Beneficial Effects of Tibolone on Blood Pressure and Liver Redox Status in Ovariectomized Rats With Renovascular Hypertension

Eduardo Hideo Gilglioni,1 Lilian Brites Campos,1 Monique Cristine Oliveira,2 Rosângela Fernandes Garcia,1 Célia Regina Ambiel,1 Ana Julia dos Reis Buzzo,1 Emy Luzia Ishii-Iwamoto,2 and Clairece Luzia Salgueiro-Pagadigorria1,2

1Department of Physiological Science, and 2Department of Biochemistry, University of Maringá, Brazil.

Address correspondence to Clairece Luzia Salgueiro-Pagadigorria, Department of Biochemistry, University of Maringá, 87020–900 Maringá, Paraná, Brazil. Email: chspagadigorria@uem.br

Estrogen deficiency is associated with aging and increases the incidence of metabolic syndrome and hypertension. In this study, the effects of tibolone, a synthetic steroid, on the cardiovascular system, liver lipid metabolism, and redox status were evaluated, in ovariectomized (OVX) rats with renovascular hypertension (two-kidneys, one-clip, OVX + 2K1C). This study encompassed direct measurements of mean arterial pressure, plasma biochemical analysis, liver lipid contents, and assessments of the mitochondrial and peroxisomal β-oxidation capacities. Additionally, the liver redox status was assayed. Tibolone significantly reduced the mean arterial pressure of OVX + 2K1C rats, albeit reducing total and high-density lipoprotein (HDL) cholesterol levels. In the liver, although exerting an undesirable inhibition of mitochondrial and peroxisomal β-oxidation, tibolone reversed steatosis. Tibolone also improved the liver redox status: the reduced glutathione contents and the activity of glucose-6-phosphate dehydrogenase were restored by this compound, which also reduced the levels of thiobarbituric acid–reactive substances and the generation of mitochondrial reactive oxygen species. So, tibolone reversed the main alterations caused by hypertension and estrogen deficiency.

Key Words: Hypertension—Liver lipid metabolism—Liver redox status—Estrogen deficiency—Tibolone.

Received May 12, 2012; Accepted September 12, 2012

Decision Editor: Rafael de Cabo, PhD

MENOPAUSE is accompanied by profound disturbances in lipid metabolism and a higher incidence of metabolic syndrome and its comorbidities. Consequently, hypertension is the most frequent disease associated with the climacteric period (1). In addition, estrogen deficiency directly contributes to the development of hypertension by mechanisms that include the overproduction of vasoconstrictor factors such as endothelin (2), the hyperactivity of the sympathetic nervous system and the renin–angiotensin system (3), and oxidative stress (1).

Tibolone is a unique pharmacological agent: in tissues, it exhibits estrogenic, progestogenic, and androgenic effects. This makes it theoretically the ideal postmenopausal hormonal replacement, but still there is a need for studies examining the effect of tibolone in frail older women (4,5). Anyway, similar to the conventional hormone replacement therapies, tibolone has proven to be effective in the treatment of several abnormalities of the metabolic syndrome, including the prevention of weight gain in postmenopausal women (6) and in animal models of estrogen deficiency (7,8).

However, studies on the effects of tibolone on blood plasma parameters of postmenopausal women have raised concerns about its cardiovascular safety, because although tibolone has been shown to have some beneficial lipid effects, including reductions in plasma triacylglycerols (TG) and lipoprotein a, this compound reduces HDL cholesterol (9). This adverse effect of tibolone has also been described in animal models of estrogen deficiency (10) and has been attributed to alterations in the hepatic metabolism of cholesterol (11).

Furthermore, a more recent study has demonstrated that tibolone reduces blood pressure in healthy and hypertensive (12) postmenopausal women. The potential mechanism for this reduction can be related to increases in nitric oxide and to reductions in the endothelin plasma levels by tibolone and its metabolites (13). These results suggest a cardioprotective action of tibolone (7,12), which is independent of its effect on lipid metabolism.

Therefore, the purpose of this work was to evaluate the effect of tibolone on blood pressure and the plasma lipid profile in an animal model of estrogen deficiency associated with renovascular hypertension. The two-kidneys, one-clip model of hypertension described by Goldblatt and colleagues (14) was used in this work. In this model, the primary cause...
of hypertension is the hyperactivity of the renin–angiotensin system, and the coexistence of other confounding factors, such as underlying lipid disturbances, was avoided.

Besides, considering the adverse hepatic effects associated with the oral administration of estrogens (15), another purpose of this work was to evaluate the effects of tibolone on liver lipid metabolism and redox status in this animal model.

This investigation is justified because recent studies performed by our team (16) have revealed that, in isolated perfused liver, tibolone inhibits mitochondrial fatty acid β-oxidation, which is a common cause underlying drug-induced hepatotoxicity. Although this effect of tibolone on perfused liver has been observed only at toxic concentrations (100 µM) that could probably not be reached in humans treated with the recommended dose of tibolone (2.5 mg/kg), it occurred after a single passage through the liver. Therefore, in an animal model of estrogen deficiency associated with hypertension, the liver could be more susceptible to drug-induced injury and this effect could manifest even at therapeutic doses in animals treated for a prolonged period.

Methods

Materials

The apparatus for mitochondrial incubation was custom built in the workshops of the University of Maringá. All of the substrates used, as well as enzymes, coenzymes, adenosine diphosphate, 2,4-dinitrophenol, phenylmethylsulfonyl fluoride, sodium dodecyl sulfate, o-phthalaldehyde, horseradish peroxidase, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF), and urethane, were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium heparin was obtained from Roche (Rio de Janeiro, Brazil). Crystalline tibolone was obtained from New Cham S.P.A. (Italy). Kits from Gold Analisa were used for plasma analysis. Rat insulin enzyme-linked immunosorbent assays (ELISA) kits were used for the radioimmunoassays of plasma insulin. All reagent-grade chemicals were obtained at the highest available grade.

Animals

Groups of female Wistar rats (8 weeks old) were randomly designated for surgical procedures of sham operation (control), bilateral ovariectomy (OVX), or ovariectomy plus renovascular hypertension (OVX + 2KIC). The rats had free access to tap water and a standard laboratory diet (Nuvilab, São Paulo, Brazil). The food consumption and animal weights were measured throughout the entire experimental period (36 days). All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (certification number: 056/2011).

Surgical Procedures

For the surgical procedures, the animals were anesthetized with a mixture of xylazine–ketamine (10–50 mg/kg intraperitoneally). A single 15-mm peritoneal incision was made to perform both the removal of the ovaries and the induction of renovascular hypertension. The latter one was based on the constriction of the left renal artery through the application of a silver clip (internal diameter: 0.2 mm), with consequent reduction of the blood flow, following the Goldblatt 2K1C (two-kidney, one-clip) model (14). Sham-operated (control) rats underwent the same surgical procedure but without clipping the renal artery or removing the ovaries.

Treatment of Animals

Twenty-one days after the surgical procedures, the OVX + 2KIC groups were randomly subdivided into two groups: untreated rats (OVX + 2K1C) and animals treated with daily oral doses of tibolone (0.04 mg/kg, OVX + 2K1CT) suspended in 1% Arabic gum (final volume of 500 µL), by oral gavage, for a period of 15 days. All other groups (control, OVX, and OVX + 2K1C) received the same volume (500 µL) of the vehicle. The treatment of the animals was initiated 21 days after surgical procedures—the period required for the establishment of both conditions, estrogen deficiency and hypertension—as could be confirmed by the uterine atrophy and parallel experiments of pressure measurements, respectively. The animals were treated daily for 15 days, after which period, the effects of tibolone on blood pressure could be evidenced.

Mean Arterial Pressure Measurements and Plasma Biochemical Analysis

After ending the treatment, the overnight-fasted female rats were anesthetized with a combination of pentobarbital–urethane (50–600 mg/kg, intraperitoneally). A polyethylene cannula was inserted into the carotid artery and connected to a mercury manometer for the direct measurement of mean arterial pressure. The same cannula was used to collect blood for biochemical analyses.

Uterine atrophy was used as a marker of a well-established condition of estrogen deficiency, whereas the existence of atrophy of the left kidney and hypertrophy of the right kidney was used to evaluate the success of the hypertension induction surgery.

After the liver removal, retroperitoneal, uterine, mesenteric, and subcutaneous fats were collected and the ratio between the sums of the weights of these tissues per 100 g of body weight was used as the adiposity index.

Total cholesterol, HDL cholesterol, triacylglycerols (TG), and fasting glucose levels were analyzed by standard methods (Gold Analisa). Very low–density lipoprotein (VLDL) cholesterol was calculated by Friedewald’s equation and low-density lipoprotein (LDL) cholesterol was determined by subtracting the HDL and VLDL levels...
from the total cholesterol level. The fasting plasma insulin and glucose concentrations were used to calculate insulin resistance using the homeostasis model assessment index according to the following formula: [insulin (µU/mL) x glucose (mM)]/22.5 (17).

Liver Histochemical Analysis and Determination of the Total Lipid Content

Fragments of the liver were processed and stained with Sudan III for lipid detection. The total lipid levels in the liver were measured by gravimetry (18) and expressed in grams per 100 g of wet liver weight.

Isolation of rat liver mitochondria and peroxisomes.—

Rat liver mitochondria were isolated as described by Bracht and colleagues (19) and peroxisomes were isolated according to the method described by Natarajan and colleagues (20). The protein concentrations were determined according to the method of Lowry and colleagues (21) using bovine serum albumin as a standard.

Oxygen uptake by mitochondria oxidizing fatty acids.—

Oxygen uptake by intact mitochondria that were oxidizing fatty acids was determined polarographically, at 37°C, using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). The mitochondria (0.6–1.2 mg/mL) were incubated in 2 mL (final volume) of a specific incubation medium (22). The rate of oxygen consumption was expressed as nmol/min·mg of mitochondrial protein.

Peroxisomal fatty acyl–coenzyme A oxidase and catalase activities.—

The peroxisomal fatty acyl–coenzyme A (CoA) oxidase activities were measured fluorimetrically using a modification (23) of the method described by Small and colleagues (24). The assay was based on the determination of hydrogen peroxide (H₂O₂) production in a reaction catalyzed by exogenous peroxidase, which was coupled to the oxidation of DCFH-DA into the highly fluorescent compound DCF. After the addition of the peroxisome-enriched fraction (0.3 mg protein/mL), the reaction was initiated with the addition of the substrate, octanoyl-CoA or palmitoyl-CoA (final concentration of 30 µM). The increase in fluorescence (excitation, 503 nm; emission, 529 nm) was recorded for a period of 10 minutes, and the activity of fatty acyl-CoA oxidase was expressed as picomoles DCF produced/min·mg of peroxisomal protein.

Mitochondrial reactive oxygen species generation.—

The mitochondrial reactive oxygen species (ROS) generation was monitored by the oxidation of DCFH-DA into the fluorescent compound DCF in the presence of H₂O₂. Mitochondria (1 mg protein/mL) were preincubated with 5 mM each of glutamate and malate and 15 µM DCFH-DA. After 3 minutes, 600 µmol/L ADP was added, and the fluorescence was recorded for 5 minutes (excitation, 503 nm; emission, 529 nm). The mitochondrial ROS generation was expressed as picomoles DCF produced/min·mg of mitochondrial protein (26).

Redox state evaluation.—

The glutathione (GSH) levels were determined fluorimetrically (excitation, 350 nm; emission, 420 nm) in the supernatant, using o-phthalaldehyde according to the method described by Hissin and Hilf (27). The results were defined as the index of oxidative stress and expressed as micrograms GSH/mg of protein.

Liver malondialdehyde concentrations were measured by direct spectrophotometry and were used as biomarkers of lipid peroxidation and oxidative stress (28). The results were expressed as nanomoles of malondialdehyde/mg protein using a molar extinction coefficient for malondialdehyde of 1.56 × 10⁵/M·cm.

The activities of the following antioxidant enzymes were measured in the supernatants obtained by centrifuging liver homogenates at 15,000g: glutathione reductase (GSSG-Red), glutathione peroxidase (GSH-Px) (30), superoxide dismutase (SOD) (31), and glucose-6-phosphate dehydrogenase (G6PD) (32).

Treatment of data.—

The data in Figures and Tables are presented as the means ± standard deviations (SD). The areas under the curves (AUCs) of body weight were calculated using the GraphPad Prism program. The data were analyzed using Student’s t test or analysis of variance (ANOVA). Statistical analyses were performed with ANOVA using the Statistica or GraphPad Prism program. The level of significance was set at p < .05.

RESULTS

General Features

In Figure 1, Panel A, the growth curves of the four animal groups studied, from the day of the surgical procedures of ovariecomy and/or hypertension induction until the end of the experimental period (36 days) are presented. In Panel B are presented the areas under the curves (AUCs) obtained from these results. As can be seen, the OVX and OVX + 2K1C rats presented significantly higher weight gain than did the control rats. However, in animals treated with tibolone (OVX + 2K1C), the weight gain observed was significantly lower and reached values similar to those found in control rats.

As shown in Table 1, these variations in the body weight occurred in the absence of alterations in the adiposity index or food intake.

The mean arterial pressures of the animals are presented in Figure 2. As expected, the mean arterial pressure in OVX + 2K1C rats was significantly higher (28%) than
in control and OVX rats. Treatment with tibolone significantly reduced the blood pressure by 15% \((p < .001)\) and, in OVX + 2K1CT animals, these values no longer differed from those found in the control and OVX animals.

The ratios between the weights of the right and left kidneys were calculated, by sampling, in batches of animals. The control and OVX rats presented ratios close to 1.0 \((0.9711 \pm 0.032, n = 9; \text{ and } 1.01 \pm 0.04, n = 7, \text{ respectively})\), whereas in OVX + 2K1C rats, these values increased to \(3.179 \pm 0.54, n = 8\) and did not differ significantly from those of OVX + 2K1CT \((3.167 \pm 0.85, n = 6)\).

As shown in Table 2, the four groups of animals presented normal plasma levels of glucose and insulin and, as demonstrated by the homeostasis model assessment (HOMA) index, normal peripheral insulin sensitivity. In the lipid profile, the only changes observed between these animal groups were the plasma levels of total cholesterol and HDL cholesterol in OVX + 2K1CT rats, which were 13% \((p < .05)\) and 34% \((p < .001)\) lower, respectively, when compared to control and OVX rats and 18% and 40% lower, respectively, when compared to OVX + 2K1C rats \((p < .001)\).

Liver Histochemical Analysis and Total Lipid Contents

The liver histologic sections of the control (a), OVX (b), OVX + 2K1C (c), and OVX + 2K1CT (d) rats stained with Sudan III are presented in Figure 3. In contrast to the control rats, livers from OVX and OVX + 2K1C rats contained considerable amounts of lipid inclusions (in orange, Figure 3), whereas the OVX + 2K1CT rats showed similar results as control.

The results of the liver total lipid quantification performed by the gravimetric method are presented in Figure 4. In agreement with previous experiments, the livers from female control rats presented normal total lipid levels of 5.25% \((33)\), whereas significantly higher liver lipid levels were found in the OVX and OVX + 2K1C rats \((p < .001)\), reaching 6.6% of liver weight. This undesirable increase was completely reversed by treatment with tibolone and, in the OVX + 2K1CT rats, the liver lipid levels no longer differed from those in the control groups.

Liver Mitochondrial \(\beta\)-Oxidation Measurements

To investigate whether these alterations in the liver lipid contents could be the result of a defective liver fatty acid disposal, the peroxisomal and mitochondrial \(\beta\)-oxidation capacities were evaluated.

The capacities of the isolated liver mitochondria to oxidize octanoyl- and palmitoyl-acyl-CoA derivatives in the presence of l-carnitine. In another series of experiments, palmitoyl-1-carnitine was used. The \(\beta\)-oxidation capacity was determined in the presence of 100 \(\mu\)M 2,4-dinitrophenol. Under these conditions, no differences were observed in the oxidation of octanoyl-CoA by the mitochondria of OVX

Table 1. General Features

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX + 2K1C</th>
<th>OVX + 2K1CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiposity index (g/100 g of body weight)</td>
<td>4.42 ± 0.57</td>
<td>5.09 ± 1.22</td>
<td>4.45 ± 0.82</td>
<td>4.00 ± 0.70</td>
</tr>
<tr>
<td>Food consumption (g/day)</td>
<td>19.39 ± 4.01</td>
<td>21.07 ± 4.75</td>
<td>18.26 ± 4.10</td>
<td>19.93 ± 3.23</td>
</tr>
</tbody>
</table>

Notes: Adiposity index (g/100 g of body weight) and food consumption (g/day) of sham-operated (control), bilateral ovariectomized (OVX), OVX + two-kidney, one-clip untreated (OVX + 2K1C), and two-kidney, one-clip tibolone-treated (OVX + 2K1CT) groups. The values are expressed as the mean ± standard deviation of 5 to 8 individual experiments per group.
and OVX + 2K1C groups compared to the control rats. Treatment with tibolone led to a reduction in the ability of the mitochondria to oxidize this medium-chain fatty acid. In OVX + 2K1CT rats, the oxidizing capacity reached values slightly, but not significantly, lower than that of control animals, but which were significantly lower (20%) compared to the OVX + 2K1C and OVX rats (p < .05).

With respect to palmitate, the mitochondrial β-oxidation capacity did not differ among these four animal groups whether the substrate was presented as the palmitoyl-CoA derivative in the presence of carnitine or as palmitoyl-L-carnitine.

Peroxisomal β-Oxidations and CAT Activities

The peroxisomal capacities for the oxidation of these two fatty acids were also assessed in this study, and the results are presented in Figure 6, Panel A. Contrary to the mitochondrial system, the peroxisomal fatty acid β-oxidation system is not associated with a phosphorylating system. In the rat liver, the peroxisomal system is responsible for the generation of 35% of all H2O2 formed, which accounts for 20% of total oxygen consumption. To maintain the equilibrium between the production and scavenging of ROS, peroxisomes concentrate the bulk of the cellular CAT activity (34). Therefore, the peroxisomal CAT activity is described in Figure 6, Panel B.

When octanoyl-CoA was used as a substrate, liver peroxisomes from OVX and OVX + 2K1C rats showed significantly lower and higher β-oxidation capacities, respectively, compared to control rats. Tibolone treatment significantly reduced this capacity compared to OVX + 2K1C rats, which reached values close to those of the control group and were significantly higher than those of OVX rats. With palmitoyl-CoA as the substrate, although a clear increase in the oxidizing capacity was observed in OVX + 2K1C rats, this capacity differed significantly only compared to control rats.

Although the CAT activity (Figure 6, Panel B) in the liver peroxisomes from OVX rats was 50% lower (p < .001) compared to the control groups, the CAT activities in OVX + 2K1C and OVX + 2K1CT rat liver peroxisomes were close to those found in control rats.

The Liver Redox State

Several biomarkers of the liver redox status are presented in Figure 7, panels A through F. The GSH levels (Panel A) were significantly reduced in OVX and OVX + 2K1C animals compared to control animals, but which were significantly lower (20%) compared to the OVX + 2K1C and OVX rats (p < .05).

With respect to palmitate, the mitochondrial β-oxidation capacity did not differ among these four animal groups whether the substrate was presented as the palmitoyl-CoA derivative in the presence of carnitine or as palmitoyl-L-carnitine.

Table 2. Metabolic Profile

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX + 2K1C</th>
<th>OVX + 2K1CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia (mg/dL)</td>
<td>95.3 ± 7.4</td>
<td>93.8 ± 4.45</td>
<td>91.5 ± 8.14</td>
<td>92.8 ± 6.04</td>
</tr>
<tr>
<td>Insulinemia (ng/dL)</td>
<td>0.38 ± 0.07</td>
<td>0.50 ± 0.14</td>
<td>0.48 ± 0.14</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.21 ± 0.36</td>
<td>2.47 ± 0.51</td>
<td>2.68 ± 0.23</td>
<td>2.61 ± 0.15</td>
</tr>
<tr>
<td>Total cholesterol†</td>
<td>75.3 ± 9.74</td>
<td>77.0 ± 9.94</td>
<td>80.4 ± 10.72</td>
<td>65.8 ± 10.49*</td>
</tr>
<tr>
<td>HDL cholesterol‡</td>
<td>53.5 ± 12.70</td>
<td>52.2 ± 9.35</td>
<td>57.8 ± 11.96</td>
<td>34.8 ± 10.19**</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>13.97 ± 3.41</td>
<td>15.59 ± 3.62</td>
<td>18.81 ± 5.83</td>
<td>18.20 ± 2.20</td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>8.01 ± 1.31</td>
<td>9.19 ± 1.47</td>
<td>8.68 ± 1.11</td>
<td>9.03 ± 1.09</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>41.03 ± 5.63</td>
<td>45.95 ± 7.36</td>
<td>41.70 ± 5.20</td>
<td>47.03 ± 10.51</td>
</tr>
</tbody>
</table>

Notes: Glycemia (mg/dL), insulinemia (ng/dL), homeostasis model assessment index = HOMA index, total cholesterol (mg/dL), high-density lipoprotein cholesterol = HDL cholesterol (mg/dL), low-density lipoprotein cholesterol = LDL cholesterol (mg/dL), very low-density lipoprotein cholesterol = VLDL cholesterol (mg/dL), and triglycerides (mg/dL) of control, OVX, OVX + 2K1C, and OVX + 2K1CT groups. Values are expressed as mean ± standard deviation of 5–10 experiments. The letters indicate the statistical significance as revealed by ANOVA (*total cholesterol: *p < .05 vs. control and OVX and **p < .001 vs. OVX + 2K1C; †HDL cholesterol: *p < .001 vs. control, OVX, and OVX + 2K1C).
be the result of changes in mitochondrial ROS generation. In fact, as shown in Panel B, in OVX and OVX + 2K1C rats, the mitochondrial ROS generation was significantly higher than that observed in control and OVX + 2K1CT rats. The SOD activity (Panel C), in turn, was increased in the livers of OVX and OVX + 2K1C rats compared to the livers of control and OVX + 2K1CT rats in parallel with the increase in ROS generation. The activity of GSH-Px (Panel D), the major cytosolic H$_2$O$_2$ scavenger, was decreased in the livers of OVX + 2K1CT rats compared to control and OVX + 2K1C rats. G6PD (Panel E), which provides reduced NADP (NADPH) to restore the GSH level, was decreased in the livers of OVX rats and further decreased in the livers of OVX + 2K1C rats compared to control and OVX + 2K1CT rats. Finally, the GSSG-Red activities (Panel F) were similarly and significantly increased in the livers of OVX, OVX + 2K1C, and OVX + 2K1CT rats compared to control rats.

It would be expected that the higher mitochondrial ROS generation, associated with the increased SOD activity in OVX and OVX + 2K1C rats and the decreased activity of peroxisomal CAT in the livers of OVX rats, would lead to higher cytosolic concentrations of H$_2$O$_2$ and the formation of hydroxyl radicals and concomitant lipid peroxidation. Therefore, the lipid peroxidation levels were assessed in the livers of these animals using thiobarbituric acid--reactive substance (TBARS) measurements. As shown in Figure 8, the liver TBARS levels did not differ in OVX and OVX + 2K1C rats compared to control rats. However, in the livers of OVX + 2K1CT rats, the TBARS levels were significantly decreased compared to the other groups.

**DISCUSSION**

The results presented in this work revealed that the treatment of hypertensive OVX rats with therapeutic doses of tibolone efficiently reduced the blood pressure to normal levels and reversed negative aspects of the liver redox status with the restoration of the ROS-scavenging GSH system. This effect of tibolone could be attributed, at least in part, to its effects in reducing mitochondrial ROS generation in the liver and in restoring the G6PD activity in these animals. Although tibolone exerted the undesirable effect of inhibiting the mitochondrial and peroxisomal $\beta$-oxidation of the medium-chain octanoate, it completely reversed hepatic steatosis.

The beneficial effect of tibolone in reducing the blood pressure of hypertensive OVX rats, as demonstrated in this study, is in accordance with that found in healthy and hypertensive postmenopausal women (12). These effects could not be attributed to an improvement in the lipid profile as suggested previously (35). Actually, our results revealed...
GilGlioni et al. that tibolone exerted an adverse effect on the plasma lipid profile, reducing levels of both total and HDL cholesterol without interfering with the TG levels and the results are in agreement with those described by Henriques and colleagues (7) and Farish and colleagues (9). This effect on cholesterol homeostasis, which was initially attributed to an androgenic action of tibolone (7,9), appeared to be, instead, the result of alterations evoked in the hepatic metabolism of cholesterol (11). In fact, more recent studies have revealed that OVX rats treated with tibolone exhibit significantly increased concentrations of cholesterol and trihydroxy acids in bile (15). In this aspect, therefore, tibolone appears to exert an estrogen-like effect, as oral estrogen therapy is also associated with alterations in the bile composition, resulting in increased concentrations of biliary cholesterol (36).

In this way, a potential mechanism through which tibolone reduced the mean arterial pressure could be related to increases in nitric oxide and to reductions in the endothelin plasma levels by tibolone and its metabolites (37,38).
Studies performed on postmenopausal women have shown that, similar to estrogen, tibolone reduces plasma levels of endothelin (13).

Another beneficial effect of tibolone was the marked reduction in the weight gain by hypertensive OVX + treated rats. Our results were similar to those found by Pytlík (8) and Henriques and colleagues (7) in OVX rats. Studies performed in postmenopausal women have provided contradictory results with respect to the effects of tibolone on weight gain, ranging from reductions (39) to the absence of an effect (40) or even to increases in body weight (41).

Interestingly, the tibolone-induced reduction in body weight gain that was found in this work, contrary to some reports, was not associated with a reduction in food intake (7,8) or in the adiposity index (42). However, in the liver, tibolone reversed hepatic steatosis. This result was surprising because recent studies performed in our laboratory (16) demonstrated that tibolone inhibits mitochondrial fatty acid oxidation in isolated perfused liver. This inhibition occurs only at supratherapeutic concentrations but requires only a single passage through the liver. Our hypothesis that, with prolonged treatment under estrogen-deficient conditions, this effect could manifest at therapeutic concentrations was confirmed in this study. This phenomenon, associated with a decreased peroxisomal β-oxidation at least of octanoate, could be a contributing factor to fatty acid accumulation in the liver.

Therefore, the reduction in the total lipid accumulation in the livers of animals treated with tibolone was unexpected. One possibility is that tibolone reduced lipid synthesis in the liver. De novo fatty acid synthesis in the liver is regulated by at least two transcription factors: sterol-regulatory-element-binding protein 1c (SREBP-1c) and the nuclear peroxisomal proliferator-activated receptor γ (PPAR-γ). The transcription factor SREBP-1c upregulates acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS), which are the enzymes involved in fatty acid synthesis (43). Estrogen, in turn, decreases the expression of SREBP-1c and its downstream targets, ACC1 and FAS (44). Therefore, it could be expected that, during estrogen deficiency, lipid metabolism is diverted to free fat acids synthesis, thereby favoring hepatic TG accumulation (43,45). Thus, the effect of tibolone might result from an inhibition of lipid synthesis, a possibility that should be investigated in the future.
pools of lipids (48) are all known to be associated with oxidative stress. Because of this, the liver redox status was evaluated in this work.

The cellular redox status depends on the balance between ROS generation and the capacity of scavenging it by enzymatic and nonenzymatic antioxidant defense systems. The assessment of possible alterations of this balance, as well as oxidative damage, through measurements of TBARS, as performed in this study, allowed an evaluation of the general cellular redox status and led to very interesting results.

The hepatic GSH levels, the main cellular nonenzymatic antioxidant system, decreased to similar levels in OVX and OVX + 2K1C rats and were restored by the treatment of hypertensive OVX rats with tibolone. It must be stressed that the addition of the hypertensive phenotype to OVX rats did not produce further alterations either in the liver GSH contents or in the majority of the parameters indicative of the liver redox status evaluated in this study, as discussed in the following paragraphs.

These alterations observed in the GSH levels could largely be attributed to variations in the mitochondrial ROS generation, which was increased in OVX and OVX + 2K1C rats and which returned to normal levels in animals treated with tibolone. The higher ROS generation observed in OVX rats is not surprising. It is known that estrogens can directly act on the mitochondrial estrogen receptors and regulate a number of genes involved in mitochondrial functions. Studies have demonstrated that estrogens inhibit mitochondria-dependent ROS generation (49). Although there has been no consensus to date about the participation of the estrogen receptors in this response (50), the fact that tibolone inhibited the generation of ROS by the mitochondria suggests the possibility of the participation of these receptors.

The alterations in the activities of the antioxidant enzymes evaluated in this work merely reflect or are adaptive responses to the alterations in the redox status. The first line of defense against mitochondrial ROS is provided by SOD which converts the superoxide anion (O$_2^*$) to H$_2$O$_2$. The expression of this enzyme is controlled by ROS, especially H$_2$O$_2$ (51), which can explain its higher activity in OVX and OVX + 2K1C rats. Unlike the free radical superoxide anion, H$_2$O$_2$ is a more stable molecule and can diffuse across biologic membranes. In the cytosol, H$_2$O$_2$ can generate dangerous products, such as the hydroxyl radical (52). However, in the cytosol, GSH-Px is the major ROS scavenger, eliminating the H$_2$O$_2$ that escapes from mitochondria and peroxisomes. As this enzyme consumes GSH, the elimination of hydrogen peroxide is another factor that contributes to the reductions in GSH levels observed in OVX and OVX + 2K1C rats, in which mitochondrial ROS generation increased.

Another factor that could have contributed to the oxidative stress observed in OVX and OVX + 2K1C rats was the decreased activity of G6PD. This rate-limiting enzyme of the pentose phosphate pathway is the major intracellular source of NADPH generation (34), which serves as a cofactor for the GSSG-Red enzyme. Therefore, by restoring the GSH levels, G6PD is a critical modulator of the cellular redox potential. GSH, in turn, is required for ROS scavenging either directly or indirectly via the GSH-Px enzyme. It has long been known that estradiol stimulates G6PD activity (53). Therefore, the restoration of the G6PD activity in hypertensive OVX rats treated with tibolone suggests an estrogen-like action. The expression of GSSG-Red is also controlled by the liver redox status (51), and the activity of this enzyme was increased in OVX and OVX + 2K1C rats. Interestingly, however, the treatment with tibolone and the subsequent improvement in the liver redox status was not accompanied by a reduction in the activity of this enzyme. This phenomenon could reflect a direct stimulatory effect of tibolone on the enzymatic activity. Previous work in our laboratory (16) has revealed that tibolone exerts a prooxidant effect in isolated perfused liver at supratherapeutic doses (100 µM), leading to a reduction in the hepatic GSH levels and an increase in TBARS levels. Among the antioxidant enzymes analyzed in this study, GSSG-Red was the only one that was directly activated by tibolone.

It must be highlighted that, in OVX + 2K1C rats, the increase in hepatic peroxisomal β-oxidation was not...
accompanied by a proportional increase in the peroxisomal CAT activity, which could be another factor contributing to the alterations in the redox status in this animal group. In this aspect, tibolone exerted one positive effect: in OVX + 2K1CT rats, the peroxisomal oxidation of octanoyl-CoA was reduced compared with OVX + 2K1C rats, whereas the CAT activity remained similar to that from OVX + 2K1C rats.

All the alterations observed in OVX and OVX + 2K1C rats, in particular the increased mitochondrial generation of ROS, the decreased activity of G6PD, and the disproportional increase in peroxisomal oxidation compared with CAT activity (seen in OVX + 2K1C rats), could lead to hepatic oxidative damage. However, although the TBARS levels were slightly increased in livers from OVX and OVX + 2K1C rats, the results were not statistically significant. In OVX + 2K1CT rats, the reduction in the hepatic peroxisomal octanoyl-CoA and the maintenance of CAT activity compared to OVX + 2K1C rats, associated with the decreased mitochondrial ROS generation and the restoration of G6PD activity, could explain the reduced TBARS levels found in hypertensive OVX rats treated with tibolone.

In summary, in a general way, the addition of the hypertensive phenotype to the estrogen deficiency did not induce further alterations in the liver redox status. These results are not surprising, because 70% of the blood flow to the liver is provided by the portal vein. Therefore, this organ must be less susceptible to the oxidative damage associated with hypertension and estrogen deficiency appears to be the major factor responsible for enhanced liver oxidative stress (54).

To our knowledge, this study is the first to demonstrate the beneficial effects of tibolone on blood pressure in an animal model of estrogen deficiency associated with hypertension, which involved the hyperactivity of the renin–angiotensin system, and in the absence of any confounding factors. In addition, the impact of estrogen deficiency and hypertension on liver lipid metabolism and redox status was evaluated, and the improvement of several parameters was evaluated following treatment with tibolone. This animal model will allow a complete investigation of the role of peroxisomal oxidation and PPAR-γ, as well as its impact on renal morphological aspects and function. These results could, in the near future, help to direct the choice of therapeutic strategies in hypertensive women.

**Funding**

Conselho Nacional de Pesquisa Científica e Tecnológica (CNPq 475704/2010); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES 014/10).

**Conflict of Interest**

There are no potential conflicts of interest between us and any other researcher or institution.

**References**

22. Garland PB, Shepherd D, Nicholls DG, Yates DW, Light PA. Interactions between fatty acid oxidation and the tricarboxylic acid system.
32. Tian WN, Pignatere IN, Stanton RC. Signal transduction proteins that associate with the platelet-derived growth factor (PDGF) receptor mediate the PDGF-induced release of glucose-6-phosphate dehydrogenase from permeabilized cells. J Biol Chem. 1994;269:14796–14805.
38. Seeger H, Kloosterboer HJ, Studen M, Wallwiener D, Mueck AO. In vitro effects of tibolone and its metabolites on human vascular coro-
39. Dedoegilu EN, Erenas M, Yoruk P. Effects of hormone therapy and tibolone on body composition and serum leptin levels in postmeno-
40. Gol M, Ozsener S, Uretmen FSS, Oztekin K, Bilgim TTO. Does tibo-
48. Park J, Choe SS, Choi AH, et al. Increase in glucose-6-phosphate dehydrogenase in adipocytes stimulates oxidative stress and inflam-
52. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signal-
53. Ibiin SE, Randall R, Han P, Musey PI. Modulation of hepatic glucose-
6-phosphate dehydrogenase activity in male and female rats by estro-