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The trophic effect of ouabain on retinal ganglion cells is mediated by IL-1β and TNF-α

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ABSTRACT

Ouabain is a steroid hormone that binds to the enzyme Na^+, K^+ – ATPase and stimulates different intracellular pathways controlling growth, proliferation and cell survival. IL-1β and TNF-α are pleiotropic molecules, conventionally regarded as pro-inflammatory cytokines with well-known effects in the immune system. In addition, IL-1β and TNF-α also play important roles in the nervous system including neuroprotective effects. Previous data from our group showed that ouabain treatment is able to induce an increase in retinal ganglion cell survival kept in mixed retinal cell cultures. The aim of this work was to investigate if IL-1β and TNF-α could be mediating the trophic effect of ouabain on retinal ganglion cells. Our results show that the trophic effect of ouabain on retinal ganglion cell was inhibited by either anti-IL-1β or anti-TNF-α antibodies. In agreement, IL-1β or TNF-α increased the retinal ganglion cells survival in a dose-dependent manner. Accordingly, ouabain treatment induces a temporal release of TNF-α and IL-1β from retinal cell cultures. Interestingly, TNF-α and IL-1β regulate each other intracellular levels. Our results suggest that ouabain treatment triggers the activation of TNF-α and IL-1β signaling pathways leading to an increase in retinal ganglion cell survival.

1. Introduction

Ouabain is a steroid hormone that in higher concentrations (μM range) binds to the Na^+, K^+ – ATPase and specifically inhibits this enzyme. Na^+, K^+– ATPase is the key ion-motive enzyme in all eukaryotic cells. It effectively controls intracellular calcium and other cation levels through secondary cation exchangers and antiporters (e.g. Na^+/Ca^{2+}, Na^+/H^+) [1,2]. IL-1β and TNF-α are pleiotropic molecules, conventionally regarded as pro-inflammatory cytokines, eliciting relevant effects in the immune system. Nevertheless, it is well known that these molecules also play important roles in the nervous system including neuroprotective effects [3,4].

Lower concentrations (nM range) of ouabain stimulate different intracellular pathways, controlling cell growth, proliferation and survival [5]. Ouabain treatment activates intracellular pathways including (but not limited to): protein kinase C (PKC), calcium calmodulin kinase, the Ras/Raf/MAPkinase cascade, EGFR and Src [5].

Previous data from our group showed that treatment with veratridine (a drug that induces Na^+ influx) or with PMA (a classical activator of PKC) increased the survival of retinal ganglion cells (RGC) [6,7]. We also showed that ouabain treatment increases RGC kept in mixed retinal cell cultures; an effect mediated by the activation of PKC [8] Src, EGF receptor, PKC delta and JNK [9].

IL-1 plays a key role as a brain injury mediator. In ischemia, trauma, Alzheimer's disease, Parkinson's disease and multiple sclerosis the levels of IL-1 are increased and in association with the extension of the lesions [10,11]. TNF-α is a typical inducer of apoptosis and is involved in immune pathologies and neurological dysfunctions [12].

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Nonetheless, IL-1β and TNF-α may also play a role as neuroprotective mediators depending on their concentrations and on the profile of the molecules concomitantly released [12,13].

Through its effect on Na^+, K^+ – ATPase ouabain modulates the production of IL-1β and TNF-α by mononuclear cells [2]. For this reason, the present work investigated if the trophic effect of ouabain on retinal ganglion cells involved IL-1β and TNF-α.

2. Materials and methods

2.1. Chemicals

Medium 199 and fetal calf serum were purchased from GIBCO (Gaithersburg – USA). Glutamine, penicillin, streptomycin, poli-L-ornithine, tetramethylbenzidine (TMB) sodium nitroprusside and horseradish peroxidase (HPR) were obtained from SIGMA (St. Louis-USA). Trypsin was purchased from Worthington Biochemical (New Jersey-USA). Entellan and bovine serum albumin were obtained from Merck (Darmstadt-Germany) and dimethyl sulphoxide (DMSO) was purchased from Mallinckrodt Baker (Dublin-Ireland). IL-1β, TNF-α and their respective antibodies were purchased from PeproTech (New Jersey–USA). Anti-actin antibody came from Santa Cruz Biotechnology (Texas-USA). Secondary antibodies were obtained from: anti-mouse GE (Buckinghamshire-UK); anti-rabbit Bio-Rad (USA) and anti-goat Santa Cruz (Texas-USA). Elisa kits for Rat TNF-α and rat IL-1β were purchased from R&D Systems (Minneapolis-USA).

2.2. Peroxidase injection and retinal cultures

Within the first 24 h after birth Lister Hooded rats were anesthetized by hypothermia. One μL of a solution of 10% HRP in 2% DMSO was then injected into each superior colliculus. After 16 h of HRP injections animals were killed, their retinas dissected, treated with 0.1% trypsin and dissociated by trituration. Cells were plated in coverslips and kept in complete 199 medium for 4 h or 48 h at 37 °C in an atmosphere of 5% CO_2/95% air [14]. The presence of HRP in the cytoplasm of RGC was revealed according to the protocol of Mesulan [15]. Cells were fixed with a mixture of aldehydes in sodium phosphate buffer (0.1 M), the coverslips were washed in phosphate buffer and reacted with TMB. All procedures using animals were approved by the local committee for animal care and experimentation (CEPA-projects #00196-10).

2.3. Cell counting

RGC were counted using a microscope at a magnification of 400×, under bright-field. As an internal control for the variable percentage of RGC labeled with HRP in distinct experiments, the number of labeled cells at 4 h in culture was taken as 100% and the results were reported as percentage of control. Independently from the number of labeled cells, the 48 h survival was always the same (40%–60%). All data were expressed as mean ± standard error of the mean from experiments performed at least in duplicate and each experiment was repeated at least three times.

2.4. Western blotting

The levels of TNF-α, IL-1β and actin were determined by western blot analysis using procedures previously described [16]. Actin was used as a loading control protein. Membranes were incubated with goat anti-actin antibody (1:1250), mouse anti-TNF-α antibody (1:2000) or rabbit anti- IL-1β antibody (1:1500). Membranes were washed in Tris buffer solution and then exposed to HRP-conjugated secondary anti-goat (1:17000), anti-rabbit (1:15000) or anti-mouse IgG antibodies (1:10000) at room temperature for 60 min. Detection was performed on L-PixChemiMolecular Imaging by Loccus biotecnologia (Brazil) using chemiluminescence system (Luminata). The density of protein bands was analyzed by densitometry with Image J. The mean value for the control was set as 100%.

2.5. ELISA

Measurements of TNF-α and IL-1β were done on cell-free medium supernatants using ELISA kits, in accordance with the manufacturer's instructions.

2.6. Statistical analysis

The overall statistical analysis was first obtained by one-way analysis of variance (ANOVA). Newman–Keuls comparison test was used to assess statistical significance for all pairs of multiple groups of data. To analyze two experimental groups we used Student's *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Ouabain effect on RGC survival is mediated by IL-1β and TNF-α

To investigate whether ouabain effect on RGC survival was mediated by IL-1β and TNF-α we used neutralizing anti-IL-1β (0.1 μg/mL) and anti-TNF-α (0.1 μg/mL) antibodies. Fig. 1(A) shows that anti-IL-1β as well as anti-TNF-α totally abolished the trophic effect of ouabain. These results indicate the functional overlay of these two cytokines.

In order to ascertain the possible individual action of IL-1β and TNF-α in the RGC survival after 48 h in culture, we performed dose-dependent experiments. Fig. 1(B) shows that the number of RGC in control cultures, after 48 h, was reduced to approximately 50% of its initial value. Treatment with IL-1β, however, in the range of 2.5–5.0 ng/mL or with TNF-α at 0.5–1.0 ng/mL, increased RGC survival. The maximal effect was obtained with 5 ng/mL IL-β and 0.5 ng/mL TNF-α. At higher levels the trophic effect was lost for both cytokines.

Considering that cytokines alone did not elicit a 100% RGC survival we wondered if the concomitant treatment with IL-1β and TNF-α could show a synergetic effect. So, our next step was to evaluate if the combined treatment with low doses of both cytokines would increase the survival of RGC.

Fig. 1(C) shows that neither IL-1β 2.5 ng/mL nor TNF-α 0.25 ng/mL could alter the survival pattern in our cultures compared to 48 h-control, where the number of RGC was reduced to approximately 50% of its initial value. However, the combined treatment with IL-1β 2.5 ng/mL and TNF-α 0.25 ng/mL remarkably increased the RGC survival, and all RGC initially plated were still alive after 48 h.

The next step was to assay the kinetics of release and action of these two cytokines. To clarify this matter we treated cell cultures for 24 h alternating the presence of each cytokine in the medium. We performed 4 h and 48 h controls, as well as cytokines controls in their optimal concentrations. Besides, cultures were treated with one cytokine for the first 24 h and with another the remaining 24 h. To alternate the cytokine treatment it was necessary to wash our cultures, so that we also have created washed controls.

Treatment with IL-1β in the first 24 h and TNF-α in the remaining 24 h did not improve the survival of RGC (Fig. 1 D). We, thus, infer that TNF-α is required at early stages to exert its retinal neuroprotective effect. At the same time when culture was treated first with TNF-α and then with IL-1β in the remaining 24 h there