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

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

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

a key contributor to HGP (44). GNG is controlled at the transcriptional level by unidirectional key enzymes, phosphoenolpyruvate-caboxykinase (PEPCK), fructose 1,6 bisphosphatase and glucose-6 phosphatase (G6Pase) the activities of which increase during exercise (10). PEPCK, the most studied gluconeogenic enzyme, plays an important role during exercise 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 increase in hepatic PEPCK mRNA levels and enzyme activity (14, 44). Among the well-described regions of PEPCK gene promoter, the glucocorticoid response unit (GRU) and two cAMP regulatory element (CRE) are involved in exercise-related gene expression, since their mutation substantially decreased hepatic PEPCK mRNA levels (14, 29). However, the molecular mechanisms controlling PEPCK gene expression in response to exercise is still not fully understood. Recently, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) has been shown to play a pivotal role in hepatic GNG regulation and PEPCK gene transcription in response to food deprivation (33, 46). An increase in PGC-1α mRNA levels has been reported recently in exercised mice (19), however its role in hepatic GNG is unknown.

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

derived 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.

The present study focused on prolonged and intense exercise to further understand glucose homeostasis in very demanding physiologic situations. Because plasma IL-6 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 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 intense exercise; 2) to determine if IL-6, can directly modulate gluconeogenic genes mRNA abundance, independently of exercise and glucoregulatory hormones. Therefore, we studied liver IL-6 signaling and gluconeogenic genes mRNAs at the end of one bout of exhaustive 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 recombinant IL-6 injection in resting animals.

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 ± 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 $\mu g.kg$ 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

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.

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.

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 µl buffer (10X), 2 µl dNTP (2.5 mM each dNTP), 0.5 µl oligo-dT primer (50 µM), 0.2 µl RNase inhibitor (20 units.µl^{-1}), 2 µl $MgCl_2$ (25 mM), 0.25 µl Euroscript reverse transcriptase (50 U.µl^{-1}), 1.05 µl RNase free water and 3 µl template mRNA.

Real-time PCR

The PCR reactions were carried out in a 20µl final volume with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 2 µl of cDNA 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 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 calibrator. Five housekeeping genes were necessary to validate the normalization using genorm software (43). These were Hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophylin A (CycA), acidic ribosomal binding protein (ARBP), actin ß and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Primers characteristics are provided as supplemental data. The final quantification was performed using the geometric averaging of the five quantifications. In each experiment, all samples were treated at the same time for mRNA extraction, reverse transcription and PCR reaction.

Western blotting

Liver sections were homogenized in ice-cold lysis buffer (20 $\mu l.mg^{-1}$) containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 $\mu l.ml^{-1}$ 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 Tris-buffered-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 radish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology, Santa 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, 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 incubated overnight with anti STAT-3 antibody or C/EBPβ (Cell Signaling Technology, Beverly, MA, USA) and treated as described above. The bands obtained were quantified using a densitometer (GS-700, Bio-Rad, Marnes-la-Coquette, France).

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).

Blood glucose

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

IL-6 protein

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

Hormones

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

Statistical analysis

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

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).

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).

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).

Liver response to recombinant IL-6

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 ($P<0.01$ and $P<0.05$ respectively) (fig 5B and C).

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-1α mRNA levels (fig 6B and C).

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 ± 3.42, 13.03 ± 2.94 and 14.03 ± 1.69 for Cont, 3 $\mu g.kg^{-1}$ and 10 $\mu g.kg^{-1}$ groups, respectively) and corticosterone was unchanged (252.27 ± 38.24 $ng.ml^{-1}$, 254.81 ± 35.12 $ng.ml^{-1}$ and 247.48 ± 49.16 $ng.ml^{-1}$ for Cont, 3 $\mu g.kg^{-1}$ and 10 $\mu g.kg^{-1}$ groups, respectively).

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

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.

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 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 increase in circulating IL-6 can induce an active IL-6 signaling in the liver has never been assessed. When IL-6 binds to its receptor, signal transduction can involve STAT-3 and/or C/EBPß transcription factors to promote gene transcription (1). In the present study we show 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-activated transcription factors phosphorylation followed the same time course. When phosphorylated, both transcription factors are translocated in the nucleus (1), and to determine if transcription factors activation was effective in altering transcription in our experiment, we 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 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

specific of IL-6 and we studied IL-6R and C/EBPß, two IL-6-inducible genes in the liver in vivo (2, 36). We found that mRNA levels for both genes were transiently activated at the end 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 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 described in exercising human (12). IL-6 increases endogenous glucose production during exercise by an unknown, hormone-independent mechanism (11) and because we found an active signaling in the liver, we hypothesized that IL-6 could act directly on hepatic tissue to modulate HGP during prolonged and intense exercise.

As expected in an exhaustive running exercise, plasma glucose was slightly lower at the end of exercise and gluco-regulatory hormones measurement showed a drop in 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 decrease in hepatic glycogen content (95%) and a substantial increase in liver key gluconeogenic enzymes mRNA, PEPCK and G6Pase. PEPCK is regulated at the 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 PGC-1α mRNA, a key regulator of fasting-induced hepatic GNG (46). Taken together, these data confirm that our model of long lasting exercise is a challenge for glucose homeostasis and that HGP is very dependent on GNG at the end of exercise. Muscle IL-6 gene transcription starts early during exercise, but IL-6 release in the plasma is delayed (17, 25). 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

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

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 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-induced plasma concentration. To avoid post-absorptive GNG blockade by insulin, animals were fasted overnight. Injections resulted in a rise in circulating IL-6, with final plasma concentrations flanking concentrations observed at the end of exhaustive running exercise. As expected, we found a dose dependent increase in hepatic SOCS-3 and IL-6R mRNA levels 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 deprivation, increased hepatic PEPCK mRNA levels result from both increased gene transcription and mRNA stabilization through AMPc (16). In the present work, we did not 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 changes in PGC-1α and G6Pase mRNA abundance were found. Since G6Pase catalyses the 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 (approximatively 14 hours). In this situation, gluconeogenesis accounts for only 40-50% of hepatic glucose output (24, 27), whereas glycogenolysis is fully induced after 4 to 6 hours food deprivation (6). Therefore, a full activation of PEPCK gene is not expected, whereas G6Pase was probably already activated, and no additive effect of IL-6 injection was found. An increase in liver PGC-1α mRNA levels at the end of 1 hour exercise as been previously 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

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

Bibliography

1. **Akira S.** IL-6-regulated transcription factors. *Int J Biochem Cell Biol* 29: 1401-1418, 1997.
2. **Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, and Kishimoto T.** A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *Embo J* 9: 1897-1906, 1990.
3. **Banzet S, Koulmann N, Sanchez H, Serrurier B, Peinnequin A, Alonso A, and Bigard X.** Contraction-induced interleukin-6 transcription in rat slow-type muscle is partly dependent on calcineurin activation. *J Cell Physiol* 210: 596-601, 2007.
4. **Bergmeyer H.** *Methods of enzymatic analysis*. New york: Academics, 1983.
5. **Blumberg D, Hochwald S, Brennan MF, and Burt M.** Interleukin-6 stimulates gluconeogenesis in primary cultures of rat hepatocytes. *Metabolism* 44: 145-146, 1995.
6. **Cahill GF.** Fuel Metabolism in Starvation. *Annual Review of Nutrition* 26: 1-22, 2006.
7. **Christ B, Nath A, and Jungermann K.** Mechanism of the impairment of the glucagon-stimulated phosphoenolpyruvate carboxykinase gene expression by interleukin-6 in rat hepatocytes: inhibition of the increase in cyclic 3',5' adenosine monophosphate and the downstream cyclic 3',5' adenosine monophosphate action. *Hepatology* 26: 73-80, 1997.
8. **Coker RH and Kjaer M.** Glucoregulation during exercise : the role of the neuroendocrine system. *Sports Med* 35: 575-583, 2005.
9. **Coker RH, Simonsen L, Bulow J, Wasserman DH, and Kjaer M.** Stimulation of splanchnic glucose production during exercise in humans contains a glucagon-independent component. *Am J Physiol Endocrinol Metab* 280: E918-927, 2001.
10. **Dohm GL, Kasperek GJ, and Barakat HA.** Time course of changes in gluconeogenic enzyme activities during exercise and recovery. *Am J Physiol* 249: E6-11, 1985.
11. **Febbraio MA, Hiscock N, Sacchetti M, Fischer CP, and Pedersen BK.** Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. *Diabetes* 53: 1643-1648, 2004.
12. **Febbraio MA, Ott P, Nielsen HB, Steensberg A, Keller C, Krustrup P, Secher NH, and Pedersen BK.** Hepatosplanchnic clearance of interleukin-6 in humans during exercise. *Am J Physiol Endocrinol Metab* 285: E397-402, 2003.
13. **Febbraio MA and Pedersen BK.** Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *Faseb J* 16: 1335-1347, 2002.
14. **Friedman JE.** Role of glucocorticoids in activation of hepatic PEPCK gene transcription during exercise. *Am J Physiol* 266: E560-566, 1994.
15. **Fujimoto M and Naka T.** Regulation of cytokine signaling by SOCS family molecules. *Trends Immunol* 24: 659-666, 2003.
16. **Hanson RW and Reshef L.** Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* 66: 581-611, 1997.
17. **Helge JW, Stallknecht B, Pedersen BK, Galbo H, Kiens B, and Richter EA.** The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. *J Physiol* 546: 299-305, 2003.
18. **Hill M and McCallum R.** Altered transcriptional regulation of phosphoenolpyruvate carboxykinase in rats following endotoxin treatment. *J Clin Invest* 88: 811-816, 1991.
19. **Hoene M, Lehmann R, Hennige AM, Pohl AK, Haring HU, Schleicher ED, and Weigert C.** Acute regulation of metabolic genes and insulin receptor substrates in the liver of mice by one single bout of treadmill exercise. *J Physiol* 587: 241-252, 2009.
20. **Howlett K, Febbraio M, and Hargreaves M.** Glucose production during strenuous exercise in humans: role of epinephrine. *Am J Physiol* 276: E1130-1135, 1999.

21. **Inoue H, Ogawa W, Asakawa A, Okamoto Y, Nishizawa A, Matsumoto M, Teshigawara K, Matsuki Y, Watanabe E, Hiramatsu R, Notohara K, Katayose K, Okamura H, Kahn CR, Noda T, Takeda K, Akira S, Inui A, and Kasuga M.** Role of hepatic STAT3 in brain-insulin action on hepatic glucose production. *Cell Metab* 3: 267-275, 2006.
22. **John-Alder HB, McAllister RM, and Terjung RL.** Reduced running endurance in gluconeogenesis-inhibited rats. *Am J Physiol* 251: R137-142, 1986.
23. **Kamimura D, Ishihara K, and Hirano T.** IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol* 149: 1-38, 2003.
24. **Katz J and Tayek JA.** Gluconeogenesis and the Cori cycle in 12-, 20-, and 40-h-fasted humans. *Am J Physiol Endocrinol Metab* 275: E537-542, 1998.
25. **Keller C, Steensberg A, Pilegaard H, Osada T, Saltin B, Pedersen BK, and Neufer PD.** Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *Faseb J* 15: 2748-2750, 2001.
26. **Kjaer M.** Hepatic metabolism during exercise. In: *Exercise metabolism*, edited by Hargreaves MS, LL. Champain, IL: human kinetics, 1998, p. 73-97.
27. **Landau BR, Wahren J, Chandramouli V, Schumann WC, Ekberg K, and Kalhan SC.** Contributions of gluconeogenesis to glucose production in the fasted state. *J Clin Invest* 98: 378-385, 1996.
28. **Livak KJ and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
29. **Nizielski SE, Arizmendi C, Shteyngarts AR, Farrell CJ, and Friedman JE.** Involvement of transcription factor C/EBP-beta in stimulation of PEPCK gene expression during exercise. *Am J Physiol* 270: R1005-1012, 1996.
30. **Ostrowski K, Rohde T, Zacho M, Asp S, and Pedersen BK.** Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol* 508 (Pt 3): 949-953, 1998.
31. **Park EA, Roesler WJ, Liu J, Klemm DJ, Gurney AL, Thatcher JD, Shuman J, Friedman A, and Hanson RW.** The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol* 10: 6264-6272, 1990.
32. **Pedersen BK and Febbraio MA.** Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* 88: 1379-1406, 2008.
33. **Puigserver P and Spiegelman BM.** Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* 24: 78-90, 2003.
34. **Ramadoss P, Unger-Smith NE, Lam FS, and Hollenberg AN.** STAT3 targets the regulatory regions of gluconeogenic genes in vivo. *Mol Endocrinol* 23: 827-837, 2009.
35. **Ramji DP and Foka P.** CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 365: 561-575, 2002.
36. **Saito M, Yoshida K, Hibi M, Taga T, and Kishimoto T.** Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. *J Immunol* 148: 4066-4071, 1992.
37. **Sherman WM.** Metabolism of sugars and physical performance. *Am J Clin Nutr* 62: 228S-241S, 1995.
38. **Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B, and Pedersen BK.** Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537: 633-639, 2001.

519 39. **Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, and Klarlund**
520 **Pedersen B.** Production of interleukin-6 in contracting human skeletal muscles can account
521 for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529 Pt 1: 237-242, 2000.
522 40. **Stith RD and Luo J.** Endocrine and carbohydrate responses to interleukin-6 in vivo.
523 *Circ Shock* 44: 210-215, 1994.
524 41. **Tsigos C, Papanicolaou DA, Kyrou I, Defensor R, Mitsiadis CS, and Chrousos**
525 **GP.** Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. *J*
526 *Clin Endocrinol Metab* 82: 4167-4170, 1997.
527 42. **Turcotte LP, Rovner AS, Roark RR, and Brooks GA.** Glucose kinetics in
528 gluconeogenesis-inhibited rats during rest and exercise. *Am J Physiol* 258: E203-211, 1990.
529 43. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and**
530 **Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric
531 averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.
532 44. **Wasserman DH and Cherrington AD.** Hepatic fuel metabolism during muscular
533 work: role and regulation. *Am J Physiol* 260: E811-824, 1991.
534 45. **Wasserman DH, Spalding JA, Lacy DB, Colburn CA, Goldstein RE, and**
535 **Cherrington AD.** Glucagon is a primary controller of hepatic glycogenolysis and
536 gluconeogenesis during muscular work. *Am J Physiol* 257: E108-117, 1989.
537 46. **Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford**
538 **J, Kahn CR, Granner DK, Newgard CB, and Spiegelman BM.** Control of hepatic
539 gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413: 131-138, 2001.
540
541
542
543

Legends

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, $P<0.05$.

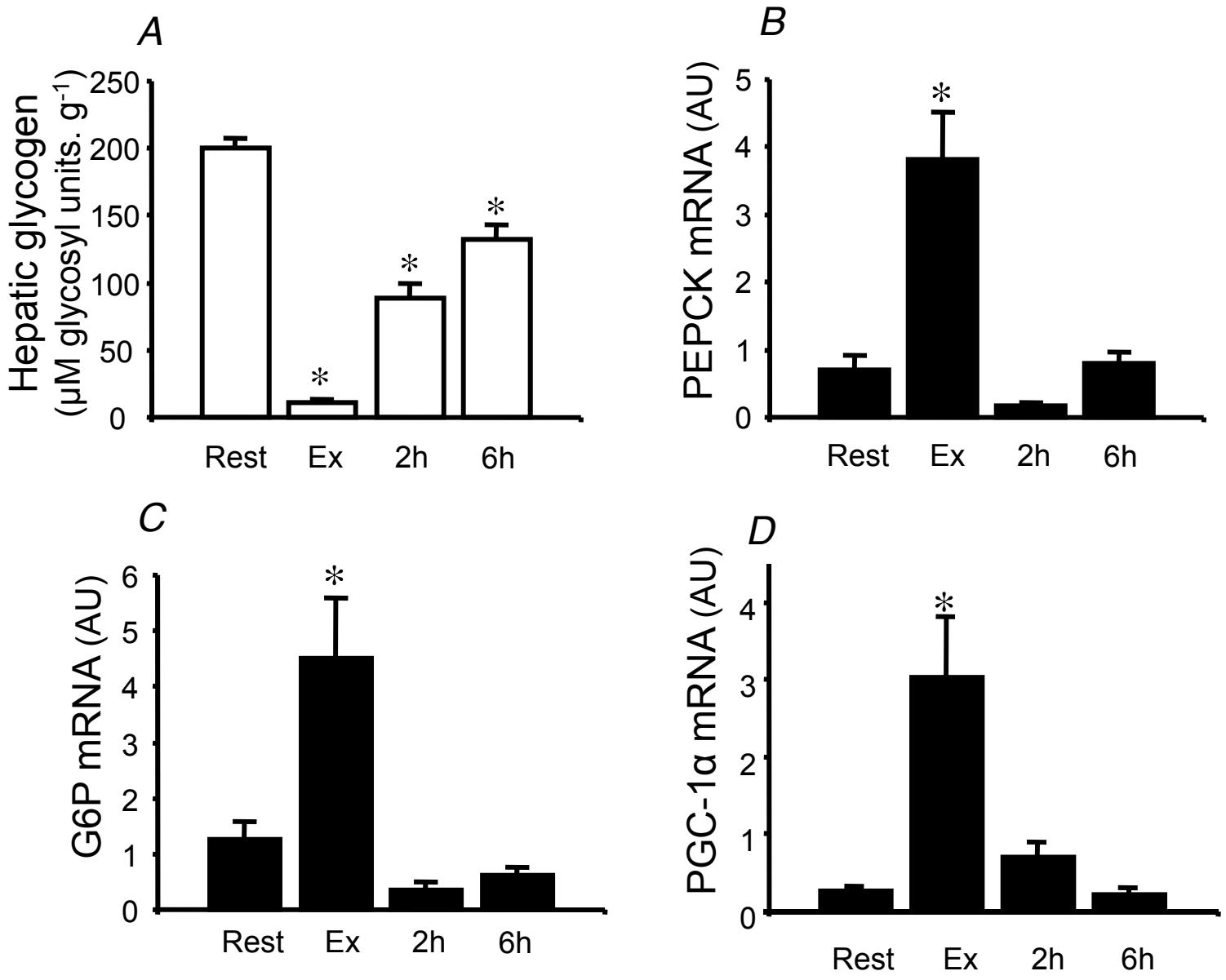
Figure 6: Liver gluconeogenic genes mRNA levels in response to recombinant IL-6 injection.

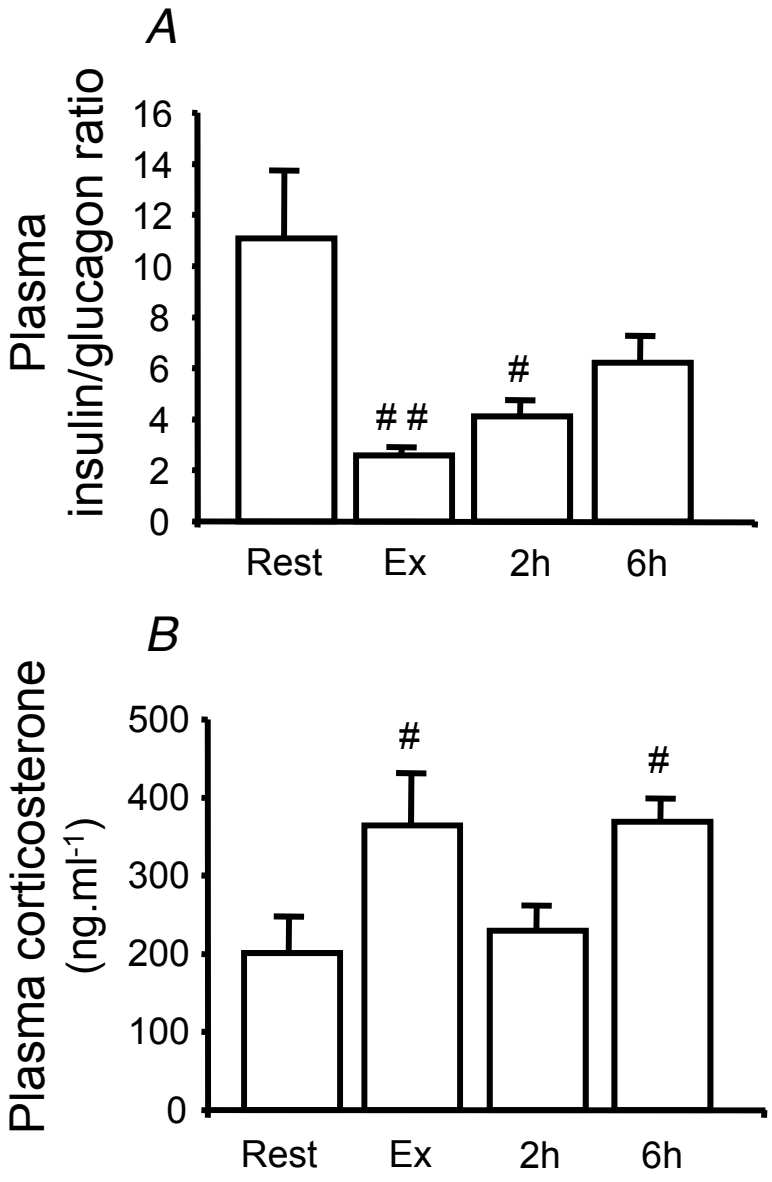
Liver PEPCK (*A*), G6P (*B*), PGC-1α (*C*) mRNA levels were measured in resting fasted rats 1 hour after recombinant IL-6 injection (3 or 10µg.kg bodyweight^{-1}) or an equal volume of PBS buffer (Cont).

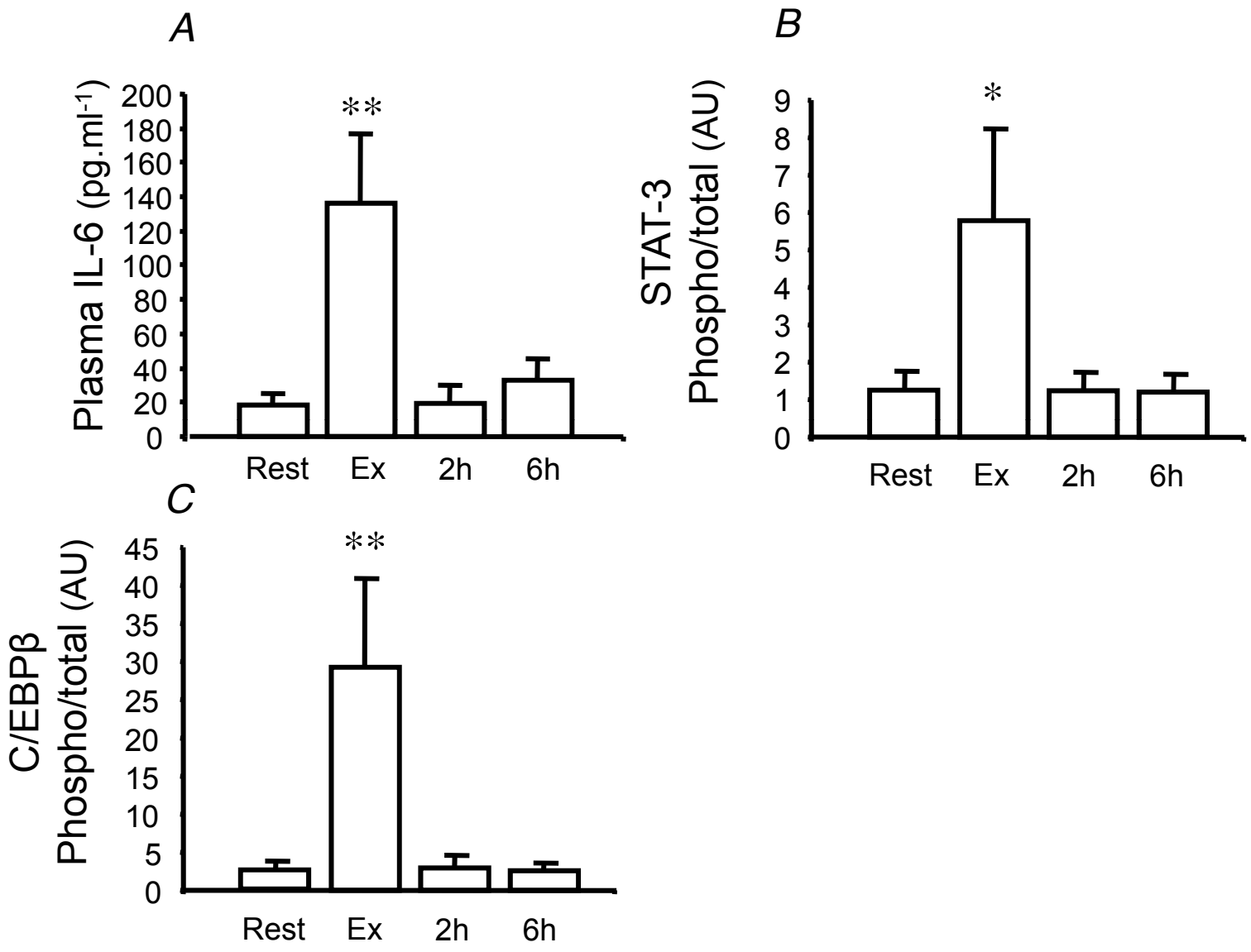
* : significantly different from Cont group, $P<0.05$; ** : significantly different from Cont group, $P<0.01$.

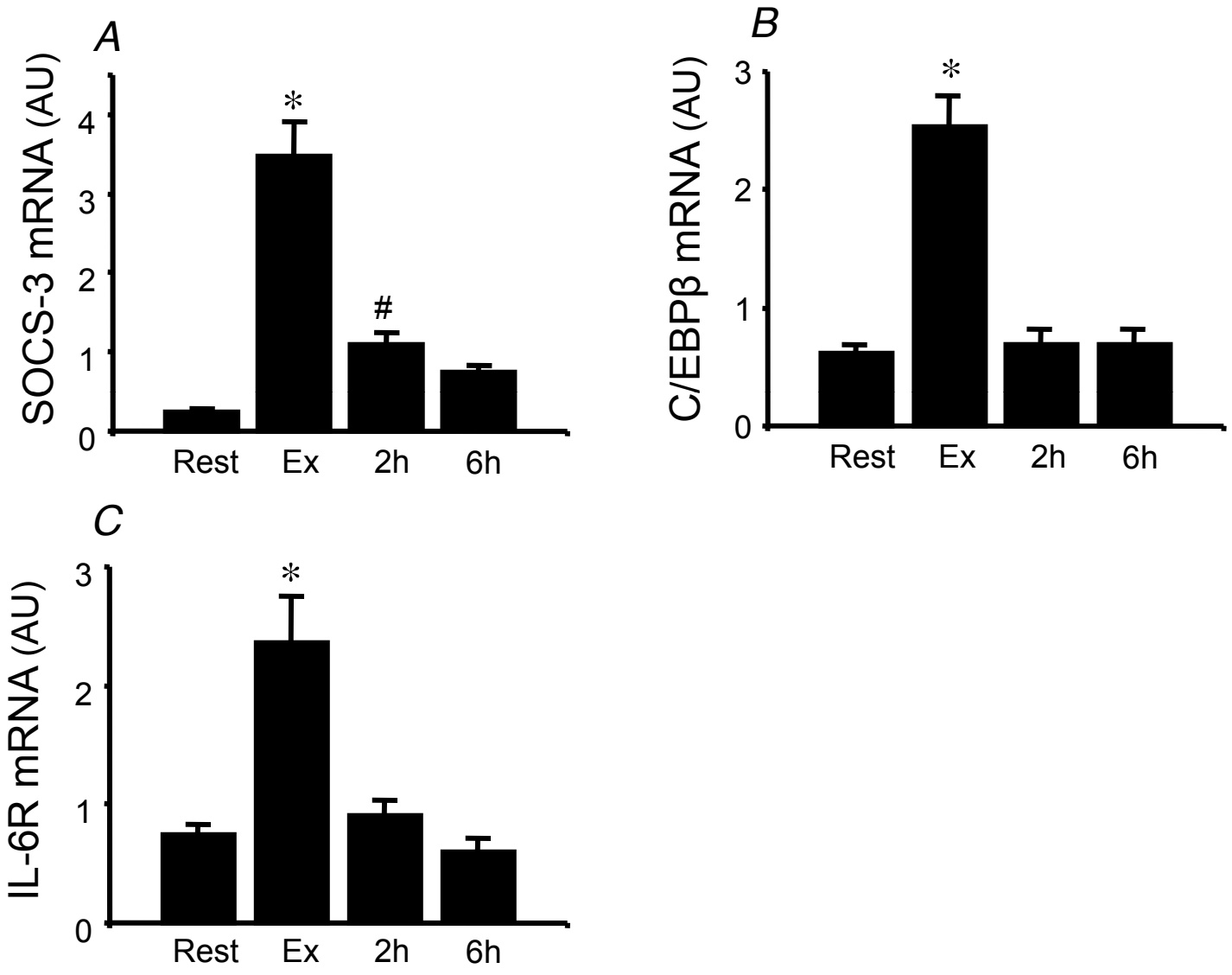
Figure 7: Association between IL-6 responsive genes and PEPCK mRNA in liver in response to exercise and to recombinant IL-6 injection.

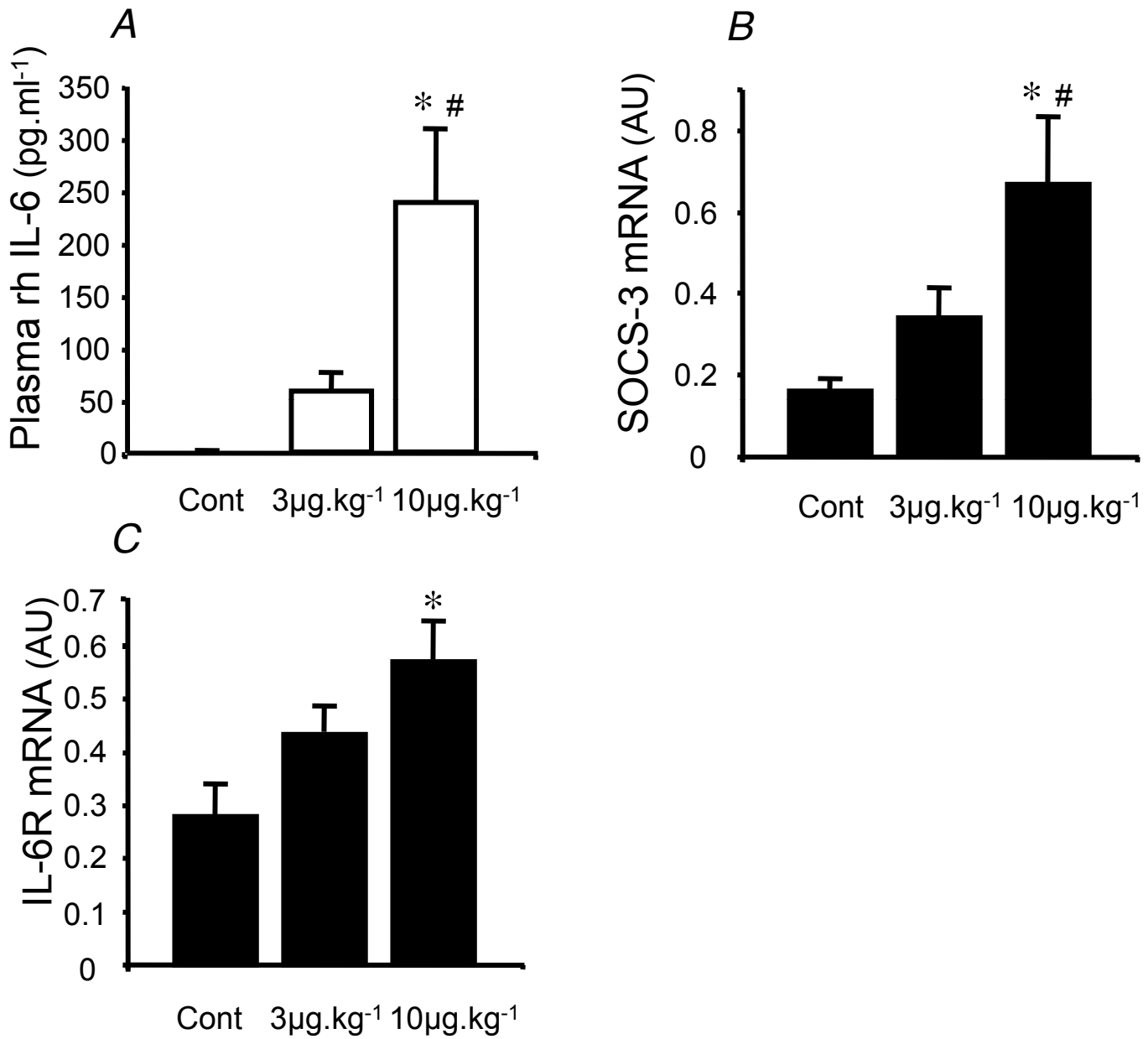
590 Scatterplot showing SOCS-3 or IL-6R against PEPCK mRNA in liver from Cont and Ex
591 groups in exercise experiment (*A* and *B*), and in response to injection of 3 or 10μg.kg
592 bodyweight^{-1} recombinant IL-6, or an equal volume of PBS buffer (*C* and *D*)

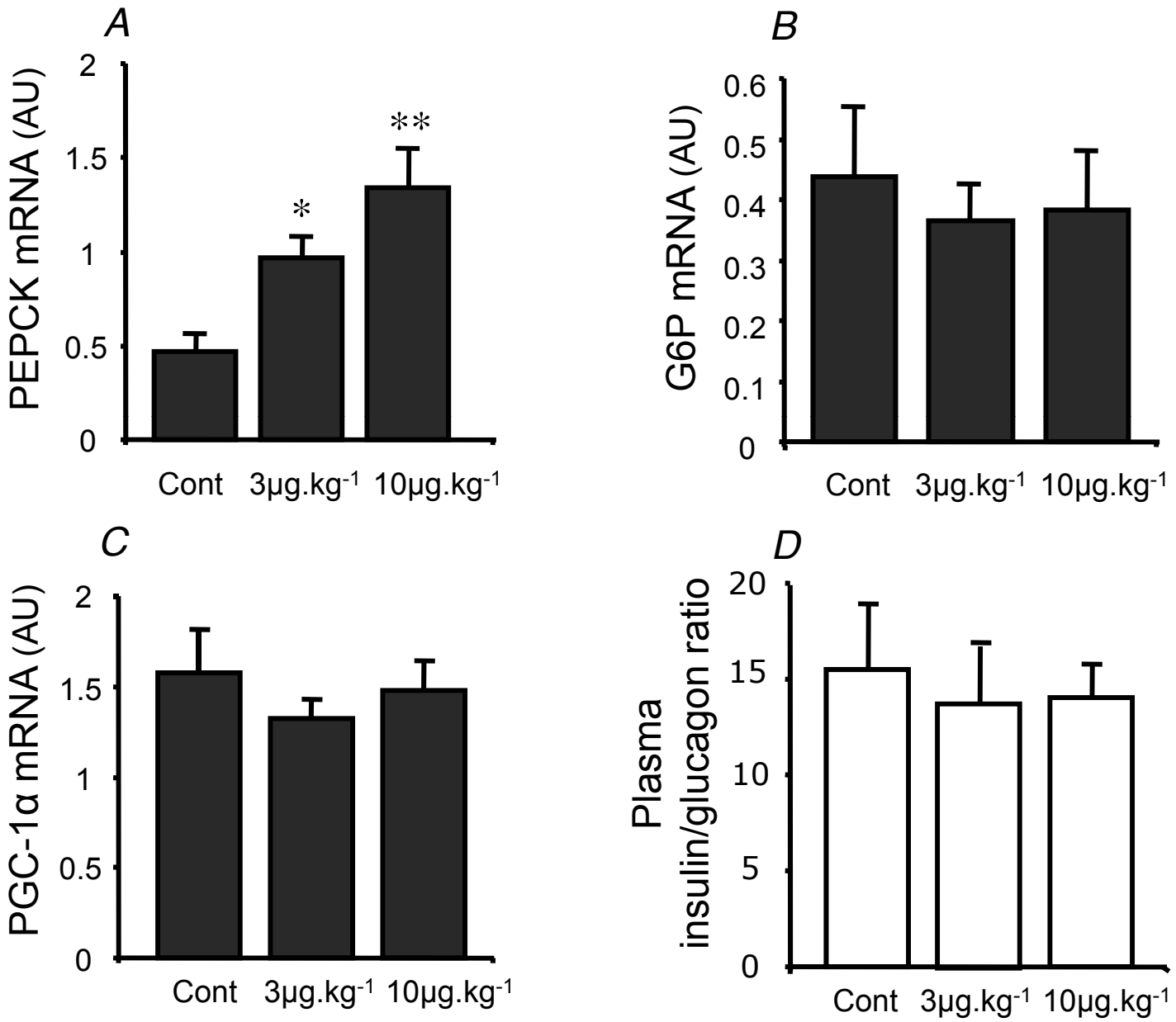

A
Hepatic glycogen
(μM glycosyl units. g-1)
250
200
150
100
50
0
*
*
*
Rest
Ex
2h
6h
B
PEPCK mRNA (AU)
5
4
3
2
1
0
*
Rest
Ex
2h
6h
C
G6P mRNA (AU)
6
5
4
3
2
1
0
*
Rest
Ex
2h
6h
D
PGC-1α mRNA (AU)
4
3
2
1
0
*
Rest
Ex
2h
6h

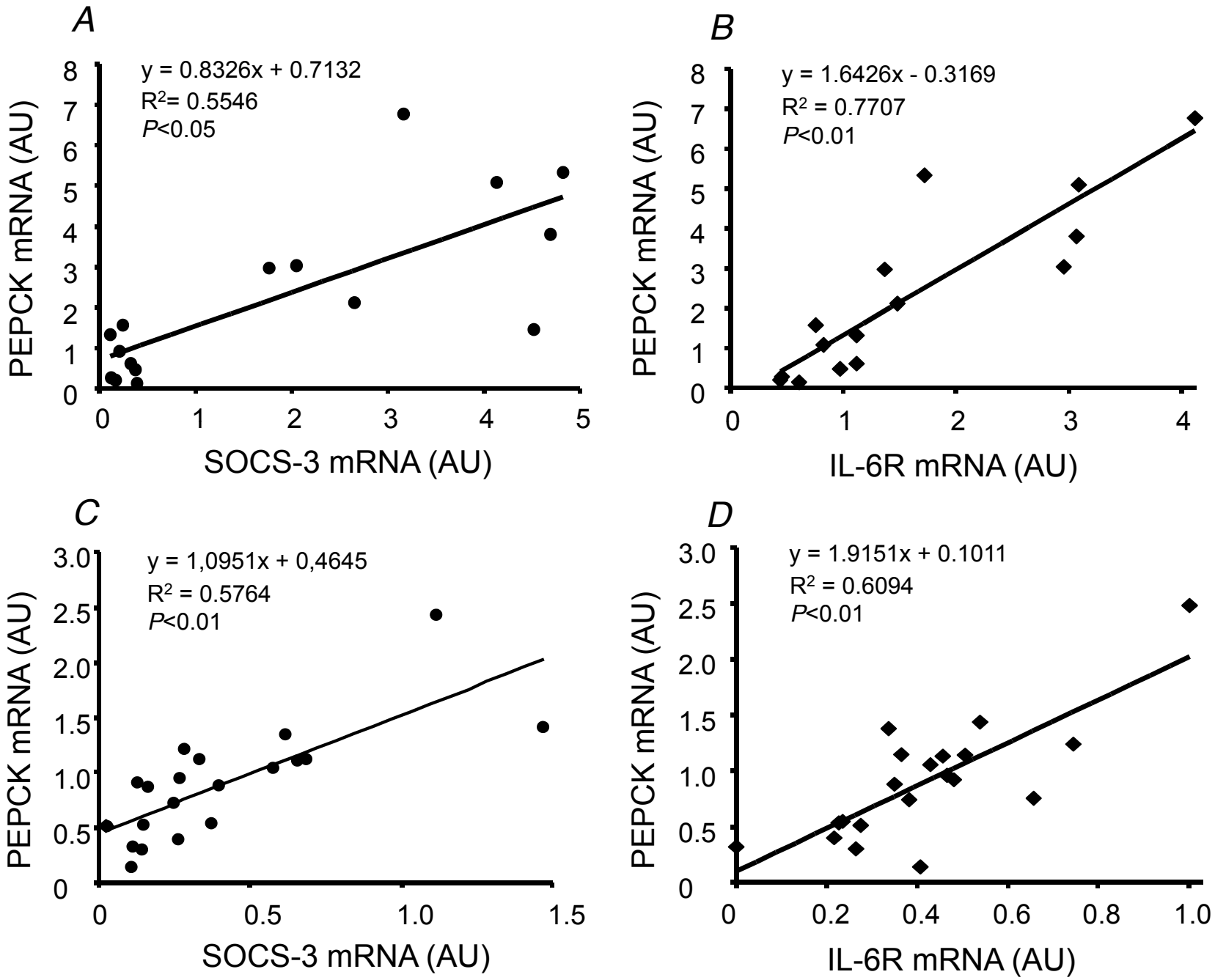

A
Plasma insulin/glucagon ratio
16
14
12
10
8
6
4
2
0
##
#
Rest
Ex
2h
6h
B
Plasma corticosterone (ng.ml-1)
500
400
300
200
100
0
#
#
Rest
Ex
2h
6h


A
Plasma IL-6 (pg.ml-1)
200
180
160
140
120
100
80
60
40
20
0
**
Rest
Ex
2h
6h
B
STAT-3
Phospho/total (AU)
9
8
7
6
5
4
3
2
1
0
*
Rest
Ex
2h
6h
C
C/EBPβ
Phospho/total (AU)
45
40
35
30
25
20
15
10
5
0
**
Rest
Ex
2h
6h


A
SOCS-3 mRNA (AU)
0
1
2
3
4
*
#
Rest
Ex
2h
6h
B
C/EBPβ mRNA (AU)
0
1
2
3
*
Rest
Ex
2h
6h
C
IL-6R mRNA (AU)
0
1
2
3
*
Rest
Ex
2h
6h


A
Plasma rh IL-6 (pg.ml-1)
350
300
250
200
150
100
50
0
* #
Cont
3µg.kg-1
10µg.kg-1
B
SOCS-3 mRNA (AU)
0.8
0.6
0.4
0.2
0
* #
Cont
3µg.kg-1
10µg.kg-1
C
IL-6R mRNA (AU)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Cont
3µg.kg-1
10µg.kg-1


A
PEPCK mRNA (AU)
2
1.5
1
0.5
0
*
**
Cont
3μg.kg-1
10μg.kg-1
B
G6P mRNA (AU)
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont
3μg.kg-1
10μg.kg-1
C
PGC-1α mRNA (AU)
2
1.5
1
0.5
0
Cont
3μg.kg-1
10μg.kg-1
D
Plasma
insulin/glucagon ratio
20
15
10
5
0
Cont
3μg.kg-1
10μg.kg-1


A
y = 0.8326x + 0.7132
R2= 0.5546
P<0.05
PEPCK mRNA (AU)
SOCS-3 mRNA (AU)
B
y = 1.6426x - 0.3169
R2 = 0.7707
P<0.01
PEPCK mRNA (AU)
IL-6R mRNA (AU)
C
y = 1,0951x + 0,4645
R2 = 0.5764
P<0.01
PEPCK mRNA (AU)
SOCS-3 mRNA (AU)
D
y = 1.9151x + 0.1011
R2 = 0.6094
P<0.01
PEPCK mRNA (AU)
IL-6R 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