Does the aging skeletal muscle maintain its endocrine function?

Running head: Muscle-derived interleukin-6 in elderly humans

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Abstract

Contracting skeletal muscles produce and release the cytokine interleukin (IL)-6 and this release is augmented by the presence of low muscle glycogen. Since muscle metabolism in elderly subjects relies on glycogen more than younger subjects, it is possible that aging is associated with an altered production of muscle-derived IL-6 during exercise. To test the relation between aging and muscle-derived IL-6, seven healthy elderly males, mean age 70 ± 1 (SEM) yr and six healthy young males, mean age 26 ± 2 (SEM) yr performed three hours of dynamic knee-extensor exercise at 50% of maximal work load (W_{max}). IL-6 mRNA and glycogen in muscles were analysed and the IL-6 release were estimated before, during and after the exercise. Although the absolute work load in the elderly was less than half of that in the young, 41.1 ± 3.1 W and 92.5 ± 4.0 W, respectively, the muscle glycogen utilization after three hours of exercise did not differ between groups, 238.7 ± 52.4 and 245.2 ± 74.0 mmol/kg muscle in elderly and young, respectively. This could explain that the IL-6 release and the IL-6 mRNA amplification increased during exercise with no difference between groups, two-way ANOVA-P = 0.50 and 0.45, respectively.

In conclusion, elderly healthy people maintain the capacity to produce and release IL-6 in response to dynamic exercise, with no difference compared to young individuals furthermore, glycogen utilization expressed in changes of glycogen related to muscle mass was equal in elderly and young subject at 50 % of W_{max}.

Keywords: Interleukin-6, exercise and aging

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Introduction

Plasma interleukin (IL)-6 levels increase up to 100 fold in response to exercise (42) (12;39-41;50;59). It has recently been shown that IL-6 is released from contracting skeletal muscles during dynamic knee extensor exercise and that this release may be responsible for the total increase in the systemic concentration (61). Studies in rats (26) (30) and humans (24;28;57;61) (10;15;45) demonstrate elevated levels of IL-6 mRNA in contracting skeletal muscle. Evidence exists that it is the muscle fibers per se, which are the source of muscle-derived IL-6 (14;16;44).

When muscle glycogen was depleted prior to exercise in one leg, this leg released IL-6 one hour prior the non-glycogen depleted leg (61). Furthermore, the transcription rate of the IL-6 gene in muscle nuclei (28), and in total muscle IL-6 mRNA increased when exercising with lower muscle glycogen compared with a trial where the same subjects exercised with normal muscle glycogen content (10;28;61), therefore low muscle glycogen has been suggested as a signal for IL-6 release from contracting muscles. This statement has been strengthened by a study showing an inverse correlation between high IL-6 release during exercise and muscle glycogen content at the end of exercise (21) and by the finding that training reduces the contraction-induced IL-6 mRNA expression in skeletal muscle (17).

Regular exercise offers protection against all cause mortality, primarily by protection against atherosclerosis and type 2-diabetes (3). In addition, physical training is effective in the treatment of patients with ischemic heart disease (25), hypertension (46) and hyperlipidemia (31).

Over the past decade, there has been much focus on the role of inflammation in the pathogenesis of atherosclerosis and its complications. Whereas most clinicians previously regarded atheroma as a bland lesion, the current notion that inflammation and immune response contribute to atherogenesis has created increased interest (32).

During ageing circulating levels of a number of cytokines increase. Thus, increased plasma levels of TNFα (6;7;11;36), IL-6, IL-1ra (11) and sTNFR (5;6;8) have been demonstrated. In addition ageing is also associated with increased levels of acute phase proteins such as C reactive protein (CRP) and Serum Amyloid A (SSA)(2). Elevated levels of circulating IL-6 and TNFα have been associated with several disorders. Thus, increased levels of TNFα and IL-6 have been observed in obese individuals, in smokers and in non-insulin dependent diabetes mellitus (68) and levels of IL-6 have been shown to predict all-cause mortality as well as cardiovascular mortality (20;69). Furthermore, plasma concentrations of IL-6 and TNFα have been shown to predict the risk of myocardial infarction in several studies (52;53;64), and recently it was shown that the CRP level is a stronger predictor of cardiovascular events than the LDL cholesterol level and that CRP adds prognostic information to that conveyed by the Framingham risk score (54). It has been proposed that IL-6 is the mediator that links the acute phase response to visceral obesity, insulin resistance and atherosclerosis (72). High levels of IL-6 in patients with metabolic syndrome may be explained by the fact that IL-6 is produced in adipose tissue (18;35). Adipose tissue also produces and releases TNFα (65). However, in contrast to IL-6, the available data

suggests that TNFα plays a mechanistic role in insulin resistance. Thus, TNF-α down-regulates GLUT-4 and inhibits insulin receptor activity (23). Since TNF-α can trigger IL-6 release, one theory holds that adipose tissue derived TNF-α actually is the „driver" behind the metabolic syndrome and that TNF-α rather than IL-6 should be placed in the center as the cytokine that induces insulin resistance and thereby initiates diabetes type 2 and atherosclerosis.

It is possible that exercise mediates its beneficial health effects by inducing an anti-inflammatory environment. The cytokines, which are present in the circulation following exercise is IL-6 and classical anti-inflammatory cytokines such as IL-10 and IL-1ra (39;43).

There has been much debate on how to classify the cytokine IL-6. Tilg et al suggested that IL-6 should be classified as an anti-inflammatory cytokine (63).

The finding that rhIL-6 infusion enhances levels of IL-10 and IL-1ra (60) and inhibits endotoxin-induced TNFα production lends support to the idea that IL-6 has anti-inflammatory effects (56).

The fact that exercise alone inhibited endotoxin-induced TNFα increase in the circulation (56) and that TNF-α overexpression returned to normal levels after exercise in the TNF-α receptor knockout model (27) indicates that exercise mediates anti-inflammation.

Given the biological profile of IL-6, it would be important to know whether the aging skeletal muscle is able to produce IL-6. The purpose of the present study, therefore, was to test whether elderly subjects maintain their capability of producing and releasing IL-6, and whether this could be explained by an age related change in muscle glycogen metabolism.

Matherials and Methods

Subjects: Seven healthy elderly males and six healthy young males (whose characteristics are shown in table 1. participated in the study. All subjects were recruited through earlier studies in our department. Medical history, physical examination, clinical chemistry profile, complete blood count, cell differential count and CRP, and electrocardiogram demonstrated that the subjects were completely healthy. The study was in accordance with the Helsinki II Declaration and approved by the regional research ethical committee (No. 01-216/00). All subjects gave their written consent before participation.

Table 1. Subject characteristic

	Young (n = 6)	Elderly (n = 7)
Age, yr	25.7 ± 1.7	69.7 ± 1.2*
Bodyweight (kg)	78.1 ± 3.2	78.9 ± 2.6
BMI, kg m^{-2}	22.3 ± 0.7	24.1 ± 0.9
QF, kg	5.1 ± 0.2	3.7 ± 0.1*
Workload, W	92.5 ± 4.0	41.1 ± 3.1*

Values are shown as mean ± SEM
QF: M. quadriceps femoris in both legs (MRI)
*, P ≤ 0.05, vs. young

Studies of the effect of aging on metabolism or immune function during exercise are complicated by large between-group differences in VO_{2max}, which raises the question of how to best standardize the exercise intensity. In the present study, an exercise model was chosen which was not dependent upon the cardiorespiratory capacity and therefore useful in studies including elderly untrained subjects. Therefore, in this study we chose to investigate the two groups when performing exercise at same relative percentage of maximal workload.

The subjects performed 3 h of dynamic two-legged knee extensor exercise at 50 % of maximal workload (W_{max}) on a modified Krogh cycle ergometer as previously described (1). At least one week before the trial a two-legged knee extensor exercise test was performed to determine W_{max}. Resistance load was increased every two minutes until a cadence of 60 extensions/min could no longer be maintained. The highest workload that could be maintained for two minutes was set as the maximum workload. After ½ hour rest the subjects performed 2 h of two-legged knee extensor exercise at 50% of W_{max} to familiarise themselves with the apparatus.

The evening before the experiment, all subjects were provided with a fixed diet to ensure that there was no difference in diet between the two groups. The subjects reported to the laboratory the following day after an overnight fast. Catheters were placed in the femoral vein and artery of one leg under local anaesthesia (lidocaine 20 mg/ml). Blood samples were drawn into pre-cooled tubes containing EDTA at 0, 30, 60, 90, 120 and 180 min of exercise and at 60 and 120 min post-exercise. Blood samples were immediately spun at 4°C and plasma was isolated and stored at -80°C until analyses were performed. Blood flow in the femoral artery at each time point was measured with Doppler ultrasound technique as described in details previously (51). In addition, muscle biopsies were obtained from the vastus lateralis at 0, 30, 90 and 180 min of exercise and at 120 min post-exercise using the percutaneous needle biopsy technique with suction. Biopsy samples were obtained from the left leg (0 and 90 min during exercise and 120 min post-exercise) and from the right leg (30 and 180 min). Muscles were cleaned of connective tissue and blood, and quickly frozen in liquid nitrogen for later analysis.

IL-6 measurements: Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used (Quantikine HS, R&D systems, Minneapolis, MN, USA) to measure plasma IL-6. According to R&D systems the detection limit is less than 0.094 pg/ml. All samples were run in duplicates and mean values were used. Net IL-6 release was calculated according to Fick's principle (70) by multiplying the arterial-femoral venous (a-fv) difference by blood flow.

Measurements of catecholamines: High Performance Liquid radioimmunoassay Chromatography described in detail elsewhere (4), was used to determine systemic concentrations of adrenaline and noradrenaline.

Measurements of lactate: Plasma lactate were measured in arterial and venous blood using an automatic analyser (Cobas Fara, Roche, France) and release was calculated according to Fick's principle.

IL-6 mRNA analyses: Total RNA was isolated from 9-86 mg of muscle tissue by a modified guanidinium thiocyanate phenol-chloroform extraction method adapted from Chomczynski and Sacchi (9) as previously described (48). Final pellets were dissolved in 0.1 mM EDTA (2 μl/mg wet weight). Reverse transcription reactions were carried out on 11 μl sample using the Superscript II Rnase H-system (Gibco-BRL) in a reaction volume of 20 μl. All samples were diluted to a final volume of 150-200 μl with nuclease free water.

β-actin mRNA and IL-6 mRNA levels were determined by real-time PCR, which determines the amount of cDNA amplification after each PCR cycle. With

real-time PCR a probe, in addition to the forward and reverse primer, is used. The probe, located between the primers, has a fluorescent dye attached to the end, which is split off during amplification by a 5' nuclease. The splitting of the probe results in emission of light from the dye, which can be measured by the machine, hereby giving a measure of the amount of amplification of cDNA that has been performed during each PCR cycle.

The IL-6 primers and probe sequences used were designed by Starkie et al (57). An 81-bp fragment was amplified using the IL-6 forward primer: 5'-GGTA-CATCCTCGACGGCATCT-3', and the IL-6 reverse primer: 5'-GTGC-CTCTTTGCTGCTTTCAC-3'. The flourescent IL-6 probe: 5'-FAM-TGT-TACTCTTGTTACATGTCTCCTTTCTCAGGGCT-TAMRA-3' was included in the PCR reaction. We used the pre-developed assay reagents from Applied Biosystems for β-actin mRNA determination. β-actin was measured as a reference gene in a multiplex reaction with IL-6. The use of different dyes with different wave length emission patterns attached to the end of the probes gives the opportunity to measure two genes of interest in the same reaction well. The presence of more than one primer and probe set did not affect the amplification of neither IL-6 nor β-actin. All PCR-reagents were obtained from Applied Biosystems. A reaction volume of 100 µl was made up for each sample with 1x MasterMix, 900 nm IL-6 forward primer, 300 nm reverse primer, 100 nm IL-6 probe, 1x B-actin mix (primers and probe), 10 to 15 µl of sample and made up to a final volume of 100 µl with water. Each sample was run in triplicates in a reaction volume of 25 µl.

Muscle glycogen content: Frozen muscle samples (10-20) mg were freeze dried, dissected free of connective tissue, weighed and hydrolysed in 1 M HCL. Glycogen concentrations were determined by standard enzymatic technique with fluorimetric detection (38). The energy expenditure from muscle glycogen utilization was calculated as:
Total energy expenditure from glycogen utilization =

$Post_{glycogen} - Pre_{glycogen}$ (g/kg_{muscle}) x Muscle mass (kg)(MRI)) x 16 KJ/g x 180 g/mol

Muscle glycogen utilization and effect of work load: To investigate whether elderly were more dependent on muscle glycogen as an energy source, the energy from glycogen oxidation compared with total mechanical work performed was calculated by dividing the energy from muscle glycogen utilization after 3 h of exercise with the total mechanical work performed during 3 h of exercise.

Muscle mass estimation: The volume of m. quadriceps femoris (QF) was measured by Magnetic Resonance Imaging (MRI) (Siemens 1.5 tesla magnet). Twenty-eight T1 weighed scans (TR = 900 ms) were acquired with a slide thickness of 3 mm and an interslice thickness of 12 mm. View field was 400 x 400 mm with a resolution of 256 x 256 (pixel size = 1.56 x 1.56 mm). Scicon Images for Windows (Scion Corporation, Frederick, Maryland, USA) were used to analyse MRI images. For quantification the first image used was at mid patella and the last image just above trochanter major. The use of trochanter major as an endmark for QF does, however, not always include the full length of m. rectus femoris, but due to the increased noisiness of the images in this area this procedure gave the best reproducibility. Muscle volume was calculated by multiplying

the area of QF in each slide by the distance between the slides and summed for all images and the muscle mass was calculated assuming a muscle density of 1.04 kg/l (22).

Statistical methods: SYSTAT version 8.0 (SPSS Inc., Chicago, USA) was used as software. Plasma IL-6 and IL-6 mRNA values were not normally distributed and were therefore square root transformed. Absolute changes in response to exercise were evaluated by analysis of variance (ANOVA) for repeated measurements (model parameter = time + age + time x age). If a significant (time x age) was found, a two-sample t test (Bonferoni-adjusted) for independent groups was used to detect age-related differences in absolute changes from baseline levels. If only time turned out to be significant, age group was pooled and a paired t-test (Bonferoni-adjusted) was performed to detect changes from baseline levels. A P-value < 0.05 was considered as significant.

Results:

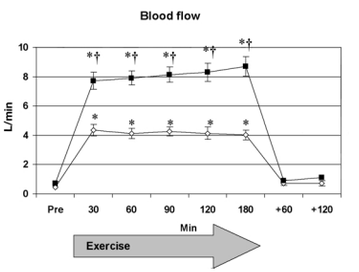

Figure 1: Blood flow in femoral artery in 7 elderly subjects (◊) and 6 young controls (■) measured before, during and after (+60 and +120) 3 h of dynamic knee-extensor exercise. Data are presented as mean and S.E.M.
*, P < 0.05 vs. pre-ex
†, P < 0.05 vs. elderly subjects

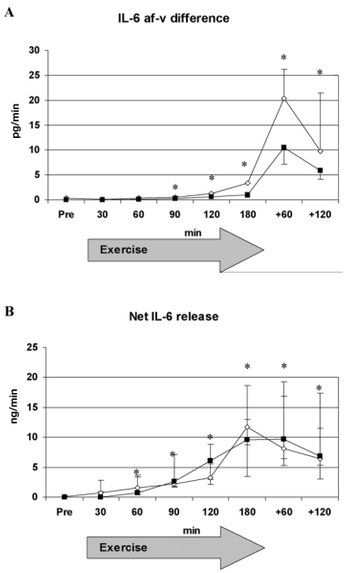

Figure 2: IL-6 data for 7 elderly subjects (◊) and 6 young controls (■) before, during and after (+60 and +120) 3 h of two-legged dynamic exercise. Data are presented as median and quartiles. A, IL-6 a-vf differences for elderly and young subjects. B, net release of IL-6 from elderly and young subjects (Fick's principle: blood flow x a-fv differences).
*, P < 0.05 vs. pre-ex (groups pooled).

Blood flow: The blood flow increased in both groups, being more pronounced in the young subjects (Two-way ANOVA for repeated measurements, P<0.001)(Fig. 1).
Plasma IL-6: The a-v difference for IL-6 increased in the end of exercise in both groups (P < 0.05) (Fig. 2A). When the net IL-6 release was calculated there was a gradual increase during exercise (P< 0.05). However, over time there was no difference between the groups (Fig. 2B). Furthermore, when the net IL-6 release was related to the QF muscle mass (MRI) there was no differ-

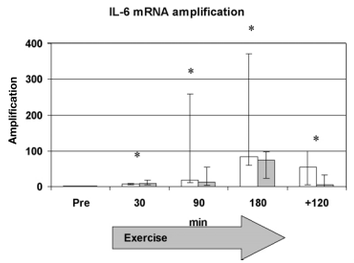

Figure 3: IL-6 mRNA in 7 elderly subjects (open) and 6 young controls (solid) before, during and after (+120) 3 h of two-legged dynamic exercise measured by real time PCR. Data are presented as median and quartiles.
*, P < 0.05 vs. pre-ex (groups pooled)

ence among groups (data not shown).
Muscle IL-6 mRNA: The IL-6 mRNA level in the muscle increased during exercise (P<0.05). However, there appeared to be large inter individual variations and the difference between groups did not reach statistical significance (P = 0.2) (Fig. 3).
Muscle Glycogen Content: The muscle glycogen content decreased during exercise with no difference between groups, either before or during the exercise (Fig. 4A). After 3 h of exercise the glycogen utilization was 238.7 ± 52.4 and 245.2 ± 74.0 mmol/kg muscle in elderly and young, respectively. When energy from glycogen utilization was calculated, there was no difference between groups (Fig. 4B).
Effect of mechanical work: When the total energy from muscle glycogen breakdown was related to the total mechanical energy performed, it was demonstrated that the elderly had a higher (P <0.05) glycogen breakdown, indicating that the elderly individuals may rely more on glycogen breakdown than the young subjects (Fig. 4C).
Lactate release: There was a small but significant increase in lactate release over time (P = 0.002) with no difference between groups. After 30 min of exercise the release peaked in the elderly group to 1.06 ± 0.21 mmol/min and in the young group after 120 min of exercise to 0.35 ± 0.27, (Fig. 5).

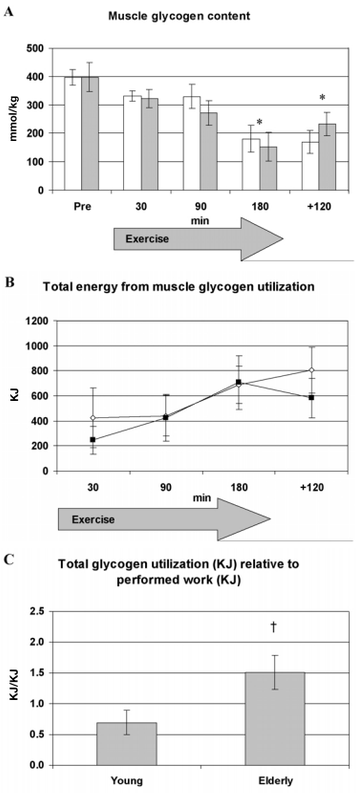

Figure 4: Muscle glycogen data in 7 elderly and 6 young males. Glycogen was measured in the quadriceps muscles. Data are presented as mean and S.E.M. A, muscle glycogen content (mmol/kg) in the vastus lateralis from 7 elderly (open bars) and 6 young (solid bars) before, during and after (+120) 3 h of two-legged dynamic exercise. B, energy from total quadriceps muscle glycogen utilization in 7 elderly (◊) and 6 young (■) males during and after (+120) 3 h of two legged dynamic exercise. C, Relation between energy from total glycogen utilization in the quadriceps muscle and energy used to mechanical work performed after 3 h of two-legged dynamic exercise in 7 elderly and 6 young males.
*, P < 0.05 vs. pre-ex (groups pooled)
†, P < 0.05 vs. young controls

Catecholamines: Adrenaline and noradrenaline increased (P<0.01) as expected during exercise, with no difference between the two age groups, although there was a tendency to higher levels in the young subjects after three hours of exercise (Table 2).

Discussion

The present study is the first to determine the effect of aging on IL-6 release from contracting skeletal muscles. Although the absolute work load and the muscle mass in the elderly was less than that in the young controls, the net IL-6 release and the IL-6 mRNA in muscle did not differ between groups. These data suggest that elderly subjects maintain a capability to produce and release muscle-derived IL-6 and that IL-6 release is not related to absolute workload, muscle mass or age.

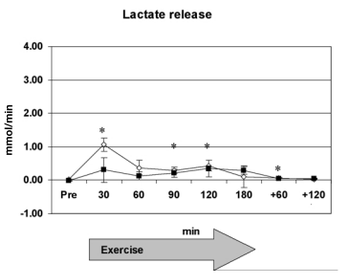

Figure 5: Lactate release before, during and after 3 h of two-legged dynamic exercise in 7 elderly humans (◊) and 6 young controls (■), (Fick's principle: blood flow x a-fv differences). Data are presented as means and S.E.M.
*, P < 0.05 vs. pre-ex (groups pooled)

Previous findings have demonstrated a relationship between muscle-derived IL-6 release and muscle glycogen concentration (15;21;28;37;61). In the present study the decline in muscle glycogen content during exercise did not differ between groups but the glycogen breakdown per mechanically energy performed after three hours of exercise was significantly higher in the elderly, which demonstrates that the elderly were more dependent on mobilization of glycogen as an energy source during exercise. This finding is likely to be related to a lower training degree of old versus young subjects. Thus, it has been demonstrated that during exercise untrained muscles metabolise more carbohydrate relative to fat compared with trained muscles (55). The calculations in relation to energy turnover from glycogen use were based on the assumption that all glycogen utilization was oxidised. Although, in the present study, we did not measure muscle lactate accumulation, the fact that the peak lactate release was 1.06 mmol/min and 0.35 mmol/min for elderly versus young, respectively, and the fact that this release peaked after 30 min in the elderly after

Table 2. Catecholamines measured in arterial blood.

	Young (n = 6)			Elderly (n =7)		
	Pre	180 min	+ 120 min	Pre	180 min	+ 120 min
Adrenaline (nmol/l)	0.22 ± 0.05	5.16 ± 1.93	1.01 ± 0.29	0.33 ± 0.08	1.73 ± 0.61	0.35 ± 0.05
Noradrenaline (nmol/l)	0.97 ± 0.13	7.95 ± 1.20	1.35 ± 0.17	1.56 ± 0.20	7.27 ± 1.35	2.46 ± 0.32

Values are mean ± SEM

which it declined, makes this assumption valid since it indicates that lactate production was negligible and unlikely to contribute to anaerobic glycogenolysis and/or affect IL-6 production.

In this study, the groups differed with regard to both maximal workload performance as well as muscle mass. However, the difference between groups with regard to maximal workload performance was more pronounced than the difference in muscle mass. This may be explained by an overestimation of muscle mass in the elderly group, as a result of a higher water and fat content in aging muscles (19;49;66).

It has been debated whether catecholamines stimulate IL-6 production during exercise (62).

However, the exercise-induced increase in plasma IL-6 could not be mimicked by epinephrine infusion. Although epinephrine induced a small increase in IL-6 and may, therefore, partly influence the plasma levels of IL-6 during exercise, it could not account for the massive increase in IL-6 during exercise (58).

As skeletal muscle is the major source of IL-6 during exercise (61) it is not likely that adrenaline is a major stimulator of muscle-IL-6, although it stimulates adipose tissue IL-6 production (29).

In the present study there was no significant difference between the two age groups with regard to the neither the catecholamine responses nor the IL-6 release. Therefore, the present study does not really shed much light on the role of epinephrine in the regulation of muscle-IL-6.

IL-6 has been placed in the center of modern internal medicine as the link between inflammation, obesity, stress and coronary heart disease (72). Given the finding that during exercise skeletal muscles produce and release large amounts of IL-6 into the systemic circulation and given the many beneficial effects of physical exercise on health, it is possible that during moderate regular exercise, IL-6 may mediate some of these effects. Recently, it was demonstrated that IL-6 knockout mice develop late onset obesity and impaired glucose tolerance (71). Furthermore, mice bearing IL-6-producing tumours lose weight (34) and rhIL-6 infusion to healthy young and elderly people as well as people with type 2 diabetes induces lipolysis and fat oxidation (47;67).

We therefore suggest that muscle-derived IL-6 may work in a hormone like fashion mediating exercise-induced lipolysis and anti-inflammation (13;33;43). These findings further classify skeletal muscle as an endocrine organ.

In conclusion, the present study demonstrates that aging skeletal muscle maintain its endocrine functions and specifically demonstrates that healthy elderly subjects maintain a normal capability of producing and releasing IL-6 from contracting muscle compared to young subjects.

Acknowledgments

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