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Forum Review Article

Zinc Fingers as Biologic Redox Switches?

Klaus-D. Kröncke[1] and Lars-Oliver Klotz[2]

Abstract

Despite zinc ions being redox inert in biologic systems, zinc-finger structures act as redox-sensitive molecular switches controlling several crucial cellular processes. Oxidative or nitrosative stress, *via* modification of zinc finger cysteine thiols, leads to a release of Zn^{2+} from these structures, causing not only a loss of zinc-finger function but also an increase of cytoplasmic or nuclear free Zn^{2+} that may, in turn, stimulate and interfere with cellular signaling cascades. A signaling cascade stimulated by exposure of cells to zinc ions or to stressful stimuli that are reported to cause an intracellular release of zinc ions involves phosphoinositide 3′-kinases and the Ser/Thr protein kinase Akt, resulting in an inactivation of transcriptional regulators of the FoxO family. Possible modes of action of zinc ions to stimulate this signaling cascade and consequences of stimulation are discussed. Moreover, we present an overview on human diseases or disorders characterized by an intracellular Zn^{2+} dyshomeostasis. *Antioxid. Redox Signal.* 11, 1015–1027.

Introduction

Next to iron, zinc is the second most abundant transition metal in living organisms. Because of its fully occupied *d* shell, zinc is always present as Zn^{2+} in biologic systems and—although it is a crucial cofactor of several oxidoreductases—does not change its oxidation state. In proteins, cysteine, histidine, aspartic acid, and glutamic acid serve as high-affinity ligands to Zn^{2+} (for review, see ref. 67), establishing both catalytic zinc-binding sites and sites that serve the purpose of stabilizing a defined protein structure. Whereas aspartic acid and glutamic acid are ligands often found at catalytic sites, cysteines are much more frequent at sites of structural significance. Histidines are found in both types of Zn^{2+}-binding sites (75).

The term "zinc finger" was first coined to describe a 30-amino acid, ninefold repeated cysteine- and histidine-rich sequence motif of the eukaryotic transcription factor IIIA (for review, see ref. 41). In this so-called classic zinc finger, Zn^{2+} is at the center of a tetrahedral coordination motif established by two cysteines and two histidines, thus creating an independent structural domain with a two-stranded antiparallel ß-sheet and a short α-helix. The term "zinc finger" is now somewhat more generally used to describe zinc-binding sites with four ligands that may be either cysteines or histidines, independent of whether the "zinc finger"–harboring proteins interact with DNA, in addition to serving as factors in transcription, DNA replication or DNA repair. Several such proteins are also involved in signal transduction, the regulation of apoptosis and proliferation, and in general metabolism. This overview explores how—despite the earlier-mentioned lack of redox activity of Zn^{2+} in biologic systems—zinc fingers can act as efficient redox-sensitive molecular switches ("redox switches") to affect the listed cellular processes. Moreover, intracellular signaling cascades affected by zinc ions (including Zn^{2+} released from zinc fingers) are discussed, followed by a brief overview on conditions and diseases characterized by intra- and subcellular changes in Zn^{2+} concentrations.

Zinc fingers as efficient redox-sensitive molecular switches (redox switches)

Thiolate-zinc(II)-thiolate bridges, such as those found in zinc fingers, may be regarded as substitutes for disulfide bonds, which, under the reducing cytoplasmic conditions, are difficult to establish intracellularly and probably are not very stable. Zinc-thiolate motifs, therefore, assist protein folding in bringing different parts of a protein together and, compared

[1]Institute of Biochemistry and Molecular Biology I and [2]Environmental Health Research Institute (IUF), Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany.

with disulfides, are advantageous under the reducing conditions in cells. It is thus not surprising that up to 10% of the human proteome are potentially zinc-binding proteins. Why zinc and sulfur? Zn^{2+}, in contrast to other transition metal ions such as Co^{2+}, Ni^{2+}, or Cd^{2+}, is abundant and readily available.

Furthermore, zinc ions do not undergo redox cycling, in contrast to iron or copper ions. Moreover, Zn^{2+} is a borderline soft metal forming more stable complexes with the soft sulfur-based ligands than many other transition or heavy metals. *Vice versa*, sulfhydryl ligands provide Zn^{2+}-complex formation constants about 2 orders of magnitude higher than those of the corresponding nitrogen-containing ligands (80).

Last, the Zn^{2+}-S moiety allows zinc to be both tightly bound and yet available, as a modification of the sulfur entails ejection of Zn^{2+} from zinc fingers or related structures. It is this property and the reversibility of binding and releasing zinc ions depending on the respective redox conditions that renders zinc fingers efficient redox switches.

Many different zinc-binding motifs with cysteine and histidine ligands in proteins have now been identified. In addition to zinc fingers, these motifs comprise RING finger, treble clef finger, zinc ribbon, gag knuckle, B-box, LIM, PHD or TAZ domains (49, 68). Most of the metal-binding motifs detected among putative human zinc-binding proteins have four ligands, and ~97% of these four-ligand binding motifs contain at least two cysteine residues (Fig. 1) with Cys_4 and Cys_2His_2 being the most common types (2). In contrast, >70% of the three-ligand zinc-binding motifs do not contain a cysteine, implying that they are unlikely to be capable of acting as redox switches.

If thiolates in zinc fingers are oxidized or modified otherwise, Zn^{2+} may be released from the coordination site, resulting in secondary effects, such as intracellular signaling. Second, destruction of the zinc finger leads to a conformational change of the protein. Zinc fingers represent the most common motif encountered in eukaryotic DNA-binding proteins, and disruption of one or of several zinc fingers in a transcription factor causes loss of DNA-binding activity of the factor, resulting in either an attenuated or an enhanced transcription rate, depending on whether the respective protein acts as a transcriptional activator or repressor. Several examples are found in the literature of oxidative or nitrosative stress causing a loss of DNA-binding activity of zinc-finger transcription factors (for reviews, see refs. 51, 97, and 98). Interestingly, oxidative stress appears to result in irreversible modifications of zinc fingers, whereas cells are capable of restoring the activity of zinc-finger transcription factors lost under nitrosative stress (54).

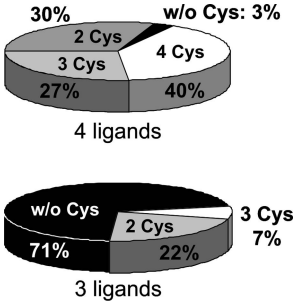

FIG. 1. Distribution of metal-binding motifs types detected in putative human zinc-binding proteins. Only motifs with four and three zinc ligands are shown. Modified from (2).

Disruption of zinc-thiolate motifs in proteins serving as redox switches has been shown to affect the chaperones Hsp33 and Hsp40, respectively, the activities of which are either enhanced or inhibited (for reviews, see refs. 34 and 50). As zinc fingers are widely distributed in different proteins, their disruption might affect different specific cellular processes like signal transduction, the regulation of apoptosis, differentiation and proliferation, *etc*., but also the general metabolism of the cell.

Compounds causing ejection of Zn^{2+} from zinc fingers

Stable complexation of Zn^{2+} in zinc-finger structures is, to a great extent, realized by the cysteine residues being present in their reduced form. As a consequence, all compounds that oxidize thiols in zinc fingers to disulfides, sulfenic acid, sulfinic acid, or sulfonic acid, as well as thiol-reactive compounds such as *N*-ethylmaleimide (NEM), 2,2′-dithiopyridine (DTDP), aldehydes, or reducible selenium compounds like selenocystine or ebselen, may disrupt zinc-finger structures, resulting in release of Zn^{2+}. In addition, thiophilic metal ions like the soft metals Cd^{2+}, Hg^{2+}, and Pb^{2+}, have been shown to be able to displace Zn^{2+} from its binding sites. If histidine contributes to zinc binding, histidine-reactive compounds such as diethylpyrocarbonate (DEPC) may also cause the release of Zn^{2+} from zinc fingers (for reviews, see refs. 31, 53, and 99).

Zinc release from metallothionein

Metallothioneins are the most abundant intracellular Zn^{2+}-storage proteins, which also function as intracellular redox sensors (for recent reviews, see refs. 46, 66, and 67). Mammalian metallothioneins are composed of 61 to 68 amino acids, including 20 cysteines, which bind Zn^{2+} into Zn_3S_9- and Zn_4S_{11}-type of zinc/thiolate clusters, in which the Zn^{2+} ions are tetrahedrally coordinated by four thiolate ligands. By using cells transfected with a fusion protein consisting of metallothionein sandwiched between two mutant green fluorescent proteins, NO-mediated zinc release from metallothionein has been demonstrated by using the FRET technique (76).

However, intracellular proteins containing zinc fingers have repeatedly been shown to be *S*-nitrosated after applying nitrosative stress *in vitro* (56, 60, 63). It is thus reasonable to assume that all compounds and probably all conditions that have been found to result in a Zn^{2+} release from metallothionein will also lead to a release of Zn^{2+} from zinc-finger proteins. At present it is not clear how much of the Zn^{2+} released intracellularly under conditions of cell stress derives from metallothionein and how much from zinc-finger proteins. This ratio probably depends on the cell type or the cellular stress conditions or both. Although disruption of zinc-finger domains usually results in a direct modulation of the respective protein function, it is not clear whether a Zn^{2+} release from metallothionein results in more than the indirect effect of increasing the concentration of intracellular protein-unbound zinc.

Perturbation of the Cellular Zinc Homeostasis

The intracellular Zn^{2+} concentration of a typical eukaryotic cell has been calculated to be in the range of ~200 μM, but because of an excess of complexing ligands, free Zn^{2+}

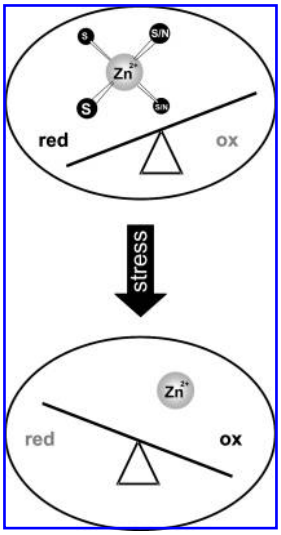

FIG. 2. Conditions leading to intracellular zinc release. Under physiologic conditions, the cytoplasmic milieu of the cell is reducing, and most of the Zn^{2+} in cells is bound by proteins. In zinc fingers, cysteine thiols or imidazole nitrogen atoms of histidine residues (or both) act as zinc ligands. If the cytoplasmic milieu becomes more oxidative, zinc-finger thiols can be oxidized and the Zn^{2+} released from zinc-finger structures.

(also referred to as "labile," "rapidly exchangeable," "easily available," or "loosely bound" Zn^{2+}) in cells under physiologic ("normal") conditions is in the pico- to low-nanomolar range (48, 74). Usually this low concentration of free zinc is tightly controlled by the cell and is thought to be in constant equilibrium with many intracellular Zn^{2+}-binding proteins (46), the most prominent being the metallothioneins.

However, it should be understood that free zinc within cells is not devoid of ligands, although the nature of these ligands varies. One of the zinc-binding ligands probably is glutathione (GSH), which, as the main intracellular low-molecular-mass thiol in cells, usually is present in millimolar concentrations. As a consequence of cysteine thiols being among the predominant ligands of intracellular Zn^{2+}, shifting the redox balance to a more oxidative intracellular milieu increases the availability of free zinc (Fig. 2) and thus affects the cellular zinc homeostasis (for reviews, see refs. 53 and 67).

In the last few years, application of several oxidants as well as of NO to cells has repeatedly been found to lead to an increase of intracellular free Zn^{2+} (53). Endogenous high-output NO production by cytokine-activated endothelial cells *via* inducible NO synthase (iNOS) results in an intracellular zinc release predominantly in the nuclei of the cells (86). In addition, exposing murine lung but not aortic endothelial cells to media containing low oxygen concentrations causes an acute increase of intracellular (predominantly intranuclear) free Zn^{2+}, which can be inhibited by an NO synthase inhibitor (9).

Similarly, in an animal model of acute hypoxic pulmonary vasoconstriction, an intracellular Zn^{2+} release can be observed in the pulmonary endothelial cells of isolated perfused rat lungs, which also can be inhibited by an NO synthase inhibitor (9). This suggests that changes of the intracellular zinc homeostasis in lung endothelia during hypoxia critically depend on NO synthesis, probably by the endothelial NO synthase (eNOS).

Another method of shifting the intracellular redox balance is to deplete cells from GSH. However, simply inhibiting GSH biosynthesis by treating fibroblasts with the specific and irreversible γ-glutamylcysteine synthetase inhibitor BSO for 24 h does not lead to a detectable intracellular zinc release. In contrast, acute changes of the cellular redox balance induced by exposure to diamide, which rapidly oxidizes thiols (preferentially GSH) to disulfides without producing free radicals to any great extent, results in a rapid, strong, and transient intracellular Zn^{2+} release (77). BSO pretreatment significantly shifts the effective diamide concentration to lower concentrations, implying that indeed GSH is important for intracellular Zn^{2+} homeostasis (Fig. 3).

A transient translocation of Zn^{2+} into the nucleus has been found in human myeloid HL-60 leukemia cells during phorbol ester–induced differentiation into macrophages (26). The mechanism was postulated to involve protein kinase C-β, which requires bound zinc. However, phorbol esters are well-known activators of NADPH oxidase, catalyzing the production of superoxide anion radicals ($O_2^{\cdot -}$), which in turn could cause oxidative zinc release. Thus, the exact mechanism of the transient Zn^{2+} release under these conditions is not clear to date. However, zinc release from PKC has been postulated to be a common event during activation by phorbol esters or reactive oxygen species (ROS) (45).

Photooxidative stress and cellular zinc homeostasis

Exposure of cells to ultraviolet (UV)-A (320–400 nm) radiation results in intracellular ROS formation, including singlet oxygen ($^1O_2$) and hydrogen peroxide (38, 39). Irradiation of fibroblasts with subtoxic doses of UV-A light leads to a transient increase of intracellular free Zn^{2+} (77). In contrast, exposure to biologically equivalent doses of UV-B (280–320 nm) does not lead to a Zn^{2+} release under subtoxic conditions *in vitro*, nor does irradiation with infrared (IR) light (760–1,400 nm). The UV-A–induced transient Zn^{2+} release can

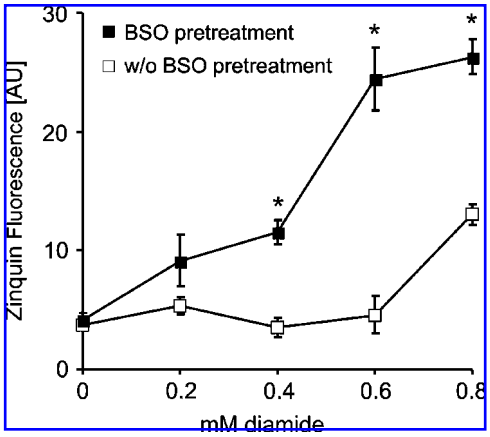

FIG. 3. Effects of depleting intracellular glutathione on the intracellular zinc homeostasis. Fibroblasts pretreated or not for 24 h with 3 m*M* specific and irreversible γ-glutamylcysteine synthetase inhibitor BSO were additionally incubated with up to 0.8 m*M* diamide for 1 h. Cells were subsequently stained for intracellular free Zn^{2+} with 10 μM ethyl ester of the Zn^{2+}-specific fluorophore zinquin, examined under a fluorescence microscope, and photographed. Electronic pictures were processed, and the fluorescence quantified as described (77). Treatment with diamide results in a rapid increase in intracellular zinquin fluorescence at a diamide concentration of 0.8 m*M*, reflecting intracellular Zn^{2+} release. Depleting intracellular glutathione with BSO does not lead to a detectable intracellular Zn^{2+} release, but significantly shifts the effective diamide concentration to the left. *$p < 0.0004$.

be mimicked by intracellular singlet oxygen generation as well as by application of the cell-membrane permeant peroxide *tert*-butyl hydroperoxide. In contrast, exogenous application of even up to 3 m*M* H_2O_2 to fibroblasts does not lead to any detectable intracellular Zn^{2+} release (77), which may be due to the quite short half-life of extracellular H_2O_2.

A well-known method of generating $O_2^{\cdot-}$ intracellularly is by applying quinones that undergo intracellular flavoenzyme-catalyzed reduction at the expense of NAD(P)H. This results in the formation of semiquinones, which easily transfer an electron to O_2, yielding $O_2^{\cdot-}$ and the original quinone, the whole process being known as redox-cycling. Formation of $O_2^{\cdot-}$ is the beginning of a cascade that generates H_2O_2 and hydroxyl radicals ($OH^{\cdot}$). All of these ROS are able to oxidize thiols. However, only toxic concentrations of several quinones lead to an increase of intracellular free Zn^{2+}, whereas subtoxic concentrations do not, at least not in fibroblasts (77). It is thus presently unclear which of the ROS generated during UV-A irradiation are predominantly responsible for the intracellular Zn^{2+} release (Fig. 4).

Intracellular localization of the released zinc

Disturbing the cellular zinc homeostasis by oxidative or nitrosative stress usually results in cytoplasmic zinc release. However, a nuclear zinc release has been found in some cases. In cytokine-activated endothelial cells, NO-dependently accumulated free zinc was identified predominantly in the nuclei in round spots, probably nucleoli (86). Similar results were found after application of ROS to fibroblasts (77). Exposure of HaCat keratinocytes to nitrosative stress resulted in release of zinc that was found predominantly in perinuclear regions and in vesicles (53). Treatment of these cells with zinc ions in the absence of stress resulted in the accumulation of free zinc in the nucleus, predominantly in nucleoli. In HaCat cells with a mutated *EVER2* gene, the concentration of free zinc in nucleoli was found to be even significantly higher (59). This suggests that in human keratinocytes, EVER2, a protein with unknown function, modulates the influx of zinc to nucleoli. In conclusion, subcellular localization of free zinc appears to vary with the cell type and the type of stress applied.

Signaling Effects of Zinc

A dysregulation of intracellular zinc homeostasis (*e.g.*, resulting from the oxidation-induced release of Zn^{2+} from zinc binding proteins) will cause a shift in subcellular Zn^{2+} distribution, allowing novel interactions between zinc ions and various proteins, peptides, or small ligands. This may not only directly affect metabolic circuits by inactivation of crucial enzymes, but such a Zn^{2+} dysbalance will also result in a signaling dysbalance caused by a local surplus of Zn^{2+} interfering with cellular signaling networks. By and large, three modes of interaction of Zn^{2+} with cellular signaling networks may be discerned:

1. Zn^{2+} may compete with or substitute for metal ions crucial for the activity of signaling proteins (Fig. 5A); for

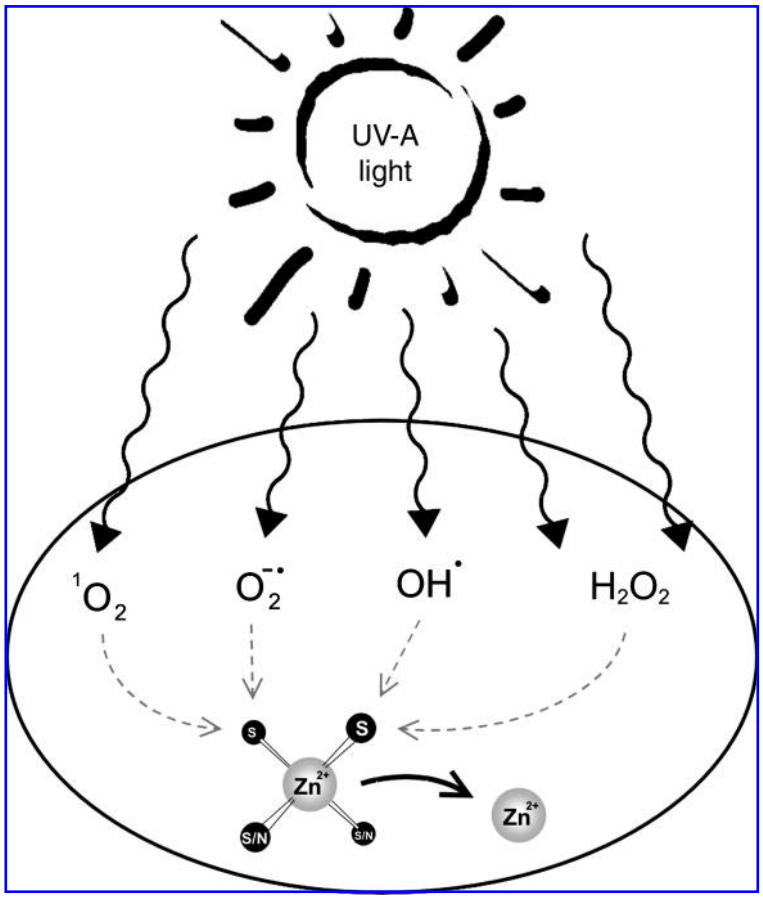

FIG. 4. Effects of exposure of cells to UV-A on intracellular zinc homeostasis. H_2O_2 and superoxide anion radicals ($O_2^{\cdot-}$) are the major diffusible species generated during UV-A irradiation in cells. Singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot}$) are far more reactive with much shorter half-lives and attack nonselectively almost all types of biomolecules. It is presently unknown which of the reactive oxygen species is/are predominantly responsible for the transient UV-A–induced intracellular Zn^{2+} release.

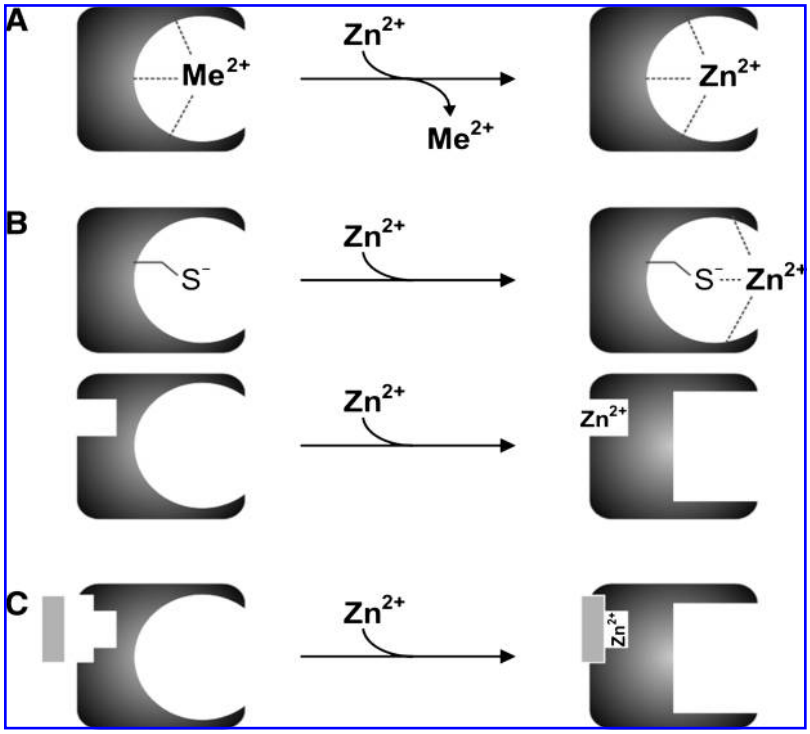

FIG. 5. Modes of interaction of Zn^{2+} with signaling proteins. (**A**) Zn^{2+} may compete with or substitute for metal ions crucial for the activity of signaling proteins. (**B**) Zn^{2+} may directly interact with signaling enzymes, resulting in either activation (*e.g.*, if the enzyme is a zinc enzyme present in its apoform), or inactivation of the enzyme (*e.g.*, if access of substrate to the active site is prevented by zinc binding). Both the binding to active sites (*upper*) or protein sites that allow for an allosteric effect of the metal ion on enzyme activity (*lower*) may be envisioned. (**C**) Zn^{2+} can enhance or attenuate the binding of ligands or regulators to proteins.

example, Zn^{2+} was demonstrated to interfere with Ca^{2+}/calmodulin signaling (61).

2. Zn^{2+} may directly interact with signaling enzymes, resulting in either activation or inactivation of the enzyme. Both the binding to active sites or protein sites that allow an allosteric effect of the metal ion on enzyme activity to occur may be envisaged (Fig. 5B). It was shown that Zn^{2+} can directly activate or (at higher concentrations) inhibit neuronal calcium/calmodulin-dependent protein kinase-II but may also interfere with binding of Ca^{2+}/calmodulin to the kinase (61).
3. Zn^{2+} can be envisioned either to enhance or to attenuate the binding of ligands to proteins (Fig. 5C), as was demonstrated for inhibition of an RNase by a hydroxyurea derivative being enhanced in the presence of Zn^{2+}, presumably by mediating inhibitor-RNase interaction (65).

The most prominent signaling effect of Zn^{2+} belongs to the second category: binding of Zn^{2+} to the metal-responsive transcription factor-1 (MTF-1) stimulates transcriptional activation of gene expression, including MTF-1–dependent metallothionein gene expression (47, 57, 62). However, several other signaling effects of Zn^{2+} have been described, some of which have been summarized elsewhere (10) and include signaling modules affected by exposure to zinc, such as receptor tyrosine kinase–dependent cascades [*e.g.*, the activation of the classic mitogen-activated protein kinases (MAPKs), ERK-1 and ERK-2, *via* epidermal growth factor receptor (EGFR) and MAPK/ERK kinase (MEK)-1/2 (83, 100), or cascades culminating in NF-κB– (37, 105) or Nrf2- (14) dependent effects].

Here we provide an overview of a signaling cascade that has been recently discovered to be stimulated in cells exposed to Zn^{2+}. Although such an exposure of cells to exogenous Zn^{2+} may not be directly comparable to an intracellular local release of zinc ions, it is a well-established model of inducing signaling effects that may well occur after the release of Zn^{2+} from intracellular stores to an extent rendering these effects experimentally accessible. Conclusions regarding the involvement of phosphatases in zinc-induced signaling were drawn from experiments with cells exposed to Zn^{2+} that were recently also demonstrated to hold true for signaling induced by Zn^{2+} release from intracellular stores (see later).

Zinc-induced phosphoinositide 3′-kinase/Akt signaling

We focus here on the activation of the phosphoinositide 3′-kinase (PI3K)/Akt signaling cascade by Zn^{2+}, which (although also considered a classic stress-response cascade, in that it mediates antiapoptotic and cytoprotective effects) is involved in the control of gene expression and insulin signaling and may thus provide a partial explanation for some insulin-like effects of Zn^{2+} that were identified previously. Exposure of cells, organs, or organisms to zinc ions has long been known to elicit insulin-like effects. For example, gluconeogenesis was demonstrated to be attenuated in isolated rat hepatocytes (92) and in rat renal cortex slices exposed to Zn^{2+} (81). Furthermore, the livers of rats exposed to sublethal doses of zinc salts had twice as much glycogen after 30 days as did those of rats held under control conditions (78). It was further demonstrated that Zn^{2+} prevents lipolysis in rat adipocytes (69) and stimulates glucose uptake in rat (21, 69) and murine adipocytes (91). It is hypothesized here that stimulation of the PI3K/Akt signaling cascade by Zn^{2+} contributes to these effects and that Zn^{2+} is capable of imitating insulin by stimulating this cascade in the absence of insulin.

The classic type Ia PI3Ks are typically activated *via* receptor tyrosine kinases (RTK) after stimulation of cells with growth

factors and catalyze the phosphorylation of inositol phospholipids in the 3′-position, thus generating membrane-bound 3′-phosphoinositides that serve the recruitment both of 3′-phosphoinositide–dependent kinases (PDK) and of the serine/threonine kinase Akt to the cell membrane, thus facilitating the activation of Akt by phosphorylation at Thr-308 and Ser-473. Downstream targets of Akt include the forkhead box transcription factors FoxO1a, FoxO3a, and FoxO4, phosphorylation of which by Akt results in inactivation and nuclear exclusion (7, 27). FoxO proteins are potent transactivators of genes involved in glucose metabolism [*e.g.*, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK)], and inhibition of hepatic glucose production by insulin was demonstrated to be mediated by Akt-dependent phosphorylation and inactivation of FoxO1a (6, 106). Modulators of PI3K-dependent FoxO inactivation (see Fig. 6) include SIRT1 (mammalian silent mating-type information regulation-2 homologue), a deacetylase connected with FoxO proteins, and PTEN (phosphatase and tensin homologue deleted on chromosome 10), a lipid phosphatase that cleaves off the 3′-phosphate from 3′-phosphoinositides, thereby inactivating the lipid products of PI3K and acting as an antagonist of PI3K.

Exposure to zinc salts elicits an activation of Akt in various cell types, including human skin fibroblasts, human hepatoma cells, and human cervix carcinoma cells (3, 36, 70, 95), thus imitating insulin in that respect. Interestingly, activation of Akt is similarly found in several cell types exposed to Cu^{2+}, which appears to be an even more potent stimulator of PI3K/Akt signaling than is Zn^{2+} (73). In both cases, activation of Akt occurs *via* PI3K, as PI3K activity is enhanced in cells exposed to Zn^{2+} (3, 20, 36, 55) or Cu^{2+} (73), and Akt activation is prevented by PI3K inhibitors (3, 36, 73, 95, 102).

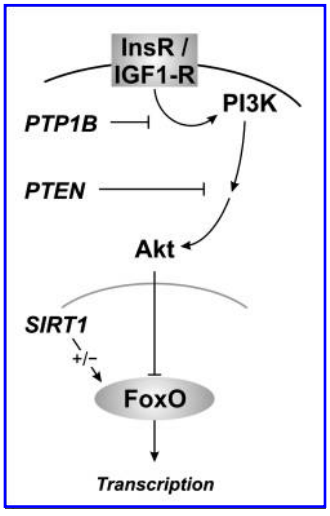

FIG. 6. Schematic representation of phosphoinositide 3′-kinase (PI3K)/Akt signaling. Stimulation of receptor tyrosine kinases such as the insulin receptor (InsR) or insulin-like growth factor-1 receptor (IGF1-R) results in recruitment and stimulation of PI3K and the production of 3′-phosphoinositides. PI3K-dependent activation of the serine/threonine kinase Akt may be counteracted by protein tyrosine phosphatases like PTP1B at the level of receptor tyrosine phosphorylation or by lipid phosphatases such as PTEN at the level of dephosphorylation of 3′ phosphoinositides (see text for further details). Akt phosphorylates and thereby inhibits FoxO transcription factors, the activity of which is also modulated by the NAD^+-dependent deacetylase SIRT1.

Regarding the Zn^{2+}-induced signaling cascade upstream of the PI3K/Akt module, it was demonstrated for human bronchial epithelial cells that PI3K/Akt activation occurs *via* Src-dependent EGFR stimulation (102). Additionally, the Zn^{2+}-induced release of active matrix metalloproteases (MMPs) to cleave heparin-binding EGF off the membrane was found to cause Zn^{2+}-induced EGFR activation (101). No primary targets of Zn^{2+} upstream of Src or MMP and heparin-binding EGF release have been clearly defined that would initiate the cellular signaling cascade leading to Akt activation, but several hints exist in the literature as to the nature of potential target structure(s) of Zn^{2+}, as discussed later.

Mechanisms involved in activation of the PI3K/Akt cascade by Zn^{2+}

A comparison of the capability of several metal ions of (patho-)physiologic relevance in human cells revealed that of all tested ions, only Cu^{2+}, Zn^{2+} and Cd^{2+} [and to some extent also Ni^{2+} (19)] were capable of stimulating Akt (5, 95). In all cases, activation occurs in a PI3K-dependent fashion, as it was abolished in the presence of PI3K inhibitors (5, 95). Although H_2O_2 (96) as well as several other ROS, including peroxynitrite (40) and singlet oxygen (84), were demonstrated to cause Akt activation in cells, the formation of ROS cannot be a prerequisite for activation of the PI3K/Akt cascade by metal ions, as both zinc and cadmium ions are present in biologic systems exclusively in their divalent forms and do not undergo any redox cycling. As expected, the exposure to neither of these ions leads to the formation of detectable levels of ROS in HeLa cells, whereas exposure to Cu^{2+} or Fe^{2+} (with the latter not causing Akt activation under the conditions chosen) does (5). Interestingly, of all metal ions tested, only those with a substantial affinity to thiol(ate)s cause a significant Akt phosphorylation. Of the proteins involved in PI3K/Akt signaling, members of the protein tyrosine phosphatase (PTPase) family are known targets of thiol-reactive compounds. The PI3K/Akt cascade is modulated by phosphatases of the PTPase family at the level of receptor tyrosine kinases, the tyrosine phosphorylation of which is reversed by PTPases like PTP1B, and at the level of lipids generated by PI3K, the level of which is controlled by PTEN (see earlier and Fig. 6). Almost all PTPase family phosphatases harbor a cysteine residue with an unusually low pK_a at their active sites (44, 72), which therefore is present predominantly in the thiolate form at physiologic pH, rendering it an exquisite target for various oxidants and electrophiles such as H_2O_2 (42, 93), peroxynitrite (89), or singlet oxygen (94). The role of PTPases in oxidant-induced PI3K/Akt signaling has been extensively discussed elsewhere (4). Zinc ions as well as copper ions are capable of inhibiting isolated PTPases, including PTP1B and human vaccinia H1-related phosphatase (VHR) [(28, 29, 35) and references therein]. Several examples exist of signaling effects elicited by attenuation of PTPase activity in cells exposed to Zn^{2+}; various PTPases were inhibited in Zn^{2+}-treated airway epithelial cells, including PTPases controlling EGFR phosphorylation (90). Inhibition of those PTPases by Zn^{2+} resulted in a net activation of EGFR (90). Similarly, insulin-receptor/

insulin-like growth factor receptor tyrosine phosphorylation was achieved in cultured glioma cells exposed to Zn^{2+} *via* inhibition of PTPases (28). PTEN protein levels are lost in Zn^{2+}-treated airway epithelial cells, resulting in a Zn^{2+}-induced Akt activation (103). The loss of PTEN was demonstrated to be due to proteasomal degradation rather than a direct enzyme inhibition (103); furthermore, PTEN mRNA levels were shown to be downregulated after stimulation of cells with Zn^{2+} (103).

Consequences of metal ion–induced activation of the PI3K/Akt cascade

Several Akt substrates are phosphorylated in cells exposed to Zn^{2+}or Cu^{2+}, such as glycogen synthase kinase-3 (GSK-3) and FoxO transcription factors (95). Both GSK-3 and FoxO proteins are known players in carbohydrate metabolism. Akt inactivates GSK-3 by phosphorylation (Ser-21 and Ser-9 in GSK-3α and GSK-3β, respectively), thereby stimulating glycogen synthesis because GSK-3 phosphorylates and inactivates glycogen synthase (17). Similarly, FoxO transcription factors are inactivated by Akt-dependent phosphorylation, resulting in nuclear exclusion of phosphorylated FoxO proteins and an attenuated expression of FoxO target genes, such as those of the gluconeogenesis enzymes PEPCK and G6Pase (6, 7). Both Zn^{2+} and Cu^{2+} imitated insulin in causing the nuclear exclusion of an overexpressed FoxO1a-EGFP fusion protein in human hepatoma cells (5, 95), suggesting that expression of FoxO target genes is downregulated under these conditions.

In summary, the PI3K/Akt cascade, in addition to its role in regulating apoptosis and proliferation, is a major mediator in insulin signaling, and a prominent role of PTPases in the activation mechanism of the cascade by Zn^{2+} is hypothesized. Activation of Akt by Zn^{2+} (like insulin) results in modulation of FoxO-family transcription factors in cultured mammalian cells. Zn^{2+}- (and Cu^{2+}-) induced activation of PI3K and Akt as well as the resulting regulation of downstream effectors not only is an example of ligand (*e.g.*, insulin)-independent activation of a signaling cascade by stressful stimuli but also may provide a molecular explanation for the insulin-mimetic effects of these ions. The insulin-mimetic activities of Zn^{2+} may be exploited pharmacologically in the future: insulin-mimetic low-molecular-mass Zn^{2+} complexes are indeed being designed for use in humans (71, 82), some of the complexes were demonstrated to initiate insulin-like signaling (*i.e.*, PI3K-dependent activation of Akt and phosphorylation of GSK-3 as well as GLUT4 translocation to the cell membrane) in cultured adipocytes (8, 71). Further work is required to answer the question whether the application of Zn^{2+} complexes will be a suitable alternative to presently available insulin-mimetic therapeutic approaches.

Signaling effects of intracellularly released Zn^{2+}

It was hypothesized earlier that PTPase inactivation or downregulation of PTPase levels may be a major mediator of Zn^{2+}-induced effects on PI3K/Akt-dependent signaling. This hypothesis is the result of data from experiments with cells exposed to exogenous Zn^{2+} or to membrane-permeant zinc complexes. In line with these data, it was recently demonstrated with human mast cells that Zn^{2+} is released from intracellular stores on stimulation of cells by crosslinking the high-affinity IgE receptor. As a result of zinc release, cellular stress–signaling cascades are stimulated, including ERK and JNK mitogen-activated protein kinases, the duration of activation of which is shortened and prolonged by addition of a Zn^{2+}-chelator and by a zinc ionophore, respectively (104). These authors also demonstrated that general cellular PTPase activity is significantly reduced in stimulated cells because of an intracellular Zn^{2+} release (104). Figure 7 summarizes these findings on Zn^{2+} release on exposure of cells to stressful stimuli, and this very Zn^{2+} affects signaling processes by interfering with protein tyrosine phosphatase activity.

A severe consequence of signaling induced by intracellularly released Zn^{2+} was demonstrated for neuronal cells: release of Zn^{2+} from cellular binding sites induced by an exposure to peroxynitrite initiated an activation of $p38^{MAPK}$, resulting in apoptosis and cell death (107). Similarly, an important role of intracellularly released Zn^{2+} in inducing cell death was demonstrated for a neuron/microglia co-culture system in which activated microglial cells not only caused intraneuronal zinc release but also induced zinc-dependent neurotoxicity, likely involving ASK1, a stress kinase upstream of $p38^{MAPK}$ (43). In summary, Zn^{2+} derived from intracellular stores or binding sites is capable of eliciting signaling effects in various cell types. Both the exact pathways targeted and consequences of signaling cascades being stimulated appear to vary with the cell type affected. Finally, a major conclusion regarding methods of zinc-induced signaling analyses may be drawn from the reported data: the same pathways demonstrated to be stimulated by intracellularly released zinc ions were also shown to be activated in cells exposed to exogenous Zn^{2+}, suggesting the suitability of the latter approach for the general analysis of zinc signaling.

Diseases and Disorders Characterized by an Intracellular Zinc Release

Whereas eukaryotic cells under physiologic conditions maintain a highly reducing redox environment with GSH/GSSG ratios of approximately 100:1 (1, 33), disorders associated with oxidative or nitrosative stress, such as neurodegenerative diseases, acute or chronic inflammatory reactions, or cancer, are often characterized by high levels of oxidizing species and an impaired antioxidant defense, which results in a disturbed intracellular redox balance. Neurons appear to be particularly vulnerable to attack by ROS, as their GSH content is low, their membranes contain high amounts of polyunsaturated fatty acids, and brain metabolism requires substantial quantities of O_2. The brain contains high levels of Zn^{2+} in synapses of cells in certain brain areas, but the role of zinc in modulating neurophysiological processes is not yet fully understood (for review, see ref. 23). To date most studies investigating an intracellular zinc release in human diseases have been performed with brain disorders. In addition, infusion of various NO donors into the brain has been shown to trigger Zn^{2+} release (16, 24).

Traumatic brain injury

In animal models of traumatic brain injury (TBI), an increase of free Zn^{2+} has been found several hours after the impact (18, 32, 87). However, it is unclear at present whether this Zn^{2+} is synaptically released from adjacent neurons or whether the intracellular free Zn^{2+} is released from cytosolic

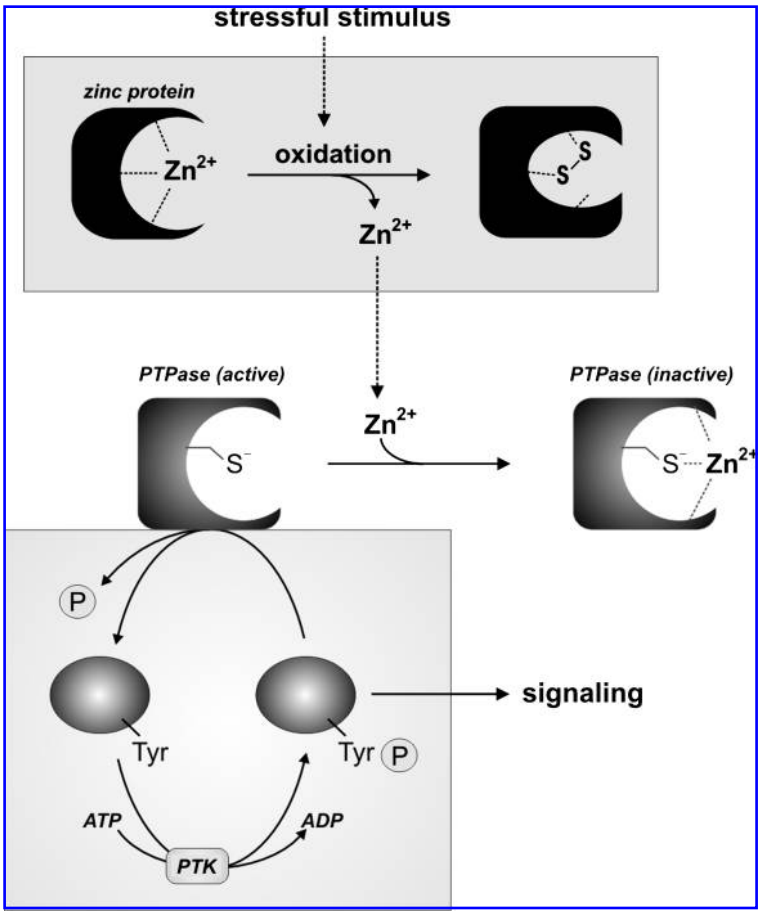

FIG. 7. Signaling induced by zinc released from zinc proteins. Stressful stimuli that are paired with a generation of reactive oxygen species will cause the oxidation of proteins, including zinc proteins with Zn^{2+} chelated by cysteinyl thiols, resulting in the local release of Zn^{2+}. Phosphotyrosine phosphatases (or protein tyrosine phosphatases, PTPases) are susceptible to inhibition by Zn^{2+} as their active-site cysteine thiolate avidly interacts with soft ions such as Zn^{2+}. PTPases act as counterparts of protein tyrosine kinases (PTKs): the latter catalyze phosphorylation of tyrosyl residues, whereas the former catalyze phosphotyrosyl dephosphorylation. Inactivation of PTPases will cause equilibria to shift toward a net increase in tyrosyl residue phosphorylation. As many signaling proteins are activated by tyrosine phosphorylation, this will result in enhanced signaling. In the case of PI3K/Akt signaling, receptor tyrosine kinases are examples of proteins activated by tyrosine phosphorylation.

proteins within the cells because of oxidative or nitrosative stress, or both (for review, see ref. 24).

Acute lung injury is commonly seen in comatose victims with TBI. In rats subjected to TBI, the levels of the proinflammatory cytokines TNF-α and IL-8 were elevated in the lung for several days. In parallel, pulmonary free Zn^{2+} was significantly increased (108).

Cerebral ischemia

Ischemic stroke is one of the most pervasive life-threatening neurologic conditions. It has repeatedly been shown that zinc synaptically released from a subset of glutamatergic terminals of neurons after ischemia likely promotes translocation and accumulation of Zn^{2+} in neighboring neurons. However, oxidative or nitrosative stress as well as acidosis during or after ischemia may additionally promote release of intracellularly bound Zn^{2+} (for review, see ref. 25) and references therein].

Alzheimer's disease

Alzheimer's disease (AD), the most common senile dementing disorder, is characterized pathologically by the accumulation of Aß-amyloid protein, neurofibrillary tangles, and neuropil threads. Lesions present in the brain of AD patients typically show characteristics of severe oxidative stress (*e.g.*, ROS-mediated protein oxidation, lipid peroxidation, and DNA damage) (for review, see ref. 13). Zinc, copper, and iron ions have been implicated as possible pathogenic agents in AD because of high concentration gradients of these metals in the cortex, hippocampus, and the cortical vasculature, brain regions that are severely affected in AD. All three cations are significantly elevated in AD senile plaques (64). The Zn^{2+} in these plaques has been characterized as "histochemically reactive" (88). Free Zn^{2+} was also found to be highly concentrated in the somata of numerous individual neurons in the hippocampus of AD patients, which has never been found in human, apparently healthy, subjects (88). These results suggest a disease-driven oxidative stress–induced Zn^{2+} release in AD patients (for reviews, see refs. 12 and 15).

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a neuropsychiatric complication of acute or chronic liver failure. In fulminant hepatic failure, astrocyte swelling contributes to the development of a clinically overt cerebral edema, whereas HE in chronic liver disease is characterized by a low-grade cerebral edema with consequences for astrocyte function and glioneuronal communication. The mechanisms involved in HE still remain to be defined. Nonetheless, it is well recognized that NH_3/NH_4^+, which after liver failure is only insufficiently metabolized *via* the hepatic urea cycle, is a major factor in HE pathogenesis, in that astrocytes represent a major target of its CNS toxicity. One consequence of astrocyte swelling is oxidative and nitrosative stress (for reviews, see refs. 79 and 85). In cultured

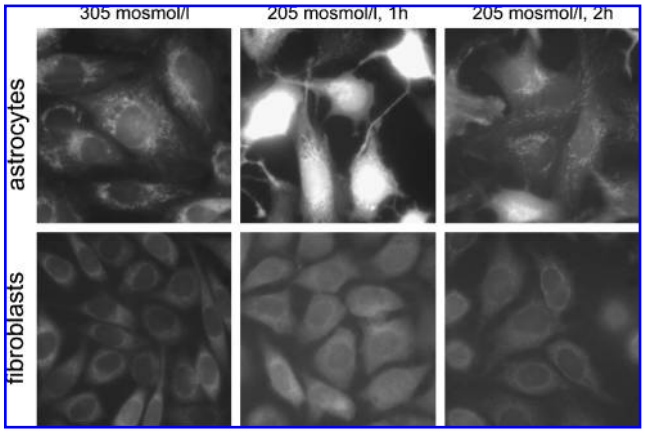

FIG. 8. Effects of hypoosmolarity on the intracellular zinc homeostasis in astrocytes and fibroblasts. The human astrocyte cell line MOG-G-CCM and the murine fibroblast cell line L929 were exposed to normoosmotic (305 mOsm/L) or hypoosmotic (205 mOsm/L) DMEM for 1–24 h and subsequently stained for 30 min with 10 μM Zn^{2+}-specific fluorophore zinquin ethyl ester. Live cells were then examined under a fluorescence microscope. Although hypoosomolarity induces a rapid and reversible intracellular Zn^{2+} release in the astrocyte cell line with a maximum at about 1 h, this effect is not found in the fibroblasts.

astrocytes, hypoosmolarity induces a rapid, transient (Fig. 8) and reversible intracellular Zn^{2+} release (55). This zinc release in astrocytes depends on Ca^{2+}-dependent enzymatic NO synthesis, presumably derived from Ca^{2+}-activated neuronal NO synthase (nNOS). In contrast, hypoosmolarity does not induce any intracellular Zn^{2+} release in fibroblasts, a cell type that lacks nNOS (Fig. 8). NH_3 and TNF-α both induce astrocyte swelling, and both, like diazepam, also induce an intracellular Zn^{2+} release in astrocytes (55). Studies investigating a possible release of Zn^{2+} in the brain during HE must still be performed.

Other diseases or disorders

Several proteins involved in the human DNA-repair system contain zinc fingers or related zinc-thiolate motifs, the integrity of which is indispensable for the proper function of these proteins. Among these are XPA, RPA, DNA damage-recognition proteins of the nucleotide excision-repair pathway, the single-strand repair protein PARP, and the breast and ovarian cancer susceptibility factor BRCA1, which is required for transcription-coupled repair. Damage of zinc fingers in DNA-repair proteins has been suggested to be a molecular mechanism in carcinogenesis (for reviews, see refs. 11 and 99).

In addition, the tumor suppressor p53 contains a zinc finger essential for DNA binding. P53 plays a dual role within cells, both as a regulator of cell-cycle arrest under conditions of mild DNA damage and as a trigger for apoptosis after severe DNA damage. The gene encoding p53 is frequently mutated in a wide variety of human cancers, and most of these mutations are missense mutations scattered in the region of the DNA-binding domain, emphasizing the role of sequence-specific DNA binding as the main biochemical property of p53 in tumor suppression. Various conditions like oxidative or nitrosative stress have been shown to disrupt the zinc finger in p53, but whether this is a significant mechanism in carcinogenesis is not known (30, 52).

Aging is a universal phenomenon characterized by pathologic features such as oxidative stress, alterations in cell metabolism, accumulation of misfolded proteins, and nucleic acid damage. In the brain, aging is associated with progressive neuronal loss, cognitive impairment, and enhanced susceptibility to neurologic diseases. It has been suggested but not yet proven that zinc establishes a link between oxidative stress and brain aging (for review, see ref. 22).

First evidence has been provided that the zinc homeostasis in a cell might represent a common cellular target for different papillomaviruses (58). Whether this holds true for other viruses as well is not known.

Conclusions

Although primarily demonstrated for the brain, it can be hypothesized that almost all inflammatory reactions accompanied by oxidative or nitrosative stress or both and resulting in a severe shift of the intracellular redox balance to a more oxidative state will lead to a cellular zinc dyshomeostasis with increased intracellular concentrations of non–protein-bound Zn^{2+}. Further work on causes and consequences of a disturbed zinc homeostasis in cells and organs will have to answer several open questions.

1. Do differences exist in the susceptibility of different kinds of zinc fingers to oxidative or nitrosative stress, and what are the functional consequences of these different susceptibilities?
2. What happens with zinc-finger proteins that have released zinc; are these proteins degraded, or are zinc fingers restored by the cells?
3. Why do we find free zinc in nuclei under certain conditions, sometimes predominantly in the nucleoli? Is this nuclear free zinc imported from the cytosol or from other cell organelles or released from nuclear zinc proteins, and what are the functional consequences of free zinc in these compartments?
4. What are the major targets of Zn^{2+} in cellular signaling networks? Although much is known about Zn^{2+} activating signaling cascades, we are only at the beginning of understanding whether free intracellular Zn^{2+} acts as a significant signaling species during human disorders or diseases or whether intracellular Zn^{2+} release is just an epiphenomenon accompanying a disturbed cellular redox balance.

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Abbreviations

AD, Alzheimer's disease; EGFR, epidermal growth factor receptor; G6Pase, glucose-6-phosphatase; GSH, glutathione; GSK, glycogen synthase kinase; HE, hepatic encephalopathy;

IR, infrared; MAPK, mitogen-activated protein kinases; MMP, matrix metalloproteases; MTF-1, metal-responsive transcription factor-1; NOS, nitric oxide synthase; PDK, 3′-phosphoinositide–dependent kinase; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositide 3′-kinase; PTPase, protein tyrosine phosphatase; PTPEN, phosphatase and tensin homologue deleted on chromosome 10; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; SIRT1, mammalian silent mating–type information regulation-2 homologue; TBI, traumatic brain injury; UV, ultraviolet; VHR, vaccinia H1–related phosphatase.

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Address reprint requests to:
Dr. Klaus-D. Kröncke
Institute of Biochemistry and Molecular Biology I
Heinrich-Heine-University of Düsseldorf
Universitätsstr. 1
D-40225 Düsseldorf
Germany

E-mail: kroencke@uni-duesseldorf.de

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