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Retinoic acid increases glucocorticoid receptor phosphorylation *via* cyclin-dependent kinase 5

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ABSTRACT

Glucocorticoid receptor (GR) function is modulated by phosphorylation. As retinoic acid (RA) can activate some cytoplasmic kinases able to phosphorylate GR, we investigated whether RA could modulate GR phosphorylation in neuronal cells in a context of long-term glucocorticoid exposure.

A 4-day treatment of dexamethasone (Dex) plus RA, showed that RA potentiated the (Dex)-induced phosphorylation on GR Serine 220 ($_{pSer220}$GR) in the nucleus of a hippocampal HT22 cell line. This treatment increased the cytoplasmic ratio of p35/p25 proteins, which are major CDK5 cofactors. Roscovitine, a pharmacological CDK5 inhibitor, or a siRNA against CDK5 prevented RA potentiation of GR phosphorylation.

Furthermore, roscovitine counter-acted the effect of RA on GR sensitive target proteins such as BDNF or tissue-transglutaminase.

These data help understanding the interaction between RA- and glucocorticoid-signalling pathways, both of which have strong influences on the adult brain.

Introduction

Glucocorticoid hormones (GC), end-products of the hypothalamic-pituitary-adrenal axis, are a major component of the endocrine response to stress. While short-term exposure to stress affects brain function to facilitate the adaptation to environmental challenges, long-term exposure may increase the allostatic load and ultimately cause diseases (McEwen, 2012). In the central nervous system for instance, prolonged stress elicits various harmful effects particularly in the hippocampus (Alfarez et al., 2002; Conrad, 2006; Herbert et al., 2006; Krugers et al., 2006; McEwen and Milner, 2007; Sousa et al., 2008). The GC-dependent activation of glucocorticoid or mineralocorticoid receptors (GR and MR, respectively) elicits their nuclear translocation and homodimerisation. GR dimers then interact with target gene promoters on glucocorticoid-responsive elements (GRE) to regulate the gene transcription (Nicolaides et al., 2010). Importantly, GR subcellular localization, transcriptional activity and turnover are largely modulated by several GC-induced phosphorylations on GR serines or threonines (Bodwell et al., 1991; Galliher-Beckley and Cidlowski, 2009; Ismaili and Garabedian, 2004; Orti et al., 1989). Phosphorylation of Serine 220 (Ser220), Ser212 and Ser234 of mouse GR are the modifications the most frequently studied. The phosphorylation of Ser220 ($_{pSer220}$GR) has been implicated in the chronic effects of GC through modulation of GR-dependent transcriptional activity (Adzic et al., 2009b; Chen et al., 2008; Wang et al., 2002) while the phosphorylation of Ser212 affects non-genomic activity. The phosphorylation of Ser234 has been implicated in the acute effects of GC through the enhancement of nuclear $_{pSer220}$GR export (Galliher-Beckley and Cidlowski, 2009). The two major families of kinases responsible of GR N-terminal phosphorylation are the mitogen-activated protein kinases (MAPK) and the cyclin-dependent kinases (CDK) (Krstic et al., 1997).

Retinoic acid (RA) is crucial for foetal brain development but also for adult brain plasticity (Shearer et al., 2012). For instance, RA modulates hippocampus plasticity through various processes such as neurite outgrowth (Etchamendy et al., 2001; Maden, 2007; Mingaud et al., 2008; Shearer et al., 2012).

We hypothesised that opposite effects of RA and GC in neuronal cells could be explained by a RA-dependent modification of GC signalling pathway. Recently, we demonstrated that an interaction exists between

Abbreviations: GC, glucocorticoids; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinases; PI3K, Phosphoinositide 3-kinase; RA, retinoic acid; Dex, dexamethasone; BDNF, brain-derived neurotrophic factor; TrkB, Tropomyosin receptor kinase B; $_{pSer220}$GR, phosphorylated GR on serine 220; tTG, tissue transglutaminase 2; FBS, foetal bovine serum; Sgk1, Serum and Glucocorticoid-regulated Kinase 1; ERK, extracellular-signal-regulated kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Arc, activity-regulated cytoskeleton-associated protein.

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RA and GC pathways with, for instance, consequences on tissue transglutaminase 2 (tTG) and brain-derived neurotrophic factor (BDNF) expression, two neuronal target proteins (Brossaud et al., 2013) involved in cerebral plasticity, as well as on the morphology of neuronal cells (Roumes et al., 2016). The mechanisms involved in the interactions between RA and GC signalling pathways have been investigated in very few studies and only in non-neuronal-cells (Subramaniam et al., 2003; Toth et al., 2011; Wang et al., 2004).

RA is known to be able to activate several kinase cascades such as p38 MAPK, extracellular-signal-regulated kinase p44/42 (Erk1/2), PI3K or CDK cascades (Bour et al., 2007). Thus, we investigated here whether the interaction between RA and GC in neurons could be the consequences of an indirect modification of GR activity by RA *via* a modification of the GR phosphorylation particularly on $_{pSer220}$GR. This led us to focus on the implication of a cyclin-dependent kinase, CDK5, in the RA-dependent effects. Indeed, CDK5 and its neuron-specific activators p35 and p25 are involved in $_{pSer220}$GR regulation particularly in the hippocampus (Adzic et al., 2009b; Galliher-Beckley and Cidlowski, 2009; Kino, 2007). Furthermore, CDK5 plays an important physiological and pathological role in neuronal activity in other brain regions (Cruz et al., 2003; Kumazawa et al., 2013; Patrick et al., 1999).

We show that: i) Ser220 phosphorylation status is modified both by RA and by a GR agonist, dexamethasone (Dex), and ii) CDK5 plays a key role in the effect of RA on GC signalling.

1. Materials and methods

1.1. Cell cultures

As we had previously shown interactions between GC and RA in neurons (Brossaud et al., 2013) only after long-term treatment, we used here the same experimental design to investigate the mechanisms implicated in this interaction. Thus cells were treated 4 d by RA and/or Dex.

For experiments, an hippocampal cell line, HT22, were cultured for 4 days (4 d) in Dulbecco's Modified Eagle Medium with antibiotics and pyruvate supplemented with 10% charcoal-depleted foetal bovine serum (FBS) as previously described (Brossaud et al., 2013). The cells were seeded in plates (3000 cells/cm^2 for protein analysis, or 1500 cell/cm^2 for transfection or BDNF secretion experiments). Treatments consisted in vehicle (control condition), RA (final concentration 10^{-6} M), Dex (final concentration 10^{-6} M) or RA/Dex (final concentrations 10^{-6} M for both). RA and Dex stock solutions (Sigma Aldrich, St. Louis, MO, USA; 17.5 and 10 mmol/L, respectively) were diluted into ethanol:dimethylsulfoxide (DMSO, 50:50) (VWR International, West Chester, PA, USA) added as vehicle in controls.

Roscovitine, a CDK5 inhibitor (Cell Signaling, Saint-Quentin-en-Yvelines, France) stock solution (2.8 mM) was prepared into ethanol:DMSO 50:50 and added to the culture medium to a 20 μM final concentration. K252a, a Tropomyosin receptor kinase B (TrkB) inhibitor (Abcam, Cambridge, UK) stock solution (2 mM) was prepared into ethanol:DMSO 50:50 and added to the culture medium to a 100 nM final concentration. When needed, roscovitine was added for 2 d (after 2 d of Dex & RA treatments at the change of medium time-point) and k252a was added during all the 4d of the Dex & RA treatments.

1.2. Animals

Twenty-weeks old C57BL/6 J male mice (Janvier, France) had *ad libitum* access to food and water from their arrival in our animal house to the day they were killed and maintained on a 12-h light/12-h dark cycle. A first group (n = 3) received a control diet offering 2.9 kcal/g and 5 UI/g of retinol. A second group (n = 3) was exposed to the same diet than the control group but enriched with 25 UI/g retinol (SAFE, Augy, France). Just before being sacrificed, animals were exposed to a novel environment in order to have increased glucocorticoid levels. This experiment had been approved by the French national ethic committee for animal experimentation (no 5012047-A). At sacrifice, hippocampi from 3 mice of each group were dissected and kept frozen at −80 °C until protein extraction and Western blot analyses.

1.3. siRNA CDK5 transfection

siRNA targeting the mouse CDK5 and scrambled siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) were added to the culture medium (free of FBS but with RA/Dex 10^{-6} M) for 8 h after 2d of RA/Dex treatment at the change of the medium time point, with lipofectamine 2000 Reagent (Invitrogen, Carlstbad, CA, USA). At the end of siRNA transfection, the medium were removed and replaced by culture medium RA/Dex for the rest of the 4d treatment.

1.4. Real-time PCR analyses

Real-time quantitative PCR was performed using the LightCycler 480II system (Roche) as described previously (Brossaud et al., 2013). Specific primers were: forward TCAGAGCGGAATGTTCTGTTG and reverse GCGGTCTGGAATGAGAAGTGA for Serum and Glucocorticoid-regulated Kinase 1 (Sgk1). The specificity of the PCR was validated following MIQE (Minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin et al., 2009). The GAPDH housekeeping gene was used as reference gene for relative quantification and GAPDH was proved not to vary among the conditions in our experiments. Specific primers for GAPDH were: forward GAACATCATCCCTGCATCCA and reverse CCAGTGAGCTTCCCGTTCA. The results were normalized by the ratio of the relative concentration of the target to that of GAPDH in the same sample.

1.5. Western blots

Protein extraction, electrophoresis and transfer were performed as described (Brossaud et al., 2013). Briefly, cell pellets were crushed in a lysis buffer (Tris HCl pH 7.5 20 mM, EDTA 1 mM, MgCl2 5 mM, DTT 1 mM, NaOV 1 mM, NaF 1 mM, and a protease mix inhibitor (Sigma P2714)) on ice (incubation 30 min). Cytoplasmic and nuclear cells fractions were obtained using a centrifugation at 2500*g* during 5 min at 4 °C. The supernatants were centrifuged (13,000*g*, 20 min, 4 °C) and final supernatants were used as cytoplasmic fraction. The pellets were collected in 50 μL of the lysis buffer, sonicated few seconds and centrifuged (10,000*g*, 10 min, 4 °C). The supernatants were used as nuclear fraction.

Primary antibodies were diluted as follows: rabbit anti-GADPH (Sigma Aldrich) 1:2500; rabbit anti-histone H3 (Cell Signaling), 1/1000; rabbit anti-GR total (Santa Cruz Biotechnology) 1:10,000; rabbit anti-GR phosphorylated on serine 220 (Cell Signaling) 1:500; mouse anti-tTG 2 (Abcam) 1:500; mouse anti-CDK5 (SantaCruz Biotechnology) 1:500; rabbit anti-p35/p25 (Santa Cruz Biotechnology) 1:500.

The results of protein quantifications were always expressed related to GAPDH or histone for cytoplasmic or nuclear proteins, respectively.

1.6. Plasmid DNA transfection and luciferase assay

Cells were transfected 18 h before the end of the 4 d experiment using Lipofectamine 2000 Reagent (Invitrogen), with i) either 500 ng specific reporter vectors pTAL (control condition) or pGRE (Clontech, Mountain View, CA, USA) each containing a GRE cis-acting DNA response element upstream of the firefly luciferase reporter gene, and ii) 25 ng of phRL-TK vector encoding the *Renilla reniformis* luciferase (Promega, Charbonnières, France). The cells were lysed on day 4 and the enzymatic activities of the two separate reporter enzymes (renilla and firefly) were measured with the Dual-Luciferase Reporter Assay