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ORIGINAL ARTICLE

Relationship Between the Neuroprotective Effects of Insulin-Like Growth Factor-1 and 17β-Oestradiol in Human Neuroblasts

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Insulin-like growth factor-1 (IGF-1) and oestrogens interact with each other as neuroprotective factors. We have previously demonstrated that 17β-oestradiol protects against β-amyloid and oxidative stress toxicity and increases the amount of cell cholesterol in human foetal neuroblasts (FNC). The present study aimed: (i) to assess the protective effects of IGF-1 in FNC cells; (ii) to investigate the relationship between IGF-1 and 17β-oestradiol; and (iii) to determine whether cholesterol was a major mediator of the effects of IGF-1, similarly to 17β-oestradiol. We found that IGF-1 effectively exerts neuroprotective effects in FNC cells. We also demonstrated that the IGF-1 receptor (IGF-1R) pathway is needed to maintain oestrogen-mediated neuroprotection. Finally, we found that, opposite to 17β-oestradiol, IGF-1 did not cause a significant increase in cell cholesterol. These findings indicate that a cross-talk between IGF-1 and 17β-oestradiol occurs in FNC cells. In particular, the activation of the IGF-1R cascade appears to be fundamental to warrant 17β-oestradiol-mediated neuroprotection, even though cell cholesterol does not play a major role as an effector of this pathway.

Key words: IGF-1, nervous system, neuroprotection, 17β-oestradiol, cholesterol.

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There is strong evidence that the insulin-like growth factor (IGF) system (namely IGFs, IGF receptors, IGF binding proteins) is represented in the central nervous system (CNS) and plays an important neuroprotective role by favouring neuronal development, metabolism, survival and regeneration (1–4). Similarly, experimental evidence strongly supports a neurotrophic and neuroprotective role of oestrogens both *in vitro* and *in vivo* (5–7). Furthermore, a strong link between oestrogen receptors (ERs) and the IGF-1 receptor (IGF-1R) occurs in the brain (8). Many neurons and astrocytes express both receptors and 17β-oestradiol is able to activate IGF-1R and its signalling pathways, leading to the activation of Akt that, in turn, regulates synaptic plasticity (9) and favours neuronal survival (10,11). On the other hand, IGF-1 can regulate the transcriptional activity of ERs. Indeed, IGF-1 may activate ERs in the absence of oestrogens (12,13), inducing ER-mediated gene expression in different cell types including neuronal cells (14–16).

We have previously demonstrated that, in human foetal neuroblast long-term cell cultures (FNC cells), the neuroprotective effects of oestrogens are, at least in part, mediated by seladin-1 (SELective AD INdicator-1) (17,18). This protein, which has been found to be down-regulated in brain regions affected by Alzheimer's disease (AD) (19), confers resistance against ß-amyloid-mediated toxicity and oxidative stress by acting as an anti-apoptotic factor via the inhibition of caspase-3 activation (19). Furthermore, it was subsequently demonstrated that the *seladin-1* gene corresponds to the gene encoding 3ß-hydroxysterol Δ24-reductase (DHCR24), which catalyses the reduction of the Δ24 double bond of desmosterol to produce cholesterol (20). There is evidence that the amount of cell cholesterol may be a critical factor for CNS homeostasis. One of the mechanisms of β-amyloid toxicity is the generation of membrane pores permeable to toxic calcium ions. This mechanism is enhanced in cholesterol-depleted membranes, whereas it is markedly reduced in cholesterol-enriched membranes (21,22). Accordingly, reduced membrane lipids in the cortex of AD transgenic mice have been detected (23). Furthermore, the amount of cell cholesterol may also affect amyloidogenesis. Indeed, a moderate decrease in cholesterol levels, similar to that occurring in the nerve cells membrane of AD patients, has been shown to increase β-amyloid production (24–26).

In a previous study, we have demonstrated that FNC cells express IGF-1R and IGF-1. These cells also secrete IGF-1 and its release is increased by 17β-oestradiol. Finally, we found that IGF-1 induces

cell growth and significantly increases the expression level of seladin-1 (27). The present study aimed: (i) to investigate on the neuroprotective effects of IGF-1 in FNC cells; (ii) to determine whether these effects are, at least in part, related to the amount of cell cholesterol; and (iii) to identify the presence of a cross-talk between IGF-1 and 17β-oestradiol in this cell model.

Materials and methods

Cell cultures

The primary neuroblast long term cell culture FNC was established, cloned and propagated *in vitro* from human foetal olfactory neuroepithelium (28). FNC cells synthesise both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons (29). In addition, they display neuroendocrine features (30). FNC cell cultures were propagated at 37 °C in 5% CO_2 in Coon's modified Ham's F-12 medium (Sigma, St Louis, MO, USA), 10% foetal bovine serum (FBS) (HyClone, Logan, UT, USA) and antibiotic solution (penicillin 100 IU/ml; streptomycin 100 μg/ml) (PAA, Colbe, Germany). The B4 clone, showing the highest levels of expression of neuronal and olfactory markers (28), was used in the present study.

Viability assays

Cell viability after β-amyloid exposure was determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS; Promega, Milan, Italy) assay, as described previously (18). Briefly, cells were seeded in 96-well plates for 24 h in growth medium. Recommended instructions for β-amyloid (Calbiochem, Darmstadt, Germany) preparation were observed: the lyophilised peptide was solubilised in 50 mM Tris-HCl (pH 9.0) and then diluted to the appropriate concentration with calcium- and magnesium-free PBS. Successively, the peptide was incubated at 37 °C for 48 h to allow aggregation. Thereafter, the cells were exposed to 10 nM IGF-1 or 10 nM 17β-oestradiol (Sigma) in the presence or in the absence of 1 μM of the ER antagonist ICI 182 780 (Sigma) or 1 μg/ml of the IGF-1R antagonist JB-1 (Sigma) for 48 h. During the last 20 h of incubation, cells were treated with 1 μM β-amyloid or 100 μM H_2O_2. Multiwell dishes were analysed using an enzyme-linked immunosorbent assay plate reader (Seac-Radim, Moncalieri, Italy) and optical density (OD) at 490 nm was considered directly proportional to the number of living cells. Each experimental point was performed in six replicates and the results were expressed as the OD/well (mean ± SE of three different experiments).

To assess the resistance against H_2O_2-mediated oxidative stress, viable cells were also determined by Trypan blue dye exclusion test, as described previously (18). Briefly, the cells were cultured in 25-cm^2 flasks in growth medium in the presence or in the absence of 10 nM IGF-1 for 48 h. Cells were then treated with 100 μM H_2O_2 during the last 20 h of incubation. Subsequently, cells were stained with Trypan blue dye for 1 min. Blue-positive and white-negative cells were counted in ten × 20 fields and the results were expressed as mean ± SE percentage of dead/total cells in three different experiments.

Cell proliferation

FNC cell growth was evaluated by the ^{3}H-thymidine incorporation assay. Cells were cultured in 24-well plates at 5×10^4 cells/well for 48 h, in the presence of 10 nM IGF-1. During the last 20 h of incubation, cells were treated with 100 μM H_2O_2 and pulsed with 1 μCi ^{3}H-thymidine. Cells were then harvested and samples were analysed using a β-counter. Each experimental point was performed in six replicates and results were expressed as counts per minute/well (mean ± SE of three different experiments).

Cell cholesterol measurement

The amount of cell cholesterol in control FNC cells and in cells treated with 10 nM IGF-1 (in the presence or in the absence of 1 μM ICI 182 780) or 10 nM 17β-oestradiol (in the presence or in the absence of 1 μg/ml JB-1) for 48 h was determined by gas chromatography-mass spectrometry (GC-MS) as described previously (22), with minor modifications. Briefly, cells were maintained in medium without FBS for 6 h, then lysed in 1 N NaOH and the cell lysate was used for cholesterol quantification or frozen for storage at −80 °C until cholesterol measurement. Aliquots corresponding to 10 μg of protein, as determined by Bradford staining, were used. After the addition of stigmasterol (1000 ng) as an internal standard, sterols were extracted with n-hexane, derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide at 10% in trimethyl-chlorosilane and automatically injected in a Hewlett-Packard GC-MS system (Hewlett-Packard, Palo Alto, CA, USA). A six-point calibration curve in the 25–1000 ng cholesterol range was used for cholesterol quantification. The peak area ratios were calculated using the signals at *m/z* 458 and 484 for cholesterol and stigmasterol, respectively. Each point was performed in duplicate and the results represent the mean ± SE of three experiments.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Nucleospin® RNAII (Macherey-Nagel, Duren, Germany) with DNAse treatment in accordance with the manufacturer's instructions. The concentration was determined spectrophotometrically with Nanodrop® ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The amount of ERα, ERβ and IGF-1R mRNA was determined by real-time RT-PCR using pre-developed assays (Hs00174860_m1; HS00230957_m1 and Hs00609566_m1 respectively; Applied Biosystems, Foster City, CA, USA). According to the comparative C_t (threshold cycle) method, the amount of target mRNA was normalised to the endogenous reference 18S RNA (Hs99999901_s1) and also relative to an internal control and was calculated by the $2^{-\Delta\Delta Ct}$ method. The results were expressed as the mean ± SE mRNA fold variations compared to the control. All measurements were carried out in triplicate and three experiments were performed.

Statistical analysis

Statistical differences were analysed using one-way ANOVA or Student's t-test. When the Student's t-test was used, significance was adjusted for multiple comparisons of means using Bonferroni's approximation.

Results

Effects of IGF-1 on cell viability and proliferation

The presence of the expression and secretion of IGF-1 and of the expression of IGF-1R in FNC cells was described previously (27). In the present study, to determine whether IFG-1 exerts neuroprotective effects in FNC cells, cells were exposed to toxic insults, such as β-amyloid and H_2O_2. β-amyloid (1 μM) significantly decreased cell viability compared to control cells, as assessed by the MTS assay (Fig. 1A). However, in cells pre-treated with IGF-1 (10 nM), the toxic effect of β-amyloid was effectively counteracted. Similarly, when

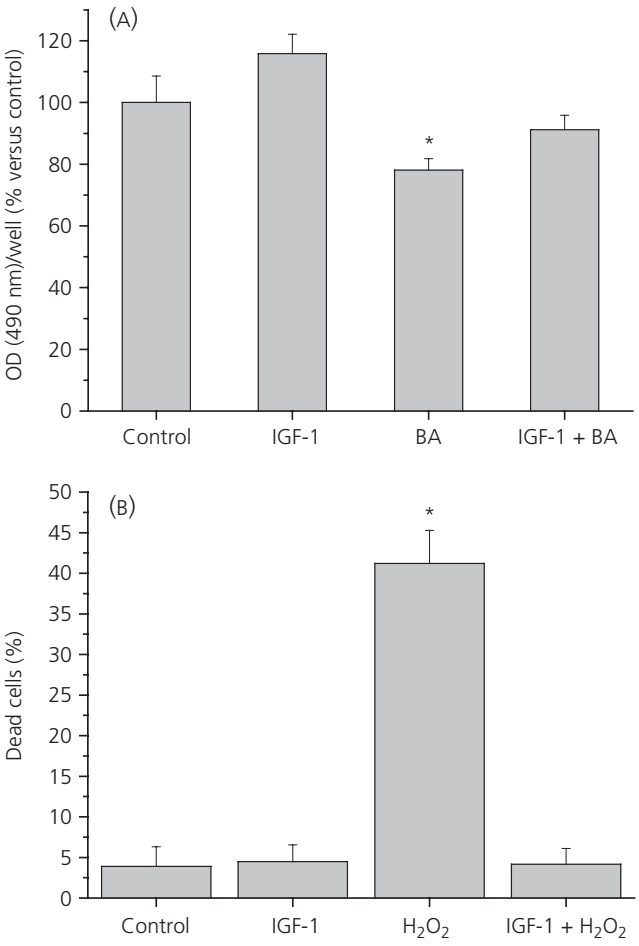

Fig. 1. (A) Effect of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against β-amyloid (BA) (1 μM, 20 h)-induced toxicity in human foetal neuroblasts (FNC). *P < 0.05 versus control cells, cells treated with IGF-1 or with IGF-1 + BA. OD/well = optical density (absorbance at 490 nm)/well; n = 3, six replicates/experiment. (B) Effect of IGF-1 (10 nM, 48 h) against H_2O_2 (100 μM, 20 h)-induced toxicity. *P < 0.05 versus control cells, cells treated with IGF-1 or IGF-1 + H_2O_2. n = 3, cells were counted in ten 20× fields.

the cells were exposed to H_2O_2 (100 μM H_2O_2), the number of dead cells significantly increased, as shown by Trypan blue dye exclusion test experiments, whereas pre-treatment with IGF-1 was able also to significantly prevent cell death (Fig. 1B).

With regard to cell proliferation, we observed a significant reduction, upon exposure to H_2O_2, as assessed by ^{3}H-thymidine incorporation (77.5 $\pm$ 7% reduction versus control cells; mean $\pm$ SE). This inhibitory effect was significantly reversed in cells that were pre-exposed to IGF-1 (Fig. 2).

Effects of IGF-1 on cell cholesterol

We had previously demonstrated that cell cholesterol is an essential mediator of the neuroprotective effects of oestrogens, which increase the synthesis of the neuroprotective factor seladin-1 (17,18). IGF-1 is also able to increase the expression of this factor (30). To determine whether cell cholesterol may be a possible factor

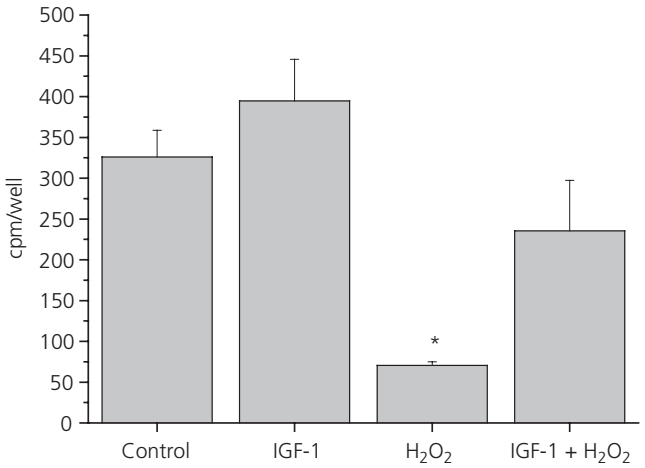

Fig. 2. Role of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against the inhibitory effect of H_2O_2 (100 μM, 20 h) on cell proliferation. cpm (counts per minute)/well, as measured by a β-counter. *P < 0.05 versus control cells, cells treated with IGF-1 or IGF-1 + H_2O_2. n = 3, six replicates/experiment.

mediating the protective effects of IGF-1 in nerve cells, we measured the amount of cholesterol in control FNC cells and in cells treated with 10 nM IGF-1 for 48 h, by GC/MS. We found that, although in cells exposed to IGF-1 there was a trend toward an increased amount of cholesterol, there was no significant difference versus control cells (11.2 $\pm$ 7.8%; mean $\pm$ SE, percentage increase versus control).

Cross-talk between IGF-1 and 17β-oestradiol in FNC cells

To determine whether a cross-talk between IGF-1 and 17β-oestradiol occurs in FNC cells, similar to that described in other cell models (8), we followed various experimental approaches. First, we investigated whether IGF-1 affects the expression levels of ERs and/or 17β-oestradiol affects the expression of IGF-1R. Accordingly, we treated the cells with IGF-1 (10 nM) or with 17β-oestradiol (10 nM) for 48 h. Significantly higher levels of ERα mRNA, but not of ERβ, were detected in cells exposed to IGF-1 compared to untreated cells (Fig. 3). Conversely, treatment with 17β-oestradiol did not affect the amount of IGF-1R mRNA.

Subsequently, we determined whether and how oestrogen and IGF-1 signalling cascades interact with each other by using the ER antagonist ICI 182 780 and the IGF-1R antagonist JB-1. The two antagonists did not show any effect *per se* on cell viability (Fig. 4). In a first set of experiments, we determined whether the two antagonists effectively counteracted the protective effects of the corresponding agonists against H_2O_2-induced toxicity. We confirmed that both 17β-oestradiol (10 nM) and IGF-1 (10 nM) were able to prevent oxidative stress toxicity, although we demonstrated that the protective effect was lost in the presence of ICI 182 780 (1 μM) and JB-1 (1 μg/ml), respectively (Fig. 4). Next, we investigated whether the protective effects of IGF-1 were maintained in the presence of the ER antagonist and, simultaneously, whether 17β-oestradiol-mediated protection was maintained in the presence

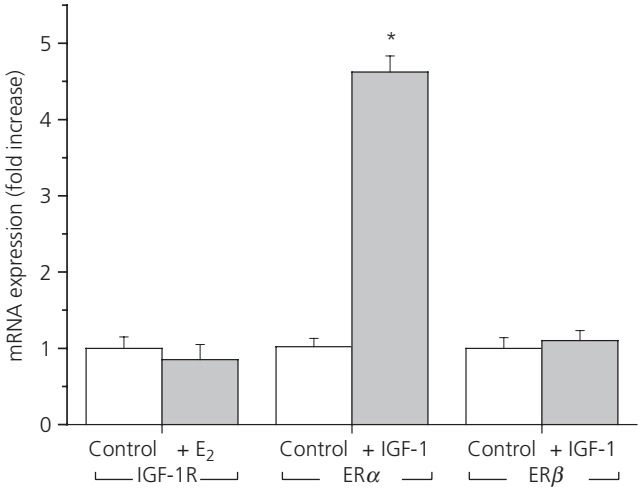

Fig. 3. Effect of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) on the expression of oestrogen receptor (ER)α and ERβ and of 17β-oestradiol (E_2) (10 nM, 48 h) on IGF-1 receptor (IGF-1R), as assessed by quantitative reverse transcriptase-polymerase chain reaction. *P < 0.05 versus control. n = 3, three replicates/experiment.

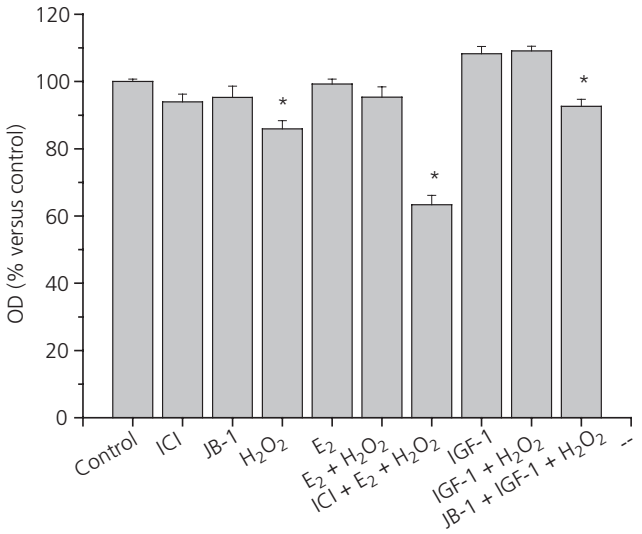

Fig. 4. Cell viability experiments, showing that the protective effects of 17β-oestradiol (E_2) (10 nM, 48 h) and insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against H_2O_2 (100 μM, 20 h)-induced toxicity are lost in the presence of the corresponding antagonists, ICI 182 780 (ICI) (1 μM, 48 h) and JB-1 (1 μg/ml, 48 h). *P < 0.05 versus control cells. n = 3, six replicates/experiment.

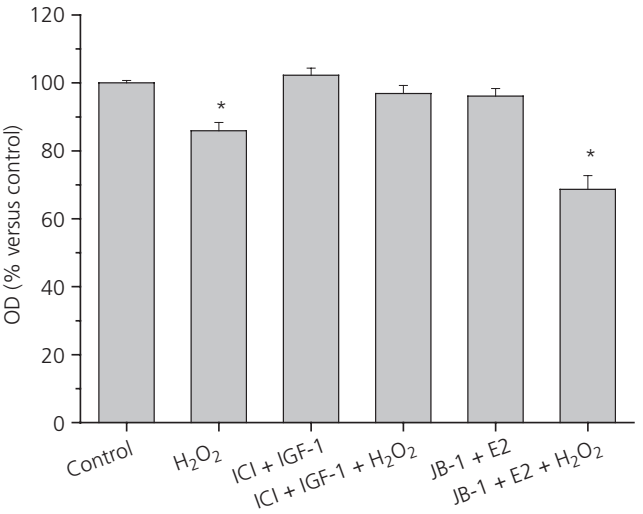

Fig. 5. Cell viability experiments, showing that the protective effects of insulin-like growth factor-1 (IGF-1) (10 nM, 48 h) against H_2O_2 (100 μM, 20 h)-induced toxicity are maintained in the presence of ICI 182 780 (ICI) (1 μM, 48 h), whereas the protective effects of 17β-oestradiol (E_2) (10 nM, 48 h) are lost in the presence of JB-1 (1 μg/ml, 48 h). *P < 0.05 versus control cells. n = 3, six replicates/experiment.

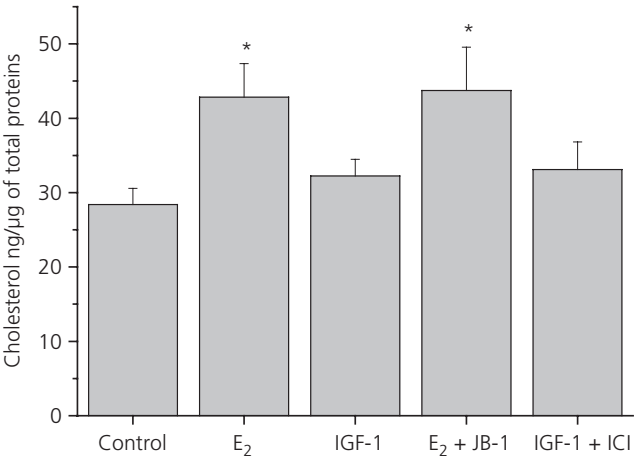

Fig. 6 Cell cholesterol amount (measured by gas chromatography-mass spectrometry) in control cells, cells treated with 17β-oestradiol (E_2) (10 nM, 48 h), insulin-like growth factor-1 (IGF-1) (10 nM, 48 h), E_2 + JB-1 (1 μg/ml, 48 h) and IGF-1 + ICI 182 780 (ICI) (1 μM, 48 h). *P < 0.05 versus control cells. n = 3, two replicates/experiment.

of the IGF-1R antagonist. We found that the exposure of FNC cells to IGF-1 and ICI 182 780 or to 17β-oestradiol and JB-1 did not affect *per se* cell viability. IGF-1 readily counteracted the toxic effect of H_2O_2 in the presence of ICI 182 780, thus indicating that the ER signalling pathway is not necessary to warrant the protective effects of IGF-1. Conversely, the protective effect of 17β-oestradiol against oxidative stress toxicity was lost in the presence of the IGF-1R antagonist JB-1, thus suggesting that the IGF-1R signalling cascade is involved in oestrogen-mediated neuroprotection in FNC cells (Fig. 5).

To determine whether the loss of the protective effect of 17β-oestradiol in the presence of JB-1 and, conversely, the maintenance of the protective effect of IGF-1 in the presence of ICI 182 780 might be a result, at least in part, of a different amount of cell cholesterol, we measured cholesterol in cells subjected to different treatments. As previously demonstrated (17), we found a significant increase of cholesterol amount in cells exposed to 17β-oestradiol (Fig. 6). In cells treated with 17β-oestradiol + JB-1, the level of cell

cholesterol was not significantly different from the level measured in cells treated with 17β-oestradiol alone. This result, together with the finding that JB-1 prevented the neuroprotective effect of 17β-oestradiol, suggests that cholesterol *per se* is not sufficient to confer oestrogen-mediated neuroprotection. Furthermore, the amount of cholesterol in cells treated with IGF-1 or with IGF-1 + ICI 182 780 did not differ from the amount detected in control FNC cells. These data further support an important role for cell cholesterol in affecting ER-mediated, but not IGF-1R-mediated, neuroprotection in FNC cells.

Discussion

IGF-1 is known to play an important neuroprotective role by favouring neuronal proliferation and survival (8). Accordingly, there is evidence that the IGF system may be altered in AD, the most common neurodegenerative disease in the elderly. IGF-1 gene polymorphisms have been associated with a greater risk for the onset of the disease through their effects on IGF-1 levels (31). Indeed, it has been demonstrated that lower levels of intracerebral IGF-1 in AD patients, together with the development of IGF-1 resistance, determine β-amyloid accumulation as a consequence of a reduced clearance (32–34). Furthermore, IGF-1 has also been associated with the preferential activation of the non-amyloidogenic α-secretase processing of amyloid precursor protein (35).

In the present study, we first demonstrated that IGF-1 exerts protective effects in FNC. These cell cultures were established, cloned and propagated several years ago, starting from GnRH-secreting cells isolated from human foetal olfactory epithelium. FNC cells show unique features because they synthesise both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons (28,29). These human neuronal precursors represent a more appropriate *in vitro* model for targeting studies related to neurodegenerative diseases compared to animal cell or tumoural cell lines models, which are usually employed. We have previously shown that FNC cells are a suitable cell model to study the role of IGF-1 in neuroprotection. Indeed, these cells express both IGF-1 and IGF-1R and release IGF-1 in the culture medium (27). In the present study, we observed that IGF-1 is able to prevent β-amyloid- and H_2O_2-induced cell toxicity, as assessed by cell viability assays. Furthermore, we demonstrated that IGF-1 effectively counteracts the inhibitory effect of H_2O_2 on cell proliferation.

To determine whether the protective effects of IGF-1 in FNC cells might be related to an optimal amount of cell cholesterol, as suggested by our previous finding that IGF-1 increases the expression of the *seladin-1* gene (27), we measured cholesterol levels in control FNC cells and in cells exposed to IGF-1. We found that cell cholesterol was not significantly increased by IGF-1. This finding suggests that, in contrast to our previous experimental data showing that cell cholesterol is an essential mediator of the neuroprotective effects of oestrogens in FNC cells (17), IGF-1-mediated neuroprotection does not appear to involve cholesterol as a major factor, at least in this cell model.

Despite the different role of intracellular cholesterol, our present and previous findings indicate that both oestrogens and IGF-1 exert neuroprotective effects in FNC cells, thus suggesting the presence of a cross-talk between these hormones. Some studies addressed this issue by using specific receptor antagonists. One study clearly showed that rat hippocampal cell death induced by *in vivo* kainic acid administration was prevented by either 17β-oestradiol or IGF-1. However, none of these hormones was able to prevent cell death also when animals were treated with the IGF-1R antagonist JB-1 or with the ER antagonist ICI 182 780, respectively (36). Other studies showed that ICI 182 780 or JB-1 blocked the induction of neurogenesis or neurite outgrowth induced by IGF-1 or 17β-oestradiol, respectively, both *in vitro* and *in vivo* (37,38). In an *in vivo* model of global ischaemia-induced neuronal cell death in rats, JB-1 abolished the pro-survival effects of 17β-oestradiol (39). To assess the presence of a cross-talk between oestrogens and IGF-1 in our human neuronal cell model, we also performed additional cell viability experiments after oxidative stress, using different combinations of 17β-oestradiol, IGF-1, ICI 182 780 and JB-1. Our results indicated that the IGF-1R signalling cascade is involved in the neuroprotective effects of oestrogens, although the ER signalling pathway is not necessary for IGF-1-mediated neuroprotection in FNC cells. This suggests that the activation of one or more factors that are associated to the IGF-1R pathway is sufficient to exert neuroprotection.

Furthermore, to determine whether variations in cell cholesterol amount might be, at least partially, responsible for the loss of the protective effect of 17β-oestradiol in the presence of JB-1, as well as the maintenance of the protective effect of IGF-1 in the presence of ICI 182 780, we measured cholesterol levels in cells subjected to different treatments. We found that JB-1 did not blunt the stimulatory effect of 17β-oestradiol on cell cholesterol (17), whereas IGF-1 or IGF-1 + ICI 182 780 did not cause any change compared to control cells. Overall, these findings support a pivotal role of the IGF-1R pathway in mediating the neuroprotective effects of oestrogens. However, unlike the ER pathway, cell cholesterol does not appear to play a major role in mediating the neuroprotective effects elicited by the activation of the IGF-1R cascade, which are likely the result of other mechanisms, such as the IGF-1-induced anti-apoptotic effects (1–3). Therefore, although an optimal amount of cell cholesterol appears to be essential, as previously reported (17), this is not sufficient to warrant the neuroprotective effects of oestrogens, which appear to involve the activation of multiple pathways.

Finally, in the present study, we demonstrated that IGF-1 up-regulates the expression of ERα, yet not of ERβ, whereas 17β-oestradiol does not affect the expression of IGF-1R. These data, together with our previous observation that 17β-oestradiol stimulates the release of IGF-1 in FNC cells (30), lead us to hypothesise a model in which: (i) 17β-oestradiol and IGF-1 mutually interact in protecting nerve cells and (ii) a reinforcement mechanism is provided by the presence of an autocrine loop in which ERα activation stimulates the release of IGF-1, which in turn induces the expression of ERα.

Overall, the data reported in the present study appear to support the hypothesis that the interaction between IGF-1 and oestrogens

in neuroprotection may be relevant also in defining possible strategies against neurodegenerative diseases, and primarily against AD. With regard to this issue, although clinical trials for the prevention/treatment of AD with oestrogens have been performed (40,41), no trial so far has addressed the use of IGF-1. However, the hypothetical use of this growth factor in the treatment of CNS pathologies, including AD, has been proposed several times in the last 20 years (42,43). It is known that the cholinesterase-inhibitor donezepil increases plasma levels of GH and IGF-1 in AD patients (44) and that the lack of a response to this drug is associated with lower levels of IGF-1 (45). Accordingly, an enhancement in spatial learning and memory after peripheral administration of GH in rats displaying AD-like deficiences has been recently reported and it is very likely to be a result of the increased release of IGF-1 (46). A phase II trial, aiming at evaluating the effects of GHRH in healthy older men and women and in those with mild cognitive impairment, is currently recruiting participants (http://www.clinicaltrials.gov, trial no. NCT00257712). These interventional studies, together with the basic science, are expected to provide further information and to enhance our knowledge about the neuroprotective effects of IGF-1 and its possible role against neurodegenerative diseases.

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