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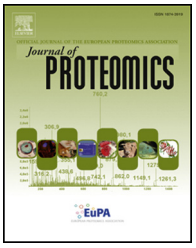

Identification and quantification of the basal and inducible Nrf2-dependent proteomes in mouse liver: Biochemical, pharmacological and toxicological implications

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ABSTRACT

The transcription factor Nrf2 is a master regulator of cellular defence: Nrf2 null mice ($Nrf2^{(-/-)}$) are highly susceptible to chemically induced toxicities. We report a comparative iTRAQ-based study in $Nrf2^{(-/-)}$ mice treated with a potent inducer, methyl-2-cyano-3,12-dioxooleana-1,9(11) dien-28-oate (CDDO-me; bardoxolone -methyl), to define both the Nrf2-dependent basal and inducible hepatoproteomes. One thousand five hundred twenty-one proteins were fully quantified (FDR <1%). One hundred sixty-one were significantly different ($P < 0.05$) between WT and $Nrf2^{(-/-)}$ mice, confirming extensive constitutive regulation by Nrf2. Treatment with CDDO-me (3 mg/kg; i.p.) resulted in significantly altered expression of 43 proteins at 24 h in WT animals. Six proteins were regulated at both basal and inducible levels exhibiting the largest dynamic range of Nrf2 regulation: cytochrome P4502A5 (CYP2A5; 17.2-fold), glutathione-S-transferase-Mu 3 (GSTM3; 6.4-fold), glutathione-S-transferase Mu 1 (GSTM1; 5.9-fold), ectonucleoside-triphosphate diphosphohydrolase (ENTPD5; 4.6-fold), UDP-glucose-6-dehydrogenase (UDPGDH; 4.1-fold) and epoxide hydrolase (EPHX1; 3.0-fold). These proteins, or their products, thus provide a potential source of biomarkers for Nrf2 activity. ENTPD5 is of interest due to its emerging role in AKT signalling and, to our knowledge, this protein has not been previously shown to be Nrf2-dependent. Only two proteins altered by CDDO-me in WT animals were similarly affected in $Nrf2^{(-/-)}$ mice, demonstrating the high degree of selectivity of CDDO-me for the Nrf2:Keap1 signalling pathway.

Biological significance

The Nrf2:Keap1 signalling pathway is attracting considerable interest as a therapeutic target for different disease conditions. For example, CDDO-me (bardoxolone methyl) was investigated in clinical

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trials for the treatment of acute kidney disease, and dimethyl fumarate, recently approved for reducing relapse rate in multiple sclerosis, is a potent Nrf2 inducer. Such compounds have been suggested to act through multiple mechanisms; therefore, it is important to define the selectivity of Nrf2 inducers to assess the potential for off-target effects that may lead to adverse drug reactions, and to provide biomarkers with which to assess therapeutic efficacy. Whilst there is considerable information on the global action of such inducers at the mRNA level, this is the first study to catalogue the hepatic protein expression profile following acute exposure to CDDO-me in mice. At a dose shown to evoke maximal Nrf2 induction in the liver, CDDO-me appeared highly selective for known Nrf2-regulated proteins. Using the transgenic Nrf2$^{(-/-)}$ mouse model, it could be shown that 97% of proteins induced in wild type mice were associated with a functioning Nrf2 signalling pathway. This analysis allowed us to identify a panel of proteins that were regulated both basally and following Nrf2 induction. Identification of these proteins, which display a large magnitude of variation in their expression, provides a rich source of potential biomarkers for Nrf2 activity for use in experimental animals, and which may be translatable to man to define individual susceptibility to chemical stress, including that associated with drugs, and also to monitor the pharmacological response to Nrf2 inducers.

1. Introduction

Maintenance of a stable intracellular environment is a prerequisite for normal physiological function. In a manner, somewhat analogous to the immune system, mammalian cells exhibit both innate and adaptive properties that allow them to withstand and respond to a variety of stress stimuli including environmental, dietary and, more recently, pharmaceutical-induced stresses. At the core of this cellular defence strategy is the Keap1:Nrf2 signalling pathway, which regulates expression of a battery of antioxidant proteins and enzymes involved in a variety of mechanisms that function to counter noxious stimuli. In the absence of stress, the transcription factor Nrf2 is retained in the cytoplasm through interaction with its inhibitor protein, Keap1, which targets Nrf2 for ubiquitination and proteasomal degradation. Thus, Nrf2 is rapidly recycled with a half-life of approximately 20 min [1]. Upon exposure to stress stimuli, such as reactive oxygen species and electrophiles, Nrf2 is stabilized and able to translocate to the nucleus where it transactivates target genes that possess an antioxidant responsive element (ARE) in their promoter regions. The precise mechanism through which the Keap1:Nrf2 interaction is disrupted is not fully understood, but the widely accepted 'hinge and latch' model [2] envisages that the function of the Keap1 dimer is disrupted by direct modification of sensitive cysteine residues, preventing Nrf2 ubiquitination and blocking access to Keap1 binding sites for newly synthesized Nrf2 molecules. Whilst Keap1-targeted ubiquitination results in highly efficient degradation of Nrf2, it is clear that low levels of Nrf2-mediated signalling do still occur under basal conditions, as evidenced by studies on Nrf2$^{(-/-)}$ mice. Although mice deficient in Nrf2 appear phenotypically normal, examination at the molecular level shows clear differences in gene expression profiles, confirming a constitutive role for Nrf2 in the orchestration of cellular defence [3,4].

In the context of chemical stress, Nrf2$^{(-/-)}$ mice are more vulnerable to the deleterious effects of chemicals toxic to the liver, as well as to several other organs. The animals show enhanced susceptibility to the hepatotoxicity associated with paracetamol [5,6], carbon tetrachloride [7] and ethanol [8], as well as drug-induced injury to the lungs [9,10] and colon [11].

With respect to liver, basal differences between wild type and Nrf2$^{(-/-)}$ mice have been shown both by gene microarray studies and by targeted protein analysis [4,12,13]. More recently, we conducted a global protein expression analysis using iTRAQ-based proteomics and identified two discrete pools of hepatic proteins which display differential expression profiles in wild type and Nrf2$^{(-/-)}$ mice: cytoprotective proteins and proteins involved in lipid metabolism [3]. Pathway analysis confirmed that the cytoprotective proteins found to be down-regulated in Nrf2$^{(-/-)}$ mice were predominantly phase II drug metabolizing enzymes or those involved in the glutathione system. In contrast, proteins involved in lipid metabolism were primarily over-expressed in Nrf2$^{(-/-)}$ mice, indicating an unexpected negative regulation of the fatty acid synthetic pathway.

2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) was synthesised for its anti-inflammatory properties via the modification of the A and C rings of oleanolic acid [14]. CDDO was found to potently inhibit nitric oxide production, and analogues including CDDO methyl ester (CDDO-me) and the imidazole derivative (CDDO-im) were subsequently synthesised with the aim of optimising potency and bioavailability [15,16]. A link between Nrf2 induction and CDDO treatment was first identified in a study in which the synthetic triterpenoid was shown to potently induce the phase II response in mouse embryonic fibroblasts [17], a response that was abolished in Nrf2-deficient cells. In a later study, CDDO and its derivatives were shown to induce Nrf2 protein levels in vitro along with mRNA levels of the Nrf2 target gene haem oxygenase 1 (*Ho-1*) [18]. Furthermore, the Nrf2 target NAD(P)H dehydrogenase [quinone] 1 (*Nqo1*) was subsequently found to be transcriptionally activated in CDDO-im and CDDO-me treated mice, with induction seen in the liver, lung and small intestine after a single dose [19].

Recently, CDDO-me (under the name bardoxolone methyl) has undergone clinical evaluation for the treatment of chronic kidney disease in diabetic patients [20]. Whilst the therapeutic benefit was promising, the development was terminated in phase III due to a high incidence of adverse reactions [21]. Nevertheless, there remains considerable interest in this class of compounds, particularly in the field of cancer chemotherapy [22]. The precise mechanism by which CDDO and its derivatives