-Oxidation of Free Fatty Acids is Required for Maintenance of Translational Control of Protein Synthesis in Heart


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Abbreviated Title: Translational control of protein synthesis by FFA

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ABSTRACT

The study described herein investigated the role of free fatty acids (FFAs) in the maintenance of protein synthesis in vivo in rat cardiac and skeletal muscle. Suppression of FFA $\beta$-oxidation by methyl palmoxirate caused a marked reduction in protein synthesis in the heart. The effect on protein synthesis was mediated in part by changes in the function of eukaryotic initiation factors (eIFs) involved in the initiation of mRNA translation. The guanine nucleotide exchange activity of eIF2B was repressed, phosphorylation of the $\alpha$-subunit of eIF2 was enhanced, and phosphorylation of 4E-BP1 and S6K1 was reduced. Similar changes in protein synthesis and translation initiation were not observed in the gastrocnemius following treatment with methyl palmoxirate. In heart, repressed $\beta$-oxidation of FFA correlated, as demarcated by changes in ATP/AMP ratio and phosphorylation of AMP-activated kinase (AMPK), with alterations in the energy status of the tissue. Therefore, the activation state of signal transduction pathways that are responsive to the cellular energy stress represents one mechanism whereby translation initiation may be regulated in cardiac muscle.

Keywords: translation initiation, gastrocnemius muscle, AMP-activated protein kinase
INTRODUCTION

Previous studies have suggested a role for free fatty acids in modulation of the initiation of mRNA translation in muscles composed primarily of oxidative fibers. Those studies show that protein synthesis is stimulated in association with enhanced translation initiation in rat hearts perfused with medium containing palmitate whereas the fatty acid is without effect in perfused preparations of rat skeletal muscle (19; 31). These observations suggest that long-chain fatty acids facilitate protein synthesis solely within oxidative fibers. Support for this suggestion comes from the observation that hypophysectomized rats, which have both decreased serum insulin and FFA concentrations, have ribosome profiles indicative of reduced translation initiation rates, in both skeletal muscle and heart. In contrast, diabetic-hypophysectomized rats, which have low serum insulin but elevated FFA concentrations, have similarly altered ribosome profiles in non-working (i.e. not actively contracting) skeletal muscle, but not in the heart (30). To explain these observations, it has been proposed that the relatively greater ability of the heart to metabolize FFA allows for maintenance of cardiac intracellular energy stores during insulin deficiency (10). This in turn permits protein synthesis to be maintained at, or near, control values in heart compared to fast-twitch skeletal muscle which predominantly depends upon glucose to maintain energy stores.

It is well recognized that unlike non-working glycolytic muscle fibers, in which glucose is the preferred substrate, the working cardiac muscle utilizes FFAs as its principle substrate (8). Moreover, FFA oxidation in the myocardium is greatly increased during pathological conditions such as diabetes (32). In light of these facts, one could surmise that it is the ability of oxidative fibers, relative to glycolytic fibers, to efficiently synthesize ATP from FFAs that allows them to maintain protein synthetic rates in the presence of reduced glucose uptake, as occurs with diabetes and starvation. Based on this premise, inhibition of β-oxidation would be expected to reduce the energy status and thus produce an energetic stress within working oxidative fibers, but not within resting glycolytic fibers.
As the development of energetic stress is closely mirrored by a fall in protein synthesis, it has been suggested that energy status is an important determinant of the protein synthetic process (3). The protein 5’ AMP-activated protein kinase (AMPK) is particularly sensitive to the energy status of a tissue and is recognized as a cellular energy sensor (16). AMPK responds to changes in the ratio of ATP/AMP as well as phosphocreatine/creatine and its activation has been correlated with the suspension of various anabolic processes (17; 20). For example, a recent study reported that skeletal muscle protein synthesis and mRNA translation initiation are inhibited *in vivo* in response to the artificial activation of AMPK using the chemical 5-aminoimidazole-4-carboxamide 1-[D]-ribonucleoside (AICAR) (2).

Translational control of protein synthesis is mediated primarily at the stage of initiation of mRNA translation. The proteins that mediate this process are referred to as eukaryotic initiation factors (eIFs). For initiation to occur in most eukaryotic systems, a complex including eIF2-bound GTP transports an initiator methionyl-tRNA (tRNA$_{Met}$) to the 40S ribosomal subunit where GTP is hydrolyzed to GDP. For subsequent rounds of initiation to occur the guanine nucleotide exchange protein, eIF2B, must catalyze the exchange of GDP for GTP on eIF2 (27). Phosphorylation of the α-subunit of eIF2 (eIF2α) results in the sequestration of eIF2B and thereby prevents guanine nucleotide exchange (18). Another mechanism whereby translation initiation may be regulated is through the association of mRNA to the 40S ribosomal subunit. This process initially requires that a 7-methylguanosine capped mRNA be bound by the cap binding protein, eIF4E, (18). Availability of eIF4E is regulated by the eIF4E binding proteins (4E-BPs) (14). Finally, a correlation exists between the activity of the 40S ribosomal protein S6 kinase (S6K1) and the capacity to synthetize protein (5; 6). This correlation stems from the fact that the translation of transcripts that contain a terminal oligopyrimidine sequence (TOP mRNA), which often encode components of the translational apparatus such as ribosomal proteins and translation factors, is increased when S6 is phosphorylated (15).

Alterations in the function of eIFs can occur in response to environmental, nutritional, and/or hormonal signals. Insulin, for example, has been shown to modulate several eIFs in
skeletal muscle, reviewed by (1; 23) but surprisingly, research focusing on the role of FFAs in the translational control of protein synthesis in heart or skeletal muscle, or any other tissue, is extremely limited. Therefore, the objective of the study described herein was to investigate the role of FFA oxidation in the control of protein synthesis in heart and skeletal muscle in vivo.

MATERIALS AND METHODS

Animal care. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University, College of Medicine. Male Sprague-Dawley rats (~200g) were maintained on a 12 hour light: dark cycle with a standard diet (Harlan-Tekland Rodent Chow, Madison, WI) and water provided ad libitum.

Inhibition of \( \beta \)-oxidation. \( \beta \)-oxidation of FFAs was inhibited through the administration of methyl palmoxirate (R.W. Johnson Pharmaceutical Research Institute, Spring House, PA), a known inhibitor of carnitine palmitoyltransferase I (CPT I) (35). The protocol for methyl palmoxirate administration has been described previously (35) and was modified slightly. To ensure that fatty acids were being used as the major energy substrate, animals were food deprived for 21 hours, weighed, and randomly received either a single bolus of methyl palmoxirate (25mg/kg body weight suspended in 0.5% carboxymethylcellulose) or 0.5% carboxymethylcellulose via oral gavage. Animals were returned to their cages and allowed free access to water only until administration of metabolic tracer as described below.

Administration of metabolic tracer and sample collection. A flooding dose (1.0 mL/100 g body weight) of L-[2,3,4,5,6-\(^{3}\)H] phenylalanine (150 mM, containing 3.70GBq/L) was administered via tail vein injection 170 min after methyl palmoxirate administration for the measurement of protein synthesis (11). Animals were killed by decapitation 10 min later. Trunk blood was collected and centrifuged at 1800 x g for 10 min at 4°C to obtain serum. After excision, a portion of the gastrocnemius or heart were homogenized in seven volumes of buffer
consisting of 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.4), 100 mM KC1, 0.2 mM EDTA, 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N’, N’-tetraacetic acid, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 0.5 mM sodium vanadate. An aliquot (0.5mL) of the homogenate was used for the measurement of muscle protein synthesis as described below. The remainder of the homogenate was immediately centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was used for analysis of mRNA translation initiation factors as described below. The remaining tissue was used to assess eIF2B activity as described below.

**Serum measurements.** Serum free fatty acids were analyzed using a commercial colorimetric kit (Wako Chemicals, Richmond, VA). Serum insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO). Serum glucose concentrations were measured using an automated glucose oxidase-peroxidase method (YSI Model 2300 analyzer, Yellow Springs, OH).

**Measurement of muscle protein synthesis.** Fractional rates of protein synthesis were estimated from the rate of incorporation of radioactive phenylalanine into total mixed muscle protein as described previously (22). The elapsed time from injection of the metabolic tracer until homogenization of the muscle was recorded as the actual time for radiolabeled phenylalanine incorporation.

**Analysis of translation control regulatory mechanisms.** Phosphorylation of 4E-BP1, S6K1, and eIF2α was evaluated in 10,000 x g supernatants by protein immunoblot analysis as described previously (12; 13; 22). The guanine nucleotide exchange activity of eIF2B was assessed by the exchange of [3H]GDP bound to eIF2B for non-radioactively labeled GDP as described previously (21).

**Measurement of tissue adenine nucleotides.** Adenine nucleotides were measured in tissue samples that had been frozen in situ from animals administered a lethal dose of Nembutal Sodium (Abbott Laboratories, North Chicago, IL). Adenine nucleotide concentrations were determined as described previously (24).
Analysis of AMP-activated Protein Kinase phosphorylation. Phosphorylation of the catalytic subunit of AMPK was evaluated in 10,000 x g supernatants by protein immunoblot analysis using a phosphospecific (Thr 172) AMPK antibody.

Statistical Analysis. Data are expressed as the mean ± SEM. All data were analyzed by the InStat Version 3 statistical software package (GraphPad Software Inc., San Diego, CA). Statistical significance was assessed using a two-tailed Student’s t-test unless stated otherwise. *P* values of less than 0.05 were considered statistically significant.

RESULTS

To examine the contribution of β-oxidation of FFAs to the maintenance of protein synthesis in cardiac and skeletal muscle, male Sprague Dawley rats were treated with methyl paloxirate, a known inhibitor of CPT1. The effectiveness of the treatment was assessed by measuring plasma FFA concentrations. As shown in Table 1, methyl paloxirate treatment resulted in an almost 3-fold increase in plasma FFAs. These results are consistent with methyl paloxirate inhibiting the β-oxidation of FFAs and thus reducing their clearance from the blood. As reported previously (26), serum glucose, but not serum insulin, concentrations were significantly decreased following methyl paloxirate administration. This decrease in serum glucose concentrations is consistent with Randle’s hypothesis that FFA oxidation reduces glucose oxidation in the heart (29).

The effect of treatment with methyl paloxirate on protein synthesis in cardiac and skeletal muscle was examined by measuring in vivo rates of protein synthesis using the flooding dose method (11). In animals treated with methyl paloxirate, cardiac protein synthesis was reduced to 64% of the control value (Fig. 1). In contrast, protein synthesis was unaltered in the gastrocnemius following methyl paloxirate administration. These results support the concept that FFAs are positive regulators of protein synthesis specifically within working muscles composed primarily of oxidative fibers.
Regulation of mRNA translation can occur through changes in the rate of translation initiation, translation elongation, or both. Multiple mechanisms exist for regulating translation initiation including modulation of eIF2B activity, assembly of the eIF4F complex, and S6K1 activity. To examine whether or not methyl palmoxirate treatment altered eIF2B activity, tissue extracts were assayed for guanine nucleotide exchange activity using eIF2•[^3]H]GDP as a substrate. As shown in Fig. 2, eIF2B activity was significantly repressed following methyl palmoxirate administration in the heart but not in the gastrocnemius. Thus, eIF2B activity paralleled the changes in protein synthesis observed following treatment with methyl palmoxirate. To gain further insight into the regulation of eIF2B activity under these conditions, the phosphorylation status of eIF2α was measured by protein immunoblot analysis using an antibody that recognizes the protein only when it is phosphorylated on Ser^51. As shown in Fig. 3, treatment with methyl palmoxirate resulted in a significant increase in the relative phosphorylation of eIF2α specifically within the heart. These results suggest that the decrease in eIF2B activity observed in rats treated with methyl palmoxirate was due, at least in part, to increased phosphorylation of eIF2α.

The most well characterized mechanism for regulating assembly of the eIF4F complex involves changes in eIF4E association with 4E-BP1, an event that is modulated by the phosphorylation status of 4E-BP1. Thus, hyperphosphorylation of 4E-BP1 prevents it from associating with eIF4E, allowing eIF4E to bind to eIF4G and form the active eIF4F complex (13). As shown in Fig. 4, treatment with methyl palmoxirate resulted in a marked reduction in the phosphorylation status of 4E-BP1 in the heart. There was however, no significant change in 4E-BP1 phosphorylation in the gastrocnemius. Phosphorylation of 4E-BP1 in response to nutrients or hormones depends on the activity of a protein kinase referred to as the mammalian target of rapamycin, mTOR (reviewed by 34). In addition to 4E-BP1, mTOR also controls the activity of the ribosomal protein S6 kinase, S6K1, by promoting its hyperphosphorylation (7). As observed for 4E-BP1, treatment with methyl palmoxirate reduced the amount of S6K1 present in hyperphosphorylated forms in the heart but not in the gastrocnemius (Fig. 5). Thus, in
addition to a reduction in eIF2B activity, the reduction in protein synthesis in the heart by treatment with methyl palmoxirate was also associated with a redistribution of 4E-BP1 and S6K1 into hypophosphorylated forms.

The most well characterized mechanism for regulating translation elongation involves modulation of the phosphorylation status of translation elongation factor eEF2. In this regard, phosphorylation of eEF2 is associated with reduced rates of protein synthesis under a variety of conditions (4). To assess whether or not treatment with methyl palmoxirate altered protein synthesis in the heart through the step in translation elongation mediated by eEF2, the phosphorylation status of the protein was examined by protein immunoblot analysis using an antibody that recognizes the protein only when it is phosphorylated on Thr^{56}. The relative phosphorylation of eEF2 on Thr^{56} was unaffected by treatment with methyl palmoxirate (data not shown). Although possible effects on the activity of eEF1 cannot be excluded, the results suggest that methyl palmoxirate treatment does not regulate translation elongation through the step involving eEF2.

It has been proposed that FFAs maintain protein synthetic rates in the heart by acting as oxidative substrates. Thus, deprivation of this energy source, as occurs during methyl palmoxirate-mediated inhibition of FFA oxidation, would be expected to lower the energy status of the heart. As depicted in Figure 6A, the energy status in the heart, as monitored by the ATP/AMP ratio, was significantly reduced following methyl palmoxirate administration whereas the nucleotide ratio was unchanged in the gastrocnemius. AMPK has been shown to be activated during energy deficit conditions (17; 20) and its activation is demarcated by phosphorylation at Thr^{172} (16). As shown in Fig. 6b, there was a significant increase in the phosphorylation of AMPK on Thr^{172} in the heart following treatment with methyl palmoxirate. In contrast, there was no significant change in AMPK phosphorylation in the gastrocnemius.

DISCUSSION
Previous studies have suggested a role for FFAs in modulation of the initiation of mRNA translation. Furthermore, those studies indicate that the role is tissue specific; being limited to working muscles composed primarily of oxidative fibers. However it is only recently, with the development of pharmacological agents that inhibit fat metabolism, that it has become possible to investigate the role of FFAs on the translational control of protein synthesis in vivo.

To our knowledge this study is the first to demonstrate in vivo that protein synthetic rates in the heart are markedly reduced in response to decreased FFA oxidation. This suggests that an inhibition of mRNA translation occurs at the stage of initiation and/or elongation when FFA oxidation is inhibited or availability of FFAs becomes limiting. As eEF2 phosphorylation was unaltered following methyl palmoxirate administration, FFA oxidation would appear not to modulate translation elongation. This result is in keeping with previous in vitro studies demonstrating that a decrease in free (i.e. nonpolysome-associated) ribosomal subunit content, indicative of inhibited elongation/termination, does not occur in hearts perfused without FFAs (30). In the aforementioned perfusion studies, the authors observed an accumulation of free ribosomal subunits, suggestive of an inhibition of translation initiation, which led them to hypothesize that FFAs modulate translation initiation in the heart.

Our results support the hypothesis that FFAs modulate translation initiation in the heart and, to our knowledge, are the first to demonstrate that multiple mechanisms underlie this modulation in vivo. The inhibition of eIF2B activity in the hearts of methyl palmoxirate-treated indicates an impairment in the transport of met-tRNA_{Met} to the 40S ribosomal subunit. As the phosphorylation of eIF2α was enhanced under these conditions, the decrease in eIF2B activity most certainly results, at least in part, from competitive inhibition mediated by phosphorylated eIF2. Changes in allosteric regulation may have also contributed to the aforementioned changes in eIF2B activity in this study. It has been demonstrated previously that eIF2B may be regulated allosterically by NADP⁺ (9) and initial observations from our laboratory indicate that NADP⁺/NADPH ratios are altered in the hearts, but not the gastrocnemius, of methyl palmoxirate-treated animals (data not shown).
The phosphorylation of both 4E-BP1 and S6K1 was decreased in the hearts of methyl paloxirate-treated animals. These respective changes indicate that transport of mRNA to the 40S ribosomal subunit and synthesis of the translational apparatus itself is inhibited when FFA oxidation is inhibited. Furthermore, as 4E-BP1 and S6K1 are downstream targets of the kinase mTOR, the results indicate that an mTOR-dependant signaling pathway is modulated by FFA oxidation.

Our observation that methyl paloxirate does not have a significant effect on protein synthetic rates in the gastrocnemius supports the hypothesis that FFAs modulate mRNA translation initiation specifically within oxidative fibers. It should be noted however that whole gastrocnemius muscle was utilized in these experiments and that this muscle is composed primarily of glycolytic fibers but does contain mixed fibers as well as a small proportion of purely oxidative fibers (25). Therefore, it is not surprising that the mean synthesis value from the gastrocnemius of methyl paloxirate treated animals was slightly below that of controls.

Our results indicate that methyl paloxirate-induced inhibition of FFA \(-\)oxidation culminates in decreased ATP/AMP ratios in the heart but not in the gastrocnemius. Accordingly, AMPK phosphorylation is increased in the heart but not in the gastrocnemius following methyl paloxirate administration. Thus, an energetic stress develops within the heart, but not the gastrocnemius, following methyl paloxirate-induced inhibition of FFA \(-\)oxidation. These results are in keeping with the fact that FFAs are the primary energy source of the heart, while glucose is the principle energy source of the gastrocnemius at rest. Experiments performed in our laboratory suggest that AMPK signals through PKB to mTOR and its downstream effectors (2)]. Therefore we believe that the phosphorylation of AMPK, stemming from reduced FFA oxidation, in the heart represents a causal event in the inhibition of the mTOR-dependant signaling pathway and the subsequent inhibition of translation initiation. It should also be noted that in our previous studies involving the artificial activation of AMPK with the chemical AICAR, eIF2B activity was unaltered and the phosphorylation of eIF2\[^{\alpha}\] was actually decreased. These results are in contrast to those presented here with regards to the heart and suggest that
methyl paloxirate may induce eIF2\[\alpha\] phosphorylation through a mechanism distinct from AMPK.

Although unexplored in these studies, FFAs may also modulate protein synthetic rates in oxidative fibers in a manner that is independent of their oxidation. Based on the finding that the stimulation of protein synthesis in hearts perfused with palmitate is significantly blunted in the presence of the transcriptional inhibitor, actinomycin D, Rannels et al. (30) speculated that FFAs function as transcriptional regulators and recent in vivo studies support this hypothesis. For example, it has been demonstrated that the increased expression of various mRNAs that occurs in skeletal muscle with fasting can be prevented by inhibiting fasting-induced elevations in circulating FFAs (a relevant observation from these studies was that this inhibition is specific to slow-twitch oxidative muscles) (33). It has also been demonstrated that the expression of certain mRNAs involved in substrate metabolism is elevated in fat biopsies, independent of changes in circulating insulin, following a 5 hour lipid infusion (28). Therefore, it is possible that FFA-mediated transcriptional events also contribute to changes in protein synthesis within the heart following methyl paloxirate administration.

In summary, the results of this study indicate that the contribution of \(\beta\)-oxidation of FFAs to the maintenance of mRNA translation initiation differs in a tissue-specific manner, thus offering one explanation for the tissue-specific effects of metabolic disorders like diabetes on protein synthesis. We propose that plasma FFAs play a key role in the modulation of mRNA translation specifically within muscles composed primarily of oxidative fibers. More specifically, we hypothesize that alterations in FFA \(\beta\)-oxidation in working oxidative fibers result in the development of cellular stress. Both AMPK-dependant and -independent signaling pathways are activated by this stress, culminating in the inhibition of translation initiation. Further research will be required to explore the mechanisms whereby FFA oxidation activates intracellular signaling pathways and to elucidate the relationship between cellular stress, FFA mediated transcriptional events, and the translational control of protein synthesis.
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REFERENCES


FIGURE LEGENDS

Figure 1. Rates of protein synthesis in cardiac and gastrocnemius muscles of control and methyl palmoxirate-treated rats. Protein synthesis was measured as the incorporation of [\(^3\)H]phenylalanine into protein as described under “Materials and Methods”. Light bars, control animals; dark bars, animals administered methyl palmoxirate. Values represent means ± SEM; n = 5-13. Means not sharing a superscript are significantly different, p < 0.05.

Figure 2. eIF2B activity in cardiac and gastrocnemius muscles of control and methyl palmoxirate-treated rats. The guanine nucleotide activity of eIF2B was measured as the exchange of nonradiolabeled GDP in an eIF2•GDP binary complex for free GTP as described under “Materials and Methods”. Light bars, control animals; dark bars, animals administered methyl palmoxirate. Values represent means ± SEM; n = 5-9. Means not sharing a superscript are significantly different, p < 0.05.

Figure 3. Phosphorylation of eIF2\(\alpha\) on Serine 51 in cardiac and gastrocnemius muscles of control and methyl palmoxirate-treated rats. Phosphorylation of the \(\alpha\)-subunit of eIF2 was assessed by Western blot analysis using an anti-phospho-eIF2\(\alpha\) that specifically recognizes the protein when it is phosphorylated on Ser\(^{51}\). Total eIF2 content was measured by Western blot analysis using a monoclonal antibody that recognizes phosphorylated and unphosphorylated forms of the protein. Results of typical blots are shown as an insert to the figure. Gastroc, gastrocnemius muscle; C, control rats; MP, methyl palmoxirate-treated rats; eIF2\(\alpha\)(P), eIF2 phosphorylated on Ser\(^{51}\); eIF2, total eIF2\(\alpha\) content. Light bars, control animals; dark bars, animals administered methyl palmoxirate. Values represent means ± SEM; n = 7-21. Means not sharing a superscript are significantly different, p< 0.05.
**Figure 4.** Phosphorylation of 4E-BP1 in cardiac and gastrocnemius muscles of control and methyl paloxirate-treated rats. When subjected to SDS-PAGE, 4E-BP1 is resolved into multiple electrophoretic forms whereby the hypophosphorylated forms exhibit the greatest mobility and hyperphosphorylated forms the least. Results of a typical blot are shown as an insert to the figure. Gastroc, gastrocnemius muscle; C, control rats; MP, methyl paloxirate-treated rats; □, 4E-BP1□; □, 4E-BP1□; □ 4E-BP1□ Results are expressed as the ratio of hyperphosphorylated 4E-BP1 (□ and □ forms) to total 4E-BP1. Light bars, control animals; dark bars, animals administered methyl paloxirate. Values represent means ± SEM; n = 5–13. Means not sharing a superscript are significantly different, p< 0.05.

**Figure 5.** Hyperphosphorylated S6K1 in cardiac and gastrocnemius muscles of control and methyl paloxirate-treated rats. When subjected to SDS-PAGE, S6K1 is resolved into multiple electrophoretic forms whereby the most highly phosphorylated forms exhibit the slowest electrophoretic mobility. Results of a typical blot are shown as an insert to the figure. Gastroc, gastrocnemius muscle; C, control rats; MP, methyl paloxirate-treated rats; □, S6K1□; □ S6K1□ Results are expressed as the ratio of hyperphosphorylated S6K1 (□ and □ forms) to total S6K1. Light bars, control animals; dark bars, animals administered methyl paloxirate. Values represent means ± SEM; n = 5–13. Means not sharing a superscript are significantly different, p< 0.05.

**Figure 6.** Adenosine nucleotide ratios and AMPK phosphorylation in cardiac and gastrocnemius muscles of control and methyl paloxirate-treated rats. (a) Heart and gastrocnemius AMP and ATP contents were measured as described under “Materials and Methods”. Light bars, control animals; dark bars, animals administered methyl paloxirate. Values represent means ± SEM; n = 4–5. (b) Phosphorylation of the AMPK was assessed by Western blot analysis using an anti-phospho-AMPK antibody that specifically recognizes the protein when it is phosphorylated on Thr177. Total AMPK content was measured by Western
blot analysis using a monoclonal antibody that recognizes phosphorylated and unphosphorylated forms of the protein. Results of typical blots are shown as an insert to the figure. Gastroc, gastrocnemius muscle; C, control rats; MP, methyl palmoxirate-treated rats; AMPK(P), AMPK phosphorylated on Thr\textsuperscript{177}; AMPK, total AMPK content. Means not sharing a superscript are significantly different using a one-tailed Student’s t-test, P < 0.05.
Table I. Plasma Concentrations of Free Fatty Acids, Insulin, and Glucose in Control and Methyl Palmoxirate-Treated Rats

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<th>Group</th>
<th>Plasma Concentration</th>
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<tr>
<td></td>
<td></td>
<td>FFA (mM)</td>
<td>Insulin (ng/mL)</td>
<td>Glucose (mM)</td>
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<tr>
<td>Control</td>
<td>0.52 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.51 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Methyl Palmoxirate</td>
<td>1.45 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.72 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
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Serum free fatty acid (FFA), insulin, and glucose concentrations of control and methyl palmoxirate-treated rats were measured as described under “Materials and Methods”. Values represent means ± SEM; n = 10-13. Means not sharing a superscript are significantly different, p< 0.05.
Figure 1

Protein Synthesis (% control value)

Heart | Gastrocnemius
--- | ---
a | a'
b | a'
Figure 2

- eIF2B Activity (pmol GDP exchanged/min)
- Heart
- Gastrocnemius
Figure 3

eIF2α Phosphorylation on Ser51 (fraction of control)

Heart

Gastroc

C  MP  C  MP

eIF2α(P)  eIF2α

Heart Gastrocnemius

a  a'  a'
4E-BP1 Phosphorylation (fraction in hyperphosphorylated form)

Heart

Gastrocnemius

Figure 4
Figure 5

S6K1 Phosphorylation (hyperphosphorylated / total)

Heart Gastrocnemius

a b a' a'

Heart

Gastrocnemius

C MP C MP

β γ α
Figure 6

**A**

ATP : AMP Ratio

- **Heart**
  - a
  - b

- **Gastrocnemius**
  - a'

**B**

AMPK Phosphorylation on Thr172 (fraction of control)

- **Heart**
  - a

- **Gastrocnemius**
  - a'