Relationships between thyroid status, tissue oxidative metabolism, and muscle differentiation in bovine fetuses

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Abstract

The temporal relationships between thyroid status and differentiation of liver, heart and different skeletal muscles were examined in 42 bovine fetuses from day 110 to day 260 of development using principal component analysis of the data. Plasma concentrations of reverse-triiodothyronine (\(\text{rT}_3\)) and thyroxine (\(\text{T}_4\)) increased during development from day 110 to day 210 or 260, respectively, whereas concentration of triiodothyronine (\(\text{T}_3\)) and hepatic type-1 5′-deiodinase activity (5′D1) increased from day 180 onwards. On day 260, high \(\text{T}_4\) and \(\text{rT}_3\) and low \(\text{T}_3\) concentrations were observed together with a mature 5′D1 activity. Cytochrome-c oxidase (COX) activity expressed per mg protein increased at day 180 in masseter and near birth in masseter, rectus abdominis and cutaneus trunci muscles (\(P<0.05\)). Significant changes in citrate synthase (CS) activity per mg protein were observed between day 110 and day 180 in the liver and between day 210 and day 260 in the liver, the heart and the longissimus thoracis muscle (\(P<0.05\)). Muscle contractile differentiation was shown by the disappearance of the fetal myosin heavy chain from day 180 onwards. A positive correlation (\(r>0.47, P<0.01\)) was shown between thyroid status parameters (5′D1, concentrations of \(\text{T}_4\) and \(\text{T}_3\)) and COX activity in muscles known to be oxidative after birth (masseter, rectus abdominis) but not in liver and heart, nor in muscles known to be glycolytic after birth (cutaneus trunci, longissimus thoracis). A similar correlation was found between thyroid parameters and CS activity in liver and masseter. Results indicate that elevation

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of plasma T₃ concentrations in the last gestational trimester could be involved in the differentiation of oxidative skeletal muscles.

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Keywords: Bovine fetus; Thyroid hormone; Oxidative enzymes; Muscle differentiation; Principal component analysis

1. Introduction

Thyroid hormones (TH) play a crucial role in growth, development, and function of most vertebrate tissues, such as brain, bone, adipose tissue, and skeletal muscles [1]. They affect both tissue accretion and differentiation in the fetus by a combination of metabolic and non-metabolic mechanisms [2]. Thyroid hormones also exert critical effects on a variety of metabolic pathways, especially energy metabolism, and are key regulators of postnatal growth. Heart and skeletal muscles are important TH targets [3] especially the developing skeletal muscle [4]. In rat, TH control myosin heavy chain (MyHC) isoform transitions [5,6]. A role for TH is also suspected in coordinating the expression of contractile and metabolic muscular proteins [7].

Most studies relate to postnatal TH effects on experimental animals, mainly rodents. However, TH regulation of muscle development occurs as early as in the fetus for species which are mature at birth based on muscle biology, such as ruminants [8]. In fetal sheep, an intact thyroid gland is required for normal development and for biochemical and contractile differentiation of muscle masses [9].

Developmental and metabolic effects of TH are mediated by triiodothyronine (T₃), which is mainly produced by peripheral 5′-deiodination of thyroxine (T₄) [10]. However, the mechanisms of a TH action during fetal development of muscles are poorly documented although local interactions with the activity of the somatotrophic axis have been shown [11].

In this study, we investigated the influence of TH on tissue differentiation (heart, liver and skeletal muscles). We have examined temporal relationships between thyroid status, tissue oxidative capacity, and muscle differentiation in bovine fetuses during the two last trimesters of gestation. For this purpose, we have applied a multivariate analysis procedure [12].

2. Materials and methods

2.1. Animals and tissue samples

This study was carried out as part of a research program approved by the “Institut National de la Recherche Agronomique” (INRA, France) Ethical Committee. The study included 33 fetuses of comparable chronological age collected at day 110 (111.5 ± 6.5; n = 6), 180 (181.7 ± 5.9; n = 7), 210 (207.8 ± 4.8; n = 10), and 260 (259.6 ± 2.5; n = 10)
post-conception (p.c.). Fetuses were obtained by artificial insemination of Charolais heifers with Charolais sperm. The cows were bred and slaughtered, and fetuses were collected, according to ethical guidelines concerning animal care.

Fetal heart (H), liver (L), and samples from four skeletal muscles namely cutaneus trunci (CT), rectus abdominis (RA), masseter (MA), longissimus thoracis (LT) were collected at slaughter, snap-frozen in liquid nitrogen, and stored at −80 °C until analyzed. The rationale for sampling these muscles was that they display different contractile and metabolic properties in the adult in the following gradient from the most slow oxidative to the most fast glycolytic: MA > RA > LT > CT [13].

2.2. Hormone determinations

Blood samples from individual fetuses were collected on potassium-EDTA (1.6 mg/mL). Plasma was separated by centrifugation at 4 °C within 15 min and kept frozen at −20 °C until analysis.

Concentrations of total T4 and T3 were determined in duplicate by radioimmunoassay using commercial kits (AMERLEX-M, Ortho Clinical Diagnostics, Issy-les-Moulineaux, France). Lower limits of sensitivity were 3 ng/mL for T4 and 0.1 ng/mL for T3. Circulating concentrations of rT3 were determined using a diagnostic kit (Serono Diagnostics, Rungis, France) according to the manufacturer’s protocol. Lower limit of sensitivity was 0.009 ng/mL.

2.3. Hepatic 5′-deiodinase determination

Type-1 5′-deiodinase activity was determined from liver samples collected at slaughter and snap-frozen in liquid nitrogen. Outer-ring deiodinating activity (5′D1) was determined by quantifying the $^{125}$I released from 3,3′,5′-[125I]-T3 (rT3) as previously described [14]. In brief, frozen liver samples were homogenized in 0.01 M HEPES buffer (pH 7.0, 0.25 M sucrose, 5 mM EDTA) using a Polytron homogenizer (Brinkman Instruments Inc., Westbury, NY). After centrifugation (30 min at 2000 × g), the supernatant was incubated for 5 min in 0.1 M phosphate buffer (pH 7.0, 1 mM EDTA) in the presence of 5 mM dithiothreitol at 37 °C with approximately 80,000 cpm of $^{125}$I-rT3 (DuPont-New England Nuclear, Boston, MA) and 1 μM of unlabeled rT3 (Calbiochem, La Jolla, CA). The assay mixture contained 30–50 μg of protein. The released $^{125}$I was isolated as trichloroacetic acid (TCA)-soluble radioactivity. Protein concentration in homogenates was determined with bicinechonic acid reagent and bovine serum albumin as a standard (Pierce Chemical Co., Rockford, IL, USA). The 5′D1 activity was expressed as nmol I−/(h mg protein).

2.4. Tissue oxidative metabolism

Citrate synthase (CS; EC 4.1.3.7) activity in sonicated tissue homogenates was determined by measuring the rate of initial reaction at 412 nm by means of the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method as previously described [15]. The reaction mixture contained 0.2 mM DTNB, 50 μM acetyl-CoA, 100 mM Tris–HCl (pH 8.1), 100 μM oxaloacetate and sample in a total volume of 1 mL. The reaction was carried out at 25 °C
and initiated by the addition of oxaloacetate. Cytochrome-c oxidase (COX; EC 1.9.3.1) activity was assayed in freeze-thawed and sonicated tissue homogenates at 25 °C according to Smith and Conrad [16] with 90 μM reduced cytochrome-c as substrate and 50 mM potassium phosphate (pH 7.4). The velocity was calculated from \( V = k[S] \), in which the first order constant \( k \) is determined in the assay and \( [S] \) is set at 90 μM. For CS and COX activities, one unit of enzyme is defined as the amount which, under assay conditions, catalyzes the liberation of 1 μmol of CoA, or the oxidation of 1 μmol of cytochrome-c, respectively, per min at 25 °C. Specific activity was expressed as units per g tissue protein.

2.5. Muscle contractile differentiation

Once enzymatic assays have been performed for metabolic determination, amounts of MA, CT and RA muscles that remained at day 110 p.c. (0.96, 1.73, and 1.77 g, respectively) were insufficient for further muscle studies in the youngest animals. However, previous experiments have shown that the kinetics of fetal MyHC disappearance were similar in muscles despite differences in the absolute level of accumulation [17]. Thus, the disappearance of fetal MyHC in LT was studied as an indicator of contractile differentiation of the whole muscle mass. Fetal MyHC accumulation in fibers was measured by immunocytochemistry and immunoblotting as described by Picard et al. [18] using the F158 4C10 monoclonal antibody (Biocytex, Marseille, France). Immunocytochemistry was carried out on serial frozen muscle sections, thickness 10 μm. After incubation with the primary antibody and visualization with secondary antibody fluorescence, the sections were analyzed under an epifluorescence microscope (Labophot II, Nikon, Tokyo, Japan). The fetal MyHC positive cells were scored. A complementary immunoblotting analysis was performed after SDS-PAGE of 5 μg myofibrillar proteins as described in Picard et al. [19]. The proteins were blotted to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, USA) in a semi-dry system at 24 V for 1 h. The blot was incubated with the primary antibody and then with a secondary antibody (rabbit anti-mouse IgG, Jackson Immunotech, West Grove, Pennsylvania, USA) coupled to alkaline phosphatase. Visualization of immunoblots was performed in a solution of Nitro Blue Tetrazolium (NBT)–5 Bromo 4 Chloro Indolyl Phosphate (BCIP).

2.6. Statistical analysis

Analysis of variance of the data was made using the general linear models (GLM) procedure of SAS [20] to test the effect of age for each parameter (tissue weights, protein contents and enzyme activities). Differences were considered to be statistically significant if \( P < 0.05 \) and to show a tendency for a statistical significance if \( P < 0.10 \). Results are presented as least square means and standard errors, with three degrees of freedom.

Principal component analyses [12] were performed using the FACTOR procedure of SAS to study the relationships between the following variables: thyroid status (T4, rT3, and T3 plasma concentrations, and 5′D1 activity in the liver), oxidative metabolic activities (CS and COX activities in liver, heart, and MA, RA, LT and CT muscles), weights of the fetus and of its liver, and percentage of fetal MyHC positive fibers in LT. This statistical method is a mathematical method of reorganising information in a data set of variables, and of
individuals on which those variables were measured. It allows reducing the dimensionality of data set originally described with much large number of variables without a significant loss of information. PCA allows calculating new variables, called principal components, which account for the variability in the data. This enables to describe the information with fewer variables than originally present. Principal components are linear combinations of the original variables. The first principal component is the combination of variables that explains the greatest amount of variability in the data. The second principal component, and subsequent, describes the maximum amount of remaining variability and must be independent of (orthogonal to) the first principal component. Principal component analysis is based on the study of the covariances and the correlations between variables. The main steps of the calculations are: (i) transformation of the data into centered and normalized values, (ii) calculation of the correlation matrix of the variables, (iii) research of proper vectors, i.e., the best independent combinations of the studied variables which explain the highest proportions of the variability, and finally (iv) projections of the variables and the individuals on the proper vector basis (score plot). Results are presented in a 2D projection graph where variables near each other at the periphery of the circle are positively correlated, orthogonal variables are independent and variables separated by 180° are negatively correlated. The closer to the circle periphery, the higher is the coefficient of correlation between variables.

3. Results

3.1. Thyroid hormone status in fetuses

Mean plasma concentrations of iodothyronines in fetuses ranging from day 110 to day 260 p.c. are shown in Fig. 1. Plasma T4 was detected from day 110 p.c. onwards (Fig. 1A). Plasma T4 concentrations increased from a mean concentration of 8.7 ng/mL at day 110 p.c to 63 ng/mL (P < 0.001) at day 180 p.c. An increase was recorded between day 180 and day 210 p.c. (56%; P < 0.01). Plasma T4 concentrations increased also in the last third trimester and reached 130.5 ng/mL at the end of gestation (P < 0.01 between day 210 and day 260 p.c.).

Total T3 concentrations were barely detectable from day 110 to day 180 p.c. (0.14 ng/mL; Fig. 1B). An increase in T3 concentration was recorded during the third trimester (77% between day 180 and day 210 p.c.; P < 0.05; 31% between day 210 and day 260 p.c.; P = 0.07) to reach 0.38 ng/mL. Concentrations of T3 in the fetuses were positively correlated with fetal weight (r = 0.67; P < 0.01), fetal liver weight (r = 0.65; P < 0.01), and T4 plasma concentrations (r = 0.65; P < 0.01).

Plasma rT3 concentrations averaged 0.85 ng/mL at day 110 p.c. and increased (P < 0.01) until day 210 (Fig. 1C) and thereafter remained unchanged. At day 260 p.c., rT3 concentration had reached 1.75 ng/mL. Hepatic 5’D1 activity was barely detectable in fetuses until day 180 p.c. (Fig. 1D). There was a sharp increase in hepatic 5’D1 activity during the third trimester, i.e., between day 180 and day 210 p.c. (96%; P < 0.05) and between day 210 and day 260 p.c. (158%; P < 0.001). Hepatic 5’D1 activity was positively correlated with plasma, T4 concentration (r = 0.72; P < 0.01), T3 concentration (r = 0.53; P < 0.01) and rT3 concentration (r = 0.36; P < 0.05).
Fig. 1. Thyroid hormone status in fetuses during the second and third trimesters of gestation. (A) Thyroxine (T₄) plasma concentration; (B) triiodothyronine (T₃) plasma concentration; (C) reverse-triiodothyronine (rT₃) plasma concentration; (D) hepatic type-1 5′-deiodinase (5′D1) activity. Results are shown as least square means ± S.E. *P < 0.05, **P < 0.01, ***P < 0.001, †P < 0.10.

3.2. Differentiation of oxidative tissue metabolism and of muscle characteristics

Changes in protein concentration were tissue-specific because total protein content, calculated per unit of wet organ weight, increased with age in all tissues except in the liver where it decreased (Table 1). In the LT, it increased regularly all through gestation (P < 0.001) whereas it rose mainly between day 210 and day 260 p.c. in the heart (P < 0.001) and skeletal muscles (P < 0.01). Activities of CS and COX, mitochondrial enzymes representing the state of oxidative metabolism, were determined in oxidative tissues (liver and heart) and in skeletal muscles. Results were expressed relative to the total protein content of tissues (Tables 2 and 3, respectively). Metabolic differentiation pattern differed between liver, heart and skeletal muscles. In liver and heart, COX activity remained stable all through gestation (Table 3) but CS activity increased between day 110 and day 260 in liver and between day 210 and day 260 in heart (P < 0.05, Table 2). In skeletal muscles, COX activities were lower than those recorded in liver and heart. In LT, there was a decrease of CS activity between day 110 and day 180 but an increase in CS activity between day 210 and day 260 (Table 2). A similar increase was detected in MA (Table 2). There was a significant effect of fetal age on COX activity in MA, RA and CT (Table 3). The COX activity increased regularly in MA throughout gestation. However, in RA and CT, it significantly increased only between day 210 and day 260. In LT, no significant effect of fetal age could be detected when the activity was expressed relative to tissue protein content in spite of an increase in specific activity per g wet tissue (data not shown).

Fetal MyHC accumulation in LT averaged 100% of muscle cells throughout the second trimester of gestation (Fig. 2A and B). From day 180 p.c. onwards, it markedly decreased to
Table 1
Body weight, liver weight, and tissue protein content of bovine fetuses at different fetal ages

<table>
<thead>
<tr>
<th>Item</th>
<th>Fetal age&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANOVA (age effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 110</td>
<td>Day 180</td>
</tr>
<tr>
<td>Fetus weight (kg)</td>
<td>0.67 ± 1.38&lt;sup&gt;b&lt;/sup&gt; z</td>
<td>8.00 ± 1.27 y</td>
</tr>
<tr>
<td>Liver weight (kg)</td>
<td>0.023 ± .039 y</td>
<td>0.232 ± 0.037 y</td>
</tr>
<tr>
<td>Liver proteins&lt;sup&gt;c&lt;/sup&gt;</td>
<td>116.13 ± 3.0 z</td>
<td>101.00 ± 2.78 y</td>
</tr>
<tr>
<td>Heart proteins</td>
<td>71.55 ± 3.74 z</td>
<td>80.62 ± 3.47 y</td>
</tr>
<tr>
<td>MA&lt;sup&gt;d&lt;/sup&gt; proteins</td>
<td>45.94 ± 2.98 x</td>
<td>53.35 ± 2.76 x</td>
</tr>
<tr>
<td>RA&lt;sup&gt;e&lt;/sup&gt; proteins</td>
<td>57.69 ± 4.74 z</td>
<td>74.1 ± 4.39 yw</td>
</tr>
<tr>
<td>CT&lt;sup&gt;f&lt;/sup&gt; proteins</td>
<td>39.58 ± 3.34 y</td>
<td>47.31 ± 3.09 xy</td>
</tr>
<tr>
<td>LT&lt;sup&gt;g&lt;/sup&gt; proteins</td>
<td>31.67 ± 5.14 z</td>
<td>67.96 ± 4.76 y</td>
</tr>
</tbody>
</table>

Within each tissue, mean value with different letters are significantly different (P < 0.05).

<sup>a</sup> Days post-conception (p.c).
<sup>b</sup> Values are least means ± S.E. for n = 6 at day 110 p.c, n = 7 at day 180 p.c, and n = 10 at day 210 and day 260 p.c.
<sup>c</sup> Proteins are expressed in g per tissue total wet weight.
<sup>d</sup> Masseter muscle.
<sup>e</sup> Rectus abdominis muscle.
<sup>f</sup> Cutaneus trunci muscle.
<sup>g</sup> Longissimus thoracis muscle.
Table 2
Citrate synthase (CS) activitya in liver, heart and skeletal muscles of bovine fetuses at different fetal ages

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fetal ageb</th>
<th>Day 110</th>
<th>Day 180</th>
<th>Day 210</th>
<th>Day 260</th>
<th>ANOVA (age effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Day 110</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.8 ± 3.9c</td>
<td>z</td>
<td>34.28 ± 3.6 xy</td>
<td>31.8 ± 3 yz</td>
<td>43.1 ± 3 x</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67.3 ± 17.7</td>
<td>y</td>
<td>114.1 ± 16.4 xy</td>
<td>75.7 ± 13.7 y</td>
<td>124 ± 13.7 x</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>MAd</td>
<td>11.5 ± 3.2</td>
<td>y</td>
<td>17.4 ± 2.9 y</td>
<td>16.4 ± 2.5 y</td>
<td>41 ± 2.5 x</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>RAe</td>
<td>27.8 ± 8</td>
<td>y</td>
<td>8.4 ± 7.5</td>
<td>9.1 ± 6.3</td>
<td>27.2 ± 6.3</td>
<td>P &lt; 0.10</td>
</tr>
<tr>
<td>CTF</td>
<td>24 ± 5.3</td>
<td>y</td>
<td>13.8 ± 4.9</td>
<td>12.6 ± 4.1</td>
<td>23.9 ± 4.1</td>
<td>ns</td>
</tr>
<tr>
<td>LTg</td>
<td>41.2 ± 1.4</td>
<td>y</td>
<td>19.5 ± 1.3</td>
<td>19.5 ± 1.1 z</td>
<td>25.5 ± 1.1 x</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Within each tissue, mean values with different letters are significantly different (P < 0.05); ns, non-significant.

a CS activity is expressed as units per g protein.
b Days post-conception (p.c.).
c Values are least means ± S.E. for n = 6 at day 110 p.c, n = 7 at day 180 p.c, and n = 10 at day 210 and day 260 p.c.
d Masseter muscle.
e Rectus abdominis muscle.
f Cutaneus trunci muscle.
g Longissimus thoracis muscle.

reach 50% at the end term (P < 0.001) indicating that contractile differentiation of muscles occurred during the third term. The fetal MyHC isoform was not detected in adult samples as shown by immunoblotting of myofibrillar proteins (Fig. 2C).

3.3. Relationships between thyroid status and tissue differentiation

The method of principal component analysis was used to generate a reduced set of variables, which are called principal components and are much easier to analyze and interpret

Table 3
Cytochrome-c oxidase (COX) activitya in liver, heart and skeletal muscles of bovine fetuses at different fetal ages

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fetal ageb</th>
<th>Day 110</th>
<th>Day 180</th>
<th>Day 210</th>
<th>Day 260</th>
<th>ANOVA (age effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Day 110</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>522.5 ± 58.8c</td>
<td></td>
<td>525.3 ± 54.5</td>
<td>640.8 ± 45.6</td>
<td>655.3 ± 45.6</td>
<td>ns</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1338.3 ± 142.1</td>
<td></td>
<td>1106.4 ± 131.5</td>
<td>1221.8 ± 110</td>
<td>1391.1 ± 110</td>
<td>ns</td>
</tr>
<tr>
<td>MAd</td>
<td>105.5 ± 28.5</td>
<td>z</td>
<td>173 ± 26.4 yz</td>
<td>210.9 ± 22.1 y</td>
<td>365.8 ± 22.1 x</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>RAe</td>
<td>80.6 ± 18.9 y</td>
<td></td>
<td>82.6 ± 17.5 y</td>
<td>119 ± 14.6 y</td>
<td>167.7 ± 14.6 x</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>CTF</td>
<td>119.1 ± 27.9 y</td>
<td></td>
<td>129.8 ± 25.9 y</td>
<td>114.3 ± 21.6 y</td>
<td>218.6 ± 21.6 x</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>LTg</td>
<td>195.8 ± 57.4</td>
<td>y</td>
<td>105.6 ± 53.2</td>
<td>183.5 ± 44.5</td>
<td>107.1 ± 44.5</td>
<td>ns</td>
</tr>
</tbody>
</table>

Within each tissue, mean values with different letters are significantly different (P < 0.05); ns, non-significant.

a COX activity is expressed as units per g protein.
b Days post-conception (p.c.).
c Values are least means ± S.E. for n = 6 at day 110 p.c, n = 7 at day 180 p.c, and n = 10 at day 210 and day 260 p.c.
d Masseter muscle.
e Rectus abdominis muscle.
f Cutaneus trunci muscle.
g Longissimus thoracis muscle.
Fig. 2. Changes in fetal myosin heavy chain (MyHC) isoform in muscles with fetal age. (A) Fetal MyHC accumulation in fibers. An immunocytochemical analysis of transverse muscle sections was carried out with a monoclonal antibody raised against fetal MyHC in longissimus thoracis (LT) muscle at different stages of fetus development. Scale bar = 50 μm. (B) Mean percentage of muscle fibers expressing fetal MyHC. The number of labeled fibers was expressed as a percentage of the total number of fibers. Results are shown as least square means ± S.E. Mean value with different superscript letters are significantly different (P<0.05). (C) Changes in fetal MyHC accumulation. Visualization of fetal MyHC (200 kDa band) was performed by SDS-PAGE and immunoblotting with the monoclonal antibody against fetal MyHC. Adult muscle extract was used as negative standard.

than original ones. Of the principal component analysis which was carried out, the first two principal components were retained as they explained 60% of the standardized variance (45 and 15%, respectively). The variables and the fetuses were then plotted in a two-dimensional space (score plot, Fig. 3) defined by the two axes of the first component (x-axis) and the second component (y-axis), respectively. The most important variables accounting for the first component were: weights of the fetus and of the liver, plasma T4 concentration, 5′D1 activity in liver, COX activities in MA and RA, CS activity in MA and fetal MyHC expression in LT (Fig. 3A), i.e., all variables affected by the age of the fetuses. The first component illustrated the negative correlation between fetal MyHC expression and all the other variables cited above (r>0.63; P<0.01; Table 4). It explained three times the variability indicated by the second principal component underlying the importance of fetal age, as expected. The first component also illustrated the positive correlation (i) between rT3, T4, and T3 plasma concentrations (r>0.36; P<0.05), (ii) between T3 and T4 concentrations and 5′D1 activities (r>0.53; P<0.01), (iii) between T3 and T4 concentrations and COX activity in MA and RA on one hand (r>0.46; P<0.01; Table 4), and CS activity in MA on the other hand (r>0.40; P<0.05; Table 4). It also illustrated the positive correlation between hepatic 5′D1 and COX activity in MA, RA and CT muscles (r>0.49; P<0.01; Table 4). The second factor was explained by rT3 levels and CS activity in RA, CT and LT (Fig. 3A).
Table 4
Correlation coefficients between variables representing thyroid status\(^a\), mitochondrial enzyme activities\(^b\) and muscle contractile differentiation\(^c\)

<table>
<thead>
<tr>
<th>Item</th>
<th>r(_T3)</th>
<th>T(_3)</th>
<th>T(_4)</th>
<th>5('D)1</th>
<th>COX L</th>
<th>COX H</th>
<th>COX MA</th>
<th>COX RA</th>
<th>COX CT</th>
<th>Cox LT</th>
<th>CS L</th>
<th>CS H</th>
<th>CS MA</th>
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\(\text{\(^a\) Thyroid status was represented by hepatic type-1 5’D-deiodinase activity (5’D1) and plasma concentration of thyroxine (T\(_4\)), triiodothyronine (T\(_3\)), and reverse-T\(_3\) (rT\(_3\)).}\)

\(\text{\(^b\) Mitochondrial enzymes, citrate synthase (CS) and cytochrome-c oxidase (COX) were determined in liver (L), heart (H) and in four skeletal muscles (masseter, MA; rectus abdominis, RA; cutaneus trunci, CT; longissimus thoracis, LT).}\)

\(\text{\(^c\) Muscle contractile differentiation was followed by scoring fetal myosin heavy chain positive fibers (MyHC) in LT muscle.}\)

\(* P<0.05.\)

\(** P<0.01.\)
Fig. 3. Principal component analysis of the data. The analysis was performed with the following variables: fetus weight (FW), liver weight (LW), triiodothyronine (T3), thyroxine (T4), and reverse-T3 (rT3) plasma concentrations, hepatic type-1 5′-deiodinase (5′D1) activity, activities of citrate synthase (CS) and cytochrome-c oxidase (COX) in liver (L), heart (H) and skeletal muscles (MA: masseter; RA: rectus abdominis; CT: cutaneus trunci; LT: longissimus thoracis), and percentage of fetal myosin heavy chain (MyHC) positive fibers in the longissimus thoracis muscle (LT). The first two principal components (PC) of the analysis were retained. (A) Projection of variables in a “xy” plane defined by the axes for the first two PC (PC1 and PC2). (B) Projection of fetuses in the same “xy” plane. Numbers close to scores indicate day of gestational age: (▲) fetuses at day 110; (♦) fetuses at day 180; (/H17033); fetuses at day 210; (■) fetuses at day 260.

When projecting the individual animals on the score plot of the first two principal components, a clear discrimination among fetuses could be observed along the first (x) axis according to the age of the fetuses (Fig. 3B). The fetuses of the same gestational age were placed close together, underlying the fact that they had similar characteristics. Fetuses at day 110 and day 260 p.c. were the most discriminated along the first axis, underlying the tissue differentiation process that occurred intensively at the end of the fetal period. However, fetuses, especially at day 260 p.c., were also characterized by a high variability along the second (y) axis (Fig. 3B) which is age-independent and could be explained by high variations in CS activity in RA, CT and LT and in rT3 plasma concentration (Fig. 1).

4. Discussion

4.1. Thyroid status ontogeny

A histological study by Koneff et al. [21] has shown that the thyroid gland in bovine fetuses differentiates by day 90 p.c. It undergoes thereafter a gradual development during the third trimester of gestation, and displays a marked increase in functional activity near term coinciding with biochemical maturation [22]. In the present study, the pattern of TH
concentrations fits well with the data describing ontogeny of the bovine fetal thyroid. Plasma TH concentrations increased with gestational age as previously described. Fetal plasma T4 concentrations were similar to those recorded in Holstein fetuses [23] and a marked increase in circulating fetal T4 during the last trimester was also recorded in our study. Plasma T4 concentrations at the end-term agreed well with those recorded in newborn Charolais [24]. However, as compared to older cattle, these concentrations were more elevated [14]. This is in accordance with higher T4 concentrations observed in fetuses than in their dams during the last trimester of pregnancy [23,25]. Thus, plasma T4 concentrations would be expected to fall in the postnatal period, in spite of a transient surge immediately after birth, as shown by others in sheep and cattle [23].

Our data also revealed low plasma T3 concentrations in 260-day-old fetuses as compared to those recorded in 15-day-old Montbéliard calves [14]. They represented approximately one third of the levels found in 15-month-old Charolais cattle. Concentrations of plasma rT3 (whose biological significance has not yet been identified in the fetus) were high from the beginning of the last trimester as shown previously in human fetuses [26]. One key observation of our study is that the activity of hepatic 5′D1 increased concomitantly with the increases in plasma T4 and T3 concentrations: it rose late in the third trimester, reaching an activity comparable to that of newborn calves (our unpublished data). This result indicates that maturation of hepatic 5′D1 activity occurred in the prenatal period, as previously observed in bovine fetuses from Angus and Brahman breeds [27]. Such fetal evolution of 5′D1 activity has also been reported in the liver of fetal pigs [28].

As already shown in mammals, the pattern of serum iodothyronines in fetuses differs from that in adults. In the present study, elevated T4 and rT3 and low T3 plasma concentrations in combination with high 5′D1 activity in liver were found at the end of gestation. As 5′D1 activity is mainly responsible for T3 generation, low plasma T3 levels may reflect preferential T4 deiodination into rT3 (due to high hepatic and placental type-3 deiodinase, 5′D3) rather than an immature hepatic 5′D1 activity [26]. However, it may also be that most of the T3 produced by fetal liver would be captured by the target tissues and be implicated in the metabolic and differentiation effects of this hormone. Supporting this suggestion, an increase in T3 level in tissues (including skeletal muscles) with gestational age has been described in the fetal pig [29].

4.2. Differentiation of liver, heart and skeletal muscles

First of all, this study confirmed that the contractile and metabolic differentiation of skeletal muscles, which are partially linked to each other, mainly occurred during the last third of gestation [17]. As expected, fetal MyHC isoforms disappeared gradually from the end of the second trimester onwards. They have been likely replaced by developmental and adult MyHC isoforms during the on-going process of contractile differentiation [30]. Secondly, the ontogeny of oxidative metabolism in this study was depicted based on the activities of two mitochondrial enzymes (namely COX and CS) in liver, heart, and skeletal muscles. Activity of COX was shown to be more related to the ability of tissues to oxidize fats compared to CS activity which is not correlated to palmitate oxidation capacity across tissues and species [31]. However, CS activity is known to be a better estimate of mitochondrial volume because it is present in the mitochondrial matrix. The developmental pattern of
metabolic differentiation differed between these tissues depending on the enzyme. The heart has a higher COX activity than liver and muscles as previously described in the young calf [31]. The high and stable COX activity throughout gestation in liver and heart indicates that these key metabolic organs are metabolically active at the early stages of gestation. However, they increase their ability to oxidize nutrients (carbohydrates and fats) as reflected by the increase in CS activity with gestational ages. In the most oxidative skeletal muscle (namely masseter), activities of both COX and CS increased during the last third of gestation. Together with the demonstration that isocitrate dehydrogenase (oxidative) and lactate dehydrogenase (glycolytic) activities also increased during this period [17], this result confirms that the metabolic differentiation of skeletal muscles mainly occurs during the last third of gestation. However, muscle differentiation is fully achieved postnatally [30], especially at weaning in the bovine masseter muscle [18,32]. Interestingly, activities of COX and CS in examined tissues were not correlated with each other. The absence of correlation between CS and COX activities has already been observed during ontogenesis in bovine heart [33] and across tissues (heart, liver, muscles) and species (rats, cattle) [31]. This observation may not be surprising because CS is entirely encoded by nuclear genes while COX subunits are encoded both by nuclear and mitochondrial genes [34]. We provide here an original demonstration of a tissue-specific pattern of COX ontogeny as shown by (i) the absence of correlation between liver and heart COX activities, as previously reported [35]; (ii) the correlation of COX activity in heart with COX activity in the most oxidative muscle (masseter) only; (iii) the absence of correlation of COX activities between some skeletal muscles such as LT and RA. Supporting our hypothesis of a tissue-specificity, Bonne et al. [36] have already shown that all COX transcripts were increased from fetal to adult state in human heart and skeletal muscles, whereas little change was observed in liver. Moreover, a tissue-specific switch of COX isoforms in bovine heart and skeletal muscles has been reported during late fetal development [37,38].

4.3. Are oxidative tissues sensitive to thyroid hormones in the fetus?

Thyroid hormones are major regulators of energy metabolism through modulation of the glycolytic [39] and oxidative pathways, especially of COX activity in liver [40], heart [41], and skeletal muscles [42]. In rats, TH enhance mitochondrial ATP production, CS and COX activities in highly oxidative tissues such as soleus muscle, liver and heart but not in the glycolytic plantaris muscle [42]. Because of small increases in plasma levels of T3 (bioactive TH) during the last third of gestation, the question was arisen whether low T3 concentrations could regulate the acquisition of oxidative metabolism in liver, heart and skeletal muscles. Firstly, T3 and T4 plasma concentrations and hepatic 5′D1 activities were found to be negatively correlated with fetal MyHC accumulation, suggesting that thyroid activity may drive the disappearance of fetal myosin isoforms during muscle contractile differentiation. Supporting this hypothesis, myosin isoform transition was shown to be inhibited by hypothyroidism and accelerated by hyperthyroidism, independently of neural control and growth hormone activation [4]. Secondly, heart, liver and skeletal muscles were found to display differential T3 sensitivity for metabolic differentiation, especially for COX activity. Indeed, COX activities of liver and heart were found to be independent of plasma T3 levels and 5′D1 activity. By contrast, masseter, the most oxidative skeletal muscles was
found to be T3 sensitive based on the evolution of CS and COX which are both correlated to T3 levels and 5’D1. Activity of COX in the RA, an oxido-glycolytic muscle, was also T3 sensitive, whereas both oxidative enzymes were not correlated to T3 level in CT and LT which are much more glycolytic. A TH sensitivity of enolases expression has already been described in skeletal muscles but not in the heart [43]. Moreover, the expression of nuclear-encoded COX subunits (IV, Va and Vc) and of mitochondrial-encoded subunits (II and III) is regulated by T3 via separate pathways in a tissue-specific manner [44] in muscles but not in liver. Finally, the developmental switch of COX isoforms during rat cardiac development are not regulated by changes in TH levels [45].

The differential response of the examined tissues to T3 concentrations may be related to a differential expression of TH receptor (TR) isoforms between target tissues. Particularly, the different TR isoforms, encoded by the c-erbA-α and -β genes, are differentially expressed both quantitatively and qualitatively in cardiac and skeletal muscles [46,47]. A differential response to the altered thyroid status has been demonstrated, with heart being the least sensitive in binding capacity [48]. Moreover, muscle-specific developmental profiles have been reported for the four TR receptors in cardiac, oxidative, and glycolytic pig muscles [49]. White et al. [49] have proposed a functional role (i) for TRα isoforms in fetal myogenesis, (ii) for the TRα1/TRα2 ratio in determining cardiac and skeletal muscle phenotype and function, and (iii) for TRβ in maintaining a basal level of cellular response to TH. Further studies on TH receptor expression, in relation to age and nutritional supply to the fetus, will provide additional elements for the understanding of T3 influence on contractile and/or differentiation status of muscle tissues. However, the possibility of indirect changes in bioavailability of TH before birth via a modulation of the local somatotrophic axis and increased muscle insulin-like growth factor-I cannot be excluded [11,50].

In summary, the present study identified temporal relationships between oxidative muscle development and TH ontogeny. It therefore emphasizes the tissue-specific importance of thyroid hormones, especially in the differentiation of the most oxidative skeletal muscles in bovine fetuses. Our results suggest that elevation of circulating concentration of T3 during the last trimester of fetal development may more specifically direct metabolic differentiation of oxidative muscles.

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References


