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The protein kinase C inhibitor, Ro-31-7459, is a potent activator of ERK and JNK MAP kinases in HUVECs and yet inhibits cyclic AMP-stimulated *SOCS-3* gene induction through inactivation of the transcription factor c-Jun

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ABSTRACT

Induction of the *suppressor of cytokine signalling 3* (*SOCS-3*) gene is vital to the normal control of inflammatory signalling. In order to understand these processes we investigated the role of the proto-oncogene component of the AP-1 transcription factor complex, c-Jun, in the regulation of *SOCS-3* gene induction. We found that cyclic AMP stimulation of HUVECs promoted phosphorylation and activation of JNK MAP kinase and its substrate c-Jun. The JNK responsive element of the human SOCS-3 promoter mapped to a putative AP-1 site within 1000 bp of the transcription start site. The PKC inhibitors, GF-109203X, Gö-6983 and Ro-317549, were all found to inhibit AP-1 transcriptional activity, transcriptional activation of this minimal *SOCS-3* promoter and *SOCS-3* gene induction in HUVECs. Interestingly, Ro-317549 treatment was also found to promote PKC-dependent activation of ERK and JNK MAP kinases and promote JNK-dependent hyper-phosphorylation of c-Jun, whereas GF-109203X and Gö-6983 had little effect. Despite this, all three PKC inhibitors were found to be effective inhibitors of c-Jun DNA-binding activity. The JNK-dependent hyper-phosphorylation of c-Jun in response to Ro-317549 treatment of HUVECs does therefore not interfere with its ability to inhibit c-Jun activity and acts as an effective inhibitor of c-Jun-dependent *SOCS-3* gene induction.

1. Introduction

The suppressor of cytokine signalling (SOCS) protein family consists of eight closely related members, cytokine inducible Src homology 2 protein (CIS) and SOCS-1 to 7 [1]. The basic structure of SOCS proteins consists of a central SH-2 and a C-terminal SOCS box domain [1]. SOCS-3, in particular, has been studied extensively and is known to play a vital role in the regulation of inflammatory processes [1,2]. For example, levels of SOCS-3 protein are increased at sights of inflammation [3] and conditional deletion of the *SOCS-3* gene in hematopoietic and endothelial cells causes mice to die from severe inflammatory lesions [4]. Pro-inflammatory cytokines, such as interleukin 6 (IL-6), activate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, leading to the induction of the *SOCS-3* gene [2]. SOCS-3 protein inhibits the JAK-STAT pathway, forming part of a negative feedback loop [1]. SOCS-3 can down-regulate the JAK-STAT signalling through several mechanisms, including targeting SH-2 bound proteins for ubiquitination and proteosomal degradation, through the recruitment of an E2 ubiquitin transferase [5], competitively inhibiting JAK proteins binding to the receptor and inhibiting STAT activation through its kinase inhibitory region (KIR) [1].

It has been demonstrated that recombinant cell-penetrating forms of SOCS-3 protein can serve as an effective therapy against pathogen-derived acute inflammation [6]. Clearly, therefore, small molecule regulators of SOCS-3 gene activity could also have a similar effect in combating acute and chronic inflammation [7]. In this respect we have aimed investigations into unravelling the molecular control of *SOCS-3* gene activity and have found that induction of SOCS-3 by cyclic AMP has an anti-inflammatory effect in vascular endothelial cells [8,9]. Here, elevations in intracellular cyclic AMP lead to *SOCS-3* gene induction through the mobilisation of C/EBP transcription

Abbreviations: Cyclic AMP, 3′, 5′ cyclic adenosine monophosphate; C/EBP, CCAAT/enhancer binding protein; HUVEC, human umbilical vein endothelial cell; SOCS-3, suppressor of cytokine signalling 3; SEM, standard error of mean; EPAC, exchange protein activated by cyclic AMP; AP-1, activator protein 1; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; MAP kinase, microtubule associated kinase.

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factors β and δ through the concomitant activation of exchange protein activated by cAMP 1 (EPAC1) and the ERK MAP kinase pathway [10–12]. Further work in COS1 cells highlighted a potential role for protein kinase C isoforms α and δ, acting downstream of EPAC1 in the pathway leading to SOCS-3 induction [13]. In the current work we aim to further delineate the signalling mechanisms underlying cyclic AMP-regulated SOCS-3 induction in VECs in order to define future targets for therapeutic intervention. To this end we have investigated the mechanisms of action of the bisindolemaleimide PKC inhibitors, RO-318220 [14] Gö-6983 [15] and GF-109203X [16], which we previously determined to be effective inhibitors of cyclic AMP-induced SOCS-3 induction in COS1 cells [10]. Our results demonstrate a number of "off-target" effects of RO-318220 that, nevertheless, allowed us to identify the transcription factor c-Jun as a key regulator of cyclic AMP-induced *SOCS-3* gene induction in VECs.

2. Materials and methods

2.1. Materials

Primary antibodies to anti-total ERK, anti-phospho-ERK (Thr202/Tyr204), anti-total c-Jun, anti-phospho-c-Jun (Ser63), anti-total JNK, anti-phospho-JNK, pan-PKC and anti-β-tubulin were purchased from New England Biolabs. Anti-SOCS-3 antibody was from Santa Cruz Biotechnology. Secondary antibodies anti-rabbit, anti-goat and anti-mouse IgG conjugated with HRP were purchased from GE Healthcare. Forskolin, rolipram, 12-myristate 13-acetate (PMA), MG132, U0126, SB 202190, JNK inhibitor III, GF-109203X, GÖ-6983 and Ro-317549 were purchased from Merck/Calbiochem. The AP-1 reporter construct was provided by Professor Walter Kolch, University College, Dublin.

2.2. Cell culture and transfections

COS-1 cells were grown in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) foetal bovine serum (Sigma-Aldrich UK), 2 mM glutamine and 2% (v/v) penicillin/streptomycin (Sigma-Aldrich UK) at 37 °C in a humidified 5% (v/v) CO_2 atmosphere. Human umbilical vein endothelial cells (HUVECs) were grown in human endothelial cell growth medium 2 (PromoCell Heidelberg, Germany) at 37 °C in humidified 5% (v/v) CO_2. Cultures of 80%–90% confluent COS-1 cells grown on 12-well culture clusters were transfected with 0.125 μg *Renilla* Luciferase reporter construct (pGL4.74) plus 1.125 μg of human SOCS3-Luc promoter constructs. Plasmids were diluted in a total volume of 12.5 μl Hanks balanced salt solution (HBS; Sigma-Aldrich UK) before being added to 25 μl transfection agent 30% (v/v) DOTAP (Roche, UK) in HBS. Transfected cells were then incubated overnight at 37 °C and experiments carried out the next day.

2.3. Generation of human SOCS-3 promoter constructs

A 1.7 kbp fragment of the human SOCS-3 promoter cloned into pGL3-Basic (hSOCS3-1.7 kbp) was generously provided by Dr. Jason Mathews, University of Toronto [17]. Consecutive promoter truncates were generated with the QuikChange II Site-Directed Mutagenesis Kit (Agilent) using this promoter fragment as an initial template. The primers used were hSOCS3-1.1 kbp (forward 5′-GCCGAGGCTGGGTAGCCCCTGCTCGCGGCC-3′ and reverse 5′-GGCCGCGAGCAGGGGCTACCCAGCCTCGGC-3′), hSOCS3-1.1-T1 (forward 5′-GCCGAGGCTGGGTAGTTTCTCTGCTGCG-3′ and reverse 5′-CGCAGCAGAGAAACTACCCAGCCTCGGC-3′), hSOCS3-1.1-T2 (forward 5′-GCCGAGGCTGGGTAGGCCGGCCGCGCAGTTCC-3′ and reverse 5′-GGAACTGCGCGGCCGGCCTACCCAGCCTCGGC-3′) and hSOCS3-1.1-T3 (forward 5′-GCCGAGGCTGGGTAGGCGGGGCGCGGCGGC-3′ and reverse 5′-GCCGCCGCGCCCCGCCTACCCAGCCTCGGC-3′). The putative AP-1 site in pGL3-Basic-hSOCS3-1.1-T1 was disrupted (GTGACTAA to AAGCTTAA, generating T1-delta AP1) using QuikChange mutagenesis and the primers, forward 5′-GCTGCGAGTAAAGCTTAAACATTACAAGAAGGCCGGCCGCGC-3′ and reverse 5′-GCGCGGCCGGCCTTCTTGTAATGTTTAAGCTTTACTCGCAGC-3′.

2.4. Dual Luciferase Reporter Assays

COS-1 cells transfected with human SOCS3-Luc promoter constructs were incubated for 16 h in the presence or absence of 10 μM or 0.1 μM phorbol 12-myristate 13-acetate (PMA) (Merck, UK). In some experiments cells were co-incubated with 10 μM SB600125, 10 μM JNK inhibitor III, 10 μM SB202190, 10 μM U0126, 5 μM RO-317549, 25 μM Gö-6983, 25 μM GF-109203X. After incubation the medium was removed and the cells washed with PBS. Cells were then lysed with 250 μl of 1× passive lysis buffer (Promega, UK) and placed on a rocking platform for 20 min at room temperature. Cell lysates were collected and 20 μl samples were assayed in triplicate for luciferase activity using the Promega Dual Luciferase Reporter Assay System according to the manufacturers' protocols. Luciferase activities were measured using a BMG Labtech luminometer.

2.5. RT-PCR

HUVECs were incubated with the indicated drugs, washed with PBS, harvested by scraping into 350 μl RLT buffer (Qiagen) and then lysed with 10 passes through a 21-gauge needle attached to a 1 ml plastic syringe. RNA was then extracted from cell extracts using the Qiagen RNeasy Mini kit according to the manufacturer's protocols. RNA samples were then diluted with water to a final concentration of 5 ng/μl RNA and the RT-PCR reaction was carried out using the Qiagen One-Step RT-PCR Kit, using 0.4 mM dNTPs and 0.6 μM of each primer, according to published protocols. The primers used were, hSOCS3-Forward, 5′-CACATGGCACAAGCACAAGA-3′, hSOCS3-Reverse, 5′-AAGTGTCCCCTGTTTGGAGG-3′, actin-Forward, 5′-CTGGCACCCAGCACAATG-3′ and actin-Reverse, 5′-GCCGATCCACACGGAGTACT-3′. The RT-PCR programme consisted of 30 min at 50 °C, 15 min at 95 °C and then 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 72 °C followed by 10 min at 72 °C. The RT-PCR products were resolved on 1.5% (w/v) agarose gels for 1 h at 80 V.

2.6. Intracellular Ca^{2+} measurements

HUVECs were grown overnight and then loaded with 1 mM FURA-2. Cells were then stimulated with the indicated treatments and changes in intracellular Ca^{2+} concentration were determined as previously described [18].

2.7. Immunoblotting

Cells lysates were prepared in sample buffer (50 mM Tris–HCl, pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-Mercaptoethanol, 12.5 mM EDTA, 0.02% (w/v) bromophenol blue, 100 mM DTT). Protein samples were then separated by SDS-PAGE on 10% (w/v) gels, transferred to nitrocellulose, blocked for 1 h at room temperature in 5% (w/v) BSA, immunoblotted with antibodies specific for JNK, phospho-JNK, ERK, phospho-ERK, c-Jun, phospho-c-Jun or PKC and then developed using ECL chemiluminescence (GE Healthcare).

2.8. c-Jun activation assay

A TransAM™ AP-1/c-Jun activation kit was purchased from Active Motif. Following stimulation nuclear extracts were prepared from HUVEC cells using an Active Motif nuclear extract kit, according to