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Tamoxifen and Estradiol Interact with the Flavin Mononucleotide Site of Complex I Leading to Mitochondrial Failure*

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This study evaluated the action of tamoxifen and estradiol on the function of isolated liver mitochondria. We observed that although tamoxifen and estradiol *per se* did not affect mitochondrial complexes II, III, or IV, complex I is affected, this effect being more drastic (except for state 4 of respiration) when mitochondria were coincubated with both drugs. Furthermore, using two respiratory chain inhibitors, rotenone and diphenyliodonium chloride, we identified the flavin mononucleotide site of complex I as the target of tamoxifen and/or estradiol action(s). Tamoxifen (25 μM) *per se* induced a significant increase in hydrogen peroxide production and state 4 of respiration. Additionally, a significant decrease in respiratory control ratio, transmembrane, and depolarization potentials were observed. Estradiol *per se* decreased carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-stimulated respiration, state 3 of respiration, and respiratory control ratio and increased lag phase of repolarization. With the exception of state 4 of respiration whose increase induced by tamoxifen was reversed by the presence of estradiol, the effects of tamoxifen were highly exacerbated when estradiol was present. We observed that 10 μM tamoxifen in the presence of estradiol affected mitochondria significantly by decreasing FCCP-stimulated respiration, state 3 of respiration, respiratory control ratio, and ADP depolarization and increasing the lag phase of repolarization. All of the deleterious effects induced by 25 μM tamoxifen were highly exacerbated in the presence of estradiol. Furthermore, we observed that the effects of both compounds were independent of estrogen receptors because the pure estrogen antagonist ICI 182,780 did not interfere with tamoxifen and/or estradiol detrimental effects. Altogether, our data provide a mechanistic explanation for the multiple cytotoxic effects of tamoxifen including its capacity to destroy tamoxifen-resistant breast cancer cells in the presence of estradiol. This new piece of information provides a basis for the development of new and promising anticancer therapeutic strategies.

Selective estrogen receptor modulators, also known as tissue selective estrogens, are a class of drugs with mixed estrogen agonist/antagonist actions (1). They were originally classified as antiestrogens because of their ability to antagonize the proliferative effects of estrogens (2). It was later discovered that these compounds could antagonize the action of estrogen by binding to the estrogen receptor (ER)[2] to thereby block estradiol (E2) access (1, 3). It is now known that under certain circumstances and in certain tissues, in addition to acting as competitive inhibitors of endogenous estrogen, these same estrogen antagonists can exert estrogenic agonist properties (4–6). These findings indicate that the mechanisms regulating ER function are not the same in all cells and that a compound can only be classified as an ER agonist or antagonist in relation to a specific cell type or process.

It is known for a long time that breast cancer is an estrogen-dependent malignancy. Based on this finding, the current strategy for treatment of hormone-dependent breast cancer is to block the action of estrogen on cancer cells either by inhibiting estrogen from binding to ER using an antiestrogen (7) or preventing its synthesis using an aromatase inhibitor (8). Tamoxifen (TAM), the first clinically useful selective estrogen receptor modulators (9), has antiestrogenic effects in the breast tissue and is the standard endocrine treatment for postmenopausal women with breast cancer. Currently, the standard of care for breast cancer is 5 years of TAM (7) or an aromatase inhibitor such as anastrazole (8). However, the consequence of long term TAM therapy is drug resistance (10, 11). Although, resistance to TAM has been well documented (10, 12, 13), evidence suggests the emergence of a new form of drug resistance that develops after many years of selective estrogen receptor modulator therapy (14). A recent study showed that TAM-resistant breast cancer can be reversed by the use of low doses of E2 for a short period of time to resensitize ERα-positive tumors again to the growth inhibitory effects of antiestrogens (15). However, and as far as we know, this effect was not explored in a mechanistic point of view. We chose mitochondria as an experimental model because it is increasingly recognized that these organelles are essential for generating energy that fuels normal cellular function and, at the same time, are the major intracellular source of cytotoxic free radicals and the primary determinants of cell death (16). The unique role of the mitochondria is in supplying high energy ATP molecules, while at the same time, monitoring cellular health to make a rapid decision to initiate a programmed cell death. As such, mitochondria sit at a strategic position in the hierarchy of cellular organelles to continue the healthy life of the cell or to terminate it. Furthermore, accumulating evidence indicates that both E2 (17, 18) and TAM (19–22) are capable of modulating mitochondrial function. To achieve our goal, we evaluated several mitochondrial parameters from the respiratory chain (states 3 and 4 of respiration, respiratory control ratio (RCR), ADP/O ratio, and FCCP-stimulated respiration), oxidative phosphorylation system (transmembrane, depolarization, and repolarization potentials and lag phase of repolarization), and the capacity of

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[2] The abbreviations used are: ER, estrogen receptor; DPI, diphenyliodonium chloride; E2, estradiol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RCR, respiratory control ratio; TAM, tamoxifen; TPP^+, tetraphenylphosphonium.

mitochondria to produce hydrogen peroxide. Furthermore, to identify the specific site(s) of TAM and/or E2 action(s), we performed our experiments in the presence/absence of two respiratory chain inhibitors, rotenone and diphenyliodonium chloride (DPI), and the pure estrogen antagonist ICI 182,780.

EXPERIMENTAL PROCEDURES

Materials—TAM and E2 were obtained from Sigma. DPI was obtained from Aldrich, and ICI 182,780 was obtained from TOCRIS (UK). All of the other chemicals were of the highest grade of purity commercially available.

Animals—Male Wistar rats (3 months old) were housed in our animal colony (Laboratory Research Center, University Hospital, Coimbra, Portugal). They were maintained under controlled light (12 h day/night cycle) and humidity with free access (except in the fasting period) to water and powdered rodent chow (URF1; Charles River). Adhering to procedures approved by the Institutional Animal Care and Use Committee, the animals were sacrificed by cervical displacement and decapitation.

Isolation of Liver Mitochondria—The animals were killed by cervical displacement, and the mitochondria were isolated by conventional methods (23) with slight modifications. Briefly, the liver mitochondria were isolated in a medium containing 250 mM sucrose, 5 mM Hepes, 0.5 mM EGTA, and 0.1% defatted bovine serum albumin (pH 7.2). EGTA and bovine serum albumin were omitted from the final washing medium. The mitochondrial pellet was washed twice and suspended in the washing medium. Mitochondrial protein was determined by the biuret method calibrated with bovine serum albumin (24).

Mitochondrial Respiration—Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode (25) connected to a suitable recorder in a 1-ml thermostatted water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of the standard medium with 1 mg of protein. ICI (1 min) and/or E2 (1 min) and/or TAM (2 min) were incubated with isolates before 5 mM glutamate/2.5 mM malate or 5 mM succinate addition.

Membrane Potential ($\Delta\Psi_m$) Measurements—The mitochondrial transmembrane potential ($\Delta\Psi_m$) was monitored by evaluating transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared according to Kamo *et al.* (26) using a Ag/AgCl-saturated electrode (Tacussel, model MI 402) as reference. TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Linear 1200 recorder. The voltage response of the TPP^+ electrode to log[TPP^+] was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. The reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard medium (130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 100 μM EGTA, 5 mM Hepes, pH 7.4) containing 3 μM TPP^+. This TPP^+ concentration was chosen to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria (27, 28). The $\Delta\Psi_m$ was estimated by the following equation as indicated by Kamo *et al.* (26) and Muratsugu *et al.* (29).

$$\Delta\Psi_m\,(\mathrm{mV}) = 59\log(v/V) - 59\log(10^{\Delta E/59} - 1) \quad \text{(Eq. 1)}$$

v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium, and deflection of the electrode potential from the base line,

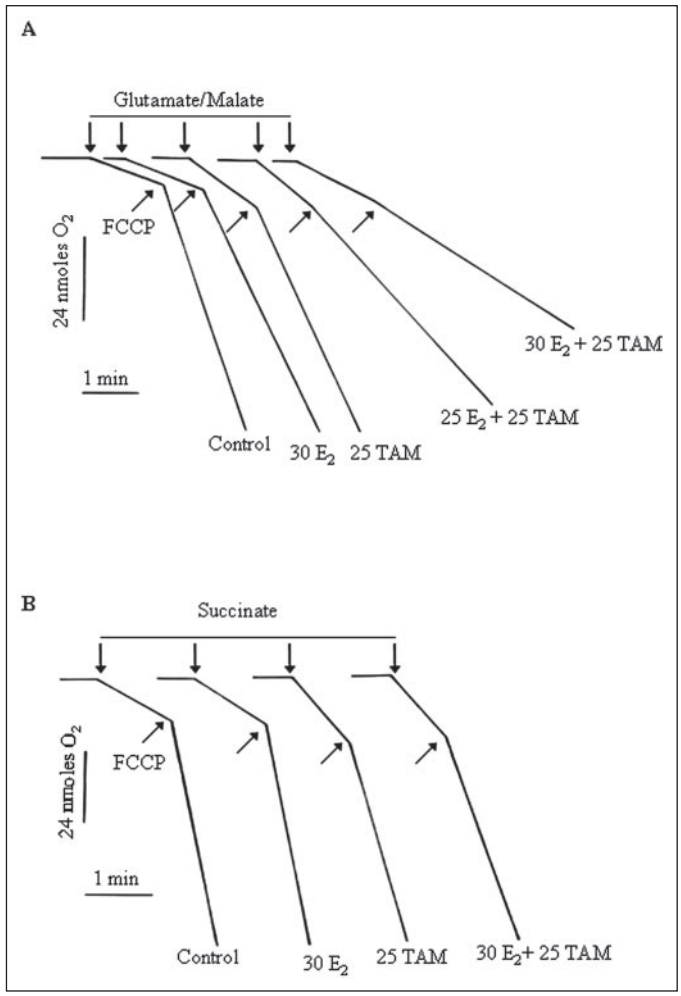

FIGURE 1. **Representative traces of the effect of TAM and/or E2 on FCCP-stimulated respiration.** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate (*A*) or 5 mM succinate (*B*). The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. The values of TAM and E2 are in μM.

respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation and that the law of mass conservation is applicable. A matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on $\Delta\Psi_m$ values. However, the overestimation is only significant at $\Delta\Psi_m$ values below 90 mV, therefore, far from our measurements. Neither TAM nor E2 affected TPP^+ binding to mitochondrial membranes on the electrode response.

Mitochondria (1 mg/ml) were energized by the addition of 5 mM glutamate/2.5 mM malate. After a steady state distribution of TPP^+ had been reached (~1 min of recording), ICI and/or E2 and/or TAM were added, and $\Delta\Psi_m$ fluctuations were recorded.

Evaluation of Hydrogen Peroxide Production—The rate of H_2O_2 production was measured fluorimetrically using a modification of the method described by Barja (30). Briefly, the mitochondria were incubated at 30 °C with 5 mM glutamate/2.5 mM malate in 1.5 ml of phosphate buffer (pH 7.4) containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid, and 6 units/ml horseradish peroxidase in the presence or absence of 1 mM ICI,

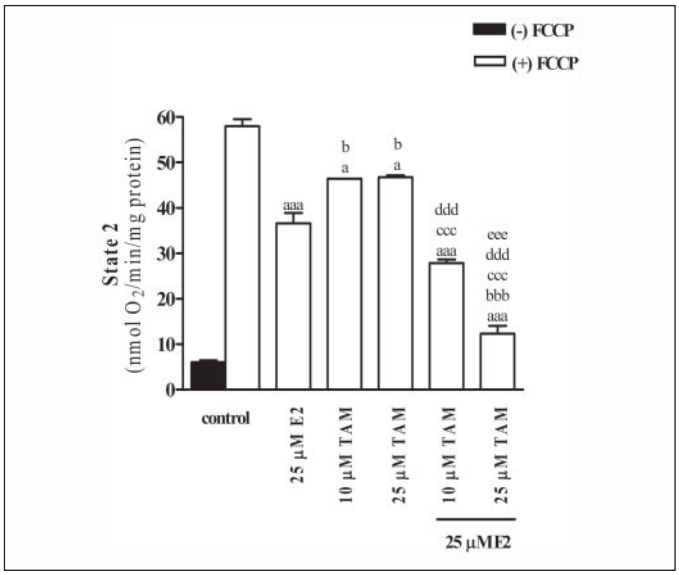

FIGURE 2. **Effect of TAM and/or E2 on FCCP-stimulated respiration.** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. *aaa*, $p < 0.001$; *a*, $p < 0.05$, when compared with FCCP-stimulated respiration. *bbb*, $p < 0.001$; *b*, $p < 0.05$, when compared with FCCP-stimulated respiration in the presence of 25 μM E2. *ccc*, $p < 0.001$, when compared with FCCP-stimulated respiration in the presence of 10 μM TAM. *ddd*, $p < 0.001$, when compared with FCCP-stimulated respiration in the presence of 25 μM TAM. *eee*, $p < 0.001$, when compared with FCCP-stimulated respiration in the presence of 25 μM E2 and 10 μM TAM. The data shown represent the means ± S.E. from six independent experiments.

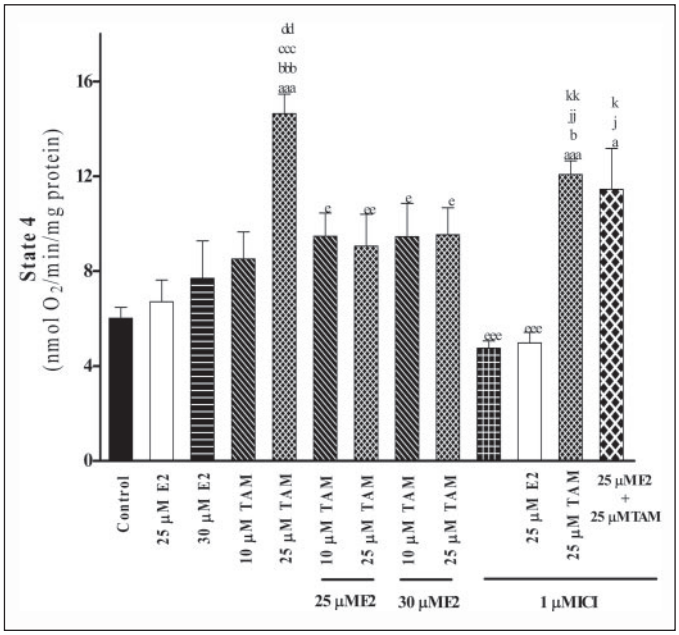

FIGURE 3. **Effect of TAM and/or E2 on state 4 of respiration (consumption of oxygen after ADP phosphorylation).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. *aaa*, $p < 0.001$; *a*, $p < 0.05$ when compared with control condition. *bbb*, $p < 0.001$; *b*, $p < 0.05$, when compared with 25 μM E2. *ccc*, $p < 0.001$; *e*, $p < 0.05$ when compared with 30 μM E2. *dd*, $p < 0.01$, when compared with 10 μM TAM. *eee*, $p < 0.001$; *ee*, $p < 0.01$; *e*, $p < 0.05$, when compared with 25 μM TAM. *jj*, $p < 0.01$; *j*, $p < 0.05$, when compared with ICI condition. *kk*, $p < 0.01$; *k*, $p < 0.05$, when compared with ICI plus 25 μM E2. The data shown represent the means ± S.E. from six independent experiments.

0.5 mM DPI, rotenone and E2 and/or TAM. The incubation was stopped at 15 min with 0.5 ml of cold 2 M glycine buffer containing 25 mM EDTA and NaOH (pH 12). The fluorescence of supernatants was measured at

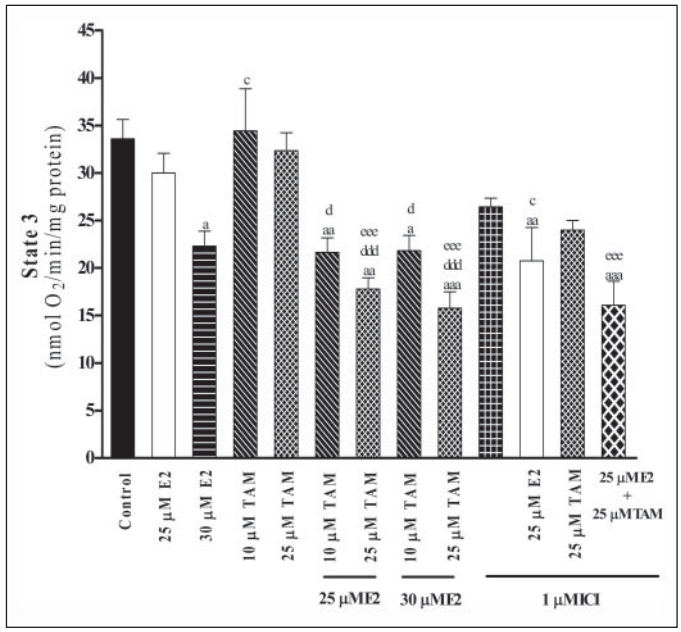

FIGURE 4. **Effect of TAM and/or E2 on state 3 of respiration (consumption of oxygen in the presence of substrate and ADP).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. *aaa*, $p < 0.001$; *aa*, $p < 0.01$; *a*, $p < 0.05$, when compared with control condition. *c*, $p < 0.05$, when compared with 30 μM E2. *ddd*, $p < 0.001$; *d*, $p < 0.05$ when compared with 10 μM TAM. *eee*, $p < 0.001$; *e*, $p < 0.05$, when compared with 25 μM TAM. The data shown represent the means ± S.E. from six independent experiments.

312-nm excitation wavelength and 420-nm emission wavelength. The rate of H_2O_2 was calculated using a standard curve of H_2O_2.

Statistical Analysis—The results are presented as the means ± S.E. of the indicated number of experiments. Statistical significance was determined using the one-way analysis of variance test for multiple comparisons, followed by the post hoc Tukey-Kramer test.

RESULTS

Mitochondrial Complex I Is the Key Target of the Deleterious Effects Induced by E2 Plus TAM—In control conditions after the addition of glutamate/malate (substrate of complex I) there was a slight increase in oxygen consumption (state 2 of respiration) caused by the activation of the mitochondrial respiratory chain (Fig. 1*A*). The addition of 1 μM FCCP, a well known respiratory chain uncoupler, promoted a significant stimulation of oxygen consumption (Figs. 1*A* and 2). The preincubation of mitochondria with 25 μM E2, 10 μM, and 25 μM TAM significantly decreased the consumption of oxygen induced by 1 μM FCCP (~37, 20, and 19%, respectively) when compared with control condition (Fig. 2). However, the coincubation of mitochondria with 25 μM E2 and TAM (10 or 25 μM) exacerbated the decrease in FCCP-induced oxygen consumption, with this effect being much more pronounced in the presence of the highest concentration of TAM (~52 and 79%, respectively) when compared with control condition (Figs. 1*A* and 2). The preincubation of mitochondria with 30 μM E2 and 25 μM TAM completely abolished FCCP-stimulated respiration (Fig. 1*A*). In contrast, when these conditions were tested in mitochondrial complex II (using succinate as substrate), we did not observe any statistical difference when we compared mitochondria without treatment with those incubated with E2 and/or TAM (Fig. 1*B*). These results indicate that mitochondrial complex I is the target of E2 and TAM.

Respiratory Chain Activity Is Drastically Affected When Mitochondria Are Coincubated with E2 and TAM—RCR is the ratio between mitochondrial respiration states 3 (consumption of oxygen in the pres-

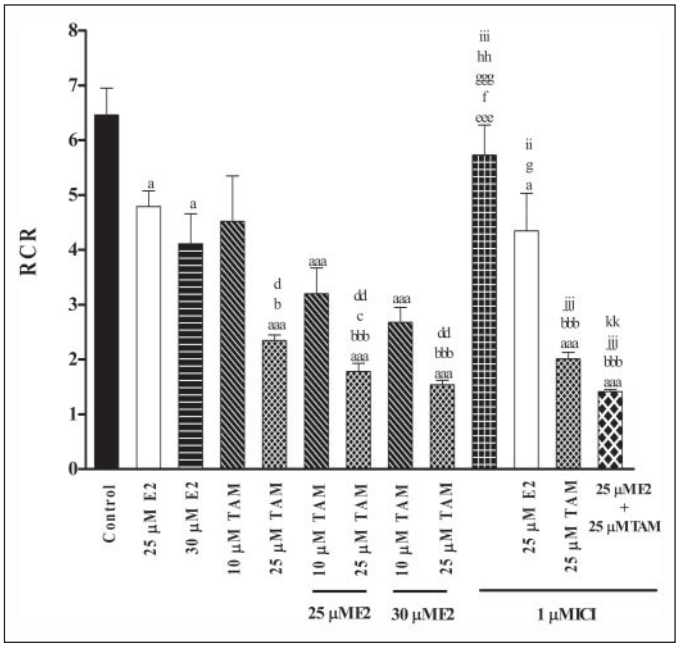

FIGURE 5. **Effect of TAM and/or E2 on RCR (ratio between states 3 and 4 of respiration).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. *aaa*, $p < 0.001$; *a*, $p < 0.05$, when compared with control condition. *bbb*, $p < 0.001$; *b*, $p < 0.05$, when compared with 25 μM E2. *c*, $p < 0.05$, when compared with 30 μM E2. *dd*, $p < 0.01$; *d*, $p < 0.05$; when compared with 10 μM TAM. *eee*, $p < 0.001$, when compared with 25 μM TAM. *f*, $p < 0.05$ when compared with 25 μM E2 plus 10 μM TAM. *ggg*, $p < 0.001$; *g*, $p < 0.05$, when compared with 25 μM E2 plus 25 μM TAM. *hh*, $p < 0.01$, when compared with 30 μM E2 plus 10 μM TAM. *iii*, $p < 0.001$; *ii*, $p < 0.01$, when compared with 30 μM E2 plus 25 μM TAM. *jjj*, $p < 0.001$, when compared with ICI conditions. *kk*, $p < 0.01$, when compared with ICI plus 25 μM E2. The data shown represent the means ± S.E. from six independent experiments.

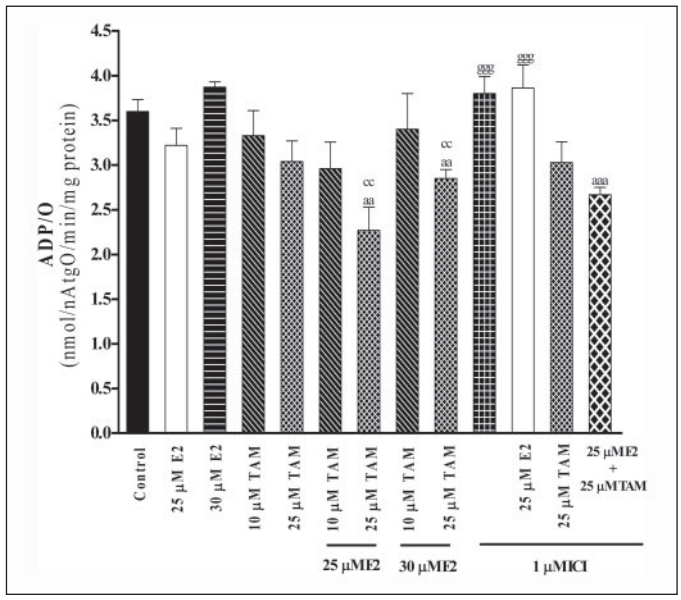

FIGURE 6. **Effect of TAM and/or E2 on ADP/O (the ratio between the amount of ADP added and the oxygen consumed during the state 3 of respiration).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. The isolates were incubated with TAM and E2 for 2 and 1 min, respectively, at 30 °C, before mitochondria energization. *aaa*, $p < 0.001$; *aa*, $p < 0.01$, when compared with control condition. *cc*, $p < 0.01$, when compared with 30 μM E2. *ggg*, $p < 0.001$, when compared with 25 μM E2 plus 25 μM TAM. The data shown represent the means ± S.E. from six independent experiments.

ence of substrate and ADP) and 4 (consumption of oxygen after ADP has been consumed). We observed that 25 μM TAM induced a significant increase in respiration state 4 (~143%) when compared with control condition (Fig. 3). However, coincubation of E2 with TAM decreased this parameter to a value similar to that of control conditions (Fig. 3). Preincubation of mitochondria with ICI, the pure estrogen antagonist, did not affect this parameter, indicating that estrogen receptors are not involved in these effects. This finding is supported by the results obtained with mitochondria incubated with ICI and TAM (25 μM) alone or in combination with E2 (25 μM) because the increase observed in respiration state 4 (~101 and 90%, respectively) is not statistically significant compared with that induced by 25 μM TAM *per se* (Fig. 3).

When we analyzed respiration state 3, we observed that 30 μM E2 induced a decrease of this parameter (~34%) when compared with control condition (Fig. 4). Similarly, coincubation of mitochondria with E2 (25 or 30 μM) and 10 μM TAM induced a decrease in respiration state 3 (~36 and 35%, respectively), and this effect was exacerbated when both E2 concentrations were present with 25 μM TAM (~47 and 53%, respectively) (Fig. 4). ICI in the presence of E2 (25 μM) alone or in combination with TAM (25 μM) induced a significant decrease in respiration state 3 (~38 and 52%, respectively) when compared with control condition (Fig. 4). However, this decrease was not statistically significant from that induced by 25 μM E2, indicating that the effect on respiration state 3 is only due to E2 action (Fig. 4).

Fig. 5 shows that 25 and 30 μM E2 induced a similar decrease in RCR (~26 and 36%) and that 25 μM TAM promoted a drastic decrease of this parameter (~64%) when compared with control condition. However, coincubation of mitochondria with 25 or 30 μM E2 and 10 μM TAM significantly potentiated the decrease in RCR (~51 and 58%, respectively), and this potentiation was more pronounced when both concentrations of estradiol were present with 25 μM TAM (~72 and 76%, respectively), when compared with control condition. Once again, ICI *per se* did not affect RCR (Fig. 5).

The ADP/O ratio, an indicator of oxidative phosphorylation efficiency, is expressed by the ratio between the amount of ADP added and the oxygen consumed during state 3 respiration. We observed that coincubation of 25 or 30 μM E2 with 25 μM TAM induced a similar decrease in the ADP/O ratio (~37% and ~21%, respectively) when compared with control condition (Fig. 6). Similar to what happened with the previous parameters analyzed, ICI did not interfere with ADP/O ratio (Fig. 6). Although we observed a decrease in ADP/O ratio when mitochondria were coincubated with ICI, 25 μM E2, and 25 μM TAM, this effect was not statistically different from that induced by E2 and TAM in the absence of ICI (Fig. 6).

Oxidative Phosphorylation System Is Drastically Affected When Mitochondria Are Coincubated with E2 and TAM—The mitochondrial transmembrane potential ($\Delta\Psi_m$) is fundamental for the phenomenon of oxidative phosphorylation, the conversion of ADP to ATP via ATP synthase. Mitochondrial respiratory chain pumps H^+ out of the mitochondrial matrix across the inner mitochondrial membrane. The H^+ gradient forms an electrochemical potential (Δp), resulting in a pH (ΔpH) and a voltage gradient ($\Delta\Psi_m$) across the mitochondrial inner membrane. $\Delta\Psi_m$ developed by mitochondria after energization with glutamate/malate was ~−220 mV (negative inside) (Fig. 7). However, the addition of TAM after mitochondria energization induced a decrease in $\Delta\Psi_m$, as shown in Figs. 7 and 8. Because the addition of E2 (25 or 30 μM) did not significantly affect $\Delta\Psi_m$, we compared the alterations promoted by the other conditions on $\Delta\Psi_m$ with that induced by E2. We observed that, with the exception of 10 μM TAM, all of the other experimental conditions induced a significant decrease in $\Delta\Psi_m$, with this effect being much more pronounced when 25 μM TAM was present with 25 and

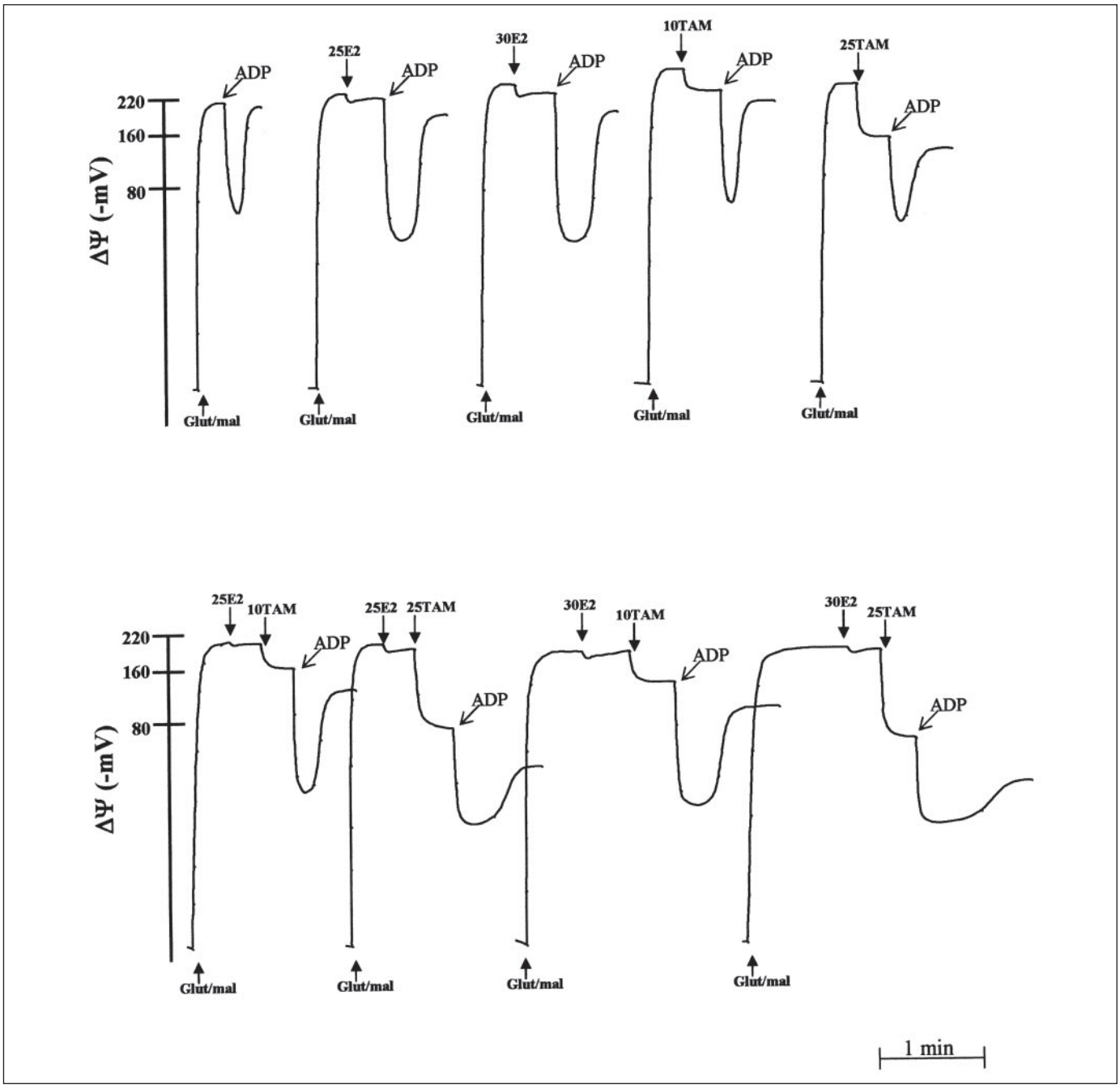

FIGURE 7. **Representative traces of the effect of TAM and/or E2 on $\Delta\Psi_m$.** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. After a steady state distribution of TPP^+ had been reached (~1 min of recording), TAM and/or E2 were added to mitochondrial suspension.

30 μM E2 (~77 and 101%, respectively) when compared with E2 alone (Fig. 7).

Although the depolarization induced by ADP was not statistically affected when we compared E2 and/or TAM effects with control condition, we observed that the repolarization potential (capacity of mitochondria to reestablish $\Delta\Psi_m$, after ADP phosphorylation) decreased similarly in the presence of 25 μM TAM (~5%), 25 μM E2 plus 10 μM TAM (~5%), 25 μM E2 plus 25 μM TAM (~8%), and 30 μM E2 plus 10 μM TAM (~8%) when compared with control conditions (Figs. 7 and 9). It should be noted that the presence of 30 μM E2 plus 25 μM TAM potentiated the decrease of repolarization potential (~11%) when compared with control condition (Fig. 9). It is noteworthy that ICI did not interfere with this parameter (Fig. 9).

The lag phase of repolarization (time necessary for ADP phosphorylation) was significantly affected by 25 and 30 μM E2 (~58 and 69%, respectively) when compared with control condition (Figs. 7 and 10). This effect of E2 was exacerbated in the presence of 10 μM TAM (~66 and 92%, respectively) and 25 μM TAM (~97 and 159%, respectively) (Figs. 7 and 10). Additionally, the presence of ICI did not induce any statistical difference in the lag phase of repolarization (Fig. 10).

Hydrogen Peroxide Production Is Potentiated When Mitochondria Are Coincubated with E2 and TAM: FMN Is the Site in Complex I Where Both Compounds Exert Their Effects—The production of H_2O_2 by mitochondria gives an indication about the propensity of mitochondria to originate and/or exacerbate oxidative stress. At basal conditions (con-

trol), we observed that incubation of mitochondria with 25 μM TAM induced an increase on H_2O_2 production (~41%) (Table 1). However, after the coincubation of mitochondria with 30 μM E2 and 25 μM TAM/mg protein, we observed a potentiation in H_2O_2 production (~84%) when compared with untreated mitochondria (Table 1). Except in the conditions where 25 μM TAM was present, rotenone pretreatment increased the levels of H_2O_2 in all of the conditions tested when compared with levels generated by mitochondria not exposed to complex I inhibitors (Table 1). In the presence of ICI, we observed that incubation of mitochondria with 25 μM TAM induced an increase in H_2O_2 production (~32%) (Table 1), this effect being exacerbated when mitochondria were coincubated with 30 μM E2 and 25 μM TAM (~88%) when compared with the untreated mitochondria (Table 1). In the presence of DPI, which irreversibly binds to the FMN site in complex I, we did not observe any statistical difference between the several experimental conditions assayed, and in all cases, the level of H_2O_2 production was very low (Table 1), indicating that both TAM and E2 exert their effects on H_2O_2 production through the FMN site of complex I.

DISCUSSION

Here we show that TAM and/or E2 act(s) directly on the FMN site of complex I leading to mitochondrial failure. These results also provide a mechanistic basis to explain how E2 treatment is capable of reversing TAM-induced resistant cancers. Resistance to TAM involves changes that prevent apoptosis and enhance cell proliferation and survival. Recently, Osipo *et al.* (15) observed that with long term therapy (5 years), a distinct phase of resistance develops where TAM stimulates growth of breast cancer but in the presence of E2 apoptosis is induced, inhibiting tumor growth. Furthermore, we also demonstrate that mitochondrial failure induced by TAM and/or E2 is independent of ER.

Changes in the structural and functional characteristics of mitochondria provide a number of primary targets for drug-induced toxicity and cell death (31). Previously, we have reported that TAM-induced alterations of mitochondrial bioenergetic capacity may be involved in its antiproliferative ER-independent activity and side effects on different cell types (32). It was shown that when mitochondria were energized

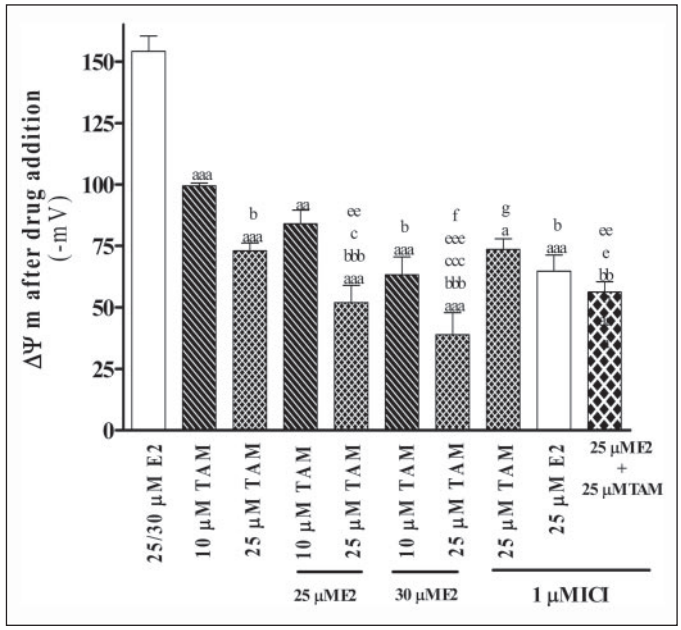

FIGURE 8. **Effect of TAM and/or E2 on $\Delta\Psi_m$.** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. After a steady state distribution of TPP^+ had been reached (~1 min of recording), TAM and/or E2 were added to mitochondrial suspension. *aaa*, $p < 0.001$; *aa*, $p < 0.01$; *a*, $p < 0.05$, when compared with estradiol. *bbb*, $p < 0.001$; *bb*, $p < 0.01$; *b*, $p < 0.05$, when compared with 10 μM TAM. *ccc*, $p < 0.001$; *c*, $p < 0.05$, when compared with 25 μM TAM. *eee*, $p < 0.001$; *ee*, $p < 0.01$, when compared with 25 μM TAM plus 25 μM E2. *f*, $p < 0.05$ when compared with 30 μM E2 plus 10 μM TAM. *g*, $p < 0.05$, when compared with 25 μM E2 plus 25 μM TAM. The data shown represent the means ± S.E. from six independent experiments.

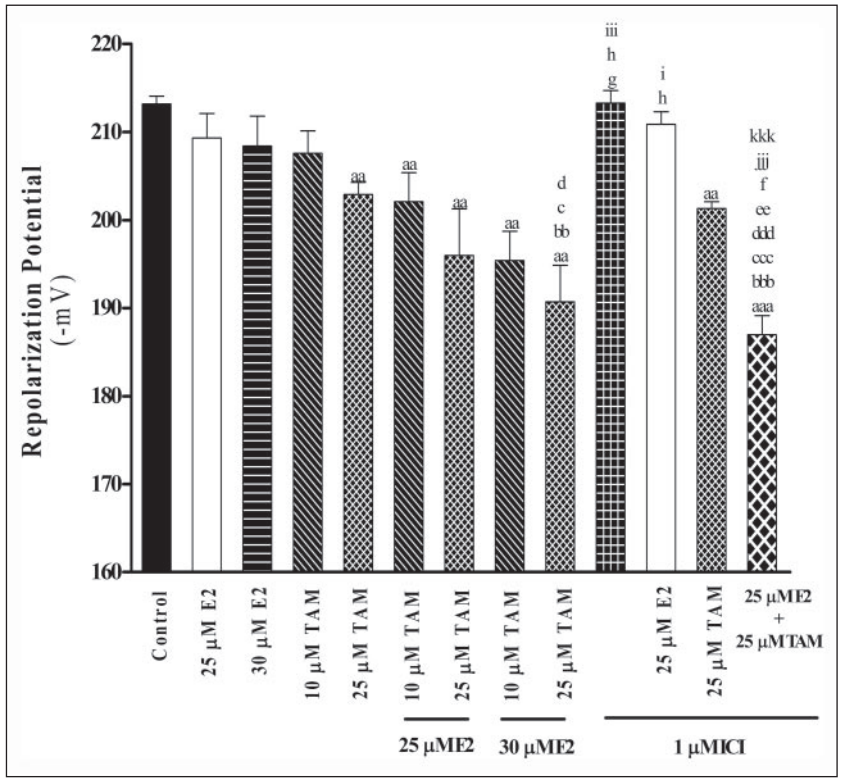

FIGURE 9. **Effect of TAM and/or E2 on repolarization potential (capacity of mitochondria to reestablish $\Delta\Psi_m$ after ADP phosphorylation).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. After a steady state distribution of TPP^+ had been reached (~1 min of recording), TAM and/or E2 and/or ICI were added to mitochondrial suspension. *aaa*, $p < 0.001$; *aa*, $p < 0.01$, when compared with control condition. *bbb*, $p < 0.001$; *bb*, $p < 0.01$, when compared with 25 μM E2. *ccc*, $p < 0.001$; *c*, $p < 0.05$, when compared with 30 μM E2. *ddd*, $p < 0.001$; *d*, $p < 0.05$, when compared with 10 μM TAM. *ee*, $p < 0.01$, when compared with 25 μM TAM. *f*, $p < 0.05$ when compared with 25 μM E2 plus 10 μM TAM. *g*, $p < 0.05$, when compared with 25 μM E2 plus 25 μM TAM. *h*, $p < 0.05$, when compared with 30 μM E2 plus 10 μM TAM. *iii*, $p < 0.001$; *i*, $p < 0.05$, when compared with 30 μM E2 plus 25 μM TAM. *jjj*, $p < 0.001$, when compared with ICI conditions. *kkk*, $p < 0.001$, when compared with ICI plus 25 μM E2. The data shown represent the means ± S.E. from six independent experiments.

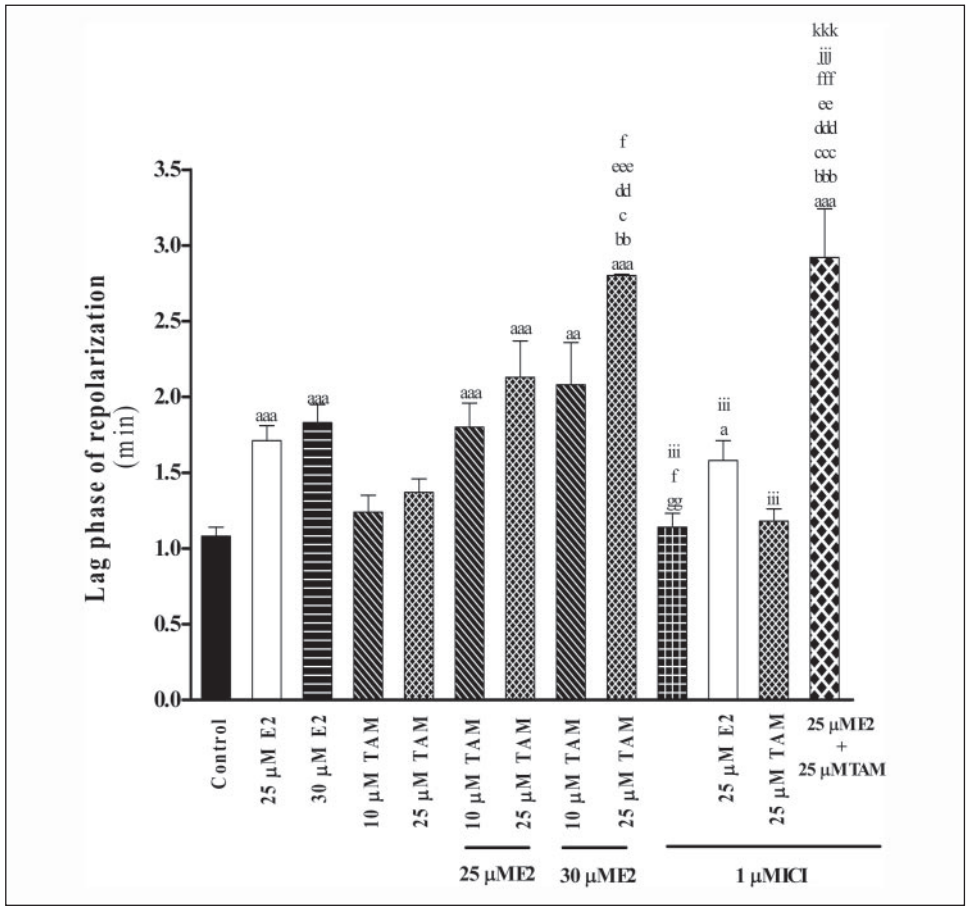

FIGURE 10. **Effect of TAM and/or E2 on lag phase of repolarization (time necessary for ADP phosphorylation).** Freshly isolated liver mitochondria (1 mg) in 1 ml of the standard medium were energized with 5 mM glutamate/2.5 mM malate. After a steady state distribution of TPP^+ had been reached (~1 min of recording), TAM and/or E2 and/or ICI were added to mitochondrial suspension. *aaa*, $p < 0.001$; *aa*, $p < 0.01$; *a*, $p < 0.05$, when compared with control condition. *bbb*, $p < 0.001$; *bb*, $p < 0.01$, when compared with 25 μM E2. *ccc*, $p < 0.001$; *c*, $p < 0.05$, when compared with 30 μM E2. *ddd*, $p < 0.001$; *dd*, $p < 0.01$; when compared with 10 μM TAM. *eee*, $p < 0.001$; *ee*, $p < 0.01$, when compared with 25 μM TAM. *fff*, $p < 0.001$; *f*, $p < 0.05$ when compared with 25 μM E2 plus 10 μM TAM. *ggg*, $p < 0.01$, when compared with 25 μM E2 plus 25 μM TAM. *iii*, $p < 0.001$, when compared with 30 μM E2 plus 25 μM TAM. *jjj*, $p < 0.001$, when compared with ICI conditions. *kkk*, $p < 0.001$, when compared with ICI plus 25 μM E2. The data shown represent the means ± S.E. from six independent experiments.

TABLE 1

Effect of TAM and/or E2 on H_2O_2 production

Freshly isolated liver mitochondria were incubated at 0.2 mg protein/ml under standard conditions as described under "Experimental Procedures." The values of H_2O_2 are given in nmol/mg protein/15 min. The data shown represent the means ± S.E. from six independent experiments. ND, not determined.

	Control	Rotenone	ICI	DPI
Untreated	2.66 ± 0.13	3.26 ± 0.28	2.00 ± 0.31	0.81 ± 0.06
10 μM E2	2.59 ± 0.14	3.40 ± 0.24	2.02 ± 0.24	0.70 ± 0.02
30 μM E2	2.17 ± 0.26	2.68 ± 0.41	1.62 ± 0.28	0.68 ± 0.07
10 μM TAM	2.12 ± 0.12	2.73 ± 0.24	1.56 ± 0.16	0.76 ± 0.03
25 μM TAM	3.74 ± 0.63[a]	3.45 ± 0.13	2.64 ± 0.55[a]	0.99 ± 0.14
10 E2 + 10 TAM	2.39 ± 0.26	2.28 ± 0.30	2.43 ± 0.41	0.75 ± 0.08
30 E2 + 25 TAM	4.88 ± 0.84[b]	4.69 ± 0.91[b]	3.75 ± 0.89[b]	1.06 ± 0.22
Catalase	2.80 ± 0.40	2.71 ± 0.29	ND	ND

[a] $p < 0.05$ when compared with the untreated condition.
[b] $p < 0.01$ when compared with the untreated condition.

with succinate only high TAM concentrations (above 40 nmol/mg protein) stimulate the rate of state 4 respiration, inhibit state 3, and uncouple the mitochondrial respiration (32). Similarly, Tuquet *et al.* (33) observed that increasing concentrations of TAM (30–90 μM) act as both an uncoupling agent and a powerful inhibitor of electron transport in liver mitochondria. In agreement with those findings, we now observed that respiration of mitochondria energized with succinate is not affected by lower TAM concentrations (10 and 25 μM) (Fig. 1*B*). In marked contrast, 25 μM TAM significantly affects mitochondrial complex I. This effect may explain the process of cell death induced by this anticancer agent in different cell types, including ER-negative breast cancer (34), lung adenocarcinoma (35), prostate cancer (36), ovarian carcinoma (37), virus (38), and bacteria (39) because functional mitochondria are needed for cell survival. Accordingly, we observed that 25 μM TAM induces a significant increase in state 4 of respiration (Fig. 3) and H_2O_2 production (Table 1) and a significant decrease in RCR (Fig. 5), $\Delta\Psi_m$ (Figs. 7 and 8) and depolarization potential (Fig. 9). Mitochondrial dysfunction induced by TAM may be an important early event in the activation of apoptosis, a critical process for the destruction of malignant cells. Recently, Gauduchon *et al.* (40) reported that hydroxytamoxifen, an active metabolite of TAM, at pharmacological concentrations inhibits cell proliferation; this inhibition is achieved by two independent events: a block at the G_1 phase of the cell cycle and the induction of apoptotic death through the activation of an intrinsic mitochondrial caspase-9-dependent pathway.

TAM usually is administered at a daily dose of 20 mg/kg of body weight, and the range of TAM serum levels is between 50 and 300 ng/ml (~0.8 μM) (34). However, steady state tissue concentrations of TAM in rats and humans, including the hepatic tissue, are 60–70 times higher than in serum (41), because of its strong partitioning in biomembranes (42), and at least 4 weeks of administration are required to reach steady state drug concentrations (34). Therefore, the estimated drug concentrations in peripheral tissues may reach values (~10–50 μM) in the range of our study.

This study also shows that E2 decreases FCCP-stimulated respiration (Figs. 1*A* and 2), state 3 respiration (Fig. 4), and RCR (Fig. 5) and increases lag phase of repolarization (Fig. 10). Natural estrogens, 17α-estradiol, E2, and estrone, at micromolar concentrations were demonstrated to inhibit mitochondrial electron transport in homogenates of rat uterus, liver, and skeletal muscle (43). Furthermore, Hagen *et al.* (44) reported that 2-methoxyestradiol, a naturally occurring metabolite of E2, inhibits mitochondrial respiration in both intact cells and submitochondrial particles and that this effect is due to inhibition of complex I

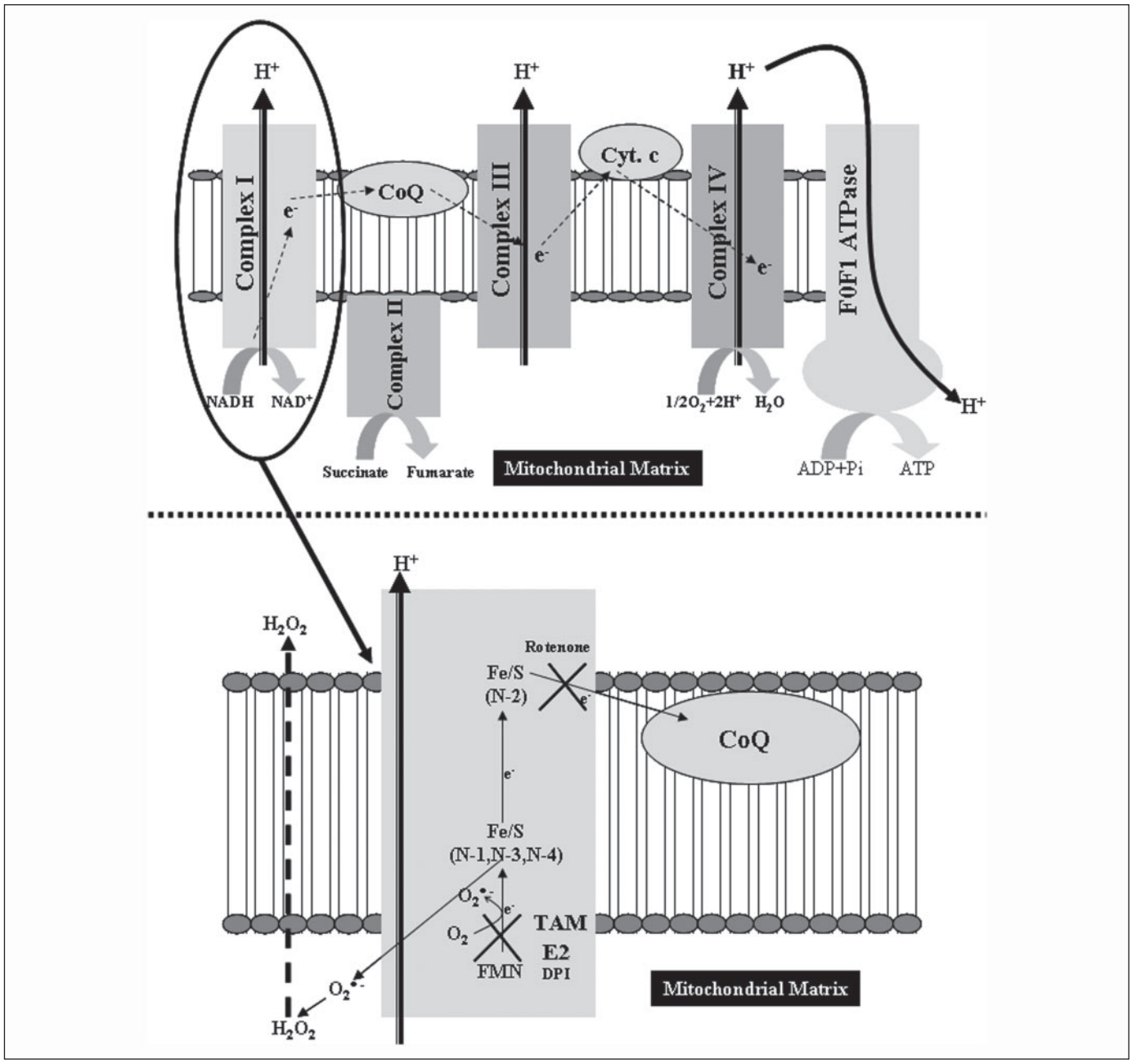

FIGURE 11. **The FMN site of complex I is the target of TAM and/or E2 action.** The *crosses* represent inhibition of electron (e^-) transfer from the iron/sulfur (*Fe/S*) center N-2 to ubiquinone (*CoQ*) by rotenone and inhibition of e^- transfer from the FMN to the iron/sulfur centers N-1, N-3, and N-4 by DPI, TAM, and E2. *Cyt. c* represents cytochrome *c*.

of the mitochondrial electron transport chain. Recently, Mishra and Shaha (45) reported that estrogen-induced spermatogenic cell apoptosis occurs via the mitochondrial pathway.

Although estrogen synthesis occurs in the mitochondria, exogenously added estrogen is also transported to this organelle. For instance, *in vivo* exposure of ovariectomized rats to tritiated E2 showed with increasing time the translocation of this hormone from the plasmalemma mainly to the mitochondria (75%) rather than the nuclei in liver, adrenal gland, and spleen tissues (46). The lipophilic property of E2 allows this molecule to easily diffuse into lipid bilayers. Because mitochondria are enriched with lipids, the organelle has the ability to act as an estrogen sink within cells.

However, the most interesting aspect of this study is that the effects of TAM and E2 are highly exacerbated when both compounds are present simultaneously. Even the lower concentration of TAM tested (10 μM), which alone does not interfere with mitochondrial function, in the presence of E2 significantly affects mitochondrial function, exacerbating the decrease in FCCP-stimulated respiration (Figs. 1*A* and 2), decreasing state 3 of respiration (Fig. 4), RCR (Fig. 5) and depolarization potential (Fig. 9) and increasing the lag phase of repolarization (Fig. 10). Furthermore, all deleterious effects induced by 25 μM TAM alone are highly exacerbated in the presence of E2. These results are in accordance with a previous study showing that the apoptotic cell death induced by TAM in GT1–7 cells is stimulated by estrogen treatment (47). Furthermore, our observations indicate a potential mechanism by which E2 is capable of reversing the TAM-induced resistance reported in the literature (15), as previously discussed.

The inhibition of respiratory complex I is known to favor ROS

generation (Fig. 11). Rat brain mitochondria that respire on complex I substrates produced a substantial level of ROS when inhibited with rotenone concentrations as low as 20 nM (48). Complex I inhibitors rotenone, piericidin A, and amytal have been used in cotreatment with estrogens to elucidate the site of estrogen action on electron transfer (43). In MCF7 cells, it was demonstrated that cotreatment with rotenone and E2 strongly inhibited ornithine decarboxylase activity by 86% (49). More recently, a study reported that treatment with 10 μM of 2-methoxyestradiol induced apoptosis in Ewing sarcoma cells through H_2O_2 production (50). Similarly, we observed that although at basal conditions E2 does not interfere with H_2O_2 production, in the presence of rotenone the production of H_2O_2 is increased (Table 1). Rotenone blocks the transfer of electrons from the iron-sulfur center N-2 to ubiquinone (51), which inhibits the flow of electrons into the ubiquinone-cytochrome bc1 complex; however, reduction of molecular oxygen to superoxide anion can still occur at the FMN site of complex I. In contrast, DPI is a potent arylating agent that reacts with flavoenzymes to form phenylate flavin adducts (52). Hence, DPI irreversibly binds with the FMN in complex I and inhibits NADH dehydrogenase activity (53). Mitochondrial NADH dehydrogenase complex is one site in which superoxide anions may be produced during the reoxidation of the FMN. (Fig. 11). The involvement of FMN site in TAM and/or E2-elicited ROS production was investigated using rotenone and DPI, which are both complex I inhibitors but block electron flow at different sites within the NADH dehydrogenase complex. Our data indicate that mitochondria incubated with DPI produce low levels of H_2O_2 (Table 1), indicating that the FMN site of complex I is the target of TAM and/or E2 effects. Accordingly, Li and Trush (54) demonstrated that diphenyleneiodonium, a DPI-related compound, inhibits superoxide production in isolated mitochondria. Furthermore, Bailey *et al.* (55) showed that incubation of untreated and ethanol-treated hepatocytes with rotenone significantly increased ROS levels, whereas pretreatment with DPI significantly attenuated ROS production. In the same line, Caraceni *et al.* (56) reported similar effects of rotenone and DPI on superoxide production induced in hepatocytes subjected to anoxia reoxygenation injury.

Furthermore, to investigate whether an ER was involved in the regulation of mitochondrial function by TAM and/or E2, we performed some experiments using the pure ER antagonist, ICI. We observed that ICI did not interfere with mitochondrial function (Figs. 3–10) and generation of ROS (Table 1). This finding is supported by previous studies reporting the existence of ER-independent actions of estradiol (57) and TAM (34).

In conclusion, our results demonstrate that TAM and/or E2 lead(s) to mitochondrial failure by acting through the FMN site of mitochondrial complex I (Fig. 11). This is an important aspect concerning cancer therapy because mitochondrial failure may contribute to arrest disease progression. Furthermore, our study provides a mechanistic basis to understand the multiple cytotoxic effects of TAM and why TAM-resistant breast cancer can revert to TAM-sensitive with the use of E2 at the right time, as previously reported by Osipo *et al.* (15). Although we used liver mitochondria as experimental model, we believe that our results can be extrapolated to breast cancer cells for several reasons: 1) TAM is extensively metabolized in the liver to give 4-hydroxytamoxifen and *N*-desmethyltamoxifen (58); 2) the metabolism of estrogens mainly occurs in liver (59); 3) mitochondria control steroidogenesis (60); and 4) mitochondria is an evolutionary conserved organelle. Altogether these data open a new and promising therapeutic window aimed at fighting tumor cell growth.

REFERENCES

1. Wijayaratne, A. L., Nagel, S. C., Paige, L. A., Christensen, D. J., Norris, J. D., Fowlkes, D. M., and McDonnell, D. P. (1999) *Endocrinology* **140,** 5828–5840
2. Lerner, L. J., Hilf, R., Turkheimer, A. R., Michel, I., and Engel, S. L. (1996) *Endocrinology* **138,** 863–870
3. Jordan, V. C. (1983) *Breast Cancer Res. Treat.* **3,** 73–86
4. Katzenellenbogen, J. A., O'Malley, B. W., and Katzenellenbogen, B. S. (1996) *Mol. Endocrinol.* **10,** 119–131
5. McDonnell, D. P. (2000) *J. Soc. Gynecol. Invest.* **7,** S10–S15
6. Sato, M., Rippy, M. K., and Bryant, H. U. (1996) *FASEB J.* **10,** 905–912
7. Early Breast Cancer Trialists' Collaborative Group. (1998) *Lancet* **351,** 1451–1467
8. Baum, M., Budzar, A. U., Cuzick, J., Forbes, J., Houghton, J. H., Klijn, J. G., and Sahmoud, T. (2002) *Lancet* **359,** 2131–2139
9. Jordan, V. C. (2003) *Nat. Rev. Drug Discov.* **2,** 205–213
10. Gottardis, M. M., and Jordan, V. C. (1988) *Cancer Res.* **48,** 5183–5187
11. Gottardis, M. M., Jiang, S. Y., Jeng, M. H., and Jordan, V. C. (1989) *Cancer Res.* **49,** 4090–4093
12. Howell, A., Dodwell, D. J., Anderson, H., and Redford, J. (1992) *Ann. Oncol.* **3,** 611–617
13. Schafer, J. M., Lee, E. S., O'Regan, R. M., Yao, K., and Jordan, V. C. (2000) *Clin. Cancer Res.* **6,** 4373–4380
14. Lewis, J. S., Cheng, D., and Jordan, V. C. (2004) *Br. J. Cancer* **90,** 944–949
15. Osipo, C., Gajdos, C., Cheng, D., and Jordan, V. C. (2005) *J. Steroid Biochem. Mol. Biol.* **93,** 249–256
16. Green, D. R., and Kroemer, G. (2004) *Science* **305,** 626–629
17. Zheng, J., and Ramirez, V. D. (1999) *Eur. J. Pharmacol.* **368,** 95–102
18. Yang, S. H., Liu, R., Perez, E. J., Wen, Y., Stevens, S. M., Jr., Valencia, T., Brun-Zinkernagel, A. M., Prokai, L., Will, Y., Dykens, J., Koulen, P., and Simpkins, J. W. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 4130–4135
19. Custódio, J. B., Dinis, T. C., Almeida, L. M., and Madeira, V. M. (1994) *Biochem. Pharmacol.* **47,** 1989–1998
20. Custódio, J. B., Moreno, A. J., and Wallace, K. B. (1998) *Toxicol. Appl. Pharmacol.* **152,** 10–17
21. Moreira, P. I., Custódio, J. B., Oliveira, C. R., and Santos, M. S. (2004) *Biochem. Pharmacol.* **68,** 195–204
22. Moreira, P. I., Custódio, J. B., Oliveira, C. R., and Santos, M. S. (2005) *Neuropharmacology* **48,** 435–447
23. Gazotti, P., Malmstron, K., and Crompton, M. (1979) *A Laboratory Manual on Transport and Bioenergetics*, pp. 62–69, Springer-Verlag New York Inc., New York
24. Gornall, A. G., Bardawill, N. C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766
25. Estabrook, R. E. (1967) *Methods Enzymol.* **10,** 41–47
26. Kamo, N., Muratsugu, M., Hongoh, R., and Kobatake, V. (1979) *J. Membr. Biol.* **49,** 105–121
27. Jensen, B. D., and Gunter, T. E. (1984) *Biophys. J.* **45,** 92
28. Wingrove, D. E., and Gunter, T. E. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44,** 1082–1087
29. Muratsugu, M., Kamo, N., Kurihara, K., and Kobatake, Y. (1977) *Biochim. Biophys. Acta* **464,** 613–619
30. Barja, J. (1999) *J. Bioenerg. Biomembr.* **31,** 347–366
31. Wallace, K. B., and Starkov, A. A. (2002) *Annu. Rev. Pharmacol. Toxicol.* **40,** 353–388
32. Cardoso, C. M., Custódio, J. B., Almeida, L. M., and Moreno, A. J. (2001) *Toxicol. Appl. Pharmacol.* **176,** 145–152
33. Tuquet, C., Dupont, J., Mesneau, A., and Roussaux, J. (2000) *Cell Biol. Toxicol.* **16,** 207–219
34. Jordan, V. C. (1990) *Breast Cancer Res. Treat.* **15,** 125–136
35. Croxtall, J. D., Emmas, C., White, J. O., Choudhary, Q., and Flower, R. J. (1994) *Biochem. Pharmacol.* **47,** 197–202
36. Bergan, R. C., Reed, E., Myers, C. E., Headlee, D., Brawley, O., Cho, H. K., Figg, W. D., Tompkins, A., Linehan, W. M., Kohler, D., Steinberg, S. M., and Blagosklonny, M. V. (1999) *Clin. Cancer Res.* **5,** 2366–2373
37. Trope, C., Marth, C., and Kaern, J. (2000) *Eur. J. Cancer* **36,** S59–S61
38. Laurence, J., Cooke, H., and Sikder, S. K. (1990) *Blood* **75,** 696–703
39. Luxo, C., Jurado, A. S., Custódio, J. B., and Madeira, V. M. (2001) *Toxicol. in Vitro* **15,** 303–305
40. Gauduchon, J., Gouilleux, F., Maillard, S., Marsaud, V., Renoir, J. M., and Sola, B. (2005) *Clin. Cancer Res.* **11,** 2345–2354
41. Lien, E. A., Solheim, E., and Ueland, P. M. (1991) *Cancer Res.* **51,** 4837–4844
42. Custódio, J. B., Almeida, L. M., and Madeira, V. M. (1991) *Biochem. Biophys. Res. Commun.* **176,** 1079–1085
43. Kuss, E., and Jutting, G. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348,** 913–920
44. Hagen, T., D'Amico, G., Quintero, M., Palacios-Callender, M., Hollis, V., Lam, F., and Moncada, S. (2004) *Biochem. Biophys. Res. Commun.* **322,** 923–929
45. Mishra, D. P., and Shaha, C. (2005) *J. Biol. Chem.* **280,** 6181–6196
46. Moats, R. K., and Ramirez, V. D. (1998) *Biol. Reprod.* **58,** 531–538

47. Hashimoto, M., Inoue, S., Muramatsu, M., and Masliah, E. (1997) *Biochem. Biophys. Res. Commun.* **240,** 464–470
48. Votyakova, T. V., and Reynolds, I. J. (2001) *J. Neurochem.* **79,** 266–277
49. Rowlands, J. C., and Casida, J. E. (1998) *Pharmacol. Toxicol.* **83,** 214–219
50. Djavaheri-Mergny, M., Wietzerbin, J., and Besancon, F. (2003) *Oncogene* **22,** 2558–2567
51. Palmer, G., Horgan, D. J., Tisdale, H., Singer, T. P., and Beinert, H. (1968) *J. Biol. Chem.* **243,** 844–847
52. O'Donnell, V. B., Smith, G. C. M., and Jones, O. T. G. (1994) *Mol. Pharmacol.* **46,** 778–785
53. Hensley, K., Pye, Q. N., Maidt, M. L., Stewart, C. A., Robinson, K. A., Jaffrey, F., and Floyd, R. A. (1998) *J. Neurochem.* **71,** 2549–2557
54. Li, Y., and Trush, M. A. (1998) *Biochem. Biophys. Res. Commun.* **253,** 295–299
55. Bailey, S. M., Pietsch, E. C., and Cunningham, C. C. (1999) *Free Radic. Biol. Med.* **27,** 891–900
56. Caraceni, P., Ryu, H. S., van Thiel, D. H., and Borle, A. B. (1995) *Biochim. Biophys. Acta* **1268,** 249–254
57. Kennedy, A. M., Shogren, K. L., Zhang, M., Turner, R. T., Spelsberg, T. C., and Maran, A. (2005) *Endocrinology* **146,** 201–207
58. Fromson, J. M., Pearson, S., and Bramah, S. (1973) *Xenobiotica* **3,** 711–714
59. Tsuchiya, Y., Nakajima, M., and Yokoi, T. (2005) *Cancer Lett.* **227,** 115–124
60. Felty, Q., and Roy, D. (2005) *J. Carcinog.* **4,** 1–18