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Review

Plasticity in cell defence: access to and reactivity of critical protein residues and DNA response elements

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Summary

Cellular and whole organ defence against pathogenic or chemical challenge is manifest as an adaptive response. Where appropriate, this may lead to induction of a cellular defence programme, thereby enhancing cell survival. When the challenge is overwhelming, the defence is breached and a switch is made to yield cell death, either by apoptosis or necrosis. Thus, a cell will defend itself where possible, but *in extremis*, it may recognise the futility of its resistance and allow itself to die. Transcription factor activation and access to the DNA regulatory elements that control a particular pattern of expression of defence genes is a major issue that may ultimately decide the fate of a cell in a changed environment. It is possible to visualise the access to the nucleus and to the genome, of paradigm gene loci or transcription factors, using a number of molecular techniques such as chromatin immunoprecipitation, *in vivo* footprinting and live/whole cell imaging. These methods are informative as to the array of transcription factors that may regulate a given gene, as well as the transitory nature of the transcriptional activation. The initial triggering of active transcription factor complexes typically occurs within the cytoplasm of the cell. Protein–protein interactions and signal transduction pathways, elucidated using a classical molecular genetics approach, have long been recognised as pivotal to the initial control of the levels and activity of transcription factors. We can now visualise modifications in critical residues of transcription factors and regulators during cellular response to chemical stress. These modifications may yield enhanced or repressed activity of transcription factors, they may be non-covalent or covalent, and they may occur in response to a variety of classes of chemicals. Such promiscuous signalling can provide plasticity in the cellular response to a wide array of chemical agents.

Key words: adaptation, proteins, stress, footprinting, transcription, iNOS, Nrf2, Keap1.

Introduction

The over-arching theme of this review is the concept that there exists a large degree of plasticity in the innate ability of a cell to defend itself, and that this plasticity occurs at multiple layers within the normal physiology of a cell, but the control of gene transcription is absolutely fundamental to this process. This control may be at the level of access to DNA response elements, as well as at the levels of cellular localisation and inherent chemical reactivity of specific transcriptional control proteins. This review attempts to describe these two issues critical to plasticity in defence, by charting a route through various experiments employing different cell and whole tissue models. Furthermore, the different experimental approaches that have been used in these studies exemplify some of the techniques that need to be used to demonstrate different aspects of plasticity in cell defence.

The concept of cellular injury and cell defence

Cellular injury is a critical component of most disease processes. In order to achieve a better understanding of the details of how the injury occurs and thereby to improve the prospect of intervening in the disease to lead to improved outcomes, it is important to understand the inherent ability of a cell to withstand injury, which is literally the means by which a cell can defend itself. The common types of disease-producing cellular stresses are shown in Table 1. It is beyond the scope of this review to deal with all of the experimental models and techniques that can be employed to investigate

Table 1. *Disease-producing cellular stresses*

Hypoxia
Immune reactions
Infection
Physical injury
Chemical injury

each of these cellular stresses. However, we focus on two of these in this review, namely infection and chemical injury, which are dealt with in detail below.

The degree to which a cell becomes stressed depends critically on the dose and duration of the stress, and the particular vulnerability or inherent resistance of the particular cell or cell types that comprise a whole organ. With respect to the specific molecular targets of cell injury, four biochemical systems are particularly vulnerable, namely the cell membrane, energy metabolism, functional/structural proteins and the genetic machinery.

In fact, whilst every cell type would be expected to possess some form of innate defence, cellular and whole organ defence against challenge typically materialises as an adaptive response (see Fig. 1). This adaptation can take the form of metabolic and structural changes, as well as the triggering of changes in the number, abundance and location of critical proteins (typically transcription factors). These changes enable the cellular injury to be reversible. Where a cell is overwhelmed by a stress, and the innate and adaptive defences are breached, irreversible injury occurs and cell death ensues, either by apoptosis or necrosis. In fact, a cell may defend itself where possible, but under extreme conditions it may allow itself to die.

Access to DNA response elements

The plasticity in the cellular response to attack arises through multiple layers of sensing machinery. These include extracellular soluble receptors, plasma membrane receptors and intracellular proteins that act as receptors for sensing cellular stress and that may operate as transcription factors or as master controllers of such proteins. Transcription factors are the ultimate molecular determinants of the phenotype of a cell. They are a series of more than 100 different proteins, which are constitutively present in a cell or are synthesised rapidly on demand, and which, by interacting with specific short recognition sequences close to the start of coding sequences of genes, can determine the quantity and diversity of gene expression for a given cell within a specific environment [a comprehensive text on transcription factors is given elsewhere (Latchman, 1999)].

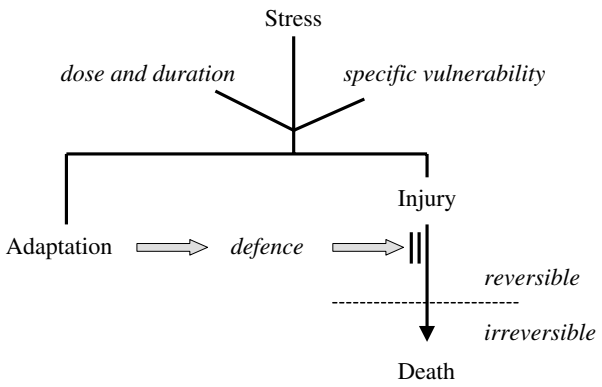

Fig. 1. Cellular response to stress.

Use of the endotoxin tolerance model to study plasticity in access to DNA response elements

The phenomenon of endotoxin tolerance

One of the most common disease-producing cellular stresses is that elicited by bacterial infection. Where the infection is due to Gram-negative bacteria such as *E. coli*, it can cause a vigorous host response, typically due to the molecular recognition of a cell wall component, lipopolysaccharide (LPS). This response materialises as a massive production of cytokine molecules, such as tumour necrosis factor-α and interleukin-1α, as well as one of the enzymes responsible for the synthesis of nitric oxide (NO) in the body, inducible nitric oxide synthase (iNOS) (Triantafilou and Triantafilou, 2002). The synthesis of these molecules represents a classical innate immune system response to deal with pathogen invasion. Unfortunately, this response can often disturb homeostasis sufficiently to cause life-threatening complications, typically referred to as septic or endotoxic shock, which is manifested as low blood pressure and reduced blood flow. Vital organs may not function properly or may fail. Interestingly, circulating leukocytes from septic patients have a limited capacity to produce cytokines in comparison to control individuals, which may represent an adaptive, protective mechanism to reduce organ injury (van Deuren et al., 1994). This is similar to the well-characterised phenomenon of endotoxin tolerance observed in monocytes/macrophages *in vitro*, in which a priming exposure to LPS elicits a refractory response in the production of TNF-α, Il-1α and iNOS upon further exposure. This was first reported in patients in 1946 by Beeson, who defined endotoxin tolerance as a reduced endotoxin-induced fever following repeated injections of typhoid vaccine (Beeson, 1946). There are a number of mechanisms that have been postulated to account for endotoxin tolerance (Cavaillon et al., 2003; Fan and Cook, 2004). One of these mechanisms implicates a transcription factor involved in processing many cellular immune and inflammatory signals, NF-kappa B, specifically a subunit of NF-kappa B called p50 [the role of p50 in endotoxin tolerance is reviewed elsewhere (Ziegler-Heitbrock, 2001)], which has the ability to bind strongly to DNA, but which does not possess the transcriptional activating properties of the p65 subunit of NF-kappa B (Schmitz and Baeuerle, 1991).

Use of in vivo footprinting to demonstrate changes in transcription factor binding in endotoxin tolerance

Using a technique known as *in vivo* footprinting it is possible

to visualise binding of NF-kappa B to its putative regulatory elements in the iNOS gene promoter and enhancer. This technique demonstrates binding of transcription factors to their regulatory elements in the chromatin in living cells, and thus it is more representative of the *in vivo* relevance of transcription factor changes than other techniques which necessitate the preparation of extracts of cells or nuclei. The technique is summarised in Fig. 2.

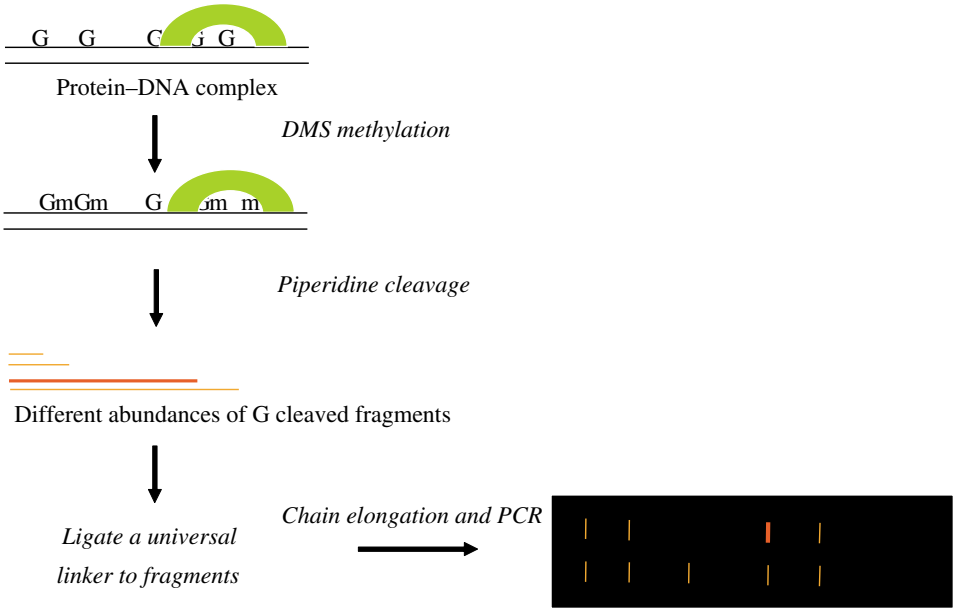

Fig. 2. *In vivo* footprinting of gene promoters. See text for details.

In brief, it depends on the variation in the susceptibility of a gene's transcriptional control regions to methylation of guanines by chemical modification with dimethylsulphate, due to the presence or absence of transcription factors. Variations in methylation can be detected by piperidine cleavage, isolation of genomic DNA G-cleaved fragments, and their ligation to a short universal double-stranded DNA sequence, which permits PCR amplification of these fragments, detection and quantification. The gene-specific promoter/enhancer fragments are resolved in a sequencing gel. Hyper- or hypo-methylations due to the presence of transcription factor are visualised as an increase or decrease in the abundance of the resolved fragments.

Using this technique, we have seen that in conditions of endotoxin tolerance, measured as a decrease in the levels of NO release, iNOS protein and iNOS gene transcription in macrophages, the same binding sites are occupied in the iNOS promoter and enhancer of desensitised macrophages and of LPS-responsive macrophages, yet the composition of NF-kappa B in the nuclei of these cells was found to be altered (Goldring et al., 1998). It appears that the presence of an overwhelming excess of transcriptionally inactive p50 homodimers on their kappa B sites in the iNOS control region in pretreated cells may block kappa B site binding by p50-p65, thereby reducing the activity of the protein complex governing iNOS transcription (see Fig. 3).

Transcription factor localisation and reactivity of critical protein residues

The defence against liver injury as a model of a whole tissue in vivo adaptive response

The liver has evolved exceptional adaptive systems to deal with chemical stress. It is therefore not surprising that most individuals can survive significant chemical stress associated with certain pharmacological agents, which are thus considered

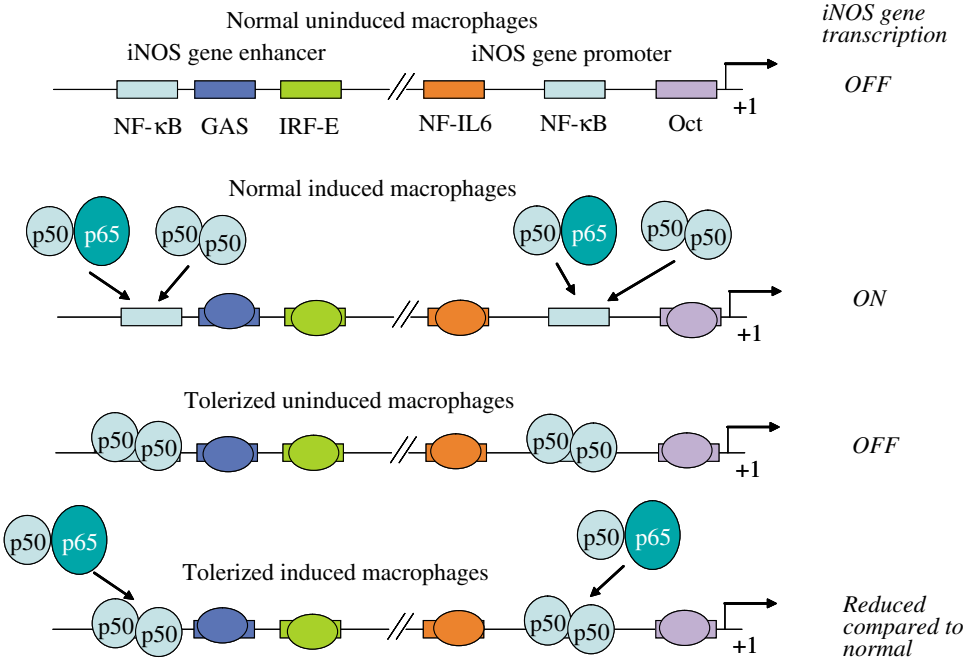

Fig. 3. NF-kappa B occupation may contribute to tolerance.

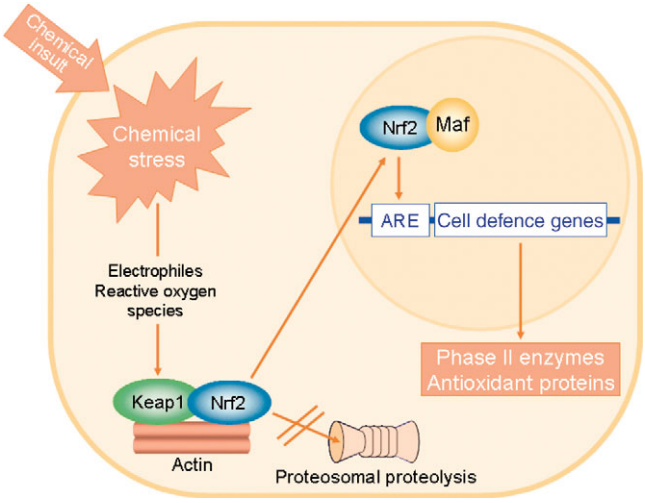

Fig. 4. Role of the Keap1-Nrf2-ARE system in the regulation of the antioxidant response. Sensing of chemical stress by Keap1 switches the fate of Nrf2 from proteasomal degradation to nuclear translocation, where it activates multiple genes involved in cellular defence.

safe at therapeutic doses (e.g. paracetamol). There is now an emerging literature that the adaptive response to chemical stress from endogenous and exogenous chemically reactive species, is activated and orchestrated by redox-sensitive transcription factors, which include Nrf2, AP-1, NF-kappa B and STAT-1. Such a response is designed to prevent protein and/or DNA damage and thus can be considered one of the earliest events in the chemical interaction of drug (metabolites) with the predominant liver cell, the hepatocyte.

The Keap1-Nrf2-ARE system is a dynamic and plastic switch necessary for the control of the defence of the liver

We will focus on the Keap1-Nrf2-ARE defence system as an excellent molecular paradigm of an adaptive tissue/cell defence transcription protein assembly. This section of the review will highlight the importance of protein localisation and chemical reactivity in determining the molecular adaptive response to stress and will define the complexity of this prototypic sense and response mechanism that is currently being revealed.

The transcription factor Nrf2 is now considered to be an important regulator of the antioxidant response element (ARE) found in the promoters of genes involved in cellular defence against electrophilic or oxidising chemical species: over 100 genes have now been shown to carry the ARE consensus sequence. In the unstressed state, Nrf2 resides in the cell cytoplasm where it associates with a repressor protein, Keap1 (Fig. 4). Although initially considered a passive inhibitor protein (Itoh et al., 1999; Zipper and Mulcahy, 2002), Keap1 is now known to play an active role in Nrf2 regulation by directing it for proteasomal proteolysis (Itoh et al., 2003; McMahon et al., 2003). Thus Nrf2 exists in a state of dynamic equilibrium with a half life of under 10 min (McMahon et al., 2004). This situation, which is analogous to the action of p53 (Harris and Levine, 2005), means that the cell is permanently primed to respond to a major chemical insult through rapid upregulation of Nrf2-driven defence proteins. Hence, the critical step in initiating a phase II response is perturbation of the interaction between Keap1 and Nrf2, as supported by the enhanced Nrf2 activity in Keap1 null transgenic mice (Wakabayashi et al., 2003) and in cells transfected with a Keap1 specific siRNA (Devling et al., 2005).

Keap1 is an unusually cysteine-rich molecule: it comprises five distinct regions, designated the N-terminal region, BTB domain, intervening region, double glycine repeat (or Kelch) domain and the C-terminal region (Fig. 5). A partial crystal structure has been solved indicating that the Kelch domain constitutes a β-propeller structure, characteristic of a multi-protein binding region (Li et al., 2004a; Li et al., 2004b; Padmanabhan et al., 2005); not surprisingly, this region is responsible for direct interaction with both Nrf2 and actin. A current model for the action of Keap1 is that it forms a homodimer and that Nrf2 is sandwiched between two Kelch domains (Fig. 6) (Dinkova-Kostova et al., 2005; Wakabayashi et al., 2004). The site of dimerisation is the BTB domain, whilst the intervening region, which is particularly cysteine-rich, forms the redox sensing part of the complex. Specifically, two cysteines (273 and 288) within the intervening region, through their proximity to basic amino acids, possess low pKa values and are thus highly chemically reactive (Zhang and Hannink, 2003). Oxidation of these cysteines results in release of Nrf2, which then translocates to the nucleus, heterodimerises with another nuclear protein (small Maf) and transactivates the ARE. Single and multiple mutations of cysteine residues in Keap1 have confirmed that C273 and C288 are critical for this protein in the repression of Nrf2 activity (Levonen et al., 2004; Wakabayashi et al., 2004; Zhang and Hannink, 2003). In addition, a further cysteine at position 151 in Keap1 has also recently been shown to be necessary for activation of Nrf2 by oxidative stress (Zhang et al., 2004b). Although a useful working model, it is likely that regulation of Nrf2 is more complex than depicted in Fig. 6. Ubiquitination (Kobayashi et al., 2004; Zhang et al., 2004a), phosphorylation (Bloom and Jaiswal, 2003; Huang et al., 2002) and nuclear

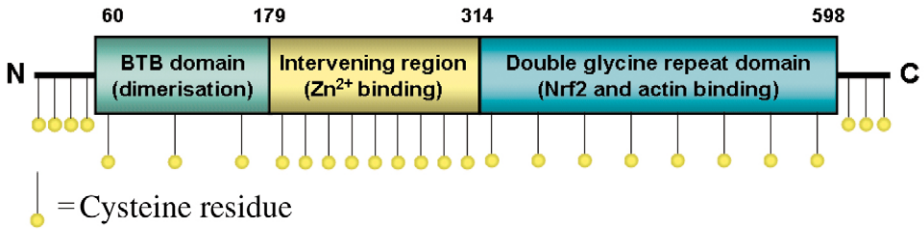

Fig. 5. Schematic representation of Keap1, indicating the relative cysteine content of the various regions.

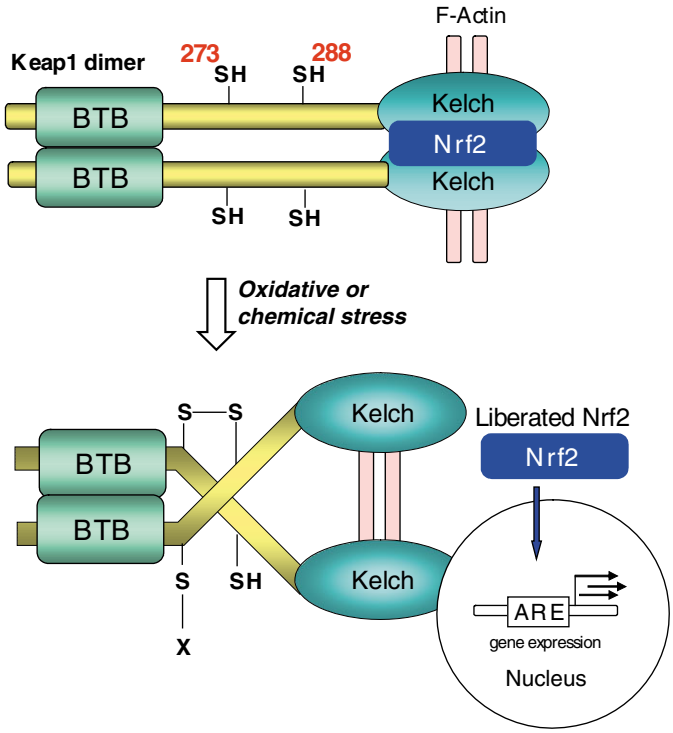

Fig. 6. Model of the activation of Nrf2 through release from the Keap1 dimer induced by oxidation (S–S) or arylation (X) of cysteine thiols 273 and 288 [adapted from (Wakabayashi, 2004)]. See text for further explanation.

shuttling mechanisms (Jain et al., 2005; Li et al., 2005) have all been implicated in Nrf2 activity. Clearly there remains uncertainty over the precise workings of the Keap1-Nrf2-ARE system; however, a consistent feature of all models proposed so far is that the Keap1 protein, and in particular its cysteine residues, plays a central role in both the detection and transduction of the activating stimulus.

Covalent modification of recombinant Keap1 has been shown for several thiol-reactive compounds including dexamethasone mesylate (Dinkova-Kostova et al., 2002), iodoacetamide (Hong et al., 2005), *N*-ethylmaleimide (Hong et al., 2005), sulphoraphane (Eggler et al., 2005), dinitrochlorobenzene (Eggler et al., 2005), xanthohumol (Dietz et al., 2005; Liu et al., 2005) and the endogenous inducer 15-deoxy-Δ12,14-prostaglandin-J2 (Eggler et al., 2005), although a consistent pattern of cysteine modification has failed to emerge. Thus there is a clear need for the systematic characterisation of Keap1 modification by inducers of Nrf2 and to relate this to the ensuing phase II response in the control of drug induced liver injury. We have developed similar tools to explore the modification of proteins involved in the Keap1-Nrf2-ARE system with the specific intention of exploring drug-derived reactive metabolites generated in the liver. We have seen that NAPQI, the reactive metabolite of paracetamol, binds to Keap1 (data not shown). Furthermore, we have demonstrated in an *in vivo* mouse model that paracetamol both induces nuclear translocation of Nrf2 (see Fig. 7) and transcriptionally activates several ARE-driven genes (Goldring et al., 2004).

Three independent laboratories have developed Nrf2 null mice (Chan et al., 1996; Itoh et al., 1997; Martin et al., 1998) and have demonstrated the pivotal role of Nrf2 for both constitutive and inducible expression of ARE-activated proteins. Nrf2 deficient strains were more susceptible to the toxic effects of model hepatotoxins, such as paracetamol (Chan et al., 2001; Enomoto et al., 2001) and the antioxidant butylated hydroxytoluene (Chan and Kan, 1999). Overall, studies utilising the Nrf2 null mouse model by ourselves (Kitteringham et al., 2005) and others (Chan et al., 2001; Chan

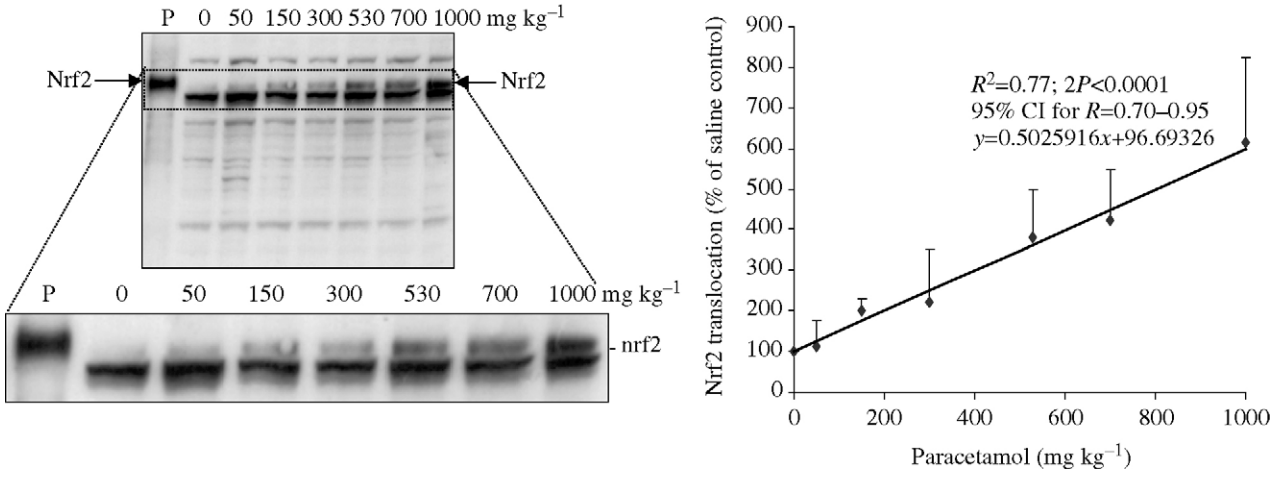

Fig. 7. Nrf2 nuclear translocation is related to the administered dose of paracetamol. Mice were treated with the indicated doses of paracetamol. After 1 h they were sacrificed, livers were removed, nuclei prepared and nuclear proteins extracted. These were separated using SDS–PAGE, alongside a recombinant mouse Nrf2 positive control, transferred to a nitrocellulose membrane, probed for Nrf2 using a polyclonal anti-Nrf2 antiserum and visualised using chemiluminescence. Each data point in the blot and in the response-curve is obtained from pooled extracts of five animals (+ s.d.).

et al., 1996; Chanas et al., 2002; Enomoto et al., 2001; Hoshino et al., 2000; Ishii et al., 2000; Itoh et al., 1997; Martin et al., 1998; McMahon et al., 2001; Nguyen et al., 2000; Pietsch et al., 2003; Thimmulappa et al., 2002) indicate that the lack of Nrf2 precludes the xenobiotic-induced enhanced expression of multiple antioxidant response proteins, but that constitutive expression of the same genes is often only marginally reduced by deletion of the Nrf2 gene. These data indicate that (i) Nrf2 is an essential redox sensor of chemical stress in the liver and (ii) that Nrf2 associated genes may define the threshold for toxicity caused by various chemical hepatotoxins.

Thus the Nrf2 pathway may play an important role in the adaptive defence of the liver against hepatotoxins, as demonstrated by the fact that a lower dose of paracetamol elicits toxicity in Nrf2 null mice, but that it can also be induced by the hepatotoxins themselves, thereby raising the threshold for toxicity following repeat exposure. We are therefore currently attempting to define intra- and intercellular signalling systems that ultimately determine bioactivation of drugs and subsequent drug induced liver injury.

Conclusions

In summary, multiple layers of adaptation of cellular transcription factors exist to respond rapidly and effectively to changes in the environment. It is possible to use a number of different cell, tissue and animal models to investigate this phenomenon experimentally. Each of these will be informative of different aspects of adaptive defence responses. These approaches demonstrate that the adaptation may depend upon accessibility to DNA and abundance, localisation and activity of transcription control proteins. Interestingly, prototypic adaptive transcription factors often display linear dose-dependent adaptation in an *in vivo* context. Finally, the crucial question that now needs to be addressed is how the adaptation relates to chemical changes in these proteins.

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