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2 **Title: Skeletal muscle ceramides do not contribute to physical inactivity-induced insulin**
3 **resistance**

4 **Running Title: Ceramides do not cause inactivity-related insulin resistance**

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26

27 **SUMMARY STATEMENT**

28 This study supports that muscle ceramide do not play a key role in insulin resistance which developed
29 early with physical inactivity.

30

31 **ABSTRACT**

32 Physical inactivity increases the risk to develop type 2 diabetes, a disease characterized by a state of
33 insulin resistance. By promoting inflammatory state, ceramides are especially recognized to alter
34 insulin sensitivity in skeletal muscle. The present study was designed to analyze, in mice, whether
35 muscle ceramides contribute to physical inactivity-induced insulin resistance. For this purpose, we
36 used the wheel lock model to induce a sudden reduction of physical activity, in combination with
37 myriocin treatment, an inhibitor of *de novo* ceramide synthesis. Mice were assigned to 3 experimental
38 groups: voluntary wheel access group (Active), a wheel lock group (Inactive) and wheel lock group
39 treated with myriocin (Inactive-Myr). We observed that 10 days of physical inactivity induces
40 hyperinsulinemia and increase HOMA-IR. The muscle ceramide content were not modified by
41 physical inactivity and myriocin. Thus, muscle ceramides do not play a role in physical inactivity-
42 induced insulin resistance. In skeletal muscle, insulin-stimulated Akt phosphorylation and
43 inflammatory pathway were not affected by physical inactivity whereas a reduction of GLUT4 content
44 was observed. Based on these results, physical inactivity-induced insulin resistance seems related to a
45 reduction in GLUT4 content rather than defects in insulin signaling. We observed in inactive mice that
46 myriocin treatment improved glucose tolerance, insulin-stimulated Akt, AMPK activation and GLUT4
47 content in skeletal muscle. Such effects occur regardless of changes in muscle ceramide content. These
48 findings highlight that myriocin could be a promising drug to improve glucose tolerance and insulin
49 sensitivity.

50 INTRODUCTION

51 Physical inactivity is now recognized as a global pandemic promoting the development of numerous
52 chronic diseases including coronary heart diseases, type 2 diabetes, cancer or dementia (Booth et al.,
53 2017; Pedersen, 2009). Each year, non-communicable chronic diseases kill 36 million people
54 worldwide, including 17.3 million of deaths due to cardiovascular diseases and 1.3 million due to
55 diabetes (Lee et al., 2012). Nine million of these deaths occur before 60 years old, while over 5.3
56 million deaths could be averted every year if all inactive people performed only 15 to 30 min/day of
57 moderate physical exercise (Lee et al., 2012). In addition to morbidity and premature mortality, the
58 economic burden of physical inactivity for national governments is estimated worldwide to 53.8
59 billion US dollars for health-care systems, and 13.7 billion US dollars for productivity losses (Ding et
60 al., 2016).

61 For the World Health Organization, a person is considered as physically inactive if he doesn't meet
62 any of these 3 criteria: 30 min of moderate-intensity physical activity on at least 5 days every week, 20
63 min of vigorous-intensity physical activity on at least 3 days every week, or an equivalent combination
64 achieving 600 metabolic equivalent (MET)-min per week (Hallal et al., 2012). Two main experimental
65 approaches are used to understand the metabolic disorders related to this deleterious behavior (Pierre
66 et al., 2016): 1) physical inactivity, induced by the reduction of the daily number of steps performed
67 (from 10000 to less than 5000) in humans (Knudsen et al., 2012; Krogh-Madsen et al., 2010; Reynolds
68 et al., 2015) and the wheel locked model in rodents (Roberts et al., 2012); 2) immobilization, induced
69 by hindlimb unloading in rodents and bed rest in humans. Even if the latter represents an interesting
70 approach to explore metabolic changes occurring with dramatic reduction of skeletal muscle activity
71 (Bergouignan et al., 2011), it can be considered as too extreme compared to what physically inactive
72 people really experience (Pierre et al., 2016; Roberts et al., 2012). In the present study, we thus choose
73 the wheel lock model to study physical inactivity in mice.

74 The physiological mechanisms responsible for the development of chronic diseases related to physical
75 inactivity has been deeply explored during the last decade (Booth et al., 2017; Gratas-Delamarche et
76 al., 2014; Pedersen, 2009; Pierre et al., 2016). Among the proposed mechanisms, insulin resistance
77 associated to chronic hyperinsulinemia is considered as a key triggering event promoting lipid storage
78 and obesity (Softic et al., 2012; Stumvoll et al., 2000), but also tumor growth and cancer (Tsujimoto et
79 al., 2017). Insulin resistance is clinically defined as the inability of a known quantity of exogenous or
80 endogenous insulin to increase glucose uptake and utilization. In humans, several studies reported that
81 a sudden reduction of physical activity causes whole-body insulin resistance after only few days
82 (Knudsen et al., 2012; Krogh-Madsen et al., 2010; Reynolds et al., 2015). In this context, the drastic
83 reduction of energy expenditure rapidly occurring in skeletal muscle is currently considered as the
84 primary event promoting insulin resistance (Booth et al., 2017). The related gain of fat mass and the
85 chronic low grade inflammatory state observed in inactive people are generally proposed as

86 responsible for insulin resistance development (Booth et al., 2017; Pedersen, 2009). However, insulin
87 resistance occurs after few days of physical inactivity whereas systemic inflammatory markers (e.g.
88 TNF- α , IL-6) or visceral fat mass generally increase after several weeks in both humans and rodent
89 experiments (Hamburg et al., 2007; Krogh-Madsen et al., 2010; Olsen et al., 2008; Rector et al.,
90 2008). Thus, adipose tissue and inflammation processes do not appear to be the culprits of early
91 insulin resistance.

92 Physical inactivity, whatever the experimental model, induces a shift in fuel metabolism in favor of
93 carbohydrate oxidation and in detriment of lipid oxidation, resulting in an accumulation of
94 intramuscular lipids (Bergouignan et al., 2006; Laye et al., 2009; Momken et al., 2011). Lipid
95 accumulation in skeletal muscle is well known to be related with insulin resistance, especially due to
96 their conversion into ceramides (Samuel and Shulman, 2012). Ceramides are bioactive mediators
97 involved in cell responses to stress, and their increase in skeletal muscle is known to induce insulin
98 resistance through the inhibitory phosphorylation of proteins of the insulin pathway including insulin
99 receptor substrate-1/2 (IRS1/2) and phosphatidylinositol-3-kinase, (PI3-kinase) (Chavez and
100 Summers, 2003). Ceramides are synthesized through both stimulation of sphingomyelinase-mediated
101 hydrolysis of membrane sphingomyelin, and *de novo* synthesis pathway consisting of the condensation
102 of palmitoyl-CoA with serine (Hannun and Luberto, 2000). Whereas accumulation of intramuscular
103 ceramides has been extensively explored in the context of obesity (Schmitz-Peiffer, 2010; Ussher et
104 al., 2010), their role in the onset of insulin resistance related to physical inactivity remains poorly
105 understood.

106 We hypothesized that physical inactivity rapidly increases ceramide synthesis in skeletal muscle,
107 which in turn would induce insulin resistance. Therefore, the present study was designed to analyze, in
108 mice, whether inhibition of ceramide synthesis prevents insulin resistance observed after a short period
109 of physical inactivity. For this purpose, we used: 1) the wheel lock model to study the effect of
110 physical inactivity; 2) myriocin, an inhibitor of *de novo* synthesis of ceramides (Salaun et al., 2016).

111

112 **MATERIAL AND METHODS**

113 All procedures described below were approved by the French Ministry of Higher Education and
114 Research in accordance with the local committee on Ethics in Research of Rennes (veterinary service
115 of health and animal protection, authorization 01259.03).

116 *Animal care and protocol.* To mimic the effects of physical inactivity in mice, we used the wheel lock
117 model first developed by Frank Booth's research group (Roberts et al., 2012). Twenty-two male
118 C57BL/6 mice were obtained at weaning (3 weeks) and allowed to acclimatize for 1 week. The
119 animals were housed in temperature controlled room ($21 \pm 2^\circ\text{C}$) with a 12-h:12-h light/dark cycle and
120 received standard rodent chow and water *ad libitum*. After 1 week, mice were separated into individual
121 cages all equipped with a voluntary running wheel outfitted with a lap counter (IntelliBio, Nancy,

122 France). Food intake and covered distance were daily noticed whereas body weight was recorded
123 every week. The distance daily covered increased the first two weeks and remained unchanged during
124 the next 4 weeks. At the end of this period, mice were assigned to 3 experimental groups with equal
125 mean and standard deviation for daily covered distance: voluntary wheel access group (Active, n=7), a
126 wheel lock group (Inactive, n=7) and wheel lock group treated with myriocin (Inactive-Myr, n=8).
127 Myriocin was daily injected (0.3 mg/kg, i.p) as previously described (Hojjati et al., 2005; Lee et al.,
128 2010). In the same schedule, Active and Inactive groups received saline vehicle. After 10 days of
129 physical inactivity, mice were sacrificed in an overnight fasting state. Mice were anesthetized with a
130 ketamine-xylazine-butorphanol cocktail. Adipose tissue, *rectus femoris* (RF) and right *tibialis anterior*
131 (TA) muscles were removed, and then immediately frozen in liquid nitrogen. Left *tibialis anterior*
132 muscles were removed, and then submitted to an *ex vivo* insulin sensitivity test. Immediately following
133 tissue harvest, mice were euthanized via exsanguination of the heart. Intracardiac blood was collected
134 into dry tubes and centrifuged (1,500 g, 10 min) for serum sampling.

135 *Glucose tolerance assessment.* Oral glucose tolerance test (OGTT) was performed on the morning one
136 day before sacrifice. Mice were fasted for 6h before the test. Then, glucose was administrated by oral
137 gavage (1 g/kg body weight). Glucose values were obtained at rest and 15, 30, 45, 60, 90, 120 min
138 after glucose gavage from tail blood samples. Glucose level was determined using a glucose meter
139 (Freestyle Papillon Vision). Wheels were locked during the whole time of fasting and OGTT.

140 *Ex vivo muscle insulin sensitivity test.* As previously described (Tardif et al., 2011), left *tibialis*
141 *anterior* muscles were longitudinally divided in two strips (20-25 mg) and each strip was pre-
142 incubated for 30 min in 3 mL of modified Krebs Ringer buffer (120 mM NaCl, 4.8 mM KCl, 25 mM
143 NaHCO₃, 2.5 mM CaCl₂, 1.24 mM NaH₂PO₄, 1.25 mM MgSO₄, 8 mM D-Glucose, 2 mM sodium
144 pyruvate, 2 mM HEPES, pH 7.4) saturated with a 95% O₂ and 5% CO₂ mix at 37°C under stirring.
145 One of the two strips was stimulated with 20 nM insulin for 30 minutes, then muscle samples were
146 frozen in liquid nitrogen until analysis. Muscle insulin sensitivity was assessed by measuring the
147 phosphorylation state of Akt on serine 473, an intermediate of the insulin pathway.

148 *Serum parameters.* Glucose concentration was performed using Automated Beckman Coulter
149 (Beckman Coulter, Brea, CA). Serum insulin concentrations were measured by enzyme-linked
150 immunosorbent assay (ELISA) according to manufacturer's instructions (Millipore, St Louis, MO,
151 USA). Insulin sensitivity was determined by calculating HOMA-IR according to the following
152 formula (Matthews et al., 1985): $HOMA-IR = [fasting\ glucose\ (mmol/l)] \times [fasting\ insulin\ (\mu U/ml)] / 22.5$.
153

154 *Quantification of muscle triglycerides.* Muscle triglycerides were determined by using DiaSys kit
155 (Diagnostic System, Grabels, France) following a preliminary organic phase extraction according to
156 Bligh & Dyer's method (Bligh and Dyer, 1959). Briefly, 30 mg of right *tibialis anterior* samples were

157 crushed with 300 μ L of 150 mM sodium chloride. Then 150 μ L of muscle homogenates were
158 extracted with 600 μ L of a methanol-chloroform mixture (1:1, v/v). The organic layers were collected
159 after centrifugation (10,000g for 10 min) and dried under nitrogen. Dry samples were reconstituted in
160 37.5 μ L of isopropanol/acetonitrile/water mixture (2:1:1, v/v/v) and 10 μ L were analyzed according to
161 the manufacturer recommendations.

162 *Quantification of muscle sphingolipids and ceramides.* Sphingolipids were extracted from \approx 30 mg of
163 homogenized *tibialis anterior* muscles using acidified cyclohexane/isopropanol mixture (60:40, v/v,
164 0.1% formic acid) and purified on NH₂ SPE cartridges (silica gel cartridges, 100 mg) to obtain distinct
165 fractions of ceramides and sphingomyelins (Bodennec et al., 2000). The sphingolipid fractions were
166 then quantified by UHPLC-ESI-MS/MS using an Acquity H-Class UHPLC system (Waters, Milford,
167 MA) combined with a Waters Xevo TQD triple quadrupole mass spectrometer. Lipid extracts were
168 injected onto a C18 BEH column (2.1 mm x 50.0 mm, 1.7- μ m particles; Waters) held to 43°C to
169 separate all species of ceramides and sphingomyelins with two different LC elution gradients. For
170 ceramide species separation, the gradient started at 95% of eluent B (mobile phase of water and of
171 methanol with 1% formic acid and 5 mM ammonium formate), up to 98% in 4 min, then rapidly
172 decreased to 95% for 0.1 min, and was maintained for 2 min. For sphingomyelin species separation,
173 the gradient started at 95% of eluent B, up to 99% for 6 min, then rapidly decreased to 95% for 0.1
174 min, and was maintained for 1.4 min. Multiple reaction-monitoring mode in positive electrospray
175 ionization was used to quantify each species of ceramides and sphingomyelins. The source heater
176 temperature hold at 150°C, and the capillary voltage was set at 3.2 kV. The flow rate of desolvation
177 gas was of 650 l/h at 350°C, and the cone voltage varied from 26–58 V. Argon was used as the
178 collision gas, and collision energies varied from 12–40 eV. Data analyses were performed by Mass
179 Lynx software version 4.1 (Waters, Manchester, UK). Sphingolipid quantification was possible with
180 calibration curves constructed by plotting the peak area ratios of analyses to the respective internal
181 standard against concentration using a linear regression model. The quantification measurements were
182 performed using the TargetLinks software (Waters).

183 *RNA extraction and quantitative real-time PCR.* Total RNA extraction from frozen *rectus femoris* was
184 performed with Trizol® (Invitrogen, France) according to the manufacturer's instructions. The RNA
185 quality and quantity were assessed by FlashGel DNA System® and Nanodrop® spectrophotometry,
186 respectively. Reverse transcription was performed on a T100 Thermal Cycler (Bio-Rad) with iScript™
187 cDNA synthesis kit (Bio-Rad) from 1 μ g total RNA. Real time PCR experiments were done on a
188 CFX96 Real Time System (Bio Rad). Samples were analyzed in duplicate in 10 μ l reaction volume
189 containing 4.8 μ l IQ™ Sybr® GreenSuperMix (Bio-Rad), 0.1 μ l of each primer (100 nM final) and 5 μ l
190 of diluted cDNA. The following primer sequences were used: GLUT4: (F:
191 GCCTGCCCGAAAGAGTCTAA and R: CATTGATGCCTGAGAGCTGTTG); PPIA (F:
192 CGTCTCCTTCGAGCTGTTG and R: CCACCCTGGCACATGAATC); HPRT (F:

193 AGGCCAGACTTTGTTGGATTT and R: CAGGACTCCTCGTATTTGCAG); RPL19 (F:
194 CAATGCCAACTCTCGTCAACAG and R: CATCCAGGTCACCTTCTCGG). GLUT4 was
195 normalized using three reference genes (PPPIA, HPRT, RPL19) according to geNorm analysis
196 (Vandesompele et al., 2002).

197 *Western Blotting.* Cytosolic protein extraction was performed from *rectus femoris* muscle in cold lysis
198 buffer containing 10 mM Tris-HCl, pH 7.4, 0.5 M sucrose, 50 mM NaCl, 5 mM EDTA, 30 mM
199 Na₄P₂O₇, 1% NP-40, 0.25% sodium deoxycholate, 50 mM NaF, 100 μM sodium orthovanadate and
200 proteases inhibitors cocktail (Sigma P8340, 5 μl/ml). The samples were homogenized using a Polytron
201 homogenizer at 4°C. Each sample was then incubated on ice for 30 min followed by 3 x 10 s of
202 sonication. The homogenates were then centrifuged at 12,000g for 12 min at 4°C. The protein
203 concentration of the supernatant was determined by a Lowry assay using bovine serum albumin (BSA)
204 as standard. Samples were then diluted in SDS-PAGE sample buffer [50 mM Tris-HCl, pH 6.8, 2%
205 SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue], and heated 5 min at 95°C
206 until analyses. Fifty micrograms of proteins were resolved on 12.5% SDS-PAGE. The proteins were
207 transferred at 240 mA for 90 min onto a 0.2-μm nitrocellulose membrane. Membranes were blocked
208 with 5% BSA or nonfat dry milk in TBST (Tris-buffered saline - 0.05% Tween-20) for 1 h at room
209 temperature. Membranes were incubated overnight at 4°C with appropriate primary antibodies: AKT
210 (1:1000, Cell Signaling), p-AKT^{Ser473} (1:1000, Cell Signaling), AMPK (1:1000, Cell Signaling), p-
211 AMPK^{Thr172} (1:1000, Cell Signaling), p65 (1:1000, Cell Signaling), p-p65^{Ser536} (1:1000, Cell
212 Signaling), IRS-1 (1:1000, Cell Signaling), pIRS-1^{Ser302} (1:1000, Cell Signaling), STAT3 (1:1000, Cell
213 Signaling), p-STAT3^{Ser727}, IκBα (1:1000, Cell Signaling), GLUT4 (1:1000, Abcam), and α-actin
214 (1:700, Sigma Aldrich). Thereafter, membranes were washed with TBST and incubated for 1 h at
215 room temperature with infrared dye-conjugated secondary antibodies (LI-COR, Lincoln, NE, USA).
216 After washing, blots were captured using the Odyssey Imaging System (LI-COR). All blots were
217 scanned, densitometric analysis of the bands was conducted using GS-800 Imaging densitometer and
218 QuantityOne software. Phosphospecific signal was normalized to the total signal to estimate the ratio
219 of activated markers.

220 *Statistical analysis.* Data are presented as mean ± SEM. Normality and equality of variances were
221 checked using a Kolmogorov-Smirnov and Fischer test, respectively. A one-way analysis of variance
222 (ANOVA) was performed to compare each parameter between the 3 experimental groups. When
223 appropriate, the Fisher LSD test was used as a post-hoc analysis. If normality and/or equal variance
224 tests failed, we checked the significance using one-way ANOVA on ranks (Kruskal-Wallis). When
225 appropriate, the Dunn's test was used as a post-hoc analysis. For all statistical analyses, the
226 significance level was set at 0.05. Data were analyzed using the statistical package GraphPad Prism
227 version 6.02 for Windows (GraphPad Software, La Jolla, California).

228

229 RESULTS

230

231 *Physical activity levels, body weight, food intake and visceral fat mass.* During the 10 days of wheel
232 lock, active mice exhibited a mean daily physical activity levels of 3.90 ± 0.78 km/day. Daily food
233 intake was lower in both Inactive and Inactive-Myr mice compared to Active mice (-7.9% and -7.6%,
234 $p=0.007$ and 0.01 , respectively, Table 1). After 10 days of wheel lock, body weight significantly
235 increased only in Active mice. No significant difference of body weight was observed between the 3
236 experimental groups before and after the 10 days of physical inactivity. Visceral fat mass did not differ
237 between the 3 experimental groups at the end of the protocol (Table 1).

238 *Muscle triglycerides and ceramides content.* We first observed that muscle triglyceride (TG) content
239 tend to increase in Inactive mice ($p=0.09$, Fig. 1A) whereas it increase in the Inactive-Myr group
240 ($p=0.027$, Fig. 1A). Contrary to our hypothesis, physical inactivity did not modify total, saturated and
241 unsaturated ceramides content in muscle (Fig. 1B). Individual ceramides species ranging from C16:0
242 to C24:1 remained also unchanged after 10 days of physical inactivity (Fig. 1C and 1D). Surprisingly,
243 muscle ceramides content were not affected by 10 days of myriocin treatment (Fig. 1B, 1C and 1D).

244 *Whole-body insulin sensitivity and glucose tolerance.* The effect physical inactivity and myriocin
245 treatment on whole-body insulin sensitivity was assessed by measuring the circulating level of glucose
246 and insulin. Fasting glycemia remained unaffected with physical inactivity and myriocin treatment
247 (Fig. 2A). Serum insulin levels were higher in both Inactive and Inactive-Myr groups compared to
248 Active group ($p=0.025$ and 0.015 , respectively, Fig. 2B). Such results were associated with a higher
249 HOMA-IR index in Inactive compared to Active mice ($p=0.045$, Fig. 2C), whereas this index did not
250 differ between Inactive-Myr and Active mice ($p=0.17$, Fig. 2C). Thus, myriocin seems to prevent
251 physical inactivity-induced whole-body insulin resistance. An OGTT was also performed at the end of
252 the protocol to observe the effects of physical inactivity and myriocin on glucose tolerance. Here, we
253 did not observe significant difference in glucose concentrations (at all time points) and area under the
254 curve (AUC) between Active and Inactive mice (Fig. 2D and E). However, we reported that Inactive-
255 Myr mice exhibited an AUC significantly lower compared to both Active ($p=0.019$, Fig. 2D) and
256 Inactive mice ($p<0.001$, Fig. 2D). This effect was due to a lower glucose levels both at the beginning
257 and the end of OGTT (15 min, 30 min, 90 and 120 min, $p<0.05$, Fig. 2E). Taken together, our results
258 indicate that myriocin improves glucose tolerance.

259 *Proximal insulin signaling and GLUT4 content in skeletal muscle.* We observed a deleterious effect of
260 physical inactivity on whole-body insulin sensitivity and a beneficial effect of myriocin on glucose
261 tolerance. To investigate the mechanism regulating such effects, we first evaluated the integrity of
262 insulin signaling in skeletal muscle by measuring, *ex vivo*, basal and insulin-stimulated Akt activation.
263 We observed that insulin stimulation increased phospho-Akt levels in Active ($p=0.019$), Inactive

264 ($p=0.029$) and Inactive-Myr ($p<0.001$, Fig. 3A). Whereas the magnitude of these responses did not
265 differ between Active and Inactive mice, Inactive-Myr mice exhibited a higher muscle activation of
266 Akt in response to insulin when compared to both Active and Inactive mice ($p=0.046$ and $p=0.036$,
267 respectively, Fig. 3A). Glucose uptake is recognized to be regulated by insulin signaling and GLUT4
268 pool in skeletal muscle (Pierre et al., 2016). In the present study, we observed that physical inactivity
269 reduced GLUT4 protein ($p=0.043$, Fig. 3B and 3E), but not GLUT4 mRNA (Fig. 3C). Interestingly,
270 we observed that myriocin treatment prevent the reduction of muscle GLUT4 protein induced by
271 physical inactivity ($p=0.005$, Fig. 3B). At the mRNA level, GLUT4 was reduced by myriocin in
272 Inactive-Myr compared to Inactive mice ($p=0.031$, Fig. 3B). As AMP-activated protein kinase
273 (AMPK) is recognized to regulate GLUT4 expression and it translocation to the membrane (McGee et
274 al., 2008), we decided to measure the levels of AMPK activation in skeletal muscle. We reported that
275 AMPK activation remained unchanged in Inactive compared to Active mice (Fig. 3D and E).
276 Interestingly, we showed that AMPK activation was higher in myriocin-treated mice compared to both
277 Active and Inactive mice ($p=0.031$ and $p=0.003$, respectively, Fig. 3D and 3E). In insulin-resistant
278 rodent models, chronic hyperphosphorylation of IRS1 on Ser³⁰² has been identified as playing a key
279 role in muscle insulin resistance (Morino et al., 2008). In our experiments, no significant change was
280 observed in the phosphorylation state of IRS1 on Ser³⁰² between the 3 experimental groups (Fig. 4A
281 and 4E). Muscle insulin resistance is also related to activation of NF- κ B and IL6/STAT3
282 inflammatory signaling pathways (Gratas-Delamarche et al., 2014). Here, we reported that physical
283 inactivity combined or not with myriocin treatment did not modulate phospho-p65 and I κ B α (Fig. 4C
284 and 4E), two well-recognized markers of NF- κ B activation (Christian et al., 2016). Similarly,
285 phosphorylation of STAT3 on Ser⁷²⁷ remained unchanged in the three experimental groups (Fig. 4B
286 and 4E).

287 **DISCUSSION**

288 Muscle ceramides are known to play a key role in the development of insulin resistance during high
289 fat nutritional intake, but their role in physical inactivity-induced insulin resistance are poorly
290 understood. Our data support that early insulin resistance observed with physical inactivity is not due
291 to ceramide accumulation but could be caused by a reduction in muscle GLUT4 content. We also
292 observed that 10 days of myriocin treatment in inactive mice improve glucose tolerance, but
293 surprisingly through a mechanism that appears independent of changes in muscle ceramide content.

294 Immobilization rapidly causes both in humans and rodents a reduction in fatty acid (FA) transport to
295 mitochondria and in mitochondrial FA β -oxidation, resulting in an accumulation of intramuscular
296 lipids (Bergouignan et al., 2006; Kwon et al., 2016; Laye et al., 2009; Momken et al., 2011; Salaun et
297 al., 2016). Saturated FA oxidation, including palmitate, are particularly reduced in skeletal muscle
298 during immobilization (Bergouignan et al., 2011). Interestingly, palmitate is well identified to increase

299 the expression of serine palmitoyltransferase 2 (SPT2), a key enzyme in ceramide biosynthesis
300 (Erickson et al., 2012). Ceramides have been identified as key bioactive sphingolipids in the
301 development of muscle insulin resistance, especially the C:16 and C:18 moieties, which are the most
302 abundant in skeletal muscle (Chung et al., 2017; Perreault et al., 2018). Based on previous data from
303 our laboratory and others showing in rodents that C:16 and C:18 ceramides increase in skeletal muscle
304 after 1 or 2 weeks of immobilization (Kwon et al., 2016; Salaun et al., 2016), we hypothesized that 10
305 days of physical inactivity induced by wheel lock will also cause muscle ceramide accumulation.
306 Herein, we show that muscle ceramides are not responsible of insulin resistance induced by physical
307 inactivity. Contrary to immobilization, we observed that total and saturated ceramide levels in muscle
308 remained unchanged after 10 days of physical inactivity. This was also the case for muscle TG which
309 are increase after immobilization (3, 50), while we reported only a trend with physical inactivity. All
310 together, these results support that immobilization and physical inactivity are not equivalent models to
311 study the effect of a reduction of energy expenditure.

312 In rodents, the wheel lock model is currently the closest model to mimic human physical inactivity
313 (Roberts et al., 2012). Using this model in mice, we observed that 10 days of physical inactivity are
314 sufficient to induce hyperinsulinemia and to increase the HOMA-IR, both considered as hallmarks of
315 insulin resistance (Singh and Saxena, 2010). These results are in accordance with previous studies
316 conducted in Sprague-Dawley or OLETF rats after 7 days of wheel lock (Rector et al., 2010, 2008;
317 Teich et al., 2017). Few data are available about the effects of the wheel lock model on glucose
318 tolerance. Teich and colleagues recently observed that 7 days of wheel lock were sufficient to affect
319 glucose tolerance in young Sprague-Dawley male rats (Teich et al., 2017). In the present study, we did
320 not report an effect on glucose tolerance after 10 days of wheel lock. Our results are in accordance
321 with data obtained in humans exposed to a reduction in daily number of steps during 5 or 14 days,
322 where no change in glucose tolerance was reported (Knudsen et al., 2012; Reynolds et al., 2015). All
323 together, these results sustain that the wheel lock model is a realistic experimental set-up to study, in
324 mice, human physical inactivity and its role in chronic diseases development.

325 Skeletal muscle being responsible of 80% of whole-body glucose uptake under insulin stimulation
326 (DeFronzo et al., 1985), early insulin resistance occurring with physical inactivity is mainly attributed
327 to alterations in skeletal muscle glucose uptake (Knudsen et al., 2012; Krogh-Madsen et al., 2010).
328 Interestingly, using the wheel lock model during only 2 days, Kump and Booth observed a reduction
329 of insulin-stimulated 2-deoxyglucose uptake in skeletal muscle (Kump and Booth, 2005). Similar
330 results were also reported in rats submitted to muscle unloading after 24h (Kawamoto et al., 2016;
331 O'keefe et al., 2004). However, the mechanisms by which physical inactivity and muscle unloading
332 rapidly induce muscle insulin resistance remain unclear. Krogh-Madsen and colleagues (2010) have
333 reported that a reduction of muscle Akt phosphorylation during hyperinsulinemic euglycemic clamp
334 occurred after 2 weeks of reduction of ambulatory activity, thus supporting the idea that insulin
335 signaling was early affected by physical inactivity. However, if the clamp is the gold standard to

336 assess peripheral insulin sensitivity, it appears less appropriate to assess insulin signaling. Indeed,
337 presence of circulating hormones or cytokines (e.g. leptin, adiponectin, IL-6) and muscle contractility
338 are all factors affecting muscle insulin signaling during clamp, independently of direct action of
339 insulin. For these reasons, we decided to explore insulin-stimulated muscle Akt phosphorylation in *ex-*
340 *vivo* conditions to determine whether insulin signaling was affected with a short period of physical
341 inactivity. In this condition, we observed that Akt phosphorylation in inactive mice did not differ from
342 active animals, suggesting that insulin signaling until Akt remains unaffected after 10 days of physical
343 inactivity. To support such results, we explored the effects of physical inactivity on inflammatory
344 pathways recognized to affect insulin signaling. Here, neither NF- κ B nor IL-6/STAT3 signaling
345 pathways were affected in skeletal muscle of inactive mice. Further, in response to pro-inflammatory
346 state IRS1 is inhibited by a phosphorylation on its Ser³⁰² (Gratas-Delamarche et al., 2014; Hage
347 Hassan et al., 2016; Morino et al., 2008). In the present study, we found that physical inactivity did not
348 modify the phosphorylation state IRS1^{Ser302}. Taken together, these results indicate that, in the context
349 of physical inactivity, the onset of insulin resistance is not due an inflammatory process affecting the
350 skeletal muscle.

351 Our data support that muscle insulin signaling does not appear affected by a short period of physical
352 inactivity. In accordance with Kump and Booth (2005), we reported that 10 days of wheel lock caused
353 a significant reduction of GLUT4 pool in skeletal muscle. These results support the idea that
354 inactivity-induced muscle insulin resistance would be rather related to a decrease of muscle GLUT4
355 pool than a defect in insulin signaling. Interestingly, we observed that mRNA coding for GLUT4 was
356 not affected suggesting that the decrease of GLUT4 pool would be due to an increase of its
357 degradation. It is well established that chronic insulin stimulation causes a decrease of GLUT4 content
358 in adipocytes, mainly due to an accelerated GLUT4 degradation in the lysosomes (Liu et al., 2007; Ma
359 et al., 2014, p. 4; Sargeant and Pâquet, 1993). Similar cellular events could occur in skeletal muscle of
360 inactive. Such mechanisms need to be explored in further experiments.

361 To explore the role of ceramides in physical inactivity-induced insulin resistance, we treated inactive
362 mice with myriocin, an inhibitor of *de novo* synthesis of ceramides. As previously reported in others
363 experimental models including Zucker diabetic rats or high fat diet-fed mice (Holland et al., 2007;
364 Ussher et al., 2010), we observed that myriocin treatment improved glucose tolerance in inactive mice.
365 Interestingly, we also reported that myriocin treatment caused a significant increase of AMPK
366 phosphorylation. This result is in accordance with the data of Liu and colleagues (2013) demonstrating
367 that myriocin prolonged yeast lifespan by activating key signaling pathways controlling stress
368 resistance and energy metabolic homeostasis including AMPK signaling pathway. Thus, the beneficial
369 effect of myriocin on glucose tolerance could be mediated by AMPK (Jensen et al., 2014).
370 Interestingly, we also observed higher insulin-stimulated Akt phosphorylation in myriocin-treated
371 compared to non-treated inactive mice. Such improvement of muscle insulin signaling could be linked
372 to AMPK activation since this kinase is recognized to enhance the response of Akt phosphorylation on

373 serine 473 residue through the modulation of MTORC2 complex activity (Kleinert et al., 2016;
374 Sarbassov et al., 2005). We also observed that myriocin prevents the decrease of GLUT4 induced by
375 physical inactivity. Although AMPK has been proposed to stimulate GLUT4 transcription (Gong et
376 al., 2011; McGee et al., 2008), we observed a decrease of GLUT4 mRNA associated with AMPK
377 activation in Inactive-My compared to Inactive mice. Consequently, an AMPK-independent
378 mechanism could be responsible of the effect of myriocin on muscle GLUT4 protein. To sum up, the
379 effect of myriocin on glucose tolerance seems implicated a coordination of AMPK, Akt and GLUT4 in
380 a manner that needs to be elucidated. Contrary to our hypothesis, the benefits of myriocin treatment on
381 glucose tolerance are not associated to modulation in muscle ceramide concentrations. The previous
382 studies reporting in mice a beneficial effect of myriocin on glucose tolerance and insulin sensitivity
383 were associated to a prevention of muscle ceramide accumulation induced by high fat diet or
384 pathological genetic background (Holland et al., 2007; Ussher et al., 2010; Yang et al., 2009). Herein,
385 we found that myriocin and physical inactivity act on insulin sensitivity independently from
386 ceramides, thus highlighting the need to explore other hypothesis.
387 In summary, our results support that muscle ceramide accumulation and inflammatory pathways do
388 not play a role in physical inactivity-induced insulin resistance. The insulin resistance observed after a
389 short period of physical inactivity seems related to a reduction in muscle GLUT4 content rather than to
390 defects of muscle insulin signaling. Our findings also support that immobilization is not what inactive
391 people experience, and that biological effects obtained with this kind of models should not associated
392 with physical inactivity. Finally, our data highlight that myriocin could be a promising molecule to
393 improve glucose tolerance and muscle insulin sensitivity.

394

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401

402 **DISCLOSURES**

403 No conflict of interest, financial or otherwise, are declared by the author(s).

404

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- 620
621

	Active	Inactive	Inactive-Myr
Body weight (g)			
Before locking	25.2 ± 0.5	25.8 ± 0.2	24.8 ± 0.2
10 days post-locking	25.8 ± 0.6 ^{\$}	25.9 ± 0.2	24.5 ± 0.3
Food intake (g/day)	4.16 ± 0.05	3.83 ± 0.09 [*]	3.84 ± 0.08 [*]
Physical activity (km/day)	3.90 ± 0.78	-----	-----
Visceral fat mass (g/100 g BW)	2.12 ± 0.19	2.30 ± 0.25	2.08 ± 0.15

622

623 **Table 1. Effects of physical inactivity and myriocin treatment on body weight, food intake and**
624 **visceral fat mass.** Data are presented as mean ± SEM. Significant differences (p < 0.05) are indicated
625 as follows: comparison vs. values before wheel locking (\$), comparison vs. Active group (*)

626 **Figure 1. Effects of physical inactivity and myriocin treatment on lipids profile.** Data are
627 presented as mean \pm SEM (n=7-8/group). *A*: triglycerides content. *B*: total, saturated, and unsaturated
628 ceramide content. *C*: unsaturated species profile. *D*: saturated species profile. Significant differences
629 are indicated as follows: comparison vs. Active group (*: p<0.05)

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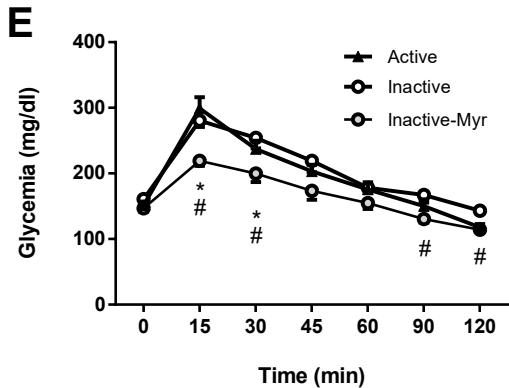
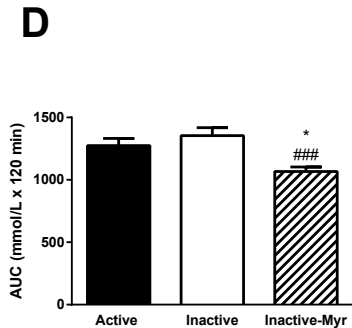
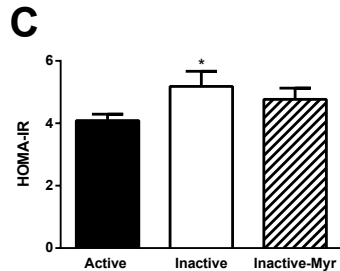
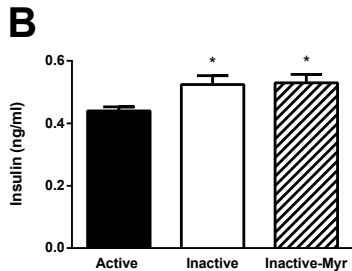
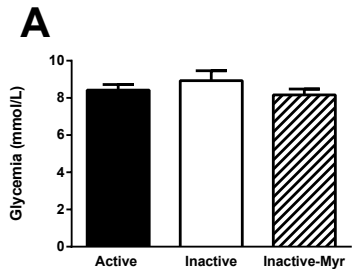
631 **Figure 2. Effects of physical inactivity and myriocin treatment on whole-body insulin sensitivity**
632 **and glucose tolerance.** Data are presented as mean \pm SEM (n=7-8/group). *A*: serum glucose
633 concentrations. *B*: serum insulin concentrations. *C*: HOMA-IR index. *D*: area under the curve (AUC)
634 for OGTT. *E*: evolution of glycemia following oral glucose load. Significant differences are indicated
635 as follows: comparison vs. Active group (*: p<0.05), comparison vs. Inactive group (###: p<0.001)

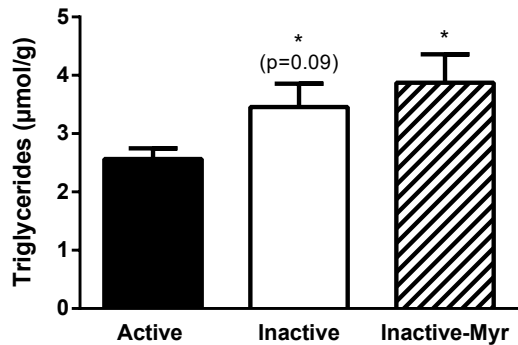
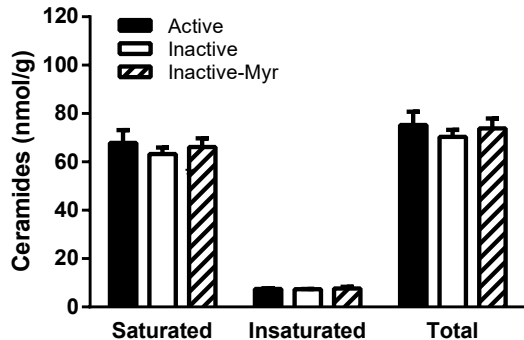
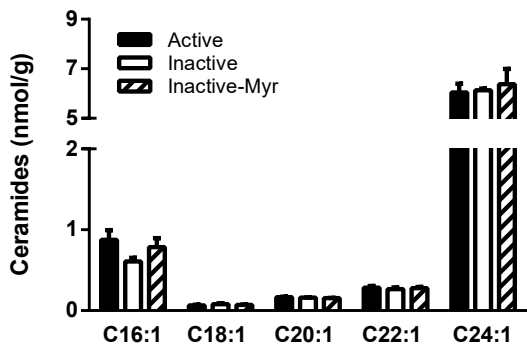
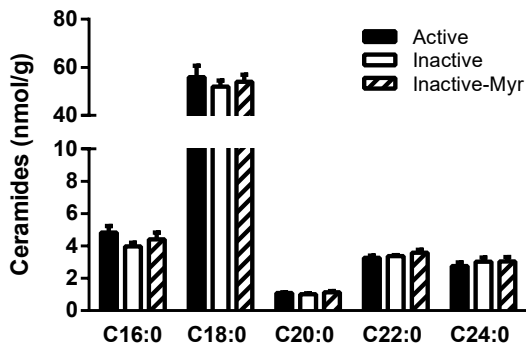
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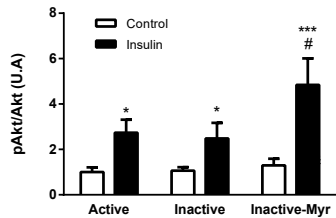
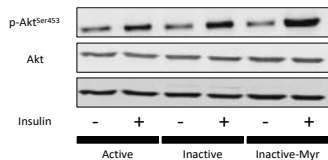
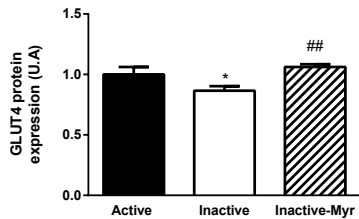
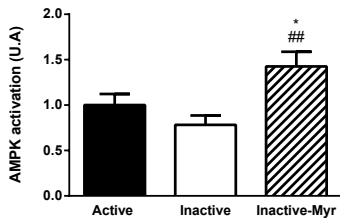
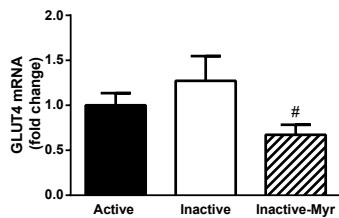
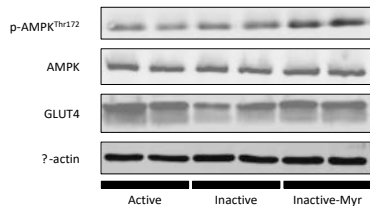
637 **Figure 3. Effects of physical inactivity and myriocin treatment on muscle insulin signaling.** Data
638 are presented as mean \pm SEM (n=7-8/group). *A*: Insulin-stimulated Akt activation in skeletal muscle of
639 different experimental groups. *B*: muscle GLUT4 protein content muscle. *C*: GLUT4 mRNA levels. *D*:
640 muscle AMPK activation. *E*: representative Western Blot of the data obtained in panel C and D.
641 Significant differences are indicated as follows: comparison vs. Active group (*: p<0.05), comparison
642 vs. Inactive group (#: p<0.05, ##: p<0.01)

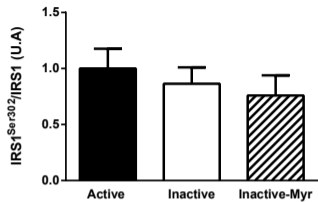
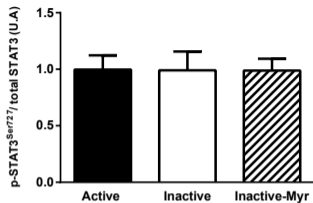
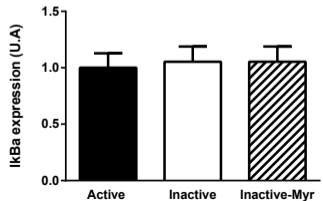
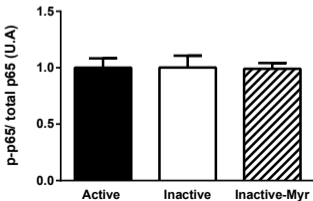
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644 **Figure 4. Effects of physical inactivity and myriocin treatment on signaling pathways involved in**
645 **muscle insulin resistance.** Data are presented as mean \pm SEM (n=7-8/group). *A*: IRS1^{Ser302} activation.
646 *B*: STAT3 activation. *C*: κ B α content. *D*: NF- κ B p65 activation. *E*: representative Western Blot
647 analyses of proteins involved in muscle insulin resistance.



A**B****C****D**

A**B****D****C****E**

A**B****C****D****E**