Tamoxifen-induced adduct formation and cell stress in human endometrial glands

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Abbreviations: CYP, cytochrome P450; GRP78, glucose regulating protein 78; NFκβ, nuclear factor κβ; PAS, periodic acid Schiff; PBS, phosphate buffered saline; SULT, sulphotransferase
Abstract

The beneficial effects of tamoxifen in the prevention and treatment of breast cancer are compromised by an increased risk of endometrial polyps, hyperplasia, and cancer. Tamoxifen is metabolized to an array of metabolites with estrogenic effects but also to reactive intermediates that may form protein and DNA adducts. The aim of this study was to investigate cellular $[^3]$H$tamoxifen adduct formation by light-microscopic autoradiography and cell stress by immunohistochemical analysis of GRP78, NF$\kappa$B and caspase 3 in human endometrial explants following short-term incubation with tamoxifen. The cellular expression of tamoxifen-metabolizing enzymes in human endometrial biopsy samples was also determined by immunohistochemistry. The results showed selective $[^3]$H$tamoxifen adduct formation in glandular and surface epithelia following incubation with a non-toxic concentration of $[^3]$H$tamoxifen (6 nM). There was also a selective expression of the endoplasmic reticulum stress chaperon GRP78 and activated caspase 3 at these sites following incubation with cytotoxic concentrations of tamoxifen (10-100 $\mu$M). The cell stress was preferentially observed in samples from women in the proliferative menstrual phase. No treatment-related expression of NF$\kappa$B was observed. Constitutive expression of the tamoxifen-metabolizing enzymes CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, and SULT2A1 in glandular and surface epithelia was demonstrated but there was a large interindividual variation. The co-localization of $[^3]$H$tamoxifen adducts, expression of GRP78, caspase 3 and tamoxifen-metabolizing enzymes in human glandular and surface epithelia suggest a local bioactivation of tamoxifen at these sites and that epithelial cells are early target sites for tamoxifen-induced cell stress.
Introduction

Tamoxifen is one of the most widely used agents for prevention and treatment of estrogen receptor-positive breast cancer but the beneficial effects are compromised by an increased risk for benign and malignant endometrial changes (Dunn and Ford, 2001). In 1998, when the National Surgical Breast and Bowel Project published the breast cancer prevention trial (BCPT), it became evident that long-term tamoxifen therapy (five years) more than doubled the incidence of endometrial cancer, from 0.9 to 2.3 cases per 1000 women (Dunn and Ford, 2001). It is generally accepted that the benefits of tamoxifen therapy far outweigh the potential risks among women who suffer from breast cancer but the prophylactic use of the drug is open to question in view of the increased risk of developing endometrial lesions.

Tamoxifen’s metabolism is highly complex. It is a pro-drug that is metabolized by cytochrome P450 (CYP) enzymes to an array of pharmacologically active metabolites with mixed agonistic and antagonistic effects on the estrogen receptors, but CYP-metabolism may also generate reactive intermediates (Phillips et al., 1999; Desta et al., 2004). CYP3A and CYP2D6 have been identified as the major enzymes involved in the principal routes of tamoxifen’s hepatic metabolism, but other CYP forms (CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C8, CYP2C9, CYP2C19) may also contribute (Desta et al., 2004; Notley et al., 2005). A local variation in the formation of pharmacologically active metabolites and reactive intermediates in the tissues due to cell specific expression of CYP enzymes might be one important reason for variability in tissue response and toxicity.

Experimental studies have demonstrated that tamoxifen is a rat liver carcinogen that forms DNA adducts in the rat liver (Phillips et al., 2005). In addition, tamoxifen protein adducts have been detected in human liver microsomes (Dehal and Kupfer, 1996). Both DNA adduct
formation and estrogenic effects have been suggested as the mechanism by which tamoxifen induces cancer in the human endometrium (Kim et al., 2004; Shang, 2006). Attempts to determine whether or not tamoxifen therapy can lead to DNA adducts in the human endometrium have yielded conflicting results (Greaves et al., 1993; Hemminki et al., 1996; Carmichael et al., 1999; Shibutani et al., 2000; Sharma et al., 2003b; Beland et al., 2004). Studies in experimental model systems suggests that sulfation of α-hydrolylated tamoxifen metabolites mediates DNA adduct formation (Shibutani et al., 1998; Kim et al., 2005a). In addition, covalent binding to proteins and to 2′-deoxyguanosine via CYP3A4 and/or CYP2D6 catalyzed formation of 3,4-dihydroxytamoxifen and subsequent bioactivation have been reported in human and rat liver microsomes (Dehal and Kupfer, 1999). The formation of protein adducts have not been reported in endometrial tissues, however.

Because tamoxifen is extensively used as a chemopreventive drug to prevent breast cancer relapse there is a need to further study the fate of tamoxifen in the human endometrium. The first objective of the present study was to investigate cellular [3H]tamoxifen adduct formation in human endometrial explants following short-term incubation with tamoxifen. Endometrial explants have a normal mixture of stromal and glandular cells and the constitutive enzyme activity is retained for at least 24 hours making them suitable as an in vitro model for studying biotransformation and cellular effects of tamoxifen (Sharma et al., 2003a). The maintenance of the normal cellular architecture and enzyme activity makes tissue explants attractive for studies using light microscopic autoradiographical techniques to visualize cell specific bioactivation of radiolabelled chemicals ( Brittebo and Brandt, 1997). Using this approach we have previously identified unforeseen sites of local bioactivation of chemicals in rodent extra-hepatic tissues and some potent and cell specific toxicants have been demonstrated (Annas and Brittebo, 1998; Granberg et al., 2003).
A marked covalent binding of reactive intermediates to tissue macromolecules may be considered as an early event in a process that can lead to cell death or carcinogenicity. The second objective was therefore to examine early onset stress protein induction in human endometrial explants following short-term incubation with tamoxifen. The endoplasmic reticulum stress chaperon glucose regulating protein 78 (GRP78) and activated caspase 3 were selected as markers of cell stress. NFκβ was selected as a marker of signalling pathways suggested to be regulated by tamoxifen (Gielen et al., 2005). The third objective was to investigate the cell specific expression of tamoxifen-metabolizing enzymes in human endometrium since a selective bioactivation of tamoxifen to reactive intermediates may be related to cell specific expression of CYP enzymes and SULT2A1.
Materials and Methods

Chemicals

Radiolabeled tamoxifen methiodide [N-methyl-³H] with a specific activity of 80 Ci/mmol and radiochemical purity of > 97 % was purchased from American Radiolabeled Chemicals, St Louis, MO, USA. Tamoxifen citrate (C₆H₉C(C₂H₅)=C(C₆H₅)C₆H₂OCH₂CH₂N(CH₃)₂)(CAS 10540-29-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Primary antibodies, rabbit anti-human CYP1A1, 1B1, 2A6, 2B6, 2C8/2C9/2C19, 2D6 and 3A4/5, were purchased from Millipore, Chemicon, Billerica, MA, USA. According to the manufacturer these antibodies do not cross-react with other CYP enzymes. Rabbit anti-human GRP78 and caspase 3 were purchased from Cell Signalling Technology, Danvers, MA, USA, and rabbit anti-human NFκB, and SULT2A1 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The secondary antibody, a biotin-labeled swine anti-rabbit IgG, was bought from DAKO Cytomation, Glostrup, Denmark. Biotin-labeled horse anti-rabbit/mouse/goat, avidin-biotin peroxidase complex (ABC), and Vector VIP substrate kit for peroxidase assay were obtained from Vector Laboratories, Burlingame, CA, USA. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Hydrogen peroxide was purchased from VWR International, Stockholm, Sweden. Schiff’s reagent was purchased from Solveco AB, Rosersberg, Sweden, and PBS from Invitrogen, Paisley, UK.

Isolation of human endometrial biopsy samples

Human endometrial biopsy samples were collected from 24 hysterectomy specimens. The surgery was performed at Uppsala University Hospital, Uppsala, Sweden. The endometrial biopsy samples were obtained from women undergoing hysterectomy on benign indications and with no signs of endometrial pathology. The menstrual cycle phase was determined from
menstrual history data and confirmed by histological staining with PAS-hematoxylin (Campbell et al., 2000). All patients were of fertile age and had regular menstruation cycles. None of the women included in the study had received hormone therapy within 3 months prior to surgery. The Ethics Committee at Uppsala University approved the use of human endometrial tissue and informed consent was obtained from each study participant. The endometrial biopsy samples were obtained immediately after surgery and transferred to ice-cold sterile PBS, where they were stored for no more than 3 hours prior to experiments or processing for immunohistochemistry.

**Autoradiographic studies on the localization of [3H]tamoxifen adducts in endometrial explants**

Endometrial biopsy samples from four women, three in the proliferative phase and one in the secretory phase, were cut into millimeter-sized pieces and incubated for 2 hours with 6 nM [3H]tamoxifen dissolved in 10 ml sterile PBS. A low non-toxic concentration (6 nM) was used to preserve the metabolic activity in the cells and to get a high level of specific radioactivity. Based on our previous experience with autoradiographic studies of covalent binding of drugs and chemicals in rodents, concentrations equivalent to the plasma concentration, approximately 370 nM, was not suitable for the autoradiographic studies (Jin et al., 2005). The vials were flushed briefly with O2 prior to the start of the incubation, which was performed by rotation in an incubation chamber with 95% oxygen/5% carbon dioxide at 37°C. Controls were incubated at 0°C. The samples were transferred to ice-cold 4% phosphate-buffered formaldehyde (pH 7) immediately after incubation and processed as described by (Granberg et al., 2003). The formalin-fixed endometrial biopsy samples incubated with [3H]tamoxifen were first extracted and dehydrated in an ethanol series as follows: 50% for 12 hours, 70% for 12 hours, 95% for 3 hours, 99.5% for 2 hours and
xylene for 30 minutes. The explants were then embedded in paraffin and sectioned at, 4 μm, on glass slides. The paraffin-embedded tissue sections were deparaffinized in xylene for 30 minutes and rehydrated using a decreasing concentration series of ethanol, 99.5 %, 95 %, 70 %, for 30 minutes at each concentration. During the extensive dehydration/rehydration procedures, the unbound and reversibly bound parent compound and its metabolites are extracted, leaving only non-extractable, most likely covalently bound, metabolites in the tissue. The non-extractable radioactivity in the tissues is therefore considered to represent [3H]tamoxifen adducts. The solvent-extracted tissue sections were dipped in NTB-2 liquid film (Kodak, Rochester, NY, USA) and stored dark at 4°C for 3-4 months before development and staining with hematoxylin and eosin. Autoradiograms were examined by light microscopy using an Olympus BH-2 RFCA microscope, and the digital images were captured with a NIKON DXM1200F camera. Autoradiograms were also examined using dark field microscopy in a Leica DMRXE microscope and the digital images were captured with an ORCAIII camera (Hamamatsu Photonics, Hamamatsu city, Japan). The digital images were processed with Adobe PhotoShop CS3.

Immunohistochemical studies on tamoxifen’s effects on the expression of activated caspase 3, GRP78 and NFκβ in endometrial explants

Endometrial biopsy samples from six women, four in the proliferative phase and two in the secretory phase were cut into millimeter-sized pieces and incubated for 4 hours with tamoxifen dissolved in 10 ml sterile PBS, within 3 hours after surgery. The use of cell culture medium was avoided because phenol red is reported to have estrogen like effects (Ernst et al., 1989). Cytotoxic concentrations (10 μM, 50 μM or 100 μM) markedly exceeding the reported plasma concentration were used and a short incubation time was selected to avoid significant cell death (Jin et al., 2005). The vials were incubated at 37 or 0°C as described above. In
addition, vehicle-treated controls were incubated at 37°C. After incubation the explants were transferred to ice-cold 4% phosphate-buffered formaldehyde (pH 7) and immunohistochemistry was performed as described below.

*Immunohistochemical studies on the constitutive expression of tamoxifen-metabolizing CYPs and SULT2A1 in endometrial explants*

The constitutive cellular expression of tamoxifen-metabolizing enzymes was examined in human endometrial explants from 14 women in the proliferative phase and 10 women in the secretory phase of the menstrual cycle. The explants were fixed in ice-cold 4% phosphate-buffered formaldehyde (pH 7) within 3 hours after surgery and the expression of CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2C8/2C9/2C19, CYP2D6, CYP3A4, and SULT2A1 was examined by immunohistochemistry as described below. The expression of all enzymes was not examined in all 24 women because of a limited amount of tissue.

*Immunohistochemistry*

Fixed endometrial samples were embedded in low-temperature paraffin and sectioned (4 µm). The slides were washed with PBS with and without 0.3% triton X-100 for 10 minutes. Endogenous peroxidase activity was quenched with hydrogen peroxide for 30 minutes. The sections were then washed in PBS and immersed in sodium citrate buffer (pH 8) for antigen retrieval by microwave treatment and again washed in PBS. Non-specific binding sites were blocked with 4% BSA in PBS for one hour at room temperature. The sections were incubated overnight in a humidified chamber with the primary antibody diluted in PBS with 4% BSA at 4°C. The primary antibodies were diluted as follows: CYP1A1 and CYP1B1 (1:1000); CYP 2A6, 2B6, 2C8/2C9/2C19 and 2D6 (1:750); CYP3A4 and SULT2A1 (1:500); caspase 3, GRP78 and NFκβ (1:100). The sections were rinsed in PBS and incubated with a secondary
antibody (biotin-coupled swine anti-rabbit biotin-labeled horse anti-rabbit/mouse/goat) at room temperature for 30 minutes (1:300 dilution in 4% BSA). ABC solution was applied and the sections were incubated at room temperature for 30 minutes and rinsed in PBS. They were then stained with Vector’s VIP substrate kit for peroxidase assay for 2-6 minutes and thereafter rinsed in ddH₂O. Subsequently the sections were dehydrated with an increasing concentration series of ethanol: 70 %, 95 %, 99.5 %, and xylene. Finally, they were mounted in Pertex.

Immunohistochemical staining was performed on at least two separate occasions for each primary antibody. Negative controls for endometrial samples were performed by omitting the primary antibody. Rodent liver was used as a positive control for CYP staining, and nasal mucosa from toxicant-treated mice was used as a positive control for GRP78 (Franzen and Brittebo, 2005). The negative controls served as references when the sections were evaluated and scored. The slides were coded and evaluated by two persons on separate occasions. The staining of stroma, glandular epithelium, surface epithelium, desquamated cells in the glandular lumen, and blood vessels were scored on a four-graded scale (0-3). The stained slides were analyzed using light microscopy and the digital images were captured in an Olympus BH-2 RFCA microscope with a NIKON DXM1200F camera. The images were processed with Adobe PhotoShop CS3.
Results

Cellular localization of \([^{3}\text{H}]\text{tamoxifen adducts}\)

Analysis of autoradiograms of solvent-extracted tissue sections from endometrial explants incubated with a low, non-toxic concentration of \([^{3}\text{H}]\text{tamoxifen}\), revealed a selective localization of radioactivity (black silver grains) in glandular and surface epithelia (Table 1, Figs. 1 and 2). Since the thin tissue sections (4 µM) had been extensively extracted with solvents, the radioactivity is considered to represent \([^{3}\text{H}]\text{tamoxifen adducts}\) bound to tissue constituents. No selective intracellular localization of silver grains was observed in the epithelial cells. The level of radioactivity in the stroma and blood vessels did not exceed the background level. The cellular localization of radioactivity was similar in the samples from all studied women, 3 in the proliferative phase and 1 in the secretory phase of the menstrual cycle. The level of radioactivity in explants incubated at 0°C did not exceed that of the background level. No signs of treatment-related morphological changes were observed in the samples. Since \([^{3}\text{H}]\text{tamoxifen}\) was labeled in the methyl-groups, adducts due to N-demethylated reactive intermediates cannot be detected.

Tamoxifen-induced cellular expression of activated caspase 3 and GRP78

Table 2 presents the compiled immunohistochemical data on the expression of caspase 3, GRP78, and NFκβ in tamoxifen-treated endometrial explants from six women, four in the proliferative phase and two in the secretory phase of the menstrual cycle. The PAS-hematoxylin staining confirmed the menstrual history data on cycle phase. The glandular epithelium and lumen content in the endometrial samples from women in the secretory phase showed an intense red PAS-positive reaction whereas endometrial samples from women in the proliferative phase showed no PAS-positive reaction in the glands. The surface epithelium was not present in all samples, but when it was seen it displayed results similar to those of the
glandular epithelium.

**Caspase 3:** Endometrial explants from all six women (nos. 5, 6, 7, 8, 9, 10) included in this part of the study showed preferential expression of activated caspase 3 in the glandular and surface epithelia and in desquamated cells in the glandular lumen following incubation with tamoxifen for 4 hours. The level of expression among glands did, however, show some heterogeneity. The desquamated cells in the glandular lumen displayed higher expression of activated caspase 3 than the epithelial cells, which showed moderate expression (Fig 3A-D). However, some scattered cells in the glandular epithelium also expressed a high level of activated caspase 3 (Fig. 3E-F). In explants from women in the proliferative phase (nos. 5, 6, 7, 8) incubation with all concentrations of tamoxifen (10-100 \( \mu \)M) at 37°C led to increased expression of activated caspase 3 in epithelial cells and in desquamated cells in the glandular lumen (Table 2). In explants from women in the secretory phase (no. 9 and 10), only incubation with the highest concentration of tamoxifen (100 \( \mu \)M) resulted in increased caspase 3 expressions in epithelial cells and in desquamated cells in the glandular lumen (Table 2). The staining of the stroma was weak or absent in all samples (Table 2). No cell specific staining was observed in endometrial explants incubated with vehicle at 37°C or with tamoxifen at 0°C (Fig. 3G-H). The negative controls, obtained by omission of primary antibody, showed no staining.

**GRP78:** Tamoxifen-treatment for 4 hours induced the expression of the endoplasmic reticulum stress chaperon GRP78 in glandular and surface epithelia in endometrial explants from three (nos. 6, 7, 8) of four women in the proliferative phase (Table 2). The level of expression among glands did, however, show some heterogeneity. The expression of GRP78 was most intense in the cytoplasm and plasma membrane of the epithelial cells, and some
glands displayed higher expression in the apical part of the cells (Fig. 4A-D). The expression of GRP78 observed in the endometrial explant from women no. 5 (proliferative phase) was not considered to be treatment-related, as GRP78 expression was also observed in the vehicle control and in explants incubated at 0°C. In explants from women in the secretory phase (no. 9 and 10), no selective tamoxifen-induced expression of GRP78 was detected in the glands. There was, however, a weak or moderate staining in a few glands (Table 2). The staining of stroma was weak or absent in all samples (Table 2). No cell specific staining was detected in the vehicle controls, except in women no. 5, or in explants incubated with tamoxifen at 0°C. The negative controls, obtained by omission of primary antibodies, showed no staining.

**NFκβ:** The expression of NFκβ following incubation of endometrial explants with tamoxifen (10-100 µM) for 4 hours showed large variation both within the samples and between women. Tamoxifen-induced expression of NFκβ was observed in the glandular epithelium in endometrial explants from two women in the proliferative phase (no. 5 and 7) (Table 2). These explants exhibited higher expression after incubation with a high concentration of tamoxifen (Table 2). The other samples (no 6, 8, 9, 10) displayed a weak or moderate staining in a few glands (Table 2). The staining of stroma was weak or absent in all samples (Table 2). No cell specific staining of NFκβ was detected in explants incubated with vehicle at 37°C or with tamoxifen at 0°C. The negative controls, obtained by omission of primary antibodies, showed no staining.

*Cellular expression of tamoxifen-metabolizing CYP enzymes and SULT2A1*

The overall data for the cellular expression of CYP enzymes and SULT2A1 in endometrial biopsy samples are presented in Table 3. The results demonstrated preferential expression of
CYP enzymes and SULT2A1 in glandular and surface epithelial cells, but with a large interindividual variation. Immunohistochemical analysis revealed moderate expression of CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, and SULT2A1 in glandular and surface epithelia in endometrial biopsy samples from some of the women included in the study (Table 3, Fig. 5). The individual expression of CYP enzymes in epithelial cells was as follows: 8 of 10 women (80%) expressed CYP1B1; 3 of 11 women (27%) expressed CYP2A6; 8 of 11 women (72%) expressed CYP2B6; 5 of 13 women (38%) expressed CYP2C8/9/19; 6 of 14 women (43%) expressed CYP2D6 and 2 of 8 women (25%) expressed SULT2A1. A strong expression of CYP1A1 and CYP1B1 was observed in blood vessel endothelium and vascular smooth muscle cells (images not shown). CYP3A4 was not detected in samples from the studied women (0 of 9 women) (images not shown). Most of the tissue sections also showed very weak CYP staining in the stroma as compared to the negative controls, indicating that a weak CYP expression at this site cannot be ruled out. No staining was observed in the negative controls obtained by omission of the primary antibodies.
Discussion

The beneficial effects of tamoxifen in the prevention and treatment of breast cancer are compromised by an increased risk of endometrial polyps, hyperplasia, and cancer (Dunn and Ford, 2001). Both estrogenic and genotoxic mechanisms have been suggested as being responsible for the tamoxifen-induced lesions in the human endometrium, but the underlying mechanisms are not fully understood (Kim et al., 2004; Shang, 2006). The present study demonstrated that short-term incubation with tamoxifen caused tamoxifen adduct formation and increased expression of the stress proteins GRP78 and activated caspase 3 in the glandular and surface epithelia of human endometrial explants whereas there was no expression of stress proteins in the stroma. Furthermore, immunohistochemical analysis of tamoxifen-metabolizing enzymes revealed a moderate, but distinct, expression of CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, and SULT2A1 in endometrial glandular and surface epithelia in some individuals. Samples from all women included in the study showed expression of some of these enzymes but the individual expression profiles displayed large variations. These findings suggest that the human glandular and surface epithelia are early sites of bioactivation and cell stress related to tamoxifen exposure. These results are in agreement with previous histological studies demonstrating specific changes in endometrial glandular epithelial cells of tamoxifen-treated women (Hachisuga et al., 2009). Furthermore, Pole and co-workers report that tamoxifen induces specific gene expression changes in primary cultured endometrial epithelial cells as compared to stromal cells (Pole et al., 2005). The short incubation time indicates that the observed up-regulation of stress proteins is most likely due to post-translational changes and not a consequence of increased mRNA transcription.
Tamoxifen is metabolized into pharmacologically active estrogenic metabolites but it may also be bioactivated to reactive metabolites that form adducts with proteins and DNA (Desta et al., 2004; Notley et al., 2005). The autoradiographic studies demonstrated a selective \[^{3}H\]tamoxifen adduct formation in the endometrial glandular and surface epithelia indicating the formation of reactive tamoxifen metabolites that become covalently bound to the tissue. These findings are in line with previous reports demonstrating that human and rat liver microsomes bioactivate tamoxifen to reactive intermediates that bind covalently to microsomal proteins (Dehal, 1996 #376). We have previously compared the tissue localization of carcinogen adducts in vivo and in vitro following short-term incubations of rodent tissue slices with radiolabeled compounds (Brittebo and Brandt, 1997). The autoradiographic in vivo-in vitro correlations in rodents are generally good suggesting that tamoxifen may also be bioactivated in human endometrial glands in vivo. Since the radiolabeled N-methyl group of tamoxifen is susceptible to oxidative demethylation by some of the CYPs that are expressed in the epithelial cells, it is possible that the bound radioactivity is due to the released one-carbon fragment. However, previous autoradiographic studies on the incorporation of radioactive formaldehyde in vivo demonstrated that radioactivity is preferentially incorporated into cells and tissues with a very rapid turnover such as the gastrointestinal epithelium and bone marrow of rodents (Johansson and Tjalve, 1978). The short incubation time used in the present in vitro study suggest a low cell turnover in the explants and an incorporation of radiolabeled formaldehyde seems less likely.

Previous studies in endometrial explants have provided conflicting evidence concerning the ability of human endometrial cells to bioactivate tamoxifen to DNA binding products (Carmichael et al., 1996; Sharma et al., 2003b; Beland et al., 2004; Kim et al., 2005b). Furthermore, studies examining the presence of tamoxifen DNA adducts in the endometrium
of tamoxifen users have yielded inconsistent results (Hemminki et al., 1996; Carmichael et al., 1999; Shibutani et al., 2000). Notably endometrial samples have a heterogeneous cellular composition consisting of glands, stroma and blood vessels etc. This study demonstrated that \[^{3}H\]tamoxifen adduct formation was confined to the glandular and surface epithelia leaving the stroma and blood vessels devoid of labeling following a short-term exposure to \[^{3}H\]tamoxifen. Analysis of DNA adducts in homogenates of the endometrial tissue may make it difficult to detect the presence of adducts in a restricted cell population due to dilution by other cell types with no adducts.

The expression of bioactivating and detoxification enzymes as well as the level of glutathione are key factors that may determine covalent binding of reactive intermediates in various cell types (Park et al., 1995). Our immunohistochemical studies demonstrated expression of CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, and CYP2D6 in endometrial glandular and surface epithelia but with a large interindividual variation. No selective expression of CYP3A4 or CYP1A1 was detected at these sites. Protein expression of CYP1B1, CYP2C9 and CYP3A4 has previously been demonstrated in glands and stroma (Sharma et al., 2003a). In addition, expression of CYP2B6, CYP2C, and CYP3A4 mRNA has been detected in homogenates of human endometrial samples (Hukkanen et al., 1998) whereas neither CYP2D6 nor CYP2A6 mRNA have been observed (Hukkanen et al., 1998; Sharma et al., 2003a). Dehal and co-workers suggested that CYP3A4, and to a lesser extent CYP2D6, can catalyze formation of 3,4-dihydroxytamoxifen and that bioactivation of this metabolite may lead to adduct formation in rat liver microsomes (Dehal and Kupfer, 1999). Studies using recombinant expressed enzymes have identified CYP3A4 (Desta et al., 2004; Notley et al., 2005) and CYP2D6 (Sharma et al., 2003a) as the major CYPs responsible for the formation of \(\alpha\)-hydroxylated tamoxifen metabolites that can be further metabolized to reactive
intermediates, but CYP1A1, CYP1B1, CYP2B6, CYP2C9, CYP2C19, CYP3A5 can also catalyze α-hydroxylation. Experimental model systems using human recombinant enzymes have demonstrated that SULT2A1-catalyzed sulfation of α-hydroxytamoxifen leads to DNA adduct formation (Shibutani et al., 1998). Furthermore, studies indicate that sulfation of α,4-dihydroxytamoxifen and α-hydro-N-desmethyaltamoxifen to reactive intermediates mediates the formation tamoxifen-DNA adducts in rats (Phillips et al., 1999). A recent study demonstrated quantifiable levels SULT2A1 mRNA in endometrial samples whereas SULT2A1 activity has not been detected in the human endometrium (Rubin et al., 1999; Singh et al., 2008). Our immunohistochemical results revealed a glandular specific expression of SULT2A1 in explants from two of eight women. The low number of human samples examined and the large interindividual variation may explain discrepancies between reports on the expression of tamoxifen-metabolizing enzymes in the human endometrium. Taken together, however, the data suggest that human glandular and surface epithelia have the ability to metabolize tamoxifen to several metabolites such as 3,4-dihydroxytamoxifen, α-hydroxytamoxifen, α,4-dihydroxytamoxifen and α-hydro-N-desmethyaltamoxifen that may be further metabolised to reactive intermediates.

Many drugs and chemicals require metabolic activation to exert toxicity and a high local level of adducts indicates potential target sites for toxicity. The expression of activated caspase 3, GRP78 and NFκβ in tamoxifen-treated endometrial explants were therefore investigated. The most striking result was obtained for caspase 3. Tamoxifen-treatment induced expression of activated caspase 3 in glandular and surface epithelia as well as in desquamated cells in the glandular lumen in explants from all studied women. The highest expression of activated caspase 3 occurred in scattered cells in the epithelium and in desquamated cells in the lumen whereas the other parts of the epithelium displayed a low or moderate expression. This
suggests an early damage in a limited number of epithelial cells leading to disruption of cell adhesion and subsequent desquamation. Activation of caspase 3 is known to play a critical role in the execution of apoptosis and previous in vitro studies have demonstrated that tamoxifen display pro-apoptotic effects in primary endometrial cell cultures (Stackievicz et al., 2001). Recent studies have also demonstrated that increasing levels of tamoxifen may switch the mode of cell death from autophagy to apoptosis and finally to necrosis in a breast cancer cell line suggesting that the toxic effects of tamoxifen are concentration-dependent (Bursch et al., 2008). No increased level of apoptosis in the endometrium has, however, been reported in tamoxifen users (Mourits et al., 2002).

Tamoxifen-treatment also induced the expression of the endoplasmic reticulum stress chaperon GRP78 in endometrial samples from three out of six women, all in the proliferative phase. This is the first association between tamoxifen and GRP78 expression in the human endometrium. GRP78 is induced by a variety of stress signals including cell specific bioactivation of toxicants, and its main function appears to be maintenance of protein integrity following proteotoxic insult (Cribb et al., 2005). The endoplasmic reticulum contains the majority of the drug-metabolizing CYP enzymes and can be a target for initial damage by reactive intermediates. The rapid up-regulation of GRP78 observed here is in line with our previous finding regarding early onset endoplasmic reticulum stress response related to a local CYP-catalyzed bioactivation of toxicants in rodents (Franzen and Brittebo, 2005). However, 17β-estradiol may upregulate GRP78 in the rodent uterus and it cannot be excluded that the observed up-regulation of GRP78 in human endometrial glands may be related to tamoxifen’s estrogenic properties (Ray et al., 2006).

NFκβ has previously been identified as one of the genes that show selective expression in
tamoxifen users (Gielen et al., 2005). The results obtained in the present study did not, however, suggest any distinct treatment-related effects on NFκβ protein expression in endometrial glands following short-term incubation with tamoxifen. It must, however, be emphasized that the short incubation time and high concentrations of tamoxifen used in the present study is very different from an in vivo situation and that an effect on mRNA level may not be reflected on the protein expression.

Human endometrium undergoes cyclic morphological changes in response to circulating estrogen and progesterone and the endometrial epithelial cell population is reported to exhibit wide intercellular heterogeneity (Campbell et al., 2000). We found that there was an increased glandular specific expression of activated caspase 3 in the proliferative endometrium exposed to 10 -100 μM of tamoxifen whereas in the secretory endometrium only the highest tamoxifen concentration (100 μM) increased the expression of activated caspase 3 in endometrial glands. Furthermore, increased expression of the stress protein GRP78 in endometrial glands was evident only in the proliferative endometrium. These observations suggest that endometrial glands are more susceptible towards tamoxifen-induced cell stress during the proliferative phase of the menstrual cycle.

In conclusion, the present study revealed a co-localization of [3H]tamoxifen adducts and expression of activated caspase 3 and GRP78 in glands and surface epithelium of human endometrial explants following a short-term incubation with tamoxifen. The results also demonstrated a selective expression of tamoxifen-metabolizing CYPs and SULT2A1 in glandular and surface epithelia in human endometrial biopsy samples, albeit with a large interindividual variation. These findings suggest that bioactivation and subsequent adduct formation of tamoxifen in endometrial glands may play a role for tamoxifen-induced lesions.
in the human endometrium and that the glandular and surface epithelia are early target sites for tamoxifen-induced cell stress.
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Footnotes

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Legends for Figures

Figure 1. Light microscopy autoradiograms showing the localization of [\(^3\)H]tamoxifen adducts in human endometrial explants

Autoradiography of solvent-extracted tissue sections demonstrates selective localization of [\(^3\)H]tamoxifen adducts (black silver grains) in glandular epithelium (A) and surface epithelium (B) in endometrial explants incubated with [\(^3\)H]tamoxifen (6 nM) for 2 hours at 37°C. There is no corresponding localization of radioactivity in explants incubated with [\(^3\)H]tamoxifen at 0°C (C, D). High intensity of black silver grains corresponds to a high level of non-extractable radioactivity in the tissue.

Figure 2. Dark field microscopy autoradiogram showing the localization of [\(^3\)H]tamoxifen adducts in human endometrial explants

Autoradiography of solvent-extracted tissue sections showing the localization of [\(^3\)H]tamoxifen adducts in glandular epithelium in endometrial explants incubated with 6 nM [\(^3\)H]tamoxifen for 2 hours at 37°C. (A) Dark-field microscopy showing white areas corresponding to a high level of non-extractable radioactivity in the tissue. (B) Corresponding light field microscopy.

Figure 3. Immunohistochemical localization of activated caspase 3 in human endometrial explants following short-term incubation with tamoxifen

Tamoxifen induces expression of caspase 3 in endometrial glands in samples from all six women included in the study. The expression in the stroma was weak or absent. A-F shows representative images of the pattern of caspase 3 expression in endometrial samples following
incubation with tamoxifen (10 μM, 50 μM or 100 μM for 4 hours). A: 50 μM, women 6; B: 100 μM, women 5; C: 10 μM, women 8; D: 10 μM, women 7; E: 100 μM, women 6; F: 100 μM, women 9. In general, desquamated cells in the glandular lumen exhibit the highest expression and glandular epithelial cells display moderate expression (A-D), but in some glands scattered epithelial cells express a high level of caspase 3 (E, F). No cell specific staining is present in tissues incubated with tamoxifen at 0°C (G: 100 μM, women 5) or vehicle (H: 0.1% ethanol, women 9).

Figure 4. Immunohistochemical localization of GRP78 in human endometrial explants following short-term incubation with tamoxifen

Tamoxifen induces expression of GRP78 in endometrial glands in three of the six women included in the study. The expression in the stroma is weak or absent. A-D shows representative images of the pattern of GRP78 expression in endometrial samples following incubation with tamoxifen (10 μM, 50 μM, or 100 μM for 4 hours). A: 100 μM, women 6; B: 100 μM, women 7; C: 50 μM, women no 8; D: 50 μM, women 6. The expression is most intense in the cytoplasm (A-D) and some glands show the highest expression in the apical part (D). No cell specific staining is present in tissues incubated with tamoxifen at 0°C (E: 100 μM, women 6) or vehicle (F: 0.1% ethanol, women 6).

Figure 5. Immunohistochemical localization of tamoxifen-metabolizing enzymes in human endometrial explants

CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, and SULT2A1 are preferentially expressed in glandular epithelial cells and surface epithelium. The figure shows representative images of samples considered as positive.
Table 1. Adduct formation in [³H]tamoxifen-treated human endometrial explants

Endometrial explants were incubated with a non-toxic concentration of [³H]tamoxifen for 2 hours at 37°C. After fixation the samples were processed for autoradiography. The autoradiograms were evaluated by light microscopy and dark field microscopy.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Glandular epithelium</th>
<th>Surface epithelium</th>
<th>Stroma</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>4</td>
<td>+</td>
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*a* The level of [³H]tamoxifen adducts exceeds background level. *b* The level of [³H]adducts is equivalent to background level.
Table 2. Expression of caspase 3, GRP78 and NFκβ in tamoxifen-treated endometrial explants

Endometrial samples were treated with 10, 50 or 100 μM tamoxifen for 4 hours at 37°C, fixed and embedded in paraffin. After fixation the samples were processed for immunohistochemistry and the tissue sections was evaluated by light microscopy.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Menstrual cycle phase</th>
<th>μM tamoxifen</th>
<th>Activated caspase 3</th>
<th>GRP78</th>
<th>NFκβ</th>
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<td></td>
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<td>100</td>
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α 3= intense staining, 2= moderate staining, 1= weak staining, 0= no staining.
Table 3. Expression of tamoxifen-metabolizing enzymes in endometrial glandular and surface epithelium

Summary of the total number of women that showed a significant expression of CYP1B1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6 and SULT2A1 endometrial glandular and surface epithelium and the distribution between proliferative and secretory phase.

<table>
<thead>
<tr>
<th>Tamoxifen-metabolizing enzymes expressed glandular and surface epithelium</th>
<th>No. of positive individuals</th>
<th>No. of positive individuals in proliferative phase</th>
<th>No. of positive individuals in secretory phase</th>
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<tbody>
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<td>CYP1B1</td>
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<td>4</td>
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<td>1</td>
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<tr>
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<td>6/14</td>
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<td>1</td>
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<tr>
<td>SULT2A1</td>
<td>2/8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a Individuals that showed a significant expression of drug metabolizing enzymes in glandular epithelium compared to other cell types were considered a
Figure 2

A

B

glands

glands