Biochemical Pharmacology 80 (2010) 410–421

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Differential effect of covalent protein modification and glutathione depletion on the transcriptional response of Nrf2 and NF-κB☆

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ARTICLE INFO

Article history:
Received 11 February 2010
Accepted 1 April 2010

Keywords:
Nrf2
Keap1
NF-κB
RNAi
Cellular stress
Transcriptional response

ABSTRACT

Liver injury associated with exposure to therapeutic agents that undergo hepatic metabolism can involve the formation of reactive metabolites. These may cause redox perturbation which can result in oxidative stress as well as protein modification leading to activation or inhibition of cellular transcriptional responses. Nevertheless, the effects of these challenges on more than one transcriptional pathway simultaneously remain unclear. We have investigated two transcription factors known to be sensitive to electrophilic stress and redox perturbation, Nrf2 and NF-κB, in mouse liver cells. Cellular stress was induced by the probes: N-acetyl-p-benzoquinineimine (NAPQI), the reactive metabolite of acetaminophen; dinitrochlorobenzene (DNCB), a model electrophile; and buthionine (S,R)-sulfoximine (BSO), an inhibitor of glutamate-cysteine ligase. NAPQI, DNCB and BSO can all cause glutathione (GSH) depletion; however only NAPQI and DNCB can covalently bind proteins. We also employed RNAi to manipulate Keap1 (the inhibitor of Nrf2), Nrf2 itself and NF-κB-p65, to understand their roles in the response to drug stress. All three chemicals induced Nrf2, but NF-κB binding activity was only increased after BSO treatment. In fact, NF-κB binding activity decreased after exposure to NAPQI and DNCB. While RNAi depletion of Keap1 led to reduced toxicity following exposure to DNCB, depletion of Nrf2 and NF-κB augmented toxicity. Interestingly, increased Nrf2 caused by Keap1 depletion was reversed by co-depletion of NF-κB. We demonstrate that Keap1/Nrf2 and NF-κB respond differently to electrophiles that bind proteins covalently and the redox perturbation associated with glutathione depletion, and that crosstalk may enable NF-κB to partly influence Nrf2 expression during cellular stress.

1. Introduction

Drug-induced liver injury (DILI) is a major cause of hospital admissions [1], and one of the principal reasons for attrition of new chemical entities [2]. For example, acetaminophen (APAP), a commonly used analgesic known to induce liver injury on overdose, accounts for the most common form of acute liver failure in the United States [3]. A proportion of the pathogenesis of DILI may occur through the generation of chemically reactive metabolites (CRMs), usually formed through oxidative metabolism of drugs by phase 1 enzymes [2]. CRMs can covalently modify critical proteins, and bind to and deplete glutathione (GSH), the predominant cellular redox buffer. This causes disturbance to the cellular redox potential yielding a more oxidising environment [4]. Although there are defense mechanisms present to counter such disturbances [5], if the chemical challenge is overwhelming and the defense is breached, a switch from cell defense to cell death is favoured either by apoptosis or necrosis (for a review, see [2]). Thus, a cell will defend itself where possible, but under extreme conditions it may allow itself to die. The ability of a cell to respond to chemical stress occurs at least partially through the activation of transcription factors. These transcription factors are constitutively present in the cell or are rapidly synthesised when needed, and they facilitate the upregulation of proteins implicated in cell defense or cell death. Two redox-sensitive transcription factors, in particular, are the focus of much interest in determining the role of transcriptional adaption to chemical stress, namely

☆ Financial support: This work was funded by the Wellcome Trust [079674/Z/06/Z], The University of Liverpool, The Overseas Research Students Award Scheme, and Pfizer UK, as part of the Centre for Drug Safety Science supported by the Medical Research Council [grant number G0700654].
Abbreviations: DILI, drug-induced liver injury; CRMs, chemically reactive metabolites; GSH, glutathione; Nrf2, nuclear factor-erythroid 2 (NF-E2)-related factor; NF-κB, NF-kappa B; ARE, anti-oxidant response element; NAPQI, N-acetyl-p-benzoquininеimine; DNCB, dinitrochlorobenzene; BSO, buthionine (S,R)-sulfoximine; RNAi, RNA interference.
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doi:10.1016/j.bcp.2010.04.004

nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf2) and NF-kappa B (NF-κB).

Nrf2 plays a crucial role in cellular defense, and is the major regulator of anti-oxidant response elements (AREs) present in the regulatory region of the majority of cytoprotective genes [6]. Under basal conditions, Nrf2 is sequestered by Keap1 in the cytoplasm [7], and is constitutively targeted for ubiquitination by the Cul3-dependent E3 ubiquitin ligase complex, which results in its subsequent proteasomal degradation [8]. Under certain stress conditions, such as exposure to electrophiles or an increase in reactive oxygen species, it is believed that Keap1 undergoes a conformational change, blocking Nrf2 ubiquitination and enabling nuclear accumulation of Nrf2 and transactivation of ARE-dependent genes.

Nuclear factor kappa-light-chain-enhancer-of activated B cells (NF-κB) comprises five members belonging to the mammalian NF-κB family, i.e., p65 (Rel A), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). NF-κB exists in the cytoplasm of unstimulated cells as homo- or hetero-dimers, bound to a family of inhibitory proteins known as IκB via non-covalent interactions. The predominant hetero-dimer present in cells is p65/p50 dimer [9]. Upon stimulation, the IκB kinase (IKK) complex which consists of a complex with two subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modifier (NEMO) [10], phosphorylates IκB-α [11]. Upon phosphorylation, IκB-α is targeted for ubiquitination and directed for proteasomal degradation [12]. This allows the release of NF-κB and its nuclear accumulation resulting in the transcription of genes involved in the inflammatory response, cell proliferation, cell survival and to a limited degree, the anti-oxidant response.

To date, there is little evidence for simultaneous regulation of Nrf2 and NF-κB within cells and little is known about the cellular consequences of such co-regulation. It may be valuable to understand what is happening to both transcription factors simultaneously to begin to understand the likely mechanisms that occur when a cell is exposed to chemical stress. The experiments presented here were designed to investigate the functional outcome of chemical stress/protein modification on the transcription factors as well being informative of how the cells actually sense these stresses. In this study, we hypothesised that the balance between both transcription factors might serve as a key cytoprotective mechanism to sense and respond to cell stress, where activation or inhibition of NF-κB, or Nrf2, or both transcription factors, may dictate the cellular response to stress.

2. Materials and methods

2.1. Materials

All chemicals and reagents were obtained from Sigma–Aldrich (Poole, UK), unless stated otherwise. Rabbit anti-mouse Nrf2 antibody was a kind gift from Professor John Hayes (Biomedical Research Centre, University of Dundee).

2.2. Cell culture and treatment

Hepa-1c1c7 cells were maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Wokingham, UK) supplemented with 584 mg/L L-gutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. For all treatments, cells were incubated with serum-free DMEM containing the indicated compound. All compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of the solvent in media of 0.1%, except for buthionine sulfoximine (BSO), which was dissolved in water.

2.3. RNA interference

Short interfering RNA (siRNA) duplexes targeting mouse Keap1, and Nrf2 were purchased from Dharmacon (Lafayette, CO, USA) and molecules targeting p65 were purchased from Invitrogen (Paisley, UK). The siRNA duplexes were as follows; si-Nrf2 sense 5′-GCA AGA AGC CAG AUA CAA A-3′, anti-sense 5′-U UUG UAU CUG GCU UCU UGC-3′, si-Keap1 sense 5′-GCU AUG ACC CGG ACA GUG A-3′, anti-sense 5′-U CAC UGU CCG GGU CAU AGC-3′, si-p65 sense 5′-UUC AUC UCC GGA GAG ACC AUU GGG A-3′, anti-sense 5′-U CCC AAU GGU CUC UCC GGA GAU GAA-3′. Hepa-1c1c7 were plated out onto 96-well plates at 1×10^4 cells/well for NF-κB siRNA validation, 96-well plates at 7×10^3 cells/well for LDH assay, 24-well plates at 5×10^4 cells/well for GSH assays and 10-cm dishes at 4×10^6 cells/dish for nuclear extractions. Cells were transfected with 10 nM of siRNA directed against Keap1 or Nrf2, and 3 nM siRNA against p65 for 48 h, using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

2.4. Preparation of cytosolic and nuclear extracts and whole cell lysates

For experiments with nuclear proteins, cells were treated with NF-κB siRNA for 48 h or treated for 1 h with the chemicals under investigation. Cytosolic and nuclear extracts were prepared using the method of Dignam et al. [13]. For whole cell lysate experiments, cells were treated with siRNA for 48 h and 6 h with DMSO. After treatments, cells were lysed with radioimmunoprecipitation assay buffer. Total protein content was measured according to the method of Bradford protein assay [14] with commercial kit (Bio-Rad, Hemel Hempstead, UK) and samples were stored at −80 °C before analysis.

2.5. Western immunoblotting

Cytosolic or nuclear extracts or whole cell lysates were resolved by denaturing electrophoresis on pre-cast 4–12% Novex bis–tris polyacrylamide gels (Invitrogen), using a 3-(N-morpholino)propanesulfonic acid running buffer. Separated proteins were transferred to Hybond nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK), which were blocked for 15 min in 10% non-fat milk (Bio-Rad) in Tris-buffered saline (TBS, pH 7.0) containing 0.1% Tween 20. Blocked membranes were probed with rabbit anti-mouse Nrf2 (1:5000) in 10 ml of 2% bovine serum albumin (BSA) for 1 h. After several washes, membranes were probed with goat anti-rabbit (1:10000) horseradish peroxidase-conjugated anti-IgG for 1 h. Western blots were visualised using enhanced chemiluminescence (PerkinElmer, Beaconsfield, UK) and Hyperfilm (GE Healthcare Life Sciences). All blots were also probed for actin (1:5000) as a loading control. Recombinant mouse His-tagged Nrf2 (+con) was loaded as a standard to confirm anti-body specificity. Western blot band volumes were quantified using TotalLab 100 software (Nonlinear Dynamics, Newcastle, UK) and normalised against actin.

2.6. NF-κB electrophorectic mobility shift assay (EMSA)

An NF-κB wild-type oligonucleotide (Wt) probe 5′ to 3′ strand: (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was 5′ end-labelled with adenosine 5′-triphosphate [γ-32P] ATP (PerkinElmer Life science, Mechelen, Belgium) using T4 polynucleotide kinase (Promega Corp, Madison WI, USA). Nuclear extracts (5 μg) were incubated with labelled oligonucleotide in a binding buffer containing (0.1 μg/μl) polydIdC (Amersham), 4% Ficoll, 20 mM HEPES, 35 mM NaCl, 60 mM KCl, NP40 0.01% and 2 mM dithiothreitol (DTT) for 20 min at room temperature. The definition of a NF-κB