

Activity of the Akt-dependent anabolic and catabolic pathways in muscle and liver samples in cancer-related cachexia

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Abstract In animal models of cachexia, alterations in the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway have been demonstrated in atrophying skeletal muscles. Therefore, we assessed the activity of proteins in this pathway in muscle and liver biopsies from 16 patients undergoing pancreatectomy for suspect of carcinoma. Patients were divided in a non-cachectic or cachectic group according to their weight loss before operation. Extracts of skeletal muscle and liver tissue from eight cachectic patients with pancreas carcinoma and eight non-cachectic patients were analysed by Western blotting using pan- and phospho-specific antibodies directed against eight important signal transduction proteins of the PI3-K/Akt pathway. Muscle samples from cachectic patients revealed significantly decreased levels of myosin heavy chain (−45%) and actin (−18%) in comparison to non-cachectic samples. Akt

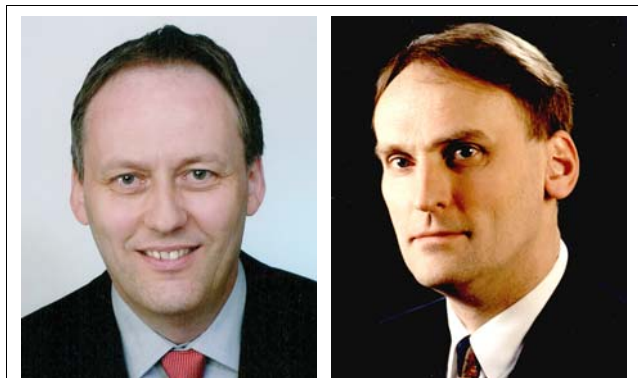
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protein level was decreased by −55%. The abundance and/or phosphorylation of the transcription factors Foxo1 and Foxo3a were reduced by up to fourfold in muscle biopsies from cachectic patients. Various decreases of the phosphorylated forms of the protein kinases mTOR (−82%) and p70S6K (−39%) were found. In contrast to skeletal muscle, cachexia is associated with a significant increase in phosphorylated Akt level in the liver samples with a general activation of the PI3-K/Akt cascade. Our study demonstrates a cachexia-associated loss of Akt-dependent signal-

ling in human skeletal muscle with decreased activity of regulators of protein synthesis and a disinhibition of protein degradation.

Keywords Cachexia · Pancreatic cancer · Skeletal muscle · Liver · Phosphatidylinositol 3-kinase · Signal transduction

Abbreviations

IR	insulin receptor
IRS1	insulin receptor substrate 1
MyHC	myosin heavy chain
PAA	polyacrylamide
PI3-K	phosphatidylinositol 3-kinase
SDS	sodium dodecyl sulphate

Introduction

The mechanisms responsible for cachexia in cancer and other chronic diseases are still poorly understood. This syndrome is always associated with a poor prognosis and is characterized by complex metabolic alterations including atrophy of skeletal muscle and adipose tissue [1]. The increased catabolism in these tissues and a systemic inflammatory response also lead to a general activation of the liver, which participates in synthesis of acute phase proteins (APP) and gluconeogenesis [2, 3]. Whether these changes are caused by humoral (host-derived) or tumor-derived factors is still a matter of controversy. Two supposedly tumor-derived factors, i.e. proteolysis-inducing factor (PIF) [4] and lipid-mobilizing factor (LMF) [5], have been isolated from the urine of cachectic cancer patients, but their clinical relevance is not firmly established. Robust evidence suggests that the pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α) participate in signalling pathways responsible for cachexia, and administration of these cytokines to experimental animals can trigger many cachectic effects [6, 7]. Cancer-related wasting of muscle and adipose tissue may be either due to decreased protein synthesis or increased protein degradation, but the precise molecular mechanisms are just beginning to be explored. A central role for muscular growth regulation in animal models plays the phosphatidylinositol 3-kinase (PI3-K)/Akt signalling pathway [8]. This important anabolic cascade is activated by the binding of insulin or insulin-like growth factor 1 (IGF-1) to its corresponding membrane receptors, which catalyse the phosphorylation of insulin receptor substrate protein 1 (IRS1). The phosphorylated IRS1 serves as a docking site for the p85 regulatory subunit of PI3-K [9]. Membrane-tethered PI3-K facilitates generation of phosphatidylinositol -3,4,5-triphosphate (PIP₃) by bringing the substrate

and enzyme into proximity. Generation of PIP₃, in turn, activates the serine/threonine kinase Akt (PKB), which then phosphorylates an array of protein substrates involved in growth, glucose homeostasis and apoptosis [10]. The linear pathway then involves activation of the mammalian target of rapamycin (mTOR), which integrates signals from nutritional status, and p70S6 kinase, which stimulates protein synthesis.

Activation of the PI3-K/Akt pathway induces muscle hypertrophy by stimulating protein translation via GSK3 β and mTOR [8, 11]. Another important feature of the PI3-K pathway has been discovered only recently, namely phosphorylation and inhibition of the pro-apoptotic Forkhead O (Foxo) group transcription factors. In two different experimental models of muscular atrophy, Foxo1 and Foxo3a were found to increase the expression of the ubiquitin-ligases MAFbx and MuRF1 [12, 13]. Due to these findings, a mechanistic link was identified from inhibition of the PI3-K/Akt pathway to the ubiquitin-dependent proteolytic machine, the proteasome, the activation of which is a hallmark of muscle wasting [14–16].

Despite these advances in the characterization of atrophy processes mainly in muscles of experimental animals, our knowledge of intracellular processes occurring in human cancer cachexia is very limited. In this context, two reports describe increased gene expression of ubiquitin and components of the proteasome pathway in skeletal muscle of patients with gastric cancer, but the presence of cachexia in this group was not investigated [17, 18]. Therefore, we decided to analyse skeletal muscle extracts from cachectic and non-cachectic patients undergoing surgery for pancreatic cancer with respect to expression and phosphorylation of proteins of the PI3-K/Akt pathway. The content of the myofibrillar protein myosin was measured as a marker of proteolysis. In addition, these analyses of peri-operatively obtained tissue samples were extended also to the liver.

Materials and methods

Patients

We obtained tissues from eight patients with cachexia (all had pancreatic carcinoma) and eight patients without cachexia (five pancreatic carcinoma and three chronic pancreatitis). Cachexia was defined as loss of more than 10% of original body weight within the last 6 months before operation. All cancer patients had ductal adenocarcinoma and were stage II according to UICC classification, except for two cases with stage IV in the cachexia group and one case with stage IV in the non-cachexia group. Two patients in the cachectic group and one patient in the non-cachectic group have been treated for diabetes.

Liver and rectus abdominis muscle biopsies were obtained during resection of the primary tumor respective to the pancreatic head in chronic pancreatitis patients, and small tissue pieces were immediately frozen in liquid nitrogen. Histology of the disease was confirmed by histopathological analysis.

Clinical data of the patients was collected in a prospectively designed database and updated during the hospital stay of the patients. The studies were approved by the ethics committees of the University of Heidelberg, Germany, and a written, informed consent was obtained before surgery.

Tissue homogenization

Frozen tissue pieces (about 200 mg) were pulverized under nitrogen cooling using the dismembrator II from B. Braun (Melsungen, Germany). The frozen powder was extracted with 2.5% *w/v* sulfosalicyl acid for 10 min and centrifuged (10 min, 10,000×*g*). The supernatant was stored frozen for further biochemical analyses. The pellet was washed in 75% ethanol and extracted in 300 μ l denaturing buffer [75 mM Tris–HCl pH 7.5, 50 mM NaCl, 2% sodium dodecyl sulphate (SDS), 0.5% Triton X-100]. The protein concentration in this total extract was determined using the BioRad DC protein assay after centrifugation (5 min at 10,000×*g*). The extracts were adjusted to the same protein concentration (2 mg/ml), supplemented with 10% *v/v* glycerol, 100 mM dithiothreitol and bromophenol blue and heated.

Myofibrillar protein analysis

Seven micrograms of protein lysate was loaded onto an 8% SDS polyacrylamide (PAA) gel containing 25% *v/v* glycerol and subjected to electrophoresis on a Mini-Protean 3 chamber (BioRad, München, Germany) for 3 h at 160 V. The gel was subsequently stained in colloidal Coomassie blue (Roth, Karlsruhe, Germany) and scanned directly after washing.

Western blot

For Western blot analysis, 30 μ g of total tissue extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; with various concentrations of PAA ranging from 7.5 to 10%). Proteins were transferred to nitrocellulose (Bio-Rad) or polyvinylidene difluoride (PVDF) membranes using tank blotting (80 V/1 h). The membranes were blocked with 4% bovine serum albumin (BSA) and incubated with antibodies of interest diluted in 2% BSA for 1 h. After washing and labelling with peroxidase-conjugated secondary antibodies, light emis-

sion after addition of electrochemiluminescence (ECL) substrate (Perkin Elmer, Rodgau-Jügesheim, Germany) was captured onto X-ray film. When alkaline phosphatase-labelled antibodies were used, light emission was detected after addition of LumiPhos (BioRad). Bound antibodies were stripped from membranes by incubation in 2% SDS+100 mM dithiothreitol. Stripped blots were washed and then probed with other antibodies of interest. To control for equal loading, all blots were finally stained with the antibody directed against moesin. Films were evaluated after scanning with the TINA densitometry program without any further processing of electronic files. For quantitative analysis, samples of both groups of patients (cachectic and non-cachectic) were evaluated in the same experiment. Densitometric values are expressed as percentages relative to an identical standard extract run on each gel, thus assuring comparability of the different blots.

Antibodies

Antibodies directed against FKHR (#9462), p70S6K (#9202), pT₃₈₉-S6K (#9206), pS₂₄₄₈-mTOR (#2971), Akt1-3 (#9272), pS₄₇₃-Akt (#9271), pT₃₀₈-Akt (#9275) and pS₉-GSK3 β (#9336) were obtained from CST (Frankfurt, Germany). Antibodies directed against FKHL1 (#sc11351), pT₃₂-FKHL1 (#sc12357), IRS1 (#sc-559) and mTOR (FRAP; #sc1549) were obtained from Santa Cruz (Heidelberg, Germany). The monoclonal anti-IR β antibody (#GR36) was purchased from Oncogene (Schwalbach/Ts, Germany), and the monoclonal antibody directed against moesin was kindly provided by R. Schwartz-Albiez (D010, DKFZ). Peroxidase- and alkaline phosphatase-conjugated secondary antibodies specific for rabbit or mouse IgG were obtained from Dianova (Hamburg, Germany).

Statistics

Results are presented as mean \pm SEM. Differences between groups or subgroups were assessed using the unpaired Student's *t* test. Differences were considered as statistically significant if *P* was <0.05. All statistical procedures were performed with the program Statistical Package for the Social Sciences (SPSS) for Windows (version 11.5, SPSS, Chicago, IL, USA). Comparisons were performed between the cachectic group (solely comprised of patients with pancreatic cancer) and the entire non-cachectic groups (Figs. 1, 2, 3, 4 and 5). As the non-cachectic group had three patients without pancreatic cancer, separate comparisons of the cachectic group and the non-cachectic patients with pancreatic cancer were performed (Table 2).

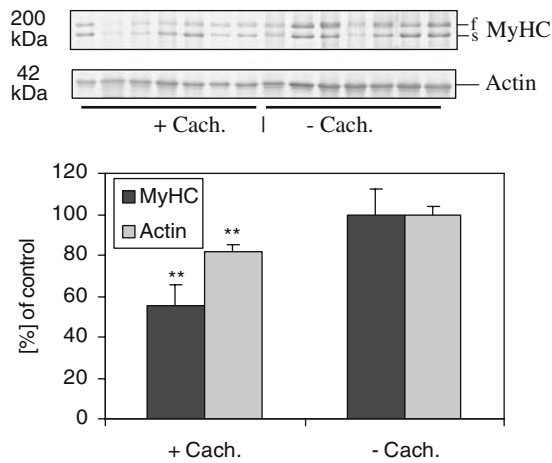


Fig. 1 Analysis of MyHC and actin levels in muscle biopsies taken from cachectic (+*Cach.*) and non-cachectic (–*Cach.*) patients. Equal protein amounts (7 μ g) of representative muscle lysates from patients with cachexia and without cachexia were separated on an 8% SDS-PAA gel and stained with Coomassie blue. The regions corresponding to the MyHC (200 kDa) and actin (42 kDa) are shown. For MyHC, the indices *f* and *s* denote the fast and slow isoforms, respectively. In the lower panel, the percentage of relative protein level is reported as mean \pm SEM, where the value from non-cachectic subjects (control group) is set to 100%. ** $P\leq 0.01$

Results

The clinical and demographic data for both the non-cachectic and cachectic group are shown in Table 1. A significant difference between the cachexia and the non-cachexia group was noted only for the relative weight loss,

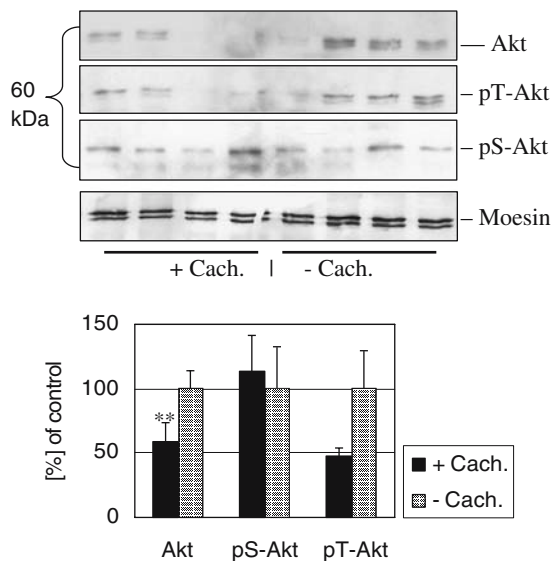


Fig. 2 Western blot analysis of Akt expression and phosphorylation in muscle biopsies of cachectic (+*Cach.*) and non-cachectic (–*Cach.*) patients. The blot shows representative lysates that were separated on a 9% SDS-PAA gel and stained with antibodies directed against all isoforms of Akt, pS⁴⁷³-Akt or pT³⁰⁸-Akt. In the lower panel, the percentage of relative protein level is reported as mean \pm SEM, where the value from non-cachectic subjects (control group) is set to 100%. ** $P\leq 0.01$

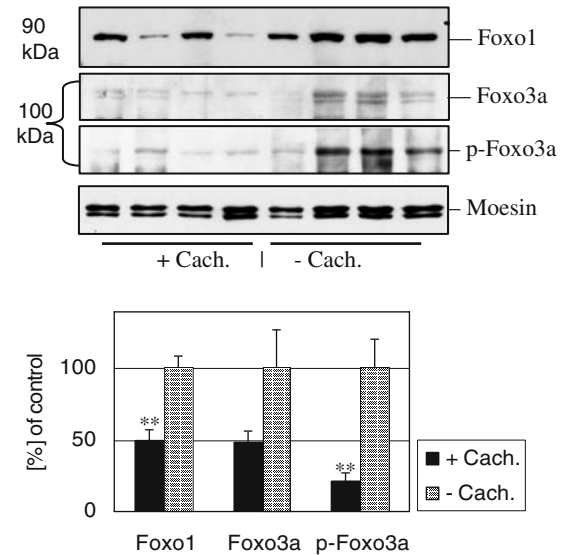


Fig. 3 Western blot analysis of expression and phosphorylation of Foxo proteins in muscle biopsies of cachectic (+*Cach.*) and non-cachectic (–*Cach.*) patients. The blot shows representative lysates that were separated on a 9% SDS-PAA gel and stained with antibodies directed against Foxo1 (FKHR), Foxo3a (FKHRL1) and pThr³²-Foxo3a, respectively. In the lower panel, the percentage of relative protein level is reported as mean \pm SEM, where the value from non-cachectic subjects (control group) is set to 100%. ** $P\leq 0.01$

which attributed patients to the respective group. The body mass index (BMI) values were similar between both groups. Determination of the maximal thickness of the rectus abdominis muscle by abdominal computed tomography (CT) scans, performed before operation, showed lower values (–22%) in cachectic (5.4 \pm 0.8 mm) compared to non-cachectic (6.9 \pm 0.9 mm) patients, which however failed to be significant. Moreover, no significant differences were

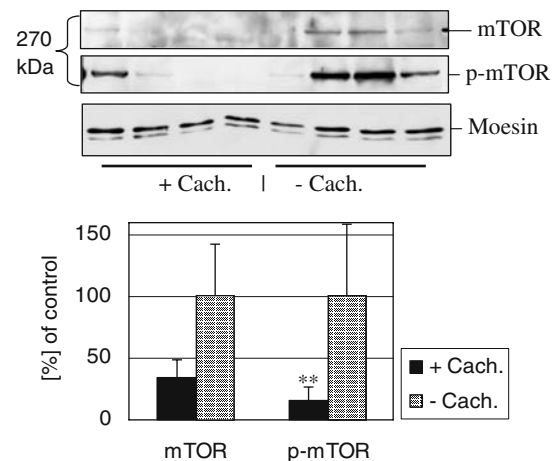


Fig. 4 Western blot analysis of mTOR expression and phosphorylation in muscle biopsies of cachectic (+*Cach.*) and non-cachectic (–*Cach.*) patients. Representative lysates were separated on a 7.5% SDS-PAA gel and stained with antibodies directed against mTOR and pS²⁴⁴⁸-mTOR. In the lower panel, the percentage of relative protein level is reported as mean \pm SEM, where the value from non-cachectic subjects as the control group is set to 100%. ** $P\leq 0.01$

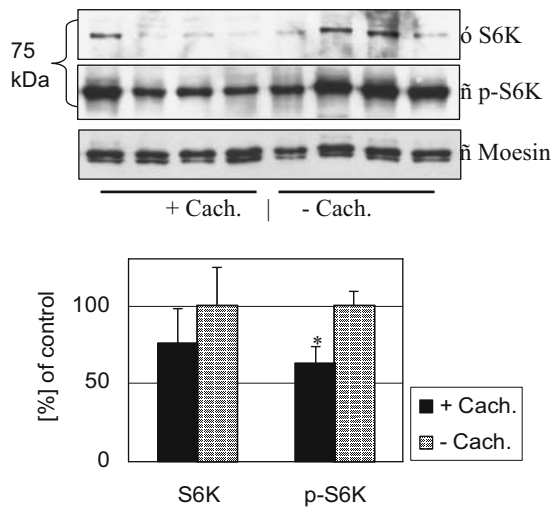


Fig. 5 Western blot analysis of expression and phosphorylation of p70S6 kinase in muscle biopsies of cachectic (+Cach.) and non-cachectic (–Cach.) patients. The blot shows representative muscle lysates from muscle that were separated on a 9% PAA gel and probed with antibodies directed against S6K and pT³⁸⁹-S6K. In the lower panel, the percentage of relative protein level is reported as mean±SEM, where the value from non-cachectic subjects (control group) is set to 100%. *P<0.05

seen in any parameter under test when patients were grouped by sex.

Myosin, actin and moesin levels

To identify differences in myosin expression between the two groups, protein extracts from skeletal muscle were separated on an 8% SDS-PAA gel and stained with Coomassie blue. The slow (type I) and fast (type II) isoforms of myosin heavy chain (MyHC) were identified by their corresponding molecular weight (200 kDa) and specific abundance in muscle. A significant (P=0.01) decrease by –45% of the total levels of both MyHC isoforms was noted in biopsies of cachectic in comparison to non-cachectic patients (Fig. 1). However, no significant difference in the ratio of fast to slow MyHC isoforms was noted between the cachectic (50% fast and slow) and non-cachectic (56% fast and 44% slow) group. Furthermore, much smaller differences (–18%) were seen in the level of

Table 1 Anthropometric and clinical data

	Non-cachexia	Cachexia	P value
N (female, male)	8 (1 f, 7 m)	8 (5 f, 3 m)	
Age (year)	53±4	62±3	NS
Weight loss (%)	5.2±1.4	12.4±1.0	0.002
BMI (kg/m ²)	24.8±1.0	23.5±1.4	NS
Bilirubin (mg/dl)	2.5±1.9	5.2±2.9	NS
CRP (mg/l)	9.0±6.6	12.2±5.1	NS

Values are mean±SEM.

Table 2 Comparison of muscle parameters between cachexia and non-cachexia within the carcinoma group

	Non-cachexia	Cachexia	P value
Number of patients	5	8	
MyHC	149.8±27.1	80.3±15.3	0.036
Akt	69.2±6.6	28.5±5.7	0.001
pT-Akt	517.4±202.9	199.0±28.7	NS
p-GSK3β	173.2±16.3	113.4±10.2	0.009
Foxo1	281.7±30.8	131.7±25.8	0.011
p-Foxo3a	198.2±61.9	44.9±11.6	0.011
mTOR	12.4±7.6	4.9±1.9	NS
p-mTOR	47.9±14.4	7.1±4.6	0.007
S6K	228.1±72.8	115.4±35.5	NS
p-S6K	132.2±18.7	74.5±7.1	0.033

Values are given in relative arbitrary units (mean±SEM).

actin (42 kDa), another abundant and important muscular contractile protein (Fig. 1).

To rule out a general effect of cachexia on protein content, we performed a Western blot using an antibody directed against moesin, an ubiquitous cytoskeletal protein [19]. Densitometric evaluation of moesin content showed that muscle and liver samples of cachectic patients contained 93.9±5.7% and 103.4±1.7% of that of non-cachectic patients, respectively (cf. Figs. 2, 3, 4 and 5). These results indicate that (1) there is no substantial difference in moesin content between both groups and (2) cachexia is not associated with non-specific protein degradation but involves rather a specific loss of the contractile protein myosin. This was confirmed by comparing the cachexia-related decrease in myosin as values given per mg total protein (–45%, see above) to values normalised for the housekeeping protein moesin, which showed an identical decrease (–44%, P=0.007).

PI3-K/Akt pathway in muscle and liver

The PI3-K/Akt pathway is stimulated by insulin and IGF1, effects believed to be diminished in cachexia. We analysed several members of this pathway by highly sensitive Western blotting with phospho-specific antibodies to determine the activation state of various components.

The IR protein was not detectable in the muscle samples but was found to be present in liver samples; there was no difference in hepatic IR levels between the two groups of patients. No differences were found in the phosphorylation of the hepatic IR, which was present only in the unprocessed holoprotein IRαβ, migrating in the SDS-PAA gel at 180 kDa, and not in the processed β subunit normally seen at 95 kDa.

IRS1 is an important substrate for the IR, and its abundance and phosphorylation are tightly regulated. The

levels of IRS1 protein, which was detectable at 175 kDa in both tissues, was lower in muscle and higher in liver samples from cachectic patients without reaching statistical significance (data not shown). As a generally accepted and functionally important phosphorylation site has not yet been established, we did not analyse phosphorylation of IRS1.

Akt is a serine/threonine kinase that is central for the PI3-K/Akt pathway and catalyses the phosphorylation of many target proteins. In muscle biopsies of cachectic patients, we found decreased Akt1-3 protein expression (45.4% of the level seen in non-cachectic group; $P=0.01$) and phosphorylation, as assessed by phospho-specific antibodies that recognize phosphorylated Thr³⁰⁸ (pT-Akt; 45.9% of the level seen in the non-cachectic group; $P=0.13$). Phosphorylation of Ser⁴⁷³ (pS-Akt) was marginally increased in samples from cachectic patients, but the difference was not statistically significant (Fig. 2). In liver samples from cachectic patients, there was a statistically insignificant increase (+73%) in the amount of Akt1-3 ($P=0.079$), and a significant twofold increase in the phosphorylation of Ser⁴⁷³ ($P=0.022$) in comparison to non-cachectic patients (data not shown).

We then determined the abundance and activation states of several substrates of Akt. Analysis of GSK3 β , which is important for glucose homeostasis, revealed a 30% reduction in phosphorylation at Ser⁹ in muscle samples from cachectic patients (data not shown). However, no differences were seen in GSK3 β phosphorylation in liver samples despite the increased Akt phosphorylation in samples obtained from cachectic patients.

Next, we examined the Forkhead (Foxo) group of proteins, which have been implicated in apoptosis and atrophy, but have not yet been characterised in human tissue extracts to date. In muscle samples, Foxo1 was identified at 90 kDa, and its abundance was reduced almost twofold ($P=0.004$) in biopsies from patients with cachexia (Fig. 3). The expression of Foxo3a (detected at 100 kDa) showed a reduction in samples from cachectic patients ($P=0.19$), but the phosphorylation of its critical residue Thr³² was more than fourfold lower ($P=0.006$) in comparison to non-cachectic patients (Fig. 3). Importantly, the relative phosphorylation of Foxo3a, calculated as the ratio of phosphorylated to total Foxo3a, was 2.7 lower in muscle biopsies from cachectic patients in comparison to non-cachectic patients ($P=0.006$). This strong reduction should lead to a disinhibition and transcriptional activation. In liver tissue, Foxo1 was reduced about twofold, and Foxo3a was not detectable (data not shown).

The major PI3-K pathway leads from Akt to mTOR, a protein kinase that is sensitive to hormonal and nutritional inputs. The large (275 kDa) mTOR protein showed the lowest level and the greatest variability of all proteins

investigated in our study of muscle tissue. In muscle biopsies from patients with cachexia, a strong but statistically insignificant decrease to 36% of control in mTOR abundance was seen, and a sixfold decrease ($P=0.01$) in phosphorylation of mTOR was found (Fig. 4). Consequently, the relative phosphorylation of mTOR was twofold lower ($P=0.048$). In the liver, an insignificant increase (+50%) in mTOR protein abundance and a twofold increase ($P=0.113$) in mTOR phosphorylation were noted (data not shown).

The final member of the PI3-K/Akt pathway investigated was ribosomal protein S6 kinase (S6K), an effector of protein synthesis. In skeletal muscle biopsies of cachectic patients, the protein level of S6K was reduced only insignificantly, but the phosphorylation of the critical residue Thr³⁸⁹ was significantly decreased to 61% of the levels seen in non-cachectic patients (Fig. 5). In liver tissue, a statistically insignificant decrease in the content of S6K ($P=0.141$) and marginal increases in Thr³⁸⁹ phosphorylation in biopsies from cachectic patients were found (not shown).

Analysis and comparison of subgroups carcinoma and pancreatitis

Because the non-cachectic control group included three patients without cancer, we also analysed the influence of cachexia when comparing only the non-cachectic patients with pancreatic carcinoma to the cachectic patients (all of whom had pancreatic carcinoma). We found that all significant differences seen in the initial analyses were unaffected by exclusion of the three non-cachectic patients with pancreatitis (Table 2). Moreover, a comparison between the non-cachectic patients with pancreatic cancer or with pancreatitis revealed no difference in any of the parameters under test (data not shown).

Discussion

The cellular mechanisms leading to cancer cachexia, especially muscle wasting, have not been elucidated in humans. In experimental models, a wealth of information about atrophy mechanisms in skeletal muscle has been obtained recently. The altered PI3-K/Akt pathway has been shown to play a central role in this process by attenuating anabolic processes together with increasing protein catabolism [11]. In the present cross-sectional study on a non-locomotor muscle, we found that the muscle wasting of cachectic cancer patients involves alterations in the PI3-K/Akt effector molecules mTOR/S6K and Foxo1/3. These changes are obviously detectable at a relatively early stage,

i.e. during surgery shortly after diagnosis. Our present comparative cross-sectional study of muscle wasting in ‘clinically’ cachectic and non-cachectic groups was crucially strengthened by the significant difference found for MyHC in the presence of virtually identical control protein levels. In fact, MyHC has been recently shown to be a primary and rather selective target of cachectic factors [20, 21]. Furthermore, the small decrease in actin levels is not surprising, as a 14-kDa cleavage product of actin has been detected in atrophic muscles of rats with diabetes or chronic uremia [22].

Notably, we found no differences in the level of IRS1, and this result is in agreement with a report on diabetic and non-diabetic pancreatic cancer patients that showed unaltered muscular levels of IR and IRS1 levels [23]. This does not exclude differences in more downstream insulin signalling events such as PI3-K-activity and glucose transport, which have been shown to be impaired in skeletal muscles of pancreatic cancer patients [24]. Of interest, we did not find differences of liver IR protein expression between the groups. However, the finding that we did not detect IR protein in the muscle biopsies may be explained by the fact that the samples were obtained peri-operatively, and thus, the influence of fasting and/or anaesthesia has to be taken into consideration.

Strong and significant differences between muscle and liver samples taken from cachectic and non-cachectic patients were detected on the level of Akt and its substrates. In cachectic muscle samples, the Akt protein content was significantly reduced. Furthermore, its serine phosphorylation was unchanged, but phosphorylation of Thr³⁰⁸ was more than twofold reduced. This is in concert with decreased phosphorylation of the Akt substrates GSK3 β and Foxo3a and is in line with experimental studies, which show that phosphorylation of Thr³⁰⁸ is critical for the activity of Akt [25].

We show in this study for the first time that the phosphorylation of Foxo3a is significantly reduced in skeletal muscles of cachectic compared to non-cachectic cancer patients. An increased phosphorylation of Foxo3a (FKHRL1) has been shown to lead to transcriptional activation [26], as demonstrated by cellular transfection experiments [27, 28] and immunohistochemical analyses of clinical samples [29]. Further studies have shown the ability of activated Foxo1/3 to induce the ubiquitin-dependent proteasome activation via expression of the ubiquitin ligases MuRF and MAFbx [12, 13]. Therefore, our present findings provide evidence that activated Foxo transcription factors play a role in human muscle wasting due to cancer cachexia. In this context, our finding of a twofold decrease in the amount of Foxo1 is somewhat surprising and needs clarification; unfortunately, no quantitative data about Foxo1 expression have been reported in the animal studies.

In comparison to other signalling proteins under study, expression and phosphorylation of mTOR showed the greatest decrease in muscle biopsies of cachectic patients. This change in phosphorylation is much more pronounced than that of Akt and is in agreement with the emerging evidence that mTOR is not a direct substrate for Akt but rather an integrator of metabolic status and that its phosphorylation involves the tuberlin–hamartin complex [30]. The final effector molecule for protein synthesis, p70S6 kinase, was reduced significantly in muscle biopsies of cachectic patients, but more modestly than mTOR, which is claimed to be the kinase phosphorylating S6K.

Detailed statistical analysis of individual ratios of phosphorylated to total protein levels revealed significant decreases in relative phosphorylation for Foxo3a and mTOR in muscle biopsies of cachectic patients, whereas the reductions in phosphorylated Akt and p70S6K are due to lower expression of the corresponding proteins. In addition to these comparisons between the two groups, we evaluated the influence of weight loss variability on the individual expression of muscle proteins under test by performing regression analyses in both groups combined: Relative weight loss showed a negative correlation with content of myosin ($r=-0.59$, $P=0.028$), phosphorylated mTOR ($r=-0.56$, $P=0.047$), phosphorylated S6 kinase ($r=-0.58$, $P=0.020$) and phosphorylated Foxo3a ($r=0.35$, $P=0.183$).

Our findings in the liver samples of cachectic patients were exciting because, to our knowledge, no intracellular data for this organ have been reported in human cachexia. Only one study in tumor-bearing mice showed strongly increased metabolic activity of the liver [31]. In this context, it has been suggested that the liver is involved in the acute-phase response and in the negative nitrogen balance of the body. Our data now show a clear trend for the activation of the PI3-K/Akt pathway in human liver samples; however, a statistical significant difference was reached only for Akt phosphorylation, partly due to a smaller sample number. This may support the concept that the liver is involved in cachexia by uptake of the amino acids released from muscles and participates in the synthesis of acute-phase proteins [3].

As expression of constitutively active Akt induces hypertrophy of skeletal muscle, it has been proposed that activation of Akt is a logical target for pharmaceutical design in treating the muscle atrophy that occurs in cachexia [32]. Unfortunately, our findings in samples from liver tissue indicate that activation of Akt in all tissues might not be a desirable effect in treating cachexia because excessive activation of inflammatory pathways might occur. The muscle-specific transcription factor Foxo3a or the protein kinase mTOR seem to be the preferential candidates for pharmacological intervention, therefore.

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