1   **Presence and regulation of D1 and D2 deiodinases in rat white adipose tissue**

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27 **Abstract**

28 Thyroid hormones regulate adipogenic differentiation, lipogenic and lipolytic metabolism and

29 mitochondrial activity in adipose tissue. T3 levels in tissues are regulated by the deiodinase

30 enzymes.

31 Objective: To study the activity and mRNA expression of the 5' outer-ring deiodinases (D1

32 and D2) and thyroid hormone concentrations in rat white adipose tissue (WAT), where only

33 D1 activity had been described.

34 Methods: Control, thyroidectomized and thyroid hormone treated rats were used. D1 and D2

35 mRNA were determined in WAT by qRT-PCR using Taqman probes, D1 and D2 activities

36 were determined using rT3 and T4 as substrates. T4 and T3 were measured by

37 radioimmunoassay in plasma, liver and adipose tissue.

38 Results: D1 and D2 mRNAs are present in epididymal rat WAT with similar abundance,

39 which are 7% of the D2 mRNA levels in BAT and 1% that of D1 in liver. The Kms in WAT

40 are 40 nM T4 for D2 and 0.35 μM rT3 for D1. Both D1 and D2 are regulated in rat

41 epididymal WAT by thyroidal status. T4 and T3 concentrations in plasma, liver and WAT

42 decreased after thyroidectomy (Tx) and recovered after treatment with T4+T3. Both D1 and

43 D2 mRNA increased in WAT from Tx rats and T4+T3 treatment inhibited them, especially

44 D2 mRNA. D1 activity did not changed with thyroidal status, while D2 activity was inhibited

45 by T4+T3.

46 Conclusions: the presence of both deiodinases in WAT suggests important roles in regulating

47 T3 bioavailability for adipose tissue function and regulation of lipid metabolism and

48 thermogenesis.

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50 **Keywords:** epididymal adipose tissue, thyroid hormones, outer-ring deiodinases.

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52 **List of abbreviations:**

53 BAT: brown adipose tissue; D1: type1 deiodinase; D2: type 2 deiodinase; D3: type3

54 deiodinase; DTT: dithiothreitol; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

55 Km: Michaelis-Menten constant; PCR: polymerase chain reaction; PTU: 6-propyl-2-

56 thiouracil; qRT-PCR: quantitative Real-time PCR; rT3: reverse triiodothyronine; T3:

57 triiodothyronine; T4: thyroxine; Tx: thyroidectomized; UCP1: uncoupling protein 1; Vmax:

58 maximal velocity; WAT: white adipose tissue.

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Introduction

Deiodinases are selenoenzymes that regulate thyroid hormone concentrations in tissues. Two isoenzymes, D1 and D2, catalyze 5'deiodination producing T3 from T4. D1 is present in liver, kidney and thyroid. D1 Km is 0.2-0.5 μM [1], is inhibited by PTU, increases in hyperthyroidism and decreases in hypothyroidism, except thyroid D1 which increases in hypothyroidism, by thyrotropin stimulation [2]. D2 is present in pituitary, brain, BAT and other tissues, is not inhibited by PTU, its Km in nanomolar range. D2 increases in hypothyroidism and produces T3 for local needs. D3, the main T3 degradation pathway, catalyzes inner ring deiodination of T4 and T3, leading to inactive metabolites.

D2 is essential for the regulation of T3 availability during specific events of development, in the ear, retina, brain or BAT. T3 is required for the differentiation program of adipocytes [3], regulating the expression of genes involved in the differentiation and metabolism of adipose tissue. Most studies demonstrating the importance of deiodinases, specifically D2, have been performed in BAT, where T3, locally produced by D2, is important for full thermogenesis, UCP1 expression and lipogenesis [4-7]. So far, no studies have been performed on D1 or D2 abundance in WAT, its hormonal regulation or potential role in different metabolic processes, though D1 activity in WAT was found when D2 was reported in BAT [8]. Some studies have shown induction of brown adipocytes in inguinal WAT depots, using UCP1 and D2 as markers [9, 10]. In this respect epididymal WAT is considered "pure" WAT, never converted into BAT even under extreme cold exposure, as opposed to inguinal WAT considered "convertible" adipose tissue [11]. In humans, WAT represents 15-20% of the body weight in lean subjects and this percentage is much higher in obese people (50%). Thus, WAT might represent one of the largest pools of thyroid hormones. In addition, if WAT can be converted into BAT (inducing UCP1), WAT represents a potential therapeutic

88 target to increase energy expenditure in obesity. Here we studied the presence and regulation

89 by thyroid status of D1 and D2 activities and mRNA levels in rat WAT.

90

91 **Materials and Methods**

92 *Animals and treatments*

93 Protocols following the European Community guidelines were approved by our ethic

94 Committee. Male Wistar rats were divided in 3 groups: Control, Tx and Tx rats treated with

95 T4+T3 (2.4 µg T4+0.4 µg T3/day/100 g body weight) as described [12]. Plasma and organs

96 were dissected out and frozen on dry ice. Epididymal WAT was carefully dissected avoiding

97 the reproductive male organs.

98 *Analytical procedures*

99 *Determination of D1 and D2 activities.* D1 and D2 activities were assayed in WAT

100 homogenates (1:8, weight/volume) using 0.32 M sucrose, 10 mM Hepes and 10 mM DTT.

101 Homogenates were centrifuged 5 minutes at 1000 rpm to separate the upper lipid cake, the

102 infranatant was used to measure deiodinase activities.

103 D1 activity: 60.000 cpm [^{125}I]-rT3/sample, 100 nM rT$_3$ and 5 mM DTT, for 1 h at 37 C

104 using 20-30 µg protein/ 100 µl [13]. D2 activity: 100.000 cpm [^{125}I]-T4/tube, 2 nM T4+ 1

105 µM T3, 20 mM DTT and 1 mM PTU/100 µl, using the same conditions and protein [14]. For

106 kinetic analysis: 2-500 nM rT3 and 5 mM DTT were used for D1 and 1-50 nM T4 and 20

107 mM DTT for D2.

108 *D1 and D2 mRNA*

109 Total RNA was extracted using the RNeasy lipid tissue (Quiagen). D1 and D2 mRNAs

110 were measured by qRT-PCR, using Taqman probes (Rn00572183m1, Rn00581867m1,

111 Applied Biosystems). After normalization to 18S rRNA, the fold-change in mRNA

112 expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

113 *Determination of thyroid hormone concentrations.*

114   Thyroid hormone concentrations were determined by RIA in plasma, liver and WAT, after

115 extraction and purificatio [15]. High specific activity T4, rT3 and T3 labelled with [^{125}I] were

116 synthesized as described [15].

117 *Statistical analysis*

118   Mean values (±SEM) are given. Significant differences were achieved by one-way

119 ANOVA analysis.

120

121 **Results**

122 *Presence and characterization of D1 and D2 in epididymal rat WAT*

123   We first identified D1 in WAT, using PCR amplification and gel separation (not shown).

124 Then, we did tritation curves using qRT-PCR using Taqman probes (**Figure 1A**), confirming

125 that D1 and D2 mRNA were present in rat epididymal WAT being D2 mRNA abundance

126 higher than D1 mRNA (approx. double). WAT D1 mRNA abundance was less than 1% of

127 liver D1 and WAT D2 mRNA abundance was about 7% of BAT D2 (Figure 1B, 1C).

128   We then analyzed D1 and D2 activities in WAT. First, the kinetic characteristics were

129 determined; for D2: Km=40 nM T4 and Vmax= 3 pmol/h/mg protein and for D1: Km= 0.35

130 µM rT3 and Vmax= 6-18 pmol/h/mg protein (C and Tx rats, respectively). D1 activity was

131 higher in perirenal WAT; D2 activitiy was similar in perirenal and epididymal WAT and

132 lower in subcutaneous WAT (results not shown).

133 *Regulation of D1 and D2 in rat WAT by thyroid status*

134   Deiodinases are regulated by thyroid status in most tissues. To study this regulation we

135 used control, Tx and T4+T3-treated rats. The thyroidal status was checked measuring T4 and

136 T3 concentrations in plasma, liver and WAT. **Figure 2** shows that T4 and T3 decreased after

137 thyroidectomy in all samples. Treatment of Tx rats with T4+T3 reverted T4 to control values

138   in plasma and liver, while WAT T4 doubled the values in control rats, pointing to a

139   preferential T4 uptake in WAT. The treatment also increased T3 in all cases, reaching control

140   values in WAT and plasma, while liver T3 concentrations did not fully recovered.

141   D1 and D2 mRNA increased in WAT from Tx rats (**Figure 1D, 1E**), and T4+T3 treatment

142   inhibited them to control values for D1, and to less than 50% of control values for D2 mRNA.

143   Opposite to the mRNA responses no variation was observed in D1 and D2 activities, except

144   for D2 activity that was inhibited in the T4+T3 group.

145

146   **Discussion**

147   D1 activity was identified in WAT in 1983 together with D2 activity in BAT [8],

148   establishing clear differences between BAT and WAT. Then, a full UCP1 expression was

149   associated to T3 produced by D2 in BAT [4] and this was recently confirmed in the D2

150   knockout mice [7].

151   Herein, we describe that D2 is also present in WAT, both at mRNA and activity levels. We

152   used epididymal WAT, the purest of all WAT locations, never converted into BAT under cold

153   exposure; in contrast, inguinal fat, is considered "convertible" adipose tissue, that is

154   transformed into BAT under several stimuli [11, 16]. Other WAT locations (perirenal,

155   periuterine) are considered a mix of BAT and WAT in terms of UCP1 induction [17]. UCP1

156   levels in epidydimal WAT are near detection limits by qRT-PCR and less than 5% those in

157   inguinal WAT (unpublished). Therefore it is unlikely that D2 could come from residual BAT

158   cells present in epidydimal WAT.

159   Despite the 1983 report, WAT D1 had not been further studied, possibly because lipids

160   cause technical difficulties, which we solved by improving the RNA isolation using a specific

161   kit to avoid lipids and specific Taqman probes. Regarding D1 and D2 activities, we

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162   characterized them in terms of their kinetic characteristics which were in the same ranges as

163   in other tissues [1].

164   Thyroid status, a main regulator of deiodinases, was studied in this work. Both deiodinases

165   mRNAs were up-regulated in hypothyroidism. WAT D2 increased as expected in

166   hypothyroidism. WAT D1 also increased, responding to hypothyroidism as thyroid D1 [2].

167   Regarding deiodinase activities, WAT D2 followed a pattern similar to D2 mRNA, as it

168   tended to increase in hypothyroidism and decreased in T4+T3-treated rats, reflecting the high

169   WAT T4 concentrations. We showed recently increased D2 mRNA in parallel to D2 activity

170   levels in human subcutaneous fat [18]. D1 activity did not changed among groups in our

171   study. Other studies have shown discrepancies between D1 and/or D2 mRNA and activity

172   levels [19], probably due to posttranscriptional modifications.

173   Recently, D1 activity in WAT was related to adiposity, increasing by high-fat diets and by

174   leptin, and decreasing under caloric restriction [20]. Other possible regulators (hormones,

175   nutrients) remain to be identified, as we have only explored the role of thyroid hormones. D1

176   and D2 may have distinct functions in WAT, regulating lipogenesis and lipolysis or the

177   expression of genes and providing the T3 required for specific functions, including the

178   conversion of WAT into BAT under specific conditions, which would increase energy

179   expenditure. Each enzyme may have specific functions.

180   *Conclusions*

181   D1 and D2 activities and mRNAs are present in rat WAT. Thyroidal status regulates D1

182   and D2 mRNA in vivo, both increasing in hypothyroid rats. No changes are found in

183   deiodinase activities, except for the D2 inhibition by T4+T3.

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187   Madrileña 2006. CIBERObn is an iniciative of ISCIII, Spain.

188   No conflict of interest is declared.

189

190   **Authors contribution**

191   Both authors contributed equally to the study. MJO wrote the manuscript.

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194   **Legends to Figures**

195

196   **Figure 1.** Ct values for 18S rRNA, D1 and D2 mRNAs from epididymal rat WAT (panel

197   A). Relative expression of D1 (panel B) and D2 mRNAs (panel C) versus liver D1 and BAT

198   D2 mRNAs, respectively. Panels D to G show D1 and D2 mRNAs and activities in WAT

199   from control (C), thyroidectomized (Tx) and T4+T3 treated thyroidectomized rats. Values

200   are means ± SEM. * $P<0.05$ vs C, # $P<0.05$ vs Tx. (n=4-5/ group).

201

202   **Figure 2.** T4 and T3 concentrations in plasma, liver and epididymal WAT from control

203   (C), thyroidectomized (Tx) and T4+T3 treated thyroidectomized rats. Values are means ±

204   SEM. *$P<0.05$ vs C, #$P<0.05$ vs Tx. (n=4-5 /group)

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206

207 **References**

208 1 Bianco AC, Salvatore D, Gereben B, et al. Biochemistry, cellular and molecular biology,

209 and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 2002;23:38-89

210

211 2 Toyoda N, Nishikawa M, Mori Y, et al. Thyrotropin and triiodothyronine regulate

212 iodothyronine 5'-deiodinase messenger ribonucleic acid levels in FRTL-5 rat thyroid cells.

213 *Endocrinology* 1992;131:389-94

214

215 3 Obregon MJ. Thyroid hormones and adipocyte differentiation. *Thyroid* 2008;18:185-95

216

217 4 Bianco AC and Silva JE. Intracellular conversion of thyroxine to triiodothyronine is

218 required for the optimal thermogenic function of brown adipose tissue. *J Clin Invest*

219 1987;79:295-300

220

221 5 Silva JE and Rabelo R. Regulation of the uncoupling protein gene expression. *Eur J*

222 *Endocrinol* 1997;136:251-64.

223

224 6 Bianco AC, Carvalho SD, Carvalho CR, et al. Thyroxine 5'-deiodination mediates

225 norepinephrine-induced lipogenesis in dispersed brown adipocytes. *Endocrinology*

226 1998;139:571-8

227

228 7 Christoffolete MA, Linardi CC, de Jesus L, et al. Mice with targeted disruption of the Dio2

229 gene have cold-induced overexpression of the uncoupling protein 1 gene but fail to increase

230 brown adipose tissue lipogenesis and adaptive thermogenesis. *Diabetes* 2004;53:577-84

231

232   8 Leonard JL, Mellen SA and Larsen PR. Thyroxine 5'-deiodinase activity in brown adipose

233   tissue. *Endocrinology* 1983;112:1153-5

234

235   9 Ukropec J, Anunciado RP, Ravussin Y, et al. UCP1-independent thermogenesis in white

236   adipose tissue of cold-acclimated Ucp1-/- mice. *J Biol Chem* 2006;281:31894-908

237

238   10 Madsen L, Pedersen LM, Lillefosse HH, et al. UCP1 induction during recruitment of

239   brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One*

240   2010;5:e11391

241

242   11 Loncar D. Convertible adipose tissue in mice. *Cell Tissue Res* 1991;266:149-61

243

244   12 Montero-Pedrazuela A, Venero C, Lavado-Autric R, et al. Modulation of adult

245   hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior.

246   *Mol Psychiatry* 2006;11:361-71

247

248   13 Ruiz de Ona C, Morreale de Escobar G, Calvo R, et al. Thyroid hormones and 5'-

249   deiodinase in the rat fetus late in gestation: effects of maternal hypothyroidism.

250   *Endocrinology* 1991;128:422-32

251

252   14 Obregon MJ, Ruiz de Ona C, Hernandez A, et al. Thyroid hormones and 5'-deiodinase in

253   rat brown adipose tissue during fetal life. *Am J Physiol* 1989;257:E625-31

254

255 15 Morreale de Escobar G, Pastor R, Obregón MJ, et al. Effects of maternal hypothyroidism

256 on the weight and thyroid hormone content of rat embryonic tissues. *Endocrinology*

257 1985;117:1890-900

258

259 16 Medina-Gomez G, Calvo RM and Obregon MJ. Thermogenic effect of triiodothyroacetic

260 acid at low doses in rat adipose tissue without adverse side effects in the thyroid axis. *Am J*

261 *Physiol Endocrinol Metab* 2008;294:E688-97

262

263 17 Frontini A and Cinti S. Distribution and development of brown adipocytes in the murine

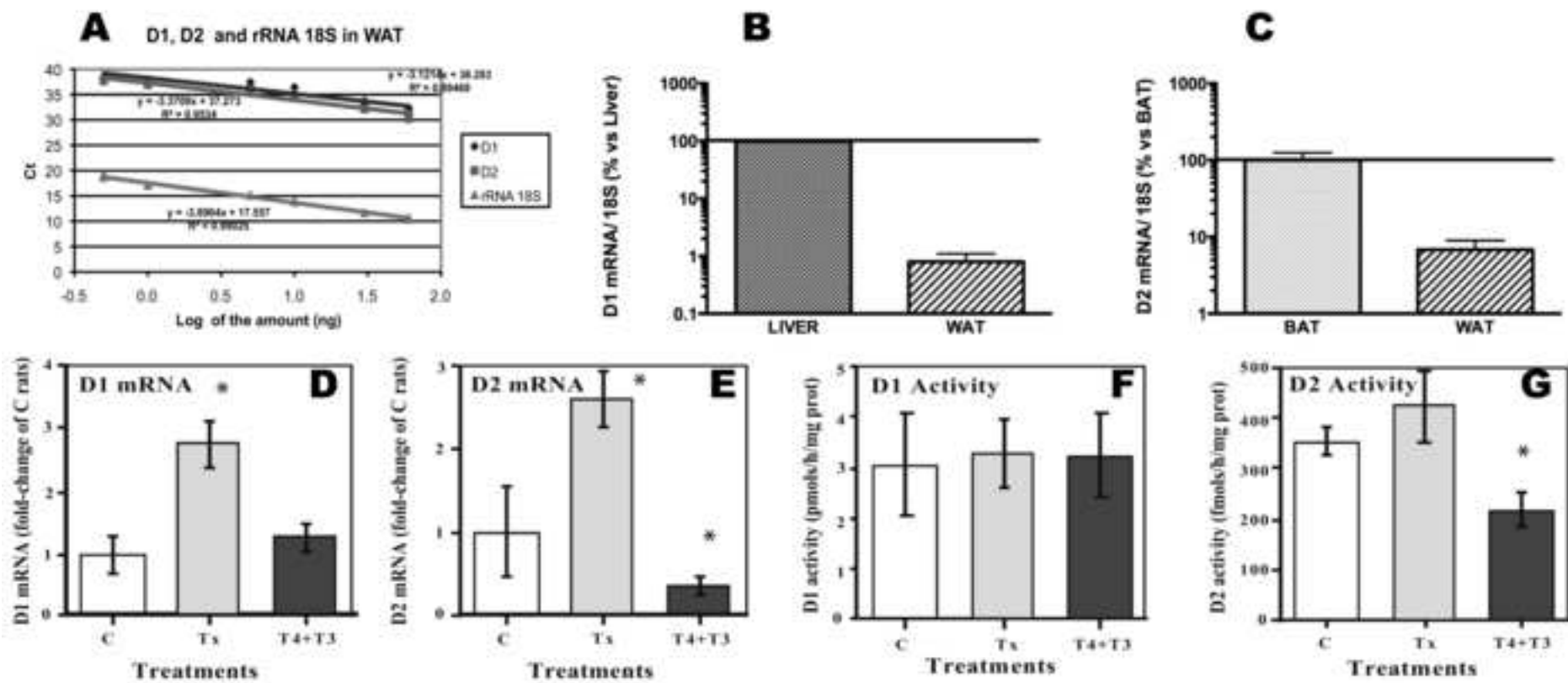264 and human adipose organ. *Cell Metab* 2010;11:253-6

265

266 18 Lado-Abeal J, Calvo RM, Victoria B, et al. Regional decrease of subcutaneous adipose

267 tissue in patients with type 2 familial partial lipodystrophy is associated with changes in

268 thyroid hormone metabolism. *Thyroid* 2010;20:419-24

269

270 19 Rodriguez-Perez A, Palos-Paz F, Kaptein E, et al. Identification of molecular mechanisms

271 related to nonthyroidal illness syndrome in skeletal muscle and adipose tissue from patients

272 with septic shock. *Clin Endocrinol (Oxf)* 2008;68:821-7

273

274 20 Macek Jilkova Z, Pavelka S, Flachs P, et al. Modulation of type I iodothyronine 5'-

275 deiodinase activity in white adipose tissue by nutrition: possible involvement of leptin.

276 *Physiol Res* 2010;59:561-9

277

278

279