TPNA10168, an Nrf-2 activator, stimulates glutathione production by inducing system Xc-, and g-glutamate cysteine ligase subunits in cultured mouse astrocytes.

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Abstract

Nuclear factor-erythroid 2-related factor 2 (Nrf-2) is a transcription factor involved in cellular defense mechanisms against oxidative stress and mediates the expression of various antioxidant-related molecules. We previously showed that TPNA10168, a novel Nrf-2 activator, has neuroprotective effects. To elucidate the mechanisms underlying the cytoprotective action of TPNA10168, its effects on the expression of glutathione biosynthesis-related molecules in cultured mouse astrocytes were examined. Cultured astrocytes were prepared from the cerebra of 1-day-old C57BL/6N mice. mRNA and protein levels were measured by quantitative PCR and immunoblotting, respectively. Treatment with TPNA10168 (30 μM) increased the astrocytic Nrf-2 protein level. Nuclear translocation of Nrf-2 and its binding to the Nrf-2 recognition DNA fragment were stimulated by TPNA10168. The mRNA and protein levels of system Xc^-, a cystine/glutamate exchange transporter, in cultured astrocytes were increased by TPNA10168. TPNA10168 also increased the expression of γ-glutamate cysteine ligase subunits (γ-GCLm and γ-GCLc). Accompanied by the increased expression of system Xc^-, γ-GCLm, and γ-GCLc, the production of astrocytic glutathione was increased by TPNA10168. The effects of TPNA10168 on the expression of system Xc^-, γ-GCLm, and γ-GCLc were reduced by the Nrf-2 inhibitor ML385 (10 μM). Rotenone (2.5 nM) induced cell death in cultured astrocytes; however, this effect was alleviated by TPNA10168. The results suggest that Nrf-2-mediated astrocytic glutathione biosynthesis is one of the mechanisms underlying the cytoprotective action of TPNA10168.

Introduction

In brain disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), and stroke, endogenous oxidants, including reactive oxygen species (ROS), are excessively generated and cause oxidative stress in nerve tissues. Because oxidative stress largely impairs the functions of neurons and glial cells, the alleviation of redox imbalance is thought to have beneficial effects against brain disorders (Gan et al. 2014; Baxter and Hardingham 2016). The Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor-erythroid 2-related factor 2 (Nrf-2) stress response pathway is a cellular defense mechanism against oxidative stress (Niture et al. 2014; Bellezza et al. 2018). Nrf-2 is a transcription factor that binds to the antioxidant response element (ARE) in the promoter regions of target genes to stimulate their transcription. Under non-stressed conditions, the transcriptional activity of Nrf-2 is suppressed by its association with the inhibitory regulator Keap1, which induces the ubiquitination-dependent degradation of Nrf-2. However, when cells are exposed to oxidants, modification of the thiol residues of the Keap1 protein dissociates Nrf-2 from Keap1 and stimulates the nuclear translocation of Nrf-2 (Bellezza et al. 2018). The activation of Nrf-2 induces the transcription of various molecules involved in cellular defense mechanisms against oxidative stress. As Nrf-2-mediated gene transcription serves as a major defense mechanism against oxidative stress-induced nerve damage, the stimulation of Nrf-2 activity is considered as a promising therapeutic approach for various brain disorders (Gan et al. 2014; Baxter and Hardingham 2016). Several Nrf-2 activators have been shown to exert cytoprotective effects in animal models of brain disorders (Kraft et al. 2004; Krämer et al. 2017; Scannevin et al. 2012; Zhang et al. 2020). In our previous

study, the Nrf-2/ARE luciferase reporter assay identified a novel Nrf-2 activator, 1-chloro-3-methanesulfinyl-6,7-dihydro-5H-2-benzothiophen-4-one (TPNA10168), in a chemical library (Izumi et al. 2018). TPNA10168 was found to protect PC12 cells and cultured mesencephalic cells from 6-hydroxydopamine-induced toxicity (Izumi et al. 2018). In a mouse model of PD, the administration of TPNA10168 reduced the degeneration of dopaminergic neurons in the substantia nigra and behavioral impairment (Inose et al. 2020). However, the mechanisms underlying the cytoprotective action of TPNA10168 have not been fully elucidated.

Astrocytes are glial cells responsible for maintaining the brain environment and are necessary for the normal function of the nerves. In defense mechanisms by brain Nrf-2, its activity has been observed to be higher in astrocytes than in neurons (Ahlgren-Beckendorf et al. 1999; Shih et al. 2003), suggesting the crucial role of astrocytes in the brain oxidative state. Glutathione is an endogenous antioxidant produced by astrocytes in the brain (Dwivedi et al. 2020; Gegg et al. 2005; Suzuki et al. 2013). Decreased brain glutathione levels have been observed in patients with neurodegenerative diseases (Sian et al. 1994; Mandal et al. 2015) and animal models of brain disorders (Anderson and Sims 2002; Javed et al. 2012; Verma and Nehru 2009). In these animal models, treatment to enhance the production of brain glutathione was found to ameliorate nerve injury (Reed et al. 2009; Anderson et al. 2004; Tanaka et al. 2002; Kahl et al. 2018). These observations suggest the cytoprotective action of increased glutathione in the brain (Dwivedi et al. 2020). In astrocytes, extracellular cystine is taken up by a cystine/glutamate exchange transporter (system Xc^-), which is a rate-limiting step in glutathione biosynthesis. After being reduced to L-cysteine, L-cysteine and L-glutamate are converted to γ-glutamyl-cysteine, a precursor of glutathione, by γ-glutamate cysteine ligase (γ-GCL). Gene analysis of system Xc^- and γGCL subunits has identified the ARE in their gene promoter regions, and their transcription can be stimulated by the activation of Nrf-2 (Chorley et al. 2012; Sasaki et al. 2002; Hirotsu et al. 2012). To understand the mechanisms underlying the cytoprotective action of TPNA10168, we examined the effects of TPNA10168 on the expression of glutathione biosynthesis-related molecules in cultured mouse astrocytes.

Materials And Methods

Isolation and culture of mouse astrocytes:

The experimental protocols followed the ARRIVE guidelines and the National Institutes of Health guidelines for the Care and Use of Animals (NIH Publication No. 8023, revised 1978) and were approved by the Animal Experiment Committee of Kobe Pharmaceutical University (approval number: 2019-17). Astrocytes were prepared from the cerebra of 1-day-old C57BL/6N mice, as described previously (Michinaga et al. 2020). As it was difficult to distinguish between males and females among the 1-day-old mice, a mixed culture of male- and female-derived astrocytes was used in the present study. Isolated cells were seeded at a density of 1×10^4 cells/cm^2 in 75 cm^2 culture flasks and cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. To remove oligodendrocyte progenitors and microglia from the protoplasmic cell layer, the culture flasks were shaken at 250 rpm

overnight after seeding for 10-14 days. The cell monolayer was then trypsinized and seeded on f15 mm glass coverslips for immunocytochemistry or 6-well culture plates for other experiments. At this stage, approximately 95% of the cells showed immunoreactivity for glial fibrillary acidic protein (GFAP). Cultured astrocytes were treated with TPNA10168 (Maybridge, Loughborough, UK; Fig. 1a) and other reagents in serum-free MEM at 37 °C.

Measurement of mRNA levels:

Total RNA was extracted from cultured astrocytes using a total RNA extraction kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). Reverse transcription and quantitative PCR were performed as previously described (Koyama et al. 2019). The following primer pairs were used:

system Xc^-,

5′-ACCATTGAAATACACATACACCCT-3′ and 5′-GTTGCAAACACCGAGCTTCC-3′;

g-GCLc, 5′-CATGAAAGTGGCCCAGAAGC-3′ and 5′-CCGCCTTTGCAGATGTCTTT-3′;

g-GCLm, 5′-CGAGGAGCTTCGGGACTGTA-3′ and 5′-CCCTGACTAAATCGGGGCTG-3′;

Nrf-2, 5′-CTCCTAGTTCTCGCTGCTCG-3′ and 5′-TGGCAACTCCAAGTCCATCA-3′;

glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

5′-CTCATGACCACAGTCCATGC-3′ and 5′-TACATTGGGGGTAGGAACAC-3′.

Serial dilutions of each amplicon were amplified in the same manner as the standard for the copy number of PCR products. The amount of mRNA was calculated as the copy number of each reverse-transcription product (equivalent to 1 μg of total RNA) and normalized to the value of GAPDH.

Immunoblotting:

Cell lysate preparation and SDS-PAGE were performed as previously described (Koyama et al. 2019). To detect Nrf-2, system Xc^-, g-GCLc, and g-GCLm proteins, the membranes were first probed with an anti-Nrf-2 rabbit antibody (1:2,000 dilution, D1Z9C cat# 12721; Cell Signaling Technology, Danvers, MA, USA), anti-system Xc^-/SLC7A11 rabbit antibody (1:2,000 dilution, cat# 98051; Cell Signaling Technology), anti-g-GCLc mouse antibody (1:2,000 dilution, clone H-5, cat# sc-390811; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), or anti-g-GCLm mouse antibody (1:2,000 dilution, clone E-4, cat# sc-55586; Santa Cruz Biotechnology), followed by incubation with peroxidase-conjugated secondary antibodies.
Labeled protein bands were detected using an enhanced chemiluminescence kit (Chemi-Lumi One L; Nacalai Tesque, Kyoto, Japan). The chemiluminescence of each band was observed using a Lumino Image Analyzer (ImageQuant LAS4000, GE Healthcare, Chicago, IL, USA), and the density of the target protein bands was quantified using ImageJ 1.45 software (US National Institutes of Health, Bethesda, MD, USA). The membranes were then stripped and re-probed with a mouse anti-β-actin primary antibody

(1:10,000 dilution, cat# MAB1501; Millipore, Burlington, MA, USA). The results are presented as the ratio of the level of β-actin protein. Whole uncropped immunoblot images obtained using these antibodies are shown in Supplementary Fig. 1.

Immunocytochemical examination:

After treatment with TPNA10168, cultured astrocytes were fixed with 3% paraformaldehyde and incubated with a rabbit antibody against Nrf-2 (1:400 dilution, D1Z9C cat# 12721; Cell Signaling Technology). To identify astrocytes, an anti-glial fibrillary acidic protein (GFAP) mouse monoclonal antibody (1:400 dilution, G-A-5; Sigma, St. Louis, MO, USA) was used. After incubation with primary antibodies, the cells were labeled with FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG. After incubation with secondary antibodies, 5 μg/mL Hoechst 33342 (cat# H341; Dojindo, Kumamoto, Japan) was used to label the nucleus. Labeled cells were observed under an epifluorescence microscope.

DNA binding activity of Nrf-2:

Nuclear extraction was performed using a nuclear extraction kit (cat# 40010; Active Motif, Carlsbad, CA, USA). After treatment with TNPA10168, the nuclear extract was prepared from cultured astrocytes according to the manufacturer's protocol. The binding of Nrf-2 protein to the DNA consensus sequence was measured using an ELISA-based assay kit (TransAM® Nrf2 Transcription Factor Assay Kit, cat# 50296; Active Motif); 2 μg of protein from astrocytic nuclear extracts was used for each treatment. The DNA binding assay was performed according to the manufacturer's protocol.

Measurement of intracellular total glutathione:

After treatment with TPNA10168 and/or rotenone, cultured astrocytes were collected in a 1.5 mL centrifuge tube with 300 μL of phosphate-buffered saline and centrifuged. The resulting cell pellets were lysed in 80 μL of 10 mM HCl containing 5-sulfosalicylic acid and centrifuged at 8000 × *g* for 10 min. The total glutathione content (sum of the reduced and oxidized forms) in the supernatant was measured using a glutathione quantification kit (Cat # T419; Dojindo). The resulting pellet was solubilized with 0.1 M NaOH, and the protein content was measured. The results are presented as glutathione content per mg protein.

Cell viability:

After treatment with TPNA10168 and/or rotenone for 48 h, astrocytes were incubated with 1 μM calcein-AM and 1 μM ethidium homodimer III (EthD-III) (Live/Dead Cell Staining Kit II, cat# D25497; Takara Bio. Inc., Kusatsu, Japan) in serum-free MEM at room temperature for 30 min and observed under an epifluorescence microscope. Cells exhibiting calcein fluorescence in the cytoplasm and EtD-III fluorescence in the nucleus were defined as living and dead cells, respectively. In each condition, more than 400 cells were observed, and the percentage of dead cells was indicated by the ratio of the total cells observed (sum of living and dead cells).

Statistical analysis:

All statistical analyses were performed using Bell Curve for Excel (version 2.20; Social Survey Research Information Co., Ltd, Tokyo, Japan). Experimental data are presented as the mean ± standard error of the mean (SEM). The results were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc test. Statistical significance was set at $P < 0.05$.

Results

Activation of Nrf-2/ARE signaling in cultured mouse astrocytes by TPNA10168:

Our previous study using a reporter gene assay in PC12 cells showed that TPNA10168 could stimulate the transcription of a reporter gene with an Nrf-2 recognition sequence (ARE sequence) (Izumi et al. 2018). Therefore, in this study, we first investigated whether TPNA10168 activates Nrf-2 in cultured mouse astrocytes. Treatment with TPNA10168 (30 μM) increased the Nrf-2 protein level in cultured mouse astrocytes at 1-3 h (Fig. 1b). However, TPNA10168 (30 μM) did not affect the Nrf-2 mRNA level in cultured astrocytes (Fig. 1c). Immunocytochemical examination did not detect obvious Nrf-2 immunoreactivity in the nuclei of untreated cultured astrocytes (Fig. 2). Treatment with TPNA10168 (30 μM) for 3 h resulted in the accumulation of Nrf-2 protein in the nuclei of cultured astrocytes (Fig. 2). The DNA binding activity of the astrocytic nuclear extract to the Nrf-2 recognition sequence was increased by treatment with TPNA10168 for 3 and 6 h (Fig. 1d).

Increased expression of system Xc^- and g-GCL subunits with TPNA10168 treatment:

The effects of astrocytic Nrf-2 activation by TPNA10168 were investigated by focusing on glutathione biosynthesis-related molecules. The treatment of mouse cerebral astrocytes with TPNA10168 (30 μM) increased system Xc^- mRNA expression in a time-dependent manner (Fig. 3a), showing an approximately 4-fold increase after 6-12 h. The mRNA levels of g-GCLm and g-GCLc were also increased following treatment with TPNA10168. The effects of TPNA10168 on the expression of these glutathione synthesis-related genes were concentration-dependent; the effects on system Xc^- and GCL subunit mRNA levels were significant at 10-30 μM and 1 μM or higher, respectively (Fig. 3b). The increase in system Xc^-, g-GCLm, and g-GCLc mRNA expression induced by TPNA10168 was reduced in the presence of 10 μM ML385, an Nrf-2 inhibitor (Fig. 3c). Accompanied by the increase in mRNA expression, the protein levels of the astrocytic system Xc^-, g-GCLm, and g-GCLc were increased by treatment with 30 μM TPNA10168 (Fig. 4).

Effects of TPNA10168 on glutathione content and rotenone-induced astrocytic cell death:

Glutathione protects astrocytes from oxidative stress (He et al. 2015; Kim et al. 2003). Therefore, the effects of the increased expression of glutathione biosynthesis-related molecules induced by TNPA10168 on astrocytic viability were examined. The total glutathione content in cultured astrocytes was increased by approximately 2-fold after treatment with 30 μM TPNA10168 for 24-48 h (Fig. 5a). Rotenone, an

inhibitor of the mitochondrial electron transport chain, induces ROS accumulation and causes cytotoxicity in cultured astrocytes (Rathinam et al. 2012; Cabezas et al. 2012). Treatment with rotenone (2.5 nM) for 48 h increased the number of cultured EtD-III-positive mouse astrocytes. (Fig. 5b). Rotenone-induced astrocytic death was decreased in the presence of 30 μM TPNA10168. Treatment with 2.5 nM rotenone for 24 h reduced the total glutathione content in cultured astrocytes (Fig. 5c); however, the difference was not statistically significant. The rotenone-induced reduction in astrocytic glutathione was alleviated by treatment with 30 μM TPNA10168.

Discussion

Nrf-2 is a transcription factor activated under oxidative conditions, which triggers cellular defense mechanisms through the transcription of various antioxidant genes. As various brain disorders, including neurodegenerative diseases, are exacerbated by oxidative stress, Nrf-2 may be a therapeutic target for the treatment of these brain disorders (Gan et al. 2014; Baxter and Hardingham 2016). Therefore, there have been attempts to identify novel compounds that activate Nrf-2 (Kraft et al. 2004; Krämer et al. 2017; Scannevin et al. 2012; Zhang et al. 2020). Our previous study showed that TPNA10168 activated Nrf-2 as potently as sulforaphane, a well-known activator, in a reporter gene assay in PC12 cells and was less cytotoxic than sulforaphane (Izumi et al. 2018). In addition, TPNA10168 had neuroprotective effects in cultured neurons and a mouse model of PD (Izumi et al. 2018; Inose et al. 2020), suggesting its effectiveness as a neuroprotective drug. In this study, TPNA10168 increased Nrf-2 protein expression in cultured mouse astrocytes (Fig. 1b). Accompanied by the increase in the Nrf-2 protein level induced by TPNA10168, nuclear translocation of Nrf-2 (Fig. 2) and its binding to the Nfr-2 recognition DNA sequence were stimulated (Fig. 1d). These observations indicated that TPNA10168 enhanced the transcriptional activity of astrocytic Nrf-2. As the increase in the Nrf-2 protein level was not accompanied by an increase in the mRNA level (Fig. 1c), Nrf-2 mRNA upregulation may not be part of the mechanisms underlying TPNA10168 action. The transcriptional activity of Nrf-2 is regulated by a post-translational mechanism mediated by protein degradation (Niture et al. 2014; Bellezza et al. 2018). In cells not under oxidative stress conditions, Nrf-2 protein is associated with its inhibitory regulator Keap1 in the cytoplasm, and its association with Keap1 leads to ubiquitin-dependent Nrf-2 degradation. Under oxidative conditions, the association with Keap1 is weakened by the modification of the binding domain, leading to the accumulation of Nrf-2 protein and the resulting stimulation of transcriptional activity. Therefore, it is possible that TPNA10168 enhances astrocytic Nrf-2 activity by affecting protein interactions with Keap1.

Glutathione, an endogenous antioxidant, is primarily produced by astrocytes in the brain (Dwivedi et al. 2020; Suzuki et al. 2013). In this study, the expression of astrocytic system Xc^-, γ-GCLm, and γ-GCLc, which are involved in glutathione biosynthesis, was increased by treatment with TPNA10168 (Figs. 3a, 3b, and 4). TPNA10168 also increased the production of astrocyte glutathione (Fig. 5a). The presence of the ARE has been observed in the gene promoter regions of system Xc^-, γ-GCLm, and γ-GCLc (Chorley et al. 2012; Sasaki et al. 2002; Hirotsu et al. 2012). Consistent with this finding, the effects of TPNA10168 on system Xc^-, γ-GCLm, and γ-GCLc mRNA expression were attenuated by the Nrf-2 inhibitor ML385

(Fig. 3c). These observations indicated that TPNA10168 increased astrocyte glutathione production by inducing glutathione synthesis-related molecules through the activation of Nrf-2. Analysis of the changes in glutathione content revealed that TPNA10168 reduced rotenone-induced astrocytic death, accompanied by an increase in glutathione content (Fig. 5b and 5c). Glutathione eliminates ROS and exerts cytoprotective effects on astrocytes (He et al. 2015; Kim et al. 2003). Therefore, an increase in glutathione production may be involved in the cytoprotective effects of TPNA10168 on cultured astrocytes.

In several types of brain disorders, including PD, AD, stroke, and traumatic brain injury, the level of glutathione in the brain is decreased (Sian et al. 1994; Mandal et al. 2015; Anderson and Sims 2002; Javed et al. 2012; Verma and Nehru 2009). The reduction of brain glutathione can impair the elimination of oxidants, including ROS, resulting in the exacerbation of nerve injury. In agreement with these observations, brain glutathione elevation has been shown to improve neurological disorders (Reed et al. 2009; Anderson et al. 2004; Tanaka et al. 2002; Kahl et al. 2018). In the brain, the glutathione-producing ability of astrocytes is higher than that of neuronal cells, and neuronal cells are protected from oxidative stress by the glutathione released from astrocytes (Dwivedi et al. 2020; Gegg et al. 2005; Chen et al. 2001; Fernandez-Fernandez et al. 2012). Therefore, the enhancement of astrocytic glutathione synthesis may be a promising treatment approach that protects astrocytes and neurons from oxidative damage in brain diseases. Nrf-2 activators, including TPNA10168, have been reported to ameliorate nerve injury in animal models of brain disorders (Kraft et al. 2004; Krämer et al. 2017; Scannevin et al. 2012; Zhang et al. 2020; Inose et al. 2020). In the brain, the Nrf-2 activity of astrocytes is higher than that of nerve cells (Ahlgren-Beckendorf et al. 1999; Shih et al. 2003), indicating that astrocytes are a possible target of Nrf-2 activators. Therefore, enhanced astrocytic glutathione synthesis may explain the neuroprotective action of TPNA10168.

Declarations

Author Contribution

YK, TK, and YI conceived and designed the study. NT, HN, and KI conducted the experiments. NT and HN analyzed the data. YK wrote the manuscript. All authors have read and approved the manuscript.

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Data Availability

Data supporting the findings of this study are provided in the Supplementary Information.

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Figures

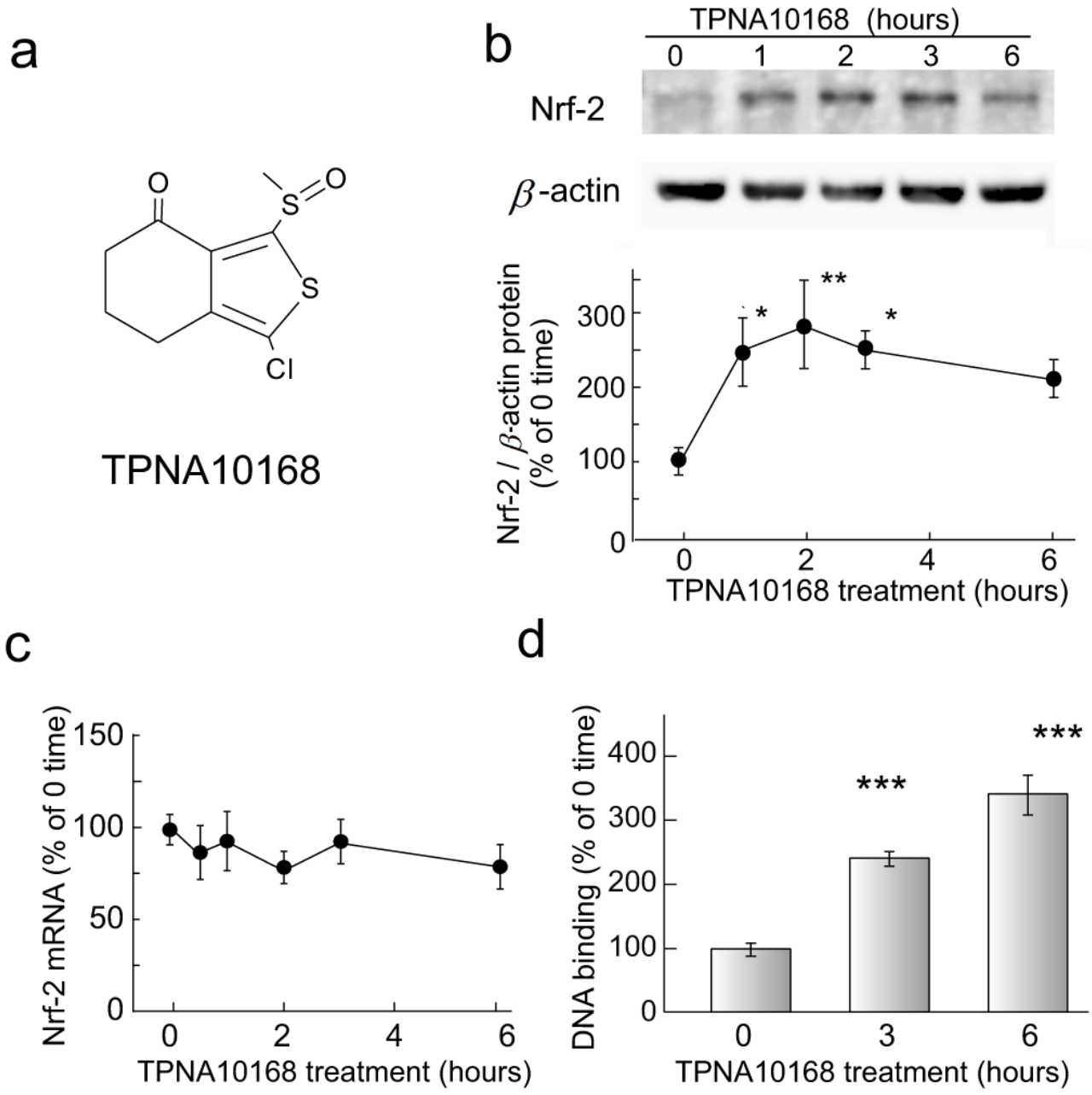

Figure 1

(a) Chemical structure of TPNA10168 (1-chloro-3-methanesulfinyl-6,7-dihydro-5H-2-benzothiophen-4-one). (b) Effects of TPNA10168 on the expression of the astrocytic nuclear factor-erythroid 2-related factor 2 (Nrf-2) protein. Cultured astrocytes were treated with 30 μM TPNA10168 for the indicated time. The expression of Nrf-2 protein was normalized to that of b-actin. Data are expressed as the mean ± standard error (SEM) of 4 different cell lysate preparations. *P < 0.05, **P < 0.01 vs. 0 h; one-way analysis of variance (ANOVA) followed by Dunnett's test. **(c) Effects of TPNA10168 on Nrf-2 mRNA expression in cultured mouse astrocytes.** Cultured astrocytes were treated with 30 μM TPNA10168 for the times indicated. The expression of Nrf-2 mRNA was normalized to that of GAPDH. Results are presented as the mean ± SEM of 8 different RNA preparations. **(d) Binding activity to the Nrf-2 recognition DNA fragment.** Cultured astrocytes were treated with 30 μM TPNA10168 for 3 and 6 h. Results are expressed as the mean ± SEM of 4 different nuclear extracts. ***P < 0.001 vs. none; one-way ANOVA followed by Dunnett's test.

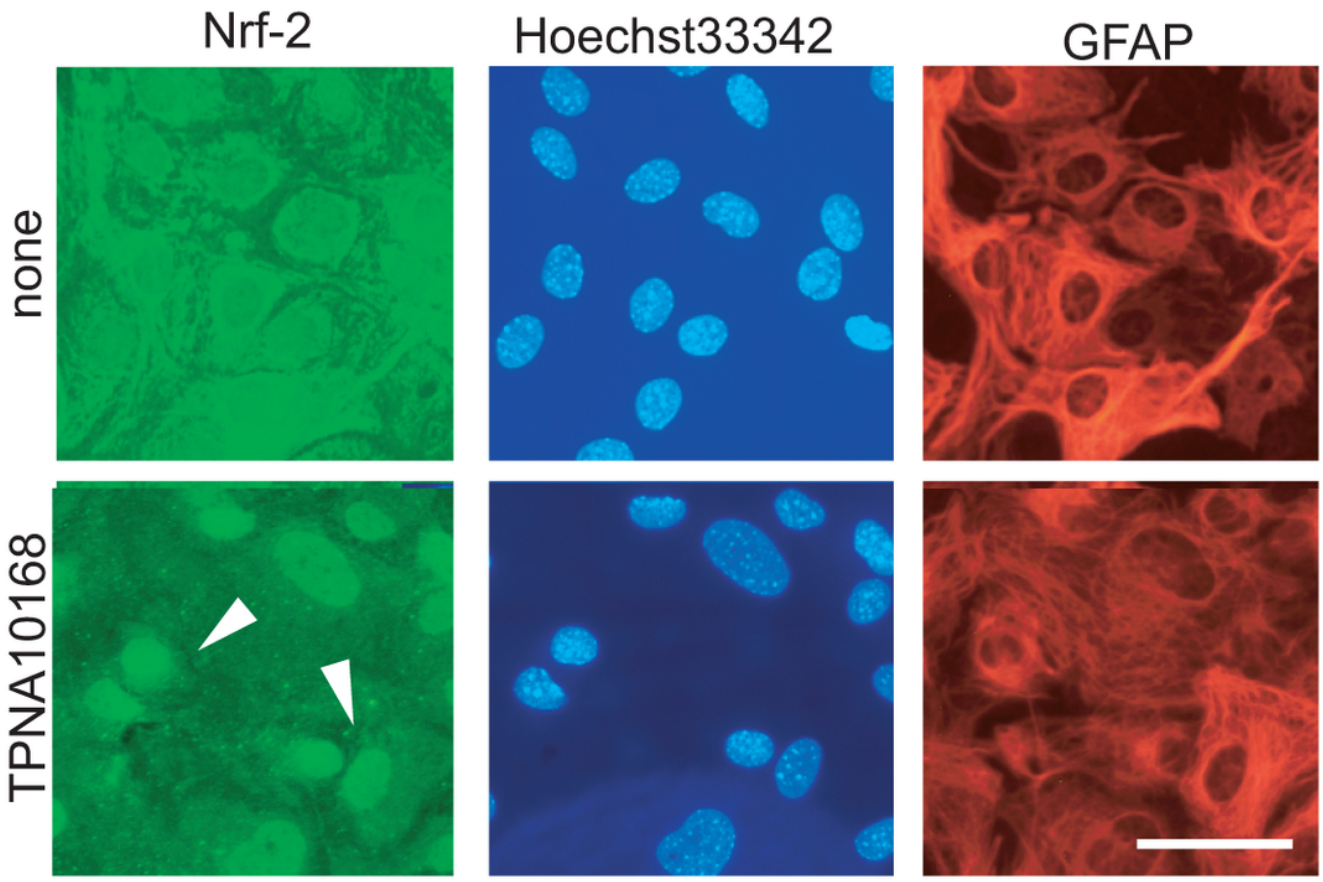

Figure 2

Immunocytochemical analysis of Nrf-2. Cultured astrocytes were treated with 30 μM TPNA10168 for 3 h. After fixation, cells were labeled with anti-Nrf-2 rabbit and anti-glial fibrillary acidic protein mouse antibodies. For counter-staining, 10 μM Hoechst 33342 was included in the secondary incubation with FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG antibodies. Typical micrographs are shown. Arrowheads indicate the nuclear location of immunoreactive Nrf-2. Bar = 20 μm.

Figure 3

(a) Effects of TPNA10168 on system Xc^- and g-GCL mRNA expression. Cultured astrocytes were treated with 30 μM TPNA10168 for the indicated time. The expression of the system Xc^- and g-GCL mRNAs was normalized to that of GAPDH. Results are expressed as the mean ± SEM of 6 different mRNA preparations. *P < 0.05, ***P < 0.001 vs. 0 h; ANOVA followed by Dunnett's test. **(b) Concentration-dependent effects of TPNA10168 on system Xc^- and g-GCL mRNA expression**. Astrocytes were treated with the indicated concentrations of TPNA10168 for 6 h (system Xc^- and g-GCLm, n = 8) or 12 h (g-GCLc, n = 4–5). Data are presented as the mean ± SEM. **P < 0.01, ***P < 0.001 vs. none; one-way ANOVA followed by Dunnett's test. **(c) Inhibitory effects of ML385 on TPNA10168.** Astrocytes were treated with 10 μM TPNA10168 in the presence or absence of 10 μM ML385 for 12 h. Data are presented as the mean ± SEM of 4 different mRNA preparations. *P < 0.05, ***P < 0.001 vs. none/control, $^{###}$P < 0.001 vs. none/TPNA; one-way ANOVA followed by Tukey's test.

Figure 4

Effects of TPNA10168 on system Xc^-, g-GCLm, and g-GCLc protein expression. Astrocytes were treated with 30 μM TPNA10168 for the indicated time. System Xc^- and g-GCL proteins were detected by immunoblotting. Representative immunoblots are shown in the upper panel. After protein detection, the same blots were re-probed with an anti-β-actin antibody. Protein bands were analyzed by densitometry. The expression of system Xc^- and g-GCL proteins was normalized to that of b-actin. Data are presented as the mean ± SEM of 4 to 6 different cell lysate preparations. *P < 0.05, **P < 0.01 vs. 0 h; one-way ANOVA followed by Dunnett's test.

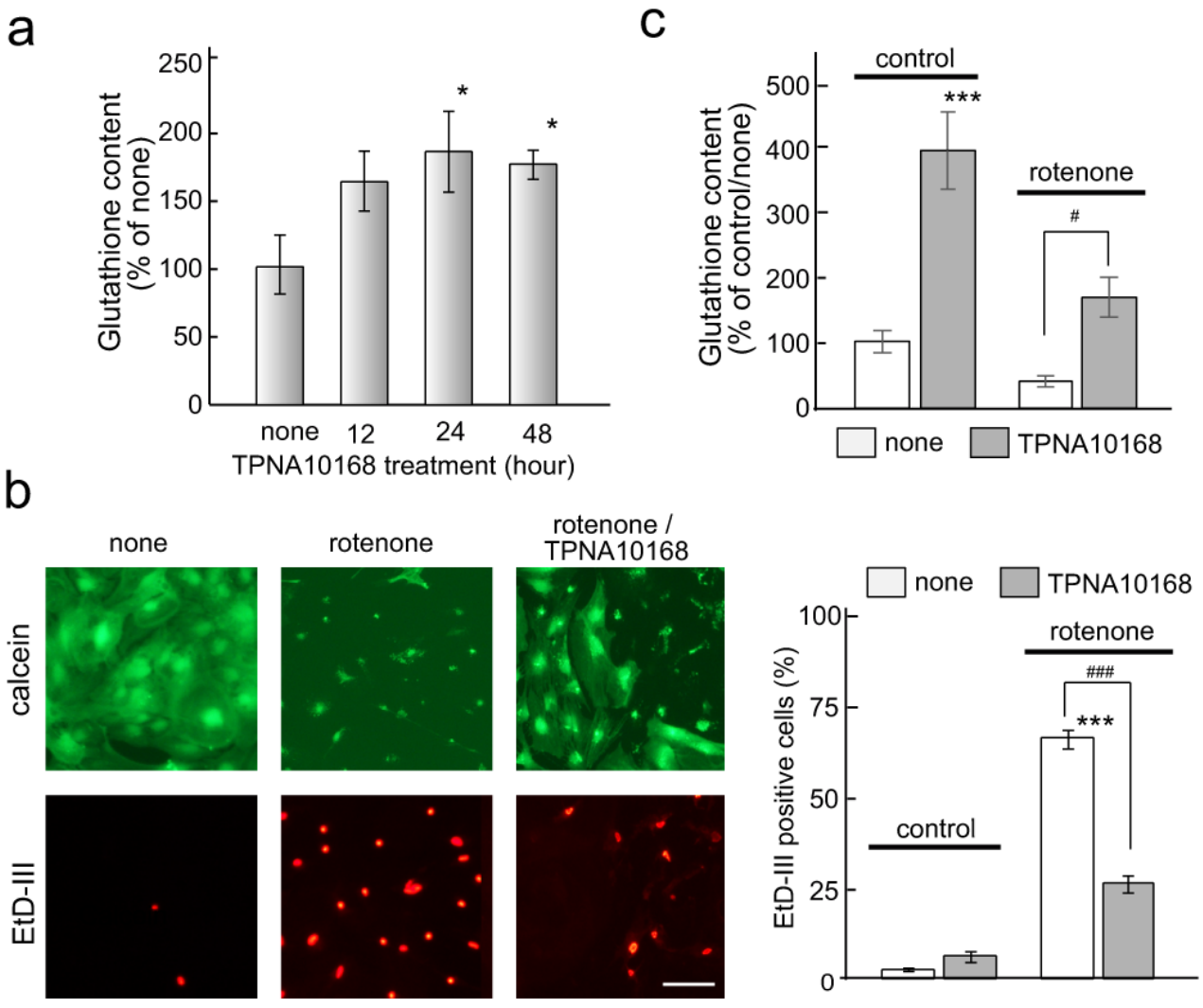

Figure 5

(a) Effects of TPNA10168 on total glutathione content. Astrocytes were treated with 30 μM TPNA10168 for 12-48 h in serum-free MEM. The total glutathione content was normalized to the total protein content. Data are presented as the mean ± SEM of 6 different cell extracts. *P < 0.05 vs. 0 h; one-way ANOVA followed by Dunnett's test. **(b) Effects of TPNA10168 on rotenone-induced cytotoxicity.** Astrocytes were treated with 2.5 nM rotenone in the absence or presence of 30 μM TPNA10168 in serum-free MEM. After 48 h, cells were stained with calcein-AM and EtD-III and observed under an inverted epifluorescence microscope. Representative images of each condition are shown. Bar = 100 μM. The quantification of dead cells is shown in the figure. Data are presented as the mean ± SEM of 4 different experiments. ***P < 0.001 vs. none/control, $^{###}$P < 0.001 vs. none/rotenone; one-way ANOVA followed by Tukey's test. **(c) Effects of rotenone and TPNA10168 on total glutathione content.** Astrocytes were treated with 2.5 nM rotenone in the absence or presence of 30 μM TPNA10168 for 24 h, and the glutathione content was

measured for each condition. Data are presented as the mean ± SEM of 6 different cell extracts. ***P < 0.001 vs. none/control, #P < 0.05 vs. none/rotenone; one-way ANOVA followed by Tukey's test.

Supplementary Files

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