Pyruvate Inhibits Clofibrate-Induced Hepatic Peroxisomal Proliferation and Free Radical Production in Rats

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In an effort to identify the effects of the 3-carbon compound pyruvate on free radical production, we measured hepatic total peroxisomal β-oxidation and catalase activity and the production of lipofuscin-like products in male Sprague-Dawley rats consuming an adequate diet supplemented with pyruvate, vitamin E, or the peroxisome proliferator and free radical enhancer clofibrate for 22 days (n = 5 in each group). Clofibrate feeding induced hepatomegaly, a fivefold increase in total peroxisomal β-oxidation activity, and a threefold increase in hepatic lipofuscin-like products ($P < .05$). Pyruvate but not vitamin E inhibited the increase in liver size by 70% ($P < .05$). Both pyruvate and vitamin E completely inhibited clofibrate-induced increases in lipofuscin-like products ($P < .05$). Pyruvate but not clofibrate or vitamin E increased plasma concentrations of the nitric oxide metabolites nitrite and nitrate ($P < .05$). We conclude that with clofibrate-induced peroxisomal proliferation and free radical production, pyruvate will inhibit peroxisomal proliferation and free radical production, inhibit free radical–induced lipid peroxidation, and enhance metabolism of nitric oxide.

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PYRUVATE, a 3-carbon compound, has been shown to have an effect on a variety of physiologic and biochemical functions. Partial isoenergetic dietary substitution of pyruvate induces enhanced arm and leg submaximal endurance capacity in normal men,[1,2] enhanced body weight and fat loss in obese women,[3,4] normalization of plasma glycemia and hepatic gluconeogenesis in non–insulin-dependent diabetics,[5] inhibition of ethanol-induced hepatic steatosis in rats,[6] and inhibition of malignancy growth in rats.[7] Infusion of pyruvate results in increased cardiac output without increased oxygen consumption in dogs,[8] and also enhances phosphorylation potential in normoxic and postischemic isolated heart.[9] The mechanisms whereby pyruvate induces these metabolic effects are not established. Although it is doubtful that pyruvate induces this myriad of physiologic responses through one mechanism, it might be reasonable to expect a common intermediate metabolic response to pyruvate metabolism. Salahudeen et al[10] have shown that pyruvate inhibits hydrogen peroxide–induced renal injury in rats by neutralizing hydrogen peroxide. Since hydrogen peroxide is the direct precursor of the metabolically active hydroxyl (OH^-) free radical, pyruvate might function to inhibit free radical production.[11]

The hypolipidemic drug ethyl-&-*p*-chlorophenoxyisobutyrate (clofibrate) induces hepatic peroxisome proliferation, with subsequent increases in production of hydrogen peroxide and free radicals.[12,13] In the present study, we evaluated the effect of pyruvate and the known antioxidant vitamin E on clofibrate-induced peroxisome proliferation and free radical production in rats.

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MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats weighing approximately 210 g were assigned to seven groups of five individually housed rats and fed a commercial liquid diet (Dyets #710027, Dyets, Bethlehem, PA) for 22 days. The groups were as follows: placebo, clofibrate, clofibrate + pyruvate, clofibrate + vitamin E, clofibrate + pyruvate + vitamin E, vitamin E, and pyruvate. The composition of the diet (g/1,000 mL) was as follows: casein 41.4, maltose dextrin 115.2, olive oil 28.4, safflower oil 2.7, corn oil 8.5, L-cystine 0.5, DL-methionine 0.3, cellulose 10.0, choline bitartrate 0.5, xanthan gum 3.0, vitamin mix 2.5, and salt mix 8.7. Pyruvate was added to the diet as sodium pyruvate (20.0 g/1,000 mL) and calcium pyruvate (17.9 g/1,000 mL) at a dosage of 10% of energy content. All diets were made isoenergetic by adding polyglucose (Polycose; Ross Laboratories, Columbus, OH) to the diets not containing pyruvate. Sodium and calcium contents of the diets were made similar by adding sodium citrate and calcium carbonate to those diets not containing pyruvate. The final composition of all diets was 47% carbohydrate, 18% protein, and 35% fat, and the energy content was 0.0042 MJ/mL. The diets were shown to be isoenergetic by bomb calorimetric analysis. To familiarize the animals with the liquid diet, all animals were fed the placebo liquid diet for 3 days, during which time the mean intake was 60 mL/d. Clofibrate (Atromid-S; Ayerst Laboratories, New York, NY) was added to the appropriate diets to a final concentration of 0.018 g/1,000 mL, or an expected dosage of 0.5 g/kg initial body weight. Vitamin E (Aquasol E drops, 50 IU/mL; Astra Pharmaceutical Products, Westborough, MA) was added to the diet to a final concentration of 2 mL/1,000 mL, or a dosage of 30 IU (20 mg)/kg initial body weight.

In an effort to ensure equal energy intake between groups, the average intake of the group with the smallest intake for a given day (rate-limiting group) was fed to the other groups of animals on the following day, and the rate-limiting group was fed ad libitum. In most instances the rate-limiting group was clofibrate + pyruvate. During the feeding period the mean dietary intake of all the animals was 1,798 ± 19 mL and the energy intake of all the groups was similar (data not shown). During the feeding period the total intake of clofibrate between groups was similar: clofibrate, 3.3 ± 0.1 g; clofibrate + vitamin E, 3.3 ± 0.1 g; clofibrate + pyruvate, 3.1 ± 0.1 g; clofibrate + pyruvate + vitamin E, 3.1 ± 0.1 g (P = NS between groups). The total intake of vitamin E was similar: vitamin E, 184 ± 5 IU; clofibrate + vitamin E, 184 ± 5 IU; clofibrate + pyruvate + vitamin E, 173 ± 4 IU (P = NS between groups).

At the end of the feeding period, after an overnight fast of 16 hours, animals were euthanized by decapitation without anesthe-

sia. The liver was obtained for immediate biochemical analysis and prepared for light and electron microscopic evaluation. Blood was obtained from the cervical stump, and plasma was frozen at −20°C for later analysis.

Biochemical Analysis

A portion of the liver was homogenized in sucrose buffer (0.25 mol/L sucrose, 0.10% ethanol, 1.00 mmol/L EDTA, and 5.00 mmol/L Trishydrochloride, pH 7.5) and analyzed for total peroxisomal β-oxidation activity,[14] catalase activity,[15] and lipofuscin-like products.[16] Plasma nitrite and nitrate concentrations were measured using a high-performance liquid chromatography technique based on the Griess reaction.[17]

Morphologic Evaluation

Sections of rat liver were processed for routine histologic analysis by light microscopy and for ultrastructural evaluation performed on a Phillips CM12 electron microscope (Eindhoven, The Netherlands). Low-magnification photographs (×3,920) were taken from three hepatocyte zones. In addition, 12 random high-magnification (×28,000) photographs of midzone hepatocytes were taken for quantification of peroxisomes and mitochondria.

Statistical Analysis

Data were analyzed by ANOVA and Tukey's test. Differences were considered significant when P was less than .05. Data are presented as the mean ± SEM for five animals in each group.

This study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

RESULTS

Animals fed clofibrate alone showed a 45% increase in liver size and a 70% increase in the liver weight to body weight ratio ($P < .05$ compared with placebo; Table 1). When pyruvate was added to the clofibrate diet, these effects of clofibrate were reduced by 70% ($P < .05$; Table 1). The protein content of the liver (milligrams per gram liver, milligrams total) was also increased by clofibrate and almost completely normalized by pyruvate ($P < .05$; Table 1). Vitamin E supplementation of the clofibrate diet had no effect on the clofibrate-induced liver size or protein changes, whereas supplementation of vitamin E to the clofibrate + pyruvate diet did not appreciably enhance the normalizing effects of pyruvate (Table 1).

The total hepatic peroxisomal β-oxidation activity (nanomoles per minute per milligram protein) is presented in Fig 1. Clofibrate induced a fivefold increase in the activity of this peroxisomal enzyme system ($P < .05$). Pyruvate reduced clofibrate-enhanced increases in β-oxidation by 75% ($P < .05$). Vitamin E neither inhibited the effects of clofibrate on this enzyme system nor enhanced the normalizing effects of pyruvate.

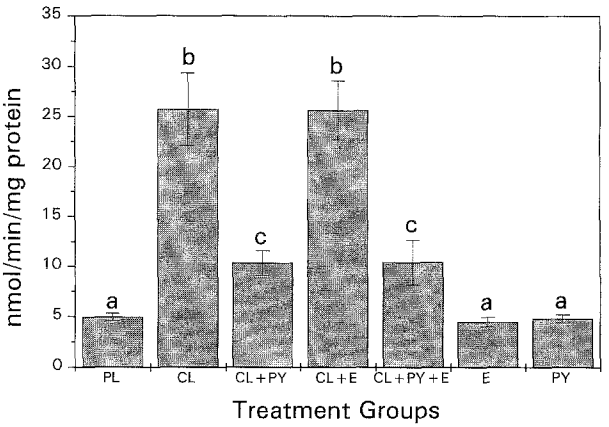

Fig 1. Hepatic total peroxisomal β-oxidation activity (nmol/min/mg protein). Treatment groups: PL, animals fed the placebo diet; CL, diet supplemented with clofibrate; CL + PY, diet supplemented with clofibrate + pyruvate; CL + E, diet supplemented with clofibrate + vitamin E; CL + PY + E, diet supplemented with clofibrate + pyruvate + vitamin E; E, diet supplemented with vitamin E; PY, diet supplemented with pyruvate. n = 5 in all groups. Different superscripts denote significant difference, $P < .05$.

Total catalase activity (Bodansky units) is presented in Fig 2. Total catalase activity increased by almost threefold in animals fed clofibrate and clofibrate + vitamin E ($P < .05$). In animals fed clofibrate + pyruvate, total catalase activity increased by only 1.5-fold ($P < .05$ *v* clofibrate and placebo). Catalase activity (Bodansky units per milligram protein, data not shown) was similar in all groups of animals.

The relative fluorescent intensity of lipofuscin-like products is presented in Fig 3. Clofibrate induced a threefold increase in lipofuscin-like products ($P < .05$), which was totally inhibited by addition of pyruvate or vitamin E to the clofibrate diet ($P < .05$).

Plasma nitrite and nitrate concentrations (micromoles per liter) are presented in Fig 4. Normal plasma concentra-

Table 1. Hepatic Parameters of Each Diet Group

Group	Liver Weight (g)	Liver Weight/Body Weight	Protein	
			mg/g Liver	mg Total
PL	7.1 ± 0.3[a]	0.024 ± 0.001[a]	160 ± 6[a]	1,130 ± 67[a]
CL	10.1 ± 0.4[b]	0.041 ± 0.002[b]	211 ± 11[b]	2,140 ± 160[b]
CL + PY	8.1 ± 0.2[c]	0.032 ± 0.001[c]	182 ± 8[c]	1,480 ± 69[c]
CL + E	10.4 ± 0.3[b]	0.041 ± 0.001[b]	204 ± 9[bc]	2,124 ± 141[b]
CL + PY + E	7.5 ± 0.3[ac]	0.029 ± 0.001[c]	172 ± 6[c]	1,298 ± 81[c]
E	6.3 ± 0.1[d]	0.024 ± 0.001[a]	199 ± 10[bc]	1,254 ± 78[ac]
PY	7.4 ± 0.5[acd]	0.025 ± 0.001[a]	170 ± 21[abc]	1,261 ± 166[ac]

NOTE. n = 5 in each group. Values with different superscripts are significantly different ($P < .05$).

Abbreviations: PL, animals fed placebo diet; CL, diet supplemented with clofibrate; CL + PY, diet supplemented with clofibrate + pyruvate; CL + E, diet supplemented with clofibrate + vitamin E; CL + PY + E, diet supplemented with clofibrate + pyruvate + vitamin E; E, diet supplemented with vitamin E; PY, diet supplemented with pyruvate.

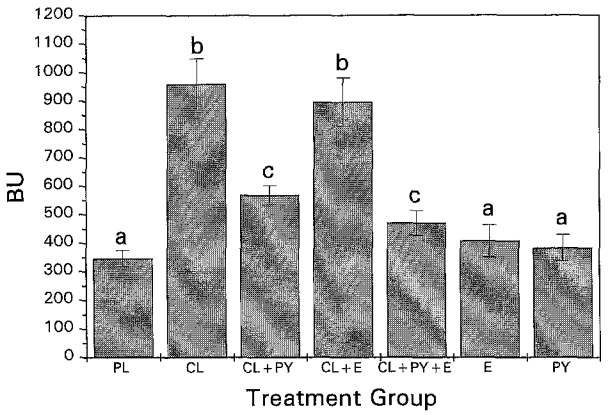

Fig 2. Hepatic catalase activity (BU, total). Treatment groups are described in Fig 1. n = 5 in all groups. Different superscripts denote significant difference, $P < .05$.

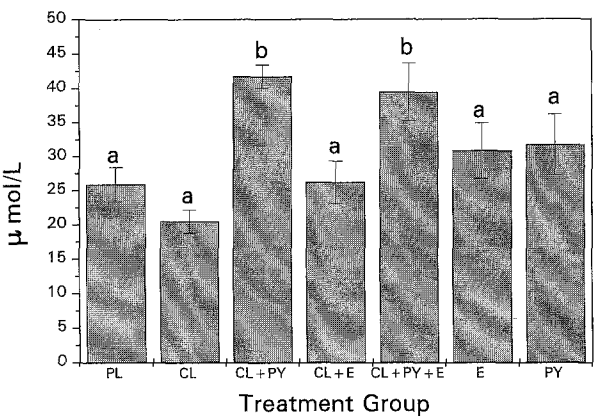

Fig 4. Plasma nitrite and nitrate concentrations (μmol/L). Treatment groups are described in Fig 1. n = 5 in all groups. Different superscripts denote significant difference, $P < .05$.

tions are typically 20 to 30 μmol/L. Placebo animals showed values within this range, and clofibrate did not alter these concentrations. With addition of pyruvate to the clofibrate diet, plasma concentrations of nitrite and nitrate increased by twofold ($P < .05$). Animals fed pyruvate alone or vitamin E showed no changes in plasma nitrite and nitrate concentrations.

Characteristic photomicrographs of a hepatocyte identifying peroxisomes and mitochondria from the placebo, clofibrate, clofibrate + vitamin E, and clofibrate + pyruvate groups are presented in Fig 5. Clofibrate induced a proliferation of peroxisomes (sevenfold to eightfold increase in number) that was reduced by pyruvate, but not by vitamin E. There were no discernible differences between groups on light microscopic evaluation.

Animals fed pyruvate alone or vitamin E alone showed no changes in any of the parameters evaluated.

DISCUSSION

The 3-carbon compound pyruvate, when consumed or infused in supraphysiologic concentrations in man and animals, can induce a multitude of beneficial metabolic effects ranging from normalization of plasma glycemia in non–insulin-dependent diabetes to inhibition of malignant growth.[1-10] In an effort to identify a mechanism whereby pyruvate might affect such diverse metabolic responses, in the present study we evaluated the possibility that pyruvate might inhibit free radical production. We chose to evaluate the effect of pyruvate on free radicals in the clofibrate-fed rat, an experimental model that results in chronic free radical production. Clofibrate feeding induced hepatomegaly with an increase in hepatic protein content, peroxisomal proliferation by electron microscopic evaluation, and a fivefold increase in peroxisomal β-oxidation activity, a marker of peroxisomal proliferation and hydrogen peroxide production.[12,13] Total hepatic catalase, which detoxifies hydrogen peroxide, increased by only twofold to threefold. Lipofuscin-like products, end products of free radical–induced lipid peroxidation,[18] increased by threefold in our clofibrate-fed animals. These findings suggestive of hydrogen peroxide production and toxic free radical metabolic effects are in agreement with previous investigations.[12,13,19].

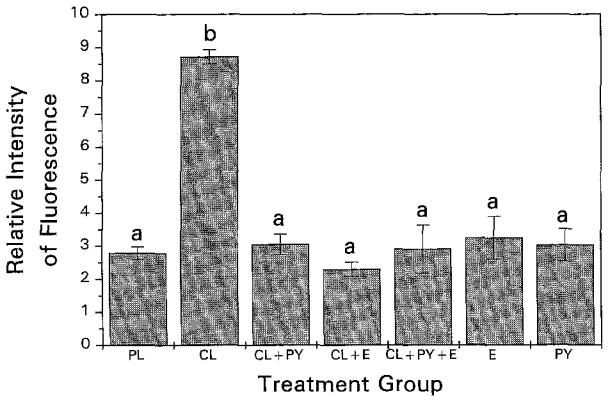

Fig 3. Hepatic lipofuscin-like products (relative intensity of fluorescence). Treatment groups are described in Fig 1. n = 5 in all groups. Different superscripts denote significant difference, $P < .05$.

When pyruvate was added to the diet as 10% of the energy intake of clofibrate-fed animals, the effects of clofibrate on peroxisomal proliferation and free radical production were altered. Clofibrate-induced changes suggestive of hepatic peroxisomal proliferation and enhanced free radical production (hepatic size, protein content, and peroxisomal β-oxidation activity) were reduced by 70% with pyruvate supplementation. Despite evidence that free radical production remained slightly elevated in clofibrate + pyruvate–treated animals, end products of free radical–induced lipid peroxidation, lipofuscin-like products, were not increased. These data suggest that pyruvate inhibited both clofibrate-induced enhanced free radical production and at least one toxic metabolic effect of free radicals.

In contrast, supplementation of clofibrate-fed animals with vitamin E had no effect on hepatic size, protein content, peroxisomal proliferation, or β-oxidation activity. These findings indicate that free radical production continued unabated with clofibrate feeding despite supplementation with vitamin E. However, the antioxidant vitamin E, as expected, did completely inhibit the effects of free radicals on lipid peroxidation.[20]

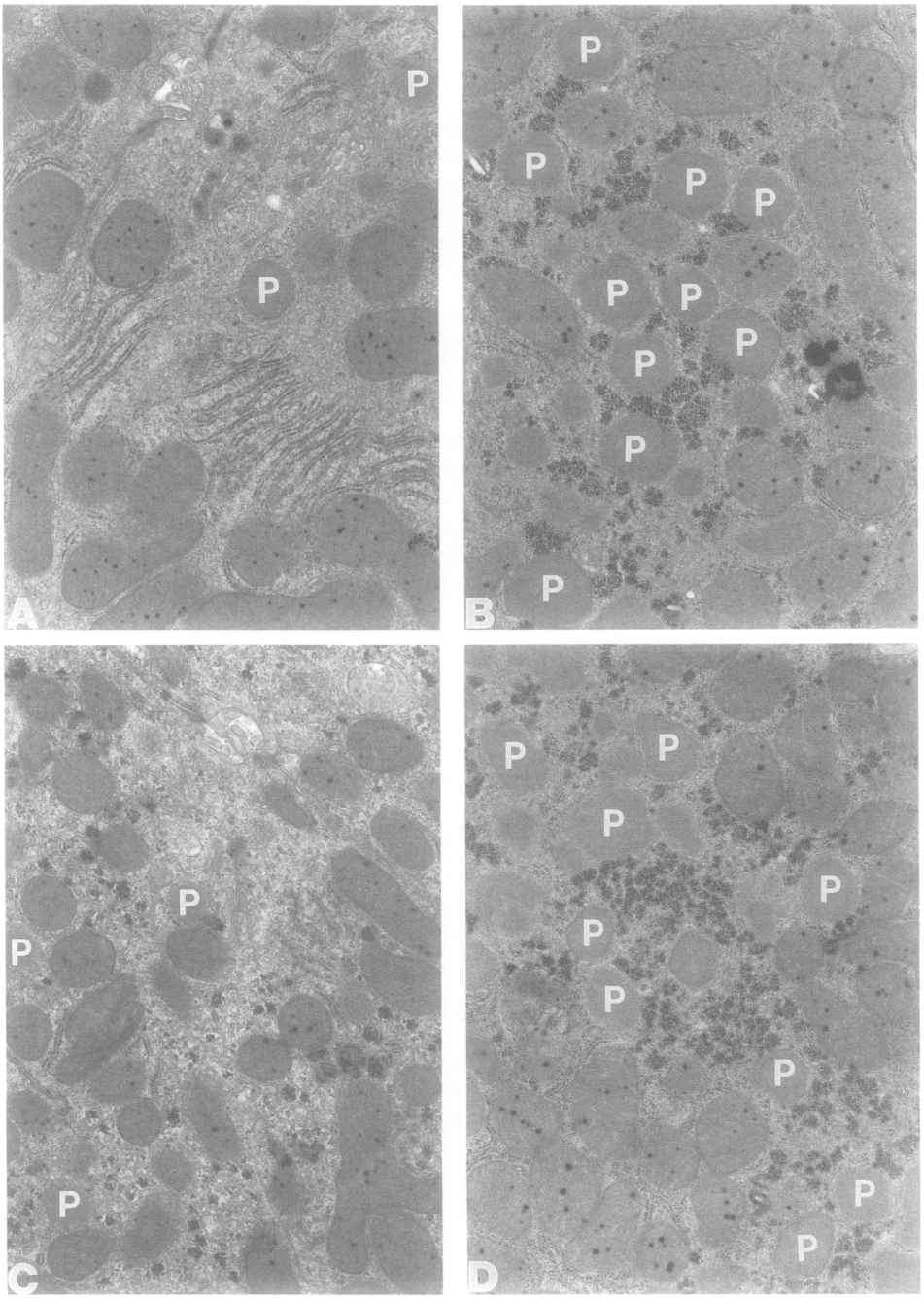

Fig 5. Electron micrograph of hepatocyte. Peroxisomes (P) identified in animals fed (A) placebo diet, (B) diet supplemented with clofibrate, (C) diet supplemented with clofibrate + pyruvate, and (D) diet supplemented with clofibrate + vitamin E. (Original magnification × 28,000.)

Nitric oxide is a short-lived free radical derived from L-arginine and synthesized by the enzyme nitric oxide synthase. Once produced, nitric oxide rapidly and spontaneously degrades to nitrite and nitrate, which can be detected in the circulation. Nitric oxide is known to interact with oxygen free radicals to neutralize them or to form the toxic radical peroxynitrite. During endotoxemia or chronic inflammation, hepatocytes express an inducible nitric oxide synthase,[21] and subsequent increases in nitric oxide are thought to protect the liver from oxygen radical–mediated injury.[22] In the present study, pyruvate in the presence of clofibrate induced an increase in plasma concentrations of nitrite and nitrate, but the mechanism and implications of this effect are unclear. One possibility is that pyruvate increases nitric oxide formation. Alternatively, the effects of clofibrate could include an increase in nitric oxide, which is not detected due to oxygen radical interaction, and pyruvate unmasks this effect by preventing oxygen radical production.

Our data suggest that the 3-carbon compound pyruvate has effects on free radical production and subsequent lipid peroxidation. In our experimental model, peroxisome proliferation was altered to a greater extent than mitochondria (Fig 5), and subsequent organelle free radical generation

might be differentially stimulated. However, pyruvate might offer promise in the control of the metabolic effects of free radicals as an inhibitor of free radical production. Vitamin E did not influence free radical generation. These findings suggest that effectors of free radicals will have individual specific effects on free radicals and/or metabolic sequelae. These effects should be identified before therapeutic intervention with effectors is considered in disease states associated with free radicals such as postischemic myocardial and intestinal injury,[23] adult respiratory distress syndrome,[24] and malignancy.[25]

The mechanism whereby peroxisomes proliferate in the hypolipidemic-drug model is not established. Proliferation varies with species,[26] and is absent in monkeys.[27] Nevertheless, the inhibitory effect of pyruvate on peroxisomal proliferation in our clofibrate rat model is intriguing. It has been suggested that increased hepatic lipid stimulates peroxisomal proliferation.[28] Thus, inhibitory effects of pyruvate on hepatic[6] and total-body lipid deposition[3,4] might be interrelated with the effects of pyruvate on peroxisomal proliferation. Recent studies have identified a peroxisome proliferator–activated receptor as a member of a novel steroid hormone receptor superfamily.[29-32] The expression of peroxisome proliferator–activated receptor has been shown to be tissue-specific, with the highest level of expression in the liver.[29] The liver is also the tissue most responsive to peroxisome proliferators.[33] Whether peroxisome proliferator–activated receptor is influenced by pyruvate is not known.

Peroxisomal proliferation has been associated with the production of hepatic carcinoma.[19] Pyruvate inhibits the growth of mammary adenocarcinoma in rats.[7] Nitric oxide has recently been shown to mediate apoptosis in macrophages[34] and to induce DNA breakage.[35] The relationship of total-body and hepatic lipid, peroxisomal proliferation, free radicals, carcinogenesis, and the effects of supraphysiologic concentrations of pyruvate remains to be elucidated.

We conclude that with clofibrate-induced peroxisomal proliferation and free radical production, pyruvate supplementation as 10% of energy intake will inhibit peroxisomal proliferation and free radical production, inhibit free radical–induced lipid peroxidation, and enhance metabolism of nitric oxide.

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