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COMPREHENSIVE INVITED REVIEW

Basic Principles and Emerging Concepts in the Redox Control of Transcription Factors

Regina Brigelius-Flohé[1] and Leopold Flohé[2]

Abstract

Convincing concepts of redox control of gene transcription have been worked out for prokaryotes and lower eukaryotes, whereas the knowledge on complex mammalian systems still resembles a patchwork of poorly connected findings. The article, therefore, reviews principles of redox regulation with special emphasis on chemical feasibility, kinetic requirements, specificity, and physiological context, taking well investigated mammalian transcription factor systems, nuclear transcription factor of bone marrow-derived lymphocytes (NF-κB), and kelch-like ECH-associated protein-1 (Keap1)/Nrf2, as paradigms. Major conclusions are that (i) direct signaling by free radicals is restricted to $O_2^{\bullet -}$ and $^{\bullet}NO$ and can be excluded for fast reacting radicals such as $^{\bullet}OH$, $^{\bullet}OR$, or $Cl^{\bullet}$; (ii) oxidant signals are H_2O_2, enzymatically generated lipid hydroperoxides, and peroxynitrite; (iii) free radical damage is sensed *via* generation of Michael acceptors; (iv) protein thiol oxidation/alkylation is the prominent mechanism to modulate function; (v) redox sensors must be thiol peroxidases by themselves or proteins with similarly reactive cysteine or selenocysteine (Sec) residues to kinetically compete with glutathione peroxidase (GPx)- and peroxiredoxin (Prx)-type peroxidases or glutathione-S-transferases, respectively, a postulate that still has to be verified for putative mammalian sensors. S-transferases and Prxs are considered for system complementation. The impact of NF-κB and Nrf2 on hormesis, management of inflammatory diseases, and cancer prevention is critically discussed. *Antioxid. Redox Signal.* 15, 2335–2381.

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[1]Department Biochemistry of Micronutrients, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany.
[2]Department of Chemistry, Otto von Guericke-University Magdeburg, Magdeburg, Germany.

"It was clear as mud but it covered the ground and the confusion made the brain go 'round."
—Harry Belafonte (27)

I. Introduction

Activation of gene transcription has for long been considered to be primarily, if not exclusively, regulated by cascades of protein phosphorylation and de-phosphorylation. Screening the reviews that were written in the nineties (18, 114, 116, 117, 492) or even during the present decade (115, 118, 431, 433) by pioneers in protein phosphatase (PP) or kinase research such as Edmund Fischer, Joseph Schlessinger, or Axel Ullrich, it is hard to detect any hint that gene activation could be driven by anything else but phosphorylation, dephosphorylation of receptors, and/or downstream signaling molecules. During the nineties, though, a second area was recognized to be intimately related to transcriptional regulation, the ubiquitin/proteasome system (69, 71, 73, 458, 480), that had been shown to either degrade transcription factors such as c-Fos and C-Jun (220, 463), p53 (420), hypoxia-inducible factor 1α (HIF-1α) (230), Nrf2 (323), β-catenin (2), and nuclear hormone receptors (342) or inhibitory cytosolic complexes of transcription factors such as the complex of nuclear transcription factor of bone marrow-derived lymphocytes (NF-κB) with its inhibitor IκB (240, 524). Other proteolytic systems such as the calpains (220, 364) and caspases (359) followed, and it became clear that these proteolytic systems complemented the phosphorylation cascades (178, 240, 359). In parallel, the impact of redox processes on transcriptional gene activation became obvious, although the main focus of the oxygen clubs and free radical associations remained the concern about the potential hazards of the reactive oxygen species (ROS) (453).

In retrospect, the reluctance to accept oxidants as mediators or modulators of regulatory processes is hard to understand. Already in the early nineties vanadate plus H_2O_2 (177) or peroxovanadium compounds were reported to mimic insulin action, were recognized to act as phosphatase inhibitors (381), and became widely used to enhance protein phosphorylation in the analysis of kinase cascades in general. That this analytical trick could have a physiological correlate could also have been guessed from publications of the seventies claiming H_2O_2 to be a second messenger of insulin signaling (81, 319, 320). However, the concept that gene transcription might be controlled by redox reactions remained dormant until an important transcription factor in eukaryotes, the nuclear transcription factor in B-cells NF-κB (19, 444, 455, 464), was shown to be activated by compounds known to trigger production of superoxide/H_2O_2 (440, 444) or by oxidants themselves, *inter alia* by H_2O_2, and inhibited by antioxidants (15, 440). Conceptually, an oxidative inactivation of phosphatases leading to enhanced signal transduction emerged as a likely mechanism (128).

Oxidative inactivation of phosphatases in signaling cascades, however, did not for long remain the only possible mechanism how oxidants could affect transcription. Microbiologists demonstrated that a direct oxidation of the transcription factor OxyR may orchestrate the transcription of defensive genes (11, 68). Other concepts followed, for example, activation of protein kinases (PKs), redox-dependent noncovalent binding of thioredoxin (Trx), thiol modification of proteins that form cytosolic complexes with transcription factors, or heterodimer formation of glutathione peroxidase (GPx)- and peroxiredoxin (Prx)-type peroxidases with transcription factors [reviewed in refs. (123, 134), see section II.D.1].

The multiple ways of redox regulations that became obvious over the last two decades lead us to presume that most, if not all, of the classical routes to transcriptional activation are modulated by redox processes or even critically depend on oxidant signals (Table 1). In this article we will briefly summarize pertinent mechanistic principles. In this context, insights from microbiology, which as usual is leading the field, will be discussed in respect to their possible relevance to the more complex mammalian systems. We then will focus on the redox-sensitive mammalian pathways of gene activation, choosing the two best investigated ones, the Nrf2 and NF-κB systems, as paradigms of redox-controlled transcriptional activation and basis for hormetic responses in higher organisms.

II. Mechanistic Principles in Redox Regulation

A. Indispensable components of regulatory circuits

As in technology in general, a biological regulatory circuit needs a minimum set of elements to adapt the metabolic system to special requirements: a signal and a sensor to switch-on the adaptive process, a transducer, a modulator of

Table 1. Mammalian Transcription Factors Regulated by Redox Events

Transcription factor systems	*Main regulatory redox events*	*Physiological consequences*	*Refs.*
AhR	Hypoxia, competition of HIF and AhR for ARNT is discussed	Inhibition of DNA binding	(119)
AP-1	Enhanced phosphorylation of c-Jun and c-Fos by activation of upstream kinases (*e.g.*, RTKs, MAPKs, PKCs and/or inactivation of PPAs)	Enhanced association of c-Jun and c-Fos with CBP/p300 and transcriptional activation (inhibition of DNA binding of c-Jun when phosphorylated by GSK3β)	(475)
	Activation of ASK-1 due to oxidation of associated Trx (or Grx)	De-inhibition of ASK-1 and activation of JNK	(415)
	Oxidant-induced dissociation of GSTπ or μ from ASK-1 and JNK	Activation of JNK	(488)
	Oxidation of Cys154 in c- Fos and Cys272 in c-Jun	Inhibition of DNA binding	(1)
	Reversal of Cys oxidation in c-Fos and c-Jun by Trx and APE1/Ref-1	Prerequisite for DNA binding	(475, 520)
β-catenin	Thiol oxidation in nucleoredoxin	Release of nucleoredoxin from dishevelled which activates β-catenin	(139)
Egr-1	Oxidation of cysteines in the 3 Zn-fingers reversible by APE1/Ref-1	Inhibition of DNA binding	(193, 467)
FOXO	Cysteine oxidation of PTP1b	Activation of Akt, phosphorylation and nuclear export of FOXO	(83)
	Intramolecular disulfide formation between Cys297 and Cys311 in Akt	Affinity increase of Akt for PP2A, de-phosphorylation and inactivation of Akt. FOXO remains in the nucleus	(83)
	Oxidative activation of JNK	Phosphorylation of FOXO, in this case prevention of nuclear export thus counteracting Akt.	(83)
GR	Zn-finger oxidation, reversible by Trx	Inhibition of DNA binding	(159)
HIF-1α	Oxidation of Fe^{2+} to Fe^{3+} in HPH	Inactivation of HPH, prevention of hydroxylation and degradation of HIF, activation of HIF	(155, 475)
	Reduction of Cys800 in HIF by APE1/Ref-1 and Trx/TrxR	Increase in transactivation capacity of HIF	(248, 293)
P53	Low oxidative activation of MAPKs	Low phosphorylation/activation of p53, induction of antioxidant and defense systems, cell growth	(412, 467, 475)
	Enhanced oxidative activation of MAPKs	Enhanced phosphorylation and activation of p53, elimination of cells with mutations by apoptosis	(412)
	Oxidation of conserved cysteines in the DNA-binding domain and Zn-fingers	Inhibition of DNA binding	(391)
	Reduction of Zn-coordinating cysteines in p53 by APE1/Ref-1 and Trx/TrxR	Maintenance of DNA-binding capacity	(54, 221)
Pax-5, −8	Oxidation of Cys45 and 57 in Pax-8, reversible by APE1/Ref-1	Inhibition of DNA binding	(55, 426, 475)
	Intramolecular disulfide formation in Pax-5, reversible by APE1/Ref-1	Inhibition of DNA binding	(476)
NF-κB	Formation of an intermolecular disulfide bond in LC8, reversible by TRP14	Enabling phosphorylation of IκBα by IKKβ followed by IκB degradation. Release and activation of NF-κB	(227)
	Reduction of Cys62 in p50 by APE1/Ref-1 and Trx/TrxR in the nucleus	Enhancement of DNA binding	(159, 228)
	Others	See section IV	
Nrf2	Modulation of Cys272 and Cys288 in Keap1	Conformational change of Keap1, prevention of Nrf2 ubiquitination, activation of Nrf2	(97)
	Modulation of Cys151	Prevention of ubiquitination and degradation of Nrf2, activation of Nrf2	(389)
	Others	See section III	
Sp1	Oxidation of cysteines in 3 Zn-fingers	Inhibition of DNA binding	(508, 519)
TTF	Oxidation of cysteines in the Zn-finger domain, reversible by APE1/Ref-1	Inhibition of DNA binding	(467, 475)
USF	Cys229 and Cys248 cross-linking by oxidants	Inhibition of DNA binding	(377)
	Interaction of HMG-1 (high mobility group protein-1) with reduced USF	Enhancement of DNA binding	(310)

ARNT, aryl hydrocarbon receptor nuclear translocator; ASK-1, apoptosis signal-regulating kinase-1; CBP, CREB-binding protein; Egr, early growth response; FOXO, Forkhead box O; GR = glucocorticoid receptor; Grx, glutaredoxin; GSK3β, glycogen synthase kinase-3β; GST, glutathione-S-transferase; HIF-1, hypoxia-inducible factor 1; HPH, HIF prolyl hydroxylase; IKK, IκB kinase; JNK, c-jun N-terminal kinase; Keap1, kelch-like ECH-associated protein-1; LC8, dynein light chain 8; MAPK, mitogen-activated protein kinase; NF-κB, nuclear transcription factor of bone marrow-derived lymphocytes; PKC, protein kinase C; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; TRP14, thioredoxin-related protein-14; Trx/TrxR, thioredoxin/thioredoxin reductase; TTF, thyroid transcription factor; USF, upstream stimulatory factor.

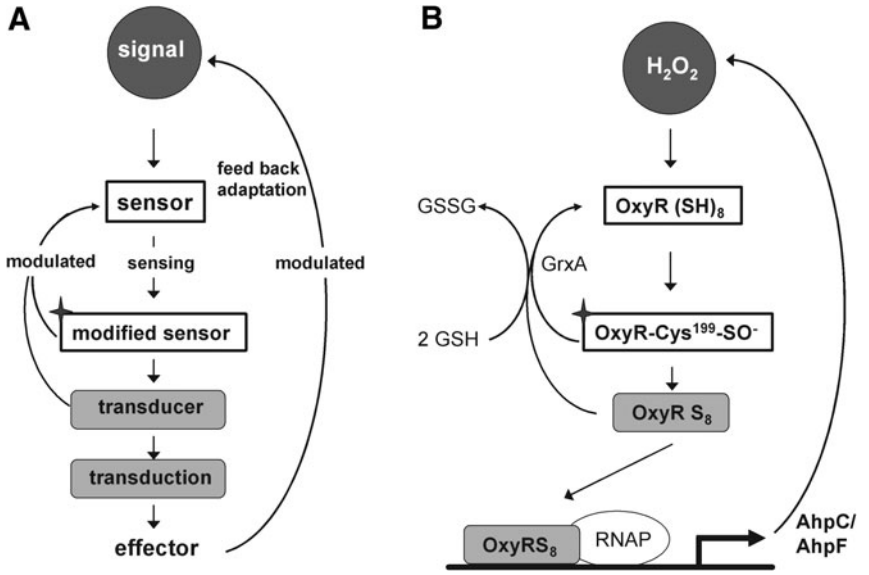

FIG. 1. Scheme of transcriptional regulation and its implementation in bacterial redox control. (A) A minimalistic scheme of a regulatory circuit with the required elements: signaling molecule (black with white letters); sensor (white box); transducer(s) (light gray); and effector (typically the target gene). Termination reactions, which are subject to modulation, are indicated by *reversed arrows*. The labeling code is maintained in all following figures, if appropriate. **(B)** Demonstrates how this scheme is used in the OxyR regulation of prokaryotes. The signaling molecule is H_2O_2 targeting reduced OxyR as sensor. OxyR is first oxidized at Cys199 (with the oxidized sensor marked by a star) and stepwised further oxidized by H_2O_2. OxyR turns into a transducer ($OxyRS_8$) and binds to its effector, the responsive element in the DNA, and allows the RNA polymerase (RNAP) to effect gene expression that removes the signal *via* enhanced alkyl hydroperoxide reductase (AhpC/AhpF) synthesis, thus terminating or modulating the sensing process. Prevention or termination of transduction is also achieved by reducing oxidized OxyR by glutaredoxin A (GrxA). The Grx system is modulated by glutathione (GSH) regeneration. In all eukaryotic systems additional transducers that are distinct from modified sensors are involved (see Fig. 2 and others).

sensitivity, an effector, and a switch-off device. As is common for biological processes, also the biochemistry of regulatory circuits is more complicated. At best in prokaryotes, simple versions resembling technical regulatory circuits appear to be realized (Fig. 1). With increasing complexity of the organism, the regulatory systems have to cross-talk with different compartments of the cell, with the entire organism, and its environment. They have no chance to operate in splendid isolation; moreover, the resting position is not an equilibrium but a snap shot of steady states of competing reactions within the metastable and open system that defines life. The complexity, thus created, has two important implications: (i) each regulatory step under consideration has to be kinetically competitive with a realm of competing reactions and (ii) the signal has to be specifically transduced to the effector despite possible side reactions and cross-talks between signaling cascades. As will become evident, these two aspects are particularly relevant in redox regulation when the technical terms of a circuit are to be translated into defined biochemical entities.

Whereas, for example, the signal/sensor interaction in cytokine signaling, that is, binding of a peptide to its receptor, is unproblematic in respect to specificity, it is enigmatic how signaling by promiscuously reacting ROS or radicals complies with the specificity requirement of a meaningful redox regulation. The other problem is raised by the abundance of superoxide dismutases (SOD), heme-based peroxidases, GPxs, and Prxs, which eliminate most of the ROS at rates that are hard to beat. SODs dismutate $O_2^{\bullet -}$ with rate constants around $10^9\ M^{-1}\ s^{-1}$ (251, 321); for peroxidases, the bimolecular rate constants k_{+1}, which defines the oxidation of the reduced enzymes by ROOH in analogy to Eq. 1 or 4, are around $10^7\ M^{-1}\ s^{-1}$ in case of heme enzymes (59); they range from 10^4 to $10^7\ M^{-1}\ s^{-1}$ for Prxs (489) and GPx-type peroxidases (487) working with sulfur catalysis; they reach $10^8\ M^{-1}\ s^{-1}$ for GPxs working with selenium catalysis (487), and rate constants beyond $10^7\ M^{-1}\ s^{-1}$ were also reported for peroxynitrite reduction by Prxs (101, 489). Whatever the signaling molecule is, it should hit the sensor selectively despite competition with a realm of extremely fast enzymes.

B. Radicals or hydroperoxides as oxidant signals in redox signaling?

At least one radical, $^{\bullet}NO$, is an accepted signaling molecule. It reversibly binds to heme prosthetic groups in guanylate cyclases, thereby triggering a broad spectrum of physiological events (136, 196, 302, 331), but signaling by $^{\bullet}NO$ is not commonly subsumed under the term redox signaling, as long as $^{\bullet}NO$ is not previously transformed to peroxynitrite, which is an oxidant signaling molecule indeed but no longer a radical (112, 430). Like $^{\bullet}NO$, the superoxide radical anion $O_2^{\bullet -}$ has the potential to bind reversibly to heme (322), but evidence on regulatory consequence of this ability is scarce. Its affinity to iron complexes is, however, widely used for redox sensing by iron sulfur [Fe-S] clusters in bacteria. The transcription factor SoxR responds to $O_2^{\bullet -}$ with transcription of MnSOD and other protective proteins (351, 518) and its binuclear (247) cluster proved to be functionally essential for superoxide sensing (182), the mechanism being a univalent oxidation of the complexed iron by $O_2^{\bullet -}$ (82).

A recent thoughtful analysis by Forman *et al.* concluded that there is neither evidence nor likelihood that redox regulation is directly mediated by any of the fast reacting oxygen-centered or other radicals that may arise from chain reactions initiated by the primary physiological radicals (134). Their persuasive, if not convincing, argument is that aggressive radicals such as $^{\bullet}OH$, $RO^{\bullet}$, or halogen radicals derived from the myeloperoxidase reaction, which react with almost all kind of biomolecules at nearly diffusion-limited rate constants

($\sim 2\times10^{10}\,M^{-1}\,s^{-1}$), simply lack the ability to modify regulatory proteins with the mandatory selectivity. Instead, they consider H_2O_2 as the key oxidant signal in redox signaling, because it can selectively oxidize particularly sensitive SH groups.

This seemingly provocative article may benefit from minor amendments, but convinces in so far as very few of the protein modifications that have up to now been identified in ROS signaling disclose sequelae of direct reactions between radicals and target proteins. An exception seems to be the H_2O_2-dependent de-repression of the repressor PerR (50, 332). In these proteins iron-co-ordinated histidines are oxidized to oxo-histidines, which reminds of a Fenton chemistry-mediated hydroxylation. Accordingly, the $^{\bullet}OH$ radical is considered the ultimately reacting ROS (82). It is, however, not a free $^{\bullet}OH$ that is sensed by the repressor protein, but rather a crypto-OH (421, 527), that is, an $^{\bullet}OH$ that is likely formed locally from an iron peroxo complex and targeted to its intimate environment, which is here the imidazole moiety of a histidine (274). More commonly, the modifications detected in oxidized sensors are easily explained by two-electron withdrawal from thiolates.

The most common one of these reactions is the oxidation of cysteine residues by H_2O_2 to sulfenic acids (Eq. 1) followed by disulfide formation with glutathione (GSH) or protein thiols (Eqs. 2 and 3). The same sequence of reactions is achieved by the primary products of lipoxygenases (LOX) or cyclooxygenases (COX), which are alkylhydroperoxides (ROOH) (Eq. 4), and also by peroxynitrite ($ONOO^-$), which is spontaneously formed from $^{\bullet}NO$ and $O_2^{\bullet-}$ at nearly diffusion-limited rates (Eqs. 5 and 6). The efficiencies of these compounds in oxidizing thiols differ, peroxynitrite being the most and H_2O_2 the least reactive one, whereas lipid hydroperoxides occupy interim positions. Also, the residue R in lipid hydroperoxides may prevent or facilitate the interaction with a particular protein thiol, thus lending some selectivity to these signaling molecules. The qualitative equivalence of H_2O_2, ROOH, and $ONOO^-$ in oxidizing protein thiols to sulfenic acids has, however, been amply demonstrated in the initial step of the catalytic cycles of Prx- and GPx-type peroxidases, which is the oxidation of the peroxidatic cysteine C_P, as shown in Eqs. 1, 4, and 5 (105, 301, 379, 380, 487, 489). Analogous reactions of exposed and dissociated thiols in other proteins cannot reasonably be doubted, whereas a radical-initiated sulfhydryl oxidation would likely yield unspecific protein damage, if not terminated by the extremely unlikely event of meeting another thiyl radical to form a disulfide bond. In short, glutathionylation or intra- and intermolecular disulfide bond formation in regulatory proteins does not result from any free radical attack, but most likely from an electron pair transition from nonradical ROS such as H_2O_2, ROOH, or $ONOO^-$. Hydroperoxides including $ONOO^-$ and not radicals themselves may, thus, be considered oxidant signals in ROS signaling, the sensing process being the oxidation of susceptible cysteine residues to cysteine sulfenic acids (123, 133, 134). The intimate downstream transduction step, the reaction of the sulfenic acid with another thiol according to Eqs. 2 and 3, respectively, is analogous to the reaction of the oxidized C_P in thiol peroxidases with their resolving cysteine C_R. This coreacting C_R is typically not dissociated and not reactive enough to sense an ROOH by itself. However, it readily reacts with the sulfenic acid if this is sterically possible. Therefore, the analogous transduction of an oxidant signal, sensed as a sulfenic acid residue (Eq. 3), appears to be exclusively determined by sterical fit, which lends further specificity to the overall signaling process.

$$H_2O_2 + \text{Prot-S}^- \rightarrow \text{Prot-SO}^- + H_2O \quad (1)$$

$$\text{Prot-SO}^- + H^+ + \text{GSH} \rightarrow \text{Prot-S-SG} + H_2O \quad (2)$$

$$\text{Prot-SO}^- + H^+ + \text{Prot'-SH} \rightarrow \text{Prot-S-S-Prot'} + H_2O \quad (3)$$

$$\text{ROOH} + \text{Prot-S}^- \rightarrow \text{Prot-SO}^- + \text{ROH} \quad (4)$$

$$ONOO^- + \text{Prot-S}^- \rightarrow \text{Prot-SO}^- + NO_2^- \quad (5)$$

$$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^- \quad (6)$$

Protein thiol modification as sensing mechanism may also be achieved by different ways (250). Theoretically, glutathionylation may result from thiol/disulfide exchange between oxidized glutathione (glutathione disulfide [GSSG]) and protein thiol (47, 48).

$$\text{GSSG} + \text{Prot-SH} \rightarrow \text{Prot-S-SG} \quad (7)$$

However, the comparatively low cellular GSSG concentrations render this reaction less likely than the direct protein SH oxidation followed by reaction with GSH (Eqs. 1 and 2). Further, disulfides can be formed by nitroso glutathione (GSNO) and protein SH groups or, inversely, from S-nitrosylated proteins and GSH (Eqs. 8 and 9) (513).

$$\text{GSNO} + \text{Prot-SH} \rightarrow \text{Prot-S-SG} + \text{HNO} \quad (8)$$

$$\text{GSH} + \text{Prot-SNO} \rightarrow \text{Prot-S-SG} + \text{HNO} \quad (9)$$

$$\text{HNO} + \text{R(SH)}_2 \rightarrow \text{RS}_2 + \text{H}_2\text{NOH} \quad (10)$$

Thereby, products derived from $^{\bullet}NO$ and oxygen with the potential of S-nitrosation, the reactive nitrogen species (RNS) (112), have to be considered as possible signaling molecules, the most likely candidates being the nitroxyl cation NO^+ and N_2O_3, whereas the nitroxyl HNO formed according to Eq. 8 might again contribute to disulfide formation (Eq. 10) (328). As in the case of ROS signaling, not the $^{\bullet}NO$ radical itself is used as thiol modifier in RNS signaling but nonradical derivatives thereof.

Apart from $O_2^{\bullet-}$, the only oxygen-centered radical that is directly used as signaling molecule appears to be molecular dioxygen itself, a bi-radical that, though, is hardly ever addressed as ROS (see section II.D.6).

C. Signals of free radical damage

The statement that free radicals are not ideal signaling molecules seemingly conflicts with the widely accepted view that the organism reacts to free radical formation and radical-driven processes with adaptive responses. As is evident from the previous section, the often used term "free radical

signaling" can claim its justification from the fact that most of the oxidant signals so far identified are indeed derived from the two important natural radicals $^{\bullet}NO$ and $O_2^{\bullet -}$. Remains the question how the organism responds if these radicals are not channeled into smooth physiological pathways for, for example, hormone or growth factor signaling, but cause tissue damage due to unbalanced production in conditions that deserve the name oxidative stress or nitrosative stress. In pathological conditions such as septicemia or reperfusion injury or any other kind of fulminant inflammation, free radical-dependent tissue damage is obvious and it seems straight forward that the organism should somehow sense the abundance of radicals. Apparently, however, the radicals themselves are not sensed either under these extreme conditions. Instead, the outcome of the radical attack is sensed by a nonradical sensing mechanism.

Protein thiols identified as likely sensors in redox signaling offer chemical options beyond thiol oxidation and thiol/disulfide exchange. If dissociated, they are powerful nucleophiles and, thus, may react with a large variety of compounds with reactive double bonds such a α, β-unsaturated aldehydes or ketones and other Michael acceptors. This huge list of compounds *inter alia* comprises well-documented products of enzymatic or free-radical-driven lipid peroxidation, the most prominent examples being 4-hydroxy-nonenal (HNE) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). Such compounds have been amply documented to alkylate particular protein thiols under oxidative or nitrosative stress and therefore may be implicated as stress signaling molecules that are sensed *via* S-alkylation. The best known example of a regulatory protein modified this way is kelch-like ECH-associated protein-1 (Keap1), which plays a pivotal role in responding to oxidative challenge with an adaptive response *via* activation of the transcription factor Nrf2 (6, 94–98, 254, 479, 481) (see section III), but analogous stress sensing has also been implicated in the NF-κB pathway (408) and in apoptosis (12).

D. Sensing and transducing proteins

As outlined, the main problem of redox signaling is seen in rendering specificity to oxidant signals. Since thiol oxidation and alkylation appear to be the prevailing sensing mechanisms in redox regulation, proteins with highly reactive thiols must be sensors of choice. Such thiols have to fulfill three requirements: they have to be surface exposed, dissociated, and kinetically competent to compete with peroxidases and, if S-alkylation is involved, also with glutathione-S-transferases (GSTs). Beyond, the resulting thiol modification must lead to a structural change of the sensor to allow specific signal transduction.

1. Thiol peroxidases as sensors. The rate constants for the oxidation of freely accessible SH groups in low–molecular-weight compounds by H_2O_2, even if extrapolated to full SH dissociation, do hardly exceed 50 $M^{-1}\,s^{-1}$ (512) and, thus, fall short by orders of magnitude when compared to those of GPx- or Prx-type peroxidases (see section II.A). Protein-bound cysteines are by no means more reactive (31, 123, 134), unless they are embedded in a micro-architecture that facilitates cleavage of the hydroperoxy bond by polarization and proton shuttling as in the thiol peroxidases (378, 487). Evolution has designed these proteins for highly efficient hydroperoxide reduction. Accordingly, they do not only deserve interest as hydroperoxide-detoxifying enzymes, but also as ideal sensors for ROOH.

In recent years, a sensor/transducer function of peroxidases has indeed been elucidated in transcriptional regulation of yeasts (135). In *Saccharomyces cerevisiae* a GPx-type peroxidase Orp1 senses H_2O_2 in being oxidized to its sulfenic acid form, as in Eq. 1. The cysteine sulfenic acid residue of Orp1 then forms a disulfide bridge with a particular thiol of the transcription factor activating protein-1 (AP-1)-like transcription factor from yeast (Yap1), thereby directly transducing the oxidant signal (86) (Fig. 2). The physiological meaning of the H_2O_2 sensing by the peroxidase is seen in transducing the oxidant signal to a defined target protein with the specificity typical for protein/protein interactions (86). In another *S. cerevisiae* strain, the 2-Cys Prx Tsa1 appears to activate Yap1 in an analogous way (357, 407), and in *Schizosaccharomyces pombe* Tsa1 is the major peroxidase that reacts with the transcription factor Pap1, which is an homolog of Yap1 (333). Also, in *S. pombe* Tsa1, upon having sensed H_2O_2, forms a disulfide bridge with the stress kinase Sty1, thereby transducing the oxidant signal to the transcription factor Atf1 (333, 497).

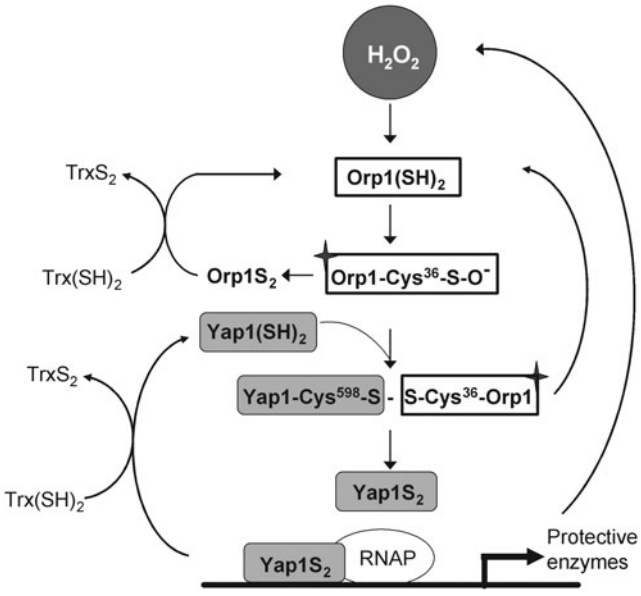

FIG. 2. Sensing and transducing the H_2O_2 signal by the Orp/AP1-like transcription factor from yeast (Yap) system in yeast. In this system the glutathione peroxidase (GPx)-type peroxidase Orp1 act as sensor for H_2O_2 and becomes oxidized at Cys^{36} to a sulfenic acid. The sulfenic acid then forms a heterodimeric disulfide bridge with Cys^{598} in Yap1, which in turn is resolved by two more thiol/disulfide exchange reactions (not shown), ultimately resulting in oxidized Yap1 ($Yap1S_2$) and reduced Orp1. Oxidized Yap1 can bind to the respective enhancer elements in the promoter of target genes, which protect against oxidative challenge. This way Yap1 acts as the transducer of the H_2O_2 signal. An alternative reaction of Orp1 sulfenic acid leads to an intramolecular disulfide bridge ($Orp1S_2$) that is reduced by thioredoxin (Trx). In this role of a Trx peroxidase Orp1 acts as a modulator of this system. Trx also terminates signaling by reducing $Yap1S_2$.

A case of H_2O_2 sensing by a Prx has also been demonstrated in *Kinetoplastida*. In these unicellular parasites a universal minicircle sequence binding protein (UMCSBP), which is a Zn-finger protein, binds to the peculiar kinetoplast DNA in a Zn- and redox-dependent manner, thereby regulating replication [reviewed in ref. (452)]. DNA binding of UMCSBP is abrogated by H_2O_2, which is sensed by the Prx-type tryparedoxin peroxidase with rate constants that, depending on species, range from 10^5 to 10^7 M^{-1} s^{-1} (489). The peroxidase directly transduces the oxidation equivalents to CxxC motifs of the Zn-finger domain. The reductive reactivation of UMCSBP depends on the trypanothione redox cascade comprising trypanothione reductase, trypanothione, and tryparedoxin, the latter being a thioredoxin-related protein (TRP) specialized for protein disulfide reduction (349).

Analogous ROOH sensing by peroxidases in mammals have not yet been reported. However, the idea that at least some of the GPx- or Prx-type peroxidases could similarly act as sensors for H_2O_2, alkyl hydroperoxides, and peroxynitrite in mammals is intriguing in several respects. It would comply with the observation that redox signaling also takes place at physiological H_2O_2 fluxes; it could explain why in some cases Prxs improve rather than inhibit signaling (75, 238), and it finally might help to explain oxidative modification of proteins with SH group reactivities that are simply not competitive enough to occur without enzymatic support (134). Mechanistically, transducing mechanisms analogous to those worked out for peroxidases of yeasts and *Kinetoplastida* would be straight forward for all 2-Cys Prxs. Upon oxidation of their peroxidatic cysteine C_P to a sulfenic acid, they can either form an internal disulfide bridge with their resolving cysteine C_R, which in the ROOH removing function is then reduced by a redoxin, or an intermolecular disulfide bridge with another protein, thus acting as thiol-modifying agent with regulatory consequences. Examples of Prxs found disulfide-linked to other proteins are abundant (93, 187, 333), but also GPx-type peroxidases devoid of a C_R can modify protein thiols, as has been demonstrated for mammalian GPx4. In shortage of GSH, the oxidized active site selenium (U_P) can selenylate proteins, a process that so far has not been implicated in transcriptional regulation, but is pivotal to the differentiation of spermatids into spermatozoa (121, 318, 428, 493) (Fig. 3).

Prxs can, however, also sense ROOH and transduce the signal in a more indirect way, just by means of their conventional role in removing hydroperoxides. Most of the mammalian Prxs, as well as the nonmammalian 2-Cys Prxs

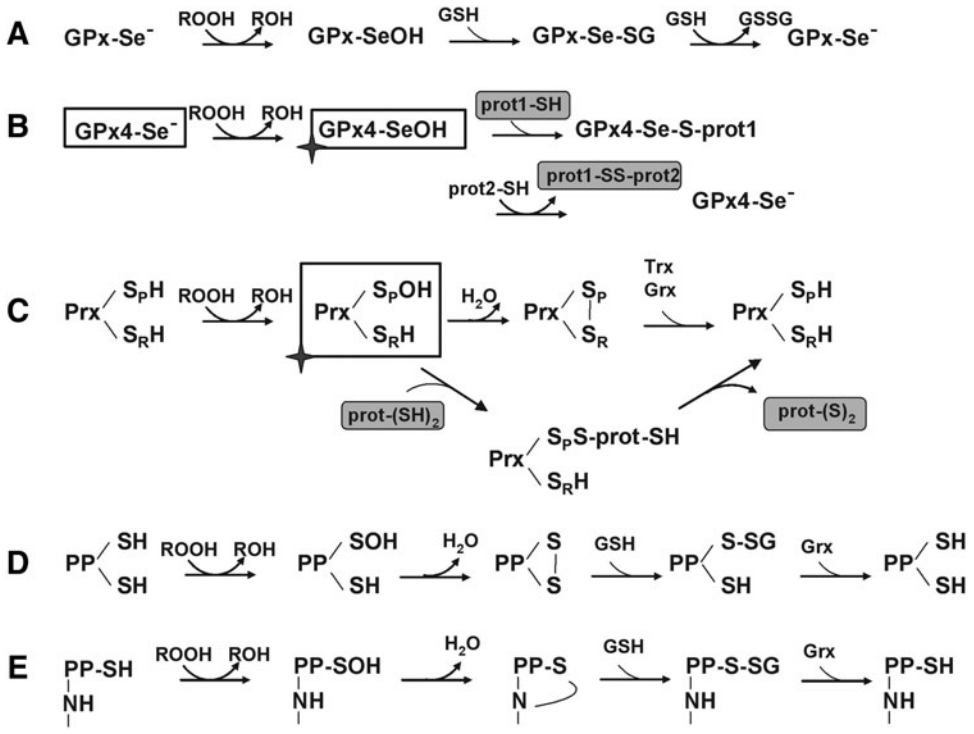

FIG. 3. Possible routes of hydroperoxide sensing by thiols or selenols. (A) General reaction scheme of GPx-catalyzed reduction of hydroperoxides. The selenol is oxidized by ROOH to a selenenic acid, which is step-wise reduced back to selenol by two molecules GSH. A role of selenols in ROOH sensing has so far not been reported. **(B)** The selenenic acid in GPx4 has been shown to react not only with GSH but also with thiol groups of other proteins. The enzyme, thus, transduces a signal to another protein target and acts as a thiol modifying agent. This mechanism is the molecular basis for the unique transformation of GPx4 into a structural protein during sperm maturation (493). Theoretically, the reaction sequence could be used in redox signaling with GPx4 as sensor and Se-S linked heterodimers as transducers. The sensor-reduced GPx4 could be regenerated by GSH (as in **A**) or by SH groups of the same or another protein (lower line). **(C)** 2-Cys peroxiredoxins (Prxs) react with a hydroperoxide at the peroxidatic cysteine (S_P) to the sulfenic acid. Oxidized S_P can then form an intra- or intermolecular S-S bridge with the resolving cysteine (S_R). This bridge is typically reduced by a redoxin (Trx, TXN, or Grx). The oxidized S_P can, however, also react with another protein, thus acting as a thiol modifying agent, as demonstrated in the redox control of yeasts (333). **(D)** Certain cysteines in protein phosphatases (PPs) react with a hydroperoxide to form a sulfenic acid. This either reacts with a second cysteine to form an intramolecular disulfide bridge in analogy to **(C)**. Alternatively **(E)**, the sulfenic acid reacts with a nitrogen in the peptide bond to a sulfenyl amide. Both forms can be reduced by GSH and Grx. The oxidized enzymes are typically inhibited. As discussed in section II.D.3, the known reaction constants for the reaction of ROOH with thiols in phosphatases are low. Therefore, the possibility that the oxidative signal is first sensed by a GPx- or Prx-type peroxidase and then transduced to a PP, as shown in **(B)** or **(C)**, respectively, merits consideration.

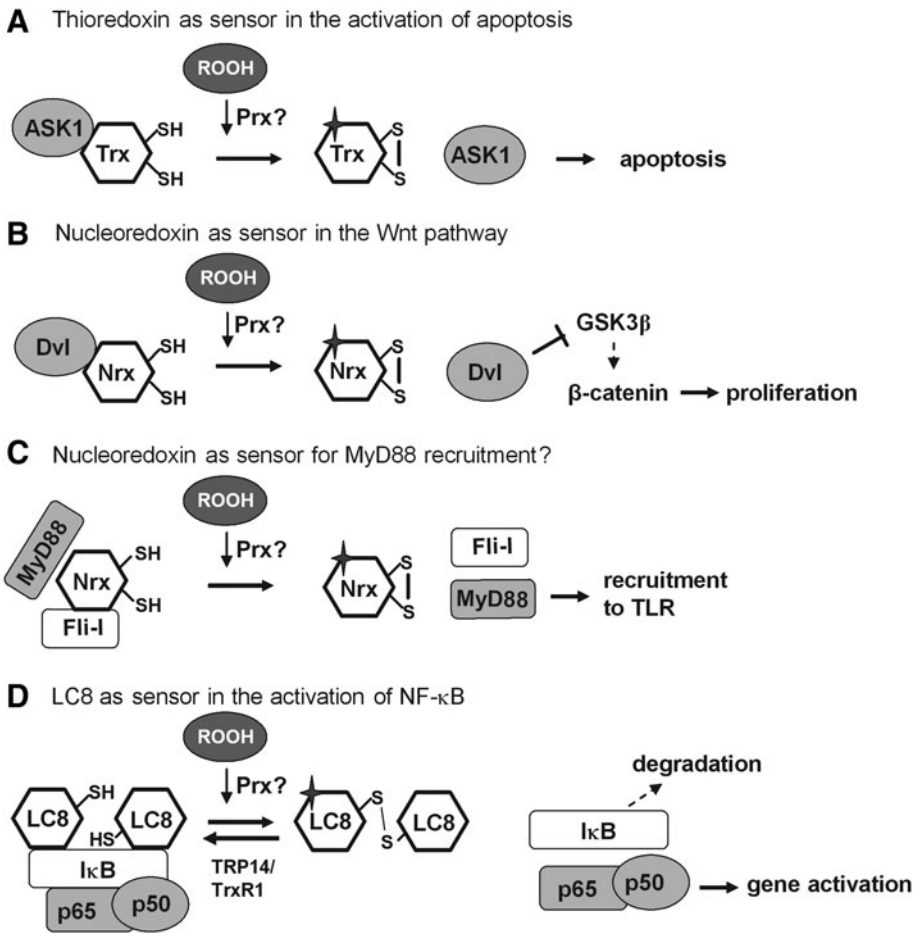

FIG. 4. Redoxins in sensing mechanisms. (A) Reduced Trx has been demonstrated to inhibit apoptosis signaling by binding (and inhibiting) apoptosis signal-regulating kinase-1 (ASK-1). Oxidation of Trx relieves the block (415). Since redoxins are excellent substrates of Prxs, a hypothetical Prx is inserted as real sensor of the system as in **(B–D)**. **(B)** Nucleoredoxin (Nrx) in the reduced state binds to dishevelled (Dvl), a phosphoprotein that transduces wingless and Int 1 (Wnt) signals from the Frizzled receptor. Upon oxidation, Nrx is released and Dvl can associate with the β-catenin degradadion complex, where it inhibits glycogen synthase kinase-3β (GSK3β) and phosphorylation, ubiquitination, and degradation of β-catenin. β-Catenin moves into the nucleus and activates genes required for proliferation (138). **(C)** Reduced Nrx associates with flightless-I (Fli-I) and myeloid differentiation factor 88 (MyD88), thereby preventing recruitment of MyD88 to the TLR4 after lipopolysaccharide stimulation (171). This way unnecessary stimulation is avoided. After removal of Nrx, MyD88 can be recruited, and the TLR mediate its signals. Although in this system oxidative processes have not yet been studied, the requirement for oxidants in TLR stimulation may be based in the need to oxidatively remove Nrx from the Fli-I/MyD88 complex, as in **(B)**. **(D)** Reduced dynein light chain (LC8) binds to IκB and prevents its degradation. Upon oxidation to an intermolecular disulfide LC8 dissociates and gives the way free for IκB degradation and activation of nuclear transcription factor of bone marrow-derived lymphocytes (NF-κB). The oxidation is reversed by the Trx homolog thioredoxin-related protein-14 (TRP14), which is reduced by cytosolic thioredoxin reductase (TrxR1). In this system either LC9 itself is the sensor or is oxidized by an upstream sensing thiol peroxidase (224).

and many nonmammalian GPxs, use Trx or related redoxins as reductants. Under high peroxide flux, they thereby reduce the steady-state of reduced redoxins that are often used as terminators of transcriptional gene activation or interrupters of signaling cascades. Inactivating reduction of OxyR (100) or Yap1 (86, 333) by glutaredoxin (Grx) or Trx, respectively, and reduction of GSH mixed disulfides by Grx (250, 292) may suffice as examples. Further, oxidation of a redoxin may promote signaling due to reversal of inhibition, as has first been shown for the apoptosis signal-regulating kinase-1 (ASK-1), which is blocked by noncovalently bound reduced Trx, but not by the oxidized one (415). Similar functions are ascribed to nucleoredoxin (Nrx) in the wingless and Int 1 (Wnt) pathway (139), and to the dynein light chain 8 (LC8)/Trx-like 14 protein couple in the NF-κB system (227) (see section IV and Fig. 4). Although metabolic regulation by Trx and redoxins, in general, has for long been a widely accepted concept (131), it appears to be still persistently overlooked that oxidation of redoxins *in vivo* does not likely occur spontaneously, but is catalyzed by Prxs, which came to stage much later (399). Although the pK_a of the exposed cysteine in their CxxC motif is quite low, their rate constants for oxidation by ROOH is usually two orders of magnitude smaller than those of the most abundant Prxs that efficiently oxidize redoxins (489, 490). It therefore appears not too risky to consider most, if not all, regulatory events that are attributed to Trx oxidation to be downstream events of hydroperoxide sensing by a thiol peroxidase, a Prx being the more likely candidate in mammals (123, 134).

Finally, a role of Prxs in sensing ROOH may be seen in their ability to switch from a peroxidase to a chaperone, as was first demonstrated in yeast (217, 333) but has also been implicated for mammalian Prx1 (403). The chaperone function of Prxs itself appears to be independent from their peroxidase activity (403), as it prevails in the peroxidatically inactive high-molecular-weight forms having their C_P oxidized to a sulfinic acid. The functional switch to the chaperone, however, is initiated *via* fast ROOH sensing by C_P. Once transformed into a chaperone, the Prx may affect many regulatory pathways, enhanced cytokine expression through Toll-like receptor-4 (TLR4)-mediated NF-κB activation being a revealing example (403).

2. Transcription factors as sensors for hydroperoxides. Certainly, also proteins that are not annotated as per-

oxidases may sense ROOH through thiol oxidation, if their cysteine residues are activated by neighboring basic residues or other mechanisms. A well-documented example is the bacterial transcription factor OxyR (100). Its Cys199 may be considered kind of C_P, as it is directly attacked by H_2O_2 with a bimolecular rate constant of $\sim 10^5\, M^{-1}\, s^{-1}$ (11), which comes close to those of peroxidases. The initial oxidation of Cys199, like in the thiol peroxidases, is followed by a disulfide formation that facilitates oxidation of the remaining cysteines to the fully oxidized active transcription factor, whereas the inactivation is achieved by Grx-catalyzed reduction by GSH (Fig. 1).

Similarly, direct sensing of organic hydroperoxides has been implicated for organic hydroperoxide resistance repressor (OhrR), a repressor of the *Ohr* gene found in many bacteria (100). Although OhrR is phylogenetically unrelated to any of the peroxidase families, it shares the mechanism with atypical 2-cysteine Prxs (281): it is oxidized by ROOH (not by H_2O_2) at its C_P, which is the arginine-coordinated Cys60 (*Pseudomonas aeroginosa* sequence). The oxidation of Cys60 to a sulfenic acid, which suffices for de-repression, leads to formation of an intramolecular disulfide bond with Cys124, which can be reduced by dithiothreitol, the natural reductant being unknown.

Any direct interaction of a hydroperoxide or any other oxidant signal with a mammalian transcription factor has so far not been convincingly demonstrated.

3. PPs and PKs as potential sensors. Phosphorylation and de-phosphorylation are key events in the regulation of enzyme activity in cellular signaling and certainly also dominate mammalian transcription factor activation. In most of the cases, the phosphorylated kinases and phosphatases represent the active modifications, whereas de-phosphorylation leads to inactivation of these enzymes. Exceptions to the rule are, for instance, glycogen synthase (GS) and glycogen synthase kinase-3β (GSK3β), which are active in the de-phosphorylated state. Protein kinases and, more importantly, PPs are widely assumed to sense H_2O_2, other oxidants, or S-alkylants, and it may be guessed that most, if not all, phosphorylation/de-phosphorylation balances are redox-controlled *via* cysteine modification (Fig. 3D, E).

In receptor protein kinases, a highly conserved MxxCW motif is considered as the general switch that, upon cysteine oxidation, starts the tyrosine phosphorylation required for catalytic activity (339). Oxidative activation is also observed in the various forms of protein kinase C (PKC) that are characterized by Zn-fingers in their regulatory domain, in which two Zn^{++} ions are tetrahedrically coordinated with six conserved cysteine and two histidine residues (77, 194, 258). The Zn-finger is assumed to work as kind of hinge wherein the Zn^{++} functions as a linchpin. Oxidation of the Zn-coordinated cysteines was shown to cause Zn^{++} release (259) and is assumed to favor the active kinase conformation, phosphorylation, binding to Ca^{++}, and phosphatidylserine and, thus, membrane recruitment of these enzymes (258, 309). Similarly, Zn^{++} release associated with activation of kinase activity due to oxidation of Zn-coordinated cysteines was reported for c-RAF (190). Since PKCs *inter alia* mediate oxidative responses such as the oxidative burst of phagocytes (see section IV.D.1), the inhibitory function of Zn^{++} in these enzymes may well explain the still mysterious antioxidant function of the ion, which by itself is redox-inert under physiological condition (49, 309, 382).

In contrast, PPs are more or less readily inhibited by cysteine oxidation/modification. PPs are specific for protein tyrosine phosphates, serine (Ser)/threonine (Thr) phosphates, or for both, and, accordingly, are classified into tyrosine PPs (protein tyrosine phosphatase [PTPs]), Ser/Thr PPs (PSPs), and dual specificity PPs (DSPs). Their mechanism of action differs and so does their sensitivity to oxidants.

PTPs are generally susceptible to oxidative inactivation. They are characterized by an HCx_5R motif that comprises the PTP loop that binds the phosphate groups of phosphotyrosines [(472); reviewed in ref. (67)]. The cysteine in this motif, due to its low pK_a value, is nucleophilic, which is a prerequisite for both substrate de-phosphorylation and oxidative inactivation. More than 100 PTPs of the human genome (486) also include the DSPs that share with the typical PTPs the characteristic HCx_5R motif and may, therefore, be suspected to be equally prone to oxidative inactivation. The DSP family not only de-phosphorylates tyrosine, Ser, and Thr residues, but also further comprises phosphatases acting on nonprotein substrates, such as phosphoinositol phospholipids (PIPs). Examples for the latter specificity are the Src homology-2 (SH2)-domain-containing inositide phosphatases (SHIPs) (149), which de-phosphorylate the membrane-bound PI3K-generated key signaling lipid $PI(3,4,5)P_3$ at position 5 to $PI(3,4)P_2$, and the phosphatase and tensin homologue (PTEN), which dephosphorylates the 3 position of both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (304). Sharing the active site motif with the PTP/DSP family, PTEN is a typical example of a redox-sensitive phosphatase (280).

A particularly reactive cysteine being unidentified in PSPs, this type of phosphatase can not *a priori* be rated as redox sensitive. However, also Ser/Thr phosphatases such as PP2A have been shown to undergo reversible oxidation. PP2A contains 10 cysteine residues in the catalytic subunit including a vicinal pair at positions 266–269, which reminds of a redoxin motif. This CxxC motif proved to be sensitive to oxidation *in vitro*, and its oxidation resulted in a decreased activity (130). In addition, PSPs can have metal ions in their active center, which are essential for their enzymatic function (449). Thus, apart from redox modification of cysteine residues, also an oxidation of the metal clusters appears conceivable.

Mechanistically, the oxidative inactivation of phosphatases *via* cysteine oxidation involves reactions known from GPx or Prx mechanisms (127). A pivotal cysteine appears to be oxidized to a sulfenic acid (Eq. 1). This unstable oxidation form typically forms a disulfide with another cysteine residue (Eq. 3), which reminds of the analogous reaction of C_P and C_R in Prx or GPx catalysis (Fig. 3). In oxidized PTEN a disulfide bridge between the catalytic Cys124 and the neighboring Cys71 was detected (275, 418). Similarly, in the DSP Cdc25 phosphatase B, a second cysteine resides in the active site, which in the oxidized form is disulfide-linked to the nucleophilic cysteine of the signature motif (51). In contrast, in PTP1B, the cysteine in the active site motif [I/V]HCxxGxxR[S/T] forms a cyclic sulfenyl-amide (419, 495) in analogy to the redox cycle of the GPx mimic ebselen (423) (Fig. 3E). As in the catalytic cycles of Prxs (378) and GPxs (487), the physiological meaning of conserving the labile oxidation state of the sulfenic acid in a more stable form is seen in the prevention of sulfur oxidation to the sulfinic or sulfonic forms that would be hard to reverse.

As in the enzymatic cycles, the oxidized phosphatases are readily reactivated by physiological thiol compounds such as GSH, Trx, Grx, or other redoxins.

However, the almost generally agreed assumption that protein kinases and phosphatases may sense oxidants *via* cysteine oxidation is overshadowed by lack of any confirming kinetic data. None has been reported for any kinase and the few available rate constants for a direct oxidation of the critical thiols in PPs by H_2O_2 do not exceed 50 M^{-1} s^{-1} (31, 87, 459) and, thus, are by far too small to corroborate any physiological relevance of the process. For three redox-sensitive PTPs (PTP1, leukocyte-common antigen-related (phosphatase) [LAR], and Vaccinia H1-related (phosphatase) [VHR]) reaction rates for oxidative inactivation were insufficient (10–20 M^{-1} s^{-1}); for three Ser/Thr phosphatases (PP2Cα, calcineurin, and lambda phosphatase) they were evidently too slow to be quantified (87). For the PTP-type Cdc25 phosphatases the rate was a bit more promising, being 15-fold higher than for PTP1B (459). In the latter case, the authors stress that the reaction of H_2O_2 with Cdc25 is 400 times faster than with GSH, which though is a poor argument, since the spontaneous reaction of GSH with H_2O_2 is physiologically irrelevant. For the relevant GPx reaction the rate constant is around 10^8 M^{-1} s^{-1}.

According to Forman *et al.* (134), the available kinetic data predict that even the nonenzymatic reaction of H_2O_2 with GSH would completely compete out the oxidation of the active site cysteine of PPs. However, since oxidation of PTP1B and other PPs, as evidenced by glutathionylation (Eqs. 1 and 2), evidently occurs under *in vivo* conditions (250, 405), oxidant sensing by PPs must be more complex. It could be envisaged that any of the numerous thiol peroxidases first senses ROOH and then, in analogy to the yeast GPx- and Prx-type peroxidases, forms a disulfide bridge with the target phosphatase that is cleaved by GSH, leaving the glutathionyl residue at the target protein. Grx has been implicated in protein glutathionylation but appears to rather de-glutathionylate (229). Alternatively, the rate constants, which were obtained *in vitro* with isolated enzymes, could be grossly misleading, as they might *in vivo* be substantially improved by protein/protein interaction within regulatory complexes. Human Prx6 may be taken as an example for a dramatic change in thiol reactivity due to association with another protein: it displays a significant GPx activity only when associated with a GSH-loaded GSTπ (439). Before the discrepancies between *in vitro* data and *in vivo* observations have not been convincingly explained, we should cautiously address redox-sensitive PPs and kinases as putative ROOH sensors.

4. Redox sensing by cytosolic inhibitory complexes of transcription factors. In eukaryotes, transcription factors are commonly sequestered in the cytosol in form of multicomponent complexes from which they have to be released for gene activation in the nucleus. The activation of the transcription factor requires modification of one or more components of the complex to enable release of the transcription factor and its nuclear import. Almost regularly, a redox modification of a component is followed by ubiquitination and proteasomal degradation of the same or another component (183). A typical example of this reaction scheme is the stress-signaling Nrf2/Keap1 system, wherein Nrf2 is the transcription factor and Keap1 the complex partner that prevents Nrf2 activation (see section III for details). In this particular case, the inhibitory protein Keap1 not only prevents transcription factor activity by keeping it in the cytosol, but also by targeting it for the proteasomal degradation pathway (254, 531).

Keap1, which again is a Zn-finger protein, is considered to be the redox sensor of the system. Its reactive cysteines may be oxidized to sulfenic acids, form disulfides, or be alkylated by electrophiles (Michael acceptors) such as HNE (201), other α,β-unsaturated aldehydes, or ketones (282, 410), a realm of phytochemicals (469) or other electrophilic xenobiotics (104). The consequence of cysteine modification in Keap1 is a dramatic conformational change resulting in a destabilization of the complex and prevention of Nrf2 degradation. As generally observed in Zn-finger proteins (see section II.D.3), oxidation of Zn-coordinated cysteines leads to release of Zinc and consecutive unfolding of the finger structure. The structural change of Keap1 then prevents ubiquitination and proteasomal degradation (see section III.C.2).

The ubiquitination process proceeds *via* the conventional scheme: a coordinated cascade of 3 enzymes, the ubiquitin-activating enzymes E1, the ubiquitin-conjugating enzymes E2, and the ubiquitin ligases E3 (Fig. 5). Of the known E3 ligases, the really interesting new gene (RING) family is the largest. The RING motif in these enzymes interacts with E2 and facilitates the transfer of ubiquitin from E2 to a lysine in the target protein (370). One subtype of the RING family is the Cullin family. Cullin-RING E3 ligases utilize 1–7 Cullin scaffolds to assemble several substrate-specific adaptors that recognize and position the target, here Nrf2, in the cullin-E3 complex for proper ubiquitination. The unmodified Keap1 serves as an adapter for the RING box protein (Rbx1)-bound Cullin-3 (Cul3)-based E3 ligase, which targets the Nrf2 within the Keap1/Nrf2 complex (80, 140, 141, 255, 372, 532). Cysteine modification in Keap1 disrupts the presentation of Nrf2 for ubiquitination.

The ubiquitination machinery, here briefly introduced as a downstream event of an oxidative Keap1 modification, has also been rated as redox sensitive. Both the ubiquitin-activating enzyme E1 and the ubiqutin-conjugating E2 were shown to be reversibly inactivated by glutathionylation due to treatment with H_2O_2 or diamide (212, 355). The ubiquitin ligases, of which about 600 different ones were identified in man, are mostly characterized by a RING finger domain, in which two Zn^{++} ions are coordinated with seven cysteine and one histidine residues. Clearly, this structural element, like the Zn-finger of PKCs and Keap1, is suggestive of a site for redox regulation, an option that, however, has so far been left unexplored (88). Similarly, the redox regulation of SUMOylation, the analogous protein modification by small ubiquitin-like modifiers (SUMO), appears not to have been investigated to any significant extend (511).

Ubiquitination followed by degradation is also used for deinhibition of transcription factors in other redox-sensitive cytosolic complexes, but the sensing step and the ubiquitinated entities as well as the regulatory principles of the systems differ substantially (Fig. 6). In the hypoxia response system it is also the transcription factor itself that is permanently ubiquitinated and degraded under normal conditions, that is, under normoxia. The redox sensor, however, is not an inhibitory protein such as Keap1 (Fig. 6A), but an enzyme: the proline hydroxylase that hydroxylates the transcription factor

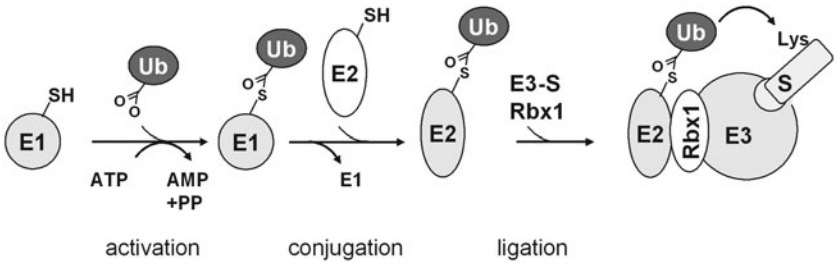

FIG. 5. Simplified scheme of ubiquitination of substrates in mammalian cells. In a first step ubiquitin is activated at the C-terminal glycine by the ATP-dependent formation of a thioester at a cysteine of the E1 enzyme. E1 transfers ubiquitin to a cysteine of the ubiquitin-conjugating enzyme E2 and is released from the complex. The ubiquitin-loaded E2 then forms a complex with the E3 enzyme to which the respective substrate (S) is bound. The really interesting new gene (RING) box protein-1 (Rbx1) targets the substrate for ubiquitination. Ubiquitin can then be passed either directly to a lysine of the RING-bearing E3-bound substrate (Rbx1) or, in case of the homologous to E6AP C terminus (HECT)-domain bearing E3, to another cysteine on E3 and from there to the substrate lysine (186).

HIF-1α and targets it for ubiquitination (Fig. 6B). Similarly, the transcription factor β-catenin in the Wnt pathway is steadily ubiquitinated and degraded under resting conditions. In this context Nrx is assumed to be the redox sensor; in the reduced state it blocks the Wnt pathway by binding to the upstream adapter protein disheveled (Dvl), while allowing nuclear import of β-catenin upon oxidation of its CxxC motif (138, 139) (Fig. 6C). In the canonical NF-κB system, the inhibitory NF-κB-binding protein IκB has to be ubiquitinated and degraded to allow nuclear import of p50/p65. IκB is targeted for ubiquitinating by phosphorylation through IκB kinase (IKK) (Fig. 6D). The redox sensor of the system might be the LC8, which in its reduced states, is bound to IκB and protects IκB from attack by IKK, but allows IκB phosphorylation and degradation upon oxidative dimerization *via* intersubunit disulfide formation (Fig. 4D and section IV.D.3) (227). For lipopolysaccharide (LPS)-induced NF-κB activation *via* TLR 4, again Nrx has been implicated as negatively regulating sensor by binding to Fli-1 (flightless), thereby preventing recruitment of the essential adaptor myeloid differentiation factor 88 (MyD88) to the receptor (171).

It is needless to state that kinetic data for component interactions in these complex systems do not exist at all and will hard to be obtained. The mentioned redox-sensitive proteins, which, in principle, can directly be oxidized by any ROOH, might therefore not be the real sensors, but, instead, be oxidized by an upstream thiol peroxidase sensing the oxidant.

5. Sensing by selenocysteine-containing proteins? A sensor's competence to sense ROOH or an alkylant selectively would certainly be enhanced if its sensing sulfur were replaced by the super-sulfur selenium (120). Many selenoproteins display signatures of redox proteins (146) and most of those with an established enzymatic function are indeed oxidoreductases with selenocysteine (Sec) as pivotal catalytic entity (122). As a rule, these seleno-enzymes are substantially faster than their homologs working with sulfur catalysis (33). Simply by chemical reasoning, therefore, selenoproteins

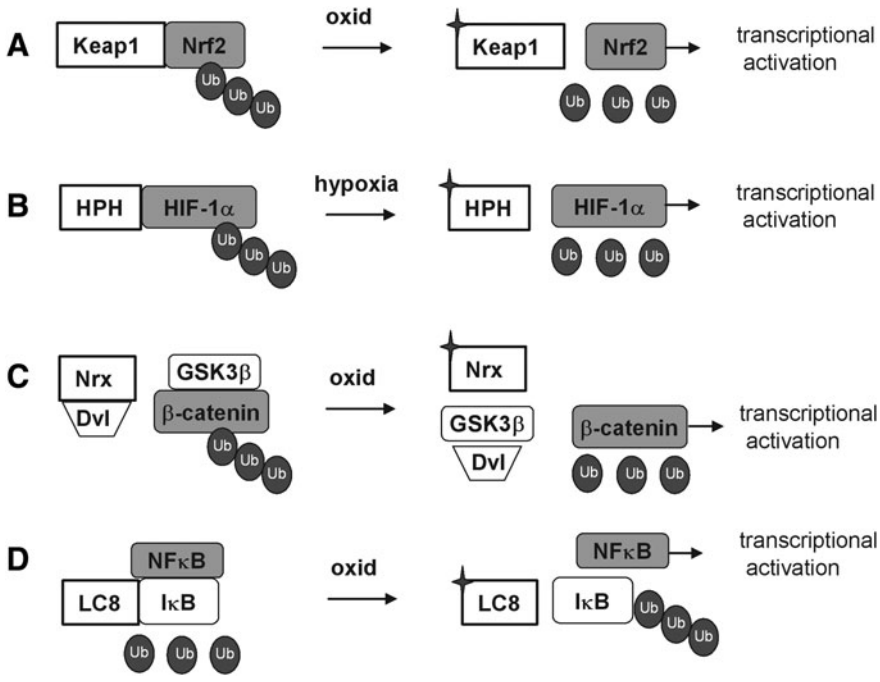

FIG. 6. Distinct roles of redox-dependent ubiquitination in the regulation of transcription factor activity. (A) In the Nrf2/kelch-like ECH-associated protein-1 (Keap1) system, oxidant sensing by Keap1 terminates the ubiquintination and degradation of Nrf2 and allows its nuclear translocation and transcriptional activation of target genes. **(B)** In the hypoxia-inducible factor 1 (HIF) system, under normoxic conditions HIF-1α is hydroxylated by HIF prolyl hydroxylase (HPH), ubiquitinated, and degraded. In hypoxia HPH is inactive, ubiquitination is prevented, and HIF-1α can translocate into the nucleus and activate gene expression. **(C)** In the Wnt/β-catenin pathway, GSK3β phosphorylates β-catenin, thereby facilitating its ubiquitination and degradation and preventing β-catenin-mediated gene expression. Dvl is captured by Nrx. Upon oxidation of Nrx, Dvl is released to inhibit GSK3β activity. β-Catenin is no longer phosphorylated and is translocated into the nucleus. **(D)** In the NF-κB system, LC8 is associated to IκB, thereby preventing its degradation and release of NF-κB. Nuclear translocation of NF-κB can take place after oxidative modification and release of LC8 and degradation of the inhibitor IκB.

would have an optimum chance to beat thiol-based redox sensors in terms of speed and specificity.

Circumstantial evidence has for long suggested that selenoproteins are relevant to transcriptional activation. In selenium-deficient animals a huge number of nonselenoproteins, now overwhelmingly known as Nrf2 targets, were found elevated since the late seventies (78, 79, 270, 395–397). The unexpected phenomenon could not be convincingly explained by GPx1 deficiency alone (396). However, at least one of the selenoproteins had to be involved in Nrf2 activation, since a complete loss of all selenoproteins by an organ-specific removal of the gene encoding selenocysteine tRNA (Trsp) (56) resulted in the induction of GST isoforms in liver, of GSTP1 and NADPH quinone oxidoreductase 1 (NQO1) in liver and macrophages (471) and of heme oxygenase-1 (HO-1) in liver (446). Also a moderate selenium deficiency in which only selenoprotein W, GPx1, and selenoprotein H and M were markedly decreased (249) led to an increase in NQO1, GSTs, sulfotransferases, and UDP-glucuronyl transferases (UGT) as well as HO-1, Prx1, sulfiredoxin-1 (Srx1), and γ-glutamylcysteine synthase (335). A firm link between selenium deficiency and Nrf2 activation was finally established by Burk *et al.* by demonstrating a strong increase in electrophile-responsive element (EpRE)-driven reporter gene activity in livers of selenium-deficient wild-type but not in $Nrf2^{-/-}$ mice (52).

Certainly, these findings do not strongly corroborate a role of selenoproteins as sensors, as they may be equally explained as adaptive response to impaired hydroperoxide metabolism. The hypothesis of a direct sensing function has not yet been established for any of the selenoproteins, but remains attractive from a chemical point of view. H_2O_2-dependent selenylation of protein thiols by GPx4 (see section II.D.1) is mechanistically equivalent to H_2O_2 sensing by thiol peroxidases and reveals that selenoproteins can in principle act as combined redox sensors and transducers (121, 123). After all, the mammalian Trx reductases, which all are selenoproteins, may be indirectly involved in ROOH sensing by regenerating Trxs as key redox regulators and reducing substrates of Prxs. These aspects certainly deserve more interest when searching for the biological roles for the more than 25 distinct mammalian selenoproteins (167).

6. Oxygen sensing. [Fe-S] clusters in proteins have for long been known to be susceptible to oxidation (314) and may therefore be considered *bona fide* sensors for oxidants. In bacteria [Fe-S]-cluster proteins have not only been reported to sense $O_2^{\bullet -}$ (see section II.B), but also molecular oxygen. The transcription factor FNR (for fumarate nitrate reduction), which is responsible for the adaptation to oxygen restriction in bacteria, directly reacts with O_2, resulting in loss of transcriptional activity. Its $[4Fe\text{-}4S]^{2+}$ cluster is thereby transformed into a $[2Fe\text{-}2S]^{2+}$ cluster (247). Mammalian [Fe-S] proteins also respond to oxidative stress, the moonlighting of aconitase to the transcription factor iron response element-binding protein 1 (IRP1) being a prominent example (74, 334).

Beyond, any of the numerous oxidoreductases using O_2 as acceptor (all EC numbers 1.1.3–1.10.3 having three in the third position as well as the oxygenase group EC 1.13) may be considered to be oxygen sensors, if their respective products have regulatory functions. Of particular importance in our context are the NADPH oxidases (NOX) that produce $O_2^{\bullet -}$ (see section IV.D.1), and the prolylhydroxylases that hydroxylate proline residues in the HIF (383, 443). In the hypoxia response, O_2 serves as substrate of prolylhydroxylases that hydroxylate specific proline residues in the α-subunits of HIF depending on O_2 pressure, thereby priming HIF for ubiquitination and proteasomal degradation (442).

E. Adjusting sensitivity by competing systems

As mentioned above, redox signaling triggered or modulated by hydroperoxides has to compete with peroxidases that reduce ROOH. In fact, it appears enigmatic how thiol oxidation in regulatory proteins can at all occur in the presence of peroxidases. The most commonly heard explanation is that all redox regulations must be events restricted to cellular microenvironments, which are hard to describe in kinetic or thermodynamic terms, an argument that is also used to explain regulatory phenomena in general (432). However, genetic tools have meanwhile revealed that redox regulation is also linked to, and controlled by, the overall cellular hydroperoxide metabolism. As a rule, suppression of Prx expression or Prx inhibition facilitates transduction through phosphorylation cascades, whereas overexpression of Prx dampens it, as compiled by Sue Goo Rhee and others (161, 198, 218, 236, 400). Similarly, overexpression of GPx1 in tissue culture inhibits tumor necrosis factor α (TNFα)-induced NF-κB activation by affecting the phosphorylation state of IκB (261). Also, overexpression of GPx4 abrogated lipid hydroperoxide- and interleukin-1 (IL-1)-induced NF-κB activation (45, 46). Most remarkably, a mouse systemically overexpressing GPx1 became insulin-resistant (277). Although these observations are open to different interpretations (218), they collectively suggest that peroxidases efficiently compete for ROOH that is needed in signaling cascades, thereby determining their sensitivity or setting the threshold at which they respond. An extreme version of this view is the flood gate theory, which assumes that hydroperoxide scavenging by Prxs has to be overcome by reversible oxidative inactivation of the latter to save ROOH for signaling (516). However, the flood gate can be opened not only by over-oxidation of Prxs but also by phosphorylation of the latter: Prx1 activity is inhibited by phosphorylation at Thr90 by cyclin-dependent kinases (CDKs), saving H_2O_2 to signal cell cycle progression (60) or at Tyr194 by a Src kinase within a growth factor receptor complex, thus enhancing platelet-derived growth factor (PDGF)- or epidermal growth factor (EGF)-triggered signaling due to local increase of H_2O_2 (514).

When being alkylated, the thiols of regulatory proteins also have to face competition. In mammals, seven classes of GSTs have been identified that efficiently compete with thiol groups in regulatory proteins for endogenous stress signals such as HNE or structurally related xenobiotics (175). Although the alkylation-susceptible SH groups in some regulatory proteins have been identified (256), the kinetics of interaction with alkylants is poorly investigated. The rate constants for the reaction of GSH with various Michael acceptors range between 1 and $350\,M^{-1}\,s^{-1}$ (410) and may be higher by a factor up to 100 for a fully dissociated thiol. Nevertheless, the GSTs, which *in toto* can make up 5% the cellular protein, represent a substantial hurdle, which has to be overcome before S-alkylation of regulatory proteins can come into play. Accordingly, damage signaling *via* S-alkylation is dampened by GSTs and facilitated by localized or general GSH depletion (175), as it

may happen in severe oxidative stress. The importance of GSTs in counteracting stress signaling through S-alkylation has been corroborated by inverse genetics. As compiled by Hayes *et al.* (175), knockout of several of the GSTs results in exaggerated response to xenobiotics and oxidative stress. Inversely, a conditioned knockout of the selenocysteyl-t-RNA gene, which disrupts the entire selenium-dependent antioxidant defense, leads to a compensatory increase of GST alpha, mu, and theta, which is likely mediated by activation of the Nrf2/Keap1 system due to increased H_2O_2, ROOH, and/or alkylating damage signals (see section II.D.5).

F. The problems beyond signals and sensors

Trying to present generally applicable concepts as to how proteins with regulatory potential may work in a complex eukaryotic system is an almost hopeless adventure. There is not any rule emerging without exceptions. As already evident from previous sections, peroxidases may act as sensors, transducers, or competitive modulators. Reduced Trx may act as inhibitor of signaling cascades by noncovalent binding as in the ASK-1 example (415), more commonly as terminator by reversing transcription factor binding to its target DNA as in the Orp1/Yap1 system (86) or as essential enhancer of transcription factor activity as in the NF-κB system. Grx is believed to play a key role in dampening phosphorylation by de-glutathionylation of counteracting phosphatases, but depending on the glutathionylated protein and the context may adapt an opposite role. Ubiquitination and proteasomal degradation facilitate import of transcription factors, if they attack their cytosolic inhibitors, but inhibit translocation, if the factor itself is degraded. We therefore refer to the following sections, which elaborate on two prototypic mammalian systems of transcription with focus on putative or established roles of redox-responsive elements.

III. Orchestrating the Adaptive Response by the Keap1/Nrf2 System

A. Discovery

Nrf2, the nuclear factor erythroid 2 (NF-E2)-related transcription factor, is a member of the Cap 'n' Collar (CNC) family of basic leucine zipper (bZIP) proteins and was first described in 1994 by Moi *et al.* as stimulator of β-globin gene expression (329). It was recognized to transduce gene activation of phase II enzymes by binding to the α, β-naphtoflavone-responsive element (210), previously described by Rushmore and Picket and called antioxidant-responsive element (ARE), because it was also activated by phenolic antioxidants such as t-butyl hydroquinone (411). A similar set of antioxidants were shown to also induce γ-glutamyl-cysteine synthetase (40, 294) and the pertinent responsive element was termed EpRE for electrophile-responsive element (330), which is identical with ARE. After it had become clear that the name-giving antioxidants *in vivo* acted as oxidants due to redox cycling or, as their oxidized counterparts, as electrophiles, the misnomer ARE should better be replaced by EpRE, but still persists in the literature.

B. The physiological context of Nrf2-dependent gene activation

Nrf2, *via* activation of EpRE (ARE), regulates the expression of proteins that collectively favor cell survival. They comprise enzymes that directly or indirectly exert antioxidant functions (5, 21, 111, 202, 264, 373, 491), molecular chaperons (265), and proteins that enhance GSH synthesis and regeneration (164, 174, 210, 276, 330, 336), phase 2 detoxication, drug metabolism (169, 210, 326, 392, 424, 479), recognition, repair, and removal of damaged proteins (191, 264, 265, 393), and nucleotide excision repair (8), further proteins that regulate the expression of other transcription factors, growth factors, and receptors (113, 163), and inhibit cytokine-mediated inflammation (6, 209, 245, 385, 503) and autophagy (394). With its broad range of target genes, Nrf2 is certainly one of the most important transcription factors that protect the organism against exogenous stressors, be they poisonous food ingredients, physical damage, or infection. In line with the appreciation of Nrf2 as dominant inducer of the adaptive response, the realm of its inducers comprises endogenous signaling molecules associated with oxidative stress such as H_2O_2, ROOH, $ONOO^-$, oxo-aldehydes, and ketones, as well as exogenous ones such as isothiocyanates, thiocarbamates, trivalent arsenicals, quinones, dithiolethiones, vicinal dimercaptanes, certain statins, and heavy metals (96, 97, 160, 460, 474). They are obviously recognized by the organism to be potentially hazardous and to be eliminated *via* enforced Nrf2-mediated gene activation. The only common denominator of these chemically heterogeneous compounds is their ability to modify cysteine residues. With these characteristics, the Nrf2 system builds the major molecular basis for an enigmatic toxicological phenomenon known as hormesis: it describes that subtoxic dosages of a poison may protect against toxic ones or even against other challenges (180, 305, 317). Thus, with a delay of some centuries the therapeutic wisdom of the ancient Philippus Theophrastus Bombastus von Hohenheim (1493–1541), who claimed that *solum dosis facit venenum* (the dose only makes the poison), has been corroborated by a scientific basis.

The protective role of Nrf2 is also demonstrated by inverse genetics. $Nrf2^{-/-}$ mice are vital and do not display any signs of an increased basal oxidation state (58). Only when challenged they have an impaired ability to respond (454). The Nrf2 system, thus, is an emergency device that comes into play upon severe challenge only. For instance, mice lacking Nrf2, when treated with dextran sulfate sodium, developed a more severe intestinal inflammation with increased aberrant crypts than controls (246, 360), which suggests a function of Nrf2 in prevention of inflammation and inflammation-mediated carcinogenesis. Activation of Nrf2 is therefore considered to contribute to phenomena such as development of tolerance against all kind of inflammatory stimuli and, accordingly, potent activators such as sulforaphane, by acting as kind of vaccine, hold great promise in chemoprevention.

However, also this coin has two sites, since Nrf2 activation may not be beneficial under all circumstances. Upregulation of enzymes metabolizing xenobiotics such as GSTs will not always improve detoxification, since these enzymes may also increase xenobiotics' toxicity, as reviewed by Hayes *et al.* (175). Also, if the xenobiotic is a drug, Nrf2 activation may negatively affect its bioavailability. Moreover, not only normal but also tumor cells may benefit from the protective function of Nrf2. In fact, tumor cells usually show a constitutively high Nrf2 activity; hence, given the physiological result thereof, that is, inhibition of autophagy and apoptosis and increase of proteasomal degradation of damaged proteins, tumors have acquired a superior survival chance.

Accordingly, activation of Nrf2 by chemo-preventive dietary compounds can reasonably be expected to inhibit tumor initiation but is likely detrimental, once the tumor has been established (10, 268). In support of this view, a selenoprotein that is an Nrf2 target, GPx2 (21), impairs inflammation-driven intestinal carcinogenesis, as convincingly demonstrated in knockout mice (109), but, as xenografts, wild-type tumor cells grew up substantially faster with than those without GPx2 (22). Finally, a persistent overactivation of the Nrf2 system, as is observed in autophagy-deficient (*Atg7* knockout) mice, causes, or contributes to, severe liver damage, as evidenced by abrogation of the liver pathology in Atg7/Nrf2 double knockout mice (257).

C. Mechanistic aspects of Nrf2 signaling

1. Basics of Nrf2 activation. As already mentioned under section II.D.4, Nrf2 belongs to the type of transcription factors that are kept in cytosolic complexes under resting conditions and is there permanently subjected to proteasomal degradation (344). Within the cytosolic complex Keap1 serves as *bona fide* sensor for oxidants or electrophiles. Modification and conformational change of Keap1 allows translocation of Nrf2 to the nucleus for transduction. The molecular details of Nrf2 and the pertinent signaling pathway have been summarized in many reviews (42, 104, 287, 348, 469) and will here be briefly recalled and amended as far as appropriate.

2. Keap1 as primary redox sensor of Nrf2 signaling. Keap1, sometimes also called INrf2 for inhibitor of Nrf2 (348), contains three characteristic domains, the broad complex/tramtrac/bric-a-brac (BTB) domain, the intervening region (IVR), and the kelch domain, also known as double glycine repeat (DGR) domain (3). The BTB domain binds Cul3 (see section II.D.4) (141) and is required for homodimerization (538), whereas the IVR contains cysteine residues that are in part Zn-coordinated (98) and are considered relevant to regulation (see below) and a nuclear export signal (NES) (498). The kelch/DGR domain is required for the interaction of Keap1 with the actin cytoskeleton (234, 235) and/or myosin VIIa (499), which immobilizes Keap1 in the cytoplasm. The kelch/DGR domain is also essential for binding of Nrf2, which interacts with Keap1 with its amino-terminal Nrf2-ECH homology-2 (Neh2) domain (208).

The structure of the kelch/DGR domain of Keap1 has recently been resolved (297, 362), which sheds new light on the Keap1/Nrf2 interaction (484) (Fig. 7). According to the hinge and latch model there are two distinct binding motifs in the Neh2 domain of Nrf2, each binding one molecule of a Keap1 homodimer (two-sites binding) (325, 484). The high affinity binding ETGE motif functions as a hinge to pin down Nrf2 to Keap1. The low affinity DLG motif functions as a latch. The link between the hinge and the latch motif is the lysine-rich central α-helix of Nrf2. In this helix six of seven lysine residues point to the same site (484). The Neh2 domain is locked in a position suitable for ubiquitination of the Lys residues (325, 485) which, hence, act as ubiquitin acceptors (532). The hinge and latch model (484), thus, complies with a substrate-presentation mechanism: presentation of Nrf2 for ubiquitination and subsequent proteasomal degradation.

The hinge and latch model can also explain how thiol-modification disturbs the presentation of Nrf2 for ubiquitination. Murine and rat Keap1 contain 25 cysteine residues, whereas human Keap1 has 27 (97, 98). Nine of these are predicted to be reactive due to their location adjacent to basic amino acids. The cysteines 257, 273, 288, and 297 lie in the IVR and, by MS analysis of tryptic peptides, were demonstrated to be most sensitive to alkylation by dexamethason mesylate

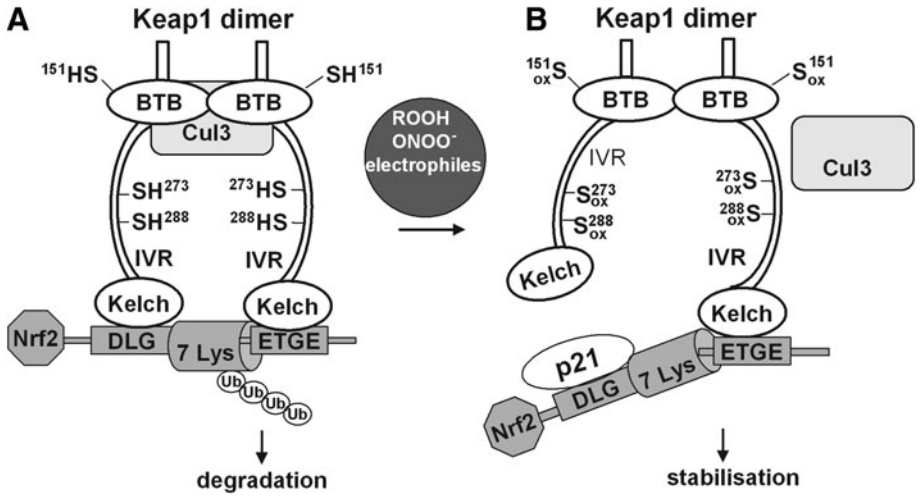

FIG. 7. Model for redox-regulated Nrf2 activation based on conformational change of Keap1. In the resting state **(A)**, Keap1 forms a homodimer *via* the broad complex/tramtrac/bric-a-brac (BTB) domains and associates to Nrf2 with its kelch domains. The Nrf2-ECH homology-2 (Neh2) domain of Nrf2 contains two Keap1 binding motifs with different affinity to the kelch domains, with ETGE representing the amino acid sequence with the higher affinity and DLG, the sequence with a much lower affinity. This two-site binding locks the central α-helix of the Neh2 domain of Nrf2 with is seven lysine residues (7 Lys) into a position suitable for ubiquitination. The intervening regions (IVR) contain the most reactive cysteine residues (Cys273 and 288) and build the adapter region for the Cullin-3 (Cul3)-based E3 ligase system. Nrf2 is polyubiquitinated and degraded by the proteasome. Under stressed conditions **(B)**, oxidative/electrophilic modification of Cys272 and 288 induces a conformational change of Keap1 resulting in the dissociation from the weaker binding DLG motif in Nrf2, whereas binding to the ETGE motif remains [hinge and latch model as proposed by (484)]. P21 interacts with the DLG motif and supports dissociation of Nrf2 from this site. In contrast, modification of Cys151 in the BTB domain does not change the conformation of Keap1 but rather disrupts BTB Cul3 interaction. By both modifications ubiquitin ligase activity is lost and newly synthesized and released Nrf2 can move into the nucleus.

in vitro (97). Further, optical monitoring of the reaction of Keap1 with dipyridyl disulfide revealed a sequential reaction of the cysteine residues, likely starting with an S-thiylation of the most reactive one and followed by thiol/disulfide exchange to finally form internal disulfides within Keap1. Competition experiments with a representative set of Nrf2 activators demonstrated that Keap1 modification capacity of these heterogeneous compounds roughly correlated with their *in vivo* activity (97). By these and many other thiol modification studies (256, 287) the hypothesis of Keap1 being the redox sensor of the Nrf2 system was corroborated. *In vitro* and *in vivo* mutation studies finally revealed that Cys273 and 288, which are the Zn-coordinated ones, are crucial for the response of Nrf2 to activators (282, 504, 531), whereas mutation of Cys151 mediates stressor-induced dissociation of Keap1 from Cul3 (389, 521) (Fig. 7).

However, the original hypothesis that the primary sensing event, that is, modification of critical cysteine residues, leads to instant release of Nrf2 has been revised: the hinge and latch model predicts that thiol modification of Keap1 changes its conformation in a way that only binding of Nrf2 through its DLG motif, the latch, is reversed, whereas Nrf2 remains attached to Keap1 by its hinge, the ETGE motif. Thereby, the presentation of Nrf2 for ubiquitination is impaired. Accordingly, the unmodified critical cysteines 273 and 288 are crucial for ubiquitination of Nrf2 (531) and the degradation of Nrf2 in the unstressed situation (254). Upon challenge, the still hinge-bound Nrf2 is stabilized by preventing ubiquitination and may finally be released by downstream events (see section III.C.3). Moreover, continuously newly synthesized Nrf2 can bypass the still Nrf2-loaded Keap1 for transduction (254).

How thiol modification leads to the conformational change is likely explained by the zinc finger nature of Keap1 (95). Zinc is bound to Keap1 with an impressive K_{ass} of 10^{11} M^{-1}. Zn binding to Keap1 evidently involves the critical cysteines, as thiylation of the latter by dipyridyl disulfide is accompanied by a stoichiometric release of Zn; by a Cys to Ala mutation, the K_{ass} is dramatically decreased, and profound conformational changes are associated with Zn release, as evidenced by shifts in tryptophan fluorescence or depression of fluorescence of a hydrophobicity probe. The Zn-stabilized structure of Keap1, thus, is disturbed by derivatization of Zn-binding cysteines and equally by Zn chelation or Zn shortage, as has been similarly discussed for other redox-sensitive zinc proteins (82, 258, 309). However, it can also be envisaged that cysteine-coordinated Zn in Keap1 contributes to the pronounced reactivity of the cysteines, thereby facilitating Nrf2 activation. The pivotal role of zinc in Keap1 function might provide another lead to explain its antioxidant effect (49) (see also section II.D.3).

Finally, the realm of Nrf2 activators raises the question how their chemical heterogeneity complies with the expectation that a sensor should sense specifically. Emerging evidence indeed reveals that groups of activators act on different sets of cysteines in Keap1, which may be translated into a specific biological effect. From these observations the term cysteine code was created and breaking this code for each Nrf2 activator will help to understand the various effects resulting from Nrf2 activation (256, 521).

3. Downstream signaling events. The essence of the events following sensing by Keap1 is that free cytosolic Nrf2 has to reach its target EpRE in the nucleus. The nuclear transport of Nrf2 is mediated by the conventional set of nuclear importin and exportin proteins (156). Typical nuclear localization signals (NLS) or NES of cargo proteins to be recognized by the import and export machinery are found in the sequence of Nrf2: two NES, one located in the leucine zipper dimerization (Zip) domain and a second one in the transactivation domain (TAD) (215, 289), and three NLS (478).

The basic requirements for nuclear traffic being evident, we first have to elaborate on the questions how free Nrf2 is generated and primed for nuclear import (Fig. 8). As mentioned in the previous section, cysteine modification of Keap1 by itself is not considered sufficient to release Nrf2 from the cytosolic complex. The view favored at present therefore is that the Nrf2 that is synthesized *de novo* bypasses cytosolic

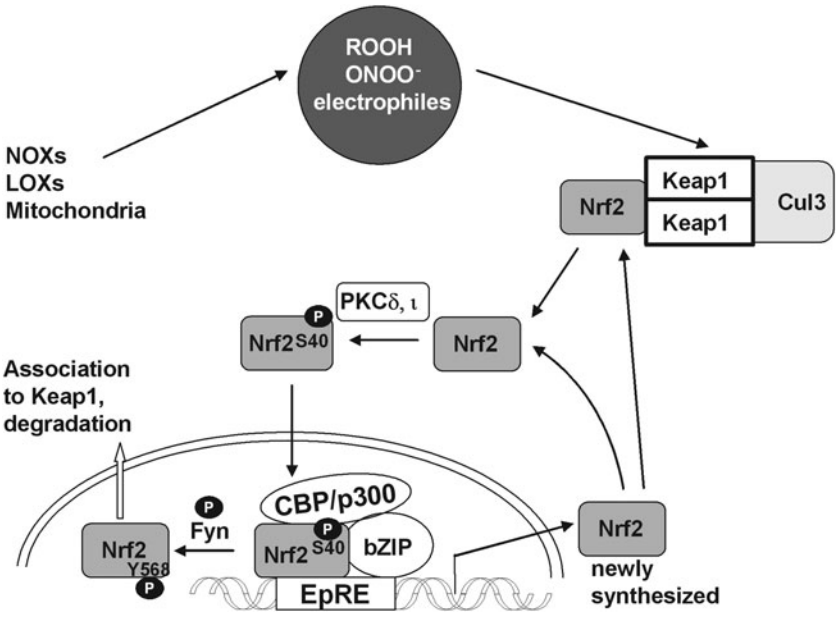

FIG. 8. Nuclear translocation of Nrf2. Activation of Nrf2 does not necessarily require activation of a specific surface receptor. Stimuli can be exogenous or endogenously produced. In response to oxidative/electrophilic stress, the interaction of Keap1 and Nrf2 is disturbed and Nrf2 is no longer degraded. Nrf2, either released from Keap1 or newly synthesized, enters the nucleus. For translocation, Nrf2 has to be phosphorylated at Ser40. The phosphorylation is achieved by PKCδ or ι and/or other kinases (see text). Phosphorylated Nrf2 enters the nucleus, forms a complex with the basic leucine zipper (bZIP) factors CREB-binding protein (CBP)/p300, and activates gene expression by binding to the electrophile responsive element (EpRE). Phosphorylation at Tyr568 by the tyrosine kinase Fyn leads to nuclear export of Nrf2 and terminates the signal. It is not known whether Nrf2 has first to be de-phosphorylated at Ser40 or whether the *bis*-phosphate is exported. Exported Nrf2 can re-associate with Keap1.

Keap1 when the latter has been modified and hence loaded with stabilized Nrf2, whereas Nrf2 meeting Keap1 under resting conditions has a poor chance to escape from degradation (287). However, since permanent wasting of Nrf2 by the unchallenged organism appears uneconomic, mechanisms to release Nrf2 from the cytosolic complex merit consideration and do indeed exist. (i) PKC phosphorylates Nrf2 at Ser40 (192), the isoforms later being identified as PKCδ (283) and PKCι (350). Ser40 is located between the DLG and the ETGE motifs, and phosphorylation inhibits binding to Keap1 and simultaneously facilitates nuclear import (347, 348). It appears, however, to be unsettled if this phosphorylation is also achieved within the Keap1/Nrf2 complex (35, 192, 350). (ii) The CDK inhibitor p21 binds to stabilized Nrf2 at Keap1 and possibly facilitates Nrf2 release (63). (iii) Prothymosin α (239, 361), and (iv) the sequestosome 1 (SQSTM1; p62) (269) interact with Keap1 in a way that displacement of Nrf2 appears unavoidable. SQSTM1 was further shown to lower Keap1 levels by increasing its rate of degradation (76). Accordingly, overexpression of SQSTM1 results in transcriptional activation of Nrf2 target genes (76, 257). Collectively, these findings suggest that also the Keap1-bound Nrf2 that has been saved from ubiquitination can be made available for nuclear import (482). It has further also been proposed that Nrf2-loaded Keap1 enters the nucleus (498).

Thus, nuclear import of Nrf2 is initiated by the key oxidative modification of Keap1 and further facilitated, *inter alia*, by PKC-dependent phosphorylation, which is also considered to be favored by thiol oxidation (Fig. 8; section II.D.3). Moreover, Nrf2 itself and, hence, its nuclear localization is redox sensitive (Fig. 9). Its activity can also be enhanced by inhibiting the nuclear export of Nrf2. The NES of Nrf2 are recognized by chromosome region maintenance (Crm1)/exportin, but in the nucleus are largely masked, probably by heterodimerization of Nrf2 with musculo-aponeurotic fibrosarcoma (Maf) proteins that retain Nrf2 in the nucleus. In consequence, the import/export balance is in favor of import even under basal conditions, which accounts for the constitutive Nrf2 activity that guarantees the basal expression of stress response genes. Whereas the NES in the bZIP domain is redox insensitive (289), oxidation of Cys183 in the NES in the TAD of human Nrf2 is discussed to inhibit the access and binding of Crm1 (288). Under oxidative stress conditions, therefore, the function of the redox-sensitive NES in the TAD is impaired and the overall weight of export signals is further decreased (287). Similarly, an oxidative modification within an NES of nuclear Keap1 can retain Nrf2 in the nucleus (498). Irrespective of the mechanism, the net effect is a nuclear accumulation of Nrf2. Cysteine modification, depending on its chemistry, may be reversible, for example, by GSH or a redoxin, and reversal of nuclear localization of Nrf2 by Trx has indeed been reported (162). Cys506 is critical for binding of Nrf2 to its responsive element and the interaction with the cofactor CREB-binding protein (CBP)/p300 (34). Apart from Cys506 also Cys119 and 235 (numbering in the mouse Nrf2) have been found to have similar functions (176). Hence, re-establishing reductive conditions in the nucleus deserves attention not only as required for DNA binding but also as shut-off mechanism of the system (see section III.C.5).

Further, nuclear export of Nrf2 is regulated by phosphorylates (Fig. 7). The Fyn kinase phosphorylates Nrf2 at Tyr568 in the nucleus which leads to the strengthening of the interaction with Crm1 and to an enhanced nuclear export of Nrf2 (213, 348). A translocation of Fyn into the nucleus is observed several hours after Nrf2 activation and requires the phosphorylation of Fyn at a so far unknown Thr residue by GSK3β. For this activity GSK3β has to be phosphorylated at Tyr216, which in contrast to the phosphorylation at Ser9 by Akt, which inactivates GSK3β (327), keeps GSK3β in the active state. The Tyr216-specific kinase is still unknown, but it is activated by H_2O_2 (214). This way GSK3β may contribute to the termination of the Nrf2 signal.

4. Nrf2-mediated transduction events. In the nucleus Nrf2 dimerizes with small Maf proteins (207) or other bZIP transcriptions factor such as c-Jun or activation transcription factor-4 (ATF4). The heterodimer binds to the Nrf2-responsive element EpRE. The Nrf2/bZIP complex then recruits the CBP and p300 (536) and initiates transcription of target genes (Fig. 8). Formation of a heterodimer with c-Fos or c-Fos-related antigen-1 (Fra-1) inhibits Nrf2 activity due to binding to the AP-1 site within the EpRE. Also, overexpression of small Maf proteins leading to Maf homodimers and possibly unproductive Maf-Nrf2 heterodimers inhibited Nrf2 activity (90). The Nrf2 gene itself carries an EpRE and is transcriptionally stimulated by Nrf2 activators (263).

Binding of Nrf2 to EpRE is competed out by the BTB and CNC homology-1 (Bach1) transcription factor (91). Oxidative conditions inactivate Bach1 and allow Nrf2 binding to EpRE (203). Inactivation of Bach1 is mediated by a redox-sensitive phosphorylation at Tyr486 by an unknown kinase, which leads to rapid export of Bach1 (243).

5. Shut-off mechanisms. Apart from nuclear export of Nrf2 (see above, section III.C.3), termination of Nrf2-mediated

FIG. 9. Organization of Nrf2. The Nrf2 protein comprises six domains (Neh1–6) with different functionalities. Neh2: Keap1 binding site; Neh4 and 5: transactivation (TA) site harbors the nuclear export signal (NES) with the redox-sensitive C183, and is required for binding of Nrf2 to its responsive element and interaction with the coactivator CBP/p300; Neh6: linker, site responsible for Keap1-independent nuclear degradation under oxidative stress (324); Neh1: contains the Cap 'n' Collar region (CNC), the redox-insensitive NES, the nuclear localization signal (NLS) with the redox-sensitive C506, and the bZIP motif; Neh3: the C-terminal domain contains Y568 and is required for transactivation (345). S40 is the phosphorylation site for PKCδ and ι; Y568, the phosphorylation site for Fyn. *Arrows* mark putative sites for casein kinases. Two novel NLSs spanning amino acids 42–53 and 587–593 identified in the mouse sequences are not included for sake of clarity (478). For numbering of different species see ref. (176).

transcription may be achieved by various mechanisms. (i) Also the nucleus contains Keap1, whose function is not fully clarified, but it may be envisaged that Keap1 also targets the nuclear Nrf2 for degradation by the nuclear proteasome (348). (ii) Polymerization of the actin skeleton is considered to be essential for retaining Nrf2 in the cytosol, thus preventing Nrf2 activity. PI3K *inter alia* regulates the response to oxidants by de-polymerization of actin, which is considered to allow Nrf2 to escape from Keap1 and to enter the nucleus together with actin (234). Re-polymerization of actin allows Nrf2 to exit the nucleus. In this context it might be revealing that glutathionylation of actin leads to depolymerization and is reversed by Grx (267, 506). (iii) Nrf2 activates transcription of its own cytosolic inhibitors such as Cul3, Rbx1, and Keap1 (242). (iv) The most efficient shut-off is certainly achieved by means of the myriad of enzymes simply eliminating the system's signals or preventing their formation.

Notably, Nrf2 targets such as Trx and Trx reductase, Srx and γ-glutamyl-cysteine synthetase synergize in improving the capacity of the entire Prx- and GPx-mediated hydroperoxide metabolism and thus eliminate oxidant signals and prevent the formation of damage signals arising from oxidative stress (125, 200). The scenario is complemented by induction of particular peroxidases such as Prx1 (200) and GPx2 (21). Similarly, the induction of many GSTs prevents activation of the system by eliminating the nonoxidant alkylants (175). Apart from eliminating Nrf2-activating signals, however, the expression of antioxidant enzymes may also interfere directly with ongoing Nrf2 transduction: murine liver *txnrd1*$^{-/-}$ cells that lack the selenoprotein Trx reductase-1 do not display any gross changes in the cellular redox homoeostasis, likely because GPxs can easily substitute for the Trx-dependent Prxs under most conditions. However, the *txnrd1*$^{-/-}$ cells displayed a pronounced persistence of Nrf2 in the nucleus. This, for the first time, points to the requirement of reduced Trx for switching off Nrf2 transduction (470) and provides a mosaic stone possibly explaining the role of selenium in Nrf2 signaling (see section II.D.5).

6. Modulation of Nrf2 function by cross-talk with phosphorylation cascades. In the previous paragraphs several phosphorylation/de-phosphorylation steps have already been mentioned that appear not to belong to the mainstream activation of the Nrf2 system. The functional relevance of phosphorylation events to the Nrf2 system has for long been established by demonstrating enhanced activation by broad-spectrum phosphatase inhibitors such as ocadaic acid (344) or genistein (213). Cross-talk of phosphorylation cascades has to be considered in particular when the Nrf2 system responds to oxidative signals produced endogenously upon activation of Toll-like or TNF receptors (TNFRs) as in infections, physical injury, or acute inflammation. Receptor activation by pathogen-derived molecules and pro-inflammatory cytokines is essentially signaled *via* phosphorylation and/or protease cascades but consistently accompanied by massive production of oxidants *via* activation of NOX and LOX or mitochondrial $O_2^{\bullet -}$ generation (see section IV.D.1). Under these conditions, therefore, the oxidant and alkylant signals, which are provided in concert with enhanced phosphorylating activities, activate Nrf2, whereas the outcome of Nrf2 activation feeds back to dampen the oxidative responses to inflammatory stimuli. In this situation the limited specificity of protein kinases and phosphatases provides ample opportunities for cross-talk between seemingly unrelated signaling pathways (298). Beyond, the oxidant conditions generally favor protein phosphorylation (see section II.D.3).

The following examples of enhanced Nrf2 activation due to phosphorylation are compiled below (Fig. 8):

(i) From the mitogen-activated protein kinase (MAPK) pathway, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) stimulate Nrf2 activity. However, site-directed mutagenesis in putative MAPK phosphorylation sites in Nrf2 did not influence Nrf2 activity (448) and Ser/Thr residues that have been identified as MAPKs' targets *in vivo* did not significantly contribute to Nrf2 activation (468). Thus, indirect mechanisms have been suggested. The assumption is corroborated by earlier findings showing that ERK-mediated phosphorylation of CBP, which binds to the Nrf2 TA in the nucleus, stimulated transactivation activity of Nrf2 (448) and that electrophiles activate JNK by the inhibition of a JNK-specific phosphatase (64).

(ii) Casein kinase2 (CK2) phosphorylates Nrf2 at several sites, which are located in the TADs. Phosphorylation at the site resulting in an Nrf2 form with a molecular weight of 98 facilitates nuclear import of Nrf2, whereas the phosphorylated form with the molecular weight of 118 appears to be degraded more readily (9, 371).

(iii) The protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) phosphorylates Nrf2, triggers nuclear import, and inhibits re-association to Keap1 (80). However, the target amino acid has not been identified yet.

(iv) As mentioned (Fig. 8), phosphorylation of Nrf2 at Ser40 by PKCδ and PKCι facilitates nuclear import of Nrf2 (283, 347, 350).

(v) Phosphorylation of the initiation factor elF2α is implicated in the enhanced translation of Nrf2 mRNA in response to the Nrf2 activators H_2O_2 and sulforaphane (387).

(vi) Several Nrf2 activators have been shown to stimulate phosphorylation of Akt, the key player of the PI3K pathway. Activation of the PI3K pathway inactivates GSK3β, in turn preventing activation of Fyn with consequences discussed in sections III.C.3 and III.C.5 (213, 214, 234, 311, 417).

Phosphorylation reactions can also lead to an inhibition of Nrf2-mediated transduction (i) by phosphorylation of Nrf2 through p38 mitogen-activated kinases in an as yet unclear way (528); (ii) by GSK3β affecting nuclear export *via* Fyn-mediated Nrf2 phosphorylation at Tyr568 (see section III.C.3) (214, 417); and (iii) by stabilizing Keap1 *via* phosphorylation at Tyr141 (216).

D. Synopsis of the Nrf2 system

In view of the complexity of the Keap1/Nrf2 system and its ramifications a wood-carved summary of the essentials might be helpful. The system responds to oxidative stress with expression of a broad range of cytoprotective enzymes. It responds to H_2O_2, organic hydroperoxides, peroxynitrite, and electrophiles that are generated by oxidative tissue damage, the common denominator of these compounds being their ability to oxidize or alkylate thiols. The *bona fide* sensor is the Nrf2 inhibitor Keap1, which sequesters Nrf2 in the cytosol in a way that it is permanently degraded by the ubiquitin/proteasome system. The sensing mechanism consists of oxidation or alkylation of critical cysteine residues in Keap1. Downstream

signaling is achieved by conformational changes of Keap1, whereby the transcription factor Nrf2 is made available for nuclear import and transduction by activation of EpRE (ARE). Termination of Nrf2 signaling is achieved by nuclear export, autoregulatory feedback loops, and re-establishing the redox homoeostasis by *de novo* synthesis of enzymes that eliminate the activating signals.

The Keap1/Nrf2 system, thus, is the only one that uses oxidants or electrophilic products of oxidative processes as primary signals. Beyond, several steps of the system, in particular the nuclear trafficking, critically depend on reversible phosphorylation and accordingly may be modulated by redox biochemistry.

Signaling through Keap1 has to overcome the competition by enzymes such as GPxs, Prxs, and GSTs, which eliminate the signaling molecules ROOH and electrophiles, respectively, and thus determine the threshold for the response. The scope of Nrf2 target genes allows an adjustment of the system's threshold and guarantees improved defense against more severe oxidative challenges. These characteristics place the system into the context of physiological adaptation to oxidative stress, as triggered in infectious diseases, acute inflammation, or injury (see section IV).

The endogenous signaling molecules of the system are mimicked by a wide range of chemicals and xenobiotics that activate the system either by generating H_2O_2 *via* redox cycling or simply by their ability to S-alkylate thiols. Such Nrf2 activators, in particular the redox-inert natural ones, are currently discussed for safe adaptation to oxidative challenges and the prevention of related diseases.

IV. NF-κB, a Key Regulator of the Immune Response

A. Discovery and definitions

NF-κB was discovered in 1986 by Sen and Baltimore (444) as a transcription factor of B lymphocytes (19). Over the years the term NF-κB has been applied to different protein complexes made up from homo- or heterodimers of five distinct proteins: p65 (RelA), RelB, c-Rel, p50, and its precursor p105, and p52 and its precursor p100 [reviewed in ref. (172)]. These proteins have an N-terminal Rel homology domain (RHD) that is responsible for binding to DNA and other proteins and harbor an NLS. As Nrf2 also NF-κB belongs to the type of transcription factors that is kept in the cytosol by complexation with inhibitors which have to be removed for activation. The family of NF-κB inhibitors (IκB) comprises IκBα, IκBβ, IκBγ, IκBε, and BCL-3. They contain 6–7 ankyrin repeats (240) that mediate binding to the RHD and interfere with its NLS function. The prevalent composition of the cytoplasmic NF-κB appears to be the p50/p65/IκBα complex.

NF-κB was the first mammalian transcription factor shown to be redox regulated (461) and suggested to be directly activated by a variety of ROS (438). Meanwhile, however, the NF-κB system has been recognized to be primarily activated by cytokines and nonoxidant foreign stressors *via* TNF, Toll-like, and other receptors.

B. The biological context of NF-κB

The pivotal role of the NF-κB system consists of the activation of an innate immune response upon challenge by micro-organisms or mimicking ligands. The main receptors of the system are TLRs, of which so far 13 distinct ones have been identified in mammals, 4 residing in endosomal vesicles, the other ones at the cell surface (262, 450). Most of the TLRs (TLR1, 2, 4–6 and 11) respond to microbial structures known as pathogen-associated molecular pattern (PAMP), the most prominent examples being the LPS, which are components of the outer membrane of Gram-negative bacteria (4, 29). The TLRs 3, 7, and 9 are specialized for recognition of double-stranded or single-stranded RNA or CpG-containing DNA, respectively, and thus can sense viral infection (4), and TLR 2 and 4 are implicated in the response to cellular damage by recognizing damage-associated molecular patterns (DAMPs) (144). NF-κB is also activated by inflammatory mediators such as IL-1β and TNFα *via* their respective receptors as well as by certain growth factor receptor tyrosine kinases (RTKs), by G-protein-coupled receptors (GPCRs) and antigen receptors (38, 147, 172, 173) (Fig. 10).

The involvement of such diverse receptor and signaling systems implies that the response to different NF-κB activators can hardly be uniform and must vary with the cell type and stimulus. However, the overall outcome of the NF-κB activation is an inflammatory response characterized by enhanced expression of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6, chemokines such as monocyte chemotactic protein-1 (MCP-1), IL-8, and macrophage inflammatory protein-1α (MIP-1α), adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM)-1, and endothelium leukocyte adhesion molecule (ELAM), growth factors, and enzymes that produce secondary inflammatory mediators such as COX-2 and induced NO synthase (iNOS). The proteins induced by NF-κB activation, thus, comprise several activators of NF-κB itself, which implies an amplification of the initial response. However, the NF-κB-dependent expression pattern, apart from the potentially self-destructive cocktail of the innate immune response, also includes self-protective proteins and inhibitors of autophagy and apoptosis (13, 38, 99, 172, 173). Collectively, therefore, the system serves to guarantee survival in a hostile environment although sometimes by using risky strategies (see section V).

The risky side of NF-κB has best been characterized in inflammation and cancer. A causal relationship between inflammation and cancer has been suspected already in the 19th century by Virchow [reviewed in ref. (17)]. Virchow's hypothesis that carcinogenesis may result from chronic inflammation has been amply confirmed for various forms of cancer. Among the inflammatory mediators, NF-κB ranks high in the list of potential culprits. NF-κB could indeed be shown to be crucial for malignant transformation (158, 300, 374). NF-κB links inflammation to carcinogenesis by at least three distinct roles: (i) By enforcing and sustaining an inflammatory response, the mutagenic potential of superoxide, H_2O_2 and lipid hydroperoxides comes into play, which can certainly not be ignored in the initiation process. (ii) In established tumors, the Janus-faced tumor-associated inflammation has to be considered. It may kill tumor cells *via* massive oxidative attack induced by TNFα (151); after all killing tumors was the name-giving activity of the TNF. On the other hand, NF-κB-mediated expression of inflammatory mediators such as TNFα, IL-1, IL-6, or prostaglandin E_2 (PGE_2), depending on the tumor type, may promote tumor growth. (iii) NF-κB activates gene expression for antiapoptotic factors such as cellular inhibitor of apoptosis proteins (cIAPs), X-linked inhibitor of apoptosis protein

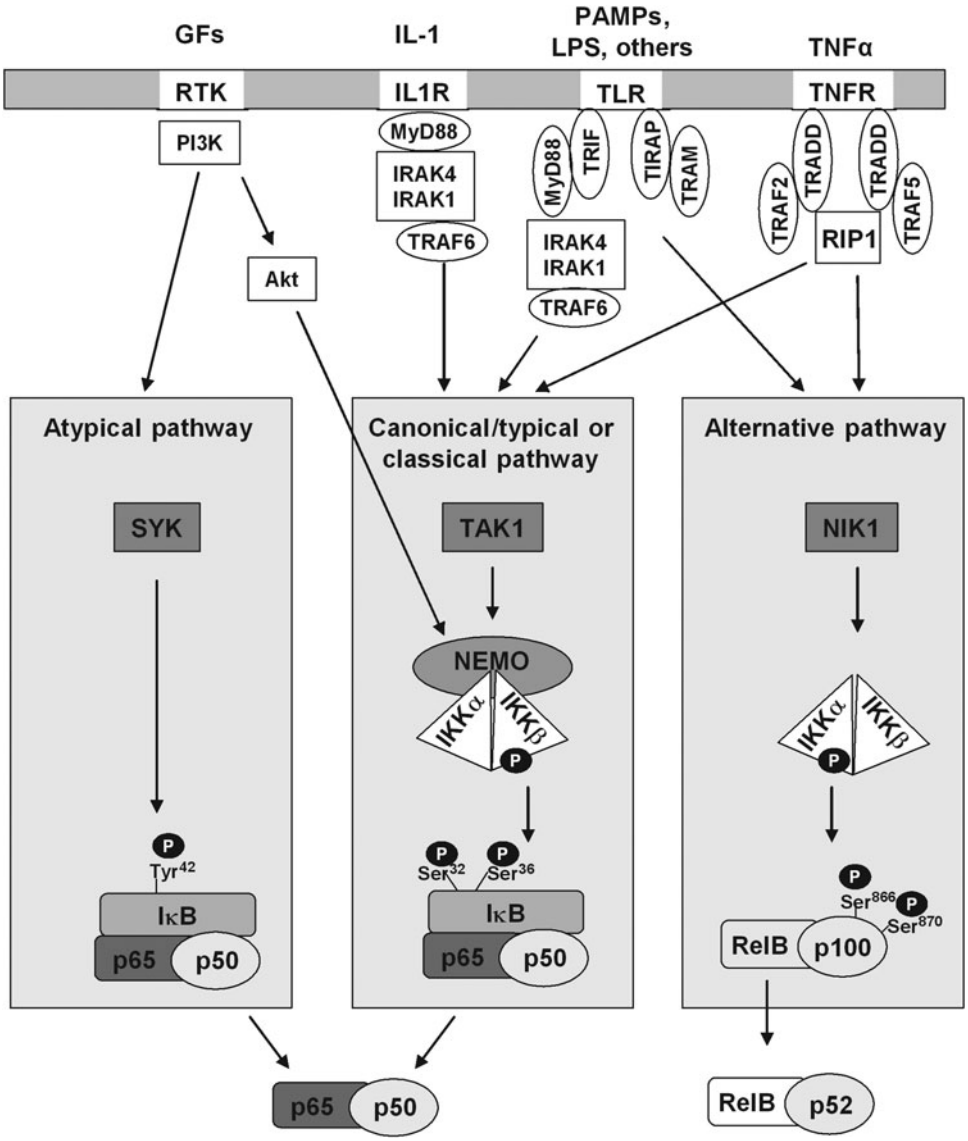

FIG. 10. Different ways to activate NF-κB. Receptor tyrosine kinases (RTK): upon growth factor binding, RTKs associate forming dimers, are autophosphorylated at multiple tyrosines, and recruit additional signaling proteins. Among recruited proteins is phosphatidyl-inositol-3-kinase (PI3K). In the atypical pathway, PI3K phosphorylates spleen tyrosine kinase (SYK), which in turn phosphorylates IκB at Tyr42, the signal for IκB degradation. Alternatively, PI3K-activated Akt can transduce signals through the typical pathway by directly phosphorylating IκB kinase β (IKKβ). Interleukin-1 receptor-1 (IL-1R1): after binding of IL-1 to the receptor, MyD88 is rapidly recruited and associates to the receptor *via* interaction of Toll/IL-1 receptor (TIR) domains present in both IL-1R and MyD88. IL-1R-associated kinase 1 (IRAK1) and IRAK4 are recruited to MyD88, where IRAK4 phosphorylates IRAK1, resulting in hyperphosphorylated IRAK1 by autophosphorylation. The kinases dissociate from MyD88. An intermediate cytosolic complex composed of the E3 ligase pellino-1, IRAK1, IRAK2, and IRAK4 is formed, whereas TNF receptor (TNFR)-associated factor 6 (TRAF6) becomes ubiquitinated, which serves as a platform for TGFβ-activated kinase (TAK1). The same complex is formed in TLR signaling (not shown) [reviewed in ref. (157)]. TAK1 then activates IKKβ. Toll-like receptors (TLR): with the exception of TLR3 all TLRs similarly to IL-1R activate TAK1 through MyD88, activation of IRAK1 by IRAK4, IRAK1 autophosphorylation, and TRAF6 recruitment. In addition, Toll-receptor-associated activator of interferon (TRIF)-related adaptor molecule (TRAM), TRIF, and TIR-domain-containing adaptor protein (TIRAP) interact with the receptor. If the typical pathway is taken, TRAF6 activates TAK1. If the signal enters the alternative pathway, NIK1 becomes activated. NIK1 directly phosphorylates IKKα, which initiates proteolytic cleavage of p100, the precursor of p52, by phosphorylation at Ser866 and 870. Finally, the RelB/p52 heterodimer can enter the nucleus. TNFR: TNF binding to TNFR1 triggers the association of the adapter protein TNFR1-associated death domain protein (TRADD) to the receptor. Then, TRAF2, TRAF5, and the kinase receptor interacting protein-1 (RIP1) are recruited. RIP1 can activate TAK1 in the typical and NIK in the alternative pathway.

(XIAP), A20, also known as TNFα-induced protein 3, Bcl-2, survivin proteins, and MnSOD (41, 188), which collectively support tumor development. The consequences of an NF-κB activation, therefore, depend on type and stage of tumor.

C. Basics of NF-κB activation

The basics of NF-κB activation consist of liberation of the free transcription factor from its cytosolic complex with the inhibitor of NF-κB (IκB) to allow nuclear import and gene activation (Figs. 10 and 11). In the absence of stimuli, NF-κB dimers are tightly associated to IκB. IκB masks the NLS in NF-κB and, thus, keeps it in the cytosol. The central event in NF-κB activation, the release of IκB from the cytosolic NF-κB complex, is typically the consequence of an IκB phosphorylation by IKK (but see below). IκB is then ubiquitinated and subsequently degraded by proteasome 26S. The IKKs form a complex consisting of IKKα, IKKβ, and IKKγ. Therein two molecules of IKKγ linked through disulfide bonds between Cys54 and Cys347 (181) form the NF-κB essential modulator (NEMO) to which IKKα and IKKβ bind in the resting state (409, 522). Conformational changes and/or ubiquitination of

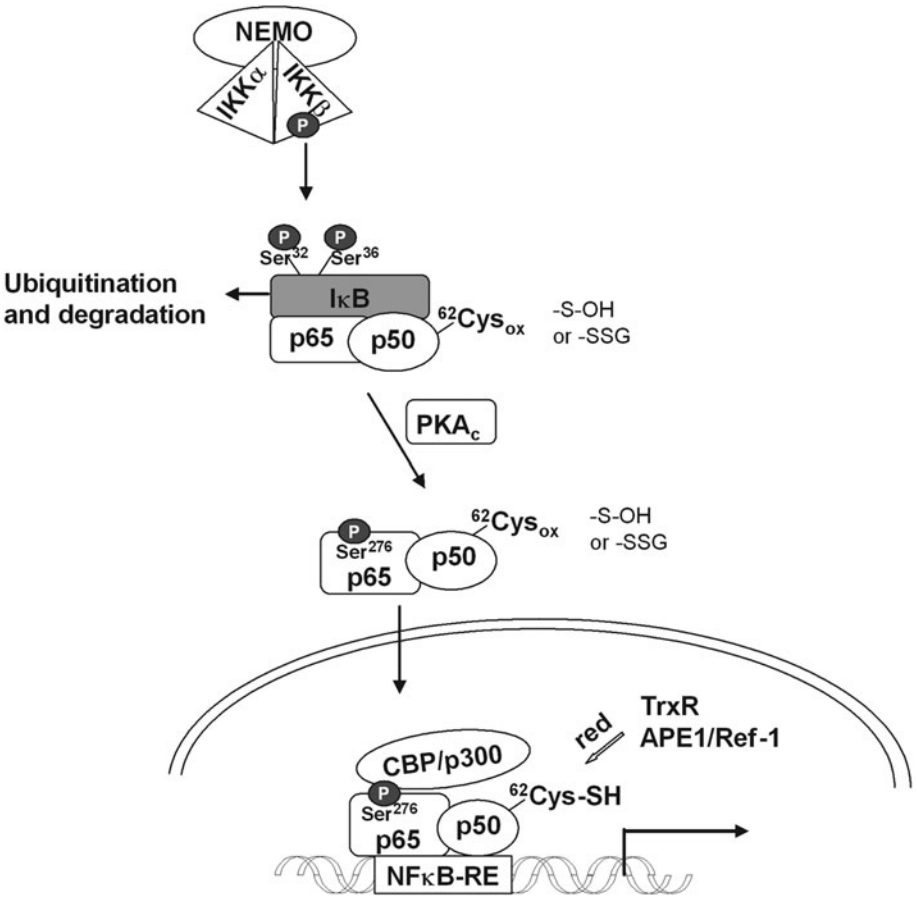

FIG. 11. Activation of NF-κB *via* the canonical pathway and putative sites for redox-regulation. TAK1, activated by IL-1R, TNFR, or TLR signaling, phosphorylates IKKβ in the IKK complex (see Fig. 10). IKKβ phosphorylates IκB at serines (Ser) 32 and 36 leading to the ubiquitination and degradation of IκB. Deliberated p65/p50 becomes phosphorylated by protein kinase A, catalytic subunit (PKAc) at Ser276 in p65, and is translocated into the nucleus. There it binds together with the coactivators CBP and p300 to the NF-κB responsive element in the promoters of target genes. For binding to DNA, cysteine 62 in p50, which can be oxidized to a sulfenic acid forming a mixed disulfide with GSH in the cytosol, has to be reduced in the nucleus by the Trx/TrxR or Grx system or by the base excision repair enzyme apurinic/apyrimidinic endonuclease/redox factor-1 (APE1/Ref-1).

NEMO due to binding to a receptor interacting protein (RIP) kinase leads to the phosphorylation/activation of IKK by an IKK kinase such as TGFβ-activated kinase (TAK1) (103, 173, 205, 425, 451, 517). With IκB phosphorylation by activated IKKs, nuclear import of NF-κB is initiated. In the nucleus NF-κB, typically the p50/p65 complex, binds to its promoter sequence together with the transcriptional coactivators CBP and p300. CBP and p300 have histone acetylase (HAT) activity. Further, NF-κB displaces histone deacetylase (HDAC) from the promoter. Thereby p65, p50 themselves, and, more importantly, histones become acetylated, the latter event leading to uncoiling of chromatin, which allows access of the transcription factor to its canonical and other promoter regions (147). Inversely, deacetylation of histones by HDACs leads to silencing of gene transcription and, further, deacetylation of NF-κB promotes its association with IκB and export from the nucleus (61).

The routes toward this largely common downstream event vary considerably with the cell type, the upstream signal and in particular with the receptor involved. The multiple channels by which NF-κB activation can be initiated are reflected in multiple activation pathways that can roughly be classified into three: the typical, the atypical, and the alternative one (147) (Fig. 10).

(i) The typical pathway (also called classical or canonical) relies on the phosphorylation of IκBα on Ser32 and 36 by TAK1-activated IKKβ (92), which creates a binding site for the β-transducing repeat containing protein (β-TrCP), the receptor subunit of an $SCF^{\beta-Trcp}$ E3 ubiquitin ligase. E3 catalyzes ubiquitination of IκB for degradation through the 26S proteasome (65, 172, 204, 296, 406). This pathway is triggered, for example, by TNF, IL-1, LPS, and other PAMPs (89) and initiates the innate immune response (38). The receptors involved are TLRs, IL-1 receptor (IL-1R), and TNFRs. They have in common that they are not receptor kinases by themselves, but work through associated kinases, as, for example, described for recruitment of the IL-1R-associated kinase (IRAK) in IL-1 signaling (312). In some cell types the genuine system receptors may be by-passed by H_2O_2 (358).

(ii) In the atypical pathway IκBα is phosphorylated at Tyr42 or on Ser residues in the PEST domain (197, 437). The phosphorylation at Tyr42 is IKK independent and, instead, catalyzed by the spleen tyrosine kinase (SYK) (473), which is activated by kinases of the PI3K pathway. Phosphorylation of IκBα at Tyr42 is followed by its dissociation and degradation. Dissociation can also be obtained by direct interaction of activated PI3K with the Tyr-phosphorylated IκB, thereby removing it from NF-κB (28). The atypical pathway is triggered by growth factors like EGF, ciliary neurotrophic factor (CNTF), or nerve growth factor (NGF) binding to RTKs, but receptor occupation can be mimicked by H_2O_2 and PP inhibitors (147). Activation of the atypical pathway is mainly T cell-specific and depends on the presence of the phosphatase SHIP-1 (149). Inhibition of SHIP-1 by either H_2O_2 or PP inhibitors maintains phosphorylation of IκBα at Tyr42 and allows activation of NF-κB. In cells lacking SHIP-1 such as Jurkat cells, H_2O_2 mainly leads to IKK-dependent Ser-

phosphorylation and degradation of IκB *via* the proteasome with only minor or absent phosphorylation at Tyr42 (149).

(iii) In the alternative pathway (also called noncanonical pathway) IKKα is activated by the NF-κB inducing kinase-1 (NIK1). Activated IKKα phosphorylates p100, resulting in its ubiquitination and processing to p52 (241, 337, 445). The pathway is activated by certain members of the TNF-receptor superfamily, such as CD40L (166), and thought to be necessary for the adaptive immune response (38). Interestingly, the alternative pathway also leads to the assembly of different NF-κB elements in the nucleus (368).

For a more detailed description of these pathways, the reader is referred to recent reviews in which also ubiquitination events are considered in more detail (147, 173, 368).

D. Redox regulation of NF-κB activation

In the years after the discovery of NF-κB, a series of studies revealed that (i) most, if not all, agents activating NF-κB trigger the formation of $O_2^{\bullet -}/H_2O_2$ *via* NOX or are oxidants by themselves such as superoxide, H_2O_2 or LOX products; (ii) in some cell lines, NF-κB activation can be triggered by H_2O_2 or organic hydroperoxides in the absence of any physiological stimulus; (iii) NF-κB activation is inhibited by a broad range of chemically unrelated antioxidants. The findings were compiled in a hypothesis paper of 1997 (128) with the above title and summarized as follows: "This complex cascade of phosphorylation and dephosphorylation is modulated by redox reactions of unknown nature in the sense that the oxidant status increases the phosphorylation and degradation of IκB. NF-κB action, however, requires a Trx-dependent reduced status in the nucleus. Upstream kinase(s) and or phosphatase(s) prone to thiolation or oxidation of vicinal SH groups are at present considered the best candidates mediating the redox regulation of NF-κB." In essence, the hypothesis has remained attractive, could be corroborated by detailed insights, and can be amended by novel system components.

Nevertheless, the seemingly convincing picture of a strict dependence of NF-κB activation on oxidants of the early nineties has since been controversially discussed. Main arguments were, for instance, that compounds labeled as antioxidants exerted effects that were unrelated with their antioxidant potential. For instance, the presumed antioxidant pyrrolidine dithiocarbamate (PDTC) inhibited IL-1-mediated NF-κB activation not due to an antioxidant function but rather by the modification of thiols in the IL-1R/IRAK1 complex (477). Similarly, an attempt to correlate the antioxidant function of N-acetyl cysteine (NAC) and PDTC with their potential to inhibit TNFα-induced NF-κB activation failed. Instead, NAC decreased the affinity of the receptor for TNFα and PDTC inhibited IκB ubiquitin ligase (168). Conflicting observations were also made with H_2O_2 itself. A critical review analyzing 40 articles on NF-κB activation by H_2O_2 alone or in conjunction with suitable cytokines revealed that H_2O_2 did not consistently lead to NF-κB activation: an activation of NF-κB was observed just in one third of the investigations, whereas in one-third H_2O_2 inhibited NF-κB activation and had no effect whatsoever in another third (358). This puzzling scenario is hard to explain. Revealingly, bolus administrations of 1 m*M* and beyond tended to inhibit NF-κB activation, and the authors recommended more physiological approaches in analyzing the problem (84, 358). The most decisive aspect of the role of H_2O_2, however, appeared to be the cellular system and the prevailing NF-κB-activating pathway (358). A regulatory function of H_2O_2 or similar oxidants in NF-κB activation has, nevertheless, to be inferred from experiments with genetically modified organisms and cells overproducing or being deficient in peroxidases. In mice deficient in GPx1, inflammatory responses, which likely are mediated by NF-κB, are consistently enhanced irrespective of being triggered by a PAMP-like LPS (211) or by viral infections (23, 24). Similarly, mice deficient in both GPx1 and GPx2 spontaneously developed an ileocolitis, when the gut was only colonized with apathogenic bacteria (108). Inversely, cells overexpressing catalase (434), GPx1 (261), GPx4 (45, 46), or Prx2 (237) displayed a dampened NF-κB activation, whereas modulation of SOD activity remained ambiguous (306, 434). Collectively, the meanwhile accumulated knowledge does no longer corroborate the concept that H_2O_2 acts as a direct activator or obligatory mediator of NF-κB activation, but rather supports the activation by other stimuli (148).

1. The source of the oxidants. In most of the schemes illustrating redox-regulated NF-κB activation, activating ROS, like a *deus ex machina*, show up in the center of an exploding star, leaving open the problems of the chemical nature and biological source of the magic activator. Intriguingly, however, a list of compounds known to induce $O_2^{\bullet -}$ formation in phagocytes already in 1985 (126) almost coincides with the one of NF-κB enhancers published by Schreck *et al.* in 1991 (438). It comprises a variety of PAMPs, antibodies, calcium ionophores, phorbolesters, substrates/products of LOX, and lipid mediators of inflammation. The two lists become practically congruent just by adding the pro-inflammatory cytokines IL-1 and TNFα to the older one. It incidentally was this pronounced similarity of activators of NOX and NF-κB enhancers that led to the concept of NF-κB activation by oxidants (128). It now corroborates that PAMPs or pro-inflammatory cytokines, when activating their respective receptors, simultaneously activate NOX and/or LOX enzymes.

The ability of calcium and PKC activators such as phorbol esters to trigger an oxidative burst in phagocytes had for long revealed a role of PKC-type enzymes in linking receptor occupation to the activation of NOX. Members of the PKC family are known to regulate many cellular processes (258) (see sections II.D.3 and III.C.3) and have meanwhile been shown to activate NOX- and LOX-type enzymes (see section IV.D.1), and to also interfere with the NF-κB system at other sites (see section IV.D.2). As mentioned in section II.D.3, they are characterized by redox-sensitive zinc finger domains. While they are activated by oxidation of the cysteine residues that are coordinated to zinc (152, 153), they may be inactivated by oxidation or alkylation of their catalytic cysteines (154). It, thus, appears conceivable that they amplify an NOX- or LOX-mediated oxidative response but contribute to its termination under stronger oxidative conditions.

Involvement of NOX has been convincingly shown in $p47^{phox-/-}$ mice. $p47^{phox}$ is an indispensable component of the phagocytic NOX complex that further contains $p22^{phox}$, $p40^{phox}$, the GTP-binding protein Rac-1, and the flavocytochrome $gp91^{phox}$ as the $O_2^{\bullet -}$ producing NOX2 (14). Accordingly, in the lungs of mice deficient in this essential activator the activation of NF-κB elicited by pathogens or LPS was impaired (253, 414). Similarly, in human aortic endothelial

cells TLR4-mediated superoxide production and NF-κB activation were shown to depend on the homologous NOX4. A knockdown of NOX4 inhibited IκB degradation and binding of p65 to DNA (365). Mechanistically, an interaction of the C-terminal region of NOX4 with the Toll/IL-1 receptor (TIR) domain of TLR4 was discussed which now is known to be linked by MyD88 (290). Also, the source of IL-1-induced $O_2^{\bullet -}/H_2O_2$ production in MCF7 cells was shown to be an NOX. The H_2O_2 thereby produced was reported to activate NIK-mediated phosphorylation on IκB probably by the inhibition of a phosphatase, since ocadaic acid mimicked the IL-1 effect (284).

The precise link between ligand occupation of TLRs and the activation of NOX systems is not yet entirely clear. In the possibly analogous growth factor signaling the activation of NOX1 is achieved by sequential activation of phosphatidylinositol-3-kinase (PI3K), formation of GTP-loaded Rac-1, and recruitment of phagocytic oxidase (phox) subunits to the membrane to assemble the active NOX complex (366). The PI3K pathway appears also to be generally activated through TLRs (313), whereby MyD88 links PI3K to the receptor (266) and PI3K produces the phosphatidyl inositol phosphates to form the platform where phox subunits can bind (106, 232) (Fig. 12). A similar sequence may, therefore, be envisaged for Rac-1 activation for NOX2 activation in phagocytes. The pivotal TLR-activated kinase for the phosphorylation of $p47^{phox}$ (30) and $p67^{phox}$ (533) in human monocytes is PKCδ, which in turn is activated by SYK and Src kinases associated with the zymosan-recognizing receptor Dectin-1, which in turn cooperates with TLR2 in the induction of inflammatory responses (142).

Novel mechanisms linking NF-κB activating receptors to NOX have recently been proposed. The TNFR proved to be coupled to NOX1 *via* riboflavin kinase (RFK), formerly known as flavokinase. RFK bridges TNFR1 and NOX1 *via* binding of the TNFR1-death-domain to the NOX subunit $p22^{phox}$. In cells deficient in RFK, TNF-induced $O_2^{\bullet -}$ production was inhibited (525). Oakley *et al.* (353) highlighted the involvement of lipid rafts in the synchronized activation of NF-κB and NOX2 by IL-1β. Both IL-1R1 and NOX2 were found colocalized in lipid rafts. Upon IL-1 stimulation MyD88 was recruited to IL-1R1 and endocytosed into endosomes together with Rac-1 and NOX2 in a caveolin-dependent manner. H_2O_2 produced in this complex facilitated TNFR-associated factor (TRAF6) association with the receptor complex, thereby building a redox-active signaling platform that was called redoxosome (354) (Fig. 13). Recruitment of receptors together with NOX enzymes into specific membrane domains could be the missing link between receptor activation and the mysterious coactivation of $O_2^{\bullet -}/H_2O_2$-producing enzymes. Compartmentalized NOX activation and redox signaling has recently been reviewed by Ushio-Fukai (494).

The NOX family is, however, not the only one of interest in the context of redox regulation of NF-κB. A steadily increas-

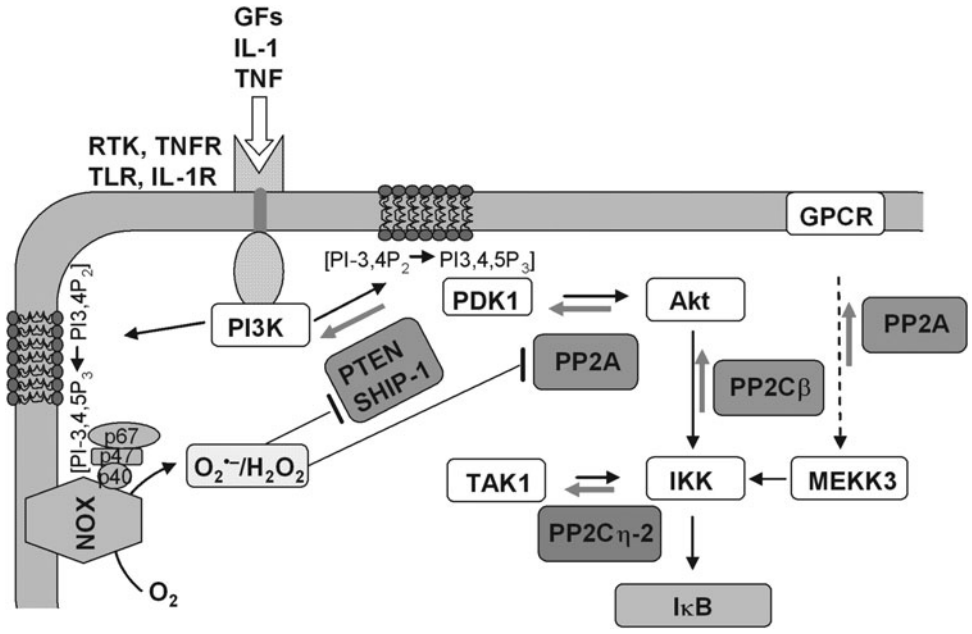

FIG. 12. Upstream kinases and redox-sensitive phosphatases in NF-κB activation. Upon binding of stimuli to their receptors, which can be RTKs, TLRs or TNFRs, PI3-kinase is recruited to the membrane and binds to RTKs *via* its Src homolog-2 (SH2) domain to autophosphorylated tyrosines (72), to TLR *via* direct interaction with the receptor and MyD88 [reviewed in ref. (290)], and to TNFR *via* riboflavin kinase (525). Once localized at the membrane, the p110 catalytic subunit of PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3′ position of its inositol residue forming PIP_3. PIP_3 recruits Akt and phosphoinositide-dependent protein kinase-1 (PDK1), the latter phosphorylating Akt at T308 and S493 in the regulatory domain. Akt thus activated phosphorylates multiple downstream targets one of them being IKKβ. Other IKKβ phosphorylating enzymes are TAK1, activated in the canonical pathway, and mitogen-activated protein kinase (MAPK) kinase kinase-3 (MEKK3), which can be activated by G-protein-coupled receptors (GPCR). PPs counteracting the involved kinases are the dual substrate phosphatases PP2A and PP2C. PP2A has been shown to colocalize with Akt and this way inhibits PDK1 action. It also reverses MEKK3 activation. PP2Cβ reverses Akt-catalyzed IKK phosphorylation and PP2Cη-2 de-phosphorylates TAK1-phosphorylated IKK. The lipid phosphatases PTEN and Src homology-2 (SH2)-domain-containing inositide phosphatase (SHIP-1) reverse the PI3K signal by removing the phosphate groups at position 3′ (phosphatase and tensin homologue [PTEN]) or 5′ (SHIP-1). All phosphatases in the scheme can be inactivated by oxidative modification of cysteine residues. The oxidizing signal comes from NADPH oxidase (NOX) enzymes that are also activated by receptor-bound PI3K there producing the inositol phospholipid products to which phagocytic oxidase (phox) subunits can bind (107, 232).

ing number of observations document that a coactivation of LOX may be equally important. Overexpression of GPx4, which preferentially reduces lipid hydroperoxides, almost abrogated IL-1β-induced NF-κB activation in endothelial cells, whereas changes in GPx1 had a minor effect (45). Similarly, lipid peroxidation and not H_2O_2 was shown to enhance the activation of NF-κB by TNF in the human endothelial cell line ECV304, whereas it appeared not to be involved in IL-1-induced NF-κB activation (43). The 5-LOX inhibitor AA861 inhibited NF-κB activation in parallel with leukotriene B_4 production in A549 cells (70). By similar approaches Bonizzi *et al.* demonstrated that NF-κB activation by IL-1β depends on 5-LOX in lymphoid cells, but not in epithelial cells, whereas it depended on NOX in monocytes (39). More recently, activation of TLR8 was demonstrated to induce the phosphorylation of the cytosolic phospholipase A2α (cPLA$_2\alpha$) and to promote 5-LOX translocation, thus stimulating the synthesis of inflammatory leukotrienes (165). The pivotal kinase involved in activating 5-LOX is likely PKCα, which has for long been known to phosphorylate cPLA2 (285).

The precise mechanisms how the activation of TNFRs or TLRs coactivates NOXs and/or LOXs likely vary with the receptor type, the stimulus, and the tissue. In any case, however, the NF-κB activation consistently occurs under conditions in which the production of $O_2^{\bullet-}/H_2O_2$ and lipid hydroperoxides is drastically enhanced, which warrants a serious consideration of the interference of these oxidants with the NF-κB-activating cascades.

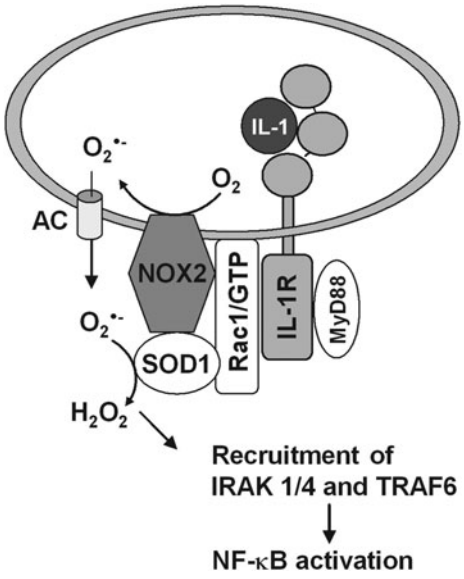

FIG. 13. Redoxosome formation in IL-1 signaling. Redoxosome (*redox* active endosomes which produce $O_2^{\bullet-}/H_2O_2$) formation has been demonstrated for the activation of NOX2 by IL-1 in MCF7 cells (354). After docking of IL-1 to IL-1R1 MyD88 is recruited as effector, and initiates endosome formation. Then, Rac-1 in its GTP-bound conformation together with superoxide dismutase 1 (SOD1) is transferred to the membrane, resulting in the recruitment of the second effector, NOX2 with all its subunits. $O_2^{\bullet-}$ produced by NOX leaves the endosome *via* anionic channels (AC) and is dismutated to H_2O_2 by SOD1. This creates an oxidative environment, which promotes docking of IRAK and TRAF6 to the receptor complex finally leading to NF-κB activation. An analogous pathway works in TNF signaling where TRADD and RIP1 are recruited instead of MyD88 and TRAF2 instead of TRAF6.

2. Modulation of NF-κB activation *via* redox-sensitive phosphorylation. As outlined NF-κB activation appears to benefit from oxidative events in the cytosol that result in increased phosphorylation of many of its components, thus facilitating nuclear import and ultimately transactivation. A general PTK/PTP imbalance causing an increased NF-κB activity is indeed observed in aged and LPS-treated rats (226). In principle, such enhanced phosphorylation state may be achieved by oxidative activation of protein kinases or oxidative inactivation of phosphatases (see section II.D.3). In particular, the p65 subunit of NF-κB requires phosphorylation at multiple sites for translocation, transactivation, and other activities (Fig. 14). These phosphorylations involve several kinases, as has been amply reviewed in refs. (147, 172, 173, 363, 501). Phosphorylation of p65 and release from IκB were, in fact, the first regulatory steps of the cascade observed (341, 343) and found to be required for DNA binding of NF-κB and the recruitment of coactivators. The possible regulation of NF-κB *via* oxidative kinase activation has lately been reviewed under thorough consideration of possible methodological artefacts and tissue specificities (363). A somewhat bold summary of this review would be that oxidative modification of kinases has so far not been convincingly shown to be involved in *in vivo* NF-κB activation, whereas oxidative inactivation is commonly observed.

The Ser/Thr kinase Akt, which itself is activated by phosphorylation at Thr308 and Ser473 *via* the PI3K pathway, phosphorylates p65 in its DNA-binding domain, the precise site being unknown (62) (Fig. 14). Akt itself is redox sensitive, being inactivated by H_2O_2 due to disulfide formation between Cys297 and 311 and reactivated by Grx (338). The cAMP-dependent kinase PKAc phosphorylates p65 in the cytosol at Ser276 (535), as do the mitogen- and stress-activated protein kinases (MSK)-1/2 in the nucleus (500). These phosphorylations facilitate release from the cytosolic complex or enhance transcriptional activity, respectively (500, 534). The activity of protein kinase A (PKA), though, is inhibited by oxidants, whereas the redox-sensitivity of the MSKs (and ribosomal S6 kinase 1 [RSK-1], see below) appears not to be investigated (363). PKC-type kinases have been reported to be activated by H_2O_2 and other oxidants (153) due to oxidation of Zn-coordinated cysteines in their N-terminal regulatory domain (152), but inactivated by cysteine oxidation/alkylation of the catalytic domains, the prevailing event at a particular phase of the activation process remaining unclear (154). PKCζ, which interacts with the NF-κB activation cascade at multiple sites, also phosphorylates Ser311 of p65 (279), thereby promoting association with the coactivator CBP (102), but, like other PKCs, also PKCζ is inactivated, for example, by peroxynitrite (252). CK2 phosphorylates p65 at Ser529 with so far unknown consequences (32, 505) and at Thr435, which decreases HDAC binding (352). Apparently, the redox-sensitivity of this kinase also remains to be investigated. IKKβ and IKKε phosphorylate p65 at Ser468, which supports nuclear import (316), whereas phosphorylation by GSK3β rather inhibited the activity of p65 (53). More recently, Ser468-phosphorylated p65 has been shown to be preferentially ubiquitinated and degraded in the nucleus, leading to the termination of NF-κB-dependent gene

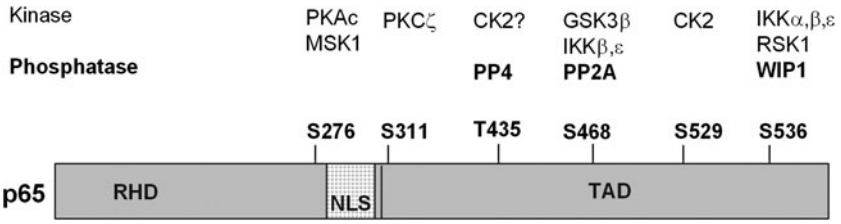

FIG. 14. Phosphorylation sites of p65 and kinases and phosphatases involved. Phosphorylation at Ser276 prevents interaction of the Rel homology domain (RHD) with the C-terminus of p65. Thereby, DNA binding and interaction with CBP and p300 is facilitated. Similarly, Ser311 phosphorylation enhances the interaction with CBP/p300. Phosphorylation at Thr435 promoter- and cell-type-specifically decreases histone deacetylase (HDAC) binding after TNF stimulation. Dephosphorylation by PP4 (526) appears to be cell type and promoter specific. Phosphorylation at Ser468 supports nuclear import. Phosphorylation at Thr505 inhibits transactivation activity due to increased association with HDAC1. The consequence of Ser529 phosphorylation is unclear; that of Ser536 affects transcriptional acitivity in a cell-specific way. For more detailed information see text (see section IV.D.2).

expression (143, 307). Finally, p65 is phosphorylated at Ser536 by RSK-1 and, more typically, by IKKs. Phosphorylation at Ser536 is widely considered to be the decisive event that facilitates the release of p50/p65 from IκB in the cytosol (16, 303, 457), although in T-cells it appears to delay nuclear import (316). However, the phosphorylation at Ser536 also plays an important role in the nucleus, where it prevents recruitment of HDAC C3 to chromatin, allows its own acetylation at Lys310 by p300 (185) and impairs affinity to nuclear IκB and, thus, export from the nucleus (36). IKKα has indeed been reported to be recruited to the nucleus together with p65 and to there phosphorylate chromatin-bound p65 (185). IKKβ, the key player in NF-κB activation (16, 416), is redox sensitive in a sense that it cannot account for an activation of NF-κB by oxidants either. IKKβ can become directly oxidized by H_2O_2 at Cys179. This cysteine residue is located between the Ser residues 177 and 181, which have to be phosphorylated for activation. In consequence, oxidation of the residue prevents phosphorylation and results in inhibition of IKKβ (260, 363). By analogy, oxidative inactivation may also be assumed for IKKα and IKKε. A seemingly contradictory report claiming an oxidative activation of IKKα and β associated with enhanced phosphorylation at Ser180 or 181, respectively (231), is likely explained by H_2O_2-mediated inhibition of phosphatases (see below). In this context it may be revealing that Cys179 of IKKβ can also be alkylated by 15d-PGJ_2. The modification of IKKβ with this bulky residue proved to be equally inhibitory, likely due to prevention of IKKβ phosphorylation. Since 15d-PGJ_2 is a product of NF-κB-induced COX2, it is considered to contribute to the resolution of inflammation *via* IKKβ modification (408).

Thus, the enhanced phosphorylation state observed during, and obligatory for, NF-κB activation cannot likely be attributed to oxidative modification of any of the kinases with the possible exception of PKCs and oxidatively activated RTKs that initiate the atypical pathway (339) (see also section II.D.3). In contrast, oxidative inhibition of PPs at each level of the activation cascade may well account for the often seen enhanced NF-κB activation under oxidizing conditions. A systematic RNAi screen of phosphatases in mouse astrocytes identified a total of 19 phosphatases regulating NF-κB transcriptional activity. In particular, the PP2A-type enzymes were found associated with the IKK, NF-κB, and TRAF2 complexes (286). Depending on the cell type, the NF-κB activation mechanism and cross-talking signaling cascades, even more and other phosphatases may come into play (Fig. 12).

The central event in the typical pathway, IKKβ activation by phosphorylation *via* TAK1, could be reversed by PP2A (92) or PP2Cη-2 (179) and that of IKKα by PP2Cβ (384). Equally, IKKβ phosphorylation by the lysophosphatidic acid-induced MAP kinase kinase kinase (MEKK3) is counteracted by PP2A (465). TNFα-mediated IKKβ activation is reversed by PP2Cα and β (466). Similarly, the phosphorylation of p65 (Fig. 14) at Ser468 is reversed by PP1/PP2A (53), whereas the nuclear Ser/Thr-specific phosphatase PP4 was shown to act on Thr435 (526). Most recently, the wild-type p53-induced phosphatase-1 (WIP1), also known as PP2Cδ, has been identified as a key phosphatase for the most important p65 phosphorylation site Ser536 and, accordingly, is considered to act as a shut-off device of the NF-κB system (66). Interestingly, NF-κB upregulates WIP1 expression, thus contributing to the termination of its activity (299). Upstream events, in particular the PI3K/Akt pathway that substantially contributes to NF-κB activation by IL-1β, TNFα, and LPS (233), offer further possibilities for oxidative interference *via* phosphatase inhibition (Fig. 12): PI3K phosphorylates phosphatidyl inositol 3,4 bisphosphate [$PIP(3,4)P_2$] to phosphatidyl inositol 3,4,5 trisphosphate [$PIP(3,4,5)P_3$]. Interference with NF-κB activation by phosphatases is possible already at these early steps, since formation of $PIP(3,4,5)P_3$ is reversed by the action of PTEN and SHIP-1. $PIP(3,4,5)P_3$ in turn activates the phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 phosphorylates Akt at Thr308 and Ser473, and its inactivating de-phosphorylation is again achieved by PP2A (291). Akt can by itself phosphorylate IKKα at Tyr23 and this phosphorylation is required for the phosphorylation of IκB (16).

Out of the numerous phosphatases that are possibly involved in counteracting NF-κB activation (298) a great deal can be inactivated by reversible or irreversible thiol oxidation, glutathionylation, or other mechanism involving oxidative processes, PTEN, SHIP-1, PP2A-, and PP2C-type enzymes being the most quoted suspects.

3. Regulation of NF-κB by redoxin systems. Trx, the prototype of the redoxins that is characterized by the WCGPC motif, has for long been recognized to interfere with NF-κB activation (128); transient cytosolic overexpression of Trx dampened NF-κB activation upon stimulation by phorbolester (429), whereas in the nucleus Trx was demonstrated to be of pivotal importance to keep the p50 subunit of NF-κB in the reduced state, which is required for DNA binding (170). These early findings for the first time revealed that the NF-κB

system responds to redox events that are at best indirectly related to the then already widely accepted redox sensitivity of the phosphorylation/de-phosphorylation balance.

In contrast to the situation in apoptotic signaling (see section II.D.1) (415), a direct interaction of cytosolic Trx with any of the components of the NF-κB system could not be corroborated. In this context Trx appears to be replaced by other redoxins (see below). Inhibition of NF-κB activation by cytosolic Trx is now likely explained by its role as substrate of Prx I and II, which compete for hydroperoxides that facilitate NF-κB activation *via* other mechanisms (398) (see section II.E). In the nucleus, however, Trx directly interacts with p50. While in the redox circuits of lower organisms reduced Trx is often used to shut off redox signaling (see Figs. 1 and 2; section II), an opposite role is adopted in the mammalian NF-κB system: it reduces critical cysteines of p50, thereby enabling DNA binding of the p50/p65 complex and initiating target gene activation. Cys62 within the N-terminal region of p50 has been identified as the pivotal residue (483), which was a surprise, since this cysteine is most easily oxidized in the cytoplasm. However, when NF-κB has entered the nucleus, Cys62 is rapidly reduced by either Trx/TrxR (169, 184, 315) or apyrimidinic endonuclease 1/redox factor 1 (APE1/Ref-1) (7, 346). Similarly, NF-κB, which is translocated to mitochondria upon stimulation by apoptotic signals, including TNFα (529), is kept active *via* reduction of p65 by Trx2 (386). The response is complex: NF-κB negatively regulates the expression of proteins for oxidative phosphorylation (OXPHOS). Simultaneously, however, Trx2 interacts with the glucocorticoid receptor, which enhances OXPHOS expression. Also, TNFα-driven mitochondrial $O_2^{\bullet -}$ formation and apoptosis is inhibited by Trx2 (386). It is further discussed that activated mitochondrial NF-κB might be translocated back to the cytosol and the nucleus to initiate transcription of antiapoptotic genes such as MnSOD (386). The mechanism of p65/Trx2 interaction in mitochondria has not yet been clarified but is thought to be analogous to that of p50/Trx1 interaction, because only oxidized p65 was found to be bound to (reduced) Trx2, which suggests the conventional disulfide reductase activity to be involved (386).

Also, Grxs, characterized by the CPYC motif, interact with the NF-κB system at several levels. Apart from de-glutathionylating kinases and phosphatases (see sections II.D.3 and IV.D.2), Grx have been implicated in direct interaction with p50 and p65. Oxidation of the critical Cys62 to a sulfenic acid followed by S-glutathionylation has been elucidated as further mechanism for inhibition of DNA binding (376). Also, S-glutathionylation of the p65 subunit inhibited its binding to DNA (388). The reacting cysteine has not been identified; it might be Cys38 in the DNA-binding loop, since this cysteine is also a target for nitrosylation (147). Both glutathionylations are reversed by Grx (376, 388).

Another redoxin, the TRP 14 (TRP14), which is characterized by a WCPDC motif, was shown to inhibit TNFα-induced NF-κB activation by the typical pathway (223) (Fig. 4D). As its congeners, TRP14 acts as a disulfide reductase, its substrate being the LC8. In thereduced form LC8 binds to IκB and inhibits its phosphorylation by IKKs and subsequent degradation. By maintaining LC8 in the reduced state, TRP14 prevents cytosolic activation of NF-κB. Exposure to oxidants or treatment with TNFα, IL-1, or LPS known to signal *via* H_2O_2 production leads to the formation of LC8 dimers in which two molecules are linked *via* a disulfide bridge (515), resulting in its dissociation from IκB (224, 227). This mode of redox-dependent inhibition of protein activities by interaction with binding partners is analogous to the association of Trx to ASK-1, an upstream activator of the c-Jun N-terminal kinase (INK) and p-38 MAPK signaling pathways (415) (Fig. 4A). In contrast, in the NF-κB system it is not the redoxin itself that blocks signaling but the redoxin substrate LC8. This seemingly tiny difference opens up the still unresolved question how regulation by TRP14/LC8 interaction is integrated into the metabolic environment. Shutting-off NF-κB signaling thus activated appears to be clarified. Oxidized TRP14 is reduced by the cytosolic form of Trx reductase TrxR1, but not by the mitochondrial TrxR2 (222, 224). Remains the questions how the activating oxidation of LC8 is achieved. Certainly, LC8 could be considered the sensor for ROOH. However, oxidative linking of two remote cysteines in a dimeric protein (see Fig. 4D) is *a priori* not likely to happen spontaneously, nor has this possibility so far been supported experimentally. TRP14 having a redox potential (−257 mV) (222) between those of Trxs and Grxs might be able to catalyze thiol/disulfide exchange both ways and thus might be the ROOH acceptor that oxidizes LC8, but the reaction of TRP14 with H_2O_2 is reportedly just four times as fast as that of Trx, which has been rated as disappointingly slow (224). The possibility that TRP14, in analogy to other redoxins, might be oxidized by an ROOH-sensing Prx has evidently been ruled out experimentally (224). We are thus left with the options that LC8 itself has the capacity to sense ROOH or is oxidized by a still unknown upstream redox sensor.

Most recently, also Nrx has been demonstrated to be a specific negative regulator of LPS-induced TLR4-mediated signaling (171). Nrx, like tryparedoxin (see section II.D.1), belongs to the redoxin subfamily that is characterized by a WCPPC motif. In mammals, Nrx appears to be the only redoxin displaying this particular motif (137). It had so far been primarily discussed as a redox-sensitive regulator of the Wnt/β-catenin pathway (Fig. 4B). In this context reduced Nrx noncovalently binds to the adaptor protein Dvl, thereby silencing Wnt-responsive signaling. Oxidation of the WCPPC motif in Nrx leads to dissociation from Dvl and, in consequence, to activation of the transcription factor β-catenin. In TLR4-dependent signaling Nrx was found to be bound to the adaptor protein Fli-1 (for flightless in *Drosophila*), and control experiments with truncated mutants revealed that the WCPPC motif is essential for binding to Fli-1 (171). Moreover, LPS-triggered TLR4 stimulation and NF-κB activation was substantially enhanced in cells from $Nrx^{-/-}$ mice, which corroborates the functional relevance of Nrx/Fli-1 interaction (Fig. 4C). In respect to the mechanism of the inhibition of TLR4 signaling by Nrx, complex formation between Fli-1 and the adaptor protein MyD88 comes into play. MyD88 is essential for NF-κB activation mediated by TLRs and IL-1R where it recruits IRAKs to the receptor complexes (Fig. 10) (219, 244, 290, 427). Fli-1 inhibits TLR signaling through binding to MyD88 (507) and sequestering the adaptor in the cytosol. This sequestration of MyD88 has now been shown to require Nrx forming a ternary complex of Fli-1, MyD88, and Nrx (171).

Whether this novel adaptor function of Nrx is redox-controlled remains to be established. It is tempting to speculate that Nrx, like in the Wnt/β-catenin pathway, is released by

oxidation, thus allowing TLR signaling to proceed. Interestingly, two more sequence-related proteins were shown to similarly bind to Fli-1: the rod-derived cone viability factor (RdCVF), expressed by photoreceptors and the chromosome 9 open reading frame 121 (C9orf121) protein (171). The former has the active site motif ACPQC, which can be rated as potentially active in disulfide reduction, in the latter the homologous sequence is RCAPS, which is incompatible with disulfide reductase activity but nevertheless could lead to the formation of mixed disulfides. However, also inactive Cys to Ser mutants of Nrx did bind to Fli-1, which implies that the active site of the redoxins is not likely involved in their interaction with Fli-1, but leaves open that redox-state-dependent structural changes affect protein/protein interaction. Alternative mechanisms, though, cannot be ruled out, since Nrx also interacts with other proteins such as, for example, HDAC6, Dvl1-3 (171), and the PP2A (137). The routes leading to oxidation and reduction of Nrx remain to be worked out. For the prototype tryparedoxin the oxidizing partner is a Prx, whereas the reduction is achieved by a typical flavin-dependent disulfide reductase mediated by the low-molecular-weight thiol trypanothione (199, 349). The search for an analogous metabolic context may be helpful in finally defining the precise role of this novel redox mediator in transcriptional activation.

E. Termination of NF-κB signaling

Out of the 39,679 publications with the keyword NF-κB (PubMed by July 29, 2010) only a vanishing proportion deals with the termination of signaling, which therefore is still poorly understood (173). This obvious lack of interest is quite surprising, since termination of the signaling cascade is pivotal to the problem why an exposure to PAMPs, DAMPs, or pro-inflammatory cytokines only exceptionally turns into a fulminant or chronic inflammation. In fact, the over 100 genes activated by NF-κB comprise also those encoding pro-inflammatory cytokines, whereby NF-κB would trigger perpetuation and even amplification of an inflammatory response, if not adequately balanced. Clearly, the organism requires tools that discriminate between an irrelevant exposure to bacterial structures or local tissue damage from systemic infections or polytrauma, respectively.

An essential way to terminate NF-κB signaling is the resynthesis of IκBs, which is controlled by NF-κB itself (128, 401). Thereby, not only the resting state of the NF-κB system can be re-established by sequestering the transcription factor in the cytosol; also nuclear degradation of p65 is initiated. In particular IκBα binds to NF-κB dimers in the nucleus and transports them back to the cytosol as inactive complex. Nuclear export of p65 *via* IκBα binding is facilitated by nitration of Tyr66 and Tyr152 and nitrosation of Cys38 on p65 (147, 367). IκBα also dissociates p65 from DNA, facilitates its proteasomal degradation in the nucleus, and, thus, terminates transcription (340, 413). Simultaneously, NF-κB activity may be terminated by binding p50/p50 dimers, which have for long been recognized to inhibit NF-κB-mediated transcription (15). Oxidation or nitrosation of the critical Cys62 of p50 also contributes to nuclear export of NF-κB (147). Finally, binding of NF-κB to IκBα and nuclear export is facilitated by deacetylation of p65 through the HDAC3. HDAC3 appears to be negatively affected by phosphorylation, nitration, or carbonylation (206, 523).

Surprisingly, IKKα, which is part of the central activation complex in the typical pathway and essential for activating the alternative pathway (see section IV.C), proved to adopt the role of a terminator in TLR4-mediated typical NF-κB signaling (271, 272). In macrophages stimulated with LPS, IKKα repressed NF-κB activity by enhancing the proteasomal degradation of p65. The biological relevance of this novel role of IKKα is evidenced by a sepsis-like response and enhanced mortality upon LPS stimulation as well as enhanced bacterial clearance in mice bearing an inactive IKKα variant (272).

The NF-κB system not only manages its own termination by *de novo* synthesis of system components that have been degraded during the activation process, but also *via* expression of other target genes. Particular interesting examples are iNOS, LOXs, and COX2. The iNOS product $^{\bullet}NO$, as long as it does not react to $ONOO^-$ by simultaneous $O_2^{\bullet -}$ formation, is clearly protective in the context of inflammation (196). The main products of 5-LOX, leukotriene B_4 and D_4, are clearly pro-inflammatory, but the lipoxins derived from 5-LOX or other LOX are currently considered to be key mediators of the resolution of inflammation (447). COX2, generally rated as the prototype of a pro-inflammatory enzyme, is, however, also Janus-faced, since some of its many products play distinct roles depending on the inflammatory phase: (i) PGE_2, being responsible for the classical signs of inflammation such as pain, swelling, and redness, also inhibits superoxide formation in chemokine-stimulated neutrophils (375). PGE_2 also interferes with NF-κB activation by selectively inhibiting nuclear import of p65, thus favoring a nuclear accumulation of inhibitory p50/p50 dimers in synovial fibroblasts (150). (ii) Prostacyclin (PGI_2) known to strongly inhibit platelet aggregation and to cause vasodilatation (496), also inhibits leukocyte adherence to the endothelial layer (44). Further, metabolically stable PGI_2 analogs (129) were shown to dampen PAMP-triggered $O_2^{\bullet -}$ formation in polymorphonuclear leukocytes (PMNs) (462) and protected rats against a lethal endotoxin dosage (435). The massive rise in PGI_2 levels consistently observed in septic conditions may therefore be re-interpreted to beneficially interfere with the deadly scenario resulting from a massive TLR4-mediated NF-κB activation rather than to kill patients (124, 295, 530). (iii) An activation of Nrf2 and a simultaneous inhibition of NF-κB activity were observed in smooth muscle cells overexpressing GPx4 or 15-LOX (20). Subsequent upregulation of HO-1 inhibited IL-1-induced NF-κB activation and expression of VCAM-1. Similarly, disruption of Nrf2 enhanced NF-κB activity, production of pro-inflammatory cytokines, and ICAM expression in the brain of mice (225). (iv) More recently, COX2-derived cyclopentenone prostaglandins, in particular 15d-PGJ_2, have been implicated in the resolution of inflammation (145). 15d-PGJ_2 is a ligand of PPARγ (195) and thereby acts as a repressor of LPS-stimulated AP-1, STAT1, and NF-κB activation (402). Being a strong electrophile, 15d-PGJ_2 can react with susceptible cysteines in a set of cytosolic and nuclear proteins (422), one of them being Keap1 (189). By targeting Keap1, 15d-PGJ_2 activates Nrf2 and, thus, initiates gene transcription with an overall anti-inflammatory result (see sections III.B and III.D) (Fig. 15).

The latest concept on resolution of inflammation implicates NOX-derived H_2O_2 as the major mediator to terminate inflammatory processes (441). This proposal obviously conflicts with the wide-spread belief that the role of the coactivation of NOXs by cytokine, TNF, and TLR occupation consist in for-

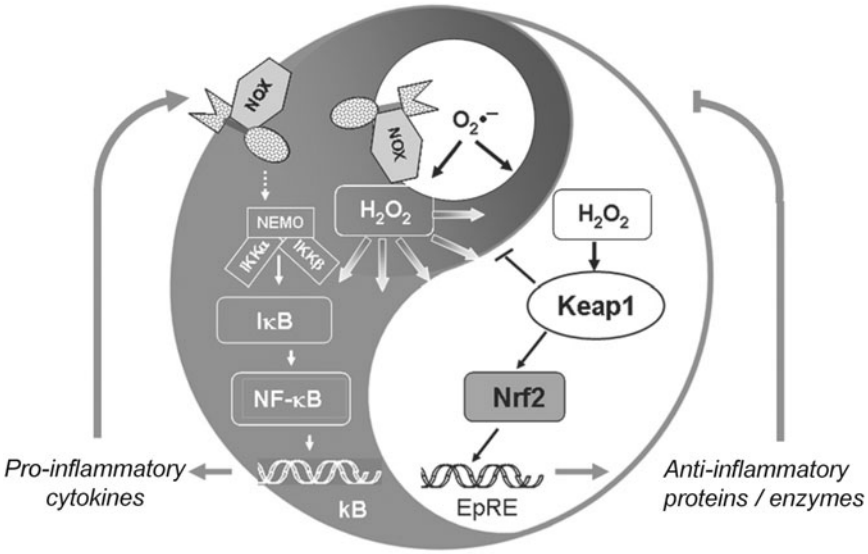

FIG. 15. NF-κB and Nrf2, the Yin and Yang of the inflammatory response. Occupation of receptors by pathogen-associated molecular patterns, damage-associated molecular pattern (DAMPs), TNFα, or IL-1 leads to superoxide formation in the redoxosome by NOX. H_2O_2 formed by SOD favors activation of NF-κB at multiple sites though enhancing protein phosphorylation, but also oxidizes Keap1 in the Nrf2 system. While NF-κB tends to enhance and perpetuate the inflammatory response by triggering the expression of pro-inflammatory cytokines, Nrf2 activation through Keap1 oxidation dampens pro-inflammatory signaling by expression of peroxidases and other anti-inflammatory proteins. As E3-ligase, Keap1 also primes IKKβ to degradation *via* ubiquitination, thereby directly interfering with NF-κB activation. For sake of clarity, only NOX-derived H_2O_2 is shown as oxidant signal. Depending on the cellular system and the inflammatory stimulus, NOX-derived H_2O_2 may be supported or replaced by mitochondrial H_2O_2, lipoxygenase products, and S-alkylating electrophiles derived there from.

tifying signaling to NF-κB. However, by means of a model of sterile lung inflammation triggered by intratracheal application of zymosan or LPS, the authors unambiguously demonstrated that inflammation was exaggerated and more progressive in mice deficient in phagocytic NOX2 as compared to wild-type mice. In parallel, whole lung NF-κB activation was dramatically increased and persisted for at least 6 days in the NOX2-deficient mice; in consequence, the release of NF-κB-dependent production of cytokines such as TNFα, granulocyte colony-stimulating factor (G-CSF), and IL-17 was similarly increased and prolonged in broncho-alveolar lavage macrophages from the deficient mice. The enhanced response was observed irrespective of $p47^{phox}$ or $gp91^{phox}$ being knocked out, and practically identical results were obtained with peripheral blood monocytes of chronic granulomatous disease patients with a defective NOX. Clearly, NOX-derived H_2O_2 in macrophages and monocytes rather inhibited than supported NF-κB activation. The mechanism remains to be established, but conceivably oxidative inhibition of NF-κB-activating kinases in these systems is more important than that of the counteracting phosphatases. Also, Keap1, the key player in Nrf2 activation, has recently been shown to down-regulate NF-κB activation by priming IKKβ to degradation *via* ubiquitination (273). More importantly, the classical role of Keap1 as sensor in the Nrf2 system in part explains the unexpected role of H_2O_2 as terminator of NF-κB activation. Nrf2 activation in NOX-deficient macrophages stimulated with zymosan was completely abrogated, whereas it was clearly detectable in control cells already 1 h after zymosan exposure (441). Moreover, $Nrf2^{-/-}$ mice behaved similar to NOX-deficient mice, and the wild-type phenotype could almost be restored in NOX-deficient mice by administration of a compound (1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole) that activates Nrf2 *via* direct, that is, H_2O_2-independent Keap1 modification (441).

In conclusion, activation of the Keap1/Nrf2 system be it by products of unspecific lipid peroxidation, cyclopentenone prostaglandins, or NOX-derived H_2O_2, appears to be the prominent mechanism to terminate the NF-κB-driven immune response.

F. Synopsis of the NF-κB system

NF-κB is a pleiotropic transcription factor that regulates expression of more than hundred genes that collectively dominate the innate and acquired immune response and associated inflammation. NF-κB is activated *via* different receptor families which signal to NF-κB by distinct phosphorylation cascades, the common denominator being release from an inhibitory cytosolic complex and nuclear import of the active transcription factor, which typically is the p50/p65 heterodimer. Receptor occupation that leads to NF-κB activation is regularly associated with activation of NOX-type enzymes, the nature of which depends on the receptor type. Concomitant activation of LOX has also been observed. Redox regulation of the activating phosphorylation cascades has been debated since the discovery of the system and remains a matter of debate 20 years thereafter. Likely this issue has to be more critically viewed under consideration of the heterogeneity of the pathways. By triggering the production of its own stimuli such as IL-1 and TNFα, the system tends to become self-amplifying, which raises the question of termination. Restoring the resting state involves nuclear export, *de novo* synthesis of cascade components that were degraded during the activation process and synthesis of enzymes that produce molecules cross-talking to antagonizing systems, in particular to the anti-inflammatory Keap1/Nrf2 system. In this context, Nrf2 activation *via* modification or oxidation of Keap1 by COX products such as 15d-PGJ_2, H_2O_2 or other oxidants arising from coactivation of NOX- and LOX-type enzymes are most attractive candidates. The NF-κB system, thus, generates the signals required for Nrf2 activation and thereby complements the hormetic response to a broad scope of exogenous challenges.

V. Loose Ends and Perspectives

It was neither our intention nor a realistic option to in-depth review what has been published on redox-dependent transcriptional gene activation since this topic became a hot issue about two decades ago. We rather tried to clarify basic chemical principles that might reasonably work in redox-regulation of biological processes and to demonstrate the relevance of these principles to two seemingly well-documented examples of redox-regulated mammalian transcription systems, the Nrf2 and the NF-κB systems, which, acting in concert, appear to be the key players in the hormetic and inflammatory responses to exogenous stressors (456). Needless to state that we ended up with a considerable number of loose ends.

Plausible regulatory circuits to adapt life to environmental oxidant changes have been worked out for procaryotes and lower eukaryotes such as yeast (333) (see sections II.A and II.D.1) and trypanosomatids (452). In these cases, not more than two dozen of solid investigations were needed to generate a concise and satisfying picture on the signaling molecules, the sensors, the transducer(s), affected genes, and modulating mechanisms (see Figs. 1 and 2). When switching to the best investigated redox-responsive mammalian transcription systems we chose as paradigms, the Keap1/Nrf2 (see section III) and the NF-κB system (see section IV), we felt confronted with a horror scenario: thousands of related original publications and hundreds of reviews were unable to generate a comprehensive and generally accepted concept and the daily increment of about 10 publications, which often conflicted with seemingly established views or opened up new perspectives, permanently forced us to revise our article. We therefore have to apologize for just delivering a snap shot of current opinions, which, beyond, is biased by a chemical/enzymological point of view.

For sure, the difficulties in understanding redox regulation in mammalian organisms are easily explained by the complexity of the systems. However, these inherent difficulties are not the only reasons for the persistent confusion. Major deficiencies one stumbles across in the field are (i) lack of chemical precision, (ii) lack of (bio)chemical kinetics, and (iii) insufficient consideration of time and spatial organization of biological processes.

To (i): The persistent reluctance to name the chemical entity supposed to exert a biological effect does not solve problems; the current talking of signaling by poorly defined chemicals such as ROS, NRS, or radical species simply widens the range of chemical reactions to unpredictability. It is, however, neither impossible nor irrelevant to define the chemical entity that is supposed to signal. As outlined in section II, signaling by radicals is rather the exception than the rule and is likely restricted to $^{\bullet}NO$ and $O_2^{\bullet -}$; the most common oxidant signaling molecule is H_2O_2; it may be a LOX product, under special conditions also $ONOO^-$, and under severe oxidative challenge an electrophilic break-down product of oxidized lipids. Knowing this, the possibly resulting chemistry is defined and its relevance under biological conditions amply documented by related enzymological studies. Making better use of the accumulated knowledge on the reaction mechanisms of, for example, thiol peroxidases and S-transferases will certainly be helpful to further define modifications of proteins relevant to regulatory processes. Another source of confusion is the uncritical use of the term "antioxidant" for a huge number of chemically unrelated compounds that have the only common denominator to react with most aggressive oxygen-centered radicals such as $^{\bullet}OH$ in physiologically meaningless *in vitro* settings. In a biological context, these compounds may indeed act as antioxidants in the chemical definition, which means compounds interfering with an oxidant-initiated free-radical-mediated chain reaction. Yet, experimental evidence supporting this concept is very scarce. More likely the antioxidants are simply reductants, S-modifying agents, redox-cyclers, cofactors, or constituents of enzymes, or display their own pharmacodynamic profile that sometimes is unrelated to redox chemistry. Facing these uncertainties, one can hardly expect conclusive data from uncritically exposing tissue cultures or animals to massive dosages of antioxidants. A revealing example is the misleading historical name ARE for EpRE, the target element of Nrf2 (see section III.B). The term "antioxidant-responsive element" was coined because the element responded to test-tube antioxidants that *in vivo* produced $O_2^{\bullet -}$ or H_2O_2 due to autoxidation or redox cycling.

To (ii): The lack of kinetic data in redox regulation is a problem indeed. It has become fashionable to declare signaling components with reactive cysteines to be sensors for H_2O_2, ROOH, peroxynitrite, or alkylants (see section II.D). These assignments, however, are overwhelmingly based on swampy grounds. After having screened the pertinent literature, we are aware of a total of 3 investigations (11, 87, 459) that documented the reactivity of such cysteines in presumed sensors that are not peroxidases by appropriate kinetic measurements: the only satisfying case was OxyR. With a rate constant of $10^5\ M^{-1}\ s^{-1}$ this sensor is kinetically competitive enough to sense H_2O_2 in the presence of competing peroxidases. In contrast, the known rate constants for oxidative inactivation of phosphatases had to be rated as noncompetitive (87, 459) (see section II.D.3). For all remaining thiol-based H_2O_2 sensors experimental data that would corroborate their kinetic competiveness are missing. The kinetoplast system (see section II.D.1) works with a tryparedoxin peroxidase as H_2O_2 sensor for which competitive rate constants have been established (349, 489, 490). The H_2O_2 sensors of the yeast systems (see section II.D.1) may by analogy be regarded as competitive, as they are related Prxs or equally efficient GPx-type peroxidases (487). A sensing function of the mammalian Keap1 is corroborated by circumstantial evidence. For the phosphatases, kinases, redoxins, and others, a direct sensing function is rather unlikely (134), which promises future surprises. Moreover, the interpretation of redox changes in regulatory proteins obtained *in vitro* is often impeded by the experimental settings in which they are generated. Tissue cultures, if not adequately supplemented, tend to be deficient in selenium and, thus, in GPxs (278) and Trx reductases (308). Being thereby deprived of both GSH- and Trx-dependent peroxidase systems, protein oxidations may occur upon H_2O_2 challenge that are physiologically irrelevant, but even well-supplemented culture cells, when suspended a medium up to 10 m*M* H_2O_2 (358), may be anticipated to have their reductive capacities (GSH and Trx pools) exhausted within minutes, allowing the excess H_2O_2 to attack protein thiols that physiologically would never be affected. In short, whereas the chemical principles of redox regulation by protein thiol modifications have quite convincingly been worked out, the

physiological relevance of individual proposals may often be questioned, because of missing kinetic data and testing conditions that favor the generation of artefacts.

To (iii): The most challenging task ahead, however, is the resolution of system kinetics at a more extended time scale. The activation of transcription factors generally operates by hit and run mechanisms: receptor occupation followed by downstream processes. Receptor activation and downstream signaling requires milliseconds to minutes; target genes expression extends into hours and days; and the consequences thereof, if cell recruitment and differentiation is involved, may take weeks. Cross-talk between different transcription factor systems already occurs in the early phases, if signaling components are shared, but regularly dominates later stages. The need of a more serious consideration of these aspects is, again, revealed by the Yin and Yang interplay of NF-κB and Nrf2 (Fig. 15). Depending on kind, strength, and persistence of a PAMP or DAMP exposure, a hormetic response without any obvious inflammatory process, a self-healing or chronic inflammation, or a deadly septic crisis may be triggered. The adaptive response to a subcritical challenge likely reflects the early and delayed counteracting activation of Nrf2. The latter, in concert with various growth factor systems, also contributes to self-healing inflammation. The switch to chronic inflammation, if not explained by persistent challenge, is poorly understood. Septic shock, finally, the prototype of an oxidative stress disease, presents as an extreme dysbalance of several transcription factor systems and may become lethal at various stages and by different pathogenic mechanisms. The early phase is undoubtedly caused by a massive overactivation of NF-κB due to PAMP or DAMP exposure with oxidative burst in phagocytes and excessive expression of adhesion factors, which, in combination with vasoconstriction and intravascular coagulation, leads to endothelial dysfunction, collapse of the microcirculation, tissue hypoxia, and multiorgan failure. Later counteracting events such as activation of the fibrinolytic system and vasodilation due to exorbitant production of PGI_2 and $^{\bullet}NO$ may resolve the circulatory block, but inevitably result in a critical drop in blood pressure, but even if the early crises of septicemia are overcome, patients tend to die weeks later in a status of immune paralysis (37). Evidently, the final outcome of a septic shock has nothing in common with the initial event which in essence is the beneficial activation of the innate immune response to cope with intruded pathogens or tissue damage. The concommittant oxidative burst will not only activate Nrf2 but also enhance signaling through all pathways that positively respond to oxidants; NF-κB not only induces circulatory collaps but also promotes TNFα-mediated apoptosis; tissue ischemia must trigger the hypoxic response; resumption of circulation may result in reperfusion injury; the neuronal and hormonal alarm that accompanies the entire process alerts a lot more signaling cascades; and with progressing time primary target cells such as PMNs and macrophages undergo apoptosis and are replaced by populations of lymphocytes responding differently to different signals, antiinflammatory cytokines becoming predominant. As net outcome, the exaggerated activation of the self-protective innate immune response turns over weeks into a life-threatening anergy of the immune system (37, 369).

A clinical exploitation of the emerging knowledge clearly demands a better understanding of the long-term consequences of a transcription factor activation, which result from multiple and poorly understood cross-talks with other systems. Nevertheless, modulation of the NF-κB or Nrf2 system has for long been, and still is, an attractive clinical perspective, in particular for the treatment of inflammatory diseases and the prevention of cancer. However, the complexity of an inflammatory response we could only briefly address, as well as the fragmentary knowledge, hampers a fast clinical implementation, and sepsis may again serve to demonstrate the inherent difficulties. Already in the eighties therapeutic success in sepsis, polytrauma, and reperfusion injury was expected from preventing tissue damage due to PAMP- or DAMP-induced oxidative burst and acute inflammatory responses (85, 404). Numerous animal experiments were performed with application of SOD alone (436) or together with catalase (510) to remove excess $O_2^{\bullet -}$, H_2O_2, or $ONOO^-$ (25), thiol- or selenium-based antioxidants (509), iron chelators to prevent Fenton chemistry (25), TNFα (132) or LPS antibodies (390, 404) or IL-1 antagonists (356, 502), mostly with encouraging results in well defined experimental settings. But none of these intervention strategies has so far been crowned by clinically success. The major reason for the failure of these strategies has to be seen in their limited feasibility under clinical conditions: first of all, at the time of clinical diagnosis, the self-amplifying inflammatory cascades are already too advanced for a meaningful intervention. Second, animal sepsis models that are designed for, for example, a precisely defined LPS dosage and an exact time point of intervention after challenge can hardly be extrapolated to the clinical situation with usually unknown onset and overlapping phases of the inflammatory process. Finally, all experimental strategies mentioned above focus on the correction of early dysregulation in septicaemia and it remains to be explored if any intervention with the early oxidant inflammatory processes can improve the overall clinical outcome. If so, a prophylactic blockade of the early inflammatory response still deserves interest in the treatment of polytrauma, where the sepsis-like syndrome develops with a predictable delay of two to 3 days.

More convincingly, hopes for better therapy of inflammation are based on the hormetic character of the NF-κB - Nrf2 interplay. It has for long been known that a sub-critical dosage of LPS induces tolerance to a lethal one (26), whereby pronounced cross-tolerance between LPS, other PAMPs, hyperbaric oxygen and inflammatory cytokines (IL-1, TNF) is observed (57). The mechanism of tolerance development is not fully understood (110). Inhibition of NF-κB transcriptional activity by p50 homodimers appears to be involved (537). However, circumstantial evidence suggests that this kind of tolerance development is, in part at least, caused by oxidative activation of the Keap1/Nrf2 system (see section III). As the latter can also be activated by natural and presumably safe electrophiles that by themselves do not necessarily induce any oxidative damage, such compounds are being considered for prophylactic treatment of high risk groups. However, this option still awaits a systematic clinical exploration.

A logical extrapolation of this hormetic concept seems to be the application of Nrf2 activators for prevention of inflammation-associated carcinogenesis. Indeed, many natural compounds activating Nrf2 such as sulfuraphane, curcumin, resveratrol, flavonoles and catechins are currently being promoted for chemoprevention. However, since evolution

has designed the Keap1/Nrf2 axis as a system that only works on demand and for a limited time, there are good reasons to be concerned about the long term effects of a permanent Nrf2 activation. Liver damage due to persistent Nrf2 activation, although so far only documented for autophagy-deficient mice (257), may be taken as a warning.

The few spot-light may suffice to demonstrate clinical spin-offs are showing up at the horizon. However, as outlined in this article, the regulatory systems are extremely complex and a lot of gray and black areas have to be filled with solid data, before reliable predictions on any interference strategy can safely be made. The steps from bench to bedside, therefore, will hardly be quick ones.

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Address correspondence to:
Dr. Regina Brigelius-Flohé
Department Biochemistry of Micronutrients
German Institute of Human Nutrition Potsdam-Rehbruecke
Arthur-Scheunert-Allee 114-116
D-14558 Nuthetal
Germany

E-mail: flohe@dife.de

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Abbreviations Used

15d-PGJ$_2$ = 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
AhR = aryl hydrocarbon receptor
AP-1 = activating protein-1
ARE = antioxidant responsive element
ARNT = aryl hydrocarbon receptor nuclear translocator
ASK-1 = apoptosis signal-regulating kinase-1
Atf1 = activating transcription factor-1, cyclic AMP dependent
ATF4 = activation transcription factor-4
Atg = autophagy-related
β-TrCP = β-transducing repeat containing protein
Bach1 = BTB and CNC homology-1
BTB = broad complex/tramtrac/bric-a-brac
bZIP = basic leucine zipper
CBP = CREB-binding protein
Cdc25 = cell division cycle 25, dual specificity phosphatase from yeast
CDK = cyclin-dependent kinase
CDNB = chloro-dinitrobenzene
c-FOS = cellular protooncogene which forms with c-jun the AP-1 transcription factor complex
cIAPs = cellular inhibitor of apoptosis proteins
CK2 = casein kinase-2
CNC = Cap'n Collar
CNTF = ciliary neurotrophic factor
COX = cyclooxygenase
CREB = cAMP response element binding protein
Crm1 = chromosome region maintenance
Cul3 = Cullin-3
Cys = cysteine
DAMP = damage-associated molecular pattern
DGR = double glycine repeat
DLG = Asp-Leu-Gly
DSP = dual specificity phosphatase
Dvl = dishevelled
ECH = erythroid cell-derived protein with CNC homology
EGF = epidermal growth factor
Egr = early growth response
ELAM = endothelium leukocyte adhesion molecule
EpRE = electrophile responsive element
ERK = extracellular signal-regulated kinase
ETGE = Glu-Thr-Gly-Glu
Fli-1 = flight less (*drosophila*)
FOXO = Forkhead box O
Fra-1 = c-Fos-related antigen 1
Fyn = membrane associated nonreceptor protein tyrosine kinase, of the Src family
G-CSF = granulocyte colony-stimulating factor
GPCR = G-protein-coupled receptor
GPx = glutathione peroxidase
GR = glucocorticoid receptor
Grx = glutaredoxin
GSH = glutathione
GSK3β = glycogen synthase kinase-3β

Abbreviations Used (Cont.)

GSSG = glutathione disulfide
GST = glutathione-S-transferase
HAT = histone acetylase
HDAC = histone deacetylase
HIF-1 = hypoxia-inducible factor 1
HNE = 4-hydroxy-nonenal
HO-1 = heme oxygenase-1
ICAM = intercellular adhesion molecule-1
IKK = IκB kinase
IL-1 = interleukin-1
iNOS = induced NO synthase
IRAK = IL-1R-associated kinase
IRP1 = iron response element-binding protein 1
IVR = intervening region
JNK = c-jun N-terminal kinase
Keap1 = kelch-like ECH-associated protein-1
LAR = leukocyte-common antigen-related (phosphatase)
LC8 = dynein light chain 8
LOX = lipoxygenase
LPS = lipopolysaccharide
Maf = musculo-aponeurotic fibrosarcoma
MAPK = mitogen-activated protein kinase
MCP-1 = monocyte chemotactic protein-1
MEKK = MAP kinase kinase kinase
MIP-1 = macrophage inflammatory protein-1
MnSOD = manganese superoxide dismutase
MSK = mitogen- and stress-activated protein kinase
MyD88 = myeloid differentiation factor 88
NAC = N-acetyl cysteine
Neh = Nrf2-ECH homology
NEMO = NF-κB essential modulator
NES = nuclear export signal
NF-κB = nuclear transcription factor of bone marrow-derived lymphocytes
NGF = nerve growth factor
NLS = nuclear localization signal
NOX = NADPH oxidase
NQO1 = NADPH quinone oxidoreductase
Nrx = nucleoredoxin
Ohr = organic hydroperoxide resistance
OhrR = organic hydroperoxide resistance repressor
Orp1 = GPx3-like protein in *Saccharomyces cerevisiae*
OxyR = transcription factor which regulates genes induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*
p300 = transcriptional coactivator with HAT activity
PAMP = pathogen-associated molecular pattern
Pap1 = transcription factor regulation hydrogen peroxide induced responses in *S. pombe*
PDGF = platelet-derived growth factor
PDK1 = phosphoinositide-dependent protein kinase-1
PDTC = pyrrolidine dithiocarbamate
PERK = PKR-like endoplasmic reticulum kinase
PerR = transcription factor regulating gene expression in response to H_2O_2
PEST = proline glutamic acid serine and threonine-rich peptide sequence
PGE_2 = prostaglandin E_2
PGI_2 = prostacyclin
Phox = phagocytic oxidase
PI3K = phosphatidyl-inositol-3-kinase
PIP = phosphoinositol phospholipid
PKAc = protein kinase A, catalytic domain
PKC = protein kinase C
PKR = protein kinase R, eukaryotic translation initiation factor-2α kinase-2
PMN = polymorpho nuclear leukocytes
PP = protein phosphatase
Prx = peroxiredoxin
PSP = protein serine/threonine phosphatase
PTEN = phosphatase and tensin homologue
PTP = protein tyrosine phosphatase
Rac = small GTPase of the family of Rac proteins
Rbx1 = RING box protein 1
RFK = riboflavin kinase
RHD = Rel homology domain
RING = really interesting new gene
RIP = receptor interacting protein
RNAP = RNA polymerase
RNS = reactive nitrogen species
ROS = reactive oxygen species
RSK = ribosomal S6 kinase
RTK = receptor tyrosine kinase
Ser = serine
SH2 = Src homology-2
SHIP = SH2-domain-containing inositide phosphatase
SOD = superoxide dismutase
SoxR = transcriptional regulator in response to superoxide generating compounds
SQSTM1 = sequestosome 1
Src = cellular and sarcoma, tyrosine kinase
Srx1 = sulfiredoxin-1
SUMO = small ubiquitin-like modifier
SYK = spleen tyrosine kinase
TAD = transactivation domain
TAK1 = TGFβ-activated kinase
Thr = threonine
TIR = Toll/IL-1 receptor
TIRAP = TIR-domain-containing adaptor protein
TLR = Toll-like receptor
TNF = tumor necrosis factor
TNFR = TNF receptor
TRADD = TNF-associated receptor death domain
TRAF = TNFR-associated factor
TRAM = TRIF-related adaptor molecule
TRIF = Toll-receptor-associated activator of interferon
TRP14 = thioredoxin-related protein-14
Trx = thioredoxin
Tsa1 = first characterized peroxiredoxin in *S. cerevisiae*
TTF = thyroid transcription factor
Tyr = tyrosine
UGT = UDP-glucuronyl transferase
UMCSBP = universal minicircle sequence binding protein
USF = upstream stimulatory factor
VCAM = vascular cell adhesion molecule
VHR = Vaccinia H1-related (phosphatase)
XIAP = X-linked inhibitor of apoptosis protein
WIP1 = wild-type p53-induced phosphatase-1
Wnt = wingless and Int 1
Yap1 = AP-1 like transcription factor from yeast