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Effects of glucocorticoids on activation of c-jun N-terminal, extracellular signal-regulated, and p38 MAP kinases in human pulmonary endothelial cells

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Abstract

Mitogen-activated protein kinases (MAPK) play a central role in signal transduction by regulating many nuclear transcription factors involved in inflammatory, immune, and proliferative responses. The aim of this study was to investigate, in human pulmonary endothelial cells, the effects of synthetic glucocorticosteroids on activation of c-jun N-terminal kinases, extracellular signal-regulated kinases, and p38 subgroups of the MAPK family.

Human microvascular endothelial cells from lung were stimulated for 2 h with either H_2O_2 (2 mM), IL-1β (10 ng/mL), or tumour necrosis factor-α (10 ng/mL). Under these conditions, a remarkable increase in the phosphorylation pattern of c-jun N-terminal kinases, extracellular signal-regulated kinases 1/2, and p38 was detected. Pretreatment for 12 h with dexamethasone (100 nM) was able to prevent phosphorylation-dependent MAPK activation in stimulated cells, without substantially affecting the expression levels of these enzymes.

Our results suggest that inhibition of MAPK signaling pathways in human pulmonary endothelial cells may significantly contribute, by interfering with activation of several different transcription factors, to the antiinflammatory and immunosuppressive effects of glucocorticosteroids.

Keywords: GCS; MAPK; H_2O_2; IL-1β; TNF-α

1. Introduction

GCS† exert their antiinflammatory and immunosuppressive effects through the binding to specific intracellular receptors that, upon ligand-dependent activation, induce or inhibit gene expression [1]. However, although these therapeutic properties may be partly dependent on increased transcription of some genes encoding anti-inflammatory proteins (e.g. lipocortin-1, secretory leukocyte protease inhibitor, interleukin-1 receptor antagonist), GCS mainly act by decreasing the synthesis of many pro-inflammatory proteins such as cytokines, chemokines, adhesion molecules, and enzymes synthesizing inflammatory mediators. Inhibition of gene expression by GCS occurs via at least three distinct mechanisms including: (i) binding of activated GR to cognate DNA sequences named negative glucocorticoid response elements, responsible for direct transcriptional repression [2]; (ii) indirect transcriptional regulation, (commonly known as transrepression), mediated by protein-protein interactions between GR and other transcription factors such as nuclear factor-κB [3,4], AP-1 [5,6], and signal transducers and activators of transcription [7,8], as well as by competition between GR and other transcription factors for binding sites on coactivator complexes such as CBP/

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†*Abbreviations:* AP-1, activator protein-1; Dex, dexamethasone; ERK, extracellular signal-regulated kinases; GCS, glucocorticosteroids; GR, glucocorticoid receptors; H_2O_2, hydrogen peroxide; HMVEC-L, human microvascular endothelial cells from lung; IL-1β, interleukin-1β; JNK, c-jun N-terminal kinases; MAPK, mitogen-activated protein kinases; Test testosterone; and TNF-α, tumour necrosis factor-α.

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p300 [2,9]; (iii) post-transcriptional effects, involving the reduction of mRNA half-life [10,11].

Moreover, recent evidences suggest that GCS may also interfere with signal transduction pathways responsible for activation of AP-1 and other transcription factors [2]. In regard to this aspect, a central role is played by the various members of the MAPK family which, upon dual phosphorylation on threonine and tyrosine residues triggered by a wide range of stimuli including cytokines, growth factors, hormones, and environmental stress (e.g. heat shock, osmotic shock, reactive oxygen metabolites, ultraviolet irradiation), become active and may in turn phosphorylate and activate many transcription factors [12,13].

In particular, the JNK subgroup includes among its substrates c-Jun [14,15], which is an AP-1 component, activating transcription factor-2, nuclear factor of activated T cells-4, and Elk-1 [16]. The ERK1/2 subfamily phosphorylates and regulates the transcription factors Elk-1, Ets1, Sap1a, c-Myc, Tal, and signal transducers and activators of transcription [12,17,18], whereas the p38 subgroup plays an important role in cellular stress, inflammation, and development by phosphorylating several different substrates such as the transcription factors Chop (also known as GADD153) and Max [12,19,20].

MAPK are remarkably expressed by alveolar and airway vascular endothelial cells, which actively participate in the inflammatory and immune responses characterizing many lung disorders such as idiopathic pulmonary fibrosis, adult respiratory distress syndrome, chronic obstructive pulmonary disease, and asthma. In fact, upon stimulation by a wide range of agents including IL-1β, TNF-α, and oxidative stress, the endothelium undergoes an activation process leading to enhanced expression of leukocyte adhesion molecules, increased production of cytokines and chemokines, as well as upregulation of class II HLA molecules [21].

Therefore, in order to further elucidate the molecular mechanisms underlying the anti-inflammatory and immunosuppressive actions of GCS, that are responsible for their therapeutic properties in the above mentioned lung diseases, we investigated the effects of Dex on activation of JNK, ERK1/2, and p38 in human pulmonary endothelial cells.

2. Materials and methods

2.1. Reagents

Dex, Test, and H_2O_2 were from Sigma (St. Louis, MO, USA). Recombinant human IL-1β and TNF-α were purchased from PeproTech (Rocky Hill, NJ, USA). Antiphospho-JNK, antiphospho-ERK1/2, and antiphospho-p38 monoclonal antibodies were from New England Biolabs (Beverly, MA, USA). Anti-(total)JNK, anti-(total)ERK1/2, and anti-(total)p38 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

HMVEC-L (Clonetics, San Diego, CA, USA) were grown on 6-well plates to 80% confluence in endothelial growth medium supplemented with 10% fetal calf serum.

Cell stimulation, occurring in the presence or in the absence of a preincubation with either Dex (100 nM, for 12 h) or Test (10^{-8} M, for 12 h), was performed by addition of either H_2O_2 (2 mM), IL-1β (10 ng/mL), or TNF-α (10 ng/mL). After 2 h, the medium was removed and HMVEC-L was processed for protein extraction and immunoblotting.

2.3. Protein extraction and immunoblot analysis

For Western blotting with the antibodies against the phosphorylated forms of JNK, ERK1/2, and p38, HMVEC-L were grown to confluence and, following stimulation, lysed in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM HEPES, pH 7.4, plus PPIM, 25 mM β-glycerophosphate, 1 M Na_3VO_4, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin), and then subjected to a 12.5% SDS-PAGE. Immunoblotting was performed on PVDF membranes (Amersham, Piscataway, NJ, USA) with the anti-phospho JNK, anti-phospho ERK1/2, and anti-phospho p38 monoclonal antibodies. After being "stripped," the membranes were re-probed with polyclonal antibodies against total (phosphorylated and unphosphorylated) JNK, ERK1/2, and p38.

Antibody binding was visualized by enhanced chemiluminescence (ECL-Plus; Amersham), and intensities of experimental bands were analyzed by computer-assisted densitometry.

3. Results

3.1. Effects of HMVEC-l stimulation on phosphorylation of JNK, ERK1/2, and p38

Evaluation of the results obtained in three independent sets of experiments showed that HMVEC-L exposure for 2 h to either oxidative stress, mimicked by H_2O_2 (2 mM), or to the pro-inflammatory cytokines IL-1β (10 ng/mL) and TNF-α (10 ng/mL), induced a marked increase in the amount of phosphorylated JNK, ERK1/2, and p38 (Fig. 1). The magnitude of the effects elicited by the three stimuli was similar, thus confirming that these MAPK subgroups are very sensitive, in HMVEC-L, to different types of induced activation. Indeed, because the monoclonal antibodies (antiphospho-JNK, antiphospho-ERK1/2, and antiphospho-p38) used in this study specifically recognize the phosphorylated, active forms of JNK, ERK1/2, and p38, respectively, the remarkable increase in the phosphorylation

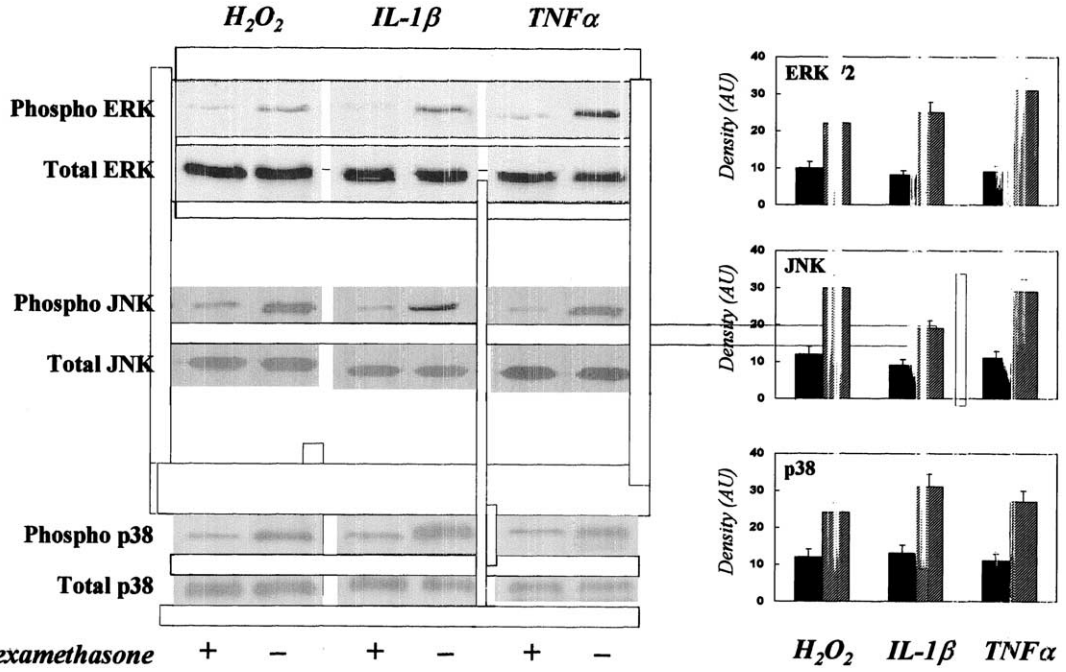

Fig. 1. Activation of MAPK ERK[1/2], JNK and p38 was inhibited by Dex. HMVEC-L were incubated for 2 h in the presence (+) or in the absence (-) of Dex (100 nM, for 12 h), with either H_2O_2 (2 mM), or IL-1β (10 ng/mL), or TNF-α (10 ng/mL). Each of these agents induced a 3–5-fold increase in the amount of the phosphorylated, active forms of ERK[1/2], JNK, and p38, detected by antiphospho-specific monoclonal antibodies. Dex exerted an almost complete inhibitory effect on MAPK phosphorylation without affecting total protein expression, as evidenced by immunoblot analysis with polyclonal, anti-(total)MAPK antibodies. Densitometric analysis of experimental bands in the presence (filled bars) or absence (dashed bars) of Dex is shown. AU: arbitrary units.

pattern of these enzymes can be considered as a reliable marker of their highly efficient activation.

3.2. Effects of pretreatment with either DEX or test on phosphorylation of JNK, ERK1/2, and p38

Pretreatment of HMVEC-L for 12 h with 100 nM Dex effectively inhibited MAPK activation. In fact, Dex was able to prevent the stimulatory actions of H_2O_2, IL-1β, and TNF-α on phosphorylation of JNK, ERK1/2, and p38 (Fig. 1). Dex exerted its effects uniquely on phosphorylation-dependent activation of JNK, ERK1/2, and p38, without affecting the total expression of these enzymes, as shown by the unchanged binding patterns of the anti-(total)JNK, anti-(total)ERK1/2, and anti-(total)p38 polyclonal antibodies (Fig. 1).

In contrast, Test did not prevent MAPK phosphorylation induced by the three distinct activating stimuli, thus providing a reliable negative control (Fig. 2).

With regard to the effects of both Dex and Test on different lung cell types, overlapping results were also obtained in normal human airway epithelial cells, cultured from bronchial mucosal biopsy samples taken from fresh surgical specimens (data not shown).

4. Discussion

The present study, performed in human pulmonary endothelial cells, shows that GCS are able to inhibit the phosphorylation of JNK, ERK1/2, and p38, induced by oxidative stress and some pro-inflammatory cytokines (IL-1β, TNF-α). Because these agents are notably involved in the pathogenesis of many inflammatory and/or immune respiratory diseases like idiopathic pulmonary fibrosis, adult respiratory distress syndrome, asthma, etc. [22], we believe that our results may provide new insights into the molecular mechanisms underlying the therapeutic effects of GCS in such disorders. In fact, MAPK enzymatic cascades constitute an intricate network of cellular pathways engaged in transducing, integrating, and amplifying a wide range of signals up to the transcriptional machinery in the nucleus [23]. As a consequence, a negative interference with the activation of JNK, ERK, and p38 may represent a key feature of GCS action. This consideration arises from the central role played by several MAPK substrates, including AP-1, activating transcription factor-2, nuclear factor of activated T cells-4, signal transducers and activators of transcription, and many others, in regulating and coordinating the expression of many genes involved in the inflammatory, immune, and proliferative processes elicited by proinflammatory cytokines, as well as by environmental stress [24]. Therefore, the blockade of MAPK signaling pathways can allow GCS to exert a very effective control of these cellular responses by targeting a crucial step in the complex events leading to transcription factor activation. This inhibitory effect of GCS seems to be quite specific, because our data demonstrate that another steroid hormone such as Test did not affect MAPK

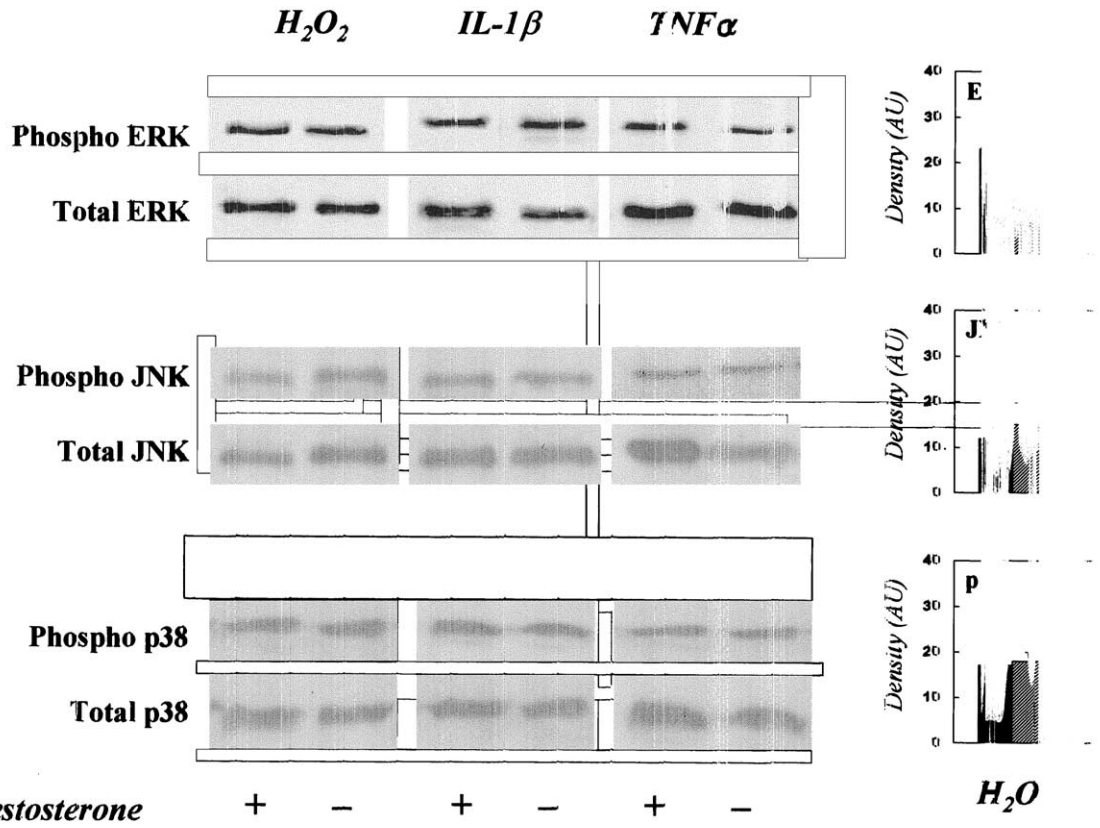

Fig. 2. Test did not prevent MAPK phosphorylation. HMVEC-L were incubated for 2 h, in the presence (+) or in the absence (−) of Test (10^{-8} M, for 12 h), with either H_2O_2 (2 mM), or IL-1β (10 ng/mL), or TNF-α (10 ng/mL). No differences in the phosphorylation levels of MAPK $ERK^{1/2}$, JNK, and p38 were detected upon pre-treatment with Test. Densitometric analysis of phosphorylated MAPK in the presence (filled bars) or absence (dashed bars) of this steroid hormone is shown. AU: arbitrary units.

phosphorylation, consistently with the results of a recent study performed in androgen-sensitive prostate cancer cell lines, which showed that dihydrotestosterone enhanced cell proliferation, but did not exert any effect on ERK signaling pathway [25].

The patterns of MAPK expression, activation, and pharmacological modulation, differ among the various cells and tissues exposed to the effects of endogenous and synthetic GCS. In particular, our results obtained in HMVEC-L confirm recent reports referring to the inhibitory action of GCS on JNK activation in other endothelial cell lines [26], fetal rat hepatocytes [27], murine macrophages [28], mouse mammary epithelial cells, HeLa, and Cos-7 cells [29]. Furthermore, a Dex-induced inhibition of the p38 stabilizing function on cyclooxygenase 2 mRNA has been very recently observed in a variety of cell types stimulated by different agents [30]. ERK activity has been also previously shown to be inhibited by GCS in Swiss 3T3 fibroblasts [31] and in a rat basophilic leukemia cell line (RBL2H3) [32], but not in murine macrophages [28] and human cultured airway smooth muscle cells [33]. This suggests that the ability of GCS to block MAPK activation is, at least in part, cell-type specific, and may depend on cell sensitivity to the intimate biochemical mechanisms responsible for GCS inhibitory action.

However, these mechanisms have not yet been elucidated, thus requiring to be deeper investigated. GCS-dependent induction of protein phosphatases catalyzing MAPK dephosphorylation does not seem to be involved, because the negative effect of Dex on JNK phosphorylation was unaffected by cell preincubation with okadaic acid or vanadate [27], that act as inhibitors of serine-threonine phosphatases or tyrosine phosphatases, respectively. In addition, GCS did not increase the expression levels of MAPK phosphatase-1, that inactivates MAPK by dephosphorylating both their phosphothreonine and phosphotyrosine residues [29]. A blockade of the signal transduction pathways located upstream of MAPK phosphorylation appears to be also unlikely, in that Dex was able to afford the same degree of JNK inhibition even when the MAPK kinase kinases (MEKK) responsible for JNK cascade activation were overexpressed [29]. Moreover, GCS were still capable of exerting their inhibitory action also on a constitutively activated JNK module [29].

Dex treatment of GR-deficient Cos-7 cells transfected with a mutant form of GR, which cannot homodimerize, bind to DNA and transactivate GRE-dependent genes, resulted in a very efficient inhibition of JNK phosphorylation, not different from that detected in cell lines expressing the wild-type GR [34]. Therefore, these findings suggest that

the negative interference of GCS with the JNK signaling system is not based on direct transcriptional regulation of GRE-dependent genes, thereby reinforcing the assumption that GCS actions may be independent from DNA binding. Furthermore, Dex did not inhibit the nuclear translocation of JNK induced by TNF-α [34], thus probably repressing JNK activation in both cytosol and nucleus.

Taken together, all these evidences do not rule out a possible inhibitory protein-protein interaction between ligand-activated, monomeric GR, and MAPK. Such a functional interaction which, however, has so far never been demonstrated, could occur either directly or indirectly, e.g. through the intervention of one or more intermediate regulatory proteins.

Whatever is the biochemical/molecular mechanism involved, suppression of MAPK activation may significantly contribute to the biological and pharmacological actions of GCS. This concept is also substantiated by recent studies that have detected an increased expression of the c-fos component of AP-1 in monocytes and T-lymphocytes of corticosteroid-resistant asthmatic patients, in comparison with corticosteroid-sensitive subjects [35]. These abnormalities were concomitant with higher levels of phosphorylated c-Jun and activated JNK. After 9 days of a daily treatment with 40 mg of prednisolone, immunohistochemical analysis of skin-biopsy specimens from a tuberculin-induced model of dermal inflammation, evidenced that phosphorylation of both JNK and c-Jun resulted to be suppressed in corticosteroid-sensitive, but not in corticosteroid-resistant asthmatics [36]; thus, corticosteroid resistance may be due, at least in part, to the inability of GCS to repress JNK activation. Consequently, JNK inhibition by GCS delineates a further level of interaction within the cross-talk between these hormones and AP-1 components, that is currently considered as very important for the pharmacological control of inflammation [37]. Furthermore, p38 inhibition by GCS may be particularly relevant to the treatment of inflammatory lung diseases such as asthma because this MAPK plays a significant role in cytokine biosynthesis [38] and NF-κB-dependent gene expression [39]. In fact, p38 activation is involved in the development and maintenance of airway eosinophil infiltration [40], a cytokine/chemokine-associated process, which can be attenuated by a selective p38 inhibitor [41].

In conclusion, we have shown that GCS are very effective in preventing phosphorylation-dependent activation of JNK, ERK1/2, and p38 kinases elicited by H_2O_2, IL-1β, and TNF-α in human pulmonary endothelial cells. Such an interference with a key-step in the signal transduction cascade mediated by MAPK modules may play an important role in the anti-inflammatory and immunosuppressive effects of GCS. A crucial target of these hormones is likely represented by the endothelial cells of lung microvessels, because the pulmonary vascular endothelium is dynamically involved in the inflammatory responses triggered by several different pathogenic stimuli. The present study may thus contribute to better understand the molecular mechanisms underlying the therapeutic action of GCS in many inflammatory and immune respiratory diseases, and can also help to explore new pharmacological strategies directly targeted to MAPK modulation.

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