Apoptotic neuronal changes enhanced by zinc chelator - TPEN in organotypic rat hippocampal cultures exposed to anoxia

Ewa Nagańska, Ewa Matyja
Department of Clinical and Experimental Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

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Abstract
Both, the neurotoxic and neuroprotective effects of zinc have been well established, but the exact mechanism of its dual abilities still remains unclear. It has been shown that zinc deficiency leads to progressive neuronal injury. Therefore a safe zinc concentration levels seem to be necessary in neuronal protection from different noxious factors. This study was undertaken to determine the effect of zinc chelating agent – TPEN on neuronal morphological changes in organotypic hippocampal culture and its effect on post-anoxic changes in this model. The study evidenced that exposition to 15 μM of TPEN induced various stages of apoptotic changes in hippocampal pyramidal neurons and enhanced the anoxia-induced neuronal apoptosis in this model. These results confirmed the hypothesis that manipulations of intracellular pool of zinc by zinc-chelating agents may be a cause of both induction and prevention of apoptotic cell death in various pathological conditions.

Key words: TPEN, apoptosis, hippocampus in vitro.

Introduction
Zinc is one of the well known neuromodulatory agents [39,40,44]. After exposition to different injuring factors zinc accumulates especially in degenerating neurones of CA1 hippocampal subfield. It has been documented that transient ischemia/hypoxia may induce the increase of extracellular zinc concentration accompanied by over-expression of zinc transporter ZnT-1 gene [19,43]. Zinc may play a casual role in various forms of apoptosis and its accumulation has been demonstrated in central neurons undergoing apoptosis during development [21]. Zinc chelating agents are thought to be responsible for decrease of neurotoxic properties of zinc [4,8].

Our previous ultrastructural studies showed the neuroprotective effect of zinc on apoptotic cell death in a model of anoxia in vitro [28].

The aim of this study was the evaluation of the effect of zinc-chelator - TPEN on the course of morphological changes in the model of organotypic hippocampal culture exposed to anoxia to answer the question if intracellular zinc deficiency could potentiate postanoxic neuronal injuries.

Material and methods
The experiments were performed on organotypic hippocampal cultures prepared from 2- to 3-day-old Wistar rats. In sterile conditions the hippocampi were
dissected out from both cerebral hemispheres, placed in dishes containing Eagle Minimal Essential Medium (MEM) and cut coronally into thin slices. The explants were placed on collagen-coated cover glasses with 2 drops of nutrient medium and sealed into the Maximow chambers. The cultures were kept at 36.6°C in a medium consisting of 20% inactivated foetal bovine serum and 80% of MEM, supplemented with glucose to a final concentration of 600 mg%, with antibiotics. The medium was renewed twice a week. On the 14-18 day in vitro the well differentiated and sensitive to anoxic injury cultures were divided into the following experimental groups: 1. cultures exposed to TPEN (N,N,N′N′-tetakis-(2-pyridylmethyl) ethylenediamine) in concentration of 15 μM; 2. cultures exposed to 20-minutes anoxia in a pure nitrogen atmosphere in flasks adapted for permanent gas flow;

**Fig. 1.** Hippocampal culture, 24 hours after exposition to 15 μM TPEN. Apoptotic bodies containing fragments of condensed chromatin and cytoorganelles. x 18 750

**Fig. 2.** Hippocampal culture, 5 days after exposition to 15 μM TPEN. Pyramidal neuron with characteristic apoptotic form of condensed chromatin in the close proximity to nuclear membrane, so-called “half-moon”. x 18 750
3. cultures exposed to 20-minutes anoxia, pretreated with TPEN (15μM); 4. control cultures grown in standard conditions. After 30 minutes, 2 and 24 hours, 3 and 5 days the cultures from experimental and control groups were processed for electron microscopy. They were rinsed in cacodylate buffer, pH 7.2, fixed in a mixture containing 0.8% formaldehyde and 2.5% glutaraldehyde for 1 hour, postfixed in 1% osmium tetroxide, dehydrated in alcohols in graded concentrations, and embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined under a JEOL XB 1500 electron microscope.

Results

The effect of TPEN on ultrastructural features in organotypic hippocampal cultures

Exposure to TPEN in 15 μM concentration led to progressive ultrastructural changes in the structure of both nucleus and cytoorganelles of piramidal neurones. After 30 minutes and 2 hours of the experiment a slight vacuolization of cytoplasm and swelling of mitochondria were observed. Numerous neurones showed extensive changes within the mitochondrial matrix with loss of mitochondrial cristae. After 24 hours following the exposure, the pyramidal neurones displayed dilatation of Golgi apparatus channels and extensive vacuolization of cytoplasm, whereas the nucleus maintained its normal appearance. Some massively damaged cells, presenting morphological criteria of necrosis and/or apoptosis were noticed. There were also neurones exhibiting typical apoptotic features with condensed cytoplasm containing numerous well preserved cytoorganelles. Numerous apoptotic bodies were seen (Fig. 1).

The most prominent apoptotic neuronal changes were observed after 5 days following the exposure to TPEN. A lot of pyramidal neurones showed the aggregation of chromatin close to the nuclear membrane, often in the form of so-called “half-moon” (Fig. 2). Frequently, the condensed chromatin formed numerous aggregations under the nuclear membrane.

Fig. 3. Hippocampal culture, 5 days after exposition to 15 μM TPEN. Neuronal cell with nucleus containing aggregations of chromatin under nuclear membrane. x 25 000

Fig. 4. Hippocampal culture, 5 days after exposition to 15 μM TPEN. Neuronal cell with nucleus containing aggregations of chromatin in the form of “cups” characteristic of apoptosis. x 18 750
Some cells, displaying marked condensation of cytoplasm and aggregation of nuclear chromatin, lacked the nuclear membrane integrity (Fig. 5). A large number of apoptotic bodies containing chromatin clumps and fragments of cytoplasm with destructed cytoorganelles were frequently observed (Fig. 6).

**Fig. 5.** Hippocampal culture, 5 days after exposition to 15 μM TPEN. Nucleus containing condensed chromatin and lack of the nuclear membrane integrity. x 18,750

**Fig. 6.** Hippocampal culture, 5 days after exposition to 15 μM TPEN. Apoptotic body containing chromatin clumps and fragments of cytoplasm with destructed cytoorganelles. x 18,750

The effect of TPEN on the development of post-anoxic morphological changes in organotypic rat hippocampal culture

Cultures exposed to 20-minutes anoxia but pretreated with TPEN in concentration of 15 μM showed a large number of cells with morphological features of both necrosis and apoptosis. A set of cells exhibited electron-dense cytoplasm with damaged organelles and disrupted cell membranes. A large number of cells, displaying marked condensation of cytoplasm and aggregation of nuclear chromatin, lacked the nuclear membrane integrity (Fig. 5). A large number of apoptotic bodies containing chromatin clumps and fragments of cytoplasm with destructed cytoorganelles were frequently observed (Fig. 6).

**Fig. 7.** Hippocampal culture, 24 hours after exposition to 15 μM TPEN and 20-minutes anoxia. Neuronal cell containing nucleus with characteristic apoptotic form of condensed chromatin. x 25,000
number of cells revealed typical apoptotic changes, especially characteristic condensation of nuclear chromatin (Fig. 7, 8). The ongoing apoptotic process was confirmed by the presence of numerous apoptotic bodies (Fig. 9). Some cells exhibited the ultrastructural features typical of both necrosis and apoptosis i.e. destruction of cytoorganelles and clumps of condensed nuclear chromatin, reflecting so-called “apoptotic-necrotic” continuum (Fig. 10).

After 5 days of observation the hippocampal cultures displayed advanced morphological changes of neuronal cells including severe vacuolisation of cytoplasm, destruction of cytoorganelles and massive condensation of nuclear chromatin with only partial preservation of the nuclear membrane (Fig. 11).

Discussion

Zinc is one of the trace elements playing an important role in the maintenance of structural and functional integrity of cells and tissues. Zinc in micromolar concentrations is necessary to maintain proper functioning of many enzymes, transcription factors and structural proteins [39,44]. The central nervous system, as well as other tissues, contains significant amounts of zinc [11]. The increasing evidence confirms the crucial role of zinc in many physiological processes but on the other hand, zinc seems to be a very important factor in the pathogenesis of different neurodegenerative diseases [20,37]. Neuroprotective and neurotoxic effects of zinc have been established in different experimental models [10,20,34]. Zinc is thought to be an endogenous modulator of synaptic activating transmitter – glutamate (GLU) through NMDA, AMPA and metabotropic glutamatergic receptors [7,13,18,31,47]. The complex effect of Zn²⁺ on many metabolic processes suggests that zinc may play a modulating role in neurodegenerative processes [7,17,26,41,45,46,48,49]. Swelling of mitochondria is one of the most prominent ultrastructural changes resulting from
postanoxic overaccumulation of zinc in postsynaptic neurones [23]. It probably follows permeability transition pore in the mitochondrial membrane [15]. Recent data shows that zinc ions from synaptic vesicles, and also of intramitochondrial origin, play an important role in pathogenesis of these changes [35,36]. Some authors point out that cellular changes resulting from the neurotoxic effect of zinc exhibit both necrotic and apoptotic features [12,16].

The reduction of zinc pool by chelating agents in physiological conditions might lead to substantial disturbances in intracellular biochemical reactions. Depletion of zinc intracellular concentration turned out to be crucial in loss of cell defence against injuring factors [32].

The present ultrastructural study demonstrated the toxic effect of zinc-chelating agent - TPEN on the pyramidal rat hippocampal neurones in vitro. The pyramidal neurones showed characteristic sequence of morphological changes typical of apoptosis, especially after 5 days since exposition. Pyramidal neurones exhibited morphological features of both early apoptotic changes with a characteristic pattern of chromatin clumping and late stages of apoptosis with formation of typical apoptotic bodies. The toxic effect of TPEN was enhanced by exposition to 20-minutes anoxia. It is consistent with our previous ultrastructural studies based on a model of anoxia in vitro which had evidenced the protective effect of ZnCl₂ on development of late postanoxic changes connected with apoptosis [28]. The neuroprotective effect of zinc is probably connected with inhibition of NMDA receptors. The regulatory role of zinc in the process of apoptotic cell death was the subject of different experimental models [2,3,22,23,28,33,51]. In physiological conditions endogenous zinc plays an inhibiting role of apoptosis [30,50], probably by inhibition of the endonucleases activity responsible for DNA degradation and by interactions with transcription factors and kinases or by its antioxidan-
tive properties [25]. Some authors emphasise the main inhibitive effect of zinc on caspase-3 [5,6,24,30,38]. On the other hand, zinc seems to have a modulatory effect on the apoptotic process by increasing the permeability of mitochondrial megachannels and causing the cascade of caspase reactions [15,42].

It has been previously documented that TPEN causes removal of zinc from zinc-dependent transcription factors. TPEN is thought to be a potentially efficient agent which prevents neuronal death due to a decrease of toxic concentrations of zinc [8,9]. However, the reduction of zinc pool by chelating agents in physiological conditions may lead to substantial disturbances in intracellular biochemical reactions.

Extensive decrease of zinc concentration leads to activation of apoptosis in different cells including neurones [1,27,29]. The exact mechanism of this effect remains unclear, but typical apoptotic changes have been observed in neurones in different experimental models, both in vivo [4,8] and in vitro [1,5,14,33]. The present study supports the opinion that the instability in intracellular zinc concentration may result in abnormality in cell death control in various pathological processes.

References
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