The Effects of Tamoxifen and Its Metabolites on Platelet Function and Release of Reactive Oxygen Intermediates

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ABSTRACT
Tamoxifen is effective in the prevention and treatment of breast cancer, but its use is associated with an increased risk of thrombosis. The mechanism for this effect is unknown. Reactive oxygen intermediates enhance platelet-dependent thrombosis, and in oncological studies, tamoxifen has been shown to increase production of reactive oxygen species. Therefore, the effects of tamoxifen and its bioactive metabolites on platelet activity and platelet reactive oxygen species were determined. Platelets were incubated with tamoxifen or the metabolites 4-hydroxy-tamoxifen (4-OH), N-desmethyl tamoxifen, or 4-hydroxy-N-desmethyl tamoxifen (endoxifen). Tamoxifen metabolites have been previously shown to possess enhanced bioactivity, and consistent with this observation, tamoxifen metabolites but not tamoxifen modestly increased platelet aggregation. These effects were similar with platelets isolated from male or female subjects. Platelet nitric oxide release or cGMP levels were not altered by incubation with tamoxifen or any of its metabolites. Incubation with tamoxifen metabolites increased stimulation-dependent platelet superoxide release [8.1 ± 1.6 arbitrary units (a.u.) for control versus 15.2 ± 3.5 a.u. for 4-OH; P < 0.01]. Coincubation with a superoxide dismutase mimetic eliminated the tamoxifen metabolite-induced enhancement of platelet aggregation. Corresponding to increased superoxide release, incubation with tamoxifen metabolites enhanced the functional activation of NADPH oxidase as determined by phosphorylation of its subunits p47phox and p67phox. In summary, incubation of platelets with the active metabolites of tamoxifen increases stimulation-dependent superoxide release through a NADPH oxidase-dependent mechanism. This results in modest changes in platelet function and seems to be consistent with previous oncological studies demonstrating tamoxifen-dependent increase in reactive oxygen species generation.

Tamoxifen, the progenitor of the selective estrogen receptor modulator class of drugs (SERMs) is widely used for the prevention and treatment of breast cancer; however, a number of studies have shown adverse effects (Pagano et al., 2001). Tamoxifen induces apoptosis in breast cancer cells through caspase-3 and JNK1 pathways, which are initiated at the cell membrane via oxidative mechanisms. These effects are thought to be related to oxygen radical overproduction occurring during tamoxifen metabolic activation (Mandlekar et al., 2000).

In the breast, tamoxifen is an estrogen antagonist, but its use is also associated with proestrogenic effects in other tissues, including increases in hepatic coagulation factor synthesis (Chang et al., 1996; Pritchard et al., 1996). Consistent with this finding, tamoxifen use is also associated with a significantly increased risk of venous thrombosis (Duggan et al., 2003). To date, the only serious adverse event clearly associated with the SERM raloxifene is a tripling of the risk of venous thromboembolic events (Cummings et al., 1999). This observation is important as the large clinical trial Raloxifene Use for The Heart is underway to study whether this SERM reduces the risk of coronary heart disease in women at high risk (Walsh et al., 1998). Because acute coronary syndromes are primarily caused by platelet-dependent aggregation, determining whether SERMs potentially alter platelet function and contribute to the process of thrombosis would be of relevance to clinical studies. Although the effects of SERMS on platelet-dependent thrombosis are not known, tamoxifen metabolites have been investigated in other vascular biological systems. They have been shown to inhibit vascular smooth muscle proliferation in an injury model and may impact lipid metabolism (Yue et al., 2000).

ABBREVIATIONS: SERM, selective estrogen receptor modulator; NO, nitric oxide; NDM, N-desmethyl tamoxifen; 4-OH, 4-hydroxy-tamoxifen; PRP, platelet-rich plasma; TRAP, thrombin receptor-activator peptide; PMA, phorbol 12-myristate 13-acetate; CSK, cytoskeletal.
Because tamoxifen’s receptor-dependent and -independent estrogenic effects may be mediated by its active metabolites (Jordan et al., 1977; Crewe et al., 1997; Stearns et al., 2003), studies were conducted to determine whether tamoxifen or its principal metabolites might influence platelet activity or alter platelet generation of reactive oxygen intermediates, specifically superoxide and nitric oxide (NO), known to modulate platelet function. In these studies, the effects of tamoxifen and N-desmethyl tamoxifen (NDM) were initially examined as these species have the highest concentrations in plasma. In addition the effect of 4-hydroxy-tamoxifen (4-OH) and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) were also studied as these species have approximately 100-fold greater potency as estrogen receptor antagonists compared with tamoxifen (Furr and Jordan, 1984; Stearns et al., 2003).

Materials and Methods

Chemicals and Reagents. Prostaglandin E1, lucigenin, sodium citrate, NaH2PO4, ADP, and dextrose were purchased from Sigma-Aldrich (St. Louis, MO). Fibrinogen was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Tamoxifen, NDM, and 2-4-hydroxy-tamoxifen (endoxifen) were obtained from Sigma-Aldrich, and 4-OH was synthesized by Dr. Ross Weatherman (Department of Medicinal Chemistry, Purdue University, West Lafayette, IN), as described in detail previously (Stearns et al., 2003).

Preparation of Platelets. Blood samples, using acid/citrate/dextrose as anticoagulant [1:9 (v/v)], were obtained from healthy male (n = 8) and female (n = 12, premenopausal) volunteers who denied having taken any drugs, medications, hormonal treatment, or supplements that could alter platelet function in the 2 weeks before blood sampling. No subjects had hypertension, hyperlipidemia, diabetes, or currently smoked. Platelet-rich plasma (PRP) was obtained after centrifugation at 150g, 20 min, 24°C.

Physiological concentrations of tamoxifen and its metabolites were chosen to be studied. The supernatant (PRP) was separated and incubated with total concentrations of tamoxifen and its metabolites, chosen to be in the range that we have observed to be present in plasma when tamoxifen is prescribed to women at the usual dose of 20 mg per day (Lee et al., 2003; Stearns et al., 2003). This approach assumes that free and bound concentrations of drugs are similar in plasma and PRP. Tamoxifen (1 μM) or the tamoxifen metabolites NDM (1 μM) or 4-OH (10 nM) or endoxifen (100 nM) were incubated with PRP for 30 min at 24°C. Some of the platelets were subjected to incubation with 17β-estradiol (0.1–10 μM) for relative comparison or vehicle control. After the addition of the equal volume of platelet washing buffer (10 mM sodium citrate 10, 150 mM NaCl, 1 mM EDTA, and 1% dextrose, pH 7.4, with prostaglandin as antiagulant), the PRP was centrifuged at 3500 rpm at 37°C.

To determine the effect of physiological concentrations of tamoxifen and its metabolites on platelet aggregation, platelets were incubated with 1 μM tamoxifen, 100 nM 4-OH, 1 μM NDM, 100 nM endoxifen, 10 μM 17β-estradiol, or vehicle control at room temperature in the absence of light exposure for 30 min. Incubated platelets were then stimulated with 20 μM TRAP, and the extent of platelet aggregation was measured in a platelet aggregometer (Model 500-CA; Chronolog Corp., Havertown, PA) as detailed previously (Freedman and Keaney, 1999).

Measurement of Platelet Superoxide Production. Lucigenin-derived chemiluminescence was used to estimate aggregation-dependent superoxide production from stimulated platelets using a lumiaaggregometer (whole blood lumiaaggregometer, model 500-CA; Chronolog Corp., Havertown, PA) as detailed previously (Freedman and Keaney, 1999). Washed platelets (4 × 10^5 platelets/μl), preincubated with lucigenin (250 μM) in HEPES buffer for 3 min, were placed into the lumiaaggregometer (aggregometer with luminescence detection). After stabilization of the signal, phorbol 12-myristate 13-acetate (PMA) (0.6 μM) stimulated superoxide production and aggregation were simultaneously measured while stirring at 1000 rpm at 37°C.

Measurement of Platelet cGMP. Briefly, washed platelets were mixed with 10–5 M volume of 3-isobutyl-1-methylxanthine. Chloracetic acid (final concentration 5% (v/v)) was added. The supernatant was extracted, acetylated, and assayed for cGMP by an enzyme-linked immunosorbent assay methodology using cGMP antiserum as described previously (Clutton et al., 2004).

Phosphorylation of Platelet Proteins. Washed platelets, incubated with [32P]orthophosphate (1 mCi/ml), were passed through PD-10 columns to remove free [32P]orthophosphate as described previously (Clutton et al., 2004). Platelet aggregation was stopped by the addition of lysis buffer. In other experiments, the lysate was centrifuged (15,000g, 5 min, 4°C) to separate the Triton X-100-soluble and -insoluble cytoskeletal (CSK) fractions. The CSK fraction was resuspended in LIPA buffer. The proteins were resolved on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with the specified primary polyclonal antibody. Immunoblots were probed with species-specific secondary antibodies coupled to horseradish peroxidase and visualized with chemiluminescent substrate (Clutton et al., 2004). Autoradiography was used to determine phosphorylation of 32P-labeled proteins. The resulting autoradiograms were analyzed by quantitative two-dimensional densitometry. Scanning densitometry was performed using NIH Image for data analysis.

Statistical Analysis. Differences between groups were determined using an unpaired Student’s t test. The effects of interventions were analyzed using a paired t test. A statistically significant difference was assumed with a value of P < 0.05. All data are expressed as the mean ± S.E.M.

Results

To determine the effect of physiological concentrations of tamoxifen and its metabolites on platelet aggregation, platelets were incubated with 1 μM tamoxifen, 100 nM 4-OH, 1 μM NDM, 100 nM endoxifen, 10 μM 17β-estradiol, or vehicle control at room temperature in the absence of light exposure for 30 min. Incubated platelets were then stimulated with 20 μM TRAP, and the extent of platelet aggregation was measured in a platelet aggregometer (Fig. 1). There was a significant increased in platelet aggregation after incubation with the tamoxifen metabolites NDM, 4-OH, or endoxifen. Platelets were also stimulated with either ADP or collagen after incubation with increasing concentrations of tamoxifen, 4-OH, NDM, endoxifen, or...
17-β-estradiol. Incubation with ADP led to similar results with statistically significant increase in aggregation for all three metabolites \((n = 3, P = 0.04; \text{Fig. 2})\). Although there was a trend toward increased collagen-induced aggregation after incubation with the three metabolites, this difference was not statistically significant \((n = 3, P = 0.1; \text{data not shown})\). There was no statistically significant change in platelet aggregation with tamoxifen using any of the platelet agonists. There was also an increase in aggregation after incubation with the superpharmacological level of 17-β-estradiol (Figs. 1 and 2). Incubation with 1 μM 17-β-estradiol did not significantly alter platelet aggregation (Fig. 2).

To determine the effect of tamoxifen and its metabolites on platelet release of reactive oxygen intermediates, the effect of tamoxifen and its metabolites on platelet NO production was studied. Platelets were incubated with 1 μM tamoxifen, 100 nM 4-OH, 1 μM NDM, 100 nM endoxifen, or 10 μM 17-β-estradiol. Platelet NO release was not significantly altered after incubation with 17-β-estradiol, tamoxifen, or any of its metabolites (Fig. 3). Increased NO production was studied. Platelets were incubated with 1 μM tamoxifen, 10 nM 4-OH, 1 μM NDM, 100 nM endoxifen, or 10 μM 17-β-estradiol. Platelet NO release was not significantly altered after incubation with 17-β-estradiol, tamoxifen, or any of its metabolites (Fig. 3). Increased NO production was studied.
is reflected by an accumulation of cGMP as a direct result of the activation of soluble guanylate cyclase. Incubation with tamoxifen or its metabolites did not significantly alter platelet cGMP levels in TRAP-stimulated platelets (Fig. 3). Thus, cGMP levels mirrors the lack of change in the platelet-derived NO as a result of incubation with tamoxifen or its metabolites.

Although platelet NO levels minimally alter platelet aggregation (Freedman et al., 1997), platelet function is known to be enhanced by the reactive oxygen species superoxide. Therefore, the effect of tamoxifen and its metabolites on platelet superoxide release was examined. Platelets were incubated with 1 μM tamoxifen, 100 nM 4-OH, NADPH oxidase, and 4-OH (n = 3, P < 0.05). Platelet superoxide release was almost eliminated after incubation with 10 μM 17-β-estradiol, but incubation with 1 μM 17-β-estradiol failed to have a significant effect. To confirm that the mild enhancement of platelet aggregation was due to the increase in superoxide release, the effect of the tamoxifen metabolites endoxifen and 4-OH on platelet aggregation and superoxide release were repeated after coincubation with the membrane-permeable superoxide dismutase mimetic tempol (1 mM). Tempol significantly decreased TRAP-stimulated platelet aggregation, restoring it to near baseline (Fig. 5b). In addition, treatment with tempol also decreased platelet superoxide release from 15.1 ± 2.9 to 3.3 ± 1.1 arbitrary units for 4-OH (n = 4, P < 0.01 compared with control). Findings were similar for endoxifen (data not shown). No change in platelet aggregation was found for tamoxifen after coincubation with tempol (n = 4, P = N.S.).

Initially identified in phagocytic cells, NADPH oxidase has since been identified as the primary producer of superoxide in vascular tissues. To determine the role of NADPH oxidase in the change in tamoxifen-dependent release of superoxide, the CSK fractions isolated from TRAP-stimulated platelets were immunoblotted with antibodies to p67-phox and p47-phox. Western blot analysis revealed that 3 min after TRAP stimulation, there was a significant increase in the amount of p67-phox present in the CSK of platelets that had been incubated with 4-OH or endoxifen (Fig. 6). In addition, incubation with NDM, 4-OH, or endoxifen led to enhanced p47-phox after TRAP stimulation. However, there was not a significant increase in either p47- or p67-phox present in the CSK fraction isolated from tamoxifen-treated platelets 3 min after TRAP stimulation (Fig. 6). Incubation with 17-β-estradiol did not alter levels of either p47- or p67-phox (data not shown).

![Figure 4](image-url)

**Fig. 4.** Representative tracings of the effect of 17-β-estradiol, tamoxifen, or tamoxifen metabolites on platelet superoxide release (arbitrary, units on y-axis, time on x-axis).

**Discussion**

Relatively little is known about the mechanism of direct effects of tamoxifen or its metabolites on platelets. The presence of the androgen receptor (Nealen et al., 2001) and both the ERα and the ERβ forms of the estrogen receptor have been shown to be present in human platelets (Khew et al., 2000; Nealen et al., 2001; Jayachandran and Miller, 2003) However, the biochemical mechanisms involved in estrogen receptor-mediated signaling in platelets remain unclear. In patients with breast cancer, an antigestrogen regimen has been shown to not change platelet-active prostacyclin or thromboxane (Marttunen et al., 1996). Despite the clear association with enhanced venous thrombosis (Duggan et al., 2003), some studies have failed to demonstrate changes in coagulation-dependent proteins known to be associated with such a risk (Love et al., 1992; Mannucci et al., 1996). This has included a lack of significant change in the markers of activated coagulation or fibrinolysis (fibrinopeptide A, prothrombin fragment 1 + 2, thrombin-antithrombin complex, and D-dimer) (Mannucci et al., 1996) and a paradoxical decrease in fibrinogen levels (Love et al., 1992). A previous study that examined platelets from women on tamoxifen exhibited decreased platelet adherence to endothelial cell matrix compared with platelets from premenopausal women (Miller et al., 1994). The differences between this study and our findings may be due to the specific patient populations (both pre- and postmenopausal) compared in the previous study (Miller et al., 1994).
pathophysiological conditions, and superoxide, in particular, is known to enhance the platelet aggregation response (Handin et al., 1987).

In the current study, tamoxifen metabolites but not tamoxifen modestly enhanced platelet function as measured by an increase in platelet aggregation. This effect did not seem to be due to altered platelet NO release as confirmed by both electrochemical detection and platelet cGMP levels. However, a statistically significant increase in superoxide release was observed with the tamoxifen metabolites and this change normalized when a superoxide dismutase mimetic was added, suggesting that change in platelet aggregation was due to the production of superoxide. Interestingly, tamoxifen, by initially partitioning into the membranes in breast cancer cells, has been shown to induce generation of transmembrane signals and an oxidative stress (Gundimeda et al., 1996).

It was notable that these effects occurred with the metabolites and not tamoxifen itself. Tamoxifen metabolism is complex and involves a number of cytochrome P450 isozymes that catalyze conversion of the parent drug to NDM via CYP3A, to endoxifen via the genetically polymorphic CYP2D6 and to 4-OH via a number of different enzymes. NDM is the tamoxifen metabolite that is present in the highest concentrations in plasma (Lee et al., 2003), and our previous data suggest formation of this metabolite is mediated by CYP3A4/5 (Stearn et al., 2003). Although not tested in this study, variation in metabolism of these metabolites might contribute to individual risk of thrombosis.

To further understand the potential of direct receptor-mediated effects, estrogen incubation was also analyzed. Estrogen has been suggested to inhibit platelet aggregation through activation of endothelial nitric-oxide synthase (Nakano et al., 1998), and a number of studies suggest a gender effect on endothelial nitric-oxide activity. However, we saw no difference in effect comparing platelets from either male or female donors. Although we observed no significant increase in platelet NO after incubation with 17-β-estradiol, we used a NO-selective electrode and cGMP levels, whereas the findings of the previous study were inferred by indirect measurements analyzing nitrate/nitrite. It is important to note that the proaggregatory changes we saw with 17-β-estradiol occurred after incubation with superpharmacological levels of estrogen, and physiological and pharmacological levels had no effect in our studies. However, the doses of tamoxifen used were within pharmacological range (Decensi et al., 1999), and the concentrations of metabolites were also chosen to be in the range that we have observed to be present in plasma when tamoxifen is prescribed to women at the normal dose of 20 mg/day (Lee et al., 2003; Stearns et al., 2003).

Previously, tamoxifen has been shown to induce apoptosis through elevation of intracellular calcium in HepG2 human hepatoblastoma cells. The role of reactive oxygen species in tamoxifen-induced apoptosis was studied in hepatoblastoma cells and caused a sustained increase in intracellular reactive oxygen species. Inhibitors of NADPH oxidase significantly blocked the tamoxifen-induced generation of reactive oxygen species (Lee et al., 2000). In phagocytic cells, superoxide is generated via a one-electron reduction of molecular oxygen by the multicomponent NADPH oxidase. The NADPH oxidase complex consists of the

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Fig. 5. a, effect of tamoxifen, tamoxifen metabolites, or 17-β-estradiol on platelet superoxide release. Platelets were incubated with 1 μM tamoxifen, 100 nM 4-OH, 1 μM 4-OH, 10 μM 17-β-estradiol, or vehicle control at room temperature in the absence of light exposure for 30 min. Incubated platelets were then stimulated with 600 nM PMA. The extent of superoxide inhibition on changes in platelet aggregation induced by tamoxifen, tamoxifen metabolites, or 17-β-estradiol. Platelets were incubated in the presence or absence of tamoxifen or its metabolites or incubation with 10 μM 17-β-estradiol or vehicle control at 37°C for 20 min. These experiments were repeated in the presence of tempol. Incubated platelets were stimulated with 20 μM TRAP, and the extent of platelet aggregation was measured in a platelet aggregometer (n = 3; *, P < 0.05 compared with control; **, P < 0.01 compared with control). b, effect of superoxide inhibition on changes in platelet aggregation induced by tamoxifen, tamoxifen metabolites, or 17-β-estradiol. Platelets were incubated in the presence or absence of tamoxifen or its metabolites or incubation with 10 μM 17-β-estradiol or vehicle control at 37°C for 20 min. These experiments were repeated in the presence of tempol. Incubated platelets were stimulated with 20 μM TRAP, and the extent of platelet aggregation was measured in a platelet aggregometer (n = 4; *, P ≤ 0.05 compared with control without tempol).
membrane-bound cytochrome $b_{558}$ (composed of the two subunits gp91-phox and p22-phox) and the cytosolic proteins p47-phox and p67-phox. Activation of this highly regulated enzyme involves the phosphorylation and translocation of the cytosolic components to the membrane-bound cytochrome $b_{558}$, where the catalytically functional oxidase is assembled. NADPH oxidase has also been identified vascular cells, and recently, specific subunits have been demonstrated in platelets (Krotz et al., 2002; Clutton et al., 2004). In the platelet, collagen activation induces NAD(P)H oxidase-dependent superoxide release, which in turn enhances availability of released ADP, resulting in increased platelet recruitment. Previous studies have demonstrated the expression of the NAD(P)H oxidase subunits p47- and p67-phox (Krotz et al., 2002). These findings are consistent with our observation that the tamoxifen metabolites, to a varying degree, alter expression of either p47- and/or p67-phox. Although a previous study showed that incubation of cultured endothelial cells with 17-$\beta$-estradiol decreased expression of the NADPH oxidase subunit gp9-phox and this effect was prevented by coincubation with the estrogen receptor antagonists tamoxifen (Wagner et al., 2001), this subunit has not been identified in platelets and estrogen did not alter either p47- and p67-phox.

In summary, tamoxifen is effective in the prevention and treatment of breast cancer, but its use is associated with an increased risk of thrombosis. The mechanism for this effect is unknown. Because reactive oxygen intermediates enhance platelet-dependent thrombosis, the effects of tamoxifen and its bioactive metabolites on platelet activity and platelet reactive oxygen species were studied. These studies showed that incubation of platelets with the active metabolites of tamoxifen increases stimulation-dependent superoxide release through a NADPH oxidase-dependent...

Fig. 6. Effect of tamoxifen and its metabolites on translocation of p47- and p67-phox to the platelet cytoskeleton. Isolated platelets were preincubated with 1 $\mu$M tamoxifen, 100 nM 4-OH, 4 $\mu$M NDM, 100 nM endoxifen, or vehicle control for 20 min and stimulated with 25 $\mu$M TRAP. The Triton X-100-insoluble fraction was analyzed by Western blot using antibodies specific for p47- and p67-phox. Densitometry was performed to quantify the amount of p47- and p67-phox protein present and expressed as the mean ± S.E.M. ($n = 3$; *, $P < 0.05$ compared with control).
mechanism. This results in enhanced platelet function and seems to be consistent with previous oncological studies demonstrating tamoxifen-dependent increase in reactive oxygen species generation.

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