Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAfbx in muscle

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Submitted 17 May 2007; accepted in final form 30 June 2007

ALTHOUGH THE NUMBER OF SMOKERS has declined steadily over the past 50 years, ~20% of US adults still smoke regularly (3, 6). One-third of these are “heavy smokers,” consuming 20 or more cigarettes daily (6). The prevalence of habitual tobacco consumption is even greater in Great Britain (6) and through-out Europe (17), as well as in the developing world (17).

Tobacco use poses a major public health problem because smoking is a major risk factor for cardiovascular disease, chronic obstructive pulmonary disease, and lung cancer (43, 57) and is associated with increased risk for other debilitating conditions, such as cataract, pneumonia, and cancers of the cervix, kidney, pancreas, and stomach (1). There is also some evidence that smoking may impair physical function (33) and probably increases the risk for sarcopenia (i.e., age-related muscle wasting) (2, 45). This suggests that smoking has direct adverse effects on muscle protein metabolism, which may lead to loss of independence and disability with advanced age. Nevertheless, the effect of smoking on muscle protein metabolism is not known.

A number of conditions in which muscle wasting occurs have been associated with a decreased rate of muscle renewal as a result of depressed muscle protein synthesis (9, 35, 37). We therefore hypothesized that habitual heavy smoking is associated with depressed muscle protein synthesis. To test this hypothesis we measured the basal, postabsorptive fractional rate of muscle protein synthesis (FSR) and whole body leucine flux (an index of whole body proteolysis) by using stable-isotope-labeled tracer techniques in heavy smokers and individuals who had never smoked. We also measured the expression of genes involved in the regulation of muscle mass [i.e., the muscle growth inhibitor myostatin (54)] and muscle atrophy F-box (MAfbx) and muscle-specific RING Finger-1 (MuRF)-1, which are associated with muscle wasting in other conditions. There were no differences between nonsmokers and smokers in plasma leucine concentration, leucine rate of appearance, and plasma concentrations of inflammatory markers, or TFN-α mRNA in muscle, but muscle protein FSR was much less (0.037 ± 0.005 vs. 0.059 ± 0.005%/h, respectively, P = 0.004), and myostatin and MAfbx (but not MuRF-1) expression were much greater (by ~33 and 45%, respectively, P < 0.05) in the muscle of smokers than of nonsmokers. We conclude that smoking impairs the muscle protein synthesis process and increases the expression of genes associated with impaired muscle maintenance; smoking therefore likely increases the risk of sarcopenia.

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nonsmokers) consumed alcohol within the recommended limits (i.e., ≤14 units/wk for women; ≥21 units/wk for men), and one subject, a smoker, consumed ≥21 units/wk but had no clinical signs of alcoholism. All subjects had normal liver function tests. Pulmonary function was assessed according to current guidelines, and the outcomes were expressed as percentages of predicted values according to age, sex, and height (10). Forced expiratory volume and forced vital capacity were measured with a dry wedge spirometer (Vitalograph, Maidhead, UK). Diffusion capacity was measured by single-breath diffusion capacity for carbon monoxide, and residual volume and total lung capacity were assessed by body plethysmography (MasterLab Jäger, Wurtzburg, Germany). The study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all subjects before their participation in the study, which was approved by the local (Copenhagen and Frederiksberg Communities, Denmark) ethics committee.

Experimental protocol. All subjects underwent a stable-isotope-labeled leucine tracer infusion study to determine leucine rate of appearance (R\textsubscript{Leu}) in plasma (an index of the whole body protein breakdown rate) and the FSR of mixed muscle protein. Subjects were instructed to adhere to their regular diet and to refrain from vigorous exercise for 3 days before the study. They arrived at the Copenhagen Muscle Research Center, at 0700, after an overnight fast. At ∼0730, a cannula was inserted into an antecubital vein for the infusion of a stable-isotope-labeled leucine tracer; another cannula was inserted into a vein of the contralateral forearm for blood sampling. At ∼0800 (t = 0 min), a baseline blood sample was obtained to determine the background enrichment of leucine and cytokine concentrations in plasma, and a muscle biopsy was taken from the quadriceps femoris muscle to determine the background enrichment in muscle protein. Immediately afterward, a primed, constant infusion of [1,2\textsuperscript{13}C\textsubscript{2}]leucine (priming dose 7.8 μmol/kg body wt, infusion rate 0.13 μmol·kg body wt \textsuperscript{-1}·min \textsuperscript{-1}) was started and maintained until the completion of the study, −2 h later. At the end of the infusion (t = 120 min), another muscle biopsy was obtained to determine the rate of muscle protein synthesis and skeletal muscle gene expression. Additional blood samples were obtained at 30, 60, 90, 100, 110, and 120 min after the start of the tracer infusion to determine the enrichment of plasma leucine and α-ketosaccharic acid (KIC; an index of intracellular free leucine enrichment (4, 24)). The tracer infusion was stopped and cannulae were removed after the second biopsy.

Sample collection and storage. Blood samples (−5 ml) were collected in preshilled tubes containing EDTA; plasma was separated immediately and stored at −70°C until final analyses. Muscle tissue (−50 mg) was obtained under local anaesthesia (lidocaine, 2%) by using the Bergström needle technique. The tissue was immediately frozen in liquid nitrogen for subsequent determination of protein-bound leucine enrichment and gene expression. The deep-frozen samples were stored at −70°C until final analyses.

Sample preparation and analyses. Plasma glucose concentration was determined on an automated glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH). Plasma insulin concentration was measured by radioimmunoassay. Enzyme-linked immunosorbent assays (ELISA) were used to determine the plasma concentrations of TNF-α, TNFR1, IL-6 (R&D Systems, Minneapolis, MN), and CRP (Roche/Hitachi, Roche Diagnostics, Mannheim, Germany).

To determine plasma leucine enrichment and concentration and plasma KIC enrichment [tracer-to-tracee ratio (TTR)]-t, a known amount of norleucine internal standard was added to plasma, proteins were precipitated, and the supernatant, containing free amino and imino acids, was collected to prepare the t-BDMS (leucine) and OPDA-t-BDMS (KIC) derivatives for analysis by gas chromatography-mass spectrometry (GC-MS; MD800, Fisons Plc, Ipswich, UK) and electron ionization and selective ion monitoring (42).

To determine the leucine enrichment in muscle proteins, frozen muscle (20–30 mg) was ground in liquid nitrogen to a fine powder and homogenized in trichloroacetic acid solution (3%, and proteins were then precipitated by centrifugation (1,500 g, 4°C for 15 min) (30, 50, 51)). Proteins were hydrolyzed in 6 N HCl (12 h at 110°C), and the liberated amino acids were purified on cation exchange columns (Dowex 50W-X8-200; Sigma, Poole, UK) (30, 50, 51). The amino acids were then converted to their NAP derivative, and the leucine TTR was determined by gas chromatography-combustion isotope ratio-mass spectrometry (GC-C-IRMS, Delta-plus XL; ThermoFinnigan, Hemel Hempstead, UK) (27, 36).

To evaluate skeletal muscle gene expression, total RNA was extracted and quantified spectrophotometrically by using the absorbances 260 and 280 nm. Precisely 1 μg total RNA was electrophoresed on a nondenaturing agarose gel containing ethidium bromide (0.5 μg/ml) to check for contaminants, RNA integrity, and equal loading. A cDNA pool was created for each sample from 1 μg of total RNA by using iScript reverse transcriptase reagents (Bio-Rad, Hemel Hempstead, UK). Gene expression analysis was performed by using a Bio-Rad iCycler. Real-time PCR for all genes was completed in duplicate by using the Bio-Rad SYBR Green supermix with 100 nM forward and reverse primers and 2 μl of a 1:5 dilution of cDNA in a 25-μl reaction. Primer sequences were as follows: myostatin forward CTA CAA CTT AAG AAA CCA TCA TTA CCA, reverse GGT TCA GAG ATC GGA TTC TCG TAT; MAFBx forward CGA CCT CAG CAG CTA TCG CAA C, reverse TTT GGT GCT ATC TGG GCC AAC AGC C; MuRF-1 forward AGT GAC CAA GGA GGA CAG TCA, reverse CAC CAG CTT TGT GGA CTT GTT; TNF-α forward CAT GTT GTA GAC CCT CCA, reverse GGT GAC CTT GGT CTT GTA G. Validation of suitability of housekeeping genes was checked by normalizing one housekeeping gene to another. The ratio of B2M to GAPDH was found to be stable; thus B2M was used for subsequent normalization. Gene changes were quantified taking into account individual primer efficiencies (34).

Calculations. The FSR of mixed muscle protein was calculated on the basis of the incorporation rate of [1,2\textsuperscript{13}C\textsubscript{2}]leucine into muscle proteins, using a standard precursor-product model as follows: FSR = ΔE\textsubscript{p}/E\textsubscript{m} × 1/t × 100, where ΔE\textsubscript{p} is the change in enrichment (TTR) of protein-bound leucine in two subsequent biopsies, E\textsubscript{m} is the mean enrichment over time of the precursor for protein synthesis (i.e., leucyl-tRNA), and t is the time between biopsies. Plasma KIC was chosen to represent the immediate precursor for muscle protein synthesis (i.e., leucyl-tRNA) (4, 24, 53). Values for FSR are expressed as percent per hour.

Leucine R\textsubscript{Leu} in plasma was calculated by dividing the tracer infusion rate by the average plasma KIC enrichment during the last 30 min of the leucine tracer infusion. The contribution of stable-isotope-labeled leucine resulting from the tracer infusion was subtracted from the calculated total leucine R\textsubscript{Leu}.

Insulin resistance was assessed by using the homeostasis model assessment of insulin resistance (HOMA-IR) as previously described (25).

Statistical analysis. All data sets were tested for normality. Differences between smokers and nonsmokers were assessed by using Student’s t-test for independent samples. Muscle gene expression data (myostatin, MAFBx, and MuRF-1) were log transformed to satisfy normality requirements, for analysis. A P value of ≤0.05 was considered statistically significant.

RESULTS

Clinical characteristics of study participants. Subjects were matched for sex, age, and body mass index (Table 1). All subjects had normal blood pressure, but pulmonary function was impaired in smokers compared with nonsmokers, as indicated by decreased forced expiratory volume (FEV\textsubscript{1}), increased residual volume, and decreased diffusion capacity (Table 1). Nonetheless, forced vital capacity (FVC) was normal, and the FEV\textsubscript{1}-to-FVC ratio was >0.70, indicating the absence of chronic obstructive pulmonary disease (11) (Table 1). Plasma...
glucose, insulin, and triglyceride concentrations, and HOMA-IR values were within the normal range (48) and not different in smokers and nonsmokers (Table 1). Plasma concentrations of CRP, TNF-α, and TNFR1 were also not different between the two groups (Table 2). Plasma IL-6 concentration tended to be greater in smokers than in nonsmokers, but the difference did not reach statistical significance (P = 0.08; Table 2).

**Muscle gene expression.** Total muscle RNA yield was not different between nonsmokers and smokers (P = 0.52). Myostatin (P = 0.036) and MAFBx (P = 0.040) mRNA concentrations were markedly elevated in the muscle of the smokers compared with those in the nonsmokers (Fig. 3), whereas muscle mRNA concentrations for MuRF-1 (P = 0.242) and TNF-α (P = 0.845) were not different between groups (Fig. 3).

**DISCUSSION**

It was our goal to discover whether smoking affects muscle protein metabolism; specifically, we tested the hypothesis that smoking depresses muscle protein synthesis. We found that the basal rate of mixed muscle protein synthesis was, in fact, markedly reduced in middle- and older-aged heavy smokers compared with age-matched individuals who had never smoked. Furthermore, we observed that the expression of genes associated with inhibition of muscle growth and muscle catabolism, namely myostatin and MAFBx, were elevated.

However, we did not discover any sign of altered whole body protein metabolism, i.e., plasma leucine concentration, an index of systemic leucine balance, and plasma leucine Ra, an index of whole body protein breakdown. Furthermore, there were no signs of increased inflammatory activity in terms of plasma concentrations of proinflammatory cytokines and CRP or increased TNF-α mRNA expression in muscle.

The techniques we used to evaluate protein turnover have been in use for 10–20 years, and the values of all measured variables obtained for our nonsmokers are in good agreement with those reported by us and other investigators. In particular, the values for mixed muscle protein synthesis are similar to those we (29, 30, 50, 51) and others (41, 52, 56) obtained for quadriceps muscle in healthy adults in the postabsorptive state.

**Table 1. Clinical characteristics of study subjects**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>63±3</td>
<td>65±3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>172±3</td>
<td>176±4</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>74±2</td>
<td>81±5</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>25.1±1.2</td>
<td>25.9±0.9</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>127±3</td>
<td>128±5</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>72±2</td>
<td>79±3</td>
</tr>
<tr>
<td>Plasma glucose concentration, mM</td>
<td>4.9±0.1</td>
<td>5.4±0.3</td>
</tr>
<tr>
<td>Plasma insulin concentration, M/U/l</td>
<td>6.7±0.9</td>
<td>6.5±0.8</td>
</tr>
<tr>
<td>HOMA-IR,</td>
<td>1.38±0.27</td>
<td>1.44±0.21</td>
</tr>
<tr>
<td>Plasma triglyceride concentration, mM</td>
<td>1.2±0.3</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Forced expiratory volume, %predicted</td>
<td>103±6</td>
<td>87±3*</td>
</tr>
<tr>
<td>Forced vital capacity, %predicted</td>
<td>105±5</td>
<td>97±6</td>
</tr>
<tr>
<td>Residual volume, %predicted</td>
<td>102±7</td>
<td>136±4*</td>
</tr>
<tr>
<td>Total lung capacity, %predicted</td>
<td>101±5</td>
<td>107±4</td>
</tr>
<tr>
<td>Diffusion capacity, %predicted</td>
<td>90±3</td>
<td>78±5*</td>
</tr>
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</table>

Values are means ± SE; n = 8 per group. *Significantly different from nonsmokers (P = 0.004).

**Table 2. Inflammatory markers in plasma**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein, mg/l</td>
<td>2.1±0.7</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>3.4±2.1</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>TNF receptor 1, pg/ml</td>
<td>720±52</td>
<td>772±106</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>1.2±0.1</td>
<td>2.2±0.4</td>
</tr>
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</table>

Values are means ± SE.
enzymes occurs independently of changes in the rate of proteolysis or vice versa (12, 15). We did not measure the rate of muscle protein breakdown (due to the investigational burden on our subjects, which we considered unwarranted at this time); this makes it impossible to estimate the true extent to which smoking affects muscle protein net balance. However, whole body protein breakdown was not different between our smokers and nonsmokers. Muscle protein breakdown normally accounts for 20–40% of a healthy person’s basal whole body protein breakdown rate (22, 31); so it is unlikely that muscle protein breakdown was markedly accelerated or markedly suppressed in smokers. Our findings are consistent with other conditions of chronic, slow muscle wasting, which is generally associated with depressed protein synthesis, and often normal or also somewhat depressed (rather than accelerated) protein breakdown (28, 35). Increased expression of key proteolytic enzymes could therefore be interpreted as increasing the susceptibility of smokers to accelerated protein breakdown, once triggered by catabolic stimuli such as injury, illness (e.g., cancer) or other conditions in which protein breakdown is accelerated (37), although this is purely speculative. It is also possible that the expected effects of smoking on muscle or whole body protein breakdown may be seen only in a diminution of the normal feeding-associated blunting of muscle protein breakdown (37). Nevertheless, our data suggest that smoking has a prosarcopenic effect, which probably predisposes smokers to an accelerated decline in physical function and loss of independence (7, 16, 44). To evaluate the overall effect of smoking on muscle maintenance, it will be necessary to investigate the effect of smoking on muscle protein metabolism during other conditions, such as, for example, during feeding or physical activity, the two major physiological regulators of muscle mass (37).

Although smoking is recognized as a major public health problem because it markedly increases the risk of cardiovascular disease and a variety of cancers (1), there are few reports suggesting that smoking may also have adverse effects on the musculoskeletal system and thus physical function. Regular tobacco use is associated with reduced bone mineral density (23) and increased risk for osteoporotic fractures (18). Smokers also have an increased risk for injury during physical activity (20) and are reportedly weaker and have poorer balance and inferior performance on measurements of integrated physical function than nonsmokers (33). The reasons for this are not entirely clear but may be linked to a smoking-related loss of muscle mass, although the evidence for this proposition is scarce. Castillo et al. (2) found that the few men and women in their study who were smokers were more likely to have sarcopenia, defined as whole body fat-free mass of ≥2.0 standard deviations below the sex-specific mean of a young reference population. Szulc et al. (45) found that smokers had lower relative appendicular fat-free mass, estimated by dual-energy X-ray absorptiometry, than did subjects who never smoked. Our results provide a potential mechanism by which habitual tobacco use could decrease muscle mass (i.e., by reducing the rate of muscle protein synthesis and increasing expression of genes associated with impaired muscle maintenance). These findings are consistent with reports in the literature that found markedly reduced fractional rates of basal muscle protein synthesis in sarcopenic elderly individuals compared with nonsarcopenic elderly persons (55) and suggests

Fig. 3. Real-time PCR gene expression analyses. Values are means ± SE; n = 5 per group. Data for myostatin, MAFBx, and MuRF-1 were log transformed to satisfy normality requirements for statistical analysis. *Significantly different from nonsmokers (P < 0.05).
that smoking induces or possibly accelerates muscle wasting.
We did not measure total body fat-free or muscle mass in our subjects, because the sample size required to detect differences in muscle mass with a cross-sectional study design (smokers vs. nonsmokers), including both men and women, was estimated to be prohibitively large (e.g., we estimated that >70 subjects would be required to detect a 10% difference in fat-free mass between smokers and nonsmokers).

The reduction of muscle protein synthesis as a result of habitual tobacco smoking could be due to a direct effect of nicotine or other toxic byproducts of cigarette smoke (e.g., acetaldehyde) or an indirect effect due to differences in lifestyle, most notably alcohol consumption (21) and physical activity (5,13) attitudes between smokers and nonsmokers. However, the increased risk of sarcopenia in smokers reported by Castillo et al. (2) and Szulc et al. (45) was present even after adjusting for physical activity and other lifestyle factors, which suggests that sarcopenia is due to smoking itself or factors directly related to smoking. We are not aware of any studies that evaluated the direct effects of cigarette smoke or its many components on muscle protein metabolism in vitro or in vivo; neither can we distinguish between a direct or indirect effect of cigarette smoking on muscle protein synthesis in the present study. However, alcohol intake was similar in our smokers and nonsmokers, and neither our smokers nor our nonsmokers engaged in regular physical activities beyond those considered part of daily living. It is therefore unlikely that small, unaccounted-for differences in physical activity patterns between smokers and nonsmokers caused marked differences in resting muscle protein synthesis rate. Even vigorous endurance exercise regimen was generally found to have no effect muscle protein synthesis or even decrease it (8,32), whereas the difference in muscle protein synthesis between our smokers and nonsmokers was of the same magnitude as that usually seen after heavy resistance exercise training (19). Thus, smoking itself was probably responsible for most of the suppression of muscle protein metabolism in our smokers. Reduced skeletal muscle blood flow in chronic smokers (38) and thus limited substrate (e.g., amino acids) delivery to the muscle may be one mechanism by which regular cigarette smoking suppresses muscle protein synthesis.

Systemic low-grade inflammation is known to contribute to the development of muscle wasting (39,40,46,49), and reduced basal muscle protein synthesis rates may be related to increased circulating cytokine concentrations (47). Nonetheless, the marked differences in muscle protein synthesis rate between our smokers and nonsmokers occurred independently of differences in systemic inflammatory markers. Chronic low-grade inflammation is therefore unlikely to be the major culprit responsible for the smoking-induced reduction in muscle protein synthesis rate and increased expression of catabolic genes.

In summary, our data highlight the importance of controlling for smoking status in studies evaluating muscle protein metabolism and indicate that smoking is a potent inhibitor of the muscle protein synthetic machinery.

ACKNOWLEDGMENTS
We thank Ruth Rousing and Hanne Villumsen for technical assistance, and the study subjects for their participation.

GRANTS
The study was supported by US National Institutes of Health Grants AR-49869 and DK-56341 (Clinical Nutrition Research Unit at Washington University), grants from the American Heart Association (05100152Z), the Danish National Research Foundation (Centre of Inflammation and Metabolism), the Danish Lung Association, the Danish Medical Research Council (no. 22-01-009), the Commission of the European Communities (LSHM-CT-2004-005272 EXGENESIS), the Pharmacist Foundation of 1991, the Legacy of Ebba Celinder, the Foundation of Managing Director Jacob Madsen & Spouse Olga Madsen, the Biotechnology and Biological Sciences Research Council UK and Cancer Research UK.

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