

## **Control of gluconeogenic genes during intense/prolonged exercise: hormone-independent effect of muscle-derived IL-6 on hepatic tissue and PEPCK mRNA.**

Sébastien Banzet, Nathalie Koulmann, Nadine Simler, Hervé Sanchez, Rachel Chapot, Bernard Serrurier, André Peinnequin, Xavier Bigard

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exercise: hormone independent effect of muscle-derived IL-6

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on hepatic tissue and PEPCK mRNA

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Banzet Sébastien, Koulmann Nathalie, Simler Nadine, Sanchez Hervé, Chapot Rachel,

7

Serrurier Bernard, Peinnequin André and Bigard Xavier.

8

Department of Human Factors,

9

Military health service research center (Centre de Recherches du Service de Santé des

10

Armées),

11

38702 La Tronche, France

12

13

Running head : IL-6 and exercise gluconeogenesis

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**Address for correspondence:**

16

Sébastien Banzet

17

CRSSA

18

Department of Human Factors

19

BP 87

20

38702 La Tronche Cedex

21

France

22

23

Phone : +334 76 63 97 67

24

Fax : +334 76 63 69 45

25

E-mail: [sbanzet@crssa.net](mailto:sbanzet@crssa.net)

26

## Abstract

27  
28 Prolonged intense exercise is challenging for the liver to maintain plasma glucose levels.  
29 Hormonal changes cannot fully account for exercise-induced hepatic glucose production  
30 (HGP). Contracting skeletal muscles release interleukin-6 (IL-6), a cytokine able to increase  
31 endogenous glucose production during exercise. However, whether this is due to a direct  
32 effect of IL-6 on liver remains unknown. Here, we studied hepatic glycogen, gluconeogenic  
33 genes and IL-6 signaling in response to one bout of exhaustive running exercise in rats. To  
34 determine whether IL-6 can modulate gluconeogenic genes mRNA independently of exercise,  
35 we injected resting rats with recombinant IL-6. Exhaustive exercise resulted in a profound  
36 decrease in liver glycogen and an increase in gluconeogenic genes mRNA levels,  
37 phosphoenolpyruvate-carboxykinase (PEPCK), glucose-6-phosphatase (G6P) and peroxisome  
38 proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), suggesting a key role for  
39 gluconeogenesis in hepatic glucose production. This was associated to an active IL-6  
40 signaling in liver tissue, as shown by signal transducer and activator of transcription (STAT-  
41 3) and CAAT/enhancer binding protein- $\beta$  (C/EBP $\beta$ ) phosphorylation and IL-6 responsive  
42 genes mRNA levels at the end of exercise. Recombinant IL-6 injection resulted in an increase  
43 in IL-6 responsive genes mRNA levels in the liver. We found a dose-dependent increase in  
44 PEPCK gene mRNA, strongly correlated with IL-6-induced genes mRNA levels. No changes  
45 in G6P and PGC-1 $\alpha$  mRNA levels were found. Taken together, our results suggest that during  
46 very demanding exercise, muscle-derived IL-6 could help increasing HGP by directly up  
47 regulating PEPCK mRNA abundance.

48  
49

50 During intense and prolonged exercise, working skeletal muscles are very dependent  
51 on carbohydrates to maintain ATP synthesis and contraction (37). In the absence of oral  
52 glucose intake, plasma glucose levels are maintained at relatively constant values through  
53 changes in hepatic glucose production (HGP). During moderate-intensity exercise, HGP is  
54 primarily controlled by portal venous insulin-to-glucagon ratio (44, 45), whereas during  
55 intense and sustained exercise, several studies have shown that hormonal changes cannot fully  
56 account for the increase in HGP, suggesting other unidentified factors are involved (9, 20). A  
57 new and original aspect of HGP control during intense exercise as been highlighted recently  
58 by a vast series of studies supporting the involvement of muscle-derived factor (for review see  
59 (32)).

60 Contracting skeletal muscles produce and release IL-6 in the plasma during prolonged  
61 sustained exercise (30, 39), and a strong relationship with low muscle glycogen content as  
62 been shown (25, 38). It has been proposed that this cytokine may be a signal of low muscle  
63 glycogen stores and, once released in the plasma, could favor hepatic glucose release (13).  
64 This hypothesis has been tested in an experiment were subjects exercised with an infusion of  
65 saline or recombinant IL-6. The rate of glucose appearance was significantly higher in the IL-  
66 6 group with no changes in gluco-regulatory hormones, suggesting IL-6 is involved in HGP  
67 increase during intense prolonged exercise (11). However, the mechanisms involved are  
68 unknown and the question was asked whether this effect was due to a direct effect of IL-6 on  
69 hepatic tissue or was secondary to IL-6-induced increase in whole-body glucose disposal (11).

70

71 Exercise-induced increase in HGP results from coordinated changes in glycogenolysis  
72 and gluconeogenesis (GNG) under complex hormonal and nervous control (8, 26). Liver  
73 glycogenolysis starts at the onset of exercise and is the main source of glucose during the first  
74 30 min of exercise (26). Thereafter, hepatic gluconeogenic flux increases and GNG becomes

75 a key contributor to HGP (44). GNG is controlled at the transcriptional level by unidirectional  
76 key enzymes, phosphoenolpyruvate-carboxykinase (PEPCK), fructose 1,6 bisphosphatase and  
77 glucose-6 phosphatase (G6Pase) the activities of which increase during exercise (10).  
78 PEPCK, the most studied gluconeogenic enzyme, plays an important role during exercise  
79 since its pharmacologic inhibition reduces endurance time in rats (22, 42). PEPCK activity is  
80 hormonally controlled at the transcriptional level (16) and exercise induces an important  
81 increase in hepatic PEPCK mRNA levels and enzyme activity (14, 44). Among the well-  
82 described regions of PEPCK gene promoter, the glucocorticoid response unit (GRU) and two  
83 cAMP regulatory element (CRE) are involved in exercise-related gene expression, since their  
84 mutation substantially decreased hepatic PEPCK mRNA levels (14, 29). However, the  
85 molecular mechanisms controlling PEPCK gene expression in response to exercise is still not  
86 fully understood. Recently, peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-  
87 1 $\alpha$ ) has been shown to play a pivotal role in hepatic GNG regulation and PEPCK gene  
88 transcription in response to food deprivation (33, 46). An increase in PGC-1 $\alpha$  mRNA levels  
89 has been reported recently in exercised mice (19), however its role in hepatic GNG is  
90 unknown.

91 IL-6 binds to a specific receptor, IL-6 receptor  $\alpha$  (IL-6R $\alpha$ ) that cooperates with a non-  
92 specific subunit, glycoprotein 130 (gp130). The signal transduction occurs through either the  
93 janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, or a  
94 Ras/extracellular signal-regulated protein kinase (ERK)/CAAT enhancer binding protein  
95 (C/EBP)  $\beta$  pathway (for review see (23)). C/EBP family members can trans activate PEPCK  
96 promoter via the CRE binding sites (16, 31). C/EBP $\beta$ , also known as nuclear factor of IL-6  
97 (NF-IL6), has been proposed to play a role in PEPCK gene transcription during exercise (29).  
98 Because this transcription factor is regulated by IL-6 at the transcriptional level and is a  
99 downstream signaling molecule of activated IL-6 receptor (reviews in (1, 35)), muscle-

100 derived IL-6 could participate in hepatic C/EBP $\beta$  activation during exercise. Whether IL-6  
101 can act directly on hepatic tissue to favor PEPCK gene transcription during exercise has to be  
102 explored.

103         The present study focused on prolonged and intense exercise to further understand  
104 glucose homeostasis in very demanding physiologic situations. Because plasma IL-6  
105 concentration is a function of exercise intensity and duration (32) and because muscle-IL-6  
106 release is concomitant of GNG increase, we hypothesized that this cytokine could act directly  
107 on hepatic tissue to help increasing HGP through GNG. Our aims were 1) to determine if  
108 there is an active IL-6 signaling in the liver of animals exposed to one bout of prolonged  
109 intense exercise; 2) to determine if IL-6, can directly modulate gluconeogenic genes mRNA  
110 abundance, independently of exercise and glucoregulatory hormones. Therefore, we studied  
111 liver IL-6 signaling and gluconeogenic genes mRNAs at the end of one bout of exhaustive  
112 exercise in rats. To evaluate the ability of IL-6 to directly activate gluconeogenic genes  
113 independently of exercise, we studied gluconeogenic genes modulation in response to  
114 recombinant IL-6 injection in resting animals.

115

## 116 **Research Design and Methods**

117

### 118 **Ethics**

119 Experiments were carried out in accordance with the Helsinki Accords for Human Treatment  
120 of Animals during Experimentation, and received prior approval from the animal ethics  
121 committee for animal research of the Centre de Recherche du Service Santé des Armées (La  
122 Tronche, France).

123

### 124 **Animals**

125 Female Wistar rats (175-200 g) were purchased from Charles River (L'Arbresle, France).  
126 They were housed at  $22 \pm 2^{\circ}\text{C}$ , on a 12–12 h light–dark period, and provided with food and  
127 water *ad libitum*. For the exercise experiment they were randomly assigned to four  
128 experimental groups: resting rats (Rest,  $n=8$ ) and 3 active groups, performing one bout of  
129 exercise and studied at the end of exercise (Ex,  $n=8$ ), 2 hours (Ex+2h,  $n=8$ ) or 6 hours  
130 (Ex+6h,  $n=8$ ) later.

131

### 132 **Exercise protocol**

133 All animals were accustomed to running on a rodent treadmill for 15-20 min per day for 5  
134 days ( $10\text{--}20 \text{ m}\cdot\text{min}^{-1}$ ;  $0^{\circ}$  grade). After 4 days at rest, active animals ran on the treadmill ( $22$   
135  $\text{m}\cdot\text{min}^{-1}$ ,  $5^{\circ}$  grade) till exhaustion.

136

### 137 **IL-6 injection**

138 Animals were fasted overnight and injected intraperitoneally with 3 or  $10 \mu\text{g}\cdot\text{kg body weight}^{-1}$   
139 human recombinant IL-6 (Biosource, Camarillo, CA, USA) and compared to control rats  
140 injected with endotoxin free PBS (Cont). Animals were sacrificed 1 hour after injection.

141

### 142 **Tissue processing**

143 Resting animals were sacrificed at different time corresponding to the other groups. After  
144 exercise, rats from Ex+2h and Ex+6h groups had free access to food and water. Animals were  
145 anaesthetized with pentobarbital (70 mg.kg body weight<sup>-1</sup> *ip*). Liver was sampled and a part  
146 was frozen in liquid nitrogen and stored at -80°C for protein and glycogen quantification,  
147 another part was placed in 1ml RNAlater (Ambion, Austin, TX, USA), kept at 4°C for 24 h  
148 and then frozen at -20°C for mRNA study. Whole blood was sampled, centrifuged and  
149 plasma was frozen at -80°C. Animals were killed by removal of the heart.

150

### 151 **RNA extraction**

152 Hepatic tissue mRNA were isolated using the MagNA Pure LC instrument (Roche Applied  
153 Science, Mannheim, Germany). 150 to 200 mg of liver were disrupted in a volume  
154 corresponding to 6,66 µl MagNA Pure LC mRNA isolation kit II lysis buffer (Roche Applied  
155 Science, Mannheim, Germany) per mg of tissue, with a Polytron mixer (Kinematica,  
156 Switzerland). Lysate was centrifuged and 50µl was transferred in a fresh tube and completed  
157 to a 300-µl final volume with lysis buffer. Extraction was then performed following the  
158 manufacturer's protocol.

159

### 160 **Reverse transcription**

161 Reverse transcription was carried out using the Reverse Transcriptase Core Kit (Eurogentec,  
162 Seraing, Belgium). The reaction was performed following the manufacturer's instructions.  
163 The reaction mix contained 1 µl buffer (10X), 2 µl dNTP (2.5 mM each dNTP), 0.5 µl oligo-  
164 dT primer (50 µM), 0.2 µl RNase inhibitor (20 units.µl<sup>-1</sup>), 2 µl MgCl<sub>2</sub> (25 mM), 0.25 µl  
165 Euroscript reverse transcriptase (50 U.µl<sup>-1</sup>), 1.05 µl RNase free water and 3 µl template  
166 mRNA.

167

### 168 **Real-time PCR**



169 The PCR reactions were carried out in a 20 $\mu$ l final volume with the LC Fast Start DNA  
170 Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 2  $\mu$ l of cDNA  
171 solution (1/20 dilution). Quantitative PCRs were performed using (Roche Applied Science,  
172 Mannheim, Germany) with 50 cycles of 95 °C for 20 s, annealing temperature (table 1) for 5 s  
173 and 72 °C for 8 s. Quantification was performed with the comparative threshold cycle method  
174 (28) using RelQuant software (Roche Diagnostics), with a pool of cDNA samples as  
175 calibrator. Five housekeeping genes were necessary to validate the normalization using  
176 genorm software (43). These were Hypoxanthine-guanine phosphoribosyl transferase  
177 (HPRT), cyclophilin A (CycA), acidic ribosomal binding protein (ARBP), actin  $\beta$  and  
178 glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Primers characteristics are provided  
179 as supplemental data. The final quantification was performed using the geometric averaging  
180 of the five quantifications. In each experiment, all samples were treated at the same time for  
181 mRNA extraction, reverse transcription and PCR reaction.

182

### 183 **Western blotting**

184 Liver sections were homogenized in ice-cold lysis buffer (20  $\mu$ l.mg<sup>-1</sup>) containing 20 mM  
185 HEPES, pH 7.4, 2 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol, 1 mM  
186 Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, 1  $\mu$ l.ml<sup>-1</sup> Protease inhibitor cocktail set III  
187 (Calbiochem, Fontenay-sous-bois, France). Samples were rotated for 1 h at 4°C and  
188 centrifuged (15,000 g, 15 min, 4°C). Protein content was determined using the BCA method  
189 (Roche/Hitachi 912 instrument, Roche Diagnostics, Mannheim, Germany).

190 Each sample (50  $\mu$ g of total proteins) was separated by SDS-PAGE (10% resolving gel).  
191 Proteins were transferred electrophoretically on nitrocellulose membranes (Hybond C-extra  
192 RPN 2020E, Amersham, Orsay, France). Membranes were blocked with non-fat milk in Tris-  
193 buffered-Saline solution added with 0.1% Tween 20 (TBST). Blots were incubated overnight  
194 at 4°C with anti-phospho-STAT-3 (Ser727) or anti-phospho-C/EBP $\beta$  (Ser105) antibody (Cell

195 Signaling Technology, Beverly, MA, USA), rinsed with TBST and incubated with horse  
196 radish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology, Santa  
197 Cruz, CA, USA) for 1h30. Blots were subjected to Enhanced chemiluminescence reagent kit  
198 (Amersham, Orsay, France) and exposed to Hyperfilm ECL RPN 3103K (Amersham, Orsay,  
199 France). Membranes were then stripped in a solution containing 60 mM Tris-HCL, pH 7.4,  
200 5% SDS and 0.07%  $\beta$ -mercapto ethanol, for 30 min at 50°C, washed with TBST, and  
201 incubated overnight with anti STAT-3 antibody or C/EBP $\beta$  (Cell Signaling Technology,  
202 Beverly, MA, USA) and treated as described above. The bands obtained were quantified  
203 using a densitometer (GS-700, Bio-Rad, Marnes-la-Coquette, France).

204

#### 205 **Liver and muscle glycogen content**

206 Liver or plantaris muscle sections (30 mg or 20 mg respectively) were disrupted in NaOH (2  
207 M). Samples were submitted to amyloglucosidase (Sigma-Aldrich, Steinheim, Germany) in  
208 acetate buffer (0.3 M) for 2 hours at 37°C to digest glycogen. Released glucose was  
209 quantified by spectrophometric measurement (340 nm) of NADH production in the presence  
210 of hexokinase and glucose-6-phosphate dehydrogenase (Sigma-Aldrich, Steinheim,  
211 Germany), as previously described (4).

212

#### 213 **Blood glucose**

214 Blood glucose was measured according to the glucose oxidase methods with Glucose GOP-  
215 PAP kit on Roche/Hitachi 912 instrument (Roche Diagnostics, Mannheim, Germany).

216

#### 217 **IL-6 protein**

218 Plasma IL-6 levels were measured using the Quantikine colorimetric sandwich ELISA kit for  
219 rat IL-6 (R&D systems, Lille, France) in exercise experimentation and sandwich ELISA kit  
220 for human IL-6 (R&D systems, Lille, France) in recombinant IL-6 injected rats.

221

## 222 **Hormones**

223 Plasma hormones were measured using BioPlex Instrument (Biorad, France) with rat endo kit  
224 (Millipore, France) for glucagon and insulin, rat stress kit for corticosterone.

225

## 226 **Statistical analysis**

227 All data are presented as means  $\pm$  S.E.M. A one factor ANOVA was used to evaluate the  
228 global effects of exercise and recovery, as well as recombinant IL-6 injection. When  
229 appropriate, the Newman-Keuls *post hoc* test was used for inter-group comparisons. Animals  
230 weight and running time values were compared using an unpaired Student *t* test. The  
231 significance level for all comparisons was set at  $P < 0.05$ .

232

233

## 234 **Results**

### 235 **Liver response to exercise**

236 *Endurance time:* Mean endurance time was 123±10 min with no differences between groups  
237 (data not shown).

238  
239 *Carbohydrate metabolism:* Plasma glucose was lower in Ex group than Rest and Ex+2h (8.25  
240 ± 0.33; 6.71 ± 0.51 and 8.26 ± 0.34 mmol.l<sup>-1</sup> respectively, data not shown) ( $P<0.05$ ). Plantaris  
241 muscle glycogen content decreased immediately at the end of exercise (Rest: 24.11 ± 0.74;  
242 Ex: 3.37 ± 0.6 μmol glycosyl units.g<sup>-1</sup>, data not shown) ( $P<0.01$ ). There was a profound  
243 decrease in hepatic glycogen in response to exercise (main effect  $P<0.001$ ), with only 5% of  
244 the resting values at the end of exercise ( $P<0.001$ ). Liver glycogen increased during recovery,  
245 but was still 35% lower in Ex+6h than in Rest group ( $P<0.001$ ) (fig 1A).

246  
247 *Gluconeogenic genes:* Prolonged running exercise induced an increase in both PEPCK and  
248 G6Pase mRNA levels (main effects  $P<0.001$ ) with a maximum at the end of exercise  
249 ( $P<0.001$ ). Mean mRNA values decreased to values measured in Rest group as soon as 2h  
250 after exercise (fig 1B and 1C). Changes in PGC-1α mRNA levels exhibited a similar  
251 evolution ( $P<0.0001$ ) (fig 1D).

252  
253 *Glucoregulatory hormones:* Exercise resulted in a prolonged decrease in plasma  
254 insulin/glucagon ratio (main effect  $P<0.01$ ), values being lower than control in Ex and Ex+2h  
255 groups ( $P<0.05$ ) (fig 2A). Plasma corticosterone concentrations increased in response to  
256 running (main effect  $P<0.05$ ), peaking at the end of exercise (fig 2B). Concentrations  
257 increased 6h after exercise ( $P<0.05$ ) (fig 2B).

258  
259 *Plasma IL-6:* Exercise induced an increase in plasma IL-6 protein (main effect  $P<0.05$ )  
260 peaking at the end of exercise ( $P<0.01$ ). Plasma IL-6 concentrations decreased to values  
261 measured in resting animals as soon as 2h after exercise (fig 3A).

262  
263 *IL-6 signaling in liver tissue:* Liver STAT-3 and C/EBPβ phosphorylation ratio significantly  
264 increased in response to exercise (global effects  $P<0.05$ ) (fig 3B and C), with a maximum at  
265 the end of exercise ( $P<0.05$  and  $P<0.01$  respectively).

266 Hepatic mRNA levels for SOCS-3 and C/EBP $\beta$ , two IL-6 responsive genes, substantially  
267 increased (main effects  $P<0.001$ ) peaking at the end of exercise ( $P<0.001$ ) (fig 4A and B).  
268 SOCS-3 mRNA levels were still slightly elevated at Ex+2h compared to resting values  
269 ( $P<0.05$ ). Interestingly, we found a 3.5 fold increase in IL-6R mRNA at the end of exercise  
270 ( $P<0.05$ ) (fig 4C).

271

272 *Correlation between PEPCK and IL-6-induced genes mRNAs in Rest an Exercise groups:* We  
273 found significant correlations between PEPCK mRNA levels and two IL-6-induced genes  
274 mRNA levels, SOCS-3 ( $R^2= 0.5546$  ;  $P<0.05$ ) (fig 7A) and IL-6R ( $R^2 = 0.7707$  ;  $P<0.01$ ) (fig  
275 7B).

276

### 277 **Liver response to recombinant IL-6**

278 *IL-6 responsive genes in liver tissue:* Recombinant IL-6 injection resulted in a dose dependent  
279 peak of recombinant IL-6 plasma levels (fig 5A) ( $P<0.01$ ) and induced an increase in both  
280 SOCS-3 and IL-6R mRNA levels ( $P<0.01$  and  $P<0.05$  respectively) (fig 5B and C).

281

282 *Gluconeogenic genes:* Recombinant IL-6 injection induced a dose dependent increase in  
283 hepatic PEPCK mRNA levels (main effect  $P<0.01$ ) (fig 6A). No effects of IL-6 injection was  
284 found on G6Pase and PGC-1 $\alpha$  mRNA levels (fig 6B and C).

285

286 *Plasma glucose and gluco-regulatory hormones:* Recombinant IL-6 had no effect on plasma  
287 glucose (data not shown). There were no changes in plasma insulin/glucagon ratio ( $15.07 \pm$   
288  $3.42$ ,  $13.03 \pm 2.94$  and  $14.03 \pm 1.69$  for Cont,  $3 \mu\text{g.kg}^{-1}$  and  $10 \mu\text{g.kg}^{-1}$  groups, respectively)  
289 and corticosterone was unchanged ( $252.27 \pm 38.24 \text{ ng.ml}^{-1}$ ,  $254.81 \pm 35.12 \text{ ng.ml}^{-1}$  and  
290  $247.48 \pm 49.16 \text{ ng.ml}^{-1}$  for Cont,  $3 \mu\text{g.kg}^{-1}$  and  $10 \mu\text{g.kg}^{-1}$  groups, respectively).

291

292 *Correlation between PEPCK and IL-6-induced genes mRNAs:* We found significant  
293 correlations between PEPCK mRNA levels and two IL-6-induced genes mRNA levels,  
294 SOCS-3 ( $R^2= 0.5764$  ;  $P<0.01$ ) (fig 7C) and IL-6R ( $R^2 = 0.6094$  ;  $P<0.01$ ) (fig 7D).

295

296

## 297 **Discussion**

298           During prolonged intense exercise, a strong relationship between muscle IL-6  
299 production and carbohydrate availability has been described (32) and IL-6 is able to increase  
300 endogenous glucose production in exercising humans (11). However, the mechanisms  
301 involved in IL-6 action are unknown. In this work, we show that: 1) prolonged intense  
302 exercise is associated to an active IL-6 signaling in the liver, involving both STAT-3 and  
303 C/EBP $\beta$  pathways; 2) Recombinant IL-6 can acutely modulate hepatic PEPCK mRNA levels  
304 independent from exercise and gluco-regulatory hormones.

305           We previously reported that exhaustive running exercise protocol in rats induces an  
306 increase in muscle IL-6 transcription and in plasma IL-6 concentration (3). This was  
307 confirmed in the present study where plasma IL-6 peaked at the end of exercise and decreased  
308 as soon as 2 hours recovery, as described in human (30). However, whether such a transient  
309 increase in circulating IL-6 can induce an active IL-6 signaling in the liver has never been  
310 assessed. When IL-6 binds to its receptor, signal transduction can involve STAT-3 and/or  
311 C/EBP $\beta$  transcription factors to promote gene transcription (1). In the present study we show  
312 that in addition to increased hepatic C/EBP $\beta$  phosphorylation, STAT-3 phosphorylation  
313 substantially raised at the end of exercise. Therefore, plasma IL-6 concentration and IL-6-  
314 activated transcription factors phosphorylation followed the same time course. When  
315 phosphorylated, both transcription factors are translocated in the nucleus (1), and to determine  
316 if transcription factors activation was effective in altering transcription in our experiment, we  
317 measured hepatic mRNA levels for IL-6-induced genes. SOCS-3, a cytokine signaling  
318 suppressor molecule, is highly induced at the transcriptional level by IL-6 (15). Here, we  
319 found an important increase in liver SOCS-3 mRNA in response to exercise, still significant  
320 after 2 hours recovery. Although commonly used to assess IL-6 activity, SOCS-3 is not fully

321 specific of IL-6 and we studied IL-6R and C/EBP $\beta$ , two IL-6-inducible genes in the liver in  
322 vivo (2, 36). We found that mRNA levels for both genes were transiently activated at the end  
323 of exercise. Taken together, concomitant elevation in plasma IL-6, the increase in its  
324 transcription factors phosphorylation and IL-6-responsive genes activation in the liver  
325 strongly suggests that prolonged intense exercise can acutely induce an active IL-6 signaling  
326 in the liver. This result is consistent with the hepato-splanchnic removal of IL-6 previously  
327 described in exercising human (12). IL-6 increases endogenous glucose production during  
328 exercise by an unknown, hormone-independent mechanism (11) and because we found an  
329 active signaling in the liver, we hypothesized that IL-6 could act directly on hepatic tissue to  
330 modulate HGP during prolonged and intense exercise.

331           As expected in an exhaustive running exercise, plasma glucose was slightly  
332 lower at the end of exercise and gluco-regulatory hormones measurement showed a drop in  
333 plasma insulin/glucagon ratio and an increase in plasma corticosterone at the end of exercise.  
334 There was a 86% decrease in plantaris muscle glycogen levels. We found both a profound  
335 decrease in hepatic glycogen content (95%) and a substantial increase in liver key  
336 gluconeogenic enzymes mRNA, PEPCK and G6Pase. PEPCK is regulated at the  
337 transcriptional level and increased mRNA levels during exercise are associated to increased  
338 enzyme activity (14, 44). Interestingly, we found a concomitant and substantial increase in  
339 PGC-1 $\alpha$  mRNA, a key regulator of fasting-induced hepatic GNG (46). Taken together, these  
340 data confirm that our model of long lasting exercise is a challenge for glucose homeostasis  
341 and that HGP is very dependent on GNG at the end of exercise. Muscle IL-6 gene  
342 transcription starts early during exercise, but IL-6 release in the plasma is delayed (17, 25).  
343 Because circulating IL-6 concentration peaks at the end of exercise when GNG becomes  
344 crucial to maintain plasma glucose levels, and because this pathway is regulated at the

345 transcriptional level, we hypothesized that IL-6 could favor HGP by modulating  
346 gluconeogenic genes mRNA levels, particularly PEPCK.

347 PEPCK control during exercise is complex and in order to understand if IL-6 is able to  
348 acutely modulate gluconeogenic genes independent of exercise-related factors, we injected 2  
349 different doses of recombinant IL-6 to inactive rats and explored hepatic PEPCK and G6Pase  
350 mRNA levels one hour after injection. Injected doses were calculated to mimic exercise-  
351 induced plasma concentration. To avoid post-absorptive GNG blockade by insulin, animals  
352 were fasted overnight. Injections resulted in a rise in circulating IL-6, with final plasma  
353 concentrations flanking concentrations observed at the end of exhaustive running exercise. As  
354 expected, we found a dose dependent increase in hepatic SOCS-3 and IL-6R mRNA levels  
355 reflecting IL-6 signaling activation in the liver. This stimulation induced a dose dependent  
356 increase in PEPCK mRNA levels, consistent with in vitro results (5). During food  
357 deprivation, increased hepatic PEPCK mRNA levels result from both increased gene  
358 transcription and mRNA stabilization through AMPc (16). In the present work, we did not  
359 measure gene transcription, however the important and rapid increase in mRNA levels  
360 suggests that PEPCK transcripts are stabilized in response exercise and IL-6 injection. No  
361 changes in PGC-1 $\alpha$  and G6Pase mRNA abundance were found. Since G6Pase catalyses the  
362 last step of glucose production, allowing glucose to leave the hepatocyte, this enzyme is  
363 common to both GNG and glycogenolysis. In our experiment, rats were fasted overnight  
364 (approximately 14 hours). In this situation, gluconeogenesis accounts for only 40-50% of  
365 hepatic glucose output (24, 27), whereas glycogenolysis is fully induced after 4 to 6 hours  
366 food deprivation (6). Therefore, a full activation of PEPCK gene is not expected, whereas  
367 G6Pase was probably already activated, and no additive effect of IL-6 injection was found.  
368 An increase in liver PGC-1 $\alpha$  mRNA levels at the end of 1 hour exercise as been previously  
369 reported (19) and we show here that this is a transient rise. Whether newly transcribed PGC-



370  $1\alpha$  is involved in PEPCK transcription during exercise is not known and need to be explored,  
371 however our results show that IL-6 effect on PEPCK gene is independent of any effect on  
372 PGC-1 $\alpha$  mRNA levels. Plasma insulin/glucagon ratio and corticosterone were unchanged in  
373 IL-6 injected rats, suggesting that gluco-regulatory hormones do not mediate IL-6 effect. We  
374 found that PEPCK mRNA levels correlated with SOCS-3 and IL-6R mRNA levels in control  
375 and exercised rats and this correlation was stronger in IL-6 injected rats, further suggesting a  
376 co-regulation by IL-6 (Fig 8A and 8B). Differences observed between the two situations may  
377 be due to the fact that hormonal factors are the main regulators of hepatic PEPCK mRNA  
378 levels during exercise.

379         Independently of the debate concerning IL-6 and insulin sensitivity, the effect of IL-6  
380 on HGP is still controversial. This cytokine has been described in vivo to whether activate  
381 (40, 41) or inhibit HGP (7, 21). These differences could be explained by very different  
382 environment in term of gluco-regulatory hormones, cytokines, gluconeogenic substrates, but  
383 also by very different stimulation times (ranging from hours to days) and injected doses. Two  
384 studies have investigated the effect of one ip injection of IL-6 in overnight fasted rodents, and  
385 found no effect on PEPCK gene transcription (18, 34). The discrepancy between these results  
386 and the increase in PEPCK mRNA we report could be explained by a substantially higher  
387 hepatic IL-6 signaling activation in previous studies. Indeed, in the first study, authors  
388 injected high IL-6 doses to mimic the same acute phase response as an acute inflammatory  
389 agent (turpentine) and stimulation time was longer (18). In the second study (34), despite  
390 similar fasting time, doses and stimulation time, the authors report a 30 fold increase in  
391 hepatic SOCS-3 mRNA whereas we found a 12 or 4.2 fold increase in exercise and injection  
392 studies respectively. Thus, it appears that acute effects of IL-6 on gluconeogenic genes are  
393 very dependent on circulating concentrations. In our experiment, animals were fasted to  
394 mimic exercise-induced variations in hormones and IL-6 was injected alone. Furthermore,

395 animals were sacrificed only 1h after injection to mimic the very transient IL-6 stimulation we  
396 observed during exercise and plasma concentrations were similar to concentrations observed  
397 in exhaustive exercise. Therefore, although we did not directly measure HGP or  
398 gluconeogenic flux, our results are consistent with the positive effect of IL-6 on HGP during  
399 prolonged exercise in human (11), and strongly suggest that IL-6 is able to directly modulate  
400 liver PEPCK mRNA to increase HGP through GNG.

401 Plasma IL-6 concentrations obtained in our IL-6-injected animals reach the highest  
402 levels described in human for intense and prolonged exercise (30). However, rats injected  
403 with 3 or 10 $\mu\text{g}\cdot\text{kg}^{-1}$  recombinant IL-6 had only a 2 and 2.7 fold increase in hepatic PEPCK  
404 mRNA (versus 5.5 at the end of prolonged intense exercise). This further confirms that  
405 muscle-derived IL-6 cannot be considered a major regulator of glucose homeostasis during  
406 exercise but rather a positive modulator of HGP, acutely supporting hormonal control in very  
407 demanding exercises (11).

408

## 409 **Conclusion**

410 Prolonged intense exercise is a very challenging physiological situation for whole-  
411 body glucose homeostasis. IL-6 has been shown to increase endogenous glucose production  
412 during exercise in human through an unknown mechanism. Here we show that exhaustive  
413 running exercise in rats induces an increase in plasma IL-6 and an active IL-6 signaling in the  
414 liver. Because HGP was very dependent on GNG at the end of exercise, and GNG is regulated  
415 at the transcriptional level, we tested IL-6 ability to modulate gluconeogenic genes mRNA  
416 abundance in resting rats. We found a dose dependent increase in PEPCK mRNA levels in  
417 response to IL-6 injection, with no changes in glucoregulatory hormones. Taken together, our  
418 results suggest that muscle-derived IL-6 could contribute increasing HGP by directly up-  
419 regulating PEPCK during intense and prolonged exercise.

420

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544

## 545 **Legends**

546

### 547 **Figure 1: Liver carbohydrate metabolism in response to exhaustive exercise**

548 Liver glycogen content (A), PEPCK (B), G6P (C) and PGC-1 $\alpha$  (D) mRNA levels were  
549 measured in resting rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after  
550 the end of exercise.

551 \* : significantly different from the other groups,  $P < 0.001$ .

552

### 553 **Figure 2: Glucoregulatory hormones in response to exhaustive exercise**

554 Plasma insulin and glucagons ratio (A) and corticosterone levels (B) were measured in resting  
555 rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.

556 # : significantly different from Rest group,  $P < 0.05$  ; ## : significantly different from Rest  
557 group,  $P < 0.01$ .

558

### 559 **Figure 3: Liver IL-6 signaling in response to exhaustive exercise**

560 Plasma IL-6 protein level (A), liver total and phospho STAT-3 (B) and C/EBP $\beta$  (C) were  
561 measured and phospho/total ratio was calculated in resting rats (Rest), at the end of exercise  
562 (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.

563 \* : significantly different from the other groups,  $P < 0.05$  ; \*\* : significantly different from the  
564 other groups,  $P < 0.01$ .

565

### 566 **Figure 4: Liver IL-6-inductible genes mRNA levels in response to exhaustive exercise**

567 Liver SOCS-3 (A), C/EBP- $\beta$  (B) and IL-6R (C) mRNA levels were measured in resting rats  
568 (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.

569 \* : significantly different from the other groups,  $P < 0.001$ ; # : significantly different from Rest  
570 group,  $P < 0.05$ .

571

### 572 **Figure 5: Plasma rh IL-6 protein levels, liver SOCS-3 and IL-6R mRNA levels in 573 response to recombinant IL-6 injection.**

574 Plasma recombinant human IL-6 protein levels (A) Liver SOCS-3 (B) and IL-6R (C) mRNA  
575 levels were measured in resting fasted rats 1 hour after recombinant IL-6 injection (3 or  
576 10 $\mu\text{g.kg bodyweight}^{-1}$ ) or an equal volume of PBS buffer (Cont).

577 \* : significantly different from Cont group,  $P < 0.05$  ; # : significantly different from 3 $\mu\text{g.kg}^{-1}$   
578 group,  $P < 0.05$ .

579

### 580 **Figure 6: Liver gluconeogenic genes mRNA levels in response to recombinant IL-6 581 injection.**

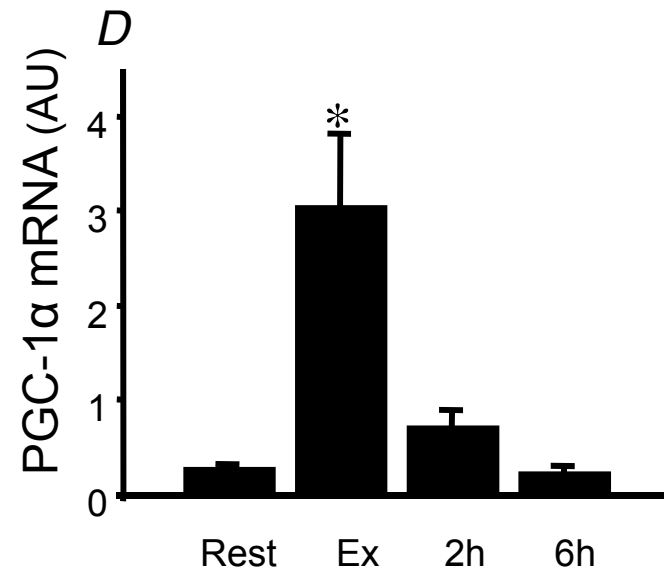
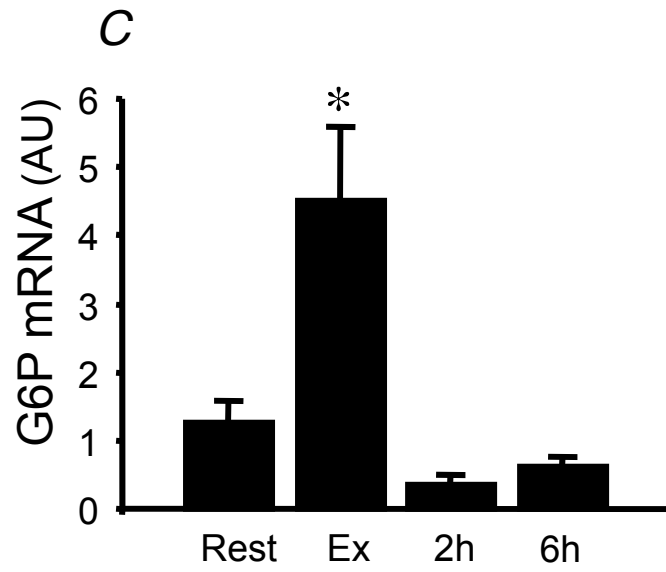
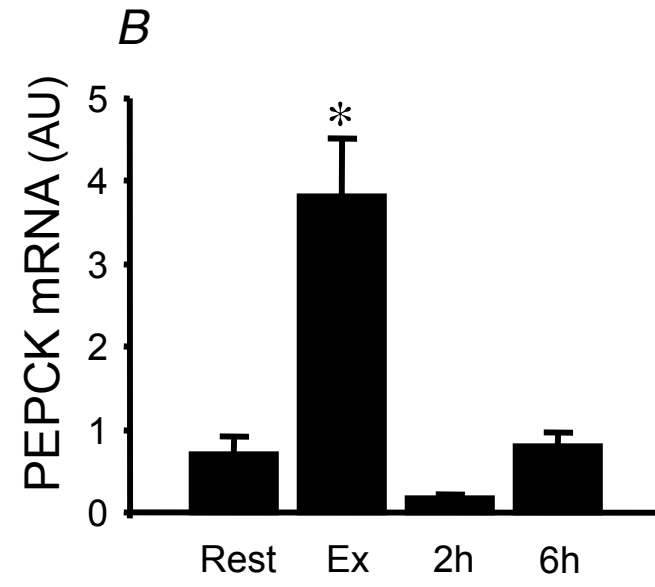
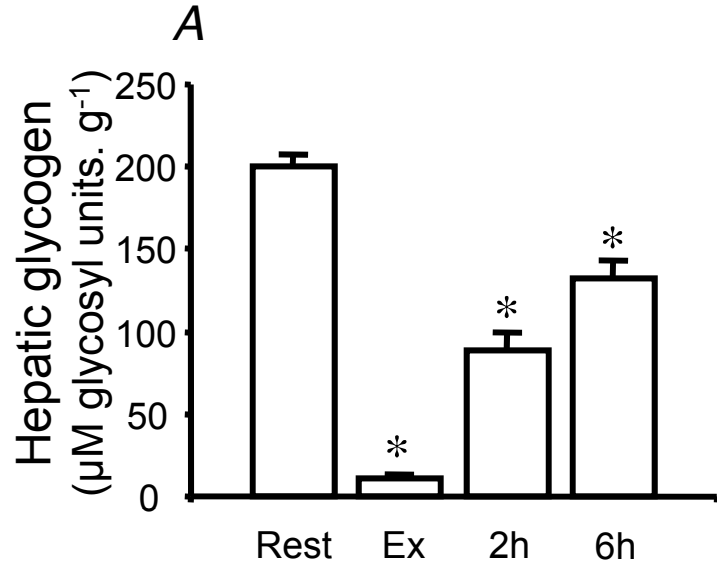
582 Liver PEPCK (A), G6P (B), PGC-1 $\alpha$  (C) mRNA levels were measured in resting fasted rats 1  
583 hour after recombinant IL-6 injection (3 or 10 $\mu\text{g.kg bodyweight}^{-1}$ ) or an equal volume of PBS  
584 buffer (Cont).

585 \* : significantly different from Cont group,  $P < 0.05$ ; \*\* : significantly different from Cont  
586 group,  $P < 0.01$ .

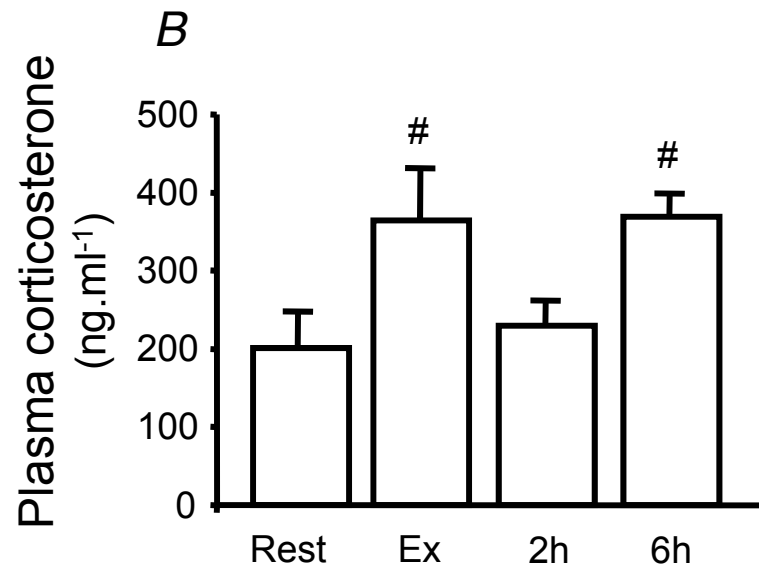
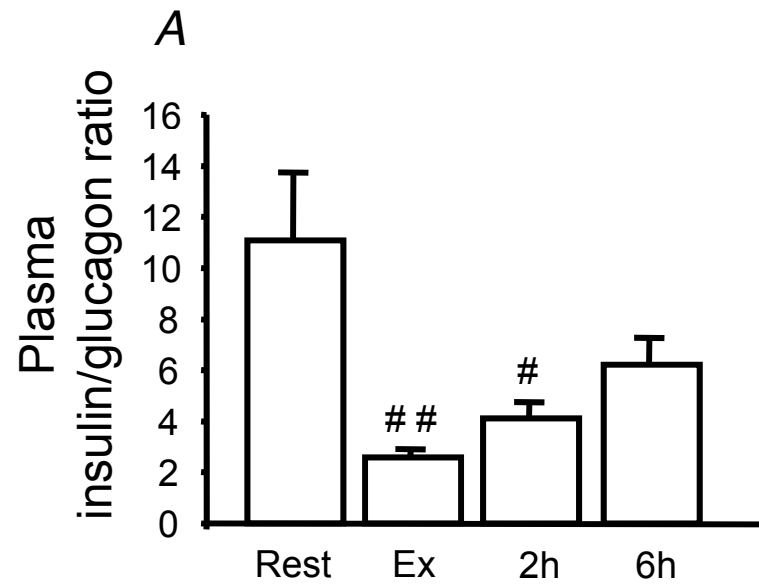
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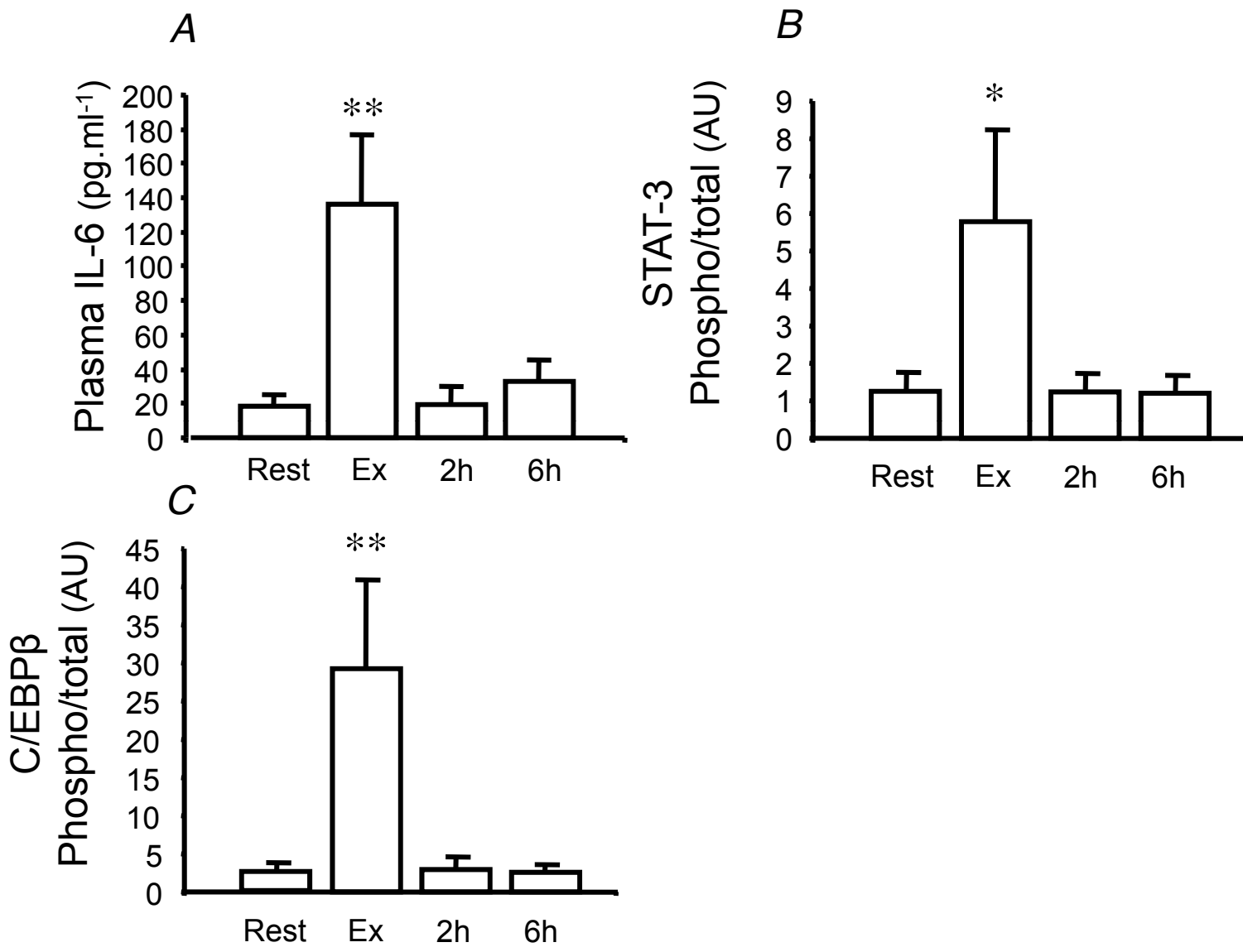
### 588 **Figure 7: Association between IL-6 responsive genes and PEPCK mRNA in liver in 589 response to exercise and to recombinant IL-6 injection.**

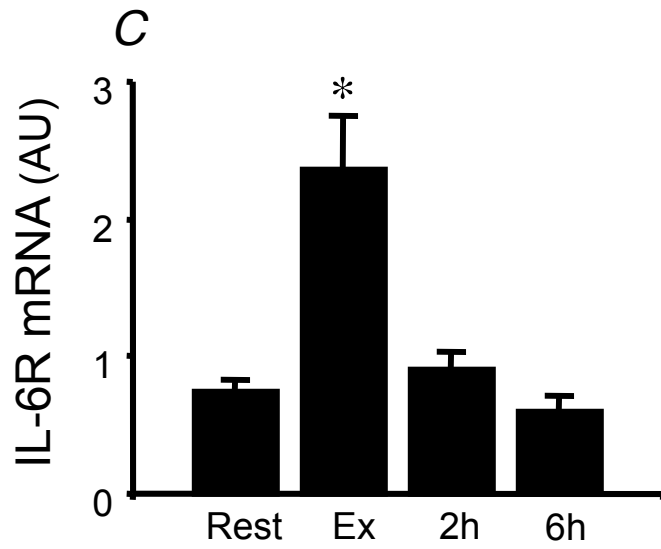
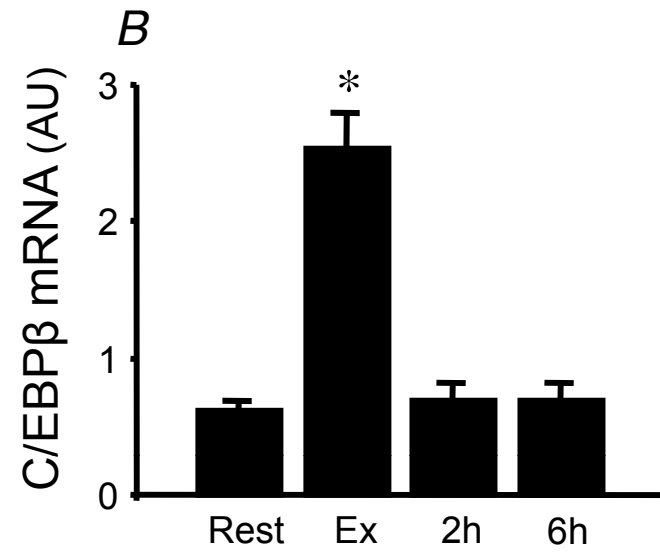
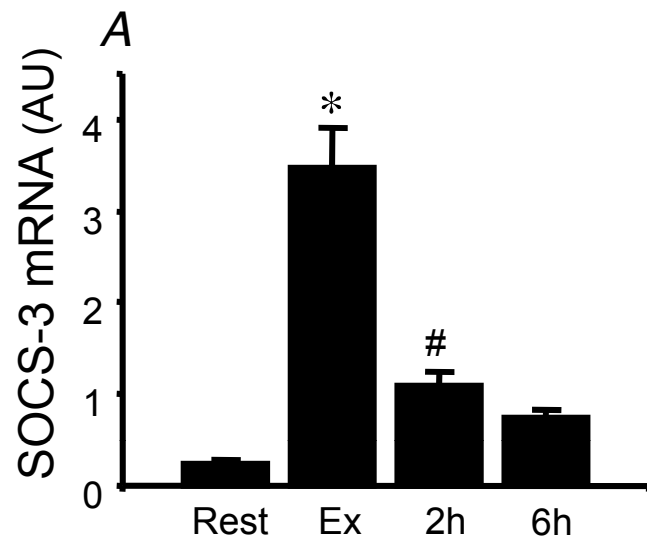
590 Scatterplot showing SOCS-3 or IL-6R against PEPCK mRNA in liver from Cont and Ex  
591 groups in exercise experiment (*A* and *B*), and in response to injection of 3 or 10 $\mu$ g.kg  
592 bodyweight<sup>-1</sup> recombinant IL-6, or an equal volume of PBS buffer (*C* and *D*)

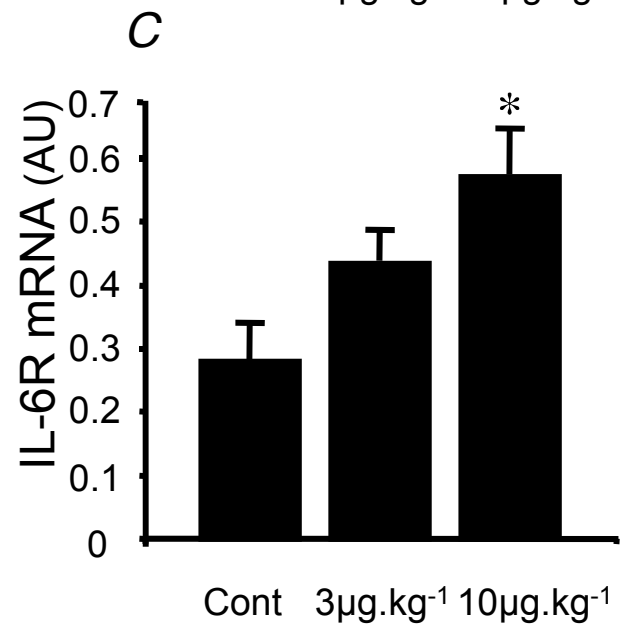
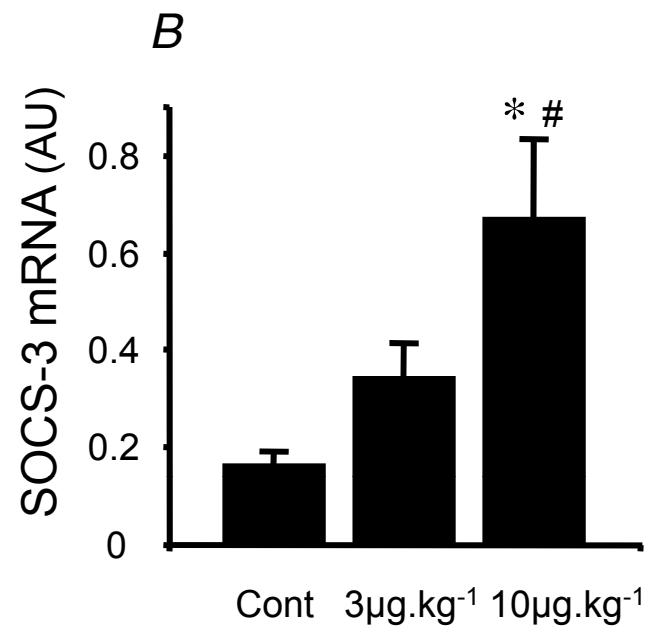
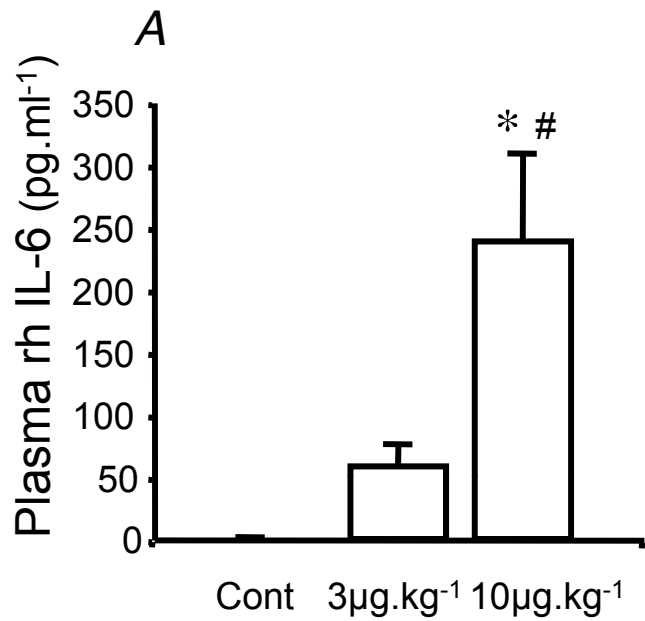


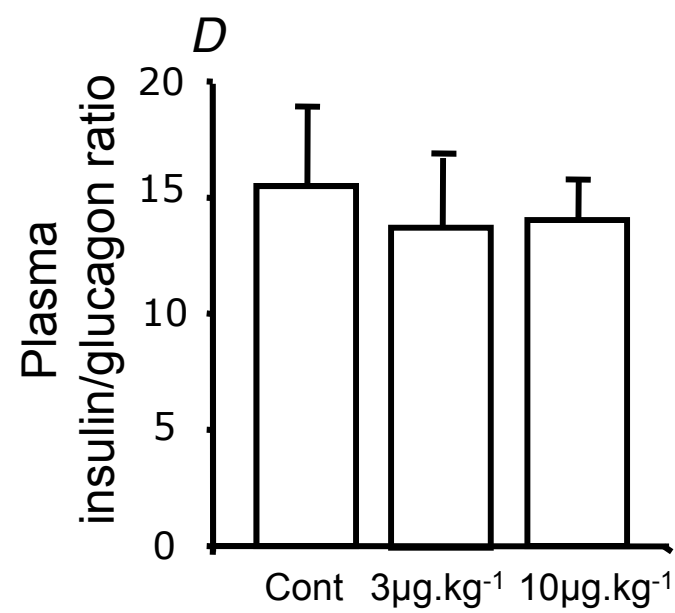
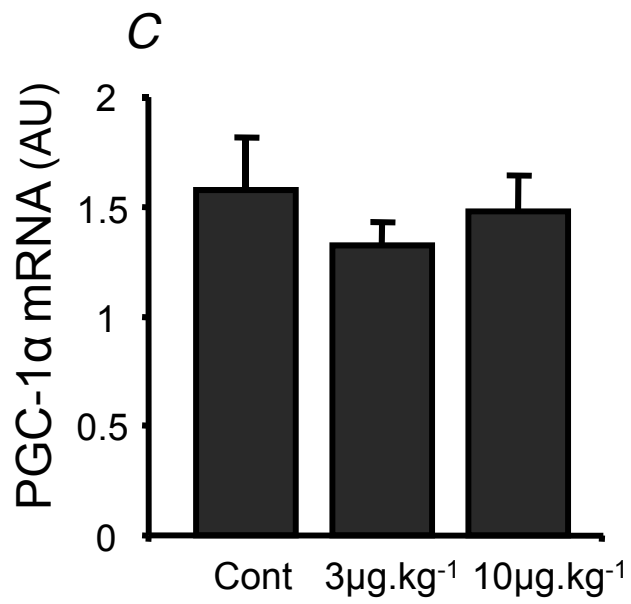
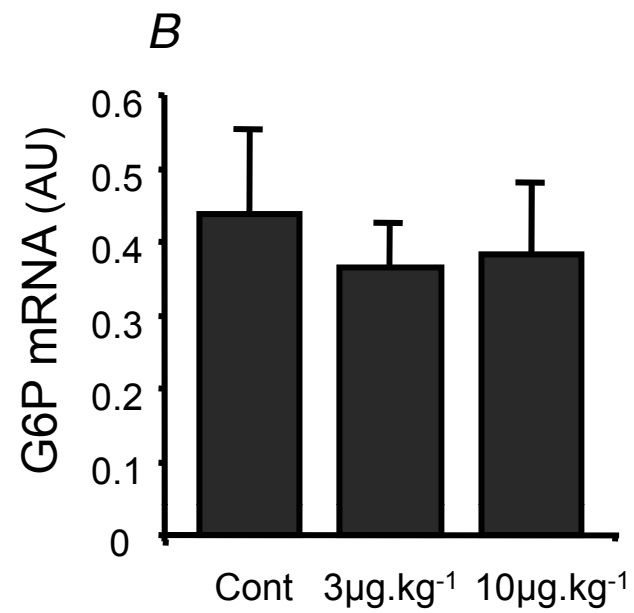
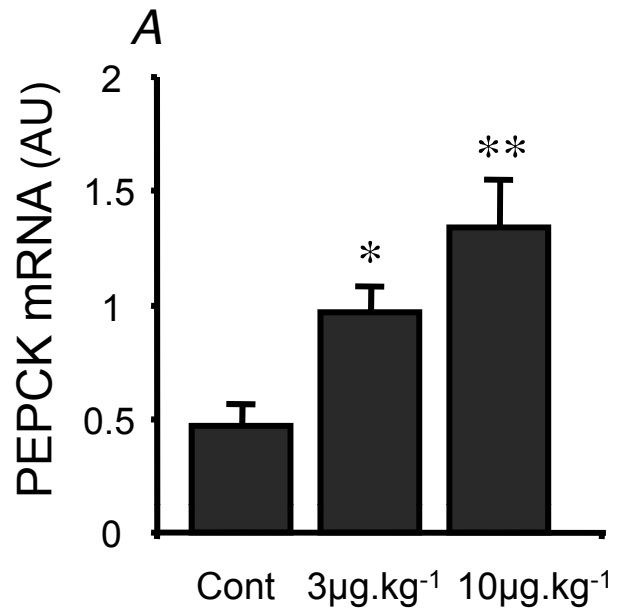


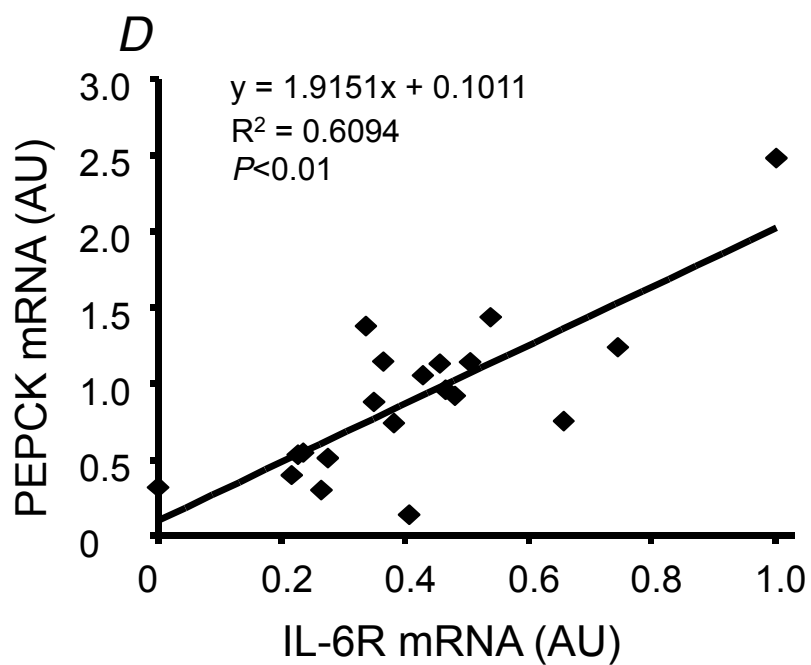
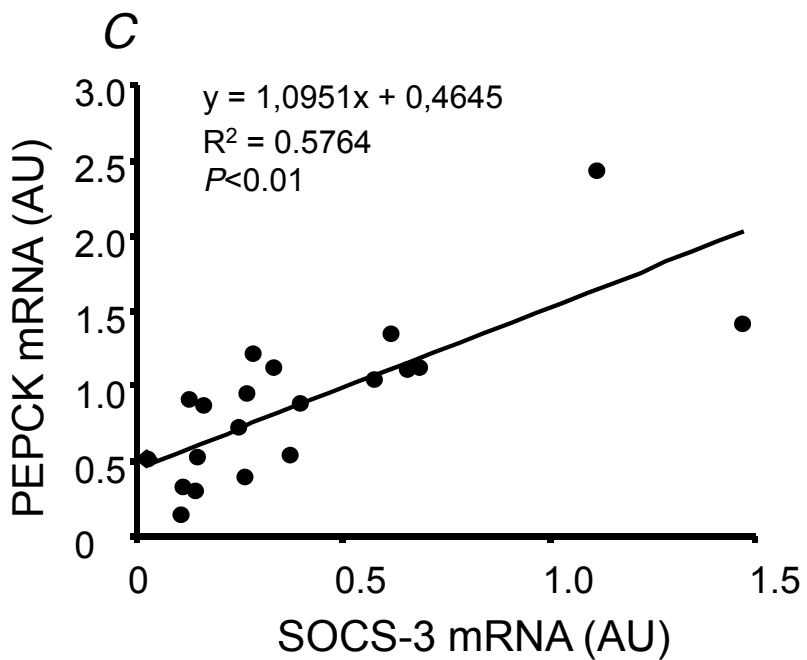
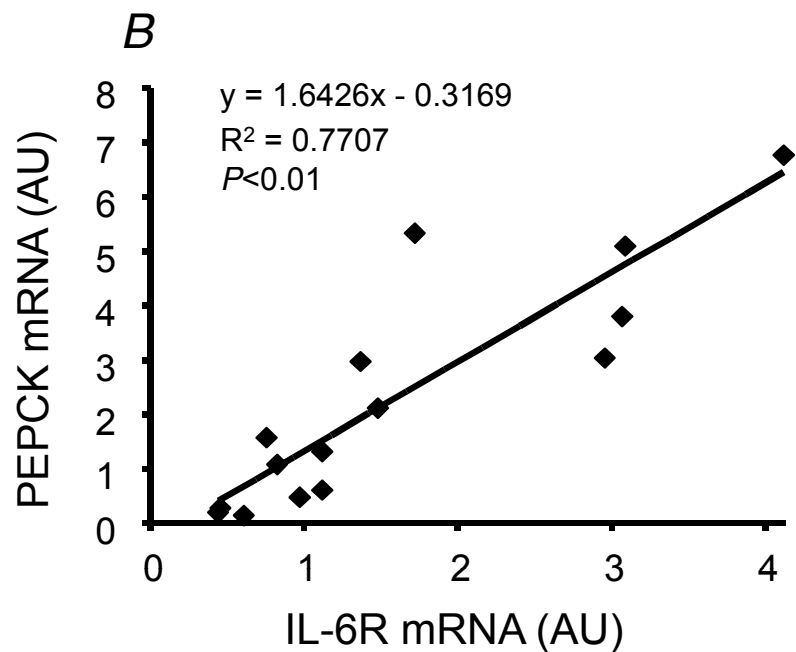
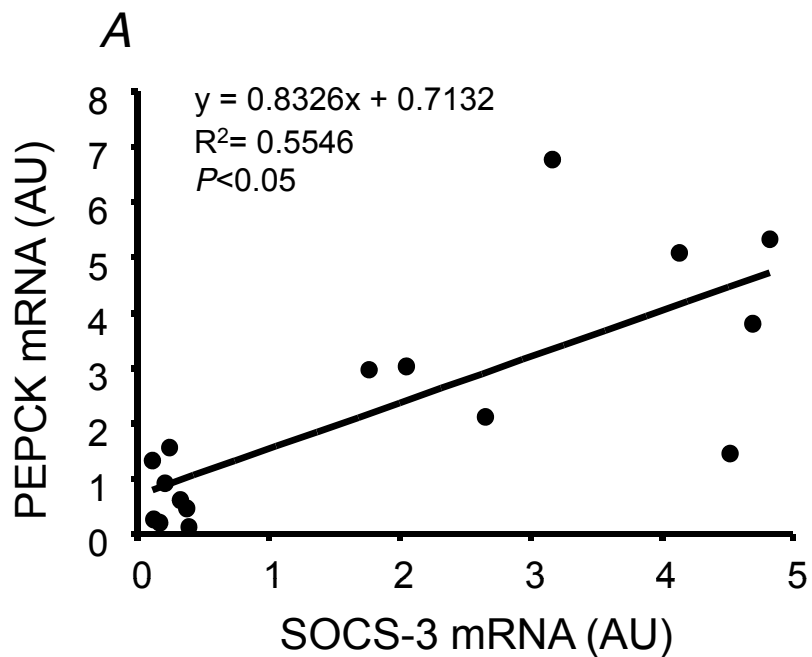












Gene	Accession number	Primers Sequences	Primers ( $\mu$ M)	MgCl <sub>2</sub> (mM)	Annealing temperature ( $^{\circ}$ C)
Actine $\beta$	NM_031144 (816-910)	F : TCAGGTCATCACTATCGGCAATG R : TTTCATGGATGCCACAGGATTC	0,6	4	58
ARBP	NM_022402 (659-732)	F : CCTGCACACTCGCTTCCTAGAG R : CAACAGTCGGGTAGCCAATCTG	0,5	4	57
CycA	M19533 (381-507)	F : TATCTGCACTGCCAAGACTGAGTG R : CTTCTTGCTGGTCTTGCCATTCC	0,4	4	58
GAPDH	NM_017008 (1556-1633)	F : CCAATGTATCCGTTGTGGATCTGAC R : GCTTCACCACCTTCTTGATGTCATC	0,5	4	57
C/EBP- $\beta$	NM_024125 (656-760)	F : TGGACAAGCTGAGCGACGAG R : TGTGCTGCGTCTCCAGGTTG	0,4	4	59
PEPCK	NM_198780 (846-952)	F : TACGGTGGGAACACTGCTTG R : ATGCCCAGGATCAGCATGTG	0,4	4	59
G6PC	NM_013098 (173-304)	F : CCATCTGGTCCACATTCAAGAG R : CAGTGTCAGGACCCACCAATAC	0,4	4	59
HPRT	S79292 (211-333)	F : CTCATGGACTGATTATGGCAGGAC R : GCAGGTCAGCAAAGAACTTATAGCC	0,4	4	60
IL-6 R	NM_017020 (696-812)	F : AAGCAGGTCCAGCCACAATGTAG R : CCAACTGACTTTGAGCCAACGAG	0,4	5	60
IL-6	E02522 (532-610)	F : TCCTACCCCAACTCCAATGCTC R : TTGGATGGTCTTGGTCCTTAGCC	0,4	3	65
SOCS-3	AF075383 (581-679)	F : CCTCCAGCATCTTTGTGCGGAAGAC R : TACTGGTCCAGGAACTCCCGAATG	0,4	4	65
PGC-1 $\alpha$	NM_031347 (2249-2364)	F : ACGCAGGTCGAATGAAACTGAC R : TGGTGAAGCAGGGTCAAAAATC	0,4	4	53

Table 1