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Neuroprotective effects of gabaergic phenols correlated with their pharmacological and antioxidant properties

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ABSTRACT

Aims: Various investigations have demonstrated the protective capacity of general anesthetics as neuroprotective agents. The effects of propofol against ischemia are known to reside in its antioxidant properties and its GABAergic activity. Other aromatic alcohols have also been reported as able to protect neurons against oxidative damage. The aim of this work is to evaluate the potential neuroprotective effect of some phenols, structurally analogues of propofol, with proven GABAergic activity. These phenols include the naturally occurring compounds thymol, carvacrol and eugenol, the synthetic product chlorothymol, and the most widely used intravenous anesthetic, propofol, as a reference compound.
Materials and methods: Taking primary cultures of cortical neurons as a suitable model to evaluate cellular protection against oxidative damage, we developed an injury model to test potential neuroprotective activity. The intracellular hydroperoxides were also determined.
Key findings: The results showed that no compound decreased cell viability at concentrations where they were active on the $GABA_A$ receptor. In neuroprotection tests, some phenols and Vit E showed a partial protective effect against the oxidative injury. These compounds induced a clear tendency to reduce H_2O_2 damage, comparing production of hydroperoxides, although these last changes were statistically non-significant.
Significance: Testing the intracellular oxidation levels suggests that this partial protection exerted by propofol, thymol and chlorothymol may be mediated in some way by their antioxidant activities. However, this neuroprotection is not completely correlated with the antioxidant capacity, but it approaches their relative pharmacological potency, which could be interpreted as a final effect that would involve both activities.

1. Introduction

The study of bioactive natural compounds has contributed significantly to the development of pharmacology and medicine through the analysis of their mechanisms of action, as well as providing models for the design of new therapeutic drugs. Numerous natural neuroactive drugs have particularly contributed to current knowledge in the field of the physiology and pharmacology of the nervous system [1]. Among the many products commonly isolated and investigated are essential oils extracted from plants and their main components such as terpenes, phenols, flavonoids, lactones, etc. In particular, many of these compounds have, among other actions, demonstrated significant activity on the nervous system as anesthetics, tranquilizers and sedatives [2–7].

The damage caused by ischemia in neurons is characterized by early death mediated by excitotoxicity or delayed death mediated by apoptosis. The vulnerability of the brain to the lack of blood supply has motivated substantial research efforts to identify pharmacological agents that can reduce brain damage [8]. Among these, general anesthetics have always been considered as logical candidates, due to their ability to reduce the metabolic rate of the brain, to antagonize glutamate-mediated excitotoxicity and enhance inhibitory synaptic transmission [9]. Potential mechanisms of neuroprotection include the inhibition of excitatory activity (as antagonists of NMDA and AMPA glutamate receptors) and the activation of inhibitory circuits (enhancing the activity of the $GABA_A$ receptor) [10–12].

It has been suggested that propofol would be an ideal anesthetic from the point of view of its possible beneficial effects on brain physiology. Various investigations have revealed the protective capacity of propofol against ischemia, which seems to lie in its antioxidant properties and its activities as an enhancer of GABA and inhibitor of glutamate release [13–15]. It has also been reported that other aromatic alcohols with intact phenolic groups, as well as various phenolic derivatives, were able to protect neurons against oxidative damage induced by

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glutamate and hydrogen peroxide because of their antioxidant properties, and that they lost this capacity when their hydroxyl groups were methylated [16,17].

The overall objective of this work is to evaluate the potential neuroprotective activity of lipophilic phenols, structurally analogues of propofol, with proven GABAergic activity [2,4]. These phenols include the naturally occurring compounds thymol, carvacrol and eugenol, the synthetic product chlorothymol, and the most widely used intravenous anesthetic, propofol, as a reference compound. The lipophilicity, membrane partition ability and "*in vitro*" antioxidant properties of all these compounds were previously studied by our group [18,19]. The correlation between their pharmacological activities and antioxidant potencies previously obtained, and the neuroprotective effects are also discussed.

2. Materials and methods

2.1. Reagents

Propofol (2,6-bis(isopropyl)-phenol), thymol (5-methyl-2-isopropyl-phenol), carvacrol (2-methyl-5-isopropyl-phenol), eugenol (2-methoxy-4-prop-2-enyl-phenol), chlorothymol (5-methyl-4-chloro-2-isopropyl-phenol), Dulbecco's minimum essential medium (DMEM), trypsin, soybean trypsin inhibitor, DNase, amino acids, bovine albumin, cytosine-arabinofuranoside, penicillin, insulin, poly-L-lysine and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and α-tocopherol (Vitamin E) were obtained from Sigma Chemical Co·(St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA (Pasching, Austria). All other reagents were of the highest analytical grade.

2.2. Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 17–18 day-old Wistar rat fetuses, as previously described [2,20]. Animals were obtained from the Animal Facility of the Instituto de Investigaciones Médicas Mercedes y Martìn Ferreyra, INIMEC-CONICET-Universidad Nacional de Córdoba (Argentina). The animals were kept under controlled temperature (22–24 °C) and light cycle. Water and pelleted food were supplied *ad libitum*. All the procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Pregnant animals were killed by cervical dislocation and fetuses were extracted. Neocortices were dissected, mechanically minced, and cells then dissociated by mild trypsinization (0.02% *w*/*v*) at 37 °C for 10 min followed by trituration in a DNase solution (0.004% *w*/*v*) containing a soybean trypsin inhibitor (0.05% *w*/*v*). The cells were re-suspended in a DMEM medium (5 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with insulin, penicillin and 10% FBS (DMEM-FBS). The cell suspension (1.6×10^6 cells/ml) was seeded in 96-well plates pre-coated with poly-L-lysine, and incubated for 6–7 days in a humidified atmosphere 5% CO_2/95% air at 37 °C. 20 μM cytosine arabinofuranoside was added after 36–48 h in culture to prevent glial proliferation.

2.3. Cellular injury model

Cell injury was quantitatively assessed by measuring the lactate dehydrogenase (LDH) released from damaged cells to the extracellular medium after 30 min or 24 h of treatment [2] by using LDH enzyme reagents from *Wiener Lab* (Rosario, Argentine) with an optimized UV method. The percentages of LDH release were calculated with respect to a sample exposed to triton corresponding to 100% of death. Cell cultures were treated with different concentrations of hydrogen peroxide (H_2O_2) [21,22] for 24 h to determine the harmful concentrations. The antioxidant effect of vitamin E (Vit E) (200 μM) was tested as a positive control of cell protection against oxidative injury. Cells were exposed to Vit E or phenolic compounds 30 min before contact with H_2O_2.

2.4. Determination of intracellular hydroperoxides

The intracellular production of hydroperoxides was assayed using DCFH-DA. The non-fluorescent DCFH-DA is permeable to the cell membrane and, by deacetylation to DCFH, is trapped inside the cell and oxidized by hydroperoxides to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [23]. The method followed in this work is that described by Perry et al. [24] with modifications. Briefly, cultures in 96-well plates were washed with HBSS at 37 °C and loaded with 10 μM DCFH-DA from a stock solution in methanol. After 20 min of loading at 37 °C, plates were washed again with HBSS, and 200 μl of NaOH 0.2 M was added to each well. The plates were shaken during 1 h and the fluorescence intensity was recorded in a fluorometer (*Fluoromax-3*, JobinYvon Inc., Edison, NJ, EEUU) set at 485 nm excitation / 520 nm emission. In all experiments, the fluorophore loading was previously confirmed by fluorescence microscopy, using a *Nikon Eclipse TE2000-U* (Tokio, Japan). Determination of intracellular hydroperoxides was carried out on 6–7 days cell cultures after 24 h of exposure to H_2O_2. Phenolic compounds or Vit E were added 30 min before the injury.

2.5. Statistical analysis

Data shown represent the mean ± standard error of mean (SEM) of n independent experiments. Statistical treatment of data was performed by one or two-way analysis of variance (ANOVA) as appropriate, using Fisher's LSD comparison method and Tukey's nonparametric test with $p < 0.05$.

3. Results

3.1. Phenol toxicity

To determine the effect of phenols on cell viability under the same conditions in which their GABAergic activities were proven [2,4], the cultures were exposed in the first assays for 30 min at different concentrations of each tested compound. The exposure time was selected taking into account the incubation time used in previous pharmacological studies. The results of LDH release indicated that none of the compounds showed significant effects on cell viability compared to the control ($p > 0.05$; one-way ANOVA) (results not shown).

3.2. Oxidative injury model

LDH release was determined in cell cultures exposed for 24 h to different concentrations of H_2O_2. Only the highest concentrations tested (400 and 600 μM) showed significant negative effects on cell viability (Fig. 1A). Thus, both these concentrations were chosen to test the protective effect of phenolic compounds. The positive control (Vit E) was able to protect cells from the effect of 400 μM H_2O_2, but only partially since it did not reach the basal value of the control cultures (Fig. 1B).

3.3. Neuroprotection test

Taking into account the injury model, the cells were initially treated with different concentrations of phenols for 24 h to assess their potential toxicity *per se* at longer times of exposure. No compound showed a decrease in cell viability until 24 h, showing similar values to controls (Fig. 2, white bars).

When cells were treated with harmful concentrations of H_2O_2 for 24 h in the presence of different phenols (added 30 min before injury), propofol (100, 250 and 500 μM), thymol (100, 250 and 500 μM) and chlorothymol (50 and 100 μM) demonstrated partial protective effects against damage by 400 μM H_2O_2, similar to those shown with Vit E. No treatment was able to protect cells from the effect of the highest concentration of H_2O_2 tested (600 μM) (Fig. 2, black and gray bars).