Inverse Relationship Between Peroxisomal and Mitochondrial β-Oxidation in HepG2 Cells Treated with Dehydroepiandrosterone and Clofibric Acid (43865)

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Abstract. **A transformed human hepatoma cell line was examined to determine if it was an appropriate model system for studying the mechanism of action of two peroxisome proliferators that lower blood lipids. Cultures of HepG2 cells were exposed to four different concentrations of either the hypolipidemic drug, clofibric acid (CLO), or the adrenal steroid, dehydroepiandrosterone (DHEA). Activities of two peroxisomal enzymes, palmitoyl-CoA oxidase and catalase, and two mitochondrial enzymes, carnitine palmitoyl-CoA transferase and succinate-INT-reductase, were measured in CLO- and DHEA-treated cells. In general, as the concentration of these hypolipidemic agents increased from 0 to 1000 μM, the specific activities of peroxisomal palmitoyl-CoA oxidase and catalase increased, and mitochondrial carnitine palmitoyl-CoA transferase and succinate-INT-reductase decreased. The activity of lactate dehydrogenase was significantly higher in the medium of cultures exposed to the 500 and 1000 μM concentration of DHEA compared with the control cultures, indicating the cytotoxic effects of this steroid at millimolar levels *in vitro*. In summary, the peroxisomal proliferators, DHEA and CLO, inversely altered peroxisomal and mitochondrial β-oxidation in HepG2 cultures, but not to the extent reported for rat hepatocytes *in vitro*. *In vitro* concentrations of DHEA greater than 500 μM adversely affected the viability of HepG2 cells. The results of this study suggest that β-oxidation in this human hepatoma cell line may not be as sensitive to hypolipidemic agents as are primary cultures of rat hepatocytes.** [P.S.E.B.M. 1995, Vol 208]

The adrenal hormone dehydroepiandrosterone (DHEA) has been shown to have many potential therapeutic properties for the treatment of atherosclerosis (1, 2), obesity (3, 4), diabetes (5), and certain types of cancer (6, 7). Clofibric acid (CLO) has been used clinically for the treatment of hyperlipidemia (8, 9). Like CLO, DHEA has been shown to reduce blood lipids (10, 11). Administration of both DHEA and CLO to rodents results in proliferation of peroxisomes and/or increased activities of peroxisomal enzymes involved in fatty acid oxidation (8, 12–14). It has been hypothesized that the hypolipidemic effects of CLO are due to increased peroxisomal fatty acid oxidation (15–21) and/or alterations in mitochondrial function in the liver (3, 4, 17–19, 22–24). Increased hepatic fatty acid oxidation induced by treatment with these hypolipidemic agents could be responsible, at least in part, for lowering blood lipids. *In vitro,* treatment of rat hepatocytes with CLO increases peroxisomal β-oxidation (17, 20, 21, 25). Previous efforts to elucidate the mech-

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anism of action of DHEA have been focused on enhanced peroxisomal β-oxidation following *in vivo* treatment of rodents with pharmacological levels of this steroid. Few studies have investigated the *in vitro* effects of DHEA on hepatocyte metabolism (26, 27). The direct effects of DHEA on human hepatocytes is not known. Although cultured rat hepatocytes are convenient models for studying the direct effects of specific agents on hepatic metabolism, animal models may not accurately predict how human hepatocytes metabolize these hypolipidemic agents (28). For this reason we used a human hepatoma cell line, HepG2, to examine the changes in peroxisomal and mitochondrial β-oxidation induced by DHEA and CLO. We used specific marker enzymes for peroxisomal and mitochondrial β-oxidation to evaluate the suitability of this human cell line for use in determining the mechanism of action of the hypolipidemic agents CLO and DHEA *in vitro*.

Materials and Methods

Cell Culture Procedures and Treatment. All cell culture medium and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. HepG2 cells (ATCC, Rockville, MD) were seeded at 2×10^6 cells per flask in T75 polystyrene disposable flasks (Corning Laboratory Science Co., Park Ridge, IL). Monolayers were allowed to grow to 70% confluency (approximately 5 days) prior to treatment in a humidified incubator (Fisher Scientific, Norcross, GA) at 37°C with an atmosphere of 95% air and 5% CO_2. Cells were maintained in Eagle's minimal essential medium (MEM; Sigma #0643) containing a final concentration of 1 m*M* sodium pyruvate, 10% fetal calf serum, 1% penicillin-streptomycin solution, and 0.2% fungisome prior to treatment. Cells were additionally supplemented during treatment with 1% fatty acid-free bovine serum albumin, 0.01 m*M* hydrocortisone, and 0.01 m*M* carnitine. At 70% confluency the cells were treated with 0, 250, 500, or 1000 μ*M* of dehydroepiandrosterone (DHEA) or clofibric acid (CLO) for 2 days prior to harvesting. Each of the eight treatment combinations were replicated four times for a total of 32 T75 flasks in this 2 × 4 factorial experiment. The factors tested were drug type (DHEA, CLO) and drug concentration (0, 250, 500, or 1000 μ*M*). These concentrations were selected based on preliminary studies with CLO in HepG2 cells (data not shown) demonstrating an effective concentration range of CLO that did not adversely affect cell viability. This is an appropriate concentration range for exposing rat hepatocytes in culture to CLO (21, 25). Concentrations of clofibrate less than 250 μ*M* did not significantly increase peroxisomal fatty acid oxidation in HepG2 cells following 48 hr of exposure. We used the DHEA concentration range of 250, 500, and 1000 μ*M* in the current study to parallel the effective concentrations of our positive control, CLO.

The DHEA and CLO were initially dissolved at a concentration of 130 m*M* in a 50:50 mixture of ethanol and dimethyl sulfoxide (DMSO) prior to adding to the medium. The final concentration of the ethanol and DMSO mixture in all cell cultures was 0.8%, including the control flasks. This level of solvent was found in pilot studies (data not shown) to have no significant effect on fatty acid oxidation in these cells. Fresh medium containing each drug was changed after 24 hr of incubation and the cells were harvested after 48 hr of treatment. The cultures were observed daily with an inverted microscope (Olympus, Lake Success, NY) for morphological changes. At the time of cell harvest, medium was removed by aspiration and the monolayer was gently washed twice with 10 ml ice cold buffer containing 0.5 *M* Tris (pH 7.4) and 0.15 *M* KCl. The cells were dislodged by scraping with a plastic scraper in 3.0 ml of buffer and transferred by pipette to a clean tube and stored on ice. After harvesting, the cells were sonicated two times with a Sonic Dismembrator sonicator (Fisher Scientific) at 15-sec intervals at a setting of 10. The disrupted cell material was divided into 0.5 ml aliquots, immediately flash frozen in liquid nitrogen, and stored at −70°C until the enzyme assays were conducted.

Assays. Protein concentration of the crude homogenate was determined according to the procedure of Bradford (29) using a commercially available assay kit (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as a standard. We were not successful in obtaining enough mitochondrial + peroxisomal protein from a T75 flask of cells via standard subcellular fraction procedures and therefore used crude homogenates of cells to conduct the enzyme assays. Changes in peroxisomal enzyme activity were determined by measuring the specific activities of catalase and palmitoyl-CoA oxidase in the crude homogenates. The activity of catalase was measured by following the disappearance of H_2O_2 at 240 nm (30). Palmitoyl-CoA oxidase activity was determined by measuring the oxidation of leuco-2,7-dichlorofluorescein (DCF) diacetate (Eastman Kodak, Rochester, NY) at 502 nm catalyzed by exogenous horseradish peroxidase and the production of H_2O_2 in the first step of β-oxidation (31). This spectrophotometric assay has been demonstrated to be much more sensitive than previous methods of measuring peroxisomal fatty acid oxidation, due to the large absorption coefficient of the dye DCF and the favorable stoichiometry of the peroxidation reaction (31).

For mitochondrial β-oxidation, we were obliged to work with flash-frozen cells and therefore could not measure β-oxidation directly since fresh tissue or cells (i.e., intact mitochondrial membranes) are required for

this procedure. For this reason, we measured the specific activities of a transporter of mitochondrial fatty acids, carnitine palmitoyl-transferase, and the inner mitochondrial membrane enzyme marker, succinate cytochrome c reductase (e.g., succinate-iodonitrotetrazolium [INT] reductase), in crude homogenates. Carnitine palmitoyl-CoA transferase activity was determined at 412 nm by measuring the release of CoA-SH from palmitoyl-CoA using Ellman's reagent (DTNB) in the presence and absence of carnitine (32). Succinate-INT-reductase was determined by incubating tissue samples with sodium succinate and INT formazan (33). After the reaction was terminated by the addition of ethyl acetate, ethanol, and trichloroacetic acid (5:5:1, vol:vol:weight), the supernatant was collected, and the reduction of INT was measured at 490 nm.

Cytotoxicity was determined by measuring the activity of lactate dehydrogenase in the cells and medium and by viewing morphological changes with an inverted microscope. Lactate dehydrogenase was measured according to Glascott *et al.* (34) by monitoring the oxidation of NADH at 340 nm in the presence of pyruvate.

Statistics. The main effects of drug type ($n = 2$), concentration ($n = 4$), and the drug by concentration interactions ($n = 8$) were analyzed by least squares analysis of variance (ANOVA) using the general linear models procedure (PROC GLM) of the SAS system (SAS, Cary, NC). Differences between the main effects and their interactions were considered significant at $P < 0.05$ (35) using the all-possible t test matrices of the least square means generated by the PROC GLM of SAS.

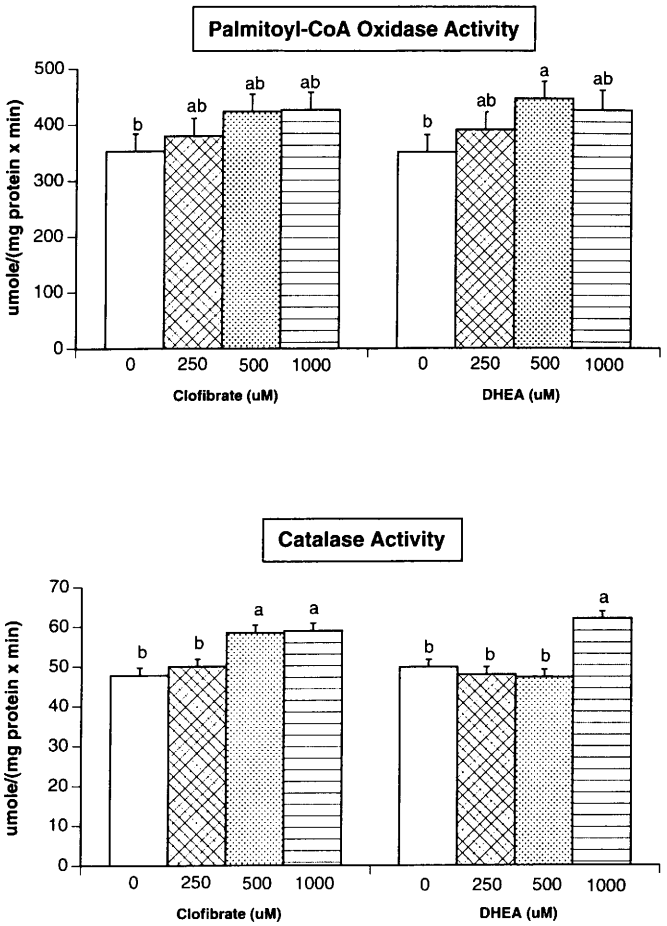

Figure 1. The effects of clofibric acid (CLO) and dehydroepiandrosterone (DHEA) on the specific activities of peroxisomal enzyme markers palmitoyl-CoA oxidase and catalase in monolayer cultures of HepG2 cells. Bars at each drug concentration represent arithmetic means + SEM. Bars sharing a common superscript are not significantly ($P < 0.05$) different.

Results

Peroxisomal Enzyme Markers. The arithmetic means of the eight treatment combinations for palmitoyl-CoA oxidase and catalase activity are presented in Figure 1. Although palmitoyl-CoA oxidase activity tended to be higher in cells treated with either drug at all concentrations, only cells treated with the 500 μM concentration of DHEA have palmitoyl-CoA oxidase activities that were significantly higher than the controls. Catalase activity was higher in cells treated with clofibrate at the 500 and 1000 μM concentrations, whereas only the 1000 μM concentration of DHEA significantly increased catalase activity compared with the control cells.

Mitochondrial Enzyme Markers. The arithmetic means of the eight treatment combinations for carnitine palmitoyl-CoA transferase and succinate-INT-reductase activity are presented in Figure 2. Carnitine palmitoyl-CoA transferase activity decreased as the concentration of both clofibrate and DHEA increased. Only those cells treated with the 1000 μM concentration of DHEA had succinate-INT-reductase activity significantly less than controls.

Evaluation of Cytotoxicity. The effects of clofibrate and DHEA treatment on percentage of total lactate dehydrogenase activity in cells and medium are presented in Figure 3. Clofibrate had no effect on lactate dehydrogenase activity. In contrast, total lactate dehydrogenase activity decreased in the cells and increased in the medium of cells exposed to the 500 and 1000 μM concentrations of DHEA. Cell cultures exposed to the 1000 μM concentration of DHEA had approximately 70% of their total lactate dehydrogenase activity present in the medium, indicating cell lysis.

This cytotoxic effect of DHEA was further supported by observations made by viewing the cells under an inverted scope. After 48 hr in culture, control cells were at approximately 90% confluency. All cells treated with clofibrate appeared normal regardless of drug concentration. The growth rate of clofibrate-treated cells was similar across drug concentrations.

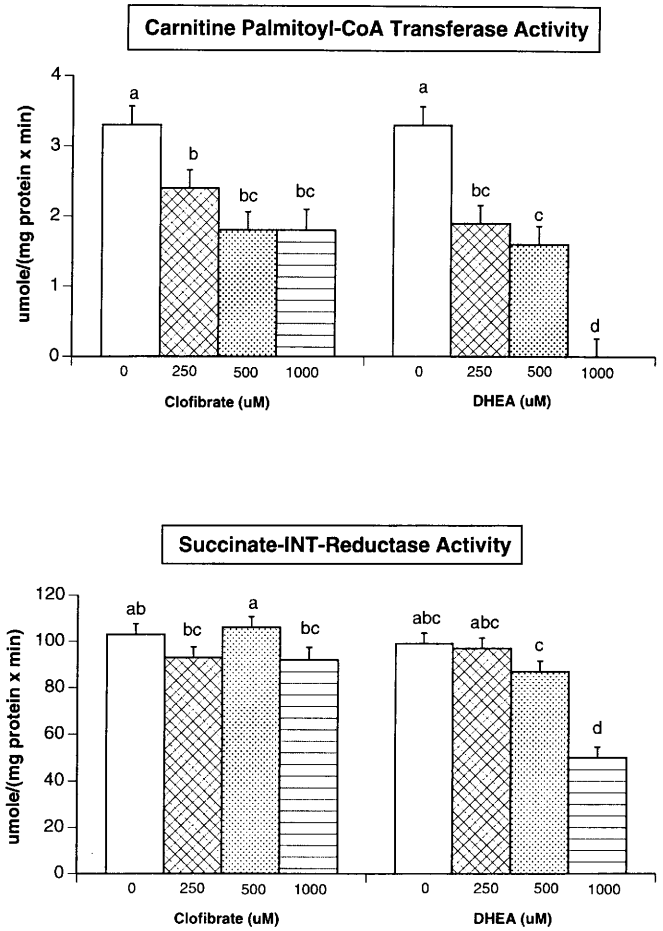

Figure 2. The effects of clofibric acid (CLO) and dehydroepiandrosterone (DHEA) on the specific activities of mitochondrial enzyme markers carnitine palmitoyl-CoA transferase and succinate-INT-reductase in monolayer cultures of HepG2 cells. Bars at each drug concentration represent arithmetic means + SEM. Bars sharing a common superscript are not significantly ($P < 0.05$) different.

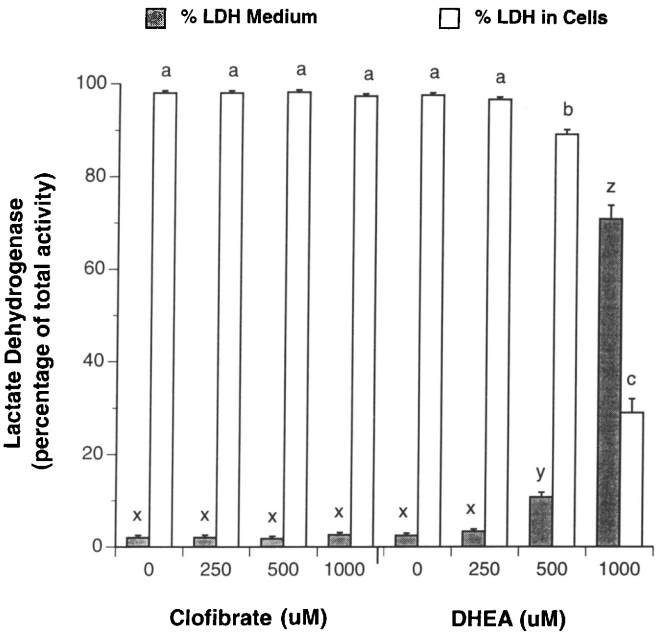

Figure 3. The effects of clofibric acid (CLO) and dehydroepiandrosterone (DHEA) on the percentage of total activity of lactate dehydrogenase (LDH) in the HepG2 cells and in the medium. Bars at each drug concentration represent arithmetic means + SEM. Clear bars (LDH activity in the cells) sharing a common superscript (a–c) are not significantly ($P < 0.05$) different. Strippled bars (LDH activity in the medium) sharing a common superscript (x–z) are not significantly ($P < 0.05$) different.

After 48 hr, cultures of cells exposed to clofibrate were at 80%–85% confluency. Cells exposed to DHEA displayed a concentration-dependent reduction in growth rate at the end of the 48-hr treatment period. Cell growth appeared to be arrested as the level of DHEA increased in the medium. Cells treated with DHEA had the following confluencies at the end of treatment: 250 μ*M*, 75%; 500 μ*M*, 60%; and 1000 μ*M*, 30%. In addition to this slow rate of growth of cells exposed to DHEA, the cells lost their normal shape and appearance as the concentration of DHEA increased in the medium. Cultures exposed to 1000 μ*M* of DHEA had a greater number of detached and anchored cells with irregular shapes. Crystals of DHEA appeared to be attached to the bottoms of the flasks treated with the 1000 μ*M* concentration of DHEA. Cells treated with clofibrate appeared to divide normally and maintained a polygonal shape for all treatment concentrations and for the duration of treatment. In contrast, DHEA-treated cells exhibited physical changes that increased with treatment concentration and duration. DHEA-treated cells appeared rounded and tended to lift off the surface of the culture flask when treated at the 500 and 1000 μ*M* concentrations.

Discussion

Some of the hypotheses that have been proposed on the hypolipidemic mode of action of DHEA *in vivo* include: (i) increased thermogenesis (12, 36–38); (ii) decreased lipogenesis and increased lipolysis in adipose tissue (3, 4, 39); (iii) decreased lipogenesis in the liver (3, 4, 11, 37); and (iv) increased mitochondrial and/or peroxisomal fatty acid oxidation in liver (3, 4, 9–14, 19, 26, 27, 36–38). Whether the effect of DHEA *in vivo* on hepatic fatty acid oxidation is due to the direct effect of this steroid on fatty acid oxidation in liver or is due to its effect on substrate availability is not known.

The major purpose of this study was to determine the direct effects of DHEA and clofibrate on markers of β-oxidation in the human hepatoma cell line HepG2. The data from this study suggest that DHEA and clofibrate tend to increase markers of peroxisomal fatty acid oxidation and decrease markers of mitochondrial fatty acid oxidation in HepG2 cells (Fig. 1 and 2). Unlike mitochondrial β-oxidation that reduces FAD^+, the first step of peroxisomal β-oxidation couples the oxidation of fatty acids with the reduction of oxygen to form H_2O_2. This lowers metabolic efficiency compared with mitochondrial respiration since 2 ATP (1

$FADH_2$) are lost per cycle of peroxisomal β-oxidation. Peroxisomes do not contain enzymes of the citric acid cycle or the electron transport chain. Consequently, the acyl-CoA and chain-shortened fatty acyl-CoAs produced from peroxisomal β-oxidation must be transported into mitochondria for ATP synthesis to occur. This transport is an energy-requiring process. Therefore, increasing the percentage of fatty acids oxidized by peroxisomes reduces the efficiency of energy transfer during lipid oxidation.

The hypolipidemic actions of DHEA and clofibrate could therefore be due to their stimulatory effects on hepatic peroxisomal fatty acid oxidation. The greater the amount of energy fuels oxidized, the lower the substrate availability for lipid synthesis. The lower the rate of hepatic lipid synthesis, the lower the rate of very low density lipoprotein (VLDL) secretion by the liver. Less VLDL in the blood could result in a reduction in plasma low-density lipoprotein (LDL) and the risk of coronary heart disease. We have previously shown that treatment of rats with DHEA decreases hepatic lipid synthesis (37) and serum triglycerides (11, 14) and cholesterol (14). In humans, Nestler *et al.* (1, 10) have shown that DHEA treatment lowers body fat and plasma LDL-cholesterol, two risk factors associated with the development of atherosclerosis (1, 2).

Many investigators have demonstrated that *in vitro* treatment of primary cultures of rat hepatocytes with hypolipidemic agents such as clofibrate (17, 20, 21, 25) and DHEA (26, 27) for 48–72 hr results in a 5- to 25-fold increase in peroxisomal enzyme activity. For example, Yamada *et al.* (26) demonstrated that primary cultures rat hepatocytes exposed to 50–250 μ*M* concentrations of DHEA had 11- and 17-fold higher levels of peroxisomal β-oxidation and carnitine acetyltransferase activities, respectively, than control cultures. Cederbaum *et al.* (22), on the other hand, demonstrated that *in vitro* treatment of isolated mitochondria with clofibrate inhibited mitochondrial respiration. Sonka *et al.* (40) and McIntosh *et al.* (41) observed that *in vitro* treatment of isolated mitochondria with micromolar levels of DHEA inhibited mitochondrial respiration in a concentration dependent fashion. Skorin *et al.* (42) also found that when rat hepatocytes were treated with mitochondrial carnitine palmitoyl-CoA transferase inhibitors, peroxisomal fatty acid oxidation increased. They proposed that this inverse relationship was due to the increase in fatty acid substrate availability for peroxisomal β-oxidation. Brady *et al.* (19) found that treatment of primary cultures of rat hepatocytes with DHEA for 4 hr increased transcription rates of markers for both mitochondrial and peroxisomal oxidation. Interestingly, the effect was much greater on the marker for peroxisomal β-oxidation (i.e., carnitine octanoyltransferase mRNA) than for the marker for mitochondrial β-oxidation (i.e., carnitine palmitoyltransferase mRNA). Similarly, Mitchell *et al.* (21) found that clofibrate treatment (1 m*M*) induced a 15-fold increase in peroxisomal palmitoyl-CoA oxidation and a 5-fold increase in mitochondrial glycerophosphate dehydrogenase activity in primary rat hepatocyte cultures. These data suggest that clofibrate, and possibly DHEA, may indeed increase hepatic fatty oxidation, with a greater percentage of fatty acids oxidized by the less energy efficient peroxisomal β-oxidation pathway.

It is known that human liver cells are less responsive to peroxisome proliferators than those obtained from mice or rats (9, 18). In addition, hepatoma cells are not identical metabolically to hepatocytes, even from within the same species. Watkins *et al.* (43) demonstrated that human HepG2 cells had 50% lower rates of peroxisomal β-oxidation compared to human hepatocytes. They also demonstrated that HepG2 cells had acyl-CoA oxidase and very long chain fatty acyl-CoA synthase activities. We found (unpublished data) that HepG2 cells have about 10-fold less peroxisomal β-oxidation activity using palmitoyl-CoA as substrate compared with rat hepatocytes. Aguis *et al.* (28) found that the oxidation of palmitate was 50% less in cultures of human hepatocytes compared with cultures of rat hepatocytes. Similarly, mitochondrial palmitate oxidation in human hepatocytes was 100 times more sensitive to the inhibitor R-etomoxir than in rat hepatocytes. Prip-Buus *et al.* (44) found that rat hepatoma cells have lower rates of oleate oxidation but higher rates of octanoate oxidation when compared with rat hepatocytes. They hypothesized that long-chain fatty acid oxidation in rat hepatoma cells is limited by high rates of lipogenesis since malonyl-CoA inhibits mitochondrial carnitine palmitoyl-CoA transferase activity. Data from the present study agree with these previous reports on the decreased rates of peroxisomal and mitochondrial β-oxidation in several rat and human hepatoma cells compared with rat and human hepatocytes.

The HepG2 cell may not have fully developed subcellular organelles, and therefore may express peroxisomal enzymes at very low levels or not at all. Hepatoma cells such as HepG2 grow very rapidly, and express many liver-specific metabolic functions including triglyceride, phospholipid, and lipoprotein synthesis (45). Intact HepG2 cells, however, are glycolytic in nature, metabolizing glucose primarily to lactate instead of CO_2 (46). This low oxidative capacity of HepG2 cells appears to also extend to oxidation of long chain fatty acids observed by Prip-Buus *et al.* (44) in rat hepatoma cells and by our study. Prip-Buus *et al.* (44) hypothesized that the high lipogenic state and subsequent maintenance of high levels of malonyl CoA, coupled with the high glycolytic activity of rat hepatoma cells, may reduced their capacity for lipid

oxidation. This observation may also explain why human hepatoma cells such as HepG2 have a reduce capacity for fatty acid oxidation.

The results obtained by treating HepG2 cells with clofibrate and DHEA, while similar in magnitude and direction, may be based on different mechanisms. In order for hepatocytes to metabolize clofibrate, this hypolipidemic agent must first join with CoA to form a thioester. If HepG2 cells have limited amounts of CoA or minimal CoA synthase activity, the ability of clofibrate to induce β-oxidation would be limited. Clofibrate treatment did not appear to be toxic at any of the levels tested. However, DHEA treatment produced morphological changes, increased lactate dehydrogenase in the medium, and caused cell death at the 500 and 1000 μM concentrations. The significant decrease in mitochondrial enzyme activity at the highest concentration of DHEA treatment may therefore be due to cytotoxicity rather than a shift in metabolism for a downregulation of mitochondrial respiration. The concentration of DHEA used for *in vitro* studies should therefore be less than 500 μM to ensure cell viability. The investigation of the gene expression of these enzymes associated with fatty acid oxidation in HepG2 cells would be an important step in determining how responsive this model is to treatment by peroxisome proliferators. Transfection of HepG2 cells with genes encoding for the limiting or missing enzymes associated with fatty acid oxidation in these cells would provide valuable insight into the mechanism of action of peroxisomal proliferators in this human hepatoma cell model system.

In summary, although DHEA and clofibrate exposure influenced peroxisomal and mitochondrial beta-oxidation as hypothesized, the magnitude of response was nowhere near that previously demonstrated in primary cultures of rat hepatocytes. For these reasons, primary cultures of human hepatocytes need to be tested, and these results compared with those found in HepG2 cells, to determine if HepG2 cells can be used as a model for studying the mechanism of action of hypolipidemic agents.

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