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Greater endogenous estrogen exposure is associated with the telomere maintenance in postmenopausal women at risk for cognitive decline

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Abstract

Longer duration of reproductive years of life and thus greater exposure to endogenous estrogen may be associated with a lower risk of age-related diseases in women. The present study examined the relationship between estimated endogenous estrogen exposure and telomere length (TL) and telomerase activity, two biomarkers of cellular aging, in a sample of postmenopausal women at risk for cognitive decline. Telomere length was measured using a quantitative PCR method and telomerase activity by TRAP (Telomere-Repeats Amplification Protocol) assay in peripheral blood mononuclear cells (PBMCs). Study subjects were 53 postmenopausal women (35 with natural and 18 with surgical menopause) receiving hormone therapy (HT) for at least one year or longer. Length of reproductive years of life, computed as the difference between age at menopause and age at menarche, was used as a proxy of duration of exposure to endogenous estrogen. Length of time on HT was the measure used for duration of exogenous estrogen exposure. We found that longer endogenous estrogen exposure was associated with greater TL (standardized $\beta=0.06$, Wald $\chi^2=3.7$, $p=0.04$) and with lower telomerase activity (standardized $\beta=-0.09$, Wald $\chi^2=5.0$, $p=0.03$). Length of reproductive years was also inversely associated with the combination of short TL and high telomerase (OR=0.78, 95% CI: 0.63, 0.97, $p=0.02$). Length of HT use was not associated with TL or telomerase activity in this study. The results suggest that the endogenous estrogens may be associated with deceleration of cellular aging. This is the first study to examine associations between endogenous estrogens, telomere length and telomerase activity.

Section: Regulatory Systems

Keywords

estrogen exposure; hormone therapy; telomere; telomerase; cardiovascular disease; cognition

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Introduction

The role of estrogen in modifying vascular disease risk in women is contentious. Menopause is associated with increased risk for ischemic heart disease and cerebrovascular disease, which collectively are the main causes of morbidity and mortality in women of developed nations. While observational studies suggested a protective role of estrogen, recent randomized controlled trials report a negative role for oral estrogen in primary and secondary prevention of cardiovascular events. Inflammation underlies the development of atherosclerosis, and as such, modulation of the inflammatory process may be a potential means of reducing cardiovascular risk. Postulated estrogen anti-inflammatory processes and antioxidant effects may hence modify risk for vascular illnesses. Estrogen's role in cognitive function is equally controversial (for review see (1)). Specifically, estrogen therapy in postmenopause has been associated with cognitive improvement as well as with decline, whereas longer endogenous exposure to estrogen has been more consistently associated with better cognitive performance (2,3). The unifying hypothesis behind the discrepant effects of estrogen in CVD and cognitive function is the "window of opportunity" theory, postulating timing of the exogenous exposure to HT as a critical predictor of its extended effects. At the same time, evolving data suggest the duration of endogenous exposure to estrogen may have an independent effect on both cardiovascular function and cognition.

Telomeres, DNA-protein complexes consisting of tandem short DNA repeats and associated proteins, protect the ends of eukaryotic chromosomes from degradation and deleterious recombination. Telomerase replenishes telomere loss by the addition of terminal DNA. Short telomeres in leukocytes have been associated in many studies with age-related diseases including cardiovascular disease, Alzheimer's disease and some cancers (4), although some studies reported association of longer telomeres and diseases (5–7).

Earlier work has linked low telomerase activity in PBMCs of healthy women with psychological stress (8). However, higher PBMC telomerase levels have been linked to early atherosclerosis development (9) and have been found in persons with established coronary heart disease (10–12) and in persons with major depression (13). Moreover, the combination of higher level of telomerase activity and shorter telomere length in PBMCs has been reported in caregivers of Alzheimer's patients (14), in persons with depression compared to controls (13) and in people with greater coronary calcification (9) and hence may mark certain diseased states. Neither telomerase activity nor the combination of telomerase with telomere length have been studied in relation to cognitive function and the literature relating telomeres with cognitive function is decidedly mixed (15–19). However, there is growing evidence of cognitive impairment secondary to cardiovascular disease (CVD) (20,21) suggesting important links and possible insights in this literature to cognitive research.

There is a consistent gender difference in telomere length with longer leukocyte telomere length in women compared with men (22,23). Longer telomeres in women have been ascribed to the ability of estrogen to upregulate telomerase and at the same time reduce oxidative stress. *In vitro*, estrogen upregulates telomerase activity in normal cells including but not limited to hematopoietic cells (24), human mesenchymal stem cells (25), endothelial cells (26), and ovarian epithelial cells (27). Oxidative stress may contribute to shorter telomeres via several potential pathways: it directly damages the G- and T-rich telomeric sequence, which is preferentially sensitive to oxidative damage (28); reactive oxygen species (ROS) lower telomerase activity (29), thus potentially compromising telomere DNA replenishing ability. Furthermore, ROS stimulate the production of proinflammatory cytokines, which potentially accelerate immune cell turnover and hence telomere loss. Estrogen has been shown to reduce oxidative stress (30,31) and therefore may indirectly affect telomere maintenance. However, the *in vivo* relationship between estrogen, telomere

length and telomerase is not well understood. Understanding these relationships would be beneficial to understanding possible mechanisms through which estrogens may influence both cardiovascular disease and cognitive function.

Here we examined associations of endogenous estrogen exposure as estimated by duration of reproductive years with telomere length and telomerase activity in a group of postmenopausal women at risk for cognitive decline, all who had been taking hormone therapy (HT) for at least one year or longer. We also examined the association between exogenous estrogen exposure, measured as length of HT use, and telomere length and telomerase activity. This was a part of a larger longitudinal study of the HT effects on brain biomarkers and current results represent baseline evaluation of reproductive biomarkers and telomere maintenance.

We hypothesized an inverse relationship of endogenous and a positive relationship of exogenous estrogen exposure and telomere length. If the telomere complex is related to cognitive functioning, similar relationships may be seen between estrogens and cognitive functioning. Analyses of telomerase were more exploratory because of mixed findings regarding the potentially adverse or beneficial nature of telomerase in cardiovascular disease development.

1. Results

Study population characteristics

Table 1 describes the general characteristics of interest in study participants categorized by reproductive length. Women with greater duration of reproductive life (>35 years) had shorter duration of HT use, later age at menopause, and a higher likelihood of natural (vs. surgical) menopause, compared with those with shorter reproductive length (≤ 35 years). However, there were no differences for age, age at menarche, BMI, number of pregnancies or live births. Endogenous estrogen exposure

Estimated endogenous estrogen exposure was positively associated with TL (standardized $\beta=0.06$, Wald $\chi^2=3.7$, $p=0.04$), adjusted for age and type of menopause. In addition, every additional year of endogenous estrogen exposure was associated with a 14% reduced odds (OR=0.86, 95% CI: 0.74, 0.99, $p=0.04$) of having shorter than median TL. Length of reproductive years differed by type of menopause ($p=0.003$) and was 31.6 vs. 35.7 years for women with surgical vs. natural menopause. However, associations between reproductive years and TL did not vary by type of menopause in stratified analysis. Adjustment for length of HT use did not substantively influence associations (OR=0.85, 95% CI: 0.72, 1.00, $p=0.06$) (table 2).

Estimated endogenous estrogen exposure was also inversely associated with PBMC telomerase activity (standardized $\beta=-0.09$, Wald $\chi^2=5.0$, $p=0.03$) in the full sample adjusted for age and type of menopause (Table 2). We also tested for any associations with the states of short or long telomeres in combination with high or low telomerase. The combination of short TL and high telomerase, but no other combination, was significantly associated with estimated endogenous estrogen exposure (Figure 1); each additional year of endogenous estrogen exposure was associated with a lower odds of having the combination of short TL and high telomerase (OR=0.78, 95% CI: 0.63, 0.97 $p=0.02$; OR=0.79, 95% CI: 0.65, 0.96 $p=0.02$, adjusted additionally for HT). Similar to the overall finding for estimated length of estrogen exposure, age at menopause was positively related to TL (standardized $\beta=0.05$, $p=0.07$) and inversely (but nonsignificantly) related to telomerase (standardized $\beta=-0.08$, $p=0.19$) (Table 2). Time since menopause was inversely, though nonsignificantly, related to

TL (standardized $\beta = -0.05$, $p = 0.07$) (Table 2). Though age at menarche was unrelated to TL, it was positively related to telomerase activity (Table 2).

Exogenous estrogen exposure

We did not find associations between HT use and telomere length or telomerase activity in the full sample and in women with natural menopause, although length of HT use was inversely associated with TL in women with surgical menopause (Table 3). The interaction between type of menopause and length of HT use was nonsignificant ($p = 0.42$). In the full sample, length of HT was not significantly related to a higher odds of short TL (OR=1.07, 95% CI: 0.94–1.21) nor to the combination of short TL and high telomerase, (OR=1.22, 95% CI: 0.97–1.53, $p = 0.09$). Simultaneous adjustment for length of HT use and duration of reproductive life did not substantively alter associations. Adjustment for vitamin intake, body mass index (BMI), HOMA (homeostatic model assessment, a method used to quantify insulin resistance and beta-cell function) had no significant influence on associations either (data not shown).

2. Discussion

Here we report the first study to examine the relationship between estimated endogenous and exogenous estrogen exposure, telomere length, and telomerase activity. Greater endogenous estrogen exposure, as measured by longer duration of the reproductive years, was related to longer PBMC telomere length and lower telomerase activity. In contrast, exogenous exposure to estrogen, as measured by length of HT use, was not associated with longer TL or telomerase.

Our finding that duration of endogenous estrogen exposure is negatively correlated with telomerase activity in this group of postmenopausal women at risk for cognitive decline may appear contradictory to reports that estrogen upregulates telomerase activity *in vitro*. However, in the present study, telomerase activity was measured several years after menopause occurred. Given that PBMC telomerase can respond rapidly (over minutes to hours) to changing physiological conditions (32), it was not surprising that telomerase activity did not show a close relationship to the estrogen status years earlier. Thus, from these data, it was not possible to directly evaluate the association of endogenous estrogens with telomerase activity, since telomerase was measured years after the greatest exposure.

High telomerase activity, however, may be a cellular response to inflammation and may thus indicate compromised health. For example, the proinflammatory cytokine IL-6 has been shown to upregulate telomerase activity in *in vitro* cultured cells (33). Elevated levels of another proinflammatory cytokine, TNF-alpha, were also reported in cells from these caregivers in response to stimulation *in vitro* (14). Thus, the inverse finding between endogenous estrogen exposure and telomerase activity may be due in part to the reported anti-inflammatory effects of estrogen. Our finding that higher telomerase is associated with fewer reproductive years is consistent with previous *in vivo* findings suggesting that telomerase may augment the proliferative capacity of lymphocytes, macrophages, and smooth muscle cell in atherosclerosis development (12,34). Thus, these data are consistent with the notion that longer reproductive years of life (and later age at menopause) could delay the development of cardiovascular disease associated with menopause.

Estimated duration of endogenous estrogen exposure was inversely associated with the state of combined short TL and high telomerase activity. Although the implications of this finding are not fully understood, we note that telomerase activity is highly regulated by a large number of mechanisms, including, in non-human model systems, DNA damage responses that act to enhance the action of telomerase on telomeres (35). Hence our finding, taken

together with previous preclinical reports (36,37) is consistent with the possibility that telomerase activity in PBMCs may increase in response to the damage signaling from too-short telomeres, although this mechanism remains to be further elucidated.

Endogenous estrogen has been associated with lower cardiovascular disease risk in women (38) and with lower risk of cognitive decline (39). Possible mechanisms involved in the protective role of estrogen include its ability to lower oxidative stress, reduce inflammation, improve vasodilation, and alter gene expression profiles in vasculature and the heart (40,41). Correspondingly, both oxidative damage and proinflammatory cytokines have been associated with telomere shortening (42,43). Our findings suggest that one possible mechanism through which endogenous estrogens may exert both cardioprotective and cognitive effects may be through deceleration of telomere shortening and hence cellular aging. The effects may be unique to endogenous hormones however. While endogenous bioavailable estradiol levels have been beneficially related to cognitive function and cardiovascular outcomes, exogenous estrogen use has been linked to earlier dementia (44) and coronary events.

The only published study examining the *in vivo* relationship between estrogens and telomere length found that women who had been on HT for more than five years had longer TL compared to age-matched women who had not used HT (45). However, the authors noted that women who exercised regularly and took daily vitamins were better represented in the group of women using HT. Both exercise and vitamin intake have been reported to be associated with longer TL (46,47). The positive correlation between TL and HT in that report therefore may be at least partially accounted for by the difference in exercise and vitamin intake between the two groups or may also be accounted for by other unmeasured variables representing good self care over the life course which may lead to residual confounding even with adjustment for these other variables. Thus, it is not clear from current data whether exogenous estrogen exposure in women is associated with TL.

There were several limitations to this study. First, all participants in this study were prescribed HT by their doctors, with at least one risk factor for cognitive decline as described in the methods, including the possibility of increased risk of cognitive decline which was reported to be associated with short TL (48). Therefore, these study results may not extend to the general population. Future studies should examine this association in women who have never used HT, without risk factors for cognitive decline. The small sample size limited our ability to explore the more complex influence of the combinations of reproductive factors, including oral contraceptive and different types of HT regimens.

As in all observational studies, we cannot rule out the possibility that the observed relationship between estrogen exposure and TL was due to some unmeasured factor related to both longer reproductive years and reduced cell aging. Socioeconomic status is a major factor which should theoretically influence both reproductive history and cell aging, but adjustment for education had little influence on our associations. Telomere length is also 30–70% genetically determined (49–51); genetic factors might ultimately be responsible for both long TL and long reproductive years. We were not able to address this issue, but longitudinal studies that examine the relationship between reproductive years and changes in telomere length over time, as well as genome-wide genetic analysis, may help address these limitations in the future.

In summary, we provide preliminary evidence that endogenous estrogen is associated with longer telomere length and lower telomerase activity in PBMCs. Larger studies are needed to draw more definitive conclusions about the relationship between estrogen, telomeres and telomerase.

3. Experimental Procedure

Study population

The participants were derived from a longitudinal 2-year study that examined the possible neuroprotective effects of randomization off of hormone therapy in postmenopausal women at risk for cognitive decline (i.e., APOE-ε4 carrier, family history of AD, hypothyroidism, or personal history or family history of mood disorder). All participants were Caucasian except for 1 Asian-American. Given that all study subjects were required to be stable HT users (taking HT for a year or longer), a substantial fraction of women in this sample (35.8%) had surgical menopause for various reasons, including fibroids and endometriosis. None of the hysterectomies were due to a gynecological cancer. Approximately 76% of study subjects had received hormonal contraceptives at some point in their lifetime though we did not have detailed information about their use.

Telomere length was measured at the time of recruitment (baseline) for 53 participants, ages 49–69, where peripheral blood mononuclear cells were available and telomerase was measured on 33 of these women where enough viable PBMCs (over 0.5 million) were available. The study was approved by Stanford University Institutional Review Board for Human Research.

Endogenous estrogen exposure and other reproductive variables data collection

Data on reproductive history, including age at menarche and menopause, parity, use of hormonal contraception during reproductive years, duration of perimenopausal transition in relation to time of start of HT use, and type of menopausal symptoms, was collected by the study physician during the screening evaluation, during which a psychiatric, physical, and neurological examination was conducted, including clinical blood chemistries to confirm postmenopausal status and general medical health. As a proxy of length of endogenous estrogen exposure, duration of reproductive life in years, was computed as the difference between age at menopause, defined as the age at which menstruation had ceased for 12 months, and age at menarche. Time since menopause was computed as the difference between current age and age at menopause, and was used to explore the separate effects of age per se and age relative to decline in endogenous estrogens (52).

All women in this study had taken HT for a year or longer and so length of HT use characterized (duration of) exogenous estrogen exposure.

Telomere length measurement

Subjects underwent blood sampling using venipuncture in a fasting state during the morning hours between 7am to 10am. All samples were processed for isolation of mononuclear cells within 1 hour of collection. 1 ml of cryopreserved PBMCs was thawed at 37 °C, washed twice with 10 ml of cold DPBS (Invitrogen, Calsbad, CA). Cell pellets were collected and DNA prepared using Puregene DNA purification Kit (QIAGEN, Valencia, CA).

Relative mean telomere length was measured from DNA by a quantitative polymerase chain reaction (qPCR) assay that compares mean telomere repeat sequence copy number (T) to a reference single copy gene copy number (S) in each sample as previously described and validated by comparison with Southern blot terminal restriction fragment (TRF) analysis (53). The T and S values were each determined by the standard curve method using a serially diluted reference DNA and the T/S ratio was derived from the T and S value for each sample. The inter-assay coefficient of variability for telomere length measurement was 3.7%. The intra-assay coefficient of variability was 2.5%.

Telomerase activity assay

1 ml of cryopreserved PBMCs was thawed at 37 °C, washed twice with 10 ml of cold DPBS (Invitrogen, Calsbad, CA) and live cells were counted using a hemocytometer with the Trypan exclusion method. 1×10^6 viable cells were pelleted and lysed with 1XCHAPS buffer as directed by the manual for the Trapeze kit (Trapeze telomerase detection kit, Millipore, Billerica, MA). Two concentrations, corresponding to 5000 and 10,000 cells were used for TRAP reactions for each sample to ensure linearity of the assay. The reaction was carried out according to the Trapeze kit manual and run on a 10% polyacrylamide-8M urea sequencing gel. The gel was exposed to a phosphorimager plate overnight and scanned on a STORM 860 (GE Healthcare, Piscataway, NJ). The 293T cancer cell line was used as a positive telomerase activity control and standard. Telomerase activity is defined as 1 unit = the amount of product from one 293T cell/10,000 PBMCs, and was quantified using the software ImageQuant 5.2 (GE Healthcare, Piscataway, NJ).

Details of telomere length and telomerase activity measurement methods are published in (54). Measurement of TL and telomerase activity was performed in a blinded fashion without knowledge of the clinical data. Finally, we defined women to have short or long TL and high or low telomerase activity based on median rather than physiologically derived cutpoints, given the small sample size.

Statistical Analysis

Using analysis of covariance (PROC GLM), we examined covariates by high and low (above or below median) years of reproductive life (median=35 years) (Table 1).

Normality of TL and telomerase activity was evaluated and found to be improved by computing the logarithms of these values. Therefore, using general linear models, we regressed the logarithms of linear telomere length and telomerase activity against duration of reproductive years and length of HT, adjusted for age and type of menopause (PROC GENMOD). Using logistic regression (PROC LOGISTIC), we also evaluated whether reproductive length was related to short TL, defined as below median TL. We additionally evaluated a model simultaneously adjusted for reproductive length and length of HT. Moreover, we evaluated models adjusted for healthy behaviors or parameters including vitamin intake and body mass index (BMI), and also adjusted for other variables including homeostasis model assessment (HOMA, a marker of insulin sensitivity) and presence of the APOE-ε4 allele, added one at a time to models. No data were available with regard to physical activity and only one woman in the sample was a current smoker and so we did not evaluate these variables. We evaluated models adjusted for education but adjustment did not influence associations. Therefore this variable was excluded from analyses.

As previously mentioned, this study included women with surgical menopause. This necessitated adjustment for type of menopause in all analyses. However, we also examined main analyses stratified by type of menopause, given the differences in the reproductive history of the two groups. The main reasons for surgical menopause are endometriosis and fibroids, conditions commonly associated with infertility and obesity, which potentially confound or alter the nature of relationships between endogenous and exogenous estrogen exposures and the outcomes of interest. We evaluated stratified models adjusted simultaneously for duration of reproductive years and years of HT use.

Finally, we employed logistic regression (PROC LOGISTIC) to evaluate associations between both reproductive years and length of exogenous HT use and the risk of having the combination of both short TL and high levels of telomerase adjusted for age as this combination may be associated with a disease state. Based on median cutpoints for TL and telomerase (for the 53 women with TL and 33 women for telomerase, 10 women (30% of

those with data on both TL and telomerase) were defined to have short TL and high telomerase. This combination state was not related to markers of insulin resistance, body mass index, vitamin use, or type of menopause.

To better assess the pattern of associations, we also evaluated the relationships of TL, telomerase activity and other markers of reproductive function, including age at menarche, age at menopause, time since menopause, and number of pregnancies. Because the possible influence of HT use on health-related outcomes may be influenced by timing of initiation, we examined associations between timing of starting HT (early or beginning in perimenopause vs. late—menopause or later), TL and telomerase.

The SAS version 9.2 statistical software(SAS Institute, Cary, NC) was used in all statistical analyses.

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Abbreviations

TL	telomere length
HT	hormone therapy

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Figure 1.

Percent with the combined state of short TL and high telomerase activity by reproductive length*

*Women with long reproductive length (>35 years) had a significantly reduced risk of the state characterized by greater than median telomerase activity and shorter than median telomere length, OR=0.04 (95% CI: 0.00–0.48), p=0.01) than women with shorter reproductive length (≤ 35 years) in a model adjusted for age and type of menopause.

Table 1

Characteristics of study population by reproductive length, N=53

	Reproductive length (years)	
	<=35 years	>35 years
Age (years, range 49–69)*	57	58
Education (years)	16	17
APOE-ε4 allele present (%)	34	42
Homeostasis model assessment (HOMA) (N=45)	0.6	0.8
<i>Lifestyle factors</i>		
BMI (kg/m ²)	26	24
Vitamin intake (%)	72	72
<i>Hormonal variables</i>		
Length HT use (years)	12	8 [†]
Duration of reproductive years of life	31	38 [†]
Age at menopause (years)	44	52 [†]
Natural (vs. surgical) menopause (%)	51	84 [†]
Time since menopause (years):	14	6 [†]
Age at menarche (years)	13	13
Number of pregnancies	3	2
Number of live births	2	2
Time since initiation of HT use (years)	2.4	0 [†]

* Least squares means from analyses of covariance, adjusted for age (continuous). Except for age variable, all analyses adjusted for age

[†] p-value, Wald test ≤ 0.05

Table 2

Standardized betas for reproductive factors, telomere length, and telomerase.

	TL (N=53)		Telomerase (N=33)	
	β	p-value*	β	p-value
Age at menarche [†]	-0.04	0.64	0.26	0.02
Pregnancies	0.10	0.17	0.04	0.73
Reproductive years	0.06	0.04	-0.08	0.03
Age at menopause	0.05	0.07	-0.08	0.19
Time since menopause	-0.05	0.07	0.05	0.19

* p-value, continuous variable. Values in bold denote significant findings (p-value<0.05)

[†] Models adjusted for age and type of menopause.

Age-adjusted standardized betas for variables characterizing hormone therapy, telomere length, and telomerase, by type of menopause.

Table 3

	TL (N=53)			Telomerase (N=33)		
	N	β	p-value*	N	β	p-value
Natural menopause [‡]	35			22		
Early initiation of HT		-0.03	0.92		-0.66	0.10
Length of HT use [‡]		0.01	0.75		-0.03	0.50
Time since HT use		-0.06	0.15		0.07	0.22
Surgical menopause	18			11		
Early initiation of HT		0.98	0.005		-1.38	0.04
Length of HT use [‡]		-0.11	0.02		0.07	0.34
Time since HT use		-0.03	0.34		0.01	0.80

* p-value, continuous variable. Values in bold denote significant findings (p<0.05).

[‡] Models adjusted for age. Additional adjustment for years of education had little substantive effect on associations.

[‡] Since all women in this study took hormone therapy (HT), length of HT use served as our proxy measure of exogenous estrogens