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TERT inhibition leads to reduction of IL6 expression induced by palmitic acid and interferes with the protective effects of tibolone in an astrocytic cell model

Running title: TERT and tibolone in astrocytes

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Abstract

Although it has been shown that telomerase has neuroprotective effects, mainly due to its non-canonical functions in neuronal cells, its role on glial cells is still unknown. There is growing evidence showing that telomerase plays an important role on inflammation, especially on the regulation of pro-inflammatory cytokine gene expression. The aim of this study was to evaluate the role of telomerase in an astrocyte cell model treated with palmitic acid (PA) and tibolone. Cell death, reactive oxygen species production and IL6 expression were evaluated under telomerase inhibition with the BIBR1532 compound in T98G cells treated with tibolone and PA, using fluorometry, flow cytometry, ELISA and qPCR. Our results showed that telomerase protein was increased by PA after 36 hours, alone or in combination with tibolone and that its activity was affected by PA. Telomerase inhibition reduced IL6 expression and it interfered with the protective effects of tibolone on cell death. Moreover, tibolone increased 707-Tyr phosphorylation in PA-treated cells. In this study, we provide novel findings about the regulation of telomerase by PA and tibolone. Telomerase was involved in inflammation by PA and in protective effects by tibolone. Therefore, we conclude that telomerase could play a dual role in these cells.

Keywords: Astrocytes; palmitic acid; tibolone; telomerase activity; TERT; telomere length; interleukin 6.

Introduction

Telomerase is a ribonucleoprotein involved in several cellular processes, with its main function being the maintenance of telomere length in dividing cells. However, non-canonical functions, such as reduction of reactive oxygen species (ROS) and cell death, mitochondrial DNA (mtDNA) protection and regulation of gene expression, have also been reported (1).

Telomerase is composed of two subunits: the telomerase reverse transcriptase catalytic subunit (TERT) and a telomerase RNA component (TERC). Human *TERT* and *TERC* genes are located in 5p15.33 and 3q26.2, respectively (2). Telomerase expression is modulated by different molecules, such as hormones (3) and inflammatory stimuli (e.g lipopolysaccharide, LPS) (4) through activation of several transcription factors (5). TERT is required by the nuclear factor kappa B (NF-kB) factor to increase the expression of cytokines, including interleukin 6 (IL-6) (6), tumor necrosis factor (TNF) and interleukin 1 beta (IL-1B) (7). The telomerase protein is regulated by post-translational modifications in order to be exported from the nucleus. For example, under conditions of oxidative stress, telomerase protein is translocated from the cell nucleus to the cytoplasm by means of a phosphorylation-dependent mechanism (8), being found in mitochondria (9) and possibly in the endoplasmic reticulum (10).

Telomeres are repetitive sequences that cap the ends of the chromosome, in order to prevent genome instability and its associated consequences (senescence or apoptosis) (11). Reduction of telomere length is associated with Alzheimer's disease (AD) (12) and other pathologies (13). Telomere shortening is triggered by the increase in oxidative stress (14) and inflammation, among other factors (15). Moreover, several clinical factors such as obesity and overweight, as well as a decline in estrogens, have also been associated with telomere length reduction in women (16-18). Interestingly, long-term hormone therapy (HT) is associated with a longer telomere length (19) and reduction of AD risk (20). Therefore, all the above factors can interact to increase the risk of developing neurodegenerative diseases (such as AD).

Obese people have high concentrations of fatty acids in serum, with palmitic acid (PA) being of particular interest (21). This saturated fatty acid is able to induce inflammation (22), oxidative stress and cell death (23) in *in vitro* models. However, the effect of saturated fatty acids on telomere length is unknown. We have previously observed that PA increases *TERT* and *TERC* gene expression and that this effect was reversed by tibolone pretreatment. Otherwise, tibolone alone induced a small increase in *TERT* expression (24). Tibolone is a synthetic steroid used as hormone replacement therapy by women in several European and Latin American countries (25). This hormone has anti-inflammatory effects (26) due to its ability to activate estrogen receptors (27).

Telomerase protein, especially TERT subunit, is upregulated in different injuries to protect neurons against oxidative stress, excitotoxicity, among other injuries (28). However, in astrocytes, the role of TERT is still unknown. Some studies have found that TERT is increased in models of ischemic brain (29) and spinal cord injuries (30). Tao and collaborators showed that TERT expression was correlated with an increase in glial fibrillary acidic protein (GFAP) expression, suggesting that TERT could be involved in glial scar formation and that it could contribute to astrocyte activation (30). Nevertheless, another study showed that TERT overexpression reduced astrocyte proliferation in a model of hypoxia and glucose deprivation (31). Thus, TERT could be involved in both detrimental and protective mechanisms in astrocytes in the brain.

Considering the canonical and non-canonical functions of telomerase, we have hypothesized that TERT is increased in cells treated with PA, in order to reduce its detrimental effects (such as cell death), or that it could be involved in the protective effects of tibolone. Therefore, we evaluated telomerase expression, protein activity and telomere length in cells

exposed to PA and tibolone. Furthermore, we inhibited telomerase function using a chemical compound and we measured cell death, ROS production, and IL6 expression, in order to understand the role of TERT in T98G cells under different stimuli. T98G is a cell line derived from a human glioblastoma tumor, which has a normal stationary phase G1 arrest (32). This cell line has been used as a glial cell model *in vitro* (33-35), and expresses some astrocyte markers, such as GFAP (glial fibrillary acidic protein), nestin, and vimentin (36).

Materials and methods

Cell culture

T98G cells (ATCC® CRL-1690™) (Manassas,VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Lonza, Walkersville, USA), supplemented with 10% fetal bovine serum (FBS) (Eurobio, France) and 10 U penicillin/10 mg streptomycin/25 ng amphotericin (Lonza, Walkersville, USA). Normal human astrocyte cells (NHA) (Lonza, Walkersville, USA) were grown in Astrocyte Basal Medium, supplemented with GA-1000, 1% recombinant human insulin, FBS, ascorbic acid solution, human epidermal growth factor (hEGF) and L-glutamine (Lonza, Walkersville, USA). Cells were kept in a humidified incubator at 37 °C and 5% CO_2.

Drug Treatments

T98G and NHA cells were deprived from serum and other supplements for 24 hours using DMEM without phenol red and L-Glutamine (Lonza, Walkersville, USA). Then, tibolone treatment was performed for 24 hours before application of 1 mM PA. T98G cells were stimulated with 70 µM tibolone (Sigma, St Louis, MO, USA) and NHA cells with 20 µM and 10 nM tibolone. Next, 1 mM PA (Sigma, St Louis, MO, USA) was added to cells for 24 hours. Tibolone was dissolved in dimethyl sulfoxide (DMSO), and PA was dissolved in 2.5%

bovine serum albumin (BSA) and 2 mM Carnitine. Controls (DMSO and BSA) were added to cells at the final concentrations used in PA and tibolone treatments (37). BIRB1532 (Santa Cruz Biotechnology, Santa Cruz, Ca, USA), an inhibitor of telomerase, was added at 100 µM before treatment with 1 mM PA or with tibolone and PA, as previously described (38). This compound was also dissolved in DMSO. BIRB1532 induces an inhibition of telomerase activity by interacting with a motif involved in DNA binding (39). Moreover, it has been observed that this compound is able to reduce *TERT* gene and telomerase protein expression (40).

Cell death and Reactive Oxygen Species production analysis

Staining with propidium iodide (PI) (Santa Cruz Biotechnology, Santa Cruz, Ca, USA) was used at 10 µg/ml to evaluate cell death. Cells were stained for 15 minutes, as previously described (37). Dihydroethidium (DHE) and 2′,7′-Dichlorofluorescin diacetate (DCFDA) (Sigma, St Louis, MO, USA) were used to quantify superoxide and hydrogen peroxide levels, respectively. Both compounds were added to cells at a concentration of 10 µM for 25 minutes. Fluorescence was detected using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) using the following parameters: PI and DHE (excitation 530 nm/ emission 590 nm), DCFDA (excitation 485 nm/ emission 540 nm). Mean fluorescence is presented in arbitrary units (a.u.) for all experiments.

Protein expression and phosphorylation analysis using flow cytometry

Flow cytometry analysis was performed to determine changes in telomerase protein expression and phosphorylation status, due to the fact that it is a very efficient technique for analyzing protein expression that allows obtaining results that are similar to western blot (41). Briefly, once the treatments were finished, cells were harvested, fixed and permeabilized

using 75% ethanol for 30 minutes. After washing the cells with 2% BSA, an overnight incubation with the primary polyclonal antibody (Invitrogen, Carlsbad, Ca, USA) was carried out. Next, cells were washed again, and 30 minutes of blocking was performed before adding the secondary antibody (Invitrogen, Carlsbad, Ca, USA) for 1 hour. Finally, cells were washed again and resuspended in 2% BSA to quantify the fluorescence with a Guava EasyCyte cytometer (Millipore, MA, USA). Antibodies and dilutions used were the following: anti-TERT (Thermo Fisher Scientific, PA511446), 1:50; anti-Phospho-TERT (Tyr707) (Thermo Fisher Scientific, PA513048), 1:50; anti-Phospho-TERT (Ser227) (Thermo Fisher Scientific, PA538817), 1:100 and anti-rabbit IgG secondary antibody, Dylight 488 (Thermo Fisher Scientific, 35553), 1:400. Parameters for flow cytometry data acquisition were: forward and side scatter plots were used to identify intact cells from debris. Doublet discrimination was performed plotting the width against the area for forward scatter. The negative control corresponded to the incubation of cells with the secondary antibody only (without the primary antibody) and 5000 cells were acquired for each sample. Mean fluorescence intensity was calculated automatically from 5000 events using the Guava Suite Software (Millipore, MA, USA) and data were normalized in order to perform statistical analysis. An increment in the fluorescence intensity indicates an increase in TERT protein expression levels.

Telomerase activity analysis

We followed the instructions for the telomeric repeat amplification (TRAP) protocol published by Herbert et al (42). Total protein was extracted using the RIPA buffer (Thermo Fisher Scientific, Waltham, Ma, USA), which contained 10 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and Thermo Scientific halt protease inhibitor cocktail 1X (Thermo Fisher Scientific, Waltham, Ma, USA). Proteins were

quantified by means of a BCA kit (Thermo Fisher Scientific, Waltham, Ma, USA) and normalized to 2 ng/µL for TRAP analysis. TRAP master mix consisted in 2X SensiFAST™ SYBR® No-ROX Master mix (Bioline, London, United Kingdom), 1 µM TS (AAT CCG TCG AGC AGA GTT) primer and 1 µM ACX (GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC) primer, 2 ng of protein and water, for a total volume of 25 µL. An initial incubation was performed at 30°C for 30 min in the dark to allow the extension of the substrate by telomerase. Later, Real-Time PCR was realized on a CFX96 Touch Real-Time System (BioRad, Hercules, CA). The PCR program consisted of one cycle at 95°C for 2 min, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Data analysis was performed based on the threshold cycle (Ct), as previous studies (43). All samples were run in duplicate and RIPA buffer was used as a negative control. Data are shown in percentage, where the value for the control sample was taken as 100% activity.

Telomere length analysis

Telomere length was analyzed following a previously used protocol (44) based on the qPCR method as previously described (45). Genomic DNA was extracted using the salting-out method (46). DNA was quantified with a Qubit dsDNA BR Assay and measured in a Qubit fluorometer (Invitrogen, Carlsbad, Ca, USA) and normalized to 10 ng/µL. The primers used were: TELG: ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T and TELC: TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA. Albumin (single-copy gene) primers were: ALBU: CGG CGG CGG GCG GCG CGG GCT GGG CGG AAA TGC TGC ACA GAA TCC TTG and ALBD: GCC CGG CCC GCC GCG CCC GTC CCG CCG GAA AAG CAT GGT CGC CTG TT (45). For PCR amplification, we used 7.5 µl of 2× SensiMix HRM (Bioline, London, UK), 20 ng of DNA, 0.8 µM of each primer and water, for a total volume of 15 µl. Real Time PCR was realized on a CFX96

Touch Real-Time System (BioRad, Hercules, CA) and Ct values were acquired and used for calculating the relative TL by means of the T/S ratio, as previously described (45). T corresponds to the Ct value for telomere signal at 74°C read and S corresponds to the Ct value for albumin signal at 88°C read (47).

Gene expression analysis

Total RNA isolation was performed with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and it was quantified by means of NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, Ca, USA) and oligo (dt) 18 (Bioline, London, United Kingdom) were used to generate cDNA from 400 ng of RNA. Quantitative PCR for gene expression analysis was performed using 2X SensiFAST™ SYBR® No-ROX Master mix (Bioline, London, United Kingdom), 400 nM of each primer (Forward and Reverse), 1 µl of cDNA and water, in a total volume of 10 µl, on a CFX96 Touch Real-Time System (BioRad, Hercules, CA). PCR program consisted in: one cycle at 95 °C for 2 min, 40 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 15 sec. Melting analysis was carried out to verify the primer specificity (48) and PCR efficiency was determined using LinRegPCR program (49). RPL27 gene was used as a normalization control, according to previous recommendations (50). Primer sequences were the following: for RPL27 gene, forward primer (ATC GCC AAG AGA TCA AAG ATA A) and reverse primer (TCT GAA GAC ATC CTT ATT GAC G); IL6 gene, forward primer (CCA CAC AGA CAG CCA CTC AC) and reverse primer (CCA GAT TGG AAG CAT CCA TC). All samples were run in triplicate. Data analysis was performed using the comparative CT method ($2^{-\Delta\Delta CT}$) (51).

IL6 secretion analysis

To quantify interleukin 6, the IL-6 Human ELISA kit (Invitrogen, Carlsbad, Ca, USA) was used. Briefly, 50 µl of supernatant were obtained from a 24-well plate and then the assay was performed following the manufacturer´s instructions. Samples (which correspond to the triplicates from three independent experiments, n=9 for each treatment), and standards were measured in duplicate in the ELISA microplate, according to manufacturer´s instructions. Absorbance was measured in a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) at 450 nm and a standard curve was used to calculate protein concentrations.

Recombinant human IL6 protein treatment

Once IL6 concentrations were determined, a treatment with a recombinant human IL6 protein (Thermo Fisher Scientific, Waltham, Ma, USA) was performed on T98G cells before 1 mM PA stimulation. Recombinant protein was reconstituted following manufacturer instructions at 100 µg/mL and different dilutions were done in DMEM medium.

Statistical analysis

All samples were analyzed in triplicate, for three independent experiments. Student's *t*, one-way ANOVA and two-way ANOVA tests were calculated using the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA). A post hoc analysis was performed using Tukey's test. A P value < 0.05 was considered significant. In the figures, all data are presented as Mean ± SEM.

Results

Telomerase protein expression, telomerase activity and telomere length analysis in T98G cell line

In this study, we aimed to evaluate the role of TERT in cells treated with tibolone and PA. First, we analyzed protein expression, using flow cytometry, in cells treated with PA, tibolone plus PA and tibolone alone **(Figure 1A, B and C)**. TERT protein expression was evaluated in T98G cells stimulated with tibolone only versus its vehicle (DMSO) for 24 hours **(Figure 1D)** and significant differences were not observed (t= 0.0118, df= 16, *P*= 0.91). Next, an analysis of two-way ANOVA was performed to evaluate the effect of time and treatment on TERT protein expression. Cells were treated for 24 hours with tibolone followed by 1 mM PA treatment for 12, 24, 36 and 48 hours. We observed an interaction between time and treatment (F (6.137) = 2.716, *P*= 0.02). The post hoc test indicated that there was an increase in protein expression at 36 hours in cells treated with PA, in comparison to 12 and 24 hours (*P*= 0.002, *P*= 0.012, respectively). In cells treated with tibolone + PA, differences were observed between 12 and 36 hours (*P*= 0.0002), 12 and 48 hours (*P*= 0.0008), 24 and 36 hours (*P*= 0.002), and 24 and 48 hours (*P*= 0.009) **(Figure 1E)**. At 36 hours, differences were found between control and PA (*P*= 0.004), and control versus tibolone + PA (*P*= 0.003). However, at 48 hours, we only observed a significant difference between control and tibolone + PA treatment (*P*= 0.007). A similar result was observed by an immunocytochemistry analysis at 48 hours after PA treatment **(Figure S1)**.

The canonical function of telomerase is to maintain telomere length. Therefore, telomerase activity and telomere length were analyzed in the current study. Cells were pretreated with tibolone following a 1 mM PA stimulus and then total protein was isolated and telomerase activity was assessed by a TRAP protocol. An ANOVA test (F (3.68) = 4.216, *P*= 0.008)

followed by a Tukey's multiple comparisons test determined that PA reduced the activity of telomerase in comparison to tibolone alone (P= 0.04); a significant difference was also observed between tibolone + PA and tibolone (P= 0.04) **(Figure 2A),** although the telomerase activity was lower in PA treatment in comparison to control, it was not statistically different. Telomere length was not affected by any treatment **(Figure 2B)**.

Effects of TERT inhibition using compound BIBR1532 on cell death and reactive oxygen species production

TERT has non-canonical functions, such as reduction of reactive oxygen species and cell death by its translocation to mitochondria (52). Our hypothesis was that TERT is associated with the reduction of the detrimental effect induced by PA or that it could be involved in the protective effects of tibolone. To investigate the role of TERT in the current model, BIBR1532, a specific inhibitor of telomerase protein (38, 39), was used alone or in combination with tibolone and PA. PI uptake test showed that TERT inhibition interfered with the protective effect of tibolone against cell death induced by PA, but it did not have any effect on PA-treated cells (ANOVA test, F (6.98) = 36.02, P= <0.0001) **(Figure 3A)**. Neither 70 µM tibolone or 100 µM BIBR1532, alone or in combination, induced a cytotoxic effect on T98G cells **(Figure S2)**. Quantification of superoxide **(Figure 3B)** did not show any effect produced by TERT inhibition, (F (5.78) = 1.927, P= 0.10). Quantification of hydrogen peroxide showed that PA and tibolone plus PA induce a reduction in its levels (P <0.0001), which were not affected by co-treatment with BIBR **(Figure 3C)**. Comparison of BIBR alone with DMEM showed that it does not affect hydrogen peroxide levels (**Figure 3C and S3)**.

Expression of *IL6* gene and protein secretion under TERT inhibition and its effects on cells T98G under palmitic acid treatment

As telomerase participates in the induction of cytokine expression, *IL6* gene expression and protein secretion under telomerase inhibition were investigated. An ANOVA test showed that the TERT inhibition interferes with the *IL6* gene expression ($F(4.39) = 4.51$, $P = 0.004$). A Tukey's multiple comparison test showed that a treatment with BIBR1532 prior to incubation with PA reduced *IL6* gene expression, in comparison with cells treated with PA alone ($P = 0.017$) **(Figure 4A)**. Expression of *IL6* was affected by BIBR1532 alone, in comparison with DMSO **(Figure S4).** An ANOVA test for IL6 secretion also showed an effect of TERT inhibition ($F(4.40) = 218.6$, $P < 0.0001$). Protein secretion was reduced by BIBR1532 (TERT inhibitor) in comparison with PA and control ($P < 0.0001$) and improved the effect of tibolone on IL6 secretion ($P = 0.0006$) **(Figure 4B)**. Differences were not observed between control and PA, however, the increase in IL6 by PA could arise with longer incubation times (such as 24 hours), similar to the observed with TERT protein expression. These data suggest that telomerase is involved in the induction of IL6 expression by PA in T98G.

The next step was to determine whether this cytokine is beneficial or detrimental for T98G cells treated with PA. We incubated cells with a recombinant IL6 protein at 58, 298 and 457 pg/mL; these concentrations were similar to those determined by ELISA in cells treated with tibolone, PA and TERT inhibitor (BIBR1532). In addition, we tested a higher IL6 concentration than those determined by ELISA (1000 pg/mL). IL6 was incubated for 24 hours before a stimulation with 1 mM PA (pre-treatment), in co-treatment with 1 mM PA, and after 1 mM PA treatment (post-treatment). We only observed an effect by IL6 pretreatment ($F(5.65) = 29.00$, $P < 0.0001$). Co-treatment and post-treatment did not affect T98G cells, in comparison with PA cells (data not shown). We found that high concentrations

of IL6 did not increase cell death by PA, but IL6 pretreatment (at 58 pg/mL) protected cells against death induced by PA (P= 0.013) **(Figure 4C).** Moreover, we found that treatment with IL6 affected TERT expression (F = 4.459, P= 0.011). A post hoc analysis determined that a treatment with IL6 (at 58 pg/mL), in comparison with DMEM, resulted in an increase in TERT gene expression (P= 0.008) **(Figure 4D).** These data suggest that IL6 has a protective effect at low concentrations and, furthermore, it puts forward the possibility that a feedback between this cytokine and the telomerase could exist.

Telomerase phosphorylation analysis

Telomerase protein has several phosphorylation sites responsible for the modulation of its protein activity and its location in cytoplasm or nucleus. In the current study, we assessed the phosphorylation status of the following sites: tyrosine 707 (a signal required to translocate it from the nucleus to cytoplasm) and serine 227 (a signal required to locate it in the nucleus) (8, 53). This analysis was carried out using flow cytometry. An ANOVA test showed an effect of treatments on Tyr-707 phosphorylation (F (2.24) = 25.51, P <0.0001). It was observed that PA increased (by a 50%) the phosphorylation levels of the Tyr-707 site, in comparison with control (P= 0.001). This effect was improved by 90% upon tibolone pretreatment when compared with control values (90 %, P <0.0001) and by 40% in comparison with PA (P= 0.013) **(Figure 5A, B)**. The phosphorylation levels of the Ser-227 site were not affected by any treatment (F (2.30) = 0.085, P = 0.92) **(Figure 6 A, B)**. Tibolone was able to induce phosphorylation in Tyr-707, in comparison with DMSO (t=3.913, df=16, P= 0.001) **(Figure 5C, D).** Tibolone alone did not induce phosphorylation of Ser-227 (t=0.1427, df=16, P= 0.89) **(Figure 6 C, D)**. These data suggest that the phosphorylation of telomerase protein, which regulates its translocation to the cytoplasm, is

modified by tibolone, a mechanism that could be involved in the protective effects of this compound in T98G cells.

Telomerase expression and Tyr-707 phosphorylation status analysis in normal human astrocytes

In order to compare the effect of PA and tibolone on T98G cells and normal human astrocytes (NHA), we focused on assessing expression and phosphorylation levels (Tyr-707 site) of the telomerase protein in NHA cells. Treatment with PA for 48 hours did not induce any change in telomerase protein expression in comparison with control or tibolone + PA ($F(3.49) = 1.205$, $P = 0.32$) **(Figure 7A and B).** Significant differences were not observed between tibolone versus DMSO ($F(2.14) = 0.1391$, $P = 0.82$) **(Figure 7C and D)**. Tyr-707 phosphorylation was affected in NHA cells ($F(3.14) = 16.97$, $P < 0.0001$). However, contrary to T98G cells, Tyr-707 phosphorylation was reduced by 22% upon PA, compared with control ($P = 0.001$). Tibolone pretreatment did not reverse this effect **(Figure 8A and B)**, although it induced an increase of 27% in phosphorylation, in comparison with DMSO ($F(2.15) = 11.46$, $P = 0.001$), at a concentration of 10 nM ($P = 0.001$) **(Figure 8C and D).**

Discussion

Telomerase has several functions in different cell types, with its expression being higher in mitotic cells (e.g somatic cells) than in postmitotic cells (such as neurons). In this context, telomerase can protect cells from apoptosis by canonical and non-canonical functions (1). The telomerase activity (its canonical function) is regulated by several molecular mechanisms, for example, regulation of gene and protein expression, protein phosphorylation and interaction with other proteins (54).

Here, we found that PA induced a reduction in telomerase activity, but this effect was not reversed by tibolone pretreatment, in spite that cells treated with tibolone had a higher activity. However, significant differences were not observed between control and PA. There are no reports showing a reduction in telomerase activity caused by a saturated fatty acid. In contrast, telomerase activity has been affected by cis-unsaturated fatty acids in previous reports (55). We observed that protein expression of TERT after 24 hours of PA treatment was not affected. Notably, telomerase activity can be modulated independently of its gene or protein expression levels (56). Nevertheless, at 24 hours after PA stimulation, *TERT* gene expression increased (24) and the protein levels were found increased after 36 hours of PA treatment **(Figure 1E)**. Such increase could arise as a compensatory mechanism due to the reduction in telomerase activity.

As TERT protein has several non-canonical functions, its increase could attenuate the detrimental effects induced by PA on cell death. Taking this into account, we aimed to evaluate the role of TERT in T98G cells using a chemical compound (BIBR1532), which is known to inhibit telomerase protein through reduction of gene and protein expression (40). Our results suggest that telomerase was involved in the protection by tibolone against cell death induced by PA. Previously, it has been demonstrated that telomerase has anti-apoptotic actions due to the regulation of several genes, such as BAX/BCL2 (57) and Caspase-3 (58), and additionally by its translocation to mitochondria (59). Therefore, we hypothesize that tibolone can induce telomerase translocation from nucleus to mitochondria, reducing the cell death. This is supported in part by our analysis of phosphorylation. We found that tibolone alone or in combination with PA increased Tyr-707 phosphorylation in T98G cells**.** Phosphorylation of this residue in telomerase protein leads to its export from the nucleus to the cytoplasm (8). Although PA also induced an increase in Tyr-707 phosphorylation, TERT

inhibition did not increase cell death. These findings suggest that tibolone might induce activation of other pathways promoting interaction of TERT with other proteins, and therefore, reduce cell death. Previously, a study demonstrated that the anti-apoptotic function of TERT is regulated by its association with Akt and HSP90 proteins (60). Interestingly, another study demonstrated that activation of estrogen receptors was involved in the phosphorylation of TERT by estradiol and raloxifene, without an increase in protein or gene expression of TERT, a mechanism that could be associated with the protection by these compounds (61).

As the study of molecular mechanisms in cell lines might have some limitations and their results should be interpreted accordingly, we aimed to extend our analysis of TERT protein expression and Tyr-707 phosphorylation to normal human astrocyte (NHA) cells (derived from fetal brain). These cells were treated under similar conditions to those of the T98G cell line. We found that, in contrast to T98G, PA reduced Tyr-707 phosphorylation in NHA cells, and although tibolone alone induced an increase in phosphorylation, the combination of PA with tibolone did not have any effect. These differences can be explained by the finding that PA did not trigger a strong effect on cell death in the NHA cells, therefore it is possible that mitochondrial integrity was not affected. On the other hand, tibolone also did not have any effects on PA actions **(Figure S5)**. It should be noted that palmitic acid oxidation in fetal astrocytes is higher than in adult astrocytes (62). In this context, the assimilation of palmitic acid might be different between NHA and T98G (cells derived from the adult brain).

We previously observed that TERT and IL6 genes were regulated by PA and tibolone in a similar manner (24). TERT is required for the induction of IL6 (6), IL1β and TNF genes by the NF-kB factor (7). Thus, we have hypothesized that TERT could be regulating IL6 expression in our model. This hypothesis was corroborated with the results of the experiments of TERT inhibition in cells exposed to PA and tibolone. Similar to other studies, we demonstrated that IL6 gene levels and protein secretion were decreased by TERT inhibition in cells under an inflammatory stimulus (6, 7). Interestingly, we observed that TERT inhibition increased the reduction of IL6 secretion by tibolone. TERT regulates IL6 expression by binding to a motif present in the IL6 gene DNA sequence (6). Our results support these findings, considering that BIBR1532 is an inhibitor that interacts with a motif located in the thumb domain, which regulates the binding of TERT to DNA or RNA by changes in its configuration (39).

IL6 is a cytokine with pleiotropic effects, which is involved in both pro- and anti-inflammatory actions (63). In astrocyte cells, IL6 is increased to carry out different actions, and depending of the inducing factor, this will be beneficial or detrimental for the brain cells. For example, in an acute insult of traumatic brain injury it was observed that IL6 is neuroprotective (64), as well as in cells exposed to LPS (an inductor of inflammation) (65). In the current cell model, we have observed that lower concentrations of IL6 are beneficial for T98G exposed to PA, although higher concentrations did not increase cell death, assessed by a propidium iodide uptake test at 24 hours of treatment. These results suggest that cells under chronic exposition to PA might have a higher IL6 expression with possible detrimental effects, especially in other cell types like neurons (66). Nonetheless, detrimental effects of IL6 will depend on whether activation of trans-signaling pathways is induced (67). Additionally, we observed that IL6 induced an increase in *TERT* gene expression in T98G

cells. TERT can regulate IL6 expression and, in turn, IL6 can modulate TERT function (activity and expression) through NF-kB/STAT3 activation (68). Therefore, this result suggests that a feedback loop between TERT and IL6 could exist. However, future studies are needed to elucidate this hypothesis.

We have evidenced that TERT participates in distinct cellular processes involved in the response to an injury with PA and to the exposure to estrogenic compounds. These results highlight the potential of targeting astrocytes for drug development in the context of the treatment of neurological diseases. Astrocytes play a crucial role in the brain because they regulate several processes associated with distinct cell types such as neurons, microglia and oligodendrocytes (69). On the other hand, activation of astrocytes is a defensive response to ameliorate acute stress in the brain, but a chronic astrocytic response is associated with diseases (70). During astrocyte activation, cytokines as IL6 are increased. In this regard, the elucidation of the mechanisms of the activation of astrocytes might provide insights about possible therapeutic targets.

In conclusion, we show for the first time how PA and tibolone modulate telomerase protein expression and activity in an astrocytic cell model. Moreover, our results demonstrate that telomerase participates in distinct cellular processes under beneficial and detrimental conditions. Future studies are needed to define the specific roles of IL6 and TERT on astrocyte cell functions and their possible feedback under inflammatory stimuli.

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Conflict of interest

The authors declare no conflict of interest.

Reference

1. Martinez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nature reviews Cancer. 2011;11(3):161-76.
2. Rebhan M, Chalifa-Caspi V, Prilusky J, Lancet D. GeneCards: integrating information about genes, proteins and diseases. Trends in genetics : TIG. 1997;13(4):163.
3. Cha Y, Kwon SJ, Seol W, Park KS. Estrogen receptor-alpha mediates the effects of estradiol on telomerase activity in human mesenchymal stem cells. Molecules and cells. 2008;26(5):454-8.
4. Gizard F, Heywood EB, Findeisen HM, Zhao Y, Jones KL, Cudejko C, et al. Telomerase activation in atherosclerosis and induction of telomerase reverse transcriptase expression by inflammatory stimuli in macrophages. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(2):245-52.
5. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene. 2001;269(1-2):1-12.
6. Ghosh A, Saginc G, Leow SC, Khattar E, Shin EM, Yan TD, et al. Telomerase directly regulates NF-kappaB-dependent transcription. Nature cell biology. 2012;14(12):1270-81.
7. Wu XQ, Yang Y, Li WX, Cheng YH, Li XF, Huang C, et al. Telomerase reverse transcriptase acts in a feedback loop with NF-kappaB pathway to regulate macrophage polarization in alcoholic liver disease. Scientific reports. 2016;6:18685.
8. Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Molecular and cellular biology. 2003;23(13):4598-610.
9. Sharma NK, Reyes A, Green P, Caron MJ, Bonini MG, Gordon DM, et al. Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria. Nucleic acids research. 2012;40(2):712-25.
10. Hosoi T, Nakatsu K, Shimamoto A, Tahara H, Ozawa K. Inhibition of telomerase causes vulnerability to endoplasmic reticulum stress-induced neuronal cell death. Neuroscience letters. 2016;629:241-4.
11. Chan SW, Blackburn EH. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. Oncogene. 2002;21(4):553-63.
12. Forero DA, Gonzalez-Giraldo Y, Lopez-Quintero C, Castro-Vega LJ, Barreto GE, Perry G. Meta-analysis of Telomere Length in Alzheimer's Disease. The journals of gerontology Series A, Biological sciences and medical sciences. 2016;71(8):1069-73.
13. Wolkowitz OM, Mellon SH, Epel ES, Lin J, Dhabhar FS, Su Y, et al. Leukocyte telomere length in major depression: correlations with chronicity, inflammation and oxidative stress--preliminary findings. PloS one. 2011;6(3):e17837.

14. Kawanishi S, Oikawa S. Mechanism of telomere shortening by oxidative stress. Annals of the New York Academy of Sciences. 2004;1019:278-84.
15. Jurk D, Wilson C, Passos JF, Oakley F, Correia-Melo C, Greaves L, et al. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. Nature communications. 2014;2:4172.
16. Kim S, Parks CG, DeRoo LA, Chen H, Taylor JA, Cawthon RM, et al. Obesity and weight gain in adulthood and telomere length. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2009;18(3):816-20.
17. Wojcicki JM, Elwan D, Lin J, Blackburn E, Epel E. Chronic Obesity and Incident Hypertension in Latina Women Are Associated with Accelerated Telomere Length Loss over a 1-Year Period. Metabolic syndrome and related disorders. 2018;16(6):262-6.
18. Shin YA, Lee KY. Low estrogen levels and obesity are associated with shorter telomere lengths in pre- and postmenopausal women. Journal of exercise rehabilitation. 2016;12(3):238-46.
19. Lee DC, Im JA, Kim JH, Lee HR, Shim JY. Effect of long-term hormone therapy on telomere length in postmenopausal women. Yonsei medical journal. 2005;46(4):471-9.
20. Imtiaz B, Tuppurainen M, Rikkonen T, Kivipelto M, Soininen H, Kroger H, et al. Postmenopausal hormone therapy and Alzheimer disease: A prospective cohort study. Neurology. 2017;88(11):1062-8.
21. Feng R, Luo C, Li C, Du S, Okekunle AP, Li Y, et al. Free fatty acids profile among lean, overweight and obese non-alcoholic fatty liver disease patients: a case - control study. Lipids in health and disease. 2017;16(1):165.
22. Gupta S, Knight AG, Gupta S, Keller JN, Bruce-Keller AJ. Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. J Neurochem. 2012;120(6):1060-71.
23. Wang Z, Liu D, Wang J, Liu S, Gao M, Ling EA, et al. Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. Journal of pineal research. 2012;52(2):253-64.
24. Gonzalez-Giraldo Y, Forero DA, Echeverria V, Garcia-Segura LM, Barreto GE. Tibolone attenuates inflammatory response by palmitic acid and preserves mitochondrial membrane potential in astrocytic cells through estrogen receptor beta. Molecular and cellular endocrinology. 2019;486:65-78.
25. Campisi R, Marengo FD. Cardiovascular effects of tibolone: a selective tissue estrogenic activity regulator. Cardiovascular drug reviews. 2007;25(2):132-45.
26. Hidalgo-Lanussa O, Avila-Rodriguez M, Baez-Jurado E, Zamudio J, Echeverria V, Garcia-Segura LM, et al. Tibolone Reduces Oxidative Damage and Inflammation in Microglia Stimulated with Palmitic Acid through Mechanisms Involving Estrogen Receptor Beta. Molecular neurobiology. 2017.
27. Guzman CB, Zhao C, Deighton-Collins S, Kleerekoper M, Benjamins JA, Skafar DF. Agonist activity of the 3-hydroxy metabolites of tibolone through the oestrogen receptor in the mouse N20.1 oligodendrocyte cell line and normal human astrocytes. Journal of neuroendocrinology. 2007;19(12):958-65.
28. Gonzalez-Giraldo Y, Forero DA, Echeverria V, Gonzalez J, Avila-Rodriguez M, Garcia-Segura LM, et al. Neuroprotective effects of the catalytic subunit of telomerase: A potential therapeutic target in the central nervous system. Ageing research reviews. 2016;28:37-45.
29. Baek S, Bu Y, Kim H, Kim H. Telomerase induction in astrocytes of Sprague-Dawley rat after ischemic brain injury. Neuroscience letters. 2004;363(1):94-6.
30. Tao X, Ming-Kun Y, Wei-Bin S, Hai-Long G, Rui K, Lai-Yong T. Role of telomerase reverse transcriptase in glial scar formation after spinal cord injury in rats. Neurochemical research. 2013;38(9):1914-20.
31. Qu Y, Duan Z, Zhao F, Wei D, Zhang J, Tang B, et al. Telomerase reverse transcriptase upregulation attenuates astrocyte proliferation and promotes neuronal survival in the hypoxic-ischemic rat brain. Stroke. 2011;42(12):3542-50.

32. Stein GH. T98G: an anchorage-independent human tumor cell line that exhibits stationary phase G1 arrest in vitro. Journal of cellular physiology. 1979;99(1):43-54.
33. Gasque P, Chan P, Mauger C, Schouft MT, Singhrao S, Dierich MP, et al. Identification and characterization of complement C3 receptors on human astrocytes. Journal of immunology. 1996;156(6):2247-55.
34. Avila-Rodriguez M, Garcia-Segura LM, Hidalgo-Lanussa O, Baez E, Gonzalez J, Barreto GE. Tibolone protects astrocytic cells from glucose deprivation through a mechanism involving estrogen receptor beta and the upregulation of neuroglobin expression. Molecular and cellular endocrinology. 2016;433:35-46.
35. Baez-Jurado E, Vega GG, Aliev G, Tarasov VV, Esquinas P, Echeverria V, et al. Blockade of Neuroglobin Reduces Protection of Conditioned Medium from Human Mesenchymal Stem Cells in Human Astrocyte Model (T98G) Under a Scratch Assay. Molecular neurobiology. 2017.
36. Belot N, Rorive S, Doyen I, Lefranc F, Bruyneel E, Dedecker R, et al. Molecular characterization of cell substratum attachments in human glial tumors relates to prognostic features. Glia. 2001;36(3):375-90.
37. Gonzalez-Giraldo Y, Garcia-Segura LM, Echeverria V, Barreto GE. Tibolone Preserves Mitochondrial Functionality and Cell Morphology in Astrocytic Cells Treated with Palmitic Acid. Molecular neurobiology. 2017.
38. Deacon K, Knox AJ. PINX1 and TERT Are Required for TNF-alpha-Induced Airway Smooth Muscle Chemokine Gene Expression. Journal of immunology. 2018.
39. Bryan C, Rice C, Hoffman H, Harkisheimer M, Sweeney M, Skordalakes E. Structural Basis of Telomerase Inhibition by the Highly Specific BIBR1532. Structure. 2015;23(10):1934-42.
40. Lavanya C, Venkataswamy MM, Sibin MK, Srinivas Bharath MM, Chetan GK. Down regulation of human telomerase reverse transcriptase (hTERT) expression by BIBR1532 in human glioblastoma LN18 cells. Cytotechnology. 2018;70(4):1143-54.
41. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry Part A : the journal of the International Society for Analytical Cytology. 2003;55(2):61-70.
42. Herbert BS, Hochreiter AE, Wright WE, Shay JW. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nature protocols. 2006;1(3):1583-90.
43. Hou M, Xu D, Bjorkholm M, Gruber A. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clin Chem. 2001;47(3):519-24.
44. Jimenez KM, Forero DA. Effect of master mixes on the measurement of telomere length by qPCR. Molecular biology reports. 2018;45(4):633-8.
45. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic acids research. 2009;37(3):e21.
46. Lahiri DK, Nurnberger JI, Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic acids research. 1991;19(19):5444.
47. Speck-Hernandez CA, Ojeda DA, Castro-Vega LJ, Forero DA. Relative telomere length is associated with a functional polymorphism in the monoamine oxidase A gene in a South American sample. J Genet. 2015;94(2):305-8.
48. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nature protocols. 2006;1(3):1559-82.
49. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research. 2009;37(6):e45.
50. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, et al. Evidence based selection of housekeeping genes. PloS one. 2007;2(9):e898.
51. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols. 2008;3(6):1101-8.

52. Haendeler J, Drose S, Buchner N, Jakob S, Altschmied J, Goy C, et al. Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. Arteriosclerosis, thrombosis, and vascular biology. 2009;29(6):929-35.
53. Chung J, Khadka P, Chung IK. Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation. Journal of cell science. 2012;125(Pt 11):2684-97.
54. Nicholls C, Li H, Wang JQ, Liu JP. Molecular regulation of telomerase activity in aging. Protein & cell. 2011;2(9):726-38.
55. Oda M, Ueno T, Kasai N, Takahashi H, Yoshida H, Sugawara F, et al. Inhibition of telomerase by linear-chain fatty acids: a structural analysis. The Biochemical journal. 2002;367(Pt 2):329-34.
56. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiology and molecular biology reviews : MMBR. 2002;66(3):407-25, table of contents.
57. Li J, Qu Y, Chen D, Zhang L, Zhao F, Luo L, et al. The neuroprotective role and mechanisms of TERT in neurons with oxygen-glucose deprivation. Neuroscience. 2013;252:346-58.
58. Zhao F, Qu Y, Xiong T, Duan Z, Ye Q, Mu D. The neuroprotective role of TERT via an antiapoptotic mechanism in neonatal rats after hypoxia-ischemia brain injury. Neuroscience letters. 2012;515(1):39-43.
59. Ahmed S, Passos JF, Birket MJ, Beckmann T, Brings S, Peters H, et al. Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. Journal of cell science. 2008;121(Pt 7):1046-53.
60. Haendeler J, Hoffmann J, Rahman S, Zeiher AM, Dimmeler S. Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. FEBS letters. 2003;536(1-3):180-6.
61. Du B, Ohmichi M, Takahashi K, Kawagoe J, Ohshima C, Igarashi H, et al. Both estrogen and raloxifene protect against beta-amyloid-induced neurotoxicity in estrogen receptor alpha-transfected PC12 cells by activation of telomerase activity via Akt cascade. The Journal of endocrinology. 2004;183(3):605-15.
62. Warshaw JB, Terry ML. Cellular energy metabolism during fetal development. VI. Fatty acid oxidation by developing brain. Developmental biology. 1976;52(1):161-6.
63. Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. Arthritis research & therapy. 2006;8 Suppl 2:S2.
64. Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernandez J, et al. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Experimental neurology. 2003;181(2):130-48.
65. Sun L, Li Y, Jia X, Wang Q, Li Y, Hu M, et al. Neuroprotection by IFN-gamma via astrocyte-secreted IL-6 in acute neuroinflammation. Oncotarget. 2017;8(25):40065-78.
66. Wei H, Chadman KK, McCloskey DP, Sheikh AM, Malik M, Brown WT, et al. Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. Biochimica et biophysica acta. 2012;1822(6):831-42.
67. Rothaug M, Becker-Pauly C, Rose-John S. The role of interleukin-6 signaling in nervous tissue. Biochimica et biophysica acta. 2016;1863(6 Pt A):1218-27.
68. Chung SS, Wu Y, Okobi Q, Adekoya D, Atefi M, Clarke O, et al. Proinflammatory Cytokines IL-6 and TNF-alpha Increased Telomerase Activity through NF-kappaB/STAT1/STAT3 Activation, and Withaferin A Inhibited the Signaling in Colorectal Cancer Cells. Mediators of inflammation. 2017;2017:5958429.
69. Kimelberg HK, Nedergaard M. Functions of astrocytes and their potential as therapeutic targets. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics. 2010;7(4):338-53.
70. Pekny M, Pekna M. Astrocyte reactivity and reactive astrogliosis: costs and benefits. Physiological reviews. 2014;94(4):1077-98.

Figure legends

Figure 1. Analysis of TERT protein expression in T98G cells under PA and tibolone treatments. Cells were treated for 24 hours with 70 µM tibolone followed by 1 mM PA treatment for 12, 24, 36 and 48 hours. Then, flow cytometry analysis was carried out to determine changes in TERT protein expression levels. Representative plot of flow cytometry for **(A)** TERT protein expression in cells treated with DMSO and 70 µM tibolone. **(B)** A shift to the right of the curve (an increment in fluorescence intensity) indicated an increase in protein expression respect to the control (Green) in cells treated with PA at 36 hours, alone (Red) or with tibolone (Blue). **(C)** A similar effect was observed at 48 after PA treatment in cells pretreated with tibolone. **(D)** Mean fluorescence intensity was calculated automatically using the flow cytometry software for each sample from 5000 events acquired; data were normalized to perform the statistical analysis of cells treated for 24 hours with DMSO and 70 µM tibolone alone. **(E)** Normalized data of mean fluorescence intensity of T98G cells treated for 24 hours with 70 µM tibolone followed by a treatment with 1 mM Palmitic acid for 12, 24, 36 and 48 hours. For all experiments, the control sample consisted in 2.5 % BSA and 2 mM carnitine. Bar graph represents Mean ± SEM of at least three independent experiments. Two-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. In the graph *** represents: <0.001, **: <0.01 and *: <0.05. TIB: tibolone, PA: Palmitic acid.

Figure 2. Telomerase activity and telomere length in T98G cells. Cells were treated for 24 hours with 70 µM tibolone followed by 24 hours of 1 mM Palmitic acid stimuli, then, protein and DNA isolation was performed, and next Telomerase activity and Telomere length were measured by qPCR. **(A)** Telomerase activity analysis**. (B)** Telomere length measurement. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test were used for statistical analysis. TIB: tibolone, PA: Palmitic acid.

Figure 3. Effects of TERT inhibition on cell death and reactive oxygen species (ROS) production. T98G cells were treated with the BIBR1532 inhibitor for 24 hours alone or with tibolone before adding 1 mM PA. Mean fluorescence represented as arbitrary units (a.u.) for: **(A)** Iodide Propidium uptake test. (**B)** Superoxide ion production (DHE: Dihydroethidium). **(C)** Hydrogen peroxide production (DFCDA: 2′,7′-Dichlorofluorescin diacetate). For all

experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. **** represents <0.0001, *** <0.001 and ** < 0.01. TIB: tibolone, PA: Palmitic acid.

Figure 4. Telomerase inhibition leads to a reduction of IL6, and its lower levels are beneficial for T98G cells and in turn increase TERT gene expression. T98G cells were treated with the BIBR1532 inhibitor for 24 hours alone or with tibolone before adding 1 mM PA. **(A)** Effects of TERT inhibition on IL6 gene expression and **(B)** IL-6 protein secretion. In this graph **** represents a *P* value <0.0001 in comparison to palmitic acid treatment. #### represents a *P* value <0.0001 in comparison to control. **(C)** Effect of recombinant IL6 on cell death assessed by PI uptake test. Cells were treated with several concentrations of IL6 for 24 hours followed by 1 mM PA. **(D)** TERT gene expression was evaluated in cells treated for 24 hours with recombinant IL6. For all experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. TIB: tibolone, PA: Palmitic acid.

Figure 5. Effects of palmitic acid and tibolone on TERT phosphorylation in Tyr-707 residue in T98G cells. Cells were treated for 24 hours with 70 µM tibolone followed by 24 hours of 1 mM Palmitic acid stimuli, then cells were fixed and incubated with antibodies for flow cytometry analysis. **(A)** Tyr-707 phosphorylation analysis in cells treated with tibolone and PA, and its **(B)** representative plot of flow cytometry data. **(C)** Phosphorylation status analysis for Tyr-707 in cells treated with DMSO and tibolone, and its **(D)** representative plots of flow cytometry data. For all experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. TIB: tibolone, PA: Palmitic acid.

Figure 6. Effects of palmitic acid and tibolone on TERT phosphorylation in Ser-227 residue in T98G cells. Cells were treated for 24 hours with 70 µM tibolone followed by 24 hours of 1 mM Palmitic acid stimuli, then cells were fixed and incubated with antibodies for flow cytometry analysis. **(A)** Ser-227 phosphorylation analysis in cells treated with tibolone and PA, and its **(B)** representative plot of flow cytometry data. **(C)** Phosphorylation status

analysis for Ser-227 in cells treated with DMSO and tibolone, and its **(D)** representative plots of flow cytometry data. For all experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. TIB: tibolone, PA: Palmitic acid.

Figure 7. Telomerase protein expression in normal human astrocytes exposed to palmitic acid and tibolone. Cells were treated with 20 µM and 10 nM Tibolone for 24 hours followed by 1 mM PA for 48 hours. Next, cells were fixed and incubated with the antibodies for flow cytometry analysis. **(A)** TERT protein expression in cells treated with tibolone (24 hours) and palmitic acid (48 hours). **(B)** Representative plot of flow cytometry data for tibolone and PA. **(C)** TERT protein expression in cells treated with DMSO and tibolone, and its **(D)** representative plot of flow cytometry data. For all experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. TIB: tibolone, PA: Palmitic acid.

Figure 8. Telomerase Tyr-707 phosphorylation status in normal human astrocytes exposed to palmitic acid and tibolone. Cells were treated with 20 µM and 10 nM Tibolone for 24 hours followed by 1 mM PA for 24 hours. Next, cells were fixed and incubated with the antibodies for flow cytometry analysis. **(A)** Tyr-707 phosphorylation analysis for tibolone (24 hours) and palmitic acid (24 hours) treatments, and its **(B)** representative plot of flow cytometry data. **(C)** Tyr-707 phosphorylation analysis for tibolone vs DMSO, and its **(D)** representative plot for flow cytometry data**.** For all experiments, control sample consisted in 2.5 % BSA and 2mM carnitine. Bar graph represents Mean and ± SEM of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. TIB: tibolone, PA: Palmitic acid.