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Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) induces initiation factor 2α phosphorylation and translation inhibition in PC12 cells

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Abstract We have investigated the effect of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on protein synthesis rate and initiation factor 2 (eIF2) phosphorylation in PC12 cells differentiated with nerve growth factor. FCCP treatment induced a very rapid 2-fold increase in intracellular Ca^{2+} concentration that was accompanied by a strong protein synthesis rate inhibition (68%). The translation inhibition correlated with an increased phosphorylation of the α subunit of eIF2 (eIF2α) (25% vs. 7%, for FCCP-treated and control cells, respectively) and a 1.7-fold increase in the double-stranded RNA-dependent protein kinase activity. No changes in the PKR endoplasmic reticulum-related kinase or eIF2α phosphatase were found. Translational regulation may play a significant role in the process triggered by mitochondrial calcium mobilization.

Key words: Calcium ion; Initiation factor 2 kinase; Mitochondrion; Permeability transition pore; PC12 cell; Translation

1. Introduction

Cellular function is acutely sensitive to alterations in the calcium contents in various compartments, including the cytosol, endoplasmic reticulum (ER) and mitochondria. Mitochondria play a role in $[Ca^{2+}]_i$ homeostasis, sequestering large quantities of Ca^{2+} via a low-affinity, high-capacity mechanism and releasing Ca^{2+}, particularly if they have previously been loaded with Ca^{2+} (e.g. due to intense cellular activity, reperfusion after ischemia or merely as a result of in vitro ageing) [1,2]. Ca^{2+} accumulation and oxidative stress can trigger the opening of a non-selective high-conductance channel in the inner mitochondrial membrane, i.e. the permeability transition pore (PTP). This opening leads to a sudden increase in permeability to solutes of molecular mass up to 1500 Da, and to a collapse of the electrochemical potential for H^+, thereby arresting ATP production and triggering production of reactive oxygen species. Depending on the nature of the PTP opening (transient or maintained), the cascade of events triggered can lead to cell apoptosis or necrosis [3–5].

It is not clear whether the PTP has the physiologic function of releasing large mitochondrial Ca^{2+} loads, however ischemia with recirculation might represent a PTP-related pathologic condition. This assumption has received strong support from studies showing that cyclosporin A (CsA), a specific blocker of the PTP in vitro, exerts a sharp anti-ischemic effect in vivo [6].

Protein synthesis is one of the cellular processes most sensitive to ischemia and a straight correlation between translation inhibition and increased phosphorylation of the α subunit of initiation factor 2 (eIF2(αP)) after transient ischemia in the rat brain has been demonstrated [7–9]. Lack of eIF2α kinase(s) activation, including the double-stranded RNA-dependent kinase (PKR), has been reported in different ischemia models [10,11]. Conversely, we have demonstrated an ischemia-induced eIF2α phosphatase inhibition in nerve growth factor (NGF)-differentiated PC12 cells [11]. However, the mechanism implicated remains to be established. Thus, to gain insight into the mechanism of the ischemia-induced eIF2α phosphorylation here we studied whether the mitochondrial dysfunction as reported in ischemia plays a role in the observed eIF2(αP) accumulation. Hence, NGF-differentiated PC12 cells were treated with the oxidative phosphorylation uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [12].

2. Materials and methods

2.1. Cell culture

PC12 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin, were maintained in a humidified 5% CO_2/95% air incubator at 37°C. Cells were subcultured either in collagen-coated 24-well plates at a density of 5×10^4 cells/well for viability assays and protein synthesis rate determination or in 6-well plates (2.5×10^5 cells/well) for the remaining experiments. Cell differentiation was performed in the presence of 100 ng/ml NGF-7S (Alomone Labs) and the trypan blue exclusion method was used to assess cell viability [11,13].

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CsA, cyclosporin A; DTT, dithiothreitol; eIF2, eukaryotic initiation factor 2; eIF2(αP), eIF2 phosphorylated on its α subunit; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NGF, nerve growth factor; poly(I·C), poly(inosinic acid·cytidylic acid); PKR, double-stranded RNA-dependent kinase; PERK/PEK, PKR endoplasmic reticulum-related kinase; PTP, permeability transition pore

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2.2. Measurement of cytosolic calcium

Differentiated PC12 cells cultured on 24-mm round collagen-coated glass coverslips were loaded with 3 μM fura-2-AM as described elsewhere [14]. After rinsing loaded cells were equilibrated in DMEM medium and coverslips were transferred to a coverglass holder filled with 300 μl of DMEM. $[Ca^{2+}]_i$ was measured as described in Fig. 1.

2.3. Measurement of protein synthesis rate

Protein synthesis rate was assayed in 24-mm diameter multi-well dishes with fresh medium containing 0.175 Ci/mmol of [^{3}H]methionine (200 μM), for 30 min at 37°C as described elsewhere [13]. All data were normalized to the number of live cells in the culture on the day of cell labelling.

2.4. Determination of eIF2α phosphorylation

Cell lysates adjusted to 35 μg of protein were resolved by horizontal isoelectric focusing slab gel (IEF) electrophoresis and analyzed by protein immunoblot with a polyclonal antibody against eIF2α (Santa Cruz Biotechnology Inc.) at a 1:200 dilution as described elsewhere [11,13]. Stained bands were scanned and quantified with an Image Analyzer using the Diversity One software package (PDI, New York, USA).

2.5. Measurement of eIF2α kinase activities

PKR activity was assayed in cell lysates adjusted to equal protein concentration (25 μg) as described previously [15,16]. Briefly, the incubation mixture contained [γ-^{32}P]ATP (32 μM, 8 Ci/mmol) and 1 μg of purified eIF2 with or without 1 μg/ml poly(inosinic acid·cytidylic acid) (poly(I·C)). Similar experiments were performed with differentiated PC12 cells after 20 h pre-treatment with rat interferon (1000 U/ml, Sigma). PKR ER-related kinase (PERK/PEK) was assayed by adding 3 μg of pure eIF2 and 20 μCi [γ-^{32}P]ATP (100 μM, 7 Ci/mmol) to the kinase immune complexes obtained by precipitating 1 mg of cell lysate (3×10^6 cells) with 10 μl of polyclonal anti-PEK peptide antibody kindly provided by Dr. Shi [17]. The kinase reaction mixtures underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by autoradiography. The radioactive phosphate incorporated to the eIF2α subunit was quantified as described above.

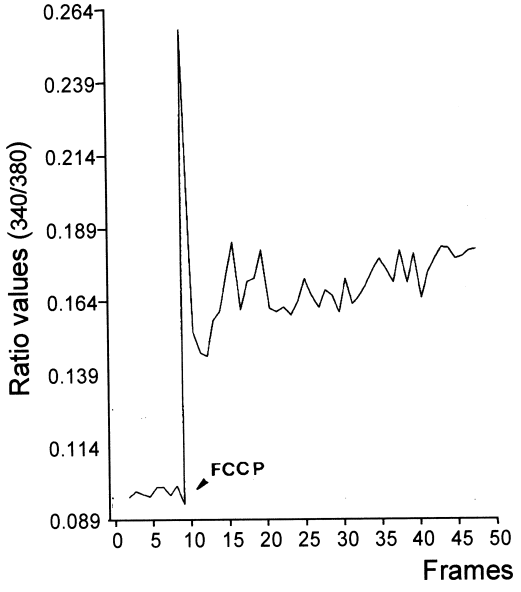

Fig. 1. $[Ca^{2+}]_i$ after FCCP treatment. $[Ca^{2+}]_i$ was measured in control cells for up to 10 min with a Nikon microscope linked to a ratio fluorescence spectrophotometer (Life Sciences resource) equipped with CONCARD software. The excitation maximum for the Ca^{2+}-bound and the free dye was 340 and 380 nm, respectively. Ratio values represent the 340/380 ratio and data were acquired each 15 s (one frame). Data acquisition was interrupted for 30 s to apply 30 μM FCCP in fresh medium and $[Ca^{2+}]_i$ was measured for 20 min. The figure shows a five cells average and it is representative of three different cultures.

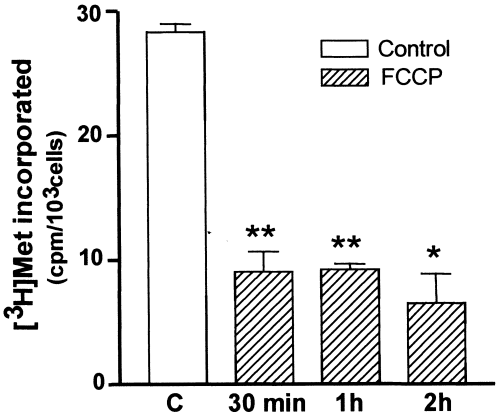

Fig. 2. FCCP effect on protein synthesis rate. Protein synthesis was measured in FCCP-treated and untreated PC12 cells, as described in Section 2. Values represent mean ± S.E.M. of 3–6 different cultures. Statistical comparisons were made by the Student *t* test. Statistical significance between control and FCCP-treated cells: $*P<0.05$, $**P<0.01$.

3. Results and discussion

To explore the potential role of mitochondrial dysfunction in translational regulation, we used the respiratory uncoupler FCCP which induces significant $[Ca^{2+}]_i$ increase through mitochondrial PTP opening [12]. As shown in Fig. 1, immediately after the addition of 30 μM FCCP, $[Ca^{2+}]_i$ levels increased 2–3-fold followed by a plateau level of at least 20 min (1.6-fold basal level). The FCCP-stimulated increased $[Ca^{2+}]_i$ in differentiated PC12 cells was similar to the one described elsewhere [14].

Protein synthesis rate was measured in PC12 cells treated with FCCP for different period of times. As shown in Fig. 2, FCCP treatment caused a strong inhibition of protein synthesis rate (68%), for at least 2 h of treatment. eIF2(αP) levels paralleled translation inhibition, they being higher in FCCP-treated cells than in control cells (Fig. 3). FCCP did not cause cell death at no times considered (not shown), this result being in agreement with those observed in cultured hippocampal neurons [18].

The FCCP-induced increased eIF2α phosphorylation is expected to be the result of a mismatch of the factor phospho/dephosphorylation mechanisms. Nevertheless, eIF2α phosphatase activity as measured by procedures we described elsewhere [11] remained unchanged following FCCP treatment (15.5 ± 0.2 vs. 14.1 ± 1.0 A.U. for control and FCCP-treated cells, respectively).

PKR, a mammalian eIF2α kinase present in most cell types, has been involved in Ca^{2+} signalling pathways [15,19,20]. The increased phosphorylation of exogenous eIF2α in the presence of poly(I·C) (Fig. 4A,B) supported the presence of PKR activity in our extracts, furthermore PKR activity was significantly enhanced by FCCP treatment. To provide additional evidence of the eIF2α kinase identity, FCCP treatment was performed in cells incubated with rat interferon. As expected, eIF2 phosphorylation in interferon-treated control cells increased due to PKR induction. PKR activity in such cells was further heightened by FCCP treatment (Fig. 4A,B). We also measured the activity of the recently characterized eIF2α kinase, PERK/PEK, involved in ER stress [17,21], but we

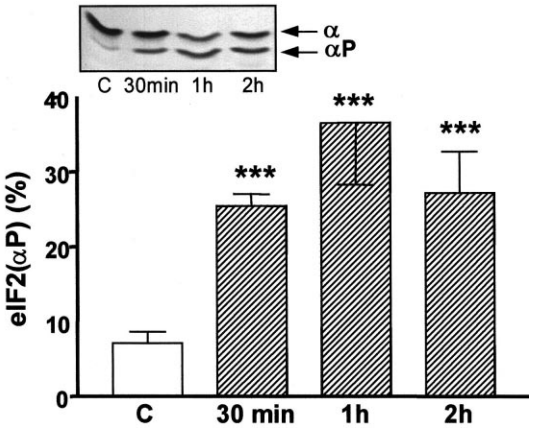

Fig. 3. FCCP effect on eIF2α phosphorylation status. Cell lysates from untreated and FCCP-treated cells were subjected to IEF electrophoresis and bands corresponding to eIF2α and eIF2(αP) were analyzed by protein immunoblot as described in Section 2. Results are expressed as the percentage of eIF2(αP) over the total eIF2 and represent mean ± S.E.M. of 3–5 different cultures. Bars as in Fig. 2. Insert: a typical experiment is shown; lane C, untreated cells. Keys: α, eIF2α; αP, eIF2(αP). Statistical significance is as in Fig. 2, $***P < 0.001$.

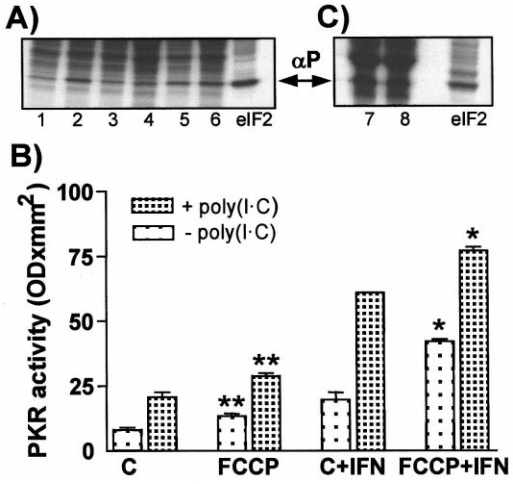

Fig. 4. FCCP effect on eIF2α kinase activities in vivo. PKR (A, B) and PEK (C) activities were assayed as described in Section 2 after a 30 min FCCP treatment. A and C show autoradiographs corresponding to a representative experiment. Lanes 1, 3–8, without poly(I·C); lane 2, with poly(I·C); lanes 1–3, 5 and 7, untreated cells; lanes 4, 6 and 8, FCCP-treated cells. Lanes 5 and 6, interferon-treated cells. eIF2, factor phosphorylated by partially purified HRI. (B) Values represent the mean ± S.E.M. corresponding to 2–4 different cultures. Keys and statistical significance as in Fig. 2.

failed to show increased PEK activity following FCCP treatment (Fig. 4C).

Calcium mobilization from the internal cell stores rather than increased cytoplasmic $[Ca^{2+}]_i$ levels is responsible for the higher eIF2α phosphorylation observed in several cell types in response to agents, such as the ionophore A23187, EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) or thapsigargin [15,19,22]. Although analyses of extracts have revealed a 2–3-fold increase in PKR activity [19,22], more recent findings in PKR-deficient mouse embryonic fibroblasts support the assumption that PKR does not mediate eIF2(αP) phosphorylation, the participation of PERK has been suggested instead [21]. As shown in Fig. 5A, EGTA treatment resulted in the expected increase in eIF2(αP) levels in the cells. The fact that EGTA and FCCP responses were additive might indicate that two different eIF2α kinases, e.g. PERK and PKR, were independently activated.

It is widely accepted that the ATP/ADP antiporter is a component of the PTP assembly, and that the redox state of two of its thiol groups plays a crucial role in PTP function [4]. FCCP is known to bind sulfhydryl groups and react with aminothiols, the direct interactions with the PTP channel proteins being the mechanism causing PTP opening [12]. In general, these reactions can be prevented by dithiothreitol (DTT), which is capable to react with phenylhydrazones such as

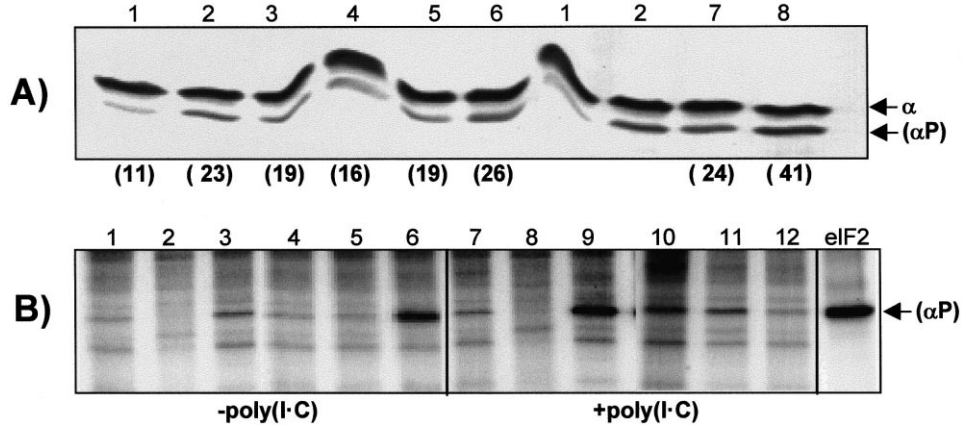

Fig. 5. Effect of several treatments on eIF2α phosphorylation in vivo (A) and PKR activity in vitro (B). (A) Differentiated PC12 cells were pre-incubated for 30 min with the different agents and incubated for an additional 30 min in the presence or absence of FCCP. eIF2(αP) levels were determined as described in Section 2 and a representative Western blot is shown. Lane 1, untreated cells; lanes 2, 4, 6 and 8, 30 μM FCCP; lanes 3 and 4, 2 mM DTT; lanes 5 and 6, 100 μM CsA; lanes 7 and 8, 5 mM EGTA. Numbers in brackets: percentage of eIF2(αP) over the total eIF2, average of 2–4 different cultures. (B) PKR activity was assayed in vitro as described in Section 2 in the presence of the agents without or with poly(I·C). Lanes 1, 4, 7 and 10, untreated cells; lanes 2 and 8, 30 μM FCCP; lanes 3 and 9, 2 mM DTT; lanes 5 and 11, 100 μM CsA; lanes 6 and 12, 5 mM EGTA. The autoradiography is representative of 2–3 different cultures. Keys as in Fig. 2.

FCCP [12]. Consistently with this mechanism, the FCCP-induced eIF2α phosphorylation in PC12 cells diminished by DTT treatment (Fig. 5A).

To further assess the implication of PTP opening in translation inhibition, we tested the effect of CsA, an inhibitor of the PTP assembly [3]. CsA treatment at concentrations that inhibit PTP opening induced a slightly lower increase in eIF2(αP) to that observed with FCCP, but both effects were not additive (Fig. 5A). CsA is a potent inhibitor of the Ca^{2+}/calmodulin-regulated protein phosphatase, i.e. calcineurin [23]. CsA might indirectly increase eIF2(αP) levels through inhibitor-1, a calcineurin substrate which specifically inhibits protein phosphatase 1 [24], one of the characterized eIF2α phosphatases [11]. The direct addition of FCCP to the kinase assay decreased eIF2α phosphorylation both in the absence and presence of poly(I·C) (Fig. 5B), indicating that FCCP-induced PKR activation does not merely represent a direct activation of PKR activity. CsA had no effect in the kinase assay, whereas DTT significantly increased PKR activity in vitro. EGTA increased eIF2α phosphorylation in vitro only in the absence of poly(I·C), which might suggest again the activation of an other eIF2α kinase distinct from PKR (Fig. 5B).

The ischemia-induced translation repression is one of the earliest events occurring during reperfusion and runs in parallel with eIF2α phosphorylation, eIF2α phosphatase inhibition and lack of PKR activation [10,11]. On the contrary, FCCP treatment increased PKR activity, this finding challenging the participation of mitochondrial calcium mobilization in the ischemia-induced translational repression.

Recent findings support the hypothesis that the ischemia-induced PTP opening is only induced when $[Ca^{2+}]_i$ exceeds a critical limit. Then, reoxygenation fails to re-establish Ca^{2+} homeostasis and subsequently cytosolic Ca^{2+} produces mitochondrial Ca^{2+} overload which in turns opens PTP, initiating the apoptotic or necrotic cascade leading to cell death [4]. On the other hand, several authors have claimed a direct role for eIF2α phosphorylation in promoting apoptosis under certain conditions, and translation inhibition can be reasonably expected to be the mechanism primarily responsible for the factor action [20]. eIF2α phosphorylation is likely to participate in the above apoptotic cascade triggered by mitochondrial PTP opening.

To our knowledge, we first report a novel effect of the uncoupler FCCP on translation. Here we present strong evidences that PKR mediates the FCCP-induced translational repression, i.e. (i) translational rate is suppressed concurrently with eIF2α phosphorylation in FCCP-treated cells; (ii) FCCP treatment increases eIF2α kinase activity as measured in cell extracts in vitro; and (iii) eIF2α kinase activity is further enhanced by both the addition of poly(I·C) to the in vitro assay and cells previously treated with interferon. Our findings support the hypothesis that translational regulation can play a significant role in the processes triggered by mitochondrial calcium mobilization.

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