RESEARCH ARTICLE

Effect of tibolone pretreatment on kinases and phosphatases that regulate the expression and phosphorylation of Tau in the hippocampus of rats exposed to ozone

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Abstract

Oxidative stress (OS) is a key process in the development of many neurodegenerative diseases, memory disorders, and other pathological processes related to aging. Tibolone (TIB), a synthetic hormone used as a treatment for menopausal symptoms, decreases lipoperoxidation levels, prevents memory impairment and learning disability caused by ozone (O_3) exposure. However, it is not clear if TIB could prevent the increase in phosphorylation induced by oxidative stress of the microtubule-associated protein Tau. In this study, the effects of TIB at different times of administration on the phosphorylation of Tau, the activation of glycogen synthase kinase-3β (GSK3β), and the inactivation of Akt and phosphatases PP2A and PTEN induced by O_3 exposure were assessed in adult male Wistar rats. Rats were divided into 10 groups: control group (ozone-free air plus vehicle [C]), control + TIB group (ozone-free air plus TIB 1 mg/kg [C + TIB]); 7, 15, 30, and 60 days of ozone exposure groups [O_3] and 7, 15, 30, and 60 days of TIB 1 mg/kg before ozone exposure groups [O_3 + TIB]. The effects of O_3 exposure and TIB administration were assessed by western blot analysis of total and phosphorylated Tau, GSK3β, Akt, PP2A, and PTEN proteins and oxidative stress marker nitrotyrosine, and superoxide dismutase activity and lipid peroxidation of malondialdehyde by two different spectrophotometric methods (Marklund and TBARS, respectively). We observed that O_3 exposure increases Tau phosphorylation, which is correlated with decreased PP2A and PTEN protein levels, diminished Akt protein levels, and increased GSK3β protein levels in the hippocampus of adult male rats. The effects of O_3 exposure were prevented by the long-term treatment (over 15 days) with TIB. Malondialdehyde and nitrotyrosine levels increased from 15 to 60 days of exposure to O_3 in comparison to C group, and superoxide dismutase activity decreased. Furthermore, TIB administration limited the changes induced by O_3 exposure. Our results suggest a beneficial use of hormone replacement therapy with TIB to prevent neurodegeneration caused by O_3 exposure in rats.

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Introduction

Tau is a microtubule-associated protein that modulates the rate of microtubule assembly and influences cell growth and shape depending on the degree of its phosphorylation (Drubin and Kirschner, 1986; Johnson and Stoothoff, 2004; Iqbal et al., 2005; Stoothoff and Johnson, 2005).

It is widely suggested that conformational changes such as truncation, cleavage, and hyperphosphorylation of Tau contribute to its abnormal processing and aggregation into bundles. Known as paired helical filaments (PHF), these structures form the aberrant neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) and a family of related neurodegenerative diseases called tauopathies (Buée et al., 2000; Sánchez et al., 2001; Binder et al., 2005; Iqbal et al., 2005; Pevalova et al., 2006).

The different states of Tau phosphorylation result from the specific activity of kinases and phosphatases (Buée et al., 2000; Johnson and Stoothoff, 2004; Ferrer et al., 2005; Zhou et al., 2009; Braithwaite et al., 2012). Glycogen synthase kinase-3β (GSK3β) is one of the principal kinases associated with physiological and pathological Tau phosphorylation (Zhou et al., 2009; Avila et al., 2012). On the contrary, Tau

dephosphorylation has been associated directly with a preponderance of protein phosphatases-2A (PP2A) activity and indirectly with phosphatase and tensin homolog deleted on chromosome 10 (PTEN) activity compared to other phosphatases (Kerr et al., 2006; Zhang et al., 2006; Qian et al., 2010; Martin et al., 2013b).

It has been suggested that the unbalance between these kinase and phosphatase activities can be the origin of Tau hyperphosphorylation and aggregation (Campos-Peña et al., 2009; Martin et al., 2013a). Also, the disruption of GSK3β, PP2A, and PTEN has been described in AD postmortem brain material (Avila et al., 2012; Martin et al., 2013a, b).

Previously, Tau phosphorylation and the formation of NFTs were thought to be prominent early events during AD pathogenesis and induced by oxidative stress (OS) (Mondragón-Rodríguez et al., 2008; Su et al., 2010). Currently, it is known that the effects of OS on the phosphorylation of Tau protein are dose- and exposure time-dependent (Mattson et al., 1997; Olivieri et al., 2000, 2001; Lopresti and Konat, 2001; Gomez-Ramos et al., 2003; Zambrano et al., 2004; Lovell et al., 2004; Poppek et al., 2006; Dang et al., 2010; Su et al., 2010; Taga et al., 2011; Petroni et al., 2012).

Different models have been proposed to study the effects of OS in neurodegeneration. It has been well established that chronic O_3 exposure increases OS markers in the hippocampus of male rats (Rivas-Arancibia et al., 2011). When inhaled, O_3 increases reactive oxygen species (ROS) that produce OS in the central nervous system (CNS) (Rivas-Arancibia et al., 1998, 2010; Dorado-Martínez et al., 2001; Santiago-López et al., 2010). OS induced by O_3 causes neuronal damage, neurodegeneration, and cell death, as well as biochemical changes as lipid peroxidation (LPO) in CNS regions such as the hippocampus (Avila-Costa et al., 1999; Dorado-Martínez et al., 2001; Farfán-García et al., 2014; Pinto-Almazán et al., 2014). Some compounds with antioxidant activity have been tested on the chronic OS induced by O_3 inhalation model, and it has been observed that they improve short- and long-term memory (Guerrero et al., 1999; Rivas-Arancibia et al., 2000; Guevara-Guzmán et al., 2009; Farfán-García et al., 2014; Pinto-Almazán et al., 2014).

Tibolone (TIB) is a steroid prescribed for the treatment of climacteric symptoms and osteoporosis that can exert antioxidant activity and diminish OS *in vitro* and *in vivo* (Vural et al., 2005; de Aguiar et al., 2008; Farfán-García et al., 2014; Pinto-Almazán et al., 2014; Stark et al., 2015). In addition to its antioxidant properties, TIB has other neuroprotective effects (Pinto-Almazán et al., 2017). Two studies had been performed to evaluate the effects of TIB on Tau phosphorylation (Pinto-Almazán et al., 2012; Neri-Gómez et al., 2017). Chronic administration of TIB decreased Tau phosphorylation through the increase in the protein content of phosphorylated GSK3β (Ser^{9}), which in turn decreased the active site availability in the hippocampus of young ovariectomized (OVX) rats (Pinto-Almazán et al., 2012). In contrast, a recent report indicates that PHF-1 (hyperphosphorylated Tau epitope in $Ser^{396/404}$) content decreased with TIB administration, without changes in the phosphorylation of GSK3β in Ser^{9} or PI3K, and decreased phosphorylated and total Akt levels in the hippocampus of aged male mice (Neri-Gómez et al., 2017).

In the model of OS induced by O_3 exposure, the administration of TIB decreases LPO, prevents neuronal death, ameliorates cholinergic deficit, and reduces cognitive and motor impairment (Farfán-García et al., 2014; Pinto-Almazán et al., 2014). Hence, these findings suggest that TIB can regulate synaptic and neuronal plasticity, axonal growth, and decrease Tau phosphorylation by GSK3β inhibition and probably by its antioxidant properties.

The purpose of this study was to assess the effects of TIB on the expression and phosphorylation of Tau, GSK3β, Akt, and phosphatases PP2A and PTEN in the hippocampus of rats exposed to O_3.

Materials and methods

Animals

Sixty male adult Wistar rats aged 8 weeks old, weighing 250–300 g, from the vivarium of the Hospital de Especialidades, CMN SXXI, Instituto Mexicano del Seguro Social were housed, five animals per cage, in acrylic boxes with free access to water and food (Purina, Minnetonka, MN, USA) and kept in a clear air room maintained under an artificial 12 hour light/dark cycle (lights on at 08:00 hours). Animals were treated by the guidelines and requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985) and those of the Ethical Committee of the Scientific Research Coordination at the Instituto Mexicano del Seguro Social (approval number R-2011-785-057). All efforts were made to minimize animal suffering and the protocol was designed to keeping the number of animals used to a minimum.

Treatments

Animals were randomly divided into ten experimental groups (n = 6). Each group received one of the following treatments: control [C] (exposed to ozone-free air plus vehicle (300 μL of pure water) by oral gavage for 60 days); control + TIB [C + TIB] (exposed to ozone-free air plus 1 mg/kg of TIB by oral gavage for 60 days); 7, 15, 30, and 60 days of ozone exposure [O_3] (daily exposure to 0.25 ppm of monitored ozone for 4 hours; Poppek et al., 2006); and 7, 15, 30, and 60 days of 1 mg/kg of TIB (Schering Plough México, Mexico City, Mexico) by oral gavage before ozone exposure [O_3 + TIB] (Farfán-García et al., 2014; Pinto-Almazán et al., 2014). Previous experience has shown that the nasogastric administration of pure water for 7–60 days does not produce relevant changes in the proteins analyzed during this study (Pinto-Almazán et al., 2012). Vehicle as well as TIB were administered 5 minutes

before placing the rats into the chamber.

Ozone exposure

Animals were daily placed in a chamber coupled to an air diffuser connected to a variable-flux ozone generator (5 L/s) (Omnialva, Mexico City, Mexico) and exposed to 0.25 ppm per hour for 4 hours. The same chamber was used to treat C group with airflow free of ozone and the groups exposed to monitored ozone. The procedure used has been described elsewhere (Rivas-Arancibia et al., 2010).

At the end of the treatment for each group (7, 15, 30 or 60 days), animals were decapitated. Brains were dissected and the hippocampus was divided: one section for western blot analysis and the other for analysis of OS markers.

Western blot analysis

Hippocampus samples were processed as previously described (Pinto-Almazán et al., 2012). The following primary antibodies were used: rabbit anti-Tau polyclonal antibody (H-150, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:1,000), which recognizes six Tau isoforms of 46-80 kDa molecular weight (total Tau) and mouse anti-Tau monoclonal antibody that recognizes phosphorylation on the Ser396 (PHF-13, Santa Cruz Biotechnology; PHF-1; diluted 1:1,000). Mouse anti-GSK3β monoclonal antibody (total GSK3β; BD Biosciences Pharmingen, San Diego, CA, USA; diluted 1:1,000); mouse anti-phosphorylated GSK3β monoclonal antibody (pSer9 GSK3β, Sigma, St. Louis, MO, USA, diluted 1:5,000); rabbit anti-Akt1/2/3 (H-136) polyclonal antibody (total Akt), rabbit anti-pAkt1/2/3 (Ser 473) polyclonal antibody (p-Akt); rabbit anti-PP2A-Cα polyclonal antibody Clone 46 (RUO) for total PP2A-Cα ; anti-goat p-PP2A-Cα/β polyclonal antibody (Tyr 307) (sc-12615) for phoshporylated PP2A-Cα; mouse anti-PTEN (A2B1) monoclonal antibody (sc-7974) for total PTEN; rabbit polyclonal antibody anti-PTEN (Ser 380) for phosphorylated PTEN; rabbit polyclonal antibody against nitrotyrosine (NT; Abcam ab42789) and mouse anti-β3 tubulin (2G10) (Santa Cruz Biotechnology; diluted 1:1,000).

After incubation with the primary antibody, membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology, diluted 1:10,000) were used. Highly precise detection of proteins from western blots was performed using an enhanced chemiluminescence system (Millipore, Billerica, MA, USA), and their intensity was quantified by densitometry (Hewlett Packard, Palo Alto, CA, USA) and densitometric analysis by KODAK 1D Image Analysis Software (Eastmond Kodak, Rochester, NY, USA). The density of each band of different primary antibodies was normalized to its loading control (tubulin).

Determination of OS markers

Hippocampus samples (subiculum, Ammon's horn and the dentate gyrus) were homogenized and processed as previously described (Farfán-García et al., 2014) at the end of the treatment in each group. Superoxide dismutase (SOD) activity was measured by the Marklund method (Marklund and Marklund, 1974). Lipid peroxidation end product malondialdehyde (MDA) was estimated using thiobarbituric acid reactive substances assay (TBARS) (Hong et al., 2000). The levels of nitrotyrosine (NT) were determined by western blot assay (Pinto-Almazán et al., 2014).

Statistical analysis

Western blot and OS data were analyzed by one-way analysis of variance with a Tukey's *post hoc* test. Prism 5.1 program (Prisma Graph Pad, La Jolla, CA, USA) was used for calculating probability values, considering values of $P < 0.05$ as statistically significant.

Results

Tau and GSK3β expression

The expression of total Tau in the hippocampus was determined by western blot assay. No changes were observed in the expression of total Tau neither in C and C + TIB nor in O_3 or O_3 + TIB groups (**Figure 1A**). However, a significant reduction of hyperphosphorylated Tau PHF-1 was observed in the C + TIB group in comparison with the C group ($P < 0.05$). Chronic exposure to O_3 for over 30 days resulted in a significant increase in the expression of PHF-1 ($P < 0.05$) (**Figure 1A**), while pretreatment with TIB diminished the expression of hyperphosphorylated Tau induced by chronic exposure to O_3. Furthermore, the ratio of hyperphosphorylated *versus* total Tau showed a significant increase in the hippocampus of rats exposed to O_3 for 30 and 60 days ($P < 0.05$), without any effect at 7 and 15 days of exposure (**Figure 1B**). In contrast, O_3 chronic exposure did not increase the ratio of hyperphosphorylated versus total Tau in any of the O_3 + TIB groups (**Figure 1B**).

Total and phosphorylated GSK3β (Ser^9) levels were assessed to determine if the changes in Tau were associated with their expression. The expression of total GSK3β in the hippocampus was not affected in any of the O_3 and O_3 + TIB groups (**Figure 2A**). Furthermore, we observed that the expression of the phosphorylated GSK3β form in C + TIB group increased when compared to C group. In contrast, the expression of the phosphorylated form of the protein decreased with respect to the time of exposure to O_3. This decrease was statistically significant over 15 days of exposure ($P < 0.05$) (**Figure 2A**). However, the expression of the phosphorylated form of GSK3β in O_3 + TIB groups was similar to that of C and C + TIB groups (**Figure 2A**). The ratio of phosphorylated *versus* total GSK3β showed a decrease in O_3 groups from day 15 to day 60 of exposure ($P < 0.05$) (**Figure 2B**). Moreover, none of the O_3 + TIB groups ratios showed any significant difference compared to C and C + TIB groups but showed statistical differences when com-

pared to the groups with the same time of O_3 exposure from day 15 (**Figure 2B**).

Akt expression

The expression of total Akt was not modified in any of the studied groups (**Figure 3A**). When the expression of phosphorylated Akt (pSer^{473}) was analyzed to determine if changes in GSK3β phosphorylation were due to Akt activity, it was noticed that the content of phosphorylated Akt decreased significantly in the O_3 groups since day 15 of O_3 exposure compared to C and C + TIB groups ($P < 0.05$). The expression of the phosphorylated form of Akt was unaltered in the O_3 + TIB groups, being similar to C and C + TIB groups (**Figure 3A**). Therefore, the ratio of phosphorylated versus total Akt diminished gradually from day 15 to day 60 of O_3 exposure ($P < 0.05$). This decrease was prevented by TIB treatment in every O_3 + TIB groups. Statistical significance was observed when compared with both the ratio of control groups and the ratio of the same exposure times of O_3 + TIB groups ($P < 0.05$) (**Figure 3B**).

Expression of PP2A and PTEN

The expression of the total protein was not affected in C, C + TIB, O_3 or O_3 + TIB groups for either PP2A (**Figure 4A**) or PTEN (**Figure 5A**). In contrast, phosphorylated PP2A levels were reduced from day 15 up to day 60 ($P < 0.05$) (**Figure 4A**) and phosphorylated PTEN from day 30 to day 60 of O_3 exposure ($P < 0.05$) (**Figure 5A**). In O_3 + TIB groups, both phosphorylated PP2A (**Figure 4A**) and PTEN (**Figure 5A**) levels were mainteined when compared with C and C + TIB groups. Consequently, the ratios of phosphorylated PP2A and PTEN forms versus total protein indicated a statistically significant decrease ($P < 0.05$) in O_3 groups when compared to controls and O_3 + TIB groups (**Figures 4B** and **5B**).

OS markers

OS markers were determined in the hippocampus of rats exposed to O_3 and treated with vehicle or TIB (**Table 1**). No statistical differences were found in the analysis of OS markers between C and C + TIB groups. However, MDA and NT levels increased significantly from 15 to 60 days of exposure to O_3 in comparison to group C ($P < 0.05$). Conversely, SOD activity decreased from 15 to 60 days of O_3 exposure ($P < 0.05$). The administration of TIB (1 mg/kg) limited the changes in the levels of these markers induced by O_3 exposure: SOD activity increased while MDA and NT levels decreased since day 15 and were maintained until 60 days in the O_3 + TIB groups, unlike the continuous increase in the O_3 groups. The values between O_3 and O_3 + TIB groups were significantly different for the same periods of O_3 exposure ($P < 0.05$).

Discussion

Our results suggest that chronic O_3 exposure induces OS, which in turn may produce hyperphosphorylation of Tau correlated with the decrease in the phosphorylation of Akt/GSK3β kinases and PP2A and PTEN phosphatases in the hippocampus of male rats. These effects can be prevented by the treatment with TIB (**Figure 6**).

Tau hyperphosphorylation induces destabilization of microtubules, compromises axonal transport, and contributes to neuronal degeneration by the formation of NFTs (Buée et al., 2000; Sánchez et al., 2001; Binder et al., 2005; Iqbal et al., 2005; Pevalova et al., 2006). The effects of OS on the phosphorylation of Tau protein appear to be dose- and exposure time-dependent. At low concentrations of H_2O_2 (< 0.1 mM) (Zambrano et al., 2004) and acute conditions of OS (Lopresti and Konat, 2001; Olivieri et al., 2001; Taga et al., 2011), OS could act as a neuroprotective mechanism through transient Tau dephosphorylation. Conversely, some reports indicate that chronic *in vitro* OS, products of OS such as 4-hydroxynonenal (4-HNE) (Mattson et al., 1997) and acrolein (Gomez-Ramos et al., 2003; Dang et al., 2010), as well as concentrations of toxic substances that exert OS, such as mercury (Hg) (< 200 nM) (Olivieri et al., 2000; Petroni et al., 2012), β-amyloid 1–42 and toxic β-amyloid 25–35 peptides (Olivieri et al., 2001), induce hyperphosphorylation of Tau in different epitopes (PS396, PHF-1, TG3 and MC1), which are associated with the formation of NFTs.

Under the last premise, we assessed the effects of chronic O_3 exposure on Tau phosphorylation. As in previous reports, we observed that chronic O_3 exposure increases OS markers (MDA and NT) in this model, with the reduction of SOD activity in the hippocampus of male rats (Rivas-Arancibia et al., 2010). In the present study, we report that chronic O_3 exposure for 30 and 60 days resulted in a significant increase in the expression of hyperphosphorylated Tau.

As stated before, hyperphosphorylation of Tau could be attributed to the unbalance between the activity of kinases and phosphatases. Physiological phosphorylation of Tau by Akt/GSK3β, direct dephosphorylation by phosphatase PP2A, and indirect dephosphorylation by PTEN may be involved in the regulation of microtubule dynamics, neuritic growth, neuronal cell proliferation, synaptogenesis, synaptic plasticity, and synaptic neurotransmission (Hall et al., 2000; Zhou et al., 2009; Martin et al., 2013a, b)

GSK3β regulates Tau hyperphosphorylation at Ser^{198}/Ser^{199}/Ser^{202} and Ser^{396}/Ser^{404} sites (Haque et al., 1999) and, while active in resting cells, it is downregulated by the Ser^{9} phosphorylation as a result of the activation of Akt, first on Thr^{308} and then on Ser^{473}, which produce Akt full activation (Piguet and Dufour, 2011; Kitagishi et al., 2012; Matsuda et al., 2013).

It was observed that chronic O_3 exposure reduced the phosphorylation of GSK3β in Ser^{9}, which correlated with the decrease of Akt phosphorylation of Ser^{473} after 30 days of exposure. These results are consistent with the increase in Tau phosphorylation at this time of exposure and suggest that

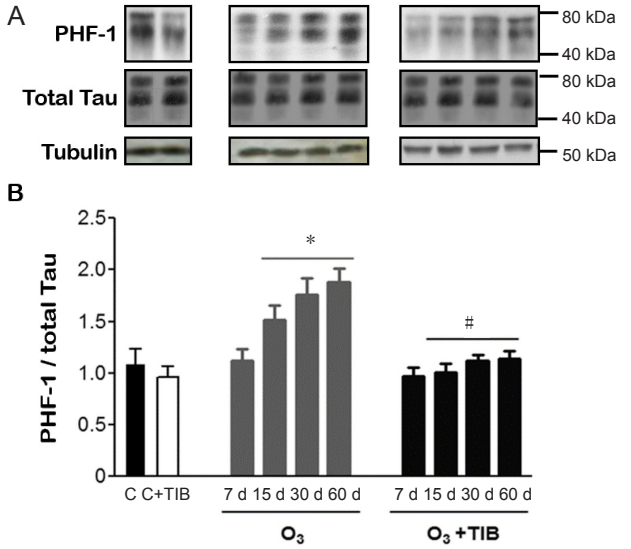

Figure 1 Effects of TIB on the hyperphosphorylation of Tau protein induced by O_3 exposure.
(A) Representative western blots of six independent assays of chronic exposure to O_3 and O_3 + TIB on the expression of phosphorylated Tau in Ser396 (PHF-1) and total Tau content in the hippocampus of rats. Animals were randomly divided into ten experimental groups (n = 6): C, Animals exposed to an air stream for 60 days; C + TIB, animals exposed to an air stream that received 1 mg/kg of TIB for 60 days. Four O_3 groups were exposed to O_3 for 7, 15, 30, and 60 days, respectively. O_3 + TIB groups received 1 mg/kg of TIB treatment for 7, 15, 30 and 60 days before O_3 exposure, respectively. (B) Densitometric analysis of PHF-1 and total Tau. Data were normalized to tubulin contents. Results are expressed as the mean ± standard error. $*P < 0.05$. *vs.* C group; $\#P < 0.05$, *vs.* O_3 groups at the same periods of time, respectively. TIB: Tibolone.

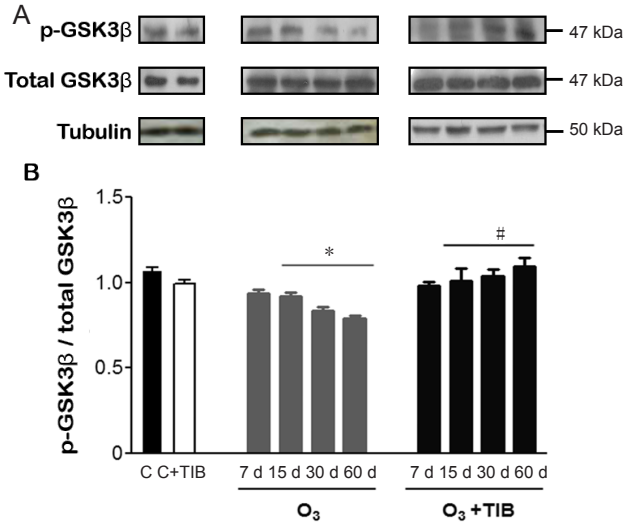

Figure 2 Effects of TIB on the phosphorylation of GSK3β protein induced by O_3 exposure.
(A) Representative western blots of six independent assays of chronic exposure to O_3 and O_3 + TIB on the expression of phosphorylated GSK3β on Ser9 (p-GSK3β) and of total GSK3β content in the hippocampus of rats. Experimental conditions were the same as described in Figure 1 (n = 6). (B) Densitometric analysis of p-GSK3β and total GSK3β. Data were normalized to tubulin contents. Results are expressed as the mean ± standard error. $*P < 0.05$, *vs.* C group; $\#P < 0.05$, *vs.* O_3 groups at the same periods of time, respectively. TIB: Tibolone; GSK3β: glycogen synthase kinase-3β.

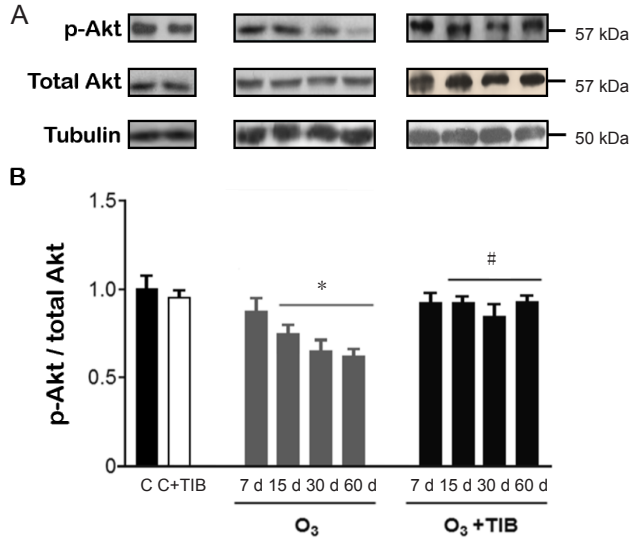

Figure 3 Effects of TIB on the phosphorylation of AKT protein induced by O_3 exposure.
(A) Representative western blots of six independent assays of chronic exposure to O_3 and O_3 + TIB on the expression of phosphorylated Akt on Ser^{473} (p-Akt) and total Akt in the hippocampus of rats. Experimental conditions were the same as described in Figure 1 (n = 6). (B) Densitometric analysis of p-Akt and total Akt. Data were normalized to tubulin contents. Results are expressed as the mean ± standard error. $*P < 0.05$, *vs.* C group $\#P < 0.05$, *vs.* O_3 groups at the same periods of time, respectively. TIB: Tibolone.

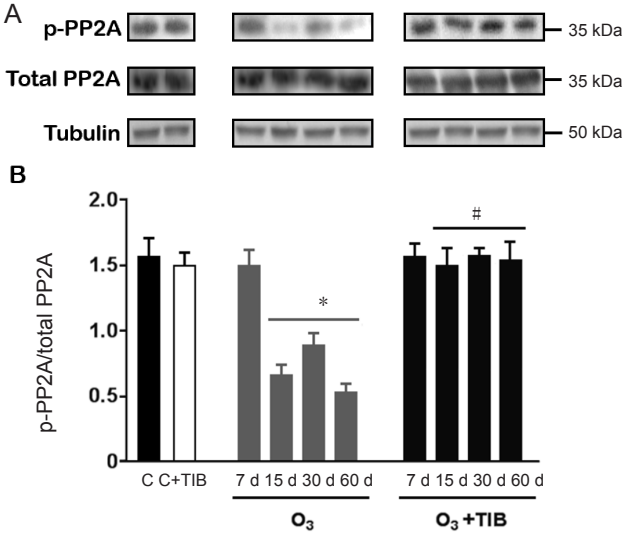

Figure 4 Effects of TIB on the phosphorylation of PP2A protein induced by O_3 exposure.
(A) Representative western blots of six independent assays of chronic exposure to O_3 and O_3 + TIB on the expression of phosphorylated PP2A on Tyr307 (p-PP2A) and total PP2A in the hippocampus of rats. Experimental conditions were the same as described in Figure 1 (n = 6). (B) Densitometric analysis of p-PP2A and total PP2A. Data were normalized to tubulin contents. Results are expressed as the mean ± standard error. $*P < 0.05$, *vs.* C group; $\#P < 0.05$, *vs.* O_3 groups at the same periods of time, respectively. TIB: Tibolone.

Table 1 Oxidative stress markers in the hippocampus of adult male rats exposed to ozone treated with vehicle or tibolone.

			O_3				O_3+TIB			
	C	C+TIB	7 d	15 d	30 d	60 d	7 d	15 d	30 d	60 d
MDA (nmol/mg protein)	1.8±0.05	2.1±0.08	2.8±0.10	3.8±0.06*	4.5±0.03*	4.8±0.05*	3.5±0.1	2.3±0.03#	2.2±0.04#	1.9±0.3#
SOD activity (U/mg protein)	70±3	75±5	67±3	55±2*	48±4*	40±4*	71±2	76±3#	81±5#	93±6#
Nitrotyrosine/tubulin (relative expression)	1.2±0.03	1.1±0.05	1.2±0.01	2.0±0.03*	2.2±0.03*	2.2±0.05*	1.2±0.05	1.1±0.04#	1.2±0.05#	1.1±0.02#

*P < 0.05, *vs.* C and C + TIB; #P < 0.05, *vs.* O_3 groups at the same periods of time, respectively. TIB: Tibolone; MDA: malondialdehyde; SOD: superoxide dismutase; d: days.

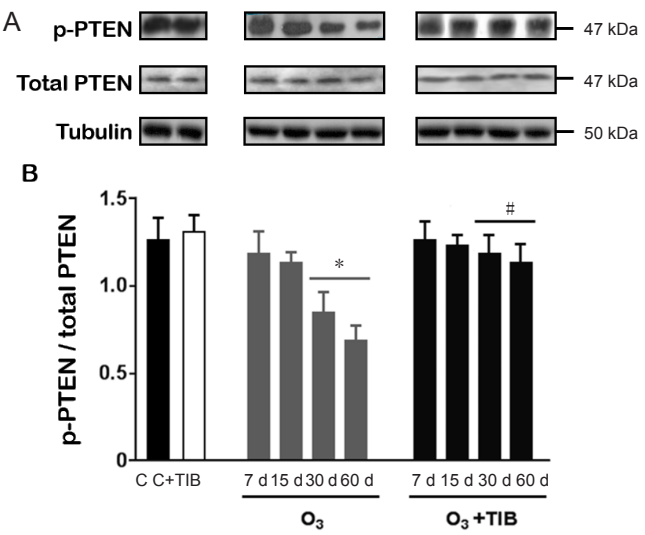

Figure 5 Effects of TIB on the phosphorylation of PTEN protein induced by O_3 exposure.
(A) Representative western blots of six independent assays of chronic exposure to O_3 and O_3 + TIB on the expression of phosphorylated PTEN on Ser380 (p-PTEN) and total PTEN in the hippocampus of rats. Experimental conditions were the same as described in Figure 1 (n = 6). (B) Densitometric analysis of p-PTEN and total PTEN. Data were normalized to tubulin contents. Results are expressed as the mean ± standard error. *P < 0.05, *vs.* C group; #P < 0.05, *vs.* O_3 groups at the same periods of time, respectively. TIB: TIB: Tibolone.

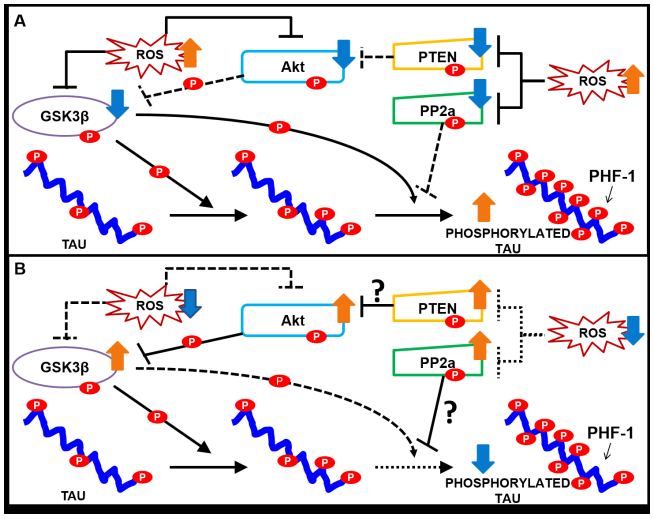

Figure 6 Neuroprotective effects of tibolone on the expression and phosphorylation of proteins involved in the formation of neurofibrillary tangles in an *in vivo* O_3 exposure model.
(A) Under O_3 exposure conditions, the phosphorylation of Tau is closely related to early pathological events. While phosphorylation of Tau Ser^{396} (PHF-1) increases in the presence of ROS, phosphorylation of Akt Ser^{473}, GSK3β Ser^{9}, PTEN Ser^{380}, PP2A Tyr^{307} decreases. (B) Conversely, TIB and its metabolites antioxidant properties diminish reactive oxygen species, which allows the phosphorylation of Akt Ser^{473}, GSK3β Ser^{9}, PTEN Ser^{380}, and PP2A Tyr^{307}. In turn, this signaling cascade prevents the increase in Tau Ser^{396} (PHF-1) phosphorylation induced by O_3 exposure.

GSK3β shows longer periods of activity without inhibition by Akt, causing the increase in Tau phosphorylation in the hippocampus of rats exposed to O_3.

For a better understanding of our results, it is important to consider that Akt is a redox-sensitive protein. At physiological pH, both cysteine residues in the activation loop (Cys^{297} and Cys^{311}) are shaped as thiols, which allow its biological effects. However, OS can produce the formation of a disulfide bond between Cys^{297}and Cys^{311} or other oxidized forms, which may reduce Thr^{308} and Ser^{473} phosphorylation in Akt, and consequently decrease its activity (Trachootham et al., 2008; Durgadoss et al., 2012; Wang et al., 2012; Tan et al., 2015). This situation could explain the decrease in Akt phosphorylation of Ser^{473} observed in our study.

PP2A is a large family of enzymes that account for the majority of brain Ser/Thr phosphatase activity. PP2A dephosphorylates Akt at Thr^{308} and Ser^{473} residues (particularly at Thr^{308}), GSK3β at Ser^{9}, and Tau at several phosphorylation sites with different efficiency (Qian et al., 2010; Hers et al., 2011; Martin et al., 2013a). PTEN is a lipid phosphatase which dephosphorylates PI(3,4,5)P3 at the 3-position of the inositol ring and decreases Akt activity (Piguet and Dufour, 2011; Martin et al., 2013a).

PP2A and PTEN are highly expressed in the brain, where they act on several substrates in different cell processes (Hall et al., 2000; Martin et al., 2013a). As Akt, the activities of these phosphatases depend on the redox state of the cell; therefore, they are considered as Cys-dependent phosphatases (CDP). At physiological pH, Cys exist as a thiolate anion, which is required for phosphatase activity. In contrast, under OS conditions, S-nitrosylation or the produced disulfide bonds inhibit their activity (Trachootham et al.,

2008).

As in previous studies, we observed a gradual decrease in the phosphorylation of both PP2A Tyr^{307} and PTEN Ser^{380} since day 15 of O_3 exposure. These results may indicate that ROS oxidize the catalytic Cys residues to the sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) acid forms that lead to irreversible phosphatase inactivation. However, further studies are needed to verify this effect.

Antioxidant (Vural et al., 2005; de Aguiar et al., 2008; Farfán-García et al., 2014; Pinto-Almazán et al., 2014; Stark et al., 2015) and neuroprotective (Qiu et al., 2009; Espinosa-Raya et al., 2012; Pinto-Almazán et al., 2012; Avila-Rodriguez et al., 2014; Beltrán-Campos et al., 2014; Neri-Gómez et al., 2017) benefits of TIB against different models of neuronal damage have been reported (Espinosa-Raya et al., 2012; Pinto-Almazán et al., 2012, 2014; Farfán-García et al., 2014; Neri-Gómez et al., 2017). In this study, we observed that chronic treatment with TIB prevented the hyperphosphorylation of Tau in contrast to the observed in the untreated O_3 groups. Consistent with our findings, it was previously reported that Tau phosphorylation on PHF-1 epitope was reduced with the chronic treatment of TIB in two other different models: young female OVX rats and aged male mice (Pinto-Almazán et al., 2012; Neri-Gómez et al., 2017).

The expression and the content of phosphorylated GSK3β, Akt, PP2A, and PTEN in the O_3 + TIB groups were similar to that observed in control groups. Previous reports have indicated that Tau phosphorylation on PHF-1 epitope was reduced and correlated with the increase in the phosphorylation of GSK3β on Ser^9 in the hippocampus and cerebellum of adult OVX rats chronically treated (two months) with TIB (Pinto-Almazán et al., 2012). In contrast, Neri-Gómez et al. (2017) reported that although a decrease in the hyperphosphorylation of Tau occurred, this was not produced through the inactivation of GSK3 by Akt phosphorylation but through induced dose-dependent changes on the content of CDK5/p35/p25 complexes. All these studies revealed that TIB can modify different signaling pathways involved in the regulation of neuroprotection mechanisms.

Although the aforementioned mechanism may explain the protective effects of TIB in this study, we propose another hypothesis. As in previous studies, we observed in this model that pretreatment with TIB can ameliorate the damage induced by chronic exposure to O_3 in the hippocampus of male rats (Farfán-García et al., 2014; Pinto-Almazán et al., 2014). Different studies have analyzed the antioxidant effects of chronic TIB treatment, demonstrating that TIB reduces *in vivo* OS by increasing the total antioxidant capacity with the increase in antioxidant enzymatic defenses, such as SOD content and activity and vitamin E (Vural et al., 2005; de Aguiar et al., 2008; Farfán-García et al., 2014; Pinto-Almazán et al., 2014). Previously, we have reported that TIB reduced lipid peroxidation and protein oxidation by increasing SOD content and activity in the chronic O_3 exposure model, preventing memory and motor deficits as well as the cholinergic system disruption (Farfán-García et al., 2014; Pinto-Almazán et al., 2014). In a recent study, Stark et al. (2015) reported that TIB did not show significant antioxidant capacity, but most of its active metabolites showed antioxidant effects. It has also been reported that TIB is rapidly metabolized after oral administration into its estrogenic metabolites (3α- and 3β-hydroxy tibolone) in rats (Verhoeven et al., 2002). Therefore, TIB metabolites could be those which exert antioxidant effects since these metabolites reach the brain at higher concentrations than TIB. In the present study, it was demonstrated that the chronic oxidative stress induced by ozone exposure was prevented by treatment with TIB, which resulted in the modulation of the activity of the PI3K/Akt/GSK3β signaling cascade and the phosphatases PP2A and PTEN, to finally observe a decrease in the hyperphosphorylation of Tau.

Some limitations of the present study are important to mention. One limitation is that the research was conducted only in male rats according to the OS model designed by Rivas-Arancibia et al. (1998). However, further research is necessary to verify if these results are gender-dependent. According to the design of the experiments, another limitation of our research is that we were not able to identify if the prevention of hyperphosphorylation of Tau induced by O_3 exposure was due to TIB itself or its metabolites. Therefore, further investigation and appropriate experiments to address this matter are required in this O_3 exposure model.

In conclusion, this work is a significant contribution to the study of the mechanisms of TIB neuroprotection against Tau neuronal damage in an OS *in vivo* model. Apparently, TIB can modulate Tau phosphorylation through various mechanisms, such as modulating the activity of kinases (GSK3β/Akt/PI3K pathway and CDK5/p35/p25 complexes) and phosphatases (PP2a and PTEN) or through the antioxidant property of its estrogenic metabolites. This study may allow scholars to develop new strategies to prevent neurodegeneration produced by OS.

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