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Synergistic neurotoxicity of oxygen-glucose deprivation and tetrabromobisphenol A *in vitro*: role of oxidative stress

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Abstract:
Background: Tetrabromobisphenol A (TBBPA) is a toxic brominated flame retardant. Previous studies have demonstrated that exposure of primary cultures of rat cerebellar granule cells (CGC) to ≥ 10 μM TBBPA induces toxicity and excitotoxicity, and the underlying mechanism may involve calcium imbalance and oxidative stress. Here we examined whether the application of TBBPA at subtoxic concentrations may exacerbate acute damage of CGC challenged with oxygen-glucose deprivation (OGD), and evaluated with fluorescent indicators the involvement of calcium imbalance, mitochondrial depolarization and oxidative stress.
Methods: Survival of CGC was assessed 24 h after OGD/TBBPA using fluorescent dyes. An OGD challenge lasting for 45, 60 or 75 min induced a duration-dependent injury to the neurons.
Results: Application of 2.5, 5 or 7.5 μM TBBPA for 45 min to normoxic and glucose-containing incubation medium did not reduce the viability of cultured CGC, but this compound exacerbated the toxic effects of OGD in a concentration-dependent way. Moreover, TBBPA had a slight effect on calcium homeostasis and mitochondrial membrane potential, but significantly activated the production of reactive oxygen species in CGC. The application of H_2O_2 at 5, 10 and 25 μM mimicked the effects of TBBPA on OGD toxicity, while 0.1 mM ascorbic acid or 1 mM glutathione ameliorated this toxicity.
Conclusion: These results suggest the involvement of oxidative stress in the synergistic neurotoxic effects of TBBPA and OGD.

Key words:
brominated flame retardants, calcium, environmental pollutant, mitochondrial membrane potential, neuronal cultures, oxidative stress

Abbreviations: BFR – brominated flame retardants, BME – basal Eagle medium, CGC – cerebellar granule cells, DCF – 2',7'-dichlorofluorescein; DCFH – 2',7'-dichlorodihydrofluorescein, DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate, DMSO – dimethyl sulfoxide, DPM – disintegrations per minute, GSH – glutathione, OGD – oxygen-glucose deprivation, R123 – rhodamine 123, ROS – reactive oxygen species, TBBPA – tetrabromobisphenol A

Introduction

Tetrabromobisphenol A (TBBPA) is a representative of the brominated flame retardants (BFR), a group of substances that are commonly used in the electronic, textile and building industries to reduce the flamma-

bility of various products. The widespread use of TBBPA has resulted in environmental pollution caused by this compound (for reviews see [2, 8]). For example, increased levels of TBBPA were found in samples of air, soil and sediments [4], and more recently in fresh waters collected in the vicinity of BFR manufacturing plants [26]. Moreover, TBBPA has been detected in fish, oysters and human body fluids [4, 36].

The results of numerous studies have demonstrated the toxic properties of TBBPA, including its immunotoxicity and endocrine-disrupting abilities [18, 27]. Experiments performed *in vivo* have shown that the acute exposure of adult mice to TBBPA results in its selective accumulation in the striatum, which is accompanied by significant behavioral effects [24]. There are also indications that TBBPA may act as a developmental neurotoxin [20], although there has been a lack of consistency in studies describing the neurodevelopmental effects of TBBPA [38]. Studies performed *in vitro* using various cell cultures demonstrated the cytotoxicity of TBBPA and have identified several putative mechanisms, including disturbances in the intracellular signaling pathways, calcium imbalance, oxidative stress and excitotoxicity in neurons [5, 22, 25, 27, 28]. In light of the above evidence, there is growing concern about the possible toxic effects of TBBPA.

Previous *in vitro* studies demonstrated the cytotoxic effects of subacute, 18–24 h exposure of cell cultures to TBBPA at concentrations exceeding 10 μM [25, 28]. Although TBBPA applied at lower micromolar concentrations does not by itself induce any detectable neuronal death, we hypothesize that such treatment might be injurious to neurons exposed simultaneously to other pathogenic conditions. Brain ischemia seems to be the most suitable model for testing this hypothesis, since the pathogenic mechanisms that participate in the ischemia-evoked neuronal death comprise excitotoxicity, calcium imbalance and oxidative stress [11, 29], and the same factors have been implicated in TBBPA toxicity for cultured neurons [28]. We speculate that the brain damage induced by ischemia might be significantly exacerbated in subjects exposed to low, nominally subtoxic doses of TBBPA. If so, such an effect should also be detectable in the well characterized *in vitro* model of the ischemic conditions.

The aim of the present study was to determine whether TBBPA applied to cultured neurons at concentrations that do not induce neuronal death, exacerbates the toxicity of the ischemia-like insult. In these experiments we used a primary cerebellar granule cells (CGC) culture as the neuronal model and the ischemia-resembling conditions of oxygen-glucose deprivation (OGD) to induce neuronal death (for review see [13]). To identify putative mechanisms of the expected potentiation of the OGD-evoked cytotoxicity by TBBPA, we studied the effects of TBBPA applied at subtoxic concentrations on ^{45}Ca uptake, intracellular Ca^{2+} concentration, mitochondrial membrane potential and free radical formation.

Materials and Methods

Animals

Primary cultures of CGC were prepared from 7-day-old Wistar rats of the outbred stock Cmd:(WI)WU. About 100 animals were used in the experiments described in this study. The use of experimental animals was approved by the Fourth Local Ethical Committee in Warszawa. In accordance with EC Directive 86/609/EEC of 24 November 1986, all efforts were made to reduce the number of animals used and to minimize their suffering.

Drugs

TBBPA was obtained from LCG Standards Sp. z o.o. (Łomianki, Poland). Hydrogen peroxide was purchased from POCH SA (Gliwice, Poland), DMSO, L-ascorbic acid, L-glutathione reduced, as well as fetal bovine serum and other materials for cell culture were purchased from Sigma Chemical Poland (Poznań, Poland). The fluorescent dyes calcein AM, ethidium homodimer-III, fluo-3 AM, rhodamine123 and DCFH-DA were obtained from Molecular Probes Inc. (Paisley, UK). All other chemicals were of analytical grade. $^{45}CaCl_2$ was produced by Polatom Sp. z o.o., Otwock – Świerk, Poland. Fresh stock solutions of TBBPA in DMSO or glutamate, H_2O_2, GSH and ascorbic acid in water were prepared for each experiment. The final concentration of DMSO was 0.5%. Two control groups were included in the experiments: "0.5% DMSO control" was a vehicle control group for TBBPA, while the "control" group received no DMSO and served for comparison with the effects of glutamate and H_2O_2.

Cell culture

A standard method of CGC culturing, as described by Schousboe et al. [30], was used, with slight modifications [40, 42]. Briefly, the cerebella were collected, chopped, tripsinized and shredded. A cell suspension was prepared in basal Eagle medium (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 4 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml). Finally, 12-well plates coated with poly-L-lysine (NUNC) were seeded at a density of 2×10^6 cells per well. The cultures were incubated under standard conditions in air with 5% CO_2 at 37°C and 95% humidity in an Assab Kebo 1970 CO_2 incubator (Kebo Biomed, Spånga, Sweden). Two days after plating, 7.5 μM cytosine arabinofuranoside was applied in order to prevent the replication of non-neuronal cells. All the experiments on CGC were performed at 7th day *in vitro*.

Acute cytotoxicity: effects of TBBPA, H_2O_2 and OGD

We carried out two main types of cytotoxicity tests. In one, cultures were incubated for 45, 60 or 75 min at 37°C in the CO_2 incubator with different concentrations of TBBPA or H_2O_2 under aerobic conditions with 5% CO_2 in 5 mM glucose-containing medium (Fig. 1A, Fig. 2A, B, Fig. 7). In the other, cultures were submitted to OGD alone, or were exposed simultaneously to OGD and TBBPA, H_2O_2, or the ROS scavengers ascorbic acid or GSH (Fig. 1B, Fig. 2C, D, Fig. 7, Fig. 8). In both cases, tests were performed by replacing the growth medium with OGD buffer (116 mM NaCl, 25 mM KCl, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, pH 7.4). TBBPA was applied at concentrations of 2.5, 5 or 7.5 μM. For the OGD treatment applied alone or in combination with exposure to TBBPA, CGC cultures were incubated at 37°C in the OGD medium without glucose and in an atmosphere containing 0% O_2, 5% CO_2 and 95% N_2 maintained by a system consisting of C-chamber, Proox Model 110 and ProCO_2 Model 120 gas controllers (BioSpherix, Lacona, NY, USA). The incubation was terminated by replacing the OGD medium with the original growth medium and culturing was continued for 24 h at 37°C under standard aerobic conditions with 5% CO_2. The cells were then fixed with 80% methanol, stained with 0.5 μg/ml propidium iodide and viable and dead neurons were counted using a fluorescence microscope (Zeiss-Axiovert, Germany). Results were expressed as the percentage of live cells. In the experiments reported in Fig. 2, we used two fluorescent dyes, calcein AM and ethidium homodimer-III, for simultaneous staining of live and dead cells, respectively. The staining and visualization procedures were exactly as described previously [19].

^{45}Ca uptake

The CGC were pre-incubated at 37°C for 10 min in Locke 5 medium containing 154 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5 mM HEPES (pH 7.4) and 5 mM glucose [40]. Then, TBBPA at different concentrations or the vehicle (0.5% DMSO) were added together with radioactive calcium (1 μCi/well). After 10 min incubation at 37°C the cells were washed with ice-cold calcium-free medium containing 2 mM EGTA, lysed in 0.5 M NaOH and radioactive uptake was measured using a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

Intracellular Ca^{2+} concentration

The CGC in the original growth medium were incubated for 30 min at 37°C in the presence of the fluorescent calcium-sensitive probe 16 μM fluo-3 AM and washed three times in Locke 5 buffer [40]. The fluorescence of cells incubated at 37°C in this buffer, supplemented with different concentrations of TBBPA, the vehicle 0.5% DMSO or with the positive control 100 μM glutamate, was recorded every 1 min for 30 min, using a microplate reader (FLUOstar Omega, Germany) at 485 nm excitation and 538 nm emission wavelengths.

Changes in mitochondrial membrane potential

The fluorescent probe rhodamine123, which is accumulated and bound inside polarized mitochondria, was used to evaluate changes in the mitochondrial membrane potential. Depolarization of mitochondria results in the efflux of rhodamine from mitochondria, and its consequently increased concentration in the cytosol causes a rise in fluorescence [3]. CGC cultures were treated with rhodamine123 (10 μM) at 37°C for 30 min. The cells were then washed with Locke 5 buffer and incubated at 37°C with different concentrations of TBBPA, 0.5% DMSO or 100 μM glutamate. Fluorescence was measured every 30 s for

32 min using a microplate reader (Fluoroscan, LabScan, Finland) at 485 nm excitation and 538 nm emission wavelengths [44].

Production of reactive oxygen species (ROS)

Formation of ROS in CGC evoked by TBBPA and OGD was evaluated using the fluorescent dye DCFH-DA. As has been described previously by others [1], DCFH-DA diffuses into cells where it is cleaved by esterases to produce free non-fluorescent DCFH which is entrapped inside the cells and may be oxidized by ROS to form the fluorescent DCF. CGC cultures were treated with 100 μM DCFH-DA at 37°C for 30 min. After washing in Locke 5 buffer, the cells were incubated with different concentrations of TBBPA, 0.5% DMSO or 100 μM glutamate at 37°C. In the experiments reported in Fig. 8, after loading with DCFH, the cells were challenged for 45 min with OGD in the presence of 5 μM TBBPA and/or 1 mM GSH or 0.1 mM vitamin C. Fluorescence of DCF was recorded every 5 min for 90 min (Fig. 6) or was measured once immediately after OGD (Fig. 8), using a microplate reader (FLUOstar Omega, Germany) at 485 nm excitation and 538 nm emission wavelengths.

Data analysis

The results are presented as the mean ± SD, with a sample size n given in legends to figures. For statistical comparisons between the analyzed groups we employed one-way ANOVA followed by Dunnett's test, two-way ANOVA followed by the Holm-Sidak test for concentration and time of OGD duration, and repeated measures ANOVA followed by one-way ANOVA for treatment and experimental time points as factors. Differences between means were considered significant at $p < 0.05$. Statistical analyses were carried out using Statistica v. 7 (StatSoft Inc.).

Results

Effects of TBBPA and OGD on CGC viability

The application of TBBPA at concentrations of 2.5, 5 or 7.5 μM had no detectable toxic effect on neurons incubated in the glucose-containing aerobic medium (Fig. 1A). OGD lasting 45, 60 or 75 min resulted in a drastic decrease in the number of surviving neurons, which was dependent on OGD duration (Fig. 1B). This OGD toxicity was additionally potentiated by the administration of TBBPA, and this effect was statistically significant when concentrations of 5 or 7.5 μM TBBPA were used.

It should be noted that a pronounced (about 30%) decrease in the number of live neurons as compared to untreated cultures was seen after aerobic incubation of CGC in the OGD buffer supplemented with glucose and containing 0.5% DMSO, the TBBPA vehicle (Fig. 1A). Since the duration of incubation, which varied from 45 min to 75 min, had no effect on the number of surviving cells, this effect was most probably caused by physical cell injury that occurred when changing the media, and not by the incubation conditions, including the presence of 0.5% DMSO.

A synergistic toxic effect of OGD and TBBPA was also observed using calcein and ethidium homodimer-III staining, when TBBPA was applied to CGC at a nominally subtoxic concentration (Fig. 2). The negligible toxicity of 7.5 μM TBBPA (Fig. 2B) in comparison with the vehicle control (Fig. 2A) contrasts with the evidently increased proportion of dead cells in CGC challenged with OGD for 45 min (Fig. 2C), and to an even greater extent with the effect of treatment with a combination of 45 min OGD and 7.5 μM TBBPA (Fig. 2D).

Changes in calcium homeostasis in TBBPA-treated CGC

We next investigated TBBPA-induced disturbances in calcium homeostasis in CGC, which may be responsible for the enhancement of OGD toxicity caused by this compound when administered at low micromolar concentrations. To evaluate calcium influx into neurons from the extracellular compartment, which could result from the activation of different calcium channels including NMDA receptors, we measured ^{45}Ca uptake (Fig. 3). TBBPA at concentrations of 2.5, 5 or 7.5 μM did not activate ^{45}Ca uptake. Rather, the lowest TBBPA concentration tended to suppress uptake, while the two higher concentrations significantly inhibited this effect.

The intracellular calcium level was then measured using the fluorescent calcium-sensitive probe fluo-3 (Fig. 4). In CGC treated with TBBPA at 2.5 and 5 μM, the observed changes in fluorescence were practically the same as in the vehicle control. However, applica-

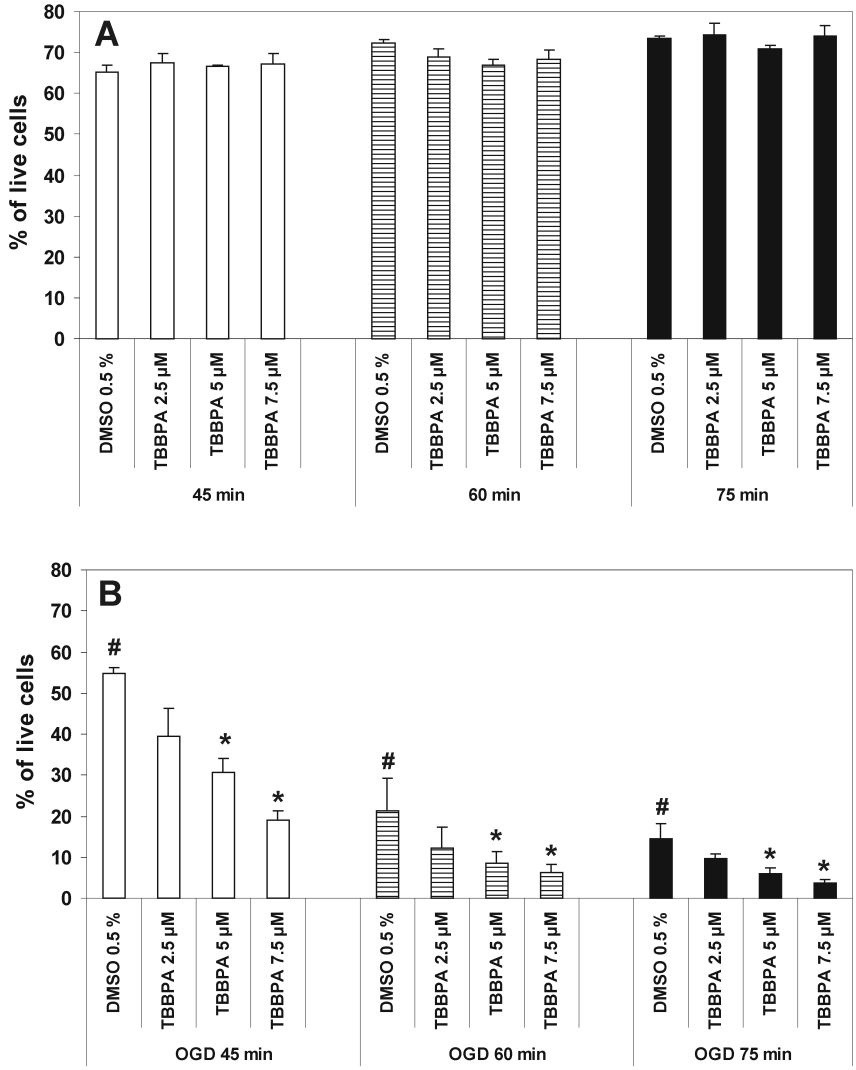

Fig. 1. TBBPA- and OGD-induced toxicity in primary cultures of rat CGC. The effect of 2.5, 5 or 7.5 µM TBBPA alone on cell viability (**A**) was studied in ionic medium containing glucose under aerobic conditions. The effect of TBBPA at the same concentrations on OGD-evoked neuronal damage was studied in anaerobic glucose-deficient medium (**B**). Control cultures were incubated in ionic medium with the vehicle (0.5% DMSO). The control for OGD was incubated in the presence of glucose under aerobic conditions (**panel A**). After incubation for the periods indicated, the cells were cultured in the original growth medium under standard aerobic conditions for 24 h, and the number of surviving neurons was determined by propidium iodide staining. Data represent the percentage change compared to untreated cultures. The means ± SD from six independent experiments (n = 6). # Effects of OGD without TBBPA significantly different from the corresponding glucose-containing aerobic control presented in **panel A**. * Effects of TBBPA significantly different from the 0.5% DMSO control, (two-way ANOVA followed by Holm-Sidak test for concentration and time of OGD duration, $p < 0.05$)

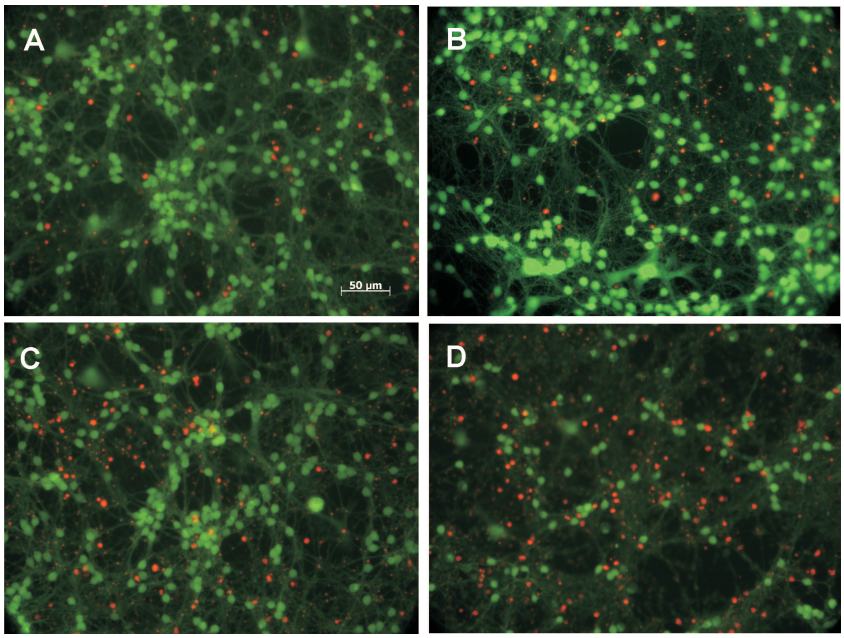

Fig. 2. Acute exposure to TBBPA at low micromolar concentration exacerbates OGD neurotoxicity in primary cultures of rat CGC. To evaluate TBBPA toxicity, the cells were incubated for 45 min in the aerobic medium containing glucose and 0.5% DMSO (**A**), or in the same medium with 7.5 µM TBBPA (**B**). OGD was induced in the anaerobic medium without glucose, with 0.5% DMSO (**C**), or with 7.5 µM TBBPA (**D**). The survival of neurons was assessed 24 h after the insult using calcein/ethidium homodimer-1 staining, which visualizes viable (green) and dead (red) neurons, respectively. Horizontal bar indicates magnification. Data from one representative experiment. The same experiments were repeated three times using separate cultures and gave consistent results

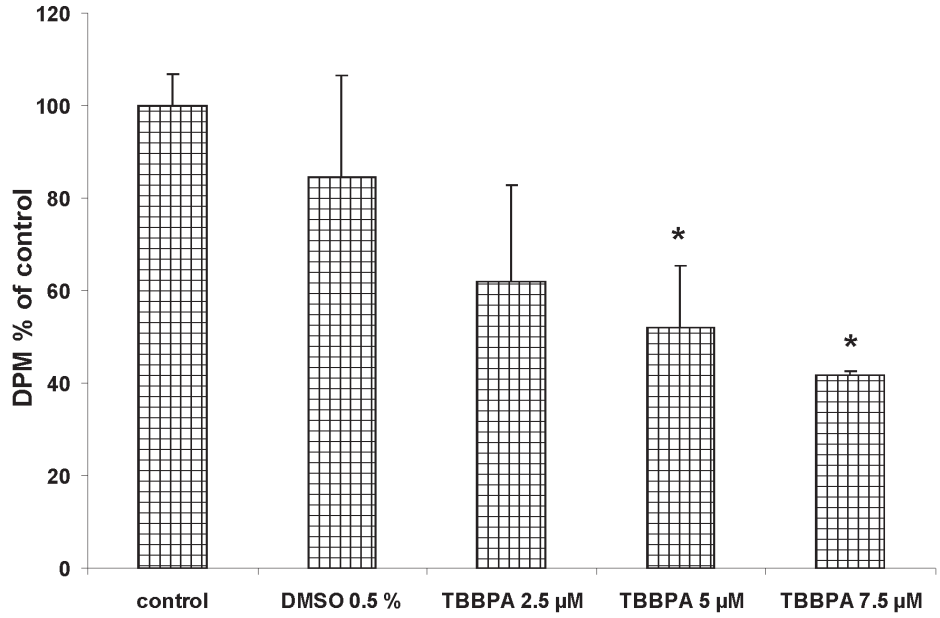

Fig. 3. Concentration-dependent effects of TBBPA on ^{45}Ca uptake in primary cultures of rat CGC. Cells were incubated for 10 min in the presence of ^{45}Ca, 0.5% DMSO or 2.5, 5 or 7.5 µM TBBPA, as indicated. Results are expressed as ^{45}Ca accumulated, measured in DPM as a percentage of the control. The means ± SD from six independent experiments (n = 6). * The means significantly different from the 0.5% DMSO control (one-way ANOVA followed by Dunnett's test, $p < 0.05$)

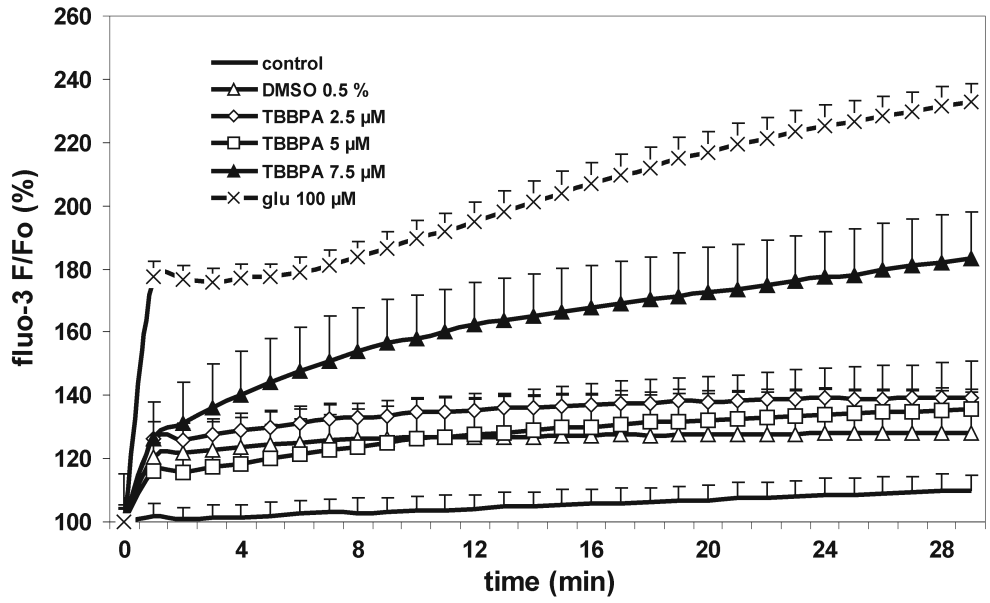

Fig. 4. Effects of TBBPA on the intracellular Ca^{2+} concentration in primary cultures of rat CGC. Following measurement of fluorescence of the fluo-3 loaded neurons incubated under steady state conditions for 60 s to obtain the basal value, 2.5, 5 or 7.5 µM TBBPA, the vehicle (0.5% DMSO) or 100 µM glutamate (glu) were administered as indicated. Changes in the intracellular Ca^{2+} concentration were evaluated by measuring fluo-3 fluorescence and expressed relative to the basal level (fluo-3 F/F_0) as a percentage. The means ± SD for six wells per treatment tested in one representative experiment (n = 6). The same experiments were repeated three times using separate cultures and gave consistent results. The results representing the effects of 7.5 µM TBBPA and the 0.5% DMSO control measured at the same time points differed significantly from the 10^{th} min to the end of the experiment. The effect of 100 µM glutamate was significantly different from the untreated control, from the first min to the end of the experiment. Repeated measures ANOVA, followed by one-way ANOVA for treatment and experimental time points as factors were used for comparisons between the analyzed groups ($p < 0.05$)

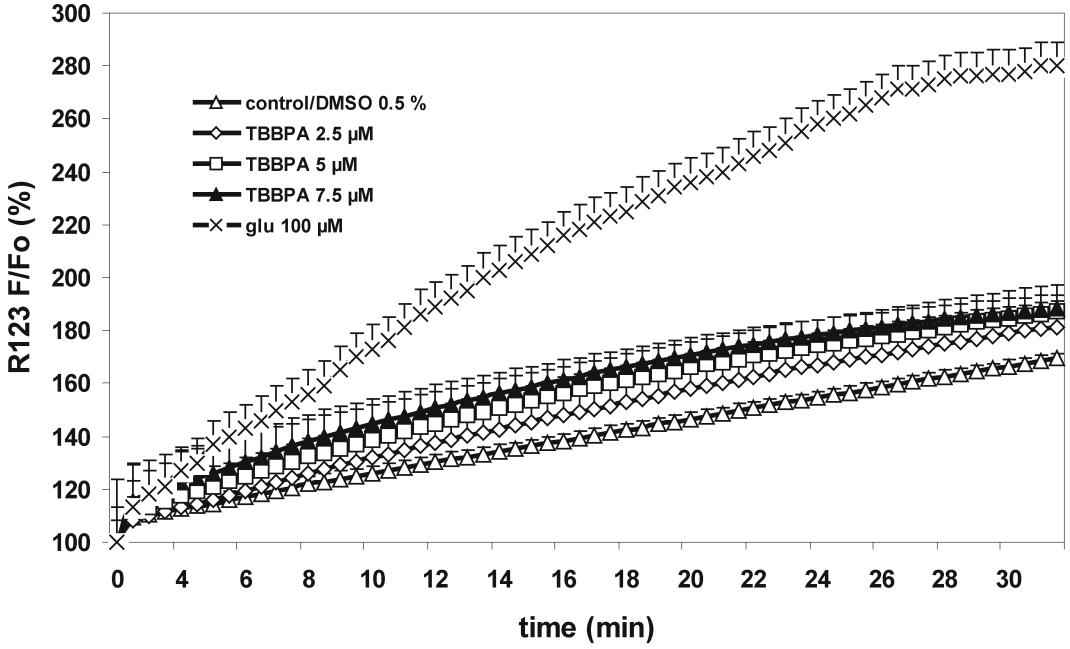

Fig. 5. Changes in the mitochondrial membrane potential in primary cultures of rat CGC exposed to TBBPA at low micromolar concentrations. The basal fluorescence of cells loaded with rhodamine 123 (R123) was measured after 60 s, then the incubation was continued without additions (control without DMSO), or following the application of 0.5% DMSO, 2.5, 5 or 7.5 µM TBBPA, or 100 µM glutamate (glu), as the positive control. Increases in R123 fluorescence reflecting a reduction in the mitochondrial membrane potential are expressed relative to the basal level (R123 F/F_0) as a percentage. The means ± SD for six wells per treatment tested in one representative experiment (n = 6). The same experiments were repeated three times using separate cultures and gave consistent results. Application of DMSO did not affect R123 fluorescence, so for clarity a common line representing the controls is shown. At all time points after 20 min of incubation, the effect of TBBPA, at all concentrations used, differed significantly from the 0.5% DMSO control, whereas the effect of glutamate differed significantly from the untreated control after 5 min and from TBBPA treatment after 9 min of incubation (one-way ANOVA, $p < 0.05$)

tion of TBBPA at 7.5 µM resulted in a significant increase in the intracellular calcium level. This was still much lower than the increase in intracellular calcium induced by 100 µM glutamate, applied as a positive control. The dynamics of these effects were also different.

Mitochondrial membrane potential in CGC treated with TBBPA

A decrease in the mitochondrial membrane potential is a sensitive marker of pathological changes which may result in cell death. Thus, we determined the effects of TBBPA on the mitochondrial membrane potential of CGC by evaluating rhodamine 123 (R123) fluorescence (Fig. 5). Application of TBBPA at concentrations of 2.5, 5 or 7.5 µM resulted in a statistically significant but relatively weak increase in the R123 fluorescence compared to the 0.5% DMSO vehicle control, which did not influence this fluorescence. This effect of TBBPA tended to be concentration dependent. It is noteworthy that the administration of 100 µM glutamate as the positive control resulted in a very pronounced increase in R123 fluorescence compared to the corresponding control without DMSO, greatly exceeding the effects of TBBPA. These results demonstrated the involvement of mitochondrial depolarization in the mechanism of excitotoxicity, and the rather stable mitochondrial membrane potential in CGC treated with TBBPA at low micromolar concentrations.

ROS production in TBBPA-treated CGC: role in cytotoxicity

ROS production reflecting oxidative stress is one of the putative TBBPA-induced pathological processes that may be implicated in neuronal death. To evaluate the effect of low micromolar concentrations of TBBPA on the production of ROS, we used the probe 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and measured accumulation of the fluorescent product 2',7'-dichlorofluorescein (DCF) in CGC (Fig. 6). TBBPA applied at concentrations of 2.5, 5 or 7.5 µM

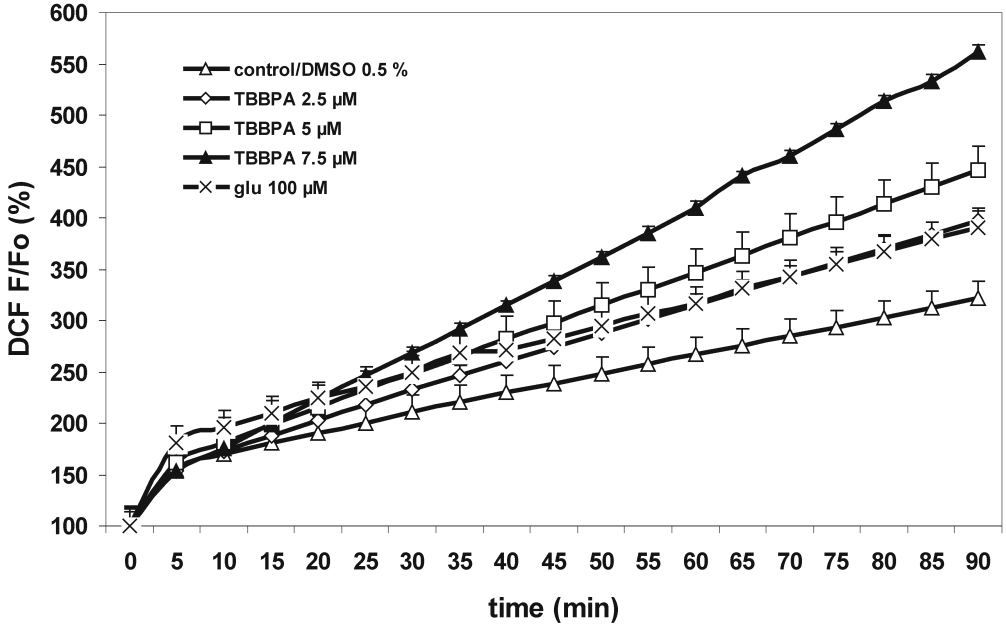

Fig. 6. Effect of TBBPA on the generation of reactive oxygen species (ROS) monitored with the fluorescent probe DCFH-DA in primary cultures of rat CGC. The basal DCF fluorescence of cells loaded with DCFH was measured after 5 min, then the incubation was continued without additions (control without DMSO), or following the application of 0.5% DMSO (vehicle control), 2.5, 5 or 7.5 µM TBBPA, or 100 µM glutamate (glu). Increases in DCF fluorescence indicating enhanced ROS production are expressed relative to the basal level (DCF F/F_0) as a percentage. The application of DMSO did not affect DCF fluorescence, so for clarity a common line representing the controls is shown. The means ± SD for six wells per treatment tested in one representative experiment (n = 6). The same experiments were repeated three times using separate cultures and gave consistent results. At all the time points after 40 min of incubation, the effects of TBBPA at all concentrations used, and that of glutamate, differed significantly from the 0.5% DMSO and untreated controls, respectively (one-way ANOVA, $p < 0.05$)

significantly enhanced DCF fluorescence compared to the 0.5% DMSO vehicle control, which did not influence this fluorescence. The application of 100 µM glutamate caused a moderate stimulation of ROS production compared to the control. The concentration-dependent rise in DCF fluorescence in TBBPA-treated CGC indicated enhanced ROS production.

The ability of TBBPA at low micromolar concentrations to induce oxidative stress in CGC suggested that it plays a role in the synergy between TBBPA and OGD in causing cytotoxicity. To test this hypothesis, we examined whether CGC under OGD conditions are more susceptible to oxidative stress than control cells, and if exogenous antioxidants can reverse this synergistic effect. First, we compared the toxic effects of hydrogen peroxide applied to CGC incubated under aerobic conditions in glucose-containing medium with its cytotoxicity under OGD (Fig. 7). Initial control experiments demonstrated a concentration-dependent increase in ROS production in CGC treated with the applied concentrations of H_2O_2 (results not shown). The application of 5, 10 and 25 µM H_2O_2 for 45 min to CGC had no effect on the number of surviving neurons when incubation was performed in the presence of glucose under aerobic conditions. However, under OGD conditions, H_2O_2 induced a significant, concentration-dependent potentiation of cytotoxicity. Thus, in our experiments, 2.5, 5 or 7.5 µM TBBPA (Fig. 1) and 5, 10 or 25 µM H_2O_2 (Fig. 7) similarly enhanced OGD toxicity. We then demonstrated that the cytotoxic effects of OGD alone or induced in the presence of 5 µM TBBPA may be almost completely reversed by application of the ROS scavengers 0.1 mM ascorbic acid or 1 mM GSH (Fig. 8A). Consistent with these findings, the accumulation of DCF (i.e., ROS production) in neurons incubated under OGD conditions was slightly reduced in the presence of 1 mM GSH (Fig. 8B). Moreover, the administration of 5 µM TBBPA under OGD increased the level of DCF, and this effect was reversed in the presence of 1 mM GSH or 0.1 mM ascorbate. Thus, the synergy between TBBPA and OGD may be prevented by antioxidants and ROS scavengers, and hydrogen peroxide mimics this effect of TBBPA, which strongly suggests that oxidative stress is involved in the mechanism underlying this phenomenon.

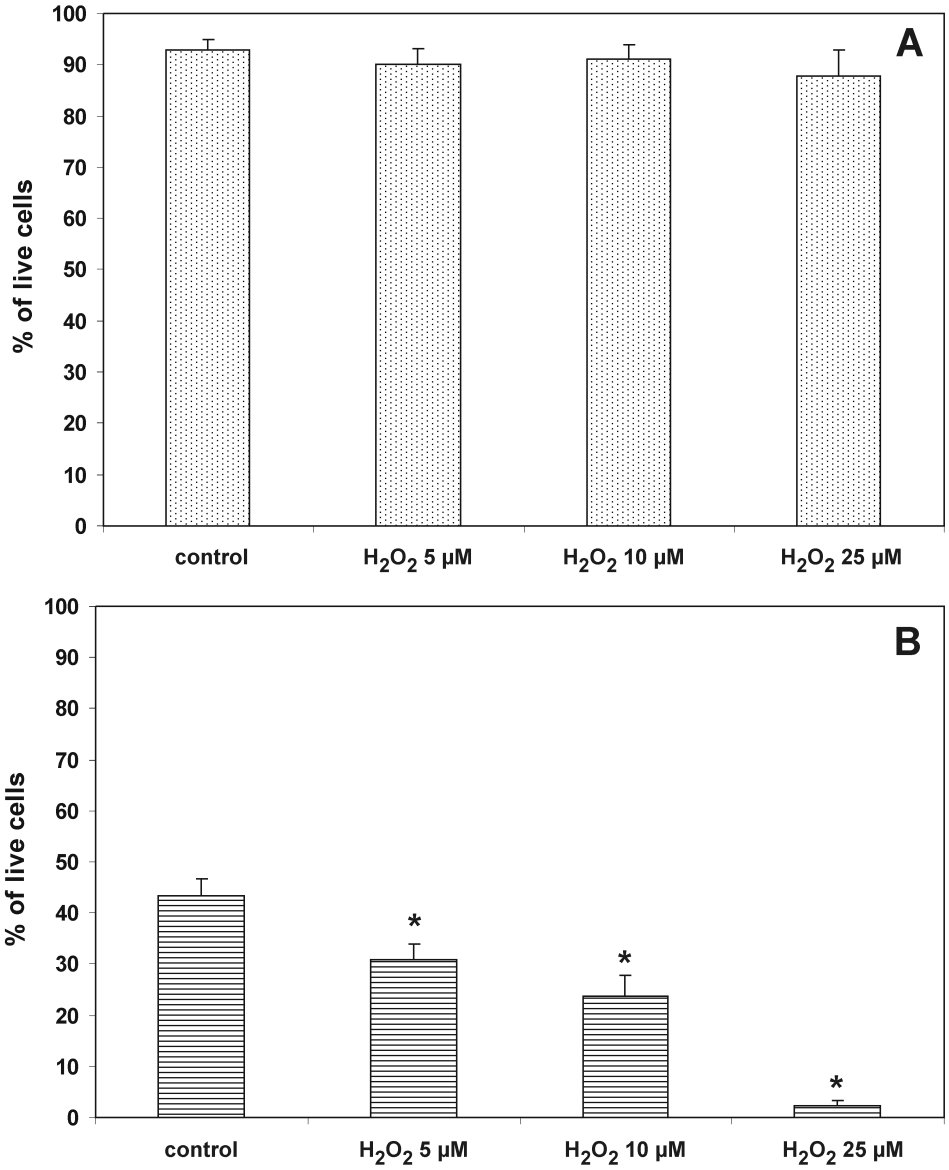

Fig. 7. Hydrogen peroxide- and OGD-induced toxicity in cultured cerebellar granule cells. The effect of 5, 10 or 25 µM H_2O_2 alone on cell viability (**A**) was studied in ionic medium containing glucose under aerobic conditions. The effect of H_2O_2 at the same concentrations on OGD-evoked neuronal damage was studied in anaerobic glucose-deficient medium (**B**). Control cultures were incubated in the ionic medium without the vehicle (0.5% DMSO). After incubation for 45 min at 37°C, the cells were cultured in the original growth medium for 24 h, and the number of surviving neurons was determined by propidium iodide staining. Data represent the percentage change compared to untreated cultures. The means ± SD from six independent experiments (n = 6). * Differences statistically significant *vs.* control (one-way ANOVA followed by Dunnett's test, $p < 0.05$)

Discussion

The present study shows that the acute application of TBBPA at low micromolar concentrations, which by itself does not induce death of *in vitro* cultured CGC, potentiates the cytotoxic effects of OGD on these cells. Moreover, our results suggest that oxidative stress induced by low micromolar concentrations of TBBPA participates in the mechanism underlying this phenomenon.

Primary cultures of rat CGC have previously been used as an *in vitro* experimental model [6], including studies concerning OGD and TBBPA cytotoxicity

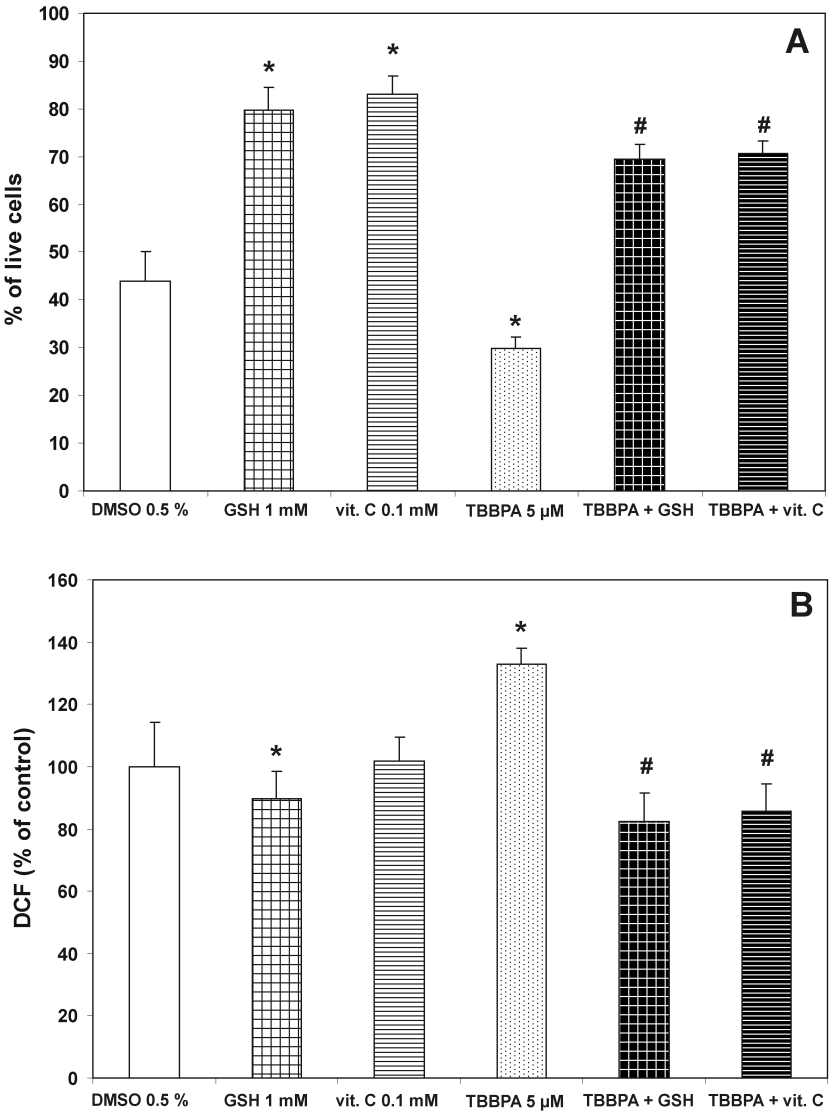

Fig. 8. Effects of the ROS scavengers glutathione and ascorbic acid on TBBPA- and OGD-induced toxicity and ROS accumulation in cultured cerebellar granule cells. The effect of 5 µM TBBPA, 1 mM glutathione (GSH) or 0.1 mM ascorbic acid (vit. C) alone or in combination, on viability of the cells exposed for 45 min at 37°C to OGD (**A**) was studied in anaerobic glucose-deficient medium. Control cultures were incubated in ionic medium with the vehicle (0.5% DMSO). After incubation for the periods indicated, the cells were cultured in the original growth medium for 24 h, and the number of surviving neurons was determined by propidium iodide staining and expressed as the percentage change compared to untreated cultures. ROS accumulation was evaluated in the cultures preincubated with DCFH-DA fluorescent probe (**B**) as described in Fig. 6. The cells loaded with DCFH were incubated under the conditions described above and DCF fluorescence was measured immediately after incubation and expressed relative to the control as a percentage. The means ± SD from six independent experiments (n = 6). * Differences statistically significant *vs.* 0.5% DMSO control. # Differences statistically significant *vs.* 5 µM TBBPA (one-way ANOVA followed by Dunnett's test, $p < 0.05$)

[28, 31, 32]. Our previous research has confirmed that the CGC model is suitable for mechanistic studies on toxicity for neurons caused by excitotoxic challenges, calcium imbalance and mitochondrial dysfunction [40, 42, 43]. To determine whether TBBPA at the nominally subtoxic low micromolar concentrations may exacerbate acute neuronal injury, we utilized OGD, which is a generally accepted *in vitro* model of the pathological conditions resembling brain ischemia. Technically, the application of OGD to cultured cells is achieved by exchanging the oxygenated growth medium for anaerobic ionic incubation medium devoid of glucose. In our present experiments, 45, 60 or 75 min OGD resulted in a significant reduction in the number of live neurons, which was dependent on the duration of these conditions. Previous studies in which CGC have been challenged with OGD used similar durations of this treatment to induce significant neuronal degeneration [14, 16, 32].

Our results show that exposure of CGC incubated under normoxic conditions with glucose to TBBPA at concentrations of 2.5, 5 or 7.5 µM for 45, 60 or 75 min had no effect on neuronal viability. These data, together with previous reports [25, 28, 41] support the notion that TBBPA at concentrations below 10 µM does not induce detectable neuronal death. However, our current study is the first to demonstrate that administration of TBBPA at these nominally subtoxic concentrations potentiates OGD toxicity in CGC in a concentration-dependent manner.

When examining the putative mechanism of TBBPA and OGD synergy we considered calcium imbalance, mitochondrial disfunction and oxidative stress. According to literature data, these processes might be common to both of toxic factors studied here [9, 14, 22, 23, 25, 27, 28, 32]. Therefore, using standard methods we measured ^{45}Ca uptake and changes in fluorescence of the probes fluo-3, R123 and DCF, which are selective for intracellular calcium concentration, mitochondrial membrane potential and ROS production, respectively [15, 27, 28, 34, 40].

We found that the application of TBBPA at concentrations of 2.5, 5 or 7.5 μM did not activate ^{45}Ca accumulation in neurons, but instead induced a slight dose-dependent inhibition of ^{45}Ca uptake. Our previous data demonstrated that in CGC acutely treated with TBBPA, the concentration threshold for inducing ^{45}Ca uptake *via* NMDA receptors and for excitotoxicity is 25 μM. It was demonstrated that in CGC, intracellular Ca^{2+} modulates the activity of K^+ channels and in this way, increased Ca^{2+} concentration may activate these channels, thus decreasing the excitability of neurons and activation of NMDA channels [37]. Consistent with this interpretation, the intracellular calcium level in CGC challenged with 2.5 or 5 μM TBBPA tended to be slightly increased, and this trend reached statistical significance at 7.5 μM TBBPA. Still, this effect represented only a fraction of the calcium transient evoked by 100 μM glutamate. Increases in intracellular calcium level in cell cultures treated with TBBPA have been previously reported [25, 28, 41]. It is known that at low micromolar TBBPA they reflect intracellular calcium release from the ryanodine sensitive stores [25, 41], and that this effect even at 10 μM TBBPA does not induce toxicity in CGC [41]. Thus, our present data show that calcium imbalance induced by TBBPA applied at 2.5, 5 or 7.5 μM is not related to excitotoxicity and its role in the potentiation of OGD toxicity by TBBPA cannot be taken into account.

A similar conclusion may also be drawn with regard to TBBPA-induced changes in the mitochondiral membrane potential. A slight but statistically significant increase in R123 fluorescence evoked by treatment of CGC with 2.5, 5 or 7.5 μM TBBPA, which reflects minor depolarization of the mitochondrial membranes, was negligible comparing to the effect of 100 μM glutamate, which was applied as a positive control. Since the release of endogenous glutamate, calcium imbalance and excitotoxicity, as well as mitochondrial pathology are known to play an essential role in OGD-induced neurotoxicity [9, 10], we believe that the additional weak effect of TBBPA on the mitochondria is irrelevant to the mechanism of TBBPA and OGD synergy.

Consequently, we focused on the potential role of oxidative stress. The ability of TBBPA to activate the formation of ROS has been observed *in vivo* in animal models [35, 39]. Previous studies by Reistad et al. [27, 28] demonstrated that TBBPA administered *in vitro* at low micromolar concentrations significantly increased the fluorescence of DCF in human neutrophil granulocytes and in CGC, which reflects the enhanced production of ROS and the generation of oxidative stress. This finding is corroborated by our present data. It is noteworthy that the intensity of ROS production evoked by 100 μM glutamate is comparable to the effect of 2.5 μM TBBPA, whereas increases of DCF fluorescence evoked by higher TBBPA concentrations were much more pronounced. This indicates that TBBPA at even low micromolar concentrations induces oxidative stress which is more pronounced than that caused by the intensive excitotoxic challenge. Thus, excessive ROS production induced by TBBPA may be a candidate for pathological factor contributing to the mechanism of TBBPA and OGD synergy.

This assumption was supported by evidence obtained using two experimental approaches. Firstly, our results suggest that CGC under OGD conditions are more susceptible to oxidative stress than control cells incubated in glucose containing normoxic medium. In these experiments the application of H_2O_2 at concentrations inducing an increase in ROS production in CGC, but not influencing neuronal viability in glucose and oxygen containing medium, resulted in pronounced exacerbation of OGD cytotoxicity. Furthermore, TBBPA and H_2O_2, two substances enhancing ROS production without reducing the viability of the CGC, when applied acutely to CGC incubated under normoxic and normoglycemic conditions, similarly potentiated OGD cytotoxicity. Hydrogen peroxide was selected for these experiments because Reistad et al. [28] showed that at least some of the TBBPA-induced ROS production in CGC is due to the formation of H_2O_2. Our other experiments demonstrated that the ROS scavengers GSH and ascorbic acid completely prevented the cytotoxic effect of OGD and considerably reduced the cytotoxicity of OGD in the presence of 5 μM TBBPA. Previously, Logan et al. [21] demonstrated that GSH and ascorbate enhance

recovery of primary spinal cord cultures after OGD, but to the best of our knowledge this issue has not been addressed using the CGC model. Reistad et al. [28] examined the effect of antioxidants on TBBPA toxicity in CGC and showed the protective effect of 50 μM vitamin E; however, this was not accompanied by an adequate reduction of ROS production. Our data support the hypothesis that oxidative stress is critical for TBBPA and OGD synergy.

The results of this *in vitro* study may not be directly related to the specific pathology of the brain *in vivo*. In the present study, TBBPA was administered *in vitro* at low micromolar concentrations. Studies conducted in highly industrialized countries like Norway and Japan have shown that the concentration of TBBPA in human serum is at the level of a few ng/g lipid weight [17, 36], which corresponds to low nanomolar concentrations [28]. Moreover, earlier *in vivo* toxicological studies using rodents have indicated that the toxicity of TBBPA is low, with toxic effects and symptoms of oxidative stress developing at doses ≥ 1 g/kg b.w. [7, 35]. The potential of TBBPA as an environmental toxin seems to be low because of its short half-life, of around 2 days [12], and the lack of its biomagnification in the food web [33]. However, more recent data have shown behavioral deficits in mice acutely treated with TBBPA at doses as low as 0.1 or 5 mg/kg b.w. [24]. Clearly, further research is needed to clarify whether in subjects with elevated plasma and brain levels of TBBPA, the course of diseases leading to neuronal damage could be aggravated, and that in cases of acute TBBPA intoxication, which never happened so far, oxidative stress should be considered as an additional target for therapy.

In summary, our study *in vitro* demonstrated a significant exacerbation of OGD-induced toxicity in CGC by TBBPA present at nominally subtoxic low micromolar concentrations. Moreover, our data indicated that TBBPA-induced oxidative stress may be instrumental in this phenomenon. Additional studies are needed to verify hypothesis that there is a potential threat of TBBPA present at even low nominally subtoxic levels when combined with brain ischemia or other neuropathological disorders.

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