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Oxidative stress impairs multiple regulatory events to drive persistent cytokine-stimulated STAT3 phosphorylation

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ABSTRACT

Although cytokine-driven STAT3 phosphorylation and activation are often transient, persistent activation of STAT3 is a hallmark of a range of pathologies and underpins altered transcriptional responses. As triggers in disease frequently include combined increases in inflammatory cytokine and reactive oxygen species levels, we report here how oxidative stress impacts on cytokine-driven STAT3 signal transduction events. In the model system of murine embryonic fibroblasts (MEFs), combined treatment with the interleukin-6 family cytokine Leukemia Inhibitory Factor (LIF) and hydrogen peroxide (H_2O_2) drove persistent STAT3 phosphorylation whereas STAT3 phosphorylation increased only transiently in response to LIF alone and was not increased by H_2O_2 alone. Surprisingly, increases in transcript levels of the direct STAT3 gene target SOCS3 were delayed during the combined LIF + H_2O_2 treatment, leading us to probe the impact of oxidative stress on STAT3 regulatory events. Indeed, LIF + H_2O_2 prolonged JAK activation, delayed STAT3 nuclear localisation, and caused relocalisation of nuclear STAT3 phosphatase TC-PTP (TC45) to the cytoplasm. In exploring the nuclear import/export pathways, we observed disruption of nuclear/cytoplasmic distributions of Ran and importin-α3 in cells exposed to H_2O_2 and the resultant reduced nuclear trafficking of classical importin-α/β-dependent protein cargoes. CRM1-mediated nuclear export persisted despite the oxidative stress insult, with sustained STAT3 Y705 phosphorylation enhancing STAT3 nuclear residency. Our studies thus reveal for the first time the striking impact of oxidative stress to sustain STAT3 phosphorylation and nuclear retention following disruption of multiple regulatory events, with significant implications for STAT3 function.

1. Introduction

The proteins of Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway, initially identified as important direct signalling components linking cytokine receptor activation to downstream transcriptional changes, have been increasingly recognised as playing major roles in cancer and pathologies associated with enhanced inflammation [1,2]. The STAT transcription factor proteins are largely inactive and cytoplasmic under normal conditions, but upon JAK-stimulated tyrosine phosphorylation, translocate to the cell nucleus to participate directly in the expression of the STAT-specific gene targets [3]. One member of the STAT family, STAT3, has attracted attention as a transcription factor mediating the gene expression changes upon stimulation by the interleukin-6 family of cytokines [4]. In this classical model of activation, STAT3 phosphorylation and nuclear localisation are usually transient, but persistent STAT3 phosphorylation and activation have frequently been linked with events of cellular injury, inflammation and cancer [3,5,6].

Although the mechanisms leading to persistent STAT3 activation remain largely unclear, several regulatory events control the magnitude and extent of STAT3 activation under control conditions. These regulatory events include the elevation of cytokine levels that promote receptor activation with downstream activation of tyrosine kinases [7], and conversely the involvement of negative regulators such as the tyrosine phosphatase T-cell protein tyrosine phosphatase (TC-PTP) [8–10] and the suppressor of cytokine signalling 3 (SOCS3) protein [11]. Notably, SOCS3 is itself a gene target of active STAT3 and its direct regulation

Abbreviations: JAK, Janus kinase; STAT, Signal Transducer and Activator of Transcription; TC-PTP, T-cell protein tyrosine phosphatase; SOCS3, suppressor of cytokine signalling 3; H_2O_2, hydrogen peroxide; LIF, leukemia inhibitory factor; OSM, oncostatin M; MAPK, mitogen-activated protein kinase; MEF, murine embryonic fibroblast; FCS, foetal calf serum; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; NES, nuclear export sequence; NLS, nuclear localisation sequence; Fn, nuclear fluorescence; Fc, cytoplasmic fluorescence; Fn/c, the nuclear to cytoplasmic ratio; SEM, standard error of the mean

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by STAT3 provides an immediate negative feedback mechanism attenuating signalling by the JAK/STAT3 pathway following cytokine activation [11].

Increased intracellular reactive oxygen species levels, leading to oxidative stress, have also been linked to disease pathologies, often promoting inflammatory processes and thus further elevating pro-inflammatory cytokine levels [12,13]. Whilst recent studies have demonstrated STAT3 activation by hydrogen peroxide (H_2O_2) as an oxidative stress trigger [14–16], the impact of oxidative stress on the magnitude and kinetics of cytokine-stimulated STAT3 phosphorylation and activation has not been evaluated. In this study, we focus on the consequences of oxidative stress on STAT3 activation by the interleukin-6 family cytokine Leukemia Inhibitory Factor (LIF). Our studies provide the first evidence that oxidative stress prolongs cytokine-driven STAT3 activation by deregulating multiple regulatory events.

2. Materials and methods

2.1. Cell culture, transfection and treatments

Wild-type (WT) murine embryonic fibroblasts (MEFs), *STAT3*$^{-/-}$ MEFs, or HeLa cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (Gibco) supplemented with 10% [v/v] foetal calf serum (FCS; DKSH Australia) and 100 U/ml penicillin/streptomycin (Gibco) then changed to serum-free media conditions 16 h before treatment with either 10 ng/ml murine recombinant LIF (Sigma), 1 mM H_2O_2 (Ajax Finechem), or a combination of 10 ng/ml LIF and 1 mM H_2O_2 (LIF + H_2O_2). For selected studies, 50 μM menadione was used to generate superoxide in place of oxidative stress initiated by H_2O_2. For protein kinase inhibitor studies, WT MEFs were pre-treated with a Mitogen-Activated Protein Kinase (MAPK) pathway inhibitor (20 μM JNK Inhibitor VIII [17]; 20 μM MEK inhibitor, UO126 [18]; 20 μM p38 inhibitor, SB203580 [19]), 0.5 μM JAK inhibitor I [20] or 10 μM Src family kinase inhibitor SU6656 [21] for 1 h prior to LIF, H_2O_2, or LIF + H_2O_2 treatment. Cells were pre-treated with the STAT3 pathway inhibitor STATTIC [22] (20 μM) 30 min prior to treatment with LIF, H_2O_2, or LIF + H_2O_2. All inhibitors were from Calbiochem.

For the delivery of anti-importin-α3 or anti-importin-β1 antibodies (Abcam) into intact cells, the Chariot peptide system was used according to the manufacturer's instruction (Active Motif) [23,24]. Briefly, antibodies (1 μg in 100 μl PBS) were mixed with 3.5 μl Chariot peptide solution and 100 μl distilled deionised H_2O, added to 150 μL of serum-free culture medium, and then preincubated with cells (12 well dishes) for 4 - 12 h prior to exposure to LIF. Transient transfection studies were carried out with the expression of myc epitope-tagged phosphatase TC-PTP [25], or Green Fluorescent Protein (GFP)-tagged nuclear export sequence (NES) and nuclear localisation sequence (NLS) constructs: GFP–Rev–NES [26], GFP-T-antigen (GFP-T-ag(114–135)) [27], GFP-pUL54(1145–1161) [28], GFP-VP3(74–121), GFP–VP3–NES mutant [29,30] or a control GFP-only construct using Lipofectamine™ LTX with Plus™ reagent and Opti-MEM according to the manufacturer's instructions (Invitrogen) prior to further treatments. In control experiments, GFP–Rev–NES cells were additionally pre-treated with 10 ng/ml Leptomycin B, a CRM1 inhibitor [31] for 60 min prior to analysis.

2.2. Cell lysate preparation and immunoblot analysis

Following treatment, cell lysates were prepared using RIPA buffer (50 mM Tris–HCl, pH 7.3, 150 mM NaCl, 0.1 mM EDTA, 1% [v/v] sodium deoxycholate, 1% [v/v] Triton X-100, 0.2% [w/v] NaF and 100 μM Na_3VO_4) supplemented with complete protease inhibitor mix (Roche Diagnostic). Protein samples, diluted with 3× protein sample buffer, were resolved by SDS-PAGE (10% [v/v] polyacrylamide gels) and the separated proteins were transferred onto PVDF membranes (Amersham Life Science). Proteins of interest were blotted using the following primary antibodies: anti-STAT3 N-terminus (BD Bioscience), anti-phospho-Y705 STAT3 or phospho-S727 STAT3 (Cell Signaling Technology), anti-TC-PTP (R & D systems), anti-α-tubulin (Sigma) and anti-c-Myc epitope (Santa Cruz Biotechnology). After incubation with horseradish peroxidase-linked secondary antibody (Thermo Scientific), immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Thermo Scientific) and quantitation carried out using ImageJ 1.38 public domain software (National Institutes of Health, USA).

2.3. Immunofluorescence, confocal laser scanning microscopy and image analysis

Samples were prepared and analysed as described previously [32]. Briefly, treated cells on coverslips were washed with cold phosphate-buffered saline (PBS) before fixation using 4% [w/v] paraformaldehyde (15 min, 37 °C) and permeabilisation in 0.2% [v/v] Triton X-100/PBS (15 min, room temperature). Non-specific binding was blocked by incubation in 1% [w/v] bovine serum albumin/PBS (30 min, room temperature), then coverslips incubated with primary antibodies [STAT3 (C-20) (Santa Cruz), Ran (BD Bioscience), importin-α3/KPNA4 or importin-β1/NTF97 (Abcam), 1:200 dilution in sterile filtered 1% [w/v] bovine serum albumin/PBS] then washed with PBS before incubation with Cy2/Cy3- (Millipore) or Alex488-coupled (Invitrogen) secondary antibodies. Nuclei were visualized using DAPI (Sigma, 1:15000 in PBS). Coverslips were mounted (Biomeda Gel Mount, ProSciTech) onto glass slides and confocal laser scanning microscopy was performed using a Leica TCS SP2 imaging system with a 100 × 1.35 NA objective. Quantitation of relative fluorescence intensities in digitised confocal images was carried out by measuring fluorescence in an area of the nucleus and cytoplasm of cells in 10 different fields (ImageJ 1.38 public domain software) from 3 independent experiments (n = 3). The values for the nuclear (F_n) and cytoplasmic (F_c) fluorescence, subsequent to subtraction of background fluorescence, enabled the specific nuclear to cytoplasmic ratio ($F_{n/c}$) to be calculated [32].

2.4. RNA preparation and quantitative real-time PCR

Samples were prepared and analysed as described previously [32]. Briefly, total RNA was extracted from MEFs using the Purelink RNA mini-kit (Invitrogen) and reverse-transcribed to cDNA using the RT High Capacity kit (Applied Biosystems) according to the manufacturer's protocols. Quantitative real-time TaqMan® PCR to determine the regulation of SOCS3 transcript levels was performed using 50 ng cDNA in a 20 μl reaction containing TaqMan® Gene Expression Master Mix and specific TaqMan® Gene Expression Assay (AssayID: SOCS3, Mm00545913_s1; Applied Biosystems). Amplification of cDNA was carried out in a 48-well Step One real-time PCR system (Applied Biosystems) using the following PCR conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The data was normalised to GAPDH (AssayID: Mm99999915_g1) in the respective samples and data quantitation was carried out using the $2^{-\Delta\Delta CT}$ method and expressed relative to the control sample. RNA isolation and expression analysis were performed on 3 independent occasions.

2.5. Statistical analysis

Statistical analysis was carried out using Graphpad Prism 5 software (Windows version 5.00, GraphPad Software) and the unpaired student *T*-test was used to compare data from control cells versus treated cells at each corresponding time point.