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

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Winther Petersen AM, Magkos F, Atherton P, Selby A, Smith K, Rennie MJ, Pedersen BK, Mittendorfer B. Smoking impairs muscle protein synthesis and increases the expression of myostatin and MAFbx in muscle. Am J Physiol Endocrinol Metab 293: E843–E848, 2007. First published July 13, 2007; doi:10.1152/ajpendo.00301.2007.—Smoking causes multiple organ dysfunction. The effect of smoking on skeletal muscle protein metabolism is unknown. We hypothesized that the rate of skeletal muscle protein synthesis is depressed in smokers compared with nonsmokers. We studied eight smokers (≥20 cigarettes/day for ≥20 years) and eight non-smokers matched for sex (4 men and 4 women per group), age (65 ± 3 and 63 ± 3 yr, respectively; means ± SEM) and body mass index (25.9 ± 0.9 and 25.1 ± 1.2 kg/m², respectively). Each subject underwent an intravenous infusion of stable isotope-labeled leucine in conjunction with blood and muscle tissue sampling to measure the mixed muscle protein fractional synthesis rate (FSR) and whole body leucine rate of appearance (Ra) in plasma (an index of whole body protein synthesis), the expression of genes involved in the regulation of muscle mass (myostatin, a muscle growth inhibitor, and MAFbx and MuRF-1, which encode E3 ubiquitin ligases in the proteasome proteolytic pathway) and that for the inflammatory cytokine TNF-α in muscle, and the concentration of inflammatory markers in plasma (C-reactive protein, TNF-α, interleukin-6) which are associated with muscle wasting in other conditions.

METHODS

Subjects. Sixteen subjects participated in this study; eight subjects (4 men and 4 women) were heavy smokers (≥20 cigarettes/day for ≥20 yr) and eight subjects (4 men and 4 women) had never smoked. All subjects were considered to be in good health after completing a comprehensive medical evaluation including a physical evaluation, standard blood tests, and pulmonary function tests. None of the subjects reported to be engaged in regular physical activities beyond those considered part of daily living. Five of the subjects (2 smokers, 3 nonsmokers) abstained from alcohol, 10 subjects (5 smokers, 5 nonsmokers) smoked cigarettes, and 4 subjects (1 smoker, 3 nonsmokers) had never smoked.

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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_{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 [1,2,3-^{13}C]leucine (priming dose 7.8 μmol/kg body wt, infusion rate 0.13 μmol·kg body wt·min^{-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-ketosocapric 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 prechilled tubes containing EDTA; plasma was separated immediately after the second biopsy. Plasma leucine enrichment was determined by gas chromatography-mass spectrometry (GC-MS). Plasma insulin concentration was determined by bio- Rad, 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 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 CGG AAA CAA TCA TTA CCA, reverse GTT TCA GAG ATC GGA TTG TAC TAT; MAFBx forward CGA CCT CAG CAG TTA CGG CAA C, reverse TTT GCT ATG AAC TGC ACC AAC AGC C, MuRF-1 forward AGT GAC CAA GGA GAA CGG, reverse CAC CAG CTG CGA GGA CAG TCA, reverse CAC CAG CTG TGT GGA CTT GT; TNF-α forward CAT GGT GTA GCA AAC CCT CA, reverse GTT GAC CTT GGT CTC 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 [1,2,3-^{13}C]leucine into muscle proteins, using a standard precursor-product model as follows: FSR = ΔE_p/ΔE_m × 1/t × 100, where ΔE_p is the change in enrichment (TTR) of protein-bound leucine in two subsequent biopsies, E_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_{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_{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 (FEV₁), increased residual volume, and decreased diffusion capacity (Table 1). Nonetheless, forced vital capacity (FVC) was normal, and the FEV₁-FVC ratio was >0.70, indicating the absence of chronic obstructive pulmonary disease (11) (Table 1). Plasma
It was our goal to discover whether smoking affects muscle 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.

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, 56) and others (41, 52, 56) obtained for quadriceps muscle in healthy adults in the postabsorptive state. This strengthens our confidence that the data obtained represent real differences likely to be due to smoking.

In light of the markedly lower rate of muscle protein synthesis in smokers than in nonsmokers, the increased expression of myostatin in our smokers is consistent with myostatin’s function as a muscle growth inhibitor (54), which is generally highly expressed in models of atrophy (14). Moreover, because myostatin inhibits muscle maintenance via satellite cell recruitment (26), it is likely that the reduced rate of muscle protein synthesis in our smokers was, at least in part, mediated by that mechanism. We also observed gene expression differences in ubiquitin ligases, which may have, in part, been mediated by the overexpression of myostatin in smokers (14) and might be taken as suggestive of increased muscle proteolysis, although there are circumstances in which increased expression of these

### Table 1. Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>63±3</td>
<td>65±3</td>
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<tr>
<td>Height, cm</td>
<td>172±3</td>
<td>176±4</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>74±2.4</td>
<td>81±5.2</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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<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>78±2</td>
<td>79±3</td>
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<tr>
<td>Plasma glucose concentration, mM</td>
<td>4.9±0.1</td>
<td>5.4±0.3</td>
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<tr>
<td>Plasma insulin concentration, M/UL</td>
<td>6.7±0.9</td>
<td>6.5±0.8</td>
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<tr>
<td>HOMA-IR,</td>
<td>1.38±0.27</td>
<td>1.4±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>
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<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>
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<tr>
<td>Diffusion capacity, %predicted</td>
<td>90±3</td>
<td>78±5*</td>
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Values are means ± SE; n = 8 per group (4 male, 4 female in each). HOMA-IR, homeostasis model assessment of insulin resistance (25). *Significantly different (P < 0.05) from nonsmokers.

### Table 2. Inflammatory markers in plasma

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<tr>
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<th>Nonsmokers</th>
<th>Smokers</th>
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<tr>
<td>C-reactive protein, mg/l</td>
<td>2.1±0.7</td>
<td>3.5±0.5</td>
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<tr>
<td>TNF-α, pg/ml</td>
<td>3.4±1.3</td>
<td>2.3±0.5</td>
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<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>
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Values are means ± SE.

Fig. 1. Mixed-muscle protein fractional synthesis rate (FSR). Values are means ± SE; n = 8 per group. *Significantly different from nonsmokers (P = 0.004).

Fig. 2. Leucine rate of appearance (Ra) in plasma. Values are means ± SE; n = 8 per group.
enzymes occurs independently of changes in the rate of protein breakdown 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
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.

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GRANTS

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