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Interleukin-6 Stimulates Lipolysis and Fat Oxidation in Humans

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Although IL-6 is a key modulator of immune function, it also plays a role in regulating substrate metabolism. To determine whether IL-6 affects lipid metabolism, 18 healthy men were infused for 3 h with saline (Con; n = 6) or a high dose (High-rhIL6; n = 6) or a low dose (Low-rhIL6; n = 6) of recombinant human IL-6 (rhIL-6). The IL-6 concentration during Con, Low-rhIL6, and High-rhIL6 was at a steady state after 30 min of infusion at approximately 4, 140, and 320 pg/ml, respectively. Either dose of rhIL-6 was associated with a similar increase in fatty acid (FA) concentration and endogenous FA rate of appearance (R_a) from 90 min after the start of the infusion. The FA concentration and FA R_a continued to increase until the cessation of rhIL-6 infusion, reaching levels approximately 50% greater than Con values. The elevated levels reached at the end of rhIL-6 infusion persisted at least 3 h postinfusion. Triacylglycerol concentrations were unchanged during rhIL-6 infusion, whereas whole body fat oxidation increased after the second hour of rhIL-6 infusion. Of note, during Low-rhIL6, the induced elevation in FA concentration and FA R_a occurred in the absence of any change in adrenaline, insulin, or glucagon, and no adverse side effects were observed. In conclusion, the data identify IL-6 as a potent modulator of fat metabolism in humans, increasing fat oxidation and FA reesterification without causing hypertriacylglyceridemia. (*J Clin Endocrinol Metab* 88: 3005–3010, 2003)

INTERLEUKIN-6 IS A biologically active substance that is not only secreted by immune cells during inflammatory conditions, but is also released by adipose tissue (1) and by contracting skeletal muscle (2) in the absence of inflammation. Although little is known about the function of this release, it is possible that IL-6 is a key modulator of lipid homeostasis and metabolism, because adipose tissue and skeletal muscle are pivotal organs in the regulation of body fat and energy metabolism.

Although inconclusive, there is evidence that IL-6 may affect lipid metabolism. Stouthard *et al.* (3) studied patients with metastatic renal cell cancer during 4 h of recombinant human IL-6 (rhIL-6) infusion and observed an increase in circulating fatty acid (FA) and whole body FA rate of appearance (R_a). Importantly, however, in this study (3) all patients experienced clinical symptoms, such as fever, and consequently increased their whole body oxygen consumption. In addition, circulating hormones such as glucagon, adrenaline, and nor-adrenaline were elevated. Therefore, the researchers could not determine whether the changes in fat metabolism were a direct effect of IL-6 and whether the response was characteristic of healthy humans. Furthermore, IL-6 increases lipolysis in human breast adipocytes (4).

Infusion of IL-6 in rats increased FA and triacylglycerol (TAG) concentrations in a dose-dependent matter (5). The hypertriacylglyceridemia was caused by increased liver secretion and not decreased clearance. Recently, Wallenius *et al.* (6) demonstrated that IL-6-deficient mice developed mature-onset obesity. In addition, when the mice were treated with IL-6 for 18 d, there was a significant decrease in body weight in transgenic, but not wild-type, mice.

Taken together, these previous studies provide a rationale for testing the efficacy of IL-6 as a lipolytic factor in healthy humans, and this was the aim of the present study. To determine whether any effect of IL-6 was direct or secondary to changes in lipolytic regulatory hormones, we chose to infuse a low and a high dose of rhIL-6. The lower dose of rhIL-6 elicits a physiological plasma IL-6 concentration that can be found in healthy individuals during prolonged exercise (7). We hypothesized that rhIL-6 infusion increases lipolysis and TAG concentration in a dose-dependent matter.

Subjects and Methods

Subjects

Eighteen healthy, active, but not specifically trained, males were recruited in the study. Each was assigned to one of three groups: saline infusion (Con), low rhIL-6 infusion (Low-rhIL6), and high rhIL-6 infusion (High-rhIL6). The characteristics of the groups were similar for Con, Low-rhIL6, and High-rhIL6: age, 23 ± 1, 24 ± 1, and 26 ± 1 yr, respectively; weight, 78 ± 2, 80 ± 2, and 77 ± 2 kg; height, 183 ± 3, 184 ± 1, and 178 ± 2 cm; and body mass index, 23.4 ± 0.5, 23.5 ± 0.8, and 24.3 ± 0.7 kg/m^2. The study was approved by the ethical committee of Copenhagen and Frederiksberg communities, Denmark, and was performed according to the Declaration of Helsinki. Subjects were informed about the possible risks and discomfort involved before giving their written consent to participate.

Abbreviations: Con, Saline infusion; FA, fatty acid; GC, gas chromatography; High-rhIL6, high rhIL-6 infusion;Low-rhIL6, low rhIL-6 infusion; R_a, rate of appearance; R_d, rate of disappearance; rhIL-6, recombinant human IL-6; TAG, triacylglycerol.

Protocol

Subjects reported to the laboratory at 0700 h after an overnight fast. They voided, changed into appropriate hospital attire, and remained supine during the entire experiment. The experimental room was kept at 24 C. They were only permitted to consume *ad libitum* water during the experiment. After 10 min the femoral arteries of both legs were cannulated, one for saline or rhIL-6 infusion, the other for blood sampling. Thereafter, a catheter was placed in a forearm vein for infusion of the stable isotopes. Immediately after an arterial sample was obtained for background enrichment, a primed constant infusion of [$^2H_5$]glycerol (0.1 μmol/min·kg; prime, 1.5 μmol/kg) was started as well as a constant infusion of [U-^{13}C]palmitate (0.015 μmol/min·kg). All isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA). For each subject the actual infusion rate was calculated from the infusate concentration multiplied by the infusion flow rate. Blood samples were taken as described in Fig. 1. Blood samples of 2.0 ml were taken for FA, palmitate, glycerol, and TAG measurements and palmitate and glycerol enrichment. A 5-ml blood sample was taken at each measurement for IL-6, insulin, glucagon, cortisol, and catecholamines. The total amount of blood that consumed during the study was 235 ml.

IL-6 infusates

The two concentrations of rhIL-6 (Sandoz Pharmaceuticals Corp., Basel, Switzerland) were infused in doses lower than those reported to be safe in other studies (3). The IL-6 doses were chosen on the basis of pilot experiments. We aimed to reach the plasma levels of IL-6 observed in healthy individuals during intense exercise (Low-rhIL6) or during infections (High-rhIL6). In the Low-rhIL6 trial, the rate of rhIL-6 infusion was 30 μg/h, and it was administered in saline. Due to the retention of rhIL-6 at the side of the container when dissolved in saline, we could not use this method during High-rhIL6. Hence, further pilot experiments were conducted with 2% human albumin added as dissolving and delivery medium. These pilot experiments revealed that a dose of 15 μg/h was required during this trial. Saline was infused during control infusion (Con).

Analysis

Plasma [U-^{13}C]palmitate and [$^2H_5$]glycerol enrichments were determined as described previously in detail (8). Plasma FA concentrations were determined by gas chromatography (GC; Autosystem XL, PerkinElmer Corp., Norwalk, CT) using heptadecanoic acid as internal standard, and ^{13}C enrichment of plasma palmitate was determined by GC-combustion-isotope ratio mass spectrometry (HewlettPackard 5890; Finnigan GC combustion III, Finnigan δ^{plus}, Finnigan MAT, Bremen, Germany). In preparation for GC and GC-combustion-isotope ratio mass spectrometry analysis, plasma samples were processed to make a methyl derivative of palmitate as described previously (8). The isotopic enrichment of palmitate was expressed as the Δ o/oo difference between $^{13}C/^{12}C$ of the sample and a known laboratory reference standard-related Pee Dee Belemnitella limestone. The methyl derivative of palmitate contains 17 carbons, of which 16 are palmitate; thus, the tracer-to-tracee ratio of palmitate was corrected by a factor 17/16. Glycerol enrichment was measured by GC-mass spectrometry (Automass II, Finnigan, Paris, France). In preparation for GC-mass spectrometry analysis, plasma samples were processed to make a trifluorobutyrate derivative of glycerol. The isotopic enrichment of glycerol was determined using electron impact ionization, selectively monitoring ions at a mass to charge ratio (m/z) of 252–256, representing the molecular ions of unlabeled (m/z = 252) and labeled (m/z = 256) derivatives, respectively. Plasma was analyzed enzymatically for glycerol and TAG on an automatic analyzer (Cobas Fara, Roche, Basel, Switzerland). Blood samples for IL-6 were measured by high sensitivity ELISA as previously described (9). Plasma insulin (Insulin RIA 100, Amersham Pharmacia Biotech, Uppsala, Sweden), glucagon (Linco Research, Inc., St. Charles, MO), and cortisol (Diagnostic Products, Los Angeles, CA) were determined by RIA, and plasma adrenaline and noradrenaline were determined by HPLC. These analyses were described in more detail previously (10, 11).

Physical analysis

Heart rate and blood pressure were measured every 60 min using electrocardiography and sphygmomanometry, respectively. Temperature was also measured at this time point via a tympanic probe. Expired pulmonary gases were collected and analyzed for oxygen consumption on-line using a CPX/D metabolic cart (Medgraphics, St. Paul, MN). Due to technical problems, indirect calorimetry data were not obtained in four subjects during the High-rhIL-6.

Calculations

The whole body R_a and rate of disappearance (R_d) of palmitate and glycerol were calculated using the steady state equation: Ra = Rd = F/E_a. F is the isotopic infusion rate (micromoles per minute per kilogram), and E_a is the arterial isotopic enrichment (tracer/tracee ratio). The FA $R_{a/d}$ was calculated by dividing the palmitate data by the fractional contribution of palmitate to the total FA concentration, on the average palmitate being 0.22 ± 0.01 of the total FA.

Statistics

All data are presented as the mean ± SE. To analyze changes over time and between groups, a two-way repeated measures ANOVA was used. If such an analysis revealed significant differences, a Newman-Keuls *post hoc* test was used to locate the specific differences. $P < 0.05$ was accepted as significant.

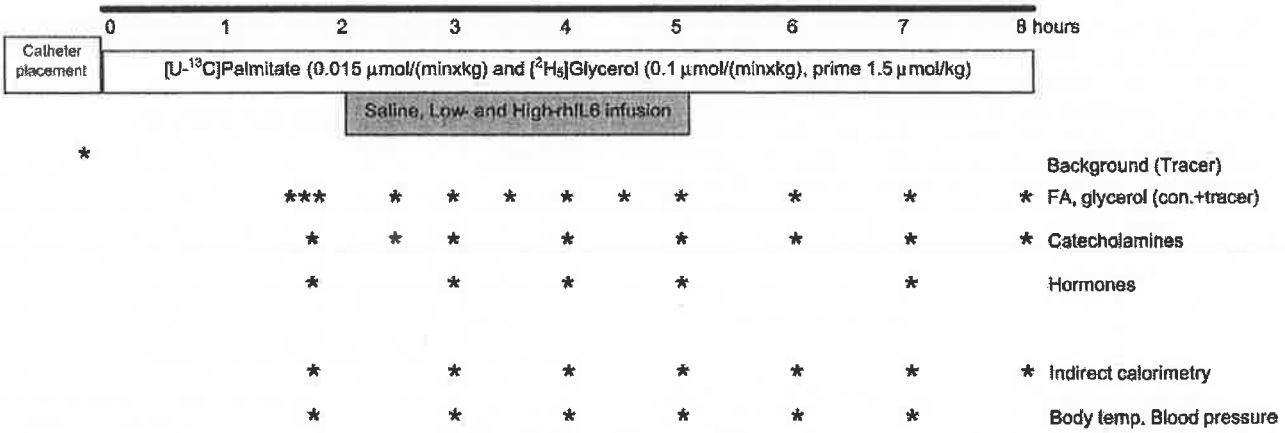

FIG. 1. Schematic of the study protocol.

Results

There were no significant differences among the trials in heart rate, body temperature, or blood pressure (Table 1). The subjects did not report any adverse effects during Con or Low-rhIL6. During High-rhIL6 all subjects experienced about 0.5 h of shivering and discomfort between 30–60 min after the rhIL-6 infusion was initiated. This effect was transient, and none of the subjects reported any severe side effects.

IL-6 and hormone concentrations (Fig. 1 and Table 2)

Arterial plasma IL-6 levels are shown in Fig. 2. During Low-rhIL6 and High-rhIL6 the mean arterial plasma IL-6 levels were 143 and 319 pg/ml, respectively. Saline infusion did not increase the concentration of IL-6. On cessation of the rhIL-6 infusion the IL-6 concentration declined rapidly, and after 1 and 2 h the concentrations were marginally elevated. The plasma hormone levels are reported in Table 2. Plasma insulin decreased over time, but there were no differences when comparing groups and no group by time interaction. Plasma glucagon levels were similar when comparing the three groups, and this hormone was not affected by either time or treatment. There were no differences in plasma cortisol concentrations when comparing the three groups at rest. Plasma cortisol concentrations did not change during Con, but increased during both Low-rhIL6 and High-rhIL6 infusion. While concentrations of plasma cortisol declined after 2 h of infusion in Low-rhIL6, they remained elevated in High-rhIL6 at 3 h of infusion. During both rhIL-6 trials, plasma cortisol levels returned to preinfusion values after 2 h of recovery. Plasma noradrenaline was not affected by time or treatment when comparing trials. In contrast, plasma adrenaline markedly increased at the onset of infusion in High-rhIL6, such that values after 60 min were greater than those in the Low-rhIL6 and Con groups. Of note, Low-rhIL6 did not affect plasma adrenaline concentrations.

Oxygen uptake and total FA oxidation (Table 3)

Oxygen uptake for both Con and Low-rhIL6 was unchanged over the entire study period, and values were not different from each other (Table 2). The respiratory exchange ratio, and thereby fat oxidation, were unchanged during Con. Low-rhIL6 did not change fat oxidation during the first hour of infusion, but thereafter fat oxidation increased and remained at the same level for the remainder of the study. After the second hour of rhIL-6 infusion energy expenditure was nearly completely covered by fat oxidation.

FA, glycerol, and TAG (Figs. 3 and 4)

The rhIL-6 infusion caused substantial changes in the FA concentration, with a similar pattern during Low-rhIL6 and High-rhIL6. A variable pattern existed during the first 90 min of rhIL-6 infusion, albeit a significant decrease was observed only after 30 and 90 min during the High-rhIL-6 compared with Con. After 2.5 h of infusion, a substantial increase in FA concentration occurred that continued until the end of the infusion for both rhIL-6 infusion rates. On cessation of rhIL-6 infusion, the FA concentration remained at virtually the level reached at the end of the rhIL-6 infusion for the next 3 h. The early changes in FA concentration were accompanied by changes in whole body FA R_a/R_d mainly during High-rhIL6. Despite a lower FA concentration after 30 and 90 min of High-rhIL6 infusion, the FA turnover rate was similar or higher (60 min) than those in the Con and Low-rhIL6 groups. After 2 h of rhIL-6 infusion a substantial similar increase in the FA turnover rate was observed for both Low- and High-IL6. The increase in FA turnover rate continued until the end of the infusion. After rhIL-6 infusion during High-rhIL6, FA turnover remained high, but FA turnover tended to decrease in the Low-rhIL6 dose. The arterial glycerol concentration was similar for all trials, except for a lower concentration after 90 min during High-rhIL6, which coincided with the low FA concentration. Substantially higher glycerol turnover rates were observed after 2 h of rhIL-6 infusion and in recovery during Low- and High-rhIL6 *vs.* Con. The patterns of changes in glycerol and FA turnover rate were similar during all trials, resulting in an FA/glycerol ratio close to 3. The arterial TAG concentration was significantly lower before treatment in Con compared with Low- and High-rhIL6 groups. The arterial TAG concentration decreased after cessation of rhIL-6 infusion for both Low- and High-rhIL6 groups, whereas the TAG concentration was unchanged in the Con group.

TABLE 1. Heart rate, blood pressure, and body temperature

	Pre	rhIL-6 infusion			5 h	6 h
		1 h	2 h	3 h		
Heart rate (beats/min)						
Con	63 ± 2	64 ± 3	63 ± 2	64 ± 3	63 ± 4	62 ± 3
Low-rhIL6	65 ± 6	68 ± 5	79 ± 5	75 ± 7	76 ± 6	74 ± 5
High-rhIL6	58 ± 2	70 ± 2	72 ± 2	72 ± 3	74 ± 4	70 ± 5
Blood pressure (mmHg)						
Con	81 ± 3	80 ± 3	82 ± 4	84 ± 5	81 ± 5	84 ± 2
Low-rhIL6	88 ± 4	86 ± 3	89 ± 4	84 ± 2	80 ± 3	87 ± 2
High-rhIL6	95 ± 3[a]	90 ± 5	88 ± 4	84 ± 4	87 ± 4	86 ± 4
Body temperature (C)						
Con	36.7 ± 0.2	36.7 ± 0.2	37.0 ± 0.2	36.9 ± 0.2	36.9 ± 0.2	36.1 ± 0.1
Low-rhIL6	36.3 ± 0.3	36.5 ± 0.3	36.6 ± 0.2	36.9 ± 0.2	37.2 ± 0.2	37.1 ± 0.2
High-rhIL6	36.7 ± 0.2	36.8 ± 0.4	37.2 ± 0.4	37.3 ± 0.2	37.1 ± 0.2	37.1 ± 0.2

Data are presented as means ± SE.
[a] Difference ($P < 0.05$) from Con.

TABLE 2. Arterial concentration of adrenaline, noradrenaline, cortisol, insulin, and glucagon

	Pre	rhIL-6 infusion				4 h	5 h	6 h
		0.5 h	1 h	2 h	3 h			
Adrenaline (nmol/liter)								
Con	0.81 ± 0.19	0.74 ± 0.20	0.76 ± 0.11	0.56 ± 0.15	0.62 ± 0.24	0.43 ± 0.08	0.30 ± 0.08	0.82 ± 0.16
Low-rhIL6	0.88 ± 0.13	0.89 ± 0.06	0.66 ± 0.08	0.68 ± 0.15	0.71 ± 0.05	0.56 ± 0.03	0.61 ± 0.05	0.92 ± 0.15
High-rhIL6	0.86 ± 0.13	1.12 ± 0.37	1.69 ± 0.32[a,b,c]	0.51 ± 0.11	0.68 ± 0.08	0.69 ± 0.24	0.49 ± 0.12	0.53 ± 0.13
Noradrenaline (nmol/liter)								
Con	1.03 ± 0.19	1.25 ± 0.21	1.11 ± 0.16	1.16 ± 0.12	0.96 ± 0.10	0.89 ± 0.08	0.67 ± 0.05	0.83 ± 0.05
Low-rhIL6	1.03 ± 0.20	1.30 ± 0.28	1.22 ± 0.30	1.10 ± 0.11	1.01 ± 0.12	0.92 ± 0.08	0.90 ± 0.09	1.13 ± 0.16
High-rhIL6	0.85 ± 0.28	1.20 ± 0.12	0.99 ± 0.11	0.86 ± 0.07	1.00 ± 0.25	1.12 ± 0.09	0.92 ± 0.27	1.16 ± 0.27
Cortisol (pg/ml)								
Con	18 ± 3		13 ± 3	10 ± 2	9 ± 2		9 ± 2[c]	
Low-rhIL6	20 ± 4		25 ± 3[a,c]	31 ± 5[a,c]	16 ± 2[a,c]		9 ± 2[c]	
High-rhIL6	19 ± 1		29 ± 3[a]	26 ± 2	31 ± 6[a,b,c]		15 ± 1	
Insulin (pmol/liter)								
Con	43 ± 9		30 ± 6[c]	30 ± 5[c]	27 ± 8[c]		28 ± 8[c]	
Low-rhIL6	35 ± 5		27 ± 3	18 ± 4[c]	22 ± 4[c]		20 ± 4[c]	
High-rhIL6	30 ± 6		38 ± 7	20 ± 5	27 ± 9		27 ± 5	
Glucagon (pg/ml)								
Con	108 ± 8		105 ± 7	114 ± 8	105 ± 5		111 ± 11	
Low-rhIL6	92 ± 7		101 ± 6	113 ± 8	114 ± 7		95 ± 3	
High-rhIL6	85 ± 17		142 ± 35	122 ± 24	135 ± 42		107 ± 31	

Data are presented as means ± SE.
[a] Difference ($P < 0.05$) from Con.
[b] Differences from Low-rhIL6.
[c] Differences from Pre.

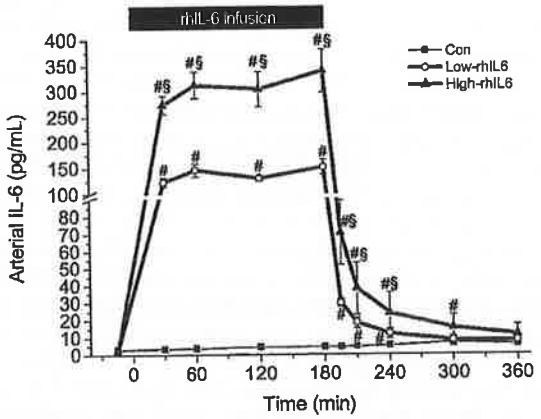

FIG. 2. Arterial plasma IL-6 before, during, and 3 h after the infusion of saline (Con) or a low or high dose of rhIL-6 (Low-rhIL6 and High-rhIL6). Data are presented as the mean ± SE. #, Difference ($P < 0.05$) from Con; §, difference from Low-rhIL6.

Discussion

The results from the present study clearly demonstrate that infusion of rhIL-6 into healthy humans increases lipolysis in the absence of hypertriacylglyceridemia or changes in catecholamines, glucagon, or insulin. These findings together with the observations that such an infusion did not result in adverse effects identify IL-6 as a novel lipolytic factor.

Unlike the High-rhIL6 treatment, Low-rhIL6 did not cause any adverse effects, and except for cortisol, no differences in hormones such as adrenaline, insulin, and glucagon were observed. Thus, the IL-6-induced lipolytic effect is not an indirect effect of adrenaline and glucagon. Despite the difference in circulating hormone concentrations and side effects when comparing the Low- and High-rhIL6, the marked enhancements in FA concentration and FA turnover were virtually the same. During both rhIL-6 infusions cortisol levels were modestly increased compared with preinfusion and Con levels. *In vivo* studies, using much higher levels than those used in the present study, suggest that glucocorticoids have no effect on (12), stimulate (13–15), or inhibit (16, 17) lipolysis, whereas in human adipocytes cortisol clearly has an antilipolytic effect (18). Furthermore, cortisol has been suggested to down-regulate β-adrenoceptor protein expression and β_3-adrenocepter-mediated adenylate cyclase activity in human adipocytes (19) and to reduce the sensitivity of adipocytes to catecholamine-stimulated lipolysis (18). Therefore, we believe that the increase in lipolysis observed in this study can be ascribed to IL-6 and not to the moderately enhanced cortisol levels.

Stouthard *et al.* (3) studied patients with metastatic renal cell cancer during 4 h of rhIL-6 infusion eliciting plasma IL-6 concentrations about 2-fold higher than those obtained using the High-rhIL6 dose of the present study. However, the increases in FA concentration and FA turnover were nearly identical to those seen in the present study. Thus, no dose-response effect on fat metabolism occurs when the IL-6 concentration is increased above 140 pg/ml. This suggests that a lower dose of rhIL-6 may exert the effect on fat metabolism, which emphasizes the potency of IL-6 as a modulator of lipolysis. Furthermore, unlike other lipolytic hormones such as adrenaline, the effect on fat metabolism persisted for hours after termination of the infusion, when the IL-6 concentration had returned to baseline values. The suggestion that IL-6 is strongly involved in fat metabolism is supported by the study by Wallenius *et al.* (6), who demonstrated that IL-6-deficient mice developed mature-onset obesity. In addition, when the mice were treated with IL-6 for 18 d, there was a significant decrease in body weight in transgenic, but not

TABLE 3. Pulmonary oxygen uptake, respiratory exchange ration, and total FA oxidation before, during, and 3 h after infusion of either saline (Con) or a low dose of rhIL-6 (Low-rhIL6)

	Pre	rhIL-6 infusion			4 h	5 h	6 h
		1 h	2 h	3 h			
Con							
Oxygen uptake (ml/min)	306 ± 18	303 ± 18	294 ± 13	295 ± 10	294 ± 9	286 ± 14	288 ± 8
RER	0.75 ± 0.02	0.73 ± 0.02	0.73 ± 0.01	0.74 ± 0.01	0.73 ± 0.01	0.74 ± 0.01	0.73 ± 0.02
Total FA oxidation (μmol · min/kg)	5.9 ± 0.5	6.5 ± 0.3	5.8 ± 0.3	5.3 ± 0.3	5.8 ± 0.2	5.8 ± 0.4	6.0 ± 0.3
Low-rhIL6							
Oxygen uptake (ml/min)	246 ± 26	262 ± 32	252 ± 29	263 ± 33	257 ± 27	262 ± 24	263 ± 29
RER	0.75 ± 0.03	0.76 ± 0.03	0.70 ± 0.02[a,b]	0.70 ± 0.04[b]	0.70 ± 0.01[a,b]	0.70 ± 0.05[b]	0.70 ± 0.04[b]
Total FA oxidation (μmol · min/kg)	5.0 ± 0.7	4.9 ± 0.4	5.6 ± 0.5[b]	5.6 ± 0.6[b]	6.1 ± 0.4[b]	6.0 ± 0.0.4[b]	6.0 ± 0.6[b]

Due to technical problems, indirect calorimetry data were not obtained during High-rhIL-6. Data are presented as mean ± SE. Total FA oxidation was determined by converting the rate of fat oxidation to its molecular equivalent, with the assumption that the average molecular weight of TAG is 860 g/mol^{-1} and multiplied by 3 to express fat oxidation in FA units, because each TAG molecule hydrolyzed three FA molecules are liberated. RER, respiratory exchange ratio.

[a] Difference ($P < 0.05$) from Con.

[b] Differences from Pre.

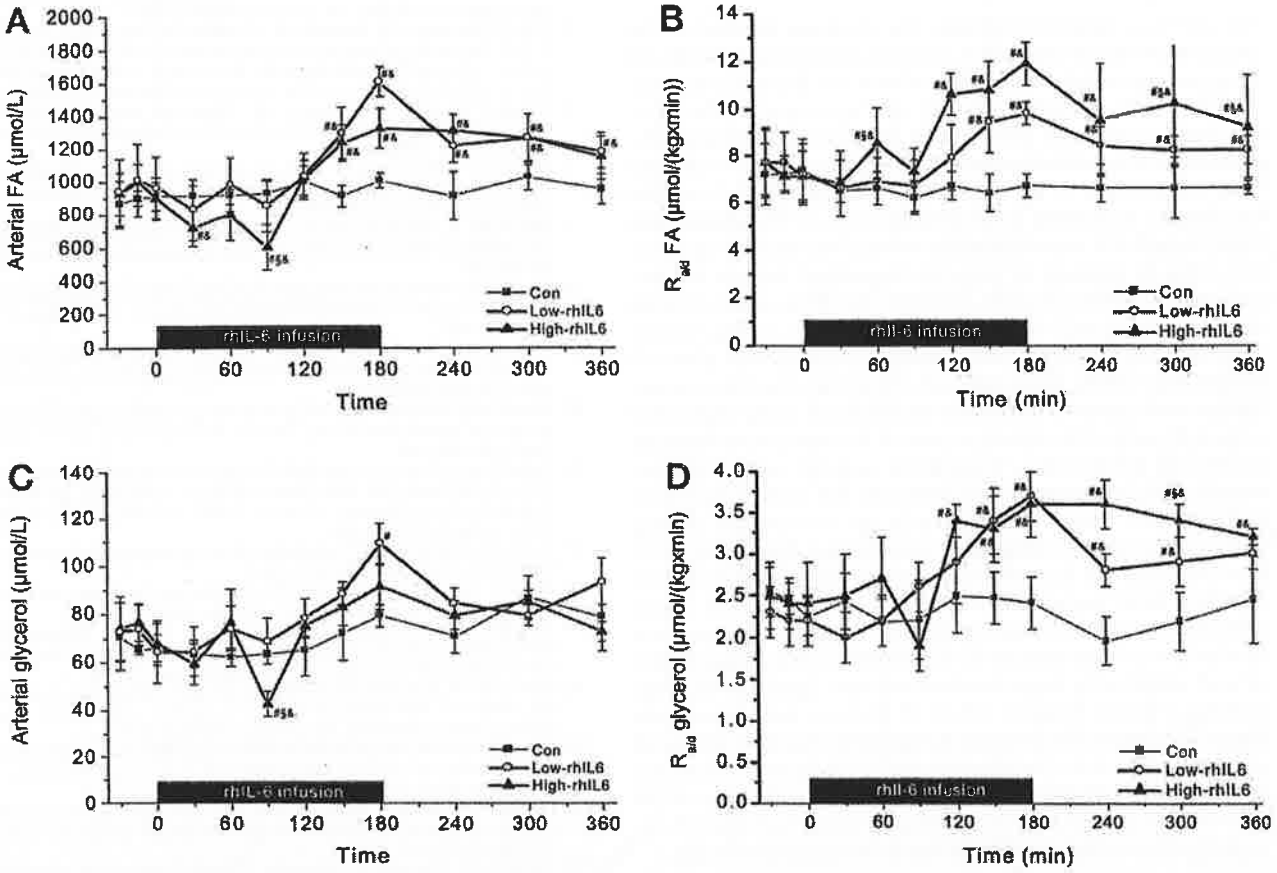

FIG. 3. Arterial FA (A) and glycerol (C) concentrations and whole body FA (B) and glycerol (D) R_a/R_d before, during, and 3 h after the infusion of saline (Con) or a low or high dose of rhIL-6 (Low-rhIL6 and High-rhIL6). Data are presented as the mean ± SE. #, Difference ($P < 0.05$) from Con; §, difference from Low-rhIL6; &, differences from preinfusion.

wild-type, mice. Studies in rats suggested that IL-6 raises TAG concentrations (5). However, in the present human study with relatively low IL-6 concentrations compared with those obtained in rats, no increase in TAG could be observed. In contrast, TAG levels were reduced after cessation of rhIL-6 infusion. Thus, the clinically negative effect of hypertriacylglyceridemia is not present with the rhIL-6 doses used in the present study.

The changes seen in FA concentration and turnover during rhIL-6 infusion suggest a complex mode of action of IL-6 on fat metabolism. During the first 90 min of rhIL-6 infusion, the FA concentration remained constant or slightly decreased,

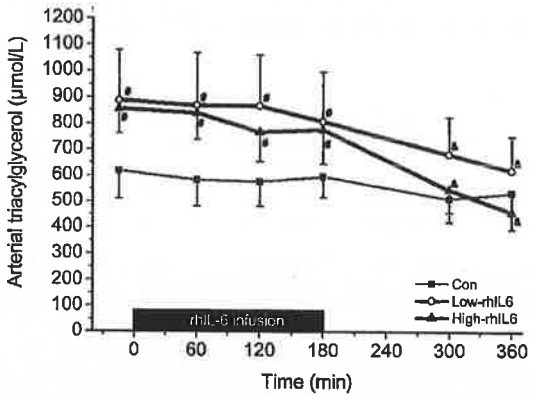

FIG. 4. Arterial TAG before, during, and 3 h after the infusion of saline (Con) or a low or high dose of rhIL-6 (Low-rhIL6 and High-rhIL6). Data are presented as the mean ± SE. #, Difference ($P < 0.05$) from Con; §, difference from Low-rhIL6.

but not in a constant fashion; the decrease seemed to be caused more by enhanced FA disappearance than by reduced FA appearance. After those first 90 min, the FA concentration and the FA R_a increased rapidly and appeared not to have reached a maximum after 3 h of infusion. In addition, the increase in FA oxidation during rhIL-6 infusion was far less than the increase in the rate of FA disappearance from the circulation, indicating a marked increase in FA reesterification. Hepatic FA reesterification to very low density lipoprotein-TAG is unlikely to play an important role in the enhanced FA reesterification, because the TAG concentration decreased with rhIL-6 infusion. On cessation of rhIL-6 infusion, the IL-6 concentration decreased rapidly, and after 3 h preinfusion levels were reached. However, the FA concentration and turnover rates were maintained at the high level seen at the end of the infusion period. Known potent lipolytic hormones, for instance, adrenaline, usually work instantaneously, and on cessation of infusion, FA metabolism normalizes fast. Thus, the changes in fat metabolism during rhIL-6 infusion are most likely not elicited directly by IL-6, but indirectly via IL-6-induced changes in other substances affecting lipid metabolism. However, when human breast adipocytes were incubated with IL-6, the glycerol release, *i.e.* lipolysis, was increased by 42%. This effect was independent of and additive to isoproterenol-induced lipolysis (4), suggesting a direct lipolytic effect of IL-6 on human adipose tissue. However, the increase in lipolysis was only observed after more then 6 h of incubation with IL-6. In conclusion, the present study identifies IL-6 as a potent modulator of fat metabolism in humans, increasing fat oxidation and FA reesterification without causing hypertriacylglyceridemia.

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Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise

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ABSTRACT

Interleukin-6 (IL-6) is produced by many different cell types. Human skeletal muscles produce and release high amounts of IL-6 during exercise; however, the cell source of origin in the muscle is not known. Therefore, we studied the protein expression of IL-6 by immunohistochemistry in human muscle tissue from biopsies obtained at time points 0, 3, 4.5, 6, 9, and 24 h in relation to 3 h of bicycle exercise performed by healthy young males (n=12) and in resting controls (n=6). The IL-6 expression was clearly increased after exercise and remained high even by 24 h, relative to pre-exercise or resting individuals. The IL-6 immunostainings of skeletal muscle cells were homogeneous and without difference between muscle fiber types. The IL-6 mRNA peaked immediately after the exercise, and, in accordance, the IL-6 protein expression within muscle cells was most pronounced around 3 h post-exercise. However, the finding that plasma IL-6 concentration peaked in the end of exercise indicates a high turnover of muscle-derived IL-6. In conclusion, the finding of marked IL-6 protein expression exclusively within skeletal muscle fibers following exercise demonstrates that skeletal muscle fibers of all types are the dominant cell source of exercise-induced release of IL-6 from working muscle.

Key words: cytokine staining • physical training • health

It is well known that the cytokine interleukin-6 (IL-6) is markedly increased during exercise. Thus, plasma IL-6 increases in an exponential fashion and peaks at the end of an exercise bout (for reference, see 1, 2). IL-6 is highly regulated by the onset of exercise and is also linked to energy availability within the working skeletal muscle, as subjects with low intra-muscular glycogen levels demonstrate a further enhanced IL-6 response independent of blood-borne substrates or hormones, which is initiated by the onset of exercise (3, 4). Furthermore, IL-6 has recently been demonstrated to have a lipolytic effect upon infusion in human subjects (5) and may thus work as a muscle-derived hormone, released in high amounts from skeletal muscle to mobilize energy as free fatty acids from adipose tissue. Although earlier studies demonstrated that IL-6 mRNA is increased in skeletal muscle biopsy samples (3, 4, 6) and that IL-6 is released by contracting muscles (7), it has not previously been documented which cell type within the muscle is responsible for the production. Whereas myoblasts have been shown to be capable of producing IL-6 (8, 9), endothelial cells (10), fibroblasts (9), and smooth muscle cells (11) have also been shown to produce IL-6 under certain circumstances. Thus, several cell types in the skeletal organ have the potential to produce IL-6. However, for IL-6 to play a role as an energy-sensing muscle-derived hormone, it must be secreted from the tissue in need of energy; the myotubes.

In an attempt to determine which cells produce the IL-6, we have isolated nuclei from human muscle biopsies obtained before, during, and after exercise and demonstrated that the nuclear transcription rate for IL-6 increased rapidly and markedly after the onset of exercise (4). Furthermore, myoblasts stimulated with ionomycin, an agent that mimics the exercise-induced increase in intracellular calcium levels, have demonstrated an increase in IL-6 mRNA (Keller et al., unpublished data). Thus, it is plausible that the muscle cells themselves are the source of IL-6 production during exercise. The aim of the present study, therefore, was to address the question of whether muscle fibers are the major producers of IL-6 in response to exercise. Muscle-derived IL-6 is a consequence of transcription and translation of the IL-6 gene in the skeletal muscle organ. Transcription has been demonstrated in human muscle already after 30 min of moderate exercise (4). The concentration of the IL-6 protein in plasma increases in an exponential fashion with duration of exercise (1). Although, the intensity of exercise is also a determining factor (12), we chose an exercise model of long duration and consequently somewhat moderate intensity. We chose ergometer bicycling as mode of exercise, as we wanted a pure concentric exercise model in order to exclude a classical acute phase response as can be elicited by muscle damage induced by eccentric exercise.

MATERIALS AND METHODS

Eighteen healthy male subjects [age median 27 years (range 20–30), height 189 cm (range 164–197), weight 80 kg (range 64–96)] participated in the study. The subjects were given both oral and written information about the experimental procedures before giving their written informed consent. The study was approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark, and was performed according to the declaration of Helsinki.

One week before the first experimental day, subjects performed a maximal oxygen uptake test (VO_2max) on an ergometer bicycle. Measurement of maximum VO_2 was performed on an electrically braked cadence independent cycle ergometer (Monark 839E, Monark Ltd., Varberg, Sweden). Subjects cycled for 3 min at 100 Watts as a warm-up followed by cycling at progressively higher work rates, increasing 50 Watts every 3 min for 9 min and then increasing the workload by 25 W every minute until subjects were unable to maintain a cadence of 60 rpm. Expired oxygen (VO_2) and carbon dioxide (VCO_2) concentrations were recorded online. Median VO_2max was median 46.7 ml/kg x min (range 41–68). At the experimental day, subjects arrived at 8 am after an overnight fast and were randomized to either exercise or rest. There was no difference between the two groups with regard to age, weight, height, or VO_2max. Subjects performed 3 h of cycling at 60% of VO_2max followed by 6 h of recovery. The following day they reported to the laboratory after an overnight fast. Prior to the exercise (0); immediately after the exercise (+3 h); and 4.5, 6, 9, and 24 h after, muscle biopsies were obtained from the vastus lateralis by using the percutaneous needle biopsy technique with suction. Control subjects rested in the laboratory for 9 h and had biopsies collected to time points 0, 3, 4.5, 6, and 9 h. They reported to the laboratory the following day, where the last biopsy was obtained at time point 24 h.

Histochemistry and immunohistochemistry

Muscle tissue was cut in 6 μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides, to be used for general histology and immunohistochemistry.

For epitope retrieval, sections were pre-incubated in Digest-ALL-3 (Pepsin solution) (Zymed Lab. Inc., San Francisco, CA, code 00-3009) for 5 min followed by incubation in 10% goat serum (Company In Vitro AS, Fredensborg, DK, code 04009-1B) in TBS (TBS: 0.05 M TRIS, pH 7.4, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet) for 30 min at room temperature.

Histochemistry: Hematoxylin and eosin (HE) stainings of the sections were performed according to standard procedures (13). Myofibrillar ATPase staining with preincubations of pH 4.6 was used to identify muscle fiber types (14).

Immunohistochemistry: Sections were incubated overnight at 4°C with monoclonal mouse anti-human IL-6 diluted 1:50 (Chemicon Int. Inc./Cymbus, Temecula, CA; code CBL2117). The primary antibody was detected by using biotinylated goat anti-mouse IgG diluted 1:200 (Sigma-Aldrich, Vallensbaek, DK; code B8774) for 30 min at room temperature followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, Glostrup, DK; code K377) prepared at manufacturer's recommended dilutions for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, Boston, MA; code NEL700A) prepared following manufacturer's recommendations. The immunoreaction was visualized by using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

To evaluate the extent of non-specific binding in the immunohistochemical experiments, control sections were incubated in the absence of primary antibody. Results were considered only if these controls were negative. For the simultaneous examination and recording of the stainings, a Zeiss Axioplan2 light microscope was used.

RNA extraction: RNA was extracted by using Trizol™ (Life Technologies) according to manufacturer's protocol. In short, 1 ml of Trizol was added to 20–50 mg of tissue and homogenized by using a Polytron (PT-MR2100, Kinematica AG, Headquarters, Littau-Lucerne, Schwitzerland) on setting 25–30 for 20–30 s. Chloroform (100 µl) was added to the samples, followed by a 5-min incubation on ice. Samples were spun at 13,000 rpm for 15 min at 4°C (Heraeus Biofuge Pico, DJB Labcare, Newport Pagnell, UK), and the upper aqueous phase was placed in a fresh eppendorf tube. The same volume of isopropanol was added, and samples were placed at –20°C for 1 h followed by centrifugation at 13,000 rpm for 15 min at 4°C. The resulting RNA pellet was washed with 500 µl of 75% ethanol in DEPC-treated water and spun at 8000 rpm for 10 min at 4°C. The pellets were dissolved in 15 µl of DEPC-treated water

Reverse transcription: Total RNA (1 µm) was reverse-transcribed in a 50 µl reaction containing 1 × Taqman RT-buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM of Oligo dT primers, 0.4 U/µl of RNase inhibitor, and 1.25 U/µl of reverse transcriptase. The reactions were run in a Perkin Elmer GeneAmp PCR system 9700 (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) with conditions at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

Samples were analyzed for IL-6 mRNA levels by real-time PCR by using an ABI PRISM 7700 sequence detector (PE Biosystems). The gene expression levels were normalized to the housekeeping gene GADPH (obtained from Applied Biosystems). Human IL-6 primers and Taqman® probes (see Table 1) were designed using the Primer Express 2.0 program.

Measurement of plasma IL-6

Blood samples for measurement of cytokines were drawn into glass tubes containing EDTA. The tubes were spun immediately at 3500 g for 15 min at 4°C (Sigma 4K15 centrifuge). The plasma was stored at –80°C until analyses were performed. High-sensitivity enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) were used to measure IL-6 in plasma; sensitivity: 0.094 pg/mL. This kit does not distinguish between soluble and receptor-bound IL-6 and therefore gives a measure of the total IL-6 content in the sample.

Statistics

IL-6 mRNA data were normally distributed after log-transformation. A one-way ANOVA with correction for multiple time-points (RM-ANOVA) was used to detect a time effect (IL-6 mRNA and plasma-IL-6), with Student-Newman-Keuls *t*-test for post hoc analysis of changes from resting values. *P* values <0.05 are considered significant. Statistical calculations were performed using Sigma Stat 3.0 (SSPS Inc., Chicago, IL).

RESULTS

IL-6 immunohistochemistry

IL-6 staining was performed at time points 0, 3, 4.5, 6, 9, and 24 h for 12 subjects who performed exercise for 3 h and at the same time points for 6 subjects at rest.

In the muscle tissue from resting volunteers, the IL-6 staining was absent. Representative data are shown for time points 0 and 6 h (one resting subject) (Fig. 1*A–B*) and for one subject before exercise (Fig. 1*C*). Right after exercise ended (3 h), the expression of IL-6 had increased significantly (Fig. 1*C*, *D*). The IL-6 levels remained significantly increased by 4.5, 6, and 9 h; whereas, by 24 h, the expression of IL-6 had clearly decreased again (Fig. 1*E–H*). However, the IL-6 levels by 24 h were still higher than those of resting muscles. The IL-6 staining appeared exclusively as a homogeneous staining of the cytoplasm of skeletal muscle fibers in all subjects who had performed exercise. There was no IL-6 staining present between muscle fibers. When myofibrillar staining was compared with IL-6 staining, it appeared that there was no difference between muscle fiber types (Type I, Type IIa, or Type 2x) with regard to IL-6 expression (Fig. 2).

IL-6 mRNA and plasma-IL-6

The IL-6 mRNA level increased ($P<0.05$) with exercise. Thus, compared with the prevalue, fold changes of IL-6 mRNA compared with pre-value were 11.0 + 2.6; 8.0+5.7-fold; 1.4+0.4; 0.4+0.2; and 1.2+0.3 at 3, 4.5, 6, 9, and 24 h, respectively—peaking at the end of exercise. Furthermore, plasma IL-6 concentrations increased significantly with time: 2.2+0.2; 18.6+2.8; 16.9+3.9; 9.5+1.5; 9.1+1.4; and 3.1+ 1.7 pg/ml at time points 0, 3, 4.5, 6, 9, and 24 h. There was no sign of muscle damage as indicated by measurement of creatine kinase and myoglobin.

DISCUSSION

The present study demonstrated that physical exercise induces expression of the IL-6 protein in the cytoplasm of muscle fibers. The finding of a marked IL-6 protein expression within skeletal muscle fibers strongly indicates that exercise-induced release of IL-6 from working muscle, the so-called muscle-derived IL-6, has its origin from muscle cells per se. The fact that IL-6 is exclusively located in the cytoplasm of muscle fibers and that there is no IL-6 staining present between muscle fibers supports IL-6 as a genuinely muscle fiber-derived factor. Although, brain (15) and peritendon tissue have been demonstrated to release IL-6 in response to exercise (16), it seems plausible that muscle fibers are the major source of IL-6 in response to exercise, given the massive protein expression in the muscle fibers in addition to muscle cells being the dominant cell type within skeletal muscle. Furthermore, it has been established that blood mononuclear cells do not contribute to the production of IL-6 during exercise (17).

Several studies have demonstrated a large increase in IL-6 gene expression in skeletal muscle biopsies (3, 4, 6). Numerous studies have demonstrated that the IL-6 mRNA in muscle biopsies peak in the end of exercise or shortly after (3, 4, 6) in accordance with the findings from this study.

Furthermore, transcription rates of IL-6 are increased in isolated myonuclei in response to exercise (4). The IL-6 mRNA kinetic fits nicely with the finding that the IL-6 protein expression within muscle fibers peaked at 6 h (3 h post-exercise). Numerous studies have also demonstrated that the plasma-IL-6 levels increase in an exponential fashion during exercise, peak at the end of exercise or shortly after, and thereafter gradually decline (1). However, it has also been demonstrated that the release of IL-6 from an exercising limb is ~17-fold higher than the amount that is accumulated in the plasma (7), thus indicating a very high turnover of IL-6. The finding of IL-6 protein accumulation within skeletal muscle fibers 3 h after the exercise further supports the idea of a high IL-6 turnover.

As discussed, we have previously demonstrated that the nuclear transcriptional rate of the IL-6 gene is remarkably rapid after the onset of exercise, with a 10- to 20-fold increase when comparing 30 min of exercise with rest. We therefore hypothesized that this rapid increase in nuclear transcriptional rate was related to a glycogen-independent mechanism, possibly the cytosolic Ca^{2+} levels, because mechanical load is a potent stimulus for liberating Ca^{2+} from the lateral sacs of the sarcoplasmic reticulum (18). Therefore, muscle cells isolated from human biopsies were harvested and grown in a culture medium until they fused into myotubes (19) and then stimulated with the Ca^{2+} ionophore ionomycin. IL-6 mRNA increased progressively over 48 h compared with preincubation levels. It is clear from an examination of the literature that there is a signaling cascade in other cell types that indeed implicates intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) as a potent signaling factor for IL-6 transcription. $Ca^{2+}{}_i$ controls a diverse range of cellular functions (20, 21). In lymphocytes, the amplitude and the duration of the $[Ca^{2+}]_i$ controls the differential activation of the proinflammatory transcriptional regulators nuclear factor κβ (NF-κB), c-Jun amino-terminal kinase (JNK), and nuclear factor of activated T cells (NFAT; 22). NF-κB and JNK are selectively activated by a large $[Ca^{2+}]_i$ rise, whereas activation of NFAT was induced by a low sustained $[Ca^{2+}]_i$. Therefore, we previously proposed (1) that during prolonged contractile activity that results in an increase in IL-6 mRNA in skeletal muscle (3, 4, 6, 23), initial IL-6 transcription occurs via a Ca^{2+}/NFAT-dependent pathway. Although NFAT in itself can lead to cytokine gene transcription, it can bind to the transcription factor AP-1, which can lead to cytokine gene transcription (24, 25). Although this pathway is likely to lead to IL-6 gene transcription during sustained muscular contractions, it is possible that large $[Ca^{2+}]_i$ transients as seen with maximal contraction can activate IL-6 via NF-κB and JNK. It is known that skeletal muscle expresses JNK and that muscle contraction markedly increases JNK activation (26). Although the degree to which IL-6 is activated in skeletal muscle by these signaling pathways is not known, it is possible that during more intense muscular activity serial activation of these various pathways gives rise to the more pronounced IL-6 response.

The biological roles of IL-6 appear to be several. The IL-6 production is modulated by the glycogen content in muscles (4, 17) and thus appears to work as an energy sensor (27). IL-6 exerts its effect on adipose tissue, inducing lipolysis and increases whole body lipid oxidation (5). The findings from the present study have further supported our hypothesis of IL-6 as a muscle-fiber derived hormone that is released from the site of energy turnover.

We found that IL-6 was produced equally by Type 1 and Type 2 muscle fibers. Given that IL-6 works as an energy-sensor, it could be expected that the slow, more metabolic active Type 1 muscle fibers would express more IL-6 than Type 2 fibers. It could be argued that IL-6 is produced by Type 1 fibers but taken up by Type 2 fibers. However, the immunohistochemical appearance does not support such a notion, as there was no gradient between the fiber types. Moreover, it has been argued that Ca plays a key role in inducing the signaling of IL-6. This leads to the question of whether the signaling pathway is the same in the two fiber types as the free Ca-concentration in the cytosol varies when the Type 1 versus the Type 2 fibers are activated. The phasic response of the

Type 2 fibers when activated may, however, be modified when they are recruited in more prolonged dynamic exercise and have a pattern that mimics the activation of the Type 1 fibers (28, 29). However, it makes sense that both fiber types express IL-6, as it is well-documented that human Type 2 fibers are recruited in prolonged dynamic exercise and are able to oxidize fat for its energy needs (30). However, single-fiber studies are required to finally decide whether IL-6 is differentially expressed by fiber types.

IL-6 is not only related to energy mobilization in response to exercise. Recent findings from our group have demonstrated an anti-inflammatory effect of exercise in humans in response to endotoxin-induced TNF-α plasma levels, an effect that can fully be mimicked by IL-6 administration alone (31). The fact that IL-6 can inhibit endotoxin-induced TNF-α demonstrates another potential benefit of muscle-derived IL-6. TNF-α has been linked to insulin resistance by inhibiting glucose uptake in skeletal muscle (32). As muscle-derived IL-6 can inhibit a TNF-α response, IL-6 may directly bring glucose uptake back to normal levels, thus reversing the detrimental effects of TNF-α. For this effect to occur, it would seem reasonable that the muscle cells themselves would secrete IL-6 in an attempt to reverse the effects of TNF-α on muscle cell glucose uptake. These speculations are in accordance with the findings from the present study. In conclusion, the present study demonstrates that skeletal muscle cells are the dominant cell source of exercise-induced muscle-derived IL-6.

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Table 1

Gene name	Forward primer	Reverse primer	Taqman® probe
IL-6	5'-GGTACATCCTCGACGGCATCT-3'	5'-GTGCCTCTTTGCTGCTTTCAC-3'	5'-TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT-3'

Fig. 1

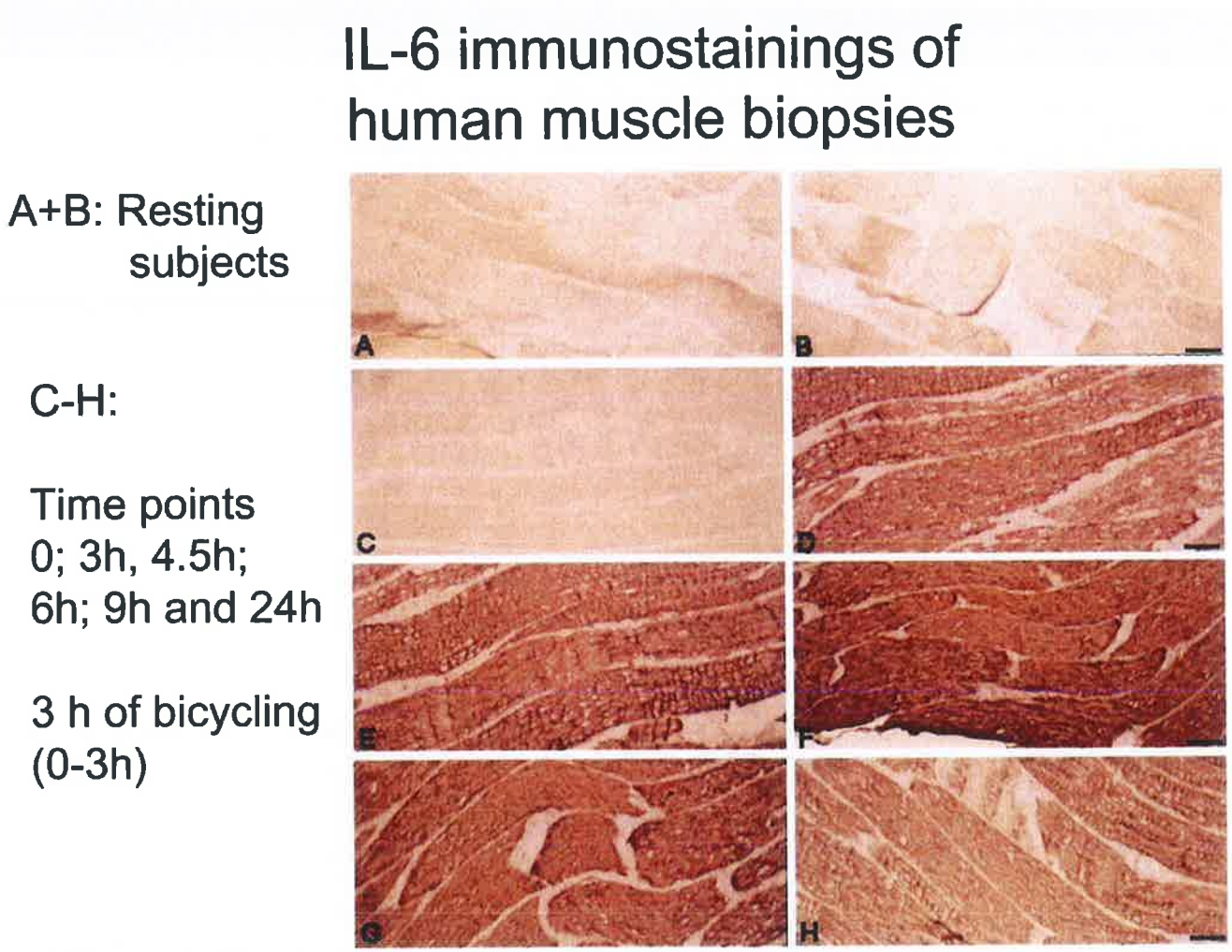

Figure 1. IL-6 expression in skeletal muscle tissue of resting subjects (***A–C***) and exercising subjects (***D–H***). ***A***, ***B***) Resting subjects at 1/2 h (***A***) and 6 h (***B***) show no significant IL-6 immunostaining. ***C***) Before exercise began, IL-6 expression was generally absent in the muscle tissue. ***D***) By 3 h, when the exercise had just ended, the muscle tissue showed significantly increased IL-6 immunoreactivity. ***E–G***) By 4.5 (***E***), 6 (***F***), and 9 h (***G***), the IL-6 expression was still significantly increased relative to that of resting muscle tissue. ***H***) By 24 h, the IL-6 levels had decreased but was still clearly higher than those of resting muscle tissue. Scale bars: ***A–H***) 50 μm.

Fig. 2

IL-6 immunostainings in post-exercise muscle biopsies - relation to fiber types

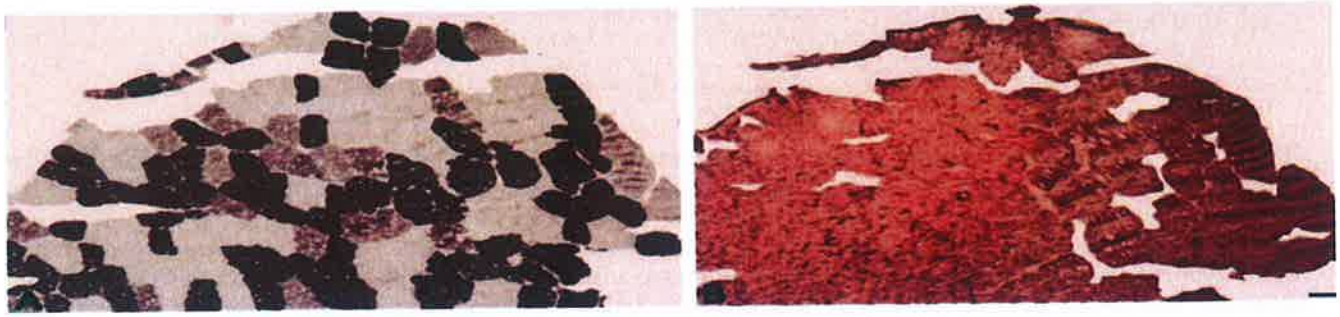

Figure 2. Neighboring sections of skeletal muscle tissue stained for myosin ATPase and IL-6. The tissue is from exercising subjects at 4.5 h. ***A)*** Histochemical identification of fiber types based on myosin ATPase staining following incubation at pH 4.6. Dark fibers are Type 1 fibers, grey fibers are Type 2a fibers and the light fibers are Type 2x fibers. ***B)*** IL-6 staining is shown on a neighboring section. It appears that there is no difference between fiber types with regard to IL-6 expression. Scale bars: (***A***, ***B***): 100 μm.

The FASEB Journal • Retraction

The article "Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise," by Milena Penkowa, Charlotte Keller, Pernille Keller, Sune Jauffred, and Bente Klarlund Pedersen, published in print as an *FJ Express* summary in *FASEB J.* 2003 Nov. 17:2166–2218, doi: 10.1096/fj.03-0311fje, and as a full-length article online at http://www.fasebj.org/content/early/2003/11/04/fj.03-0311fje, has been retracted due to a recommendation made by the Danish Committees on Scientific Dishonesty (DCSD). According to DCSD, the article "contains image manipulations and [DCSD has] therefore recommended that the authors withdraw these articles from *The FASEB Journal.*"

doi: 10.1096/fj.03-0311fjeRET

The article "Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6," by Pernille Keller, Milena Penkowa, Charlotte Keller, Adam Steensberg, Christian P. Fischer, Mercedes Giralt, Juan Hidalgo, and Bente Klarlund Pedersen, published in print as an *FJ Express* summary in *FASEB J.* 2005 July 19:1181–1183, doi: 10.1096/fj.04-3278fje, and as a full-length article online at http://www.fasebj.org/content/early/2005/06/29/fj.04-3278fje, has been retracted due to a recommendation made by the Danish Committees on Scientific Dishonesty (DCSD). According to DCSD, the article "contains image manipulations and [DCSD has] therefore recommended that the authors withdraw these articles from *The FASEB Journal.*"

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Artikel 3

Exercise induces interleukin-8 expression in human skeletal muscle

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Skeletal muscle has been recognized as an endocrine organ, and muscle cell cultures express several cytokines with potential hormonal effects. Interleukin-8 (IL-8), a chemokine, which induces angiogenesis, is expressed in working muscles; however, the cell source of origin has not been identified. We aimed to elucidate if IL-8 protein is: (1) expressed in contracting muscle fibres and (2) whether there is a release of IL-8 from exercising muscle. Seventeen healthy male volunteers were included in two independent protocols: 3 h of ergometer bicycle exercise at 60% of $\dot{V}_{O_2,max}$ ($n = 6$) or rest ($n = 5$), and 3 h of two-legged knee-extensor exercise at 60% of maximal workload ($n = 6$). Repetitive muscle biopsy samples were obtained from the vastus lateralis in all experiments. A marked increase in IL-8 mRNA was found in muscle biopsy samples obtained after exercise. A marked IL-8 protein expression was demonstrated within the cytoplasm of muscle fibres in biopsy samples obtained in the recovery phase following 3 h of bicycle exercise, and the peak occurred 3–6 h postexercise. A small transient net release of IL-8 from working muscle was found at 1.5 h of knee-extensor exercise. However, the small release of IL-8 from muscle did not result in an increase in the systemic plasma concentration of IL-8, suggesting that muscle-derived IL-8 may play a local role, e.g. in angiogenesis.

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The immunological and endocrine function of skeletal muscle is an area of increasing interest. Skeletal muscle cell cultures express several cytokines, such as interleukin (IL)-6, IL-8, interferon (IFN)-γ and tumour necrosis factor (TNF)-α (Saghizadeh *et al.* 1996; De Rossi *et al.* 2000; Alvarez *et al.* 2002). IL-8 is produced by monocytes and macrophages, as well as by most tissues (Baggiolini *et al.* 1994). IL-8 is induced by a number of stimuli, including pro-inflammatory cytokines (Hoffmann *et al.* 2002), and acts as a chemokine on neutrophils. IL-8 belongs to the CXC family of chemokines. The CXC nomenclature relates to the presence of two conserved cysteine residues, separated by one amino acid, at the amino terminus. IL-8 belongs to a subdivision of the CXC chemokines, and this subgroup has an amino acid sequence Glu-Leu-Arg (ELR) preceding the first conserved cysteine amino acid residue in the primary structure of these proteins (Baggiolini, 2001). CXC chemokines with the ELR motif are potent angiogenic factors *in vivo* (Koch *et al.* 1992; Norrby, 1996; Bek *et al.* 2002). IL-8 acts as an angiogenic factor in human microvascular endothelial cells (Heidemann *et al.* 2003). Keane *et al.* (1997) have found an association between IL-8 and angiogenesis in idiopathic pulmonary fibrosis patients. In addition, IL-8 is associated with insulin resistance and obesity (Bruun *et al.* 2002, 2003).

The plasma concentration of IL-8 increases in response to exhaustive exercise comprised of both eccentric and concentric components, such as running (Ostrowski *et al.* 2001; Nieman *et al.* 2001, 2002, 2003; Suzuki *et al.* 2003). It is unclear whether concentric exercise alone, which is not associated with an inflammatory response, results in an increased IL-8 plasma concentration (Mucci *et al.* 2000; Henson *et al.* 2000; Chan *et al.* 2004). Incremental bicycle ergometer exercise to exhaustion resulted in a small increase in plasma IL-8 concentration (Mucci *et al.* 2000), whereas plasma IL-8 concentration remained unaltered during rowing or bicycle exercise for 1 h or 2 h, respectively (Henson *et al.* 2000; Chan *et al.* 2004). Therefore, this indicates that concentric exercise alone is not related to a marked increase in IL-8 plasma concentration.

The contracting skeletal muscle responds to a 3 h treadmill run by increasing IL-8 mRNA several fold,

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concomitant with increased plasma levels of IL-8 (Nieman *et al.* 2003). Similarly, skeletal muscle IL-8 mRNA increased in response to 1 h of bicycle exercise without affecting IL-8 plasma concentration (Chan *et al.* 2004). A muscle biopsy contains several cell types in addition to myocytes. Indeed, IL-8 can be expressed by human endothelial cells (vascular tissue) *in vitro* (Zhao *et al.* 2003), and could thus serve as the cellular source of IL-8 in muscle biopsy samples. Whether myocytes are the cellular source of IL-8 in muscle biopsy samples has not been investigated. Furthermore, it is not known if the IL-8 protein is expressed in or released from skeletal muscle.

We hypothesized that: (1) concentric exercise would induce expression of IL-8 mRNA and protein in skeletal muscle cells and (2) concentrically contracting skeletal muscle would release IL-8.

Methods

Study 1. The effect of bicycle exercise on muscle IL-8 mRNA and protein expression

Bicycle ergometer exercise was chosen as the mode of exercise in this study since this type of exercise is mainly concentric and induces minimal muscle damage and subsequent inflammation. Furthermore, it has been used in several of the aforementioned studies investigating IL-8 plasma and gene-expression changes as a response to exercise, and would thus allow for a comparison of results. Eleven healthy men with a median age of 26 years (range 21–28 years), a median height of 186 cm (range 164–197 cm), and a median weight of 77.5 kg (range 65–96 kg), participated in the study. One week before the first experimental day, subjects performed a maximal oxygen uptake test ($\dot{V}_{O_2,max}$) on an ergometer bicycle. Measurement of $\dot{V}_{O_2,max}$ was performed on an electrically braked cadence independent cycle ergometer (Monark 839E, Monark Ltd, Varberg, Sweden). Subjects cycled for 3 min at 100 W as a warm-up, followed by cycling at progressively higher work rates (increasing 50 W every 3 min for 9 min), and then increasing the workload by 25 W every minute until subjects were unable to maintain a cadence of 60 r.p.m. Expired oxygen and carbon dioxide concentrations were recorded online. Median $\dot{V}_{O_2,max}$ was 50.4 ml kg^{-1} min^{-1} (range 45.9–58.4). On the experimental day, subjects arrived at 08.00 h, after an overnight fast, and were randomised to either exercise or rest. A venous catheter was placed in an antecubital vein. There was no difference between the two groups with regard to age, weight, height or $\dot{V}_{O_2,max}$. Subjects performed 3 h of cycling at 60% $\dot{V}_{O_2,max}$, followed by 6 h of recovery. The following day they reported to the laboratory after an overnight fast. Muscle biopsy samples were obtained from the vastus lateralis prior to the exercise (0), immediately after exercise (3 h), and at 4.5, 6, 9 and 24 h, using the percutaneous needle biopsy technique with suction. Control subjects rested in the laboratory for 9 h, reported to the laboratory the day after, and had biopsy samples taken at the same time points.

Study 2. The effect of knee-extensor exercise on IL-8 release from working muscles

Two-legged knee extensor exercise was chosen as the mode of exercise in this study. This mode of exercise is mainly concentric, and isolates the quadriceps muscle, and allows for catheterization of the femoral artery and vein. Therefore, it is an advantageous method for studying arterial–venous difference across the quadriceps muscle.

Six healthy physically active male subjects ranging in age from 22 to 33 years (median 26 years), with a median height of 187 cm (range 175–193 cm) and a median weight of 78.1 kg (range 70–93 kg), participated in this study. The subjects completed a trial of two-legged knee-extensor exercise on a modified Krogh cycle ergometer (Andersen & Saltin, 1985).

One week before the trial, a two-legged knee-extensor exercise test was performed to determine the maximal workload. Resistance was increased every 2 min until a kicking frequency of 60 extensions per minute could no longer be maintained. The highest workload the subject could maintain for 2 min was set as the maximum workload. After an overnight fast, the subjects reported to the laboratory at 07.30 h on the day of the trial, voided, changed into appropriate exercise attire and rested in a supine position. After resting for 10 min, a catheter was inserted in the femoral artery and the femoral vein under local anaesthesia (20 mg ml^{-1}), as previously described (Andersen & Saltin, 1985). At rest, blood was sampled from the femoral artery and the femoral vein simultaneously, and femoral arterial blood flow was measured using an ultrasound Doppler. In brief, an annular phased array transducer probe (11.5 mm diameter) was placed just below the inguinal ligament on the common femoral artery. The probe, operating at an imaging frequency of 7.5 MHz and at variable Doppler frequencies of 4.0–6.0 MHz, was used to measure the diameter of the femoral artery at rest, and the blood flow at rest and during exercise, as described and validated (Radegran, 1997). Immediately following, a muscle biopsy sample was taken from the vastus lateralis. The subjects then performed two-legged knee-extensor exercise for 3 h at ~60% of their maximum workload. Blood flow was measured using the ultrasound Doppler method, and blood was sampled from the femoral vein and femoral artery, simultaneously, after 30 min, 1.5 and 3 h of exercise, and at 2 h of recovery. Muscle biopsy samples were obtained from the vastus lateralis at 30 min, 1.5 and 3 h of exercise, and at 2 h of recovery (5 h).

Ethics

The subjects were given both oral and written information about the experimental procedures before giving their written informed consent. All studies were approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark, and performed according to the Declaration of Helsinki.

Blood analysis

In both studies, blood samples were collected at the same time points as muscle biopsy samples. Plasma was immediately separated from blood cells by centrifugation, and stored at −80°C until analysed. Quantification of plasma IL-8 was performed using an Enzyme-linked immunosorbent assay (ELISA) for IL-8 (Sanquin, Amsterdam, The Netherlands). The range and sensitivity of the assay are 1–240 pg ml^{-1} and 1 pg ml^{-1}, respectively.

Calculations of IL-8 release

The femoral venous–arterial (fv–a) IL-8 difference was calculated by subtracting the femoral arterial concentration of IL-8 from the femoral venous concentration. The fv–a was then multiplied with the blood flow of the leg (Fick principle) to get the IL-8 balance (net release/uptake) across the leg.

Muscle biopsy samples

Biopsy samples were immediately placed on an ice-cold glass plate, cleaned of connective tissue and blood, and frozen in liquid nitrogen (20–40 mg) for further analysis.

RNA isolation

Total RNA was isolated from ~20–25 mg of tissue by a modified guanidinium thiocyanate (GT)–phenol–chloroform extraction method, which was adapted from Chomczynski & Sacchi (1987), as previously described (Pilegaard *et al.* 2000).

Reverse transcription and PCR

Reverse transcription (RT) of total RNA samples was performed using the Superscript II RNase H-system (Gibco-BRL), as previously described (Pilegaard *et al.* 2000; Hildebrandt & Neufer, 2000). RT products of total RNA samples were diluted to a total of 110–220 μl, depending on the relative differences in total RNA yield among samples. Skeletal muscle IL-8 and GAPDH mRNA content were determined by PCR using predeveloped assay reagents (Applied Biosystems). GAPDH was used as an endogenous control to normalize the mRNA content of the target genes in each sample. The mRNA content of the selected genes was determined by fluorescence-based real-time PCR technology (7900 ABI PRISM Sequence Detection System, Applied Biosystems). Seven microlitres of the diluted RT product (template) were mixed with 2× TaqMan Universal Master Mix, forward primer, reverse primer, probe and nuclease-free water, to a final volume of 36 μl. The Master Mix (Applied Biosystems) contained AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, ROX as passive reference, and optimized buffer components. The final mix was added to the PCR plate and each sample in a volume of 10 (l, and was analysed in triplicate. The PCR cycle profile used was: 50°C for 2 min +95°C for 10 min + ((95°C for 15 s + 60°C for 1 min) × 50 cycles). The triplicates had a threshold cycle coefficient of variation of no more than 2%. Data were analysed by using a comparative critical threshold (Ct) method, where the amount of target normalized to the amount of endogenous control and relative to the (pre) control sample is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems). For each subject, all samples were run together, allowing relative comparison of the samples from a given subject. To determine the specificity of the IL-8 predeveloped assay reagent, the PCR-product was separated by gel (2.5% agarose) electrophoresis, stained with ethidium bromide, and visualized by UV exposure using a CCD integrating camera (Gel Doc, Bio-Rad, Hercules, CA, USA). The agarose gel electrophoresis of the PCR product resulted in a single clearly defined band between 100 and 125 base pairs.

Immunohistochemistry

Muscle tissue was cut into 6 μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides to be used for general histology and immunohistochemistry.

For epitope retrieval, sections were preincubated overnight in tris-EGTA (TEG) buffer (1.211 g Tris, 0.95 g EGTA, 1 l distilled H_2O) at 60°C, and afterwards in 0.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40) (Sigma-Aldrich) for 15 min at room temperature to quench endogenous peroxidase. Afterwards, sections were incubated in 10% goat serum (In Vitro, Denmark) in TBS/Nonidet for 30 min at room temperature in order to block nonspecific binding.

Sections were stained for myofibrillar ATPase following preincubation at pH 4.6 in order to identify muscle fibre types. Myofibrillar ATPase staining was performed according to standard procedures (Brooke & Kaiser, 1970). Other sections were incubated overnight at 4°C with the following primary antibody: mouse antihuman IL-8 diluted 1:50 (Biosource, Germany).

These primary antibodies were detected using biotinylated goat antimouse IgG diluted 1:200

(Sigma-Aldrich), followed by the streptavidin–biotin–peroxidase complex (StreptABComplex/HRP, DakoCytomation, Denmark), prepared at manufacturer's recommended dilutions, for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin–peroxidase complex (NEN, Life Science Products, USA), which were prepared following manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature. The sections stained using immunohistochemistry were always processed simultaneously, and under the same laboratory conditions.

In order to evaluate the extent of nonspecific binding in the immunohistochemical experiments, control sections were incubated in the absence of primary or secondary antibody, or in the blocking serum. Results were considered only if these controls were negative.

In order to exclude false-positive staining due to endogenous biotin, we pretreated sections sequentially with 0.01–0.1% avidin (Sigma-Aldrich) followed by 0.001–0.01% biotin (Sigma-Aldrich), each step for 20 min, before the immunohistochemistry was performed. Comparing immunohistochemistry ±biotin blocking showed that in the tissue used muscular endogenous biotin is unlikely to induce false-positive immunostainings by binding to the streptavidin used. In order to control the specificity of the primary IL–8 antibody, we preabsorbed the primary antibody with its corresponding antigenic protein. For this purpose, we used human IL-8 protein (Biosource, Germany).

Results were considered only if this preabsorption of primary antibodies resulted in negative immunostaining. For the simultaneous examination and recording of the staining, a Zeiss Axioplan2 light microscope was used.

Statistics

Content of mRNA was normalized to GAPDH mRNA levels, and results were expressed relative to the corresponding resting (pre) sample result, which was set to 1.0. All data was tested for normality. The distribution of mRNA fold-change data was normally distributed after log transformation. All other data were normally distributed. The mRNA fold-change values are presented as geometric mean ± geometric S.E.M. All other values are presented as means ± S.E.M. One-way analysis of variance (ANOVA) for repeated measures (RM) was used to analyse the main effect of time on all variables. A two-way RM ANOVA was used to evaluate the effect of exercise and time in study 1. Student–Newman–Keuls *post hoc* test was used to locate specific differences across time. Differences were considered significant at $P < 0.05$. Data were analysed using Sigma Stat 3.0 (SPSS, Inc., Chicago, USA).

Results

Study 1. The effect of bicycle exercise on muscle IL-8 mRNA and protein expression

Skeletal muscle IL-8 mRNA increased in response to exercise when compared to pre-exercise samples, and when compared to resting values (Fig. 1). A significant increase

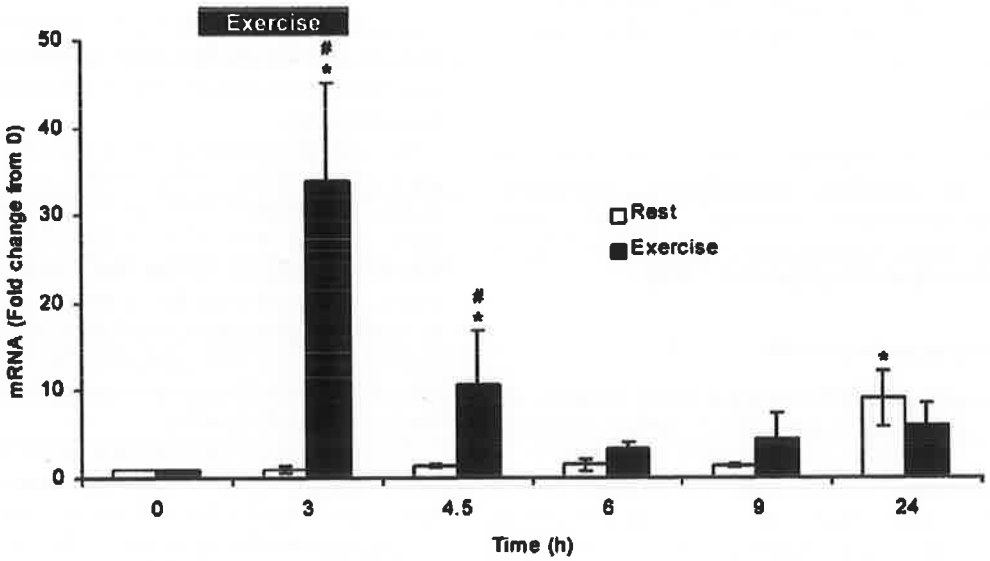

Figure 1. Muscle IL-8 mRNA in rest and exercise groups
The exercise group: pre (0 h), immediately after 3 h of bicycle exercise (3 h), and 1.5 (4.5 h), 3 (6 h), 6 (9 h) and 21 h (24 h) postexercise. The rest group: pre (0 h), after 3, 4.5, 6 and 9 h of rest, and 24 h after the presample. Values are means ± S.E.M. ($n = 6$ subjects). *Significant ($P < 0.05$) difference from pre sample (0 h), and #significant ($P < 0.05$) difference from rest group.

in IL-8 mRNA was seen at two time points in the exercise group, 3 h and 4.5 h, which were also significantly different from the rest group ($P < 0.05$). A significant increase in IL-8 mRNA compared to the pre sample was seen at 24 h in the rest group (Fig. 1). This change was not significantly different from the exercise group (Fig. 1).

The IL-8 protein was not expressed in muscle tissue before exercise ($n = 12$, Fig. 2*A*), and repetitive muscle biopsy samples at rest did not show induction of IL-8 expression ($n = 6$, data not shown). By the end of the exercise period (3 h), the immunohistochemistry for IL-8 was still not increased in the muscle tissue (Fig. 2*B*). At time points 4.5 (Fig. 2*C*), 6 (Fig. 2*E*), 9 (Fig. 2*F*, *H* and *I*) and 24 h (Fig. 2*G*), there was a strong immunoreaction for IL-8 within muscle fibres, the peak being at 6–9 h (Fig. 2*E*, *F*, *H* and *I*). At time point 4.5 h, a parallel section was stained for myofibrillar ATPase to identify muscle fibre types (Fig. 2*D*). As shown, IL-8 expression was increased similarly in type 1 and 2 muscle fibres at time point 4.5 h (Fig. 2*C* and *D*). Moreover, this IL-8 fibre-type expression pattern was observed at all the time points studied (not shown).

The plasma concentrations in the rest group and the exercise group did not change significantly over time, and the IL-8 plasma concentrations in the two groups were not significantly different from each other (Fig. 3).

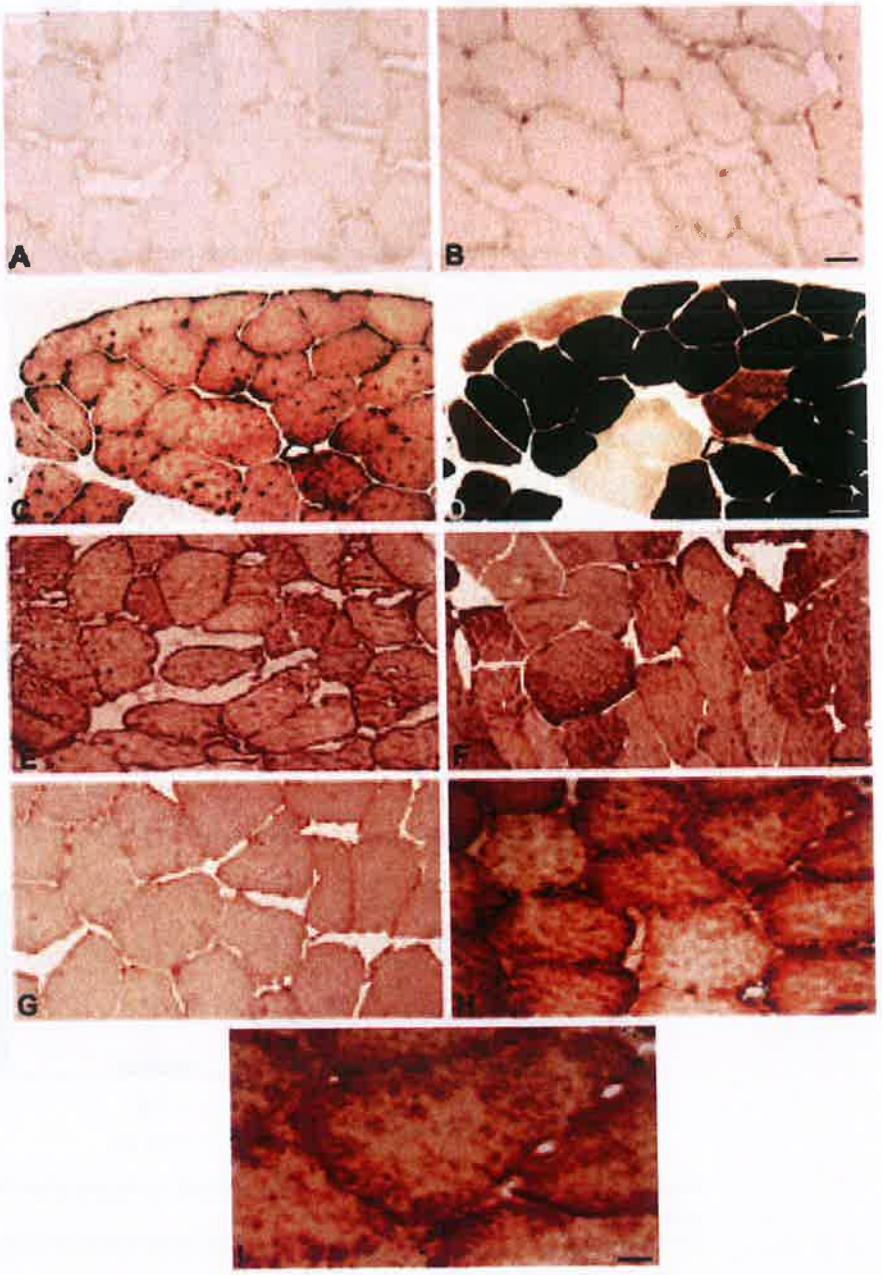

Figure 2. IL-8 immunohistochemical expression in skeletal muscle tissue before and after 3 h of bicycle exercise
A, before the exercise began, IL-8 expression was almost absent in the muscle tissue. *B*, By 3 h, when the exercise had just ended, the muscle tissue showed a comparable IL-8 expression relative to the level seen before exercise. *C*, by 4.5 h after exercise, the IL-8 expression was significantly increased in the skeletal muscle tissue, which showed a high level of IL-8 both in general in the cytoplasm and related to the cell membranes, as well as in vessels in the muscle. *D*, ATPase-stained section. This is the neighbouring section to that seen in *C*. By comparing *C* and *D*, it is seen that both muscle fibre types express IL-8 after exercise. *E* and *F*, IL-8 expression was still very high, and peaked at 6 (*E*) and 9 h (*F*) following the bicycle exercise. *G*, after 24 h, the levels of IL-8 protein had decreased again, and the staining appeared homogeneous and mildly increased in the fibres. *H* and *I*, higher magnification of skeletal muscle tissue at 9 h following exercise. As shown, IL-8 protein is mainly expressed in the cytoplasm, and it is also expressed in the membranes, including the nuclei. We also detected intermittently vascular IL-8 expression in the vessel endothelium of the muscle tissue. Scale bars: *A*–G, 50 μm; *H*, 29 μm; *I*, 14 μm.

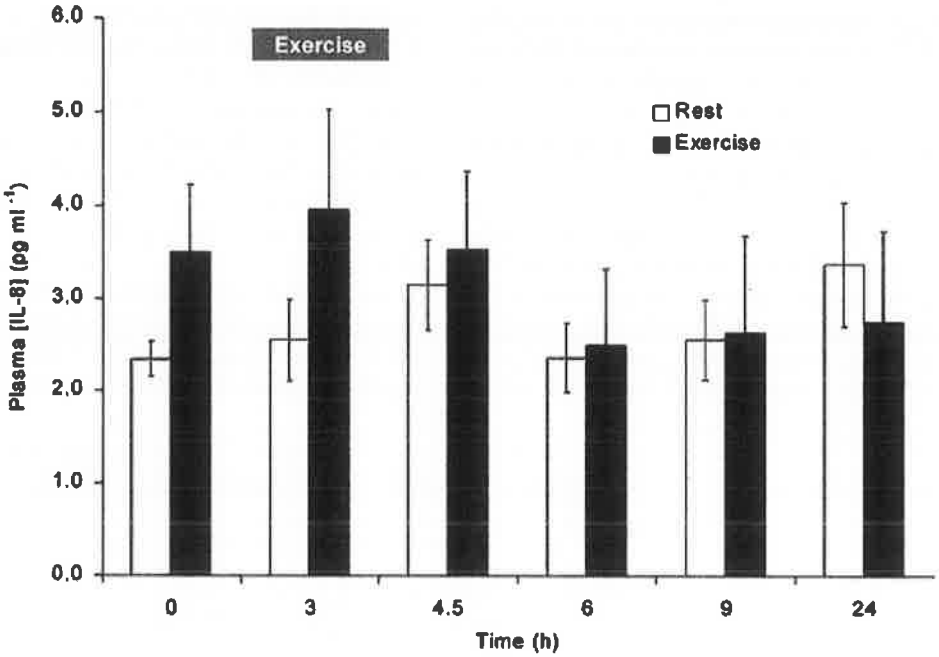

Figure 3. IL-8 venous plasma concentrations in rest and exercise groups
The exercise group: pre (0 h), immediately after 3 h of bicycle exercise (3 h), and 1.5 (4.5 h), 3 (6 h), 6 (9 h) and 21 h (24 h) postexercise. The rest group: pre (0 h), after 3, 4.5, 6, and 9 h of rest, and 24 h after the presample. Values are means ± S.E.M. ($n = 6$ subjects).

Study 2. The effect of knee-extensor exercise on IL-8 release from working muscles

IL-8 mRNA content in skeletal muscle increased in response to knee-extensor exercise (Fig. 4). At 1.5, 3 and 5 h, IL-8 mRNA was significantly increased when compared with pre-exercise ($P < 0.05$). The level of IL-8 mRNA peaked after 3 h of exercise, reaching a 225-fold increase (Fig. 4). As depicted in Fig. 5*A*, mean arterial plasma IL-8 concentration averaged 8.7 ± 2.6 pg ml^{-1} at rest, and was not altered during or after exercise. However, the leg fv–a IL-8 difference was affected by exercise

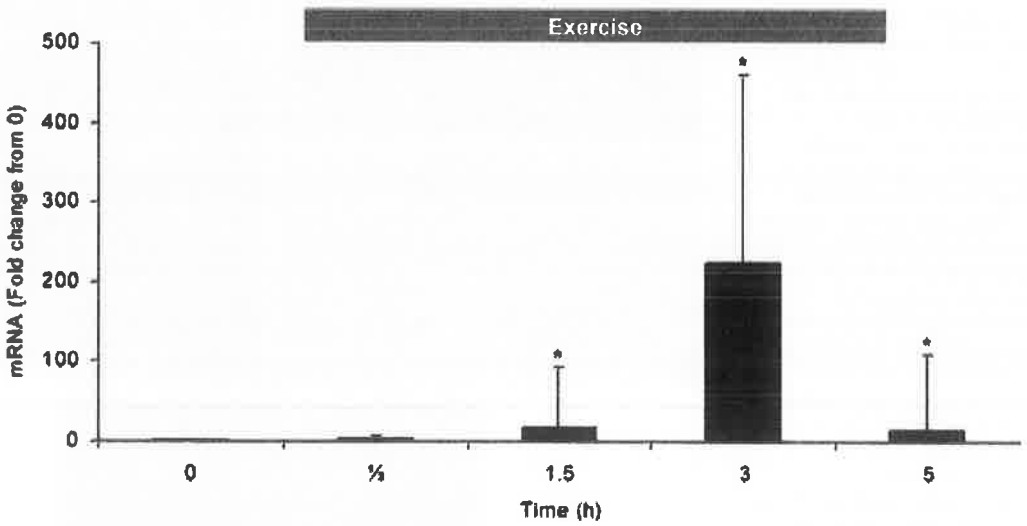

Figure 4. Muscle IL-8 mRNA before (0), during (0.5 and 1.5 h) and after 3 h of two-legged knee-extensor exercise, and 2 h after the end of exercise (5 h)
Values are means ± S.E.M. ($n = 6$). *Significant ($P < 0.05$) difference from before (0 h).

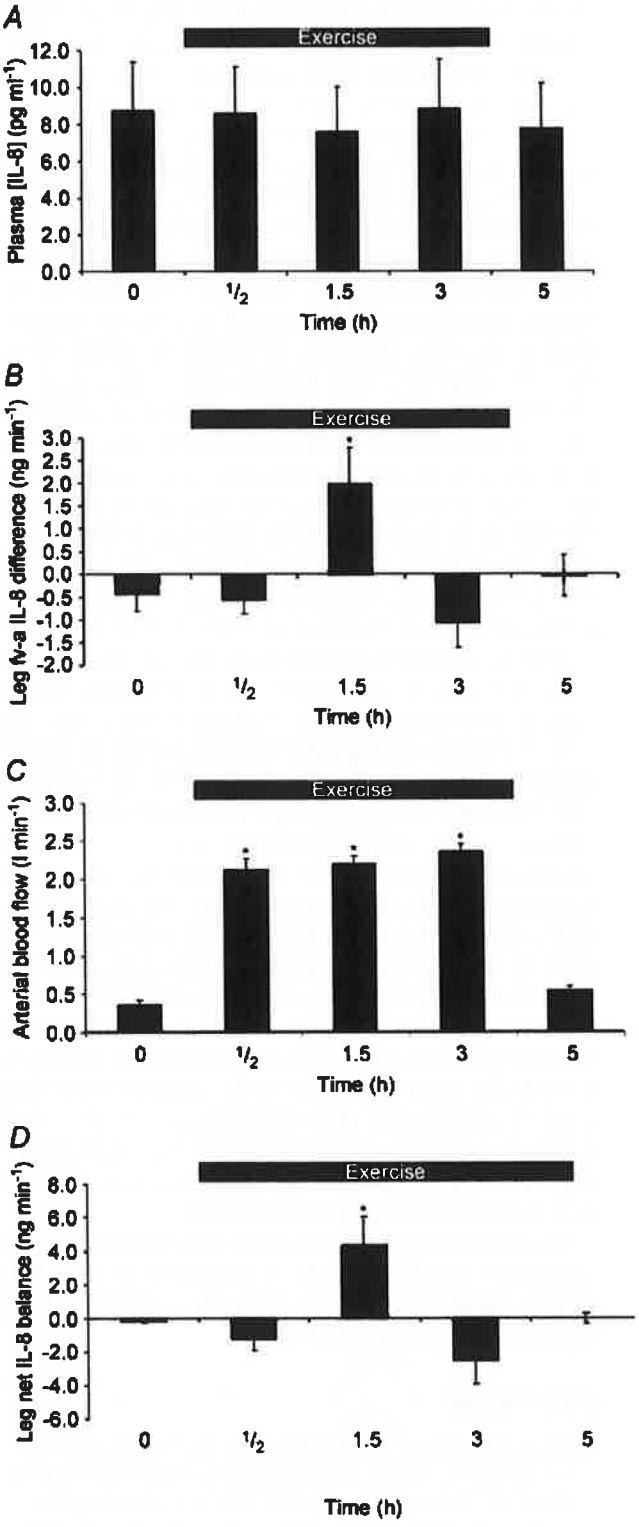

Figure 5
A, IL-8 arterial plasma concentration before (0 h), during (0.5 and 1.5 h) and after 3 h of two-legged knee-extensor exercise, and 2 h (5 h) after the end of exercise. Values are means ± S.E.M. ($n = 6$). *B*, leg plasma femoral venous–arterial (fv–a) IL-8 difference before (0 h), during (0.5 and 1.5 h) and after 3 h of two-legged knee-extensor exercise, and 2 h (5 h) post exercise. Values are means ± S.E.M. ($n = 6$). *Significant difference ($P < 0.05$) from before (0 h). *C*, leg blood flow before (0 h), during (0.5 and 1.5 h) and after 3 h of two-legged knee-extensor exercise, and 2 h (5 h) after the end of exercise. Values are means ± S.E.M. ($n = 6$). *Significant difference ($P < 0.05$) from before (0 h). *D*, leg net IL-8 balance (ng min^{-1}) values before (0 h), during (0.5 and 1.5 h) and after 3 h of two-legged knee-extensor exercise, and 2 h postexercise (5 h). Values are means ± S.E.M. ($n = 6$). *Significant difference ($P < 0.05$) from before (0 h).

(Fig. 5*B*). After 1.5 h of exercise, the venous concentration of IL-8 was slightly increased, resulting in an fv–a for IL-8 of 1.95 ± 0.8 pg ml^{-1}, which was significantly different from all other time points. None of the other time points were significantly different from the pre (0 h) value or from each other (Fig. 5*B*). Prior to exercise, mean arterial blood flow measured 351.5 ± 59.7 ml min^{-1}. Exercise increased blood flow significantly ($P < 0.05$) at all time points during the exercise period as depicted in Fig. 5*C*. Two hours post-exercise, mean arterial blood flow was reduced to 550.5 ± 59.3 ml kg^{-1}, which was not significantly different from blood flow prior to exercise ($P = 0.091$). The IL-8 balance over the working limb was calculated as previously described. Thus, following 1.5 h of exercise, a significant IL-8 net release of 5.1 ± 1.7 ng min^{-1} was observed across the thigh (Fig. 5*D*). The difference was significantly different from all other time points. No other observation deviated significantly from the pre sample.

Discussion

This is the first study to demonstrate that exercise induces expression of the IL-8 protein in the cytoplasm of skeletal muscle fibres. The finding of a marked increase of IL-8 mRNA in muscle biopsy samples during and following exercise, and IL-8 protein expression within skeletal muscle fibres in the recovery from exercise, strongly indicates that exercise *per se* stimulates muscle cells to produce IL-8. This finding is in accordance with the finding that muscle cells *in vitro* have the capacity to express IL-8 both at the mRNA and protein levels (De Rossi *et al.* 2000).

The finding of increased IL-8 mRNA and expression of IL-8 protein in skeletal muscle biopsy samples obtained during and after exercise adds to the findings of the pioneering work by Nieman and coworkers. Those authors reported increased IL-8 mRNA in skeletal muscle biopsy samples after 3 h of exhaustive treadmill running (Nieman *et al.* 2003). Treadmill running involves a substantial eccentric component leading to skeletal muscle damage and an associated inflammatory response (Brenner *et al.* 1999). It is possible that invading monocytes and macrophages represent the cell sources of IL-8 mRNA in the study by Nieman *et al.* (2003). However, our finding of a marked increase in IL-8 mRNA and IL-8 protein within muscle fibres in two independent concentric exercise

models adds strong support to previous indications that IL-8 is produced by the muscle (Figs 1, 2 and 4). In support of our finding, an increase in IL-8 mRNA following concentric exercise has recently been found by others (Chan *et al.* 2004). Concentric exercise is not associated with muscle damage or inflammation (Sorichter *et al.* 1997; Brenner *et al.* 1999), which rules out an inflammatory response being the cause of the IL-8 expression in exercising skeletal muscle in this study. Several tissues exhibit stimulus-dependent expression of IL-8. The promoter region of the IL-8 gene contains a nuclear factor (NF)-κB binding site, and binding of NF-κB is sufficient for gene transcription in all cells studied (Hoffmann *et al.* 2002). Yu and coworkers have shown intracellular Ca^{2+}-dependent activation of IL-8 gene expression in human colonic epithelial cells (Yu *et al.* 2001), and adenosine has also been shown to mediate IL-8 gene expression in mast cells (Feoktistov *et al.* 1999). Adenosine increases in the interstitial space as a response to exercise (Hellsten *et al.* 1998), and the concentration of calcium (Ca^{2+}) rises temporarily in association with skeletal muscle contraction. Adenosine and Ca^{2+} could thus represent possible pathways of IL-8 gene transcription activation in skeletal muscle as a response to exercise. However, no studies attempting to elucidate the induction pathway of IL-8 gene expression have been performed in skeletal muscle cells, which leaves the transcriptional activation mechanism unknown

Muscle biopsy samples obtained by the percutaneus needle biopsy method contain a relatively small portion of endothelial cells in the form of capillaries (Qu *et al.* 1997). Human endothelial cells express IL-8 when stimulated with IL-1 or TNF-α *in vitro* (Zhao *et al.* 2003). It is unlikely that capillary endothelial cells are the source of IL-8 mRNA, as IL-1 and TNF-α plasma concentrations are not elevated in response to concentric exercise (Pedersen *et al.* 1998). There was a slight increase in IL-8 mRNA in the control group at 24 h, which was not significantly different from the exercise group (Fig. 1). Multiple biopsy samples can induce an inflammatory response in the muscle (Malm *et al.* 2000), which might explain the slight increase in mRNA. Alternatively, it could reflect light exercise activity before the subjects returned to the laboratory the following morning.

There is a clear staining for IL-8 in the cytoplasm of the muscle cell, which indicates that myocytes are the true source of IL-8. A clear expression of the protein is seen at 4.5 h, with peaks at 6 and 9 h (Fig. 2*A–C* and *E–G*). A marked increase in the concentration of systemic IL-8 has been a consistent finding in studies with an eccentric exercise component (Nieman *et al.* 2001, 2002, 2003; Suzuki *et al.* 2003), whereas the findings are less clear in concentric exercise. Mucci *et al.* (2000) found a minimal increase (2 pg ml^{-1}) in plasma IL-8 during intense ergometer bicycling for 10–15 min, whereas neither Henson and coworkers nor Chan *et al.* found a change in plasma IL-8 after 2 h of rowing or 1 h of bicycle exercise (Henson *et al.* 2000; Chan *et al.* 2004), respectively. Possibly, the systemic increase in IL-8 as seen during exercise with an eccentric component is mainly due to an inflammatory response. In accordance, we observed no increase in the systemic IL-8 plasma concentration during or after exercise in study 1 or 2. A small and transient fv–a difference, and thus a net release of IL-8 (Fig. 5*C* and *D*), which did not result in an increase in the systemic IL-8 plasma concentration, was found at 1.5 h of exercise in study 2 (Fig. 5*A*). However, we found no release of IL-8 after 3 h (Fig. 5*D*). The finding that a high local IL-8 expression takes place in working muscle with only a small and transient release could indicate that muscle-derived IL-8 exerts its effect in an endocrine or paracrine fashion. Moreover, it is possible that an exercise factor inhibits the release of IL-8. Dexamethasone has been shown to inhibit IL-8 release in cultured epithelial cells (Chang *et al.* 2001). Plasma concentration of cortisol increases during prolonged exercise (Kjaer *et al.* 1988; Viru *et al.* 1992). It may be speculated that an exercise-related cortisol increase could function as an inhibitor of IL-8 release, and thus explain the attenuation of IL-8 release at 3 h of exercise.

IL-8-induced angiogenesis is distinct from its ability to induce inflammation (Keane *et al.* 1997). IL-8 associates with the CXC receptors 1 and 2 (CXCR1 and CXCR2) (Belperio *et al.* 2000). CXCR2 is expressed by human microvascular endothelial cells, and is the receptor responsible for IL-8-induced angiogenesis (Addison *et al.* 2000). The finding that a high local IL-8 expression takes place in working muscle, which is accompanied by only a small and transient release, indicates that muscle-derived IL-8 exerts its effect locally. The IL-8 produced by the exercising limb might elicit its response by interacting with the CXCR2 receptor present in the capillary (endothelial tissue) (Addison *et al.* 2000; Heidemann *et al.* 2003). Given that IL-8 is a potent angiogenic factor in several tissues, we suggest that a possible role of the skeletal muscle-derived IL-8 is to stimulate neovascularization.

In conclusion, the present study demonstrates that pure concentric exercise induces a marked IL-8 mRNA and protein expression within muscle fibres, without changes in plasma concentration of IL-8.

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The Journal of Physiology

RETRACTION

Retraction: Exercise induces interleukin-8 expression in human skeletal muscle

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Retraction: The following article from *The Journal of Physiology*, 'Exercise induces interleukin-8 expression in human skeletal muscle', by Thorbjorn C. A. Akerstrom, Adam Steensberg, Pernille Keller, Charlotte Keller, Milena Penkowa and Bente Klarlund Pedersen, published in *The Journal of Physiology* 563(2): 507–516, and online ahead of print on 23 December 2004 (doi:10.1113/jphysiol.2004.077610), has been retracted by agreement between Thorbjorn C. A. Akerstrom, Adam Steensberg, Pernille Keller, Charlotte Keller, Bente Klarlund Pedersen, the Editor-in-Chief, David J. Paterson, and Blackwell Publishing Ltd. The retraction has been agreed because:

- Figure 2*A* (pre-exercise) and Fig. 2*G* (24 h time point) represent the same tissue sample but the sections presented have been moved slightly and the level of colouring of the tissue section modified;
- Figure 2*A* is identical to Fig. 3*D* in the article 'Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6' by Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J, Pedersen BK, *FASEB Journal* 19(9):1181–3.

We apologize to *The Journal of Physiology* for the inconvenience. We further apologize to our colleagues for any difficulties caused by the publication of this paper.

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Experimental Physiology

Exercise-induced metallothionein expression in human skeletal muscle fibres

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Exercise induces free oxygen radicals that cause oxidative stress, and metallothioneins (MTs) are increased in states of oxidative stress and possess anti-apoptotic effects. We therefore studied expression of the antioxidant factors metallothionein I and II (MT-I + II) in muscle biopsies obtained in response to 3 h of bicycle exercise performed by healthy men and in resting controls. Both MT-I + II proteins and MT-II mRNA expression increased significantly in both type I and II muscle fibres after exercise. Moreover, 24 h after exercise the levels of MT-II mRNA and MT-I + II proteins were still highly increased and the MT-II mRNA expression reached a 15-fold increase. As expected, immunohistochemical detection of malondialdehyde (MDA) and nitrotyrosine (NITT) showed that formation of free radicals and oxidative stress were clearly increased in exercising muscle peaking shortly after the end of exercise in both type I and II muscle fibres. This is the first report demonstrating that MT-I + II are significantly induced in human skeletal muscle fibres following exercise. As MT-I + II are antioxidant factors that protect various tissues during pathological conditions, the MT-I + II increases post exercise may represent a mechanism whereby contracting muscle fibres are protected against cellular stress and injury.

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Metallothionein (MT) is a ubiquitous, low molecular-weight, metal-binding, cysteine-rich protein that accumulates under conditions of oxidative stress. MT exists in several isoforms, of which MT-I + II are coordinately regulated and are also the best characterized MTs (for review see Aschner *et al.* 1997; Hidalgo *et al.* 2001; Penkowa, 2003; Theocharis *et al.* 2003).

MT-I + II are significant antioxidant factors, which also have key anti-apoptotic functions, whereby MT-I + II protect tissues and cells during various pathological conditions (Aschner *et al.* 1997; Viarengo *et al.* 2000; Penkowa *et al.* 2000, 2001; Hidalgo *et al.* 2001, 2002; Giralt *et al.* 2002*b*; Penkowa, 2003). MT-I + II act by inhibiting reactive oxygen species (ROS)-induced degradation of DNA and tissue damage (Abel & de Ruiter, 1989; Cai *et al.* 2000; Kling & Olsson, 2000; Rana & Kumar, 2000), and by scavenging and inhibiting the formation of ROS (Thornalley & Vasak, 1985; Schwarz *et al.* 1994, 1995; Lazo *et al.* 1998; Viarengo *et al.* 2000). MT-I + II have important roles during oxidative stress by interaction with reduced glutathione (GSH)(Jiang *et al.* 1998), and by inhibiting GSH depletion (Haidara *et al.* 1999). Moreover, MT-I + II can antagonize the deleterious effects of oxidative stress on catalase (Haidara *et al.* 1999).

It has been shown that genetic MT-I overexpression as well as infusion of MT-II protects against oxidative stress and tissue injury (Schwarz *et al.* 1994, 1995; Pitt *et al.* 1997; Suzuki *et al.* 2000; Giralt *et al.* 2002*b*; Penkowa *et al.* 2002, 2003*a*). In addition, genetic MT-I + II deficiency leads to a dramatic increase in oxidative stress and cellular damage (Lazo & Pitt, 1995; Zheng *et al.* 1996; Carrasco *et al.* 2000; Suzuki *et al.* 2000; Penkowa *et al.* 2000, 2001; Giralt *et al.* 2002*a*).

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A substantial amount of data indicates that exercise is associated with an increase in the production of free radicals and ROS by skeletal muscle (Davies *et al.* 1982; Jackson *et al.* 1985). This increase appears to occur because a proportion of the molecular oxygen used in normal respiration undergoes a one-electron reduction to generate superoxide radicals (Boveris *et al.* 1972). It has been estimated that exercise can increase oxygen utilization 200-fold above resting levels in active muscle fibres and superoxide production appears to increase with this large increase in oxygen flux through muscle mitochondria during exercise. The potential consequences of the exercise-induced increase in oxidative stress have been addressed in a number of studies. Data have been presented suggesting that oxidative stress plays a role in the muscle fatigue or damage (Andrade *et al.* 1998) that accompanies some forms of exercise, although these roles remain contentious (Jackson, 1996; Patwell *et al.* 2004). Some studies report that an acute bout of exercise increases the activities of superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and catalase in skeletal muscle of rats (Ji, 1993). In humans, exercise training has been reported to increase skeletal muscle SOD activity (Jenkins *et al.* 1984) and the activities of various protective enzymes in blood (Robertson *et al.* 1991). Oxidative and other stresses to cells are also known to induce the formation of heat shock proteins (HSPs). HSPs represent important components of the cellular protective response and data also indicate that an increase in muscle HSP content occurs after exercise in rodents (Salo *et al.* 1991; Kelly *et al.* 1996; Hernando & Manso, 1997; McArdle *et al.* 2004) and in humans (Febbraio & Koukoulas, 2000; Moseley, 2000; Khassaf *et al.* 2001; Febbraio *et al.* 2002). These proteins are thought to facilitate repair from injury and to aid adaptation and remodelling of the cell to prevent the damage from recurring after a repeat of the same stress (Patwell *et al.* 2004; McArdle *et al.* 2004). Thus, intramyocelluar expression of some HSPs may counteract the muscle-damaging effects of oxidative stress.

Given that MT-I + II are induced by oxidative stress and protect against tissue damage, we suggest that these proteins might represent an alternative pathway whereby muscle fibres are protected from oxidative stress-induced damage. Little information exists with regard to MT and physical activity. In 1978 it was shown that MT synthesis increased in the rat liver after strenuous exercise (Oh *et al.* 1978), whereas a later study found that chronic exercise led to a decrease in the amounts of MT-I + II in aortic vessel tissue (Bobillier *et al.* 2001). Here we demonstrate that physical exercise, and the associated muscular oxidative stress, is accompanied by increased MT-I + II expression within human skeletal muscle fibres.

Methods

Eighteen healthy men (median age, 27 years (range, 20–30); height, 189 cm (range, 164–197); weight, 80 kg (range, 64–96)) participated in the study. The subjects were given both oral and written information about the experimental procedures before giving their written informed consent. The study was approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark, and performed according to the declaration of Helsinki.

One week before the first experimental day, subjects performed a maximal oxygen uptake test ($V_{O_2,max}$) on an ergometer bicycle. Measurement of $V_{O_2,max}$ was performed on an electrically braked cadence-independent cycle ergometer (Monark 839E, Monark Ltd, Varberg, Sweden). Subjects cycled for 3 min at 100 W as a warm-up followed by cycling at progressively higher work rates, increasing 50 W every 3 min for 9 min and then increasing the workload by 25 W every minute until subjects were unable to maintain a cadence of 60 r.p.m. Expired oxygen (V_{O_2}) and carbon dioxide (V_{CO_2}) concentrations were recorded online. Median $V_{O_2,max}$ was 46.7 ml kg^{-1} min^{-1} (range, 41–68). On the experimental day, subjects arrived at 08.00 h after an overnight fast and were randomly assigned to either exercise or rest. There was no difference between the two groups regarding age, weight, height or $V_{O_2,max}$. Subjects performed 3 h of cycling ($n = 12$) at 60% $V_{O_2,max}$ followed by 6 h of recovery. The following day they reported to the laboratory after an overnight fast. Prior to the exercise (0), immediately after the exercise (3 h), and at 4.5, 6, 9 and 24 h, muscle biopsies were obtained from the vastus lateralis using the percutaneous needle biopsy technique with suction. Control subjects rested in the laboratory for 9 h ($n = 6$), reported to t he laboratory the day after and had biopsies collected at the same time points.

Tissue processing

Muscle tissue was cut in 6-μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides, to be used for general histology and immunohistochemistry.

Sections were preincubated in 0.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (0.05 M Tris, pH 7.4 and 0.15 M NaCl with 0.01% Nonidet P-40; Sigma-Aldrich, St Louis, MO, USA) for 15 min at room temperature (20°C) to quench endogenous peroxidase. Afterwards, sections were washed in TBS/Nonidet three times for 5 min. Sections were also preincubated with 10% normal goat serum (In Vitro, DK) in TBS/Nonidet for 30 min at room temperature in order to block non-specific binding.

Histochemistry

Haematoxylin and eosin (HE) staining of the sections was performed according to standard procedures. Also, myofibrillar ATPase staining with preincubation at pH 4.6 was used to identify muscle fibre types (Brooke & Kaiser, 1970).

Immunohistochemistry

The sections stained immunohistochemically were always processed simultaneously and under the same laboratory conditions.

Sections were incubated overnight at 4°C with one of the following primary antibodies: mouse anti-horse MT-I + II diluted 1 : 50 (Dakopatts, DK); rabbit anti-rat MT-I + II diluted 1 : 500 (Gasull *et al.* 1993); rabbit anti-malondialdehyde (MDA) diluted 1 : 100 (Alpha Diagnostics, San Antonio, TX, USA) (as a marker of lipid peroxidation/oxidative stress); and rabbit anti-nitrotyrosine (NITT) diluted 1 : 200 (Alpha Diagnostics) (as a marker of peroxynitrite-induced protein nitration/oxidative stress). These primary antibodies were detected using biotinylated goat anti-mouse IgG diluted 1 : 200 (Sigma-Aldrich) or biotinylated mouse anti-rabbit IgG diluted 1 : 400 (Sigma-Aldrich) for 30 min at room temperature followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, DK) prepared according to the manufacturer's recommended dilutions for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, Boston, MA, USA) pre-pared according to the manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

In order to evaluate the extent of non-specific binding in the immunohistochemical experiments, control sections were incubated in the absence of primary antibody or in the absence of secondary antibody or in the blocking serum. Results were considered only if these controls were negative.

In order to exclude staining due to endogenous biotin, we have pretreated sections sequentially with 0.01–0.1% avidin (Sigma-Aldrich) followed by 0.001–0.01% biotin (Sigma-Aldrich), each step for 20 min at room temperature, before the immunohistochemical analysis was performed. Comparing our immunohistochemical stainings with/without specific biotin blocking showed that in the tissue, muscular endogenous biotin is unlikely to induce false-positive immunostaining by binding to the streptavidin. In order to control the specificity of the primary antibodies we have pre-absorbed the primary antibodies with their corresponding antigenic proteins. For this purpose we have used MT-I and MT-II (both from Sigma-Aldrich) proteins for the anti-MT-I + II antibodies, nitrosylated/nitrated proteins (Alpha Diagnostics) for the anti-NITT antibodies and MDA-ovalbumin (Alpha Diagnostics) for the anti-MDA antibodies. Results were considered only if this pre-absorbtion of primary antibodies resulted in negative immunostaining.

For the simultaneous examination and recording of the staining, a Zeiss Axioplan2 light microscope was used.

PCR

RNA extraction. RNA was extracted using Trizol (Life Technologies) according to the manufacturer's instructions. In short, 1 ml Trizol was added to approximately 20 mg muscle tissue and homogenized using a Polytron (PT-MR2100, Kinematica) on setting 25–30 for 20–30 s and placed on ice. Chloroform (100 μl) was added to all samples, which were then shaken vigorously and incubated for 5 min on ice. Samples were spun at 12 000 *g* for 15 min at 4°C, and the upper aqueous phase was placed in a fresh Eppendorph tube. The same volume of isopropanol was added and samples were placed at −20°C for 1 h followed by centrifugation at 12 000 *g* for 15 min at 4°C. The resulting RNA pellet was washed with 75% ethanol in diethylpyrocarbonate (DEPC)-treated water and spun at 6000 *g* for 10 min at 4°C. The pellets were dissolved in 15 μl DEPC-treated water.

Reverse transcription. Total RNA (1 μg) was reverse transcribed in a 50-μl reaction according to the manufacturer's instructions (Applied Biosystems, Taqman reverse transcription reagents) with the use of Oligo dT primers. The reactions were run in a Perkin Elmer GeneAmp PCR system 9700 at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

Analysis of gene expression levels in muscle tissue. MT-II primers and Taqman probe were designed using Primer Express 2.0. The primers and probe spanned an intron boundary to prevent amplification of genomic DNA. To ensure the specificity of the primers and probe a gel was run, where a single band of the correct size of 103 bp was obtained. MT-II: forward primer, 5′-CGCCATGGATCCCAACT-3′; reverse primer, 5′-GCAGCTTTTCTTGCACGAAGT-3′; Taqman probe, 5′-CCGCCGGTGACTCCTGCACCT-3′. The reaction conditions for the MT-II real-time PCR were:

forward primer, 50 nM, reverse primer 900 nM and Taqman probe 200 nM.

Samples were analysed for MT-II mRNA levels by real-time PCR using an ABI PRISM 7900 sequence detector (PE Biosystems). Samples were run in triplicate under standard real-time PCR conditions: 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The gene expression levels were normalized to the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH; obtained from Applied Biosystems). All reactions were run under singleplex conditions. Data were quantified and normalized using the standard curve method. A linear correlation of 0.99 was obtained for the amplification of both MT-II and GAPDH cDNA.

Statistics

Data for MT-II/GAPDH mRNA ratios were normally distributed after log-transformation. A two-way RM-ANOVA was used to detect changes over time or between groups. Student–Newman–Keul's *t* test for *post hoc* analysis was used to detect changes over time from resting values or differences between groups. $P < 0.05$ was considered significant. Statistical calculations were performed using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA). Data are presented as geometric mean ± S.E.M. of MT-II/GAPDH mRNA ratios.

Results

MT-II mRNA levels

MT-II mRNA levels in skeletal muscle biopsies increased gradually ($P < 0.05$) following one bout of exercise, reaching 11- to 15-fold increases at 24 h after exercise, whereas MT-II mRNA was hardly detectable in resting controls (Fig. 1)

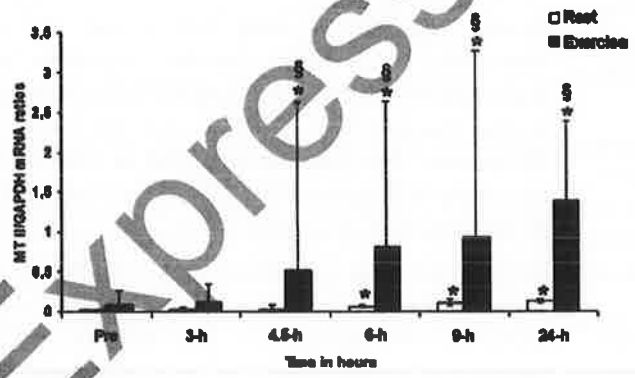

Figure 1. Muscle MT II/GAPDH mRNA ratios in response to 3 h of bicycle exercise

MT-II mRNA levels continuously increased ($P < 0.05$) in exercising subjects ($n = 6$) and by 24 h, MT-II mRNA reached a 15-fold increase. Data are presented as geometric mean ± S.E.M. of MT II/GAPDH mRNA ratios. §Significant difference between groups. *Significant change over time. MT II mRNA was on the border of detection in the resting control group ($n = 5$).

MT-I + II protein expression

Resting subjects demonstrated very low or zero MT-I + II expression in the muscle tissue (Fig. 2*A–C*). By the end of the exercise period (3 h), the immunohistochemical staining for MT-I + II was increased in all fibre types of the muscle tissue (Fig. 2*D*). However, some muscle fibres were strongly immunoreactive for MT-I + II, while others showed a smaller increase. This variety in MT-I + II staining intensity was only pronounced at 3 h when the exercise ended, and it could not be attributed to muscle fibre types, as judged by comparing myofibrillar ATPase staining (not shown) with MT-I + II immunohistochemistry in neighbouring sections. After 4.5 h and especially after 6–9 h, the levels of MT-I + II had further increased (Fig. 2*E–G*), and by then the tissue showed a very strong, homogenous MT-I + II immunostaining throughout the muscle biopsy. Accordingly, every fibre type of the muscle (Type I, Type IIa or Type 2x) displayed increases in MT-I + II. By 24 h, the levels of MT-I + II proteins had further increased (Fig. 2*H*), and all muscle fibres showed intense and homogenous MT-I + II immunohistochemical staining. There was no MT-I + II immunostaining present in between muscle fibres. Thus, exercise significantly increased MT-I + II expression levels, which remained highly increased by 24 h post exercise (Fig. 2).

Oxidative stress

In order to determine oxidative stress levels, we determined NITT and MDA immunoreactivity.

Muscle tissue from resting subjects showed no immunostaining for NITT (Fig. 3*A–C*) and MDA, while after the exercise, an increase in NITT (Fig. 3) and MDA (not shown) immunoreactivity was observed. Hence, right after the exercise at 3 h, both NITT (Fig. 3*D*) and MDA immunoreactivity clearly increased and the levels remained high until 24 h. However, NITT (Fig. 3*E*) and MDA levels peaked by 4.5 h. Accordingly, the levels of oxidative stress are high, peaking shortly after the exercise and before MT-I + II expression reached high levels.

When myofibrillar staining was compared with immunostaining for NITT and MDA in neighbouring sections, it appeared that there were no differences between muscle fibre types (Type I, Type IIa or Type 2x) with regard to oxidative stress (not shown).

Discussion

Here we demonstrate that physical exercise induces the expression of the antioxidant factors MT-I + II on the mRNA as well as the protein level within the cytoplasm of skeletal muscle fibres. Interleukin (IL)-6 gene expression is enhanced in contracting skeletal muscles (Steensberg *et al.* 2000; Keller *et al.* 2001) and IL-6 immunohistochemical staining is significantly increased in human skeletal muscle cells after exercise (Penkowa *et al.* 2003*b*). As IL-6 is a major inducer of MT-I + II (Hernandez *et al.* 1997; Carrasco *et al.* 1998; Penkowa *et al.* 2000), the increased IL-6 expression in muscle tissue in response to exercise might explain the increases in MT-I + II. Alternatively, exercise is associated with oxidative stress (Vina *et al.* 2000; Cooper *et al.* 2002), which is also a significant inducer of MT-I + II expression (Pitt *et al.* 1997; Jiang *et al.* 1998; Molinero *et al.* 1998; Penkowa *et al.* 2000; Viarengo *et al.* 2000; Ghoshal & Jacob, 2001; Hidalgo *et al.* 2001, 2002).

As control participants (the resting subjects) had biopsies taken at identical time points as the subjects who performed exercise, it is unlikely that the oxidative stress observed after 3 h exercise could be caused simply by the recurring biopsies that could induce inflammation and ROS formation by infiltrating leucocytes.

However, the observed dynamics of MT-II mRNA *versus* MT-I + II protein expression are rather surprising, as the mRNA increases should precede those of the proteins. This might be linked to the fact that the immunohistochemistry is presumably detecting all or most of the many human MT-I + II isoproteins, while the mRNA measured was only for a subtype of MT-II (subtype MT-IIa). As it is not possible to raise an antibody against either the MT-I or the MT-II isoprotein without mutual cross-reaction, this should be addressed by additional detection of the different types of MT-I mRNA as well as MT-II mRNA. On the other hand, the observed levels of mRNA *versus* proteins could be explained if human MT-I mRNA is induced and translated faster relative to MT-II mRNA. Moreover, it can not be ruled out that muscle MT-II mRNA translation is more efficient during exercise or is enhanced in the first days after exercise. The MT-II mRNA levels observed in the 24 h post-exercise samples showed a high inter-individual variation, and in support of this, MT-I + II display extensive genetic polymorphism (Hidalgo *et al.* 2001, 2002). Even though, the MT-I + II proteins did not show such variance between the participants.

These issues have to be addressed in the future, and measurements of the different types of MT-I mRNA as well as MT-II mRNA need further attention.

Regardless of the factors involved, this induction of MT-I + II by physical training may be of importance in protection against ROS and oxidative stress in the muscle, as MT-I + II are extraordinarily efficient ROS

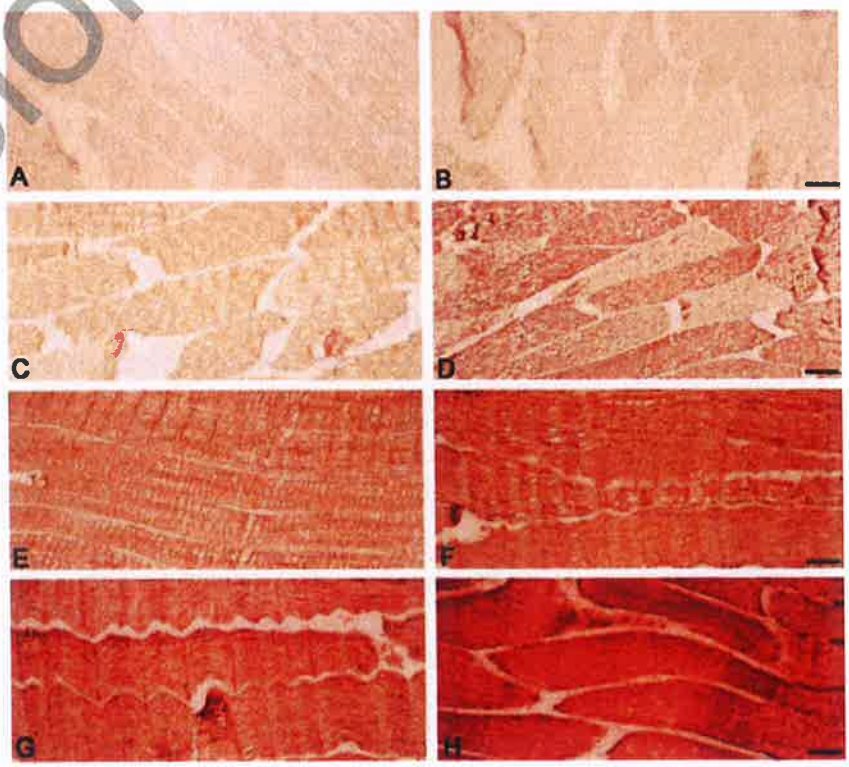

Figure 2. MT-I + II expression in muscle tissue of resting (*A–C*) and exercising (*D–H*) subjects
A and *B*, resting subjects at 0 h (*A*) and 6 h (*B*) show insignificant MT-I + II immunoreactivity. *C*, before exercise began, MT-I + II were basically absent in muscle tissue. *D*, the muscle tissue had an increased MT-I + II expression after 3 h, by which the exercise had just ended. *E–G*, by 4.5 h (*E*), 6 h (*F*), and 9 h (*G*) MT-I + II expression continuously increased relative to that seen immediately after exercise (3 h). *H*, by 24 h, the muscle tissue MT-I + II expression was further increased. Scale bars, 50 μm.

scavengers and antioxidant proteins (Thornalley & Vasak, 1985; Aschner, 1996, 1998; Molinero *et al.* 1998; Shinogi *et al.* 1999; Kumari *et al.* 2000; Rana & Kumar, 2000; Viarengo *et al.* 2000; Hidalgo *et al.* 2001, 2002; Penkowa *et al.* 2000; Penkowa, 2003; Theocharis *et al.* 2003). Accordingly, many *in vitro* studies have shown that MT-I + II inhibit ROS-induced DNA degradation and tissue damage (Schwarz *et al.* 1994, 1995; Lazo & Pitt, 1995; Lazo *et al.* 1995; Pitt *et al.* 1997; Jiang *et al.* 1998; Haidara *et al.* 1999; Cai *et al.* 2000; Kling & Olsson, 2000; Suzuki *et al.* 2000). In fact, MT-I + II could protect against ROS-induced DNA damage *in vitro* with much higher molar efficiency (almost 800-fold) when compared with glutathione (Abel & de Ruiter, 1989). Also, MT-I + II can functionally substitute for Cu/Zn-SOD in the cellular defence against oxidative stress (Tamai *et al.* 1993), and MT-I + II can compensate for Cu/Zn-SOD deficiency in mice (Ghoshal *et al.* 1999). *In vivo*, MT-I + II are very powerful antioxidants and scavengers of free radicals, and mice with genetic MT-I + II deficiency (MT-I + II knock-out (MT-I + IIKO) mice) show significantly increased levels of ROS formation and oxidative stress relative to wild-type mice, which is also reflected by increased clinical findings and disease manifestations in the MT-I + IIKO mice (Fu *et al.* 1998; Carrasco *et al.* 2000; Penkowa *et al.* 2000, 2001; Hidalgo *et al.* 2001, 2002; Giralt *et al.* 2002*a*; Trendelenburg *et al.* 2002). It is noteworthy that in MT-I + IIKO mice the levels of antioxidants catalase, Mn-SOD and Cu/Zn-SOD were increased in response to oxidative stress induced by a head trauma relative to wild-type mice (Penkowa *et al.* 2000). Thus, when MT-I + II were absent, even an increased expression of other antioxidants could not protect from increased oxidative stress levels, which indicates the protective importance of MT-I + II; while mice with MT-I overexpression (MTTg mice) are protected from oxidative stress and show very low levels of ROS (Fu *et al.* 1998; Chen *et al.* 2001; Giralt *et al.* 2002*b*; Penkowa *et al.* 2002; Molinero *et al.* 2003) relative to wild-type mice. Additionally, MT-II can be used therapeutically for *in vivo* pathological conditions with oxidative stress (Penkowa & Hidalgo, 2000; Giralt *et al.* 2002*b*; Penkowa *et al.* 2002). Also, MT-I + II were beneficial, as increases in MT-I + II production or transgenic MT-II overexpression in pancreatic islets of mice could prevent diabetes induced with streptozotocin (Ohly *et al.* 2000; Chen *et al.* 2001). Thus, transgenic MT-II overexpression could drastically reduce pancreatic islet disruption, cell death, DNA damage and depletion of nicotinamide adenine dinucleotide (NAD+) as well as clinical disease and hyperglycaemia (Chen *et al.* 2001).

In addition, overexpression of MT reduces diabetic cardiomyopathy effectively (Liang *et al.* 2002; Ye *et al.*

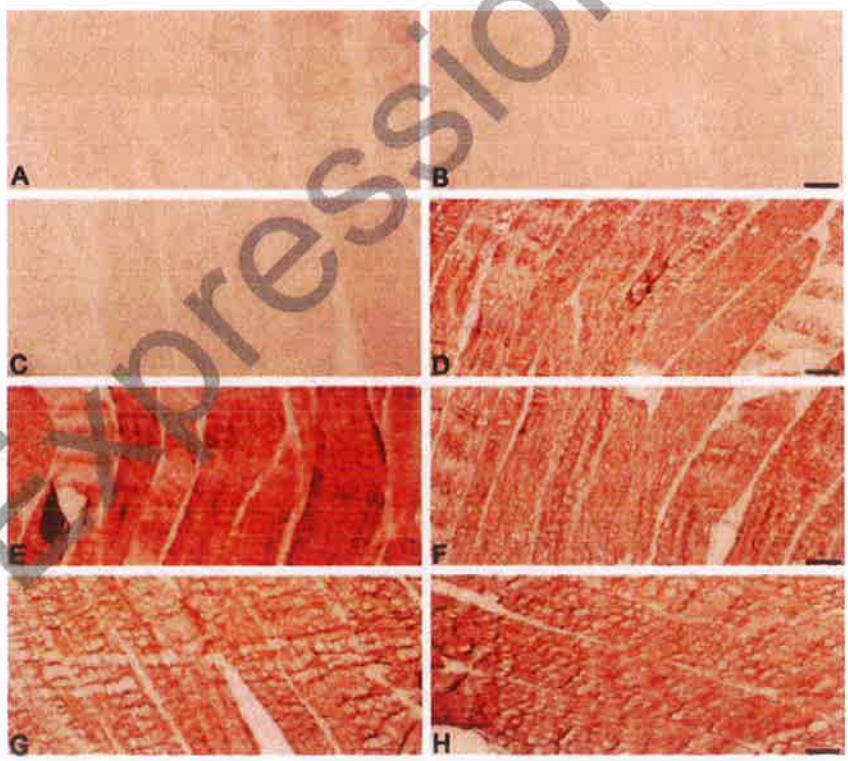

Figure 3. Immunoreactivity for oxidative stress marker NITT in muscle tissue of resting (*A* and *B*) and exercising (*C–H*) subjects
A and *B*, resting subjects at 0 h (*A*) and 6 h (*B*) show no signs of oxidative stress as NITT staining is absent. *C*, before exercise began, NITT immunoreactivity was generally absent in skeletal muscles. *D*, by 3 h when the exercise had just ended, the muscle tissue showed notably increased NITT immunostaining. *E*, by 4.5 h the NITT immunoreactivity peaked. *F–H*, by 6 h (*F*), 9 h (*G*), and 24 h (*H*), NITT levels in skeletal muscle decreased relative to those seen by 4.5 h. However, the NITT staining was still clearly increased when compared with resting muscle. Scale bars, 50 μm.

2003), and MT could eliminate the increased ROS formation in the myocytes during diabetes (Ye *et al.* 2003).

From the data presented here we can not determine the relative contribution of the increased MT-I + II to muscle total antioxidant capacity. However, during specific conditions with impaired human MT-I + II signalling, exercise-induced increases in oxidative stress are enhanced (authors' own unpublished data). Therefore it is likely that the observed increase in MT-I + II presented here is physiologically important.

As mentionend in detail above, scientific data suggest that MT-I + II are important for the tissue oxidative balance. Moreover, sections from the present study stained for other antioxidant factors (e.g. Cu/Zn-SOD, Mn-SOD, myeloperoxidase and catalase) show reduced expression levels relative to those of MT-I + II proteins after exercise. Hence, the MT-I + II increase shown here is the most pronounced antioxidant response within the time period studied after exercise. Even if that does not rule out the possibility that another antioxidant may increase even more at later time points, MT-I + II are at least manifest during the peak of exercise-induced oxidative stress.

In addition, many studies have shown that MT-I + II protect against oxidative stresses in various tissues (for review see Viarengo *et al.* 2000; Hidalgo *et al.* 2001, 2002). Accordingly, the increased MT-I + II expression observed in muscle tissue after physical exercise probably has important roles in protection against exercise-induced ROS formation and oxidative stress, which indeed were increased in the present study. In addition, the finding that non-damaging exercise induces a strong antioxidative activity may also represent yet another mechanism whereby exercise protects against chronic medical disorders such as atherosclerosis, obesity, cardiovascular diseases and diabetes (Ruderman & Schneider, 1992). In support of this, MT-I + IIKO mice develop obesity including increased obese (*ob*) gene expression, elevated fat accumulation and high concentrations of plasma leptin, all symptoms that are similar to those recorded in Zucker fatty (*fa/fa*) rats. Thus, an association between MT-I + II and energy homeostasis has been implied. Thus, mice with MT-I + II deficiency develop spontaneous obesity and hyperleptinaemia (Beattie *et al.* 1998). In conclusion, the finding that non-damaging exercise markedly increases MT-II mRNA and MT-I + II protein in human skeletal muscle fibres is likely to represent an antioxidant defence system after physical exercise, and thereby MT-I + II induction may contribute to the beneficial metabolic effects of exercise.

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Experimental Physiology

Errata

Expression of concern

Experimental Physiology has been informed by Bente Klarlund Pedersen, MD, DMSc, Professor of Integrative Medicine, Director of the Centre of Inflammation and Metabolism, Department of Infectious Diseases and CMRC Rigshospitalet at the University of Copenhagen Faculty of Health Sciences, Copenhagen, Denmark, of concerns about the validity of some of the data contained in an article published in the journal (Penkowa M, Keller P, Keller C, Hidalgo J, Giralt M, Pedersen BK. Exercise-induced metallothionein expression in human skeletal muscle fibres. Exp Physiol 2005 Jul; 90(4), 477–486; published ahead of print Jan 7, 2005, doi:10.1113/expphysiol.2004.029371) and informed us that this matter is being investigated as part of a larger investigation into a more extensive body of published work. While awaiting the outcome of our investigations and further correspondence with the authors, The Editor-in-Chief, Paul McLoughlin, wishes to notify readers of our concerns regarding this article.

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Retraction

Retraction: Exercise-induced metallothionein expression in human skeletal muscle fibres

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Retraction: The following article from *Experimental Physiology*, 'Exercise-induced metallothionein expression in human skeletal muscle fibres' by Milena Penkowa, Pernille Keller, Charlotte Keller, Juan Hidalgo, Mercedes Giralt and Bente Klarlund Pedersen published in *Experimental Physiology* **90**(4): 477–486, and online ahead of print on 7 January 2005, doi:10.1113/expphysiol.2004.029371 has been retracted by agreement between Pernille Keller, Charlotte Keller, Juan Hidalgo, Mercedes Giralt and Bente Klarlund Pedersen, the journal Editor in Chief, Paul McLoughlin, and Blackwell Publishing Ltd.

The retraction has been agreed because:

- Fig. 1, panel H, from 'Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise' by Penkowa M, Keller C, Keller P, Jauffred S, Pedersen BK, *FASEB Journal* **17**(14): 2166–2168 has been re-used as Fig. 3, panels D & F in the retracted article.

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Penkowa M, Keller C, Keller P, Jauffred S & Pedersen BK (2003). Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise. *FASEB Journal* **17**, 2166–2168.

Penkowa M, Keller P, Keller C, Hidalgo J, Giralt M & Pedersen BK (2005). Exercise-induced metallothionein expression in human skeletal muscle fibres. *Experimental Physiology* **90**, 477–486.

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Experimental Physiology

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Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6

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ABSTRACT

Contracting muscle fibers produce and release IL-6, and plasma levels of this cytokine are markedly elevated in response to physical exercise. We recently showed autocrine regulation of IL-6 in human skeletal muscle in vivo and hypothesized that this may involve up-regulation of the IL-6 receptor. Therefore, we investigated IL-6 receptor regulation in response to exercise and IL-6 infusion in humans. Furthermore, using IL-6-deficient mice, we investigated the role of IL-6 in the IL-6 receptor response to exercise. Human skeletal muscle biopsies were obtained in relation to: 3 h of bicycle exercise and rest (n=6+5), or recombinant human IL-6 infusion (rhIL-6) or saline infusion (n=6+6). We further obtained skeletal muscle samples from IL-6 knockout (KO) mice and wild-type C57/BL-6 mice in response to a 1-h bout of exercise. In exercising human skeletal muscle, IL-6 receptor mRNA increased sixfold with a peak at 6 h postexercise. Detection of the IL-6 receptor protein by immunohistochemistry revealed a pronounced staining following exercise that was primarily located at the cell membrane of the muscle fibers, whereas muscle gp130 expression and plasma levels of soluble IL-6 receptor were unaffected. Infusion of rhIL-6 to humans had no effect on the mRNA level of the IL-6 receptor, whereas there was an increase at the protein level. IL-6 receptor mRNA increased similarly in muscle of both IL-6 KO mice and wild-type mice in response to exercise. In conclusion, exercise increases IL-6 receptor production in human skeletal muscle. This effect is most prominent 6 h after the end of the exercise bout, suggesting a postexercise-sensitizing mechanism to IL-6 when plasma IL-6 is concomitantly low. Exercise-induced increases in IL-6 receptor mRNA most likely occurs via an IL-6 independent mechanism as shown in IL-6 KO mice and the human rhIL-6 infusion study, whereas IL-6 receptor protein levels are responsive to elevated plasma IL-6 levels.

Key words: gp130 • exercise • rhIL-6 infusion • mouse • IL-6 KO

In response to exercise, there is a marked increase in systemic interleukin-6 (IL-6). IL-6 plasma levels increase exponentially, with respect to duration and intensity, peaking at the end of the exercise bout (1). IL-6 is mainly regulated at the transcriptional level, possibly due

to the rapid plasma clearance of this cytokine (2). In response to exercise, skeletal muscle is a major source of IL-6, as IL-6 transcription levels, mRNA levels (3) and protein levels (4), largely increase within the muscle fibers, and the systemic increase in IL-6 can largely be accounted for by the working muscle (5).

A positive autocrine regulation of IL-6 occurs in mouse 3T3-L1 adipocytes (6) and other cell culture lines (7–9). We previously demonstrated that in human skeletal muscle in vivo, IL-6 is regulated in a positive autocrine manner (10). Possibly, this autocrine regulation of IL-6 occurs via the IL-6 receptor. The IL-6 receptor lacks an intracellular signaling domain, instead it forms a complex with the common gp130 receptor for initiation of signaling (11). The intracellular activation process occurs by association of the IL-6/IL-6 receptor complex with two gp130 receptor molecules activating intracellular signal transduction pathways (11, 12). The gp130 receptor is ubiquitously expressed, whereas expression of the IL-6 receptor is restricted (13). Expression of IL-6, the IL-6 receptor, and gp130 and a positive autocrine regulation are found in several cell types such as mature human adipose breast tissue (14), human bronchial epithelial cells (15), and hepatocytes (16). However, the IL-6 receptor is down-regulated by IL-6 treatment in monocytes (16). Thus, regulation of IL-6 and the IL-6 receptor differs between cell types. The IL-6 receptor is expressed in rat L6 muscle cell cultures, and the addition of endotoxin, which increases IL-6 levels in the media, can upregulate the IL-6 receptor (17). However, it is not known whether human skeletal muscle fibers express the IL-6 receptor.

The soluble IL-6 receptor can be generated either through differential mRNA splicing or proteolytic shedding (18, 19), although the soluble IL-6 receptor plasma levels consist mainly of the form generated by differential mRNA splicing (20). Binding of IL-6 to its soluble receptor increases the half-life of IL-6 (21). Thus, modulation of sIL-6 receptor levels may affect the IL-6 response to exercise by increasing the plasma IL-6 half-life.

The site of the IL-6 receptor position at chromosome 1q21 is a susceptibility locus for type 2 diabetes (22). Moreover, variants in the IL-6 receptor gene, which results in reduced shedding of the IL-6 receptor, are associated with higher body mass index (BMI) levels when compared with persons carrying the wild-type allele (23), although the link between reduced levels of the sIL-6 receptor and increased susceptibility to obesity is unknown (23).

IL-6 is produced in and released from adipose tissue and resting circulating levels are linked with obesity (24, 25). However, IL-6-deficient mice develop late-onset obesity (26); thus, the role of IL-6 in development of obesity and insulin resistance is controversial. Four polymorphisms exist in the IL-6 promoter, although most population-based studies focus on the G-174-C variant, where the C allele results in lower IL-6 expression than the G allele, when this polymorphism alone is considered (27). Moreover, the C-174-C genotype is associated with cardiovascular disease and all cause mortality in old humans (28), as well as insulin resistance and low energy expenditure (29). Moreover, mice that express transgenic IL-6 and the soluble IL-6 receptor develop reduced body weight with a marked decrease in body fat (30). These studies altogether suggest that regulation of the IL-6/IL-6 receptor signaling is involved in insulin resistance.

As exercise largely increases IL-6 expression in muscle and infusion of IL-6 to humans increases IL-6 mRNA levels in muscle, we hypothesized that the autocrine feedback loop of IL-6 is mediated by the IL-6 receptor. Furthermore, we suspected that IL-6 per se was a possible inducer of the IL-6 receptor production and therefore that increases in plasma IL-6 would lead to a

corresponding increase in the IL-6 receptor production. Thus, biopsies were obtained from human skeletal muscle in response to rest or exercise and rhIL-6 infusion or saline infusion to distinguish between effects mediated by exercise or IL-6 alone. Moreover, muscle samples were obtained from IL-6 knockout (KO) mice and wild-type mice in response to a swimming exercise bout, to further examine the importance of IL-6 on the regulation of the IL-6 receptor expression.

METHODS

Volunteers

Twenty-three healthy men (mean age: 24.2±0.5 years, mean height: 184±2 cm, mean weight: 79±2 kg, mean BMI: 23.3±0.3 kg/m^2) participated in the study. The volunteers had no medical history, did not use any medication, did not have any febrile illness during the fortnight preceding the study, and physical examination revealed no abnormalities. The study was approved by the Ethical Committee of the Copenhagen and Frederiksberg Communities, Denmark, and performed according to the Declaration of Helsinki. Subjects gave their informed, written consent to participate after they were informed about possible risks and discomfort.

Volunteers participated in either of the following protocols

Exercise study with muscle biopsies

Eleven subjects participated in the study (mean age: 25±1 yr, mean height: 184±3 cm, mean weight: 79±3 kg, mean BMI: 23.3±0.5 kg/m^2): six persons exercised and five persons rested. One week before the experimental day, subjects performed a maximal oxygen uptake test (VO_{2max}) on an electrically braked cadence-independent cycle ergo meter (Monark 839E, Monark Ltd., Varberg, Sweden), which averaged 47.2 ± 1.5 ml/kg/min. From this test, a workload equivalent to 60% of their maximum workload (WL_{max}) was calculated. On the experimental day, subjects arrived at 0800 h after an overnight fast and performed 3 h of cycling at the predetermined workload (180±7 Watts). Muscle biopsies were obtained before exercise (0 h) and at 3, 4.5, 6, 9, and 24 h. One piece was snap frozen in liquid nitrogen for RNA extraction; another piece was sealed in Tissue-tek and snap frozen for immunohistochemistry analysis. Blood was drawn from a catheter placed in the antecubital vein of one arm and collected at the same time-points. Muscle biopsies were obtained from the vastus lateralis using the percutaneous needle biopsy technique with suction, preceded by a local injection of lidocaine. Subjects were permitted to consume only water during the experiment.

IL-6 infusion study with muscle biopsies

Twelve subjects participated in the study (mean age: 23.5±0.5 years, mean height: 184±2 cm, mean weight: 79±2 kg, mean BMI: 23.3±0.4 kg/m^2): on the experimental day, subjects arrived at 0800 h following an overnight fast. The femoral artery of one limb was cannulated and used for the infusion. Subjects (n=6) were infused with recombinant human IL-6 (rhIL-6) for 3 h at a rate of 30 μg/hour. The rhIL-6 was administered in saline. Control subjects (n=6) were infused with saline for 3 h. Muscle biopsies were obtained from the vastus lateralis muscle before infusion, at the end of the 3-h infusion, at 6 h, and 24 h. One piece was snap frozen in liquid nitrogen for RNA extraction; another piece was sealed in Tissue-tek and then snap frozen for immunohistochemical analysis. Blood was collected before infusion (0 h), at the cessation of the 3 h infusion at 6 h and 24 h. Subjects were permitted to consume only water during the first 6 h

of the experiment, then they were given a standardized meal, and then they reported to the lab the next morning following an overnight fast.

Measurement of plasma IL-6 and sIL-6 receptor

Blood samples for measurement of cytokines were drawn into glass tubes containing EDTA. The tubes were spun immediately at 3,500 *g* for 15 min at 4°C. The plasma was stored at –80°C until analyses were performed. High-sensitivity enzyme-linked immunosorbent assay (ELISA) kits from R&D system (Minneapolis, MN) were used to measure IL-6 in plasma (HS600). The sensitivity is 0.094 pg/ml. This kit does not distinguish between soluble and receptor-bound IL-6 and therefore gives a measure of the total IL-6 content in the sample. ELISA kits from R&D system (Minneapolis, MN) were used to measure plasma levels of the soluble IL-6 receptor (DR600). This kit detects the total amount of sIL-6 receptor and has a sensitivity of 6.5 pg/ml.

Mouse study

Female C57/BL6 IL-6 KO mice (n=24) and C57/BL6 wild-type mice (n=24) were used in the study. Mice were housed with free access to water and rodent chow under 12L:12D cycles at the department of animal physiology at the Autonomous University of Barcelona, Spain. Mice were divided into 3 groups of 8 mice: (1, rest) mice were euthanized in basal conditions; (2, exercise) mice were euthanized immediately after a 1-h bout of swimming exercise; (3, exercise + recovery) mice were euthanized after a 1-h swimming exercise followed by a 3-h recovery period, in which they were allowed access to drinking water but were deprived of food. All experiments were carried out at 0800–1300 h to minimize the influence of circadian rhythms on gene expression levels. The temperature of the water was maintained at 36.5°C throughout the exercise bout. After mice were killed, skeletal muscle samples were obtained, frozen in liquid nitrogen, and stored at –80°C. Animal care and sacrificing of the mice was carried out according to the directions of the local ethical committee.

RNA extraction

RNA was extracted using Trizol (Life Technologies) according to manufacturer's protocol, as described previously (10).

Reverse transcription

Total RNA was reverse-transcribed according to manufacturer's instructions (Applied Biosystems, Taqman reverse transcription reagents) using either Oligo dT primers (used in the human exercise trial) or random hexamer primers (used in the human infusion trial and in the mouse study). The reactions were run in a Perkin Elmer GeneAmp PCR system 9700 with conditions at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

Analysis of samples

Samples were analyzed for mRNA levels by real-time PCR using an ABI PRISM 7900 sequence detector (PE Biosystems, Foster City, CA). The following genes were analyzed in humans: IL-6 receptor, gp130, GAPDH and 18S. Human IL-6 receptor primers and Taqman probe were designed using the Primer Express 2.0 program as described (31). The probe and primers of the IL-6 receptor were designed to cover both transcript variants. The human gp130 receptor was an

assay-on-demand (AOD, Applied Biosystems, Foster City, CA). GAPDH and ribosomal 18S were used as endogenous controls (Applied Biosystems). In the mice, the IL-6 receptor was detected by an AOD (Applied Biosystems). Samples were run in triplicate under standard real-time PCR conditions; 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The gene expression levels were normalized to the housekeeping gene GADPH when Oligo dT primers were used for cDNA synthesis or to 18S when hexamer primers were used for cDNA synthesis. All reactions were run singleplex and analyzed and quantitated using the standard curve method. Data are expressed as fold change from the resting control.

Tissue processing

Muscle tissue was cut in 6-μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides, to be used for immunohistochemistry.

Sections were preincubated in 3.0% H_2O_2 to quench endogenous peroxidase and afterward incubated in 10% goat serum to block unspecific background staining.

Immunohistochemistry

The sections stained by immunohistochemistry were always processed simultaneously and under the same laboratory conditions.

Sections were incubated overnight at 4°C with the primary antibodies: mouse anti-human IL-6 receptor (CD-126) IgG diluted 1:50 (Serotec, UK, code MCA822); and rabbit anti-human gp130 IgG diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA; code sc-655). The primary antibodies were detected using biotinylated goat anti-mouse IgG diluted 1:200 (Sigma-Aldrich, St. Louis, MO; code 8774) or biotinylated mouse anti-rabbit IgG diluted 1:400 (Sigma-Aldrich; code 3275) for 30 min at room temperature followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, DK, code K377) for 30 min at room temperature, prepared according to manufacturer's recommended dilutions. Afterward, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, Boston, MA; code NEL700A) prepared following manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

To evaluate the extent of nonspecific binding in the immunohistochemical analysis, control sections were incubated in the absence of primary antibody or in the blocking serum. To exclude staining due to endogenous biotin, we pretreated sections sequentially with 0.01-0.1% avidin (Sigma-Aldrich; code A9390) followed by 0.001-0.01% biotin (Sigma-Aldrich; code B4501), each step for 20 min at room temperature, before the immunohistochemistry was performed. Comparing our immunohistochemical stainings +/– specific biotin blocking, we conclude that in the used tissue, muscular endogenous biotin is unlikely to induce false-positive immunostainings by binding to the used streptavidin. To control the specificity of the anti-IL-6 receptor primary antibody, we pre-absorbed it with the corresponding antigen, that is, the IL-6 receptor peptide (Santa Cruz Biotechnology; code sc-661). Results were considered only if these controls were negative.

For the simultaneous examination and recording of the stainings, a Zeiss Axioplan 2 light microscope was used.

Statistics

Data on IL-6 receptor mRNA in the exercise study were log-transformed in order to reach a normal distribution, and data are presented as geometric means ± SEM. Data on IL-6 receptor mRNA in the rhIL-6 infusion study were normally distributed, and data are presented as means ± SEM. Data on gp130 mRNA in the exercise study were normally distributed, and data are presented as means ± SEM.

In the rhIL-6 infusion study, data on plasma IL-6 were normally distributed after log transformation and are presented as geometric mean ±95% confidence interval (CI). In the exercise study, data on plasma IL-6 and sIL-6 receptor plasma levels were normally distributed, and data are presented as means ± SEM.

For analysis of data in the human studies, a two-way repeated-measures ANOVA was used to detect changes over time or between groups. The Student-Newman-Keul's or Bonferroni's *t* test for post hoc analysis was used to detect changes over time from resting values or differences between groups.

For analysis of data in the mouse study, a *t* test was used to compare differences between groups. *P* values < 0.05 were considered significant. Statistical calculations were performed using Sigma Stat 3.0 (SPSS Inc., Chicago, IL).

RESULTS

In human skeletal muscle, IL-6 receptor mRNA increased ($P < 0.01$) in response to exercise when compared with resting persons. IL-6 receptor mRNA levels were elevated at 4.5 h and peaked at 9 h, which is 1.5 h and 6 h after the exercise ended, respectively, and remained slightly elevated at 24 h. All persons showed the same pattern of IL-6 receptor mRNA response to exercise, Fig. 1.

IL-6 receptor protein in human skeletal muscle fibers showed an increased staining of the cellular membrane of muscle fibers following exercise. The staining of IL-6 receptor protein was most prominent at 9 h; thus, the kinetics of the IL-6 receptor protein staining followed the same pattern as the IL-6 receptor mRNA levels, Fig. 2.

Although the mRNA levels for gp130 were very similar in the resting and exercising trial, there was a significant effect of exercise at the 24 h time point ($P<0.01$) (Table 1). However, protein staining of the gp130 receptor showed no differences between groups or over time in response to exercise (data not shown).

Plasma levels of the soluble IL-6 receptor were unchanged in response to exercise when compared with a resting group (Table 2).

To investigate the direct effect of IL-6, we infused rhIL-6 to humans and obtained muscle biopsies. After rhIL-6 infusion in humans, IL-6 receptor mRNA levels did not differ from levels in the control group (Table 3). However, expression of IL-6 receptor protein increased in the

rhIL-6-infused group when compared with controls. The staining of IL-6 receptor protein was localized to the cell membrane and displayed the strongest staining at the end of the 3-h infusion and at 6 h, Fig. 3. Expression of gp130 protein was not different between the rhIL-6 infusion group and the control group (data not shown).

In both IL-6 knockout mice and wild-type mice, IL-6 receptor mRNA levels increased in skeletal muscle 3 h after 1 h of swimming exercise ($P<0.01$). No differences in expression pattern were observed between wild-type and IL-6 KO mice, Fig. 4.

DISCUSSION

With these present studies, we have demonstrated that the IL-6 receptor is expressed by human skeletal muscle and that muscle contractions enhance expression of both IL-6 receptor mRNA in skeletal muscle, as well as IL-6 receptor protein on muscle fiber membranes.

Increased expression of the IL-6 receptor in muscle fibers after an exercise bout suggests that the muscle is sensitized by IL-6. The peak in IL-6 receptor production occurs several hours after the end of the exercise bout, at the time when IL-6 plasma levels are decreasing (4). Expression of the IL-6 receptor may therefore be a mechanism whereby muscle is sensitized to the effects of IL-6 when IL-6 levels are sparse.

In canine kidney cell cultures, IL-6 receptor protein appears at the cell surface ~45 min after mRNA production (32). In response to exercise, there is a significant increase in IL-6 receptor mRNA at 4.5 h, or 1.5 h after the exercise bout. Seemingly, the IL-6 receptor production is initiated after muscles stop contracting, suggesting a sensitization of muscles to the effects of IL-6. As the half-life of the IL-6 receptor protein is 2-3 h in canine kidney cells, independent of the presence of IL-6 (32), the peak of the IL-6 receptor protein presumably occurs approximately at 9 h or more, as this is the time point with the highest IL-6 receptor mRNA response. In human hepatoma cells, the IL-6/IL-6 receptor complex is internalized and degraded (33). For sustained signaling through the IL-6 receptor to occur, de novo synthesis of the IL-6 receptor is required. Thus, the continuously elevated levels of IL-6 receptor mRNA and protein after a single exercise bout may affect the IL-6/IL-6 receptor system for more than 6 h after the end of exercise.

The IL-6 receptor can be proteolytically cleaved, generating the soluble IL-6 receptor; thus, any cell type that expresses the gp130 receptor can bind IL-6 in complex with its soluble receptor for initiation of signal transduction (34). To determine whether the IL-6 response elicited following exercise also has the potential to activate other cell types that do not themselves express the IL-6 receptor, we measured plasma levels of sIL-6 receptor in response to exercise. As the plasma levels of the sIL-6 receptor were unaltered in response to exercise, this again suggests a local muscle-specific response to IL-6.

Although the mRNA for the signal-transducing part of the IL-6 receptor complex, gp130 was affected by exercise at the 24-h time-point, this effect did not seem physiologically relevant. This was also confirmed by protein staining of the common gp130, which was unaffected by exercise, and there were no differences between the resting and exercising group. This again suggests an IL-6-specific response. It may seem that gp130 availability limits IL-6 receptor signaling; however, as the gp130 is ubiquitously expressed and also serves as a receptor for many other

cytokines, there is a surplus of the gp130 receptor and it is thus highly unlikely that it will limit IL-6 receptor signaling.

To investigate the importance of IL-6 in regulation of the IL-6 receptor response, rhIL-6 was infused in healthy humans. Despite IL-6 plasma levels being higher in the infusion trial compared with the exercise trial, the IL-6 receptor mRNA levels were unaltered, suggesting that IL-6 is not the primary signal for the increase in IL-6 receptor mRNA observed in response to exercise. Thus, the exercise-induced increase in IL-6 receptor production most likely occurs via an IL-6-independent mechanism. This was further substantiated by the ability of the IL-6 KO mice to increase IL-6 receptor mRNA levels to the same extent as control mice with exercise. Conversely, IL-6 increases both IL-6 receptor mRNA and protein levels in a human myeloma cell line (35). This discrepancy may be explained by variations in the IL-6 receptor response between cell types and dose of IL-6, and the latter data may not reflect the situation observed in skeletal muscle. However, rhIL-6 infusion in humans increased IL-6 receptor protein expression in skeletal muscle, possibly owing to post-transcriptional regulation of the IL-6 receptor in response to elevated IL-6 plasma levels. Although other receptor systems show decreased half-lives of the receptors when exposed to saturating amounts of their ligands, our results showed increased IL-6 receptor protein levels following rhIL-6 infusion. This is consistent with results from canine kidney cells where IL-6 receptor half-life is independent of the presence of saturating amounts of IL-6 (32).

We have previously shown that IL-6 is produced in both type I and type II skeletal myofibers, when subjects perform 3 h of intense ergometer bicycle exercise (4), whereas another study by Hiscock et al. reported specificity to fiber type II in response to 2 h of ergometer exercise (36). The studies (4, 36) indicate that the mode and intensity of exercise determines to which degree either fiber type expresses IL-6. In the present study, we studied 3 h of ergometer bicycle exercise and saw uniform expression of the IL-6 receptor in both type I and type II fibers, suggesting that both fiber types are rendered responsive to IL-6.

The kinetics of IL-6 and IL-6 receptor expression suggest that the pathways regulating IL-6 and the IL-6 receptor are linked, thus factors initiating IL-6 transcription may be inhibiting IL-6 receptor transcription. Exercise-induced activation of IL-6 transcription is regulated by energy availability, as low intramuscular glycogen levels further enhance transcription of the IL-6 gene (3) furthermore, ingestion of carbohydrate during exercise decreases the plasma IL-6 response (37). A role for IL-6 in metabolism is suggested, as rhIL-6 infusion to humans increases lipolysis and fat oxidation (38), and as IL-6-deficient mice develop late-onset obesity and glucose intolerance (26). Thus, seemingly IL-6 serves as an energy-sensor. Several metabolic genes are trancriptionally activated in the recovery phase from exercise, presumably to rebuild energy stores (39). It would seem reasonable if activation of transcription of the IL-6 receptor was also linked with factors involved in energy sensing, as energy stores are being rebuilt in the recovery phase from exercise, when IL-6 receptor levels peak. This would render the muscle fibers more responsive to IL-6, possibly initiating glycogen synthesis.

In conclusion, muscle contractions induce post-exercise expression of IL-6 receptor mRNA and protein levels in human skeletal muscle, possibly to sensitize muscle to the decreasing IL-6 plasma levels elicited by exercise. As exercise increases IL-6 receptor mRNA both in IL-6 KO mice and wild-type mice, exercise-induced transcription of IL-6 receptor in muscle fiber most likely occurs via an IL-6-independent mechanism. This is further supported by the human rhIL-6

infusion study. However, IL-6 may enhance IL-6 receptor production at a post-transcriptional level, as infusion of rhIL-6 increased IL-6 receptor protein.

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Table 1

Gene expression levels

Gene	Study	0 h	3 h	4.5 h	6 h	9 h	24 h
Gp130	Exercise	1.0 ± 0	0.6 ± 0.1	0.6 ± 0.2	1.4 ± 0.1	1.2 ± 0.2	1.6 ± 0.3*
	Rest	1.0 ± 0	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1

Gene expression levels for gp130 in response to exercise or rest (n = 6+5), data are presented as means ± SEM. There was a significant effect when the exercising group was compared with the resting group over time (P<0.01). Differences between groups are indicated by *.

Table 2

Plasma levels of IL-6 and sIL-6 receptor

Plasma	Study	0 h	3 h	4.5 h	6 h	9 h	24 h
sIL-6R, ng/ml	Rest	25.6 ± 3.3	25.3 ±3.2	25.3 ± 3.1	26.3 ± 3.8	26.8 ± 3.8	24.4 ± 3.3
	Exercise	29.4 ± 1.0	32.0 ±2.4	30.7 ± 2.5	30.5 ± 2.4	29.4 ± 2.2	27.1 ± 2.0
IL-6, pg/ml	RhIL-6 inf.	1.5 (1.1 – 2.3)	159.0 (138 –184) *,‡	7.8 (5.1–12.5) ‡	7.4 (4.9 –11.6) ‡	NA	NA
	Placebo	2.5 (1.4–5.5)	3.9 (2.1–7.5)	5.8 (3.8 –9.3) ‡	5.5 (3.9 –7.9) ‡	NA	NA

Data on plasma levels of the sIL-6 receptor (sIL-6R) in response to exercise or rest (n=6+5) are presented as mean ± SEM. Plasma levels of sIL-6 receptor were unchanged in response to exercise, when compared with a resting control group. Plasma IL-6 levels in response to rhIL-6 infusion or saline infusion (n=6+6) are presented as geometric mean ± 95% CI. There is a significant difference between the IL-6 infusion group and the control group over time (P<0.001). Differences between groups are indicated by *Difference over time is denoted by ‡.

Table 3

Gene expression levels

Gene	Study	0 h	3 h	6 h	24 h
IL-6R	IL-6 infusion	1.0 ± 0	1.9 ± 0.5	3.1 ± 0.8	2.3 ± 0.5
	Placebo	1.0 ± 0	2.5 ± 0.5	2.8 ± 0.4	2.5 ± 0.6

Gene expression levels for the IL-6 receptor (IL-6R) in response to rhIL-6 infusion or saline infusion (n=6+6), data are fold change from the resting control and presented as means ± SEM. There was no significant difference between the two groups.

Fig. 1

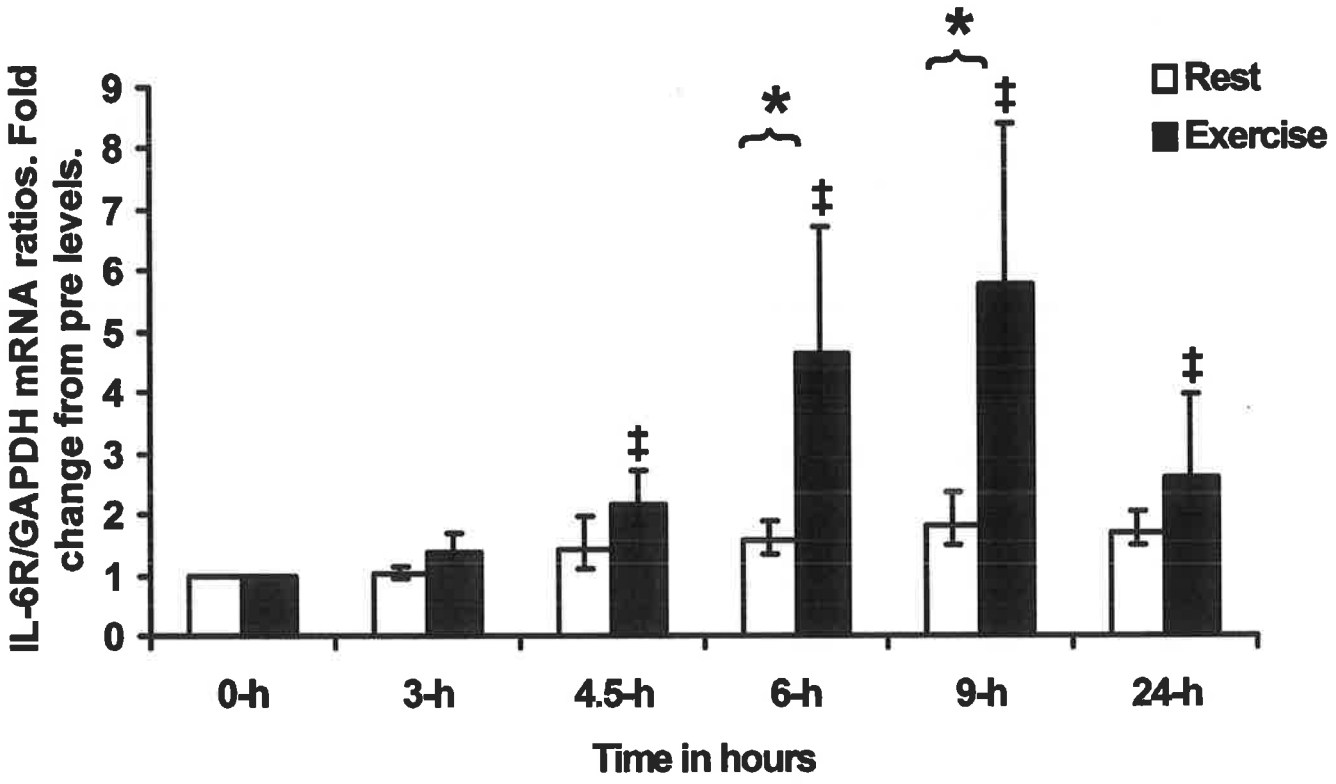

Figure 1. IL-6 receptor mRNA levels in response to exercise or rest (n=6+5). There is an increase in IL-6 receptor mRNA levels (P<0.01) in exercising persons when compared with resting persons. IL-6 receptor mRNA levels increase at 4.5 h and peak at 9 h, which is 1.5 and 6 h after the end of the exercise bout, respectively. Difference over time is denoted by ‡. Difference between groups is denoted by *.

Fig. 2

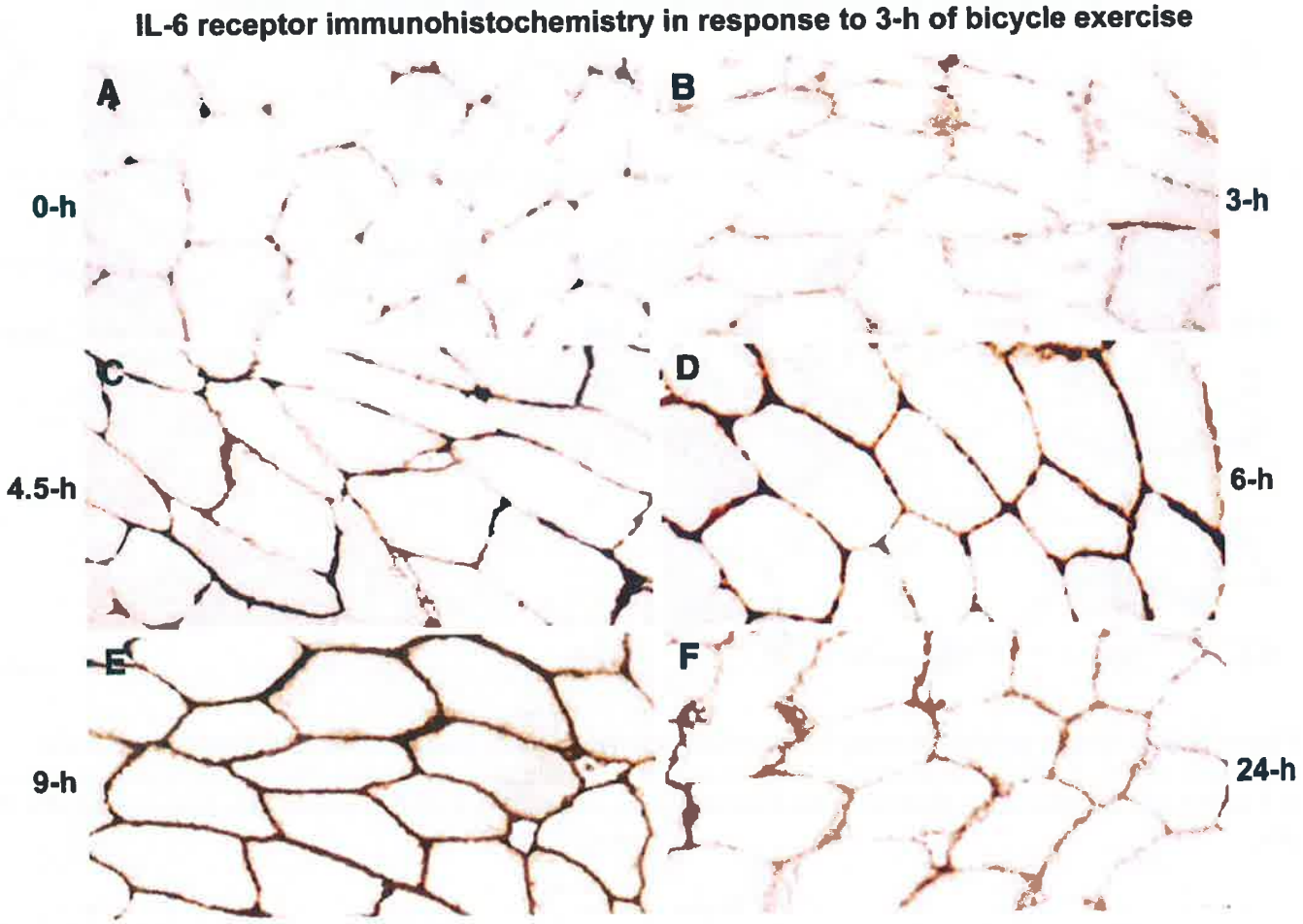

Figure 2. IL-6 receptor protein staining in human skeletal muscle in response to exercise or rest (n=6+5). The protein staining of the IL-6 receptor increases markedly in response to exercise, with staining being most prominent at 9 h. The IL-6 receptor protein is predominantly located to the cell membrane of the muscle fibers. Scale bars (*A–F*): 30 μm.

Fig. 3

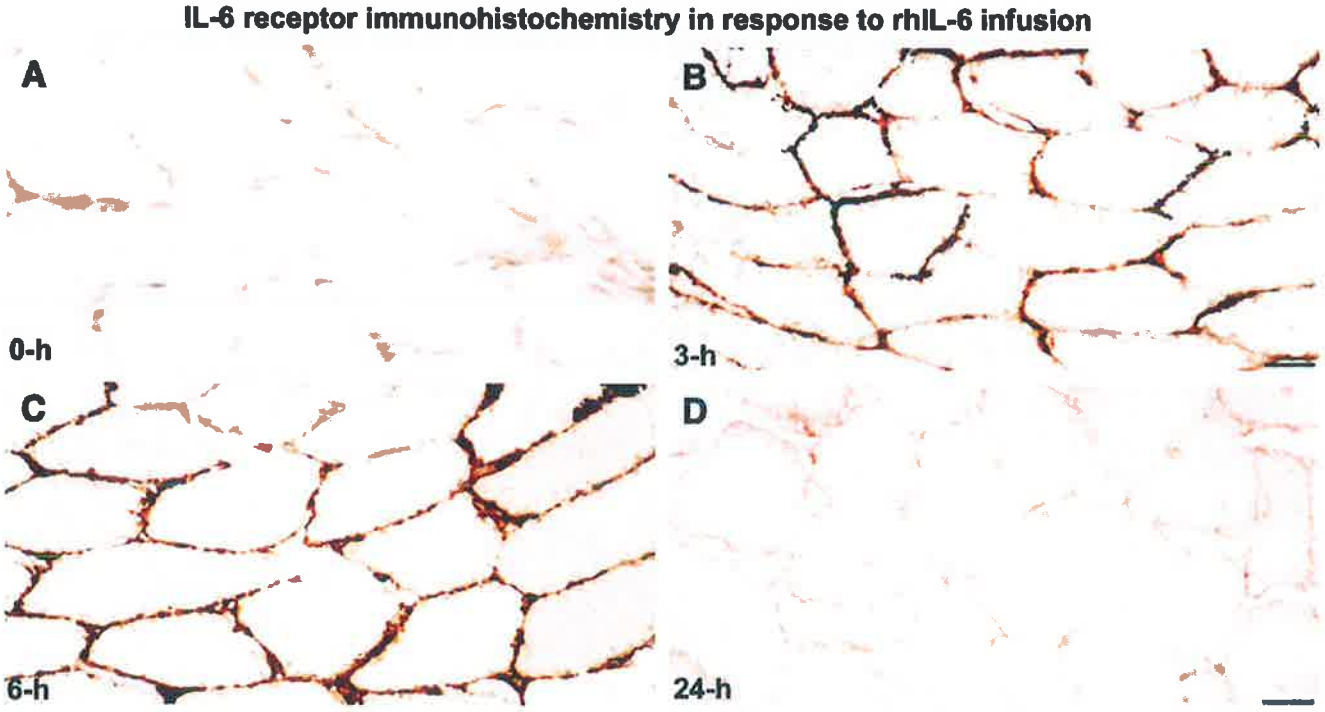

Figure 3. IL-6 receptor protein following rhIL-6 infusion (n=6+6). Protein staining of the IL-6 receptor increases in response to an rhIL-6 infusion with staining being located to the muscle fiber membranes. The staining is most pronounced at 3 and 6 h and has returned to prelevels at the 24-h time-point. ***A***) 0 h; ***B***) 3 h; ***C***) 6 h; and ***D***) 24 h. Scale bars (***A–D***): 30 μm.

Fig. 4

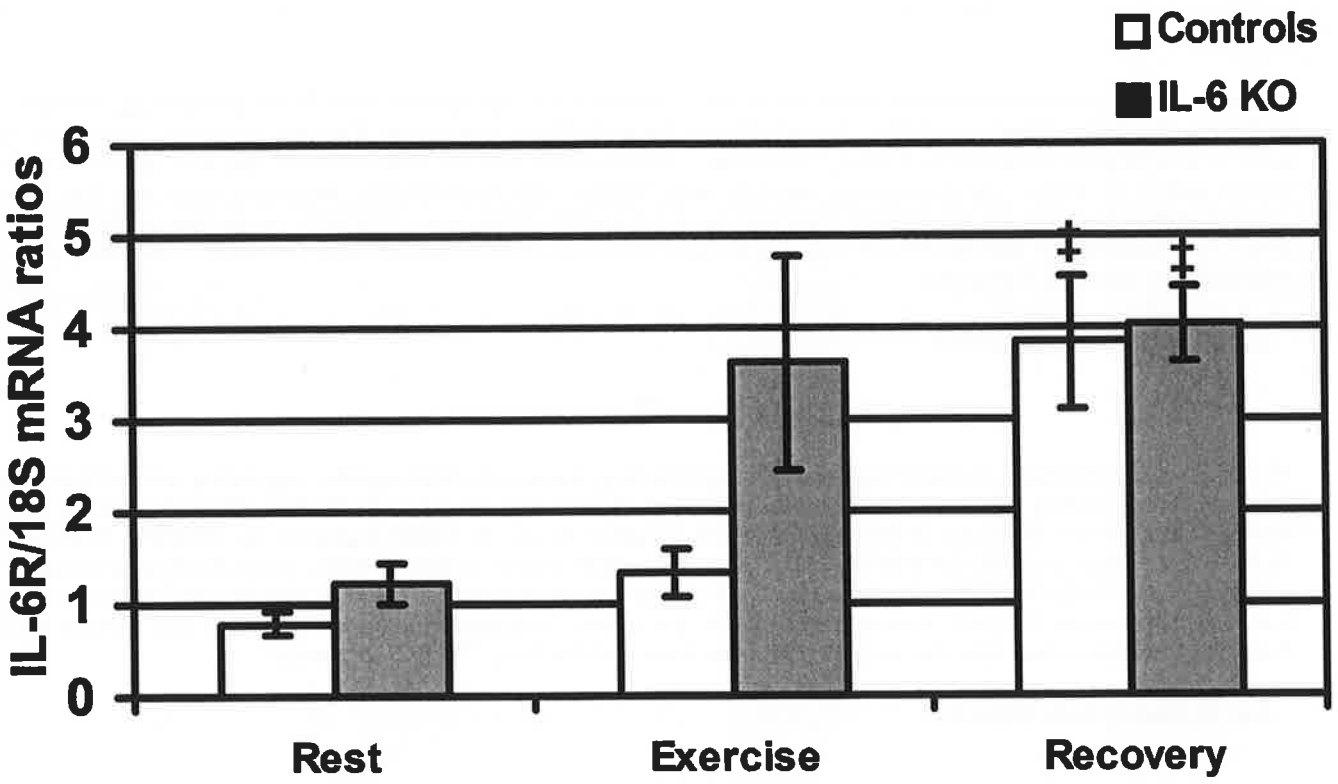

Figure 4. IL-6 receptor mRNA levels in muscle of IL-6 KO mice and wild-type mice: at rest; in response to a 1-h bout of swimming exercise; and after 3 h recovery from the swimming exercise. There is a significant increase in IL-6 receptor mRNA in response to an exercise bout in both wild-type mice and IL-6 KO mice ($P<0.01$). Difference from resting group is denoted by ‡.

The FASEB Journal • Retraction

The article "Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise," by Milena Penkowa, Charlotte Keller, Pernille Keller, Sune Jauffred, and Bente Klarlund Pedersen, published in print as an *FJ Express* summary in *FASEB J.* 2003 Nov. 17:2166–2218, doi: 10.1096/fj.03-0311fje, and as a full-length article online at http://www.fasebj.org/content/early/2003/11/04/fj.03-0311fje, has been retracted due to a recommendation made by the Danish Committees on Scientific Dishonesty (DCSD). According to DCSD, the article "contains image manipulations and [DCSD has] therefore recommended that the authors withdraw these articles from *The FASEB Journal.*"

doi: 10.1096/fj.03-0311fjeRET

The article "Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6," by Pernille Keller, Milena Penkowa, Charlotte Keller, Adam Steensberg, Christian P. Fischer, Mercedes Giralt, Juan Hidalgo, and Bente Klarlund Pedersen, published in print as an *FJ Express* summary in *FASEB J.* 2005 July 19:1181–1183, doi: 10.1096/fj.04-3278fje, and as a full-length article online at http://www.fasebj.org/content/early/2005/06/29/fj.04-3278fje, has been retracted due to a recommendation made by the Danish Committees on Scientific Dishonesty (DCSD). According to DCSD, the article "contains image manipulations and [DCSD has] therefore recommended that the authors withdraw these articles from *The FASEB Journal.*"

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Experimental Physiology

Exercise induces interleukin-8 receptor (CXCR2) expression in human skeletal muscle

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Exercise induces a marked increase in interleukin-8 (IL-8) mRNA and protein expression within skeletal muscle fibres. Interleukin-8 belongs to a subfamily of CXC chemokines containing a Glu-Leu-Arg (ELR) motif. CXC chemokines with ELR motifs are potent angiogenic factors *in vivo*, and IL-8 has been shown to act as an angiogenic factor in human microvascular endothelial cells by binding to the CXC receptor 2 (CXCR2). In the present study, we examined the expression of the interleukin-8 receptor CXCR2 in human skeletal muscle biopsies after concentric exercise. Healthy volunteers were randomized to either 3 h of cycle ergometer exercise at 60% of maximum oxygen uptake ($n = 8$) or rest ($n = 7$). Muscle biopsy samples were obtained from the vastus lateralis before exercise (0 h), immediately after exercise (3 h), and at 4.5, 6, 9 and 24 h. Skeletal muscle CXCR2 mRNA increased significantly in response to exercise (3 and 4.5 h) when compared with pre-exercise samples. Expression of the CXCR2 protein was low in skeletal muscle biopsies before exercise and at the end of the exercise period (3 h). However, at 4.5–9 h, an increase in CXCR2 protein was seen in the vascular endothelium, and also slightly within the muscle fibres, as determined by immunohistochemistry. The present study demonstrates that concentric exercise induces CXCR2 mRNA and protein expression in the vascular endothelial cells of the muscle fibres. These findings suggest that muscle-derived IL-8 may act locally to stimulate angiogenesis through CXCR2 receptor signalling.

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Skeletal muscle is acknowledged to be an endocrine organ, which produces cytokines. These may have both local and systemic effects. Skeletal muscle has the capacity to express, for example, tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8 and IL-15 (Chan *et al.* 2004). Among these cytokines, solid evidence exists that IL-6 (Pedersen *et al.* 2003*a*,*b*; Febbraio & Pedersen, 2002, 2005) and IL-8 (Nieman *et al.* 2003; Chan *et al.* 2004; Akerstrom *et al.* 2005) are regulated by exercise, at the levels of both mRNA and protein. Interleukin-6 and IL-8 are released from working skeletal muscle. Muscle-derived IL-6 (Steensberg *et al.* 2000) is released in significant quantities into the systemic circulation, whereas only a small transient net release of IL-8 is found from working muscle. This small release of IL-8 does not result in an increase in the systemic plasma concentrations of IL-8, suggesting that muscle-derived IL-8 might have a local effect (Akerstrom *et al.* 2005).

Interkeukin-8 was originally identified as a chemotactic factor secreted by activated monocytes and macrophages that promote directional migration of leucocytes (Baggiolini *et al.* 1989). However, IL-8 possesses biological functions in addition to and distinct from its role in regulating inflammatory responses. Interkeukin-8 is a member of the CXC chemokine family, which is defined by four highly conserved cysteine amino acid residues, with the first two cysteines separated by one non-conserved amino acid residue. The subfamily can be further subclassified by the presence of a characteristic three amino acid motif, Glu-Leu-Arg (ELR motif), at the NH_2-terminus before the first cysteine amino acid. The family members that contain the ELR motif (CXC

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ELR$^+$) are potent promoters of angiogenesis, and IL-8 has been shown to induce endothelial cell chemotaxis *in vitro* and to induce angiogenesis *in vivo* (Koch *et al.* 1992; Strieter *et al.* 1992, 1995; Norrby, 1996; Bek *et al.* 2002). In contrast, CXC chemokines that lack the ELR motif (CXC ELR$^-$) are inhibitors of angiogenesis (Strieter *et al.* 2005). Two homologous chemokine receptors, the CXC receptors 1 and 2 (CXCR1 and CXCR2), bind IL-8 with high affinity (Belperio *et al.* 2000). Interleukin-8 mediates its chemotactic effects via CXCR1, whereas CXCR2 is expressed by human microvascular endothelial cells and is considered to be the receptor responsible for IL-8-induced angiogenesis (Addison *et al.* 2000; Heidemann *et al.* 2003). The importance of CXCR2-mediated angiogenesis *in vivo* is further demonstrated by the lack of angiogenic activity induced by ELR$^+$ CXC chemokines in the presence of neutralizing antibodies to CXCR2 in the rat corneal micropocket assay and in the corneas of CXCR2$^{-/-}$ mice (Addison *et al.* 2000). The receptors for IL-8, CXCR1 and CXCR2, are widely expressed on normal and various tumour cells (Yang *et al.* 1997; Smith *et al.* 1994; Singh *et al.* 1999; Inoue *et al.* 2000) and bind IL-8 with high affinity (Holmes *et al.* 1991; Cerretti *et al.* 1993; Baggiolini *et al.* 1997; Wang *et al.* 1998). Angiogenic factors that are regulated in skeletal muscle by exercise include vascular endothelial growth factor (VEGF; Prior *et al.* 2004) and transforming growth factor-β (TGF-β; Gavin & Wagner, 2001).

Given that IL-8 is a potent angiogenic factor in several tissues, we propose a role for skeletal muscle-derived IL-8 in the stimulation of angiogenesis in response to exercise. In the present study, we investigated whether the IL-8 receptor CXCR2 is expressed in human skeletal muscle and whether the expression is regulated by exercise. In addition, we analysed the cellular localization of the CXCR2 protein and its colocalization with the accessory TGF-β receptor, endoglin (CD105). Endoglin is reported to be predominantly expressed on activated endothelial cells, and its expression is potently induced by hypoxia (Miller *et al.* 1999; Sanchez-Elsner *et al.* 2002; Fonsatti *et al.* 2003). Consistently, elevated levels of endoglin have been detected on vascular endothelial cells in tissues undergoing active angiogenesis, such as regenerating tissues and inflamed tissues or tumours (Wang *et al.* 1994; Krupinski *et al.* 1994; Miller *et al.* 1999; Torsney *et al.* 2002).

Methods

Regulation of CXCR2 mRNA in skeletal muscle by exercise

Subjects. Fifteen men, all non smokers, with a mean (± S.D.) age, height, weight and body mass index (BMI) of 24.9 ± 4 years, 180.9 ± 1 cm, 82.0 ± 8 kg and 24.9 ± 2 kg m^{-2}, respectively, participated in this study. All subjects had a normal medical history, and physical examination revealed no abnormalities. Eight subjects exercised, and seven control individuals rested to control for an effect of repeated muscle biopsies. There was no difference between the two groups with regards to age, weight, height, BMI or maximal oxygen uptake ($\dot{V}_{O_2max}$).

Ethics. Before the expeimental procedures, the subjects were given both oral and written information about the experimental procedures before providing their written informed consent. All studies were approved by the Copenhagen and Frederiksberg Ethics Committee, Denmark, and were performed in accordance with the Declaration of Helsinki.

Experimental procedures. . Cycle ergometer exercise was chosen as the mode of exercise in this study because this type of exercise is mainly concentric and induces minimal muscle damage and subsequent inflammation. The subjects performed two incremental maximal exercise tests to determine $\dot{V}_{O_2max}$ on a cycle ergometer (Monark 839E, Monark Ltd, Varberg, Sweden). The first one, a familiarization trial, was performed 5 days before the first experimental day; the second test was performed 2 days after the experimental day. On the experimental day, subjects arrived at 07.00 h, after an overnight fast including all kinds of beverages. The subjects rested for approximately 10 min in the supine position, after which a venous catheter was placed in an antecubital vein. Subsequently, the subjects performed 3 h of cycling at approximately 60% $\dot{V}_{O_2max}$, followed by 6 h of recovery. Muscle biopsies were obtained from the vastus lateralis prior to the exercise (0 h), immediately after exercise (3 h), and at 4.5, 6, 9 and 24 h, using the percutaneous needle biopsy technique with suction. To acquire the 24 h samples, the subjects reported to the laboratory the following day after an overnight fast. Control subjects rested in the laboratory for 9 h, reported to the laboratory the day after in a fasted state, and had biopsy samples taken at the same time points as during the exercise trial.

Biopsies were obtained by anaesthetizing the skin and the muscle fascia using lignocaine (20 mg ml^{-1}; SAD, Copenhagen, Denmark). A 5–7 mm incision was made, and the Bergström needle introduced into the muscle tissue, suction applied and three to five cuts were made. Biopsies were obtained from both quadriceps and individual biopsies were obtained with a distance of at least 5 cm. The biopsy was divided into two parts. Approximately 50 mg of the biopsy was used for RNA isolation. If present, superficial blood was quickly removed, and the biopsy was frozen in liquid nitrogen. The other part of the biopsy was prepared for histochemical analysis by mounting a small muscle piece in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and then frozen in 2-methyl-butane (Acros Organics, Geel,

Belgium) precooled in liquid nitrogen. Both samples were stored at −80°C until analysed.

The design was identical to the exercise protocol used in a former study (Akerstrom *et al.* 2005), but different subjects participated in the former and the present study.

Isolation of RNA and reverse transcription

Total RNA was isolated from skeletal muscle with TriZol (Life Technology) as described by the manufacturer. The RNA concentration was determined spectrophotometrically, and 2 μg of RNA was reversed transcribed in a 100 μl reaction according to the manufacturer's instructions using random hexamer primers (Taqman™ reverse transcription reagents, Applied Biosystems, Naerum, Denmark). The reactions were run in a Perkin-Elmer GeneAmp PCR system 9700 (Applied Biosystems) with conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

Real-time polymerase chain reaction (PCR) analysis

Real-time PCR was performed on an ABI PRISM 7900 sequence detector (PE Biosystems). Each assay included (in triplicate): a cDNA standard curve of five serial dilution points (ranging from 1 to 0.01), a no-template control, a no-reverse transcriptase control, and 150 ng (35 ng for glyceraldehyde-3 phosphate dehydrogenase (GAPDH)) of each sample of cDNA. The amplification mixture was made from 17.5 μl 2 x TaqMan Universal MasterMix, 1.75 μl of 20 x TaqMan probe, and primer assay reagents, 7.5 μl of the cDNA preparation, and 8.25 μl water to give a final volume of 35 μl. The primers and probes for CXCR2 (code, Hs00174304_m1) and GAPDH (code, Hs99999905_m1) were predeveloped TaqMan probes, and primer sets were from Applied Biosystems (AB). All assay reagents were from AB. The amplification mixtures were amplified according to standard conditions (50°C 2 min, 95°C 10 min followed by 50 cycles of 95°C 15 sec, 60°C 1 min). The relative concentrations of CXCR2 and the endogenous control, GAPDH, were determined by plotting the threshold cycle (Ct) *versus* the log of the serial dilution points. GAPDH levels were not influenced by the exercise protocol and have recently been validated for this type of study (Mahoney *et al.* 2004; Lundby *et al.* 2005). The relative expression of CXCR2 was subsequently determined after normalization to GAPDH. For CXCR2, a slope of −3.31 and a correlation coefficient (r) value of 0.99 were obtained. The corresponding GAPDH values were −3.50 for slope and 0.99 for the r^2 value. The y-intercept on the standard curves generated represents the cycle threshold value for 150 ng of sample CXCR2, which amounted to 30.5. The threshold value for 35 ng of GAPDH was 17.3.

Protein expression

Tissue processing. Muscle biopsies were sectioned in 6 μm consecutive sections on a Microm cryostat, and sections were immediately collected on glass slides, in order to be stained by immunohistochemistry.

Sections were pre-incubated in 0.5% H_2O_2 to quench endogenous peroxidase and afterwards incubated in 10% goat serum to block unspecific background staining.

Immunohistochemistry. The sections stained by immunohistochemistry were always processed simultaneously and under the same laboratory conditions. Sections were incubated overnight at 4°C with primary monoclonal mouse antihuman CXCR2/IL-8 receptor B (CXCR2) antibodies diluted 1:50 (clone 48311, code no. MAB331, RD Systems, Abingdon, UK). The primary antibodies were detected using biotinylated goat antimouse IgG diluted 1:200 (code 8774, Sigma-Aldrich, USA) for 30 min at room temperature followed by streptavidin–biotin–peroxidase complex (StreptABComplex/HRP, code K377, Glostrup, Denmark) prepared at the manufacturer's recommended dilutions for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin–peroxidase complex (code NEL700A, Perkin Elmer, Wellesley, MA, USA) prepared according to the manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature. In order to evaluate the extent of non-specific binding in the immunohistochemical analysis, control sections were incubated in the absence of primary antibody or in the blocking serum. To exclude staining due to endogenous biotin, we pretreated sections sequentially with 0.01–0.1% avidin (code A9390, Sigma-Aldrich, USA) followed by 0.001–0.01% biotin (code B4501, Sigma-Aldrich, USA), each step lasting 20 min at room temperature, before the immunohistochemistry was performed. Comparing our immunohistochemical stainings with and without specific biotin blocking showed that in the used tissue, muscular endogenous biotin is unlikely to induce false positive immunostainings by binding to the used streptavidin. For the simultaneous examination and recording of the stainings, a Zeiss Axioplan 2 light microscope was used.

Statistics

All data were normally distributed after log transformation. Data are presented as geometric means ± S.E.M. A two-way repeated-measures ANOVA was used to detect changes over time or between groups. *Post hoc* analyses (Bonferroni adjusted t test) were performed to identify specific differences across time or

between groups. Differences were considered significant at $P < 0.05$. Statistical calculations were performed using SYSTAT 8.0 software (Richmond, CA, USA).

Results

The level of CXCR2 mRNA expression in skeletal muscle biopsies increased threefold (95% confidence interval; 2.9- to 3.6-fold) in response to exercise when compared to pre-exercise values. A significant increase in CXCR2 mRNA from pre-exercise values was seen at two time points in the exercise group: 3 (immediately after exercise) and 4.5 h. Compared with the control group, the exercise group had significantly elevated CXCR2 mRNA levels at time points 3, 4.5 and 6 h ($P = 0.009$; Fig. 1).

The temporal and spatial expression of the CXCR2 protein following exercise was determined by double immunofluorescence staining of skeletal muscle biopsies for CXCR2 and the accessory TGF-β receptor, endoglin (CD105). The expression of endoglin protein was low or absent prior to and immediately after the exercise bout (0 and 3 h; Fig. 2*A* and *B*). Endoglin expression appeared to increase following exercise at time points 4.5, 6 and 9 h (Fig. 2*C*, *D* and *E*), and at 24 h the expression of endoglin was decreased to levels similar to pre-exercise values (Fig. 2*F*). The CXCR2 protein was low or absent in skeletal muscle before exercise (Fig. 2*A*) and in the early post-exercise period (3 and 4.5 h; Fig. 2*B* and *C*; $n = 12$), and repetitive muscle biopsies at rest did not induce CXCR2 expression ($n = 7$; data not shown). However, at time points 6 and 9 h (Fig. 2*D* and *E*), it appeared that there was an increased CXCR2 immunoreactivity and that the peak was at 9 h. The CXCR2 immunoreaction was seen in the microvascular endothelium, as judged by the colocalization with endoglin. In addition, the CXCR2 protein was mildly expressed within the muscle fibres and at the sarcolemma at all time points. At the time point 24 h, the expression of CXCR2 and endoglin appeared to be decreased to pre-exercise values (Fig. 2*F*).

Discussion

In the present study, we demonstrate that the IL-8 receptor CXCR2 is expressed in human skeletal muscle and that CXCR2 is regulated by acute exercise at both the mRNA and the protein level. In addition, we find that the TGF-β receptor endoglin protein (CD105) appears to be upregulated by exercise and is coexpressed with CXCR2.

Vascular endothelial cells are the major source of CD105. Other cell types, including vascular smooth muscle cells, fibroblasts, macrophages, leukaemic cells of pre-B and myelomonocytic origin, express CD105 to a lesser extent. In skeletal muscle, however, expression of CD105 is most likely to reflect endothelial cells (Duff *et al.* 2003). Assessment of microvessel density with panendothelial markers, namely CD34, CD31 and von Willebrand factor, may not be accurate, since factor VIII stains large vessels with high sensitivity and capillaries with variable and focal staining (Akagi *et al.* 2002). It is also not specific for blood vessels, since it can stain lymphatics (Guidi *et al.* 1994). Transmembrane glucoprotein CD31 (platelet endothelial cell adhesion molecule) is found on endothelial cells and many haematopoietic cells (Miettinen *et al.* 1994). Although

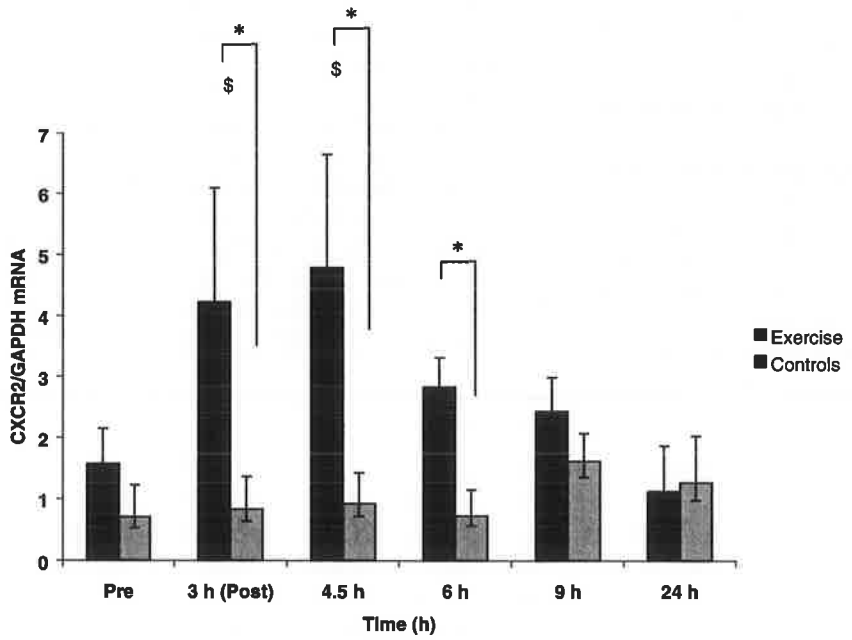

Figure 1. CXCR2 mRNA expression in muscle tissue in exercising (black bars) or resting control subjects (grey bars)
For exercising subjects, $n = 8$; for control subjects, $n = 7$. Data are expressed as geometric means ± S.E.M. There was an increase in CXCR2 mRNA levels ($P = 0.009$) in exercising subjects when compared with resting subjects (2 way ANOVA). CXCR2 mRNA levels were increased at the following time points: immediately after the exercise bout (3 h), at 4.5 and at 6 h. Differences between groups are denoted by an asterisk. $ Denotes difference from pre-exercise value.

it is a good marker for endothelial cells, it also stains blood vessels. Also, the reliability of CD31 staining has been inconsistent between laboratories (Smith-McCune & Weidner, 1994). Therefore, we determined to evaluate microvessel quantification using the CD105, a marker that is preferentially expressed only in angiogenic vessels. We found that the exercise-induced CXCR2 protein is localized primarily to the microvascular endothelial cells, as judged by CD105. Interestingly, we found that the protein expression of CD105 appeared to be upregulated by exercise. The possible regulation of TGF-β and its receptor was not the focus of this work. Previous studies have found only a modest effect of exercise on TGF-β expression in skeletal muscle (Smith-McCune & Weidner, 1994; Breen *et al.* 1996) and neither room air training nor chronic hypoxic training appeared to alter TGF-β mRNA levels significantly (Olfert *et al.* 2001). In the light of the small gene responses to exercise and the fact that hypoxic training abolished the TGF-β mRNA response to exercise yet increased muscle capillarity, the importance of the TGF-β system as an angiogenic regulator in response to or after exercise training is questionable. However, the unexpected finding in the present study that the TGF-β receptor CD105 is regulated by exercise sheds new light on the TGF-β system in exercise-induced angiogenesis.

The present study aimed to focus on the IL-8 receptor, since it was recently shown that acute exercise induces an increase in IL-8 protein expression within skeletal muscle fibres (Akerstrom *et al.* 2005). Interleukin-8 was released from the working muscle; however, no increase in the systemic plasma concentration of IL-8 was observed, suggesting that muscle-derived IL-8 may act locally. Neither IL-8 mRNA in skeletal muscle nor IL-8 arteriovenous differences across an exercising limb were evaluated in the present study. The time course of the CXCR2 mRNA response in the present study is, however, similar to the IL-8 mRNA response reported by Akerstrom *et al.* (2005) using a similar exercise protocol. Although speculative, an increase in CXCR2 expression with exercise lends some support to the hypothesis that muscle-derived IL-8 produced in response to an acute bout of exercise may act locally to stimulate angiogenesis through CXCR2 receptor signalling.

Exercise generates a powerful angiogenic stimulus within the active muscle, which leads to an increase in capillarity with training (Andersen & Henriksson, 1977; Hudlicka *et al.* 1992). Angiogenesis in skeletal muscle

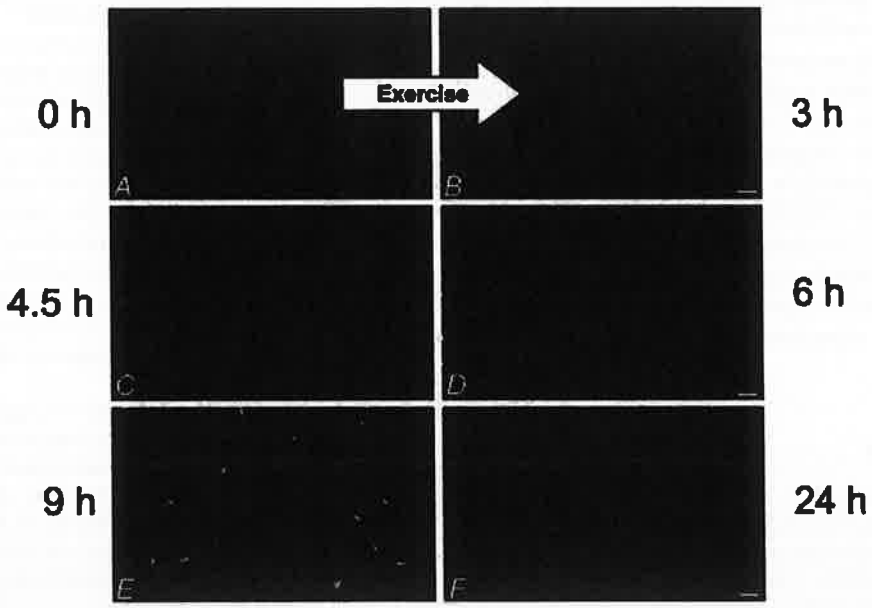

Figure 2. Double immunofluorescence staining for CXCR2 (green) and endoglin (red) in skeletal muscle biopsies before and after 3 h of ergometer cycle exercise
The expression of endoglin was weak or absent from the skeletal muscle before and immediately after exercise (*A* and *B, respectively*). However, an increased immunoreaction for endoglin was seen at time points 4.5, 6 and 9 h (*C*, *D* and *E*, respectively); by 24 h, endoglin protein expression had declined to pre-exercise levels (*F*). The expression of CXCR2 protein was low or absent from skeletal muscle biopsies before exercise (*A*) and in the early postexercise period (3 and 4.5 h; *B* and *C*, respectively). At time points 6 and 9 h (*D* and *E*, respectively), increased CXCR2 reactivity was seen, the peak being at 9 h. CXCR2 immunoreaction was seen in the microvascular endothelium, as judged by the colocalization with the endoglin (yellow). In addition, mild immunoreaction for CXCR2 was seen within the muscle fibres and on the sarcolemma at all time points. By 24 h, the CXCR2 expression had declined to to pre-exercise levels (*F*). Scale bars represent 64 μm.

in response to exercise has been ascribed to increases in blood flow and accompanying capillary shear stress and/or wall tension, and to the contraction itself (Prior *et al.* 2004). However, little is known with regard to the regulation of exercise-induced angiogenesis. The most studied angiogenic factor involved in exercise-mediated angiogenesis is vascular endothelial growth factor (VEGF). Muscle contractions induce an increase in human VEGF mRNA in muscle tissue (Gustafsson *et al.* 1999; Richardson *et al.* 1999; Jensen *et al.* 2004). The increase is greatest (two- to eightfold) shortly after the exercise bout (2–4 h) and declines with time thereafter, returning to normal values within 24 h (Jensen *et al.* 2004). Moreover, the mRNA levels of the VEGF receptors VEGF1 and VEGF2 are also increased by exercise (Gavin *et al.* 2000; Gavin & Wagner, 2002; Lloyd *et al.* 2003). The VEGF gene contains an upstream regulatory sequence that increases VEGF mRNA production when bound by the hypoxia-inducible factor (HIF-1α; Forsythe *et al.* 1996). A recent study reported that IL-8 was sufficient to sustain angiogenesis *in vivo* in HIF-1α-deficient colon cancer cells, indicating that VEGF and IL-8 represent different angiogenic pathways (Mizukami *et al.* 2005). Interleukin-8 induction can be mediated via activation of NF-κB (Mizukami *et al.* 2005). NF-κB signalling is induced by an acute bout of exercise in rat skeletal muscle (Ji *et al.* 2004, 2006). These findings suggest that, in addition to VEGF signalling, IL-8 signalling via the CXCR2 receptor may be an additional pathway of exercise-mediated angiogenesis in the skeletal muscle.

In summary, the IL-8 receptor CXCR2 is expressed in skeletal muscle biopsies, and the expression of CXCR2 mRNA and protein is transiently increased by exercise. Moreover, the CXCR2 protein is localized primarily to activated microvascular endothelium. Although speculative, these findings may support the hypothesis that muscle-derived IL-8 produced in response to exercise may act locally to stimulate angiogenesis by CXCR2 receptor signalling.

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ARTICLE

Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase

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Abstract
Aims/hypothesis Brain-derived neurotrophic factor (BDNF) is produced in skeletal muscle, but its functional significance is unknown. We aimed to determine the signalling processes and metabolic actions of BDNF.
Methods We first examined whether exercise induced *BDNF* expression in humans. Next, C2C12 skeletal muscle cells were electrically stimulated to mimic contraction. L6 myotubes and isolated rat extensor digitorum longus muscles were treated with BDNF and phosphorylation of the proteins AMP-activated protein kinase (AMPK) (Thr^{172}) and acetyl coenzyme A carboxylase β (ACCβ) (Ser^{79}) were analysed, as was fatty acid oxidation (FAO). Finally, we electroporated a *Bdnf* vector into the tibialis cranialis muscle of mice.
Results BDNF mRNA and protein expression were increased in human skeletal muscle after exercise, but muscle-derived BDNF appeared not to be released into the circulation. *Bdnf* mRNA and protein expression was increased in muscle cells that were electrically stimulated. BDNF increased phosphorylation of AMPK and ACCβ and enhanced FAO both in vitro and ex vivo. The effect of BDNF on FAO was AMPK-dependent, since the increase in FAO was abrogated in cells infected with an AMPK dominant negative adenovirus or treated with Compound C, an inhibitor of AMPK. Electroporation of a *Bdnf* expression

V. B. Matthews and M.-B. Åström contributed equally to this study.

B. K. Pedersen and M. A. Febbraio co-directed this study.

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vector into the tibialis cranialis muscle resulted in increased BDNF protein production and tropomyosin-related kinase B ($TrkB^{Tyr706/707}$) and extracellular signal-regulated protein kinase (p44/42 Thr^{202}/Tyr^{204}) phosphorylation in these muscles. In addition, phosphorylation of ACCβ was markedly elevated in the *Bdnf* electroporated muscles.
Conclusions/interpretation These data identify BDNF as a contraction-inducible protein in skeletal muscle that is capable of enhancing lipid oxidation in skeletal muscle via activation of AMPK.

Keywords Cytokines · Lipid metabolism · Metabolism · Neuropeptides · Physical activity

Abbreviations

ACCβ	Acetyl coenzyme A carboxylase β
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMPK	AMP-activated protein kinase
BDNF	Brain-derived neurotrophic factor
ERK	Extracellular signal-regulated protein kinase
FAO	Fatty acid oxidation
IHC	Immunohistochemistry
LDH	Lactate dehydrogenase
PCr	Phosphocreatine
rhBDNF	Recombinant human BDNF
TrkB	Tropomyosin-related kinase B

Introduction

Regular physical activity is known to have multiple health benefits [1]. Of note, exercise is associated with increased insulin-stimulated glucose uptake in the immediate post-exercise period [2, 3], while chronic physical activity enhances insulin sensitivity [4]. We have recently identified skeletal muscle as a cytokine-producing organ, demonstrating that the metabolic and physiological effects of exercise may also be mediated by skeletal muscle-derived humoral factors [5, 6]. Skeletal muscles have the capacity to express a number of 'myokines' and it is now clear that IL-6 [7] and IL-8 [8] production is upregulated by muscle contraction and that these cytokines may be released from contracting skeletal muscle. Muscle-derived IL-6, but not IL-8, contributes markedly to systemic levels, where it works in a hormone-like fashion and plays an important role in lipid and glucose metabolism [9, 10], principally via activation of AMP-activated protein kinase (AMPK) [10]. Recent studies from others, employing skeletal muscle-specific AKT1 transgenic [11] and muscle-specific peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) knockout [12] mice, have also observed altered metabolism in tissues such as pancreas, liver and adipose tissue. The authors of these respective papers hypothesised that muscle-derived circulatory factors or myokines may have been responsible for the observed tissue cross-talk. Indeed, myokines are now recognised as a major contributing factor that links muscular activity with health [13].

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factor family that plays a key role in regulating survival, growth and maintenance of neurons [14]. It is also known that BDNF reduces food intake and lowers blood glucose in genetically modified (*db/db*) obese mice [15–18]. It is clear, therefore, that BDNF plays a role in both neurobiology and metabolism. Recent studies have demonstrated that physical exercise can increase circulating BDNF levels in both healthy humans [19, 20] and patients with multiple sclerosis [21], although the cellular origin of this increase is unclear. There are some reports in rodents showing that *Bdnf* mRNA increases in skeletal muscle in response to contraction [22, 23], but these studies suggested that the source of the BDNF was neurons within the skeletal muscle beds. However, in a recent study, *Bdnf* mRNA was shown to be expressed in murine skeletal muscle and was increased by inhibition of histone deacetylases [24]. Since exercise can inhibit histone deacetylases [25], the possibility exists that the increase in circulating BDNF in humans during exercise may originate in the contracting muscle cells and that BDNF may be a novel contraction-induced myokine. Accordingly, we aimed to investigate whether skeletal muscle would produce BDNF in response to exercise. In addition, since skeletal muscle contains many cell types, including motor neurons, we conducted in vitro experiments to determine whether muscle cells per se produced BDNF during contraction. Having shown that BDNF is indeed produced by skeletal muscle during contraction, we next sought to investigate whether BDNF can affect metabolic processes in skeletal muscle. We hypothesised that BDNF would be produced by skeletal muscle cells in response to contraction and then released into the circulation where it would enhance insulin action in other tissues such as liver and adipose tissue.

Methods

Human in vivo experiments Eight healthy, physically active but untrained men (mean±SD age: 25±4 years, weight: 82±8 kg, height: 181±1 cm, BMI: 25±2 kg/m^2) were recruited to participate in the study, which was approved by the Ethics Committee of the University of Copenhagen. On the day of the experiment, the volunteers arrived at about 07:00 hours after an overnight fast. The participants performed 120 min of bicycle exercise at 60% of their predetermined $\dot{V}O_{2\,max}$

followed by a 24 h recovery period. Muscle biopsy samples were obtained from vastus lateralis before exercise, immediately after exercise, and 3, 5, 8, 24, 48 and 72 h into recovery using a percutaneous needle biopsy technique with suction. Samples were snap frozen before being analysed for *BDNF* mRNA and protein expression. Serum was obtained at the above mentioned time-points. Serum levels of BDNF were measured by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany). Platelet counts were determined by standard laboratory procedures.

Cell culture experiments C2C12 cells (fewer than six passages) were purchased from the American Type Culture Collection (Manassas, VA, USA). Six-well culture plates were coated with extracellular matrix from Engelbreth–Holm swarm murine sarcoma (Sigma, Castle Hill, NSW, Australia). The C2C12 myoblasts were seeded in culture plates and grown in DMEM (low modified; SAFC Biosciences, Brooklyn, VIC, Australia) +10% (vol./vol.) FBS+ 1% penicillin/streptomycin. Differentiation of the myoblasts was induced by transferring cells to medium containing 2% (vol./vol.) horse serum rather than FBS when the myoblasts were about 90% confluent. Experimental treatments commenced after 5 days of differentiation when nearly all myoblasts had fused to form myotubes. Cells were cultured at 37°C in 5% CO_2 in a humidified chamber.

Next, to determine the effect of contraction on BDNF production in skeletal muscle cells, differentiated C2C12 cells in six-well culture plates were placed in a custom-built chamber that allowed the cells to be electrically stimulated. Muscle cells were stimulated for 120 min (18 V; 1 Hz; 24 ms) at 37°C in 5% CO_2 in a humidified chamber according to methods previously described [26]. To verify that cells were contracting, we filmed the cells. We also measured the ATP and phosphocreatine (PCr) concentration in unstimulated and stimulated cells using enzymatic assays with fluorometric detection [27] and measured lactate dehydrogenase (LDH) release [27] from cells to verify that the contraction protocol was not causing cell membrane damage.

To examine whether BDNF affected phosphorylation (Thr^{172}) of AMPK and ACCβ (Ser^{79}), fully fused L6 myotubes were treated with recombinant human BDNF (rhBDNF; R&D Systems, Minneapolis, MN, USA), which is known to cross-react with cells of rat origin [28]. Cells were treated with rhBDNF for 10 and 30 min at doses of 1, 10 and 100 ng/ml. Palmitate oxidation and glucose uptake experiments were performed as previously described [10]. For adenovirus experiments, myotubes were infected with a titred adenovirus containing control vector (AdGo/GFP) or a dominant negative AMPK mutant [10]. In addition, L6 cells were also treated with 40 μmol/l of the AMPK inhibitor Compound C (Calbiochem, Kilsyth, VIC, Australia).

Ex vivo experiments Male Wistar rats (about 200 g) were fed rat chow (6% energy from fat, 21% from protein, 71% from carbohydrate; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) and water, which were available ad libitum. All experimental procedures were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. To examine palmitate metabolism, the extensor digitorum longus muscle was carefully dissected into longitudinal strips and analysed for [^{14}C]palmitate oxidation, as previously described [29] and contained in the Electronic supplementary material (ESM).

Electroporation experiments Electroporation experiments were conducted with permission from the Danish Animal Experiments Inspectorate. Animal experiments were performed on 8- to 10-week-old C57BL6/C mice (Taconic Tornbjerggaard, Lille Skensved, Denmark). The plasmids pTet-On, encoding the rtTA transactivator [30, 31], and pTetS, encoding the tS silencer [32], were both obtained from Clontech (Palo Alto, CA, USA). We used a pBI-BDNF encoding murine *Bdnf* under the control of an rtTA-dependent promoter (Geneart, Regensburg, Germany). The *Bdnf* cDNA sequence was cleaned for immunogenic motifs, and codons were optimised to ensure optimal expression efficacy. Moreover, the plasmid contained a SV40 polyadenylation downstream of the *Bdnf* sequence. All DNA preparations were performed using Qiafilter Plasmid Maxiprep kits (Qiagen, Hilden, Germany), and the concentration and quality of the plasmid preparations were controlled by spectrophotometry and gel electrophoresis. The animals were anaesthetised 15 min prior to DNA electrotransfer by i.p. injection of Hypnorm (0.4 ml/kg; Janssen, Saunderton, UK) and Dormicum (2 mg/kg; Roche, Basel, Switzerland). The plasmid solution (2.5 μg in 20 μl) was injected i.m. along the fibres into the right tibialis cranialis muscle using a 29G insulin syringe. Plate electrodes with 4 mm gap were fitted around the hind legs. Good contact between electrode and skin was ensured by hair removal and use of electrode gel. The electric field was applied using a Cliniporator (IGEA, Carpi, Italy), applying a combination of a high voltage (800 V/cm [applied voltage=320 V], 100 μs) pulse followed by a low voltage (100 V/cm [applied voltage=40 V], 400 ms) pulse. Induction of gene expression was obtained by administering drinking water containing doxycycline (doxycycline hyclate; Sigma-Aldrich, Brondby, Denmark) at a concentration of 0.2 mg/ml in distilled water [33]. The control group was injected with saline and received doxycycline in the drinking water during the entire experiment. A third group of animals (sham) underwent electroporation of an empty vector. As no differences were reported when comparing the muscles of the control with the sham group, data from sham-treated muscles are reported.

Analytical methods The RNA extraction, quantification and the mRNA abundance of *BDNF/Bdnf* and relevant controls were performed as previously described [34]. Real-time PCR was performed on an ABI PRISM 7900 sequence detector (PE Biosystems, Warrington, UK). Pre-developed TaqMan probe and primer sets for human GAPDH (Hs99999905_m1), human *BDNF* (Hs00380947_m1), mouse *Bdnf* (Mm00432069_m1) and eukaryotic 18S rRNA (4310893E) were obtained from Applied Biosystems (Scoresby, VIC, Australia). Muscle protein analyses were performed as previously described [10, 34]. Lysates were incubated overnight at 4°C in blocking buffer containing a primary antibody against BDNF (Santa Cruz Biotechnology, Heidelberg, Germany; sc-546), phospho-AMPK (Thr^{172}), phospho-ACCβ (Ser^{79}), phospho-extracellular signal-regulated protein kinase (ERK)1/2 (p44/42 Thr^{202}/Tyr^{204}), ERK1/2, phospho-TrkB ($Tyr^{706/707}$), β-actin (Cell Signaling, Beverly, MA, USA) or α-tubulin (Santa Cruz Biotechnology; sc-5546) at a final concentration of 1 μg/ml. Membranes were washed three times in washing buffer and incubated for 60 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (P0448; Dako, Glostrup, Denmark) at a 1:2,000 dilution in blocking buffer, followed by three 5 min washes in washing buffer. The protein bands were detected using Supersignal West Femto (Pierce, Rockford, IL, USA) and quantified using a CCD image sensor (ChemiDoc XRS; Biorad, Gladesville, NSW, Australia) and software (Quantity One; Biorad). BDNF in the media collected from contraction-stimulated experiments and in the plasma from *Bdnf*-electroporated and control mice were analysed using a commercially available ELISA kit (BDNF Emax; Promega, Alexandria, NSW, Australia).

For muscle immunohistochemistry (IHC), muscle tissue was cut into 7 μm consecutive sections on a cryostat, and the sections were immediately collected on glass slides. Sections were pre-incubated in 3% H_2O_2 to quench endogenous peroxidase and followed by incubation in 10% (vol./vol.) goat serum to block unspecific background staining. Sections were incubated overnight at 4°C with rabbit anti-human BDNF IgG diluted 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; code sc-20981). The primary antibodies were detected using biotinylated anti-rabbit IgG diluted 1:400 (Sigma-Aldrich, St Louis, MO, USA; code B 3275) for 30 min at room temperature followed by streptavidin–peroxidase complex (StreptABCcomplex/HRP, code K377; Dakopatts, Glostrup, Denmark) for 30 min at room temperature, prepared according to the manufacturer's recommended dilutions. The immunoreaction was visualised using 0.015% H_2O_2 in 3,3′-diaminobenzidine/TRIS-buffered saline for 10 min at room temperature. Afterwards, slides were dehydrated and mounted with cover glass for microscopy.

Statistics To examine whether there was an effect of exercise on the time-course of serum BDNF or platelets we used a two-way ANOVA for repeated measures. AUCs were compared by an unpaired *t* test. In the cell culture experiments all data were analysed from three independent experiments and paired *t* tests were used to statistically analyse the data.

Results

BDNF is increased in human contracting skeletal muscle in vivo, but is not released into the circulation There were no differences in *BDNF* mRNA levels in the exercise cohort compared with the resting cohort (Fig. 1a). This was probably due to the fact that the *BDNF* mRNA levels demonstrated a marked inter-individual variation with peak mRNA levels between 5 and 8 h for the majority of the participants. As such, we calculated the AUC over time for both groups and showed a difference ($p<0.05$) for the AUC when comparing the two groups. The antibody against BDNF protein recognises both the pro- and mature forms of BDNF. In all western blot experiments, rhBDNF was loaded into one lane as a positive control. In the human samples, the mature form of BDNF was recognised and the production appeared to increase progressively following exercise. At 24 h into recovery from exercise, BDNF protein levels were increased (about 50%; $p<0.05$) in the muscle homogenates (Fig. 1b, c). To confirm that this increase in BDNF expression was indeed increased within muscle fibres, we performed IHC experiments and showed that the increased BDNF production 24 h after exercise was indeed intramyocellular (Fig. 1d). We next aimed to ascertain whether the elevated BDNF in contracting skeletal muscle might be a source of circulating BDNF. Serum BDNF concentrations increased immediately following exercise, before returning to pre-exercise levels 60 min following the cessation of exercise (Fig. 2a). Since platelets are known to store and release BDNF [35], we examined the relationship between circulating BDNF and platelet count. The pattern of increase in platelet count (Fig. 2b) closely matched that for serum BDNF. In addition, the plasma levels of P-selectin, a marker of platelet activation, were also increased immediately following exercise (data not shown). Taken together, these results demonstrate that physical exercise increases circulating BDNF and that the increase may at least to some extent reflect in vivo release of BDNF from activated platelets. The kinetics of the exercise-induced elevation in serum BDNF levels failed to correlate with the increased production of BDNF in muscle 24 h after exercise, suggesting that the skeletal muscle is not a source of the increase in serum BDNF observed early into recovery from exercise.

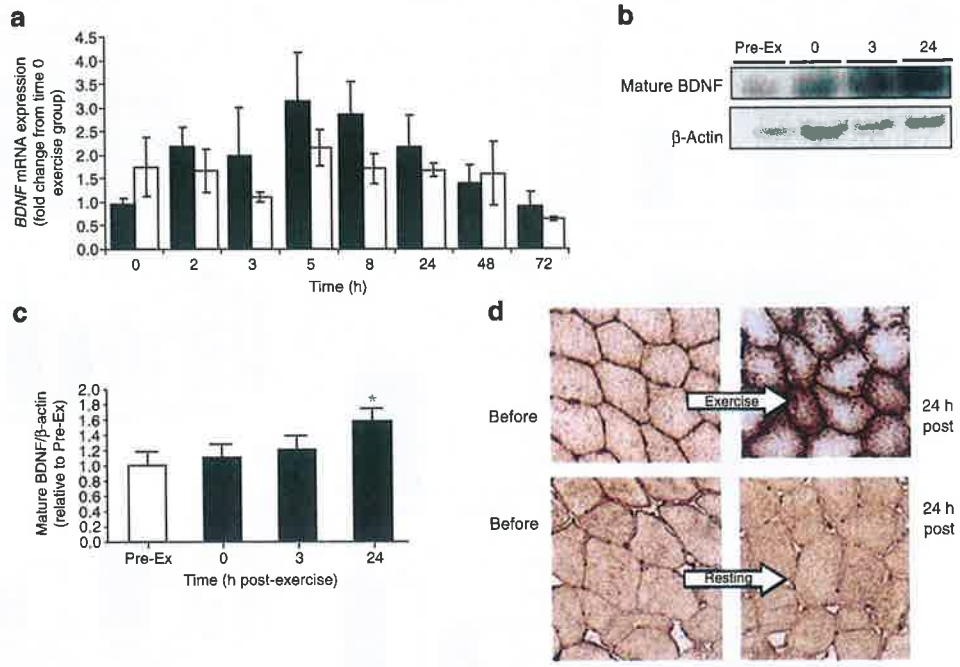

Fig. 1 BDNF is increased in contracting skeletal muscle in vivo. *BDNF* mRNA levels (**a**) and protein production in muscle tissue measured by western blot (**b**, **c**) and IHC (**d**) at time-points ranging from 0 to 72 h after 2 h of ergometer bicycle exercise of the volunteers at 60% of $\dot{V}O_{2\,max}$. *$p<0.05$, difference from pre-exercise (Pre-Ex). $n=8$ per time-point. For *BDNF* mRNA studies, black columns indicate the exercised cohort and white columns the resting cohort. Values are means±SEM

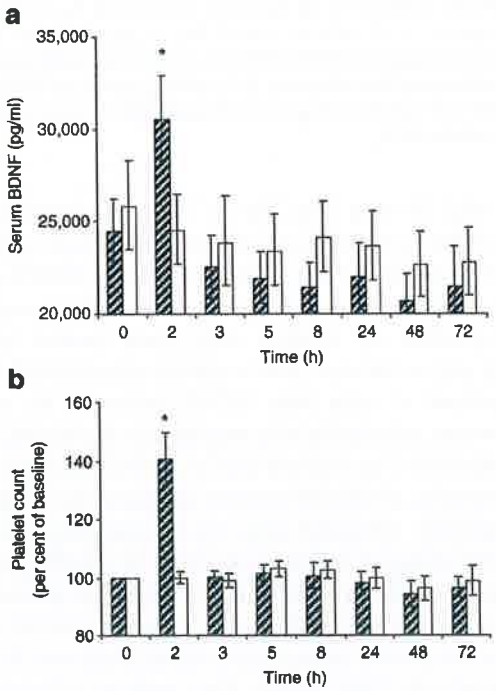

Fig. 2 Exercise results in immediate increases in serum BDNF levels and platelet numbers in volunteers. Serum BDNF levels (**a**) and platelet counts (**b**) are shown at times in relation to 2 h of ergometer bicycle exercise at 60% of $\dot{V}O_{2\,max}$ (hatched columns) or in resting controls (white columns). *$p<0.05$, difference from baseline. $n=10$ per time-point. Values are means±SEM

Muscle contraction increases BDNF levels in muscle cells in vitro Our IHC results suggested that the contraction-induced increase in BDNF protein level was increased in skeletal muscle cells. Since it was possible that BDNF was produced by other cell types within the skeletal muscle bed before being taken up by muscle cells, it was necessary to move to a cell culture model to determine whether skeletal muscle cells per se can increase BDNF levels when contracted. We established a cell culture system to contract differentiated C2C12 myotubes in vitro. We were able to visualise series contraction during the application of the electrical stimulus (ESM video clip). To demonstrate that the cells were undergoing metabolic stress characteristic of in vivo prolonged low-intensity muscle contraction, rather than cell damage, we first showed that PCr, but not ATP, stores were decreased by contraction (Fig. 3a). We also showed that contraction did not increase LDH release from cells into the media (Fig. 3b). In addition, in line with previous studies from our laboratory [34, 36], contraction increased phosphorylation (Thr^{180}/Tyr^{182}) of p38 mitogen-activated protein kinase, heat shock protein 70 protein production and *IL6* mRNA (data not shown) and the phosphorylation of AMPK (Thr^{172}) (Fig. 3c). In addition, muscle contraction increased *Bdnf* mRNA levels compared with non-contracted cells (Fig. 3d). Moreover, such a treatment increased both the pro- and mature BDNF protein levels immediately after the contraction protocol (Fig. 3e, f). These data demonstrate that BDNF is a contraction-induced protein. We were unable to detect any differences in BDNF release in the media of control compared with contraction-treated cells (data not shown).

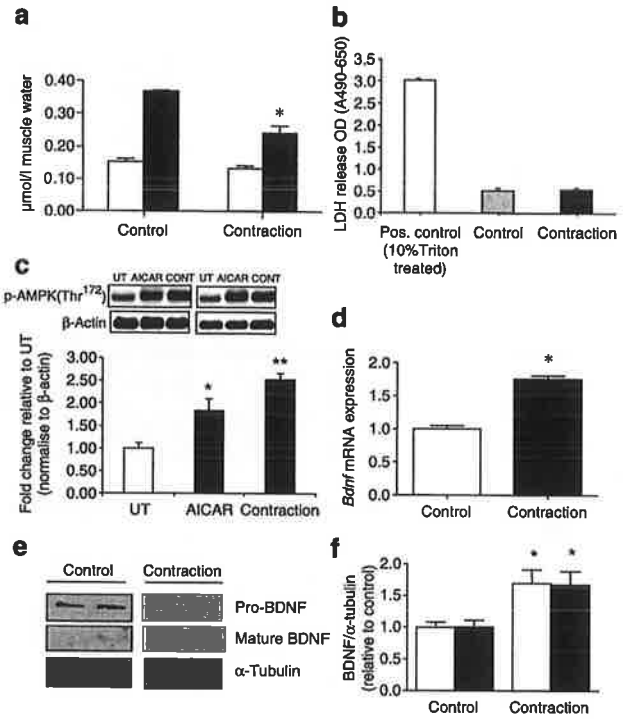

Fig. 3 *Bdnf* mRNA and protein expression is increased in contracting muscle cells. ATP (white columns) and PCr (black columns) (**a**), LDH release (**b**), phosphorylation (p-) of AMPK (**c**), *Bdnf* mRNA expression (**d**), representative immunoblot (**e**) and quantification (**f**) of pro-BDNF (white columns) and mature BDNF (black columns) protein levels in unstimulated (Control) or electrically stimulated C2C12 cells (Contraction; 18 V, 1 Hz, 24 ms) at 37°C, 5% CO_2 for 120 min. UT, untreated. *$p<0.05$ for difference from CON; $n=6$ per group. Values are means±SEM

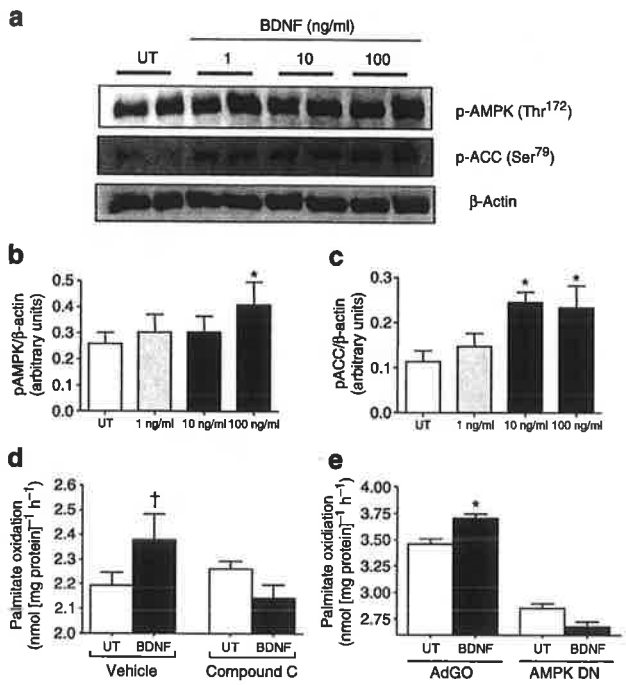

Fig. 4 BDNF increases phosphorylation (p-) of AMPK, its downstream target ACCβ (ACC) and FAO in an AMPK-dependent manner in L6 myotubes. Representative immunoblots (**a**) and quantification of phosphorylation of AMPK (Thr^{172}) (**b**) and ACCβ (Ser^{79}) (**c**) in L6 myotubes treated for 10 min with 1, 10 and 100 ng/ml rhBDNF. Palmitate oxidation in L6 myotubes treated with the AMPK inhibitor Compound C or vehicle control (**d**) or infected with an AMPK dominant negative (AMPK DN) or control virus (AdGO) (**e**). *$p<0.05$ for difference from untreated (UT) cells. †$p<0.05$ for difference from BDNF (10 ng/ml)+Compound C-treated cells. $n=6$ per group. Values are means±SEM

BDNF increases fat oxidation in an AMPK-dependent manner in L6 myotubes Having established that BDNF was a contraction-induced protein, we next sought to determine whether BDNF affected signalling pathways associated with fat oxidation in skeletal muscle. This hypothesis was based on the observation that BDNF reduces obesity in diabetic mice [18]. AMPK phosphorylates ACCβ resulting in inhibition of ACC activity, which in turn leads to a decrease in malonyl-CoA content, relieving inhibition of carnitine palmitoyl transferase 1 and increasing fatty acid oxidation (FAO). Accordingly, we treated L6 myotubes with 1, 10 and 100 ng/ml BDNF for 10 and 30 min and quantified the phosphorylation of AMPK (Fig. 4a, b) and ACCβ (Fig. 4a, c). We showed that at the two higher doses, BDNF markedly phosphorylated ACCβ, while a similar effect was seen for phospho-AMPK at the higher dose. The lack of effect of phospho-AMPK at 10 ng/ml was unexpected since phospho-ACCβ was increased. The time-dependent effects were transient as no differences were seen in phospho-AMPK or phospho-ACCβ at 30 min (data not shown), with the effects present at only 10 min (Fig. 4a–c). Next, to determine if fat oxidation was enhanced in these cells and whether any such increase was due to activation of the AMPK pathway, we performed labelled palmitate oxidation experiments. Irrespective of whether cells were treated with PBS (Fig. 4d) or infected with a control virus (AdGO) (Fig. 4e) treatment of cells with BDNF enhanced fat oxidation. However, when cells were treated with the AMPK inhibitor Compound C or infected with an AMPK dominant negative adenovirus, the BDNF-induced increase in fat oxidation was completely abrogated (Fig. 4d, e) demonstrating that the BDNF-induced increase in FAO is AMPK-dependent. Finally, we tested whether BDNF promoted glucose uptake in L6 myotubes. Unlike insulin treatment, BDNF failed to increase glucose uptake above those levels seen in vehicle-treated cells (fold change from vehicle: vehicle=1±0.04; insulin at 100 nmol/l=2.32±0.072; BDNF at 10 ng/ml=0.94±0.071; and insulin+BDNF=1.99±0.185).

BDNF increases phosphorylation of AMPK and its downstream target ACCβ and results in enhanced fat oxidation

in intact skeletal muscle ex vivo Having established that BDNF enhanced AMPK signalling and fat oxidation in vitro, we next sought to determine whether this effect was also seen in intact rodent skeletal muscle. Accordingly, experiments were performed in extensor digitorum longus muscle obtained from rats and dissected into longitudinal strips from tendon to tendon. Incubating these muscle strips in BDNF resulted in increased phospho-AMPK at 90 min (Fig. 5a, c) and phospho-ACCβ at 30 and 90 min (Fig. 5b, c). Moreover, treatment of muscle strips with BDNF for 120 min resulted in enhanced palmitate oxidation (Fig. 5d). It should also be noted that while both BDNF and the positive control 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) increased ^{14}C in the CO_2 fraction, this was not seen in the acid soluble metabolite fraction, indicating complete oxidation of the label.

Skeletal muscle overproduction of BDNF in vivo results in ERK, TrkB and ACCβ phosphorylation, but does not increase circulating BDNF levels Results from our human exercise studies and cell culture experiments suggested that BDNF was produced by muscle cells during contraction, but was not released into the circulation or media. These data, coupled with the in vitro and ex vivo observations that BDNF enhanced AMPK signalling and fat oxidation, suggested that BDNF was produced by skeletal muscle cells to act locally. To test this hypothesis in vivo, we transiently overproduced BDNF in skeletal muscle using the in vivo electroporation technique. We observed marked overproduction of both the pro- and mature form of BDNF (Fig. 6a, b). Despite this level of overproduction, we did not observe any differences in plasma BDNF when we compared these animals with saline-treated control animals (data not shown), providing further evidence that BDNF exerts its action locally and is not released into the circulation when intramuscular production is increased. We next addressed whether BDNF activated intracellular signalling in an autocrine and/or paracrine fashion. BDNF signals via a specific receptor tyrosine kinase (TrkB). Upon BDNF binding, TrkB is phosphorylated ($Tyr^{705/706}$) resulting in a signalling cascade that includes phosphorylation of ERK (Thr^{202}/Tyr^{204}). Overproduction of BDNF resulted in increased phosphorylation of both ERK (Fig. 6c) and TrkB (Fig. 6d, e) compared with sham-electroporated muscle. These data suggest that BDNF may act in an autocrine and/or paracrine fashion in skeletal muscle. Moreover, overpro-

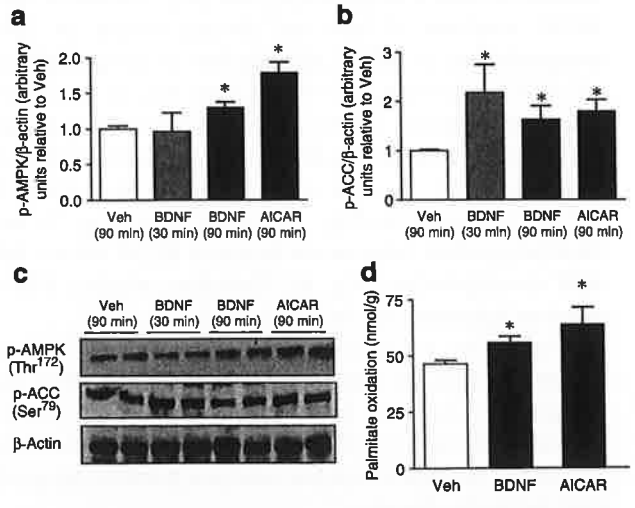

Fig. 5 BDNF increases phosphorylation (p-) of AMPK and its downstream target ACCβ (ACC) and enhances fat oxidation in isolated intact rat skeletal muscle. Quantification of phosphorylation of AMPK (Thr^{172}) (**a**) and ACCβ (Ser^{79}) (**b**) and representative immunoblots (**c**) and palmitate oxidation (**d**) in isolated rat extensor digitorum longus muscles treated for 30 and 90 min (**a**–**c**) and 120 min (**d**) with 10 ng/ml rhBDNF. *$p<0.05$ for difference from untreated vehicle control muscles (Veh). $n=5$ rats per group. Values are means±SEM

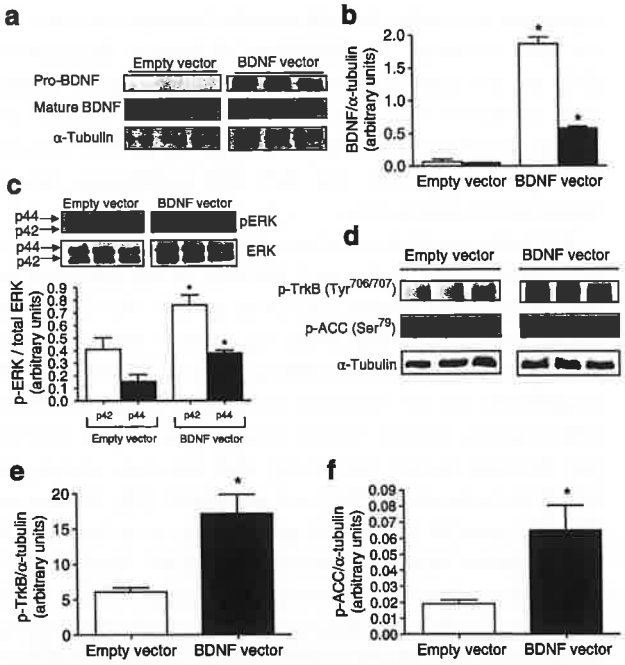

Fig. 6 Mouse skeletal muscle overexpression of BDNF in vivo increases ERK, TrkB and ACCβ (ACC) phosphorylation (p-). Representative immunoblots (**a**) and quantification (**b**) of pro-BDNF (white columns) and mature BDNF (black columns) protein expression. Representative immunoblots and quantification of phosphorylation of ERK (Thr^{202}/Tyr^{204}) (**c**), representative immunoblots (**d**) and quantification of phosphorylation of TrkB ($Tyr^{706/707}$) (**e**) and ACCβ (Ser^{79}) (**f**) in skeletal muscle that underwent in vivo electroporation with an empty vector or vector containing *Bdnf* plasmid. *$p<0.05$ for difference from empty vector. $n=3$ mice per group. Values are means±SEM

duction of BDNF in skeletal muscle in vivo markedly increased ACCβ phosphorylation (Fig. 6d, f).

Discussion

Taken together, our data demonstrate that BDNF is a protein produced in skeletal muscle cells that is increased by contraction to enhance fat oxidation in an AMPK-dependent fashion, most probably by acting in an autocrine and/or paracrine manner within skeletal muscle. Hence, we have identified BDNF as being a novel contraction-induced protein that may contribute to the multiple health benefits associated with exercise, possibly by enhancing fat oxidation in skeletal muscle.

A novel and important finding from the present study is that skeletal muscle cells per se produced BDNF and that this production is increased following contraction. Recent studies have demonstrated that *Bdnf* mRNA increases in the contracting skeletal muscle of rodents [22, 23], but these studies concluded that the likely source of the BDNF was neurons within the skeletal muscle beds. In addition, in a recent study, the mRNA of *Bdnf* was shown to be expressed in murine skeletal muscle homogenates and that this was increased by inhibition of histone deacetylases [24], but this study too could not conclude that the muscle cells produced the BDNF. By conducting muscle cell culture experiments, we show that C2C12 murine cells not only produce BDNF, but that this production can be increased by contraction.

Recently, we demonstrated a strong association between low plasma BDNF and type 2 diabetes on the one hand and both obesity and insulin resistance on the other [37]. The latter findings are supported by animal studies, which demonstrate an insulin-sensitising effect of BDNF [15–17]. Importantly, we have recently demonstrated that a different neurotrophin, namely ciliary neurotrophic factor (CNTF) can increase insulin sensitivity and decrease obesity by acting on both central [38] and peripheral [39, 40] tissues. Here we provide a potential mechanism as to how BDNF may improve metabolic disease, since we show both in vitro and in intact muscle ex vivo that, like CNTF, BDNF activates AMPK signalling and enhances FAO. Moreover, we demonstrate that the BDNF-induced increase in FAO is AMPK-dependent, at least in vitro, since either infection of cells with an AMPK dominant negative adenovirus (Fig. 4e) or treatment of cells with the AMPK inhibitor Compound C (Fig. 4d), completely attenuated the increase. It should be noted that during exercise in humans, fat oxidation is increased at the onset of contraction while the increase in BDNF in skeletal muscle does not occur until well into recovery. Therefore, it is clear that BDNF plays no role in mediating contraction-induced increases in fat oxidation. Nonetheless, our data raise the possibility that ligands that activate TrkB [41] are a possible therapeutic target for metabolic disease.

Unlike the cytokine IL-6, which is upregulated by muscle contractions, leading to altered liver [9] and adipose tissue [42] metabolism, our data suggest that contraction-induced muscle-derived BDNF does not act in a hormone-like manner. We showed that circulating levels of BDNF increased immediately after exercise and did not correlate with the elevated muscle-derived BDNF 24 h after exercise. In addition, we were unable to detect any BDNF in the media from cells that underwent contraction, even though such an intervention increased BDNF protein production. Moreover, when we overproduced the mature form of BDNF in skeletal muscle in vivo more than ten-fold via electroporation, we did not detect any increase in systemic BDNF concentration compared with control animals. It is more likely that muscle-derived BDNF works in an autocrine and/or paracrine manner within the skeletal muscle bed. This hypothesis is plausible since overproduction of BDNF resulted in marked ERK (Fig. 6c) and TrkB (Fig. 6d, e) phosphorylation, which is indicative of signalling through TrkB, which is produced in skeletal muscle and myotubes [43]. In addition, in our electroporation experiments, we detected increases in both the pro- and mature form of BDNF (Fig. 6a, b). It is well known that pro-BDNF can be processed into mature BDNF by several matrix metalloproteinases and thereby regulate activation of TrkB either by autophosphorylation or via an interaction with a neighbouring cell [44]. Consistent with BDNF treatment of cells and skeletal muscle ex vivo, overproduction of BDNF also resulted in phosphorylation of ACCβ. Whether this effect was due to paracrine/autocrine signalling or due to a direct intracellular signalling event requires further clarification. It must be acknowledged, however, that the contraction-induced increase in BDNF both in vivo (Fig. 1) and in vitro (Fig. 3) was relatively modest, whereas we increased BDNF several fold with electroporation (Fig. 6). Therefore, whether BDNF plays an important role in vivo is unclear.

In summary, we have identified BDNF as being a novel contraction-induced muscle cell-derived protein that can increase fat oxidation in skeletal muscle in an AMPK-dependent fashion. Our data, therefore, raise the possibility that BDNF analogues could be used as a possible therapy to treat metabolic disease.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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ERRATUM

Erratum to: Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase

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It has been brought to our attention that the Methods section did not include a description of a group of subjects involved in the study. The authors regret this omission. The first paragraph of the Methods should have read as follows (new material shown in red):

Human in vivo experiments Twenty healthy, physically active but untrained men (age 25.6±3.5 years, weight 78.9±9 kg, height 185±6.5 cm, BMI 21.3±2.11 kg/m^2 [mean±SD]) were randomised to either an exercise (n=10) or control (n=10) group. There was no difference between the two groups with regard to age, weight, height or $\dot{V}O_{2max}$. Subjects either performed 120 min of bicycle exercise at 60% $\dot{V}O_{2max}$ followed by a 6 h recovery period (exercise) or rested in bed for 8 h (control). Subjects also reported to the laboratory after an overnight fast at 24, 48 and 72 h after the commencement of the experimental trial. Blood was obtained at the following time points: 0, 2, 3, 5, 8, 24, 48 and 72 h. Muscle biopsy samples were obtained from vastus lateralis at time points 0, 2, 3, 5, 8, 24, 48 and 72 h using a percutaneous needle biopsy technique with suction. Samples were snap-frozen before being analysed. Serum levels were measured by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany). Platelet counts were determined by standard laboratory procedures. Data from this study are included in Figs 1a,b,d and 2a,b. Because of a lack of material, we included another eight healthy men (age 25±4 years, weight 82±8 kg, height 181±1 cm, BMI 25±2 kg/m^2 [mean±SD]). They had muscle biopsies taken immediately pre and post exercise and at 3 and 24 h after exercise. The data from these subjects are used only in Fig. 1c. The study was approved by the Ethics Committee of the University of Copenhagen.

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