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Catechol-*O*-methyltransferase: Effects of the val108met polymorphism on protein turnover in human cells

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Abstract

A single nucleotide polymorphism in the human *COMT* (catechol-*O*-methyltransferase) gene has been associated with increased risk for breast cancer and several CNS diseases and disorders. The G to A polymorphism causes a valine (val) to methionine (met) substitution at codon 108 soluble - (S)/158 membrane - (MB)-COMT, generating alleles encoding high and low-activity forms of the enzyme, $COMT^{H}$ and $COMT^{L}$, respectively. Tissues and cells with a $COMT^{LL}$ genotype have decreased COMT activity compared to $COMT^{HH}$ cells. Previously, we reported that the decreased activity was due to decreased amounts of S-$COMT^{L}$ protein in human hepatocytes. In this study, we investigated the role of S-COMT protein synthesis and turnover as determinates of reduced COMT protein in $COMT^{LL}$ compared to $COMT^{HH}$ cells. No association between S-COMT protein synthesis and COMT genotype was detected. Using a pulse-chase protocol, the half-life of S-$COMT^{H}$ was determined to be 4.7 days, which was considerably longer than expected from the half-lives of other phase 2 enzyme proteins. The half-life of S-$COMT^{L}$ compared to S-$COMT^{H}$ protein was significantly shorter at 3.0 days, but the difference was affected by the medium used during the chase period. These results suggest that increased turnover may contribute to reduced COMT activity in cells and tissues from $COMT^{LL}$ individuals. Subtle differences appear to be able to affect the stability of the S-$COMT^{L}$ protein, and this may contribute to the differences observed in epidemiological studies on the association of this polymorphism with breast cancer risk.

Keywords: Catechol-*O*-methyltransferase (COMT); *COMT* polymorphism; Protein synthesis; Protein turnover; Human breast epithelial cell lines

1. Introduction

COMT is a major inactivation enzyme for catecholamines and carcinogenic catechol estrogens. The human *COMT* gene encodes two enzymes, cytosolic soluble (S-COMT) and membrane-bound (MB-COMT), and two promoters, P1 and P2, control transcription of two different mRNAs [1]. In human tissues S-COMT is the predominantly expressed form, except in the brain where MB-COMT is highly expressed [1–3].

A G to A polymorphism in exon 4 of the human *COMT* gene encodes a val to met substitution at codon 108 (S-COMT)/158 (MB-COMT). Tissues and cells with the $COMT^{LL}$ genotype exhibit decreased COMT activity, compared to those with the $COMT^{HH}$ genotype, and the activity of the $COMT^{L}$ protein is more thermolabile compared to the $COMT^{H}$ protein [4,5]. The val108/158met *COMT* polymorphism has been positively associated with breast cancer risk in some, but not all, studies [6–14] and with a number of neuropsychiatric (reviewed in [15]) and neurodegenerative conditions, including Parkinson's disease [16,17].

Until recently the mechanism causing the decrease in COMT activity in tissues and cells with the $COMT^{LL}$ genotype was unknown. We demonstrated that the COMT genotype does not affect the kinetic characteristics of COMT activity for

Abbreviations: COMT, catechol-*O*-methyltransferase; val, valine; met, methionine; $COMT^{L}$, low-activity *COMT* allele; $COMT^{H}$, high-activity *COMT* allele; $COMT^{L}$, low-activity COMT protein; $COMT^{H}$, high-activity COMT protein; $COMT^{LL}$, low-activity COMT genotype; $COMT^{HH}$, high-activity *COMT* genotype; S-COMT, soluble COMT; MB-COMT, membrane-bound COMT; 2-OHE_2, 2-hydroxyestradiol

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methylation of catechol estrogens [18]. However, we observed that the COMT genotype does affect the amount of S-COMT protein present in cells, and it is the amount of protein that determines the level of S-COMT activity [19]. Thus, S-COMT activity (2-*O*-methylation of 2-OHE_2) and S-COMT protein levels were significantly lower in human hepatocytes from $COMT^{LL}$ individuals versus those from $COMT^{HL}$ and $COMT^{HH}$ individuals. Additionally, S-COMT activity correlated with S-COMT protein levels in hepatocyte cytosolic fractions [19]. In further support of these findings, a decrease in the levels of immunoreactive COMT protein has been observed in cells transiently expressing the met108 variant, as well as in hepatic biopsy samples [20] and postmortem human prefrontal cortex tissue samples from $COMT^{LL}$ subjects [20,21].

We hypothesized that the reduced level of S-COMT protein in $COMT^{LL}$ human cells, compared to $COMT^{HH}$, is due to altered protein turnover. The rationale for this hypothesis was based on previous reports that described thermostability differences between $COMT^{L}$ and $COMT^{H}$ proteins, suggesting altered structure [4,5]. In addition, the val108met *COMT* polymorphism is located in exon 4 and not in a promoter region, thus making it less likely to affect COMT mRNA levels. To test this hypothesis we investigated the rates of COMT protein synthesis and turnover in $COMT^{HH}$ and $COMT^{LL}$ human breast epithelial cell lines.

2. Materials and methods

2.1. Chemicals and reagents

Rabbit anti-COMT antibody, used for COMT Western blotting, was produced by Spring Valley Laboratories (Sykesville, MD, USA) using purified human recombinant S-COMT protein [18]. The rabbit anti-COMT antibody, used for COMT immunoprecipitation, was also prepared against recombinant human S-COMT and was generously provided by Dr. David Eaton (University of Washington, Seattle, WA). All other chemicals and reagents were of analytical grade and were purchased commercially.

2.2. Cells, cell culture and sample preparation

Table 1 shows the human breast epithelial cells lines used along with their reported estrogen receptor (ER) status [22]. The cell lines were obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in IMEM (Biofluids, Rockville, MD) containing 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). T47D, ZR-75-1 and MDA-MB-231 cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA) containing 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 0.2 U/ml bovine insulin and 10 μg/ml gentamiacin sulfate. MCF-10A cells were maintained in DMEM-F12 (Gibco) containing 5% horse serum (Gibco), 10 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 μg/ml gentamiacin sulfate, and 1× antibiotic–antimycotic solution (Cellgro, Herndon, VA).

Table 1
Quantitation of S-COMT Western blots in $COMT^{HH}$ and $COMT^{LL}$ human breast epithelial cell lines

	MCF-7 $COMT^{LL}$	T47D $COMT^{LL}$	ZR-75-1 $COMT^{HH}$	MDA-MB-231 $COMT^{HH}$	MCF-10A $COMT^{HH}$
ER[a] status	+	+	+	−	−
S-COMT	39.39± 19.16[b] *	32.91± 10.28 *	141.2± 67.51	6.12± 2.41 **	0.67± 0.25 **

[a] ER = estrogen receptor.
[b] Mean band intensity±SD from three separate experiments.
* $p<0.05$ compared to ZR-75-1.
** $p<0.01$ compared to ZR-75-1.

2.3. Odyssey™ Western blot analysis

Human breast cell lines were harvested, washed with 1× PBS and lysed in 1 ml of ice cold RIPA buffer [1× TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.004% sodium azide with 10 μl of each of the following added fresh to every 1 ml of 1× RIPA buffer: phenylmethylsulfonyl fluoride (PMSF) in DMSO, protease inhibitor cocktail, sodium orthovanadate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] according to the procedures provided by the manufacturer. A Bio-Rad protein assay was performed to determine protein concentrations of cell lysate samples (Bio-Rad, Hercules, CA).

Proteins in the cell lysates (15 μg of total protein) from human breast cell lines were separated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) as been previously described [18]. Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) according to the procedures provided by the manufacturer. Primary antibody (rabbit anti-human COMT) was added to the blocking buffer at a 1:10,000 dilution and secondary antibody (goat anti-rabbit IgG linked to Alexa Fluor 680) (Molecular Probes, Eugene, OR) was diluted 1:5000 in the blocking buffer. The conditions were optimized for antibody excess so that the amount of S-COMT protein detected was proportional to the amount of recombinant S-COMT and cell lysate protein per lane [18]. Immunoblotted proteins were detected using the Oydssey Imaging System (Li-Cor Biosciences). Band intensity measurements were determined using Odyssey Software, version 2.1 (Li-Cor Biosciences). Each experiment was repeated at least three times.

2.4. Determination of protein synthesis

The protocol used to measure COMT protein synthesis was adapted from [23]. The cells were plated out in 100 mm dishes and grown to 80–90% confluency. Cells in each dish were rinsed and incubated in pulse-labeling medium (RPMI 1640 lacking methionine (BioSource, Rockville, MD), 10% FBS (dialyzed overnight against PBS), 25 mM HEPES (pH 7.4), and 10 μg/ml gentamiacin) for 15 min in a 37 °C CO_2 incubator to deplete intracellular pools of methionine. Next cells were incubated with long-term labeling medium [RPMI 1640 lacking methionine (BioSource, Rockville, MD), 10% FBS (dialyzed overnight against PBS), 25 mM HEPES (pH 7.4), 10 μg/ml gentamiacin, and 1.5 mg/l unlabeled methionine] and 0.02 mCi/ml [^{35}S] methionine (MP Biomedicals, Inc., Irvine, CA) in a 37 °C CO_2 incubator. Cells were incubated for 0, 2, 4, 8 and 24 h in labeling medium. Each time point was assayed in duplicate and each experiment was repeated at least three times. At the time of harvest, the medium was removed and the plates were rinsed with 5 ml of cold 1× PBS. Cells were scraped into 1 ml of PBS and centrifuged at 4 °C for 5 min at 1000 ×*g*. The supernatant was removed and the cell pellets were frozen at −80 °C. In all the experiments utilizing [^{35}S]methionine, 100 mm dishes were placed within larger cell culture dishes containing sterile activated charcoal paper in order to absorb volatile [^{35}S]methionine break-down products.

2.5. Determination of protein turnover

This protocol to measure COMT protein turnover was adapted from [23]. The cells were plated in 100 mm dishes and grown to 10–20% confluency. To label cellular protein with [^{35}S]methionine, the cells were washed and incubated with long-term labeling medium [RPMI 1640 lacking methionine (BioSource, Rockville, MD), 10% FBS (dialyzed overnight against PBS), 25 mM HEPES (pH 7.4), 10 μg/ml gentamiacin, and 1.5 mg/l unlabeled methionine] and 0.02 mCi/ml [^{35}S]methionine (MP Biomedicals, Inc., Irvine, CA) for 16 h in a 37 °C CO_2 incubator. Two different protocols were followed during the chase period. Initially, after the 16 h labeling incubation, the long-term labeling solution was removed, cells were rinsed, and cultured in 10 ml of what is referred to as the standard chase medium, which consisted of RPMI 1640 lacking methionine (BioSource), 1 or 5% FBS (dialyzed overnight against PBS),

25 mM HEPES (pH 7.4), 10 μg/ml gentamiacin, and 15 mg/l unlabeled methionine. In the follow-up experiments, the cells were cultured in their normal growth medium containing ~15 mg/l of unlabeled methionine during the chase period. For the half-life determinations, cells were harvested at time 0 (immediately following the 16 h labeling period) and after 1, 3, 5 and 7 days. In the experiments designed to investigate only turnover (determination of day 5/day 0 pulse-chase ratios), and not half-life, cells were harvested at time 0 (immediately following the 16 h labeling period) and after incubation in chase medium for 5 days. Each time point was assayed in duplicate and each experiment was repeated at least three times. At the time of harvest, the medium was removed and plates were rinsed with 5 ml of cold 1× PBS. Cells were scraped into 1 ml of PBS and centrifuged at 4 °C for 5 min at 1000 ×*g*. The supernatant was removed and the cell pellets were frozen at −80 °C. In all the experiments utilizing [^{35}S]methionine, 100 mm dishes were placed within larger cell culture dishes containing sterile activated charcoal paper in order to absorb volatile [^{35}S]methionine break-down products.

2.6. COMT immunoprecipitation from [^{35}S]methionine-labeled cells

[^{35}S]methionine-labeled cells were lysed in 1 ml of ice cold RIPA buffer. The cell lysates (400–500 μg; equal amounts of protein used in each experiment) were combined with 5 μl of primary antibody and incubated with rotation for 2 h at 4 °C. Immunoprecipitation conditions were optimized with respect to the amount of cell lysate protein and antibody used such that the amount of labeled S-COMT and MB-COMT was proportional to the amount of [^{35}S]-labeled cell lysate protein from 250–1000 μg/sample (data not shown). Next, 20 μl of Protein A-agarose were added to cell lysates and incubated overnight at 4 °C with rotation. Immunoprecipitates were centrifuged, washed and the pellet was resuspended in 40 μl of 2× electrophoresis sample buffer [contained glycerol, 2-mercaptoethanol, SDS, 1.0 M Tris–HCl pH 6.7 and bromophenol blue (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)]. The samples were heated at 100 °C for 2–3 min and then centrifuged to pellet the agarose beads. Eluted proteins were separated via SDS-PAGE as previously described [18]. The gel was soaked in gel fixing buffer (isopropanol: water: acetic acid, 25:65:10) for 30 min, in Amplify Fluorographic Reagent (Amersham Biosciences, Piscataway, NJ) for 15 min, and then dried for 2 h at 80 °C. The dried gel was exposed to film and densitometry measurements were determined using Scion Image for Windows (Scion Corporation, Frederick, MD). Correction of band densities for growth/dilution was done by multiplying the fractional increase in total protein measured at each time point, relative to day 0, by the band density at each specific time point, Additionally, day 5/day 0 pulse-chase ratios were used to serve as a measure of protein turnover, and the ratios were calculated by dividing the mean band density of the day 5 time points by the mean band density for the day 0 time points for each cell line in each experiment.

2.7. Data analysis

Data were analyzed using Prism 3.02 (GraphPad Software, San Diego, CA). COMT protein levels among individual cell lines were compared using analysis of variance (ANOVA), with the Bonferroni post-test to compare pairs of group means. Non-linear regression analyses were used to assess the time-dependent accumulation and turnover of radiolabeled S-COMT and MB-COMT proteins. Half-lives and rate constants were derived from the equations of the non-linear regression curves for time-dependent turnover or synthesis of radiolabeled S-COMT and MB-COMT proteins. Two-tailed Student's *t* tests were used to compare rate constants, half-life determinations and pulse-chase ratios (day 5 compared to day 0) for S-COMT or MB-COMT proteins between MCF-7 and ZR-75-1 human breast cell lines. Pulse-chase ratios for S-COMT and MB-COMT proteins among individual cell lines were compared using analysis of variance (ANOVA), with the Bonferroni post-test to compare pairs of group means. Statistical significance was determined *a priori* by a two-tailed p-value<0.05.

3. Results

To confirm the findings of reduced COMT protein in $COMT^{LL}$ compared to $COMT^{HH}$ human hepatocytes [19], we extended our studies to the $COMT^{HH}$ and $COMT^{LL}$ human breast cell lines listed in Table 1. S-COMT and MB-COMT protein levels were determined using Western blot analysis, and a representative Western blot showing the amount of S- and MB-COMT protein in total cell lysates from the cell lines is shown in Fig. 1. As seen in Fig. 1, total COMT protein is represented predominantly by S-COMT in the estrogen receptor (ER) positive MCF-7, T47D and ZR-75-1 cell lines. The mean band intensities (±SD) for S-COMT from three separate experiments are presented in Table 1. Among the three ER positive cell lines, the levels of S-COMT protein in the $COMT^{LL}$ cell lines MCF-7 and T47D were significantly less compared to $COMT^{HH}$ ZR-75-1 cells ($p<0.05$). The $COMT^{HH}$ ER negative MDA-MB-231 and MCF-10A cells also had significantly less S-COMT protein compared to $COMT^{HH}$ ZR-75-1 cells ($p<0.01$). The ER positive $COMT^{LL}$ MCF-7 and T47D cells appeared to have higher levels of S-COMT compared to the ER negative $COMT^{HH}$ MDA-MB-231 and MCF-10A cells, but the differences were not significant. These results suggest that the val108met *COMT* polymorphism is not consistently associated with decreased levels of S-COMT protein in these five human breast cell lines.

MB-COMT appears to be the predominant form of COMT expressed in the ER negative cell lines. While significant differences in MB-COMT protein levels were not observed among the $COMT^{LL}$ and $COMT^{HH}$ cell lines (data not shown), we cannot be certain that this represents the total amount of MB-COMT present in the lysates since antibody excess was only confirmed for detection of different amounts of S-COMT protein present.

Since the ER positive MCF-7 ($COMT^{LL}$) contained approximately 70% less S-COMT protein compared to the ER positive ZR-75-1 ($COMT^{HH}$) cells, these lines were used to investigate the contributions of protein synthesis and turnover to the different protein levels. Preliminary experiments using cyclohexamide inhibition of protein synthesis followed by Western blot analysis indicated that the half-life of COMT

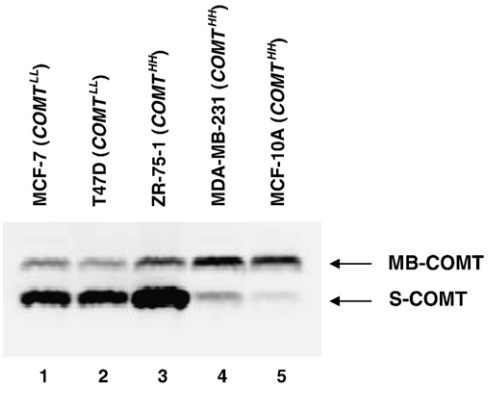

Fig. 1. Western blot analysis of S-COMT and MB-COMT in total cell lysate preparations from human breast epithelial cell lines. COMT Western blot analyses were performed as described in the Materials and methods section. Blots were visualized using an Odyssey infrared imaging system. The antibody recognizes both S-COMT and MB-COMT proteins. *Lane 1*: MCF-7 ($COMT^{LL}$); *lane 2*: T47D ($COMT^{LL}$); *lane 3*: ZR-75-1 ($COMT^{HH}$); *lane 4*: MDA-MB-231 ($COMT^{HH}$); *lane 5*: MCF-10A ($COMT^{HH}$). 15 μg of total protein were used for each cell line. Each experiment was repeated three times. Quantitation of S-COMT Western blot data is presented in Table 1.

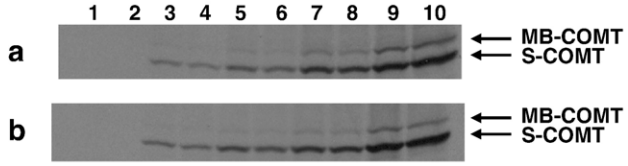

Fig. 2. Pulse-labeling analysis of COMT protein synthesis in MCF-7 and ZR-75-1 cells: time-dependent accumulation of newly synthesized COMT protein. Cells were pulsed with 20 μCi/ml [^{35}S]methionine for 0, 2, 4, 8 and 24 h and then harvested. COMT protein was immunoprecipitated using equal amounts of each radiolabeled cell lysate sample and subjected to SDS-PAGE. Gels were fixed, incubated in fluorographic reagent, dried and exposed to film. Each time point was assayed in duplicate. The upper bands on each gel are MB-COMT and the lower bands are S-COMT. Representative pulse-labeling gels: a: MCF-7 (*COMT*^{LL}); b: ZR-75-1 (*COMT*^{HH}); *lanes 1–2*: 0 h; *lanes 3–4*: 2 h; *lanes 5–6*: 4 h; *lanes 7–8*: 8 h; *lanes 9–10*: 24 h. Each experiment was repeated three times.

protein was greater than 24 h (data not shown), indicating that a pulse-chase protocol should be used. To compare the rates of COMT protein synthesis in the MCF-7 and ZR-75-1 cell lines, the cells were labeled with [^{35}S]methionine for 0, 2, 4, 8 and 24 h and harvested for analysis of COMT protein labeling using immunoprecipitation. A representative autoradiograph from the pulse-labeling analyses is shown in Fig. 2. The pulse-labeling data were quantified to assess the time-dependent accumulation of radiolabeled S-COMT protein from 0–24 h (Fig. 3). The S-COMT and MB-COMT data were fitted using non-linear regression (MB-COMT data not shown). The rate constants for COMT protein synthesis were derived from these curves and are presented in Table 2. While the time-dependent accumulation of radiolabeled S-COMT was slightly less in MCF-7 (*COMT*^{LL}) compared to ZR-75-1 (*COMT*^{HH}), no significant differences between the rate constants for S-COMT protein synthesis were observed between these two cell lines (Table 2). In addition, no significant differences in the rate constants for

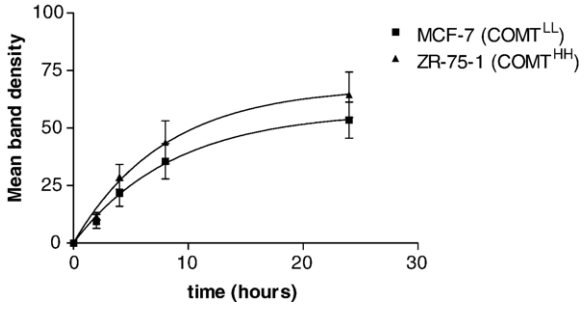

Fig. 3. Time-dependent accumulation of radiolabeled S-COMT protein in MCF-7 and ZR-75-1 cells: quantitation of pulse-labeling data. The radiolabeled S-COMT protein bands were visualized by autoradiography following incubation of gels in fluorographic reagent, vacuum drying and exposure to film. S-COMT protein levels were determined by measuring the density of the band area on scanned films using Scion Image for Windows (Scion Corporation). Data showing the time-dependent accumulation of radiolabeled S-COMT protein for MCF-7 (*COMT*^{LL}) and ZR-75-1 (*COMT*^{HH}) human breast epithelial cell lines from 0–24 h were fitted by non-linear regression and the rate constants derived from these curves are presented in Table 2. Each experiment was repeated three times. Each value is the mean±SEM of three experiments (within each experiment all time points were done in duplicate).

Table 2
Rate constants (*K*) for S-COMT and MB-COMT protein synthesis in *COMT*^{HH} and *COMT*^{LL} human breast epithelial cell lines

	MCF-7 *COMT*^{LL} *K* (h^{-1})	ZR-75-1 *COMT*^{HH} *K* (h^{-1})
S-COMT	0.11±0.04 [a]	0.13±0.03
MB-COMT	0.12±0.03	0.09±0.03

[a] Mean±SD from three separate experiments.

MB-COMT protein synthesis were detected between MCF-7 (*COMT*^{LL}) and ZR-75-1 (*COMT*^{HH}) cell lines (Table 2).

To determine COMT protein half-life, the cells were labeled for 16 h with [^{35}S]methionine and chased in the standard chase medium for 0, 1, 3, 5 and 7 days. A representative autoradiograph from the pulse-chase analyses is shown in Fig. 4a. The density of the band area for COMT proteins was measured from the scanned film in order to assess the time-dependent turnover of radiolabeled S-COMT and MB-COMT proteins. Additionally, the data were corrected for cell growth that occurred during the prolonged chase period. The S-COMT and

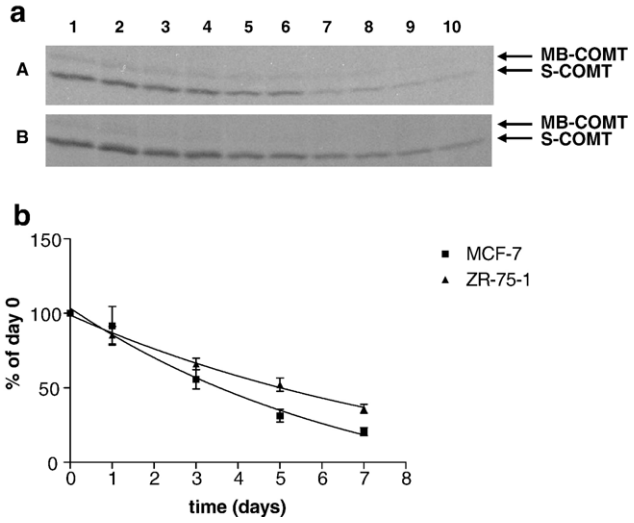

Fig. 4. Pulse-chase analysis of COMT protein turnover in MCF-7 and ZR-75-1 human breast epithelial cells cultured in standard chase medium. The cells were incubated for 16 h in the presence of 20 μCi/ml [^{35}S]methionine and then chased in the standard chase medium containing unlabeled methionine for 0, 1, 3, 5 and 7 days. COMT protein was immunoprecipitated (from equal amounts of radiolabeled cell lysate for each cell line) and subjected to SDS-PAGE. The gels were fixed, incubated in fluorographic reagent, dried and exposed to film. Each time point was assayed in duplicate and each experiment was repeated four times. (a) Representative pulse-chase gels: *A*: MCF-7 (*COMT*^{LL}); *B*: ZR-75-1 (*COMT*^{HH}); *lanes 1–2*: day 0; *lanes 3–4*: day 1; *lanes 5–6*: day 3; *lanes 7–8*: day 5; *lanes 9–10*: day 7. The upper bands represent MB-COMT and the lower bands S-COMT. (b) S-COMT protein levels were determined by measuring the density of the band area on scanned films using Scion Image for Windows (Scion Corporation). Each value is the mean±SEM of four experiments (within each experiment all time points were done in duplicate). Non-linear regression analysis (using an exponential decay function) was used to assess the disappearance of radiolabeled S-COMT over time. Half-life determinations and day 5/day 0 pulse-chase ratios (a measure of the signal intensity of radiolabeled COMT protein remaining at day 5 of the chase period compared to the signal intensity at day 0) are presented in Table 3.

Table 3
Half-life determinations for S-COMT and MB-COMT in $COMT^{HH}$ and $COMT^{LL}$ human breast epithelial cell lines

	MCF-7 $COMT^{LL}$		ZR-75-1 $COMT^{HH}$	
	Half-life (day)	Day 5/Day 0 Ratio [a]	Half-life (day)	Day 5/Day 0 Ratio
S-COMT	3.4±0.8 [b]	0.31±0.08 *	4.8±1.2	0.52±0.09
	(3.0±0.6) [c, *]	(0.31±0.08) [c, *]	(4.7±1.0) [c]	(0.47±0.09) [c]
MB-COMT	3.9±0.9	0.43±0.08	4.4±1.7	0.49±0.22
	(3.6±0.9) [c]	(0.41±0.07) [c]	(4.2±1.5) [c]	(0.43±0.20) [c]

[a] Day 5/day 0 pulse-chase ratios are a measure of the signal intensity of radiolabeled COMT protein remaining at day 5 of the chase period compared to the signal intensity at day 0.
[b] Mean±SD from four separate experiments.
[c] Half-life without correction for growth.
* $p<0.05$ compared to $COMT^{HH}$ group.

MB-COMT data were fitted using non-linear regression with an exponential decay function. The time-dependent degradation of radiolabeled S-COMT appeared faster in MCF-7 ($COMT^{LL}$) compared to ZR-75-1 ($COMT^{HH}$) cells (Fig. 4b), while no remarkable differences in the degradation of MB-COMT protein were observed (data not shown).

The half-lives for COMT protein in the MCF-7 ($COMT^{LL}$) and ZR-75-1 ($COMT^{HH}$) cells were derived from the non-linear regression curves for S-COMT or MB-COMT proteins and are shown in Table 3. The half-lives are presented with and without correction for growth. The mean half-life for S-COMT protein was shorter (approximately 30–36% less) in MCF-7 cells ($COMT^{LL}$) compared to ZR-75-1 cells ($COMT^{HH}$) ($p<0.05$ without growth correction; $p=0.10$ with growth correction). The half-life estimates for MB-COMT protein in the human cell lines are also shown; no significant differences were observed.

Using the data generated from the half-life experiments, we also assessed protein turnover in the MCF-7 ($COMT^{LL}$) and ZR-75-1 ($COMT^{HH}$) cell lines by comparing the ratios of the band densities for radiolabeled COMT protein at day 5 (end of the chase period) to the band densities at day 0 (end of the labeling period). As presented in Table 3, the day 5/day 0 ratio for S-COMT was significantly lower (approximately 34–40% less) in MCF-7 cells compared to ZR-75-1 cells ($p<0.05$, with and without growth correction). This indicated that the turnover of S-COMT protein was significantly greater in MCF-7 cells ($COMT^{LL}$) compared to ZR-75-1 cells ($COMT^{HH}$). No significant differences in the day 5/day 0 ratios for MB-COMT protein were observed between MCF-7 and ZR-75-1 cells.

Next, we extended these protein turnover determinations to three additional cell lines, ER positive T47D ($COMT^{LL}$), and ER negative MDA-MB-231 ($COMT^{HH}$) and MCF-10A ($COMT^{HH}$). The ER positive MCF-7 ($COMT^{LL}$) and ZR-75-1 ($COMT^{HH}$) cell lines were assayed along-side the three new lines to assure that new observations could be compared with our original observations. The MCF-7 and ZR-75-1 cell lines had day 5/day 0 pulse-chase ratios for S-COMT similar to those shown in Table 3 (data not shown). However, the experimental conditions were not optimal for the other cell lines. Their morphology changed from having a flattened smooth appearance to being more rounded and irregular in shape, which we interpreted as an indication that the culture conditions were having an adverse effect on their viability. Thus, we could not investigate S-COMT half-life in the other cell lines under the same experimental conditions. We did attempt a half-life comparison when culturing all five of the cell lines in their appropriate normal growth medium, instead of the standard chase medium, during the five day chase period. While pulse-chase ratios were similar to what had been observed using the standard chase medium and the mean day 5/day 0 pulse-chase ratios for S-COMT were somewhat lower in the two $COMT^{LL}$ cell lines (MCF-7, T47D) compared to the $COMT^{HH}$ cell lines (ZR-75-1, MCF-10A), when chased in their normal growth media, these differences were not statistically significant (data not shown). These results suggest that an association between the val108met *COMT* polymorphism and S-COMT protein turnover was observed under some, but not all, culture conditions. Thus, subtle factors, represented here by differences in culture medium, can affect the stability of the $S\text{-}COMT^{L}$ protein, even in the ER positive cells.

4. Discussion

Previous studies demonstrated reduced COMT activity and protein levels in cells and tissues from subjects genotypically $COMT^{LL}$ compared to those who are $COMT^{HH}$ [18–21]. The results from this study using cultured human breast epithelial cells lines, demonstrate that differences in COMT protein turnover may contribute to genotype-related differences in COMT activity and protein levels.

At the inception of this research, our thinking was that the val108met *COMT* polymorphism would most likely not be associated with changes in COMT mRNA levels since the polymorphism is located in exon 4 and not in a promoter region and since the $COMT^{L}$ protein appears less stable as indicated by its increased thermolability. We did investigate whether the polymorphism was associated with differences in steady state levels of COMT mRNA in human hepatocyte samples from six of the same subjects used in our previous study [19] as well as in the human breast cell lines. However, the overall results from these studies did not support an association between the val108met *COMT* polymorphism and COMT mRNA levels (data not shown). In further support of these findings, no significant association was observed between the val108/158met *COMT* polymorphism and total COMT mRNA levels in postmortem human prefrontal cortex tissue from 108 subjects [21].

While the val108met *COMT* polymorphism is not consistently associated with changes in protein levels in the human breast cell lines, it is important to note that there are genetic, cellular and molecular differences among these five cell lines. First, they originated from different individuals. Three of the cell lines (MCF-7, T47D and ZR-75-1) are tumorigenic and express estrogen receptors (ERs) [22,24,25]. MDA-MB-231 cells are also tumorigenic, however they are ER negative [22,24]. Finally, MCF-10A cells are non-tumorigenic, and while they weakly express ERα, are not estrogen responsive [22,26–29]. Nevertheless, we chose to investigate levels and

turnover of COMT protein in these human breast cell lines, since they express sufficient levels of COMT protein and have been previously utilized for studying the val108met *COMT* polymorphism [18].

The three tumorigenic, ER positive cell lines appeared to predominately express S-COMT, and S-COMT protein levels were lower in the $COMT^{LL}$ cell lines (MCF-7, T47D) compared to the $COMT^{HH}$ cell line (ZR-75-1). Thus, among cell lines with similarities in ER status and tumorigenicity, the val108met *COMT* polymorphism was associated with decreased levels of S-COMT protein.

Next, we turned our focus to the investigation of COMT protein synthesis and turnover employing pulse-labeling analysis using [^{35}S]methionine and immunoprecipitation. While a difference in the rate of protein synthesis could account for differences in COMT protein levels between cells, as mentioned, we did not detect a difference in the rates of COMT protein synthesis between the $COMT^{LL}$ MCF-7 and $COMT^{HH}$ ZR-75-1 cells. However, Nackley et al. [30] recently reported that different COMT haplotypes can affect COMT mRNA structure leading to differences in the amounts of translated protein. Thus, future studies on the mechanisms associated with differences in COMT protein levels will need to consider haplotypes rather than individual polymorphisms.

No differences were observed in the turnover or half-life of MB-COMT protein. We did observe that S-COMT protein in MCF-7 cells ($COMT^{LL}$) was degraded faster and had a shorter protein half-life compared to ZR-75-1 cells ($COMT^{HH}$), when cultured in standard chase medium. Protein half-lives and pulse-chase ratios were corrected for cell growth/dilution that occurred during the prolonged chase period to ensure that the disappearance of radiolabeled COMT protein was due to protein turnover and not to dilution by cellular growth and new protein synthesis; in general similar results were seen with and without correction for cell growth/dilution. Next these studies were extended to the other human breast cell lines and different culture medium was used during the prolonged chase period; however, the differences in S-COMT protein turnover between the MCF-7 and ZR-75-1 were no longer statistically significant. Thus, the val108met *COMT* polymorphism is associated with decreased levels of S-COMT enzyme activity and protein. However, significant differences in S-COMT protein turnover are detectable under some, but not all, culture conditions, and thus appear to be influenced by subtle factors.

Protein turnover has evolved to be an important and effective mechanism to modify and control cellular processes in response to changes in environmental or growth conditions [31]. Thermostability, "the ratio of enzyme activity after heating to the activity in controls before heating" [4], is often used to assess differences in protein structure or conformation [4,5]. Thermostability studies have shown $COMT^{L}$ activity to be more sensitive to inactivation by heat than $COMT^{H}$, indicating that the two forms of the enzyme may differ in structure leading to reduced stability of the $COMT^{L}$ protein under some conditions [4,5]. Along these lines, perhaps for MCF-7 cells, changes in culture conditions affect $COMT^{L}$ conformation leading to its increased turnover. Alternatively, changes in culture conditions may potentially alter protein degradation machinery. Another possibility might be that under altered culture or growth conditions, the S-$COMT^{L}$ protein associates differently with other proteins within the cell, i.e. heat shock proteins, chaperones, or ubiquitin which could potentially enhance protein instability and degradation. While we did not observe differences related to genotype for MB-COMT, a limitation to our study was that we did not investigate the half-lives of other proteins or determine whether turnover differences among other proteins were altered by the composition of the chase medium.

In addition to enhanced protein turnover, another potential explanation may be that the polymorphism induces a conformational change in the protein which may allow S-$COMT^{L}$ to aggregate within a cell. Misfolded proteins may be subject to degradation and/or aggregation [32–34]. Several polymorphisms in other proteins which result in variation in amino acid sequence have been associated with decreased protein levels and the formation of aggresomes [33,35]. Therefore, enhanced aggresome formation may be another potential mechanism for explaining the decreased levels of S-COMT protein associated with the val108met *COMT* polymorphism.

To our knowledge, the studies presented here are the first report of protein half-life determinations for S-COMT and MB-COMT in human cells. The half-lives observed for the polymorphic human S-COMT and MB-COMT proteins were considerably longer (3.0–4.8 days and 3.6–4.4 days, respectively) than those observed for other polymorphic Phase II enzymes including NQO1 (1.2–18 h) [36] and UDP-glucuronosyltransferase (0.8–12.8 h) [37].

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