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γ-Tocopheryl quinone, not α-tocopheryl quinone, induces adaptive response through up-regulation of cellular glutathione and cysteine availability via activation of ATF4

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Abstract

α-Tocopheryl quinone (α-TQ) and γ-TQ are oxidized metabolites of the corresponding tocopherol (T) isoforms, which are vitamin E homologues. Unlike α-TQ, γ-TQ functions as an arylating agent that reacts with nucleophiles such as reduced sulphydryl groups and it has unique biological properties such as high toxicity. Increasing evidence indicates that reactive oxygen species and other physiologically existing oxidative stimuli upregulate the antioxidant system, thereby triggering the adaptive response. The present study used PC12 cells and immature primary cortical cells to examine the possible adaptive cytoprotective effects of γ-TQ against oxidative stress. Pre-treatment with γ-TQ at sub-lethal concentrations resulted in cytoprotective effects against oxidative stress. γ-TQ induced a significant increase in the cellular glutathione (GSH) levels while α-TQ did not. γ-TQ did not induce any considerable change in the activity of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, whereas it increased the cellular GSH levels by facilitating the availability of cysteine through the induction of xCT, which is the core sub-unit of the x_c^- high-affinity cystine transporter system. An activating transcription factor 4 (ATF4)-small interfering RNA effectively attenuated the xCT mRNA level as well as the increase in cellular cysteine levels induced by γ-TQ, while the NF-E2-related factor (Nrf2)-small interfering RNA treatment did not. Collectively, these findings indicate that γ-TQ acts as a signal messenger to induce adaptive response through the upregulation of intracellular GSH synthesis via transcriptional activation of ATF4 in order to cope with the forthcoming oxidative insult.

Keywords: *γ-Tocopheryl quinine, adaptive response, glutathione, cystine transporter, activating transcription factor 4, amino acid response element, electrophile response element*

Abbreviations: *AARE, amino acid response element; ATF4, activating transcription factor 4; BSO, buthionine sulphoximine; BzQ, 1,4-benzoquinone; CHOP, C/EBP homologuous protein; Cumene-OOH, cumene hydroperoxide; elF2, eukaryotic initiation factor 2; EpRE, electrophile response element; ER, endoplasmic reticulum; GCL, glutamate-cysteine ligase; GCLC, GCL catalytic sub-unit; GCLM, GCL modulatory sub-unit; GSH, glutathione; HO-1, heme oxygenase-1; Keap 1, Kelch-like ECH-associated protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2-related factor 2; Ribo-L32, Ribosomal protein L32; ROS, reactive oxygen species; T, tocopherol; TQ, tocopheryl quinone; TQH_2, tocopheryl hydroquinone; TR, thioredoxin reductase; T3, tocotrienol; T3Q, tocotrienyl quinone; xCT, cystine/glutamate exchange transport system x_c^- light sub-unit; 4-HNE, 4-hydroxy-2-nonenal; 6-OHDA, 6-hydroxydopamine; 13(S)-HpODE, 13S-hydroperoxyl-9Z, 11E-octadecadienoic acid; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2.*

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Introduction

Quinones are biologically active compounds that are provided through our diet and synthesized in cells. They are involved in a wide variety of biological and chemical processes, including electron transport, photosynthesis, post-translational modification of proteins and metabolism of cellular signalling molecules such as catecholamines and antioxidants such as vitamin E [1–4]. All quinones are redox cycling agents that generate reactive oxygen species (ROS). In contrast, partially substituted quinones can function as arylating agents that react with cellular nucleophiles such as thiols present in the cysteine residues of proteins, thereby forming covalently linked quinone-thiol Michael adducts [1–4]. Arylating quinones have unique biological properties such as high cytotoxicity that are not commonly shared by non-arylating quinones and arylated thiol adducts. Recently, differences in the endoplasmic reticulum (ER) stress induction property of arylating and nonarylating quinones have been reported using arylating γ-tocopheryl quinone (γ-TQ) and non-arylating α-TQ [5].

TQ is an oxidized metabolite of vitamin E, which is one of the most potent lipid-soluble antioxidants [6]. Vitamin E occurs in nature in at least eight different isoforms: α-, β-, γ- and δ-tocopherol (T) and α-, β-, γ- and δ-tocotrienol (T3) [7]. T3s differ from their corresponding Ts only in their aliphatic tail. It has been reported that T3s exhibit higher cellular uptake in a cell culture system [8] and this accelerated cellular uptake is thought to be mediated by the aliphatic tail. Tocopheryl hydroquinone (TQH_2), a reduced form of TQ, has been shown to be capable of acting as a radical-scavenging antioxidant [9], while TQ itself is not considered to be an effective antioxidant [10]. α-TQ is a fully substituted quinone and therefore exhibits the properties of a non-arylating quinone. On the other hand, since γ-TQ is partially substituted, it has some characteristics of an electrophilic arylating quinone, including higher cytotoxicity [1,5,11].

There is increasing evidence to indicate that electrophilic lipids such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and 4-hydroxy-2-nonenal (4-HNE), which contain a reactive α,β-unsaturated carbonyl functional group that forms covalently linked quinone-thiol Michael adducts, exhibit not only cytotoxicity but also a cytoprotective effect that induces an adaptive response [12,13]. It has been suggested that electrophilic compounds could act as potential activators of the NF-E2-related factor 2 (Nrf2) and could induce the expression of phase II detoxification enzymes via the Michael addition reaction with Kelch-like ECH-associated protein 1 (Keap1), which is the cytoplasmic inhibitor of Nrf2 [14]. This modification of Keap1 results in the liberation and activation of Nrf2, which subsequently translocates into the nucleus and induces the expression of detoxification enzymes via the electrophile response element (EpRE). Furthermore, it has been reported that other transcription factors such as Nrf1, Jun-B and Jun-D can promote the expression of phase II antioxidant enzymes [15,16].

The involvement of glutathione (GSH) in the protection of cells against oxidative stress and other xenobiotic compounds has been well established [17]. The intracellular GSH content is a function of the balance between synthesis, consumption and regeneration. It has been reported that GSH synthesis is regulated by a combination of glutamate-cysteine ligase (GCL) activity, cysteine availability and GSH feedback inhibition [18] and it is thought that in most cases the elevation in GSH levels is principally due to *de novo* synthesis [19]. GCL is a heterodimer that can be dissociated under non-denaturing conditions into a catalytic sub-unit (GCLC) and a modulatory sub-unit (GCLM) [20]. It has been reported that the cystine/glutamate exchange transport system x_c^- contributes to cysteine availability, thereby maintaining the cellular GSH level [21]. This transport system is composed of a heavy sub-unit 4F2hc and a light sub-unit xCT and transport is thought to be mediated by xCT [22]. It has been reported that these GSH synthesis-related genes, namely *GCLC*, *GCLM* and *xCT*, contain the EpRE, which is responsible for induction by electrophilic agents regulated by Nrf2 [23–25]. In addition, it has been demonstrated that the transcriptional control of *xCT* is regulated by activating transcription factor 4 (ATF4), an important regulator of the eukaryotic initiation factor 2 (elF2) kinase pathway, via the amino acid response element (AARE) [26–28].

In the present study, we used PC12 cells and immature primary cultured cortical neuronal cells to explore the possible adaptive response induced by the arylating quinone γ-TQ and compared this with that induced by the non-arylating quinone α-TQ. We found that γ-TQ, not α-TQ, induced an adaptive response mediated through elevated GSH levels by the induction of *xCT* via the transcriptional activation of ATF4.

Materials and methods

Chemicals

Dulbecco's modified eagle medium: nutrient mixture F-12 Ham = 1:1 (D-MEM/F-12) and horse serum were obtained from Gibco BRL (Rockville, MD); foetal bovine serum from JRH Biosciences (Lenexa, KS); GSH and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from nacalai tesque (Kyoto, Japan); 6-hydroxydopamine (6-OHDA), cumene hydroperoxide (Cumene-OOH)

and 1,4-benzoquinone (BzQ) from Sigma-Aldrich (St. Louis, MO); α-TQ from Wako Pure Chemical Industries (Osaka, Japan); antibodies against Nrf2, C/EBP homologuous protein (CHOP) and ATF4 from Santa Cruz Biotechnology (Santa Cruz, CA); GCLC antibody from Lab Vision (Fremont, CA); heme oxygenase-1 (HO-1) antibody from Stressgen Bioreagents (Victoria, BC, Canada); mouse anti-actin monoclonal antibody (mAb) (clone C4) was obtained from Chemicon International (Temecula, CA). Natural isoforms of vitamin E were kindly supplied by Eisai Co. Ltd. (Tokyo, Japan). γ-TQ, α-tocotrienyl quinone (α-T3Q) and γ-T3Q were synthesized from their parent compounds, γ-T, α-T3 and γ-T3, respectively, according to the previous report, with a slight modification by using $CuSO_4$ instead of $FeCl_3$ [29]. The reduced form of each quinone was prepared by the reduction with sodium borohydride. Other chemicals were of the highest quality commercially available.

Cell culture and determination of cell viability

Undifferentiated PC12 cells, rat pheochromocytoma cell line, were routinely maintained in D-MEM/F-12 medium containing 10% heat-inactivated foetal bovine serum, 5% heat-inactivated horse serum and antibiotics at 37°C under an atmosphere of 95% air and 5% CO_2 [30,31]. After the cells were attached (16–18 h), they were treated with vitamin E derivatives at different concentrations for 24 h. In the experiment, vitamin E derivatives were dissolved in ethanol and an equal amount of ethanol was added as a control. The culture medium was replaced with fresh serum medium after each treatment and then stressors such as 13S-hydroperoxyl-9Z and 11E-octadecadienoic acid (13(S)-HpODE, Cayman Chemical, Ann Arbor, MI) were added to the medium as an aqueous solution. For the determination of cell viability, MTT assay was conducted for the indicated periods, as described previously [32]. The cells were incubated with 0.5 mg/ml MTT at 37°C for 2 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, by volume) and they were mixed by pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Theromo Labsystems, Helsinki, Finland).

Determination of intracellular vitamin E derivatives

Intracellular vitamin E was detected using HPLC systems with electrochemical detection (SHISEIDO, Japan) as described previously [8]. Cell samples in PBS (three times washed) were mixed with chloroform/methanol (2/1) containing 0.02% butylated hydroxytoluene at twice the volume of the samples. Then, an equal volume of 0.1 M NaCl with cell samples was added to the extract and mixed. After centrifugation for 10 min at 17 000 × g, the lower chloroform layer was evaporated to dryness under a stream of N_2, redissolved in methanol and injected into the HPLC for T, TQ and TQH_2 analysis. The uptake of vitamin E derivatives by PC12 cells was measured with an HPLC using a post-column amperometric electrochemical detector (NANOSPACE SI-1, Shiseido, Tokyo, Japan) set at 700 mV, using an ODS column (LC-18, 5 μm, 250 × 4.6 mm, Wako, Japan), a reduction column (RC-10, 4.0 mm × 15 mm, SHISEIDO, Japan) and methanol containing 50 mm sodium perchlorate as an eluent at 0.7 ml/min. In the case of TQ and TQH_2, the extraction efficiency in this procedure is as follows: 73% (α-TQ), 46% (γ-TQ), 72% (α-TQH_2) and 80% (γ-TQH_2).

Determination of cellular GSH content

Intracellular GSH content was enzymatically determined by using 5,5′-dithiobis-(2-nitrobenzoic acid), according to the method described previously [33]. GSH content was calculated using reduced GSH (Nacalai tesque) as the standard. The protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Immature primary cortical neurons

Cells were isolated from the cerebral cortex of rat foetuses (Sprague-Dawley rats, day 17 of gestation, SLC, Sizuoka, Japan) as described previously [13]. Briefly, the cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Worthington Biochemical, Lakewood, NJ) at 37°C and the cells were then plated at a final density of 2×10^5 cells/cm^2 on a polyethyleneimine-coated plate. Cells were grown in D-MEM/F-12 supplemented with 10% heat-inactivated foetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). Over 90% of the cells in the cultures were neurons, as determined by immunostaining of the astrocyte-specific marker glial fibrillary acidic protein (Upstate, Lake Placid, NY) and the neuron-specific markers microtubule-associated protein-2 (Sigma-Aldrich, St. Louis, MO) and β_{III}-tublin (Promega, Madison, WI) (data not shown). More than 6 h after plating the cultured neurons with serum medium, the cells were treated with TQ derivatives at different concentrations for 24 h. The culture medium was replaced with fresh serum medium after each treatment and then glutamate or 13(S)-HpODE was added to the medium as an aqueous solution. For the determination of cell viability using MTT, the cells were incubated with 0.5 mg/ml MTT at 37°C for 4 h.

Determination of antioxidant genes

The expression of the target gene was determined by a real-time polymerase chain reaction, as previously described [13] using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The housekeeping gene of Ribosomal protein L32 (Ribo-L32) was used as an endogenous control. The primers for amplification were as follows: *Rat Ribo-L32*, 5′-GAA ACT GGC GGA AAC CCA-3′ (forward), 5′-GGA TCT GGC CCT TGA ACC TTC-3′ (reverse). *Rat GCLC*, 5′-GGG AAG GAA GGC GTG TTT CCT-3′ (forward), 5′-GTC GAC TTC CAT GTT TTC AAG GT-3′ (reverse). *Rat GCLM*, 5′-CCA GGA GTG GGT GCC ACT GT-3′ (forward), 5′-TTT GAC TTG ATG ATT CCT CTG CTT-3′ (reverse). *Rat ATF4*, 5′-TCG ATG CTC TGT TTC GAA TGG-3′ (forward), 5′-CAA CGT GGC CAA AAG CTC AT-3′ (reverse). *Mouse xCT*, 5′-GGT CCT TTG CTG GCT TTT GTT-3′ (forward), 5′-AGC TCC AGG GCG TAT TAC GCA-3′ (reverse). *Mouse Ribo-L32*, 5′-GAA ACT GGC GGA AAC CCA-3′ (forward), 5′-GGA TCT GGC CCT TGA ACC TTC-3′ (reverse).

Enzyme asssay

GCL activity was determined using the method reported previously [31], with slight modification. 0.1 M Tris-HCl buffer, pH 8.2, containing cytosolic fractions (~150 μg protein), 10 mM KCl, 25 mM $MgCl_2$, 2 mM Na_2EDTA, 5 mM L-glutamate, 5 mM L-cysteine, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 1 unit/ml lactate dehydrogenase and 1 unit/ml pyruvate kinase, was pre-incubated at 37°C for 5 min and the reaction was initiated by the addition of ATP to a final concentration of 5 mM. The decrease of NADH was recorded at 340 nm and the results were normalized to protein content. Thioredoxin reductase (TR) activity and NAD(P)H:quinone oxidoreductase 1 (NQO1) activity was determined using the method reported previously [30,34].

Determination of intracellular cysteine

The intracellular cysteine was determined using HPLC system with electrochemical detection as described previously [35]. Treated cells were harvested, washed with ice-cold PBS and re-suspended in ice-cold 10% trichloroacetic acid. The cell samples were centrifuged and used for further analysis. The soluble supernatant was injected into the HPLC for cysteine analysis. The cellular content of cysteine was measured with an HPLC using a post-column amperometric electrochemical detector (NANOSPACE, SHISEIDO, Japan) set at 700 mV, using an ODS column (CA-5ODS 2.1 × 150 mm, Eicompak, Japan) and 100 mM phosphate buffer (pH 2.5) containing 1% methanol, 400 μg/ml SOS and 5 μg/ml EDTA as an eluent at 0.5 ml/min.

Preparation of cellular samples and Western blot analysis

Nuclear fractions of cells were prepared as described previously [36]. In the case of total cell extract samples, treated cells were suspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 5 mM EDTA, 0.5% Triton X-100 and 1 mM Na_3VO_4 with a protease inhibitor cocktail tablet), as described previously [37]. The detection of specific proteins either in the total cell extracts or in the cell fractions was carried out by Western blot analysis, following the previously described procedure [38].

Transfection of siRNA

Rat Nrf2– and ATF4–siRNA was designed and manufactured by Invitrogen, according to the current guidelines for effective knockdown by this method. The target sequences for Nrf2-siRNA are 5′-UUA AGA CAC UGU AAC UCG GGA AUG G-3′ (forward), and 5′-CCA UUC CCG AGU UAC AGU GUC UUA A-3′ (reverse), whose knockdown effect has been demonstrated previously [30,31]. In the case of ATF4–siRNA, Atf4–RSS340174 (Invitrogen, catalogue number: 10620318 and 10620319) was used. The siRNA was transfected into PC12 cells at a concentration of 20–50 pmole/10^5 cells by Lipofectamine (Invitrogen) 24 h prior to further experiments.

Primary cortical cell culture from mouse

Primary cortical cell cultures were prepared from newborn pups from wild type C57BL6J and *nrf2* mutant mice [39], as described above. Cells were used for experiments at 3 days *in vitro*.

Statistical analysis

Data are reported as mean ± SD of at least three independent experiments. The statistical significance of the difference between determinations was calculated by Student's t-test and the analysis of variance (ANOVA) using Dunnett's and Turkey tests for multiple comparisons. The calculation method was described in the figure legends. Values of $p < 0.05$ were considered significant.

Results

Induction of cytoprotective effect against oxidative stress by arylating tocopheryl and tocotrienyl quinone

In order to explore the possible adaptive cytoprotection of the arylating quinone, the cytotoxicity induced by γ-TQ and γ-T3Q was first investigated in PC12 cells. Both γ-TQ and γ-T3Q exhibited

higher cytotoxicity than other isoforms of vitamin E (Figure 1A and B). In the case of γ-TQ, significant cytotoxicity was observed at concentrations higher than 8 μM, whereas γ-T3Q was at 4 μM. Based on the above observations, 8 μM and 4 μM were considered to be the quasi-toxicological thresholds at which γ-TQ and γ-T3Q caused no appreciable cytotoxicity in PC12 cells. Consequently, concentrations below these were selected for further studies to examine a possible adaptive protective effect.

Pre-treatment of PC12 cells with sub-lethal concentrations of arylating γ-TQ for 24 h caused significant protection of PC12 cells against cell death induced by subsequent treatment with 40 μM 13(S)-HpODE (Figure 1C). In the case of γ-T3Q, a significant cytoprotective effect was also observed against 13(S)-HpODE-induced cell death (Figure 1C). It was found that the protective effect of T or T3 was lower than that of its oxidative metabolite TQ or T3Q, respectively, at the same concentrations. It was also observed that pre-treatment with the non-arylating α-type quinone resulted in a cytoprotective effect similar to that of the γ-type at the concentration used in this study. Furthermore, pre-treatment of cells with γ-TQ as well as α-TQ for 24 h caused significant protection against cell death induced by subsequent treatment with 6-OHDA or Cumene-OOH (Figure 1D).

It has been reported that the antioxidant activity of TQ is implied so far, presumably through the generation of TQH_2, since TQ is not considered to be a chain-breaking antioxidant [9,10]. In the present study, we measured the TQ and TQH_2 contents in PC12 cells cultured for 24 h in a serum medium

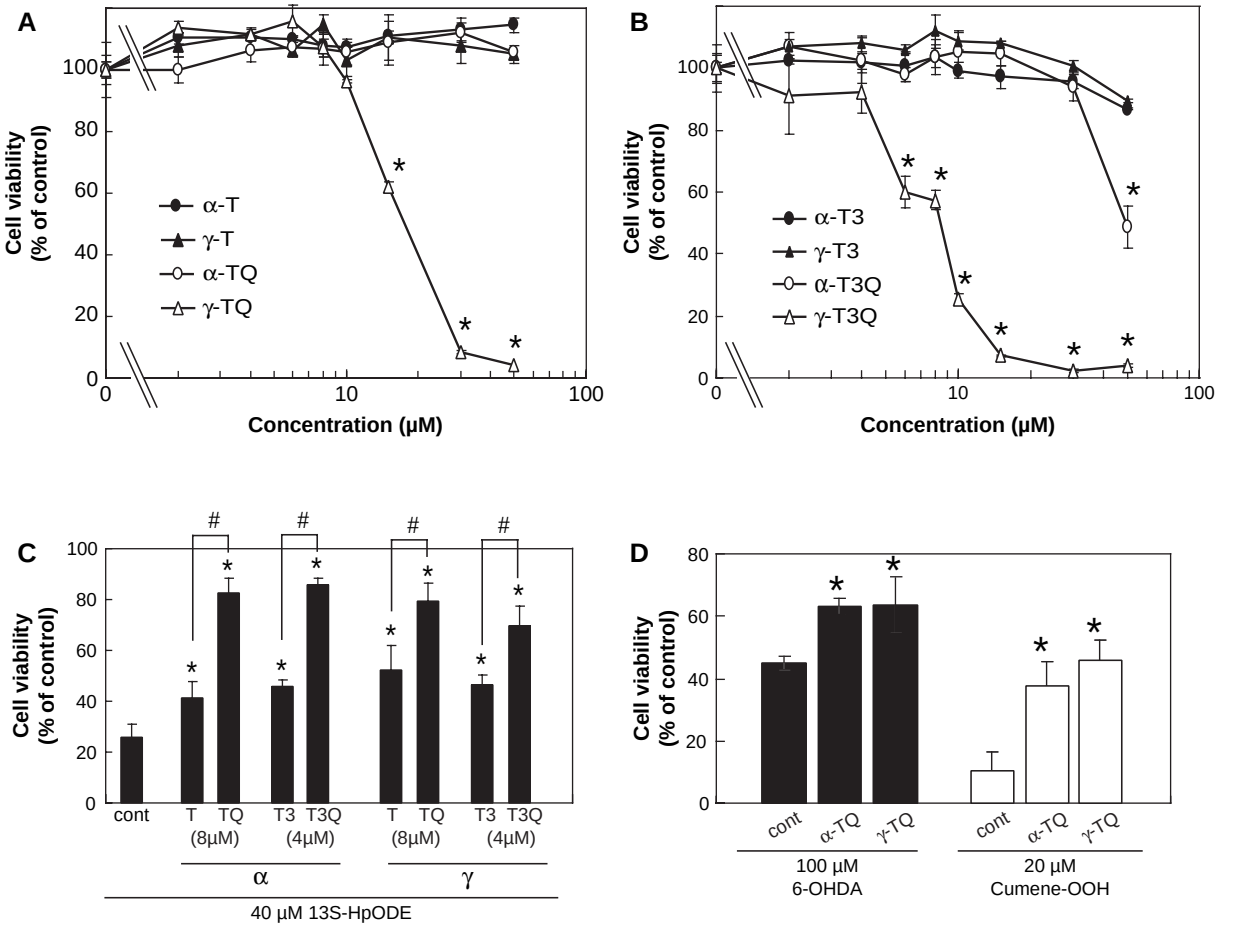

Figure 1. Cytotoxicity and cytoprotective effect of tocopherols (T), tocotrienols (T3) and their quinones against oxidative stress in PC12 cells. (A, B) Cells were treated with variable concentrations of T (A), T3 (B) and their quinones for 24 h and the viability was measured by the MTT assay, as described in Materials and methods. *Significantly different from the value at 0 μM ($p < 0.001$, Dunnett, ANOVA). (C) Cytoprotective effect induced by vitamin E isoforms against 13S-HpODE toxicity. The cells were pre-treated with vitamin E isoforms or control ethanol for 24 h followed by treatment with 40 μM 13S-HpODE for an additional 24 h (MTT assay). *Significantly different from the value of the control ($p < 0.025$, Turkey, ANOVA). # Significantly different ($p < 0.025$, Turkey, ANOVA). (D) Cytoprotective effect of TQ against 6-OHDA and Cumene-OOH toxicity. The cells were treated with 8 μM α-TQ or γ-TQ for 24 h followed by treatment with 6-OHDA or Cumene-OOH for an additional 24 h (MTT assay). *Significantly different from the value of the control ($p < 0.001$, Dunnett, ANOVA).

Table I. Cellular uptake of α-TQ and γ-TQ into PC12 cells and reduction to TQH_2.[a]

	α-TQ	α-TQH_2	γ-TQ	γ-TQH_2
control	nd	nd	nd	nd
α-TQ	1200±260	680±120	nd	nd
γ-TQ	nd	nd	530±120	660±220

(pmol/mg protein)

[a] PC12 cells were cultured with or without 8 μM α-TQ or γ-TQ for 24 h and the cellular content was measured using an HPLC system with an electrochemical detector. Mean values of cellular content are shown with standard error. nd: not detected.

containing 8 μM of α- and γ-TQ. As shown in Table I, we detected not only α- and γ-TQ (1.2 and 0.5 nmol/mg proteins, respectively) but also their corresponding reduced forms, namely, α- and γ-TQH_2 (0.7 and 0.7 nmol/mg protein, respectively). These results suggest that the generated TQH_2 take a significant role in both α- and γ-TQ-induced cytoprotective effect.

Effect of γ-TQ on the intracellular GSH contents

We attempted to determine the underlying molecular mechanisms responsible for the cytoprotective effects induced by arylating γ-TQ in PC12 cells. Since we have recently observed the pivotal role of GSH and TR1 in ROS-induced adaptive response in PC12 cells [30,31], we studied the possible involvement of GSH and TR1 in the cytoprotective effect induced by γ-TQ. In addition, we also analysed the alteration in NQO1 enzyme activity and HO-1 expression. Although no significant variation in the TR enzyme activity, NQO1 enzyme activity or HO-1 expression was observed in α- and γ-TQ-treated PC12 cells (Supplementary material, Table and Figure SA), treatment with γ-TQ, not α-TQ, at sub-lethal concentrations for 24 h significantly increased the total cellular GSH contents by up to ~4-fold (Figure 2A). A study of the effect of γ-TQ on the cellular GSH levels revealed that this compound induced GSH

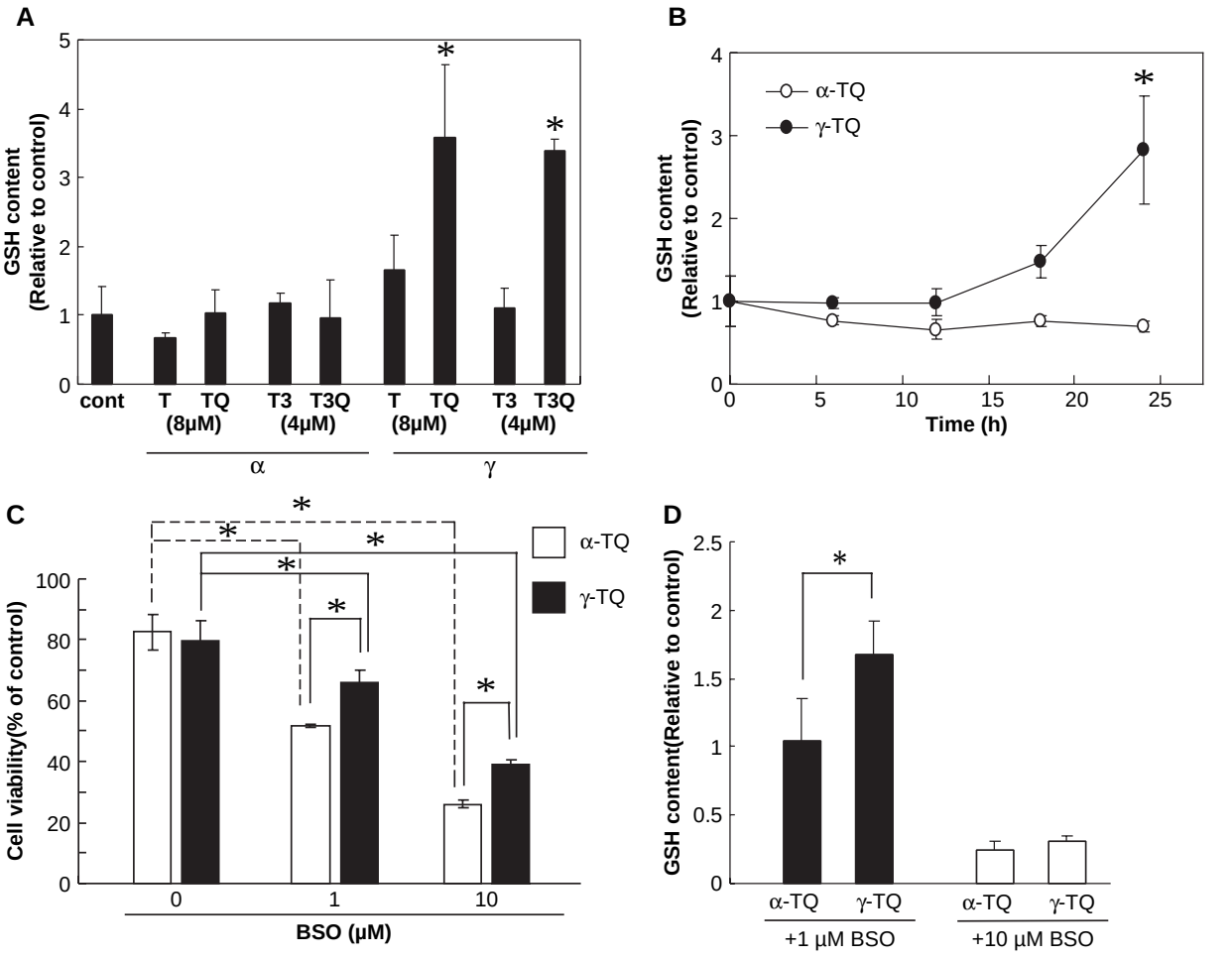

Figure 2. Alteration of cellular GSH levels by γ-TQ in PC12 cells. (A) The cells were treated with the indicated concentrations of vitamin E isoforms or control ethanol for 24 h. The cells were then harvested and the GSH levels were measured. *Significantly different from the value of the control ($p < 0.01$, Turkey, ANOVA). (B) Time-dependent variation of GSH by γ-TQ. PC12 cells were treated with control ethanol or 8 μM γ-TQ or α-TQ for the indicated times. *Significantly different from the value of the control ($p < 0.01$, Turkey, ANOVA). (C) Role of GSH in the adaptive response induced by γ-TQ. PC12 cells were exposed to control ethanol or 8 μM α-TQ or γ-TQ for 24 h in the absence or presence of the GCL inhibitor BSO, followed by treatment with 40 μM 13S-HpODE for an additional 24 h. *Significantly different ($p < 0.05$, Turkey, ANOVA). (D) Cells were exposed to control ethanol or α-TQ or γ-TQ in the presence of BSO for 24 h. *Significantly different ($p < 0.05$, *t*-test).

content in a time-dependent manner (Figure 2B). Furthermore, γ-T3Q, not α-T3Q, at sub-lethal concentrations also enhanced the cellular GSH level by up to ~3.5-fold (Figure 2A), indicating that GSH elevation was due to the functioning of both γ-TQ and γ-T3Q as arylating agents that reacted with nucleophiles. These results also suggest the involvement of the adaptive response in the cytoprotective effect induced by γ-TQ and γ-T3Q.

GSH elevation was responsible for the cytoprotection induced by γ-TQ

In order to further confirm the role of GSH elevation in the adaptive effect induced by γ-TQ, we attempted to explore the effect of buthionine sulphoximine (BSO), a specific GCL inhibitor that is widely used in a variety of cell lines [12,13,31]. Treatment of PC12 cells with BSO at 10 μM for 24 h revealed no appreciable cytotoxicity (data not shown). Pre-treatment with 1 or 10 μM BSO together with α- or γ-TQ effectively reduced the cytoprotection against oxidative stress induced by 13(S)-HpODE (Figure 2C). It was found that γ-TQ-pre-treated cells were resistant to BSO co-treatment in comparison with α-TQ-treated cells. In accordance with this cytoprotective effect, γ-TQ-treated cells were resistant to the decrease in cellular GSH induced by the BSO treatment (Figure 2D). These results suggest the role of cellular GSH elevation on the cytoprotective effect induced by γ-TQ.

Cytoprotective effects and GSH elevation induced by γ-TQ in immature primary cortical neurons

In order to explore the possible adaptive protective effects of γ-TQ on neuronal cells, we further applied TQ derivatives to immature cortical neuronal cells. It has been observed that cell death in these cultures is caused by glutamate-induced oxidative stress and

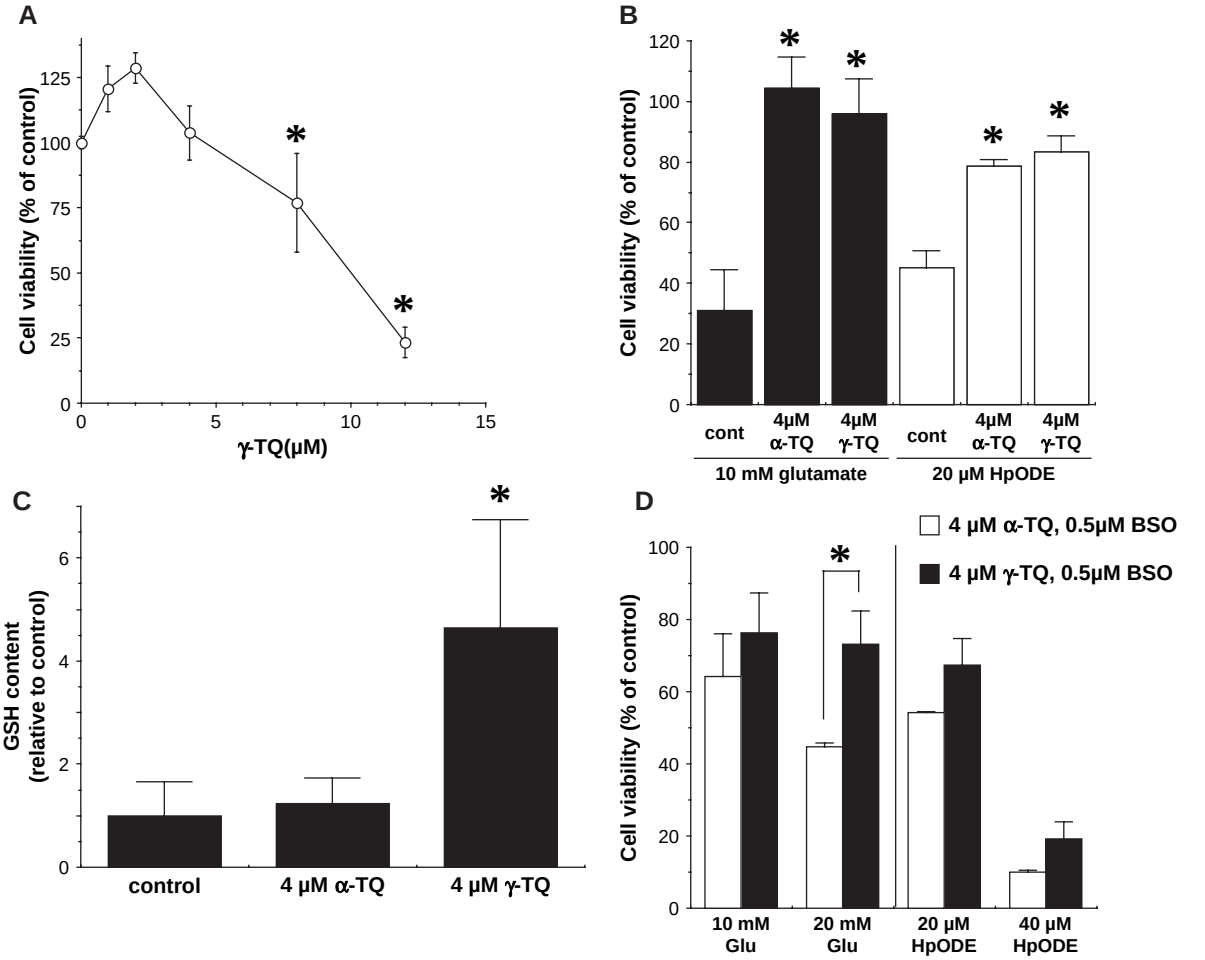

Figure 3. γ-TQ-induced adaptive protection against glutamate and 13S-HpODE in immature primary cortical neurons. (A) Cytotoxicity induced by γ-TQ in cultured cortical neurons. Cells were treated with γ-TQ for 24 h and subjected to an MTT assay. *Significantly different from the value at 0 μM ($p < 0.05$, Dunnett, ANOVA). (B) Cultured cortical neurons were pre-treated with control ethanol or 4 μM α-TQ or γ-TQ for 24 h, followed by treatment with 10 mm glutamate or 20 μM 13S-HpODE for an additional 24 h (MTT assay). *$p < 0.05$ in comparison with the control (Dunnett, ANOVA). (C) Alteration in cellular GSH levels by γ-TQ. Cultured cortical neurons were treated with control ethanol or α-TQ or γ-TQ for 24 h. *$p < 0.05$ in comparison with the control (Turkey, ANOVA). (D) Role of GSH in the adaptive response induced by γ-TQ. Cultured cortical neurons were exposed to control ethanol or α-TQ or γ-TQ in the presence of BSO for 24 h, followed by treatment with glutamate or 13S-HpODE (MTT assay). *Significantly different ($p < 0.05$, *t*-test).

that oxidative glutamate toxicity is initiated by the competition of glutamate with the cystine uptake system regulating the synthesis of GSH [13]. Pre-treatment of cultured neuronal cells with γ-TQ at sub-lethal concentrations (4 μM, Figure 3A) for 24 h conferred a significantly protective effect against cell death induced by subsequent treatment with 10 mm glutamate (Figure 3B) and 20 μM 13(S)-HpODE (Figure 3B). α-TQ treatment also resulted in a cytoprotective effect similar to that of the γ-TQ treatment (Figure 3B). In the case of primary cortical neurons, elevation of cellular GSH was also induced by γ-TQ treatment and this increase was 4.7-fold (Figure 3C). We found that in comparison with α-TQ-pre-treated cells, γ-TQ-pre-treated cells were significantly resistant to the BSO co-treatment only when the oxidative stress was induced by 20 mM glutamate (Figure 3D).

The underlying molecular mechanism of cellular GSH elevation induced by γ-TQ

In order to further explore the underlying molecular mechanisms of GSH elevation induced by γ-TQ, we investigated the effect of γ-TQ on the expression of *GCLC* and *GCLM* mRNAs and on the enzyme activity of GCL. The adaptive concentration of 15d-PGJ_2 increased *GCLM* and *GCLC* sub-unit expression as well as GCL enzyme activity by ~2.4-, 2.5- and 1.5-fold, respectively [31]. On the other hand, γ-TQ did not induce any remarkable change in *GCLM* and *GCLC* expression or in GCL enzyme activity (Supplementary material, Figures SB, C and Table). We further explored the possible involvement of facilitated cysteine availability in γ-TQ-treated cells. We found that γ-TQ induced a dramatic 3.0-fold enhancement in cellular cysteine content (Figure 4A). It has been reported that cysteine availability is regulated by the cystine/glutamate exchange transport system that is composed of xCT [21]. It was observed that γ-TQ, not α-TQ, significantly increased the gene expression of the xCT sub-unit by ~4.8-fold at 8 h in PC12 cells (Figure 4B) as well as in primary cortical neuronal cells (Figure 4C). In order to confirm the role of xCT, we carried out an inhibition study using glutamate, which is known to inhibit xCT-mediated cystine uptake [13,26]. Co-treatment of cells with glutamate and γ-TQ effectively attenuated the increase in cellular cysteine (Figure 4D) as well as GSH (Figure 4E) induced by γ-TQ, suggesting a pivotal role for xCT induction in adaptive GSH elevation induced by γ-TQ.

Involvement of ATF4 in cysteine elevation and the subsequent adaptive response induced by γ-TQ

In order to elucidate the possible involvement of a transcription factor in *xCT* induction, we first investigated the effect of Nrf2-siRNA against *xCT* elevation induced by γ-TQ. In contrast to *GCLC* and *GCLM* induction by 15d-PGJ_2 [31], the γ-TQ-induced expression of *xCT* was not significantly attenuated in cells treated with Nrf2-siRNA (Figure 5A). The role of Nrf2 on *xCT* induction by γ-TQ was also investigated using the cells derived from nrf2−/− mouse. Cortical cells from nrf2−/− mouse (Nrf2 KO) showed higher sensitivity against γ-TQ than nrf2+/+ mouse (WT) (Figure 5B). In the case of WT, significant induction of *xCT* was observed at 32 μM γ-TQ (Figure 5C), in which a slight but significant decrease of cell viability was observed (Figure 5B). In accordance with the result obtained in WT, Nrf2 KO treated with cytotoxic concentration of 16 μM γ-TQ resulted in significant induction of *xCT* (Figure 5C). All these findings strongly suggested that Nrf2 appeared to take a less predominant role in the γ-TQ-induced *xCT* elevation.

We further studied the possible involvement of ATF4, which has been implicated as the protein that interacts with the AARE to induce xCT expression [26]. In accordance with a previous report [5], γ-TQ significantly induced ATF4 protein in PC12 cells (Figure 6A), suggesting a possible involvement of the ATF4 pathway in the adaptive response. In accordance with ATF4 induction, slight but significant induction of CHOP protein at adaptive concentration of γ-TQ was observed (Figure 6A). PC12 cells treated with ATF4-siRNA exhibited a marked decline of the constitutive mRNA level (Figure 6B). Moreover, the γ-TQ-induced ATF4 protein was significantly reduced in cells treated with ATF4-siRNA (Figure 6C). In ATF4-siRNA-treated cells the γ-TQ-induced expression of *xCT* was found to be significantly attenuated (Figure 6D). Consequently, the enhanced cysteine and GSH levels induced by γ-TQ were markedly decreased by ATF4-siRNA treatment (Figure 6E and F). Collectively, these findings suggested that ATF4 take a significant role in the γ-TQ-induced GSH elevation.

Involvement of Nrf2 in xCT elevation induced by BzQ

In order to determine whether the observations described above apply to other arylating quinones or were specific to γ-TQ, we analysed the effect of arylating BzQ, a major oxidative product of environmental-polluting aromatic hydrocarbons [5]. BzQ exhibited cytotoxicity at concentrations higher than 3 μM (Figure 7A). Based on this observation, 3 μM BzQ was selected to examine a possible adaptive effect. In accordance with arylating γ-TQ, BzQ at sub-lethal concentrations significantly increased the gene expression of the xCT sub-unit by ~3.5-fold at 12 h in PC12 cells (Figure 7B). In contrast to γ-TQ-induced *xCT* elevation, BzQ-induced xCT mRNA transcript was completely abolished in cells treated with Nrf2-siRNA (Figure 7B). Since the BzQ-induced expression of *xCT*

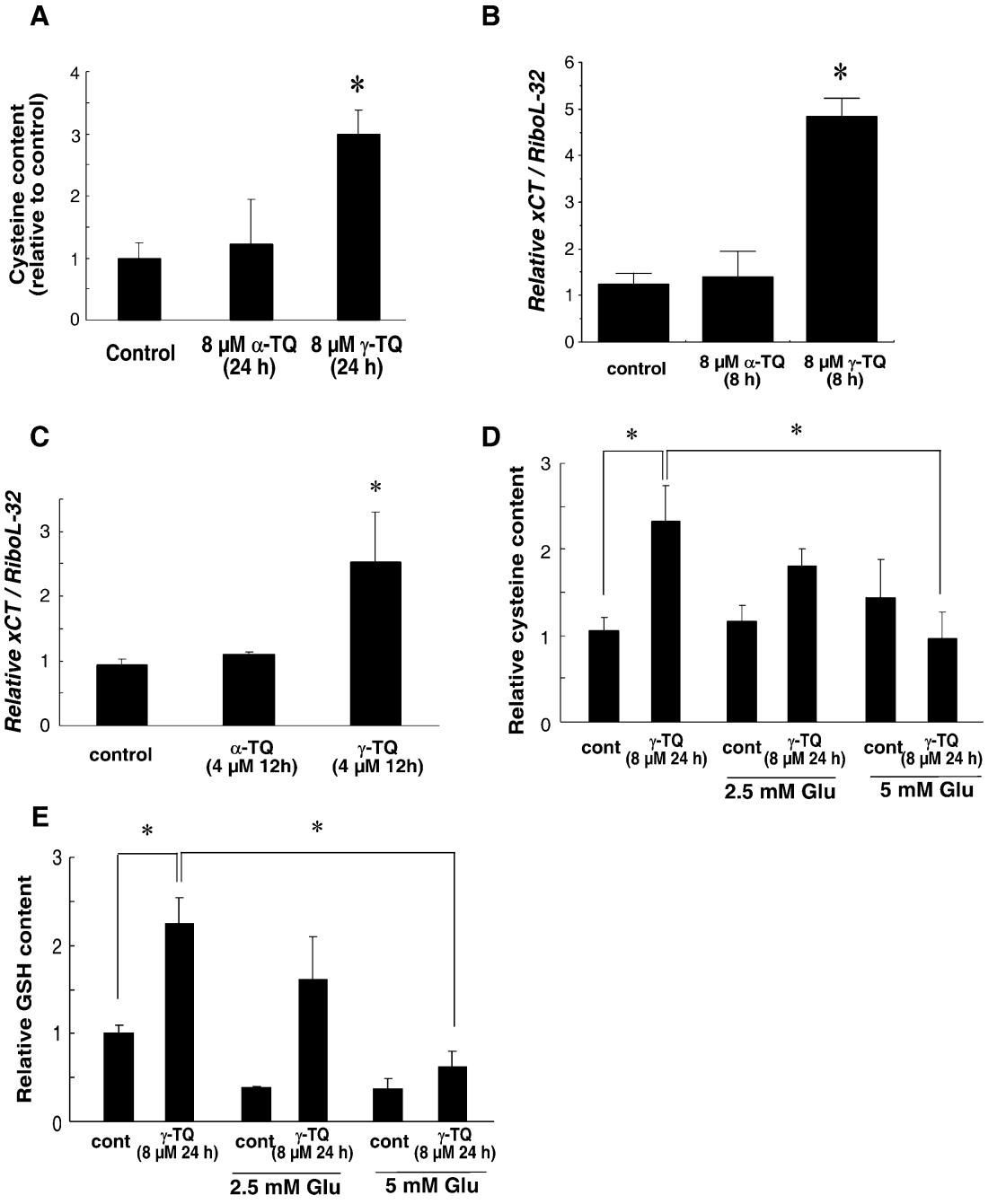

Figure 4. Alteration in the intracellular cysteine level and *xCT* expression by γ-TQ. (A) The cysteine level was measured, as described in Materials and methods. $^{*}p < 0.001$ in comparison with the control cells (Turkey, ANOVA). (B) Treated cells were harvested for RNA isolation and real-time PCR analysis. The expression levels of *xCT* were normalized to that of *Ribo-L32* mRNA and are shown relative to those of the control under static conditions. $^{*}p < 0.001$ in comparison with the control cells (Turkey, ANOVA). (C) Immature primary cortical neuronal cells were treated and harvested for RNA isolation and real-time PCR analysis. $^{*}p < 0.001$ in comparison with the control cells (Turkey, ANOVA). (D, E) PC12 cells were exposed to control ethanol or γ-TQ in the absence or presence of the xCT inhibitor glutamate for 24 h. The cells were then harvested and the cysteine (F) and GSH (G) levels were measured. *Significantly different ($p < 0.05$, Turkey, ANOVA).

was not significantly attenuated in cells treated with ATF4-siRNA, it was suggested that γ-TQ specifically induced adaptive response via the activation of ATF4.

Discussion

TQs are redox cycling compounds, but only partially substituted quinones, which are arylating electrophiles, can form adducts with nucleophiles and are therefore ideally suited for separating the redox cycling properties from the adduct-forming properties in explaining biological effects [1,5,40–42]. In the present study, we clearly demonstrated that arylating γ-TQ induced an adaptive response through the upregulation of GSH via the increase in cysteine availability in an ATF4-dependent manner.

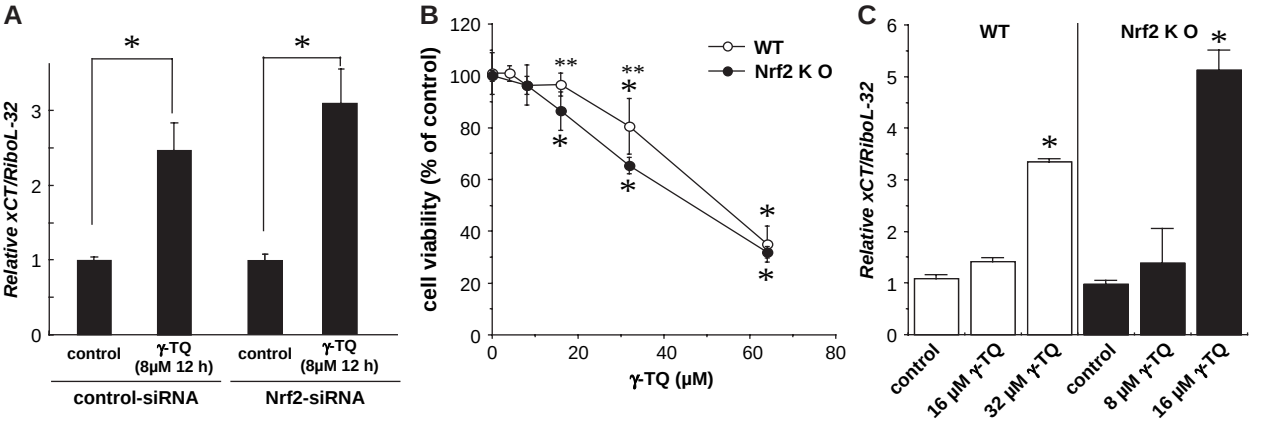

Figure 5. Lesser predominant role of Nrf2 in *xCT* expression induction by γ-TQ. (A) Effect of Nrf2-siRNA on γ-TQ-induced xCT mRNA expression. PC12 cells were pre-treated with Nrf2-siRNA or a non-specific RNA sample for 24 h followed by treatment with control ethanol or γ-TQ for an additional 12 h. *Significantly different ($p < 0.05$, Turkey, ANOVA). (B) Cytotoxicity induced by γ-TQ in cultured cortical cells from nrf2 +/+ (WT) and nrf2 −/− (Nrf2 KO) mouse. Cortical cells derived from WT and Nrf2 KO mouse were treated with γ-TQ at various concentrations for 24 h and subjected to an MTT assay. *Significantly different from the value at 0 μM ($p < 0.05$, Dunnett, ANOVA). (C) Cortical cells derived from WT and Nrf2 KO mouse were treated with control ethanol or γ-TQ for 12 h. *$p < 0.05$ in comparison with the control cells (Dunnett, ANOVA).

The basic leucine zipper transcriptional regulator ATF4 is integral to the elF2 kinase pathway that regulate translation under different stress conditions such as viral infection, nutrient limitation, UV irradiation and ER stress [27,28]. In the case of the ER stress response, phosphorylation of elF2α via pancreatic ER kinase (PERK) activation is the central mediator of this translational response. Phosphorylation of elF2α is postulated to attenuate global protein translation, while a paradoxical upregulation in selected transcripts including ATF4 has been reported [27,28,43]. It has been demonstrated that the arylating quinone γ-TQ induces cell death via ER stress by activating the PERK signalling pathway including elF2α, ATF4 and CHOP [5]. In the present study, we observed slight but significant induction of the

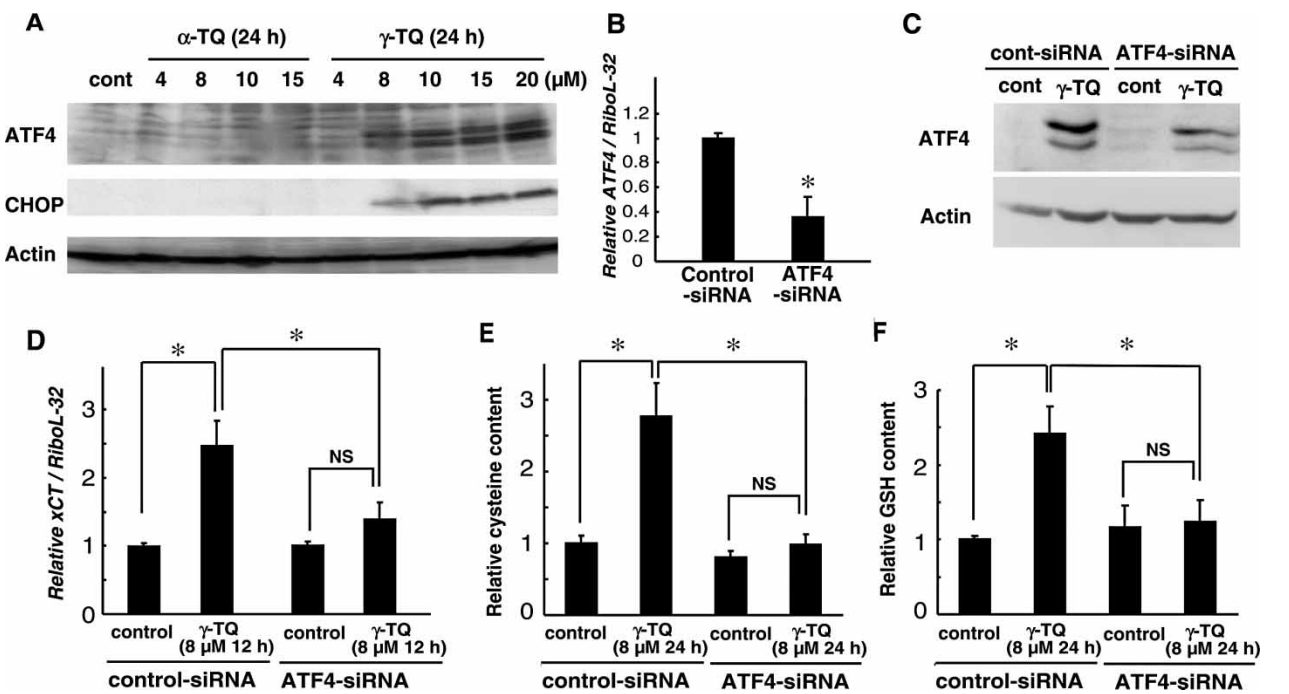

Figure 6.ATF4-dependent elevation of xCT expression, cellular cysteine and GSH induction by -TQ. A Effect of -TQ on the ATF4 and CHOP levels. PC12 cells were treated with - or -TQ for 24 h and subjected to Western blot analysis 50 g protein per lane. B Effect of ATF4-siRNA on the constitutive expression of ATF4. PC12 cells were treated with ATF4-siRNA or a non-specific RNA sample for 24 h. Significantly different p0.05, t-test. CF The elevations of ATF4 protein, xCT mRNA expression, cellular cysteine and GSH induced by -TQ were abolished in cells treated with ATF4-siRNA. PC12 cells were pre-treated with ATF4-siRNA or a non-specific RNA sample for 24 h followed by treatment with -TQ for an additional 6 h C, 12 h D or 24 h E, F. The cells were then harvested and the levels of ATF4 protein C, xCT expression D, cysteine E and GSH F were determined. Significantly different p0.05, Turkey, ANOVA. NS: not significant.

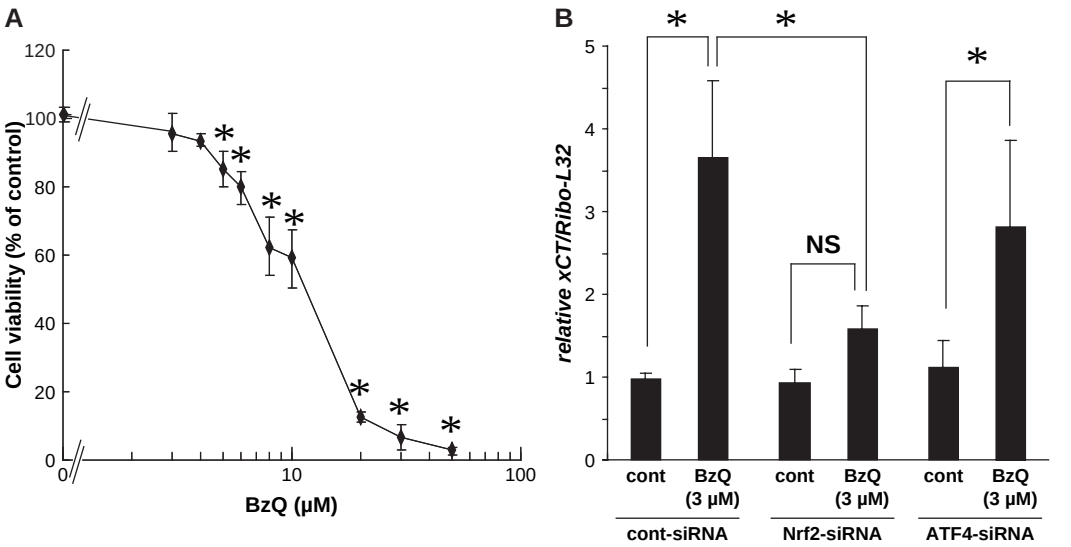

Figure 7. Nrf2-dependent elevation of *xCT* expression induction by BzQ. (A) Cytotoxicity induced by BzQ in PC12 cells. Cells were treated with BzQ for 24 h (MTT assay). *Significantly different from the value at 0 μM ($p < 0.05$, Dunnett, ANOVA). (B) The elevation of *xCT* mRNA expression induced by BzQ was abolished in cells treated with Nrf2-siRNA. PC12 cells were pre-treated with Nrf2-siRNA, ATF4-siRNA or a non-specific RNA sample for 24 h followed by treatment with control ethanol or 3 μM BzQ for an additional 12 h. *Significantly different ($p < 0.05$, Turkey, ANOVA). NS: not significant.

CHOP protein as well as the ATF4 protein at adaptive concentrations of γ-TQ, although the induction levels of these proteins were relatively lower than that of toxic conditions (Figure 6A). It is well known that the ER stress response has properties like a double-edged sword: it can induce cell death as well as a set of adaptive pathways leading to the improvement of ER dysfunction [44]. Collectively, these findings suggest that the adaptive response induced by γ-TQ is mediated through the ER stress response. It has been reported that ER stress can be triggered by disparate perturbations in normal ER function such as the accumulation of unfolded, misfolded or excessive protein; ER lipid or glycolipid imbalances; or changes in the redox or ionic conditions of the ER lumen [44]. It is interesting to investigate the molecular mechanisms by which arylating γ-TQ can induce the ER stress response.

Due to its reactive α,β-unsaturated carbonyl functional group, 15d-PGJ_2 is able to react with cellular DNA and proteins, including Keap1, which is a cytoplasmic inhibitor of Nrf2, through the Michael addition reaction [14,45]. On the other hand, the role of Nrf2 in the PERK signalling pathway has been reported [43,46,47]. Nrf2 is a substrate of PERK and ER stress induces Nrf2 nuclear translocation in a PERK-dependent manner [43,46]. It has also been reported that ATF4 interacts with Nrf2 in a cell type-dependent manner [47]. In fact, we observed that treatment with γ-TQ induced the accumulation of nuclear Nrf2 (Supplementary material, Figure SD). However, we could not observe the involvement of Nrf2 in GSH elevation induced by γ-TQ (Figure 5). In addition, HO-1 induction was also not observed in γ-TQ treated cells (Supplementary material, Figure SA); this was not the case for Nrf2-activators such as 15d-PGJ_2 and 4-HNE [30,31]. The details of the different induction mechanisms of γ-TQ and 15d-PGJ_2 are still unclear. It has also been reported that Nrf2 activation could be regulated by several upstream protein kinase pathways, including mitogen-activated protein kinases, protein kinase C and phosphatidylinositol 3-kinase, which modify either Keap1 or Nrf2 through phosphorylation and disassociate Nrf2 from Keap1 [30,48,49]. It has also been known that the induction of gene expression through the Nrf2-EpRE signalling pathway is regulated via interaction with other transcriptional factors such as a small Maf protein after translocation to the nucleus [14]. Therefore, it appears that the activation of different kinase pathways and/or binding to partner proteins in the nucleus is considered as candidates for the difference between γ-TQ and 15d-PGJ_2.

Furthermore, arylating BzQ-induced expression of *xCT* was significantly attenuated in cells treated with Nrf2-siRNA (Figure 7B). Collectively, these findings suggest that γ-TQ specifically induce adaptive response via the activation of ATF4. In contrast to our study using PC12 cells, it has been reported that cytotoxicity of arylating BzQ against N2A cells is lower than that of γ-TQ [5]. It has been reported that BzQ can induce cell death via ER stress response [5]; however, the detailed molecular mechanism of BzQ-induced adaptive response has not been investigated. γ-TQ is more lipophilic than BzQ and it is thought γ-TQ, but not BzQ, might distribute to microsomal membrane fraction similar to lipophilic vitamin E of which cellular distribution is linearly correlated with

lipid contents [8]. It appears that the different lipid solubility is considered as a reason for the difference between γ-TQ and BzQ.

Cornwell's research group previously demonstrated the nature of γ-TQ, including the fact that γ-TQ has concentration-dependent cytotoxic as well as antioxidative effects [1,5,29,40]. In the present study, we detected not only α- and γ-TQ (1.2 and 0.5 nmol/mg protein, respectively) but also their corresponding reduced forms, namely, α- and γ-TQH_2 (0.7 and 0.7 nmol/mg protein, respectively) in PC12 cells cultured for 24 h in a serum medium containing 8 μM of quinone. Therefore, the cytoprotective effects observed in the α-TQ-treated cells were primarily ascribed to α-TQH_2 generated inside the cells. In addition, it is thought that the generated γ-TQH_2 also plays a significant role in the protective effect resulting from γ-TQ treatment. It is considered that numerous antioxidants with different molecular weights, solubilities and localizations form a network called a cellular antioxidant system and that an increase in the levels of TQH_2 and GSH might contribute to this system. In fact, BSO treatment decreases the cellular GSH levels, thereby weakenig cell tolerance against oxidative stress. In the presence of BSO, γ-TQ treated cells with an increase in the levels of both TQH_2 and GSH exhibited a greater cytoprotective effect than that of α-TQ treated cells in which only the level of TQH_2 was increased (Figure 2C and 3D). These results indicate the beneficial effects of the γ-TQ treatment; this treatment resulted in an increase in the levels of both TQH_2 and GSH as compared to the α-TQ treatment. Furthermore, we attempted to examine the effect of ATF4 knockdown on the cytoprotective effect induced by γ-TQ; however, the expected decrease in cytoprotection was not observed (data not shown). We observed a high cytotoxicity of γ-TQ in siRNA-treated cells (significant decrease in cell viability was observed at 2 μM, data not shown) and this may be one of the reasons for the discrepancy. Furthermore, it is considered that the formation of TQH_2 might complicate this experimental condition.

Although both γ-TQ and γ-TQH_2 were detected in γ-TQ-treated cells, it is known that the amount of γ-TQ relative to its precursor γ-T found in living tissues is much lower than that of α-TQ relative to α-T found in tissues, even in animals whose diet was supplemented with γ-T [50]. The TQ content of chicken liver tissues is reported as follows: α-TQ, 1.47 pmol/mg protein and γ-TQ, 0.085 pmol/mg protein [51]. Therefore, it is thought that the cellular concentrations in TQ-treated PC12 cells were considerably higher than those in the tissues *in vivo*. TQ is produced via the free radical-mediated oxidation of T. Our observations suggest that at the initial time of oxidative stress, generation of γ-TQ, not α-TQ, serves as a feedback that transmits an SOS signal and warns cells of the oxidative status, thereby triggering early response enzymes or proteins to cope with the forthcoming oxidative insults. However, in this study, there are still considerable differences between the concentration of TQ in the cells and that in the tissues *in vivo*. Further studies on the identification of the direct target of γ-TQ and the reaction site are necessary to clarify the physiological significance of the γ-TQ-induced adaptive response.

In conclusion, the present study demonstrated that γ-TQ, not α-TQ, induced adaptive responses that increased the cellular GSH levels via the upregulation of cysteine availability in an ATF4-dependent manner. The present results indicate the possibility that antioxidants can function as an oxidative stress sensor. The physiological significance of such beneficial effects of lipid oxidation products and the significance of antioxidants should be explored in a future study.

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Supplementary materials

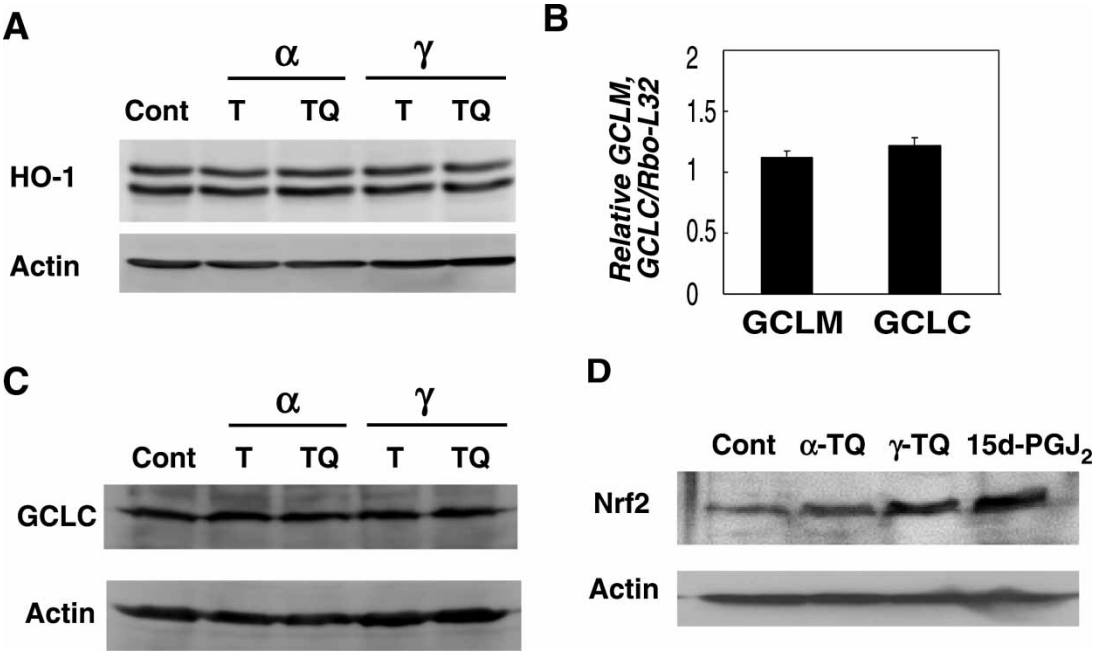

Figure S1. (A) Effects of γ-TQ on the HO-1 level. PC12 cells were treated with 8 μM vitamin E derivatives for 24 h and subjected to Western blot analysis (25 μg protein per lane). (B, C) Effects of γ-TQ on the GCL level. PC12 cells were treated with 8 μM γ-TQ for 8 h (A) or 24 h (B, 25 μg protein per lane). (D) Effect of γ-TQ on the nuclear level of Nrf2. PC12 cells were treated with 8 μM γ-TQ, 8 μM γ-TQ, or 7.5 μM 15d-PGJ_2 for 8 h and the nuclear fractions were extracted for Western blot analysis (50 μg protein per lane).

Table. Effect of TQ on TR, NQO and GCL enzyme activity.[a]

	TR	NQO1	Total NQO	GCL
Control	17±3.1	110±19	140±21	29±2.3
α-TQ	19±4.5	120±28	160±31	37±4.3
γ-TQ	22±5.6	120±14	150±20	30±5.0

(μmol/min/mg protein)

[a] PC12 cells were cultured with or without 8 μM α-TQ or γ-TQ for 24 h and harvested for enzyme activity analysis. Mean values of enzyme activity are shown with standard error.