Diet-induced modulation of mitochondrial activity in rat muscle

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**Diet-induced modulation of mitochondrial activity in rat muscle.** *Am J Physiol Endocrinol Metab* 293: E1169–E1177, 2007. First published August 21, 2007; doi:10.1152/ajpendo.00263.2007.—Growing evidence supports the theory that mitochondrial dysfunction is an underlying cause of intramyocellular lipid (IMCL) accumulation and insulin resistance. Here, we hypothesized that high dietary fat (HF) intake could trigger changes in mitochondrial activity such that fatty acid oxidation is impaired in muscle and contributes to an elevation in intramyocellular lipid (IMCL) levels. Muscle mitochondrial activity was determined in vivo through measurement of the F1F0 ATP synthase flux, the terminal step in the oxidative phosphorylation process. An initial study comparing rats on normal chow diet with rats on an HF diet revealed strong correlations between muscle ATP synthesis rates, IMCL levels and whole body glucose tolerance. Results obtained from two latter studies showed multiphasic responses to dietary intervention. Initially, the ATP synthesis rates decreased as much as 50% within 24 h of raising the fat content in the diet to 60% of the caloric intake. These rates eventually returned to normal values after 2–3 wk on the HF regimen, seemingly to prevent further IMCL accumulation. Subsequently, 31P magnetic resonance spectroscopy (MRS) was employed to investigate the existence of changes in mitochondrial activity that may lead to an accumulation of intracellular fatty acyl-CoA and harmful metabolites, such as diacylglycerol, that disrupt insulin signaling (16, 35). Furthermore, excess dietary fat may also play a crucial role in the development of mitochondrial dysfunction, as supported by a recent study showing downregulation of genes involved with oxidative phosphorylation and mitochondrial biogenesis in response to high-fat feeding (39).

Building upon these findings, it has been suggested that restoration of mitochondrial activity could help improve insulin sensitivity through enhanced fat oxidation. This type of treatment was shown to be effective on obese patients undergoing therapy involving physical activity (11, 24). Although divergent data occasionally suggest a dissociation between skeletal muscle adaptations and mitochondrial function (28), maximal enzyme activities do not necessarily reflect actual flux through metabolic pathways and consequently may not be sufficiently sensitive to detect the subtle changes in bioenergetic status. In addition, these studies rely on tissue biopsies to measure enzyme activities, allowing only a selected few time points, and often making longitudinal studies unfeasible.

Conversely, 31P magnetic resonance spectroscopy (MRS) provides a noninvasive means to monitor the energetic status of the cell by measuring intracellular phosphorylated metabolites [e.g., ATP and creatine phosphate (PCr)]. However, because of efficient regulation mechanisms, levels of energy metabolites in muscle under nonischemic and resting conditions frequently remain unchanged, making them insensitive indicators for the characterization of a pathological state. Accordingly, it is conceivable that a direct assessment of specific metabolic fluxes would offer a better alternative, with a dynamic range potentially wide enough for drug profiling studies. In this respect, 31P magnetization transfer offers the unique possibility to noninvasively determine certain reaction rates without disturbing the chemical equilibrium, as is often the case when labeled substrates are used. These qualities make this technique particularly effective in measuring reaction rates in vivo such as the one catalyzed by the mitochondrial F1F0 ATP synthase, the terminal step in the oxidative phosphorylation process. Since mitochondrial ATP production is coupled to substrate utilization via a stoichiometric relationship (40), it is reasonable to assume that any change in the ATP synthesis rate will reflect a change in oxidative phosphorylation.

In this context, the primary aim of this study was to investigate the existence of changes in mitochondrial activity that have also shown subtle and early defects in mitochondrial oxidative capacity (i.e., due to mitochondrial dysfunction and/or mitochondrial loss), which may lead to an accumulation of intracellular fatty acyl-CoA and harmful metabolites, such as diacylglycerol, that disrupt insulin signaling (16, 35).

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may occur in a diet-induced obesity rat model of insulin resistance. To do so, serial measurements were performed to 1) verify whether muscle ATP synthesis turnover is associated with other relevant markers of insulin resistance, specifically intracellular fat accumulation and glucose tolerance, 2) determine the conditions under which diet-induced changes in muscle ATP synthesis rate are readily reversible, and, finally, 3) assess the compliance of mitochondria in response to high lipid exposure during short-term and long-term high-fat diet regimens.

METHODS

Animals

All experiments were performed in male rats anesthetized with 2% isoflurane administered via a face mask during NMR data acquisition. Dietary interventions involved controlled modulation between normal chow diet (NC, 4.5% crude fat, Pico Lab Rodent Diet 20 no. 5053c; Lab Diet, Brentwood, MO) and high-fat diet (HF; diet no. D12492, 60% fat cal; Research Diets, New Brunswick, NJ). All experimental procedures were carried out with the approval of the Novartis Institutional Animal Care and Use Committee.

Study Protocols

Study 1. In the initial study, the interactions between muscle ATP synthesis rates, whole body glucose tolerance, and intramyocellular lipid (IMCL) levels were investigated. To make these comparisons, six Sprague-Dawley rats were fed the HF diet for 10 wk, a situation known to induce peripheral insulin resistance, and were tested against six age-matched rats fed the NC diet.

\[
\text{ISI}_{\text{composite}} = \frac{1}{\left(\text{fasting glucose} \times \text{fasting insulin}\right)/\text{mean glucose}_{\text{OGTT}} \times \text{mean insulin}_{\text{OGTT}}} \quad (22)
\]

In Vivo NMR Setup

All in vivo MR measurements were performed on a Bruker Avance 7.0 T/30 cm wide-bore instrument (Bruker Medical, Billerica, MA) equipped with 20-cm id actively shielded gradient insert. For each time point, two 13-min \( ^{31} \text{P} \) saturation transfer spectra (i.e., ATP\( ^{31} \text{P} \) saturated and unsaturated spectra), six 3.5-min \( ^{1} \text{H} \) inversion recovery receiver mode and tuned to 300.31 (1H) and 121.57 (31P) MHz. Acquisition parameters were used: nonselective detection of the in-tramitochondrial resonance (CHESS water suppression, 256 scans).

Measurement of muscle ATP synthesis rate. The unidirectional ATP production can be assessed noninvasively as initially described for measurements in Escherichia coli (6) and subsequently in the skeletal muscle in rat (5). In this procedure, the ATP\( ^{31} \text{P} \) NMR peak is nullified with a saturating radio frequency (rf) pulse, and a reduction in the Pi peak is observed as a result of the transfer of the saturated spins between ATP\( ^{31} \text{P} \) and the Pi pool (via the \( F_{1}F_{0} \) ATPase reaction). In contrast to the heart, the glycolytic contribution to ATP synthesis [through e.g., glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-phosphoglycerate kinase reactions] can be considered negligible in skeletal muscles for which the \( \text{Pi} \rightarrow \text{ATP} \) flux is predominantly due to mitochondrial ATP\( ^{31} \text{P} \) synthesis rate (5).

\[
M_{r}/M_{0} = 1/(1 + kT_{1}) \quad (1)
\]

where \( k \) is the rate constant describing the loss of magnetization from the Pi, and \( T_{1} \) is the longitudinal (spin-lattice) relaxation time for the Pi nucleus (17).
Despite the relatively fast reaction rate between PI and ATP\gamma, it is important that the spin-lattice relaxation time (i.e., observed $T_{1\text{obs}}$) be evaluated on an individual basis in the presence of continuous ATP\gamma saturation for accurate measurement. While applying an inversion recovery (IR) pulse during the mixing period, the apparent $T_{1\text{obs}}$ is related to $T_1$ by the following equation:

$$1/T_{1\text{obs}} = 1/T_1 + k$$  \hspace{1cm} (2)

A 2,000-ms sech inversion pulse was applied at inversion delays (TI) of 299, 799, 1,749, 2,999, 4,499, and 5,999 ms from the 90° detection pulse to invert all PI spins during the ATP\gamma saturation and accurately determine $T_{1\text{obs}}$ for all PI metabolites. Each IR spectrum was constituted of 32 averages, leading to a total experimental time of $\sim$20 min. Typical results from an NC rat are summarized in Fig. 1. Individual $T_{1\text{obs}}$ values were calculated using a nonlinear least square fitting method based on the following equation:

$$M = M_0 \left[1 - 2e^{-T_1/T_{1\text{obs}}}ight]$$ \hspace{1cm} (3)

The decrease in PI signal ($\Delta M$) used to determine the kinetics of ATPase activity (i.e., the rate constant $k$) was computed by simultaneously solving Eqs. 1 and 2, yielding the following equation (17):

$$k = 1/T_{1\text{obs}} \times \Delta M/M_0$$ \hspace{1cm} (4)

where $\Delta M = M_0 - M_z$.

The unidirectional ATP synthesis flux was then calculated by multiplying the constant $k$ by the PI concentration ([PI]) extrapolated from the baseline NMR spectrum (comparing peak integrals from PI and ATP\gamma) and using concentrations ([ATP]) as obtained biochemically from calf muscles of 35 normal rats (i.e., [ATP] = 4.39 ± 0.53 mmol/g (2)). Since changes in the $\Delta$P signal contribute, for the most part, to changes in the calculated value of muscle ATP synthesis rate (i.e., only small variations in $T_{1\text{obs}}$ are expected), it was verified that any changes in the muscle ATP synthesis rate were actually matched with changes in corresponding $\Delta$P/ATP\gamma ratios (results not shown).

### Data Analysis

All 31P spectra were processed using XWIN NMR Suite (version 3.2, Bruker Biospin, Karlsruhe, Germany). After applying a 20-Hz line-broadening filter, phasing, and baseline correcting, peak areas were assessed for signals from PI (4.9 ppm), PCr (0 ppm), and all three ATP (ATP\gamma: −2.4, ATP\alpha: −7.4, and ATP\beta: −15.9 ppm). To gain sensitivity in the saturation transfer experiment, the saturation-induced change in PI peak area ($\Delta P_i$) was assessed after subtracting the ATP\gamma saturated spectrum from the unsaturated control spectrum. IMCL spectra were processed using the Nuts-PPC software package (Acorn NMR, Fremont, CA). Once spectra were line broadened, phased, and baseline corrected, peak areas for total creatine (tCr: 3.02 ppm), EMCLTA (methylene peak at 1.5 ppm), and IMCLTA (methylene peak at 1.3 ppm) were determined using a line-fitting procedure. IMCLTA content was then expressed as a percentage of tCr content.

### Statistics

Where applicable, intergroup comparisons were made using a two-tailed, nonpaired Student’s t-test or a two-way repeated-measures analysis of variance and simultaneous pairwise multiple comparison procedures (Holm-Sidak method). Data are presented as means ± SE, and $P < 0.05$ was considered statistically significant.

### RESULTS

#### Study 1: Relationships Between Muscle Mitochondrial Function and Whole Body Glucose Tolerance

Figure 2 displays the variations observed between a rat on the HF diet for 10 wk and an age-matched NC control. For each rat, the presented spectrum shows the difference observed between the unsaturated and the ATP\gamma-saturated spectra. Although saturation of the ATP\gamma peak was maximally effective in both cases, the loss of PI signal was clearly greater for the NC rat than the rat fed the HF diet. Analysis of signal quality showed SNR values consistently in the range of 10–12 for rats fed an NC diet [using SNR = (2.5 × peak height)/peak-to-peak noise]. Assuming an SNR with a threshold value of 3 or greater for accurate measurement, this would theoretically give a dynamic range of $\sim$75% for assessment of the muscle ATP synthesis flux between a nonpathological and a pathological model. For relatively constant PI relaxation times ($T_{1\text{obs}}$), this would translate into ATP synthesis flux values between $\sim$3 and $\sim$18 mmol·g$^{-1}$·min$^{-1}$. Ten weeks on the HF diet resulted in a $\sim$35% decrease in ATP synthesis rate (Fig. 3). A positive linear correlation with a slope of $\sim$1 with 10 ± 7% deviation from the mean was found during a test-retest study, indicating an excellent intraindividual reproducibility of ATP synthesis.

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**Fig. 1.** Typical in vivo spectra from rat muscle obtained for the determination of the ATP synthesis rate by steady-state saturation transfer (A) and the spin-lattice relaxation time $T_{1\text{obs}}$ in the presence of continuous ATP\gamma saturation (B). Arrows indicate the position for selective irradiation. The difference spectrum on the left was produced by subtraction of the ATP\gamma saturated spectrum ($M_z$) from the control spectrum ($M_0$). The inversion recovery (IR) delay was adjusted to yield optimal signal-to-noise ratio for the range of $T_{1\text{obs}}$ observed.
rate measurements (data not shown). Overall, actual rates obtained for muscle ATP synthesis in rats at rest are in excellent agreement with recent human data (36). After the 10-wk regimen, rats fed the HF diet also exhibited significantly higher body weights (data not shown) and IMCL levels (Fig. 3), supporting an obesity phenotype. At this time, an OGTT was also performed to determine whether the diet change triggered an insulin-resistant state. Areas under the curves (AUCs) for both glucose and insulin excursion data doubled ($P < 0.05$) in HF-fed rats relative to their NC-fed counterparts (Fig. 3), indicative of significant HF diet-induced glucose intolerance, a prediabetic condition. As expected, the HF

Fig. 2. Typical $^{31}$P magnetization transfer difference spectra obtained from age-matched rats, one fed a normal chow diet (left) and the other one a high-fat (HF; right) diet for 4 wk. Area under the $\Delta P_i$ peak is much smaller for the HF-fed rat, likely indicative of a decrease in its muscle mitochondrial activity. Spectra were normalized to ATP$\beta$ peak assuming muscle ATP concentrations were constant between rats.
diet also induced a significant impairment of whole body insulin sensitivity, as estimated under fasting by the Matsuda index (Fig. 3).

**Study 2: Effect of Diet Manipulation on Muscle ATP Synthesis**

Figure 4 illustrates the time-course variations observed both in muscle ATP synthesis rates and in IMCL levels while the fat content in the diet was modulated. Upon induction of the HF diet, a drastic drop in mitochondrial activity was observed (~38% decrease in muscle ATP synthesis rate, \( P < 0.05 \)). The changes in ATP synthesis rate measured after 5 days on the HF diet were accompanied by a sharp increase, almost fourfold, in IMCL storage. This change is likely to be a result of both an increased fatty acid uptake and decreased utilization (i.e., due to a defect in lipid oxidation). At day 21, the ATP flux appeared to return to normal values, and, even though dietary fat ingested remained elevated, IMCL stores were maintained at levels similar to those measured at day 5. Returning to the NC diet resulted in a rapid decline of IMCL contents (i.e., back to baseline levels in less than 2 days) without a concomitant change in muscle ATP synthesis rates. Two days later (day 25), the variables analyzed showed some divergence: muscle ATP synthesis drastically decreased by 27% while IMCL contents remained low. Also, no increase in body weight was observed during this 5-day transition period, suggesting that caloric intake was kept relatively low immediately after switching back to the NC diet (Fig. 4). This disconnect was only temporary, and within 2 wk the inverse relationship between the ATP synthesis rate and IMCL levels was again observed.

**Study 3: Short-Term vs. Long-Term Effects of HF Diet on the IMCL-ATP Synthesis Relationship**

Here, we examined in more detail the time-course variations in muscle mitochondrial activity of rats on the HF diet for 5 wk. Unpublished in-house data demonstrate relative steadiness in muscle ATP synthesis rates and IMCL contents in rats on an NC diet up to 20 wk of age that were comparable to the baseline values measured in this study. Therefore, by comparing the time-course changes with the baseline values, each rat served as its own control, thus eliminating the need for comparisons with animals on an NC diet. Figure 5 shows a typical \(^1H\) spectrum series obtained in vivo from a single rat illustrating IMCL changes over the course of the study. Thanks to repeated measurements performed at relatively short time intervals, several phases in the response could be clearly identified during this period of altered diet both in terms of IMCL and muscle ATP synthesis rates (Fig. 6). After only 24 h on the HF diet (phase I), rats responded in an acute fashion with drastic lowering of the muscle ATP synthesis rate (~45%, \( P < 0.05 \) vs. baseline) and a concomitant increase in IMCL content (~3-fold, \( P < 0.05 \) vs. baseline). Beyond the first day (phase II), rats gradually regained mitochondrial activity while accumulating IMCL at a much slower pace, reaching maximum after 7 days (IMCL/tCr ratio of ~2.8, \( P < 0.05 \) vs. baseline). More succinctly, the increment in IMCL contents decreased as the ATP synthesis rate gradually returned toward baseline levels. During the subsequent 2 wk (phase III), both the ATP synthesis rate and IMCL contents remained constant, indicating the existence of a new metabolic steady state. This temporal equilibrium was characterized by an ATP flux slightly below the baseline value (~20%, not significant) in conjunction with relatively high IMCL levels. However, beyond 3 wk on the HF diet, the steady state gave way to a slow but stable decrease in muscle ATP synthesis rate with levels reaching ~60% of the baseline value at week 5 (~0.05). Concurrently (from weeks 3 to 5), IMCL contents regularly increased to levels six- to sevenfold greater than the baseline values. Body weight gain in these animals remained steady despite such variations (Fig. 6).
DISCUSSION

The primary purpose of this work was to assess whether alterations in diet regimen influence muscle mitochondrial activity and whether those effects are associated with changes in lipid storage and whole body glucose tolerance. Although the data do not suggest causality, they do imply a direct association between these factors.

The diet-induced reduction in the rate of ATP synthesis fits nicely with a recent report (14), which showed a significant decrease in the respiratory capacity of the hindleg muscles from isolated mitochondria of adult rats under similar feeding conditions. Of note, these same authors had earlier revealed a completely contradictory picture in young rats (i.e., 30 days of age), in which high-fat feeding resulted in increased mitochondrial capacity to employ lipids as metabolic fuel (15). This parallels the accepted notion that adult rats are more prone to obesity than younger rats, which may benefit from adaptive thermogenesis to counteract diet-induced obesity (34).

Whether high-fat feeding elicits a decline in the metabolic activity of adult rats through a decrease in mitochondrial mass and/or specific activity or by some other unknown mechanism, in a similar fashion to the aging process (1) remains to be determined.

Results obtained from the second experiment illustrated the high compliance of the mitochondrial system with variations in fat availability. The initially observed decrease in mitochondrial capacity to employ lipids as metabolic fuel (15). This parallels the accepted notion that adult rats are more prone to obesity than younger rats, which may benefit from adaptive thermogenesis to counteract diet-induced obesity (34). Whether high-fat feeding elicits a decline in the metabolic activity of adult rats through a decrease in mitochondrial mass and/or specific activity or by some other unknown mechanism, in a similar fashion to the aging process (1) remains to be determined.

Results obtained from the second experiment illustrated the high compliance of the mitochondrial system with variations in fat availability. The initially observed decrease in mitochondrial activity revealed itself to be transient, and consequently IMCL levels steadied as the ATP synthase flux rate recovered to baseline values. Speculatively, this may indicate that excessive fat storage can be temporarily barred by efficient oxidative phosphorylation despite augmented availability of circulating fat. This would imply that any new fatty acid entering the cell would be oxidized, preventing further lipid accumulation within the cell. This points to an adaptive mechanism that would allow the rat to still effectively counteract obesity at this time. This theory is in agreement with data showing significant increases in enzyme activity for fatty acid oxidation in muscles of adult rats maintained on a high-fat diet for 4 wk (27).

These should require time, one can speculate that the sharp rise in IMCL contents during this transition period results from both a lipid mass effect and a transient decrease in mitochondrial activity, the latter being directly or indirectly caused by inhibitory lipid intermediates. This may also partially explain why short-term consumption of a high-fat diet often results in only a mild impairment of whole body glucose tolerance, a defect likely to be hepatic in origin (9, 21). It also reconfirms the notion that, beyond the relative amount and nature of ingested fat (see example in Ref. 47), the length of the regimen obviously plays a crucial role as a dietary factor affecting the metabolic phenotype of muscle in a sustained fashion. Thus, depending on the model required, adult rats would need to be maintained on a high-fat diet for a minimum of 4 wk before showing clear signs of muscle insulin resistance (21).

The return to a normal chow diet prompted a rapid decline of IMCL contents, returning to baseline levels in less than 2 days, without concomitant change in muscle ATP synthesis rates. This supports the previous assertion that fully efficient oxidative phosphorylation attacks previously stored lipids (i.e., IMCL contents in the form of lipid droplets) as main substrates when new lipids entering muscle cells are scarce, thus returning the situation to one similar to that depicted under baseline conditions. However, 2 days later, the data showed some divergence: muscle ATP synthesis drastically decreased by 27% while IMCL contents remained low. One possible explanation is that mitochondrial activity temporarily disconnects itself from lipid substrates by some unknown mechanism in times of short supply. As such, there was no change in body weight during the 5-day transition period, suggesting that caloric intake did not increase shortly after switching back to the normal chow diet. In other words, muscle fat oxidation decreased while lipids were, at that time, scarce as metabolic fuels. The lack of information on plasma glucose, insulin, and lipid profiles certainly limits our interpretation of these data, and additional studies would be needed to support such an assumption. However, one should note that this disconnect was only temporary, as within 2 wk the inverse relationship be-
latter study support the possible existence of an intimate relationship between substrate (i.e., fat) availability and efficiency of utilization by the “burning factory” (i.e., muscle), as previously observed in healthy (4), prediabetic (30), and type 2 diabetic patients (36). Although the underlying molecular mechanism responsible for the initial inhibitory effect of intracellular lipids on oxidative phosphorylation remains to be elucidated, these results unambiguously demonstrate the capacity of the mitochondrial system to recover and preserve its function as long as ~3 wk with chronic exposure to high-fat food. These data are well aligned with previous results obtained from DIO (diet-induced obese) rodent models showing that at least one month on a high-fat diet is required to generate significant impairment in glucose tolerance (Ref. 13 and unpublished data), muscle insulin sensitivity (12), or muscle insulin signaling (31). This suggests an adaptive mechanism, which could, in part, explain why elevated dietary fat does not necessarily result in a drastic drop of oxidative enzyme activities (8, 42). It is possible that such a mechanism involves the mitochondrial uncoupling protein-3, which when induced under high-fat conditions (46) could protect mitochondria against lipid-induced oxidative damage (25) and help restore fatty acid oxidation to a normal level. However, this new equilibrium, which is usually not associated with insulin resistance in rats, appeared to be a transient phenomenon, since prolonging the high-fat diet beyond 3 wk resulted in a progressive degradation of muscle mitochondrial function and a steady increase in fat storage, as was recently shown (7). As suggested above, this latter transition phase may be associated with the appearance of an insulin resistance phenotype.

Assuming no change in physical activity, the fate of the extra ATP generated in response to an increase in mitochondrial activity (as observed for example during phase II in study 3) is unclear. Our hypothesis is that ATP production through non-oxidative glycolysis is decreased whereas proper mitochondrial activity is restored, which would preserve the balance between energy demand and supply. On the contrary, this pathway is not completely suppressed during insulin infusion in non-insulin-dependent diabetic patients (45). Such a hypothesis warrants further investigation.

To our knowledge, apart from indirect calorimetry, which is more global and provides little dynamic range, there is no gold standard against which the ATP synthesis rate measured by $^{31}$P saturation transfer can easily be validated. As mentioned earlier, maximal activity of the ATP synthase, which can only be determined in vitro, may not necessarily match with the actual flux measured in vivo. The same holds true for tricarboxylic acid cycle enzymes such as succinate dehydrogenase or citrate synthase. Since a reduction in mitochondrial density is associated with insulin resistance (26), one could consider measuring muscle cardiolipin, a known tissue marker of mitochondrial capacity, from tissue extracts (23, 43), except that the exact biological function of cardiolipin remains elusive. Conversely, muscle mitochondrial activity depends not only on the density and functionality of mitochondria but also on the supply of oxygen and substrate. In the present studies, ATP synthesis rate measurements were restricted to a resting state, i.e., dealing with basal metabolism. The fact that resting energy expenditure contributes to two-thirds of daily energy expenditure was the main justification for choosing this context. As a matter of fact, body weight regulation (and to some extent insulin resis-

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**Fig. 6.** Short- and long-term response to HF feeding in muscle ATP synthesis rates, IMCL levels, and body weights in male Wistar rats. Note the different phases (II, III, and IV) reflecting various metabolic states, phase I corresponding to the 24-h period after switching to the HF diet. Data obtained from study 1 ($\circ$) were added to the figure to show continuity, while time was scaled to maintain resolution of earlier measurements. Data are presented as means ± SE. *P < 0.05 vs. baseline; #P < 0.05 vs. 21 days.
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