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Phosphorylation status of glucocorticoid receptor, heat shock protein 70, cytochrome *c* and Bax in lymphocytes of euthymic, depressed and manic bipolar patients

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Summary Bipolar disorder (BD), a severe mental illness, has been correlated with alterations in glucocorticoid receptor (GR) signaling. Since it is phosphorylated GR that contributes to receptor function and determines its transcriptional activity, the Ser211 being a biomarker for activated GR *in vivo*, it is pertinent that we seek to determine the putative role of the total phosphorylation status of GR and site-specific phosphorylation at serine 211 (S211) in BD and their possible association with parameters of apoptosis. In lymphocytes from 48 BD patients under multiple psychotropic therapy and 20 healthy subjects, we measured whole cell GR, total GR phosphorylation, and phosphorylation of GR at serine 211 in nucleus, using immunoprecipitation, phosphospecific antibody and Western-blot analysis. Cytosolic cytochrome *c* and Bax and whole cell HSP70 were determined by immunoblot analysis. One-way ANOVA statistical analysis was carried out. Total phosphorylated GR was lower ($P < 0.001$) while the GR S211 was higher ($P < 0.001$) in all BD patients as compared to healthy subjects. HSP70 was reduced in euthymic ($P < 0.05$), depressed ($P < 0.001$) and manic ($P < 0.001$) as compared to healthy subjects. Cytochrome *c* was higher in all-patient groups as compared to healthy subjects, however without reaching statistical significance ($P > 0.05$). Bax levels were lower in the cytosolic fraction of all three BD groups. We provide the first evidence of altered GR phosphorylation joined with signs of apoptosis in lymphocytes of BD patients and suggest that the phosphorylation status of GR may play a role in the pathophysiology of bipolar disorder.

1. Introduction

Bipolar disorder is a psychiatric disease known to be triggered under stressful life events. Among the primary features of

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bipolar disorder (BD) patients is the modulation of glucocorticoid receptor function, reflected by a hyperactivity of the hypothalamus–pituitary–adrenal (HPA) axis and glucocorticoid insensitivity (Watson et al., 2004; Daban et al., 2005). Neuropsychological impairment in BD patients has recently been correlated with abnormal GR function (Watson et al., 2006).

Glucocorticoids mediate their effects via their intracellular glucocorticoid receptor alpha (GRα) and its isoform GRβ, both important regulators of HPA axis negative feedback and glucocorticoid sensitivity (Hollenberg et al., 1985; Bamberger et al., 1995; Kino et al., 2003). Glucocorticoids bind to GRα, which resides in the cytoplasm in an inactive state bound to heat shock proteins and immunophilins. After GC binding, GR undergoes conformational changes, dissociates from the heat shock proteins and, as a dimer, translocates into the nucleus, where it binds to glucocorticoid response elements (GREs) on DNA in the promoter of target genes, resulting in stimulation or suppression of the transcription of responsive genes (known as transactivation effect) (Beato et al., 1995; Beato and Sanchez-Pacheco, 1996; Kino et al., 2003; Dostert and Heinzel, 2004). The GC–GR complex may also interact with other coactivators and corepressors, attracting histone acetyltransferases (HATs) or histone deacetylases (HDAC). The GC–GR complex may prevent other transcription factors, via protein–protein interactions, such as activator protein-1 (AP-1) and nuclear factor kB (NF-kB), from activating their target genes being known as transrepression effect (Blanco et al., 1998; McKenna et al., 1999; De Bosscher et al., 2003). The complexity of the GC–GR signaling cascade is further revealed by the potential of G-protein coupled receptor (GPCR) (which is activated by extracellular compounds) to influence GR transcriptional activity, the existence of isoform GRβ (which is a potent endogenous inhibitor of GC–GRα activity in humans), as well as the demonstration of multiple translational GR isoforms (Bamberger et al., 1995; Kino et al., 2005; Lu and Cidlowski, 2005; Chrousos and Kino, 2005; Duma et al., 2006). Finally, the ubiquitin–proteasome pathway is another important regulator of GR turnover and GR-mediated transactivation (Deroo et al., 2002).

Phosphorylation is a very important post-translational modification of GR, which is regulated by various cellular enzymes (kinases and phosphatases). It is a process involving the consumption of several molecules of ATP, which serve as the donor of phosphate groups. The N-terminal domain of human GR contains five phosphorylation sites at amino acids serine 131 (S131), serine 141 (S141), serine 203 (S203), serine 211 (S211) and serine 226 (S226). The phosphorylation status of GR affects the GR evoked transcriptional activity, the GR stability and nucleo-cytoplasmic shuttling, resulting in enhanced or inhibited GR-induced gene expression, thus influencing GC sensitivity (Ismaili and Garabedian, 2004). Accumulating evidence indicates that phosphorylation of GR at subsets S203, S211 and S226 is activated by the cognate ligand (GCs) as well as by the mitogen activated kinases (MAPKs), such as the extracellular signal-regulated kinase (ERK), the c-jun-N-terminal kinase (JNK), the p38 mitogen activated protein kinase (p38 MAPK) and the cyclin-dependent kinases (CDKs). Since MAPKs respond to a variety of cellular stimulus (growth factors, stress events, cytokines and mitogens), it is evident that alterations in cellular events may, in response, recruit multiple kinases at a given site and may coordinate differently the signaling cascades and receptor N-terminal phosphorylation, thus rendering the GR differentially responsive to GCs (Ismaili and Garabedian, 2004; Miller et al., 2005). More important, the transcriptional activity of GR and GR-dependent functions, such as apoptosis and stress response, have been correlated with alterations in the phosphorylation status of the Ser-211 residue, a site characterized as a biomarker of GR function (Wang et al., 2002; Miller et al., 2005; Kino et al., 2007; Chen et al., 2008). Conceivably, the phosphorylation status of GR is of particular importance in the glucocorticoid-mediated activity.

In spite of the many different processes taking place at the molecular level that define the overall GC activity/sensitivity, much of the research effort in BD is limited to only a few parameters involved in GR signaling cascade. The reduction in glucocorticoid responsiveness observed in BD patients has been ascribed to either reduced GR number and/or function as determined in lymphocytes and brain tissue (Webster et al., 2002; Juruena et al., 2003; Pariante, 2004; Perlman et al., 2004; Daban et al., 2005; Matsubara et al., 2006). A diminished ability of the GR to bind to DNA in lymphocytes from BD patients has also been reported (Spiliotaki et al., 2006). The integrity of GRα/GRβ gene structure excluded their possible involvement in the GC-insensitivity accompanying BD disease (Moutsatsou et al., 2000).

Due to the key role of GR phosphorylation in defining GR-activity and target gene expression (Chen et al., 2008), we sought to ascertain a role of the total phosphorylation status of GR and site-specific phosphorylation at S211 in lymphocytes of BD patients. As pGR–S211 has been associated with apoptosis and stress response in lymphoid cells (Miller et al., 2005; Miller et al., 2007), we considered it important to assess key regulatory proteins in the control of apoptosis such as HSP70, cytochrome *c* and Bax in the same cells (Li et al., 2000; Garrido et al., 2001; Parcellier et al., 2003; Yenari et al., 2005; Garrido et al., 2006; Arya et al., 2007).

2. Materials and methods

2.1. Subjects

Demographic and clinical characteristics from 48 patients and 22 healthy control subjects are presented in Table 1. BD patients were diagnosed by trained psychiatrists using the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1997), case-note and medication review, the 21-item Hamilton Depression Rating Scale (HDRS) (Hamilton, 1967), and the Young Mania Rating Scale (YMRS) (Young et al., 1978). These included both outpatients and inpatients of the Department of Psychiatry, Eginition Hospital, Medical School, Athens, Greece. Fourteen depressed bipolar patients (DSM-IV criteria for BDI or BDII with major depression; HDRS score $\geq$17), 14 manic bipolar patients (DSM-IV criteria for BDI with a manic episode; YMRS score $\geq$15), and 20 euthymic bipolar patients (DSM-IV criteria for BDI or BDII, HDRS score $\leq$7, and YMRS score $\leq$6) were recruited to participate in this study.

Patients' medication is summarized in Table 2 and had been unchanged for 6 weeks prior to participation. The majority of patients were on combined therapy with 33 taking at least one mood stabilizer, 18 taking at least one antidepressant and 29 taking at least one antipsychotic.