Nuclear Magnetic Resonance Spectroscopy Studies of Mitochondrial Oxidative Phosphorylation Activity and Insulin Resistance in the Elderly

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Running Title: Mechanism of insulin resistance in the elderly

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Key Words: insulin resistance, type 2 diabetes mellitus, lipolysis, mitochondria, oxidative phosphorylation, nuclear magnetic resonance spectroscopy

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ABSTRACT

Background Insulin resistance is a major factor in the pathogenesis of type 2 diabetes in the elderly but the mechanism responsible for it is unknown.

Methods In order to examine this question we studied healthy, lean elderly (age: 70±2 years, n=15) and young (age: 27±2 years, n=13) subjects matched for: BMI, fat mass and activity. Hyperinsulinemic-euglycemic clamps, in combination with infusions of [6,6-$^{2}H_{2}$]glucose, were performed to assess liver and muscle insulin sensitivity. ^{1}H NMR studies were performed to assess intramyocellular and intrahepatic triglyceride content. Rates of whole body and subcutaneous fat lipolysis were assessed by measuring rates of [$^{2}H_{5}$]glycerol turnover in combination with microdialysis measurements of glycerol release from subcutaneous fat. ^{13}C/^{31}P nuclear magnetic resonance spectroscopy studies were performed to assess rates of mitochondrial oxidative and phosphorylation activity in muscle.

Results Elderly subjects were markedly insulin resistant compared to the control subjects, which could be attributed to ~40% reduction ($P<0.002$ versus young) in insulin stimulated muscle glucose metabolism. These change were associated with ~45% increase in intramyocellular triglyceride content ($P=0.035$ versus young) and ~225% increase in intrahepatic triglyceride content ($P=0.036$ versus young). These increases in tissue triglyceride content were associated with ~40% reduction in both mitochondrial oxidative and phosphorylation activity (both $P<0.004$ versus young). There were no differences in basal or insulin suppression of systemic or localized rates of lipolysis.

Conclusions These data support the hypothesis that insulin resistance in skeletal muscle of the elderly is due to increased intramyocellular lipid, which in turn can be attributed to an age associated reduction in mitochondrial oxidative and phosphorylation activity.

Type 2 diabetes is the most common chronic metabolic disease in the elderly with a prevalence of ~23% among Americans 60 years of age or older[1]. Estimates of the economic burden of type 2 diabetes is ~$100 billion/year, of which a substantial proportion can be attributed to persons with diabetes in this age group[2]. Epidemiological studies have shown that the transition from the normal state to overt type 2 diabetes in aging is typically characterized by deterioration in glucose tolerance[3,4] which is due to impaired insulin stimulated glucose metabolism in skeletal muscle[5,6]. Recent studies measuring muscle triglyceride content by biopsy[7] or intramyocellular lipid content (IMCL) by ^{1}H NMR[8-10] have shown a strong relationship between intramuscular fat content and insulin resistance in muscle. Similar relationships have been established for hepatic insulin resistance and hepatic steatosis[11-15]. Increases in the intracellular concentration of fatty acid metabolites in turn have been postulated to activate a serine kinase cascade leading to increased insulin receptor substrate-1 (IRS-1)/insulin receptor substrate-2 (IRS-2) serine phosphorylation and decreased insulin stimulated IRS-1/IRS-2 tyrosine phosphorylation resulting in decreased IRS-1/IRS-2 associated phosphatidylinositol 3-kinase (PI 3-kinase) activity in muscle[16-18] and liver[13], respectively. These changes in turn result in reduced glucose transport activity[16] and glycogen synthesis[19,20] in muscle and reduced suppression of glucose production by insulin in the liver[11-15]. In the present study we examined whether insulin resistance in the elderly is associated with similar increases in intramyocellular and/or liver triglyceride content, assessed by ^{1}H NMR spectroscopy, in combination with hyperinsulinemic-euglycemic clamps to assess liver and muscle insulin sensitivity. Since increases in intramyocellular and intrahepatic triglyceride content could occur secondary to increased fatty acid delivery from lipolysis and/or decreased rates of mitochondrial oxidative phosphorylation activity we also examined these processes. Rates of whole body and

subcutaneous fat lipolysis were assessed by measuring rates of [$^{2}H_{5}$]glycerol turnover in combination with microdialysis measurements of glycerol release from subcutaneous fat. Rates of *in vivo* mitochondrial oxidative and phosphorylation activities were assessed in skeletal muscle using a novel $^{13}C/^{31}P$ NMR technique.

METHODS

Subjects All subjects were screened to be in excellent health, non-smoking, sedentary, body mass index (BMI) <25 m^2/kg and taking no medications. Sixteen elderly (ages: 61-84 years) volunteers were screened with a 3-hour Oral Glucose (75g) Tolerance Test (OGTT) and underwent Dual-Energy X-ray Absorptiometry (DEXA) scanning (Hologic QDR-4500 W, Hologic, Bedford, MA) to assess lean body mass and fat mass[21]. One elderly subject was excluded from participating in the study because of an abnormal glucose profile. Thirteen young (ages: 18-39 years) volunteers, who had no family history of diabetes or hypertension were matched to the older subjects for BMI, fat mass and habitual physical activity, assessed using an activity index questionnaire[22]. Each subject then underwent a complete medical history and physical examination, and blood tests to verify normal: blood and platelet count, electrolytes, aspartate amino transferase, alanine amino transferase, blood urea nitrogen, creatinine, prothrombin time, partial prothrombin time, cholesterol, and triglyceride. In addition all subjects were screened for any metal implants, body piercing, and claustrophobia. Subjects then underwent ^{1}H NMR studies to assess liver and muscle triglyceride content as described below and then randomized to undergo either a hyperglycemic-euglycemic clamp study, to assess liver, muscle and fat insulin responsiveness, and/or ^{13}C/^{31}P NMR studies to assess muscle mitochondrial oxidative phosphorylation activity. Written consent was obtained from each subject after the purpose, nature, and potential complications of the studies were explained. The protocol was approved by the Yale University Human Investigation Committee.

Diet and Study Preparation: Subjects were instructed to eat a regular, weight maintenance diet containing at least 150g of carbohydrate per day for three days prior to admission for either the clamp or ^{13}C/^{31}P NMR studies. All subjects were instructed not to

perform any exercise other than normal walking for the 3 days prior to the study. To minimize changes in ovarian hormonal effects on glucose metabolism, the pre-menopausal female subjects were studied during the follicular phase (day 0-12) of the menstrual cycle[23]. Subjects were admitted to the Yale-New Haven Hospital General Clinical Research Center (GCRC) the evening before the clamp or $^{13}C/^{31}P$ NMR study and the subjects remained fasting with free access to regular drinking water until the completion of the study the following day.

Analytical Methods

Metabolites and Hormones: Plasma glucose and lactate concentrations were measured using a YSI STAT 2700 Analyzer (Yellow Springs, CA). Plasma concentrations of insulin, glucagon, growth hormone, and leptin were measured using double-antibody radioimmunoassay kits (Linco, St. Louis, MO). Plasma fatty acid concentrations were determined using a microfluorimetric method[24]. Urine nitrogen content was measured at the Mayo Medical Laboratories (Rochester, MN). Microdialysate glycerol was measured using an enzyme linked colorimetric determination of 0.5 μl samples by a CMA 600 microdialysis analyzer (CMA 600 Microdialysis, N. Chelmsford, MA)[25]. Ethanol concentrations were determined enzymatically using an YSI 2700 STAT Analyzer (YSI, Yellow Springs, CA)[25]. Gas chromatography mass spectrometer (GC-MS) analysis of enrichment of [6,6-^{2}H]-glucose and [$^2H_5$]glycerol in plasma were performed using a Hewlett-Packard 5971A Mass Selective Detector (Wilmington, DE) as previously described[25].

Body Composition: Fat and lean body mass were determined in all subjects using Dual-Energy X-ray Absorptiometry (DEXA) scanning (Hologic QDR-4500 W, Hologic, Bedford, MA) with the subject in the supine position and all clothes removed. Fat and lean body mass in each arm, each leg, the trunk, and the head were calculated as previously described[21].

^{1}H NMR spectroscopy of intramyocellular and intrahepatic triglyceride content: On a separate day, after a 12 hour fast, all subjects were transported by wheelchair to the Yale Magnetic Resonance Center (MRC) and localized ^{1}H NMR spectra of the soleus muscle and liver were acquired on a 2.1T Biospec Spectrometer (Bruker Instruments Inc., Billerica, MA) as previously described[25].

Hyperinsulinemic-euglycemic Clamp: In order to determine rates of fasting glucose production and glycerol turnover a 3-hour baseline infusion was initiated at 6 a.m. (-180 min) with a primed (3.0 mg/kg) continuous (0.05 mg/kg/min) intravenous infusion of [6,6-^{2}H]-glucose (99% ^{2}H enriched) and a continuous infusion of [$^2H_5$]glycerol (0.03 mg/kg/min, 99% ^{2}H enriched). An intravenous catheter was inserted retrogradely into a hand vein, which was kept warm in a hot box at 55°C, and during the last 40 min of the basal period arterialized blood was drawn at 10-minute intervals for the measurement of plasma glucose and insulin levels, [6,6-^{2}H]glucose and [$^2H_5$]glycerol isotope enrichment. At time zero the hyperinsulinemic-euglycemic clamp was initiated with a primed-continuous infusion of insulin at a rate of 20 mU/m^2-min (Novolin-R U-100, Novo Nordisk, Princeton, NJ) and plasma glucose concentrations were maintained at 100±5 mg/dl with a variable infusion of glucose (20 gram/dL) as previously described[26]. During the last 40 min of the clamp period blood was collected for determination of plasma glucose and insulin levels, and [6,6-^{2}H]glucose and [$^2H_5$]glycerol isotope enrichment for determination of glucose and glycerol turnover rates[25-27].

Respiratory exchange measurements: Rates of whole body energy expenditure, glucose and fat oxidation were calculated from measurements of rates of oxygen consumption and carbon dioxide production during the last 30 min of the baseline period and during the last 30 minutes of the clamp with continuous indirect calorimetry using a Deltratrack Metabolic Monitor (Sensormedics, Anaheim, CA) as described[21].

Adipose tissue microdialysis: Localized rates of *in vivo* lipolysis were assessed with microdialysis probes (CMA/60, CMA, Microdialysis, Solna, Sweden) inserted into the fat deposits in two locations on the abdomen, 4-6 cm below the umbilicus, as previously described [25,28,29].

$^{13}C/^{31}P$ NMR measurements of mitochondrial oxidative/phosphorylation activity: Rates of mitochondrial oxidative capacity, tricarboxylic acid (TCA) flux, were assessed by ^{13}C NMR spectroscopy as previously described[30]. Briefly ^{13}C NMR spectra were acquired for 20-min before and during a 120-min [2-^{13}C] acetate infusion (350 mmol·l^{-1} sodium salt 99% ^{13}C enriched, Isotech, Miamisburg, OH) at an infusion rate of 2.9 mg/kg/min. TCA cycle flux was assessed by monitoring the ^{13}C incorporation into glutamate in skeletal muscle from [2-^{13}C] acetate and modeling the data as previously described[30]. Rates of mitochondrial phosphorylation activity, ATP synthase flux, were assessed in the same subjects by ^{31}P NMR spectroscopy performed at 36.31 MHz using a flat concentric probe made of a 9-cm diameter inner coil (for ^{31}P) and a 13-cm outer coil tuned to proton frequency for scout imaging and shimming as previously described[30].

Statistical Analyses: Statistical analyses were performed using the Stat View package (Abacus Concepts, Berkeley, CA). To detect statistical differences between young and older subjects unpaired student t-tests were performed for independent samples with a P-value of <0.05 considered significant. Non-normally distributed data (i.e. area under the curve data) were log transformed. All data are expressed as means ± SEM.

RESULTS

Subject Characteristics: Young and elderly subjects had similar body weight, BMI, fat mass, % fat mass, and lean body mass (Table 1) and had similar fasting plasma concentrations of glucose (young: 90±3 mg/dL, elderly: 94±3 mg/dL, P=0.50), insulin (young: 9± 1 μU/mL, elderly: 10±1 μU/mL, P=0.28), glucagon (young: 55±3 pg/mL, elderly: 61±6 pg/mL, P=0.35), growth hormone (young: 1.9±0.5 ng/mL, elderly: 1.2±0.2 ng/mL, P=0.19), and leptin (young: 5.3±1.7 ng/mL, elderly: 8.3±2.2 ng/mL, P=0.31).

Oral Glucose Tolerance Test: The elderly subjects had a tendency for slightly higher plasma glucose concentrations (Figure 1a) and significantly higher plasma insulin concentrations (P=0.02 for area under the curve for the elderly versus the young subjects, Figure 1b) during the oral glucose tolerance test. Basal plasma fatty acid concentrations (Figure 1c) also tended to be higher in the elderly subject (P=0.08) compared to the young subjects but suppressed similarly following glucose ingestion.

Hyperinsulinemic-Euglycemic Clamps: Basal rates of glucose production were similar in the young (N=7) and elderly subjects (N=11) (Figure 2) and suppressed completely in both groups during the hyperinsulinemic-euglycemic clamp. In contrast rates of glucose infusion required to maintain euglycemia (elderly: 4.00±0.42 and young: 6.23±0.64 mg/kg LBM/min, P=0.0002) and insulin stimulated rates of peripheral glucose uptake (Figure 2) were ~40% lower (P<0.002) in the elderly subjects compared to the young subjects during the clamp. Basal energy expenditure (elderly: 1489±42 kcal/24hours, young: 1660±79 kcal/24 hours, P=0.055) and respiratory quotient (elderly 0.74±0.01, young: 0.78±0.02, P=0.059) both tended to be lower in the elderly subjects compared to the young subjects. There were no differences in basal rates of: glucose oxidation (elderly: 0.65±0.19, young: 0.96±0.19 mg/kg LBM/min, P=0.33), fat oxidation

(elderly: 0.98±0.09 mg/kg fat mass/min, young: 0.93±0.19 mg/kg fat mass/min, P=0.82) or insulin-stimulated rates of: glucose oxidation (elderly: 1.58±0.29 mg/kg LBM/min, young: 2.03±0.79 mg/kg LBM/min, P=0.35), fat oxidation (elderly: 3.17±0.87 mg/kg fat mass/min, young: 2.54±0.79 mg/kg fat mass/min, P=0.89), energy expenditure (elderly: 1586±101 kcal/24hours, young 1686±109 kcal/24 hours, P=0.54) or respiratory quotient (elderly 0.87±0.04, young: 0.89±0.03, P=0.65).

Whole Body and Localized Rates of Glycerol Metabolism: Basal rates of glycerol turnover (elderly 0.98±0.28 mg/kg fat mass per min vs. young 0.93±0.19 mg/kg fat mass per min, P=0.1876) and insulin suppression of glycerol turnover during the clamp (elderly 0.52±0.07 mg/kg fat mass per min vs. young 0.56±0.08 mg/kg fat mass per min, P=0.8653) were similar in the young and elderly subjects. Consistent with this finding the interstitial glycerol concentrations, assessed by microdialysis, decreased by a similar degree (elderly: 46±7% vs. young: 48±6%, P=0.8155) during the hyperinsulinemic-euglycemic clamp in both groups.

^{1}H NMR assessment of Intramyocellular (IMCL) and Intrahepatic Triglyceride Content: The IMCL content in the soleus muscle, as determined by ^{1}H NMR spectroscopy, was increased by ~45% (P=0.035) in the elderly subjects (N=15) compared to the young subjects (N=11) (Figure 3a) and there was a inverse correlation between IMCL and insulin stimulated muscle glucose metabolism (r=0.56, P=0.07). Intrahepatic triglyceride content was also increased by 225% (P=0.036) in the elderly subjects (N=14) compared to the young subjects (N=8) (Figure 3b).

^{13}C and ^{31}P NMR Spectroscopy of Mitochondrial Oxidative/Phosphorylation Activity: Rates of mitochondrial oxidative activity (TCA cycle flux), assessed by ^{13}C NMR (Figure 4a) and phosphorylation activity (ATP synthase flux), assessed by ^{31}P NMR, (Figure 4b) in skeletal

muscle were both ~40% lower (both $P<0.006$) in the elderly subjects (N=9) compared to the young subjects (N=8). In contrast mitochondrial energy coupling, assessed by the ratio by ATP synthase flux to TCA cycle flux, was similar in the groups (young: 0.082±0.009, elderly: 0.069±0.011, $P= 0.38$).

DISCUSSION

In this study we found that healthy lean elderly subjects were severely insulin resistant compared to BMI-fat mass-activity matched younger subjects which could be attributed to an ~40% decrease in insulin stimulated skeletal muscle glucose metabolism. Using ^{1}H NMR spectroscopy, to measure intramyocellular triglyceride content, we found that insulin resistance in muscle was accompanied by an ~50% increase in intramyocellular lipid content in the elderly subjects compared to the young subjects. Recent studies in man[8-10] and rodents[11-13] have demonstrated a strong relationship between intramyocellular content with insulin resistance in skeletal muscle and the data in the present study suggests a similar role for fatty acid induced insulin resistance in skeletal muscle in the elderly. Surprisingly there was no detectable hepatic insulin resistance in the elderly subjects despite a greater than twofold increase in hepatic triglyceride content. This result contrasts with recent studies that have demonstrated a strong relationship between hepatic triglyceride content and hepatic insulin resistance[13-15]. These differences may be due to the relatively lower intrahepatic triglyceride concentration observed in the elderly subjects compared to the insulin resistant diabetic subjects examined in the previous studies.

In order to assess whether the increase in intramyocellular and hepatic triglyceride content in the older subjects was due to increased delivery of fatty acids we measured whole body and localized rates of lipolysis. Rates of whole body lipolysis were similar in the young and elderly subjects during the basal state and suppressed equally during the hyperinsulinemic-euglycemic clamp. Consistent with this finding we found that insulin suppression of peripheral lipolysis, assessed by microdialysis, was also similar in both groups. Taken together these data suggest that insulin resistance was confined mostly to skeletal muscle and that increased basal

rates of peripheral lipolysis, and/or defects in insulin suppression of lipolysis, do not play a major role in causing the increased intramyocellular and intrahepatic triglyceride content in the elderly.

In order to assess whether decreased mitochondrial activity might be a contributing factor to the increased triglyceride content in muscle we also assessed rates of mitochondrial oxidative and phosphorylation activity in skeletal muscle using a novel $^{13}C/^{31}P$ NMR technique[30]. Using this approach we found that rates of TCA cycle flux and ATP production were similarly reduced by ~40% in muscle of the elderly subjects compared to the young subjects. These are the first *in vivo* studies to assess mitochondrial oxidative and phosphorylation activity in muscle of elderly subjects and the results are consistent with histologic studies demonstrating disruption and loss of mitochondrial cristae in muscle of the elderly [31]. These results are also consistent with a previous *in vitro* study, which found decreased state III (activated) mitochondrial respiration in isolated mitochondria obtained from elderly subjects[31]. However these *in vitro* measurements were performed in muscle strips, obtained from orthopedic and chronic fatigue syndrome patients, under artificial substrate conditions that do not reflect *in vivo* conditions. Taken together our results suggest that insulin resistance in the elderly is related to increases in intramyocellular fatty acid metabolites due to an age-associated reduction in mitochondrial oxidative phosphorylation activity. The similarity in mitochondrial energy coupling, assessed by the ratio between ATP synthase flux and TCA cycle oxidation, suggests an age associated reduction in mitochondrial number and/or function as opposed to an acquired defect in mitochondrial energy coupling. This observation is consistent with recent studies demonstrating an accumulation of mutations in mitochondrial DNA control sites with aging that are responsible for mitochondrial replication[32]. Since oxidative phosphorylation is the major source of energy in most organs,

including the brain, these data support the hypothesis that a decline in oxidative energy production may also have an important role in senescence[33,34]. Furthermore since mitochondria play a critical role in mediating glucose induced insulin secretion[35] similar age associated reductions in beta cell mitochondrial function, in the setting of peripheral insulin resistance, might explain the very high prevalence of diabetes in the elderly.

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Table 1

Body Composition

	Age (years)	Body Weight (kg)	Fat Mass (kg)	% Fat Mass (% Body Weight)	Lean Body Mass (kg)	BMI (kg/m^2)
Young (n=13)	27±2	71±4	19.9±2.5	28±3	54±5	23.8±1.1
Elderly (n=15)	70±2	70±3	20.1±1.7	29±2	49±3	25.1±0.5
P-value	<0.0001	0.69	0.93	0.77	0.28	0.28

Figure Legends

Figure 1: Plasma concentrations of: **(a)** glucose, **(b)** insulin and **(c)** fatty acid before and after an oral glucose tolerance test in young and elderly subjects. *P=0.02 for area under the curve (AUC) for insulin.

Figure 2: Basal and insulin stimulated rates of muscle glucose metabolism in young and elderly subjects.

Figure 3: **(a)** Intramyocellular lipid content (IMCL) and **(b)** intrahepatic triglyceride content in young and elderly subjects.

Figure 4: Rates of muscle mitochondrial: **(a)** oxidative activity (TCA cycle) and **(b)** phosphorylation activity (ATP synthase flux) in young and elderly subjects.

Figure 1a

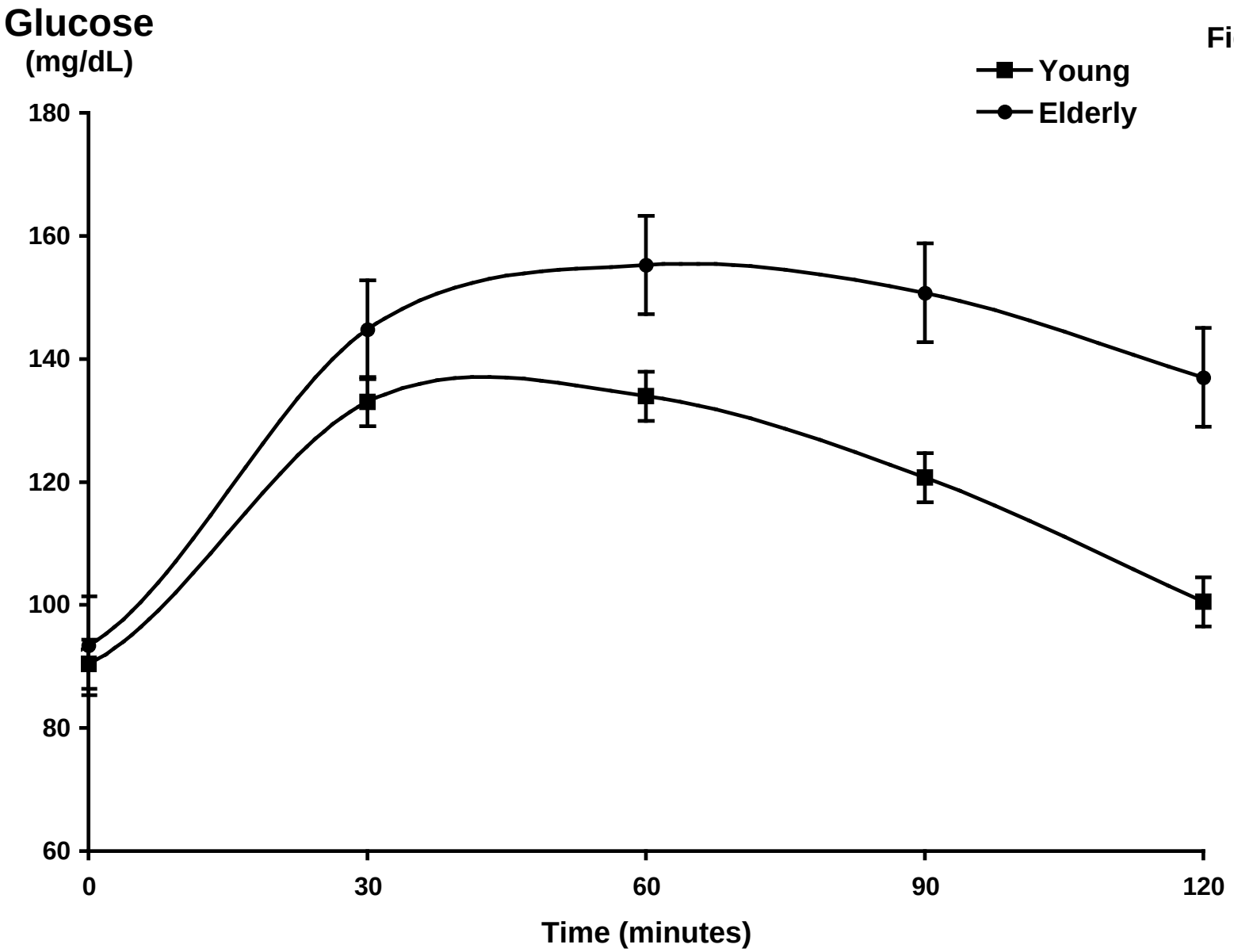

Glucose
(mg/dL)
Young
Elderly
180
160
140
120
100
80
60
0
30
60
90
120
Time (minutes)

Figure 1b

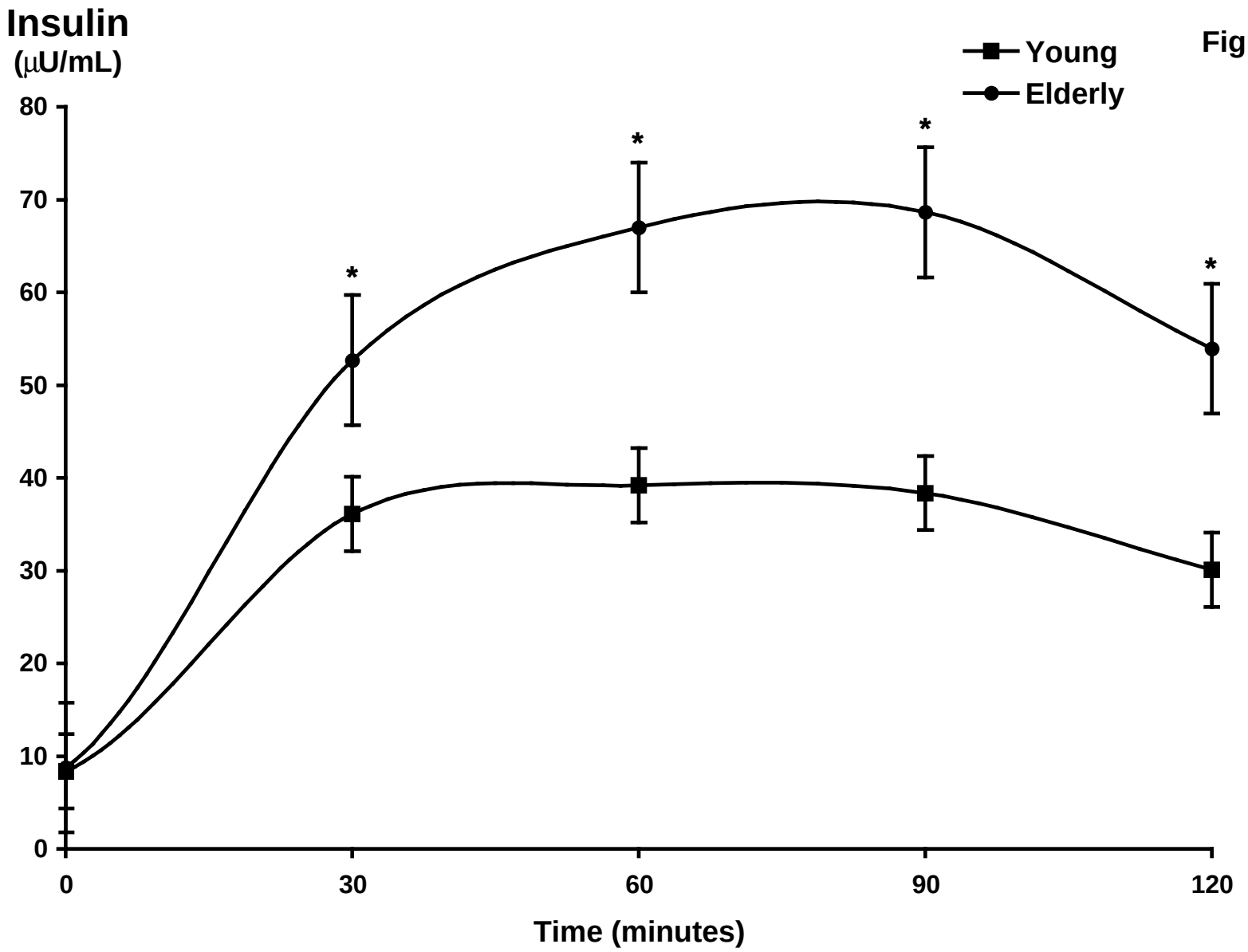

Insulin
(μU/mL)
Young
Elderly
80
70
60
50
40
30
20
10
0
*
*
*
*
0
30
60
90
120
Time (minutes)

Figure 1c

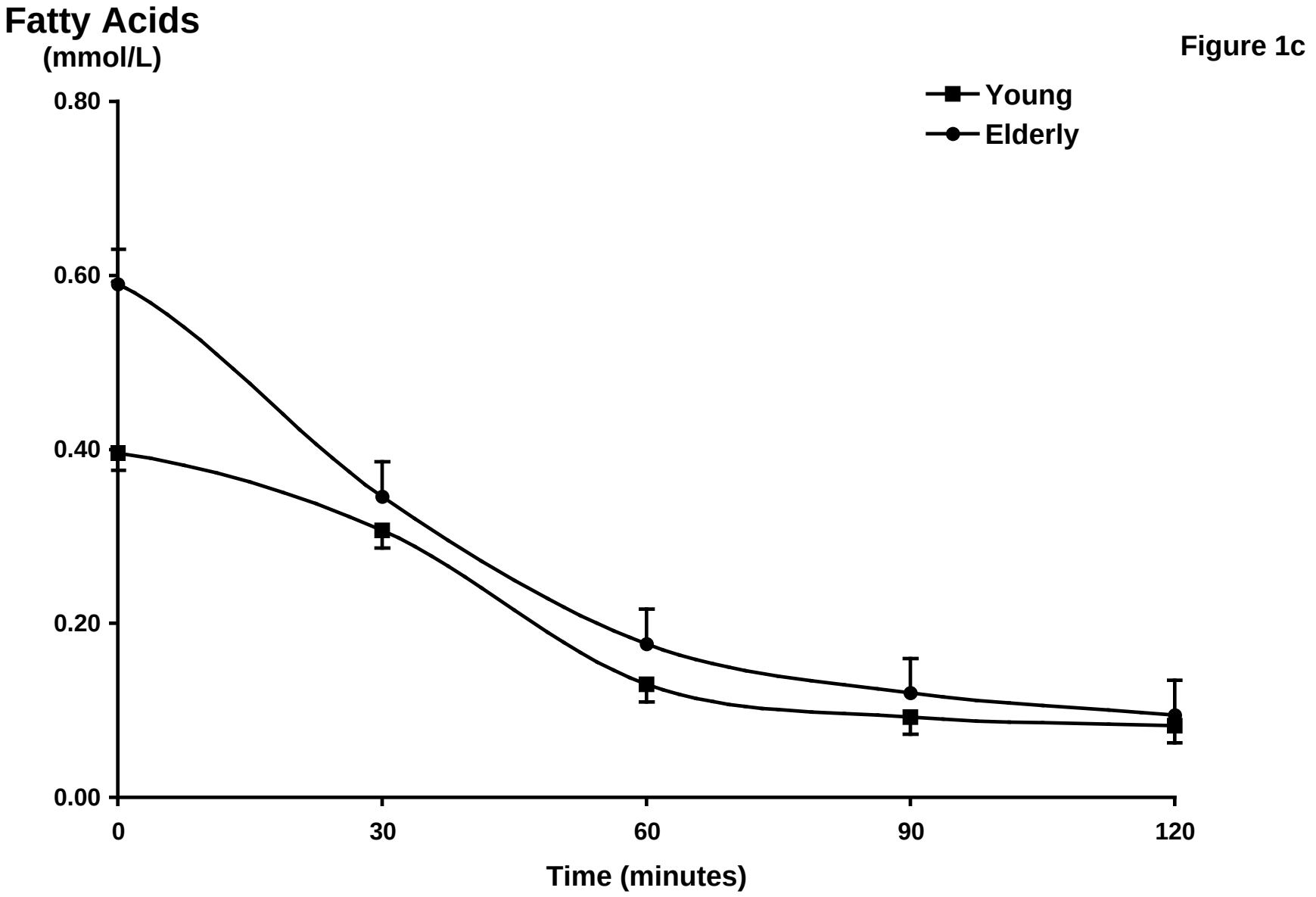

Fatty Acids
(mmol/L)
Young
Elderly
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
Time (minutes)

Figure 2

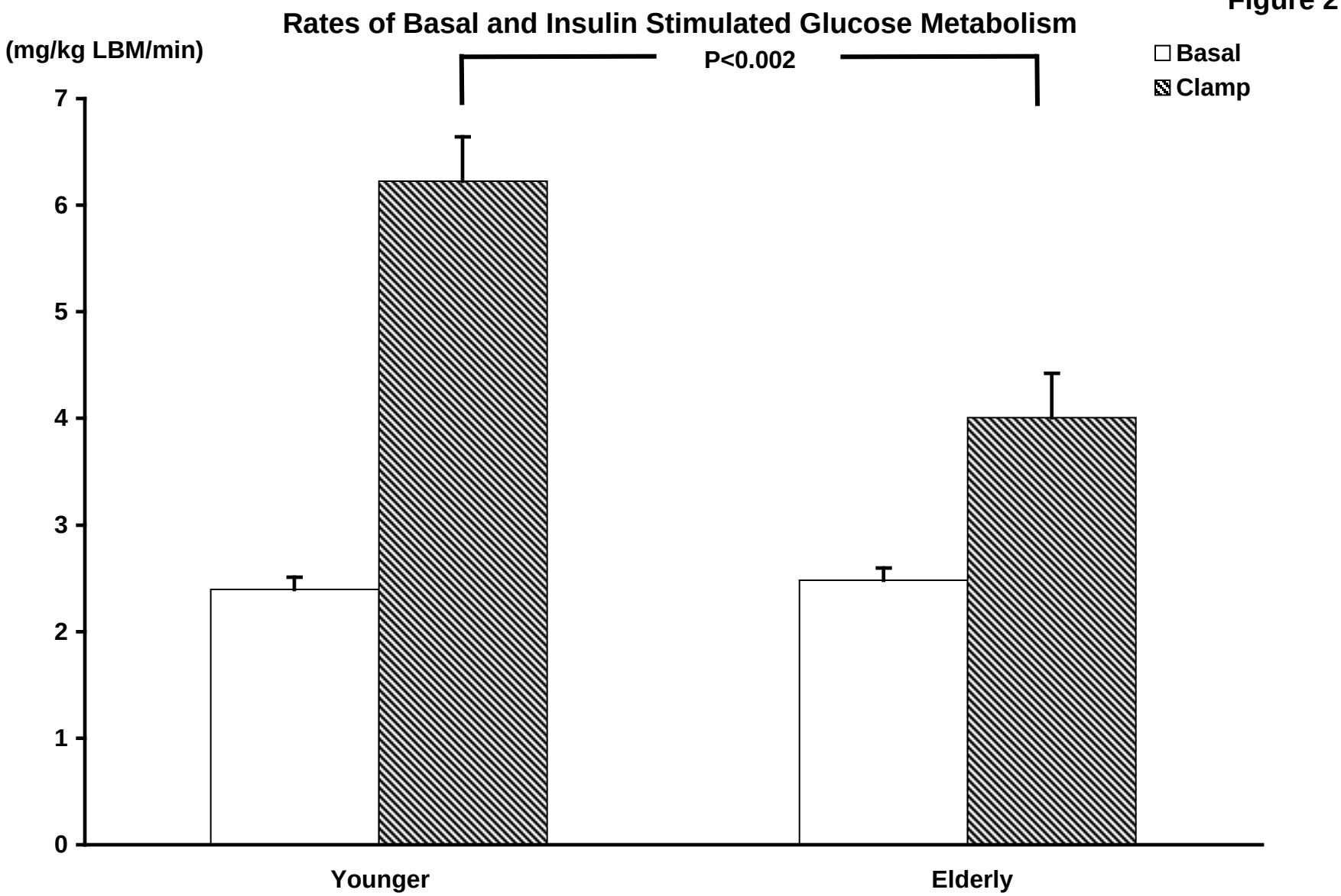

Rates of Basal and Insulin Stimulated Glucose Metabolism
(mg/kg LBM/min)
P<0.002
Basal
Clamp
7
6
5
4
3
2
1
0
Younger
Elderly

Figure 3a

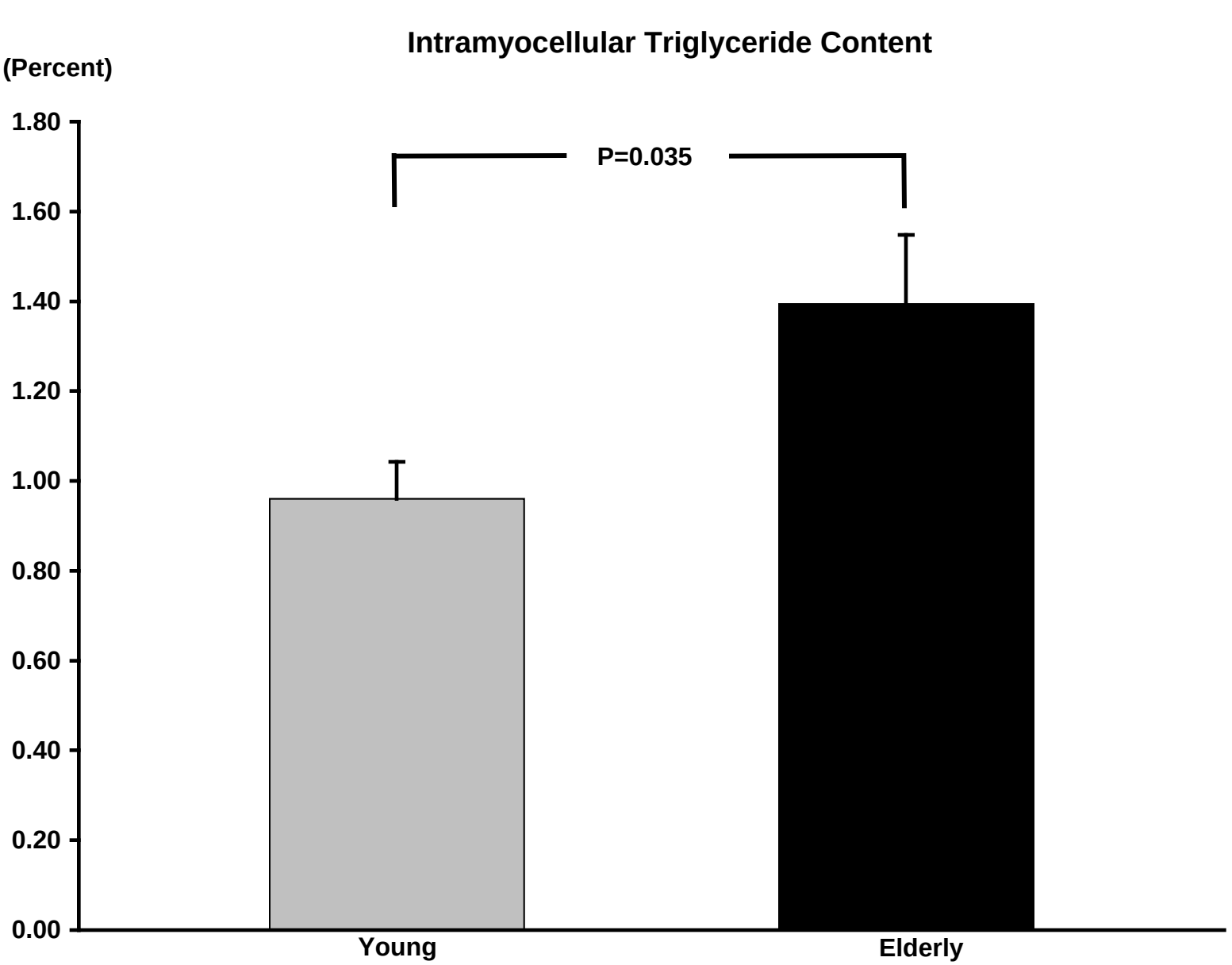

Intramyocellular Triglyceride Content
(Percent)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P=0.035
Young
Elderly

Figure 3b

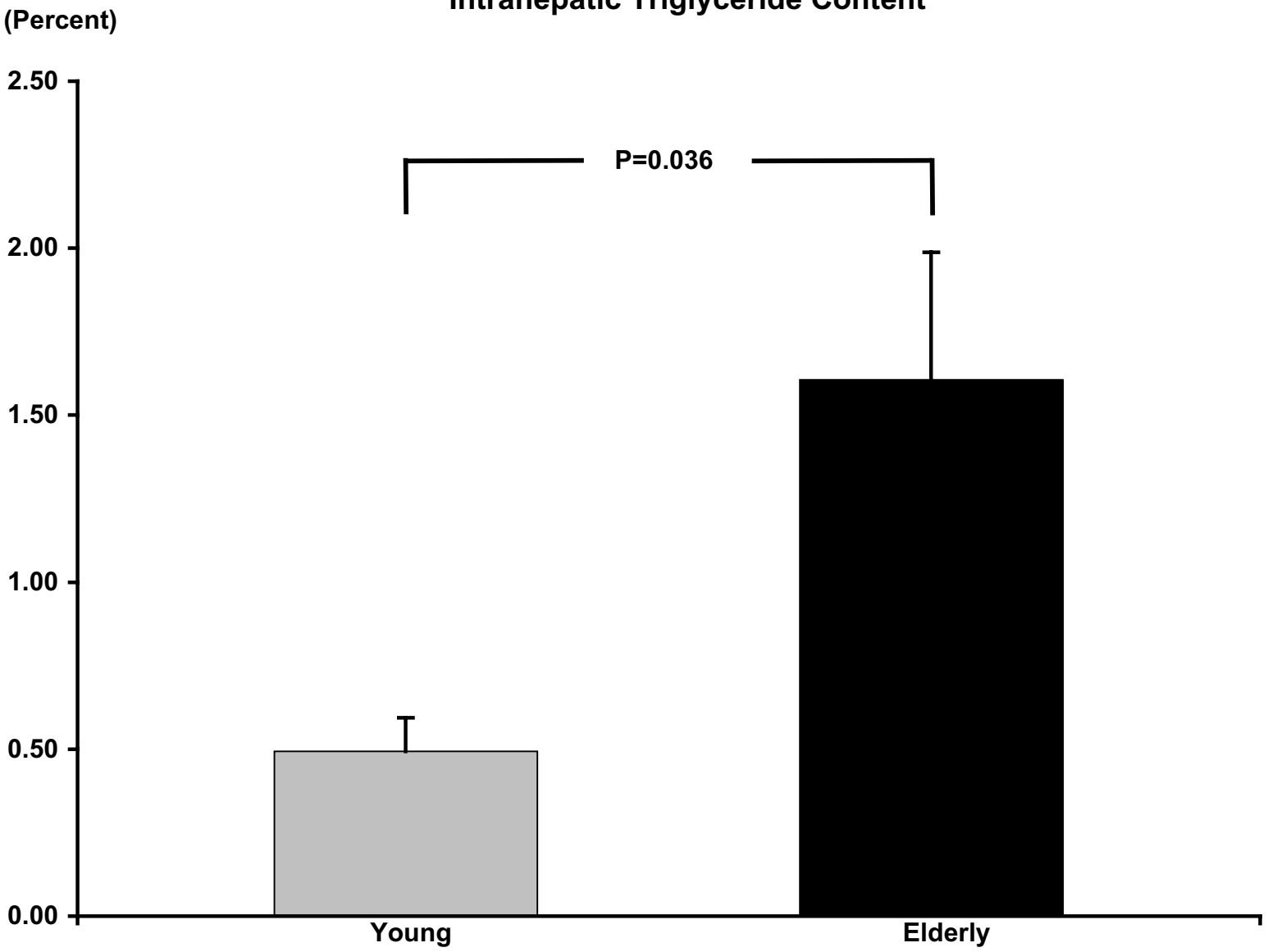

Intrahepatic Triglyceride Content
(Percent)
2.50
2.00
1.50
1.00
0.50
0.00
P=0.036
Young
Elderly

Figure 4a

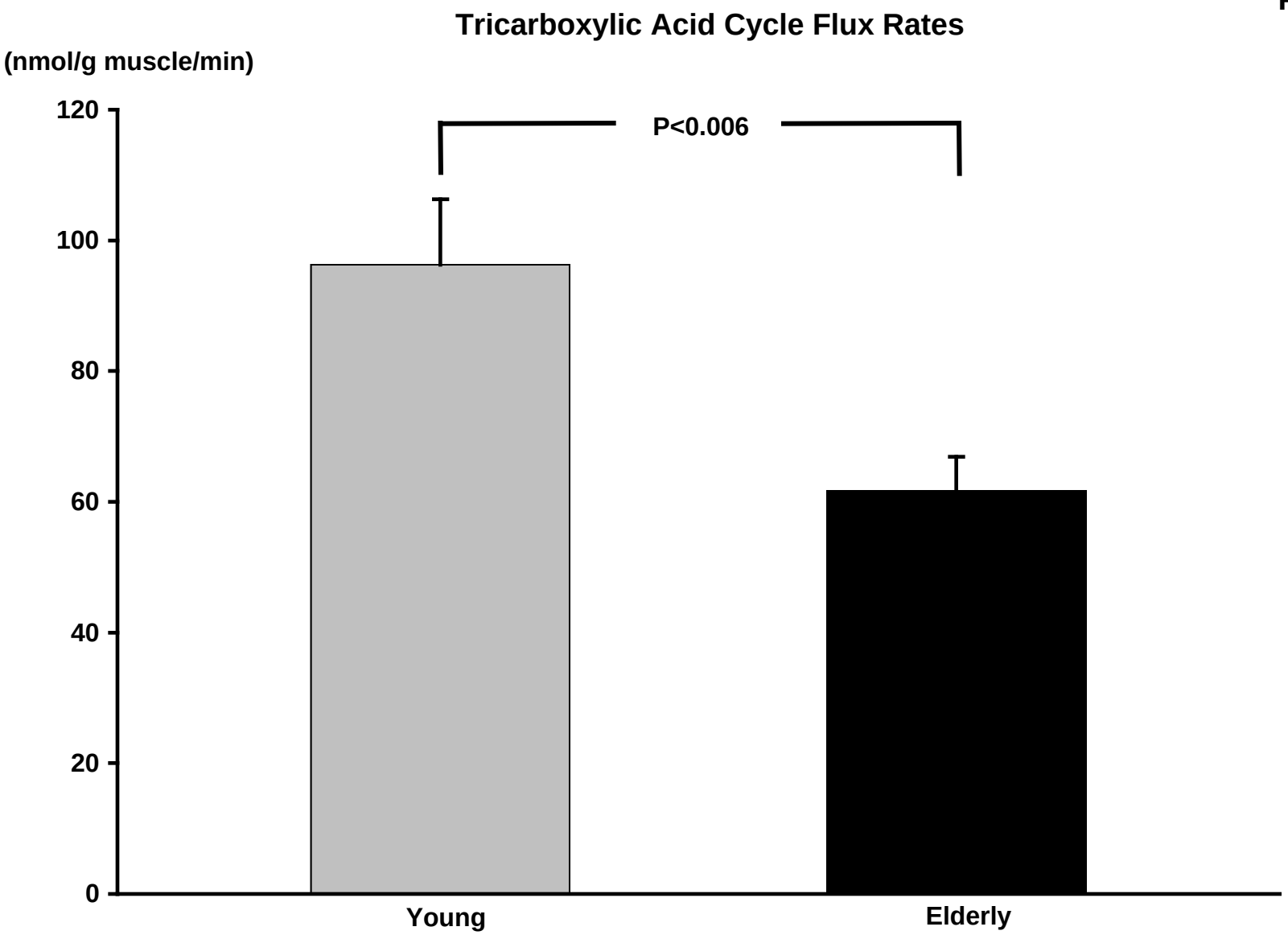
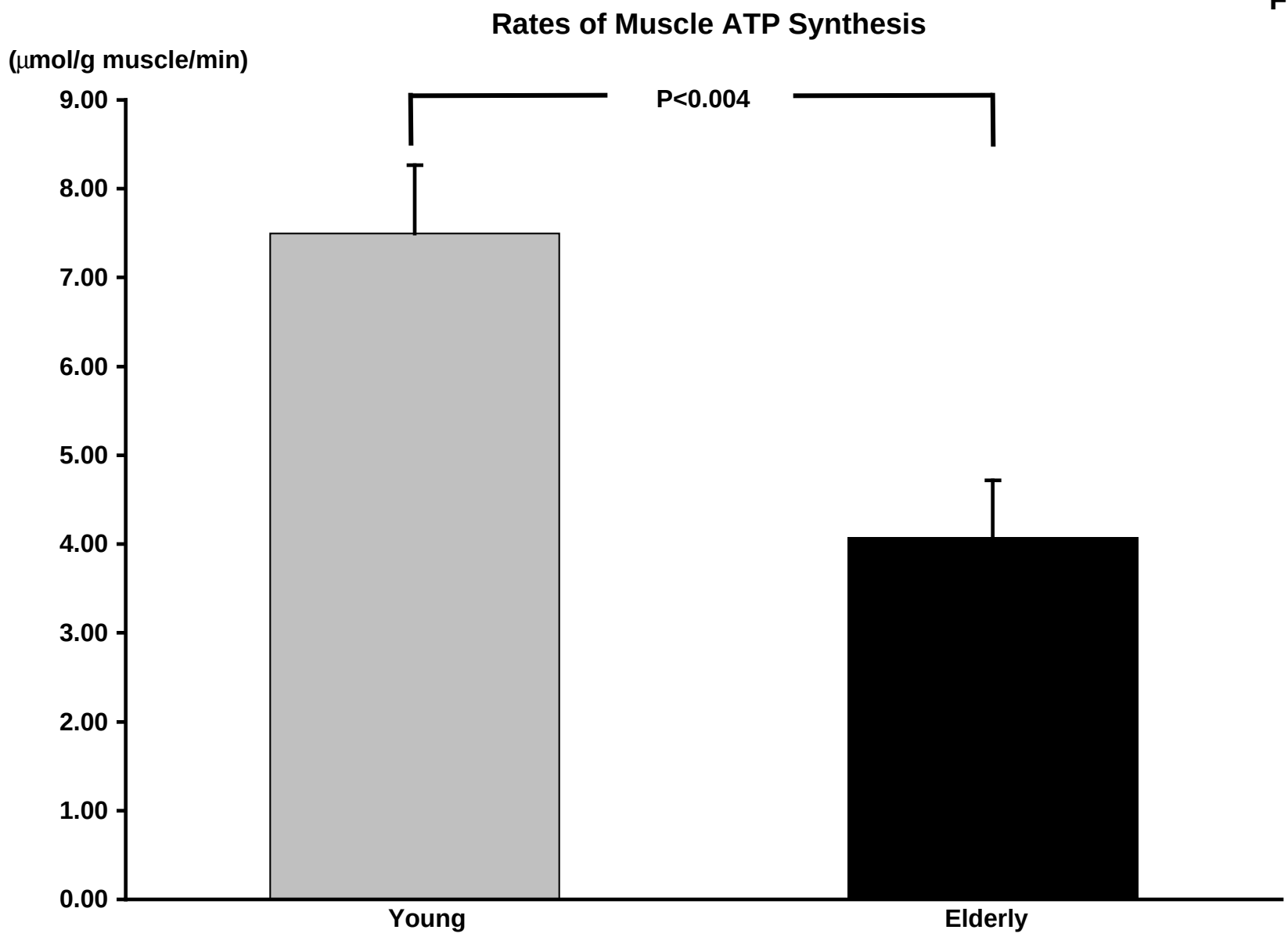

Tricarboxylic Acid Cycle Flux Rates
(nmol/g muscle/min)
120
100
80
60
40
20
0
P<0.006
Young
Elderly

Figure 4b


Rates of Muscle ATP Synthesis
(μmol/g muscle/min)
P<0.004
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Young
Elderly