Fasting rapidly increases fatty acid oxidation in white adipose tissue of young broiler chickens

Emmanuelle Torchona, Rodney Ray, Matthew W. Hulver, Ryan P. McMillan, and Brynn H. Voy

Department of Animal Science, University of Tennessee, Knoxville, TN, USA; Department of Human Nutrition, Foods and Exercise, Virginia Tech, Blacksburg, VA, USA; The Metabolic Phenotyping Core at Virginia Tech, Blacksburg, VA, USA

ABSTRACT
Upregulating the fatty acid oxidation capacity of white adipose tissue in mice protects against diet-induced obesity, inflammation and insulin resistance. Part of this capacity results from induction of brown-like adipocytes within classical white depots, making it difficult to determine the oxidative contribution of the more abundant white adipocytes. Avian genomes lack a gene for uncoupling protein 1 and are devoid of brown adipose cells, making them a useful model in which to study white adipocyte metabolism in vivo. We recently reported that a brief (5 hour) period of fasting significantly upregulated many genes involved in mitochondrial and peroxisomal fatty acid oxidation pathways in white adipose tissue of young broiler chickens. The objective of this study was to determine if the effects on gene expression manifested in increased rates of fatty acid oxidation. Abdominal adipose tissue was collected from 21 day-old broiler chicks that were fasted for 3, 5 or 7 hours or fed ad libitum (controls). Fatty acid oxidation was determined by measuring and summing 14CO2 production and 14C-labeled acid-soluble metabolites from the oxidation of [1-14C] palmitic acid. Fasting induced a progressive increase in complete fatty acid oxidation and citrate synthase activity relative to controls. These results confirm that fatty acid oxidation in white adipose tissue is dynamically controlled by nutritional status. Identifying the underlying mechanism may provide new therapeutic targets through which to increase fatty acid oxidation in situ and protect against the detrimental effects of excess free fatty acids on adipocyte insulin sensitivity.

KEYWORDS
adipose; fasting; fatty acid oxidation

Introduction

Obesity compromises the health of children and adults worldwide by promoting Type 2 diabetes, cardiovascular disease, and other disorders. The fundamental feature of obesity is excess storage of energy as triglycerides in white adipose tissue. Storage occurs in mature white adipocytes, which are characterized by a large central lipid droplet and comprise the majority of adipose tissue. During lipolysis, fatty acids are hydrolyzed from triglycerides and released into the circulation as non-esterified fatty acids (NEFA) for oxidation by muscle and other tissues. Plasma NEFA levels are normally maintained within a controlled range through a balance of utilization, mobilization and re-esterification into triglycerides. Failure to adequately store fatty acids or to control lipolysis elevates circulating NEFA, which can reduce insulin release, diminish insulin sensitivity in skeletal muscle, and impair cardiac efficiency.1,2 Both insulin resistance and obesity are associated with dysregulated lipolysis and elevated NEFA levels.3

Even adipocytes, which are continually exposed to high rates of fatty acid flux, are subject to insulin resistance and inflammation induced by excessive free fatty acids.4-6 In vitro and in vivo studies show that increases in both exogenous and endogenous fatty acids can initiate proinflammatory responses in adipose tissue through activation of c-Jun N-terminal kinase (JNK) and other pathways that impair adipocyte insulin sensitivity.6-8 Adipocytes can remove fatty acids by either partitioning them into storage or oxidizing them internally in mitochondria and peroxisomes through β-oxidation. The physiological importance of β-oxidation in white adipocytes typically has been dismissed because oxidation rates are relatively low compared to tissues like skeletal muscle.9,10 Recently, however, there has emerged a growing awareness that fatty acid oxidation in white adipose tissue plays an important role in metabolic homeostasis.11-14 Genetic and pharmacological models of chronic lipolysis demonstrate that increasing oxidation in white adipose depots is effective in buffering tissue NEFA levels and preventing metabolic stress and insulin resistance in...
adipose tissue. In these models, enhanced oxidation is achieved through tissue remodeling that includes both induction of white adipose browning and stimulation of mitochondrial biogenesis, both of which increase the depot’s overall oxidative capacity. In liver, muscle and other tissues, fatty acid oxidation is controlled more specifically through transcriptional regulation of carnitine palmitoyltransferase 1 (Cpt1) and other key genes in the β-oxidation pathway. Little is known about the corresponding regulation of this pathway in white adipocytes. However, forced overexpression of Cpt1 in 3T3-L1 adipocytes confers some of the same metabolic benefits as white adipose remodeling.

Domestic broiler (meat-type) chickens are a valuable model for focused studies of white adipocyte biology because avians appear to lack brown adipocytes. In addition, relative rates of de novo fatty acid synthesis in adipose tissue are comparable between avians and humans (unlike rodents and humans), with liver being the primary lipogenic organ in both species. This similarity may be particularly relevant to β-oxidation because fatty acid synthesis is required for the production of at least one endogenous ligand for peroxisome proliferator activated receptor α (PPARα), a key transcriptional mediator of this pathway. Using this model, we discovered that a period of fasting that was brief (5 hours), but sufficient to stimulate lipolysis, upregulated expression of genes involved in fatty acid oxidation, such as carnitine palmitoyl transferase 1 (Cpt1), in abdominal adipose tissue. The relatively rapid nature of these effects suggests that fatty acid oxidation in white adipose tissue is acutely and dynamically coupled to free fatty acid supply, which may play an important role in mitigating NEFA levels and maintaining healthy adipocyte metabolism. The purpose of the study described here was to functionally confirm predictions based on previous microarray experiments, to characterize the efficiency of fatty acid oxidation, and to define the temporal relationship with lipolysis in vivo.

Materials and methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech. Cobb500 broiler chicks were housed under standard conditions with free access to water and feed. On each of 2 consecutive days chicks that were 20–21 d of age, were weighed and assigned to one of 4 weight-balanced treatment groups: ad libitum fed (controls), or fasted for 3, 5 or 7 hours. Feed was removed at the same time of day for all fasted birds. After the treatment period, blood was collected by venipuncture and transferred to serum separation (SST) tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Serum was collected after centrifugation and immediately stored at −80°C. After venipuncture, chicks were euthanized by cervical dislocation.

Serum glucose and NEFA

Serum glucose concentration was determined using a commercial kit based on the glucose oxidase method (Cayman Chemical, Ann Arbor, MI). Non-esterified fatty acid levels in the serum were measured using a commercially available colorimetric assay kit (Wako Chemicals, Neuss, Germany).

Preparation of adipose tissue homogenates, fatty acid oxidation and citrate synthase activity assays

Approximately 50 mg samples of freshly-harvested abdominal adipose tissue were harvested into tubes on ice containing a modified sucrose-EDTA buffer (250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 1 mM ATP, pH 7.4.). Tissue pieces were then minced with scissors and buffer was added to a 20-fold diluted (wt:vol) suspension. Minced tissue pieces were homogenized in a Potter-Elvehjem glass homogenizer for ~30 seconds at 150 rpm with a motor-driven teflon pestle. Homogenates were kept on ice for immediate assay of fatty acid oxidation. Fatty acid oxidation rate was determined based on the oxidation of 1–14C palmitate (Perkin-Elmer, Waltham, MA), as previously described. Complete oxidation was measured by production of 14CO2 and incomplete oxidation by production of 14C-labeled acid-soluble metabolites (ASM). Total fatty acid oxidation was determined by the sum of complete and incomplete oxidation. Citrate synthase activity was measured spectrophotometrically based on the rate of reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), upon exposure to acetyl CoA at 412 nm. Homogenate protein content was measured using a Bicinchoninic acid assay (Life Technologies, Grand Island, NY).

Statistical analysis

Data were analyzed using R (version 3.1.3). All data were analyzed for normality and homogeneity of variance prior to statistical testing. Effects of treatment were determined by fitting a linear mixed-effects model, adjusting for random effects of day. Differences between treatments were identified in post-hoc analyses by performing pairwise contrast analyses.
using the R package lsmeans. Pearson’s correlation coefficients and associated p-values were calculated in R to identify relationships between variables. A p-value > 0.05 was used as the criterion for statistical significance for all analyses.

Results and discussion

The relatively brief period of fasting was sufficient to exert a modest but significant decline in glycemia (Fig. 1A, p = 0.027). Serum glucose levels after 5 and 7 hours of fasting were reduced by ~12% relative to fed controls (p < 0.05). Fasting also significantly increased lipolysis (p = 0.024) as reflected by serum NEFA levels (Fig. 1B). After seven hours of fasting, serum NEFA levels were approximately 47% greater than those of fed controls (0.31 ± 0.04 vs. 0.21 ± 0.04 mg/ml, respectively). There were no significant differences in NEFA levels between either the 3 or 5 hour fasting groups compare with fed birds (p > 0.05). Although not measured in this study, we expect that increased lipolysis was due to a significant rise in circulating glucagon, the primary regulator of lipolysis in avians. Glucagon levels increased significantly after 5 hours in birds used in our previous study, and in others evaluating the effects of acute fasting in broiler chickens.

Effects of fasting on fatty acid oxidation were determined by measuring the oxidation of 14C-labeled palmitate in adipose tissue homogenates. In this assay, the production of 14CO2 reflects complete fatty acid oxidation through the TCA cycle, while 14C-labeled acid soluble metabolites (ASM) are molecules of acylcarnitine that reflect incomplete oxidation of fatty acids. As shown in Figure 2A, fasting significantly and progressively increased the rate of complete fatty acid oxidation (p < 0.001). After five hours of fasting, 14CO2 production rate increased by 31% over control levels (p = 0.05, 5 hr. fasting vs. fed control). By seven hours fasting, oxidation increased an additional 30% (1.95 +/- 0.56 vs. 1.49 +/- 0.17 nmol/mg protein/hr., 7 and 5 hours fasting, respectively) and was upregulated by 71% compare with the fed group (p < 0.001). It is worth noting that the degree of induction (~71%) of fatty acid oxidation that we observed after 7 hours of fasting is comparable to the difference in oxidation rates of adipose tissue from genetically selected lean vs. obese lines of chickens, which differ in fatness by ~10-fold. This level of induction may therefore be sufficient to affect adiposity. The rate of ASM production (Fig. 2B) was similar between groups except for a slight reduction after 5 hours fasting, but the overall effect of treatment was not significant (p = 0.167). Effects of fasting on complete but not incomplete palmitate oxidation were reflected in a significantly increased 14CO2/ASM ratio (Fig. 2C; p0.005), which reflects the efficiency of β-oxidation. Complete fatty acid oxidation includes both β-oxidation and metabolism of the resultant acetyl-CoA molecules through the citric acid cycle. Accordingly, 14CO2 production and citrate synthase activity were significantly correlated across all treatments (r = 0.619, p < 0.001). As shown in Figure 2D, 7 hours of fasting significantly increased citrate synthase activity by ~23% relative to fed controls (p = 0.012). These results demonstrate that fasting rapidly upregulates fatty acid oxidation in visceral white adipose tissue, confirming predictions based on our previous microarray study. Increased oxidation precedes appearance of increased serum NEFA, suggesting that oxidation was induced early in the course of lipolysis.

Fatty acid oxidation in white adipocytes is therapeutically relevant because elevated free fatty acids induce adipocyte stress and inflammation that impair insulin sensitivity. Enhancing β-oxidation in 3T3-L1 adipocytes through constitutive overexpression of Cpt1 prevents fatty acid-induced JNK activation, inflammation, insulin resistance and oxidative stress. Conversely, pharmacological inhibition of fatty acid oxidation exacerbates cytokine release, JNK activation and impaired insulin action. Fatty acid oxidation may also promote insulin sensitivity through metabolic effects that support adipocyte glucose metabolism. Griesel et al., reported that inhibiting fatty acid oxidation...
acid oxidation in vivo through acute treatment with the Cpt1 antagonist etomoxir suppressed expression of GLUT4 in adipose tissue, suggesting that oxidation of fatty acids feeds back to maintain glucose uptake.

Studies in vivo have focused primarily on mitochondrial biogenesis as a means to increase adipocyte oxidative capacity. Chronic lipolysis induced by sustained activation of β3-adrenergic receptors (β3-AR) with the highly selective agonist CL-316,243 (CL) increases mitochondrial number in white adipose depots of mice. Activation of the β3-AR induces an initial inflammatory response that precedes and is necessary for the subsequent increase in fatty acid oxidation, which manifests within approximately 6 d of agonist infusion. By contrast, we observe increased oxidation within a few hours of feed withdrawal and coincident with the onset of lipolysis. Based on our previous studies, this response is not associated with activation of inflammatory signaling through JNK or NFκB, although we did observe an increase in phosphorylation of P38MAPK after 5 hours of fasting. Endogenous stimulation of lipolysis through fasting may elicit different downstream metabolic adaptations than pharmacological induction using CL. Induction of lipolysis with CL has been shown to induce adipocyte inflammation, whereas fasting inhibited inflammation, despite comparable degrees of lipolysis. Agonism of β3-AR also induces ER stress that is independent of fatty acid mobilization. Differences in the timing (hours vs. days) and underlying mechanisms (expression of oxidative enzymes vs. mitochondrial biogenesis) of increased fatty acid oxidation between these 2 models may therefore result from divergent effects on inflammation. Alternatively, transcriptionally-mediated upregulation of the β-oxidation pathway may represent a first line of adipocyte defense against elevated intracellular free fatty acids. If the response is sufficient, fatty acid levels are controlled, a significant inflammatory response is prevented, and the cascade of mitochondrial biogenesis is not induced. If the lipolytic stimulus is strong or sustained and overwhelms the capacity provided by rapid transcriptional control, a second layer of defense is provided by mitochondrial biogenesis. Additional experiments with more fine-scale, parallel measurements of lipolysis, oxidation and inflammation will be important to test this possibility.

Whether white adipose fatty acid oxidation is sufficient to directly impact obesity through energy expenditure is questionable, although some models demonstrate an inverse relationship with adiposity. Even if adipose fatty acid oxidation is insufficient to cause weight loss, augmenting this pathway may be a strategy to reduce adipocyte size, an important determinant of insulin sensitivity and secreted adipokine profiles. Adipocyte size is positively correlated with insulin resistance and macrophage infiltration of adipose tissue, including in early childhood. Enhancing fatty acid oxidation may therefore be a therapeutic target for attenuating the progression of obesity-prone children to insulin resistance.

One limitation of this study is that the responsible cell type(s) within adipose tissue cannot be determined. Mature adipocytes are assumed to be responsible because they comprise the majority of mass in an adipose depot, and because they are the cell type in which intracellular fatty acids rise during lipolysis. Other cell types in the

Figure 2. Fatty acid oxidation and citrate synthase activity in abdominal adipose tissue. Mean ± std. dev.; n = 8, 4, 8 and 7 for control, 3 (3 Hr), 5 (5 Hr) and 7 (7 Hr) hours fasting, respectively; 'p < 0.05, relative to fed control. (A) CO2 production (nmol/mg protein/hr); (B) acid soluble metabolites (ASM; nmol/mg protein/hr), (C) ratio of CO2: ASM production, and (D) citrate synthase activity, μmol/min/mg protein.
In fact, expression of Cpt1 was shown to be higher in stromal vascular cells than in mature human adipocytes.16 Fractionation experiments will be valuable in evaluating the relative contributions of adipocytes and other cell types in future studies. It is worth noting that these data were obtained in the equivalent of a juvenile model, in which adipose tissue is progressively expanding through both hypertrophy and hyperplasia. Whether comparable effects would be seen in adult adipose tissue, which may be less metabolically active, remains to be determined. Finally, the period of fasting that is necessary to induce this pathway in different species may vary due to meal patterns and sensitivity to lipolytic stimuli. Broiler chickens eat almost continually due to consequences of selection for growth. Accordingly, lipolysis may be induced more rapidly than in rodents or other animals that consume food in bouts. We predict that the time required to induce lipolysis, rather than the specific duration of feed withdrawal, is the important temporal variable.

In summary, we have demonstrated for the first time that a brief period of fasting induces fatty acid oxidation in abdominal white adipose tissue. Induction precedes increased plasma NEFA levels, suggesting that it is an adaptive response to increase intracellular free fatty acids. These data may represent an endogenous, homeostatic mechanism through which adipocytes protect their metabolism during daily fluxes in energy status. In light of recent studies highlighting metabolic benefits of fatty acid oxidation in white adipocytes, it will be important to further understand the underlying mechanisms that upregulate this pathway in our model.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Brynn H. Voy http://orcid.org/0000-0003-1923-9110

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