

HUMAN NUTRITION

Title: Effect of pork ingestion on postprandial mitochondrial protein synthesis and inflammation in healthy weight, overweight, and obese adults - **NPB # 16-012**

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Date Submitted: 5 Oct 2017

revised

Industry Summary:

Obesity is associated with numerous chronic diseases. Many obese individuals, however, do not display overt disease. The skeletal muscle is an important tissue for keeping many nutrients in the blood in balance (e.g. blood sugars and fats). However, aspects of skeletal muscle metabolism become dysregulated with fat mass gain resulting in systemic complications (i.e. diabetes). Muscle mitochondria are responsible for several processes including maintaining cellular energy stores.

Maintenance of the proteins in the mitochondria represents an essential component of muscle health that has not been thoroughly studied in participants with a wide range of fat masses. Moreover, it has not been established if skeletal muscle mitochondrial proteins are nutritionally modulated in humans in response to a meal-like amount of protein. In healthy, normal-weight adults, mitochondrial protein synthesis is stimulated by amino acid administration intravenously. However, muscle inflammation in obese individuals may prevent optimal mitochondrial protein synthesis. Therefore, the main objectives of this study were 1) to determine mitochondrial protein synthesis rates at basal and after consuming a meal-like amount of lean pork at breakfast; and 2) to establish the systemic and muscle inflammatory response to the consumption of lean pork. Both of these objectives were determined in participants across a wide range of body compositions.

Main findings:

- 1) Basal rates of mitochondrial protein synthesis were similar among all participants regardless of their BMI score and body composition.
- 2) Consuming a meal-like quantity of lean pork was a potent stimulus to increase the mitochondrial protein synthetic response in all participants regardless of their BMI score.
- 3) Systemic and muscle inflammation was increased by eating pork in obese individuals only
- 4) The obesity related inflammation response (systemic and muscle) did not influence the stimulation of the mitochondrial protein synthetic response.

In summary, pork ingestion strongly stimulates mitochondrial protein synthesis in young men and women across a wide range of body compositions, which has important implications for muscle health. Further, neither systemic, nor muscle, inflammation appear to influence, or modulate, the postprandial mitochondrial protein synthetic response to lean pork ingestion. Obese individuals

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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experience an inflammatory response to lean pork ingestion, but more research is needed to determine if this is related to specific food components or simply related excess fat mass.

Keywords: Mitochondria, protein synthesis, inflammation, obesity, muscle, insulin

Scientific Abstract:

Context:

Excess fat mass may diminish the anabolic potency of protein-rich food ingestion to stimulate muscle protein sub-fractional synthetic responses. However, the impact of adiposity on mitochondrial protein synthesis rates (MPS) after protein-rich food ingestion has not been thoroughly examined *in vivo* in humans.

Objective:

We compared basal and postprandial MPS and markers of muscle inflammation (Toll-like receptor 4 [TLR4] and myeloid differentiation primary response protein 88 [MyD88] protein content) in young adults with different BMIs.

Methods:

10 normal-weight (NW; BMI 22.7 ± 0.4 kg/m²), 10 overweight (OW; BMI 27.1 ± 0.5 kg/m²), and 10 obese (OB; BMI 35.9 ± 1.3 kg/m²) adults received primed continuous L-[ring-¹³C₆]phenylalanine infusions, blood sampling, and skeletal muscle biopsies before and after the ingestion of 170 g of pork.

Results:

Pork ingestion increased muscle TLR4 and MyD88 protein content in the OB group ($P < 0.05$), but not in the NW or OW groups. Basal MPS were similar between groups ($P > 0.05$). Pork ingestion stimulated MPS ($P < 0.001$) (0-300 min) in the NW (2.5 ± 0.6 -fold above baseline values), OW (1.7 ± 0.3 -fold), and OB groups (2.4 ± 0.5 -fold) with no group differences ($P > 0.05$).

Conclusions:

Protein-dense food ingestion promotes muscle inflammatory signaling only in obese adults. However, the consumption of a dinner-sized amount of protein strongly stimulated a postprandial MPS response irrespective of BMI. Our data suggest that alterations in postprandial mitochondrial protein synthesis are unlikely to contribute to compromised muscle macronutrient metabolism witnessed with obesity.

Introduction:

Obesity is a disease characterized by impaired postprandial macronutrient metabolism. Specifically, obese individuals have elevated plasma inflammation [e.g. tumor necrosis factor α (TNF α), Interleukin 6 (IL-6), non-esterified fatty acids (NEFA)], which has been linked to altered muscle metabolism including insulin resistance. Moreover, muscle inflammation has been implicated in impairing mitochondrial function and inducing insulin resistance in rodents. Currently, there are little data in humans that describe the interaction between inflammation, adiposity, and the stimulation of postprandial mitochondrial protein synthesis in response to protein dense food ingestion.

The proposed research aimed to assess mitochondrial protein synthesis before and after the ingestion of lean pork in participants with a wide range of body mass indices (BMI). Impairments in the feeding-induced synthesis of mitochondrial proteins over time may diminish muscle quality or metabolic function and ultimately limit quality of life. Results from this research will provide the evidence that consuming high-quality pork at breakfast represents an effective strategy for maintaining the metabolic health of the skeletal muscle.

Objectives:

Primary objective #1. Determine the postabsorptive and the postprandial mitochondrial protein synthetic response to the consumption of 6 oz pork in healthy weight, overweight, and obese adults.

Primary objective #2. Assess circulating and skeletal muscle inflammation and non-esterified fatty acids before and after the consumption of pork in healthy weight, overweight, and obese adults.

Materials & Methods:

Participants and ethical approval

Ten normal-weight (NW), 10 overweight (OW), and 10 young obese volunteers (OB) were recruited to participate in this study. The groups were counterbalanced for age and sex. The characteristics of these participants have been described in detail elsewhere (2). This study represented an extension of our previous work (2) to include measurements of the systemic and muscle inflammatory responses and mitochondrial protein synthesis measurements before and after food ingestion *in vivo* in humans. Participant characteristics are reported in **Table 1**. Participants were classified as *insufficiently active* according to a Godin Leisure-Time Exercise Questionnaire (GLTEQ: < 14 units (15)) and deemed healthy based on responses to a routine medical screening questionnaire. Each participant was informed of the study purpose, experimental procedures, and all of its potential risks prior to providing written consent to participate. The study was approved by the Institutional Review Board at University of Illinois at Urbana-Champaign and conformed to standards for the use of human participants in research as outlined in the seventh revision of the Declaration of Helsinki.

Pretesting

Participants reported to the laboratory on two separate occasions for screening sessions to assess height, weight, and body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Bedford, MA, USA). Participants were also screened for diabetes risk using an oral glucose tolerance test.

Infusion protocol

Participants were instructed to refrain from physical activity, analgesic drugs, and alcohol for three days prior to the experimental infusion trial. The evening prior to the trial, all participants consumed a standardized meal of the same composition (providing ~30% of estimated total daily energy expenditure and containing 50% of energy of carbohydrate, 25% energy of fat, and 25% energy of protein). On the trial days, participants reported to the laboratory in the morning after an overnight fast and a Teflon catheter was inserted in an antecubital vein for baseline blood sample collection. Subsequently, a primed ($2 \mu\text{mol}\cdot\text{kg}^{-1}$) continuous infusion of L-[ring- $^{13}\text{C}_6$]phenylalanine ($0.05 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) was initiated ($t=-180 \text{ min}$), which was passed through a $0.2 \mu\text{mol}$ filter, and maintained until the end of the trial. A second Teflon catheter was inserted in a contralateral dorsal hand vein and kept patent with a 0.9% saline drip for repeated arterialized blood sampling using a heated blanket. Biopsy samples of the *vastus lateralis* were collected in the postabsorptive-state at $t=-120$ and 0 min of the infusion trials. Subsequently, participants consumed 170 g ground lean pork loin (containing 36 g protein, ~3 g leucine, and 3 g fat) and 300 mL of water enriched to 4% with L-[ring- $^{13}\text{C}_6$]phenylalanine according to the phenylalanine content of pork to minimize disturbances in isotopic equilibrium during the infusion ($t=0$). Additional muscle biopsies were collected at 120 and 300 min after pork ingestion. Arterialized blood samples were drawn every 30 or 60 min during the post-absorptive and postprandial-states. Blood samples (8 ml) were collected in EDTA-containing tubes and centrifuged at $3000\times g$ at 4°C for 10 min. Aliquots of plasma were frozen and stored at -80°C until subsequent analysis. Biopsies were collected from the *vastus lateralis* (15 cm above the patella) with a Bergström needle under local anesthesia (2% lidocaine). The postabsorptive muscle biopsies were randomly obtained from one leg and the postprandial biopsies from the contralateral leg. All muscle biopsy samples were freed of any visible adipose, connective tissue and blood tissue, frozen in liquid nitrogen, and stored at -80°C until subsequent analysis.

Plasma analyses

Plasma NEFA, IL-6, TNF α , and C-reactive protein (CRP) concentrations were determined according to manufacturer's instructions using a commercially available enzyme-linked immunosorbent assays (NEFA: Abcam; IL-6: R&D systems; TNF α , insulin, and CRP: Alpco diagnostics; USA). Plasma leucine and phenylalanine concentrations and L-[ring- $^{13}\text{C}_6$]phenylalanine enrichments were measured by GC/MS analysis using electron impact ionization (Agilent 7890A GC/5975C; MSD, USA) as previously described (2). Amino acid concentrations were quantified using the AMDIS software package (v. 2.71, NISTTM, USA) and standards with known concentrations.

Mitochondrial protein synthesis measurements

Mitochondrial protein-enriched fractions were extracted from ~100 mg of wet muscle tissue using a Dounce glass homogenizer on ice in ice-cold homogenizing buffers supplemented with a Complete Mini, protease inhibitor and phosphatase cocktail tablets (PhosSTOP, Roche Applied Science, Germany) and differential centrifugation method as described in detail previously (16). Mitochondrial-enriched protein pellets were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before the mitochondrial protein-bound enrichments were determined by LC/MS/MS analysis (5500 QTRAP, Sciex, USA) as described previously (2). The L-[ring- $^{13}\text{C}_6$]phenylalanine mitochondrial protein-bound enrichments were determined by multiple reaction monitoring (MRM) at m/z 166.0 \rightarrow 103.0 and 172.0 \rightarrow 109.0 for unlabeled and labeled L-[ring- $^{13}\text{C}_6$]phenylalanine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis.

Western blotting analysis

An aliquot of muscle homogenate representing the sarcoplasmic fraction, which was isolated during the mitochondrial protein extractions, was used for Western Blot analysis. Total protein concentrations of each sample were determined by Bradford assay (Bio-Rad), and then equal amounts of protein (80 μg) were separated by SDS-PAGE before being transferred to polyvinyl difluoride membranes. After blocking, membranes were incubated in primary antibodies overnight at 4°C to determine the total protein content of Toll-like receptor 4 (TLR4: R&D systems, USA) and myeloid differentiation factor 88 (MyD88: Cell Signaling, USA). In addition, total protein content and phosphorylation status of Akt at Thr308 (Akt/PKB: Cell Signaling, USA), and the 160 kDa Akt Substrate (AS160: Cell Signaling, USA) were also determined. Membranes were then incubated with appropriate secondary antibodies, and protein content was detected using West Femto Maximum Sensitivity substrate (SuperSignal, Thermo Scientific, USA) and the ChemiDoc-It² Imaging System (UVP, USA). Bands were quantified using ImageJ software (NIH) and then normalized to either α -tubulin (Abcam, USA; TLR4, MyD88) or total protein (Akt, AS160).

Calculations

The fractional synthetic rates (FSR) of mitochondrial protein were calculated using standard precursor-product methods by dividing the increment in tracer enrichment in the mitochondrial protein by the enrichment of the plasma free precursor pool over time.

Statistics

Differences in plasma insulin, NEFA, IL-6, TNF α and CRP, muscle TLR4 and MyD88, and mitochondrial protein synthesis rates were tested by two-factor (group \times time) repeated measures analysis of variance (ANOVA). Demographics, body composition, level of habitual physical activity (GLTEQ), and net area under the time curve (AUC) for plasma insulin, NEFA, IL-6, TNF α and CRP were analyzed using one-factor (group) ANOVA. Any data not conforming to ANOVA assumptions were transformed prior to statistical analysis. In addition, Mauchly's test of sphericity was also performed and Greenhouse-Geisser or Hunyh-Feldt corrections were applied as appropriate. When significant effects were detected in the ANOVA, Tukey's post-hoc tests were performed to locate the differences between means for all significant main effects and interactions. For all analyses, differences were considered significant at $P < 0.05$. All calculations were performed using IBM SPSS Statistics Version 20. All data are expressed as means \pm SEMs.

Results:

Primary objective #1:

Basal mitochondrial protein synthesis rates (**Figure 1**) were not different among the NW, OW, and OB groups ($P=0.91$) Pork ingestion increased cumulative postprandial mitochondrial protein synthesis rates measured over the 0-300 min postprandial period (**Figure 1 inset**) in the NW (2.5 ± 0.6 -fold above baseline values), OW (1.7 ± 0.3 -fold), and OB groups (2.4 ± 0.5 -fold; all $P<0.05$). However, the postprandial mitochondrial protein synthetic response determined during the early postprandial period (0-120 min) increased in the NW (2.6 ± 0.4 -fold above basal; $P=0.01$) and OB groups (2.3 ± 0.6 fold above basal; $P=0.03$) but not in the OW group (1.4 ± 0.3 , $P=0.62$) after pork ingestion. There was no significant feeding-induced stimulation of mitochondrial protein synthesis rates during the late postprandial period (120-300 min) in any of the groups (all $P>0.05$).

Primary objective #2:

Plasma variables

Plasma leucine (**Figure 2A**) and phenylalanine (**Figure 2B**) concentrations increased after pork ingestion ($P<0.001$) and did not differ between groups (both, $P>0.05$). Plasma NEFA concentrations were not different between groups at basal (both, $P>0.05$) and decreased after pork ingestion in all groups (**Figure 2C**; $P<0.05$). However, plasma NEFA concentrations (**Figure 2C**) decreased earlier in the NW group ($t=60$ min; $P=0.02$), whereas the plasma NEFAs concentrations decreased later in the OW and OB groups of the postprandial period (120 min; both $P<0.05$). Moreover, plasma NEFA concentrations increase above basal values after pork ingestion at $t=300$ min in the NW group ($P<0.001$) and tended to increase in the OW group ($P=0.06$) but not in the OB group ($P=0.23$). Plasma IL-6 concentrations (**Figure 3A**) were elevated throughout the basal and postprandial period in the OB group ($P=0.03$) and tended to be elevated in the OW ($P=0.16$) as compared with the NW group. However, pork ingestion increased plasma IL-6 concentrations in all groups (all, $P<0.05$). Plasma TNF α concentrations (**Figure 3B**) were not different among groups at basal ($P>0.05$). At 300 min, plasma TNF α was greater in the OB ($P=0.04$) but not the OW group ($P=0.98$) as compared to NW group. Plasma CRP concentrations (**Figure 3C**) were greater at all time points in the OB group ($P<0.001$) when compared with the NW and OW groups (all, $P>0.05$).

Muscle inflammation

The relative concentrations of total muscle TLR4 protein were greater in the OB as compared with the NW and OW groups at basal (both, $P=0.05$; **Figure 4A**). After pork ingestion, total muscle TLR4 protein was greater in the OB group at 120 and 300 min of the postprandial period when compared to the NW and OW groups (all, $P<0.05$). In addition, there was a trend for increased TLR4 protein content after pork ingestion in the OB group at 300 min as compared to the NW group ($P=0.14$). No changes were observed in total muscle TLR4 protein content in the NW or OW groups (both $P>0.05$). At baseline, total muscle MyD88 protein content (**Figure 4B**) was greater in the OW and OB compared to the NW group (both $P<0.05$). Total MyD88 protein content increased above basal values after pork ingestion in the OB group at 300 min ($P=0.001$) of the postprandial phase with no observed differences in total MyD88 protein in either the NW or the OW groups (all $P>0.05$).

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

Our data demonstrated that greater fat mass does not alter basal rates of mitochondrial protein synthesis. Moreover, we also demonstrate that pork ingestion strongly stimulated postprandial mitochondrial protein synthesis rates across the range of BMIs. Indeed, we witnessed an obesity-specific increase in muscle inflammation (TLR4 and MyD88) after the ingestion of pork. This increase may be related to the increase in TNF α concentrations in this group. Despite this, it appears that elevated systemic and muscle inflammation with obesity is unrelated to mitochondrial protein synthesis rates during either the basal or the post-meal period. Moreover, lean pork ingestion at breakfast did not modulate postprandial inflammation in healthy weight or overweight young adults.

Pork producers can benefit from these findings in that pork ingestion demonstrates a strong ability to stimulate maintenance of important aspects of muscle health in young adults. This work also provides evidence that inflammation in obesity may be coincidental with regard to protein metabolism. The benefit to the pork producers is that we show that eating high-quality protein from pork helps the body in maintaining muscle health in a wide-range of young adults. Thus, our data supports the regular incorporation of pork-based products into a healthy eating pattern to support muscle metabolic health. Future work needs to identify what aspects of meal ingestion are responsible for the increased inflammation in obesity after meal ingestion.

Appendix A**Table 1.** Participant characteristics

Variable	Normal-weight	Overweight	Obese
<i>n</i> (females)	10 (5)	10 (5)	10 (5)
Age (y)	24 ± 1	26 ± 2	27 ± 3
Ht (m)	1.73 ± 0.03	1.70 ± 0.02	1.71 ± 0.03
Wt (kg)	68.5 ± 3.5	78.6 ± 2.2	106.0 ± 5.0†‡
BMI (kg/m ²)	22.7 ± 0.4	27.1 ± 0.5	35.9 ± 1.3
% Body fat	22.2 ± 1.8	29.1 ± 1.4†	35.3 ± 1.8†‡
Waist Circumference (cm)	77.2 ± 2.5	89.9 ± 2.5†	110.6 ± 3.8†‡
Physical Activity (GLTEQ score)	7.7 ± 3.0	2.5 ± 1.7	2.8 ± 2.0
HOMA-IR	1.36 ± 0.17	1.25 ± 0.11	5.82 ± 0.81†‡

† $P < 0.05$ vs. NW. ‡ $P < 0.05$ vs. OW. Data are Mean ± SEM.

Appendix B

Mitochondrial Protein Synthesis Rates

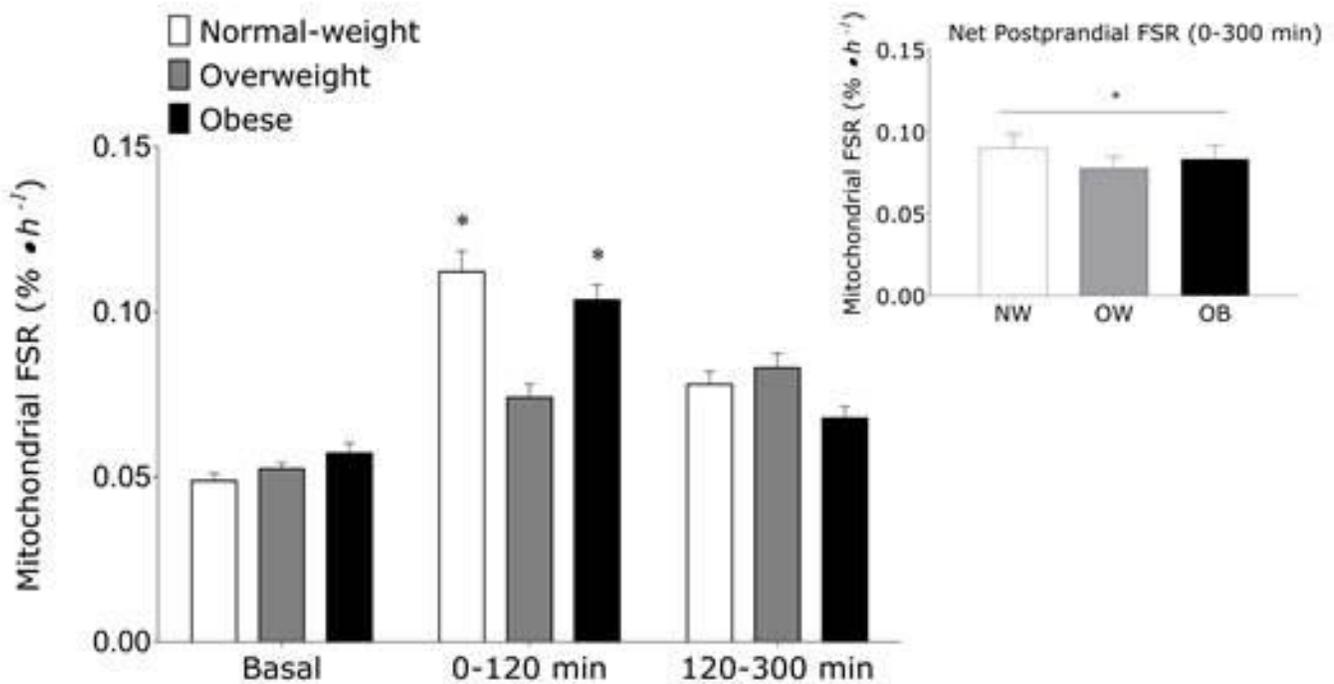


Figure 1. Skeletal muscle mitochondrial protein fractional synthesis rates (FSR) in the basal-state and after (0-120 and 120-300 min) pork ingestion ($n=10$ per group). Inset shows the cumulative postprandial (0-300 min) mitochondrial protein synthetic response. * $P<0.05$ vs. basal. NW, normal weight; OW, overweight; OB, obese. Data are mean \pm SEM.

Appendix C

Plasma Metabolites

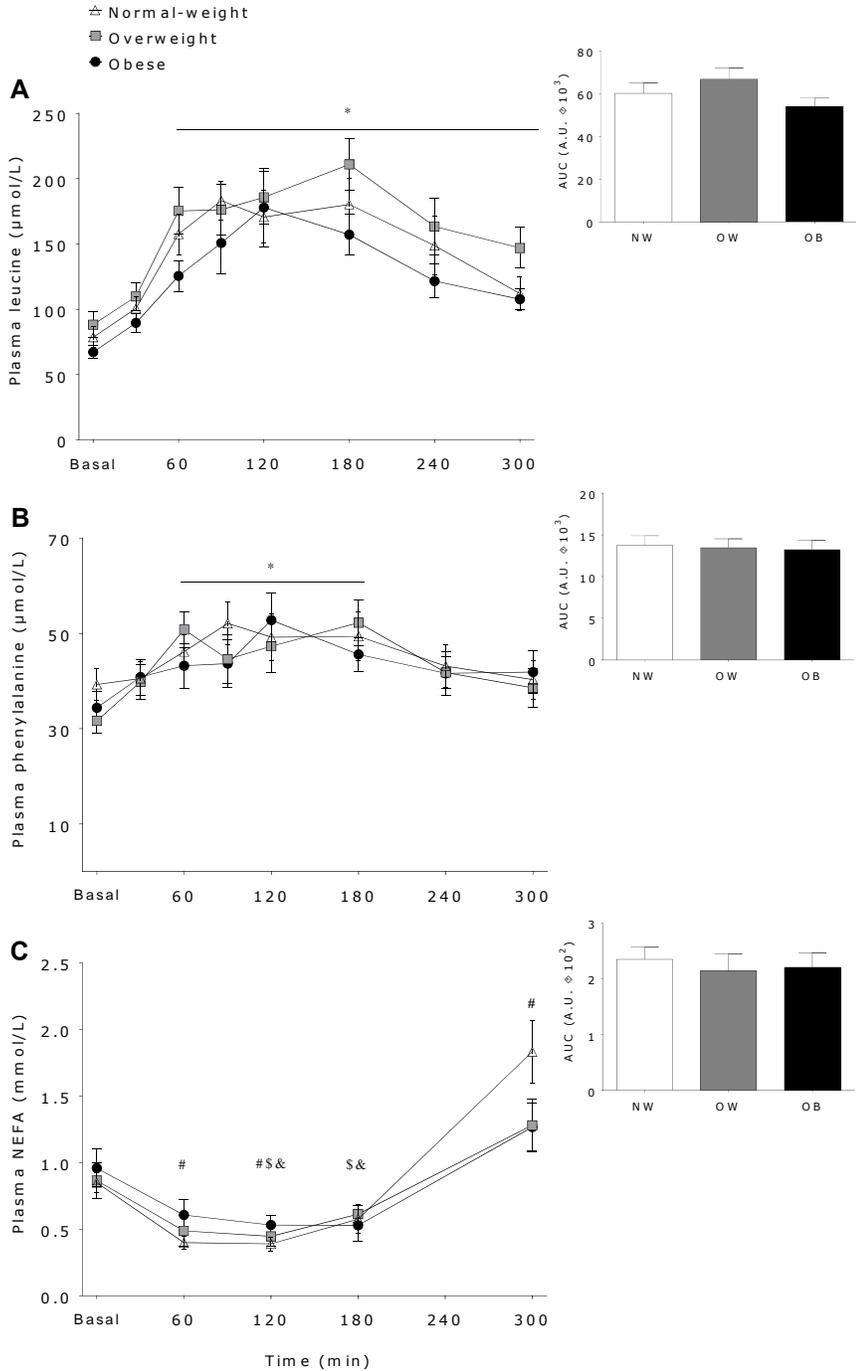


Figure 2. Plasma leucine (**A**), phenylalanine (**B**), and non-esterified fatty acids (NEFA; **C**) concentrations in the basal state and after consumption of pork ($n=10$ per group). Inset shows the area under the time curves (arbitrary units). * $P<0.05$ vs. basal in all groups. # $P<0.05$ vs. basal in NW. \$ $P<0.05$ vs. basal in OW. & $P<0.05$ vs. basal in OB. NW, normal weight; OW, overweight; OB, obese. Data are mean \pm SEM.

Appendix D

Plasma Inflammation

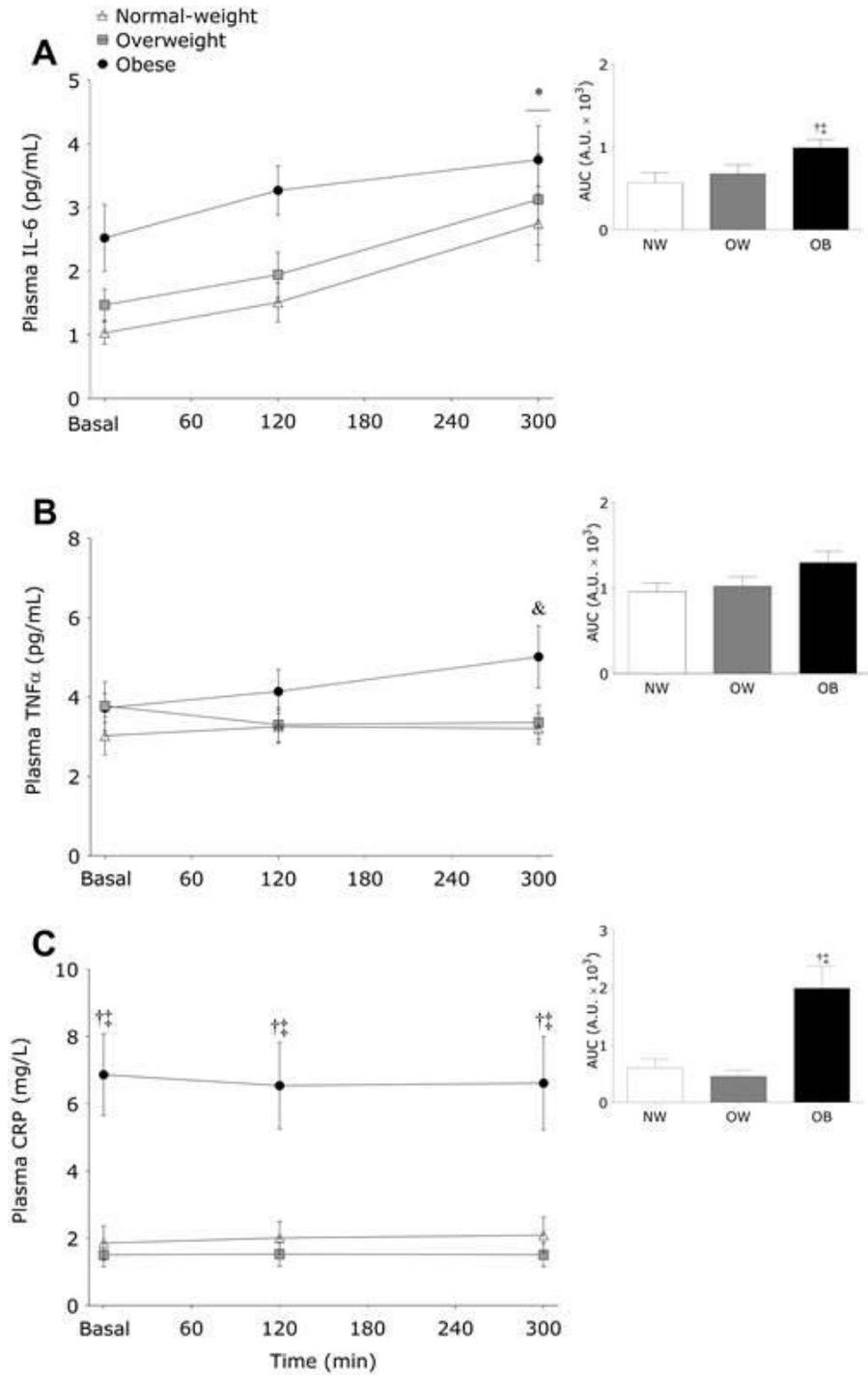


Figure 3. Plasma Interleukin 6 (IL-6; **A**), Tumor Necrosis Factor α (TNF α ; **B**), and C-reactive protein (CRP; **C**) concentrations in the basal state and after consumption of pork ($n=10$ per group). Inset shows the area under the time curves (arbitrary units). * $P<0.05$ vs. basal. & $P<0.05$ vs. basal in OB. † $P<0.05$ vs. NW. ‡ $P<0.05$ vs. OW. NW, normal weight; OW, overweight; OB, obese. Data are mean \pm SEM.

Appendix E

Muscle Inflammation

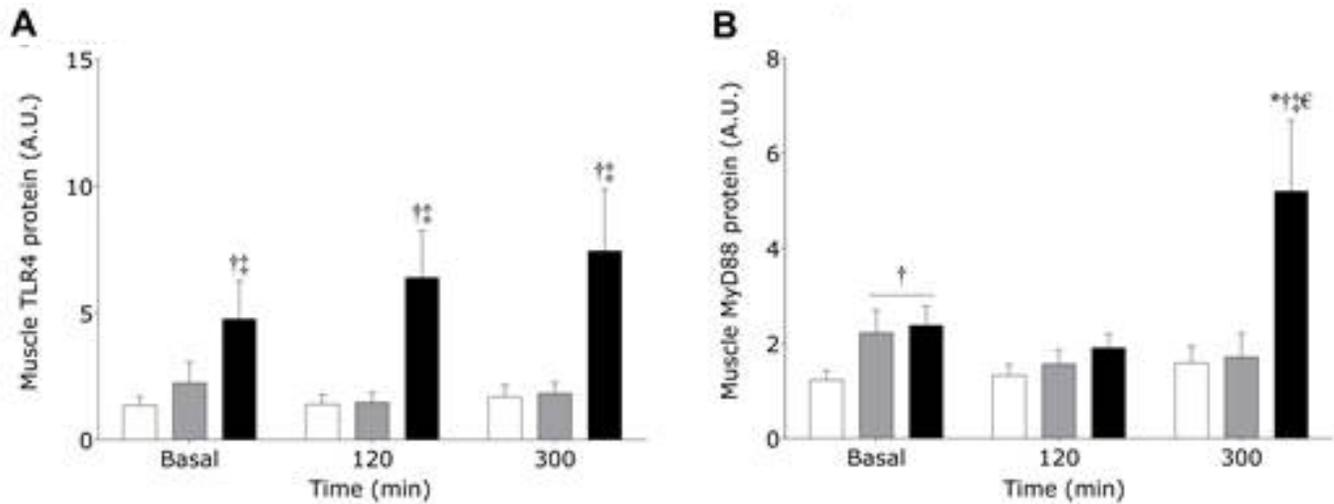


Figure 4. Protein content for toll-like receptor 4 (TLR4; **A**), myeloid differentiation factor 88 (MyD88; **B**) at basal and after the ingestion of pork ($n=10$ per group). * $P<0.05$ vs. basal. † $P<0.05$ vs. NW. ‡ $P<0.05$ vs. OW. € $P<0.05$ vs. OB. Data are mean \pm SEM.