Tamoxifen Induces Oxidative Stress and Mitochondrial Apoptosis via Stimulating Mitochondrial Nitric Oxide Synthase

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Abstract
Tamoxifen is an anticancer drug that induces oxidative stress and apoptosis via mitochondria-dependent and nitric oxide (NO)–dependent pathways. The present report shows that tamoxifen increases intramitochondrial ionized Ca2+ concentration and stimulates mitochondrial NO synthase (mtNOS) activity in the mitochondria from rat liver and human breast cancer MCF-7 cells. By stimulating mtNOS, tamoxifen hampers mitochondrial respiration, releases cytochrome c, elevates mitochondrial lipid peroxidation, increases protein tyrosine nitration of certain mitochondrial proteins, decreases the catalytic activity of succinyl-CoA:3-oxoacid CoA-transferase, nitration of certain mitochondrial proteins, decreases the mitochondrial lipid peroxidation, increases protein tyrosine nitration and inhibition of NOS activity prevented the apoptosis induced by tamoxifen. [Cancer Res 2007;67(3):1282–90]

Introduction
Tamoxifen is a cancer chemotherapeutic agent of a drug family known as estrogen receptor (ER) modulators. Tamoxifen exerts ER antagonistic and agonistic properties in various cells and tissues (1–3). Many cancer chemotherapeutic agents (4), including tamoxifen (5), exert their anticancer properties by inducing apoptosis through mechanisms that involve mitochondria. Therapeutic doses of tamoxifen are 150 to 300 µg·d·kg−1 body weight with an average therapeutic blood concentration of 120 ng/mL that is equal to 0.3 µmol/L (6). Treatment of isolated rat liver mitochondria with 20 to 100 µmol/L tamoxifen, concentrations 60 to 300 times greater than the therapeutic, inhibited mitochondrial permeability transition pore opening (7). Likewise, these high concentrations of tamoxifen increased the mitochondrial respiration and proton permeability and decreased mitochondrial transmembrane potential (ΔΨ) and oxidative phosphorylation (8, 9), typifying uncoupling mitochondria. The present study tested the effect of submicromolar concentrations of tamoxifen on mitochondria.

Tamoxifen induces apoptosis in both ER-positive and ER-negative cells via nitric oxide (NO)–dependent pathways (10). Tamoxifen increased the NO synthase (NOS) activity in 10T1/2 murine fibroblasts (11) and in human erythroleukemia K562 cells undergoing apoptosis induced by tamoxifen (10). Moreover, exogenously added NO-potentiated tamoxifen-induced apoptosis and inhibition of NOS activity prevented the apoptosis induced by tamoxifen. We have shown that mitochondria possess a NOS [mitochondrial NOS (mtNOS)] that generates NO in response to elevation of intramitochondrial ionized Ca2+ concentration ([Ca2+]m; refs. 12, 13). NO generated by mtNOS regulates mitochondrial bioenergetics via reversible regulation of cytochrome oxidase activity (12, 13). mtNOS-derived NO also generates peroxynitrite that induces oxidative stress and apoptosis (13). The present study investigated the function of mtNOS for oxidative stress and apoptosis induced by tamoxifen. It was found that tamoxifen increases [Ca2+]m that stimulates mtNOS activity of isolated rat liver mitochondria and human breast cancer MCF-7 cells. It was found that tamoxifen also induces oxidative stress and release of cytochrome c from mitochondria by mechanisms resembling mtNOS-derived peroxynitrite formation.

Materials and Methods
Mitochondria and mitochondrial subfraction preparations. Liver mitochondria were purified from female Sprague-Dawley rats as described (14). The purity of the mitochondria was verified by measuring the cytochrome a content at 605 to 630 nm using the extinction coefficient of 12 mmol/L·cm−1·cm−1 (14). Only mitochondria with <5% impurity were used in this study. Broken mitochondria were prepared from freshly isolated intact mitochondria by freeze-thawing followed by hypoosmolar shock as described (12, 14). Mitochondria and cytoplasm of MCF-7 cells were isolated as described (15). Briefly, after washing cells twice in PBS, protease inhibitor cocktail (aprotinin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and leupeptin (10 µmol/L each); refs. 13, 15) was added and cells were homogenized with 20 to 30 strokes with a glass homogenizer while visualizing microscopically to avoid overhomogenizing that damages the mitochondria. The homogenate was centrifuged at 1,000 × g and the pellet was discarded. The supernatant was centrifuged at 10,000 × g for 30 min and the crude cytoplasmic (supernatant) and mitochondrial (pellet) fractions were separated. The crude cytoplasmic fraction was centrifuged at 100,000 × g and the cytoplasm (supernatant) was collected. Mitochondria were purified from crude mitochondria fraction by 20% Percoll purification at 100,000 × g followed by rinsing twice in cold buffer.

Incubation procedure for liver mitochondria. Unless mentioned otherwise, intact or broken mitochondria [1 mg protein in 100 µL HEPES buffer (100 mmol/L; pH 7.10)] were incubated with 100 µmol/L Nω-nitro-L-arginine (L-NMMA; to inhibit mtNOS; ref. 14) or 100 µmol/L glutathione monoethyl ester (GME; to supplement mitochondria with glutathione; ref. 16) for 30 min on ice. After this incubation time, tamoxifen (0.1 or 0.5 µmol/L) or equal volume of its solvent (ethanol) was added and mitochondria were incubated for 20 min at room temperature with occasional gentle shaking. When used, horse heart cytochrome c (800 pmol; ref. 17) was added immediately before tamoxifen.

Incubation procedure for MCF-7 cells. Cells were cultured in phenol red–free DMEM. At 80% confluence, cells were passaged using 5% trypsin and 0.03% EDTA and seeded at 1 × 106 cells/mL. At ~80% confluence, cells were treated with tamoxifen (0.1–10 µmol/L; ref. 18) in the absence or presence of 1 mmol/L L-arginine (L-Arg) to inhibit NOS (19). Cells were incubated for 24 h in the presence of tamoxifen (0.1–10 µmol/L), L-arginine (1 mmol/L), or tamoxifen + L-arginine at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested by trypsinization and 1 × 106 cells were resuspended in 1 mL cold saline and subfractionated as described (15). Broken mitochondria were prepared as described (12, 14).

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(References have been omitted from this text and are available upon request.)
presence of L-nitroarginine methyl ester (L-NAME; 100 μmol/L; NOS inhibitor) for 12 to 72 h. Mitochondrial oxygen consumption. Liver mitochondria samples were suspended in a final volume of 1 mL HEPES buffer, respiration was supported by K+-succinate (0.8 mmol/L), and the oxygen consumption was determined using a Clark-type oxygen electrode (Harvard Apparatus, Holliston, MA) as described (14).

NOS activity. NOS activity was determined by citrulline assay, NO-sensitive electrode (World Precision Instrument, Sarasota, FL; Apollo 4000), and NO chemiluminescence analyzer (Sievers 280i, General Electric, Boulder, CO) as described (14). For citrulline assay, liver mitochondria were incubated as under incubation procedure for liver mitochondria, whereas buffer was supplemented with 30,000 to 50,000 cpm i-[3H]arginine. At the end of the incubation, mtNOS activity was terminated by adding ice-chilled stop solution [2 mmol/L EDTA and 1 mmol/L unlabeled l-citrulline in 20 mmol/L Na+-acetate buffer (pH 5.00)]. Samples were run through Dowex columns prepared as described (14), and mtNOS activity was measured by using chemiluminescence assay and expressed as pmol NO (NOx)/10⁶ cells or pmol NO/μg mitochondrial protein. Traces are representative of three to four experiments. Columns, mean of four to six experiments; bars, SE. *, P < 0.05; **, P < 0.01, significantly different from control.

Figure 1. Tamoxifen increases mtNOS activity. A, effect of tamoxifen [0.1 μmol/L (Tam 0.1) or 0.5 μmol/L (Tam 0.5)] or its solvent [control (Ctrl)] on mtNOS activity of rat liver mitochondria (black columns) and the inhibitory effect of L-NMMA (white columns). mtNOS activity was determined by citrulline assay and expressed as counts per minute L-[3H]citrulline per milligram mitochondrial protein per minute. B, effect of 0.5 μmol/L tamoxifen (Tam 0.5) or its solvent [control (Cont)] on mtNOS activity of broken liver mitochondria and the effect of L-NMMA (+ L-NMMA) or EGTA (+ EGTA); mtNOS activity was measured with a NO-sensitive electrode and expressed as picoampere. Arrowhead, addition of broken mitochondria to NO electrode chamber. C, effect of tamoxifen on succinate-supported (0.8 mmol/L K+-succinate; arrowhead) oxygen consumption of intact and broken liver mitochondria. D, NOS activity of MCF-7 cells (whole cell) and the mitochondria of MCF-7 cells (mitochondria) treated with tamoxifen (3 μmol/L, 48 h) in the absence (black columns) or presence (white columns) of L-NAME. NOS activity was measured by using chemiluminescence assay and expressed as pmol NO (NOx)/10⁶ cells or pmol NO/μg mitochondrial protein. Traces are representative of three to four experiments. Columns, mean of four to six experiments; bars, SE. *, P < 0.05; **, P < 0.01, significantly different from control.
interfere with the signal detected by the NO electrode. To measure NOS activity in MCF-7 cells, their cytoplasm or mitochondria, samples were injected into the purge vessel containing vanadium chloride (0.6% in 1 mol/L HCl) thermostated at 95°C and the chemiluminescence of the NO released was measured using the NO analyzer (Sievers 280i). The vessel was depleted of oxygen by purging with N₂ at least 20 min before injecting the samples and during the entire measurement. NaN₃O₂ was used to prepare the standard curve.

\[ \text{Ca}^{2+}_{\text{m}} \text{ determination.} \] \[ \text{[Ca}^{2+}_{\text{m}} \text{]} \text{ was done at 675 to 685 nm in the presence of Arsenazo III (5 μmol/L) by using an Aminco DW-2000 spectrophotometer (17, 19), by a Ca²⁺-sensitive electrode (Accumet-Fisher Scientific, Hanover Park, IL; ref. 20) and by fluorometry using the Ca²⁺-sensitive fluorescent probe, fura-2 (21, 22). In the former assay, carbonyl cyanide m-chlorophenylhydrazone (cccp; 1 μmol/L) was added to the intact mitochondria samples to collapse the Δψ and allow the \[ \text{Ca}^{2+}_{\text{m}} \] to equilibrate with extra mitochondrial buffer. The same approach was taken with the Ca²⁺ electrode, except 50 μmol/L antimycin A (AA) was added to collapse the Δψ because cccp interfered with the signal detected by the electrode. The \[ \text{Ca}^{2+}_{\text{m}} \] was also measured in broken mitochondria. No cccp or AA was added to the broken mitochondria preparation. Standard curves were obtained by using known concentrations of Ca²⁺ added to the broken mitochondria suspensions. Tamoxifen or L-NMMA at the concentrations used in this study did not interfere with the Ca²⁺ signal detected by either method.

Both \[ \text{Ca}^{2+}_{\text{m}} \] detection assays are very sensitive; however, they require larger amounts of mitochondria than can be obtained from cultured cells. Therefore, the Ca²⁺-sensitive fluorescent probe fura-2 was used and a series of experiments was conducted to establish a sensitive assay to measure \[ \text{Ca}^{2+}_{\text{m}} \] in very small amounts of mitochondria. Fura-2 (the active form) was excited from 320 to 400 nm and emission was collected at 510 nm. A clear peak and a sharp isosbestic point were detected at 352 and 362 nm, respectively. Next, isolated mitochondria were loaded with the membrane-permeable fura-2/acetoxymethylester (fura-2/AM; 15 min followed by twice wash in cold buffer), excited from 320 to 400 nm, and emission was collected at 510 nm. Identical peak and isosbestic point of 352 and 362 nm were detected. To do a highly sensitive \[ \text{Ca}^{2+}_{\text{m}} \] measurement, fura-2-loaded mitochondria were excited at dual wavelengths of peak minus isosbestic point (352–362 nm) and emission was detected at 510 nm. A \[ \text{Ca}^{2+}_{\text{m}} \]-dependent fluorescence was successfully obtained. This sensitive dual-wavelength excitation fluorometric assay was used to test the \[ \text{Ca}^{2+}_{\text{m}} \] of mitochondria of MCF-7 cells.

Mitochondrial Δψ. The Δψ of intact liver mitochondria was supported by K⁺-sucinate (800 μmol/L) and measured at 511 to 533 nm in the presence of 10 μmol/L safranin as described (13, 17).

Cytochrome c release and lipid peroxidation. Cytochrome c was determined by Western blot using monoclonal anti–cytochrome c antibody (eBioscience, San Diego, CA) described (13, 17). Lipid peroxidation (LPO) was determined by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm as described (13).

Protein tyrosine nitration. Samples were lysed in 100 μL lyses buffer containing 133 μmol/L NaCl, 50 μmol/L Tris-HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 0.1% NP40, and protease inhibitors (10 μmol/L each): PMSF, pepstatin A, leupeptin, and aprotinin. Proteins (20 μg) of each sample were separated by SDS-PAGE on a 10% gel and blotted onto nitrocellulose membrane, probed with monoclonal anti-nitrotyrosine antibody (Alexis Biochemicals, San Diego, CA) or monoclonal anti–cytochrome oxidase subunit VIc antibody (Molecular Probes, Eugene, OR) as loading control, and visualized with alkaline phosphatase.

Activity of succinyl-CoA:3-oxoacid CoA-transferase. Liver mitochondria samples were thrice frozen-thawed in liquid nitrogen followed by addition of ice-cold H₂O (four times the volume of mitochondria suspension) containing protease inhibitors (leupeptin, aprotinin, pepstatin-A, and PMSF; 10 μmol/L each) to fully rupture mitochondrial membranes (14). The suspension was centrifuged at 100,000 × g at 4°C for 30 min and the supernatant [soluble fraction (SF)] was collected. One

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**Figure 2.** Tamoxifen, \[ \text{Ca}^{2+}_{\text{m}} \], and Δψ of liver mitochondria. A, effect of tamoxifen (0.1 μmol/L (Tam 0.1) or 0.5 μmol/L (Tam 0.5)) or its solvent (Cont) on \[ \text{Ca}^{2+}_{\text{m}} \] of intact mitochondria in the absence or presence of L-NMMA. The Δψ was collapsed by AA to allow \[ \text{Ca}^{2+}_{\text{m}} \] efflux. Where indicated, 5 mmol/L EGTA (Neg) or its solvent (Cont) was present in the buffer. Traces are representative of three to four experiments. Columns, mean of four to six experiments; bars, SE. **, **, P < 0.01, significantly different from control.
Tamoxifen Stimulates mtNOS activity of intact isolated rat liver mitochondria and that this effect of tamoxifen was prevented with L-NMMA. Figure 1B shows that NO formation by broken mitochondria was increased by tamoxifen and that this effect of tamoxifen was prevented when mtNOS was inhibited with L-NMMA, or [Ca$^{2+}$]$_{in}$ was removed by EGTA. An L-NMMA- and EGTA-sensitive increase in NO formation of broken mitochondria was also observed for 0.1 μmol/L tamoxifen (data not shown). These results indicate that tamoxifen stimulates mtNOS activity of rat liver mitochondria. NO is the pharmacologic competitive antagonist of O$_2$ (i.e., NO reversibly decreases mitochondrial respiration by competing with O$_2$ for the O$_2$ binding site of cytochrome oxidase; ref. 34). Several reports have shown that NO produced by mtNOS decreases mitochondrial O$_2$ consumption (12, 13). Thus, the present study tested the effect of tamoxifen on mitochondrial oxygen consumption. Figure 1C shows that tamoxifen decreased the oxygen consumption of intact liver mitochondria. Some studies suggested that tamoxifen increased the oxygen consumption of mitochondria (7–9). This can be explained by considering that those studies used 20 to 100 μmol/L tamoxifen, which is 60 to 300 times >0.3 μmol/L, the submicromolar concentration of tamoxifen (6). Tamoxifen is highly lipophilic and high concentrations of lipophilic compounds readily increase mitochondrial respiration by perturbing the mitochondrial membrane integrity and uncoupling the mitochondria. Figure 1C shows that the decrease of mitochondrial oxygen consumption was prevented when mtNOS was inhibited by L-NMMA. This finding indicates that tamoxifen decreased the mitochondrial oxygen consumption by stimulating mtNOS activity. Figure 1C shows that tamoxifen also decreased the respiration of broken mitochondria and that this effect of tamoxifen was prevented when [Ca$^{2+}$]$_{in}$ was chelated by EGTA. This finding together with those presented in
Fig. 1B strongly suggest that tamoxifen increases mtNOS activity by increasing $[\text{Ca}^{2+}]_{\text{im}}$.

Next, the effect of tamoxifen on NO formation by human breast cancer MCF-7 cells and their mitochondria was tested. Figure 1D shows that whereas tamoxifen did not increase the cellular NOS activity in MCF-7 cells, tamoxifen caused a dramatic increase (>500%) in the NOS activity of MCF-7 mitochondria. These findings indicate tamoxifen increases the mtNOS activity in human breast cancer cells.

Mitochondria contain sufficient substrates and cofactors that mtNOS requires, and elevation of $[\text{Ca}^{2+}]_{\text{im}}$ per se stimulates the mtNOS activity (12, 13, 30, 31). Because tamoxifen increased the mtNOS activity and this effect of tamoxifen was inhibited when $[\text{Ca}^{2+}]_{\text{im}}$ was removed, the present study tested whether tamoxifen increases $[\text{Ca}^{2+}]_{\text{im}}$. Figure 2A shows that tamoxifen increased the $[\text{Ca}^{2+}]_{\text{im}}$ stored in intact liver mitochondria. Figure 2A also shows that L-NMMA did not alter the effect of tamoxifen on $[\text{Ca}^{2+}]_{\text{im}}$, indicating that the increased $[\text{Ca}^{2+}]_{\text{im}}$ efflux was not due to decreasing the $\Delta\psi$ by mtNOS-derived NO (12, 19). To rule out possible direct effect of tamoxifen on Ca$^{2+}$ transport system of intact mitochondria, the effect of tamoxifen on broken mitochondria was tested. Figure 2B shows that tamoxifen increased the Ca$^{2+}$ concentration in broken mitochondria and that L-NMMA did not alter this effect of tamoxifen. Elevation of $[\text{Ca}^{2+}]_{\text{im}}$ neutralizes the negative charges of the inner mitochondrial membrane and decreases the $\Delta\psi$. Thus, the present study tested the effect of tamoxifen on $\Delta\psi$. Figure 2C shows that tamoxifen decreased the $\Delta\psi$ and that this effect of tamoxifen was prevented when $[\text{Ca}^{2+}]_{\text{im}}$ was chelated by EGTA. This finding further supports the findings presented in Fig 2A and B and indicates that tamoxifen increases the $[\text{Ca}^{2+}]_{\text{im}}$ in liver mitochondria.

To detect $[\text{Ca}^{2+}]_{\text{im}}$ in small amounts of mitochondria obtained from MCF-7 cells, the Ca$^{2+}$-sensitive fluorescent probe fura-2 was used (21, 22). Fura-2 (the active form) in the absence (no Ca$^{2+}$) or presence of Ca$^{2+}$ (1–50 μmol/L) was excited and emission was collected at 510 nm (35), and a clear peak and a sharp isosbestic point were detected at 352 and 362 nm, respectively (Fig. 3A). Next, liver mitochondria were loaded with membrane-permeable fura-2AM, excited, and emission was collected at 510 nm. Identical peak and isosbestic points of 352 and 362 nm were detected (Fig. 3B). To do highly sensitive $[\text{Ca}^{2+}]_{\text{im}}$ measurement, fura-2-loaded mitochondria were excited at dual wavelengths of peak minus isosbestic point (352–362 nm) and emission was collected at 510 nm. A $[\text{Ca}^{2+}]_{\text{im}}$-dependent increase in fluorescence was observed when $[\text{Ca}^{2+}]_{\text{im}}$ was increased by loading mitochondria with Ca$^{2+}$ (Fig. 3C). No autofluorescence was observed when mitochondria not loaded with fura-2 were excited in the absence or presence of 1 to 10 μmol/L Ca$^{2+}$ (data not shown). Figure 3C also shows that the

![Scheme](https://example.com/scheme.png)

**Scheme.** Mitochondria, mtNOS, and tamoxifen. Mitochondria consist of the inner (IM) and the outer membrane (OM), matrix, and the intermembrane space (IMS). The respiratory chain consists of four complexes (I–IV), coenzyme Q ($Q_0$; ubiquinone), and ATP synthase also called complex V, which are embedded in the IM. Electrons ($e^-$) flow down the chain to complex IV and reduce O$_2$ to H$_2$O. Coupled to the electron flow, protons (H$^+$) are pumped from the matrix into the IMS. The proton extrusion establishes a transmembrane potential ($\Delta\psi$; negative inside) and an electrochemical gradient ($\Delta\psi$; alkaline inside) across the coupling membrane. The $\Delta\psi$ is the driving force for mitochondria to take up Ca$^{2+}$. Whereas mitochondria take up relatively large quantities of Ca$^{2+}$, intramitochondrial ionized Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_{\text{im}}$) is maintained very low by mechanisms, including precipitation of the $[\text{Ca}^{2+}]_{\text{im}}$ to nonionized calcium pools, the matrix electron-dense granules. Mitochondria generate NO through the mtNOS. mtNOS is Ca$^{2+}$ sensitive and elevation of $[\text{Ca}^{2+}]_{\text{im}}$ stimulates the mtNOS activity. mtNOS-derived NO reversibly inhibits mitochondrial respiration by competing with $O_2$ for the $O_2$ binding site on complex IV. mtNOS-derived NO also reacts with superoxide anion ($O_2^-\cdot$) to produce peroxynitrite (ONOO$^-\cdot$). Peroxynitrite induces cytochrome $c$ release, elevates LPO, and nitrates tyrosine residues of susceptible proteins, including SCOT. Tamoxifen affects intramitochondrial calcium homeostasis by shifting the mitochondrial nonionized/ionized calcium equilibrium in the favor of the ionized form. Elevated $[\text{Ca}^{2+}]_{\text{im}}$ stimulates mtNOS that increases intramitochondrial ONOO$^-\cdot$ that induces oxidative stress and mitochondrial apoptosis.
A fluorescent signal was fully abolished when mitochondria were depleted of \([\text{Ca}^{2+}]_{\text{m}}\) by collapsing the \(D_w\), indicating that the signal was solely from the \([\text{Ca}^{2+}]_{\text{m}}\). Similar abolishment of fluorescent signal was observed when \([\text{Ca}^{2+}]_{\text{m}}\) was chelated by EGTA (data not shown). Using this assay, the present study tested the effect of tamoxifen on \([\text{Ca}^{2+}]_{\text{m}}\) of MCF-7 cells. As shown in Fig. 3D, tamoxifen, indeed, increased the \([\text{Ca}^{2+}]_{\text{m}}\) in the mitochondria of MCF-7 cells. Different Y axes scales have been used in Fig. 3. Figure 3A and B where single-wavelength excitation spectra were used have same scales that are different from those used in Fig. 3C and D where excitation was done at dual wavelengths of 352–362 nm. As shown, 5 \(\mu\)mol/L \(\text{Ca}^{2+}\) caused an increase in fluorescence of \(\sim 0.1\) unit on Fig. 3B, whereas 4 \(\mu\)mol/L \(\text{Ca}^{2+}\) generated an increase in fluorescence of \(\sim 1\) unit in Fig. 3C, indicating at least one order of magnitude increased sensitivity when dual-wavelength excitation was used. The scale of Fig. 3C is different from Fig. 3D because 1 mg rat liver mitochondria was used in Fig. 3C, whereas Fig. 3D used 10 \(\mu\)g mitochondria of human epithelial breast cancer cells.

Mitochondria take up and accommodate large amounts of calcium very rapidly; however, \([\text{Ca}^{2+}]_{\text{m}}\) is maintained very low by precipitating the \([\text{Ca}^{2+}]_{\text{m}}\) to the matrix electron-dense granules.
Mitochondria maintain a dynamic intraorganelle calcium homeostasis (i.e., \([\text{Ca}^{2+}]_m\) precipitates to and releases from the matrix granules; refs. 37, 38). Drugs, hormones, or pathologic conditions alter the balance between precipitation of \([\text{Ca}^{2+}]_m\) to the granules and release of \(\text{Ca}^{2+}\) from the granules (36–38). For example, vasopressin decreases the matrix calcium granules in the mitochondria of neurons (38). Hypoxia increases the matrix granules and decreases the \([\text{Ca}^{2+}]_m\) (37), and overexpression of protein kinase C-\(\gamma\) significantly decreases \([\text{Ca}^{2+}]_m\) (39). Findings presented in the present study suggest that tamoxifen shifts the balance between \([\text{Ca}^{2+}]_m\) and matrix granules in favor of \([\text{Ca}^{2+}]_m\); elevated \([\text{Ca}^{2+}]_m\) stimulates mtNOS activity and increases mitochondrial NO formation (Scheme).

Mitochondria are one of the main cellular producers of \(\text{O}_2\). Between 2% and 5% of the entire electrons flowing down through the mitochondrial inner membrane leaks out and generates \(\text{O}_2\) (29, 40). NO reacts with \(\text{O}_2\) with the nearly diffusion-controlled rate constant of \(1.9 \times 10^{10}\) mol/L s\(^{-1}\) to produce peroxynitrite (41). Within the mitochondria, mtNOS-derived NO use up to 15% of the entire \(\text{O}_2\) generated by the mitochondrial inner membrane (33) and produces peroxynitrite (13, 30, 31, 42) with the extremely rapid rate of \(9.5 \times 10^{-8}\) mol/L s\(^{-1}\) (33). Results presented in this study indicate that tamoxifen increases mitochondrial NO formation. Thus, it was tested whether tamoxifen increases mitochondrial peroxynitrite.

**Tamoxifen releases cytochrome \(c\) and elevates LPO.** Release of cytochrome \(c\) from the mitochondria is one of the key events during most forms of apoptosis, including that induced by tamoxifen (43). Stimulation of mtNOS increases mitochondrial peroxynitrite that releases cytochrome \(c\) from the mitochondria (13). mtNOS stimulation also increases LPO that is a widely used peroxynitrite biomarker (13, 44). Figure 4A and B shows that tamoxifen released cytochrome \(c\) from liver mitochondria and increased LPO and that those effects of tamoxifen were prevented when mtNOS was inhibited. These findings suggest that tamoxifen released the cytochrome \(c\) and elevated LPO by stimulating mtNOS. To test whether these effects of tamoxifen were mediated by peroxynitrite, mitochondria were supplemented with glutathione. Supplementing mitochondria with glutathione prevented the apoptosis induced by tamoxifen (45) and decreased the oxidative damage induced by peroxynitrite (46–48). Elegant work of Nakamura et al. (46) has shown that glutathione reverses the peroxynitrite-induced oxidative damage by converting peroxynitrite to S-nitrosating species (47). Figure 4A and B shows that supplementing mitochondria with glutathione prevented tamoxifen-induced release of cytochrome \(c\) and increase of LPO. Supplementing cells with cytochrome \(c\) prevents apoptosis induced by tamoxifen (45) and supplementing mitochondria with cytochrome \(c\) prevents elevated LPO induced by mtNOS-derived peroxynitrite (13). Figure 4B shows that supplementing mitochondria with cytochrome \(c\) prevented tamoxifen-induced LPO elevation. These findings strongly suggest that tamoxifen increases peroxynitrite in liver mitochondria.

The present study also tested whether tamoxifen elevates LPO in MCF-7 cells. Figure 4C shows that whereas tamoxifen did not increase LPO in the cytoplasm of MCF-7 cells, there was a substantial increase in LPO of mitochondria of MCF-7 cells. Tamoxifen-induced increase in mitochondrial LPO was fully abolished when MCF-7 cells were treated with a NOS inhibitor.
Tamoxifen Stimulates mtNOS

These findings strongly suggest that tamoxifen increases peroxynitrite in the mitochondria of MCF-7 cells.

**Tamoxifen increases protein tyrosine nitration and decreases SCOT activity.** Peroxynitrite readily reacts with tyrosine residues to produce nitrotyrosine. Determination of protein tyrosine nitration has been widely used as a reliable peroxynitrite biomarker (49). Figure 5A shows that tamoxifen increased the tyrosine nitration of certain liver mitochondrial proteins and that this effect of tamoxifen was prevented when mtNOS was inhibited. This finding further suggests that tamoxifen increases mitochondrial peroxynitrite via stimulating mtNOS. Figure 5A shows that tamoxifen decreased the activity of SCOT and that this effect of tamoxifen was prevented when mtNOS was inhibited. This finding suggests that the 50-kDa tyrosine-nitrated protein shown in Fig. 5A is SCOT. Figure 5A and B also shows that supplementing mitochondria with glutathione or cytochrome c prevented tamoxifen-induced increase in protein tyrosine nitration and decrease in the SCOT activity, which confirms the results presented in Fig. 4A and B and further indicate tamoxifen increases mitochondrial peroxynitrite.

**Tamoxifen induces aggregation of mitochondria.** Mitochondrial movement and morphology alter under different conditions (51). During early stages of apoptosis, mitochondria form aggregates, and mitochondrial aggregation precedes the release of cytochrome c (52). It has been shown that peroxynitrite causes mitochondrial aggregation followed by release of cytochrome c (53). Because the results presented thus far strongly suggest that tamoxifen increases mtNOS-derived peroxynitrite formation, the present study tested whether tamoxifen induces mitochondrial aggregation. Figure 6 shows that tamoxifen induced extensive aggregation of isolated liver mitochondria and that this effect of tamoxifen was prevented when mtNOS was inhibited or when were supplemented with glutathione. These novel findings suggest that tamoxifen-induced mitochondrial aggregation was mediated by mtNOS-derived peroxynitrite. Figure 6 also shows that supplementing mitochondria with cytochrome c prevented mitochondrial aggregation. This finding confirms the previous reports (27, 52, 53) and further suggests that the release of cytochrome c precedes the aggregation of mitochondria.

Taken together, the present study shows that submicromolar concentrations of tamoxifen stimulate mtNOS activity by elevating [Ca\(^{2+}\)](51), (Scheme). Tamoxifen-induced mtNOS stimulation increases mitochondrial peroxynitrite, which causes oxidative and nitritative stress. NO generated in the cytoplasm exerts antiapoptotic properties by mechanisms involving caspase S-nitrosation, whereas NO produced within the mitochondria becomes proapoptotic by generating peroxynitrite (42). Findings presented in the present study strongly suggest that tamoxifen increases mitochondrial peroxynitrite.

These findings suggest a pivotal role for mtNOS in oxidative stress and apoptosis induced by tamoxifen and propose a novel mechanism for the action of a widely used cancer chemotherapeutic agent.

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