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

Serrurier Bernard, Peinnequin André and Bigard Xavier.

Department of Human Factors,
Military health service research center (Centre de Recherches du Service de Santé des
Armées),
38702 La Tronche, France

Running head : IL-6 and exercise gluconeogenesis

Address for correspondence:

Sébastien Banzet

CRSSA

Department of Human Factors

BP 87

38702 La Tronche Cedex

France

Phone : +334 76 63 97 67

Fax : +334 76 63 69 45

E-mail: sbanzet@crssa.net

Abstract

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

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

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

Exercise-induced increase in HGP results from coordinated changes in glycogenolysis and gluconeogenesis (GNG) under complex hormonal and nervous control (8, 26). Liver glycogenolysis starts at the onset of exercise and is the main source of glucose during the first 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-caboxykinase (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 γ coactivator-1α (PGC-

87   1α) 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-1α 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 α (IL-6Rα) 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) ß pathway (for review see (23)). C/EBP family members can trans activate PEPCK

96   promoter via the CRE binding sites (16, 31). C/EBPß, 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/EBPß 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

Research Design and Methods

Ethics

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

Animals

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

Exercise protocol

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

IL-6 injection

Animals were fasted overnight and injected intraperitonealy with 3 or 10 µg.kg body weight^{-1} human recombinant IL-6 (Biosource, Camarillo, CA, USA) and compared to control rats injected with endotoxin free PBS (Cont). Animals were sacrificed 1 hour after injection.

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^{-1} *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 RNA*later* (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^{-1}), 2 µl MgCl$_2$ (25 mM), 0.25 µl

165   Euroscript reverse transcriptase (50 U.µl^{-1}), 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 20µl final volume with the LC Fast Start DNA

170   Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 2 µl 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), cyclophylin A (CycA), acidic ribosomal binding protein (ARBP), actin ß 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

Western blotting

184   Liver sections were homogenized in ice-cold lysis buffer (20 µl.mg^{-1}) containing 20 mM

185   HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM

186   Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 µl.ml^{-1} 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 µg 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/EBPβ (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 cheluminescence 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% β-mercapto ethanol, for 30 min at 50°C, washed with TBST, and

201 incubated overnight with anti STAT-3 antibody or C/EBPβ (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 ± 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

Results

Liver response to exercise

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

Carbohydrate metabolism: Plasma glucose was lower in Ex group than Rest and Ex+2h (8.25 ± 0.33; 6.71 ± 0.51 and 8.26 ± 0.34 mmol.l^{-1} respectively, data not shown) ($P<0.05$). Plantaris muscle glycogen content decreased immediately at the end of exercise (Rest: 24.11 ± 0.74; Ex: 3,37 ± 0.6 µmol glycosyl units.g^{-1}, datanot shown) ($P<0.01$). There was a profound decrease in hepatic glycogen in response to exercise (main effect $P<0.001$), with only 5% of the resting values at the end of exercise ($P<0.001$). Liver glycogen increased during recovery, but was still 35% lower in Ex+6h than in Rest group ($P<0.001$) (fig 1A).

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

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

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

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

266 Hepatic mRNA levels for SOCS-3 and C/EBPß, 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-1α 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 ± 2.94 and 14.03 ± 1.69 for Cont, 3 µg.kg^{-1} and 10 µg.kg^{-1} groups, respectively)
289 and corticosterone was unchanged (252.27 ± 38.24 ng.ml^{-1}, 254.81 ± 35.12 ng.ml^{-1} and
290 247.48 ± 49.16 ng.ml^{-1} for Cont, 3 µg.kg^{-1} and 10 µg.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

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/EBPß 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/EBPß transcription factors to promote gene transcription (1). In the present study we show

312   that in addition to increased hepatic C/EBPß 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/EBPß, 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-1α 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-1α 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   (approximatively 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-1α 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   1α 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-1α 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 10µg.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

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.

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Legends

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Figure 1: Liver carbohydrate metabolism in response to exhaustive exercise
Liver glycogen content (*A*), PEPCK (*B*), G6P (*C*) and PGC-1α (*D*) mRNA levels were measured in resting rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.
∗ : significantly different from the other groups, *P*<0.001.

Figure 2: Glucoregulatory hormones in response to exhaustive exercise
Plasma insulin and glucagons ratio (*A*) and corticosterone levels (*B*) were measured in resting rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.
: significantly different from Rest group, *P*<0.05 ; ## : significantly different from Rest group, *P*<0.01.

Figure 3: Liver IL-6 signaling in response to exhaustive exercise
Plasma IL-6 protein level (*A*), liver total and phospho STAT-3 (*B*) and C/EBPβ (*C*) were measured and phospho/total ratio was calculated in resting rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.
∗ : significantly different from the other groups, *P*<0.05 ; ∗∗ : significantly different from the other groups, *P*<0.01.

Figure 4: Liver IL-6-inductible genes mRNA levels in response to exhaustive exercise
Liver SOCS-3 (*A*), C/EBP-β (*B*) and IL-6R (*C*) mRNA levels were measured in resting rats (Rest), at the end of exercise (Ex), 2 hours (2h) and 6 hours (6h) after the end of exercise.
∗ : significantly different from the other groups, *P*<0.001; # : significantly different from Rest group, *P*<0.05.

Figure 5: Plasma rh IL-6 protein levels, liver SOCS-3 and IL-6R mRNA levels in response to recombinant IL-6 injection.
Plasma recombinant human IL-6 protein levels (*A*) Liver SOCS-3 (*B*) and IL-6R (*C*) mRNA levels were measured in resting fasted rats 1 hour after recombinant IL-6 injection (3 or 10μg.kg bodyweight^{-1}) or an equal volume of PBS buffer (Cont).
∗ : significantly different from Cont group, *P*<0.05 ; # : significantly different from 3μg.kg^{-1} group, *P*<0.05.

Figure 6: Liver gluconeogenic genes mRNA levels in response to recombinant IL-6 injection.
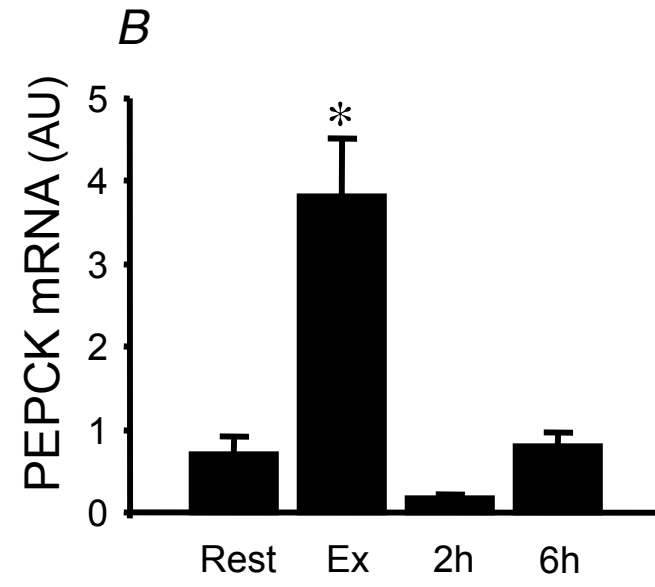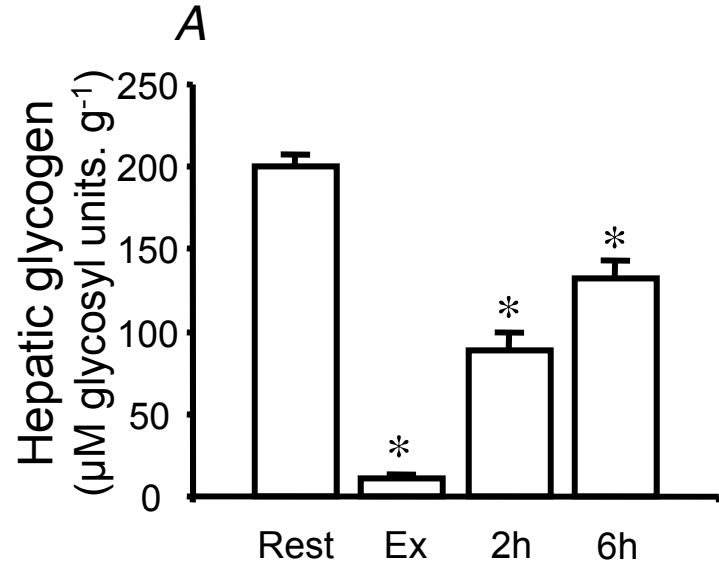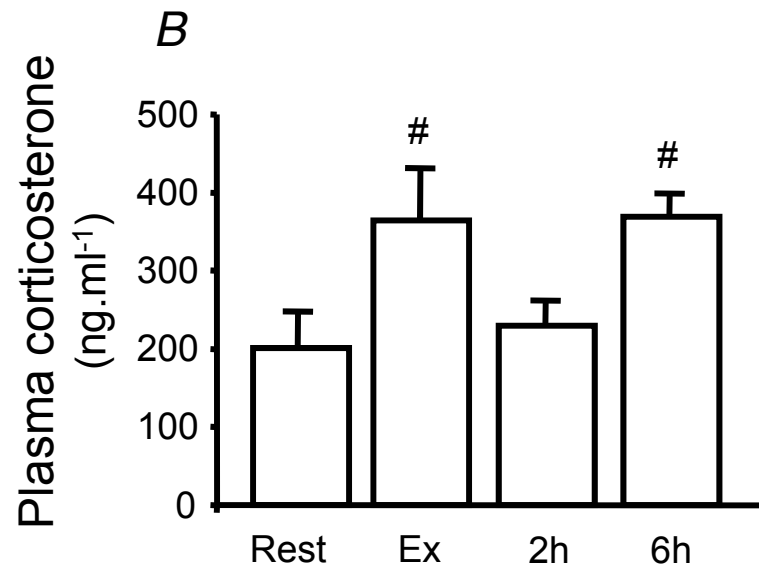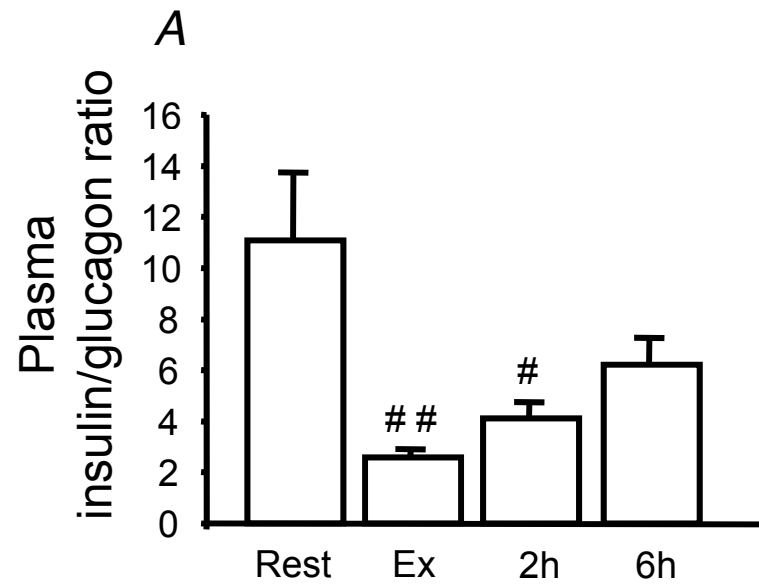Liver PEPCK (*A*), G6P (*B*), PGC-1α (*C*) mRNA levels were measured in resting fasted rats 1 hour after recombinant IL-6 injection (3 or 10μg.kg bodyweight^{-1}) or an equal volume of PBS buffer (Cont).
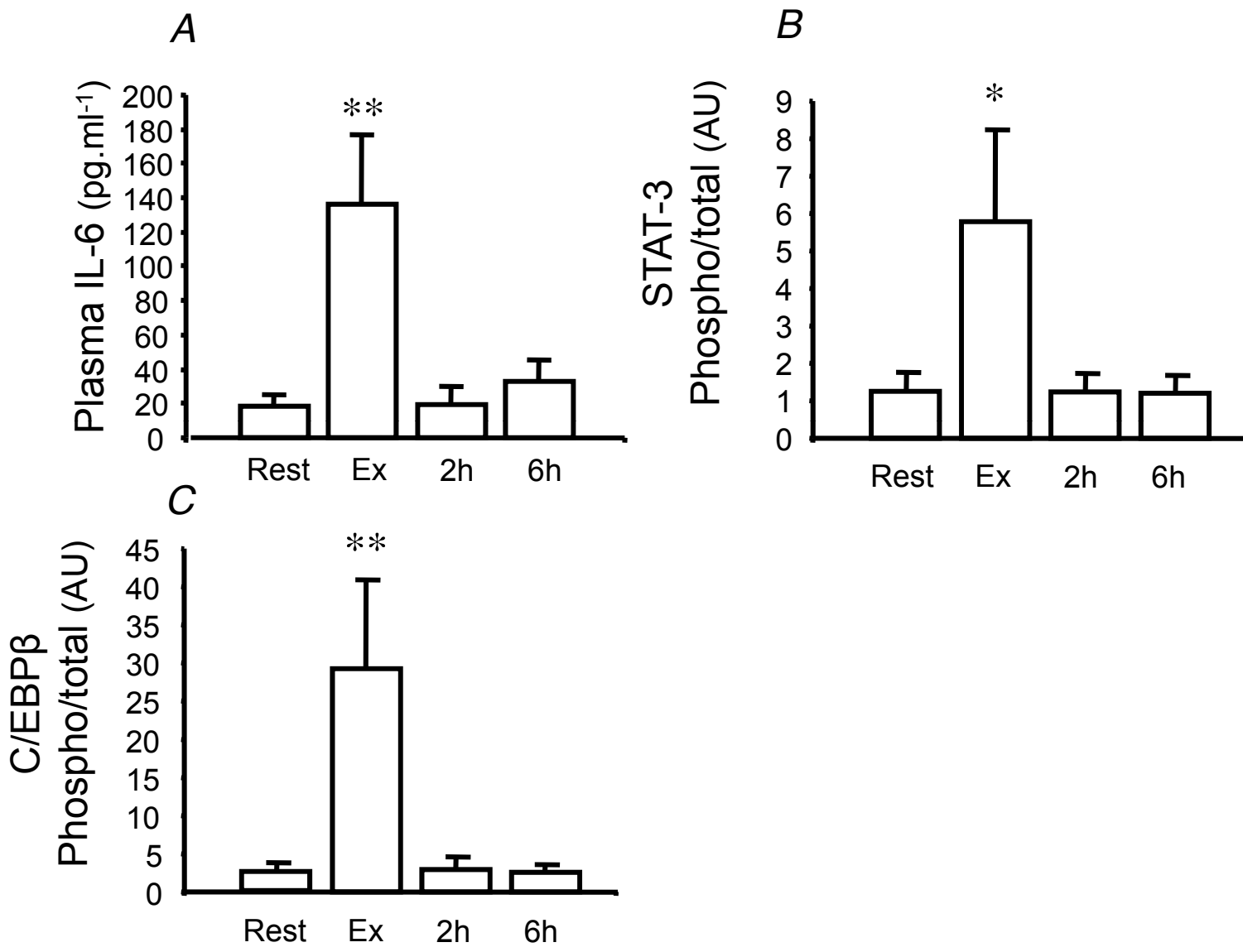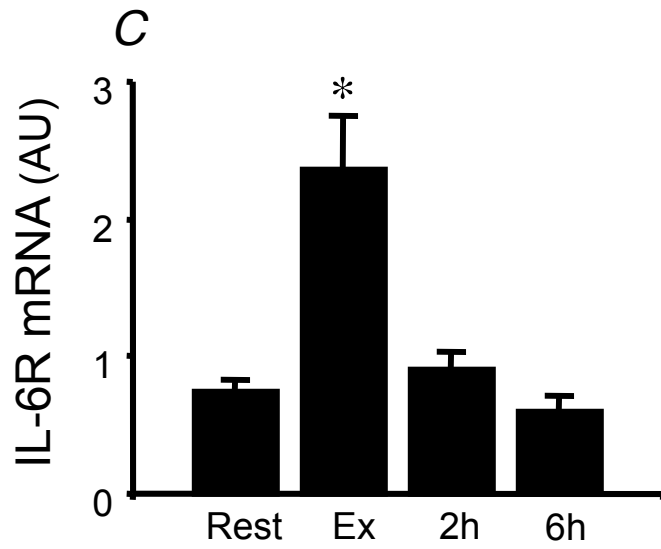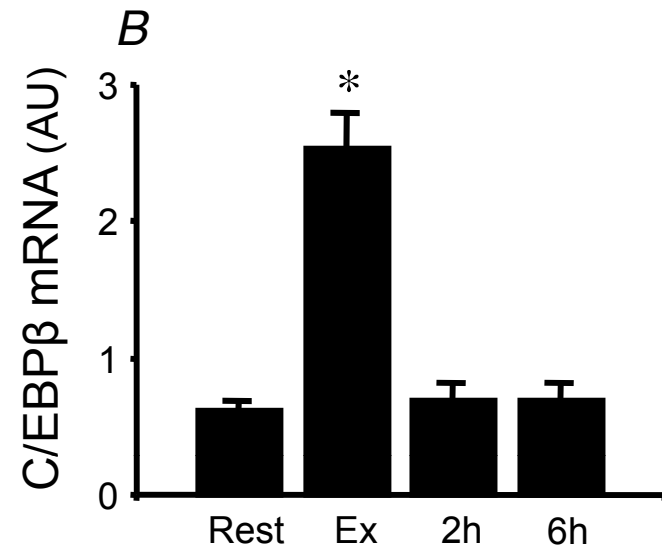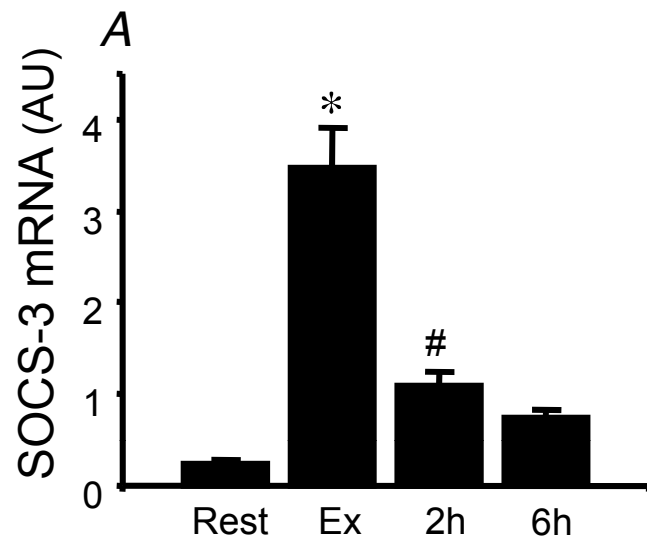∗ : significantly different from Cont group, *P*<0.05; ∗∗ : significantly different from Cont group, *P*<0.01.

Figure 7: Association between IL-6 responsive genes and PEPCK mRNA in liver in response to exercise and to recombinant IL-6 injection.
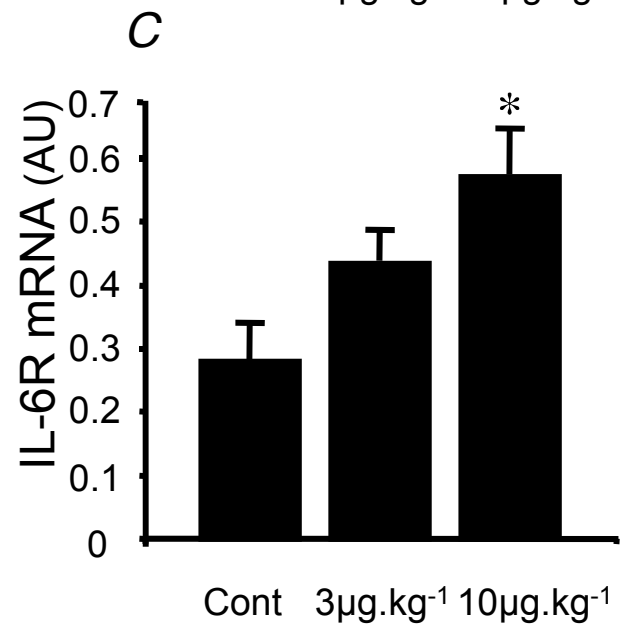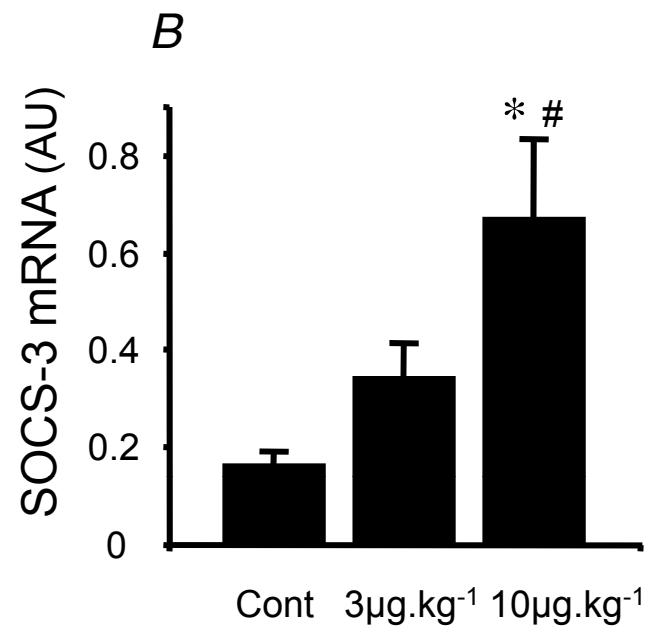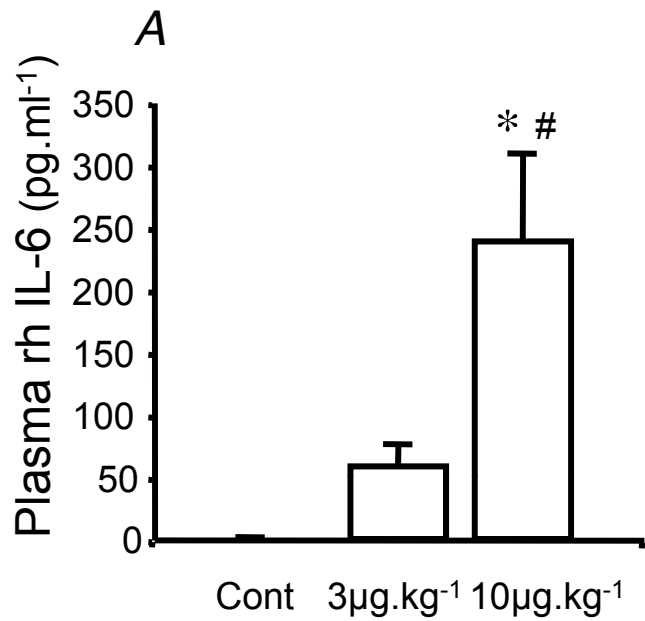
590   Scatterplot showing SOCS-3 or IL-6R against PEPCK mRNA in liver from Cont and Ex
591   groups in exercise experiment (*A* and *B*), and in response to injection of 3 or 10μg.kg
592   bodyweight^{-1} recombinant IL-6, or an equal volume of PBS buffer (*C* and *D*)
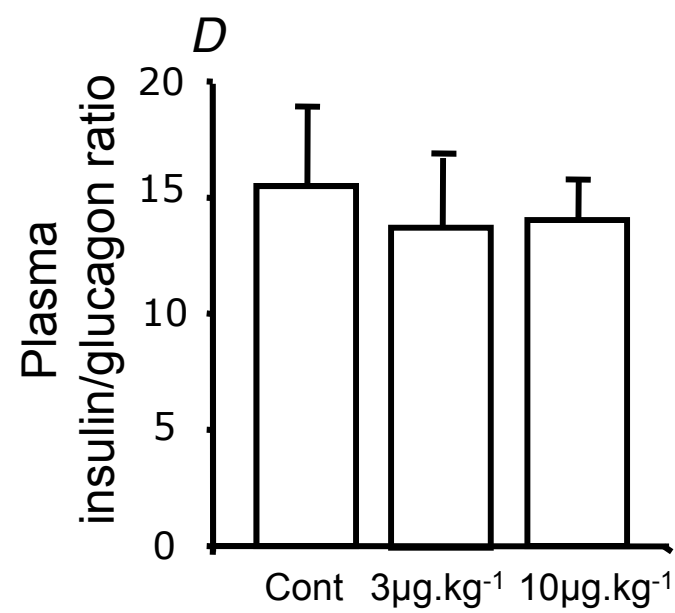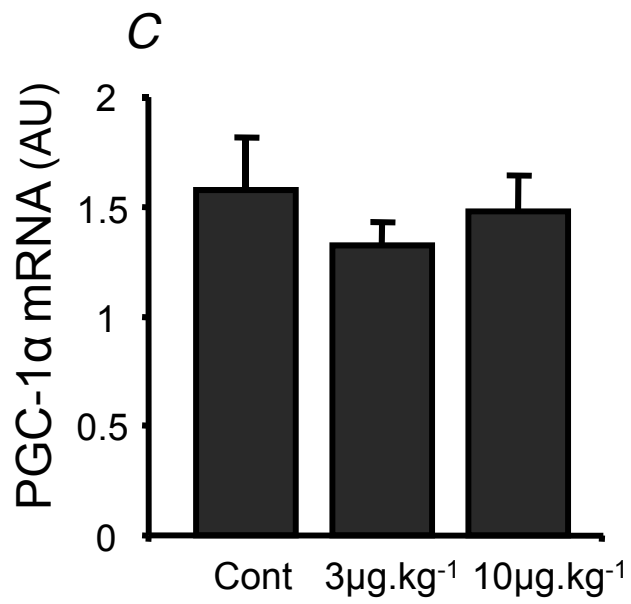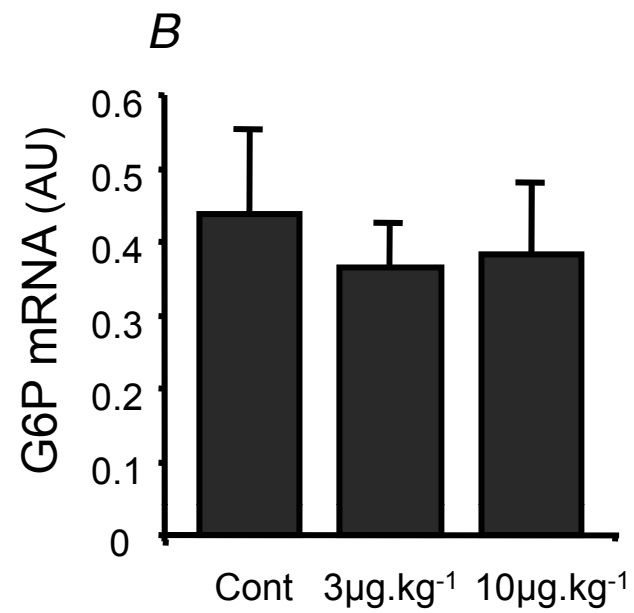
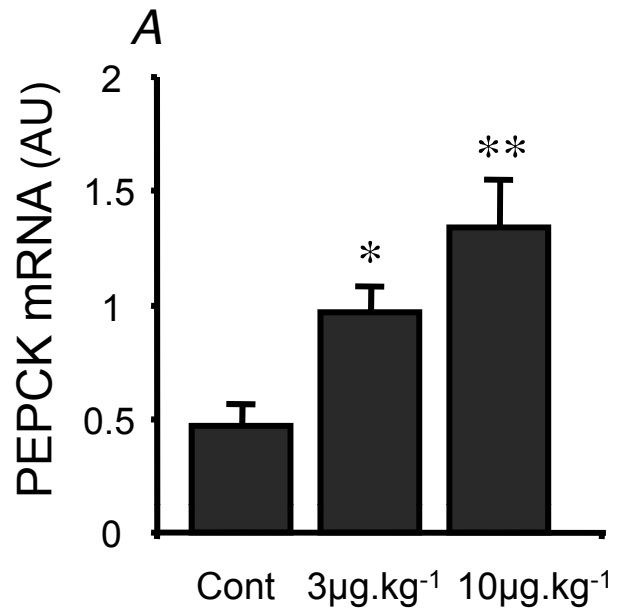*A* — Plasma IL-6 (pg.ml⁻¹) across Rest, Ex, 2h, 6h. ** above Ex.

B — STAT-3 Phospho/total (AU) across Rest, Ex, 2h, 6h. * above Ex.

C — C/EBPβ Phospho/total (AU) across Rest, Ex, 2h, 6h. ** above Ex.

A

$y = 0.8326x + 0.7132$
$R^2 = 0.5546$
P<0.05

(x-axis) SOCS-3 mRNA (AU)
(y-axis) PEPCK mRNA (AU)

B

$y = 1.6426x - 0.3169$
$R^2 = 0.7707$
P<0.01

(x-axis) IL-6R mRNA (AU)
(y-axis) PEPCK mRNA (AU)

C

$y = 1,0951x + 0,4645$
$R^2 = 0.5764$
P<0.01

(x-axis) SOCS-3 mRNA (AU)
(y-axis) PEPCK mRNA (AU)

D

$y = 1.9151x + 0.1011$
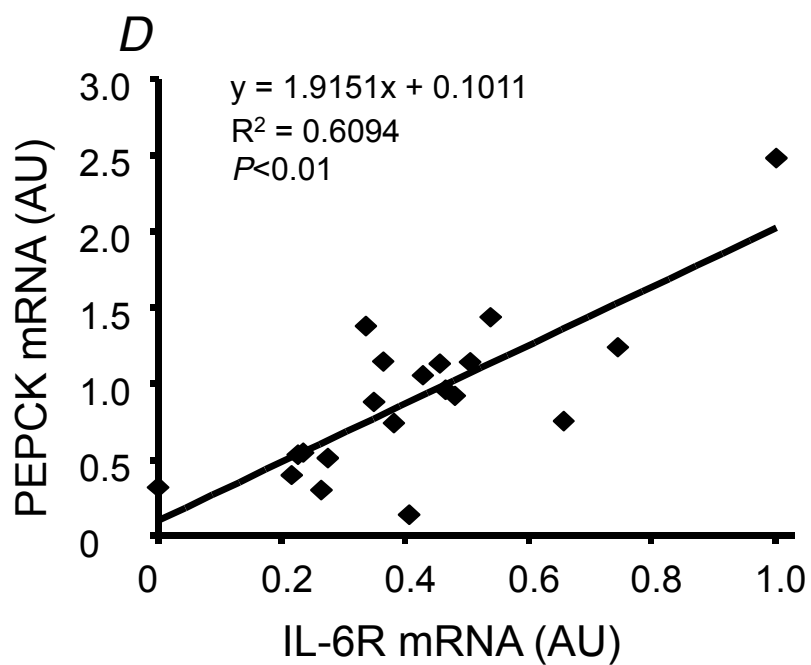$R^2 = 0.6094$
P<0.01

(x-axis) IL-6R mRNA (AU)
(y-axis) PEPCK mRNA (AU)

Gene	Accesion number	Primers Sequences	Primers (μM)	MgCl$_2$ (mM)	Annealing temperature (°C)
Actine β	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- β	NM_024125 (656-760)	F : TGGACAAGCTGAGCGACGAG R : TGTGCTGCGTCTCCAGGTTG	0,4	4	59
PEPCK	NM_198780 (846-952)	F : TACGGTGGGAACTCACTGCTTG R : ATGCCCAGGATCAGCATGTG	0,4	4	59
G6PC	NM_013098 (173-304)	F : CCATCTGGTTCCACATTCAAGAG R : CAGTGTCCAGGACCCACCAATAC	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 : TCCTACCCCAACTTCCAATGCTC R : TTGGATGGTCTTGGTCCTTAGCC	0,4	3	65
SOCS-3	AF075383 (581-679)	F : CCTCCAGCATCTTTGTCGGAAGAC R : TACTGGTCCAGGAACTCCCGAATG	0,4	4	65
PGC-1α	NM_031347 (2249-2364)	F : ACGCAGGTCGAATGAAACTGAC R : TGGTGGAAGCAGGGTCAAAATC	0,4	4	53

Table 1