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Short communication

Neuroprotective effect of thyrotropin-releasing hormone against excitatory amino acid-induced cell death in hippocampal slices

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Abstract

Thyrotropin-releasing hormone (TRH) and some of its stable analogues have recently been shown to improve functional recovery after neurologic dysfunctions, such as brain trauma and epilepsy, in both animals and humans. The exact mechanism by which TRH produces its neuroprotective effects is still uncertain. The present study provides the first evidence that TRH exerts a neuroprotective effect against *N*-methyl-D-aspartate (NMDA)-mediated excitotoxicity in rat hippocampal slices. TRH concentration dependently reduced NMDA toxicity by a mechanism that was highly sensitive to the protein kinase C blocker, bisindolilmaleimide. Delayed application of TRH, during NMDA exposure, still produced neuroprotection

Keywords: TRH (thyrotropin-releasing hormone); Neuroprotection; NMDA (*N*-methyl-D-aspartate); Hippocampus; Excitotoxicity

1. Introduction

Although evidence about the effect of interaction of thyrotropin-releasing hormone (TRH) with glutamate transmission in vitro is still controversial (Renaud et al., 1979; Kasparov et al., 1994; Stocca and Nistri, 1995; Koenig et al., 1996), particular attention has been paid to the anticonvulsant properties of TRH in epilepsy (Inanaga et al., 1989; Ujihara et al., 1991) and especially to its beneficial effect in the treatment of severe head and spinal cord injury (Latronico et al., 1993; Faden, 1996), which are pathological conditions associated with abnormal activation of excitatory amino acid pathways (Lipton and Rosenberg, 1994). In fact, of the substances that may be released in response to trauma, excitatory amino acids are responsible for major cell injury via activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Faden et al., 1989; Zhang et al., 1996).

We report here that TRH decreases neuronal vulnerability to NMDA toxicity in acutely dissected rat hippocampal slices. This experimental model, which is highly sensitive to glutamate injury (Garthwaite and Garthwaite, 1989), is particularly suited to investigations of both excitotoxicity and potential neuroprotective strategies. TRH was proposed to improve neurological recovery after brain trauma by modulating a number of secondary injury factors (Faden, 1996). We, here, add evidence of a direct anti-excitotoxic mechanism of TRH by which it may modulate central nervous system injury.

2. Materials and methods

2.1. NMDA-mediated toxicity in rat hippocampal slices

Hippocampal slices from 8-day old Sprague–Dawley rats (Charles River) were prepared according to Garthwaite and Garthwaite (1989). Briefly, slices cut at a thickness of 0.5 mm by a 752M Vibroslice (Campden Instruments, UK) were submerged in 2 ml of a Krebs–Ringer solution containing 11 mM glucose, equilibrated with 95% O_2–5% CO_2 (pH 7.4), and preincubated at 37°C for 30 min. Then, 30 μM NMDA were added and the incubation was carried

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out for 30 min. At the end of this period, slices were washed and further incubated in fresh buffer for 90 min in order to allow irreversibly damaged neurons to become visibly necrotic while giving reversibly damaged cells time to recover. TRH (Protirelin, TRH-tartrate, Xantium, Lederle, Catania, Italy) and/or bisindolilmaleimide (Sigma, Milan, Italy) were added to slices during the preincubation period and were present throughout the entire experiment. Because of the nature of experiments, this model cannot be used to establish whether the test drug prevents cell death or alters the time course of neurodegeneration, which, in vivo, may last for many days. Slices were fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Then, they were transferred to fresh phosphate buffer solution overnight and finally embedded in epoxy resin (Pizzi et al., 1996a). Semi-thin (1 μm) sections were cut in the plane of the slices and stained with methylene blue and azur II. Adjacent cells were counted in cell fields taken from CA1, CA3 and the dorsal blade of the dentate gyrus in each slice. The fields measured 1.5×10^4 μm^2. Living neurons appeared homogeneous and compact with a blue cytoplasm and a brighter nucleus while lesioned cells were oedematous and contained white vacuoles and a dark

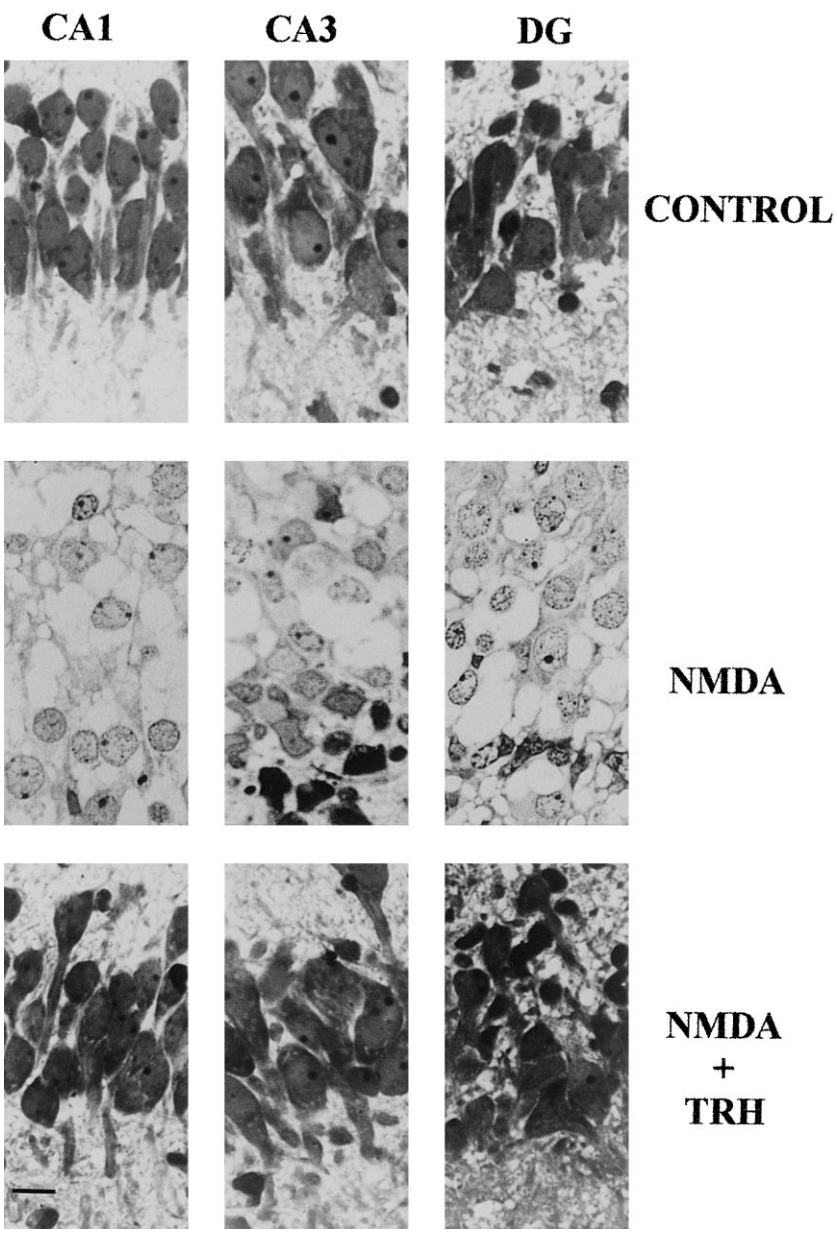

Fig. 1. Excitotoxic effect of NMDA in rat hippocampal slices: prevention by TRH. Sections were exposed to vehicle (control), 30 μM NMDA (NMDA), or 30 μM NMDA + 10 μM TRH (NMDA + TRH). Cell viability was evaluated in CA1, CA3 and dentate gyrus (DG) of each hippocampal slice. Scale bar: 10 μm.

shrinking nucleus. The percentage of cell survival was calculated as the ratio of the number of living cells to the total number of cells.

3. Results

3.1. TRH counteracts NMDA-mediated neurotoxicity in rat hippocampal slices and cerebellar granule cells

A 30-min application of 30 μM NMDA to hippocampal slices caused a specific loss of cells in almost all the neuronal layers. At least 70% of pyramidal neurons belonging to the CA1 and CA3 regions and 90% of granule cells of the dentate gyrus became acutely necrotic (Fig. 1). Application of TRH to the slices 30 min before NMDA addition resulted in a concentration-dependent prevention of the NMDA-mediated injury (as shown in Fig. 2). The regions most sensitive to neuroprotection were CA3 and dentate gyrus. In these regions, the peptide had a significant protective effect even at concentrations as low as 1 μM (35% increase in cell survival). Complete prevention of NMDA toxicity was obtained with TRH at 100 μM. As shown in Fig. 2, the delayed application of 100 μM TRH, i.e, during the last 15 min of exposure to NMDA, still had a significant protective effect. No effect was observed when TRH was applied after NMDA treatment (data not shown).

3.2. TRH neuroprotection relies on protein kinase C activation

The TRH receptors have been reported to be positively coupled to phospholipase C, leading to inositol phosphate production and protein kinase C activation (Canonico et al., 1988). To test for the possible involvement of protein kinase C activation in TRH-mediated neuroprotection, hippocampal slices were pretreated with 1 μM bisindolilmaleimide, a selective inhibitor of protein kinase C activity, and then were exposed to NMDA alone or in the presence of TRH. As shown in Fig. 2, bisindolilmaleimide significantly prevented TRH-mediated neuroprotection in all three hippocampal areas without modifying, per se, the NMDA-mediated toxicity (data not shown).

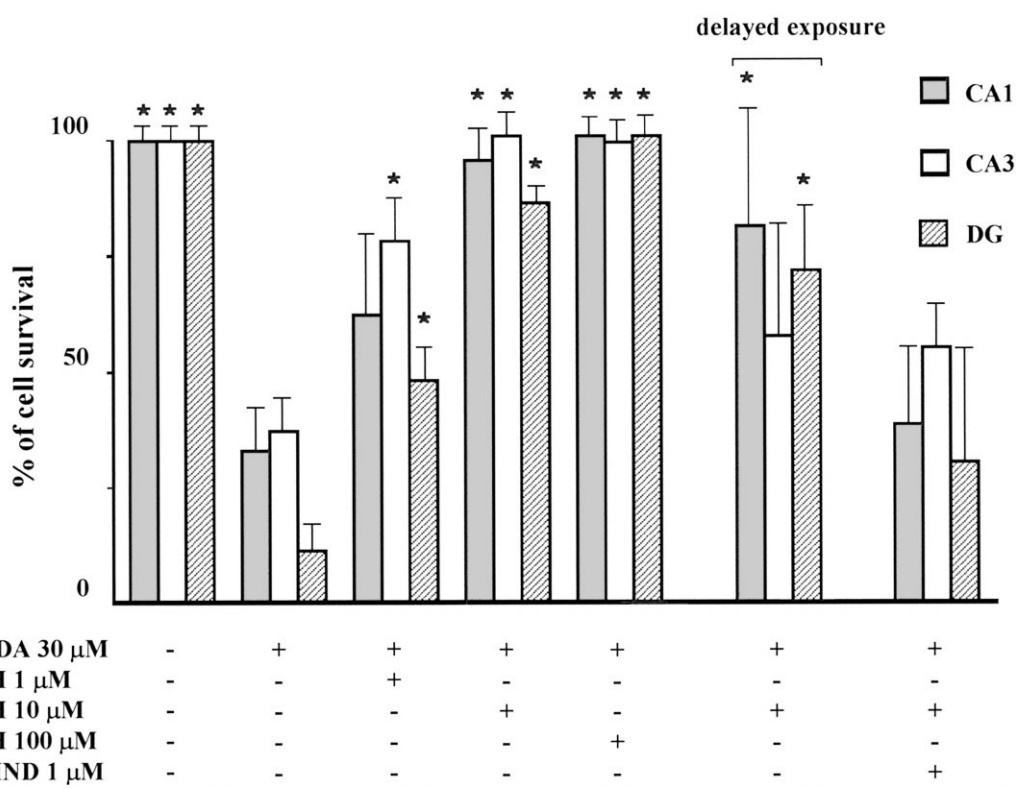

Fig. 2. Effect of different concentrations of TRH on NMDA (30 μM)-induced cell loss in rat hippocampal slices: blockade by protein kinase C inhibitors. TRH was added, at the indicated concentrations, from the preincubation period. In the delayed exposure experiment, slices were treated with NMDA and after 15 min, they were coexposed to 10 μM TRH for an additional 15 min. Last triplet of columns represents the effect of protein kinase C blockade on TRH-mediated neuroprotection. Cell viability was evaluated in CA1, CA3 and dentate gyrus (DG) of each hippocampal slice. Columns represent the means ± SEM of two experiments run in quadruplicate. Statistical significance of the differences was analysed by Kruskal–Wallis non-parametric ANOVA with adjustment for multiple comparisons. $^{*}P < 0.01$ vs. corresponding slices treated with NMDA alone.

4. Discussion

In a prospective randomized, placebo-controlled study, TRH was shown to reduce the occurrence of an unfavourable outcome of brain trauma, by reducing the duration of impaired consciousness and by increasing the recovery rate from the most common neuropsychological deficits (Latronico et al., 1993; Faden, 1996).

Multiple mechanisms for TRH neuroprotection have been proposed. These include enhancement of cerebral noradrenaline metabolism (Keller et al., 1974), antagonism of the opioid system, leukotrienes and platelet-activating factors as well as improvement of cerebral blood flow, ion homeostasis and cellular bioenergetic state (Faden, 1996).

The present study, by providing the first evidence that TRH inhibits glutamate-mediated cell loss, supports the use of TRH in the treatment of brain injury. In fact, after brain trauma, the extracellular levels of glutamate increase rapidly and are thought to be involved in secondary delayed injury of brain tissue, via the activation of NMDA receptors (Faden et al., 1989; Zhang et al., 1996). We found that TRH protected against NMDA-mediated neurotoxicity in rat hippocampal slices. The effect of TRH was particularly evident at 1 μM in the dentate gyrus cells and reached a maximum at 100 μM in all the hippocampal areas. This range of concentrations overlapped that found to be neuroprotective in the telencephalon of rats injected with TRH for the treatment of central nervous system trauma (Soube et al., 1982). The delayed application of TRH, during the last 15 min of NMDA exposure, still resulted in neuroprotection while no significant effect was elicited by its addition in the post-lesion period. The time course of the observed neuroprotection could be related to the clinical efficacy shown by TRH, when administered within 12 h from brain trauma (Latronico et al., 1993).

The effect of TRH appeared to depend on protein kinase C activation. The contribution of protein kinase C to neuroprotection is still controversial. Stimulation of protein kinase C was found to promote both protective (Koh et al., 1991; Lucas et al., 1994; Lin et al., 1997) and neurotoxic effects (Mattson, 1991). Among the possible mechanisms by which activation of protein kinase C is neuroprotective in cultured neurons are negative modulation of NMDA receptor function (Courtney and Nicholls, 1992; Pizzi et al., 1996b), blockade of both Ca^{2+} influx and intracellular Ca^{2+} mobilization, as well as protection against oxidative stress (Davis and Maher, 1994). Further investigations are required to clarify the exact mechanism by which protein kinase C leads to neuroprotection. In order to do this, more complex experimental models should be used, such as cerebral slices and injured brain.

Given the important role of NMDA receptor-mediated neurotoxicity in acute brain insults, this study indicates that preservation of cell viability may be the major mechanism by which TRH promotes functional neuronal recovery in brain trauma and suggests that TRH may be effective in other neurologic diseases associated with excitotoxic cell death.

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