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FOOD RESTRICTION AND STIMULATION OF MONOOXYGENATION OF *p*-NITROANISOLE IN PERFUSED RAT LIVER

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Abstract—This study assessed the effects of food restriction on the metabolism of model monooxygenase substrates in the perfused rat liver. Female Sprague–Dawley rats had access *ad lib.* to a Purina 5001 nonpurified diet (control) or were given 65% of the intake of controls for 3 weeks. Livers were perfused with oxygenated Krebs–Henseleit buffer using a non-recirculating system, and the rates of monooxygenation of *p*-nitroanisole and 7-ethoxycoumarin were measured. The results indicate that food restriction stimulated *p*-nitroanisole O-demethylation from 2.9 ± 0.2 to 4.6 ± 0.5 μmol/(g·hr) when saturating concentrations of *p*-nitroanisole were infused. Concomitantly, the ratio of β-hydroxybutyrate to acetoacetate (B/A) and the rates of ketogenesis (B + A) were increased significantly by food restriction. Further, *p*-nitroanisole (200 μmol/L) increased hepatic malate concentration nearly 3-fold in liver extracts from food-restricted rats. However, infusion of either a low concentration of *p*-nitroanisole (50 μmol/L) or 7-ethoxycoumarin (200 μmol/L) did not alter these parameters. On the other hand, food restriction did not alter rates of monooxygenation in isolated microsomes supplemented with excess NADPH. Taken together, these data support the hypothesis that high concentrations of *p*-nitroanisole increased monooxygenation in food-restricted rats by stimulating fatty acid oxidation, which elevates the mitochondrial NADH/NAD^+ ratio. This, in turn, increases the availability of reducing equivalents in the form of NADPH by a malate-pyruvate exchange system, leading to increased drug metabolism.

Key words: food restriction; monooxygenation; malate; fatty acid oxidation; rats

Food restriction extends life span and significantly retards the onset of physiological changes and pathologies associated with aging [1, 2]. Food restriction also diminishes the formation of spontaneous and chemically induced tumors [3, 4]; however, little is known about the biochemical and molecular mechanisms involved.

Recently, studies by Feuers [2] and others suggested that food restriction may alter hormone receptors or change hormone levels. These modifications could lead to changes in substrates for intermediary metabolism, alter enzyme levels and modify metabolic efficiency. Food restriction has been reported to increase hepatic microsomal metabolism and detoxification of drugs and carcinogens [5]. Moreover, Wall *et al.* [6] reported recently that reducing food intake increases the capacity of the liver to detoxify benzo[*a*]pyrene, a model polycyclic aromatic hydrocarbon prevalent in the environment. In addition, studies carried out using isolated hepatocytes indicate that metabolism of benzo[*a*]pyrene is inversely correlated with lipid content of the liver [7]. Taken together, these studies suggest that food restriction may prevent tumors by affecting biotransformation of xenobiotics in the liver. Surprisingly, few studies carried out to date have considered the complex relationship between mixed-function oxidation and changes that would be expected to occur in intermediary metabolism in intact cells following food restriction. Since many carcinogens require activation via monooxygenation, the purpose of this study was to evaluate the effect of food restriction on the metabolism of model monooxygenase substrates in the perfused rat liver.

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MATERIALS AND METHODS

Experimental animals. Female Sprague–Dawley rats (21 days old) were given a nonpurified diet, Purina 5001 rat chow *ad lib.* for 1 week before random assignment to control and food-restricted groups. Control animals were maintained on diet for an additional 3 weeks, while food-restricted rats were fed 65% of food ingested by controls over the same time period. Both control and food-restricted rats were fed once daily in the late afternoon to maintain the normal feeding pattern, while water was given *ad lib.* Experiments were performed between 9:00 and 10:00 a.m. on the next day. All animals were housed individually in suspended, wire-bottom cages to minimize coprophagy. Experimental procedures were approved by an Institutional Animal Care and Use Committee.

Monooxygenation in perfused liver. Details of the liver perfusion technique have been described elsewhere [8]. Briefly, livers were perfused at 37° with Krebs–Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen:carbon dioxide mixture (95:5) in a nonrecirculating system. The fluid was pumped through the liver via a cannula placed in the vena cava and flowed past a Clark-type oxygen electrode before being discarded. The bile duct was cut, allowing bile to mix freely with the effluent perfusate. To assess monooxygenation, *p*-nitroanisole (50 or 200 μmol/L) dissolved in Krebs–Henseleit buffer was infused. Free and total *p*-nitrophenolate were measured spectrophotometrically at 436 nm in samples of effluent perfusate collected at 4-min intervals. Rates of conjugation were calculated from total minus free *p*-nitrophenolate [9]. For some experiments, a stock solution of 7-ethoxycoumarin was dissolved in *N,N*-dimethylformamide and was added to Krebs–Henseleit bicarbonate buffer to give a final concentration of 3.2 mmol/L *N,N*-dimethylformamide and 200 μmol/L 7-ethoxycoumarin when infused into the liver. *N,N*-Dimethylformamide had no effect on mixed-function oxidation under these conditions [10]. The fluorescence of unknown samples was compared with authentic 7-hydroxycoumarin standards incubated under identical conditions [11]. Lactate, pyruvate, glucose, acetoacetate and β-hydroxybutyrate in the effluent perfusate were assayed by standard enzymatic techniques [12]. Rates were calculated from influent minus effluent concentration differences, the flow rate and the liver wet weight.

Microsomal monooxygenation. Hepatic microsomes were prepared by standard techniques of differential centrifugation [13]. *p*-Nitroanisole *O*-demethylase activity was determined in microsomal pellets resuspended in 0.15 mol/L KCl. Assays were performed at 37° in 25-mL Erlenmeyer flasks containing 5 mmol/L $MgCl_2$, 0.05 or 0.2 mmol/L *p*-nitroanisole, microsomes (11–13 mg of protein/mL), and an NADPH-generating system consisting of 0.4 mmol/L $NADP^+$, 30 mmol/L isocitrate, and 0.2 U of isocitrate dehydrogenase, in a final volume of 2.0 mL of 0.18 mol/L potassium phosphate buffer, pH 7.4. Incubations were initiated by the addition of an NADPH-generating system [14] and were terminated after 5 min by the addition of 0.5 mL of 0.6 mol/L perchloric acid. After centrifugation to remove denatured protein, 1.0 mL of supernatant was mixed with 0.1 mL of 12 mol/L NaOH. This mixture was recentrifuged, and *p*-nitrophenol was measured spectrophotometrically ($\varepsilon 436 = 7.11 \cdot \text{mmol/L}^{-1} \cdot \text{cm}^{-1}$) in the supernatant [15]. Microsomal 7-ethoxycoumarin *O*-deethylase activity was assayed fluorometrically using the NADPH-generating system described above with 7-ethoxycoumarin (0.1 mmol/L) as substrate, as described in detail elsewhere [16]. Protein was determined by the biuret reaction [17].

Malate measurement in liver tissue. Malate was measured in $HClO_4$ extracts prepared from livers that were freeze-clamped during perfusion of *p*-nitroanisole (50 or 200 μmol/L) or 7-ethoxycoumarin (200 μmol/L). Malate was measured enzymatically by standard fluorometric procedures [18].

Statistical analyses. Statistical analyses were performed using Student's *t*-test [19]. Results are given as means ± SEM, and the criterion for significance was $P < 0.05$.

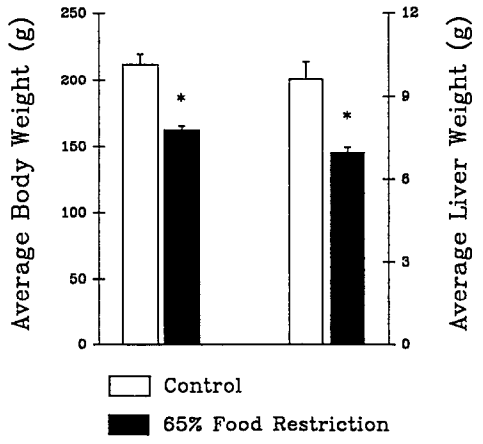

Fig. 1. Influence of food restriction on body (left columns) and liver weights (right columns). Female rats (21 days) were maintained on *ad lib.* or food-restricted diets (65% of food consumed by controls) for 3 weeks as described in Materials and Methods. Values are means ± SEM, N = 5. Key: (*) $P < 0.05$ for the comparison of food-restricted vs control.

RESULTS

Animal growth. Female Sprague–Dawley rats in the 80–100 g weight range were fed either a control nonpurified diet or a food-restricted diet (65% of food ingested by controls) for 21 days. Control animals weighed 190–210 g and food-restricted animals weighed 140–160 g at the end of the 3-week period. The average liver weights were 9.6 ± 0.6 g in the control group and 6.9 ± 0.1 g in the food-restricted group (Fig. 1). All animals appeared healthy.

Effect of food restriction on hepatic drug metabolism in perfused liver. Rates of *p*-nitroanisole O-demethylation and 7-ethoxycoumarin O-deethylation in perfused livers from control and food-restricted rats are depicted in typical experiments in Fig. 2. Maximal rates of *p*-nitrophenol production were elevated significantly ($P < 0.05$) by nearly 2-fold in the food-restricted group compared with controls when 200 μmol/L *p*-nitroanisole was infused (Fig. 2A). Rates of conjugation of *p*-nitrophenol (glucuronide + sulfate formation) after O-demethylation of *p*-nitroanisole were elevated significantly ($P < 0.05$) from 2.1 ± 0.2 to 3.7 ± 0.4 μmol/(g·hr) in the food-restricted compared with the control group. In striking contrast, metabolism of 7-ethoxycoumarin was not altered by the diet (Fig. 2B). Similarly, *p*-nitrophenolate production was not affected by food restriction when a low concentration of *p*-nitroanisole (50 μmol/L) was infused (Fig. 3). Oxygen uptake was stable during the entire perfusion

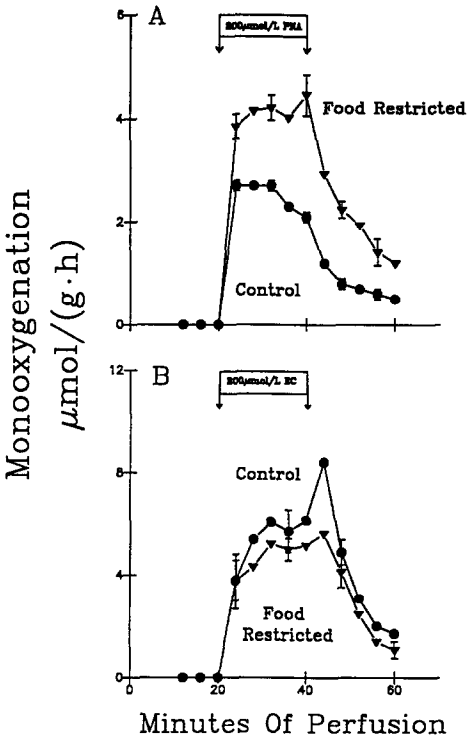

Fig. 2. Effect of food restriction on hepatic drug metabolism in perfused livers. Livers from control or food-restricted rats were perfused with Krebs–Henseleit buffer as described in Materials and Methods. After 20 min, *p*-nitroanisole (panel A; PNA; 200 μmol/L) or 7-ethoxycoumarin (panel B; EC; 200 μmol/L) was infused for 20 min as indicated by the horizontal bar and arrows. Samples of effluent perfusate were collected for determination of *p*-nitrophenolate and 7-hydroxycoumarin. Rates of monooxygenation of PNA and EC were calculated from influent minus effluent concentration differences, the flow rate and liver wet weight. Experiments were repeated four times in each group. Values are means ± SEM, N = 4.

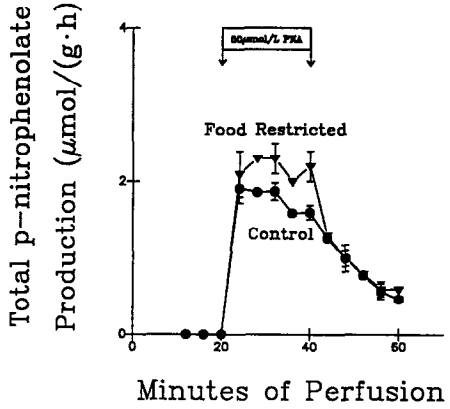

Fig. 3. Effect of food restriction on monooxygenation using submaximal substrate concentrations in perfused liver. Livers from control or food-restricted rats were perfused with nonsaturating concentrations of *p*-nitroanisole (50 μmol/L) as described in Materials and Methods, using experimental designs depicted in Fig. 2. Values are means ± SEM, N = 4.

period (data not shown), indicating that cells were not damaged under these conditions by the drugs studied.

Effect of food restriction on microsomal drug metabolism. Monooxygenation in the intact cell is influenced by at least two factors in addition to the supply of substrate—the activity of components of the monooxygenase pathway (i.e. cytochrome P450) and the availability of cellular NADPH. NADPH supply is an important rate-controlling factor for drug metabolism in intact cells. To investigate whether food restriction stimulated drug metabolism by increasing cytochrome P450 activity or by influencing NADPH supply, O-demethylation of *p*-nitroanisole and O-deethylation of 7-ethoxycoumarin were studied in microsomes isolated from control and food-restricted rats with an NADPH-generating system (Table 1). In contrast to data obtained with 200 μmol/L *p*-nitroanisole in perfused liver, monooxygenation of *p*-nitroanisole and 7-ethoxycoumarin in microsomes supplied with excess NADPH was not altered by food restriction. Thus, the increase in *p*-nitroanisole metabolism observed in the perfused liver with 200 μmol/L *p*-nitroanisole following food restriction cannot be explained at the level of the endoplasmic reticulum. Therefore, it is reasonable to hypothesize that food restriction stimulated monooxygenation of *p*-nitroanisole by increasing NADPH supply rather than by increasing cytochrome P450 activity under these conditions.

Changes in intermediates during mixed-function oxidation in perfused liver. To investigate whether food restriction affected intermediary metabolism, β-hydroxybutyrate and acetoacetate were measured. Following 20 min of perfusion with Krebs–Henseleit bicarbonate buffer, *p*-nitroanisole was infused into livers from both control and food-restricted rats. The ratio of β-hydroxybutyrate to acetoacetate (B/A), an indicator of the mitochondrial pyridine nucleotide redox state, was increased significantly ($P < 0.05$) when *p*-nitroanisole was infused into livers from the food-restricted group, but was actually diminished in livers from control rats (Fig. 4). Also, the rate of ketone body production (acetoacetate + β-hydroxybutyrate) was increased from basal values of 23.8 ± 2.3 to 37.3 ± 3.7 μmol/(g·hr) by *p*-nitroanisole in the food-restricted group. However, 7-ethoxycoumarin did not affect either the B/A ratio or the rate of ketone body production (data not shown).

Movement of reducing equivalents from mitochondria to the cytosol occurs via a malate-pyruvate exchange system [20]; therefore, malate was measured in liver extracts from control and food-restricted rats perfused with *p*-nitroanisole or 7-ethoxycoumarin. Perfusion with *p*-nitroanisole (200 μmol/L) significantly increased malate content nearly 3-fold in liver extracts from food-restricted rats (Table 2). However, perfusion with low concentrations of *p*-nitroanisole (50 μmol/L) did not

Table 1. Effect of food restriction on hepatic microsomal drug metabolism*

Treatment	*p*-Nitrophenol production nmol/(min · mg protein)		7-Hydroxycoumarin production nmol/(min · mg protein)
	p-Nitroanisole		7-Ethoxycoumarin
	(50 μmol/L)	(200 μmol/L)	(100 μmol/L)
Control	7.2 ± 0.5	10.5 ± 0.5	0.7 ± 0.1
65% Food restricted	8.4 ± 1.0	12.6 ± 0.6	0.8 ± 0.1

* Values are means ± SEM, N = 4. Hepatic microsomes were prepared and incubated with *p*-nitroanisole (50 or 200 μmol/L) or 7-ethoxycoumarin (100 μmol/L) as described in Materials and Methods, and rates of monooxygenation were determined.

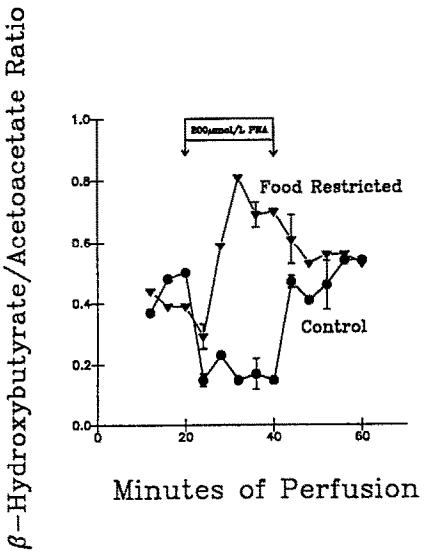

Fig. 4. Effect of food restriction on β-hydroxybutyrate/acetoacetate ratio. Livers from control or food-restricted rats were perfused with *p*-nitroanisole (200 μmol/L) as described in Materials and Methods, using experimental designs depicted in Fig. 2. Ketone bodies were measured in perfusate sample as described in Materials and Methods. Values are means ± SEM, N = 4.

alter malate content (Table 2). Further, 7-ethoxycoumarin (200 μmol/L) also did not affect malate content in the liver. In the presence of 7-ethoxycoumarin, malate content was 26.2 ± 2.8 μmol/kg wet liver wt in the control group and 24.3 ± 5.5 in the food-restricted group. Thus, increased metabolism of *p*-nitroanisole was observed only when hepatic malate content was elevated.

DISCUSSION

The present study was designed to determine how food restriction modulates monooxygenation of model drug substrates. The results demonstrate that the effects of food restriction on drug metabolism vary, both with the type and concentration of substrate.

In previous work from this laboratory, 200 μmol/L of *p*-nitroanisole was shown to be a saturating concentration for this reaction in the perfused liver. O-Demethylation of a saturating concentration of *p*-nitroanisole was increased by food restriction, whereas metabolism of a low, limiting dose of *p*-nitroanisole (50 μmol/L) and another structurally unrelated substrate, 7-ethoxycoumarin, was not altered by diet under these conditions (Fig. 2). Therefore, an important question about mechanism arises: why does food restriction have different effects on monooxygenation of different substrates or the same substrate at different concentrations? Since drug metabolism via hepatic cytochrome P450-dependent monooxygenation requires both cytochrome P450 and NADPH supply, we evaluated the effect of food restriction on these systems. In microsomes from livers of control or food-restricted rats, rates of *p*-nitroanisole and 7-ethoxycoumarin metabolism, assayed in the presence of excess NADPH, were not altered by food restriction (Table 1); therefore, it seems plausible to propose that food restriction regulates monooxygenation by affecting cofactor supply but only under some conditions.

The supply of NADPH is an important rate-determining factor for mixed-function oxidation of *p*-nitroanisole in intact cells [21]. Rates of mixed-function oxidation of a variety of substrates are regulated in large part by the availability of NADPH generated via both cytosolic and mitochondrial oxidations [16, 22], but predictions are not easy to make. For example, inhibition of the oxidative enzymes of the pentose phosphate pathway by fasting or by 6-aminonicotinamide had no effect on rates of *p*-nitroanisole O-demethylation [23]. In contrast, agents that uncoupled oxidative phosphorylation or inhibited mitochondrial respiration reduced rates of mixed-function oxidation in perfused livers from both fed and fasted rats [22, 23]. Thus, mitochondria most likely serve as a major source of NADPH for mixed-function oxidation [16]. Since the inner mitochondrial membrane is impermeable to NADPH [24], it is generally accepted that reducing equivalents are transferred from mitochondria to cytosol via

Table 2. Hepatic malate content from control and food-restricted rats perfused with *p*-nitroanisole*

Treatment	Malate (μmol/kg wet liver)		
		p-Nitroanisole	
	Basal	50 μmol/L	200 μmol/L
Control	50.2 ± 11.0	37.6 ± 7.5	32.9 ± 11.0
65% Food restricted	64.0 ± 21.0	42.6 ± 4.4	93.9 ± 15.1†

* Values are means ± SEM, N = 4. Livers from 65% food-restricted or control rats were perfused as described in Materials and Methods. *p*-Nitroanisole (50 or 200 μmol/L) infusion was initiated after 20 min of perfusion. Livers were frozen rapidly by freeze-clamping with tongs chilled in liquid nitrogen after 26 min of perfusion, when rates of *p*-nitroanisole metabolism were maximal. Cellular malate was determined as described in Materials and Methods.

† $P < 0.05$ for the comparison of food-restricted vs control.

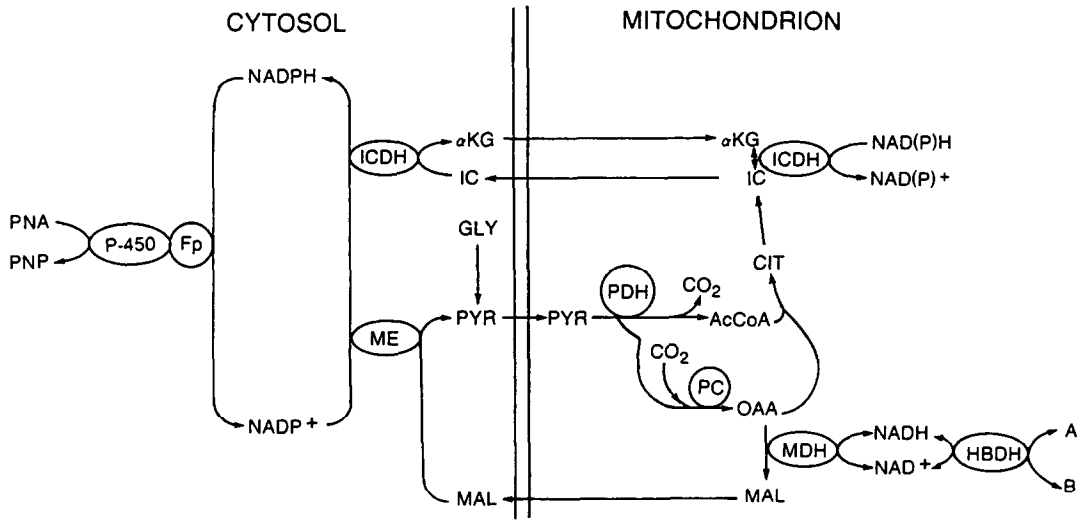

Fig. 5. Scheme depicting pathways of malate metabolism leading to NADPH generation. Abbreviations: P450, cytochrome P450; Fp, NADPH cytochrome P450 reductase; ICDH, isocitrate dehydrogenase; ME, "malic" enzyme; PYR, pyruvate; IC, isocitrate; αKG, α-ketoglutarate; MAL, malate; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CIT, citrate; AcCoA, acetyl CoA; OAA, oxaloacetate; MDH, malate dehydrogenase; GLY, glycogen; PNA, *p*-nitroanisole; PNP, *p*-nitrophenol; A, acetoacetate; B, β-hydroxybutyrate; and HBDH, β-hydroxybutyrate dehydrogenase.

substrate shuttle mechanisms [25, 26]. Two shuttle mechanisms for the generation of cytosolic NADPH have been proposed. One system involves participation of α-ketoglutarate, isocitrate, and $NADP^+$-dependent isocitrate dehydrogenase located in the mitochondrial and cytosolic compartments. The other system involves the movement of reducing equivalents from mitochondria to the cytosol via malate-pyruvate exchange. NADPH may be generated by a malate shuttle that involves the carboxylation of pyruvate via pyruvate carboxylase to form oxalacetate, reduction to malate, egress of malate into the cytosol, and NADPH production via malic enzyme. Therefore, increasing flux over metabolic pathways can increase NADPH production, leading to stimulation of drug metabolism (Fig. 5). On the other hand, fatty acids are oxidized primarily via β-oxidation in the mitochondria where they generate NADH, which can be converted to NADPH via an energy-dependent transhydrogenase. In this study, glycolytic rates (lactate + pyruvate), glycogenolysis (lactate + pyruvate + glucose) and the ratio of lactate/pyruvate (cytosolic NADH redox state) were not altered by food restriction in perfusions with either *p*-nitroanisole or 7-ethoxycoumarin (data not shown). Further, if fatty acid oxidation were affected, increased production of β-hydroxybutyrate and acetoacetate should occur. Indeed, it was observed that rates of ketone body production were increased about 60% by food

restriction when a high concentration of *p*-nitroanisole (200 μmol/L) was infused. Also, the ratio of β-hydroxybutyrate to acetoacetate, an index of mitochondrial NADH redox state, was increased significantly ($P < 0.05$) by *p*-nitroanisole only in the food-restricted group (Fig. 4). In addition, oxygen uptake was increased by infusion of *p*-nitroanisole in both control and food-restricted rats (data not shown). Concomitantly, high levels of *p*-nitroanisole increased malate content nearly 3-fold in liver extracts from food-restricted rats (Table 2). These parameters were not affected by low concentrations of *p*-nitroanisole or 7-ethoxycoumarin. Based on these results, we hypothesize that *p*-nitroanisole O-demethylation increases fatty acid oxidation in food-restricted rats, which, in turn, elevates the mitochondrial NADH/NAD^+ ratio allowing mitochondrial oxaloacetate to accept reducing equivalents from NADH. The malate formed enters the cytosol where it reacts with malic enzyme to form NADPH and pyruvate. Pyruvate then enters the mitochondria, and an energy-requiring carboxylation regenerates mitochondrial oxaloacetate to complete the cycle. Subsequently, the rate of NADPH production is elevated, which stimulates *p*-nitroanisole monooxygenation (Fig. 5).

It is known that food intake and nutrients affect steady-state concentrations of NADPH and supply active intermediates utilized in drug metabolism in intact cells [22, 27]. Data obtained from microsomes (Table 1) indicated that monooxygenation of *p*-nitroanisole and 7-ethoxycoumarin was not altered by food restriction when incubated with an NADPH-generating system. Therefore, it is likely that the results observed in perfused liver are due to changes in intermediates rather than to some other nutrient or non-nutrient dietary component that was also fed at a reduced level.

An interesting question arising from this work is why a high concentration of *p*-nitroanisole (200 μmol/L) stimulated fatty acid metabolism only in food-restricted rats, whereas 7-ethoxycoumarin did not affect fatty acid oxidation in either food-restricted or control rats. Zaleski *et al.* [7] demonstrated that in striking contrast to the results with benzo[*a*]-pyrene, metabolism of 7-ethoxycoumarin, a more hydrophilic substrate, is not altered by food restriction and high-fat diet treatment. Also, they demonstrated that 7-ethoxycoumarin has a low affinity for triacylglycerol. Thus, we hypothesize that 7-ethoxycoumarin does not affect fatty acid oxidation, which, in turn, increases cofactor by a malate-pyruvate exchange system since it is a more hydrophilic substrate. The evidence that malate levels, the ratio of β-hydroxybutyrate to acetoacetate (B/A), and rates of ketogenesis (B + A) were not altered by food restriction with perfusion of 7-ethoxycoumarin strongly supports this hypothesis. On the other hand, it has been reported that energy restriction may alter hormone receptor structure or number.* These modifications then, in turn, change levels of substrates for intermediary metabolism, alter enzyme concentration, and modify metabolic efficiency.* For example, Feuers *et al.* [2] demonstrated that the activities of glycolytic enzymes are decreased in response to caloric restriction. Likewise, enzymes associated with fatty acid synthetase are reduced.* Thus, food restriction most likely alters fatty acid metabolism, favoring increased β-oxidation in the presence of *p*-nitroanisole.

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