EXPERIMENTAL STUDY

***In vitro* study of the effect of raloxifene on lipid metabolism compared with tamoxifen**

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Abstract

Objective: Tamoxifen and raloxifene, selective estrogen receptor modulators, decrease serum concentrations of total cholesterol; however, the effect of these drugs on triglyceride metabolism is unclear. In the present study, we investigated the *in vitro* effect of raloxifene on lipid metabolism and compared it with that of tamoxifen.

Design and Methods: Intracellular concentrations of total cholesterol and triglyceride in HepG2 cells were measured by an enzymatic method after tamoxifen or raloxifene treatment with or without oleic acid and with or without very low density lipoprotein.

Results: Intracellular concentrations of total cholesterol and triglyceride without oleic acid or very low density lipoprotein were not significantly different after treatment with tamoxifen or raloxifene. In contrast, although raloxifene with oleic acid did not increase the intracellular concentrations of triglyceride, tamoxifen treatment in the presence of oleic acid or very low density lipoprotein significantly increased ($P < 0.05$) the triglyceride concentrations.

Conclusion: The present study suggests that raloxifene does not increase intracellular triglyceride in the presence of oleic acid or very low density lipoprotein, in contrast to tamoxifen. Therefore, raloxifene might be safer than tamoxifen for treating patients with unstable triglyceride levels or a history of hypertriglyceridemia.

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Introduction

Tamoxifen, a non-steroidal antiestrogenic drug, is one of the most widely used hormonal treatments for the management of breast cancer (1–4). In addition, several studies have shown that tamoxifen reduces the incidence of events related to ischemic heart disease (5–8). Moreover, paradoxically tamoxifen is also known to have weak agonistic effects in a number of physiological systems including bone and lipid metabolism (9, 10). The mechanism responsible for these effects has not been established but may involve beneficial changes in blood lipids. There have been numerous reports over the past decade about the effect of tamoxifen on serum lipid profiles; these showed an average decrease of 13% in total cholesterol and an average decrease of 19% in low density lipoprotein (11). In contrast, the effect of tamoxifen on triglyceride levels is unclear. Tamoxifen caused an increase in serum triglycerides in a few susceptible patients (12–16). We have already determined the effects of tamoxifen on

lipid metabolism, especially triglyceride metabolism. Because the possibility exists that a marked increase in triglycerides will not only cause fatal pancreatitis but is also closely related to an elevated risk of coronary heart disease (17, 18), the effect of tamoxifen on triglyceride metabolism should not be ignored.

Raloxifene, a benzothiophene derivative that binds to the estrogen receptor, is a selective estrogen receptor modulator like tamoxifen, that is used to treat and prevent osteoporosis in postmenopausal women (19, 20). Preliminary data have shown that raloxifene can reduce the risk of breast cancer without adversely affecting the endometrium in women being treated to prevent osteoporosis (21). As a result, raloxifene is being compared with tamoxifen in a new breast cancer prevention trial, P-2 (NSABP, P-2). In addition, a few available clinical and laboratory studies have demonstrated that raloxifene decreases serum concentrations of total cholesterol without raising triglycerides (19, 20, 22, 23).

However, the mechanisms of action of raloxifene and tamoxifen on lipid metabolism are still not known.

In the present study, we investigated the effect of raloxifene on lipid metabolism compared with that of tamoxifen *in vitro*, using HepG2 cell lines derived from human hepatoma cells.

Materials and methods

Materials

Minimum essential medium (MEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), L-glutamine, and penicillin/streptomycin were purchased from GIBCO Laboratories (Grand Island, NY, USA). Bovine serum albumin (BSA) (essentially fatty acid-free), sodium pyruvate (NaPy), and oleic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). Human purified very low density lipoprotein (VLDL) was purchased from Biodesign International (Kennebunk, ME, USA). 4-Hydroxytamoxifen (4-OHT) was a generous gift from Zeneca Pharmaceutical (Macclesfield, Cheshire, UK) and raloxifene was a generous gift from Eli Lilly and Company (Indianapolis, IN, USA, originally named Keoxifene).

Cell cultures

HepG2 cells obtained from American Type Culture Collection (ATCC no. HB-8065, Rockville, MD, USA) were routinely grown in MEM supplemented with 10% FBS, 1 mmol/l NaPy, 2 mmol/l L-glutamine, 1% NEAA, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. The cells were grown in 75-mm² flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed 3 times weekly and the cells were subcultured weekly with a split ratio of 1:3 after trypsinization (0.25% trypsin – 0.03% EDTA in phosphate-buffered saline (PBS)). At confluency, cells were plated out in 24-well plates at a density of 2×10^4 cells/cm² for experiments.

Experimental protocols

Cell experiments and measurement of intracellular lipids

HepG2 cells were preincubated overnight in serum-free MEM supplemented with 1% BSA. The following day, after removing the media, the cells were incubated for an additional 24 h in 1 ml media containing the appropriate compounds (raloxifene and 4-OHT) with or without free fatty acid (approximate 18 µmol/l oleic acid). Oleic acid was dissolved in 1% BSA. We also carried out another experiment with or without VLDL (50 µg/ml). The concentration of these compounds was 10^{-6} mol/l. Both compounds were dissolved in 100% ethanol and added to media at a 1:1000 dilution. At the end of the incubation period, the cells were washed 3 times with 1 ml ice-cold PBS, and the soluble cell protein was dissolved in 1 ml 0.1 mol/l NaOH and

measured using the method of Bradford (24) with BSA as the standard.

To determine the intracellular triglyceride and total cholesterol, after washing 3 times with cold PBS, the cells were treated with 1 ml hexane/isopropanol (2:1) for 30 min at room temperature. These samples were transferred to the test tubes. The wells were washed with 1 ml hexane/isopropanol, and the washing solutions were also transferred to the corresponding test tubes. The organic solvent was removed under nitrogen, and the lipids were resuspended in 95% ethanol. Cellular concentrations of triglyceride and total cholesterol were measured by an enzymatic kit (TG-555 and TC-555; Kyowa Medics, Tokyo, Japan). Intracellular concentrations of total cholesterol from the cells, which were incubated only with these compounds but without oleic acid and without VLDL, were measured.

Statistics

All data were expressed as the mean $\pm$ standard error of the mean. Data were analyzed using both the paired and unpaired Student's *t*-test. The results were considered to be significant when $P < 0.05$.

Results

Both 4-OHT and raloxifene reduced intracellular concentrations of total cholesterol in HepG2 cells at concentrations ranging from 10^{-7} to 10^{-5} mol/l ($P < 0.005$ and $P < 0.01$ respectively; Fig. 1); however, there was no significant difference between the two groups. Neither 4-OHT nor raloxifene changed the intracellular concentration of triglyceride in the absence of oleic acid (Fig. 2). In the presence of oleic acid, raloxifene produced no changes in the intracellular concentration of triglyceride, whereas 4-OHT increased the intracellular concentration of triglyceride at concentrations ranging from 10^{-7} to 10^{-5} mol/l ($P < 0.05$; Fig. 2). Moreover, in the presence of VLDL raloxifene did not increase the intracellular concentration of triglycerides, whereas 4-OHT did at a concentration of 10^{-6} mol/l ($P < 0.05$; Fig. 3).

Discussion

Tamoxifen has been shown to have a beneficial effect on lipid profiles, especially cholesterol metabolism, in numerous clinical and laboratory studies (5–11). However, it is controversial whether or not tamoxifen affects triglyceride metabolism. Several clinical studies have indicated that tamoxifen raises serum triglyceride levels in susceptible patients (12–16). Moreover, tamoxifen induces the development of a fatty liver without a gain in body weight (25). Oleic acid and VLDL have already been reported to increase triglyceride synthesis and the intracellular lipid content of HepG2 cells (26).

Figure 1 Intracellular concentration of total cholesterol in HepG2 cells treated with 4-OHT or raloxifene. Both 4-OHT (○) and raloxifene (●) reduced intracellular concentrations of total cholesterol at doses higher than 10^{-7} mol/l. * $P < 0.005$; ** $P < 0.01$ vs control.

Figure 2 Intracellular concentration of triglyceride in HepG2 cells treated with 4-OHT in the presence (○) or absence (□) of oleic acid, and with raloxifene in the presence (●) or absence (■) of oleic acid. Raloxifene produced no changes in the intracellular concentration of triglyceride following treatment with oleic acid, whereas tamoxifen in the presence of oleic acid increased the intracellular concentration of triglyceride. * $P < 0.05$ vs control.

Figure 3 The effect of 4-OHT and raloxifene on intracellular concentration of triglyceride in HepG2 cells treated with 50 µg/ml very low density lipoprotein (VLDL). * $P < 0.05$ vs control.

In the present study, both 4-OHT and raloxifene significantly reduced intracellular cholesterol concentrations in HepG2 cells. These data are in agreement with numerous clinical studies, in which these agents decreased serum total cholesterol (11). Tamoxifen and raloxifene also did not produce changes in intracellular triglyceride concentrations in the absence of oleic acid or VLDL, whereas tamoxifen increased intracellular triglyceride concentrations following treatment with oleic acid or VLDL. Most importantly, raloxifene did not change the triglyceride concentration in HepG2 cells in the presence of oleic acid or VLDL. Since we could not detect triglyceride in medium by the enzymatic method, the secretion of triglyceride from hepatocytes might be small. These data suggest that tamoxifen but not raloxifene might increase serum triglyceride and/or cause the development of fatty liver in susceptible patients in those ingesting a high-fat diet, although we did not carry on the experiment of triglyceride secretion from HepG2 cells. Therefore, raloxifene might potentially be a safer treatment in patients with unstable triglyceride levels or a history of hypertriglyceridemia. It might also be important to advise patients to ingest less fat when taking tamoxifen.

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