination (between 27 and 13% recovery at term and $53.5 \pm 4.5\%$ at day 20). IOP treatment did not affect either UCP or COII mRNA expression or mitochondrial oxidative capacity, as assessed by mitochondrial protein content and cytochrome oxidase activity.

DISCUSSION

The present study shows that nuclear T₃ content increases in IBAT, brain and liver between days 18 and 20 of gestation. Our results are in agreement with published data of T₃ and T₄ concentrations in fetal tissues, which also increase during this period, coinciding with the onset of active fetal thyroid function (31–33). Interestingly, both cellular (31) and nuclear T₃ levels are higher in fetal IBAT than in the other tissues. On the other hand, IBAT 5'D-II reaches maximal levels on day 20 of fetal life (18). Moreover, on day 20, the concentration of T₃ binding sites is also higher in IBAT nuclei than in liver (17) or brain nuclei (34). Taken together, these results suggest a general profile of early maturation in the thyroid hormone status of IBAT.

As to the effects of IOP administration during gestation, the low levels of 5'D activities in IBAT and brain of IOP-treated fetuses indicate that IOP crosses the placenta and reaches the fetus. In contrast, liver 5'D was not modified by IOP administration. It has been reported that, in adult rats, hepatic or renal 5'D-I are 100-fold less sensitive to IOP inhibition than 5'D-II from pituitary gland or cerebral cortex (10). On the other hand, low sensitivity to other inhibitory agents has been described for

Fig. 2. Dose-response curves. Inhibition of the binding of [¹²⁵I]T₃ by increasing quantities of T₃ or nuclear extracts from IBAT. T₃ concentrations in the standard curve ranged from 2.5 to 80 fmol/tube. Nuclei were isolated from IBAT of 20-d-old fetuses and extracted with ethanol. Increasing quantities of the ethanolic extracts (from 100 to 600 μ L per sample tube, corresponding to 20–120 μ g of DNA in the nuclear fraction) were dried and the residues were resuspended in the assay buffer for T₃ radioimmunoassay. Nonspecific binding was estimated in parallel incubations in the absence of specific antibody. B is the bound [¹²⁵I]T₃ corrected for nonspecific binding and expressed as percentage of total [¹²⁵I]T₃ added. Each point is the mean $\pm$ SD of three measurements. ○, T₃ standard curve; ♦, IBAT nuclear extracts.

Table 1. Effect of IOP treatment on IBAT nuclear T₃ content and plasma T₃ concentration in adult rats*

	Controls	IOP†
IBAT nuclear T ₃ content (fmols T ₃ · mg DNA ⁻¹)	328.7 $\pm$ 12.3	216.6 $\pm$ 27.7‡
Plasma T ₃ (nM)	0.93 $\pm$ 0.005	0.65 $\pm$ 0.005§

* Values are means $\pm$ SEM of eight adult male rats per group.

† IOP was injected twice daily (10 mg in 500 μ L of alkaline isotonic saline, subcutaneously) for 2 d; animals were killed 12 h after the last injection. Control animals received the vehicle solution with identical timing.

‡ $p < 0.01$.

§ $p < 0.001$ vs control group (by analysis of variance).

the fetal or neonatal forms of liver 5'D (35, 36). However, in the absence of direct data of IOP concentration in fetal tissues, we cannot rule out the possibility that the lack of effect of IOP administration on fetal liver 5'D-I may be produced by decreased transport and/or increased metabolism of IOP in fetal liver.

Our results demonstrate that IOP administration at days 18 and 19 of gestation does not block the characteristic rise in T₃ levels in IBAT nuclei during this period, despite the high sensitivity of IBAT 5'D-II to inhibition by IOP. Previous reports have described that the pattern of UCP expression during fetal development is not modified in fetuses from IOP-treated dams (16). The present results further extend these studies by showing that neither mitochondrial oxidative capacity nor UCP or COII

Table 2. Effect of IOP treatment on body and IBAT weights, IBAT 5'D activity, and on the functionality of IBAT mitochondria in fetuses at term*

	Controls	IOP†
Body weight (g)	6.1 ± 0.1	5.4 ± 0.2‡
IBAT weight (mg)	34.0 ± 1.8	32.0 ± 2.0
5'D activity (fmol I ⁻ · h ⁻¹ · mg protein ⁻¹)	122.0 ± 17.4	16.9 ± 5.7§
Mitochondrial protein (mg · g IBAT ⁻¹)	29.5 ± 4.6	25.8 ± 3.9
Cytochrome oxidase activity (nmol · s ⁻¹ · IBAT ⁻¹)	54.5 ± 7.4	53.0 ± 5.5
COII mRNA (%)	100 ± 25	122 ± 23
UCP mRNA (%)	100 ± 24	96 ± 29

* Values are means ± SEM of fetuses from 5–8 different dams/group.

† IOP group correspond to 22-d-old fetuses obtained from dams injected twice daily with IOP (10 mg in 500 µL of alkaline isotonic saline, subcutaneously) from d 18 to 21 of gestation. Control fetuses were obtained from dams receiving the vehicle solution with identical timing.

‡ $p < 0.05$.

§ $p < 0.01$ vs control (by analysis of variance).

|| Results are expressed as percentages of control values.

expression are impaired in IBAT from IOP-treated fetuses at term. It is known that UCP expression is dependent on T₃ (37). Moreover, the mRNA levels of mitochondrial-encoded cytochrome oxidase subunits are regulated by T₃ (38). Reiter *et al.* (19) have demonstrated that, in adult animals, the rise in UCP expression in response to cold stimulation is inhibited by IOP administration. Present results demonstrate that IOP treatment in adult rats led to a significant decrease in the IBAT nuclear T₃ content. Thus, it can be concluded that the discrepancies in the effects of IOP on UCP expression in fetuses and adults may be explained by the fact that IOP does not decrease the nuclear T₃ content of fetal IBAT.

Our results indicate that IOP treatment produced an increase in the nuclear T₃ content in brain and liver from 20-day old fetuses. In contrast, in adult animals, IOP administration produced a decrease in the nuclear T₃ content in IBAT (present results) as well as in brain, liver, and pituitary gland, due to the inhibition of T₄-to-T₃ conversion (12). The high T₃ concentration in nuclei from IOP-treated fetuses could result from: 1) a decrease in T₃ degradation, and/or 2) an increase in total T₃ production. Regarding the first possibility, it has been described that IOP administration decreases T₃ degradation when studied in thyroidectomized adult rats transiently injected with T₃ (39). However, as T₃ degradative pathways are decreased in hypothyroidism, it is not clear that IOP effects in thyroidectomized rats are generalizable to other situations, such as fetal life. The second possibility would result if IOP inhibition of T₃ production in tissues such as IBAT and brain was compensated by T₃ overproduction in others (perhaps fetal liver). Inner-ring deiodinase (5-D), which converts T₄ to rT₃ and T₃ to 3,3'-diiodothyronine, is active in fetal tissues and placenta (40, 41). 5-D is also inhibited by IOP *in vitro* (42). Therefore, the probable inhibition of 5-D by IOP treatment of pregnant dams might result in decreased T₃ metabolism and increased T₄ availability for the 5'D-I of fetal liver. The consequence would be increased liver T₃ production. Further experiments are needed to clarify the factors involved in the different responses to IOP administration in fetal and adult life.

Finally, cholecystographic contrast agents, such as IOP and ipodate, are often used in the treatment of Graves' disease, because they produce a fast decrease in plasma T₃ concentration (43, 44). A combined treatment with ipodate plus methimazole has been reported in one case of Graves' hyperthyroidism during pregnancy: at delivery, the mother was clinically euthyroid, but the newborn presented mild hyperthyroid status, which improved with neonatal ipodate therapy (45). Our present results

provide new insights into the differential effects of these radiographic contrast agents upon thyroid status during fetal life, at least in the rat, in which IOP treatment does not depress and may even enhance the nuclear thyroid hormone content in several fetal tissues.

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