

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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6	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
14	
15	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 26	E-mail: sbanzet@crssa.net

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Abstract

28 Prolonged intense exercise is challenging for the liver to maintain plasma glucose levels. Hormonal changes cannot fully account for exercise-induced hepatic glucose production 29 (HGP). Contracting skeletal muscles release interleukin-6 (IL-6), a cytokine able to increase 30 endogenous glucose production during exercise. However, whether this is due to a direct 31 effect of IL-6 on liver remains unknown. Here, we studied hepatic glycogen, gluconeogenic 32 genes and IL-6 signaling in response to one bout of exhaustive running exercise in rats. To 33 determine whether IL-6 can modulate gluconeogenic genes mRNA independently of exercise, 34 35 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, 36 37 phosphoenolpyruvate-carboxykinase (PEPCK), glucose-6-phosphatase (G6P) and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), suggesting a key role for 38 39 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-40 3) and CAAT/enhancer binding protein- β (C/EBP β) phosphorylation and IL-6 responsive 41 genes mRNA levels at the end of exercise. Recombinant IL-6 injection resulted in an increase 42 43 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 44 45 in G6P and PGC-1 α 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 46 regulating PEPCK mRNA abundance. 47

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During intense and prolonged exercise, working skeletal muscles are very dependent 50 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 changes in hepatic glucose production (HGP). During moderate-intensity exercise, HGP is 53 primarily controlled by portal venous insulin-to-glucagon ratio (44, 45), whereas during 54 intense and sustained exercise, several studies have shown that hormonal changes cannot fully 55 account for the increase in HGP, suggesting other unidentified factors are involved (9, 20). A 56 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 sustained exercise (30, 39), and a strong relationship with low muscle glycogen content as 61 62 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). 63 64 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-65 66 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 67 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

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

a key contributor to HGP (44). GNG is controlled at the transcriptional level by unidirectional 75 key enzymes, phosphoenolpyruvate-caboxykinase (PEPCK), fructose 1,6 bisphosphatase and 76 77 glucose-6 phosphatase (G6Pase) the activities of which increase during exercise (10). PEPCK, the most studied gluconeogenic enzyme, plays an important role during exercise 78 79 since its pharmacologic inhibition reduces endurance time in rats (22, 42). PEPCK activity is hormonally controlled at the transcriptional level (16) and exercise induces an important 80 increase in hepatic PEPCK mRNA levels and enzyme activity (14, 44). Among the well-81 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-1 α (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-1a 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-6R α) 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 Ras/extracellular signal-regulated protein kinase (ERK)/CAAT enhancer binding protein 94 (C/EBP) ß pathway (for review see (23)). C/EBP family members can trans activate PEPCK 95 96 promoter via the CRE binding sites (16, 31). C/EBPB, also known as nuclear factor of IL-6 (NF-IL6), has been proposed to play a role in PEPCK gene transcription during exercise (29). 97 Because this transcription factor is regulated by IL-6 at the transcriptional level and is a 98 99 downstream signaling molecule of activated IL-6 receptor (reviews in (1, 35)), musclederived IL-6 could participate in hepatic C/EBPß activation during exercise. Whether IL-6
 can act directly on hepatic tissue to favor PEPCK gene transcription during exercise has to be
 explored.

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

117 **Ethics** 118 Experiments were carried out in accordance with the Helsinki Accords for Human Treatment 119 120 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 121 122 Tronche, France). 123 Animals 124 125 Female Wistar rats (175-200 g) were purchased from Charles River (L'Arbresle, France). 126 They were housed at $22 \pm 2^{\circ}$ 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 127 experimental groups: resting rats (Rest, n=8) and 3 active groups, performing one bout of 128 exercise and studied at the end of exercise (Ex, n=8), 2 hours (Ex+2h, n=8) or 6 hours 129 (Ex+6h, *n*=8) later. 130 131 **Exercise protocol** 132 133 All animals were accustomed to running on a rodent treadmill for 15-20 min per day for 5 davs (10-20 m.min⁻¹; 0° grade). After 4 days at rest, active animals ran on the treadmill (22 134 m.min⁻¹, 5° grade) till exhaustion. 135 136

137 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.

141

142 Tissue processing

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

150

151 **RNA extraction**

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

159

160 **Reverse transcription**

Reverse transcription was carried out using the Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium). The reaction was performed following the manufacturer's instructions. The reaction mix contained 1 μ l buffer (10X), 2 μ l dNTP (2.5 mM each dNTP), 0.5 μ l oligodT primer (50 μ M), 0.2 μ l RNase inhibitor (20 units. μ l⁻¹), 2 μ l MgCl₂ (25 mM), 0.25 μ l Euroscript reverse transcriptase (50 U. μ l⁻¹), 1.05 μ l RNase free water and 3 μ l template mRNA.

167

168 **Real-time PCR**

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

182

183 Western blotting

Liver sections were homogenized in ice-cold lysis buffer (20 μ l.mg⁻¹) containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 1 μ l.ml⁻¹ Protease inhibitor cocktail set III (Calbiochem, Fontenay-sous-bois, France). Samples were rotated for 1 h at 4°C and centrifuged (15,000 *g*, 15 min, 4°C). Protein content was determined using the BCA method (Roche/Hitachi 912 instrument, Roche Diagnostics, Mannheim, Germany).

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

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

204

205 Liver and muscle glycogen content

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

significance level for all comparisons was set at P < 0.05.

234 **Results**

235 Liver response to exercise

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

238

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.1⁻¹ 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).

246

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-1 α mRNA levels exhibited a similar evolution (P < 0.0001) (fig 1D).

252

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).

258

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).

262

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).

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

271

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

peak of recombinant IL-6 plasma levels (fig 5A) (P<0.01) and induced an increase in both

SOCS-3 and IL-6R mRNA levels ($P \le 0.01$ and $P \le 0.05$ respectively) (fig 5B and C).

281

Gluconeogenic genes: Recombinant IL-6 injection induced a dose dependent increase in hepatic PEPCK mRNA levels (main effect P < 0.01) (fig 6A). No effects of IL-6 injection was

found on G6Pase and PGC-1 α mRNA levels (fig 6B and C).

285

286 Plasma glucose and gluco-regulatory hormones: Recombinant IL-6 had no effect on plasma

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)

and corticosterone was unchanged $(252.27 \pm 38.24 \text{ ng.ml}^{-1}, 254.81 \pm 35.12 \text{ ng.ml}^{-1} \text{ and}$

290 $247.48 \pm 49.16 \text{ ng.ml}^{-1}$ 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).

296

297 **Discussion**

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

305 We previously reported that exhaustive running exercise protocol in rats induces an increase in muscle IL-6 transcription and in plasma IL-6 concentration (3). This was 306 307 confirmed in the present study where plasma IL-6 peaked at the end of exercise and decreased as soon as 2 hours recovery, as described in human (30). However, whether such a transient 308 309 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/EBPß phosphorylation, STAT-3 phosphorylation substantially raised at the end of exercise. Therefore, plasma IL-6 concentration and IL-6-313 activated transcription factors phosphorylation followed the same time course. When 314 phosphorylated, both transcription factors are translocated in the nucleus (1), and to determine 315 if transcription factors activation was effective in altering transcription in our experiment, we 316 317 measured hepatic mRNA levels for IL-6-induced genes. SOCS-3, a cytokine signaling suppressor molecule, is highly induced at the transcriptional level by IL-6 (15). Here, we 318 319 found an important increase in liver SOCS-3 mRNA in response to exercise, still significant after 2 hours recovery. Although commonly used to assess IL-6 activity, SOCS-3 is not fully 320

specific of IL-6 and we studied IL-6R and C/EBPB, two IL-6-inducible genes in the liver in 321 322 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 transcription factors phosphorylation and IL-6-responsive genes activation in the liver 324 325 strongly suggests that prolonged intense exercise can acutely induce an active IL-6 signaling in the liver. This result is consistent with the hepato-splanchnic removal of IL-6 previously 326 327 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.

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

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

347 PEPCK control during exercise is complex and in order to understand if IL-6 is able to acutely modulate gluconeogenic genes independent of exercise-related factors, we injected 2 348 349 different doses of recombinant IL-6 to inactive rats and explored hepatic PEPCK and G6Pase mRNA levels one hour after injection. Injected doses were calculated to mimic exercise-350 induced plasma concentration. To avoid post-absorptive GNG blockade by insulin, animals 351 352 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 increase in PEPCK mRNA levels, consistent with in vitro results (5). During food 356 deprivation, increased hepatic PEPCK mRNA levels result from both increased gene 357 358 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 suggests that PEPCK transcripts are stabilized in response exercise and IL-6 injection. No 360 361 changes in PGC-1a 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 common to both GNG and glycogenolysis. In our experiment, rats were fasted overnight 363 (approximatively 14 hours). In this situation, gluconeogenesis accounts for only 40-50% of 364 hepatic glucose output (24, 27), whereas glycogenolysis is fully induced after 4 to 6 hours 365 366 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. An increase in liver PGC-1 α mRNA levels at the end of 1 hour exercise as been previously 368 reported (19) and we show here that this is a transient rise. Whether newly transcribed PGC-369

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 PGC-1 α mRNA levels. Plasma insulin/glucagon ratio and corticosterone were unchanged in 372 373 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 and exercised rats and this correlation was stronger in IL-6 injected rats, further suggesting a 375 376 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 also by very different stimulation times (ranging from hours to days) and injected doses. Two 383 studies have investigated the effect of one ip injection of IL-6 in overnight fasted rodents, and 384 found no effect on PEPCK gene transcription (18, 34). The discrepancy between these results 385 386 and the increase in PEPCK mRNA we report could be explained by a substantially higher hepatic IL-6 signaling activation in previous studies. Indeed, in the first study, authors 387 388 injected high IL-6 doses to mimic the same acute phase response as an acute inflammatory agent (turpentine) and stimulation time was longer (18). In the second study (34), despite 389 390 similar fasting time, doses and stimulation time, the authors report a 30 fold increase in hepatic SOCS-3 mRNA whereas we found a 12 or 4.2 fold increase in exercise and injection 391 392 studies respectively. Thus, it appears that acute effects of IL-6 on gluconeogenic genes are very dependent on circulating concentrations. In our experiment, animals were fasted to 393 mimic exercise-induced variations in hormones and IL-6 was injected alone. Furthermore, 394

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

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

408

409 **Conclusion**

Prolonged intense exercise is a very challenging physiological situation for whole-410 body glucose homeostasis. IL-6 has been shown to increase endogenous glucose production 411 412 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 at the transcriptional level, we tested IL-6 ability to modulate gluconeogenic genes mRNA 415 abundance in resting rats. We found a dose dependent increase in PEPCK mRNA levels in 416 response to IL-6 injection, with no changes in glucoregulatory hormones. Taken together, our 417 results suggest that muscle-derived IL-6 could contribute increasing HGP by directly up-418 419 regulating PEPCK during intense and prolonged exercise.

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545 Legends

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

548 Liver glycogen content (*A*), PEPCK (*B*), G6P (*C*) and PGC-1 α (*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.

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

553 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.

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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 β (*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.
- *: significantly different from the other groups, P < 0.05; **: significantly different from the 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- β (*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.

- * : significantly different from the other groups, P < 0.001; # : significantly different from Rest 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 10μ g.kg bodyweight⁻¹) or an equal volume of PBS buffer (Cont).
- 577 *: significantly different from Cont group, P < 0.05; #: significantly different from $3\mu g.kg^{-1}$ 578 group, P < 0.05.
- 579

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

- 582 Liver PEPCK (*A*), G6P (*B*), PGC-1 α (*C*) mRNA levels were measured in resting fasted rats 1 583 hour after recombinant IL-6 injection (3 or 10 μ g.kg bodyweight⁻¹) or an equal volume of PBS 584 buffer (Cont).
- *: significantly different from Cont group, P < 0.05; **: significantly different from Cont group, P < 0.01.

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.

- Scatterplot showing SOCS-3 or IL-6R against PEPCK mRNA in liver from Cont and Ex groups in exercise experiment (*A* and *B*), and in response to injection of 3 or 10μ g.kg bodyweight⁻¹ recombinant IL-6, or an equal volume of PBS buffer (*C* and *D*)









Rest

Ex

2h

6h







Gene	Accesion	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-1a	NM_031347 (2249-2364)	F : ACGCAGGTCGAATGAAACTGAC R : TGGTGGAAGCAGGGTCAAAATC	0,4	4	53

Table 1