ROS

RESEARCH ARTICLE

Effect of Etazolate on ROS Production after *t*BHP-Induced Oxidative Stress in Oligodendroglial 158N Cell Line

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ABSTRACT | Oligodendrocytes are the key cells for the myelin synthesis in the central nervous system (CNS). They are sensitive to oxidative stress because of their lipid-rich membranes and their limited antioxidant defense against an excessive production of reactive oxygen species (ROS). Hence, antioxidant strategies are required for myelin protection. We have previously shown that etazolate has neuroprotective and remyelinating activities, and we hypothesized that part of etazolate effect is due to its antioxidant activity. The aim of our study was to test the antioxidant potential of etazolate, in 158N oligodendroglial cell line subjected to *tert*-butyl hydroperoxide (*t*BHP) in vitro. We developed a model of *t*BHP-induced oxidative stress in 158N oligodendroglial cell line and used WST-1 assay and FACS analysis to evaluate cell viability and ROS production. *t*BHP caused cell death and ROS production in 158N oligodendroglial cells, and etazolate did not protect cells at the concentration range of 0.02 to 200 µM. At the neuroprotective and remyelinating concentration of 2 µM, etazolate did not reduce ROS production, while *N*-acetylcysteine (NAC), a potent antioxidant compound, counteracted *t*BHP effects. In conclusion, etazolate does not exert an antioxidant activity in vitro, and the previously reported antioxidant activity of etazolate in vivo might be related to an indirect effect.

KEYWORDS | *N*-Acetylcysteine; Antioxidants; *tert*-Butyl hydroperoxide; Etazolate; Flow cytometry; Oligodendrocytes; 158N oligodendroglial cell; Oxidative stress; Reactive oxygen species

ABBREVIATIONS | *t*BHP, *tert*-butyl hydroperoxide; CAT, catalase; CM-H_2DCFDA, carboxy-dichlorodihydrofluorescein diacetate; CNS, central nervous system; DCF, dichlorofluorescein; FACS, fluorescence-activated cell sorting; GSH, the reduced form of glutathione; GSSG, glutathione disulfide; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; PDE, phosphodiesterase; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBI, traumatic brain injury

CONTENTS

1. INTRODUCTION

The central nervous system (CNS) is the part of the nervous system that includes brain and spinal cord and it is composed of grey and white matter. The grey matter consists mainly of neuronal bodies and dendrites, while the white matter consists mainly of axons that are mostly myelinated. CNS in general has a relatively limited potential for anaerobic metabolism which makes it especially sensitive to hypoxia and oxidative stress [1]. In particular, it has been shown that the brain tissue is more sensitive to oxidative stress, in comparison to the spinal cord or the peripheral nervous system [2, 3]. The most important effect of reactive oxygen species (ROS) damage is reported to be in oligodendrocytes, which are the key cells for myelin synthesis in the CNS.

Oligodendrocytes are among the most sensitive cells to oxidative stress; they can be easily damaged by both hypoxia and ROS, especially during their terminal differentiation phase and while generating myelin sheaths. Oligodendrocyte precursor cells (OPCs) are more sensitive to oxidative stress than mature oligodendrocytes [4, 5]; the mature cells are better protected by a higher level of glutathione [6] and by the switch from an oxidative to a glycolytic metabolism [7]. Nonetheless, if antioxidant systems are not properly regulated, oligodendrocytes are left vulnerable to oxidative and free radical damage [8]. Overall, oxidative stress is a critical player in the pathophysiology of demyelination as reported in different studies [4, 9–15].

There are different in vitro models for reproducing an oxidative stress [16, 17]. A fundamental condition is that the redox balance has to be pushed in favor of the pro-oxidant systems. The ROS production can be stimulated by different methods, for example, exposing cells to γ-irradiation, to elevated oxygen tension (hyperoxia), to extracellular superoxide ($O_2^{\bullet-}$) and H_2O_2, or by using free radical-generating compound, such as paraquat, menadione, and *tert*-butyl hydroperoxide (*t*BHP). We are particularly interested in *t*BHP, a membrane-permeant oxidant compound that generates free radicals resulting in lipid peroxidation and depletion of intracellular glutathione [18, 19]. For this reason, *t*BHP has been extensively used as an oxidative stress-inducing agent to study the effect of free radicals in different cell types and regions of the brain [20–23]. Low doses of *t*BHP cause mostly apoptosis, while higher concentrations lead to both necrosis and apoptosis in the brain [24, 25]. In fact, French and collaborators reported an arrest of oligodendrocyte maturation at the low doses of *t*BHP [26]. These results suggest that oxidative stress directly interferes with the program of oligodendrocyte differentiation impairing myelin protection and repair after demyelination. Hence, therapeutic antioxidant strategies are required for white matter protection.

It has been shown that etazolate, a pyrazolopyridine derivative compound, promotes neuroprotection in vitro [27], and after experimental traumatic brain injury (TBI) [28, 29]. We have recently shown that etazolate enhances remyelination and oligodendrocyte maturation after experimental demyelination [30]. Interestingly, the same compound was described to exert an antioxidant effect in vivo in experimental models of depression attenuating the increase of oxidative markers in the brain [31, 32]. Chronic etazolate treatment also restored the al-

tered oxidative-nitrosative stress following experimental TBI [33] and in a model of Parkinson's disease [34]. We therefore hypothesized that the remyelinating effect of etazolate may be in part due to its antioxidant effect. However, it is not known yet if the observed beneficial effects of etazolate are due to a direct or indirect antioxidant effect.

In this study, we investigated the potential antioxidant effect of etazolate in an experimental model of oxidative stress in vitro. We developed a model of *t*BHP-induced oxidative stress using the 158N oligodendroglial cell line and we assessed cell viability and ROS production. The effects of etazolate, particularly at the neuroprotective and remyelinating concentration, and *N*-acetylcysteine (NAC), a potent antioxidant [35, 36], were analyzed on cell death and ROS production. While NAC reduced these two parameters, etazolate did not protect cells from oxidative stress suggesting that the antioxidant potential of etazolate described previously is not due to a direct antioxidant activity.

2. MATERIALS AND METHODS

2.1. 158N Oligodendroglial Cell Line Culture

The 158N oligodendroglial cell line, which preserves oligodendrocyte characteristics and expresses myelin proteins [37–39], was kindly provided by Dr. S.M. Ghandour (CNRS UMR 7357, Strasbourg, France). Cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% heat inactivated fetal bovine serum (HI-FBS, Gibco, NYC, NY, USA), L-glutamine 2 mM, sodium pyruvate 1 mM, penicillin (0.1 U/ml)/streptomycin (0.1 µg/ml) and 0.5 µg/ml fungizone. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. For fluorescence-activated cell sorting (FACS) analysis, cells were seeded at a density of 8×10^5 cells/well in medium with 5% FBS. The medium was changed after 48 h of culture with fresh medium containing 1% FBS. After 72 h of culture in total, cells were trypsinized and they underwent the FACS protocol. For cell viability (WST-1) analysis, cells were seeded in 96-well microplates at a density of 5×10^3 cells/well in medium with 5% FBS. After 72 h of culture, *t*BHP was added to the cells in medium with 1% FBS, and the plate was incubated for 2 h in a humidified atmosphere (37°C, 5% CO_2). To evaluate *t*BHP cytotoxicity, a concentration-effect study (10, 20, 50, and 100 µM) has been performed.

2.2. In Vitro Model of *t*BHP-Induced Oxidative Stress

The compound *t*BHP (458139, Sigma-Aldrich, St. Louis, MO, USA) was used as oxidative stress-inducing agent in 158N cell line [26]. For WST-1 analysis, 10, 20, 50, or 100 µM *t*BHP was added to cells and incubated for 2 h, while for FACS analysis, a higher concentration of *t*BHP (100 µM) was added to cells and incubated for 30 min in a humidified atmosphere (37°C, 5% CO_2). In order to validate the model, the cell viability and ROS production were evaluated following treatment with 5 mM NAC (A7250, Sigma-Aldrich) treatment. Once the model was validated, we tested the potential antioxidant effect of etazolate (0438, Tocris, Bristol, UK).

2.3. Pharmacological Treatments

2.3.1. NAC Treatment against tBHP-Induced Oxidative Stress

For evaluation of the effect of NAC on ROS production and cell viability by FACS analysis, cells were trypsinized and 5 mM NAC was added to cells in suspension in 0.1 M phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free) and incubated for 1 h. After 30 min following cell suspension, 100 µM *t*BHP was added in the same tube for further incubation (30 min at 37°C, 5% CO_2).

2.3.2. Etazolate Treatment against tBHP-Induced Oxidative Stress

For evaluation of the effect of etazolate on ROS production and cell viability by FACS analysis, 2 µM etazolate was tested using three different protocols: (a) protocol with 1 h etazolate incubation—cells were trypsinized and etazolate was added and incubated for 1 h. After 30 min, 100 µM *t*BHP was added and incubated for another 30 min; (b) protocol with 24 h of etazolate pre-treatment—etazolate pre-treatment started on adherent cells, 24 h before the trypsinization of cells; (c) protocol with 24 h of pre-treatment and 1 h of etazolate incubation—etazolate pre-treatment started on adherent cells, 24 h before the trypsinization of cells. Then, cells were tryp-

sinized and etazolate was added to cells in suspension in 0.1 M PBS (Ca^{2+} and Mg^{2+} free) for 1 h. After 30 min, 100 µM *t*BHP was added (for 30 min at 37°C, 5% CO_2).

2.4. Cell Viability Assay by WST-1

WST-1 assay (11644807001, Roche, Basel, Switzerland) is based on the enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases present in viable cells. After incubation, medium including *t*BHP was removed and cell viability evaluation was performed according to the manufacturer's protocol which was provided with the assay kit.

2.5. Flow Cytometric Analysis (FACS)

158N cells were trypsinized and collected for analysis with a BD FACSCanto™ II flow cytometer. For each analysis, 10,000 cells were counted. We considered only the population of cells devoid of aggregates and debris. In order to evaluate cell viability and ROS production, a double staining with propidium iodide (PI) and carboxy-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was performed [40]. Cells were incubated with 1.25 µM of CM-H_2DCFDA probe for 30 min at 37°C in the dark. Excess probe was washed out and cells were suspended in PBS (Ca^{2+} and Mg^{2+} free) and incubated for 10 min with 1 µg/mm of PI at room temperature. DCF fluorescence was recorded in the FL-1 (530/30 nm) channel together with PI fluorescence in FL-2 (585/42 nm).

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used for comparison among multiple groups when data followed a normal distribution. Otherwise, the Kruskal-Wallis with Dunn's post-hoc test was performed, which does not require the assumption of normal distributions. To test for normality, the d'Agostino–Pearson test was used. The criterion for statistical significance was $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed with the software GraphPad Prism6 (San Diego, CA, USA).

3. RESULTS

The aim of this study was to assess the potential antioxidant effect of etazolate on oligodendrocytes using the 158N cell line following an experimental model of oxidative stress in vitro. We first developed an in vitro model of *t*BHP-induced oxidative stress.

3.1. Response of 158N Cell Line to *t*BHP-Induced Oxidative Stress: Effect of Etazolate and NAC

We treated the 158N cell line with different concentrations of *t*BHP (10, 20, 50, and 100 µM) for 2 h. As shown in the **Figure 1A**, cells were susceptible to *t*BHP with a significant cytotoxicity observed at the highest concentration. Cell viability was in fact reduced by 60% at 100 µM *t*BHP. Etazolate (0.2 and 2 µM) did not show any beneficial effect on *t*BHP-induced cytotoxicity, whereas NAC treatment showed a marked increase in cell viability (**Figure 1**). Etazolate at concentrations up to 200 µM (concentration range of 0.02, 0.2, 2, 20, and 200 µM) did not protect against *t*BHP-induced cytotoxicity (data not shown).

3.2. NAC Treatment Counteracted *t*BHP-Induced Oxidative Stress in the 158N Cell Line

To test the effect of NAC and etazolate on ROS production, we used FACS analysis. The concentration of 100 µM *t*BHP was chosen to induce ROS production in 158N cells. This method requires the preparation of cells in suspension in contrary to the use of adherent cells to study *t*BHP cytotoxicity via WST-1 assay.

The ROS production was evaluated by assessing redox sensitive intracellular conversion of H_2DCFDA to DCF via FACS (**Figure 2**). *t*BHP induced a 20-fold increase in the DCF fluorescence compared to control group ($p < 0.0001$) and NAC was able to reduce *t*BHP-induced ROS production by decreasing the DCF fluorescence by 50% compared to *t*BHP control ($p < 0.01$) (**Figure 2c**). In our experimental oxidative stress condition, NAC treatment showed a protective effect indicating that our protocol and analysis are appropriate for studying the potential antioxidant effect of etazolate in the 158N cell line.

Cell death was also evaluated by the presence of PI^+ cells through FACS. In control, *t*BHP-treated, or

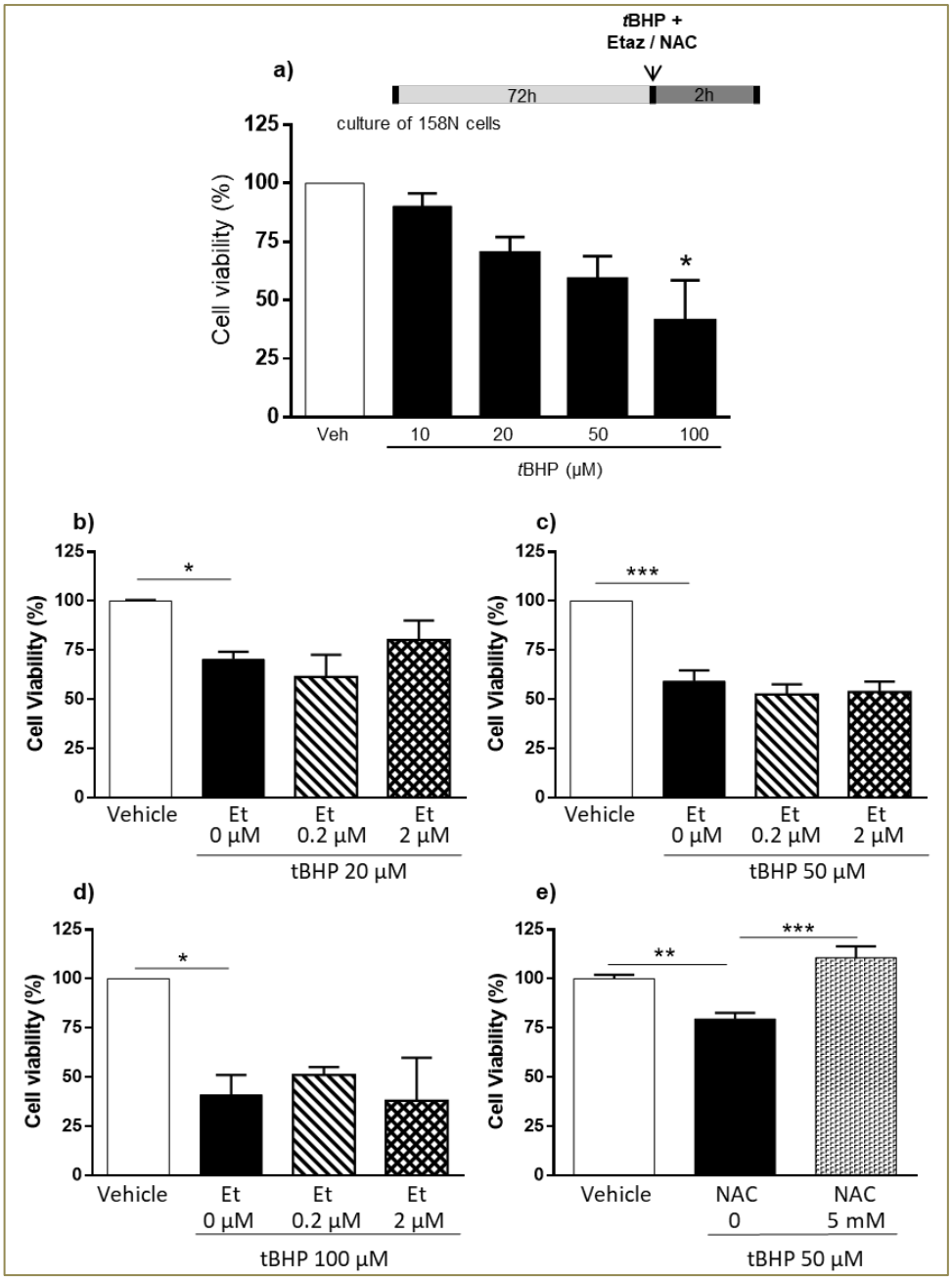

FIGURE 1. Validation of a model of oxidative stress induced by *t*BHP in the 158N cell line and effects of etazolate and NAC on cell toxicity induced by *t*BHP. Cells were incubated with different concentrations of *t*BHP (10, 20, 50, and 100 µM) for 2 h in the absence or presence of etazolate (0.2 and 2 µM) or NAC (5 mM). Then medium was removed, and cell viability was evaluated by WST-1 assay. Cells were treated with *t*BHP at concentrations of 10, 20, 50 and 100 µM (a). Etazolate was tested at different concentrations (0.2 and 2 µM) in cells treated with *t*BHP at 20 (b), 50 (c), and 100 µM (d). NAC was tested in cells treated with *t*BHP at 50 µM (e). Results are expressed as percentage of cell viability compared to that of the vehicle group. Values are the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

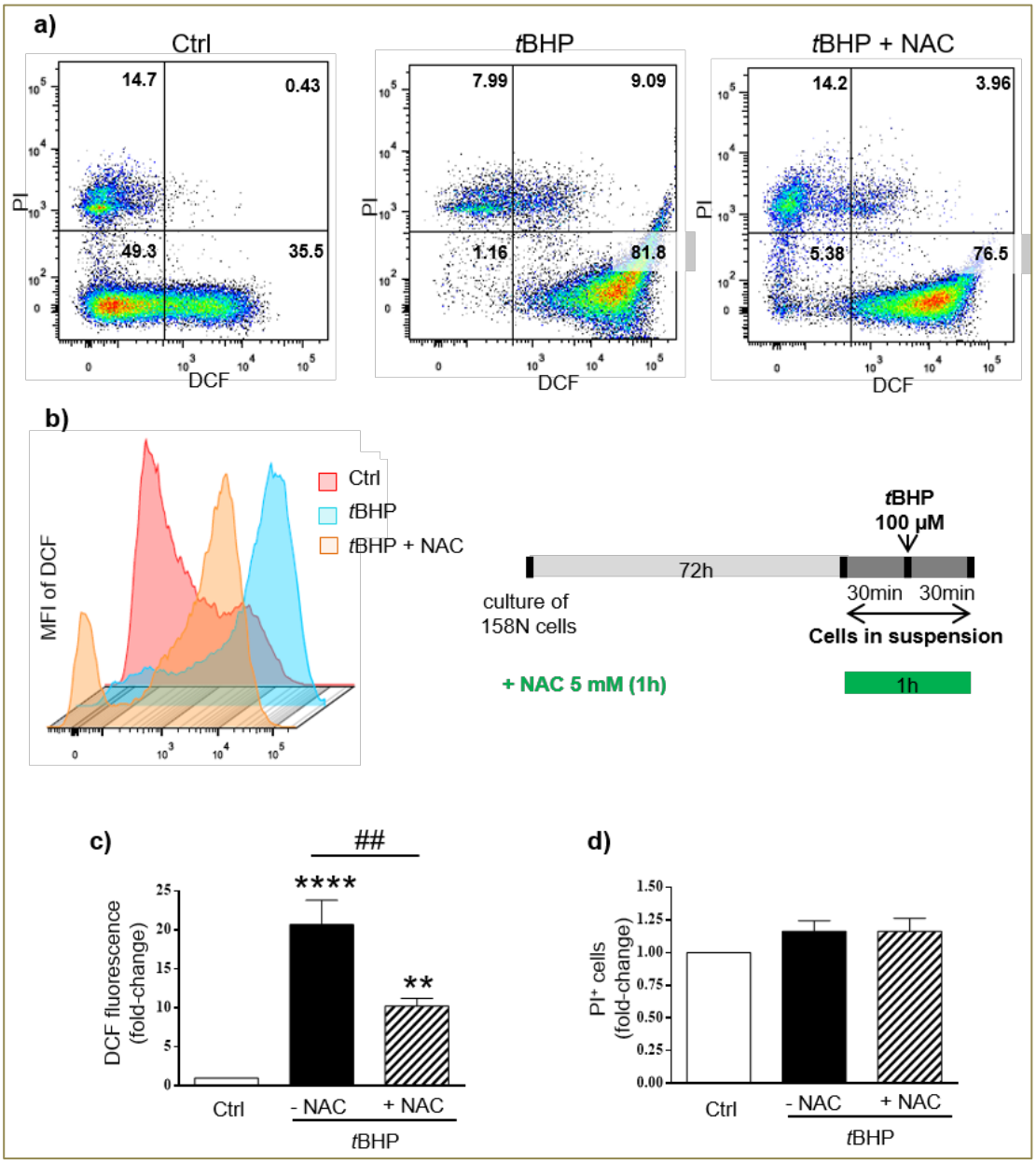

FIGURE 2. NAC decreased the level of intracellular ROS in the 158N cell line in a model of *t*BHP-induced oxidative stress. Treatment with NAC at 5 mM was performed for 1 h in cells in suspension. Thirty min prior to analysis, 100 µM *t*BHP was added. The intracellular ROS level was detected by DCFH-DA fluorescent probe. (a) Representative FACS plots of DCF-stained (FITC channel) and PI-stained (PE channel) cells were obtained from control (Ctrl), *t*BHP-treated (*t*BHP), and *t*BHP/NAC-treated (*t*BHP + NAC) cells. (b) Representative FACS analysis of the mean fluorescence intensity (MFI) of DCF measured on DCF^+/PI^- cells. (c) Quantitative analysis of the DCF fluorescence expressed as geometric mean. (d) Quantitative analysis of the percentage of PI^+ cells. All results are expressed as fold change compared to control group. Values are the mean ± SEM of five experiments. **, $p < 0.01$; ****, $p < 0.0001$ compared to control group; ##, $p < 0.01$ compared to *t*BHP group.

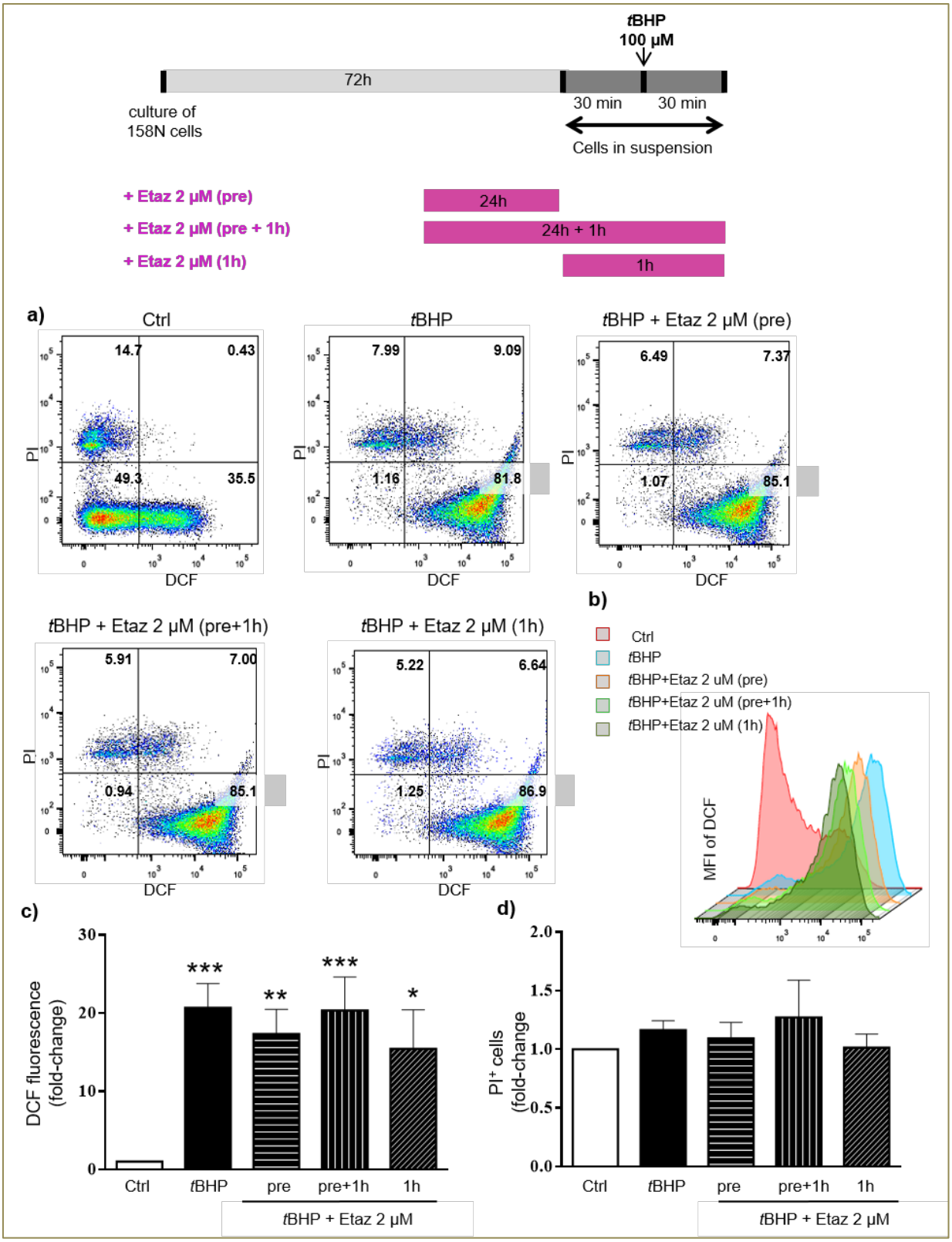

FIGURE 3. Effect of etazolate on the cell death and the level of intracellular ROS in the 158N cell line after *t*BHP-induced oxidative stress. Etazolate at 2 µM was tested using three different protocols: (i) etazolate in pre-treatment was added 24 h before the addition of *t*BHP (100 µM *t*BHP + 2 µM Etaz, pre); (ii) etazolate in pre-treatment was added 24 h before the addition of *t*BHP and during the 1 h *t*BHP incubation (100 µM *t*BHP + 2 µM Etaz, pre + 1 h); and (iii) etazolate was added during the 1 h *t*BHP incubation (100 µM *t*BHP + 2 µM Etaz, 1 h). The intracellular ROS level was detected by DCFH-DA fluorescent probe. (a) Representative FACS plots of DCF-stained (FITC channel) and PI-stained (PE channel) cells were obtained from control (Ctrl), *t*BHP-treated (*t*BHP) and *t*BHP /etazolate-treated cells with the three tested protocols. (b) Representative FACS analysis of the mean fluorescence intensity (MFI) of DCF measured on DCF^+/PI^- cells. (c) Quantitative analysis of the DCF fluorescence expressed as geometric mean. (d) Quantitative analysis of the percentage of PI^+ cells. Results are expressed as the fold change compared to that of the control group. Values are the mean ± SEM of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control.

*t*BHP/NAC-treated cells, a low number of PI^+ cells were present, excluding *t*BHP-induced cell death in these conditions analyzed by FACS (**Figure 2d**) probably due to the short period of *t*BHP incubation with 158N cells in suspension.

3.3. Etazolate Did Not Decrease the Intracellular ROS Production Induced by *t*BHP

We used FACS to assess ROS production after *t*BHP-induced oxidative stress in order to better analyse the potential antioxidant effect of etazolate (**Figure 3**). The concentration of 2 µM was chosen, given that etazolate at this concentration is able to exert neuroprotective and remyelinating effect [27, 30]. Treatment with *t*BHP induced a 20-fold increase in the DCF fluorescence compared to control group ($p < 0.001$) and etazolate was devoid of any effect neither in pre-treatment protocol nor without pre-treatment protocol (**Figure 3c**).

Cell death was also assessed by the presence of PI^+ cells. In control, *t*BHP-treated, or *t*BHP/etazolate-treated cells, the number of PI^+ cells remained the same (**Figure 3d**), thus excluding cell death in all conditions analyzed by FACS, as already observed above (**Figure 2d**). In addition, etazolate by itself did not exert a significant effect on cell viability and ROS production in both protocols without *t*BHP (**Figure 4**).

4. DISCUSSION

Oxidative stress is detrimental for myelin protection and repair after demyelination, requiring the necessity of therapeutic antioxidant strategies for white matter protection. The aim of this study was to test the antioxidant effect of etazolate on isolated oligodendrocytes, and we clearly showed that etazolate was devoid of any effect in a model of *t*BHP-induced oxidative stress in vitro in spite of promising antioxidant effects described in vivo.

Etazolate, a pyrazolopyridine derivative compound, is known to have multiple mechanisms of action, including being (i) a positive allosteric modulator of the $GABA_A$ receptor, (ii) an adenosine receptor antagonist, (iii) a phosphodiesterase (PDE) inhibitor selective for the PDE4 isoform, and (iv) an α-secretase activator [27, 41–43]. Etazolate was previously shown, in part by our team, to exert neuroprotective and remyelinating effects and to be anti-inflammatory and antioxidant in vivo [28–34, 44–47]. In particular, Jindal and colleagues demonstrated that etazolate has an antioxidant effect in an in vivo model of chronic unpredictable stress depression in mice attenuating the increase of oxidative markers in the brain, particularly, the lipid peroxidation and the nitrosative level. In the same model, etazolate treatment normalized the decreased level of the reduced form of glutathione (GSH) and the decreased activity of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), in the brain of depressed animals [31]. The etazolate antioxidant effect was also described in a model of olfactory bulbectomy-induced depression in mice [32] and in an impact accelerated TBI model [33]. A recent study also showed that etazolate treatment normalized the ratio of GSH to glutathione disulfide (GSSG) and the activity of glutathione peroxidase (GPx) in the hippocampus, and it could prevent motor dysfunction,

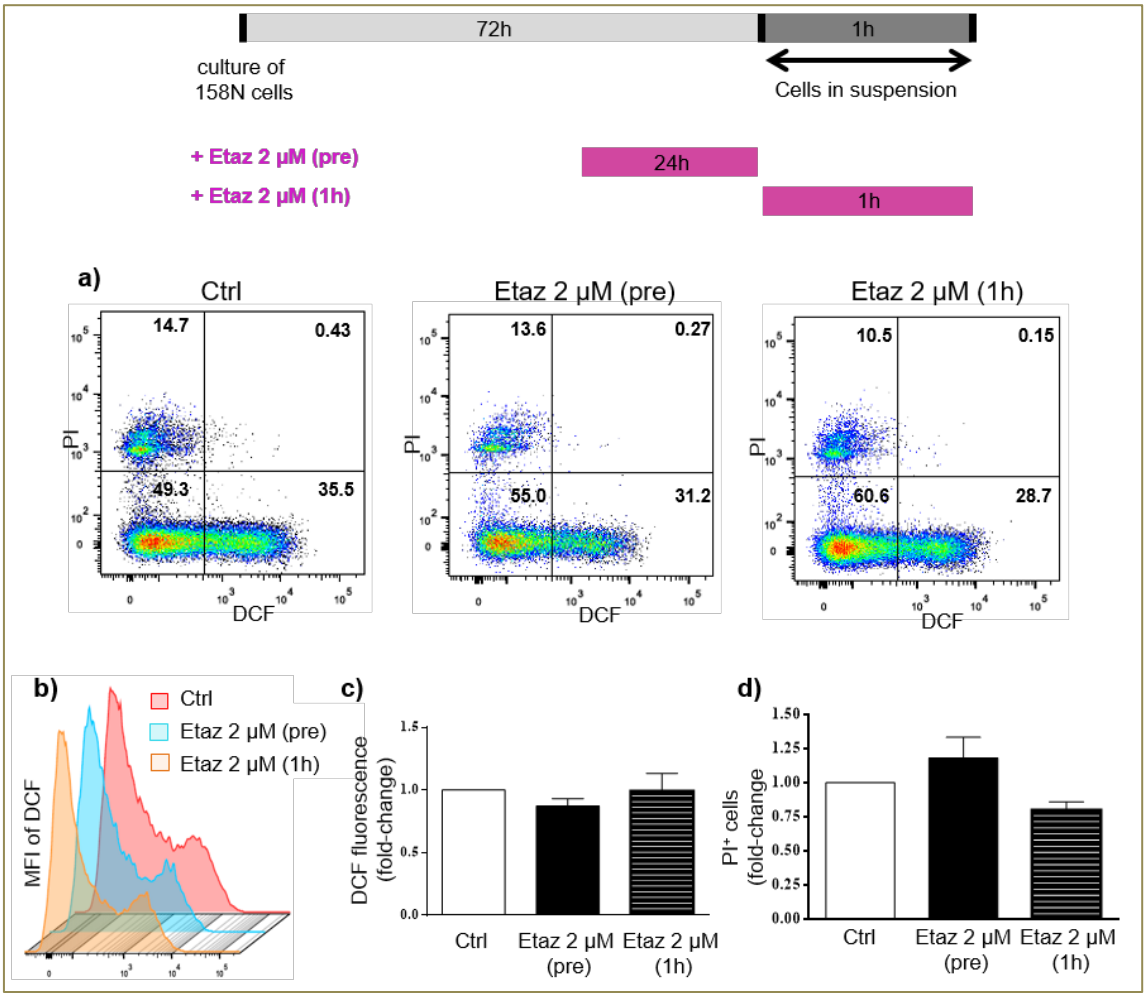

FIGURE 4. Effect of etazolate on the level of intracellular ROS in the 158N cell line without *t*BHP treatment. Etazolate at 2 µM was tested using two different protocols: (i) etazolate in pre-treatment was added 24 h before the trypsination of cells (Etaz 2 µM, pre) and (ii) etazolate was added on cells in suspension for 1 h before the FACS analysis (Etaz 2 µM, 1 h). The intracellular ROS level was detected by DCFH-DA fluorescent probe. (a) Representative FACS plots of DCF-stained (FITC channel) and PI-stained (PE channel) cells were obtained from control (Ctrl) and etazolate-treated cells with the two tested protocols. (b) Representative FACS analysis of the mean fluorescence intensity (MFI) of DCF measured on DCF^+/PI^- cells. (c) Quantitative analysis of the DCF fluorescence expressed as geometric mean. (d) Quantitative analysis of the percentage of PI^+ cells. Results are expressed as the fold change compared to that of the control group. Values are the mean ± SEM of five experiments.

depressive-like behavior, and learning and memory deficits in a rat model of Parkinson's disease [34]. The same group also demonstrated that etazolate can prevent oxidative stress and histones acetylation in a rat model of post-traumatic stress disorder [48].

Despite the aforementioned promising results in vivo, the antioxidant effect of etazolate has never been tested directly on oligodendrocytes, which are cells particularly vulnerable to oxidative stress. In fact, oligodendrocytes are one of the cell types with

the highest metabolism in the brain, since proper myelination requires large amounts of oxygen and adenosine triphosphate (ATP). Myelination requires that oligodendrocytes first construct and then maintain an extensive plasma membrane, and for this, a vast supply of both precursor molecules and oxidative substrates is required [5]. A high metabolism, with high-energy consumption, means also large amounts of toxic by-products, such as ROS. Oligodendrocytes are also highly sensitive to oxidative stress because of their lipid-rich membranes where unsaturated long-chain fatty acids are unusually concentrated [26]. In addition to their relatively poor antioxidant production, oligodendrocytes are even more susceptible to oxidative stress due to the need of iron as a cofactor for myelin production [49, 50]. They are the most iron-loaded cells of the CNS and the intracellular accumulation of iron can stimulate the conversion of hydrogen peroxide to hydroxyl radicals—the most reactive free radicals [51, 52]. Therefore, oligodendrocytes can be easily damaged and they are particularly sensitive to both hypoxia and oxidative stress, especially during their terminal differentiation phase and while generating myelin sheaths. During the process of myelin production and remyelination, the peroxisomal fraction of oligodendrocytes increases the volume and density, in accordance with metabolic requirements, thus representing an important independent ROS source [53–55].

In order to evaluate the antioxidant potential of etazolate, we first developed an in vitro model of oxidative stress induced by *t*BHP in the 158N oligodendroglial cell line. *t*BHP is a membrane-permeant oxidant compound extensively used as an oxidative stress-inducing agent in different cell types and tissues [20–23, 25, 26, 56]. As expected, *t*BHP was able to increase intracellular ROS (FACS analysis) and induce cell death (WST-1 assay) in the 158N cell line in which NAC treatment was able to reverse both ROS production and cytotoxicity. NAC is known to be a powerful antioxidant [57, 58]. It acts as a scavenger of free radicals and it is considered a treatment option for different disorders resulting from generation of free oxygen radicals [57]. It showed positive effects in in vivo models of diseases characterized by white matter alterations, such as experimental autoimmune encephalomyelitis [59], ischemia-induced neonatal white matter injury [60], and traumatic brain injury [61].

Our results showed that etazolate was devoid of any antioxidant activity in our model. In fact, etazolate was not able to reduce ROS production at 2 μM, a concentration able to exert neuroprotective and remyelinating effects [27, 30]. Our data clearly show the discrepancy between our in vitro results and in vivo reports. It remained to investigate if the antioxidant effect of etazolate observed in vivo (0.5 mg/kg/day and 1 mg/kg/day) [31–34] is a direct or an indirect effect through etazolate's anti-inflammatory activity via PDE4 inhibition. In fact, a recent study showed that another PDE4 inhibitor, FCPR16, at the concentrations of 12.5–50 μM protects SH-SY5Y cells against oxidative stress reducing ROS production in vitro [62].

In our in vitro model, etazolate was tested only on isolated oligodendrocytes excluding the in vivo systemic response. Moreover, the potential antioxidant activity of etazolate has been tested in a model where *t*BHP induces a remarkable production of ROS (20-fold increase). It is possible that ROS production in this model is too high to be reversed by etazolate. Nevertheless, NAC is able to counteract *t*BHP-induced ROS production in our model. As a future direction, it could be interesting to test the effect of etazolate on different oxidative stress parameters, including the activity of the antioxidant enzymes SOD and CAT and the levels of intracellular GSH/GSSG.

5. CONCLUSION

In our model of *t*BHP-induced oxidative stress using the 158N oligodendroglial cell line, etazolate does not exert an antioxidant activity in vitro. The previously reported antioxidant activity of etazolate in vivo might be related to an indirect effect.

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