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Raloxifene affects fatty acid oxidation in livers from ovariectomized rats by acting as a pro-oxidant agent

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HIGHLIGHTS

- The effects of raloxifene on fatty acid oxidation were examined in ovariectomized rats.
- Ketogenesis was reduced and $^{14}CO_2$ production from fatty acids was stimulated.
- Mitochondrial and peroxisomal β-oxidation of fatty acids were inhibited.
- The peroxidase-catalized reaction of raloxifene with H_2O_2 led to NADH oxidation.
- The pro-oxidant effect of raloxifene can perturb important liver metabolic processes.

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ABSTRACT

Estrogen deficiency accelerates the development of several disorders including visceral obesity and hepatic steatosis. The predisposing factors can be exacerbated by drugs that affect hepatic lipid metabolism. The aim of the present work was to determine if raloxifene, a *selective estrogen receptor modulator* (SERM) used extensively by postmenopausal women, affects hepatic fatty acid oxidation pathways. Fatty acids oxidation was measured in the livers, mitochondria and peroxisomes of ovariectomized (OVX) rats. Mitochondrial and peroxisomal β-oxidation was inhibited by raloxifene at a concentration range of 2.5–25 μM. In perfused livers, raloxifene reduced the ketogenesis from endogenous and exogenous fatty acids and increased the β-hydroxybutyrate/acetoacetate ratio. An increase in $^{14}CO_2$ production without a parallel increase in the oxygen consumption indicated that raloxifene caused a diversion of NADH from the mitochondrial respiratory chain to another oxidative reaction. It was found that raloxifene has a strong ability to react with H_2O_2 in the presence of peroxidase. It is likely that the generation of phenoxyl radical derivatives of raloxifene in intact livers led to the co-oxidation of NADH and a shift of the cellular redox state to an oxidised condition. This change can perturb other important liver metabolic processes dependent on cellular NADH/NAD^+ ratio.

1. Introduction

Raloxifene is a *selective estrogen receptor modulator* (SERM) of the benzothiopene class (Snyder et al., 2000) that has been used extensively to preserve the beneficial effects of estrogen in postmenopausal women (Delmas et al., 1997; Hochner-Celnikier, 1999). Because estrogens are important regulators of metabolic homeostasis and lipid metabolism (Chen et al., 2009; Nemoto et al., 2000; Campbell and Febbraio, 2001; Foryst-Ludwig and Kintscher, 2010), their deficiencies have been demonstrated to accelerate the development of visceral obesity (Carr, 2003; Poehlman et al., 1995), insulin resistance, type 2 diabetes, dyslipidaemia (Stevenson et al., 1993), hepatic steatosis (non-alcoholic fatty liver disease — NAFLD, Hewit et al., 2004; Mu et al., 2009), hypertension and cardiovascular diseases (Mendelson and Karas, 1994). The cellular mechanisms by which estrogen deficiency induces deregulation of liver metabolism, including hepatic steatosis, have not been completely elucidated (Hewit et al., 2004; Nemoto et al., 2000). Most of the evidence of the role of estrogens in liver metabolism has resulted from the measurement of enzyme expression in ovariectomized (OVX) rats or aromatase-deficient animals in which estrogens were administered to the animals. Under these conditions, estrogen regulates the activity and the expression of the key enzymes involved in glucose transport, glycolysis, the citric acid cycle, the mitochondrial respiratory chain and fatty acid oxidation (Chen et al., 2009; Nemoto et al., 2000; Campbell and Febbraio, 2001; Foryst-Ludwig and Kintscher, 2010).

It has been observed that certain drugs can precipitate or exacerbate steatosis and steatohepatitis by accentuating the predisposing factors, including those factors associated with estrogen deficiency

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(Farrel, 2002; Mu et al., 2009). The steatotic phenotype of PPARα (peroxisome proliferator-activated receptor)-null mice, for example, is exacerbated by etomoxir, which abolishes lipid oxidation by inhibiting long-chain fatty acid transport into the mitochondria (Djouadi et al., 1998; Farrel, 2002; Mu et al., 2009). Tamoxifen (TAM), the most well-known SERM, acts as an inhibitor of fatty acid β-oxidation and oxidative phosphorylation (Berson et al., 1998; Tuquet et al., 2000) and it has demonstrated to induce steatosis, steatohepatitis and cirrhosis in some women during the treatment of breast cancer (Oien et al., 1999; Pratt et al., 1995).

Raloxifen, on the other hand, is used in the menopausal period, in which there is an increased prevalence of lipid metabolism disturbances (Hewit et al., 2004; Mu et al., 2009). Nevertheless, few studies have been conducted concerning the effects of RLX on lipid metabolism in female animals or humans, particularly during their menopausal period. The purpose of the present work was thus to examine the effects of RLX on fatty acid metabolism in an experimental model of estrogen deficiency in rats.

The evaluation of the metabolism of a medium-chain and a long-chain fatty acid can help in the understanding of the mechanisms implicated in the possible metabolic alterations, since there are differences in the enzymatic systems responsible for the entry of these fatty acids into the liver cells (Guo et al., 2006), the transformation to acyl-CoA (McGarry and Brown, 1997; Eaton, 2002), the dependence from L-carnitine for the acyl-CoA entry into the mitochondria and in the enzymes that catalize the first steps of mitochondrial β-oxidation (McGarry and Brown, 1997). Moreover, both the medium- and long-chain fatty acids are also oxidized in the peroxisomes (Grum et al., 1994; Mannaerts et al., 1979; Piot et al., 1998; Reddy and Mannaerts, 1994).

For these reasons, in this work the oxidation of octanoate (medium-chain fatty acid) and palmitate (long-chain fatty acid) was assessed in the perfused livers and in isolated mitochondria and peroxisomes from ovariectomized (OVX) rats. The capacity of raloxifene in the induction of the peroxidase-dependent catalytic oxidation of H_2O_2 was also measured.

2. Materials and methods

2.1. Materials and chemicals

Raloxifene, ATP, ADP, NADH, phenylmethylsulfonyl fluoride (PMSF), o-phthalaldehyde (OPT), horseradish peroxidase type IV, 2′,7′-dichlorofluorescein diacetate (DCFH), 2′,7′-dichlorofluorescein (DCF), 2,4-dinitrophenol (DNP), fatty acid-free bovine serum albumin (BSA), L-carnitine, thiobarbituric acid, 2,5-diphenyloxazole (PPO), 2,2-p-phenylene-bis-5-phenyloxazole (POPOP), [^{14}-C]-octanoate and [^{14}-C]-palmitate were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA). All other reagents were of the best available grade.

2.2. Animals

For ovariectomy surgery, rats weighing 130–160 g (6 weeks of age) were anaesthetised with ketamine plus xylazine (50 and 5 mg/kg i.p., respectively). Female rats in metestrus were used as controls (Marcondes et al., 2002). The animals were housed in polycarbonate cages and their environment was controlled for a 12:12 h light–dark cycle starting at 06:00 AM, at 20–23 °C. All animals had free access to a standard rodent diet (Nuvilab®, São Paulo, Brazil) and tap water. The experiments were conducted three weeks after the ovaries were removed. All experiments were conducted in adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (certified n. 079/2008). The body weight and food intake of the rats were assessed each morning. Overnight-fasted animals were anaesthetised for blood collection by cardiac puncture. The plasma glucose concentrations were determined using a glucose analyser (Optium®). The total cholesterol and triacylglyceride levels were analysed by standard methods (kits of Gold Analisa®).

2.3. Liver perfusion

The non-recirculating perfusion technique described by Scholz and Bücher (1965) was used. For the surgical procedure, the rats were anaesthetised by i.p. injection of sodium pentobarbital (50 mg/kg). The perfusion fluid was a Krebs/Henseleit bicarbonate buffer (pH 7.4) saturated with an oxygen/carbon dioxide mixture (95/5%). The fluid was pumped through a temperature-regulated (37 °C) membrane oxygenator prior to entering the liver via a cannula inserted into the portal vein. The perfusion flow was constant in each individual experiment, and it was adjusted to be between 28 and 32 ml/min, depending on the liver weight. Raloxifene (25 μM), octanoate (50 μM), palmitate (0.3 mM), fatty acid-free bovine serum albumin (50 or 150 μM), traces of [1-^{14}C]octanoate (6.7 nCi/ml) or [1-^{14}C]palmitate (1.7 nCi/ml) were dissolved in the perfusion. The oxygen concentration in the venous perfusate was monitored with a Teflon-shielded platinum electrode. Samples of the effluent perfusion fluid were collected in 2–4 min intervals and analysed for acetoacetate, β-hydroxybutyrate and $^{14}CO_2$ content. Acetoacetate and β-hydroxybutyrate were measured by standard enzymatic procedures (Mellanby and Williamson, 1974; Williamson and Mellanby, 1974). Carbon dioxide production was measured by trapping $^{14}CO_2$ in phenylethylamine (Scholz et al., 1978). The radioactivity was measured by liquid scintillation spectroscopy. The following liquid scintillation solution was used: toluene/ethanol (2/1) containing 5 g/l 2,5-diphenyloxazole (PPO) and 0.15 g/l 2,2-p-phenylene-bis-5-phenyloxazole (POPOP). The metabolic rates were calculated from the input–output differences and the total flow rates and were referenced to the weight of the liver.

2.4. Isolation of mitochondria and peroxisomes

The livers were homogenised in a medium containing 0.2 M mannitol, 0.075 M sucrose, 1.0 mM Tris (pH 7.4), 0.2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mg% (w/v) fatty acid-free bovine serum albumin (BSA) (Bracht et al., 2003a). The homogenate was fractionated by sequential centrifugations at 536 × *g* and 7080 × *g* for 10 min. After two wash cycles by suspension and centrifugation at 6392 × *g*, the final mitochondrial pellet was suspended in a small volume of medium to yield a protein concentration of 70–80 mg/ml.

For peroxisomes isolation (Natarajan et al., 2006), the livers were excised and homogenised in 8 volumes of a medium containing 230 mM mannitol, 70 mM sucrose, 3 mM HEPES and 1 mM EDTA (pH 7.4). The homogenate was first centrifuged at 600 × *g* for 10 min, and then, the mitochondria were pelleted by centrifugation at 15,000 × *g* for 5 min. The post-mitochondrial supernatant was then centrifuged at 39,000 × *g* for 10 min to isolate the fraction including peroxisomes, which was resuspended and homogenised in 250 mM sucrose containing 1 mM EDTA and 10 mM Tris HCl (pH 7.3). This suspension was centrifuged at 15,000 × *g* for 10 min and the supernatant was again centrifuged at 39,000 × *g* to isolate the peroxisomes, which were resuspended at a final protein concentration of approximately 6–15 mg/ml. Protein concentrations were determined according to the method of Lowry et al. (1951) using BSA as a standard.

2.5. Oxygen consumption by mitochondria oxidising fatty acids and NADH

The incubation medium contained 2.0 mM potassium phosphate monobasic, 10 mM HEPES (pH 7.2), 0.1 mM EGTA, 130 mM potassium chloride, 5 mM magnesium chloride, 0.1 mM 2,4-dinitrophenol (DNP), 2.5 mM L-malate, 50 mg% fatty acid-free BSA and mitochondrial preparation (0.6–1.2 mg/ml) (Garland et al., 1969). The reaction was initiated by the addition of either 20 μM palmitoyl-CoA + 2.0 mM L-carnitine or 20 μM octanoyl-CoA + 2.0 mM L-carnitine. Mitochondria that had been disrupted by freeze-thawing were used as the source of NADH-oxidase. NADH (1.0 mM) was added to 20 mM Tris–HCl (pH 7.4) medium to start the reaction (Bracht et al., 2003b). RLX was added to the incubation medium 5 min before substrate addition at a concentration range of 2.5–25 μM. RLX was initially dissolved in dimethylsulphoxide (DMSO), and the final concentration of the solvent was 0.5% (v/v). Control reactions were performed to exclude the interference of DMSO.

2.6. Peroxisomal and mitochondrial fatty acyl-CoA oxidase activity

The fatty acyl-CoA oxidase activity was measured according to Small et al. (1985) with modifications (Taguchi et al., 1996). The assay mixture contained 11 mM potassium phosphate buffer (pH 7.4), 40 mM aminotriazole, 0.04 mg/ml horseradish peroxidase, 104 μM DCFH-DA and peroxisomes or mitochondria (approximately 0.3 mg/ml). Triton X-100 (0.02%) or L-carnitine (2 mM) was included in the reaction medium for assays with peroxisomes and mitochondria, respectively. The reaction was initiated by the addition of 30 μM octanoyl-CoA or palmitoyl-CoA. Raloxifene was added at 10 and 25 μM concentrations. The increase in fluorescence (excitation, 503 nm; emission, 529 nm) was recorded over a period of 10 min, and the activity of fatty acid acyl-CoA oxidase was expressed as nmol DCF produced/min mg of protein. The rates of H_2O_2 production were calculated from the linear regression analysis of the curves after subtracting the values of the blank curves.

2.7. NADH oxidation induced by reaction of raloxifene and H_2O_2 catalysed by peroxidase

The reaction mixtures contained 0.1 M Tris–HCl/1.0 mM EDTA buffer (pH 7.4), raloxifene (0.25–2.0 μM), H_2O_2 (25 μM), NADH (200 μM) and horseradish peroxidase (HRP) type VI-A (0.1 μM). The reactions were initiated by the addition of H_2O_2, and the oxidation of NADH was measured spectrophotometrically at 340 nm (Chan et al., 1999).