Molecular mechanisms of the metabolic adaptation to physical exercise: Acute response of the liver and the role of interleukin-6

Molekulare Mechanismen der metabolischen Anpassung an körperliche Aktivität: Akute Antwort der Leber und die Rolle von Interleukin-6

DISSERTATION

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Summary

Understanding the molecular mechanisms that determine the beneficial metabolic response to physical exercise may open up new therapeutic options to prevent and improve Type 2 Diabetes and related metabolic disorders. The focus of this thesis was on the liver as the main regulator of energy homeostasis and on interleukin-6 (IL-6) because it is released from the working muscle and could signal directly to the liver.

A single bout of non-exhaustive endurance exercise caused a rapid and strong transcriptional response of several key regulators of glucose and fatty acid metabolism in the liver of mice that was more pronounced than in the working muscle at this early time point. The amount of insulin receptor substrate-2 protein was increased and insulin signalling, induced by glucose-stimulated endogenous insulin secretion, was amplified. Different stress-responsive pathways were transiently activated in the liver, most notably, mitogen-activated protein kinase (MAPK) signalling, as evidenced by the increased phosphorylation of c-Jun terminal kinase and an extracellular signal related kinase isoform and induction of MAPK target genes. IL-6-type cytokine signalling and p53 were also activated. Exercise-induced oxidative stress was not the stimulus for the hepatic stress response, because it could not be blocked by an antioxidant-enriched diet. It could, however, be linked directly or indirectly to the fall in plasma glucose, since the levels of hepatic gene expression correlated to glucose concentrations after the bout of exercise. Activation of AMPK and the depletion of hepatic glycogen confirmed that the exercising liver experienced acute energetic stress.

IL-6-deficient (IL-$6^{-/-}$) mice showed reduced endurance capacity, which might be caused by impaired activation of IL-6-dependent pathways resulting in reduced energy supply during exercise. However, plasma glucose, insulin and free fatty acid (FFA) levels and the decrease of hepatic glycogen were similar in exercised IL-$6^{-/-}$ and wildtype mice and the induction of gluconeogenic enzymes and other metabolic regulators studied was not impaired. The gluconeogenic response to fasting was also intact in IL-$6^{-/-}$ mice. Training-associated adaptations to regular physical activity, the improvement of glucose disposal and the relative increase in exercise endurance by four weeks of training, were also similar in the IL-$6^{-/-}$ mice. In contrast, IL-$6^{-/-}$ mice had a mild metabolic phenotype in the sedentary, fed state. The lack of IL-6 in the liver appeared to cause a weaker suppression of glucose production in response to insulin, leading to higher glucose and insulin and lower FFA levels. On the other hand, the IL-$6^{-/-}$ mice in our study had a persistently lower weight gain, accompanied by a slightly improved glucose tolerance.

To conclude, the results from this thesis reveal that the liver responds intensely and acutely to physical exercise and that IL-6 is not essential for this acute metabolic response of the liver.

Publications related to this thesis

Hoene M, Lehmann R, Hennige AM, Pohl AK, Häring HU, Schleicher ED, Weigert C (2009) 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.

Hoene M, Franken H, Fritsche L, Lehmann R, Pohl AK, Häring HU, Zell A, Schleicher ED, Weigert C (2010) Activation of the mitogen-activated protein kinase (MAPK) signalling pathway in the liver of mice is related to plasma glucose levels after acute exercise. Diabetologia 53: 1131-1141.

Fritsche L*, **Hoene M***, Lehmann R, Ellingsgaard H, Hennige AM, Pohl AK, Häring HU, Schleicher ED, Weigert C (2010) IL-6 deficiency in mice neither impairs induction of metabolic genes in the liver nor affects blood glucose levels during fasting and moderately intense exercise. Diabetologia (Epub). *These authors contributed equally.

Hennige AM, Heni M, Machann J, Staiger H, Sartorius T, **Hoene M**, Lehmann R, Weigert C, Peter A, Bornemann A, Kroeber S, Pujol A, Franckhauser S, Bosch F, Schick F, Lammers R, Häring HU (2008) Enforced expression of protein kinase C in skeletal muscle causes physical inactivity, fatty liver and insulin resistance in the brain. J Cell Mol Med (Epub).

Hu C*, **Hoene M***, Zhao X, Häring HU, Schleicher ED, Lehmann R, Han X, Xu G, Weigert C. Lipidomics analysis reveals efficient storage of hepatic triacylglycerides enriched in unsaturated fatty acids after one single bout of exercise in mice. *Submitted.* *These authors contributed equally.

Hoene M and Weigert C (2008) The role of interleukin-6 in insulin resistance, body fat distribution and energy balance. *Obes Rev* 9: 20-29. Review.

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Table of abbreviations

Akt/PKB	Serine/threonine protein kinase/protein kinase B
AMPK	AMP-activated kinase
APS	Ammonium persulfate
ATF3	Activating transcription factor 3
bp	Basepairs
cAMP	Cyclic AMP
c-Fos	FBJ osteosarcoma oncogene
c-Jun	Jun oncogene
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleotide 5'-triphosphate
ECL	Enhanced chemiluminescence
EDL	Extensor digitorum longus
EDTA	Ethylendiamintetraacetate
ERK	Extracellular signal related kinase
Fasn	Fatty acid synthase
FFA	Free fatty acids
G6Pase	Glucose-6-phosphatase
GADD	Growth arrest and DNA damage-inducible
GLUT	Glucose transporter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hmox1	Heme oxygenase1
HPLC	High pressure liquid chromatography
i.p.	Intraperitoneal
IGFBP1	Insulin-like growth factor binding protein 1
IL-6	Interleukin-6
IL-6$^{-/-}$	Interleukin-6 knockout mice
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
Mdm2	Murine double minute 2
MT1	Metallothionein 1
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDK-4	Pyruvate dehydrogenase kinase-4
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
POD	Peroxidase

qPCF	quantitative PCR
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
sIL6R	Soluble IL-6 receptor
SOCS3	Suppressor of cytokine signalling 3
SOD	superoxide dismutase
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA buffer
TG	Triglycerides
TNF-α	Tumor necrosis factor-α
trp53inp1	p53 inducible nuclear protein 1
Tyr	Tyrosine
U	Enzyme unit
v/v	Volume/volume
w/v	Weight/volume
WG	White gastrocnemius
WT	Wildtype

1 General introduction and objectives

The beneficial effects of physical activity are numerous and not restricted to physical health. With the prevalence of a sedentary western lifestyle, however, the improvement of glucose and lipid metabolism might be viewed as its most important benefit. The incidence of obesity and with it, of type 2 diabetes mellitus (T2D) is growing worldwide (Zimmet et al., 2005), increasingly also in the young (Li et al., 2006) and in the developing world (Ramachandran et al., 2004). It is a consequence of excessive energy intake and/or insufficient physical activity, in interaction with additional environmental and genetic factors (Booth and Lees, 2007). The hallmarks of T2D are impaired insulin action, reduced insulin secretion and hyperglycaemia. The main actions of insulin are to increase the uptake of glucose by skeletal muscle and adipose tissue, to promote glycogen synthesis in the liver and lipogenesis in adipose tissue, and to inhibit lipolysis and hepatic glucose production. Insulin resistance is a state where the response of the main insulin sensitive tissues is impaired, leading, primarily, to elevated concentrations of glucose and free fatty acids (FFA). From a molecular point of view, insulin action is impaired when the signal transmission via the insulin signalling cascade is not powerful enough to bring about the metabolic downstream effects (White, 2002).

Obesity is associated with a low-grade inflammation of adipose tissue and the accumulation of liver fat which have both been implicated as high risk factors for the development of insulin resistance (Hotamisligil, 2006; Kirchhoff et al., 2007). Physical activity can not only reduce obesity but also prevent and improve impaired glucose tolerance and T2D. Lifestyle interventions comprising exercise programs have even been shown to be superior to pharmaceutical agents in their effectiveness to reduce the T2D incidence (Knowler et al., 2002; Tuomilehto, 2009). However, more patients are willing or able to adhere to a pharmaceutical than to a lifestyle intervention. On the other hand, a significant percentage of obese subjects appears to be resistant to standard interventions (Thamer et al., 2007). This explains why research on physical exercise is being performed with the ambitious aim to decipher the molecular mechanisms that mediate the beneficial metabolic effects of exercise on a cellular level: It could help to develop new therapeutic strategies that are more effective or more specific in their action.

Being the tissue most obviously involved and most easily accessible, the molecular response to physical exercise has best been studied in the skeletal muscle. The muscle responds acutely to physical exercise by increasing the uptake and oxidation of FFA and glucose, which leads to a transient improvement of insulin sensitivity in this tissue already after a single bout of exercise (Richter et al., 1982). More lasting structural and metabolic adaptations occur in response to regular exercise training, mediated by changes in gene expression and in protein levels which lead not only to im-

provements in exercise endurance and performance, but also in insulin sensitivity (Mikines et al., 1989; Pilegaard et al., 2000).

The increased need for energy of the contracting muscle requires a tight interplay between the muscle and the fuel-supplying tissues. This is clearly a challenging metabolic situation for the liver as the main producer of glucose and regulator of fuel homeostasis. However, little is known about the acute response of the liver on a molecular level and on how it is regulated. Factors that are released from the muscle and reach the circulation could act as signalling molecules to the liver and other tissues. The cytokine interleukin-6 (IL-6) has classically been known mainly as an inflammatory cytokine till it was shown to have many properties of such an “exercise factor” (Pedersen et al., 2003) that could mediate the beneficial effects of exercise.

The main objectives of this thesis were to characterize the molecular response of the liver to physical exercise and to assess whether IL-6 has a role in mediating this response.

2 Experimental procedures

2.1 Material

2.1.1 Chemicals, consumables and laboratory equipment

All chemicals were from Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany), except for those listed below. All chemicals were p.a. or better quality.

Tab. 1. Chemicals

Compound	**Manufacturer**
Acrylamide solution 30 %, Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Agarose, peqGold	Peqlab, München,Germany
Bradford reagent, BIO RAD Protein Assay	Bio-Rad, Freiburg, Germany
DNA ladder Generuler 100 bp	MBI Fermentas, St. Leon-Rot, Germany
Protein A-Sepharose (Suspension)	Bio-Rad, Freiburg, Germany
SDS-PAGE molecular weight standard, high range	Bio-Rad, Freiburg, Germany
Trasylol	Bayer Vital, Leverkusen, Germany

Tab. 2. Enzymes, kits and miscellaneous materials

Item	**Manufacturer**
COMPLETE protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
DNeasy Blood & Tissue Kit	Qiagen, Hilden, Germany
dNTPs	Peqlab, München, Germany
ECL Hyperfilm	GE Healthcare, Freiburg, Germany
First Strand cDNA Synthesis Kit	Roche Diagnostics, Mannheim, Germany
Insulin RIA	Linco Research, St. Charles, Missouri
Glucagon RIA	Linco Research, St. Charles, Missouri
LightCycler 480 Multiwell Plates 96, white	Roche Diagnostics, Mannheim, Germany
Micro tube EDTA-vessels, 1.3 ml	Sarstedt, Nümbrecht, Germany
MinElute PCR Purification Kit	Qiagen, Hilden, Germany
Sodium-heparin capillaries, 50µl	Hirschmann, Eberstadt, Germany
Nitrocellulose membrane Protran 0.45 µm	Schleicher&Schuell, Dassel, Germany
Pwo polymerase	Peqlab, München, Germany

RNeasy Fibrous Tissue Mini Kit	Qiagen, Hilden, Germany
Stainless Steel Beads, 5 mm	Qiagen, Hilden, Germany
UVette UV cuvettes	Eppendorf, Hamburg, Germany
Vitamin A/E kit (reagents and HPLC column)	Chromsystems, München, Germany
λ-protein phosphatase (PPase)	New England BioLabs, Beverly, MA, USA

Tab. 3. Injectables

Solution	**Manufacturer**
0.9 % NaCl	B. Braun, Melsungen, Germany
20 % D-glucose	B. Braun, Melsungen, Germany
Insuman Rapid, 40 U/ml	Aventis, Frankfurt, Germany

Tab. 4. Laboratory and mouse-specific equipment

Device	**Manufacturer**
Bicycle computers BC 906	Sigma, Neustadt a.d.W., Germany
Blotting apparatus	Hölzel, Dörfen, Germany
Developer machine Curix 60	Agfa, Köln, Germany
Ebio plus 6668 analyser with lactate electrodes	Eppendorf, Hamburg, Germany
Electrophoresis chamber for SDS-PAGE	in-house technical services
Glucometer Accu-Check Aviva	Roche, Mannheim, Germany
HPLC system with Pump L-6200, autosampler and UV detector L-4000	Hitachi, Mannheim, Germany
LightCycler 480	Roche, Mannheim, Germany
Mouse Accupacer treadmill with motorized grade adjust	Hugo Sachs Elektronik, March-Hugstetten, Germany
pH-Meter	inoLab WTW, Weilheim, Germany
Spectrophotometer BioPhotometer plus	Eppendorf, Hamburg, Germany
Table centrifuges Biofuge pico and fresco	Heraeus, Hanau, Germany
Thermal cycler Mastercycler 5330	Eppendorf, Hamburg, Germany
Heating block Thermomixer comfort	Eppendorf, Hamburg, Germany

Additional instruments used are specified in the respective parts of the methods section.

2.2 Primers

Oligonucleotides were synthesised by TIB molbiol (Berlin, Germany) and primer assays were from Qiagen (Hilden, Germany).

Tab. 5. Genotyping primers

Primer name	Sequence
sense IL-6	ttc cat cca gtt gcc ttc ttg g
antisense IL-6	ttc tca ttt cca cga ttt ccc ag
antisense NEO	ccg gag aac ctg cgt gca atc c

Tab. 6. Primer sets and cycling conditions for real-time PCR

Gene symbol	Primer sequences	Product length	$MgCl_2$ to:	Cycling conditions
Angptl4	sense: caa aac agc aag atc cag ca antisense: ttg gaa gag ttc ctg gca gt	246 bp	3 mM	denaturing: 95°C, 15s annealing: 66°C, 10s elongation: 72°C, 16s
COX1	sense: gcc ttt cga gaa tac cac ga antisense: agg ttg gtt cct cag atg tg	233 bp	4 mM	denaturing: 95°C, 15 annealing: 55°C, 10s elongation: 72°C, 10s
G6Pase	sense: gtc gac tcg cta tct cca ag antisense: gca atg cct gac aag act cc	528 bp	4 mM	denaturing: 95°C, 15s annealing: 66°C, 10s elongation: 72°C, 21s
IRS-1	sense: gat agc gag gct gag caa ga antisense: cac cac gga gtc atc cac tt	429 bp	5 mM	denaturing: 95°C, 15s annealing: 68°C, 10s elongation: 72°C, 17s
PEPCK	sense: cac ctc ctg gaa gaa caa gg antisense: cta cgg cca cca aag atg at	161 bp	4 mM	denaturing: 95°C, 15s annealing: 61°C, 10s elongation: 72°C, 15s
PGC-1α	sense: atg tgt cgc ctt ctt gct ct antisense: atc tac tgc ctg ggg acc tt	179 bp	3 mM	denaturing: 95°C, 15s annealing: 66°C, 10s elongation: 72°C, 8s
SOCS3	sense: gct ggc caa aga aat aac ca antisense: agc tca cca gcc tca tct gt	224 bp	4 mM	denaturing: 95°C, 15s annealing: 66°C, 10s elongation: 72°C, 9s
β-Actin	sense: agc cat gta cgt agc cat cc antisense: ctc tca gct gtg gtg gtg aa	227 bp	4 mM	denaturing: 95°C, 15s annealing: 69°C, 10s elongation: 72°C, 10s
28S-RNA	sense: cca gta ctt cac tcc tgt ct antisense: tct aag agt gag caa cga cg	194 bp	3 mM	denaturing: 95°C, 15s annealing: 61°C, 10s elongation: 72°C, 8s

Tab. 7. Commercial primer assays used for real-time PCR

gene symbol	Assay name	Cycling conditions and manufacturer
ATF3	Mm_ATF3_1_SG	QuantiTect Primer Assays (Qiagen, Hilden, Germany), according to standard conditions
c-Fos	Mm_Fos_1_SG	
c-Jun	Mm_Jun_1_SG	
DUSP1	Mm_Dusp1_1_SG	
DUSP4	Mm_Dusp4_1_SG	
DUSP6	Mm_Dusp6_1_SG	
Fasn	Mm_Fasn_1_SG	

FosB	Mm_Fosb_1_SG	
GADD45b	Mm_Gadd45b_1_SG	
GADD45g	Mm_Gadd45g_1_SG	
Hmox1	Mm_Hmox1_1_SG	
IGFBP1	Mm_Igfbp1_1_SG	
IRS-2	Mm_LOC384783_1_SG	
MT1	Mm_Mt1_1_SG	
p21	Mm_Cdkn1a_SG	
SOD1	Mm_Sod1_1_SG	
SOD2	Mm_Sod2_1_SG	
Trp53inp1	Mm_Trp53inp1_1_SG	

2.2.1 Antibodies

Tab. 8. Primary Antibodies

Antibody	**Dilu-tion**	**Donor**	**Manufacturer**
Akt/PKB	1:1000	mouse	BD Biosciences, San Diego, CA, USA
AMPK α	1:1000	rabbit	Millipore, Schwalbach, Germany
ERK1/2 (p44/p42)	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
IGFBP1	1:500	goat	Santa Cruz, Santa Cruz, CA, USA
IRS-1	1:1000	rabbit	Millipore, Schwalbach, Germany
IRS-2	1:1000	rabbit	Millipore, Schwalbach, Germany
JNK	1:500	mouse mAb	BD Biosciences, San Diego, CA, USA
Mdm2	1:200	mouse mAb	Santa Cruz, Santa Cruz, CA, USA
p38 MAPK	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
p53	1:1000	mouse mAb	Santa Cruz, Santa Cruz, CA, USA
PEPCK	1:5000	rabbit	Santa Cruz, Santa Cruz, CA, USA
phospho-Thr-172 AMPK α	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-ERK	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-JNK	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-p38 MAPK	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-Ser-166 Mdm2	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-Ser-473 Akt/PKB	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-Tyr 705 STAT-3	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
phospho-tyrosine, P-Tyr-100	1:1000	mouse mAb	Cell Signaling, Frankfurt, Germany
STAT-3	1:1000	rabbit	Cell Signaling, Frankfurt, Germany
β-Actin	1:1000	mouse mAb	Cell Signaling, Frankfurt, Germany

Tab. 9. Secondary Antibodies

Antibody	Dilution	Manufacturer
Anti rabbit IgG POD, goat	1:3000	Santa Cruz, Santa Cruz, CA, USA
Anti mouse IgG POD, goat	1:3000	Santa Cruz, Santa Cruz, CA, USA
Anti goat IgG POD, donkey	1:3000	Santa Cruz, Santa Cruz, CA, USA

2.3 Buffers, solutions and gels

Stock solutions and 1x dilutions were prepared in deionized water, if not indicated otherwise, and the pH was adjusted with 1 N NaOH or HCl.

Tab. 10. Buffers, solutions and gels

Buffer, solution or gel	Composition
10x Blotting buffer	480 mM Tris, 390 mM glycin, 0.4 % (w/v) SDS.
10x NET-G	1.5 M NaCl, 50 mM EDTA, 500 mM Tris, 0.5 % (v/v) Triton-X100, 2.5 % (w/v) gelatine, pH 7.4. Stored at 4°C.
10x Phosphatase inhibitor	100 mM NaF, 100 mM Na_3VO_4, 100 mM β-glycerolphosphate, 50 mM $Na_4P_2O_7$. Stored at -20°C.
10x Running buffer	0.025 M Tris, 0.19 M glycine, 0.1 % (w/v) SDS, pH 8.3.
1x Blotting buffer	100 ml 10x blotting buffer, 200 ml Methanol, 700 ml water. Stored at 4°C.
50x TAE buffer	1.25 M Tris base, 5.71 % glacial acetic acid, 50 mM EDTA. pH will be 8.0.
5x Lämmli buffer	10 % (w/v) SDS; 1 % (w/v) bromphenolblue, 50 % (v/v) glycerol, 0.5 % β-mercaptoethanol in 1M Tris/HCl pH 6.8.
7.5 % acrylamide separating gel	20 ml water, 10 ml acrylamide separating gel buffer, 10 ml 30 % acrylamide, 270 µl APS solution, 66 µl TEMED.
Acrylamide separating gel buffer	1.5 M Tris, 2 % (w/v) SDS, pH 8.8.
Acrylamide stacking gel	7.05 ml water, 2.55 ml acrylamide stacking gel buffer, 1.35 ml 30 % acrylamide, 115.5 µl APS solution, 15 µl TEMED.
Acrylamide stacking gel buffer	0.5 M Tris; 2 % (w/v) SDS, pH 6.8.
APS solution	10 % (w/v) ammonium persulfate.
ECL solution A	880 µl of 0.5 M Luminol in DMSO, 864 µl of 0.5 mol p-iodophenol in DMSO in 100 ml of 0.1 M Tris. Stored at 4°C.
ECL solution B	0.0075 % H_2O_2 in 0.1 M Tris/HCl pH 9.35. Stored at 4°C.
HNTG buffer	20 mM HEPES, 10 mM NaF, 150 mM NaCl, 1 % (v/v) Triton-X100, 10 % (v/v) , pH 7.5.
Lysis buffer stock	50 mM Tris, pH 7.6, 150 mM NaCl, 1 % (v/v) Triton-X100. Stored at 4°C. Add 1x protease and phosphatase inhibitors shortly before using.
Ponceau S staining solution	0.1 % (w/v) Ponceau S, 1 % (v/v) glacial acetic acid.

Sample loading dye for gel electrophoresis	0.1 % (w/v) bromphenolblue, 0.1 % (v/v) xylenecyanol, 60 % (v/v) glycerol in 2x TAE buffer.
Stripping buffer	66 mM Tris, 0.5 % (v/v) β-Mercaptoethanol, 2 % (w/v) SDS, pH 6.8.

Additional solutions are described in the respective parts of the methods section.

2.4 General animal procedures

2.4.1 Mouse models and genotyping

Only male mice were used in all experiments. IL-6$^{-/-}$ (B6.129S2-Il6tmlKopf/J) mice, maintained on a C57Bl/6J-background, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The generation of IL-6$^{-/-}$ mice has been described previously (Kopf et al., 1994). Further details on this animal model are given below (4.1.6). Male C57BL/6 mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) or The Jackson Laboratory (Bar Harbor, ME, USA). Whenever knockout and WT mice were directly compared, age-matched animals were ordered together.

Genotyping was performed on liver tissue of sacrificed mice. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) from a piece weighing 10-20 mg according to the manufacturer's protocol. The DNA was eluted in a volume of 200 µl. The primer sequences were as suggested by the breeder. Three primers are used together in one PCR reaction, allowing simultaneous identification of both the wildtype and the disrupted IL-6-allele. The sense IL-6 and antisense IL-6 primer pair amplifies a fragment of 174 bp from the wildtype allele. The sense IL-6 and antisense NEO primer pair amplifies a fragment of 380 bp from the neomycin-disrupted allele.

PCR reaction mixture:

DNA eluate	0.5 µl
10x Pwo buffer	3 µl
dNTP mix (2.5 mM each)	2 µl
primer sense IL-6 (10 µM)	2 µl
primer antisense IL-6 (10 µM)	2 µl
primer antisense NEO (10 µM)	2 µl
Pwo polymerase	1 µl
PCR-grade H_2O	ad 50 µl

PCR program:

DNA polymerase activation at 95°C for 5 minutes.

30 cycles of:

Denaturation	95°C	15 seconds
Primer annealing	61°C	1 minute
Extension	72°C	1 minute

Final extension at 72°C for 2 minutes

Agarose Gel Electrophoresis

20 µl of the PCR reaction were mixed with 1/6 sample volume of 6x loading dye and separated on horizontal 1 % (w/v) agarose gels containing 0.2 µg/ml ethidium bromide in 1x TAE buffer at 100 V. Bands were visualized under UV light and the molecular weight of DNA determined through comparison with a DNA ladder.

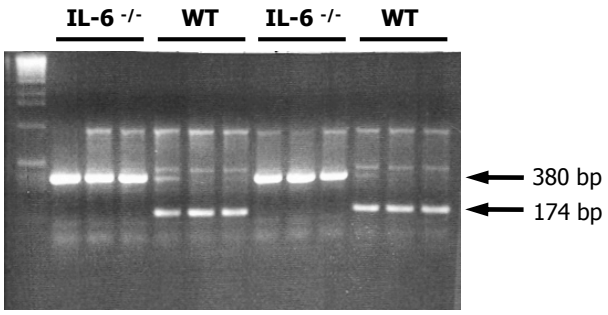

Fig. 1. Genotyping of IL-6$^{-/-}$ and WT mice. A product of 174 bp is amplified from the wildtype allele and a 380 bp fragment from the knockout allele in one PCR reaction with 3 primers.

2.4.2 Animal care

The mice were housed in the animal facility of the Interfaculty Institute of Biochemistry of the Eberhard Karls University, Tübingen. The animals were kept either in groups of 3-6 animals or, in all experiments involving voluntary exercise, singly. Cages were type IV (EBECO, Castrop-Rauxel, Germany) with elevated lids in order to accommodate running wheels where needed. If not stated otherwise, the animals had free access to standard chow Ssniff R/M-H (Ssniff, Soest, Germany) and tap water. Drink was still available during periods of food withdrawal. Mice are nocturnal animals that display the highest voluntary wheel-running activity in the first hours of the light phase (Harri et al., 1999). In order to perform the treadmill-exercise experiments in the animals' most active phase, the mice were kept under an inverted light-dark cycle (dark period 9:30-21:30 h, light period 21:30-9:30 h) and all treadmill sessions and metabolic tests took

place between 10:00 and 14:00 h. All animal experiments were conducted in accordance with the guidelines of laboratory animal care and were approved by the local governmental commission for animal research. Upon arrival, the mice were allowed to acclimatise for a minimum of 2 weeks before performing the experiments.

2.4.3 Treadmill exercise

The treadmill was a 4-channel Mouse Accupacer treadmill with a motorized grade adjust (Hugo Sachs Elektronik, March-Hugstetten, Germany) (Fig. 2A). Depending on the experimental schedule, 2 or 4 mice were exercised at the same time. Where appropriate, equal numbers of knockout and wildtype mice were exercised simultaneously. All mice designed to partake in treadmill-exercise experiments (including the future sedentary controls) were habituated to treadmill running 2-3 times, the last habituation taking place no later than one week before the single bout of endurance exercise or the first training session. The animals were first placed on the idle treadmill belt and allowed to familiarize with the new environment. At 5° uphill inclination, the speed was then gradually increased to 5 m/min and the mice were kept running for 10 min. Mice attempting to rest were encouraged to continue running by gently tapping on their back. Each treadmill session was commenced with a warm-up period of 5 min at a speed of 5 m/min and the same inclination as for the main run. The details for the treadmill sessions are given below in the specific experiment's sections. In order to increase the workload without letting the mice run too quickly, which could structurally damage the muscles, the treadmill was always set at an uphill inclination.

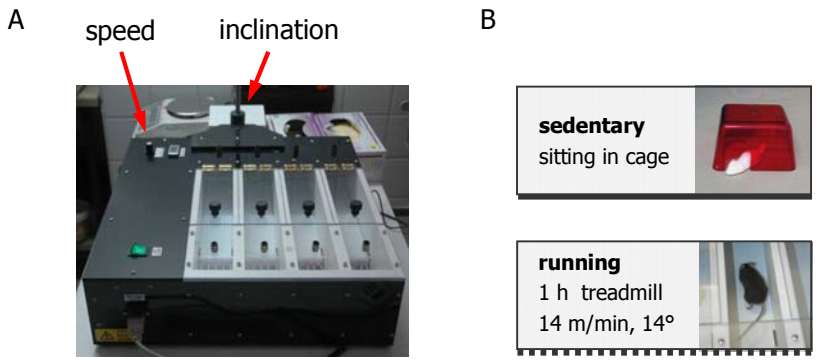

Fig. 2. **A** Picture showing the electric treadmill used to exercise the mice. The arrows indicate where the inclination and belt speed can be set. **B** An illustrated outline of the protocol for a single bout of exercise. The white mouse did not participate in the experiment.

2.4.4 Voluntary wheel-running

The provision of running wheels is an accepted model of voluntary exercise in mice (Allen et al., 2001). To provide optimal conditions for a high voluntary activity,

WodentWheels (Rodipet, Düsseldorf, Germany) with a large diameter of 20 cm and a solid plastic base were used in this study. The voluntary wheel-running activity of mice is higher in wheels of greater diameter and a solid base, in contrast to wheels having a small diameter and metals rods (Banjanin and Mrosovsky, 2000). For experiments that involved voluntary exercise, all mice were singly housed. Plastic houses were given to mice of the sedentary control group as environmental enrichment. The distance travelled in the running wheels was monitored by help of a bicycle computer as shown in (Fig. 3).

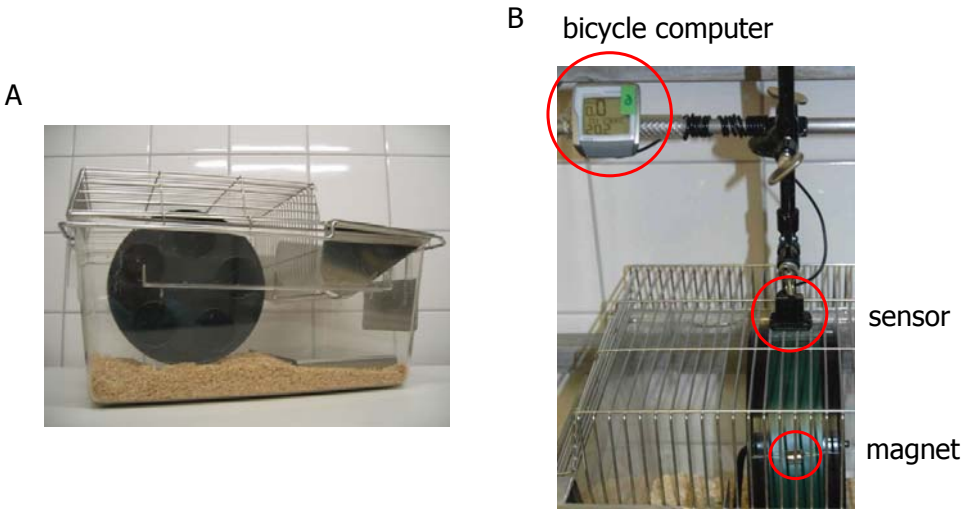

Fig. 3. **A** Image showing a cage equipped with a running wheel. **B** The voluntary wheel-running activity was assessed with a bicycle computer-based system.

2.4.5 Glucose tolerance test

In order to determine glucose tolerance, mice had to be fasted for 16 h during the light phase, from 18:00 to 10:00. The fasted mice first weighted and a small cut was made at the end of the tail to measure glucose and insulin. Plasma glucose from the tail vein was measured with a glucometer that requires less than 1 µl of blood per measurement. By lightly running along the tail from root to tip with two fingers, enough blood could be collected for insulin analysis. Approximately 50 µl were drawn into sodium-heparin-coated capillaries and transferred to plastic cups by blowing out the capillaries with a pipette. These samples were stored and processed for RIA analysis as specified below. A bolus of 2 g glucose per kg body weight was then administered by injecting an adequate volume of 20 % glucose solution i.p.. The blood glucose concentrations were assessed 15, 30, 60, and 90 min after the injection. Before each measurement, dried blood was wiped from the tail with a damp cloth and the first drop of blood was discarded.

2.4.6 Insulin sensitivity test

Insulin sensitivity was tested in fed mice by injecting 1.5 U insulin per kg body weight intraperitoneally (i.p.). Human recombinant insulin was diluted in sterile 0.9 % NaCl solution to a concentration of 0.15 U/ml. To prevent insulin loss by adhesion to the plastic tube, insulin dilutions were only used on the same day. The solution was maintained at room temperature before injecting it into the mice. Blood was collected as described for the glucose tolerance test to determine the starting levels of insulin and glucose before the test. Glucose was further measured from tail vein plasma 15, 30, 45 and 60 min after the administration of insulin.

2.4.7 Fasting

The effects of fasting for 16 h were studied in WT and IL-$6^{-/-}$ mice aged 11-15 weeks (mean: 12 weeks). The mice were fasted from 18:00 till 10:00 the next day, covering mainly the light phase. This protocol is feasible for mice because their regular food consumption during the physically less active phase is still considerable (Harri et al., 1999). The fed control mice had free access to food till being sacrificed.

2.5 Exercise protocols

2.5.1 Single bout of exercise

WT and IL-$6^{-/-}$ mice of 12 weeks age were compared in this experiment. The animals started to run on the treadmill at 5 m/min and 5° uphill slope. After 5 min of warm-up, speed and inclination were increased to 14 m/min and 14° and the mice continued to run for 60 min. Immediately after the bout of exercise, the mice were sacrificed for the collection of blood and tissues. A total of n=8 mice per group were studied in two independent experiments. The experimental procedure is illustrated in (Fig. 2B).

2.5.2 Acute exercise and recovery

Mice aged 12 weeks ran 60 min at 14 m/min and 14° uphill inclination on the treadmill and were either sacrificed immediately afterwards or placed back in their cages with food and drink for 3 h of recovery. Sedentary controls rested in their cages. Mice of all groups had no access to food for the last 60 min before they were sacrificed, either because of running or because of food withdrawal, as illustrated in (Fig. 4). The experiment was repeated three times, resulting in a total number of n=12 animals per group. WT and IL-$6^{-/-}$ mice were directly compared in this experiment.

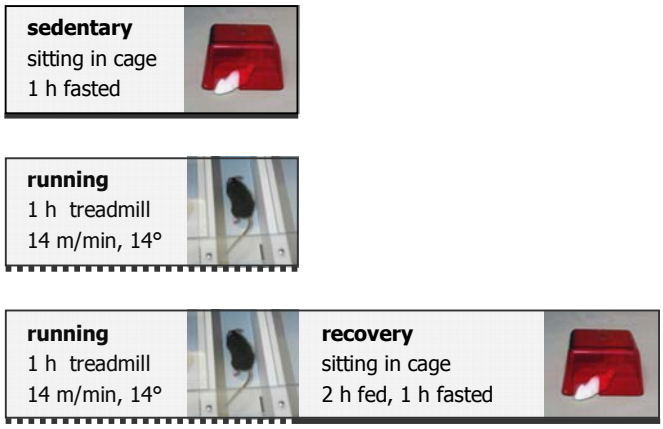

Fig. 4. A scheme depicting the experimental protocol for acute exercise and recovery. The white mice did not participate in the experiment.

2.5.3 Glucose stimulation after acute exercise

Only WT mice were studied in this experiment. The mice were subjected to an acute bout of exercise as described above (2.5.1) or rested sedentary in their cages, with free access to food. Immediately after having completed the run, the exercised mice were either sacrificed or injected i.p. with a 2 g/kg body weight bolus of glucose, given as a sterile 20 % glucose solution. The sedentary mice either received a similar glucose stimulus or were sacrificed without previous treatment. The glucose stimulation lasted 30 minutes in which the animals had no access to food. For each group, n=6 mice of an age of 12-20 weeks (average: 15 weeks) were studied in two independent experiments.

2.5.4 Antioxidant treatment and acute exercise

Before an acute bout of exercise, the mice were pre-fed with either a control diet or an antioxidant-enriched diet. Both diets were based on the standard chow C1000 manufactured by Altromin (Lage, Germany) and were isocaloric. The control diet was devoid of vitamin C and contained 149 U/kg vitamin E. The antioxidant diet contained 100 mg/kg vitamin C and 2000 U/kg vitamin E. In contrast to humans, mice are able to synthesise vitamin C de novo. For this reason, a small amount was given only to the antioxidant-fed group to ensure equal and sufficient availability of this vitamin. The dietary supplementation started when the mice were 7 weeks old and lasted for four weeks, allowing for an enrichment of the lipophilic vitamin E in the tissues. The group size was n=6 and only WT mice were used in this experiment.

2.5.5 Short-term training and running endurance

WT and IL-6$^{-/-}$ mice were compared in groups of n=6. All mice were accustomed to treadmill running as described above (2.4.3). The intervention started at an age of 9 weeks. After a first treadmill training, the exercising mice received running wheels. The exercising mice were also trained after 1 and 2 and weeks of intervention. Each training session was a run till exhaustion. The protocol for the training sessions was as follows:

Training protocol

warm-up: 5 m/min, 20° slope: shortly, till all mice were running

run: 10 m/min, 20° slope: for 5 min

14 m/min, 20° slope: for 5 min

18 m/min, 20° slope: till exhaustion, but for a total maximum of 60 min

The endurance capacity of the sedentary mice was tested in week 3 at slightly more moderate conditions than the training sessions. For a direct comparison of the endurance capacity of the sedentary and trained groups, the exercising mice were subjected to the same test protocol in week 4.

Endurance test protocol

warm-up: 5 m/min, 18° slope: shortly, till all mice were running

run: 10 m/min, 18° slope: for 5 min

14 m/min, 18° slope: for 5 min

18 m/min, 18° slope: till exhaustion, but for a total maximum of 60 min

One week after the last endurance test, glucose tolerance was assessed. Three days later, insulin sensitivity tests were performed. The mice were sacrificed three to five days after the last test, at an age of 16 weeks. To exclude acute effects of voluntary running, the wheels were withdrawn from the exercising mice 16 h before sacrification. Because food intake immediately before sacrification might cause a high variability in plasma insulin and glucose levels, all mice were starved for the last 60 min. The timeline for the whole experiment is shown in (Fig. 5). The mice were weighted immediately before starting the intervention and before the first metabolic test and the weight gain during this period was calculated.

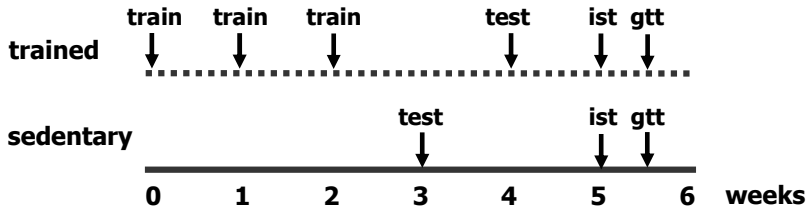

Fig. 5. Timeline for the short-term training and running endurance experiment. The arrows indicate the time points when the mice were subjected to training protocol (run), endurance test (test), insulin sensitivity test (ist) or glucose tolerance test (gtt).

2.5.6 Long-term voluntary exercise and western diet

A total of 8 groups of n=4 WT or IL-6$^{-/-}$ mice were studied. At 10 weeks age, the animals were assigned randomly to the following interventions:

1. sedentary, control diet
2. sedentary, western diet
3. exercising, control diet
4. exercising, western diet

Sedentary mice received plastic houses while exercising mice were provided with running wheels throughout the experiment. The control diet was Teklad global 14 % protein rodent diet Nr. 2014 (Harlan Teklad, Eystrup, Germany). Because the diet consumed by many humans in the western world is characterized by an excessive content of sucrose and animal fat, a western diet was created by enriching the control diet with 5 % each of sucrose and fat from lard. The content of metabolisable energy was only 10 % higher in the western diet.

	Control diet	Western diet
specification:	2014	2014+ 5 % fat, + 5 % sucrose
metabolisable energy:	12.99 kJ/g	14.35 kJ/g
fat	4 % (w/w)	9.4 % (w/w)
sugar	5.72 % (w/w)	11.4 % (w/w)

Before the intervention was started and every second week afterwards, the mice were weighted. Glucose tolerance tests were performed after 10 and 28 weeks, but the results could not be interpreted due to small sample size and high inter-individual vari-

ability. The experiment was terminated after a total of 31 weeks, when the mice were 9 months old. For the last 16 h before sacrification, running-wheels and food were withdrawn.

2.6 Blood and tissue collection and analysis

The mice were killed by decapitation after having been anaesthetised by i.p. injection of ketamine (150 mg/kg body weight) and xylazine (10 mg/kg body weight). Trunk blood was collected, immediately transferred to EDTA-vessels and placed on ice. The samples were spun down in a table centrifuge for 10 min at 4°C and 9,000 g to remove the blood cells and aliquots of the plasma were stored at -20°C. The capillary blood collected for insulin analysis was processed accordingly.

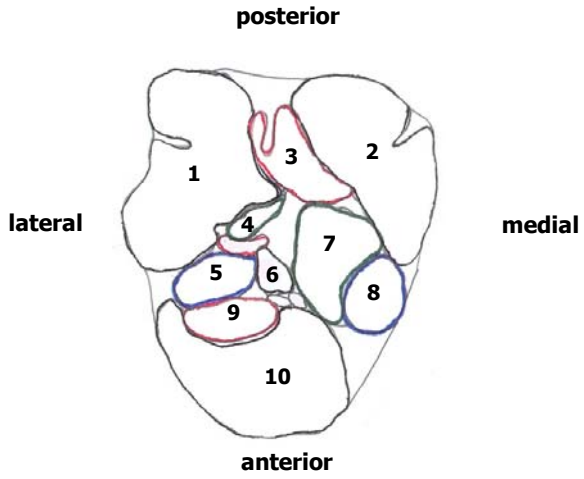

Fig. 6. Cross-section of the lower mouse hind limb showing the location of the different muscles studied. **1** Gastrocnemius medialis, **2** Gastrocnemius lateralis, **3** Plantaris, **4** Soleus, **5** Peroneus longus, **6** Peroneus brevis, **7** Flexor digitorum lateralis, **8** Tibialis posterior, **9** Extensor digitorum longus (EDL), **10** Tibialis anterior (referred to as tibialis). The following muscles were used in this study: Soleus, tibialis anterior, EDL and WG (the white parts of gastrocnemius lateralis and medialis).

Starting with the liver, all tissues were isolated as quickly as possible and either prepared for immediate protein analysis, as described below (2.8), or flash-frozen in liquid nitrogen. The liver was stored away in pieces of different sizes in order to avoid having to thaw the frozen tissue, which could lead to a significant degradation of glycogen and RNA. The muscles from the lower hind limb were dissected away from tendon to tendon. With the exception of the gastrocnemius, all muscles were isolated as a whole. The medial and lateral parts of the gastrocnemius muscle were dissected free of the red-coloured central tissue to obtain the white gastrocnemius (WG). A cross-section of the lower hind limb is shown in (Fig. 6). The following muscles were used in this study: For mRNA analysis, soleus, tibialis anterior and WG. The tibialis anterior will be further referred to shortly as tibialis. For protein analysis, EDL and WG. Glycogen content was

assayed in the WG. Because of their small size, soleus and EDL from one animal could not be simultaneously assessed for mRNA and protein. The soleus is, in mice, the hind limb muscle with the highest content of slow, oxidative type I fibres (Wang and Kernell, 2001) and was preferentially employed for the comparison of gene expression between liver and muscle. All tissues were stored at -80°C till further processing.

2.6.1 Blood parameters

Plasma glucose concentrations were quantified in capillary blood taken from the tail tip with a glucometer. Lactate levels were determined in tail blood using an Ebio plus 6668 analyser (Eppendorf AG, Hamburg, Germany). All other metabolites were measured in the EDTA-plasma collected after decapitation. FFA and TG concentrations were analyzed by a fully automated enzymatic method on the ADVIA 1650 multi analyzer (Siemens Health Care Sector, Fernwald, Germany) in the central laboratory of the University Hospital, Tübingen. Vitamin E was measured with a HPLC-based kit for Vitamin A/E comprised of reagents and column from Chromsystems (München, Germany) according to the manufacturer's protocol. IL-6 and leptin were measured in EDTA-plasma by a mouse Luminex kit (Linco Research, St. Charles, MO, USA) in a cooperation with the University Hospital Zurich. High sensitivity RIAs were used to determine glucagon and insulin levels, as specified by the manufacturer. Insulin was measured either in plasma from capillary samples drawn from the tail vein or in the EDTA-plasma collected after decapitation. Glucagon levels were assessed in a separate plasma fraction obtained from 120 µl of blood that was immediately mixed with a protease inhibitor, 2.5 µl of Trasylol (Bayer Vital, Leverkusen, Germany), in 1.3 ml EDTA-vessels.

2.7 Glycogen content

Glycogen levels were determined by a modification of the procedure described by (Chan and Exton, 1976). Whole muscles (15-100 mg) or liver pieces (approximately 30 mg) were handled on dry ice and weighted without being allowed to thaw. The frozen tissue was placed in 250 µl of 30 % KOH and solubilised for 15 min at 90°C. Glycogen was precipitated by adding 0.2 volumes 1 M Na_2SO_4 and 3 volumes 100 % ice-cold ethanol and centrifugation for 30 minutes at 10,000 g and 4°C. The precipitate was washed twice with 1 ml of 70 % ethanol and dried for 2 h at 40°C. Dry samples were hydrolyzed in 300 µl 1 M HCl by heating to 90°C for 60 min. The samples were cooled down on ice and neutralized by adding an equal volume of 1 M NaOH. The samples were centrifuged for 10 minutes at 10,000 g and glucose concentration in the cleared solution was determined enzymatically using the ADVIA 1650 system (Siemens Health Care Sector, Fernwald, Germany). Glycogen contents are given in mg of detected glucose per g of tissue.

2.8 Protein analysis

2.8.1 Tissue lysates

Immediately after isolation, liver pieces or whole muscles were homogenized in ice-cold lysis buffer in a Dounce homogenizer. After solubilisation on ice for 30 min, the homogenates were clarified by 3 rounds of centrifugation (10 min at 12,000 g) at 4°C. Protein concentration was determined in the cleared lysates and aliquots were stored at -80°C or used immediately for western blot analysis.

2.8.2 Bradford assay for protein concentrations

Protein concentrations in tissue lysates were determined according to the method of Bradford (Bradford, 1976). The Bradford reagent concentrate was diluted 1:5 with de-ionised water and filtered through a Whatman filter paper. The diluted reagent was stored at 4°C. For each assay, 10 µl of the sample were mixed with 200 µl of diluted reagent and incubated for 10 min at room temperature. The extinction was measured at 595 nm against a blank. BSA solutions ranging from 0.05 to 0.5 µg/µl were used as protein concentration standards. If necessary, the samples were diluted with water to fall into the standard range.

2.8.3 Immunoprecipitation

IRS proteins were immunoprecipitated to visualize tyrosine phosphorylation. Tissue lysates containing 1 mg of protein were mixed with 30 µl of protein A sepharose solution and 2.5 µg antibody against IRS-1 or IRS-2 and incubated on a rotor at 4°C for 4 hours. Immunoprecipitates were spun down at 4°C, 4,000 g for 30 seconds and the pellets washed and centrifuged twice with 250 µl of HNTG buffer supplemented with phosphatase inhibitors. The pellet was mixed with 25 µl of 5x Lämmli and incubated at 95°C for 5 min which lead to displacement of the proteins from the beads. The complete liquid fraction was loaded on a gel and subjected to western blot analysis.

2.8.4 Dephosphorylation assay

For the dephosphorylation of IRS-2, cleared tissue lysates containing 150 µg of protein were incubated with 100 U of λ-PPase and the supplied buffer at 30 °C for 30 min. Control samples were similarly incubated with phosphatase buffer only. The samples were then separated by SDS-PAGE and analysed by western blotting.

2.8.5 SDS-PAGE and western blotting

The 7.5 % separating gel was prepared, poured immediately into the gel cassette and topped with water. After polymerization of the gel, the water was decanted. The stacking gel was poured on top and allowed to polymerize. A comb was placed into the liquid stacking gel to define sample pockets. Aliquots from tissue lysates containing 200 µg protein were mixed with an adequate amount of 5x Lämmli buffer and heated to 95°C for 5 min. The cooled-down samples were loaded and proteins were separated by running the gel overnight at 50 mV. Molecular weights were determined by comparison to a protein molecular weight standard run on the same gel.

For western blot analysis, the proteins in the polyacrylamide gel were transferred onto nitrocellulose membrane in a semi-dry electro blot. A transfer sandwich was assembled out of filter paper, nitrocellulose immobilizing membrane and gel, all pre-soaked or rinsed in transfer buffer. Protein transfer was performed for 2 hours at 0.8 mA/cm^2. The outcome of PAGE and blotting procedure was controlled by staining the membrane with Ponceau S solution for approximately 1 minute. After washing briefly in H_2O, the total cell protein could be seen in distinct lanes. Where appropriate, the blot was cut into strips for individual analysis with different antibodies.

The membrane was blocked for 3x15 minutes at room temperature in NET-G. Incubation with the primary antibody, diluted in NET-G, was carried out overnight at 4°C. The blot was then washed three times with NET-G, followed by incubation with the appropriate POD-coupled secondary antibody for 1 hour and washing as before. The POD substrate was freshly made by mixing equal parts of ECL solutions A and B. Membranes were incubated for 3 minutes with the POD substrate and exposed on X-ray films for an appropriate period of time. The X-ray films were developed in an Agfa Curix 60 processor (Kodak, Stuttgart).

2.8.6 Stripping antibodies from nitrocellulose membranes

To directly relate the amounts of a protein to its phosphorylation, both were assayed on the same membrane. This was achieved by incubating first with the phospho-specific antibody, stripping off this antibody and re-probing the membrane with the protein-specific antibody. The membranes were stripped by placing them in stripping buffer for 30 min at 56 °C. The stripped membranes were blocked with NET-G before the second round of immunodetection.

2.9 Gene expression analysis

2.9.1 Purification of mRNA from tissues

Total RNA was extracted with the RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Frozen, whole muscles and pieces of liver tissue (about 10 mg) were quickly homogenized in a TissueLyser (Qiagen, Hilden, Germany). The samples were placed in 2 ml plastic tubes pre-loaded with an appropriate amount of lysis buffer and a steel bead and shaken for 2 min at 20 Hz (liver) or 4 min at 30 Hz (muscles). RNA concentration and quality were assessed by diluting the eluate 1:20 – 1:40 in HPLC-grade water and measuring the absorption at 260 and 280 nm in a spectrophotometer against a water blank.

2.9.2 Microarray and pathway analysis

Equal amounts of total RNA from 4 mice per group were pooled, amplified with the MessageAmp Kit II (Ambion, Austin, Texas, USA) and hybridised to a MouseRef-8 v1.0 Expression BeadChip array (Illumina, San Diego, California, USA) by the customer service program of the Microarray Facility Tübingen. The service also included image processing, quality control of the arrays, scaling and normalization of signal values. Pathway analysis was performed in a cooperation with the Center for Bioinformatics of the University Tübingen. An automated access to the KEGG database (Kanehisa et al., 2008) was implemented to retrieve information about the association of the inspected genes to signalling pathways.

2.9.3 Reverse transcription and real-time PCR

Reverse transcription of mRNA was performed using random hexamer primers with the First strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Mannheim, Germany). 1 µg of total RNA was reverse transcribed in a volume of 20 µl. cDNA aliquots of 2 µl were then submitted to real-time quantitative PCR with the LightCycler 480 system using 96 well-plates (Roche Diagnostics, Mannheim, Germany). Real-time quantitative PCR (qPCR) allows for the quantification of starting amounts of cDNA by measuring the amount of double-stranded DNA at each amplification cycle. To this end, SYBR® green is included in the PCR reaction, a dye that emits a fluorescent signal of a defined wavelength upon binding to double-stranded DNA. The fluorescence measured during each cycle of amplification is plotted as a curve and the LightCycler software determines the cycle number at which the amplification curve crosses a defined threshold. This threshold value is compared to a standard curve included in the same run to de-

termine the starting amount of cDNA in the sample. As a quality control, a DNA melting analysis was performed after each run to identify potential non-specific PCR products.

The following conditions were used for pairs of primers in combination with reagents from the FastStart DNA-MasterSYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). Primer sets used and cycling conditions have been established in the central laboratory of the University Hospital Tübingen and are listed in the materials section (Tab. 6).

FastStart PCR reaction mixture:

cDNA	2 µl
SYBR green mix	2 µl
sense and antisense primers (20 µM)	0.5 µl each
$MgCl_2$ (25 mM)	1.6 - 4 µl (primer-specific)
PCR-grade H_2O	ad 20 µl

FastStart PCR program:

Amplification

Initial activation/denaturation at 95°C for 10 minutes

50 cycles of:

Denaturation	95°C	15 seconds
Primer annealing	(primer-specific °C)	10 seconds
Extension	72°C	(primer-specific)

Melting

Denaturation	95°C	5 seconds
Start melting at	(annealing + 2°C)	10 seconds
Gradually increase to	98°C	

The following conditions were used for QuantiTect primer assays in combination with the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany):

QuantiTect PCR reaction mixture:

cDNA	2 µl
QuantiFast reagent	10 µl
QuantiTect primer mix	2 µl
PCR-grade H_2O	ad 20 µl

QuantiTect PCR program:

Amplification

Initial activation/denaturation at 95°C for 3 minutes

40 cycles of:

Denaturation	95°C	3 seconds
Combined annealing/extension	60°C	30 seconds

Melting

Denaturation	95°C	5 seconds
Start melting at	62°C	10 seconds
Gradually increase to:	98°C	

The standards for real-time PCR were generated by purification of DNA from a previous run using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) as indicated by the manufacturer. The DNA concentration in the eluate was determined by measuring the absorption at 260 nm of a 1:20 dilution in HPLC-grade water. A standard stock solution of 5 µg/µl DNA was prepared in PCR-grade water and used directly or stored in aliquots at -20°C. A series of 1:10 dilutions of the standard stock was prepared in PCR-grade water as follows:

Standard dilution series:

standard 1: 2 µl stock solution + 18 µl water

standard 2: 2 µl standard 1 + 18 µl water

standard 3: 2 µl standard 2 + 18 µl water

standard 4: 2 µl standard 3 + 18 µl water

proceeding accordingly till standard 9

Only the dilutions 4-9 were used for the standard curve. The mRNA contents are expressed as arbitrary units. Data shown in this work are non-normalized; however, normalization to β-actin mRNA content (liver) or 28S mRNA content (muscle) was always performed as a control and gave results comparable to the non-normalized values. β-actin mRNA was slightly upregulated by acute exercise in muscle (not shown).

2.10 Statistical analysis

Densitometric analysis of western blots was performed with the Gelscan Professional V5.1 Software (BioSciTec GmbH, Frankfurt a.M., Germany). The resulting values are shown as arbitrary units. Phosphorylation intensities were related to the amount of the corresponding protein determined by re-probing the same membrane, where appropriate. The levels of β-actin protein were routinely analyzed to visually confirm that similar amounts of protein had been loaded to the gel.

Means ± SEM were calculated and groups of data were compared using student's t-test using statistical software package JMP 4.0 (SAS Institute, Cary, NC, USA). Logarithmic regression analysis was also performed with the JMP 4.0 Software using standard settings. The statistical significance was set at $p < 0.05$.

3 The exercising liver

3.1 Introduction

At first sight, the organ most intensely affected by exercise is the skeletal muscle. It burns energy – carbohydrates, amino acids and fat – to perform mechanical work. The acute responses of the myofibres culminate in alterations of fuel metabolism, most notably, in an increase in fatty acid oxidation and an improvement of glucose uptake (Hayashi et al., 1997). At the same time, adaptive responses are initiated that enable the muscle to carry out similar work more efficiently in the future. These adaptations include increased muscle mass, protection against structural damage and improvement of oxidative metabolism (Holloszy and Coyle, 1984). While glucose is the main source of energy for short periods of contractile activity, sustained exercise requires the utilization of fatty acids as main fuel source, since glucose storage is limited and muscle protein is valuable. Therefore, the hallmark of endurance exercise is a shift in the energy utilization of the muscle towards fatty acids (Horowitz and Klein, 2000). With increasing exercise intensity, however, and especially in the untrained state, the oxidation of fatty acids is limited by oxidative capacity and oxygen supply. Glucose must then be provided by the liver, the main producer of glucose and the key regulator of glucose homeostasis, to supply both the working muscle and the glucose-dependent tissues with energy. For this reason, exercise is a strong metabolic challenge for the liver, a condition similar to fasting. In both states, two mechanisms contribute to hepatic glucose output, glycogenolysis and gluconeogenesis (for a review, see (Wahren and Ekberg, 2007)). Glycogenolysis, the breakdown of hepatic glycogen, is initiated first and can only be sustained till the glycogen stores are depleted. With proceeding exercise, gluconeogenesis, the synthesis of glucose from lactate, glucogenic amino acids and glycerol, increases. At the same time, fatty acid oxidation and uptake are upregulated in the liver in order to provide sufficient energy in the form of ATP to fuel gluconeogenesis. According to a recent study in mice, acute exercise strongly lowers the energy charge, the ratio of ATP to AMP in the liver, while the ATP level is preserved in the muscle (Camacho et al., 2006). This suggests that the liver might in fact be a very hard-working organ during physical activity, especially during an intensive bout of endurance exercise performed in the untrained state.

3.1.1 Regulation of signalling pathways and transcription in the working muscle

The stimuli to which the muscle responds are very diverse and range from the transduction of mechanical force (Chambers et al., 2009; Sasai et al., 2010) and hypoxia (Wagner, 2001) to systemic factors like catecholamines. Important intracellular messengers are Ca^{2+}, changes in pH and in the ratio of ATP to AMP and reactive oxygen species (ROS). The pathways that can be activated by exercise in the muscle include AMP-activated kinase (AMPK) (Hardie, 2004; Musi et al., 2003), Ca^{2+}/calmodulin-dependent kinases (Rose et al., 2006), serine/threonine protein kinase/protein kinase B (Akt/PKB) (Sakamoto et al., 2003), atypical protein kinase C (Perrini et al., 2004; Richter et al., 2004), and mitogen-activated protein kinase (MAPK) (Aronson et al., 1997) signalling. These pathways are involved both in the acute metabolic and in the adaptive response of the muscle. AMPK has specific importance as a sensor and protector of the cellular energy status. When AMP levels are high and ATP levels are low, AMPK is active and inhibits ATP-consuming and stimulates ATP-consuming processes (Hardie et al., 2006). An important role has been assigned to the transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) that is induced by AMPK and several other pathways and coordinates the transition towards a more oxidative phenotype with a higher content of mitochondria (Hood et al., 2006; Lin et al., 2002; Pilegaard et al., 2003).

The transcriptional response depends on the duration and intensity of exercise (Dudley et al., 1982; Hildebrandt et al., 2003) but also on substrate availability (Pilegaard et al., 2005) and predominant fibre type of the muscle studied (Hildebrandt et al., 2003). While some genes are upregulated quickly, the response of most metabolic genes peaks in the first hours of the recovery phase (Leick et al., 2007; Pilegaard et al., 2000). Cumulative effects of transient elevations in gene expression during the recovery phase eventually lead to a functional adaptation which, therefore, also depends on the frequency of training (Dudley et al., 1982; Neufer and Dohm, 1993). Among the late responding genes, many are involved in mitochondrial biogenesis and vascularisation, for example carnitine palmitoyltransferase 1a (Hildebrandt et al., 2003), uncoupling protein 3 (Jones et al., 2003) and vascular endothelial growth factor (Hiscock et al., 2003). Although not as prominently as after resistance exercise, the transcription of myogenic factors like MyoD is also elevated in the recovery phase after endurance exercise (Yang et al., 2005). The genes that respond more quickly to exercise are acute regulators of glucose and lipid metabolism, like hexokinase II (O'Doherty et al., 1996), glucose transporter type 4 (Glut4) (Kraniou et al., 2000) and lipoprotein lipase (Hildebrandt et al., 2003) or are involved in the oxidative defence, such as heme oxygenase1 (Hmox1) (Essig et al., 1997).

3.1.2 ROS generation and stress response in the muscle

Stress can be imposed on the muscle in the form of structural damage to the myofibres, especially during excentric exercise like downhill running. However, concentric exercise protocols like uphill running also exert significant stress on the working muscle through an increased generation of ROS (Khassaf et al., 2003; Smolka et al., 2000). The primary ROS species generated are superoxide (O_2^-) and nitric oxide (NO) which further react to form H_2O_2 and free oxygen radicals (Jackson, 2005). Superoxide accumulates due to elevated rates of mitochondrial respiration and increased xanthine oxidase activity during exercise (Sjodin et al., 1990) that exceeds the antioxidant capacity of the cell. NO is a signalling molecule synthesized from arginine by nitric oxide synthases which reacts with superoxide to peroxynitrite, a strong oxidizing agent that can lead to depletion of thiol groups, damage to the DNA and nitration of proteins (Powers and Jackson, 2008). As a countermeasure, genes involved in antioxidant defence or DNA repair are upregulated (Essig et al., 1997; Khassaf et al., 2001). However, ROS signalling also plays an important role in the acute and adaptational metabolic response of the muscle and the administration of anti-oxidative vitamins to counteract oxidative stress has even been shown to inhibit beneficial effects of exercise, namely, mitochondrial biogenesis in mice (Ristow et al., 2009) and the improvement of insulin sensitivity in humans (Gomez-Cabrera et al., 2008).

3.1.3 Exercise-induced MAPK signalling in the muscle

An important pathway that mediates cellular responses to oxidative stress and other aversive stimuli is MAPK signalling (Kyriakis and Avruch, 2001). Exercise has been shown to activate the MAPKs extracellular signal related kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK) in the muscle (Goodyear et al., 1996). The different MAPKs are regulated in distinct ways, JNK being the most stress-responsive form (Sakamoto and Goodyear, 2002) that is best activated by excentric, damaging exercise in humans (Boppart et al., 1999). However, all three MAPKs can be activated as well by mechanical damage to the muscle (Aronson et al., 1998) as by non-damaging exercise of low intensity (Goodyear et al., 1996; Widegren et al., 1998). Oxidative stress has been shown to activate ERK, JNK, and p38 MAPK signalling in a dose- and time-dependent manner in cultured skeletal myoblasts (Kefaloyianni et al., 2006). The action of MAPKs includes transcriptional upregulation of, for instance, the early stress genes FBJ osteosarcoma oncogene (c-Fos) and Jun oncogene (c-Jun) by JNK (Kramer and Goodyear, 2007). The activation of p38 has been suggested to improve insulin-independent glucose uptake via upregulation and enhanced translocation of the transporter Glut4 to the plasma membrane (Ito et al., 2006; Wright, 2007). Several findings also assign a role to p38 in exercise-induced expression of PGC-1α and in mitochondrial biogenesis (Akimoto et al., 2005; Pogozelski et al., 2009; Wright et al., 2007). ERK 1/2 activation has been shown to acutely enhance fatty acid uptake (Turcotte et al.,

2005) and oxidation (Raney and Turcotte, 2006) in rodent muscle. Thus, MAPK signalling is involved in the exercise-induced regulation of gene transcription and has acute effects on glucose and lipid metabolism in the working skeletal muscle.

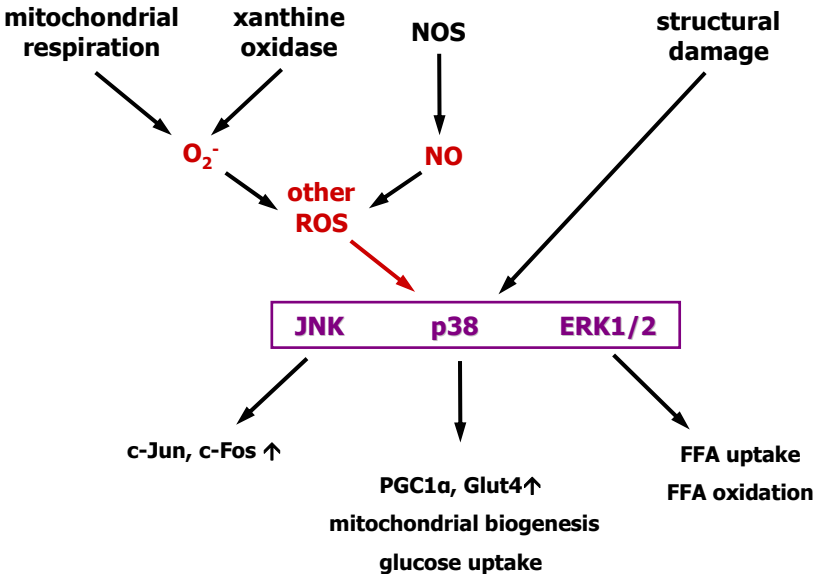

Fig. 7. Oxidative stress and activation of MAPKs in the working muscle. Depending on the exercise protocol, ROS generation or structural damage induce the activation of MAPKs in the contracting muscle. Activated JNK, p38 and ERK1/2 have different regulatory functions on gene transcription, on glucose and on fatty acid metabolism.

3.1.4 Signalling pathways and transcriptional response induced in the liver by physical exercise

In contrast to the muscle, which has extensively been studied both in humans and rodents, comparatively little is known about the molecular response of the liver. This is probably not only due to the less obvious involvement of the liver in physical exercise but also to the more difficult accessibility of this tissue in humans. For the latter reason, most data stems from rodent animal models. Some studies assessed the effects of several weeks of exercise training on hepatic transcription, predominantly in animals that were obese or hyperglycaemic (Colombo et al., 2005; Fiebig et al., 2002; Lee et al., 2006). According to these studies, long-term exercise mainly affects transcription and activity of factors involved in lipogenesis (Colombo et al., 2005; Lee et al., 2006). Exercise after fasting and refeeding for different lengths of time decreased fatty acid synthase (Fasn) mRNA and activity in healthy mice (Griffiths et al., 1996). Interventions in humans show significant reductions in the lipid content in the liver (Johnson et al., 2009; Schafer et al., 2007), an adaptive response to regular exercise that essentially contributes to the overall beneficial effect of exercise.

Few data have been published on the response of the liver to a single bout of exercise. These studies show the downregulation of lipogenic enzymes in diabetic mice (Fiebig

et al., 2001) and the upregulation of adiponectin receptor1 and forkhead box O1 (Foxo1) expression (Huang et al., 2007) as well as increased activities of gluconeogenic enzymes (Dohm et al., 1985; Nizielski et al., 1996) in healthy mice. There is also evidence for a stress response (Gonzalez and Manso, 2004) and increased production of ROS (Alessio and Goldfarb, 1988; Liu et al., 2000; Radak et al., 1996) in the liver of exercised rodents.

3.1.5 Aims of the study

Exercise is a strong metabolic stimulus for the liver because it has to provide energy for the working muscle and maintain glucose homeostasis. It is therefore quite surprising that comparatively little is known about the acute molecular response of the exercising liver, especially from lean, healthy mice without pre-treatment. Most data available so far stem from obese or diabetic mice and they point to both an acute hepatic response and an adaptation of the liver to regular exercise, shown mainly for lipid metabolism. The molecular mechanisms and pathways that mediate the acute effects of exercise in the liver are still unclear. Another unsolved question is whether moderate exercise induces a stress response in the liver that is mediated by the generation of ROS, similar to the muscle.

We aimed to characterize the acute metabolic response of the liver to physical exercise, to compare it to the muscle and to identify the signalling pathways that mediate this response. To this end, we subjected young, untrained mice to a single bout of treadmill exercise. The exercise protocol, a run of 60 min at 14 m/min and 14° uphill slope, was designed not to be structurally damaging to the muscles and to be demanding from a metabolic point of view. We assessed various metabolic parameters to ensure that the mice ran at a high aerobic intensity without, however, being exhausted. Immediately after the bout of exercise, we analyzed the levels of gene expression and activation states of signalling molecules in the liver and in different muscles. We also assessed metabolic consequences of the acute hepatic response by administering a glucose bolus to the exercised mice and analyzing components of the insulin signalling cascade.

Because the first results pointed to an acute stress response of the liver to physical exercise, we further compared the activation kinetics of MAPK and other stress-responsive signalling pathways in liver and muscles from mice either immediately after a single bout of exercise or after a 3 h recovery phase. To assess whether oxidative stress could be the stimulus for this hepatic response, we counteracted the generation of ROS by pre-feeding mice during 4 weeks with an antioxidant-enriched diet before a bout of exercise.

3.2 Results

3.2.1 Response of plasma metabolites and hormones to 1 h of moderately intense endurance exercise

The acute bout of exercise was performed as described in the methods section (2.5.1). Running on the treadmill for 1 h at 14 m/min and 14° uphill slope led to a slight but significant increase in plasma lactate concentrations (Fig. 8) as compared to levels measured in the same animals before commencing the run, from 2.37 ± 0.23 to 3.34 ± 0.34 mM. In parallel, plasma glucose levels dropped significantly during exercise (Fig. 8), from 144 ± 6 to 104 ± 11 mg/dl. Glucose levels were similar between sedentary mice and mice before running (150 ± 13 vs. 144 ± 6 mg/dl).

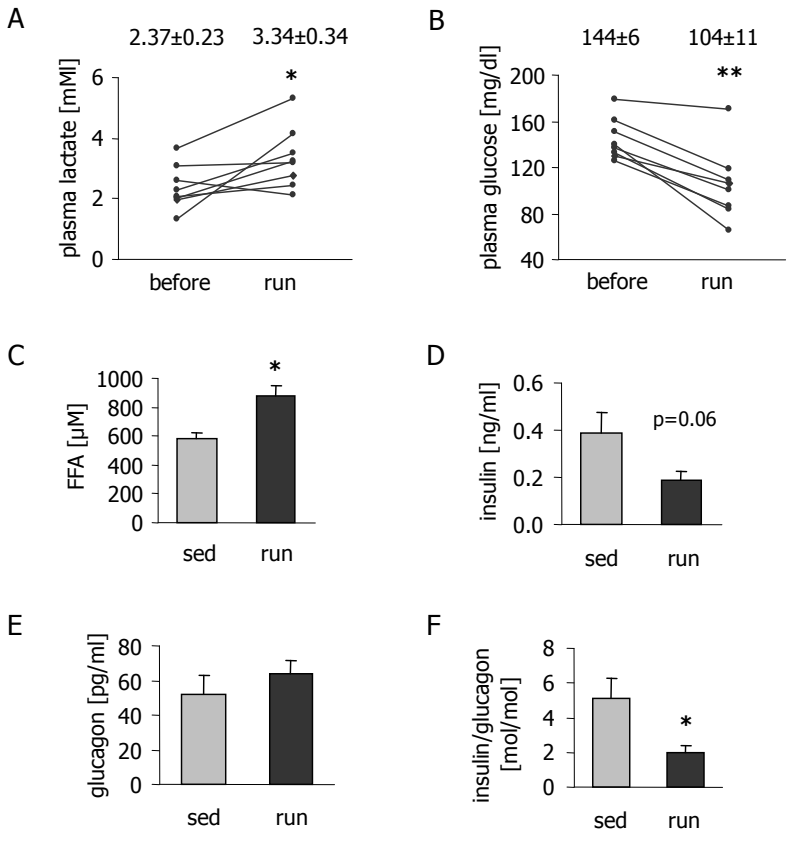

Fig. 8. Effect of the bout of exercise on plasma metabolites and hormones. Mice were exercised as described in the methods section. **A** Plasma lactate and **B** glucose concentrations measured in the same animals immediately before and after a single bout of running (run). Plasma levels of **C** FFA, **D** insulin and **E** glucagon and **F** the molar ratio of insulin to glucagon in sedentary control mice (sed) and in exercised mice after running (run) (n=8, mean ± SEM; *p<0.05, **p<0.005 vs. sedentary mice or the same mice before running).

The FFA levels were higher in the exercised group, 880 ± 188 compared to 580 ± 212 µM in sedentary mice. There was a trend towards lower insulin levels after running, 0.19 ± 0.1 vs. 0.39 ± 0.25 ng/ml, p=0.06. Although the glucagon levels did not rise significantly (64 ± 23 vs. 52 ± 32 pg/ml), the insulin/glucagon ratio was significantly reduced after running (Fig. 8).

3.2.2 Microarray analysis of global gene expression in muscle and liver in response to a single bout of exercise

To compare the transcriptional response of muscle and liver to a single bout of exercise, we performed an analysis of global gene expression in sedentary and exercised mice with the help of the Microarray Facility and the Center for Bioinformatics of the University of Tübingen. We chose the soleus muscle from the lower hind limb which is the muscle with the highest percentage of slow, oxidative type I fibres in mice. This facilitates the translation to human muscle because in general, murine muscles contain a lower percentage of type I fibres than their human counterparts. Regarding the reliance on oxidative metabolism, the soleus is also the muscle with most similarities to the liver. Equal amounts of RNA from 4 animals per group were pooled; therefore, the resulting values are group averages but do not allow for statistical evaluation. To select from the great amount of data generated by microarray analysis, only probes that showed an increase of at least 100 % or a decrease of at least 50 %, i.e. a 2-fold change, were considered to be differentially regulated. In the liver, a total of 526 genes met this requirement. Of these, 352 (67 %) were increased and 184 decreased. Markedly less transcripts, only 162, were differentially regulated in the soleus, with 72 (44 %) increased and 90 decreased (Tab. 11). The number of genes with a more than 10-fold change in expression was also higher in the liver, a total of 32 as compared to 3 in the muscle. Thus, the transcriptional response of the liver to a single bout of endurance exercise was much more extensive than in the muscle, and the percentage of induced transcripts was also higher.

Tab. 11. Number of transcripts differentially regulated in liver and soleus muscle after 1h of acute exercise

	Increased		**Decreased**	
	≥ 10-fold	≥ 2-fold	≥ 10-fold	≥ 2-fold
Liver	22	352	10	184
Soleus muscle	1	72	2	90

To further concentrate the data from global gene expression analysis, all differentially expressed transcripts were matched with the KEGG database (Kanehisa et al., 2008) that assigns genes to known pathways. With this strategy, 17 pathways could be identified that included four or more genes differentially expressed in the liver of exercised

mice. In the soleus muscle, in contrast, no single pathway included more than four regulated genes. Among the pathways found to be regulated by these criteria were a number that are directly involved in metabolic processes, among others, the insulin signalling pathway, glycolysis/gluconeogenesis and fatty acid metabolism (Tab. 12).

The highest number of regulated genes, a total of 16, was found in the MAPK signalling pathway. Most of these appeared to be upregulated after exercise (Tab. 13), among others, the MAPK targets FBJ osteosarcoma oncogene (c-Fos) and Jun oncogene (c-Jun), which belong to a family of transcription factors that dimerise via a leucine zipper structure, growth arrest and DNA-damage-inducible (GADD) 45g, and the Heat shock protein 2. GADD proteins are stress sensors that have been shown to activate MAPK signalling and play a role in cell cycle progression and DNA repair (Liebermann and Hoffman, 2008). Both upstream kinases and phosphatases of MAPK were also upregulated in the liver after the exercise bout, most notably, three members of the dual-specificity phosphatases (DUSP) family which are isoform-specific negative MAPK regulators (Camps et al., 2000).

Tab. 12. Signalling pathways affected in the liver after 1h of acute exercise

Pathway	**Count differentially expressed genes**
MAPK signalling pathway	16
Cytokine-cytokine receptor interaction	8
Insulin signalling pathway	7
PPAR signalling pathway	7
Focal adhesion	6
Arachidonic acid metabolism	5
Toll-like receptor signalling pathway	5
Wnt signalling pathway	5
Adipocytokine signalling pathway	5
VEGF signalling pathway	5
JAK-STAT signalling pathway	4
Calcium signalling pathway	4
GnRH signalling pathway	4
Glycolysis/Gluconeogenesis	4
p53 signalling pathway	4
Axon guidance	4
Fatty acid metabolism	4

Among the pathways most affected by physical exercise in the liver were several that are known to be stress-inducible. These were, besides MAPK signalling, cytokine receptor interaction, janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling, and the p53 pathway (Tab. 12). Thus, the microarray data hinted that the liver responds strongly to physical exercise, with an emphasis on firstly, metabolic processes and secondly, the cellular stress response.

Tab. 13. Hepatic genes that were differentially expressed in the liver according to microarray analysis and could be matched to the MAPK pathway in KEGG

Symbol	Full gene title	Regulation
c-Fos	FBJ osteosarcoma oncogene	up
c-Jun	Jun oncogene	up
DUSP4	Dual specificity phosphatase 4	up
DUSP6	Dual specificity phosphatase 6	up
DUSP8	Dual specificity phosphatase 8	up
GADD45a	Growth arrest and DNA-damage-inducible 45 alpha	down
GADD45g	Growth arrest and DNA-damage-inducible 45 gamma	up
Hspa2	Heat shock protein 2	up
Il1b	Interleukin 1 beta	up
Mapk11	Mitogen activated protein kinase 11	down
Mapk8	Mitogen activated protein kinase 8	up
Mapkapk2	Mitogen-activated protein kinase-activated protein kinase 2	up
Mapkapk3	Mitogen-activated protein kinase-activated protein kinase 3	up
Ppm1b	Protein phosphatase 1B, magnesium dependent, beta	down
Rac2	RAS-related C3 botulinum substrate 2	down
Rasa2	RAS p21 protein activator 2	up

3.2.3 The acute effect of physical exercise on the transcription of metabolic regulators is more pronounced in the liver than in the skeletal muscle

We employed real-time PCR to quantify the mRNA levels of several genes that are known to respond to physical exercise in the skeletal muscle and to compare this response to the liver. Besides the oxidative soleus, samples from the predominantly glycolytic WG muscle were analyzed. In line with the expected metabolic adaptation of the liver to the 1 h treadmill exercise, the expression of the liver-specific gluconeogenic enzyme glucose-6-phosphatase (G6Pase) was increased in the livers of exercised mice. The expression of another important gluconeogenic enzyme, Phosphoenolpyruvate carboxykinase (PEPCK) was, in contrast, unchanged (Fig. 9A). The transcriptional co-activator PGC-1α was 3-fold upregulated in the liver and only slightly, not significantly, in either muscle studied (Fig. 9B). PGC-1α is also induced during fasting in the liver where it contributes to the induction of gluconeogenesis (Michael et al., 2001; Yoon et al., 2001a). The mRNA content of pyruvate dehydrogenase kinase 4 (PDK4) was 4.8-fold increased in the liver and only 1.6-fold in both muscles, reaching significance in the WG (Fig. 9C). The expression of the insulin receptor substrate (IRS)-2 was upregulated 5.1-fold in the liver and 3.4- and 2.4-fold in soleus and WG, respectively (Fig. 9D).

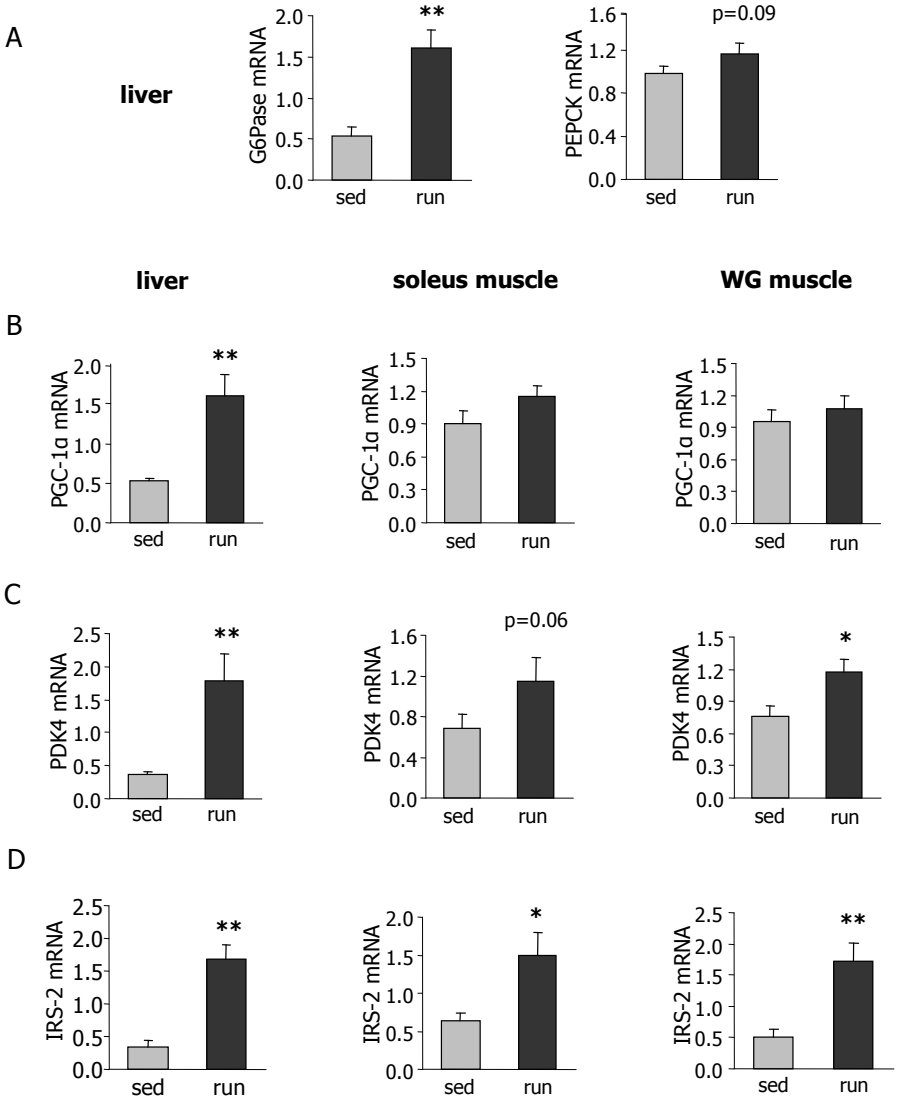

Fig. 9. Expression of genes related to glucose metabolism in liver and muscle. mRNA levels of **A** the gluconeogenic genes G6Pase and PEPCK in the liver and of **B** PGC-1α, **C** PDK4 and **D** IRS-2 in liver, soleus and WG muscle of sedentary (sed) mice or after a single bout of treadmill exercise (run). Values are shown as arbitrary units (n=8 for liver and WG, n=4 for soleus, mean ± SEM; **p*<0.05, ***p*<0.005 vs. sedentary).

After having studied known regulators of glucose metabolism, we next turned towards lipid metabolism and mitochondrial biogenesis. The expression of fatty acid synthase (Fasn) was 2-fold lower after exercise in the liver, while it remained unchanged in either muscle (Fig. 10A). The mRNA content of angiopoietin-like 4 (Angptl4), also known as fasting-induced adipose factor, was increased about 2-fold in all three tissues studied (Fig. 10B). Finally, we also assessed the expression of cytochrome c oxidase (COX) 1, whose upregulation makes part of the response to regular exercise training in the muscle, but found no differential expression immediately after the bout of running in neither the liver nor the muscles studied.

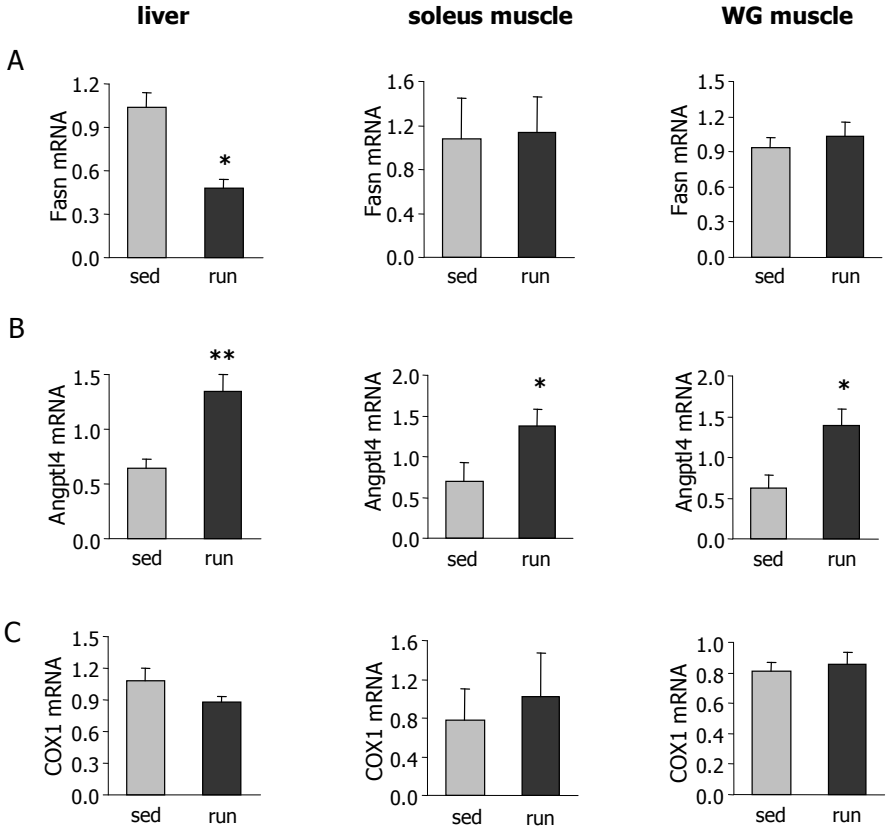

Fig. 10. Expression of genes related to fat metabolism in liver and muscle. The mRNA levels of **A** Fasn, **B** Angptl4 and **C** COX1 in liver, soleus and WG muscle of sedentary (sed) mice or after a single bout of treadmill exercise (run). Values are shown as arbitrary units (n=8 for liver and WG, n=4 for soleus, mean ± SEM; **p*<0.05, ***p*<0.005 vs. sedentary).

3.2.4 Acute effects of exercise on insulin signalling in the liver

Because of the acute response of metabolic genes in the liver that was even more pronounced than in the muscles, we suspected that as a physiological consequence, the response of the liver to a glucose challenge might also be altered. It is well-known that not only regular training, but also a single bout of exercise can at least acutely improve whole-body glucose disposal. While the mechanisms underlying the improvement of both insulin-independent (Richter et al., 2003) and –dependent (Frosig and Richter, 2009) glucose uptake in the muscle after acute exercise have been studied in detail, the effects on the liver are much less clear.

Insulin signalling was induced by the administration of glucose. This resembles, in contrast to the administration of insulin in this situation, a physiological setting, namely, food consumption following physical exercise. An i.p. glucose load, 2 g/kg body weight, was applied to mice immediately after 1 h of exercise. The animals were killed either directly after exercising, or after 30 min of glucose application. Again, exercise led to a slight fall in plasma insulin and glucose and to increased FFA levels (Fig. 11). As a response to the glucose load, insulin levels were increased, with significantly lower

levels in the previously exercised mice. In the exercised group, this rise in insulin was sufficient to bring FFA levels down to those of sedentary mice. Glucose levels, on the other hand, were elevated in both groups that received the i.p. load, and even higher in previously exercised animals, probably because of a preferential clearance of FFA.

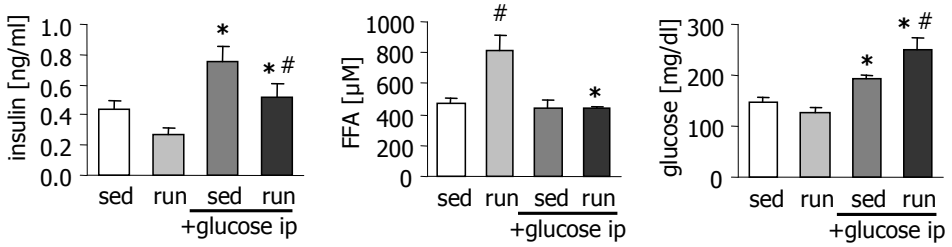

Fig. 11. Administration of a glucose load immediately after a single bout of exercise. **A** insulin, **B** FFA and **C** glucose concentrations in the plasma of sedentary (sed) and exercised (run) mice immediately after running or 30 min after having received an i.p. glucose load of 2 g/kg body weight (n=6, mean ± SEM; **p*<0.05 vs. the respective group without glucose, # *p*<0.05 vs. sedentary mice of the same group).

We then looked at the molecular components of the insulin signalling cascade in the liver. The insulin receptor substrates (IRS) are adaptor proteins that become tyrosine phosphorylated upon recruitment to the activated insulin receptor. The kinase Akt/PKB, another central player in the insulin signalling pathway, is activated upon phosphorylation at serine 473. Indeed, Akt/PKB phosphorylation was induced upon glucose stimulation and this effect was amplified by previous exercise (Fig. 12A). While no changes in IRS-1 levels occurred (Fig. 12B,D), both mRNA and protein of IRS-2 were increased after running in the liver (Fig. 12C,D). The relative tyrosine phosphorylation of the IRS isoforms, obtained by normalizing the phosphorylation signal to the protein amount, was only enhanced to a slight degree after exercise plus glucose in the case of IRS-1 (Fig. 12E) and not significantly altered in the case of IRS-2 (Fig. 12F). The visible increase in total IRS-2 tyrosine phosphorylation after exercise, with and without glucose, relates to a higher amount of protein. We also observed an enhanced mobility of IRS-1 and IRS-2 after exercise which indicates an additional regulation of both proteins on the post-transcriptional level, other than tyrosine phosphorylation. Treatment of liver extracts from sedentary and exercised mice with λ phosphatase resulted in a similar electrophoretic mobility and density of IRS-2 (Fig. 12G). Thus, serine/threonine phosphorylation seems to generate a pool of differently phosphorylated IRS-2 subspecies in sedentary mice that form a diffuse band in the western blots. Taken together, the acute regulation of IRS proteins and the enhancement of glucose-induced Akt/PKB phosphorylation indicate that physical exercise leads to an immediate improvement of insulin signalling in the livers of mice.

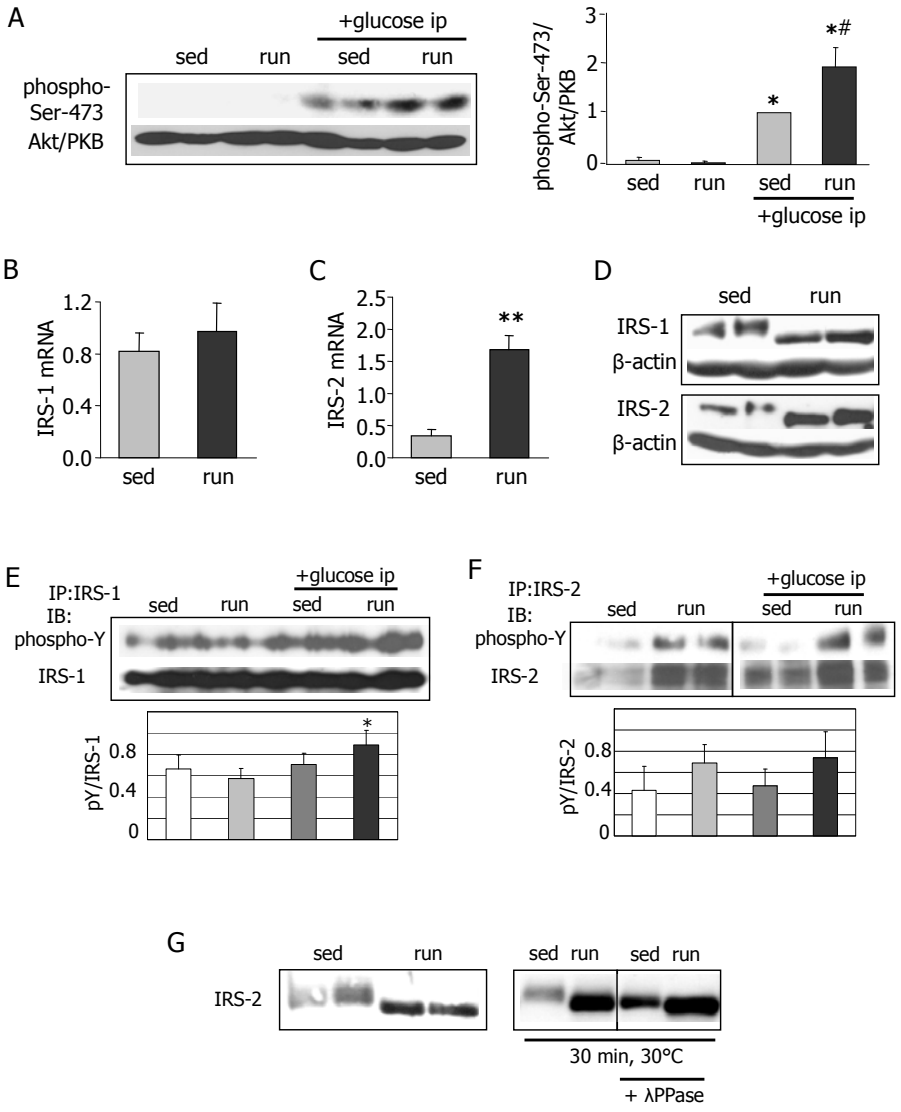

Fig. 12. Effects of exercise and glucose administration on hepatic insulin signalling. **A** Western blots of phospho-Ser-473 and protein of Akt/PKB and the relative densitometric quantification. Sedentary (sed) or exercised (run) mice were studied immediately after completing a single bout of exercise or 30 min after an i.p. glucose load. Values are shown as arbitrary units (n=6). Levels of **B** IRS-1 mRNA, **C** IRS-2 mRNA and **D** IRS proteins in livers of sedentary and exercised mice (n=8). Detection of tyrosine phosphorylation and protein of **E** IRS-1 and **F** IRS-2 after immunoprecipitation. Western blots from two representative samples per group and relative densitometric quantification (n=6). **G** Liver protein extracts from sedentary and exercised mice were left untreated, incubated with buffer, or incubated with buffer and λPPase before immunoblotting for IRS-2. Values are shown as arbitrary units (n=6, mean ± SEM; **p*<0.05, ***p*<0.005 vs. the respective group without glucose; #*p*<0.05 vs. sedentary).

3.2.5 Exercise induces a transient stress response in the liver of mice that is more pronounced than in the muscle

Based on the microarray and pathway analyses, we next focussed on the stress response of the liver and compared it to the muscle. We exercised mice as before and included a third group of mice that was sacrificed 3 h after running, in addition to the

sedentary mice and to the group studied immediately after exercise. The protocol is described in the methods section (2.5.2). This allowed us to additionally assess the early recovery phase following a single bout of exercise and to compare the kinetics of muscular and hepatic responses. To exclude acute effects of food consumption in this setup and ensure equal feeding conditions between all groups, both the recovering and the sedentary mice were starved in the last hour before sacrification. The recovery group had free access to food for the first 2 h after running and the plasma glucose levels rose to 130 ± 5 mg/dL, compared to only 112 ± 6 mg/dL in the mice studied immediately after the bout of exercise.

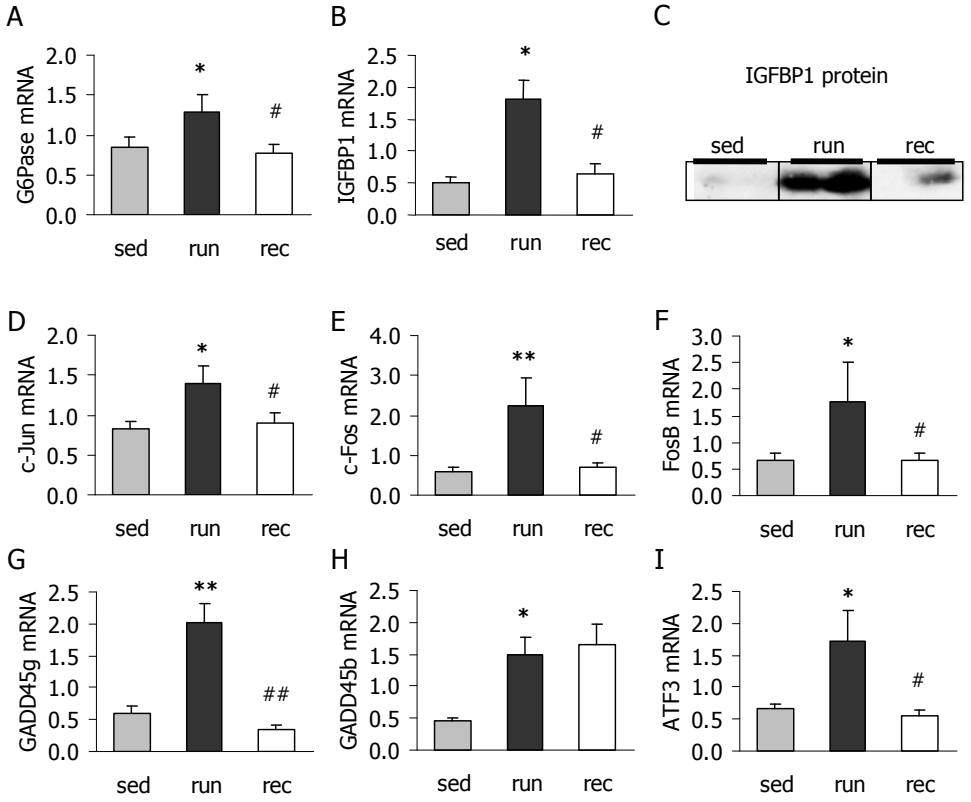

Fig. 13. Transient upregulation of metabolic and stress-responsive genes in the liver. Levels of **A** G6Pase and **B** IGFBP1 mRNA and of **C** IGFPB1 protein. Levels of **D** c-Jun, **E** c-Fos, **F** FosB, **G** GADD45g, **H** GADD45b and **I** ATF3 mRNA in livers of sedentary (sed) mice, immediately after a single bout of treadmill exercise (run) or after 3 h of recovery (rec). Values are shown as arbitrary units (n=10-12, mean ± SEM; **p*<0.05, ***p*<0.005 vs. sedentary; #*p*<0.05 vs. run).

To characterize the metabolic response of the liver in this experiment, the expression of genes that play a role in glucose metabolism was studied first. The mRNA of G6Pase (Fig. 13A) was upregulated immediately after exercise and returned to basal levels after the recovery phase. Moreover, the mRNA of insulin-like growth factor binding protein 1 (IGFBP1), which like G6Pase responds to low plasma glucose, was transiently upregulated (Fig. 13B). The IGFBP1 protein levels in the liver were transiently increased as well (Fig. 13C). Next, the stress response as the primary finding of the pathway analysis was studied. In accordance with the microarray data, qPCR analysis

confirmed that the mRNA of c-Jun (Fig. 13D) and c-Fos (Fig. 13E) was increased after running. After 3 h of recovery, the levels had returned to basal. The same kinetics could be found for two other transcription factors of the Jun-Fos family, FosB (Fig. 13F) and activating transcription factor 3 (ATF3) (Fig. 13G) and for GADD45g (Fig. 13H). In contrast, GADD45b (Fig. 13I) was also upregulated following exercise but remained elevated during the recovery phase. When analyzing the expression of the same stress-responsive genes in the soleus muscle, three different kinetics could be observed. Some did not respond to exercise (c-Jun and GADD45g, Fig. 14A,D), some showed a transient induction similar to the liver (c-Fos and GADD45b, Fig. 14B,E), and a third group remained elevated till recovery (FosB and ATF3, Fig. 14C,F).

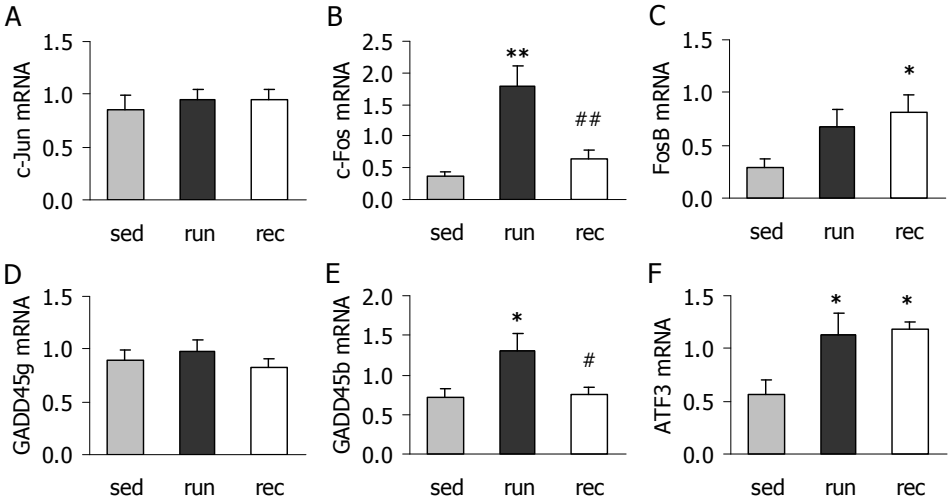

Fig. 14. Transient upregulation of stress-responsive genes in the muscle. mRNA levels of **A** c-Jun, **B** c-Fos, **C** FosB, **D** GADD45g, **E** GADD45b and **F** ATF3 in soleus muscles of sedentary (sed) mice, immediately after a single bout of treadmill exercise (run) or after 3 h of recovery (rec). Values are shown as arbitrary units (n=8, mean ± SEM; **p*<0.05, ***p*<0.005 vs. sedentary; #*p*<0.05 ##*p*<0.005 vs. run).

3.2.6 Differential activation of MAPKs in the liver after acute exercise

Having verified the expression of MAPK pathway target genes by qPCR, we also assessed the activation status of different MAPKs in the liver and in two muscles, in the WG and in the EDL. The most striking finding was a strong increase of ERK phosphorylation after exercise in the liver. Interestingly, this signal appeared between 44 and 50 kDa and could not be matched to either p42 or p44 based on immunoblots for ERK protein and the phosphorylation corresponding to p42/44 appeared to be even weaker at this time point (Fig. 15A). Liver JNK phosphorylation was increased after exercise as well (Fig. 15C) and both ERK and JNK activation disappeared after recovery. The phosphorylation of p38 MAPK was not increased in the liver (Fig. 15B).

In both the WG and EDL muscle, there was a slight increase of p44 ERK phosphorylation in the recovery phase (Fig. 15D) and similarly, of p42 (not shown). There was, however, no band that corresponded to the strong ERK phosphorylation seen in the liver. As in the liver, p38 MAPK was not activated in the muscles (Fig. 15E). JNK phosphorylation (not shown) could not be detected in the muscles, either.

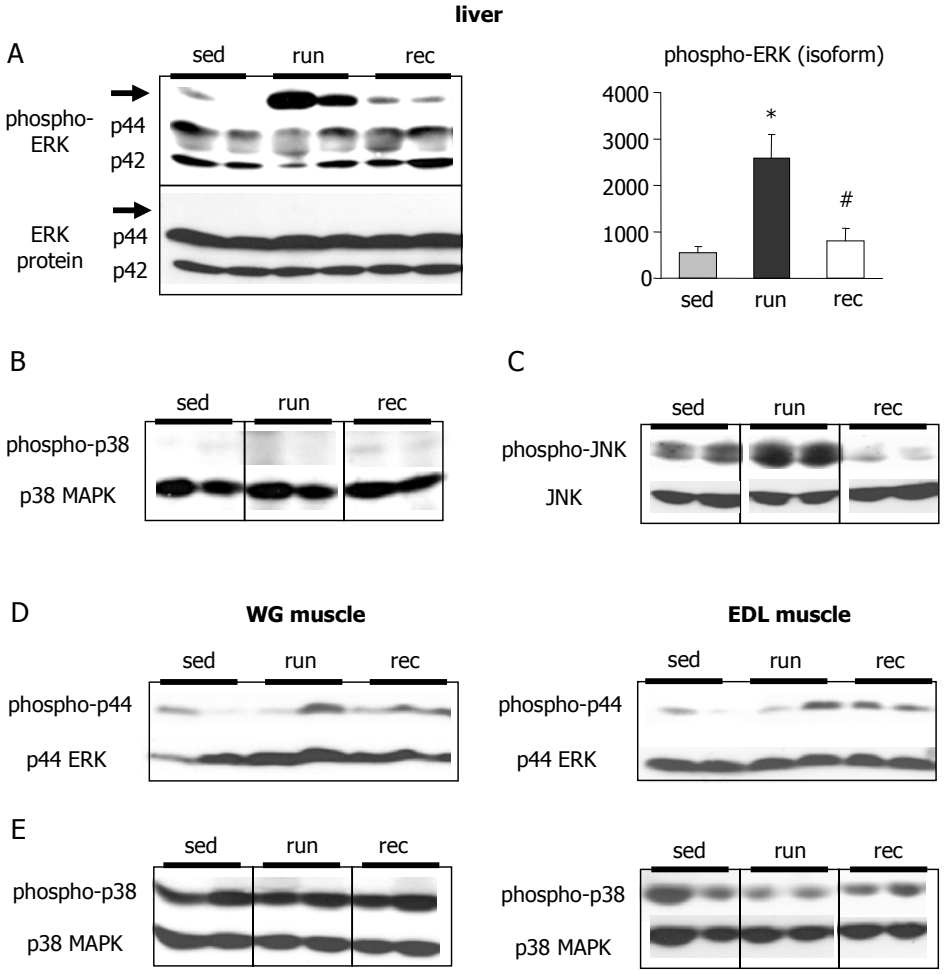

Fig. 15. MAPK activation in liver and muscle. A Detection of phosphorylated ERK and of ERK protein in livers from sedentary (sed), exercised (run) and recovered (rec) mice; western blots with two representative samples per group and the densitometric quantification is shown as arbitrary units (n=6, mean ± SEM; **p*<0.05 vs. sedentary; #*p*<0.05 vs. run). The arrow indicates the position of phosphorylated ERK. Detection of **B** phosphorylated p38 and p38 protein and **C** phosphorylated JNK and JNK protein in liver extracts. **D** Detection of phosphorylated ERK and ERK protein in WG and EDL muscles. **E** Detection of phosphorylated p38 and p38 protein in WG and EDL. Shown are representative blots with lysates from two individual animals per group.

The differential phosphorylation of p42/44 and the ERK isoform in the livers of exercised mice might be caused by the actions of isoform-specific MAPK phosphatases such as the DUSP family members. As shown by qPCR analysis, there was a significant and transient upregulation of DUSP members in the liver (Fig. 16). One of these, DUSP6, is known to have a high selectivity towards ERK (Camps et al., 2000; Owens

and Keyse, 2007) and this could explain the lack of phosphorylation of ERK1/2 in the liver immediately after the bout of exercise.

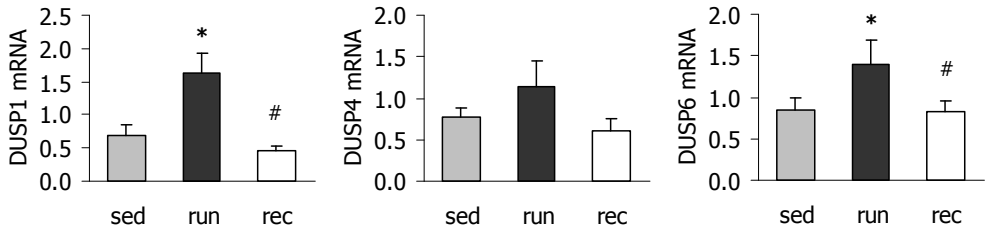

Fig. 16. Transient upregulation of DUSP isoforms in the liver. Expression of **A** DUSP1, **B** DUSP4 and **C** DUSP6 in livers of sedentary (sed) mice, immediately after a single bout of treadmill exercise (run) or after 3 h of recovery (rec). Values are shown as arbitrary units (n=12, mean ± SEM; **p*<0.05 vs. sedentary; #*p*<0.05 vs. run).

3.2.7 Antioxidants do not prevent the hepatic stress response

Next, we sought to identify the stimulus that induces the stress response in the liver. Oxidative stress is known to play a role in the response of the muscle to non-damaging exercise (Morton et al., 2009) and reactive oxygen species have been reported to be produced in the liver during exercise (Alessio and Goldfarb, 1988; Liu et al., 2000; Radak et al., 1996). In order to assess whether oxidative stress could be responsible for the hepatic stress response, we used antioxidants to counteract the generation of oxidative stress. Before an acute bout of exercise, mice were fed a diet containing 2,000 U Vitamin E per kg diet, 10 times more than the control diet. The antioxidant diet additionally contained a small amount of vitamin C (100 mg/kg diet) that was not present in the control diet to ensure a sufficient supply with this vitamin that can usually be synthesised by rodents. The dietary supplementation lasted four weeks and resulted in almost 2-fold higher vitamin E plasma concentrations, 14.2 ± 2.3 µM in the supplemented versus 7.8 ± 2.0 µM in the control group (Fig. 17A).

Mice of both dietary groups were exercised once for 1 h and the livers were prepared for mRNA and protein analysis. There was no increase in the mRNA of the anti-oxidative enzymes superoxide dismutase (SOD) 1 and 2 (Fig. 17B,C). In the livers of the control-fed animals, there was a trend for an exercise-induced increase in Hmox1 mRNA that did not reach significance due to a high inter-individual variability (Fig. 17D) and a significant increase of metallothionein (MT) 1 (Fig. 17E). The mRNA of neither Hmox1 or MT1 was elevated when the mice had been pre-fed with antioxidants.

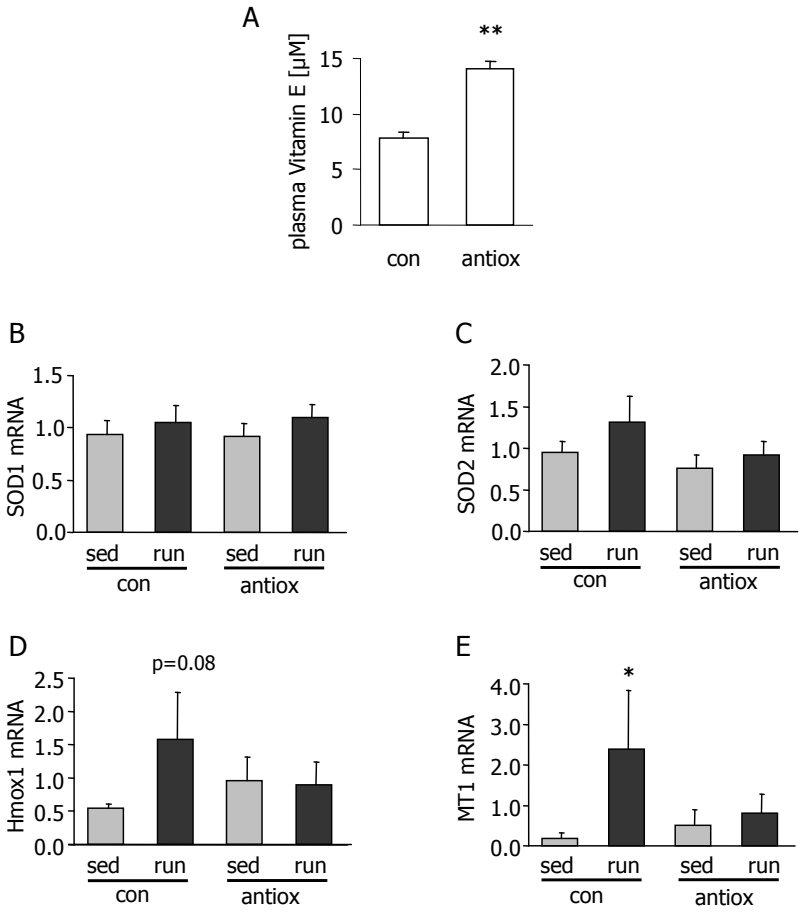

Fig. 17. Antioxidant supplementation. **A** Vitamin E levels in the plasma of mice fed 4 weeks with either control (con) or antioxidant-enriched diet (antiox). **B-E** Expression of anti-oxidative genes in livers of sedentary (sed) mice or immediately after a single bout of treadmill exercise (run) following 4 weeks supplementation with control or antioxidant diet: **A** SOD1, **B** SOD2, **C** Hmox1 and **D** MT1. Values are shown as arbitrary units (n=6, mean ± SEM; **p*<0.05 vs. sedentary).

Thus, although the 1 h treadmill exercise only caused a response of some anti-oxidative genes, it appears that it did induce oxidative stress in the liver and this was prevented by the antioxidant supplementation. However, c-Fos (Fig. 18A) and GADD45g (Fig. 18B) were induced regardless of whether the mice had been supplemented with antioxidants or not. The phosphorylation of the ERK isoform was also induced as strongly in the presence as in the absence of antioxidants (Fig. 18C). These results suggested that oxidative stress was not the stimulus for the activation of MAPKs in the exercising liver.

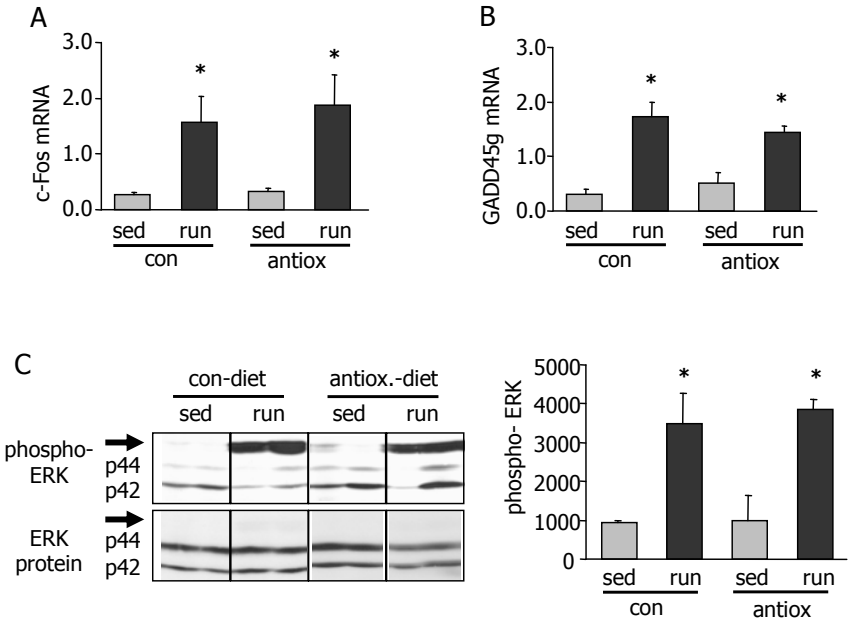

Fig. 18. Hepatic stress response and antioxidant treatment. Expression of **A** c-Fos and **B** GADD45g mRNA in livers of sedentary (sed) mice or mice immediately after a single bout of treadmill exercise (run) that had been pre-fed with control (con) or antioxidant-enriched (antiox) diet for 4 weeks. **C** Detection of ERK phosphorylation and protein in liver extracts. The arrows indicate the position of phosphorylated ERK. Shown are representative blots with lysates from two individual animals for each group and the densitometric quantification. Values are shown as arbitrary units (n=6, mean ± SEM; **p*<0.05 vs. sedentary).

3.2.8 The hepatic stress response is related to low glucose levels after exercise

The supplementation with antioxidants to counteract oxidative stress indicated that the hepatic stress response to physical exercise is not caused by oxidative stress. Because endurance exercise is accompanied by massive changes in plasma metabolites and hormones, it is conceivable that the same stimuli that induce metabolic responses in the liver also might trigger the stress response. The enhanced uptake and utilization of energy by the working muscle leads to a fall in the circulating glucose concentration which is counteracted by the liver that produces and releases glucose. Thus, the less glucose is available, the more are hepatic factors that contribute to glucose production, like G6Pase (Fig. 13) or PGC1α (Fig. 9), required to be upregulated. There was, as expected, a strong negative correlation of PGC1α mRNA in the liver to the plasma glucose levels after exercise (Fig. 19A). A similar correlation was found for IGFBP1 (Fig. 19B), a plasma protein that is secreted from the liver during physical exercise to neutralize the insulin-like activity of insulin-like growth factor 1 (Anthony et al., 2001; Lewitt et al., 1991) (Fig. 19B). In contrast, the mRNA of Fasn, a regulator of fatty acid metabolism that was downregulated during physical exercise (Fig. 10A), was neither correlated to post-exercise glucose (Fig. 19C) nor FFA levels (not shown). Interestingly, the induction of stress-responsive genes was also inversely correlated with glucose levels after

running, for example, c-Fos (Fig. 19D), GADD45g (Fig. 19E) ATF3 (Fig. 19F), JunB, and c-Jun (not shown). This relationship was specific for the liver since no significant correlation existed between plasma glucose and the transcripts significantly upregulated in the soleus muscle after the bout of exercise, namely, c-Fos (Fig. 19G), ATF3 (Fig. 19H) and GADD45b (Fig. 19I).

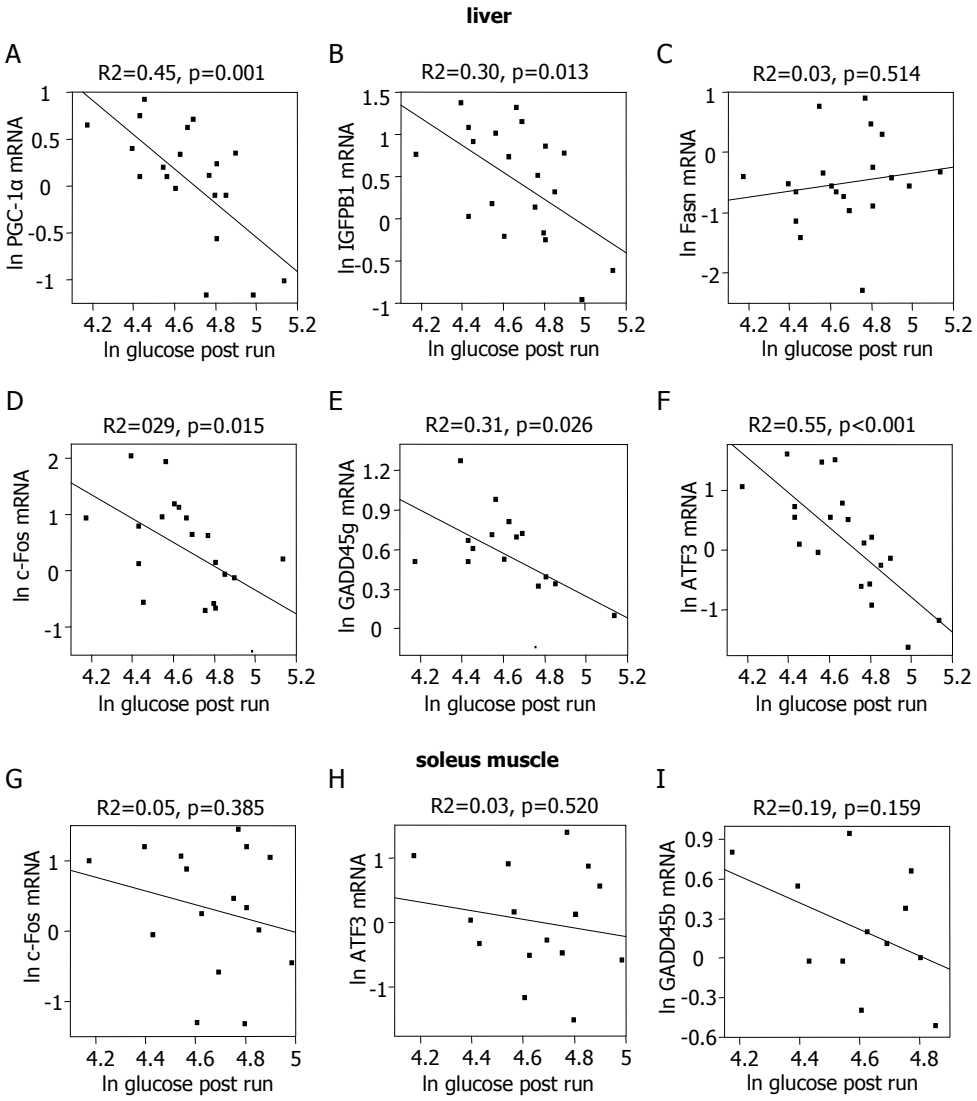

Fig. 19. Relation of the mRNA levels of metabolic and stress-responsive genes in liver and muscle to glucose concentrations immediately after a bout of exercise. Plots of log-transformed mRNA levels against log-transformed plasma glucose concentrations after a bout of exercise for **A-F** the liver an **G-I** the soleus muscle of exercised mice. **A:** PGC-1α, **B:** IGFPB1, **C:** Fasn, **D** and **G:** c-Fos, **E:** GADD45g, **F** and **H:** ATF3, and **I:** GADD45b. (n=20).

Because the exercise protocol did not only cause a fall of glucose, but also a drop in insulin levels and an elevation of circulating FFA, these factors were tested as well for possible correlations with the hepatic stress response. The mRNA levels of c-Fos, GADD45g or ATF3 could not be correlated to FFA (Fig. 20A) nor, with the exception of GADD45g, to insulin plasma levels (Fig. 20B). Moreover, there was no correlation to the plasma glucose levels before the bout of exercise (not shown). Thus, of all meta-

bolic changes studied, only the fall in plasma glucose could be related to the hepatic stress response, and insulin does not play a role as a transmitter of this signal.

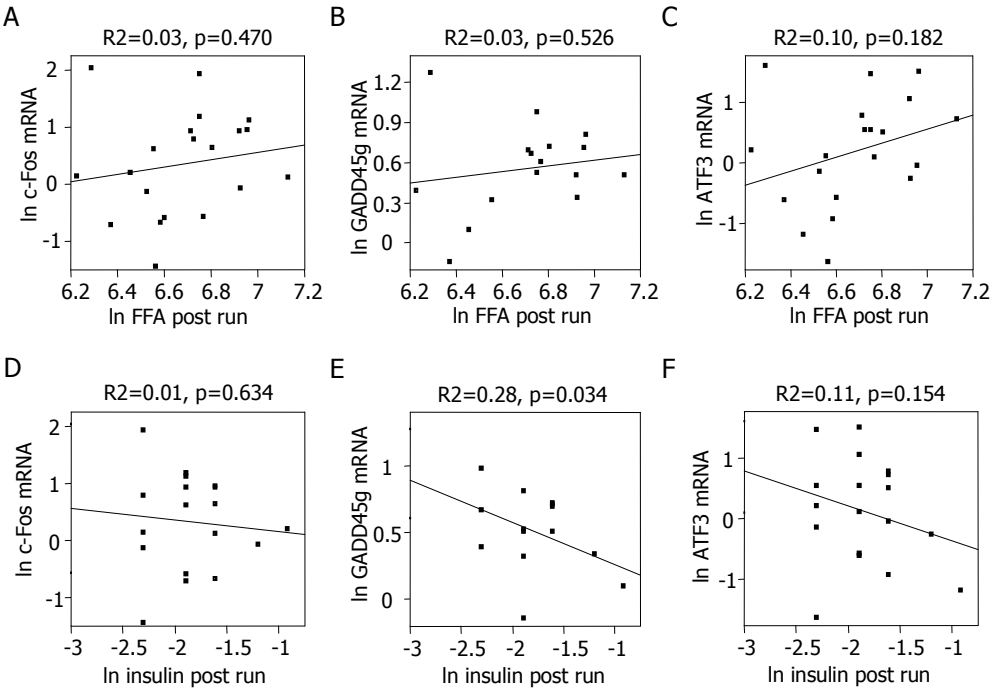

Fig. 20. Relation of the mRNA levels of stress-responsive in the liver to the concentrations of FFA and insulin immediately after a bout of exercise. Plots of log-transformed hepatic mRNA levels against log-transformed concentrations of **A-C** plasma FFA and **D-F** plasma insulin after a bout of exercise. **A** and **D:** c-Fos, **B** and **E:** GADD45g, **C** and **F:** ATF3 mRNA. (n=20).

3.2.9 Exercise induces p53 signalling in the liver

Also based on the pathway analysis, we investigated the activation of another stress-responsive pathway, of p53 signalling, in the liver. There was a transient increase of p53 inducible nuclear protein 1 (trp53inp1) mRNA after exercise (Fig. 21A) and an induction of another p53 target gene, p21, in the recovery phase (Fig. 21B). Accordingly, we also found a greater amount of p53 protein after running (Fig. 21C). Because p53 levels are mainly regulated via degradation of the protein, we further sought to identify the mechanism by which it could be stabilized. The ubiquitin ligase murine double minute (Mdm) 2 targets p53 for proteasomal degradation (Feng et al., 2004). Phosphorylation of Mdm2 at Serine 166, which is required for its interaction with p53, was enhanced only in the recovery phase, when p53 levels started to decline once again (Fig. 21D). Because this phosphorylation is controlled by p42/44 ERK as well as Akt/PKB (Malmlof et al., 2007), we compared the respective phosphorylation patterns and found that the increased phosphorylation of Akt/PKB in the recovery phase paralleled Mdm2 phosphorylation (Fig. 21D).

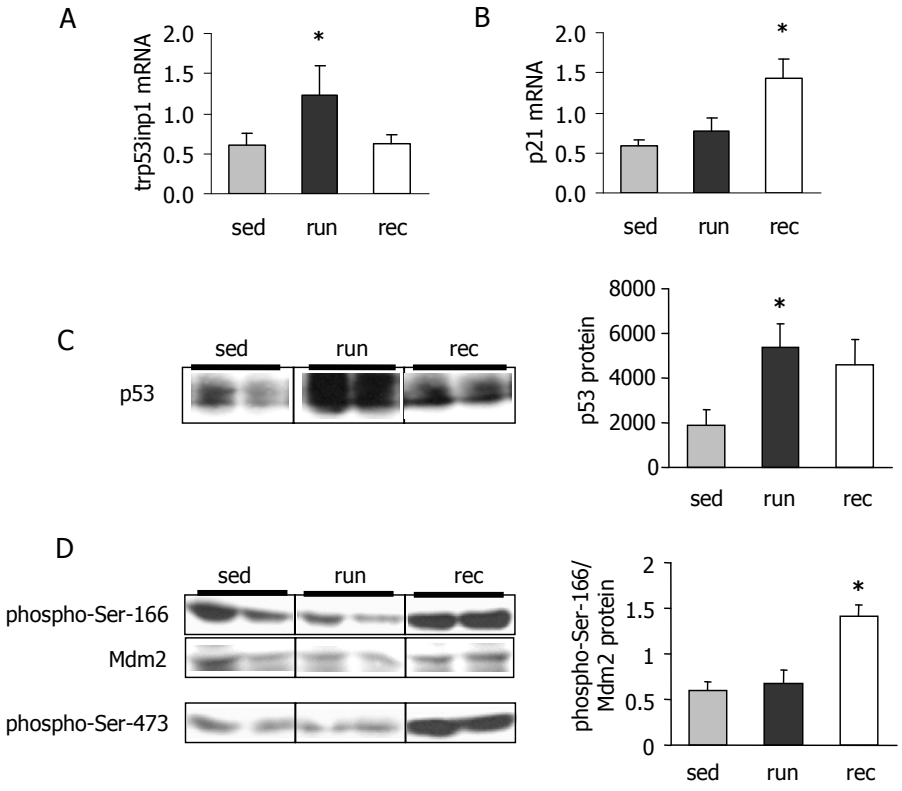

Fig. 21. Activation of p53 signalling in the liver of mice after a single bout of exercise and recovery. Expression of **A** trp53inp1 and **B** p21 mRNA in livers of sedentary (sed), exercised (run) and recovered (rec) mice (n=12, mean ± SEM). **C** Detection of p53 protein in liver extracts. Shown are representative blots from two individual animals per group and the densitometric quantification (n=6, mean ± SEM). **D** Detection of phosphorylated serine-166 of Mdm2, of Mdm2 protein and of phosphorylated serine-473 of Akt/PKB. Shown are representative blots with lysates from two individual animals from each group and the relative densitometric quantification of Mdm2 phosphorylation. Values are shown as arbitrary units (n=6, mean ± SEM; **p*<0.05 vs. sedentary).

3.2.10 Exercise induces the AMPK and JAK/STAT-3 signalling pathways in the liver

A key sensor of cellular energy status with important regulatory functions for the metabolism of the working skeletal muscle is AMPK (Richter and Ruderman, 2009). It is activated by conditions that increase the AMP/ATP ratio and exercise has been shown to create such a condition of elevated AMP and lowered ATP levels in the liver (Camacho et al., 2006). We therefore compared the activation status of AMPK in liver and skeletal muscle of sedentary and exercised mice. The phosphorylation of threonine 172, indicating AMPK activation, was clearly increased in WG muscle and even more prominently in the liver after 1 h of running (Fig. 22A). In line with a state of energetic stress, glycogen was almost completely depleted in the liver after running (Fig. 22B). The amount of glycogen in the WG muscle, in contrast, was not significantly lowered (Fig. 22B) by the exercise protocol. The observed activation of AMPK could be an im-

portant link between the energy state of the liver and its molecular response to physical exercise.

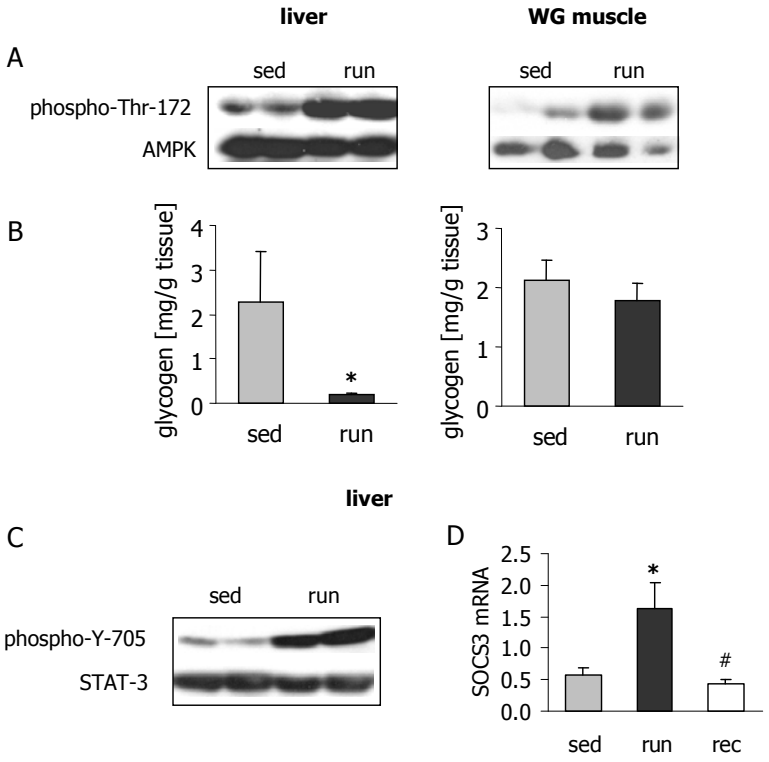

Fig. 22. Activation of the AMPK and JAK/STAT-3 signalling pathways in the liver of exercised mice. A Detection of phosphorylated threonine-172 of AMPK and of AMPK protein in liver and WG muscle of sedentary (sed) mice or following a single bout of exercise (run). Representative blots of two samples per group are shown. **B** Glycogen content in liver and WG muscle (n=20 for liver, n=12 for WG, mean ± SEM). **C** Detection of phosphorylated tyrosine-705 of STAT-3 and of STAT-3 protein in the livers of sedentary (sed) or exercised (run) mice. **D** Expression of SOCS3 mRNA in the livers of sedentary mice (sed), immediately after running (run) or following 3 h of recovery (rec). Values are shown as arbitrary units (n=12, mean ± SEM *p<0.05 vs. sedentary, # p<0.05 vs. run).

As suggested by the microarray analysis, the bout of exercise also affected cytokine signalling (Tab. 12), another pathway that could play a role in the acute transcriptional response of the liver. The JAK/STAT pathway is the principal signalling mechanism for a wide array of cytokines. All cytokines belonging to the IL-6 family signal via the transcription factor STAT-3 that is activated by phosphorylation (Heinrich et al., 1998). We found an increased phosphorylation of tyrosine 705 of STAT-3 (Fig. 22C) and a transient upregulation of its target gene suppressor of cytokine signalling (SOCS) 3 (Fig. 22D) in the liver, clearly showing an induction of STAT-3 signalling by physical exercise.

3.3 Discussion

Physical exercise is a strong stimulus not only for the working muscle but also for the liver as the tissue mainly responsible for glucose production and homeostasis. In spite of this important role, little is known about the acute effects of exercise on the liver at the molecular level. The exercise protocol used herein to study the response of the liver to a single bout of exercise, 1 h of treadmill running at 14 m/min and 14° uphill slope, was designed to have a strong metabolic impact without being exhaustive. The lower glucose levels after running indicated enhanced uptake and utilization of glucose by the skeletal muscle. Together with a slight increase in plasma lactate levels, this suggested that the animals were indeed running at an high aerobic intensity, but still below the maximal lactate steady state (Ferreira et al., 2007). This is a physiologic setting that resembles recreational endurance exercise in humans. The first step to characterize the molecular response of the liver was a microarray analysis of whole-genome expression. The assignment of the differentially regulated genes to pathways indicated that metabolic and stress-inducible processes responded most to acute physical exercise.

3.3.1 Acute regulation of metabolic genes and components of the insulin signalling pathway in the liver after exercise

The first focus of the study were genes known to be exercise-responsive in the muscle since these could also be relevant for the adaptation of the hepatic metabolism to exercise. The expression of PDK4 and PGC-1α was markedly upregulated in the liver. Both proteins are important for the regulation of the hepatic glucose metabolism. PDK4 phosphorylates and inactivates the pyruvate dehydrogenase complex, thus inhibiting glucose oxidation in liver and muscle (Watt et al., 2004) and promoting gluconeogenesis in the liver (Sugden and Holness, 2003). Accordingly, there was also a strong increase of hepatic G6Pase mRNA. In contrast to PEPCK, G6Pase is necessary not only for gluconeogenesis but also for the release of glucose derived from glycogen into the bloodstream. Glycogenolysis already provides glucose before the onset of gluconeogenesis (Kjaer, 1998; Wahren and Ekberg, 2007). The upregulation of PEPCK was only weak and might peak later during exercise, after the depletion of hepatic glycogen stores, when gluconeogenesis is the only source of glucose. In fact, a recent study has shown an increase in hepatic PEPCK mRNA in rats after exhaustive exercise (Banzet et al., 2009). Acute exercise also upregulated IRS-2 and Angptl4. The mRNA of Fasn, which is required to store away excess glucose in the fed state, was downregulated. Remarkably, the regulation of metabolic genes in the liver after 1 h of physical exercise resembled the response to long-term fasting in rodents (Kersten et al., 2000; Kubota et al., 2008; Wu et al., 2000; Yoon et al., 2001a) in all points studied.

Another interesting finding is the generally more pronounced response of the liver immediately after the bout of exercise if compared to the muscle. This was already evident from the microarray analysis, where the number of genes upregulated was almost 5-fold higher in the liver than in the muscle, and the number downregulated was 2-fold higher. Among the genes already known to respond to exercise in the muscle that were verified by qPCR, the change was more pronounced in the liver for all except Angptl4. The possibility exists that the transcriptional response could be more pronounced in other skeletal muscles. However, the results were similar for the oxidative soleus and the predominantly glycolytic WG muscle.

The following considerations could explain the more pronounced response of the liver immediately after a bout of exercise: Firstly, the energetic challenge has been shown to be much more demanding for the liver than for the muscle in an exercise protocol similar to ours (Camacho et al., 2006), and this warrants an acute metabolic response in the liver. Secondly, the muscle has to spare ATP for contractile work and might therefore postpone the nucleotide-consuming process of RNA synthesis. Thirdly, the main response of the muscle could be directed towards long-term, rather than acute metabolic adaptations. In fact, the expression of several exercise-regulated genes has been shown to peak in the muscle only after several hours of recovery and not immediately after exercise (Hildebrandt et al., 2003; McGivney et al., 2009; Yang et al., 2005). A direct comparison between the expression of PGC1α in liver and different muscles shown in the second part of this work (Fig. 34) supports the idea that the kinetics of the transcriptional responses are different between liver and skeletal muscle: The transcriptional co-activator was upregulated immediately after a bout of exercise in the liver and only after 3 h of recovery in the muscle.

The transcriptional changes discussed so far are very likely to play a role in the immediate function of the liver during physical exercise, to provide energy for the working muscle. We further found evidence for an enhancement in insulin signal transduction that could help the liver to refill its energy stores more quickly after a bout of exercise. IRS-2 is known to respond to fasting for 12 h or more in the liver of rodents and plays an important role in insulin signalling immediately after refeeding (Kubota et al., 2008). After running for 1 h, there was an increase in the amount of both IRS-2 mRNA and protein in the liver, which again highlights the analogy between endurance exercise and long-term fasting. We could also provide evidence for the regulation of serine/threonine phosphorylation of IRS-2 in the liver by the bout of exercise and these modifications might further contribute to an altered insulin signalling. By applying an i.p. glucose load to a subset of mice directly after the bout of running, an improvement of hepatic insulin signalling could further be demonstrated. The administration of glucose led to endogenous insulin secretion, to a slightly enhanced tyrosine phosphorylation of IRS-1 and to a significantly higher PKB/Akt phosphorylation in the liver of previously exercised mice, in spite of lower insulin levels in this group. This improvement of insulin signalling was not reflected by lower plasma glucose levels 30 min after the glucose load which might, however, be explained by an impairment of insulin action on glucose

uptake in muscle and adipose tissue immediately after exercise due to the increased free fatty acid and catecholamine levels (Devlin et al., 1989; Kjaer et al., 1990; Yki-Jarvinen et al., 1991). These observations suggest that the liver contributes to the improvements in whole-body insulin sensitivity and glucose metabolism that are associated with physical exercise. It is likely that similar to the muscle, beneficial effects occur in the liver both acutely and with long-term exercise training. Indeed, recent results show that aerobic exercise training without weight loss improves hepatic insulin sensitivity (van der Heijden et al., 2009) and insulin-induced suppression of hepatic glucose production (Coker et al., 2009).

3.3.2 Transient stress response in the liver after exercise

The pathway analysis of the microarray data showed that besides metabolism, stress-related signalling was most intensely affected in the liver immediately after the single bout of treadmill exercise. Further studies were performed to determine the kinetics of this stress response. The transcription factors c-Jun, c-Fos, FosB and ATF3 were transiently upregulated in the liver, as was GADD45g. Similar kinetics were displayed by two genes known to be induced by states of low glucose and insulin, the gluconeogenic G6Pase and IGFBP1, which has been shown to be released into the circulation during long-term exercise (Anthony et al., 2001) and plays a role in glucose homeostasis (Wheatcroft and Kearney, 2009). The mRNA of GADD45b was also increased after exercise in the liver and remained elevated till 3 h of recovery. In the soleus muscle, in contrast, the pattern of regulation of the same stress-related genes was very different: c-Jun and GADD45g were not induced at all, c-Fos and GADD45b were transiently elevated and FosB and ATF3 were elevated in the recovery phase. Notably, FosB levels were significantly upregulated only after recovery, but not immediately after the bout of exercise. Thus, the stress response of the muscle was clearly less pronounced than in the liver, but the patterns of upregulation could also indicate that the response of the muscle peaks at a later time point, between the cessation of exercise and 3 h of recovery. This was also indicated by the activation status of MAPKs. In the muscle, there was only a slight increase in p42/44 ERK phosphorylation after exercise, but this increase was still visible in the recovery phase. In contrast, both ERK and JNK were clearly activated in the liver immediately after exercise. The phosphorylated form of ERK recognised by the anti- phospho-Thr-202/Tyr-204 antibody in the liver is not identical to ERK1 or ERK2 and the molecular mass, which can be estimated from the immunoblots, is between 44 and 50 kDa. Since the commercially available anti-p42/44 ERK antibodies we used has been raised against a sequence in the C-terminal region of p44 ERK, the ERK isoform must differ in this region from p42/44 ERK. Several isoforms of p42/44 ERK have been reported, such as the 46 kDa ERK1b isoform with a 26 amino acid insertion between residues 340 and 341 of p44 ERK1 (Yung et al., 2000). ERK1b would fulfil some criteria for being the phosphorylated ERK detected in the liver of mice after acute exercise, but further studies are necessary to clarify the identity of

this isoform. Of note, ERK1b could be regulated differently from p42/44 ERK. It has been suggested in particular that ERK1b could still be activated when p42/44 ERK is under tight phosphatase regulation (Yung et al., 2000). The DUSP family of phosphatases are important regulators of MAPK activity that might be involved in this differential regulation of the ERK isoforms, but also for the termination of MAPK activation in the recovery phase. For instance, DUSP6 has a high selectivity towards ERK (Camps et al., 2000; Owens and Keyse, 2007). DUSP1 and DUSP6 mRNA were significantly upregulated immediately after running and this might provide an explanation for the lack of phosphorylation of ERK1/2 in the liver of the exercised mice.

Besides MAPK, p53 signalling was also transiently induced in the livers of exercised mice. This was evident by an increased amount of p53 protein and of the mRNA of its target genes trp53inp1 and p21. The enhanced phosphorylation of Akt/PKB in the recovery phase points to a possible negative feedback control to prevent further p53 protein activation via Mdm2. Our finding of hepatic p53 activation is noteworthy because this tumour suppressor has been shown to regulate the balance between glycolytic and aerobic metabolism not only in cancer cells but also in the muscle (Matoba et al., 2006) and might play a similar role in the exercising liver. Physical exercise induces p53 activation in the muscle and mice deficient for p53 have a reduced endurance capacity (Saleem et al., 2009). The mechanism by which p53 improves oxidative capacity seems to be via an increase in mitochondria, which are deficient in the muscles of p53 knockout mice (Park et al., 2009). In states of low energy like fasting and physical exercise, when AMPK is active, it can directly phosphorylate and thereby activate p53 (Thoreen and Sabatini, 2005).

3.3.3 Regulators of the hepatic response to exercise

The novel findings of acute responses of the liver to physical exercise inevitably lead to the question by which stimuli they are triggered. An overview of possible mechanisms is given in (Fig. 23). Endurance exercise can vastly reduce glycogen content and this is known to be accompanied by a significant decrease in hepatic energy charge (Camacho et al., 2006) and activation of AMPK (Kelly et al., 2004; Wojtaszewski et al., 2002). Although we did not attempt to quantify adenine nucleotide levels, it is clear from the activation of AMPK and from the almost complete depletion of glycogen that our exercise protocol must as well have evoked energetic stress in the liver. Moreover, glucagon levels were slightly increased by our exercise protocol and glucagon signalling is also capable to lower the energy state and thereby activate AMPK in the liver (Berglund et al., 2009). Since alterations in the energy charge of the muscle depend on the exercise protocol and were not detected even after exhaustive treadmill running (Camacho et al., 2006), it could be argued that the stronger transcriptional response in the liver might be related to the more pronounced AMPK activation in this tissue. However, the contraction-induced activation of calcium-dependent pathways in the working muscle can potentiate AMPK activation, even in the absence of an altered energy

charge (Birnbaum, 2005; Hurley et al., 2005). Thus, the mechanisms leading to AMPK activation appear to be different in the liver and the working muscle and it is unclear if this could explain the different temporal pattern of the transcriptional response in liver and muscle, for example, of the AMPK target gene PGC-1α. The metabolic responses of liver and muscle during exercise are certainly regulated by a multitude of factors. Altered substrate availability, namely, the increase in plasma free fatty acids and the decline in plasma glucose and in hepatic but not in muscle glycogen could be some of them. In addition, changes in hormone levels during physical exercise, especially the lowered insulin/glucagon ratio and the rise in catecholamine levels, could modulate not only the hepatic metabolism (Horowitz and Klein, 2000) but also the stress response.

As mentioned above, ROS are known inducers of the stress response in the muscle during physical exercise and there is evidence for increased ROS generation also in the liver. We therefore tested the hypothesis that oxidative stress is also the trigger of hepatic MAPK signalling by pre-supplementing mice with a diet enriched in vitamin C and vitamin E for 4 weeks before a single bout of exercise. Similar strategies have been shown to be successful in the prevention of exercise-induced oxidative stress in human muscle (Gomez-Cabrera et al., 2008; Ristow et al., 2009) or in the reduction of oxidative stress in mice (Pratico et al., 1998). Although the induction of antioxidant enzymes after the exercise bout was quite variable, there was a significant upregulation of MT1 and a trend for an induction of Hmox1 in the livers of control-fed mice that was absent in the antioxidant-fed group. Thus, it appears that the applied exercise protocol did cause some degree of oxidative stress which could be prevented by pre-feeding the antioxidant diet. However, there were no differences in the expression of the genes coding for the MAPK signalling molecules c-Fos and Gadd45g which suggests that oxidative stress is neither responsible for the upregulation of these genes nor for the activation of ERK.

Although the link is less obvious, energetic stress might be involved not only in the metabolic response of the liver to exercise but also in the activation of MAPK signalling. The expression of PGC-1α and IGFBP1 was inversely related to circulating glucose levels after the bout of exercise and a similar relationship was evident for MAPK signalling molecules like c-Fos, GADD45g, and ATF3. With the exception of GADD45g, none of these correlated with insulin levels. The concentrations of circulating FFA were elevated after exercise, in parallel to the fall in glucose, but they did not correlate to the expression of any stress-responsive gene. Some MAPK signalling proteins, namely, c-Fos, ATF3, and GADD45b, were not only induced in the liver but also in the muscle. However, none of them could be related to the fall in circulating glucose. Thus, it is clear that, similar to the activation of AMPK, different signals underlie the stress responses in liver and muscle.

We can only speculate about the exact mechanisms that link the fall in circulating glucose to MAPK signalling and the hepatic stress response. While insulin alone was no good predictor, its antagonist glucagon or the net effects of a decrease in insulin and

an increase in glucagon level might play a role. We only could measure glucagon concentrations in a subset of eight exercised mice, which is not sufficient to study any correlations, but the lower glucose concentrations after exercise might be a good correlate for glucagon action. Even when peripheral glucagon concentrations are not increased after exercise, the liver can still be exposed to higher levels of glucagon in the portal vein (Wasserman et al., 1993), sufficient to induce a hepatic response (Wasserman et al., 1989). The activation of glucagon receptors increases cAMP levels, leading to activation of protein kinase A, which activates ERK1/2 (Jiang et al., 2001) and triggers several metabolic changes in the liver that culminate in an enhanced glucose output (Jiang and Zhang, 2003).

Stimulation of alpha- and beta-adrenoceptors by catecholamines also leads MAPK activation and exercise is known to induce plasma norepinephrine and epinephrine concentrations (Christensen and Galbo, 1983). However, strategies designed to specifically address the function of hepatic adrenergic stimulation during exercise, e.g. hepatic adrenoreceptor blockade in dogs (Coker et al., 1997) or inhibition of hepatic innervation in humans (Kjaer et al., 1993), do not support an important role for catecholamines in stimulating hepatic glucose output. Of course, this does not exclude the possibility that catecholamines are involved in the activation of hepatic MAPK signalling during exercise. Moreover, evidence exists that independent of pancreatic hormones and catecholamine action, the fall in plasma glucose concentrations has stimulatory effects on the liver sufficient to enhance glucose output (Coker et al., 2001; Coker et al., 2002). According to these data, signalling molecules or metabolites released by the muscle or the decrease in glucose concentrations per se might regulate the hepatic response to exercise. A glucose sensing system involving glucokinase, Glut2 and KATP-channels is used by pancreatic β- and α-cells (Rorsman et al., 2008) and by some nutrient-sensitive hypothalamic neurons (Mountjoy and Rutter, 2007). Knockout mouse models demonstrate a role of glucokinase and the glucose transporter Glut2 in hepatic glucose sensing, too, at least in states of high glucose availability (Burcelin et al., 2000; Dentin et al., 2004).

The muscle could also directly communicate with the liver, by the release of signalling molecules such as the cytokine IL-6 (Febbraio and Pedersen, 2005). IL-6 type cytokines have been shown to activate ERK1/2, JNK and p38 MAPK by a cascade involving JAK and Ras (Heinrich et al., 2003). We found evidence for increased cytokine signalling via JAK/STAT-3 in the liver, as evidenced by increased phosphorylation of STAT-3 and increased expression of its target gene SOCS3, which might favour this hypothesis. More sophisticated experiments are, however, required to verify this link, and we performed a separate study to analyse the role of IL-6 during physical exercise.

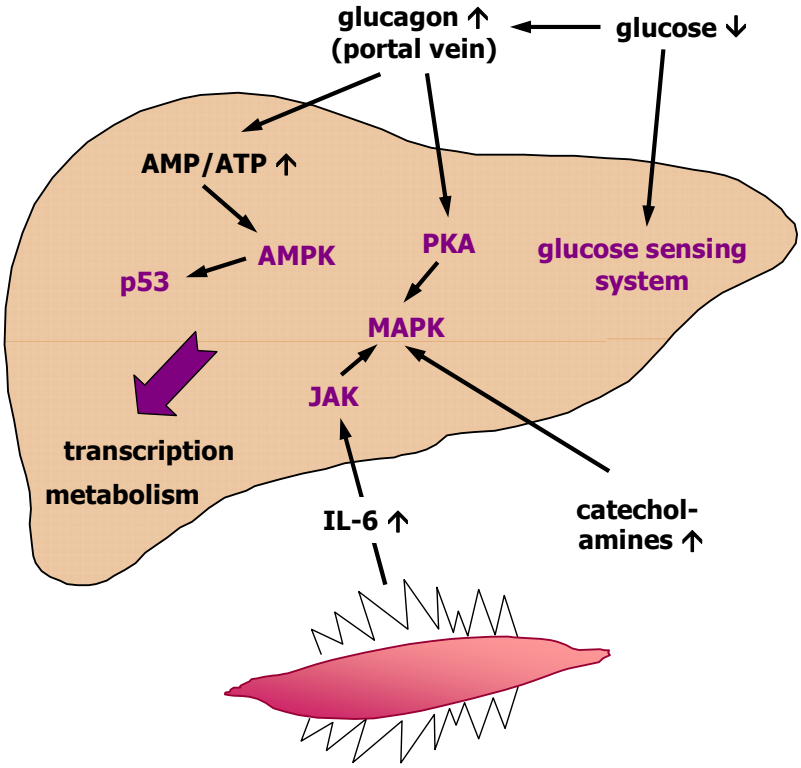

Fig. 23. Signals and pathways that could mediate the acute molecular response of the liver to physical exercise. Exercise elevates the ratio of AMP to ATP in the liver, thereby activating AMPK which in turn increases p53 activity. Either directly or via increased glucagon concentrations in the portal vein, the fall in circulating glucose could activate hepatic MAPK signalling. Catecholamines could also activate MAPKs and IL-6 or other cytokines released from the muscle could induce the JAK/STAT-3 pathway in the liver.

3.3.4 Conclusions and outlook

This thesis work shows that metabolic genes and components of the insulin signalling cascade are considerably regulated and that an acute stress response is induced in the liver of mice during physical exercise. The more pronounced acute metabolic response of the liver, as compared to the muscle, reflects the strong involvement of the exercising liver as the main regulator of fuel homeostasis. Although we could show that the hepatic stress response is linked to the fall in plasma glucose levels, it is still an important aim to identify the exact stimulus that induces MAPK and the other signalling pathways that are activated in the exercising liver. It would further be interesting to determine if MAPK signalling is linked to the acute and chronic adaptation of glucose and lipid metabolism in the liver, similar to the muscle. To understand the hepatic response to exercise is particularly important in the light of the acute enhancement of insulin signalling that could also be observed in the liver of exercised mice and the power of regular exercise to counteract insulin resistance and obesity. It is unclear how directly the data obtained in mice can be translated to humans. Since the relative size of the liver and its glycogen stores are smaller in humans than in mice, it could be argued that the

human liver is also less important as a supplier of glucose during physical exercise. On the other hand, this could require an even faster transition to gluconeogenesis and the metabolic challenge might be even more pronounced for a comparatively small liver that has to support a great amount of muscle mass. A detailed knowledge of the molecular response of the liver might help to determine which exercise protocols are best suited to acutely improve hepatic glucose metabolism and possibly, to design exercise interventions that could specifically train the liver.

4 The role of interleukin-6 in mediating the metabolic adaptations to physical exercise

4.1 Introduction

Physical exercise requires a tightly regulated interplay between skeletal muscle, liver and adipose tissue to ensure fuel supply to the muscle and glucose homeostasis. Molecules that are released from the working muscle could act to directly transmit the metabolic needs of the contracting myofibres and increase glucose production in the liver and lipolysis in adipose tissue. The search for such an “exercise factor” (Pedersen et al., 2003) has been pursued with specific emphasis because its action could, ideally, mimic exercise and open up new treatments for diseases associated with physical inactivity.

IL-6 is an important regulator of inflammatory processes and of the immune response (Van Snick, 1990) and has traditionally been viewed as a pro-inflammatory cytokine. The levels of IL-6 are elevated in obese and type 2 diabetic subjects and it is still not entirely clear if this is only a consequence or also a cause of the chronic low-grade inflammatory state seen in these conditions (Mooney, 2007; Pedersen and Febbraio, 2007). However, since the first study reporting an increase of circulating IL-6 in response to exercise in humans (Northoff and Berg, 1991), it has increasingly been viewed as candidate exercise factor and evidence exists that it could be an important regulator of glucose and fatty acid metabolism.

4.1.1 IL-6, its receptors and signalling pathways

Mouse IL-6 is a single polypeptide chain of 187 amino acids. It shares 42 % homology to the human cytokine that is also active on murine cells (Kamimura et al., 2003; Van Snick, 1990). The IL-6 receptor complex consists of two membrane glycoproteins: the specific receptor subunit IL-6R and gp130 that serves as a common signal transducer unit for the IL-6-type cytokines IL-6, IL-11, leukaemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1 (Boulay et al., 2003). The IL-6R, which is not involved in signal transduction, is expressed both as a membrane-bound protein of 80 kDa and as a soluble form that can be generated by alternative splicing or by limited proteolysis (shedding) of the membrane-bound receptor (Novick et al., 1989; Rose-John and Heinrich, 1994). The transmembrane and cytoplasmic domains lacking in soluble (s) IL-6R are not required for the formation of the signalling complex. Unlike most other soluble receptors, sIL-6R exhibits agonistic properties (Jones and Rose-

John, 2002) and has been shown to mediate IL-6 transsignalling to cells that do not express the membrane-bound receptor (Mackiewicz et al., 1992; Peters et al., 1998).

Two major signalling pathways are activated by IL-6: the JAK/STAT and the MAPK cascade (reviewed in (Heinrich et al., 2003)). Briefly, IL-6 first binds to its specific receptor. The IL-6/IL-6R-heterodimer then associates with two gp130 molecules, thereby inducing homodimerization of gp130 and intracellular signal transduction (Murakami et al., 1993). The cytoplasmic domain of gp130 has no intrinsic kinase action but contains a region for the association of JAKs (Hibi et al., 1990) which are brought into spatial proximity and transactivated upon dimerization of the receptors (Stahl et al., 1994). JAK activation then leads to phosphorylation of several residues of gp130 and to the recruitment of different signalling proteins. All IL-6-type cytokines activate, via gp130, the transcription factor STAT-3 which translocates to the nucleus to activate the transcription of target genes such as c-Fos, JunB, cyclin D1, CCAAT/enhancer binding protein beta, p21 and acute-phase genes (Heinrich et al., 1998). STAT-3 also potently upregulates SOCS3, a negative feedback regulator of gp130 signalling (Starr et al., 1997). The receptor-kinase complex also activates SH2 domain-containing tyrosine phosphatase that interacts with additional factors to activate Ras and trigger the MAPK cascade. ERK1/2, JNK, and p38 MAPK have all been shown to be activated by the IL-6 family of cytokines (Heinrich et al., 2003)). Besides JAK/STAT and MAPK signalling, an additional pathway that can be activated by the IL-6 family of cytokines is the PI3K cascade that leads to the activation of Akt/PBK (Heinrich et al., 2003).

4.1.2 The role of IL-6 as an (anti-) inflammatory cytokine

A hallmark of IL-6 common to all cytokines is its secretion by and action on a broad range of different cells. During both inflammatory and non-inflammatory conditions, IL-6 is released from endothelial cells, fibroblasts, keratinocytes, osteoblasts, myocytes, adipocytes, pancreatic β cells, and other tissues (reviewed by (Kamimura et al., 2003; Van Snick, 1990)). Moreover, IL-6 is produced by cells of the immune system, mainly by monocytes/macrophages at inflammatory sites. Its plasma concentration in healthy resting humans is typically around 1 pg/ml (Bruunsgaard et al., 1997; Starkie et al., 2001). During sepsis, IL-6 plasma levels can increase up to 1000 fold—an acute inflammatory state that is associated with high lethality (Friedland et al., 1992). More moderate increases in the systemic levels of IL-6 are found in a number of chronic low-grade inflammatory conditions (Gabay, 2006). IL-6 is a central player in the regulation of inflammation, haematopoiesis, immune responses and host defence mechanisms (Akira et al., 1993).

The scope of action of the pleiotropic cytokine includes upregulation of acute phase proteins in the liver (Moshage, 1997), induction of growth and differentiation in B-cells (Kishimoto and Hirano, 1988), activation of T-cells, stimulation of haematopoiesis (Van Snick, 1990), differentiation of macrophages and dendritic cells (Mitani et al., 2000),

and leukocyte recruitment (Kamimura et al., 2003). On the other hand, IL-6 has been shown to be essential for the attenuation of inflammation (Xing et al., 1998), to promote the synthesis of anti-inflammatory cytokines and to downregulate inflammatory ones (Steensberg et al., 2003a). While tumour necrosis factor α (TNF-α) infusion can stimulate IL-6 production in humans (Plomgaard et al., 2005), IL-6 attenuates the endotoxin-induced increase in TNF-α (Starkie et al., 2003). Therefore, IL-6 has been classified as a both pro- and anti-inflammatory cytokine (Kamimura et al., 2003). However, constant IL-6 elevation clearly is a pathologic state, and mice chronically overproducing IL-6 in skeletal muscle (Franckhauser et al., 2008) or IL-6-sIL-6R double transgenic mice (Peters et al., 1997), both of which display reduced body weight, are not a suitable model to study the effects of transient increases of circulating IL-6 that are associated with physical exercise.

4.1.3 Obesity and the release of IL-6 from adipose tissue

Obesity is accompanied by a slight but significant systemic elevation of IL-6, TNF-α and other inflammatory markers and by an infiltration of macrophages into the adipose tissue (Cottam et al., 2004; Curat et al., 2006; Trayhurn and Wood, 2004). Therefore, it is discussed as a chronic sub-inflammatory disease. Similar to TNF-α, IL-6 received particular attention when its circulating levels were directly linked to insulin resistance (Pickup et al., 1997; Pradhan et al., 2001). However, the connection between IL-6, obesity and the metabolic syndrome has been discussed controversially (Carey and Febbraio, 2004; Mooney, 2007; Pedersen and Febbraio, 2007). Some studies showed a clear relationship between elevated IL-6 levels and the presence of insulin resistance or type 2 diabetes (Fernandez-Real et al., 2001; Pickup et al., 1997; Pradhan et al., 2001). However, several findings support the perception that elevated IL-6 levels and increased fat mass are no independent risk factors for the development of insulin resistance, due to quantitative release of IL-6 from adipose tissue (Carey et al., 2004; Corpeleijn et al., 2005; Kopp et al., 2003). Adipose tissue has been shown to contribute to 10 – 35 % of circulating IL-6 plasma levels in resting, healthy humans (Mohamed-Ali et al., 1997). The adipocytes themselves can produce and secrete IL-6 (Bastard et al., 2000; Sopasakis et al., 2004), but they may only contribute to a fraction of total IL-6 released while the bulk is produced by immune cells within the adipose tissue (Fain et al., 2004; Fried et al., 1998).

4.1.4 The IL-6 response to acute exercise

Physical exercise is accompanied by an increase of plasma IL-6, the magnitude of which depends on duration, intensity and muscle mass involved. In humans, running for 1 h increases IL-6 levels 4- to 9-fold, and dramatic increases have also been reported, for instance, elevations between 43- and 128- fold after a marathon run (reviewed by (Fischer, 2006)). Systemic IL-6 levels rise in an exponential fashion, peak at

the cessation of an exercise bout, and drop quickly after (Febbraio and Pedersen, 2002)). IL-6 has a short half-life in the circulation of 2 minutes for the radiolabelled protein in mice (Montero-Julian et al., 1995).

The IL-6 response to exercise is not related to muscle damage (Toft et al., 2002) or activation of circulating monocytes (Moldoveanu et al., 2000; Starkie et al., 2001) and not accompanied by increases of TNF-α and other inflammatory cytokines (Petersen and Pedersen, 2005; Ullum et al., 1994). Working skeletal muscle itself is the main contributor to the IL-6 increase in humans (Steensberg et al., 2000) and interstitial concentrations within the muscle can be highly elevated without the occurrence of a significant rise in circulating levels (Rosendal et al., 2005). Increased levels of IL-6 mRNA in the muscle and protein in the circulation have also been found in mice after physical exercise (Jonsdottir et al., 2000). Some of the IL-6 released during exercise may also stem from peritendinous tissue (Langberg et al., 2002). Subcutaneous adipose tissue, on the other hand, does not contribute to IL-6 secretion during exercise, but in the recovery phase following prolonged exercise increased output of IL-6 was reported (Lyngso et al., 2002a).

Both IL-6 mRNA (Ostrowski et al., 1998) and protein (Penkowa et al., 2003) are produced in human skeletal muscle fibres in response to exercise. Endurance training for 10 weeks leads to elevated basal IL6-R mRNA levels (Keller et al., 2005), suggesting a sensitizing effect of exercise on the muscle, while exercise-induced IL-6 expression in muscle cells is decreased after the same period (Fischer et al., 2004b). Besides the effect of training, IL-6 production and release from working muscle are regulated by energy substrate availability in a complex manner. In exercising humans, high muscular glycogen levels reduce contraction-induced transcription and release of IL-6 (Keller et al., 2001; Steensberg et al., 2001) as well as IL-6 receptor transcription (Keller et al., 2005), while ingestion of carbohydrate results in decreased plasma IL-6 levels but not skeletal muscle IL-6 mRNA (Nieman et al., 1998; Starkie et al., 2001). Suppression of lipolysis by ingestion of nicotinic acid increases circulating IL-6 in humans, both in the resting state and during prolonged moderate-intensity exercise (Holmes et al., 2004b). In addition, hypoxia seems to increase the IL-6 response to exercise in humans (Lundby and Steensberg, 2004).

The molecular mechanisms shown to influence IL-6 production and release are as diverse as the metabolic modulators. AMPK may be the mediator of the effects of glycogen reduction and glucose availability on IL-6 expression in human muscle (Akerstrom et al., 2006; Chan et al., 2004). The release of IL-6 from myotubes can be induced by ROS in an NFκB-dependent manner (Kosmidou et al., 2002) and antioxidant supplementation reduced the exercise-induced rise in IL-6 levels in some, but not in all, human studies (Davison et al., 2007; Teixeira et al., 2009; Vassilakopoulos et al., 2003) by abrogating IL-6 release from the contracting muscle (Fischer et al., 2004a). Treatment with the Ca^{2+} ionophore ionomycin stimulates IL-6 release from murine soleus muscle ex vivo (Holmes et al., 2004a) and from cultured myocytes in a p38-dependent

manner (Chan et al., 2004). IL-6 can also regulate its own production, since infusion of recombinant IL-6 in humans elevated IL-6 mRNA in skeletal muscle (Keller et al., 2003) and, as shown for muscle cells in vitro, Ca^{2+} and stabilization of IL-6 mRNA play a role in this positive feedback loop (Weigert et al., 2007).

4.1.5 The role of IL-6 in regulating glucose and lipid metabolism

The fact that the secretion of IL-6 from the myofibres is related to the energetic intensity of exercise and not dependent on structural damage suggested metabolic rather than inflammatory actions of IL-6 during or shortly after physical exercise. Indeed, IL-6 has been shown to modulate the energy metabolism of liver and adipose tissue, as well as of the muscle itself. Both the fever-inducing (Leon, 2002) and the weight-lowering effect of IL-6 are exerted at the level of the central nervous system, but it is not known whether IL-6 produced during physical exercise does reach the brain. Intracerebroventricular but not i.p. injection of IL-6 increases energy expenditure (Wallenius et al., 2002b) and decreases body fat in rats (Wallenius et al., 2002a). A graphical overview of the predicted actions of IL-6 that support its role as an exercise factor that regulates glucose and fat metabolism is shown below (Fig. 24).

4.1.5.1 Auto- and paracrine effects of IL-6 on the working muscle

The ~100-fold rise of interstitial IL-6 levels within minutes after the commencement of exercise (Rosendal et al., 2005) suggests that skeletal muscle itself could be an important target for IL-6. According to recent findings, IL-6 may promote fuel uptake and utilization in the working muscle. As shown by in vitro studies, IL-6 enhances basal and insulin-stimulated glucose transport in human and rat L6 myotubes and in human muscle strips (Al Khalili et al., 2006; Carey et al., 2006; Glund et al., 2007; Weigert et al., 2006). Additionally, IL-6 treatment increases fatty acid oxidation in isolated rat muscle (Bruce and Dyck, 2004) and cultured myotubes (Al Khalili et al., 2006; Carey et al., 2006). IL-6-induced activation of AMPK could be an important mediator of the IL-6 effects on skeletal muscle metabolism. Activation of AMPK by IL-6 has been found in cultured cells and human skeletal muscle strips in vitro and was diminished in muscle, liver and adipose tissue of IL-6-deficient mice in response to exercise (Al Khalili et al., 2006; Carey et al., 2006; Kelly et al., 2004). The mechanism by which IL-6 could activate AMPK seems to be via cAMP generation and elevation of the AMP/ATP ratio (Kelly et al., 2009).

4.1.5.2 Acute effects of IL-6 on lipid metabolism

The lipolytic properties of IL-6 have been examined in various model systems, not always with definitive results. Adipose tissue and adipocytes cultured in the presence of IL-6 show increased lipolysis (Petersen et al., 2005; Trujillo et al., 2004) and IL-6 infu-

sion in humans increases free fatty acid concentrations and whole body fat oxidation (Lyngso et al., 2002b; van Hall et al., 2003). In contrast, infusing IL-6 during low-intensity exercise to concentrations found during moderate-intensity exercise did not elevate lipolysis or fatty acid oxidation in humans (Hiscock et al., 2005). The mechanisms that could underlie the lipolytic effect of IL-6 are not clear. Infusion of recombinant IL-6 to humans increases the mRNA, but not the protein levels of adipose tissue hormone-sensitive lipase (Watt et al., 2005). In principle, IL-6 could exert lipolytic activity by inducing other hormones. However, infusion of the recombinant cytokine to moderate levels below 50 pg/ml did not alter the concentration of insulin, cortisol, glucagon or catecholamines in humans (Hiscock et al., 2005; Krogh-Madsen et al., 2006; Tsigos et al., 1997).

4.1.5.3 Acute effects of IL-6 on glucose homeostasis

The regulation of hepatic glucose production by IL-6 is still a matter of debate. While IL-6 infusion during exercise has been shown to increase endogenous glucose production and turnover in humans (Febbraio et al., 2004) and a single dose of IL-6 increased fasting blood glucose levels in resting subjects (Tsigos et al., 1997) other studies failed to show enhanced glucose output from the liver (Carey et al., 2006; Steensberg et al., 2003b). The data concerning the action of IL-6 on gluconeogenesis and glycogenolysis, the two mechanisms of hepatic glucose production, are also ambiguous. Injection of human recombinant IL-6 into rats upregulated PEPCK mRNA in the liver (Banzet et al., 2009) and in vitro, IL-6 increased gluconeogenesis in rat hepatocytes (Blumberg et al., 1995). IL-6 has also been shown to elevate glycogenolysis in cultured hepatocytes (Ritchie, 1990) and in rat muscle ex vivo (Kelly et al., 2009). On the other hand, evidence for an inhibitory effect of IL-6-type cytokine signalling on hepatic gluconeogenesis exists. In cultured hepatocytes, IL-6 interfered with glucagon-induced upregulation of PEPCK (Christ et al., 2000). Mice with a liver-specific STAT-3 deficiency had increased expression of gluconeogenic enzymes and the liver-specific expression of a constitutively active form of STAT-3 repressed their transcription (Inoue et al., 2004). Further studies indicated that STAT-3 could repress the regulatory promoter regions of gluconeogenic genes in vivo (Ramadoss et al., 2009). Thus, it is not clear whether IL-6 acts as a repressor or inductor of gluconeogenesis, and only the latter function would fit into the profile of an exercise factor.

4.1.5.4 IL-6 and insulin action

The role of IL-6 in the development of insulin sensitivity is still a matter of debate. Elevating IL-6 levels in mice for several days by overexpression (Franckhauser et al., 2008) or continuous infusion (Klover et al., 2003) inhibits insulin signalling in the liver (Klover et al., 2003) and insulin-stimulated glucose uptake in the muscle (Franckhauser et al., 2008), while acute IL-6 application does not impair insulin signalling and glucose

disposal in liver and muscle in rats (Rotter, V et al., 2004). Thus, acute and chronic elevation of IL-6 might have contrasting effects (Nieto-Vazquez et al., 2008).

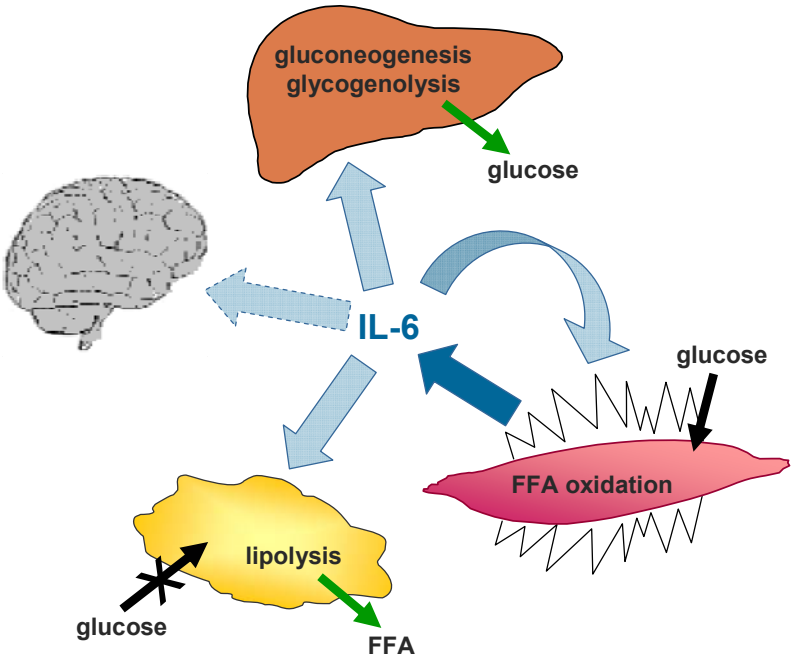

Fig. 24. Predicted actions of IL-6 as an exercise factor. The working muscle releases IL-6 into the circulation. IL-6 could signal to the adipose tissue to increase lipolysis and to the liver to increase gluconeogenesis and glycogenolysis. The influence of IL-6 on insulin-mediated glucose uptake could be tissue-specific, with inhibitory effects on the adipose tissue and stimulatory effects on the muscle. Some of the action of IL-6 could also be mediated via the central nervous system.

Moreover, the action of IL-6 on insulin signalling may be different in liver and fat on one hand and muscle on the other. IL-6 appears to have adverse effects on insulin action in liver and adipose tissue as demonstrated in animals and in cell culture studies, even though not all reports did support this (Carey et al., 2006; Rotter, V et al., 2004; Stouthard et al., 1996). In contrast, IL-6 could sensitize myotubes for the effects of insulin as evidenced by enhanced insulin-dependent glycogen synthesis and glucose uptake (Al Khalili et al., 2006; Carey et al., 2006; Weigert et al., 2005). Acute IL-6 application into mice has differential effects on IRS-1 phosphorylation in liver and muscle (Weigert et al., 2006), which provides a possible explanation for such a tissue-specific action.

It has also been hypothesized that IL-6 acts directly on the insulin-secreting β-cells. However, neither in vitro nor in vivo data argue for a role of IL-6 in β-cell apoptosis and only in vitro experiments with rodent islets, but not in vivo studies in humans could find an acute effect of IL-6 on the release of insulin (reviewed by (Kristiansen and Mandrup-Poulsen, 2005)). Recent data suggest that not the β- but the glucagon-secreting α-cells are the main target of IL-6, on which a proliferative action of IL-6 has been shown

both in human islets ex vivo and in mice in response to a high-fat diet (Ellingsgaard et al., 2008).

4.1.6 Data from IL-6-deficient mice

Whole-body IL-6 knockout (IL-$6^{-/-}$) mice have been used as a model system to study the metabolic and molecular consequences of IL-6 deficiency. The original knockout strain has been created by Kopf and co-workers (Kopf et al., 1994) and all data mentioned herein stem from mice that have been bred to a C57BL/6 background, except where stated otherwise. Disruption of the IL-6 gene was achieved by introducing a neomycin resistance cassette through homologous recombination into the second exon, which is the first coding exon (Kopf et al., 1994), and absence of IL-6 mRNA and protein production has reproducibly been confirmed (Ellingsgaard et al., 2008; Kopf et al., 1994). Mice deficient for IL-6 develop normally (Kopf et al., 1994) and a comprehensive analysis performed in mice backcrossed to a BALB.B background detected no effect of IL-6 deletion on feeding, exploratory, or anxiety-related behaviours (Swiergiel and Dunn, 2006). Early studies focused on the immunological role of IL-6 and found impairments in the hepatic acute-phase response, T and B cell function, and defence to some bacteria and viruses (Bernad et al., 1994; Kopf et al., 1995). Although a role for IL-6 has been found in regulating satellite cell-mediated hypertrophic muscle growth (Serrano et al., 2008), the relative muscle weight is not lower and myofibre cross-sectional area increases in response to muscular overload in IL-$6^{-/-}$ mice (White et al., 2009).

Contrasting evidence exists to whether or not a loss of IL-6 predisposes mice to adiposity. One study reported increased weight gain and fat mass in IL-$6^{-/-}$ mice starting at 6 months age (Wallenius et al., 2002b), while another reported an impaired weight gain of IL-$6^{-/-}$ mice fed a high-fat diet (Di Gregorio et al., 2004). Only the first, but not the second study detected impaired insulin sensitivity and elevated plasma leptin levels in the aged IL-$6^{-/-}$ mice. Both groups used WT littermates as controls. Another group reported that glucose tolerance is normal in young, lean animals but impaired after 18 weeks of high-fat feeding in mice lacking IL-6, despite a slightly lower body weight (Ellingsgaard et al., 2008). Of note, the same laboratory that reported mature-onset obesity also detected reduced endurance capacity (Faldt et al., 2004) and a higher respiratory exchange rate (RER) in sedentary IL-$6^{-/-}$ mice. RER is the ratio of CO_2 to O_2 and a higher RER indicates a relatively higher utilization of carbohydrates since more oxygen is needed for the oxidation of fat than of carbohydrates. An impaired capacity to oxidate fatty acids could in principle explain both the impaired running endurance and increased accumulation of fat in the mice lacking IL-6. However, RER was normal during exercise in young IL-$6^{-/-}$ mice (Wernstedt et al., 2006) and it has to be kept in mind that an elevation of RER does occur secondary to obesity (Goldsmith et al., 2010).

4.1.7 Aims of the study

The finding that IL-6 concentrations rise transiently in response to physical exercise suggested a role for this cytokine in the regulation of energy homeostasis because it might signal from the contracting muscle to the liver and other peripheral tissues. In support of a metabolic function, it is now clear that the myocytes themselves produce IL-6 and that the exercise-induced increase in plasma IL-6 is not related to muscle damage. However, previous data concerning the modulation of fatty acid and glucose metabolism by IL-6 are controversial and the few studies using mice deficient for IL-6 as a model system have added even more complexity. The aim of this thesis was to identify mechanisms by which IL-6 deficiency could alter metabolism and exercise performance.

The following specific questions were addressed in IL-$6^{-/-}$ mice:

1. Does IL-6 act as a “training factor” that mediates the beneficial effects of regular physical exercise on glucose metabolism? The experimental approach was to subject mice to a 4-week exercise programme and to assess their running endurance on an electric treadmill as well as glucose tolerance and insulin sensitivity.

2. Can voluntary exercise alter the effects of IL-6 deficiency on weight gain? To this end, WT and IL-$6^{-/-}$ mice were fed with 2 diets differing in energy content while having access to running wheels for 7 months.

3. Does IL-6 deficiency affect the acute metabolic and transcriptional response to a single bout of exercise? This question was assessed by subjecting young, untrained mice to a single bout of non-exhaustive treadmill exercise and analyzing plasma metabolites, mRNA content and protein levels and phosphorylation states in liver and muscle.

4. Is IL-6 required for the induction of gluconeogenesis by fasting? The gluconeogenic response of IL-$6^{-/-}$ and WT mice was compared after a 16 h fast.

4.2 Results

4.2.1 Does IL-6 act as a "training factor"?

4.2.1.1 Mice lacking IL-6 respond to exercise training despite having a persistently lower running endurance

An important piece of evidence in support of a role for IL-6 as an exercise factor is the observation that IL-6$^{-/-}$ mice have an impaired endurance capacity (Faldt et al., 2004). We wanted to verify this previous finding and further compare the effects of a 4-week exercise training in young IL-6$^{-/-}$ and WT mice. The training routine consisted of voluntary wheel-running combined with forced exercise on an electric treadmill once per week, as described in the methods section (2.5.5).

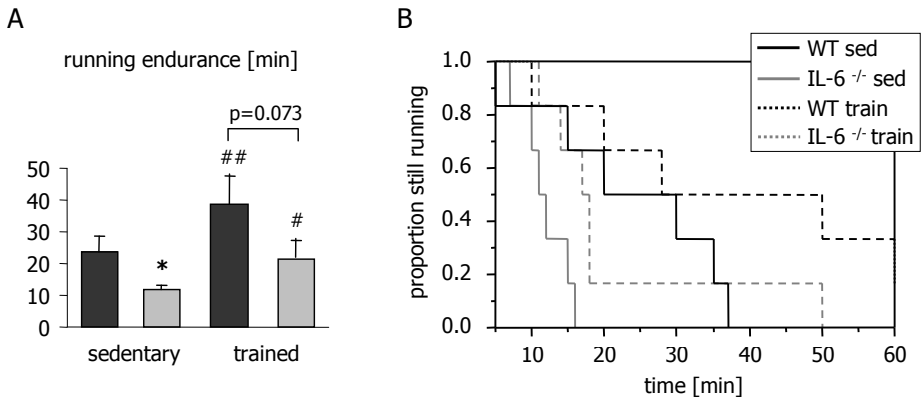

Fig. 25. Running endurance and effects of exercise training. WT (black bars) or IL-6$^{-/-}$ (grey bars) mice were subjected to a combined voluntary and forced training routine for 4 weeks or remained sedentary. The running endurance on an electric treadmill was then assessed as described in the methods section (2.5.5). The results are shown as **A** histogram and **B** Kaplan-Meier survival curve. The endurance test was discontinued after 60 min, when some of the trained WT mice were still running. (n=6, mean ± SEM; *p<0.5 vs. WT, #p<0.05, ##p<0.005 vs. sedentary WT or IL-6$^{-/-}$ mice of the same group).

The average distance run per day was similar, 8.85 ± 1.7 km in WT and 7.00 ± 1.72 km in IL-6$^{-/-}$ mice. Sedentary, untrained WT mice ran for an average of 23.7 ± 5.1 min till exhaustion, and this was significantly improved to more than 38 min in the trained group (Fig. 25A). Of note, this value underestimates the endurance of trained WT mice because the experiment was stopped after 60 min, when a proportion of trained WT mice was still running (Fig. 25B). The endurance capacity of IL-6$^{-/-}$ mice was only half that of WT mice, 11.8 ± 1.3 min, in the untrained state. Training also improved the endurance of the IL-6$^{-/-}$ mice to 21.3 ± 5.8 min. Thus, IL-6 is not mandatory for a training-induced improvement of running endurance. However, the time till exhaustion still was approximately half as long in the trained IL-6$^{-/-}$ as in the WT mice and this difference might have reached significance if the WT mice had been allowed to keep on running

for more than 60 min. This suggests that IL-6 could act as an “exercise factor”, but not as a “training factor”.

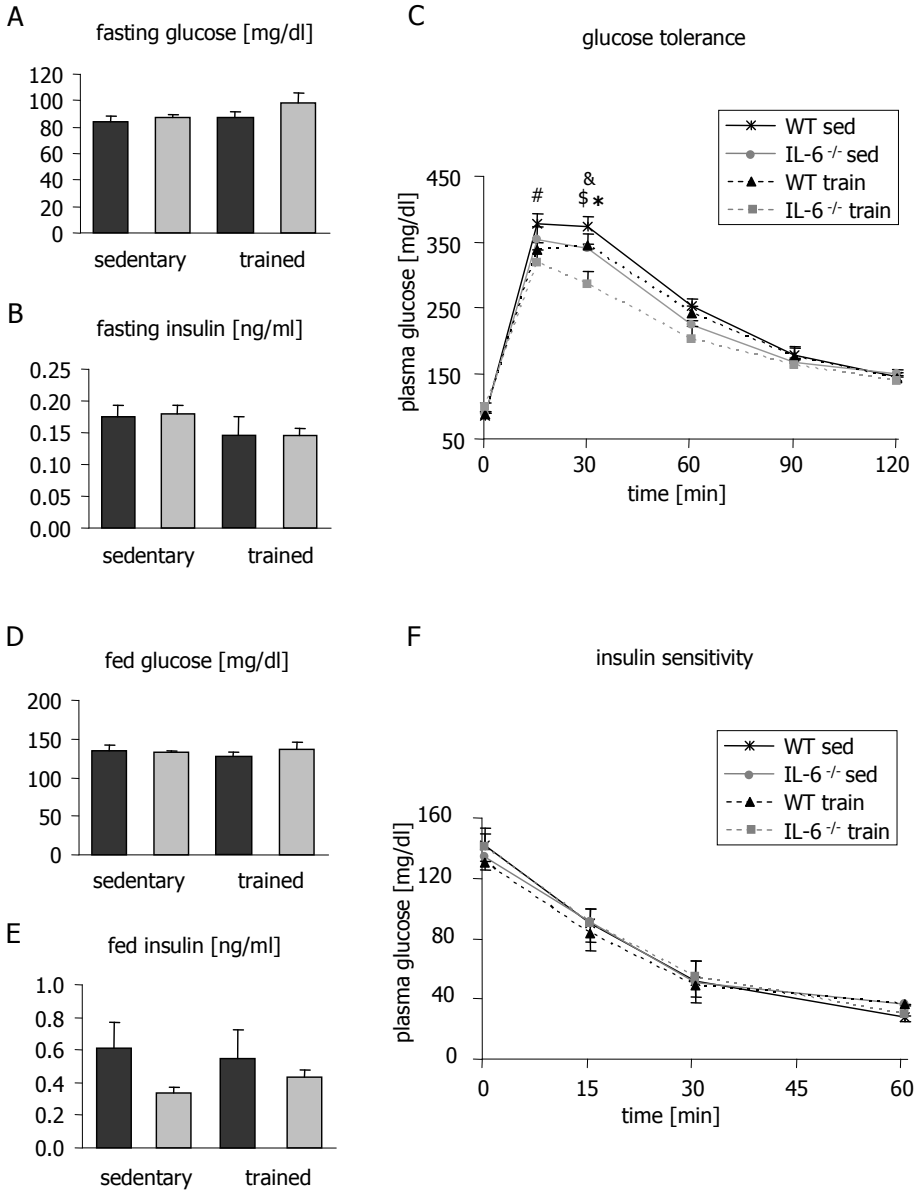

Fig. 26. Glucose metabolism of trained and sedentary WT and IL-6$^{-/-}$ mice. Plasma levels of **A** glucose and **B** insulin in 16 h fasted WT (black bars) and IL-6$^{-/-}$ (grey bars) mice after 4 weeks of exercise training. **C** Plasma glucose levels during a glucose tolerance test. 2 g glucose per kg body weight were given i.p. to fasted mice and glucose was measured during the following 120 min. Plasma levels of **D** glucose and **E** insulin in fed mice. **F** Plasma glucose levels during an insulin sensitivity test. 1.5 U insulin per kg body weight were applied i.p. to fed animals and glucose levels measured for the following 60 min. (n=6 (insulin, glucose), n=5-6 (glucose tolerance) or n=4-5 (insulin sensitivity), mean ± SEM; #p<0.05 trained vs. sedentary WT mice, $p<0.05 trained vs. sedentary IL-6$^{-/-}$ mice, *p<0.05 sedentary IL-6$^{-/-}$ vs. WT, &p<0.05 trained IL-6$^{-/-}$ vs. WT.)

4.2.1.2 Effects of 4 weeks voluntary exercise and endurance training on the metabolism of IL-6-deficient mice

We also studied the effects of the 4-week exercise training on glucose metabolism, by performing glucose tolerance and insulin sensitivity tests. The tests were performed one week after the last treadmill session in 16 week-old mice that still had access to running wheels, as described in the methods section (2.5.5). The basal levels of glucose or insulin were similar between all groups in the fasted state (Fig. 26A,B). However, the glucose tolerance was slightly improved in both groups of exercised mice, and this difference was significant at 15 min after glucose administration for WT and at 30 min for IL-6$^{-/-}$ mice (Fig. 26C). Moreover, IL-6$^{-/-}$ mice had a slightly, but visibly better glucose utilization and the concentrations were significantly lower in both trained and sedentary IL-6$^{-/-}$ than in the corresponding WT mice 30 min after glucose administration. In the fed state, there were no differences in plasma glucose (Fig. 26D) and no significant differences in insulin levels (Fig. 26E). The insulin sensitivity was also similar between WT and IL-6$^{-/-}$ as well as between trained and sedentary animals (Fig. 26F).

Four weeks of exercise did not lead to a lower weight gain in the trained mice. The final weight was 26.2 ± 0.9 and 26.4 ± 0.5 g for sedentary and trained WT mice and 26.4 ± 0.8 and 25.4 ± 0.5 g for sedentary and trained IL-6$^{-/-}$ mice, respectively. The increase in body weight was significantly reduced in IL-6$^{-/-}$ when compared to WT mice, both for the sedentary and the trained group (Fig. 27A). Circulating levels of triglycerides (Fig. 27B) and FFA (Fig. 27C) were similar between all groups in the 1 h fasted mice.

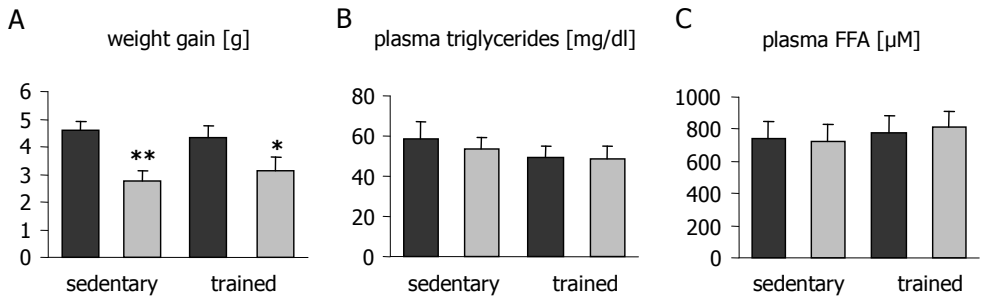

Fig. 27. Weight gain and lipid metabolism. WT (black bars) and IL-6$^{-/-}$ (grey bars) mice were subjected to 4 weeks of exercise training and **A** the weight gain during this period was monitored. Levels of **B** plasma triglycerides and **C** plasma FFA as measured after 1 h of fasting at the end of the experiment. (n=6, mean ± SEM; *p<0.05, **p<0.005 vs. WT).

Although we found some evidence that the 4-week training intervention resulted in a better glucose tolerance in the trained mice, this effect was weak and similar in IL-6$^{-/-}$ and WT mice. We next performed a long-term experiment where we combined voluntary exercise with a metabolic challenge in the form of an energy-rich diet.

4.2.2 Can voluntary exercise alter the effects of IL-6 deficiency on weight gain?

4.2.2.1 Mice lacking IL-6 have an enhanced voluntary wheel-running activity when fed a western-style diet

The results from the 4-week exercise experiment suggested a better glucose utilization and an attenuated weight gain in mice lacking IL-6. Because the short-time intervention pointed towards only slight metabolic alterations in the IL-$6^{-/-}$ mice, we next combined training for a longer time with a metabolic challenge, a western-style diet that contained 10 % more fat and carbohydrates than the control diet, to tease apart subtle effects of IL-6 deficiency. The experiment was commenced at an age of 10 weeks and lasted till the mice were 9 months old.

We monitored the daily wheel-running activity and found, unexpectedly, that IL-$6^{-/-}$ mice ran significantly more than WT mice when fed the western diet (Fig. 28A), an average of 13.3 ± 1.2 vs. 8.7 ± 0.9 km per day during the first ten weeks (Fig. 28B). WT and IL-$6^{-/-}$ mice fed the control diet (Fig. 28C) ran an average of 10.6 ± 0.4 and 7.7 ± 2.1 km, respectively, per day (Fig. 28D). Of note, both groups fed the western diet ran more than the corresponding group fed the control diet during the first ten weeks of the experiment. There was, however, a clear decrease in the daily running distance for both IL-$6^{-/-}$ and WT mice fed the more energy-rich diet throughout the experiment. For this reason, the average distance run over the entire experiment was only 10.2 ± 0.6 km/day for IL-$6^{-/-}$ and 5.5 ± 0.6 km/day for WT mice fed the western diet. Under the control diet, IL-$6^{-/-}$ mice ran an overall average of 9.8 ± 0.7 km/day and WT mice ran 7.4 ± 2.1 km/day.

The time course of daily running activity (Fig. 28) also reveals two interesting details: Firstly, an increase in the running activity during the first two weeks of the experiment, when the animals had to become accustomed to the running wheels. Secondly, a drop in the running activity after week 10, when we performed glucose tolerance tests. Another test was performed in week 28, when we stopped monitoring weight and running distance. Because of the small number of 4 mice per group, the results from these tests were not conclusive. There was, again, a trend towards an improved glucose utilization in the IL-$6^{-/-}$ mice, but this could not be separated from the effects of body weight (data not shown).

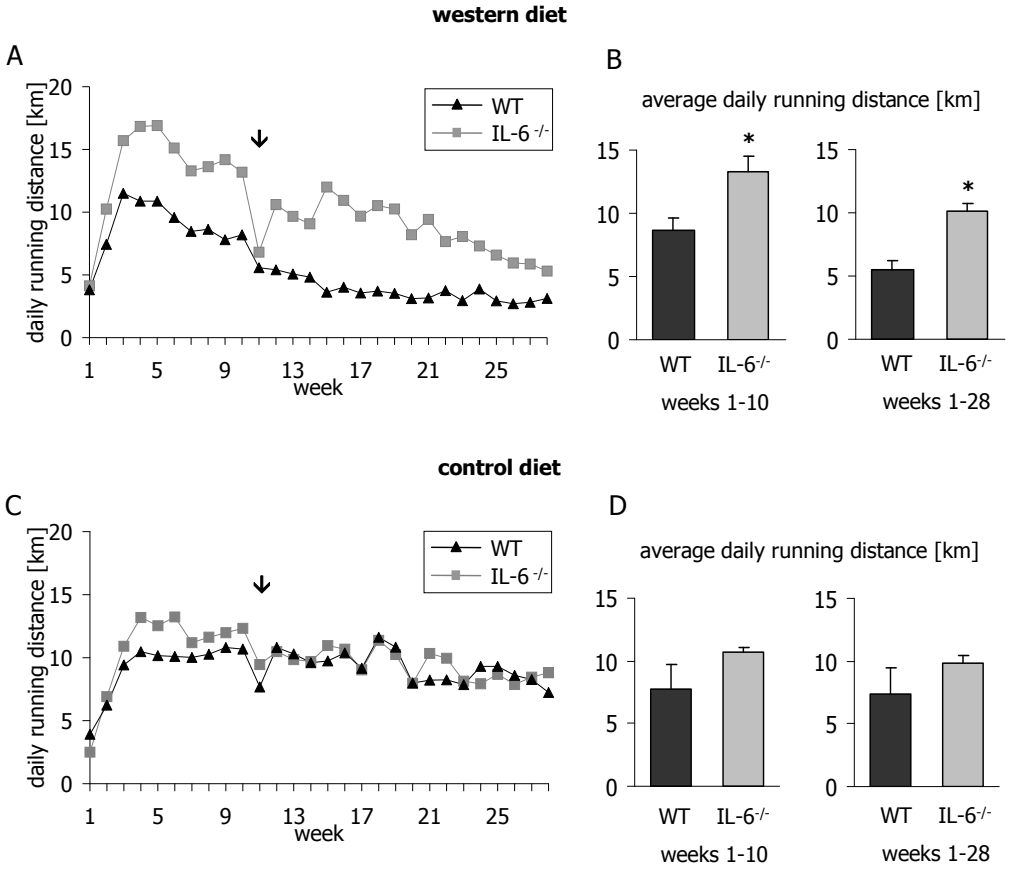

Fig. 28. Voluntary wheel-running activity of WT and IL-6$^{-/-}$ mice fed western or control diet. WT (black bars) and IL-6$^{-/-}$ (grey bars) mice were fed with control or energy-enriched (western) diet and provided with running wheels for voluntary exercise or remained sedentary. The running distance was constantly monitored and the average distance run per day was calculated for every week of the experiment, shown separately for animals receiving **A** western and **C** control diet. **B**, **D** The average daily running for weeks 1-10 and weeks 1-28 of the experiment is also shown (n=4, mean ± SEM; *p<0.05 vs. WT). The arrows mark the time point when glucose tolerance tests were performed.

4.2.2.2 Metabolic effects of a long-term intervention with western-style diet and voluntary exercise in IL-6$^{-/-}$ and WT mice

Although only slightly more rich in energy, the western diet led to a visibly increased weight gain over the entire 28 weeks, in both sedentary and exercising WT and IL-6$^{-/-}$ mice (Fig. 29A) when compared to the standard control diet. Thus, the average body weight of all groups of mice fed the western diet was significantly higher than that of the respective control groups at the end of the experiment (Fig. 29B). Although the exercising mice gained slightly less weight than the sedentary animals, this effect was not significant for either subset, given the small sample size of only 4 mice per group. As in the shorter training experiment, IL-6$^{-/-}$ mice gained less weight than WT mice, and this resulted in a trend towards a lower final body weight for both exercising and sedentary IL-6$^{-/-}$ mice fed the western diet (Fig. 29B).

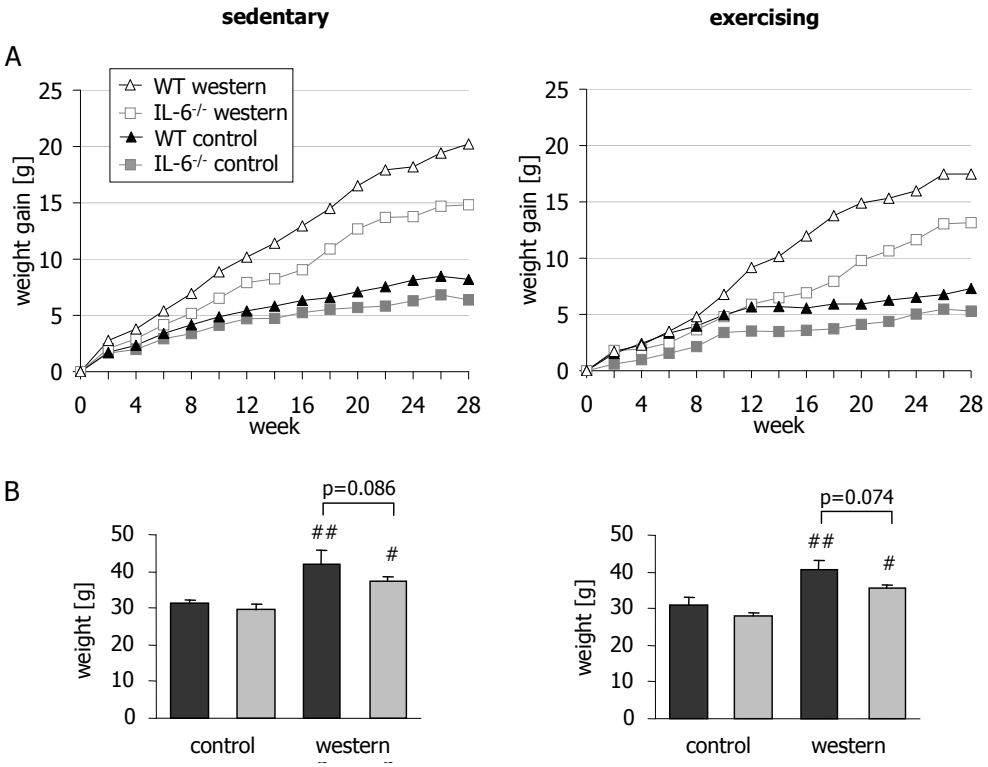

Fig. 29. Weight gain and final body weight of sedentary and exercising WT and IL-6$^{-/-}$ mice. WT (black bars) and IL-6$^{-/-}$ (grey bars) mice were fed with control or energy-enriched (western) diet and provided with running wheels for voluntary exercise or remained sedentary. The body weight was recorded every second week and **A** the time course of weight gain and **B** the body weight at the end of the experiment are shown (n=4, mean ± SEM; #p<0.05, ##p<0.005 vs. the corresponding group fed control diet).

The differences in body weight were also reflected by several parameters of metabolism in the plasma of fasted mice after 28 weeks of voluntary exercise and dietary intervention. Most prominently, circulating levels of leptin were increased in all mice fed the western diet (Fig. 30A). Similar to leptin, insulin levels clearly reflected the differences in body weight and showed at least a trend towards being elevated in all groups fed western diet, compared to the respective group fed control diet (Fig. 30B). The plasma glucose concentrations were also higher with the western diet and this effect was significant for sedentary WT and for exercising IL-6$^{-/-}$ and WT mice (Fig. 30C). The glucose levels were not reduced in IL-6$^{-/-}$ mice despite their lower body weight, which could be explained by the slightly lower insulin levels in these animals. The elevation of insulin by the western diet was less prominent in IL-6$^{-/-}$ than in the WT mice. In line with the trend towards a reduced body weight of western diet-fed IL-6$^{-/-}$ mice, the leptin concentrations also tended to be lower than in the corresponding WT mice, and this was true both for sedentary and exercising animals.

The long-term intervention was successful in causing pronounced effects of energy consumption but not of voluntary exercise. Only the glucose levels in the control-fed WT mice were significantly lowered by wheel-running, from 103 ± 5 mg/dl in the seden-

tary to 89 ± 2 mg/dl in the exercising control-fed mice. No other significant improvements could be observed as a consequence of voluntary running. It is likely that regular forced exercise training is needed as a stronger stimulus to bring about the expected beneficial metabolic effects like lower body weight and plasma glucose and insulin levels.

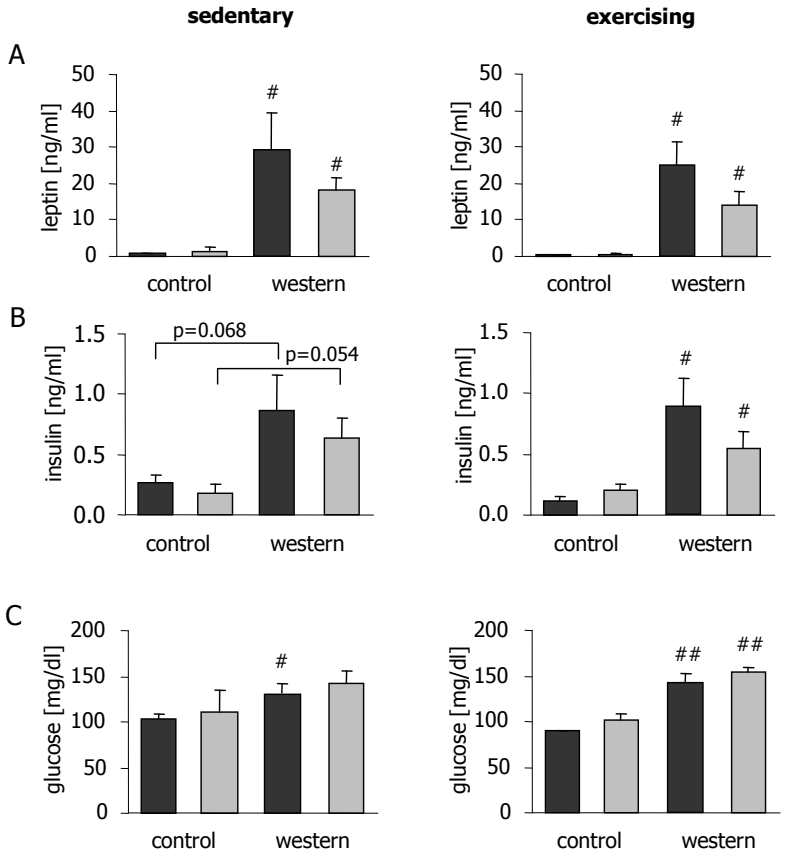

Fig. 30. Plasma metabolites and hormones in 9 month-old WT and IL-6$^{-/-}$ mice after 7 months of voluntary exercise and dietary intervention. WT (black bars) and IL-6$^{-/-}$ (grey bars) mice were fed with control or energy-enriched (western) diet and provided with running wheels for voluntary exercise or remained sedentary. Plasma from 16 h-fasted animals was analyzed for **A** leptin, **B** insulin and **C** glucose (n=3-4, mean ± SEM; #p<0.05, ##p<0.005 vs. the corresponding group fed control diet).

4.2.3 Does IL-6 deficiency affect the acute metabolic and transcriptional response to a single bout of exercise?

4.2.3.1 Plasma metabolites and tissue glycogen content

The long-term study revealed that the main chronic effect of IL-6 deficiency is an attenuation of weight gain. Because this effect, which was stronger than the effect of exercise, might have lead to all metabolic alterations of the IL-6$^{-/-}$ mice observed after the training interventions, long-term experiments might not be a feasible approach to study

the consequences of IL-6 deficiency. Moreover, it appears that acute rather than chronic consequences of the lack of IL-6 production during physical exercise account for the reduced running capacity of IL-6$^{-/-}$ mice since training enhanced glucose disposal and running endurance independent of IL-6 availability. Such acute alterations could be impairments in fuel (i.e., glucose and FFA) mobilization from liver and adipose tissue or in fuel availability and oxidation in the muscle.

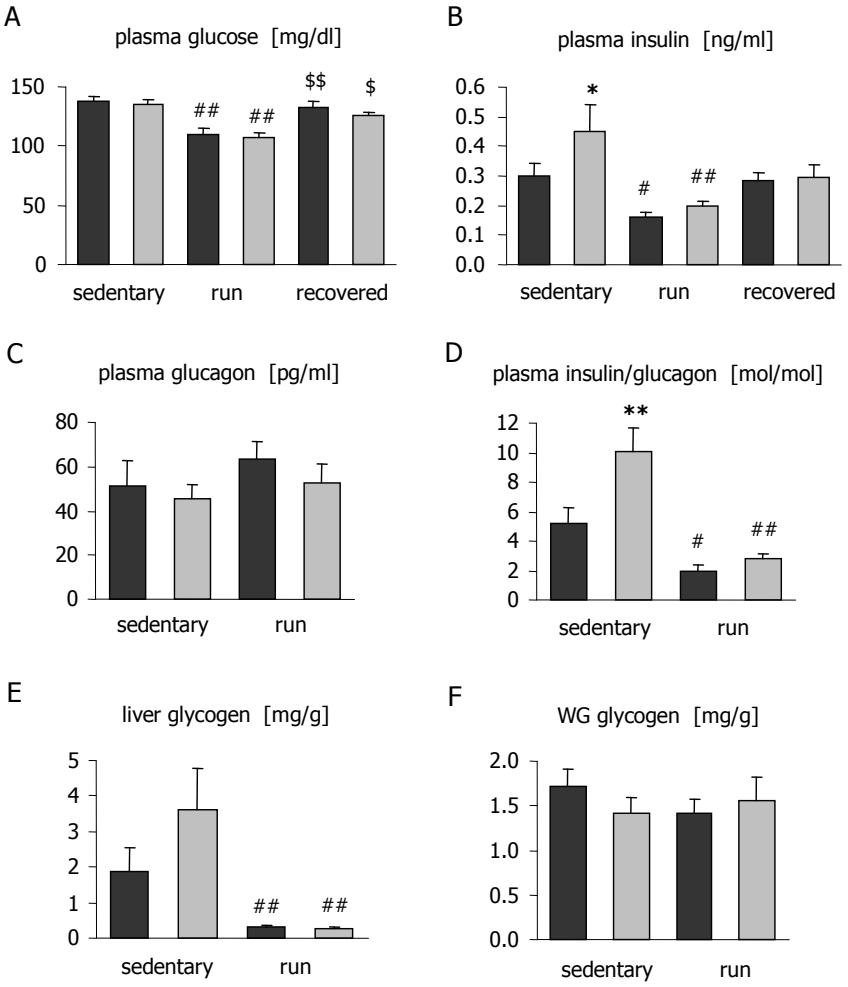

Fig. 31. Effect of a single bout of exercise on glucose metabolism of IL-6$^{-/-}$ and WT mice. Plasma levels of **A** glucose and **B** insulin were quantified in WT (black bars) and IL-6$^{-/-}$ mice (grey bars) that either rested in their cages (sedentary) or were studied immediately after a single bout of treadmill exercise (run) or 3 h later (recovered). **C** Glucagon was measured and **D** the molar ratio of insulin to glucagon was calculated for sedentary mice and mice directly after running and the amount of glycogen was determined in **E** the liver and **F** the WG muscle (n=12 (recovered) and n=20 (sedentary, run) for glucose, insulin and liver glycogen; n=8 for glucagon and insulin; n=12 for WG glycogen; mean ± SEM; *p<0.05, **p<0.005 vs. WT; #p<0.05, ##p<0.005 vs. sedentary and $p<0.05, $$p<0.005 vs. run WT or IL-6$^{-/-}$ mice, respectively).

To detect differences which could explain the reduced endurance capacity, we studied the IL-6$^{-/-}$ mice after an acute exercise challenge. Previously untrained, young IL-6$^{-/-}$ and WT mice were subjected to a single bout of treadmill running for 1 h at 14 m/min

and 14° uphill slope. Immediately after the cessation of running or following 3 h of recovery, the previously exercised mice were sacrificed. Mice resting in their cages that were fasted for 1 h served as sedentary controls. The treadmill protocol was less demanding than the one used to exercise mice till exhaustion and could be completed by both WT and IL-6$^{-/-}$ mice without obvious difficulty (details are given in the method sections, 2.5.2 and 2.5.5).

All parameters of glucose and fat metabolism measured after running where similar in IL-6$^{-/-}$ and WT mice. The treadmill run acutely led to a significant reduction of blood glucose (Fig. 31A) and insulin levels (Fig. 31B). After 3 h of recovery, during which the animals had free access to food for the first 2 h, glucose levels were significantly elevated above the levels immediately after running and similar to values in sedentary mice. Glucagon levels were not significantly elevated by this exercise protocol (Fig. 31C), but there was a strong decrease in the ratio of insulin to glucagon after the exercise bout (Fig. 31D). In addition, there was a prominent and significant reduction of the glycogen stores, almost to depletion, in the liver (Fig. 31E). The glycogen levels of the WG muscle, in contrast, were not reduced by this exercise protocol (Fig. 31F).

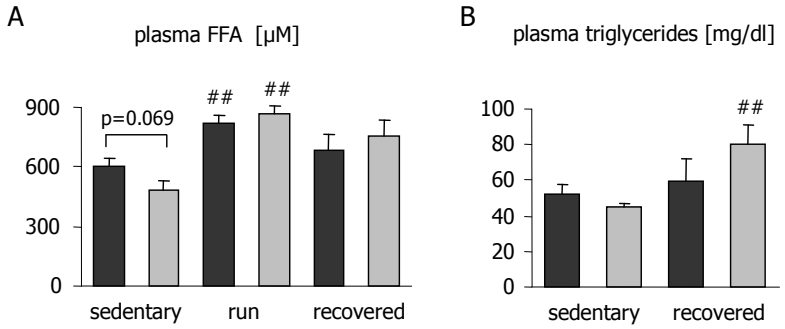

Fig. 32. Effect of a single bout of exercise on the lipid metabolism of IL-6$^{-/-}$ and WT mice. WT (black bars) and IL-6$^{-/-}$ mice (grey bars) either rested in their cages (sedentary) or were studied immediately after a single bout of treadmill exercise (run) or 3 h later (recovered). Plasma levels are shown for **A** FFA and **B** triglycerides which were only quantified in sedentary and recovered mice (n=12 (recovery) or n=20 (sedentary, run), mean ± SEM; ##p<0.005 vs. sedentary WT or IL-6$^{-/-}$ mice, respectively).

As discussed in the first part of this work, the 1 h treadmill exercise was an intense but not exhaustive metabolic stimulus and led to a moderate, significant increase in circulating lactate from 2.37 ± 0.23 to 3.34 ± 0.34 mM in the WT mice. The increase in lactate levels was similar in IL-6$^{-/-}$ mice, from 2.05 ± 0.17 before to 2.72 ± 0.17 mM after running. This suggests that IL-6$^{-/-}$ and WT mice relied on glucose as fuel for the muscles to a similar extent and fits well to the concentration of circulating glucose (Fig. 31A) which was reduced similarly in mice with and without IL-6 by the bout of exercise.

Differences between IL-6$^{-/-}$ and WT mice were only apparent in the sedentary state. Insulin levels were significantly higher in sedentary IL-6$^{-/-}$ mice and fell to the same level as in WT mice after running and recovery (Fig. 31B). Accordingly, the ratio of in-

sulin to glucagon was also significantly higher (Fig. 31D), amplified by slightly lower glucagon levels in the sedentary IL-6$^{-/-}$ mice (Fig. 31C). The amount of hepatic glycogen in the sedentary state tended to be higher in mice lacking IL-6, although this difference did not reach significance (Fig. 31E). The amount of glycogen in the liver after running was not different in IL-6$^{-/-}$ mice, and neither were glycogen levels in the WG muscle before and after running (Fig. 31F).

The bout of exercise also led to an acute elevation of FFA levels in the plasma (Fig. 32A) and this increase was as prominent in the mice deficient for IL-6. In the sedentary state, however, there was a trend towards lower concentrations of FFA in the knockout mice. Triglycerides were significantly elevated in the recovery phase compared to the sedentary state only in IL-6$^{-/-}$ mice (Fig. 32B). Because the standard method used for triglyceride quantification in our laboratory cannot discriminate between triglycerides and free glycerol and the latter can be elevated immediately after physical exercise, we did not include this time point in the analysis.

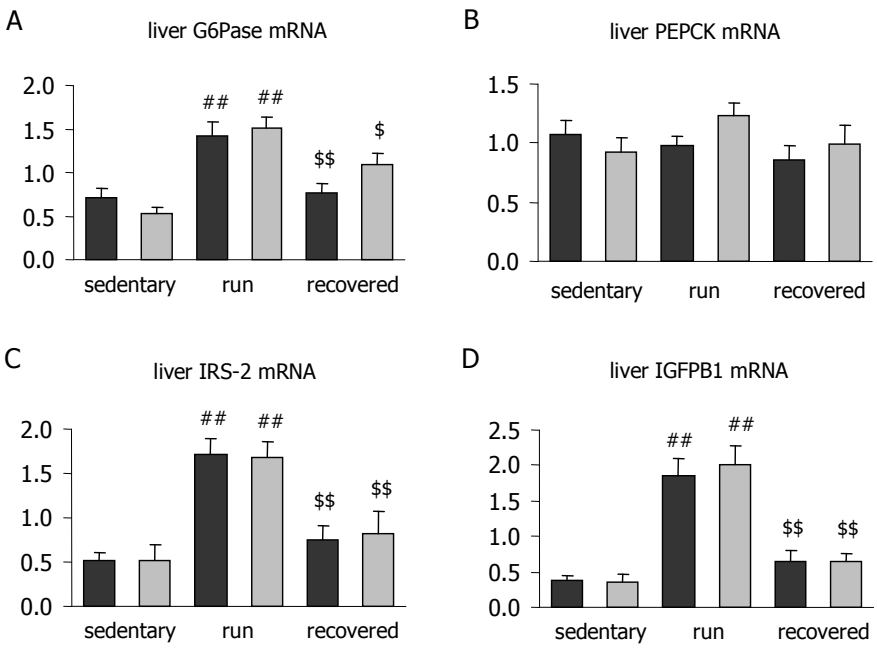

Fig. 33. Impact of a single bout of exercise on the hepatic transcription of genes related to glucose metabolism in IL-6$^{-/-}$ and WT mice. WT (black bars) and IL-6$^{-/-}$ mice (grey bars) either rested in their cages (sedentary) or were studied immediately after a single bout of treadmill exercise (run) or 3 h later (recovered). Shown are the hepatic mRNA levels of **A** G6Pase, **B** PEPCK, **C** IRS-2, and **D** IGFBP1 in arbitrary units (n=12 (recovery) or n=20 (sedentary, run), mean ± SEM; ##p<0.005 vs. sedentary and $p<0.05, $$p<0.005 vs. run WT or IL-6$^{-/-}$ mice, respectively).

4.2.3.2 Expression of key regulators of glucose and fat metabolism

The similar levels of plasma glucose after the bout of exercise pointed to a similar liberation of glucose from the livers of WT and IL-6$^{-/-}$ mice. Real-time PCR was employed next to assess whether this was also reflected by a similar induction of regulators of

glucose metabolism and homeostasis in the liver. Exercise caused a significant and transient induction of the gluconeogenic gene G6Pase, and this occurred to the same extent in IL-6$^{-/-}$ as in WT mice (Fig. 33A). Similarly, IRS-2 (Fig. 33C) and IGFBP1 (Fig. 33D) were upregulated in livers of mice with and without IL-6 after running and had almost returned to baseline values after 3 h of recovery. The levels of PEPCK mRNA were not elevated by our exercise protocol (Fig. 33B).

The impaired endurance capacity of the IL-6$^{-/-}$ mice could also be caused by a diminished oxidation of FFA. The transcriptional co-activator PGC-1α plays an important role in mitochondrial biogenesis. Levels of PGC-1α mRNA were elevated in the liver immediately after running, but there was no difference between WT and IL-6$^{-/-}$ mice (Fig. 34A). In the tibialis and in the soleus muscle, PGC-1α levels were prominently elevated in the recovery phase and this response was also similar in mice lacking IL-6 (Fig. 34B and C).

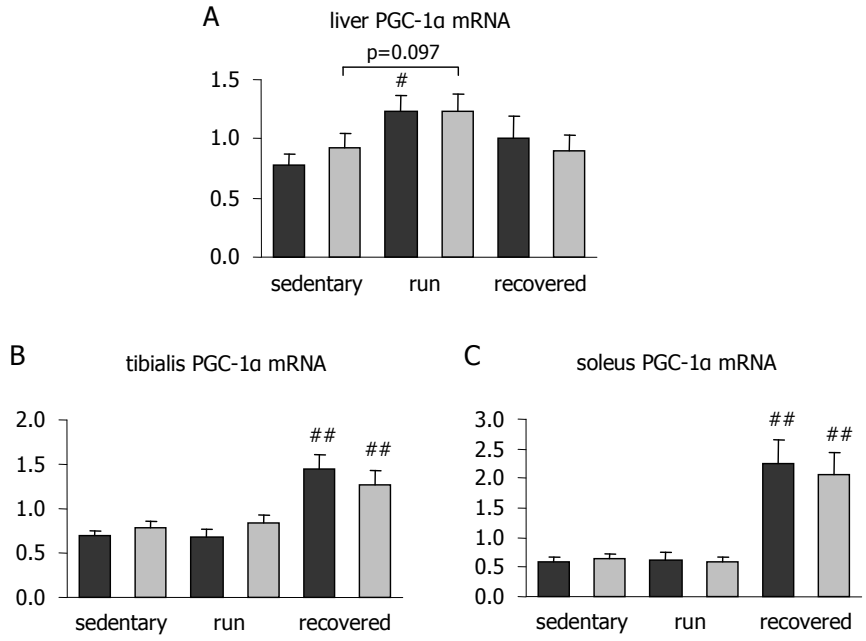

Fig. 34. Regulation of PGC-1α expression by acute exercise in liver and muscles of IL-6$^{-/-}$ and WT mice. WT (black bars) and IL-6$^{-/-}$ mice (grey bars) either rested in their cages (sedentary) or were studied immediately after a single bout of treadmill exercise (run) or 3 h later (recovered). The mRNA levels of PGC-1α were quantified in **A** liver, **B** tibialis muscle and **C** soleus muscle. Values are shown as arbitrary units (n=12, mean ± SEM; #p<0.05, ##p<0.005 vs. sedentary WT or IL-6$^{-/-}$ mice, respectively).

4.2.3.3 Exercise-induced STAT-3 signalling in liver and muscles of IL-6$^{-/-}$ and WT mice

We also verified if the applied exercise protocol was indeed capable to induce IL-6-dependent cytokine signalling. Plasma levels of IL-6 were elevated in the WT mice after running, from 7.3 ± 0.6 to 11.4 ± 1.4 pg/ml, and decreased to 8.8 ± 1.4 pg/ml after recovery (Fig. 35A). Of note, the concentrations measured in sedentary mice were

close to the non-specific background determined by measuring plasma of IL-6$^{-/-}$ mice. Thus, the true magnitude of the running-induced increase in IL-6 levels might still be higher. SOCS3 mRNA was transiently upregulated in the liver after running (Fig. 35B). However, SOCS3 induction also occurred in the livers of IL-6$^{-/-}$ mice, arguing against an IL-6-specific effect (Fig. 35B). We detected no alteration of SOCS3 mRNA levels in the two muscles studied, neither in the glycolytic tibialis (Fig. 35C) nor in the oxidative soleus (Fig. 35D).

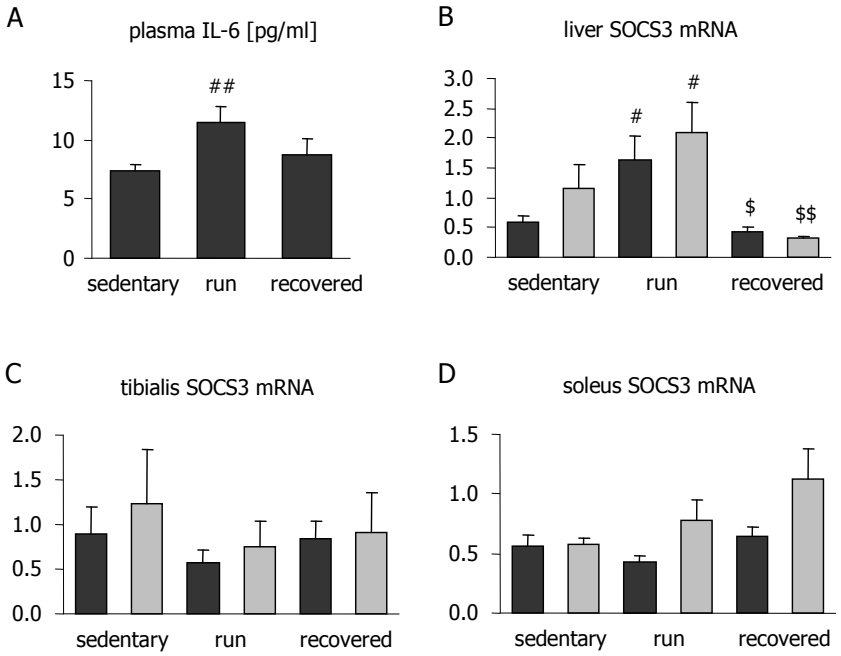

Fig. 35. Exercise-induced IL-6 production and SOCS3 expression in liver and muscles. WT (black bars) and IL-6$^{-/-}$ mice (grey bars) either rested in their cages (sedentary) or were studied immediately after a single bout of treadmill exercise (run) or 3 h later (recovered). **A** Plasma levels of IL-6 were quantified in WT mice only. The mRNA levels of SOCS3 in **B** liver, **C** tibialis muscle and **D** soleus muscle are shown as arbitrary units. (n=12 (liver, tibialis) or n=7-8 (soleus), mean ± SEM; #p<0.05, ##p<0.005 vs. sedentary, $p<0.05, $$p<0.005 vs. run WT or IL-6$^{-/-}$ mice, respectively).

Activation of STAT-3, as evidenced by phosphorylation of tyrosine-705, was increased in the liver and in three different muscle (soleus, EDL and WG) of WT mice immediately after the bout of exercise (Fig. 36A). To determine whether this effect depended on IL-6, we compared WG muscles from WT and IL-6$^{-/-}$ mice. STAT-3 phosphorylation was increased in muscles of exercised WT mice, where it could be detected immediately after exercise as well as recovery, but not in muscles of IL-6$^{-/-}$ mice (Fig. 36B). In the liver of WT mice, STAT-3 was also activated. However, when comparing the phosphorylation of STAT-3 in livers of exercised IL-6$^{-/-}$ mice (not shown), we could not determine if it was different from the WT, due to a high inter-individual variability. Taken together, exercise led to STAT-3 activation in skeletal muscles of WT mice only, but SOCS3 was not induced. In the liver, SOCS3 was induced independent of IL-6.

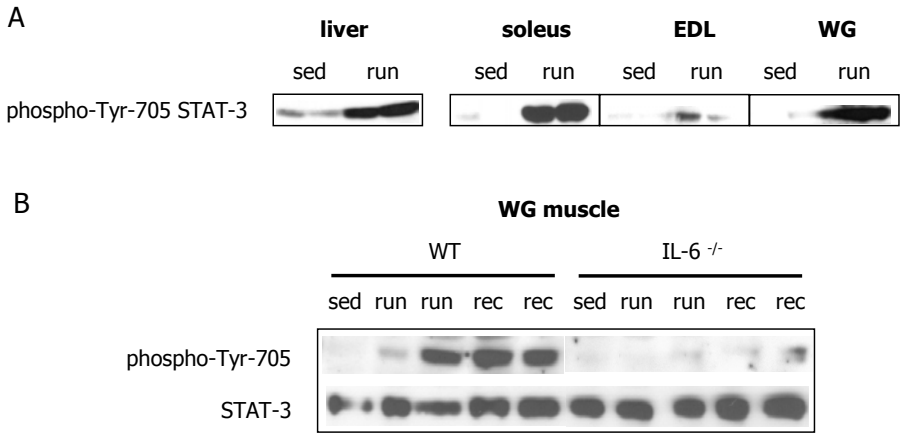

Fig. 36. Exercise-induced STAT-3 phosphorylation in liver and muscles from IL-6$^{-/-}$ and WT mice. A Tissue lysates from liver and soleus, EDL, and WG muscles of sedentary (sed) WT mice or immediately after running (run) were immunoblotted for phosphorylated tyrosine-705 of STAT-3. Shown are two representative samples per group. **B** Detection of phosphorylated tyrosine-705 and of STAT-3 protein in WG muscles of WT and IL-6$^{-/-}$ mice in the sedentary state (sed), immediately after a single bout of treadmill exercise (run) or after 3 h of recovery (rec). Shown is one sample from sedentary and two each from run and recovered mice.

4.2.4 Induction of gluconeogenesis by fasting in the livers of IL-6$^{-/-}$ and WT mice

Because the acute gluconeogenic response of the liver to physical exercise appeared not to be affected by IL-6 deficiency, we tested whether this is also the case for fasting, another physiologic situation that requires hepatic glucose production. After 16 h without food, plasma glucose (Fig. 37A), insulin (Fig. 37B) and triglycerides (Fig. 37D) were equally reduced in mice of both genotypes. Fasting significantly increased FFA levels in both WT and IL-6$^{-/-}$ mice (Fig. 37C). Slight metabolic alterations were only visible in the fed state, when the circulating glucose levels were higher in IL-6$^{-/-}$ mice, and this was paralleled by a tendency towards higher insulin levels.

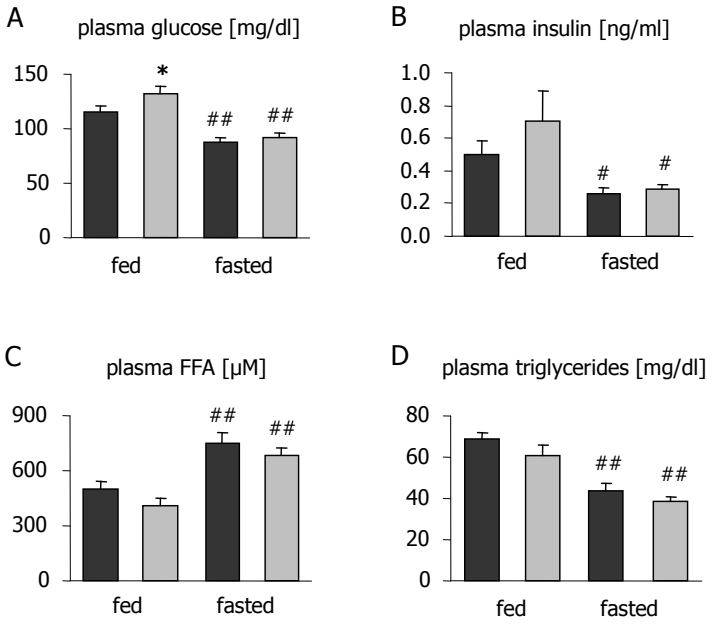

Fig. 37. Effects of fasting on glucose and lipid metabolism of IL-6$^{-/-}$ and WT mice. Plasma levels of **A** glucose, **B** insulin, **C** FFA and **D** triglycerides of fed or 16 h fasted WT (black bars) and IL-6$^{-/-}$ (grey bars) mice (n=12 (glucose, insulin, triglycerides) or n=6 (FFA), mean ± SEM; *p<0.05 vs. WT; #p<0.05, ##p<0.005 vs. fed mice of the same genotype).

Fasting also caused an increase in the amount of PEPCK (Fig. 38A) and IRS-2 (Fig. 38B) protein in the liver of WT mice. This increase occurred, similarly, in the livers of IL-6$^{-/-}$ mice. However, the amount of PEPCK was higher in the IL-6$^{-/-}$ mice in the fed state. The mRNA of G6Pase and PEPCK was not differentially expressed after 16 h of fasting (not shown) and the western blots for G6Pase protein (not shown) were inconclusive due to the detection of several bands by the antibody. The phosphorylation of STAT-3 was not increased in the liver by fasting (Fig. 38C) and the amount of SOCS3 mRNA was not elevated above the fed state (Fig. 38D), neither in WT nor in IL-6-deficient mice. A trend towards lower levels of STAT-3 phosphorylation was visible in mice lacking IL-6, both in the fasted and in the fed control group. Fasting for 16 h also induced PGC-1α expression in the liver, and this occurred not only in the WT, but also in the IL-6$^{-/-}$ mice (Fig. 38E). In the fed control group, but not after fasting, a slight difference was observable for the IL-6$^{-/-}$ mice which had higher PEPCK protein levels. Taken together, no activation of IL-6-dependent signalling could be seen after 16 h of fasting, and no differences in the response of hepatic regulators of glucose homeostasis were detected between IL-6$^{-/-}$ and WT mice.

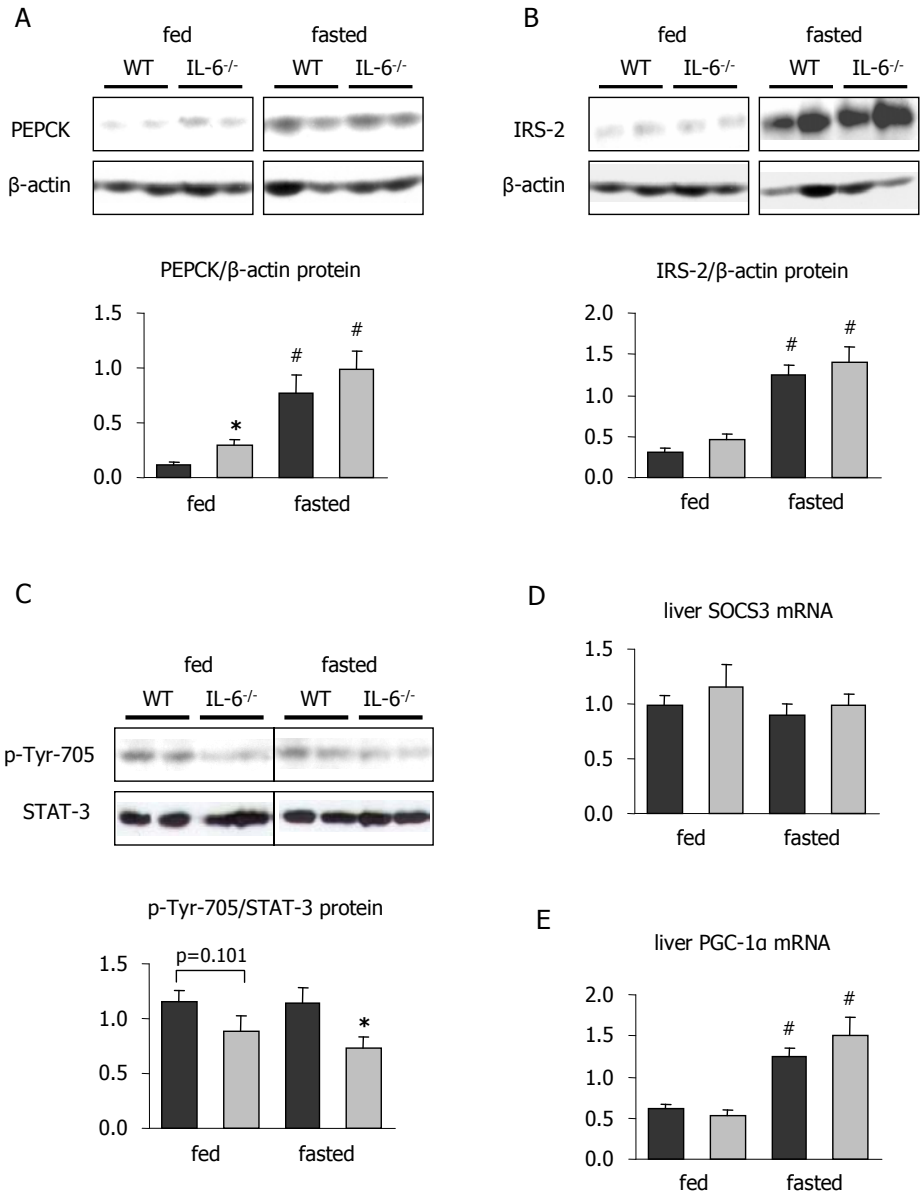

Fig. 38. Molecular response of the liver of IL-6$^{-/-}$ and WT mice to fasting. Levels of **A** PEPCK protein, **B** IRS-2 protein and **C** tyrosine-705 phosphorylation and protein of STAT-3 in livers of fed or 16 h fasted WT (black bars) and IL-6$^{-/-}$ (grey bars) mice. Shown are western blots from two representative samples and histograms with the densitometric quantification. The mRNA content of **D** SOCS3 and **E** PGC-1α in the liver is shown in arbitrary units (n=12, mean ± SEM; *p<0.05 vs. WT, #p<0.05 vs. fed mice of the same genotype).

4.3 Discussion

The results from this thesis provide evidence that although IL-6 deficiency in mice results in reduced running endurance, IL-6 it is not a key mediator of the acute metabolic response to physical exercise or of the long-term effects of exercise training on endurance performance and glucose metabolism. In the following, 1. the attenuated weight gain of the IL-6$^{-/-}$ mice in these study, 2. the impaired exercise capacity and 3. the mild metabolic phenotype of these mice in the sedentary, fed state will be discussed.

4.3.1 Attenuated weight gain in the IL-6-deficient mice

One important aim of this study was to clarify whether or not IL-6 deficiency alters body weight gain in interaction with dietary energy content and an exercise protocol. As indicated by the first experiment, 4 weeks of training, and confirmed in a second one, a "lifestyle intervention" for 7 months, IL-6$^{-/-}$ mice constantly gained less weight. This observation is in line with two earlier studies that reported a lower body weight in young and old mice lacking IL-6 (Di Gregorio et al., 2004; Ellingsgaard et al., 2008) and in contrast to another that observed increasing obesity in aged IL-6$^{-/-}$ mice (Wallenius et al., 2002b). The latter study also reported elevated leptin levels and impaired glucose tolerance in the aged, obese IL-6$^{-/-}$ mice. In our hands, the increase in plasma leptin and insulin levels by feeding a western-style diet was less pronounced, and glucose tolerance was improved in the mice lacking IL-6. This suggests that firstly, IL-6 deficiency per se does not cause impaired glucose tolerance and secondly, the improved glucose metabolism of the IL-6$^{-/-}$ mice in our study was most likely a consequence of the reduced weight gain.

To determine the origin of the differences in body weight regulation of IL-6$^{-/-}$ mice studied in different laboratories would require a direct comparison of the different strains and, if the differences persist, a concise genetic (McPherson, 2007) and possibly, also microbiotic (Musso et al., 2010) analysis. While performing such an elaborate approach does not appear to be reasonable, hints on the mechanisms causing the higher or lower body weight might already be detectable on the level of phenotype. For instance, the lower body weight of the IL-6$^{-/-}$ mice in our study could be related to a reduced food intake or to increased locomotor activity. Although these parameters were not assessed, the enhanced voluntary wheel-running activity of the IL-6$^{-/-}$ mice could indicate that these mice were generally more physically active, possibly also in the absence of running wheels, and this could explain the decreased weight gain. Of note, the sedentary mice had been provided with plastic houses for environmental enrichment, and this could have fostered locomotor activity. A high degree of physical activity in the sedentary groups of mice would also explain why we only observed a small effect of exercise

training on glucose metabolism and weight gain in the 4-week intervention and no effect in the 7-month intervention.

Differences in animal care, for instance, environmental enrichment or the number of mice housed per cage, differences in the gut microbiome (Musso et al., 2010) or genetic drift in the respective C57BL/6 background strain could interact with IL-6 deficiency to modulate locomotor activity. Of note, a previous report of unaltered locomotor activity in IL-6$^{-/-}$ mice (Wernstedt et al., 2006) stems from the same laboratory that observed maturity-onset obesity in these mice. The animals used in our experiments and in the previous study in which IL-6$^{-/-}$ mice tended to have a lower body weight (Di Gregorio et al., 2004) were purchased from Jackson laboratories while the group that detected obesity obtained WT mice from a different breeder (Wallenius et al., 2002b). To conclude, IL-6 deficiency by itself does not lead to obesity. It appears that additional endogenous or exogenous factors influence the body weight of IL-6$^{-/-}$ mice.

4.3.2 How could the impaired running endurance of IL-6$^{-/-}$ mice be explained?

Looking at IL-6 as an “exercise factor”, the most striking consequence of IL-6 deficiency that has previously been described (Faldt et al., 2004) and could be confirmed in this study was a greatly diminished endurance capacity. When forced to run continuously on a treadmill, IL-6$^{-/-}$ mice were only half as enduring as WT animals. We attempted to identify metabolic and molecular mechanisms that could provide an explanation for this impairment. Based on what is known on the metabolic effects of IL-6 and assuming that it acts as an exercise factor that increases energy supply, it could be expected that IL-6$^{-/-}$ mice have lower plasma levels of FFA or glucose, that muscle glycogen stores are more quickly depleted or that the mobilization of glucose from the liver is impaired. However, IL-6 deficiency had no effect on the changes in plasma metabolites caused by a single bout of exercise in young, previously untrained mice.

We first focussed on glucose metabolism. The fall in circulating glucose and the rise in lactate were not more pronounced in IL-6$^{-/-}$ mice, and animals of neither genotype relied on muscle glycogen as a source of glucose, while hepatic glycogen stores were equally depleted in both groups. Thus, the metabolite concentrations provided no hint that IL-6$^{-/-}$ mice relied more on glucose than on FFA as a fuel source or that the glucose supply was impaired. Accordingly, there was no difference in the upregulation of the gluconeogenic enzyme G6Pase and of PGC1-α, an upstream regulator of gluconeogenesis (Yoon et al., 2001a) in the liver. Two other genes that are typically induced by energetic stress in the liver, IRS-2 and IGFBP1, were also induced by exercise independent of IL-6. Similarly, IL-6 deficiency had no effect on glucose levels and on the induction of gluconeogenesis after a 16 h fast, a metabolic situation comparable to endurance exercise that also caused upregulation of IRS-2 and PGC-1α in WT and IL-6$^{-/-}$ mice alike.

We confirmed that the applied exercise protocol was sufficient to cause IL-6 release from skeletal muscle, as indicated by an increase of circulating IL-6 and of STAT-3 phosphorylation in muscles of WT, but not of IL-6$^{-/-}$ mice. For the liver, we could not determine whether exercise activated STAT-3 only in WT or also in IL-6$^{-/-}$ mice. However, exercise clearly increased SOCS3 mRNA in the liver independently of endogenous IL-6, indicating the involvement of additional regulators such as growth hormone (Davey et al., 1999) or other cytokines. It is not clear why SOCS3 was elevated in the liver, but not in the muscle. This could be due to different sensitivities of the SOCS3 response in liver and muscle (Weigert et al., 2006) or to different kinetics. The upregulation of SOCS3 generally occurs at an early timepoint and could already have returned to baseline in the muscles after 1 h of exercise.

If IL-6 is not essential for hepatic glucose production during moderately intense endurance exercise or fasting, it is still unclear why IL-6$^{-/-}$ mice have an impaired running endurance. The finding that IL-6 is not necessary for the induction of gluconeogenesis during moderate endurance exercise does not preclude a role for IL-6 during more strenuous challenges. In a study in rats, treadmill running till physical exhaustion lead to a pronounced increase in STAT-3 phosphorylation and G6Pase, PEPCK and PGC-1α expression in the liver. This is an overall stronger effect than in our exercised mice, where PEPCK was not, and PGC-1α mRNA was only slightly increased. Plasma levels of IL-6 rose from 20 to above 120 pg/ml in these rats, and it is likely that the exercise till exhaustion caused tissue damage and inflammatory processes that contributed to this increase. We did not attempt to measure IL-6 levels after exhaustion and they were not reported by a previous study on running endurance in mice (Faldt et al., 2004). Moreover, comparing absolute IL-6 levels between different studies is difficult. It appears that our levels of 7.3 ± 0.6 pg/ml in sedentary mice are rather low, since others reported 23.7 ± 7.6 pg/ml (Di Gregorio et al., 2004) or even 57.2 ± 15 pg/ml (Wallenius et al., 2002b) in young, sedentary C57BL/6 mice. However, the latter measurements were performed with ELISA assays while we and another group (Ellingsgaard et al., 2008) detected much lower levels of IL-6 using a bead-based system. Reports on IL-6 levels in exercising mice are generally rare, probably due to the insufficient sensitivity of commercially available ELISA kits. Another factor could complicate the comparison of studies from different laboratories: Exercise-induced IL-6 release in humans can be lowered by vitamin C and E administration (Fischer et al., 2004a) and the content of these vitamins varies substantially between different commercial rodent diets.

IL-6 could also be required as a regulator of fat metabolism during physical exercise. Firstly, the mitochondrial oxidation of fatty acids could be reduced in the IL-6$^{-/-}$ mice. If the animals are forced to perform the same amount of work as WT animals, this would have to be compensated by a higher utilisation of glucose. However, we did not see the expected effects, depletion of muscle glycogen or increased lactate accumulation. The hepatic glycogen stores, which appeared to be slightly higher in the sedentary IL-6$^{-/-}$ mice and dropped to the same levels as in the WT after the bout of exercise, are the only faint evidence for a possibly higher utilization of glucose instead of fat. Monitoring

the RER of the exercising mice might help to answer this question, although this approach has been taken in a previous study in IL-6$^{-/-}$ mice without producing definitive results (Faldt et al., 2004). The finding of a similar elevation of FFA levels by the single bout of exercise in IL-6$^{-/-}$ and WT mice suggests that IL-6 is not an important regulator of lipolysis during moderately intense endurance exercise. The two main triggers of adipose tissue lipolysis during physical exercise are the rise in catecholamine and the fall in insulin concentrations, while other hormones and cytokines have been shown to play only a minor role (Horowitz and Klein, 2000). Insulin levels clearly dropped and although we did not attempt to measure them, catecholamine levels were certainly elevated, not only by exercise itself (Christensen and Galbo, 1983), but also by the stress that was imposed on the animals by forcing them to run on the treadmill. The action of these strong regulators of lipolysis could have concealed more subtle effects of IL-6 deficiency.

It is also conceivable that chronic rather than acute consequences of the lack of IL-6 contribute to the impaired endurance capacity. The production and release of IL-6 from the contracting muscle could be a signal that is required to induce adaptation processes in the muscle itself and in organs like the liver, and this training response might be impaired in the IL-6$^{-/-}$ mice. However, we could obtain no evidence that IL-6 was the mediator of two important adaptive responses to physical exercise, improvement of physical performance and glucose metabolism. Mice lacking IL-6 always had a lower running endurance but the relative increase in running capacity by 4 weeks of exercise training was similar to WT mice. Moreover, glucose disappearance in a glucose tolerance test was slightly improved by exercise, independent of IL-6. At the molecular level, structural and functional adaptations in the muscle are initiated by delayed transcriptional responses, an important player being PGC-1α (3.1.1), and the increase in PGC-1α mRNA in the recovery phase was also not impaired by IL-6 deficiency.

It is likely that other than metabolic effects underlie the reduced running endurance of IL-6$^{-/-}$ mice. Impaired heart function could be such a factor, since IL-6 has been shown to induce cellular hypertrophy in cardiomyocytes (Szabo-Fresnais et al., 2010). However, heart-to-body weight ratio and training-induced cardiac hypertrophy are normal in IL-6$^{-/-}$ mice (Kaminski et al., 2007). Central administration of IL-6 to rats activates the hypothalamic-pituitary-adrenal axis (Lenczowski et al., 1999) and although basal and stress-induced corticosterone levels are not significantly different in IL-6$^{-/-}$ mice (Wallenius et al., 2002b), stimulatory effects of IL-6 on sympathetic nerve activity could play a role during highly intensive physical exercise. Interestingly, the hypertensive response to acute stress has been found to be attenuated in IL-6$^{-/-}$ mice and it appears that this effect is independent of hormonal changes (Lee et al., 2004).

Taken together, the reduced exercise capacity of IL-6$^{-/-}$ mice is not reflected by defects in the metabolic response to non-exhaustive exercise or in the adaptation to exercise training. Effects of IL-6 on glucose and fat metabolism may become apparent at more strenuous exercise intensities or when the action of other regulators of glucose and fat

metabolism is less dominant. Further studies may also reveal actions of IL-6 that are not related to metabolism and could explain the reduced endurance capacity of the IL-6$^{-/-}$ mice.

4.3.3 Mild metabolic phenotype of IL-6$^{-/-}$ mice in the fed state

While there were no obvious differences in the metabolic response of the IL-6$^{-/-}$ mice to physical exercise or fasting, young, sedentary IL-6$^{-/-}$ mice showed slight metabolic alterations in the fed state. They tended to have lower plasma FFA concentrations and higher levels of plasma glucose, plasma insulin and hepatic glycogen. This effect was not always apparent and most obvious in the fasting experiment, possibly because the animals in this experiment experienced the lowest level of stress, which led to generally lower glucose levels when compared to other experiments. Moreover, it cannot be expected to be observable in older animals, when the body weight and as a consequence, the insulin levels of IL-6$^{-/-}$ mice are lower than in the WT mice.

It could be assumed that the lower FFA levels in the fed IL-6$^{-/-}$ mice reflect reduced IL-6 mediated lipolysis in the sedentary state. However, a more likely explanation is that the elevated insulin levels in these mice led to reduced lipolysis and enhanced FFA clearance. The higher insulin concentrations could also cause the increased amount of glycogen in the livers of the fed IL-6$^{-/-}$ mice. A possible explanation for higher insulin levels in fed IL-6$^{-/-}$ mice was provided by a recent finding: Insulin can act on the hypothalamus to activate IL-6 expression in non-parenchymal intrahepatic cells, which leads via STAT-3 to the suppression of gluconeogenic enzymes (Inoue et al., 2006). This negative regulatory mechanism of gluconeogenesis is lacking in IL-6$^{-/-}$ mice, as indicated by lower STAT-3 phosphorylation and higher amount of PEPCK protein in the liver in the fed state (Inoue et al., 2006). This could lead to higher levels of glucose and in turn, of insulin.

Physical exercise is known to lower postprandial lipaemia (Petridou et al., 2004) and it is therefore interesting to note that the 3 h of recovery after the bout of exercise, which included 2 h of refeeding, increased plasma triglyceride levels more strongly in the IL-6$^{-/-}$ mice. Fatty meals increase circulating IL-6 levels and, similar to exercise, secretion by the skeletal muscle (Corpeleijn et al., 2005). This suggests that IL-6 might also be a regulator of postprandial metabolism, a fascinating aspect that has received little attention so far.

4.3.4 Conclusions and outlook

Taken together, IL-6 deficiency due to whole-body knockout caused a mild metabolic phenotype but no obvious metabolic defects in response to a bout of treadmill running that could explain the reduced endurance capacity of these mice. Since the analyses

were performed only after moderately intense exercise, we cannot exclude that IL-6 could play a role as a regulator of glucose or fat metabolism at more exhaustive conditions. However, increasing the work load is unlikely to be a feasible experimental approach. A standardized running protocol of higher intensity or longer duration might not be completed by all mice and could increase the inter-individual variability. Exercising the mice till exhaustion might also lead to wrong conclusions, especially if non-metabolic actions of IL-6 affect running endurance. Moreover, it could lead to tissue damage and inflammation, and IL-6 deficiency is known to affect the response to hepatic injury (Cressman et al., 1996).

It is still unclear what mechanism underlies the impaired running endurance of the IL-6$^{-/-}$ mice and whether its origin is peripheral, central, or both. It is important to keep in mind that IL-6$^{-/-}$ mice are not a defined model of acute, exercise-specific muscular IL-6 deficiency. Adaptation to a chronic lack of IL-6, the overlay of chronic and acute effects of IL-6 deficiency and the simultaneous involvement of several tissues and the central nervous system could all play a role. Thus, the most promising approach might be to isolate the central and peripheral as well as the acute and chronic effects of IL-6, for instance, by intracerebroventricular administration of IL-6-neutralising antibodies prior to a bout of exercise. Even if IL-6 is not essential for the metabolic response to moderately intense exercise, it is still important to understand its function as an exercise factor because it could be an important link between the working muscle and the immune system (Petersen and Pedersen, 2005) and the induction of the hepatic stress defence (Sato et al., 1995).

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