Involvement of IGF-II receptors in the antioxidant and neuroprotective effects of IGF-II on adult cortical neuronal cultures

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A B S T R A C T

Insulin-like growth factor-II (IGF-II) is a naturally occurring peptide that exerts known pleiotropic effects ranging from metabolic modulation to cellular development, growth and survival. IGF-II triggers its actions by binding to and activating IGF (IGF-I and IGF-II) receptors. In this study, we assessed the neuroprotective effect of IGF-II on corticosterone-induced oxidative damage in adult cortical neuronal cultures and the role of IGF-II receptors in this effect. We provide evidence that treatment with IGF-II alleviates the glucocorticoid-induced toxicity to neuronal cultures, and this neuroprotective effect occurred due to a decrease in reactive oxygen species (ROS) production and a return of the antioxidant status to normal levels. IGF-II acts via not only the regulation of synthesis and/or activity of antioxidant enzymes, especially manganese superoxide dismutase, but also the restoration of mitochondrial cytochrome C oxidase activity and mitochondrial membrane potential. Although the antioxidant effect of IGF-I receptor activation has been widely reported, the involvement of the IGF-II receptor in these processes has not been clearly defined. The present report is the first evidence describing the involvement of IGF-II receptors in redox homeostasis. IGF-II may therefore contribute to the mechanisms of neuroprotection by acting as an antioxidant, reducing the neurodegeneration induced by oxidative insults. These results open the field to new pharmacological approaches to the treatment of diseases involving imbalanced redox homeostasis. In this study, we demonstrated that the antioxidant effect of IGF-II is at least partially mediated by IGF-II receptors.

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1. Introduction

Insulin-like growth factors (IGF-I and IGF-II) are naturally occurring peptides with known pleiotropic effects as growth factors. These endocrine, paracrine and autocrine effects range from metabolism to development, including neurogenesis and cellular maturation, proliferation, survival and growth. The majority of its actions are mediated primarily by insulin receptors, IGF-I receptors (IGF-IRs) and, although less understood, IGF-II/mannose-6-phosphate receptors (IGF-IIRs) [1]. Furthermore, several IGF-binding proteins (IGFBPs) may enhance or abrogate the effects of IGF [2]. IGF-II, a 67 amino acid peptide hormone that displays extensive interspecies homology [3,4], is synthesised at high levels during embryonic development. However, in adults, there is a decrease in its synthesis, although IGF-II remains the most abundant insulin-like peptide in the adult brain, primarily expressed in the choroid plexus, the septum and the hypothalamus [5]. IGF-IIR is also the most abundant insulin-like receptor in the adult brain. The physiological actions of IGF-II in adults are not well defined, although some studies have demonstrated an association of these peptides with cognitive processes, such as memory [6]. Insulin-like growth factor-II is involved in the regulation of intracellular calcium levels [7]. In a previous ex vivo study, the authors demonstrated the neuroprotective effects of IGFs (I and II) as antioxidants via the regulation of mitochondrial membrane potential (MMP) [7,8]. Moreover, a previous report by our group indicated that administration of low doses of IGF-II to old animals (24-month-old Wistar rats) induced neuroprotective effects and decreased the levels of oxidative stress markers in the hippocampus and the cortex. These effects were attributed to IGF-II, as IGF-I plasma levels remained unchanged in these experimental animals [9].

However, glucocorticoids, such as corticosterone (CORT), increase free-radical generation and impair mitochondrial function [10,11].

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More specifically, high CORT doses increase ROS production, reduce the GSH/GSSG ratio, the enzyme activities of complexes I and IV and MMP in mitochondria isolated from mice [12]. In neuronal cultures, similar alterations in mitochondrial function have also been reported [13,14]. Furthermore, mitochondrial dysfunction impairs energy production [12] and calcium storage capacity and increases ROS production, leading to oxidative damage [13,14], reducing the neuronal survival rate [10] and contributing to an enhanced neurotoxicity of kainate. In fact, mitochondrial dysfunction has been reported as a putative mechanism underlying the pathophysiology of some neurological and neuropsychiatric disorders [12,15,16]. In this sense, prolonged kainate. In fact, mitochondrial dysfunction has been reported as a putative mechanism underlying the pathophysiology of some neurological and neuropsychiatric disorders [12,15,16]. In this sense, prolonged exposure to high levels of glucocorticoids, such as those observed during stress responses, impairs both neuroplasticity and cognition and can facilitate the neuronal damage induced by several insults, including ischemic stroke and oxidative stress, increasing the susceptibility to develop neuropsychiatric and neurodegenerative disorders [15,17].

Recently, IGF-II has been linked to neuropsychiatric conditions associated with high glucocorticoid levels, such as depression. Along these lines, decreased mRNA levels of IGF-II have been reported in animal models of stress [18,19] and endogenous depression [18]. Conversely, pharmacological treatments that exhibit antidepressant-like properties (desipramine and dicholine succinate) induced the up-regulation of IGF2 gene expression [19,20]. These recent studies strongly suggest putative roles of IGF-II in the development of and recovery from glucocorticoid- and stress-related disorders.

Altogether, in the present study, we hypothesised that IGF-II, primarily via the IGF-IRs, protects cortical neurons against neurotoxic oxidative damage associated with increased brain corticosteroid levels. This study may contribute to the development of a new therapeutic strategy in which IGF-II may be targeted for the treatment of neurodegenerative and neuropsychiatric disorders. To achieve this goal, we used an “ex vivo” model in which inhibitors of IGF-II and the IGF receptors (IGFRs) I and II were administered to adult cortical neuronal cultures under CORT-induced oxidative stress conditions. Under these experimental conditions, we measured several oxidative stress parameters, particularly ROS production in whole cells and total antioxidant status (TAS), lipid hydroperoxide (LOOH) levels, superoxide dismutase (SOD) and glutathione peroxidase (GPX) antioxidant activity and PKC expression in cell homogenates. In addition, mitochondrial membrane potential (MMP) and cytochrome c oxidase (COX) activity were also measured to evaluate mitochondrial function.

2. Material and methods

2.1. Preparation of cortical neuronal cultures

Cultures of cortical neurons from adult rats were prepared according to a previously published procedure with minor modifications [21]. After animal decapitation, the cortex was removed, and slices of approximately 0.5 mm were transferred to tubes containing Hibernate™-A/B27™ supplement (B27) (Life technologies, Spain) and papain at the appropriate concentration and incubated for 30 min in a 30 °C water bath with gentle shaking. Then, the slices were triturated 10 × 3 times. The cells obtained from the sections were suspended in OptiPrep gradient medium (SIGMA, Spain) and centrifuged at 800 × g for 15 min. The fractions containing neurons were collected, suspended in Hibernate™-A/B27 and centrifuged twice at 200 × g for 2 min. The pellets were then resuspended in growth culture medium (Neurobasal™-A/B27 containing glutamine, FGF2 and penicillin/streptomycin, Life Technologies, Spain). For cell culture, 6- and 24-well plates were precoated with 100 μg/mL of poly-n-lysine. For immunohistochemical studies, glass coverslips (12 mm diameter) were pretreated with polyethyleneimine (1/500 v/v in ddH2O) followed by 2.5% FBS. Approximately 500,000 (6-well plates) or 125,000 (24-well plates) cells were seeded. The neurons were then incubated at 37 °C in 5% CO2, and after 1 h, the wells were rinsed twice with Neurobasal™-A and filled with growth culture medium. The media were replaced with fresh media (half volume) every 4 days. The experiments were performed after 12 days of growth.

This model has some strengths and limitations. The model does not include all factors that can be found in a complete living organism. This could be considered as a negative, but at the same time, it could be regarded as an important advantage. For instance, in the present study, we used serum-free media, and therefore those factors associated with the IGF system, such as IGFBPs [22,23] and other proteases, such as Htra1 [19], that could influence the interaction of IGF with its receptors or modulate the bioavailability of IGF are not present. The absence of these factors in the cultures allows us to more accurately interpret the results.

All experimental procedures were performed in accordance with European animal research laws (European Communities Council Directive 2010/63/EU) and the Spanish National Guidelines for Animal Experimentation (Real Decreto 1201/2005 and Ley 32/2007). All animal procedures were approved by the Institutional Animal Care and Use Committee of Malaga University.

2.2. Cellular treatment

In order to avoid the antioxidant properties of B27, this supplement was removed from the culture media, and the cells were maintained under this condition for 24 h before the experiments were performed. The cell cultures were exposed to: 10 μM CORT; 100 ng/mL IGF-II; 10 μM CORT in the presence of 100 ng/mL IGF-II; 10 μM CORT and 100 ng/mL IGF-II in the presence of 20 ng/μL IGF-I analogue (JB1, Bachem, Switzerland) or 5 ng/μl anti-IGF-IIR (R&D Systems, USA) and 10 μM CORT in the presence of 100 ng/mL IGF-II with 20 ng/μL JB1 and 5 ng/μl anti-IGF-IIR. The incubation time was 2 h, and the temperature was 37 °C. All of the measurements were performed immediately after treatment, except for the measurement of neurodegeneration, which was evaluated 24 h after cell treatment (pre-rinsed with B27-free medium).

2.3. Cortical cell viability

Viability was determined by quantifying the release of the intracellular enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) [24]. The LDH levels were measured in cell-free culture supernatants using a commercial spectrophotometric assay kit (Randox Laboratories Ltd., UK) adapted to a Cobas Mira Autoanalyzer (ABX Diagnostics, France). The results are expressed as the percentage of LDH released into the medium relative to total LDH (medium and cells lysed using Triton X™-100).

2.4. Preparation of homogenised cells from cortical neuronal cultures

The cells were suspended in buffer containing 10 mM HEPES, pH 7.4, 10 mM KCl, a protease inhibitor cocktail (Roche, Spain) and phosphatase inhibitors (SIGMA, Spain), incubated at 0 °C for 20 min and homogenised in the presence of 0.01% digitonin. The protein concentrations were determined via quantification of UV 280 nm absorbance using a Nanodrop™ (Thermo Scientific NanoDrop Products, USA). COX activity, LOOH levels and TAS (see below) were determined in the homogenates [25]. Western blot analyses were performed using the same homogenates without incubation in digitonin.

2.5. Determination of intracellular levels of ROS

Cellular ROS production was estimated by measuring O2•− production via flow cytometry using a dihydroethidium (DHZ) probe according to a previously published procedure [25,26]. Prior to the end of the incubation period, the cells were labelled with 5 μM DH2 in B27-free medium for 1 h at 37 °C. The cells were then washed and immediately
analysed via flow cytometry using the 620 nm long pass filter (FL3) of an EPICSXL flow cytometer (Beckman-Coulter, USA). Ten thousand events (cells) were recorded and evaluated using WINMDI 2.8 software (http://www.cyto.purdue.edu/flowcyt/software/Winmd1.htm). Incubation in 100 μM Menadione for 2 h was used as an oxidative stress control.

2.6. Markers of oxidative stress

Oxidative stress was evaluated by measuring the cellular level of lipid peroxidation, i.e., LOOH and the TAS in cell homogenates. The LOOH levels were regarded as the extent of lipid peroxidation, as LOOH comprises the intermediates of ROS-induced oxidation of phospholipids, glycolipids and cholesterol [27]. LOOH was measured using the FOX2 method [28] and adapted to a Cobas Mira Autoanalyser [29,30]. The LOOH level was calculated relative to a hydrogen peroxide standard curve. The LOOH levels were expressed as nmol/mg of protein. The TAS, i.e., the total enzymatic and nonenzymatic antioxidant capacity, was evaluated in cells using a commercial TAS kit (Randox Laboratories Ltd., UK) adapted to a Cobas Mira Autoanalyser that measures the formation of the radical cation ABTS$^+\bullet$ at 600 nm using ABTS$^+$ in the presence of a peroxidase (metmyoglobin) and H$_2$O$_2$ [25,31]. The final values were expressed as μmol/mg of protein.

2.7. Electrophoresis and Western blot

The samples were resuspended in (5X) polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled at 100 °C for 3 min using a thermo-block. The samples were then loaded (15 μl of protein/well) on a 12% polyacrylamide gel and subjected to a constant current of 0.8 mA/cm$^2$ for 45 min). After blocking, the membrane was incubated in various primary antibodies (produced in rabbit) at different dilutions (anti-GPX1/2,-pPKC and -SOD-2, 1:200, and anti-β-actin, 1:500; Santa Cruz Biotechnology, Spain) for 12 h at 4 °C, followed by incubation for 1 h in the anti-rabbit IgG alkaline phosphatase conjugated secondary antibody (Sigma, Spain) at a 1:10,000 dilution. The final colour reaction was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The Western blots were digitised using a flatbed scanner (HP Scanjet 5500c, Hewlett-Packard, Spain) and analysed using ImageJ software (US National Institute of Health; http://imagej.nih.gov/ij/).

2.8. Measurement of mitochondrial function markers

As mitochondrial function markers, we chose MMP and COX activity. MMP was evaluated using the lipophilic cationic probe 5,5,6′,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimizidolcarbocyanine iodide (JC-1) according to a previously described procedure [32,33]. JC-1 is a lipophilic carbocyanine that exists in a monomeric form and accumulates in mitochondria. In the presence of a high MMP, JC-1 reversibly forms aggregates that, after excitation at 488 nm, fluoresce in the orange/red channel (FL2—590 nm). A collapse of the MMP provokes a decrease in the number of JC-1 aggregates (see cells incubated in valinomycin) and a subsequent increase in monomers that fluoresce in the green channel (FL1—525 nm). This phenomenon is detected as a decrease in the orange/red fluorescence and/or an increase in the green fluorescence. The distribution of monomeric and aggregated JC-1 was determined via fluorometry to quantify the MMP and via fluorescence microscopy to analyse the intracellular heterogeneity of the mitochondria. The cells were incubated in 1 μg/mL JC-1 in B27-free medium for 20 min at 37 °C. Then, the cells were rinsed twice with B27-free medium and observed under an inverted fluorescence microscope (Leica DMIL with a 50-W HBO Mercury Short-Arc Lamp) using FITC (490/515) and TRITC (550/580) filter sets. Images were captured using a cooled Nikon Digital Sight DS-5Mc camera (Nikon Europe B.V., Spain). The images were acquired at 20X magnification and processed using ImageJ software. The fluorescence intensity was determined using a FLUOSTar Galaxy spectrophotometer (BMG, Lab Technologies, Germany) at 490/530 nm (for JC-1 monomers) and 525/590 nm (for JC-1 aggregates). The fraction of aggregated JC-1 was quantified as FL2 average/(FL2 average + FL1 average), which provided the most reproducible estimate of MMP. This ratio was found to be insensitive to a 10-fold variation in the JC-1 concentration. To completely deplete the MMP, the potassium ionophore valinomycin (1 μM) was used as a control.

COX (EC 1.9.3.1) activity in cell homogenates was assessed using a COX assay kit (Sigma, Spain) adapted to a Cobas Mira Autoanalyser [34]. One unit was defined as the oxidation of 1.0 μmol of ferrocytochrome c per minute at pH 7.0 and 37 °C.

2.9. Assessment of neurodegeneration in cell culture

Neurodegeneration of the cultured cortical neurons was measured using Fluoro-Jade B$^+$ (FJ) dye according to a previously published procedure [35]. The cell culture plates were fixed using cold ethanol (−20 °C) for 30 min and washed three times with distilled water. The cells were treated with the dye (final concentration of 0.0004% FJ in 0.1% acetic acid) and gently shaken for 30 min in the dark at RT and then washed twice with distilled water. Finally, the fluorescence intensity was determined using a FLUOSTar Galaxy spectrophotometer (BMG, Lab Technologies, Germany) at 485/530 nm.

2.10. Double immunocytochemical staining of cortical neurons

Fixation was accomplished by treating the plate with methanol previously chilled to −20 °C and incubating at −20 °C for 20 min. The wells were washed with PBS, and the coverslips were removed and incubated in a primary antibody solution containing the following: 1:15 goat anti-IGF-II (R&D Systems, USA) and 1:250 rabbit anti-NeuN (Millipore, Spain) antibodies in PBS, 3% BSA and 0.02% sodium azide at 4 °C overnight. Then, the coverslips were incubated in a fluorescent secondary antibody solution containing a mixture of 1:500 Alexa Fluor$^+$ 488 anti-goat (Life Technologies, Spain) and 1:500 Alexa Fluor$^+$ 568 anti-rabbit (Life Technologies, Spain) in PBS for 1 h at room temperature in the dark. The coverslips were mounted using Fluoromount$^+$ (Sigma, Spain). Images were acquired using an Olympus BX51 epifluorescence microscope at 40X magnification and processed using ImageJ software (US National Institute of Health; http://rsweb.nih.gov/ij/).

2.11. Statistical analysis

Statistical differences were determined using one-way ANOVA. Pairwise comparisons were performed using a post hoc Bonferroni t test. Statistical significance was considered to be p < 0.05. For data in which the measured units are arbitrary, the respective values represent the percentage relative to the control value.

3. Results

3.1. ROS production in cell cultures

To evaluate the protective effect of IGF-II on neurons we used a model of adult rat cortical neuronal cultures incubated in a pathophysiological concentration of CORT (10 μM). We investigated the intracellular production of ROS, the antioxidant effect of a low concentration of IGF-II and the role of IGF-IR in these effects.

Under pathophysiological conditions, such as severe stress, plasma COR levels in rats increase up to 1–2 μM [12,23,36]. In vitro, concentrations of CORT between 1 and 10 μM or even higher (50 μM) are
considered stress-relevant concentrations [37,38]. In our study, we intentionally treated cortical neurons with a high dose of CORT (10 μM) to attain a clear increase in ROS production (Fig. 1a) in 2 h without notable necrosis or cytotoxicity, as shown in the survival curve (Fig. 1b).

The low concentration of IGF-II was chosen based on the results of previous experiments [9,39,40] that demonstrated the capacity of IGF-II to induce neuroprotection by reducing oxidative stress in the cortex and the hippocampus of aged rats. In addition, in “in vivo” studies, similar concentrations of endogenous IGF-II were found in damaged brain tissue; also, such concentrations were used in further experiments to investigate the effect of IGF-II on brain function and their ability to induce neurogenesis and neuroprotection [17,41–43]. In the present study, we determined the minimal nontoxic IGF-II concentration based on the survival curve (Fig. 1c).

As IGF-II activates both the IGFRs, I and II, at different affinities [44], in order to clarify the influence of each individual subtype in the ultimate effect, we performed a set of experiments using selective IGFR inhibitors [6,45].

To assess the influence of IGFR inhibitors in the absence of IGF-II from the incubation media, a set of experiments was performed, and neither AB nor JB1 resulted in any effect on intracellular ROS production. These treatments were excluded from the present study. The control cells treated with IGF-II did not display any difference in ROS production compared to untreated cells (Fig. 1d).

Treatment with CORT at 10 μM produced an increase in the fluorescence of DH2 (a ROS-superoxide indicator) to a level significantly higher than that of the control (CO) cells (a 40% increase). Addition of 100 ng/mL of IGF-II to the CORT-treated cells restored the ROS levels to those found in the CO cells (Fig. 2a and b). The addition of specific inhibitors of the IGF-II receptor (anti-IGF-II antibody, AB) or the IGF-I receptor (JB1, a potent inhibitor of IGF-I receptor autophosphorylation) did not alleviate the effect of the low concentration of IGF-II (Fig. 2a and c), but co-incubation of both inhibitors completely abolished the antioxidant effect of IGF-II (Fig. 2a and d).

3.2. Oxidative stress cellular markers

In agreement with the above findings, the TAS, i.e. the fraction of the antioxidant pool available for further anti-ROS activity, was significantly lower in cortical neurons incubated in CORT compared to the CO cells. Addition of the low concentration of IGF-II to the incubation media restored the TAS found in cultures incubated in CORT to that of the CO cells (Fig. 3a). Co-incubation of the CORT- and IGF-II-treated cells with JB1 or AB did not reverse the antioxidant effect of IGF-II; although some effect could be detected after the addition of AB, the decrease in the protective effect produced by IGF-II did not reach statistical significance. Nevertheless, the co-incubation of IGF-II with both inhibitors fully abolished its antioxidant effect.

The occurrence of oxidative stress and lipid membrane damage by ROS in cortical neurons was confirmed based on the cellular levels of lipid hydroperoxides (LOOHs). The results were very similar to those mentioned above. The cellular concentration of LOOH was significantly higher in CORT-treated neurons than in CO neurons (Fig. 3b). Addition of the low concentration of IGF-II to the culture medium restored the LOOH levels to control levels. Individually adding the inhibitor JB1 or AB to the CORT- and IGF-II-treated cells did not alter the LOOH levels, but co-incubation of both inhibitors dramatically increased the levels of LOOH to the same value as that of the CORT-treated cells.

3.3. Expression of antioxidant enzymes

In the present study, we measured the concentrations of enzymes involved in maintaining the intracellular redox equilibrium. The level of mitochondrial antioxidant enzyme superoxide dismutase (SOD-2, MnSOD), which rapidly catalyses the dismutation of superoxide to...
H$_2$O$_2$, was significantly decreased in cells treated with CORT for 2 h compared to the CO cells (up to 57% less than the CO cells) (Fig. 4a). Co-incubation of the cells with IGF-II and CORT partially alleviated this decrease in the MnSOD levels compared to CO cells (up to 30% less than the CO cells, compared to 57% as shown above) (Fig. 4a). The addition of the IGFR inhibitor JB1 or AB individually to the CORT- and IGF-II-treated neurons, did not significantly affect the levels of MnSOD compared to the CORT- and IGF-II-treated neurons (21% and 36% less than the CO cells, respectively). However, co-incubation of both inhibitors (JB1 and AB) significantly decreased the MnSOD levels similar to those found in the CORT-treated cells (60% less than the CO cells). The expression of GPX, an enzyme that neutralises H$_2$O$_2$ and LOOH using GSH [46], was significantly enhanced (70% more than the CO cells) in cortical neurons treated with CORT (Fig. 4b). This considerable increase was completely alleviated by incubating the cells in the low concentration of IGF-II. The beneficial effect of IGF-II on the damage induced by CORT was only partially prevented by incubation in either the inhibitor JB1 or AB. However, co-incubating the cells with both inhibitors totally abolished the protective effect of IGF-II.

3.4. Mitochondrial function in neurons

Mitochondria are the major source of physiological and pathological cellular ROS. To assess mitochondrial function, we evaluated oxidative...
phosphorylation by measuring the MMP [47] and mitochondrial COX activity, which is the rate-limiting step of the mitochondrial respiratory chain [48].

Mitochondria from healthy control cells exhibiting high membrane potential (which allows JC-1 to spontaneously form aggregates) emit orange-red fluorescence (Fig. 5c arrows). After incubation in CORT, the mitochondria become partially depolarised, decreasing JC-1 complex formation and thus emitting yellow-green fluorescence. Co-incubation of the cells with CORT and IGF-II prevented the damage induced by CORT alone, which resulted in the recovery of the orange-red fluorescence. No significant changes in the fluorescence were detected after incubation of the cells in CORT, IGF-II and either JB-1 or AB compared to the cells treated with CORT and IGF-II. Co-incubation with both inhibitors together (JB-1 and AB) partially shifted the fluorescence to yellow-green, indicating MMP levels similar to those found in the CORT-treated cells.

Analysis of the JC-1 labelling pattern revealed intracellular heterogeneity of mitochondria, which indicated diverse membrane potential and spatial localisation (see the insert of Fig. 5c). Again, we detected the distribution pattern and alterations induced by CORT (e.g. yellow-green mitochondria near the cell soma; see arrow in Fig. 5c and the insert) that were not apparent when the cells were co-incubated with IGF-II. No relevant changes in the mitochondrial fluorescence pattern were detected upon incubation of the cells in CORT, IGF-II and either JB-1 or AB, whereas co-incubation with both inhibitors produced the same pattern as that of the CORT-treated cells.

Pre-treatment with valinomycin, which dissipates the potassium gradient and diminishes the MMP, displayed a dramatic effect on the red fluorescence (Fig. 5c medium panel), which became very faint. In contrast, valinomycin treatment caused a slight but detectable increase in the green fluorescence intensity. As a result, the overlay images displayed the majority of mitochondria exhibiting green fluorescence.

To obtain a more precise quantification of the fluorescence intensity as an indicator of MMP, we measured the JC-1 fluorescence using a spectrofluorometer and calculated the ratio of FL2/(FL1 + FL2), which reflects the average MMP of all mitochondria inside the cells. We found that after CORT treatment, MMP was significantly decreased, and this ratio was restored to the CO level by treatment with a low concentration of IGF-II (Fig. 5a). No changes were detected upon incubation in CORT, IGF-II and either JB-1 or AB, whereas co-incubation with both inhibitors restored the MMP to that of the CORT-treated cells (Fig. 5a).

When we analysed COX activity as a marker of respiratory chain function, we found that cortical neurons incubated in CORT displayed a significant decrease in the COX activity (Fig. 5b). The COX activity returned to the CO level when the cells were incubated in a low concentration of IGF-II. When the cells were incubated in CORT in the presence of IGF-II and the inhibitor JB1, COX activity was slightly increased. However, incubation of the cells in CORT in the presence of IGF-II and the inhibitor AB significantly increased COX activity. Co-incubation of both JB1 and AB resulted in a reversion of COX activity to a level near that of the CORT-treated cells.

3.5. Neurodegeneration

As increased ROS production and mitochondrial dysfunction play crucial roles in major neurodegenerative disorders, we examined neuronal degeneration using the polyanionic stain FJ to specifically detect neurodegeneration independent of the mode of insult; this probe is specific to a late stage of the degenerative process [35]. Experimentally, our model did not allow us to study prolonged incubation periods, as adult neuronal cultures require antioxidants (B27™ supplement) in the culture media. Thus, only short periods (24–48 h) in the absence of antioxidants can be used to avoid spontaneous degeneration of the neurons.

A large and significant increase in the FJ fluorescence intensity of the CORT-treated cells was detected compared to the CO cells (Fig. 6). Addition of 100 ng/mL of IGF-II to the culture medium completely alleviated this increase. The addition of each inhibitor (JB1 or AB) did not exert any change in the effect of IGF-II, whereas the addition of both inhibitors together returned the fluorescence level to that of the CORT-treated cells.

**Fig. 4.** Expression of antioxidant enzymes GPX and MnSOD. Representative Western blots (upper panels) and quantifications after normalising with β-actin (lower panels) demonstrate the IGF-II recovery from the imbalance of the amount of antioxidant enzymes triggered by CORT. (a) Increase in the level of MnSOD after CORT + IGF-II co-incubation. (b) IGF-II restores GPX levels modified by CORT. Data were combined from 3 to 4 independent experiments and presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to CO; *p < 0.05, **p < 0.01, ***p < 0.001 compared to CORT. Data in graphs represent percentage of CO value.
3.6. Modulation of PKC activation

To confirm the presence of the IGF-IIR in the cultures of the analysed adult cortical neurons, we further performed IGF-IIR/NeuN double immunostaining experiments. Double immunostaining analysis confirmed the neuronal expression of the IGF-IIR. The cellular distribution of IGF-IIR + immunoreactivity was primarily located in the soma and, to a lesser extent, in the cellular projections (Fig. 7a).

We also chose to examine PKC activation because this enzyme is activated by IGF-II, glucocorticoids and ROS [36,49,50]. To assess the activation of PKC, we analysed the expression of its phosphorylated form (pPKC). CORT treatment resulted in an increase of pPKC expression (Fig. 5).
which was alleviated by treatment with a low concentration of IGF-II. Treatment with each inhibitor (JB1 or AB) did not exert any change in the effect of IGF-II on pPKC expression, whereas treatment with both inhibitors together re-established the level of pPKC expression to that of the CORT-treated cells (Fig. 7b).

4. Discussion

We and others have recently reported that IGF-II and IGF-I replacement therapy exert neuroprotective antioxidant effects on pathophysiological processes [9,51], such as acute hypoxia, cerebral ischemia and aging [9,52,53]. For the most part, the effects of IGF are primarily mediated via the IGF-IR and, although less known, the IGF-IIR [54]. The aim of this study was to determine the influence of the IGF-IIR on the antioxidant effects of IGF-II in the brain on CORT-induced oxidative damage.

The primary outcome of the present study is the finding that IGF-II displayed antioxidant effects on neuronal cultures from the adult rat cortex when exposed to ROS-mediated damage induced by glucocorticoids, and this effect is mediated in part by the IGF-IIR. These findings are supported by the following experimental results: a) the levels of ROS, in particular O$_2^\bullet-$, produced by incubation of neurons in a high concentration of CORT were significantly higher than those found in the CO neurons. Moreover, co-treatment of the cells with CORT and a

Fig. 6. Neurodegeneration in adult cortical neuronal cultures. FJ fluorescence increases after incubation of adult neurons with 10 μM CORT. Co-incubating the cells with CORT + IGF-II completely reverted this increase. The use of inhibitors (JB1 or AB) did not exert any change when incubated individually with IGF-II, whereas the use of both inhibitors together abolished the protective effect developed by IGF-II against the CORT induced damage. Kainic acid (1 mM) was used as control. Data were combined from 2 to 3 independent experiments and presented as mean ± SEM of percentage of CO value. &p < 0.05, ***p < 0.001 compared to CO; ***p < 0.001 compared to CORT.

Fig. 7. IGF-II receptor expression in neuronal cells and modulation of PKC activation. (a) The presence of IGF-IIR was confirmed in adult neuronal cell cultures by simple and merged images of double fluorescent immunostaining for IGFII-R (Alexa Fluor™ 488) and NeuN, a neuronal marker (Alexa Fluor™ 568). Detailed distribution of immunoreactivity is indicated by arrowheads and magnified image. (b) IGF-II restores to CO levels the pPKC amount increased by CORT incubation. Data were combined from 2 to 3 independent experiments and presented as mean ± SEM. &p < 0.05 compared to CO; *p < 0.05, **p < 0.01 compared to CORT. Data after normalising were presented as percentage of CO value.
low concentration of IGF-II reduced the ROS level to that of the CO cells; b) TAS, defined as the fraction of the antioxidant pool available for further anti-ROS activity, is significantly reduced in the neurons incubated in CORT compared to the CO neurons. Again, co-incubation with IGF-II alleviated the reduction in TAS; and c) the LOOH level, considered as a reliable initial biomarker of lipid membrane damage, was significantly higher in CORT-treated cells, and co-treatment with IGF-II completely abrogated this increase in the LOOH level, returning the LOOH level near that of the CO cells. Moreover, the detected increase in the LOOH levels after CORT treatment led to an enhancement in hydroperoxide-reducing enzyme (GPX) levels, and this increase was inhibited by IGF-II treatment. Similar effects of IGF-II on these oxidative markers have also been reported in the aged brain [9].

The present results suggest that the oxidative stress induced by CORT and the reduction in oxidative damage associated with IGF-II treatment as performed in our study could be closely associated with mitochondrial activity. It is well understood that mitochondria are the major source of physiological and pathological cellular ROS and contribute to the oxidative damage found in several neuropathological conditions related to enhanced glucocorticoid expression [13]. In our study, we also reported some changes in mitochondrial antioxidant defence caused by CORT treatment, leading to the aforementioned oxidative damage. First, when the cells were incubated in CORT, we detected a decrease in the expression of mitochondrial SOD, which rapidly catalyses O₂⁻, the predominant species that leads to the formation of other ROS [55,56]. Co-incubation of IGF-II and CORT significantly increased the expression of the protective enzyme MnSOD and, once again, this effect was mediated by both IGFR-I and II. Secondly, in agreement with Lee et al. [57], we detected decreases in MMP and COX activity, which are associated with mitochondrial dysfunction, as well as an increase in ROS production after incubation in CORT. A decrease in the MMP alters the energy and calcium metabolism of neurons, as suggested by the cellular distribution and morphology of the mitochondria (see the insert of Fig. 5c). We found that healthy mitochondria (emitting orange-red fluorescence) appeared to be uniformly distributed inside the cells, whereas impaired mitochondria (emitting yellow-green fluorescence) returned to the cell soma, perhaps for destruction or repair [12,58]. IGF-II can restore the MMP levels not only via its interaction with the IGF-IR, as suggested in previous studies [7,8,51,59,60], but also via activation of the IGF-IR [9,40]. When we inhibited the IGF-IR, IGF-II-mediated increase in the MMP was not abolished, but the effect of IGF-II on the MMP completely reverted to the levels of CORT treatment alone when both IGFRs were inhibited, stressing the influence of the IGFR-II on the MMP. We also detected a decrease in COX activity in the CORT-treated cells, while IGF-II restored COX activity to the levels of the CO cells, in agreement with a previous study by our group [9,40]. The effects of IGF-II on COX activity displayed a peculiar pattern of interaction that requires further examination to be clarified.

Excessive amounts of ROS and mitochondrial dysfunction are among the major mechanisms that trigger neurodegeneration [61]; in fact, we detected a large increase in the fluorescence intensity of FJ, a neurodegeneration marker, in cells incubated in CORT after an additional recovery period of 24 h post-treatment. Once again, IGF-II decreased the FJ fluorescence intensity, and patterns of the effect of IGF-II on FJ staining and ROS production were very similar, indicating a relationship between the antioxidant effect of IGF-II via inhibition of ROS production and neurodegeneration. However, these findings do not exclude the possibility that IGF-II could produce an increase in the activity of the endosomal–lysosomal system to degrade abnormal intracellular proteins induced by ROS [62–64].

Although many biological effects of IGF-II are mediated by the IGF-IR, and classically, the IGF-IIR receptor is considered to act as a “clearance receptor” to stabilise the local IGF-II concentration, presently, there is evidence strongly suggesting that the IGF-IR is involved in some of the IGF-II-mediated biological actions [1,6,65]. Interestingly, in our experiments, the neuroprotective antioxidant effects of IGF-II in cortical neurons were also mediated via the IGF-IIR. The individual blockade of the IGF-IR or the IGF-IIR using specific inhibitors (JB1 or AB, respectively) did not abolish the IGF-II protective effect, demonstrating that both the IGF-IR and the IGF-IIR contribute to the antioxidant properties of IGF-II. When both IGFRs were blocked simultaneously, the antioxidant effect of IGF-II was completely abolished, emphasising that both receptors are responsible for the antioxidant properties of IGF-II. These results strongly support the well-known antioxidant role of the IGF-IR [66–68]. However, these results also demonstrate for the first time a strong involvement of the IGF-IIR in these effects. Although IGF-II can also interact with insulin receptor at a very low affinity, these receptors are expressed at very low levels in adult neuronal tissue [42,69]. Furthermore, if some of the antioxidant effects detected in our experiments were due to an interaction between IGF-II and insulin receptors, these effects should be maintained after the blockade of both IGFRs, but this was not the case in our experimental model.

The specific signalling pathway associated with the activation of the IGF-IIR remains largely unclear at present. The actions of the IGF-IIR could be mediated by G-protein activation calcium modulation [65], GSK3 [6], MAP kinase [70] and PKC activation [54,65]. Recently, it has been demonstrated that some of the effects of CORT on neurons are mediated by PKC phosphorylation [36,71], suggesting that both IGF-II and CORT may share a common intracelluar pathway involving PKC. In agreement with the above findings, in our study, CORT and oxidative stress activate PKC, as reported by others [36,49,50], and IGF-II treatment restores the levels of PKC to those of the CO cells. However, clarification of the specific contribution of this PKC-mediated mechanism will require further studies.

Experimental evidence demonstrated that mitochondria are the major source of physiological and pathological cellular ROS and contribute to the oxidative damage found in several neuropathological disorders associated with enhanced glucocorticoid levels in tissues. Furthermore, it has been reported that an increase in glucocorticoid levels induces oxidative stress [12,14,72] and affects mitochondrial function, including decreased MMP, increased cell vulnerability, induction of apoptosis and impaired neuronal plasticity and neurogenesis [12,13,31,73]. All of these cellular abnormalities could contribute to the development of neuropsychiatric and neurodegenerative disorders associated with stress [15,74]. Moreover, these results may explain the finding that increased IGF-II expression serves as a defence mechanism in pathological conditions associated with an elevated CORT concentration, such as depression [20]. Moreover, the mitochondrial antioxidant properties of IGF-II could likewise explain the neuroprotective effect of IGF-II treatment in neuropsychiatric diseases [17,19].

The presence of the antioxidant effect of IGF-II could explain responses of nervous system tissue to IGF-II under oxidative damage, in which an increase in the levels of IGF-II were detected under pathologic conditions, such as ischemia, hypoxia [52,75], amyotrophic lateral sclerosis [76,77] and traumatic brain injury [78], in an attempt to counteract the increase in oxidative damage found in these diseases. In agreement with these findings, we recently detected an antioxidant effect of IGF-II treatment on the brains of aged rats [9]. Moreover, it has been recently determined that mitochondrial dysfunction and high levels of intracellular ROS could trigger neurodegeneration. The neuroprotective effect of IGF-II, based in its aforementioned antioxidant properties, could support the finding of increased levels of IGF-II in neurodegenerative diseases, such as Alzheimer and Parkinson disease [15,79,80]. In addition, Dore et al. found that IGF-II alleviated the mitochondrial neurotoxicity of β-amyloid and indicated that the activation of the IGF-IR was responsible for this effect [39]. They were unable to determine the role of the IGF-IIR because they did not use any antagonists or inhibitors of IGFR-I or -II in that study. However, these findings do not exclude the possibility that IGF-II could induce increased activity of the endosomal–lysosomal system (mediated via the IGF-IIR) to degrade abnormal intracellular proteins induced by ROS [62–64].
In summary, IGF-II could also act as an antioxidant [9,81] to alter the mechanisms of neuroprotection [42] and neurogenesis [17,41] and, ultimately, contribute to neuronal plasticity [62,82,83], especially under conditions in which cognitive deficits are associated with pathologies or disorders involving impaired redox homeostasis, such as aging, stroke, Alzheimer disease and other neuropsychiatric diseases [84].

5. Conclusion

Based on our analysis of the antioxidant and neuroprotective effects of IGF-II, a tentative explanation can be offered. After an oxidative insult, IGF-II acts through IGF Rs (both the IGF-IR and the IGF-IR) to promote an improvement in mitochondrial function, increasing the levels of MnSOD and COX activity and the MMP. The recovery of mitochondrial function may restore the redox status and calcium turnover, thus decreasing ROS production, membrane lipid peroxidation (primarily the phospholipid cardiolipin), cytochrome c release, apoptosis, neuronal damage and neurodegeneration. These results open the field to new pharmacological approaches to treat neuroprotective and neurodegenerative diseases.

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References

possible roles of IGF binding proteins (IGFBPs) in the mediation of IGF-II activity, Endocrinology 140 (1999) 520–532.


