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Zinc and the modulation of redox homeostasis

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Abstract

Zinc, a redox inactive metal, has been long viewed as a component of the antioxidant network, and growing evidence points to its involvement in redox-regulated signaling. These actions are exerted through several mechanisms based on the unique chemical and functional properties of zinc. Overall, zinc contributes to maintain the cell redox balance through different mechanisms including: i) the regulation of oxidant production and metal-induced oxidative damage; ii) the dynamic association of zinc with sulfur in protein cysteine clusters, from which the metal can be released by nitric oxide, peroxides, oxidized glutathione and other thiol oxidant species; iii) zinc-mediated induction of the zinc-binding protein metallothionein, which releases the metal under oxidative conditions and act per se scavenging oxidants; iv) the involvement of zinc in the regulation of glutathione metabolism and of the overall protein thiol redox status; and v) a direct or indirect regulation of redox signaling. Findings of oxidative stress, altered redox signaling, and associated cell/tissue disfunction in cell and animal models of zinc deficiency, stress the relevant role of zinc in the preservation of cell redox homeostasis. However, while the participation of zinc in antioxidant protection, redox sensing, and redox-regulated signaling is accepted, the involved molecules, targets and mechanisms are still partially known and the subject of active research.

Introduction

Multiple biological macromolecules and physiological cell events involve zinc as a structural component or as a major regulator. As a consequence, zinc is a metal that is essential for several aspects of normal human development [1] and health [2].

It is difficult to build a comprehensive list of the biological actions of zinc. This is due in part to the large number of proteins and enzymes that contain zinc, which explains the relevance of zinc in numerous cell processes. Furthermore, different zinc biological actions can superimpose, and the regulation of zinc at different levels/molecules can converge in one biological action. Fig. 1 shows a very general list of major zinc biological actions. Based on the Protein Data bank it was recently estimated that zinc is a component of more than 2700 enzymes, including hydrolases, transferases, oxido-reductases, ligases, isomerases and lyases [3]. Aproximately in 70% of these enzymes, zinc has a catalytic function, but it can also have a structural role, act as a substrate, or as a regulator of enzyme activity [3]. This large number of zinc enzymes explains the requirement of zinc in DNA, RNA, protein and lipid synthesis. Zinc also has a major role in the preservation of genomic stability [4].

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This action involves, among other factors, the antioxidant effects of zinc, its participation in DNA repair and in the DNA damage response, and in the synthesis of molecules (e.g. methionine) that are required for DNA methylation [4]. Zinc can participate in neurotransmission [5], being stored and released from vesicles located at the synaptic endings of select glutamatergic neurons. This review will subsequently discuss aspects of other listed roles of zinc in biological systems: a second messenger action of rapidly available zinc pools, indirect and direct modulation of gene transcription, the regulation of cell redox homeostasis and redox-sensitive signals, and the requirement of zinc to preserve tubulin polymerization dynamics and function.

Zinc only exists in biological systems as Zn^{2+} given its complete d shell. However, zinc deficiency is often associated with a condition of oxidative stress. This can be explained by several mechanisms underlying zinc actions which will be discussed in this review. Overall, a large amount of zinc proteins that are modulated by or contain zinc can directly or indirectly affect the cell redox balance. The events involved in the regulation by zinc of the cell oxidant/antioxidant balance are multiple and interconnected, and although not completely understood, they are driving intense and challenging new research. Some of those mechanisms involve: i) the modulation of oxidant production and oxidative damage by cellular zinc availability [6]; ii) the capacity of zinc to reversibly bind to cysteine and histidine residues in zinc protein motifs which are proposed to act as redox switches [7]; iii) the direct and indirect involvement of the main cellular zinc binding protein, metallothionein (MT), which can per se scavenge oxidants, or release zinc in a redox-regulated manner [8, 9]; iv) the regulation by zinc of glutathione (GSH) metabolism, and of the overall thiol redox status [10]; v) a direct or indirect capacity of zinc to regulate the activity of proteins involved in cell signaling. Although zinc release from proteins can potentially modulate different cell signals and events, this review will focus on those related to the maintenance of cell redox homeostasis.

Cellular zinc pools

Eukaryotic cells contain large amounts of zinc (approximately 100 μM). Intracellular zinc pools can be classified in: the zinc tightly bound to macromolecules, zinc enclosed in high amounts inside vesicles (e.g. synaptic vesicles in neuronal glutamatergic terminals); a pool of exchangeable zinc, and a pool of zinc bound to MT. These pools are tightly regulated by specific transport proteins, zinc responsive transcription factors, and by MT and other zinc-sensing proteins that can dynamically bind and release zinc [11].

Zinc can be found in proteins either as a structural component, or in the catalytic site of select enzymes. Zinc is also part of particular protein domains that allow selective protein-protein interactions, and interactions between the protein and other macromolecules such as DNA and RNA (reviewed in [12]). Proteomic analysis looking for proteins containing zinc binding patterns and domains observed that approximately 10% of proteins in eukaryotic cells contain zinc [13].

Most of cellular zinc is bound with high affinity to proteins and other cellular components including GSH, cysteine, histidine and diphosphate molecules. On the other hand, the amount of cellular exchangeable zinc (also named “free”, “labile”, “loosely bound” zinc) is relatively very small. The use of probes that can be targeted to different cellular compartments has provided a significant advancement in the knowledge of the cellular localization and availability of the free zinc pools. Using an excitation ratiometric fluorescent biosensor based on carbonic anhydrase, Bozym et al. [14] measured 5–10 pM zinc in the cytoplasm and nucleus of resting PC12 rat pheochromocytoma cells. In primary cultures of rat hippocampal neurons, mitochondria were found to be a relevant reservoir of

zinc, containing 180 nM zinc in the resting state [15]. Mitochondrial zinc is released to the cytosol when cells are stimulated with glutamate, and taken up by mitochondria upon glutamate and exogenous zinc supplementation, revealing a rapidly available mitochondrial zinc pool that could be important in signaling events [15]. In pancreatic 3 cells, while the measured cytosolic zinc concentration is 0.4 nM, in the secretory vesicles free zinc ranges between 1 and 10 μM [16]. In HeLa cells, the concentration of zinc in the endoplasmic reticulum and Golgi is very low (0.9 and 0.6 pM, respectively), increasing when cytosolic zinc increases, and being dynamically affected by variations in calcium homeostasis [17]. Fluctuations in cellular labile zinc are observed in association with cell apoptosis [18], meiosis [19], and proliferation [20]. With regard to the later, intracellular zinc concentrations vary in PC12 cells at different stages of the cell cycle [20], an event that is affected when zinc availability is low [21].

The above evidence shows the existence of different pools of free zinc that can vary dynamically, supporting a major role for the readily available zinc pools in the regulation of cell signaling, cell function and fate. In this regard and as discussed below, zinc is increasingly viewed as a second messenger. Given that free zinc concentrations vary with changes in the oxidant/antioxidant cell balance, this zinc pool can behave as a redox sensor.

The antioxidant actions of zinc

The occurrence of oxidative stress in association with a condition of zinc deficiency, and the prevention of oxidative damage by zinc supplementation has been observed in different cells and tissues [22–31]. A decrease in zinc availability is associated with an increase in cellular oxidants [6, 32], alterations in the antioxidant defense components [26, 28, 33], and increased tissue oxidation parameters [10, 25, 28, 30]. These and other similar findings generated the concept of zinc as part of the cell/tissue antioxidant defense network. The underlying mechanisms to such “antioxidant action” could encompass both cell or tissue-specific mechanisms, and others that are of general nature. Fig. 2 summarizes described mechanisms through which zinc can exert antioxidant actions in biological systems.

Zinc in the modulation of oxidant production

Zinc deficiency is associated with high steady state levels of nitric oxide (NO) and H_2O_2 in PC12 cells, H_2O_2 in human IMR-32 neuroblastoma cells [34], and of nitric oxide (NO) in glioblastoma cells [30]. There is very limited information on the mechanisms underlying a direct inhibitory effect of zinc on oxidant production. We recently described that the modulation by zinc of the N-methyl-D-aspartate (NMDA) receptor (NMDAR) affects NO and superoxide anion production in neuronal cells, as summarized in Fig. 2. The NMDAR is inhibited by zinc [35, 36], being highly sensitive to minor changes in zinc concentrations [37, 38]. Zinc binds to two independent low affinity and high affinity sites [39, 40] located inside and outside, respectively, of the receptor channel pore. NMDAR activation leads to the opening of the pore and to the transport of calcium and other cations from the extracellular space into the cytosol. The incubation of PC12 cells, differentiated into a neuronal phenotype, in zinc depleted medium leads to a rapid increase in the concentration of cellular calcium [6]. This increase is prevented by the specific NMDAR antagonist MK-801, which also prevents the increase in ROS and RNS. Accordingly, increases in calcium were also associated with zinc deficiency in human neuroblastoma IMR-32 cells [41] and in hippocampal slices from zinc deficient mice [42]. In zinc deficient neuronal cells, the NMDAR-dependent increase in calcium promotes the activation of NADPH oxidase via activation of protein kinase C, and the calcium-calmodulin-dependent activation of nitric oxide synthase [6]. The activation of these enzymes leads to an increased production of ROS and RNS in the zinc deficient neuronal cells (Fig. 3) [41]. The activation

of NADPH oxidase as a consequence of zinc deficiency was also observed in hepatic stellate cells [32].

The capacity of neurons to respond to small changes in extracellular zinc levels with a regulated production of NO, superoxide anion and derived species, which acting as second messengers, can be of relevance in the regulation of physiological processes. On the other hand, if zinc levels are chronically low, overactivation of the NMDAR can lead to oxidative stress and to alterations in neuronal function and fate. The inhibition of the NMDAR by zinc is one example of an indirect "antioxidant" action exerted through the regulation of a receptor protein, of intracellular calcium levels, and subsequently of oxidant production. The capacity of zinc to modulate the activity of numerous proteins could underlie an indirect action of zinc modulating ROS and RNS generation.

Zinc-redox active metals interactions

Another indirect mechanism to regulate oxidative damage of cell components and oxidant generation, is the capacity of zinc to compete with redox active metals (iron, copper) for membrane binding sites. Membrane-associated iron and copper can catalyze the generation of radicals from lipid peroxides. Thus, replacement of these metals by the redox inactive zinc would prevent the formation of highly reactive oxidants. While not affecting membrane physical properties, zinc preferentially binds to negative charges in liposomes, preventing Fe^{2+}-initiated lipid oxidation [43]. The competition with Fe^{2+} for membrane binding sites as a mechanism of zinc-mediated antioxidant action is supported by a positive correlation between the degree of lipid oxidation and the amount of Fe^{2+} bound to the membrane, which is ultimately determined by the concentration of zinc (Fig. 4A). In support of this mechanism, zinc is not effective preventing lipid oxidation initiated by lipid- or water-soluble azo compounds or by ultraviolet radiation [43]. Zinc also prevents the oxidation of negatively charge liposomes triggered by aluminum. Although without redox capacity, aluminum can enhance Fe^{2+}-induced lipid oxidation through the promotion of changes in membrane physical properties that increase the efficiency of the oxidative chain reaction [44]. On the other hand, zinc is not effective preventing Fe^{2+}-induced oxidative inactivation of the enzymes glutamine synthase and glucose-6-phosphate dehydrogenase [44]. Fe^{2} rapidly oxidizes, via hydroxyl radical formation, specific aminoacids in the active sites of both enzymes irreversibly inactivating them [45, 46]. Thus, at least in these particular enzymes, zinc is unable to compete with Fe^{2} for protein binding sites located in the catalytic domain.

Very importantly, zinc and hydrophilic ((−)-epicatechin) and lipophilic (3-tocopherol) antioxidants act synergistically in the prevention of Fe^{2+}–triggered liposome oxidation [43] (Fig. 4B). These findings support a role for zinc as a component of the cell antioxidant network through the protection of membrane lipids from redox active metals-induced oxidation.

Free zinc, zinc-thiol interactions, and associated antioxidant effects

As further discussed below, the capacity of zinc to bind to thiol groups is another relevant mechanism which contributes to its antioxidant action. Zinc binds to sulfhydryl groups and protects them from oxidation. Zinc bound to thiols can be released by NO, H_2O_2, oxidized GSH, and other oxidant species, and participate in antioxidant protective responses. In this regard, a decrease in the cellular reduced environment is associated with an increase in the cellular content of labile zinc [47]. On the other hand, metals with affinity for thiols (e.g. Cd^{2+}, Pb^{2+}, Hg^{2+}) [48] can compromise the amount of zinc bound to thiols which is readily available.

The pool of free zinc released under different conditions, including oxidative stress, is relevant in sustaining the cell redox homeostasis through different mechanisms including the capacity of zinc: to regulate antioxidant protective responses (e.g. transcription factor NF-E2-related factor 2 (Nrf2) [49], to prevent the binding of redox active metals to target molecules [43]; to prevent the activation of oxidant-generating molecules [6], and to upregulate the synthesis of the cysteine-rich thionein, which generate additional thiol groups with capacity to interchange zinc or to act as a direct oxidant scavenger [50]. In support of the above, zinc deficiency both in cells in culture and in animal models increases the susceptibility to metal–induced oxidative stress [51]; causes the activation of redox signaling [52], and reduces the capability of cells to respond to prooxidant stressors by upregulating cellular GSH [53]. As discussed below, a role for zinc in the upregulation of antioxidant genes via Nrf2 can be a major mechanism underlying the antioxidant actions of zinc [53–55].

The above evidence support the concept that, although redox inactive, zinc is a major component of the cell antioxidant network that maintains homeostasis. This review will subsequently focus on the interactions of zinc with protein and non protein (GSH) thiols, and on the relevance of these interactions to the cell redox balance and to redox signaling.

The interactions of zinc with protein thiols and redox sensing

Ten percent of the human genome is estimated to encode for zinc proteins [56], although this number is proposed to be significantly underestimated [12]. The interactions of zinc with protein ligands occur at the imidazole nitrogen in histidine, carboxylate oxygen in aspartic and glutamic acid, and thiol sulfur in cysteine. The nature, dynamic, and influence of these interactions on the functional role of zinc has been thoroughly reviewed before [12]. This review will only address the interactions of zinc with thiols given that they are central to the proposed role for zinc in redox sensing.

Different arrangements of zinc binding domains exist in proteins. Among them, zinc finger domains have major physiological relevance, as demonstrated by their presence in 3% of the genes in the human genome [57]. Protein zinc finger domains contain zinc coordinated tetrahedrally to cysteine and histidine residues, obtaining a chemical distinct identity through variations in select aminoacids that provide specificity in the recognition of DNA and RNA [58]. Besides the classical involvement of these protein domains in the regulation of transcription, DNA replication and repair, several other relevant regulatory functions have been described. Among them, zinc fingers have been proposed to act as biological redox switches sensing the cell redox status and triggering select responses (reviewed in [7]). A similar role is proposed for several other zinc binding domains, such as ribbons, gag knuckle, treble clef finger, and RING fingers, with a vast majority having cysteine as a coordinating aminoacid.

Zinc is highly abundant, has no redox capacity, its association with sulfur in cysteines is stable under the reducing environment of the cell, and the nature of sulfur-zinc binding allows a rapid association/dissociation of the metal. All these characteristics support the redox sensitive regulatory capacity of labile zinc [7]. Although zinc is not redox active, the redox nature of thiol groups that upon oxidation causes zinc release from MT, and essentially from any protein containing cysteine-zinc clusters [59], confers zinc a redox activity.

As summarized in Fig. 5, several molecules can release zinc from sulfur binding domains in MT and other zinc proteins, and this can have direct and indirect consequences on protein function and on the regulation of cellular processes. The reaction of NO, H_2O_2, oxidized GSH, and other oxidant species with zinc sulfur clusters can release zinc [9, 60] and confer a

dynamic nature to thiol-bound zinc [61]. However, this may not occur in all oxidative modifications of zinc clusters. In this regard, while nitrosative stress-induced damage of the 1alpha,25-dihydroxyvitamin D(3) (1alpha,25(OH)(2)D(3)) receptor (VDR) and retinoid X receptor (RXR) zinc fingers was repaired in breast adenocarcinoma cells, oxidative stress (H_2O_2, $O_2^{\cdot -}$, tert-butyl hydroperoxide, singlet oxygen)-mediated damage caused their irreversible destruction [62]. Metals can also release zinc from zinc fingers. Replacement of zinc from zinc fingers by different metals (e.g. Al^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}) has been described for several proteins (reviewed in [63]). Such replacements, can either not affect or modify the protein function (e.g. altered binding of transcription factors to DNA), or lead to cysteine oxidation.

MT, a family of low molecular weight and cysteine-rich proteins (20 cysteine residues from a total of 61–68 aminoacids), is the most abundant zinc storage protein. Although MT mostly binds zinc, it can also bind several other metals (e.g. $Cu^{1+/2+}$, Cd^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+}, Co^{2+}). MT contains zinc cluster domains with varying binding affinity and is a major regulator of cell free zinc content [59]. Cysteine thiolates in MT bind zinc tetrahedrally forming Zn_3S_9 and Zn_4S_{11} clusters. It has been widely demonstrated that MT zinc can be interchanged between the 3 and 3 domains of MT, and also between MT and proteins (enzymes, transcription factors) that contain zinc clusters (reviewed in [59]) The zinc-thiol interactions in MT are redox regulated and zinc can be released upon reaction with NO, H_2O_2, and other oxidant species (reviewed in [9]). The low molecular weight, cysteine-containing, and abundant GSH can also be a source of rapidly available zinc. Oxidized GSH interacts with MT through an interchange zinc-thiol/disulfide causing zinc release [64]. The presence of interchangeable zinc in MT, the multiple situations and molecules that promote zinc release from MT, and findings that MT dynamically translocates within cell compartments that require additional zinc amounts [65, 66] point to a major role of MT as a source of rapidly available zinc. This and the direct antioxidant action of MT is further supported by findings in MT I and II knock out mice. Genetic deficits of MT I and II increase the susceptibility to the deleterious effects of both zinc deficiency and toxicity [67]. Furthermore, embryonic cells from MT I and II knock out mice are more susceptible to oxidative stress [68].

Oxidation and other modifications of zinc-thiolate clusters may not affect protein function, exert a regulatory action on the protein activity, or cause a loss of protein function/activity. The released zinc would be available to modulate cell redox homeostasis, cell signaling, and as a consequence to regulate different cellular events including the upregulation of antioxidant defenses, cell proliferation, survival and differentiation [21, 54, 63].

Zinc, redox sensing, and cell signaling

There is a general agreement that zinc can have an important role in the modulation of cell signaling (Reviewed in [11, 69]). However, the underlying mechanisms, the zinc pools involved, the effects of zinc in different cell types, and other aspects of this modulation are complex in nature and need further investigation. For example, cellular oxidative stress and upregulation of select signaling cascades (e.g. protein kinase B (Akt)) are caused by both zinc deficiency and excess ([21, 70]. This review will subsequently focus on potential targets underlying the influence of zinc on redox signaling.

The interactions of zinc with ROS and RNS in the regulation of cell signaling can occur at different levels (summarized in Fig. 6). As previously discussed, modifications of zinc-thiol bonding by ROS and RNS can release zinc [9], which may or may not affect the activity of the protein. If the protein is a transcription factor, it can modulate its capacity to bind to DNA (e.g. transcription factors Sp1, Egr-1, glucocorticoid receptor) [71]; if the protein is a

kinase or phosphatase involved in signaling, it can activate or inhibit the pathway; in the case of tubulin, thiol oxidation can affect the nuclear transport of signaling proteins into the nucleus. The released zinc can also act at different levels, including a direct modulation of signaling cascades, the regulation of GSH homeostasis, and the activation/inhibition of kinases and phosphatases (Fig. 6).

RNS, ROS, Zn and signaling

It has been proposed that NO-mediated release of zinc bound to sulfur clusters, can be one of the mechanisms through which NO can regulate cell signaling (Reviewed in [72]). In vitro exposure to NO sources causes nitrosylation of cysteine thiols, disulfide formation, and zinc release from MT [60], and other zinc binding proteins [73]. Once zinc is released, the capacity of the protein to regulate transcription can be modified. For example, NO-mediated zinc release from LAC9, a bacterial transcription factor containing zinc finger motifs, causes the inhibition of LAC9 binding to DNA [60]. The in vitro treatment with NO donors of the VDR and RXR receptors also reversibly affects their binding to DNA 1alpha,25(OH)(2)D(3) response elements [73].

A major target of zinc-dependent regulation is the Kelch-like ECH-associated protein (Keap1)/Nrf2 stress sensing system. Nrf2 is activated by electrophiles, and also by other biotic and physical stimuli (e.g. heat, U.V., sheer stress) [74]. Nrf2 activation is modulated by Keap1, which is a sensor of environmental stress. Under basal conditions, Nrf2 resides in the cytosol bound to Keap1, interaction that favors Nrf2 degradation by the proteasome. Under stress conditions this downregulation decreases, Nrf2 levels increase, and its nuclear translocation and binding to DNA antioxidant response elements promotes the expression of antioxidant and drug metabolizing genes [75, 76]. It has been recently shown that Keap1 senses stress through three different elements that respond to NO, zinc and alkenals, respectively [49]. Zinc released from proteins would act as an intracellular second messenger mediating Nrf2/Keap1 sensing of environmental stressors, while changes in basal zinc levels are proposed to physiologically modulate Nrf2/Keap1 [49]. In line with this, we observed in zinc deficient IMR-32 neuroblastoma cells and embryonic day 19 brain, a decreased expression of the catalytic and modulatory glutamate cysteine ligase (GCL) subunits, coincident with a decreased nuclear Nrf2-DNA binding. GCL subunit expression is in part regulated by Nrf2 [53]. Consistent with this, adult rats fed severely zinc deficient diets present in the olfactory epithelium a low expression of GSH S-transferase [77], another Nrf2-regulated protein [78].

Increasing evidence point to a NO/zinc-regulation of G-protein coupled receptors (GPCR). Recently, the zinc finger-containing protein RGSZ2 has been proposed as a redox switch transforming a redox signal into a zinc signal [79]. RGSZ2 binds to nNOS inhibiting NO production, and also binds, through its interaction with the HINT1 protein, to the GPCR mu-opioid receptor (MOR). MOR can activate nNOS and cause a transient increase in NO generation which leads to zinc release [80]. Not only MOR activation, but activation of other GPCR, causes a NO/nNOS-dependent release of zinc from RGSZ2, which leads to the recruitment of the redox-sensing proteins protein kinase C (PKC) gamma and Raf-1 to the GPCR and their subsequent activation [79].

MT is a major regulator of zinc homeostasis, acting as a zinc reservoir that upon thiol oxidation by NO, H_2O_2, oxidized GSH, and other thiol reactants (reviewed in [9]), makes zinc available for different cellular targets. The interaction NO/oxidants with zinc also occurs at the level of MT expression. NO triggers a MT-dependent transient release of zinc in the nucleus, which leads to an increased expression of MT I and II [66]. NO causes the nuclear translocation of transcription factor MTF-1 [81], which binds to metal responsive elements present in the promoter of MT I and II, leading to increased MT expression [82].

This effect is proposed to be triggered by NO-mediated release of zinc from MT, rather than through a direct effect of NO on MTF-1 [81]. In vitro and in vivo studies indicate that MT can act as a free radical scavenger [9]. In vitro, MT scavenges hydroxyl radical, superoxide anion, and peroxynitrite [83], preventing hydroxyl radical-and superoxide anion-induced DNA damage [84, 85], and being 800-fold more efficient than GSH in preventing hydroxyl radical-mediated DNA degradation [85]. In vivo, mice overexpressing MT are protected from pro-oxidant conditions, such as ischemia/reperfusion [86] and doxorubicin cardiotoxicity [87]. Although a free radical scavenging action of MT is supported by numerous studies, its physiological relevance is still controversial.

Zinc and glutathione

Besides the capacity of oxidized GSH to mobilize zinc from MT and other zinc-thiol clusters, zinc per se can affect GSH metabolism. GSH is involved in the detoxification of oxidants, both enzymatically and non-enzymatically; as both carrier and storage of cysteine, in the elimination of xenobiotics through direct conjugation, and acting as a buffer of intracellular redox homeostasis [88, 89]. In cells, GSH steady state levels are regulated through its synthesis, utilization and export. Interactions between zinc and GSH metabolism are relevant to the protection of cells against oxidative stress-mediated damage. For example, NO protects endothelial cells against damage induced by H_2O_2, in association with a zinc-dependent activation of transcription factor Nrf2 [55], which upregulates GCL expression and GSH synthesis [54, 55].

A role for zinc on GSH metabolism is suggested by the frequent finding of GSH deficits in association with zinc deficiency in different cells and tissues [23, 28, 32–34]. GSH concentration was lower in the brain of embryonic day 19 fetuses from rat dams fed marginal zinc diets compared to controls [53], and in zinc deficient neuronal cells [34, 53]. In both systems, decreased levels of GSH are associated with a decreased expression of GCL, the enzyme that catalyzes the rate-limiting step of de novo GSH synthesis. The protein and mRNA levels of the GCL catalytic and modulatory subunits were lower in zinc deficient fetal brain and neuronal cells, in association with an impaired Nrf2 nuclear translocation [53]. GCL expression is regulated by transcription factors Nrf2, AP-1 and NF-3B, [90]. Given the presence of a zinc sensing cluster in Keap1/Nrf2 [49], a decrease in labile zinc as a consequence of zinc deficiency could explain the associated decrease in Nrf2 activation. Furthermore, zinc deficiency also inhibits NF-3B transcriptional activity [91, 92]. Besides a regulation at the transcriptional level, zinc deficiency also causes an increased cleavage of the full length GCL catalytic subunit [53]. This cleavage is catalyzed by caspase 3, a zinc enzyme that is activated in conditions of zinc deficiency in different cells and tissues [93]. Thus, zinc can regulate GSH synthesis by modulating GCL both at transcriptional and posttranslational levels. Nevertheless, utilization, recycling, export, and availability of substrates for synthesis could also contribute to GSH deficits in zinc deficiency.

Thus, the modulation of cellular GSH by zinc, and the relevance of the balance oxidized/reduced GSH in the release of zinc from MT points to a major role of GSH/zinc interactions in the physiological regulation of redox homeostasis. Overall these interactions can: i) have an effect on the cell thiol redox status, ii) modulate redox sensitive signaling, and iii) determine the capacity of cells to respond to oxidative stressors.

Phosphatases

Although zinc released from sulfur clusters can affect the activity of multiple proteins (e.g. enzymes, receptors, transcription factors), this review will focus on those that are involved in redox signaling. Cell signaling is largely driven by phosphorylation/dephosphorylation reactions which can mediate both activation and inactivation of signaling proteins.

Phophatases cleave phosphate groups from Ser/Thr or Tyr residues in proteins. Zinc can directly bind to phosphatases activating or inhibiting their activity. Indirectly, oxidative stress associated with decreased zinc availability, could inhibit the activity of redox sensitive phosphatases. For example, the physiological production of H_2O_2 triggered by the binding of ligands to select receptors (e.g. epidermal growth factor and insulin receptor), inhibits phosphatases allowing a longer phosphorylation of signaling intermediates, and a more prolonged activation of the cascade.

Protein tyrosine phosphatases (PTPs) can be both directly regulated by zinc and by changes in the redox state. PTP1B, one example of this type of regulation, contains a cysteine group in its catalytic site in a conserved sequence $HCX_5R(S/T)$ [94]. The environment within this motif decreases the pKa of the cysteine group giving it a higher nucleophilicity and higher susceptibility to oxidation. The oxidation of this cysteine to sulfenic acid, which is rapidly converted into sulphenyl-amide, leads to a major conformational change that renders the binding site unavailable to substrates [95]. The formation of the sulphenyl-amide prevents further irreversible oxidation to sulphonic acid, turning cysteine redox changes reversible and a mechanism of dynamic enzyme modulation [95]. PTP1B was initially described to be inhibited by micromolar concentrations of zinc [96]. However, it was subsequently shown to be also inhibited by nanomolar zinc concentrations, being the IC_{50} values for PTP1B and SHP-1 (PTP1C) 17 and 93 nM, respectively [97]. These findings support a role for zinc in the in vivo modulation of PTP1B. Thus, while zinc deficiency could inhibit PTP1B as a consequence of increased H_2O_2 production [6], physiological decreases in cellular zinc levels would provide a direct tonic modulation of PTP1B activity by zinc. Other PTP, the receptor PTP beta, is inhibited by zinc at picomolar concentrations [98]. The above evidence points to a physiological role for zinc in the modulation of PTPs involved in signaling modulation. In this regard, the inhibition of PTP1B by zinc can enhance the cellular response to insulin, which is supported by the described insulin-like actions of zinc [99, 100].

The human dual specificity phosphatase YVH1 or DUSP12 present a unique mechanism of intertwined regulation by zinc and redox state [101]. YVH1 is proposed to regulate major cellular processes including cell survival, ribosome biogenesis and cellular DNA content [102, 103]. YVH1 possesses a phosphatase catalytic domain containing a redox sensitive cysteine group with a similar structure and catalytic dynamics to that of PTPs [94], and a zinc-coordinating C terminal domain. In conditions of oxidative stress, the formation of a disulfide bond ejects zinc from the zinc-coordinating domain, and at the catalytic site causes YVH1 inactivation [101]. The zinc domain is proposed to act as a redox sensor in conditions of oxidative stress to prevent the irreversible YVH1 inactivation [101]. When oxidant levels return to basal levels, the restoration of zinc to its binding motif is needed for complete enzyme activation.

Other important phosphatase regulated by zinc and involved in cell signaling is the Ser/Thr phosphatase PP2A [104]. Although the involved mechanisms are not completely understood, indirect evidence points to a regulatory action of zinc on PP2A. PP2A activity is inhibited by 1 μM zinc in vitro [104], and this inhibition is proposed to underlie the increase in the Ser/Thr phosphorylation of Akt in cardiac myoblasts treated with zinc [105]. This mechanism could explain the cardioprotective actions of a zinc ionophore in reperfusion injury [106]. The release of zinc at synaptic terminals is proposed to cause tau hyperphosphorylation through the zinc-mediated inhibition of PP2A [107]. Of significant relevance for Alzheimer's disease, hyperphosphorylated tau accumulates in neurofibrillary tangles, in association with alterations in brain zinc homeostasis [108].

MAPKs

The family of mitogen activated kinases (MAPKs), which include ERK1/2 (extracellular signal-regulated kinases), ERK5, p38 and JNK (Jun N-terminal kinases), is one of a select group of intracellular signaling proteins linking receptor activation to nuclear and cytosolic targets. While ERK1/2 are sensitive mostly to mitogenic signals, p38 and JNK respond to stress stimuli. Zinc can indirectly modulate the activity of the MAPKs. The modulation of MAPKs by zinc is supported by findings in animal and cell models of zinc deficiency, as well as in mice with deficits in zinc transporters. In the rat brain and neuronal cells zinc deficiency differentially affects ERK1/2, p38 and JNK1/2 [109, 110].

Zinc can influence ERK activity through several mechanisms (reviewed in [111]). Those mechanisms include the previously described capacity of zinc to inhibit phosphatases that can dephosphorylate ERK (e.g. PP2A) [104, 112], zinc-mediated inhibition of kinases that phosphorylate receptors which activate ERK [113], a direct activation by zinc of receptors (e.g. GPR39) with ERK1/2 as a downstream target [114]; and the activation by zinc-dependent enzymes which generate active ligands (eg. pro-brain-derived neurotrophic factor (BDNF) conversion to BDNF by metalloproteases [113]) that trigger ERK1/2-associated cascades. An indirect regulation of ERK by zinc can also occur through the previously discussed activation of select GPCR leading to the sequential nNOS activation, NO generation, zinc release, Raf-1 recruitment to the GPCR and to the activation of Raf-1/MEK/ERK [79]. In support of a physiological role for zinc on ERK modulation, ERK1/2 is inhibited in conditions of zinc deficiency in fibroblasts [115], human neuroblastoma IMR-32 cells [21, 110], primary cultures of cortical neurons [21], and in the rat developing brain [109]. Furthermore, in knockout mice for the zinc transporter Znt3, the phosphorylation of ERK1/2 is decreased in association with an increase in ERK1/2-directed tyrosine phosphatase activity [116]. ERK inhibition can in part explain the negative impact of zinc deficits on cell proliferation and survival [21, 93, 115, 117–121], and as a consequence on development [1]. On the other hand, zinc release as a consequence of GSH depletion-induced oxidative stress causes increased ERK1/2 activation through the inhibition of ERK1/2 phosphatase activity [112].

MAPKs p38 and JNK are activated by zinc deficiency. Zinc deficiency causes high levels of p38 and JNK phosphorylation and/or activity in association with oxidative stress in different cells and tissues including testes [122], developing rat brain [109], and human neuroblastoma cells [110]. Antioxidant compounds and enzymes that prevent zinc deficiency-induced oxidant increase and GSH decrease also prevent p38 and JNK activation, and the downstream activation of transcription factor AP-1 [34, 110]. Furthermore, inhibitors of the NMDA receptor not only prevent zinc deficiency-induced and NMDA-mediated production or reactive oxygen and nitrogen species, but also AP-1 activation [6]. This indicates that the increased oxidant production occurring as a consequence of decreased cellular zinc is the main factor triggering p38 and JNK activation. The regulation of p38 and JNK by oxidant species is not completely understood, but in part involve the activation of upstream MAPK kinase kinases (e.g. ASK-1 [123]), and the inactivation of MAPK phosphatases (e.g. MKP-1 [124]). On the other hand, zinc supplementation also differentially regulates MAPKs. In T-lymphocytes zinc increases p38 phosphorylation, while not affecting ERK and JNK. Different mechanisms were proposed to be involved in p38 activation by high zinc levels, including the inactivation of phosphatases and a direct or indirect activation of upstream kinases [125].

Thus, the complex regulation of MAPKs by zinc will depend, among other factors, on the extent of cellular zinc decrease or increase, on the cell type, on the existence of different stimuli, and on the particular cell network of kinases and phosphatases.

Protein kinase C

PKC constitutes a family of Ser/Thr protein kinases involved in signaling pathways that modulate important cellular processes, e.g. growth, cell death, differentiation, response to stress. PKC is a redox sensitive kinase (reviewed in [126]). PKC contains cysteine-rich regions in both the catalytic and regulatory domains, the later containing two pairs of zinc fingers in the C1 region [127, 128]. Zinc fingers participate in the interactions of PKC with membrane diagylglycerol, and of its mimetic phorbol esters, and consequently, in the regulation of PKC cellular localization and activity. In this regard, the oxidation of PKC's zinc fingers by oxidants leads to zinc release and to the loss of zinc finger structure. As a consequence, PKC is activated in a cofactor-independent manner given the release of a self-inhibitory mechanism. Phorbol esters and mild pro-oxidant conditions cause zinc release from 3T3 and insect cells, which was identified to originate from PKC [129]. In fact, activated PKCalpha contains less zinc than its resting form. The incubation of recombinant peptides encompassing different PKC protein regions with 1.3-diolein, phorbol esters and oxidants causes a stoichiometric release of zinc [129].

Stressing the relevance of zinc in PKC regulation, not only oxidants but also zinc availability can affect PKC activity. In zinc deficient 3T3 cells, a low cytosolic classical PKC activity was observed which was not due to the translocation of the enzyme to the membrane [130]. However, a decrease in labile and total zinc causes different effects on PKC isoforms, downregulating PKCalpha (protein levels and activity), and causing a caspase-dependent cleavage and activation of PKCdelta [130]. The later could be potentially involved in zinc deficiency-associated induction of apoptotic cell death [130].

Given the multiple signaling pathways regulated by PKC, the redox/zinc-mediated regulation of PKC activity could be central to an indirect action of zinc in the modulation of redox signaling, e.g. MAPKs. In this regard, activation of PKC leads to a downstream activation of ERK1/2 which provides an additional and indirect level of zinc-mediated regulation of ERK1/2 [131]

Zinc and tubulin thiols: regulation of NF-3B and NFAT nuclear transport

A role for zinc in the regulation of cell signaling is also related to the preservation of tubulin polymerization dynamics through the maintenance of an adequate intracellular thiol redox status. Microtubules are major components of the cell cytoskeleton, being essential for multiple aspects of cell physiology including structural and transport functions. Microtubules are dynamically assembled through the reversible polymerization of 3 and 3 tubulin subunits. Tubulin contains 20 reduced cysteines per 33 dimer, 8 in the 3 and 12 in the 3 subunit [132] which makes it highly susceptible to oxidation. In this regard, tubulin oxidation by peroxynitrite, H_2O_2, and other oxidizing agents [133–135] impairs tubulin polymerization into microtubules which can be repaired by the thioredoxin reductase [134] and the GSH/glutaredoxin reductase systems [135]. Tubulin oxidation can have deleterious consequences on cell and tissue functions, including loss of the intestinal barrier selective permeability [136], increased permeability of the vascular endothelium [137], and alterations in the transport of cellular components which, for example, can affect the modulation of gene transcription [109].

Zinc deficits cause alterations in tubulin polymerization dynamics both in adult and fetal rat brain, and in neuronal cells in culture [51, 91, 92, 138–140]. Given the rapid increase in neuronal ROS and RNS when cells are cultured in zinc deficient medium, the involvement of thiol oxidation in the observed tubulin polymerization alterations was highly feasible. In fact, simultaneous incubation of IMR-32 cells in zinc deficient media and in the presence of N-acetyl cysteine or 3-lipoic acid prevented the alterations in tubulin polymerization kinetics

and the formation of tubulin oligomers with molecular weights higher than 100 kDa [10]. Similar findings were observed when cytosolic fractions from zinc deficient IMR-32 cell, rat cortical neurons and embryonic rat brain were treated with the disulfide reducing agent tris(2-carboxyethyl)phosphine. Tubulin oxidative modifications as a consequence of neuronal/brain zinc deficits involved the formation of disulfides, but not protein carbonylation or nitration [10].

Microtubules actively participate in the axonal transport of cellular components and in the cross-talk between the synapse and the nucleus [141]. A direct consequence of zinc deficiency-induced tubulin alterations in neuronal cells is an altered nuclear transport and transactivating activity of transcription factors NF-3B [91, 92] and NFAT [41]. Supplementation of IMR-32 cells with N-acetyl cysteine or 3-lipoic acid, which prevented tubulin oxidation and altered polymerization, restored the nuclear transport of NF-3B and the expression of NF-3B-dependent genes [10]. Tubulin oxidation was not observed in the liver from zinc deficient embryos, which points to the brain as a particular target of these alterations. In this regard, tubulin oxidation and its functional consequences can in part explain the adverse effects of zinc deficiency on brain development through a derangement of key developmental events, such as neurogenesis and neuronal apoptosis [142, 143].

Conclusions

Given its unique chemical characteristics, zinc is an abundant metal playing multiple roles in biological molecules and cellular events. The frequent association of oxidative stress to zinc deficiency originated the concept that zinc could be part of the antioxidant defense system. A large number of continuously growing evidence currently supports direct and indirect roles for zinc in the regulation of oxidant production, a critical relevance of thiol-bound zinc regulating the pool of free zinc which can be released by NO, H_2O_2 and other oxidants, and the involvement of zinc in redox signaling. The release of free zinc pulses capable of regulating cell signaling, and as a consequence cell function and fate, is of high physiological relevance and points to zinc as a central second messenger.

Alterations in zinc homeostasis with an associated deregulation of redox signaling can have significant adverse consequences given the multiple pathways involved and events affected. In the developing nervous system, zinc deficiency causes oxidative stress, impairs GSH metabolism, causes tubulin oxidation, and disrupt redox-sensitive signaling. These alterations could underlie the adverse effects of zinc deficiency on brain development, and on the observed alterations in behavior, cognition and motor performance. Furthermore, impaired Nrf2 activation as a consequence of decreased zinc availability can increase the susceptibility of neurons and of other cells to oxidant stressors. Deregulation of redox-sensitive signals (e.g. ERK) involved in the control of cell proliferation, can explain the adverse effects of zinc deficiency on tissues that undergo periods of rapid cell growth, such as the skin, the immune and reproductive system. Oxidative damage of cellular components, and redox-mediated alterations in the patterns and extent of proliferation and apoptotic cell death during development may lead to altered organ cellularity, organization and connectivity, increasing the risk for diseases later in life.

It is evident that the “antioxidant” actions of zinc, and the capacity of zinc to regulate redox signaling will depend on several factors including the particular cell/tissue, the levels of available zinc, and the array of particular signaling molecules and stimuli in the target cell/tissue. This rapidly growing field of research will help elucidate many aspect of zinc involvement in redox signaling that are currently unknown.

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Abbreviations

Akt	protein kinase B
BDNF	brain-derived neurotrophic factor
ERK	extracellular signal-regulated kinases
GCL	glutamate cysteine ligase
GSH	glutathione
GPCR	G-protein coupled receptors
Keap1	Kelch-like ECH-associated protein
JNK	Jun N-terminal kinases
MAPKs	mitogen activated kinases
MOR	mu-opioid receptor
MT	metallothionein
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
Nrf2	transcription factor NF-E2-related factor 2
PKC	protein kinase C
PTPs	protein tyrosine phosphatases
RXR	retinoid X receptor
VDR	1alpha,25-dihydroxyvitamin D(3) (1alpha,25(OH)(2)D(3)) receptor

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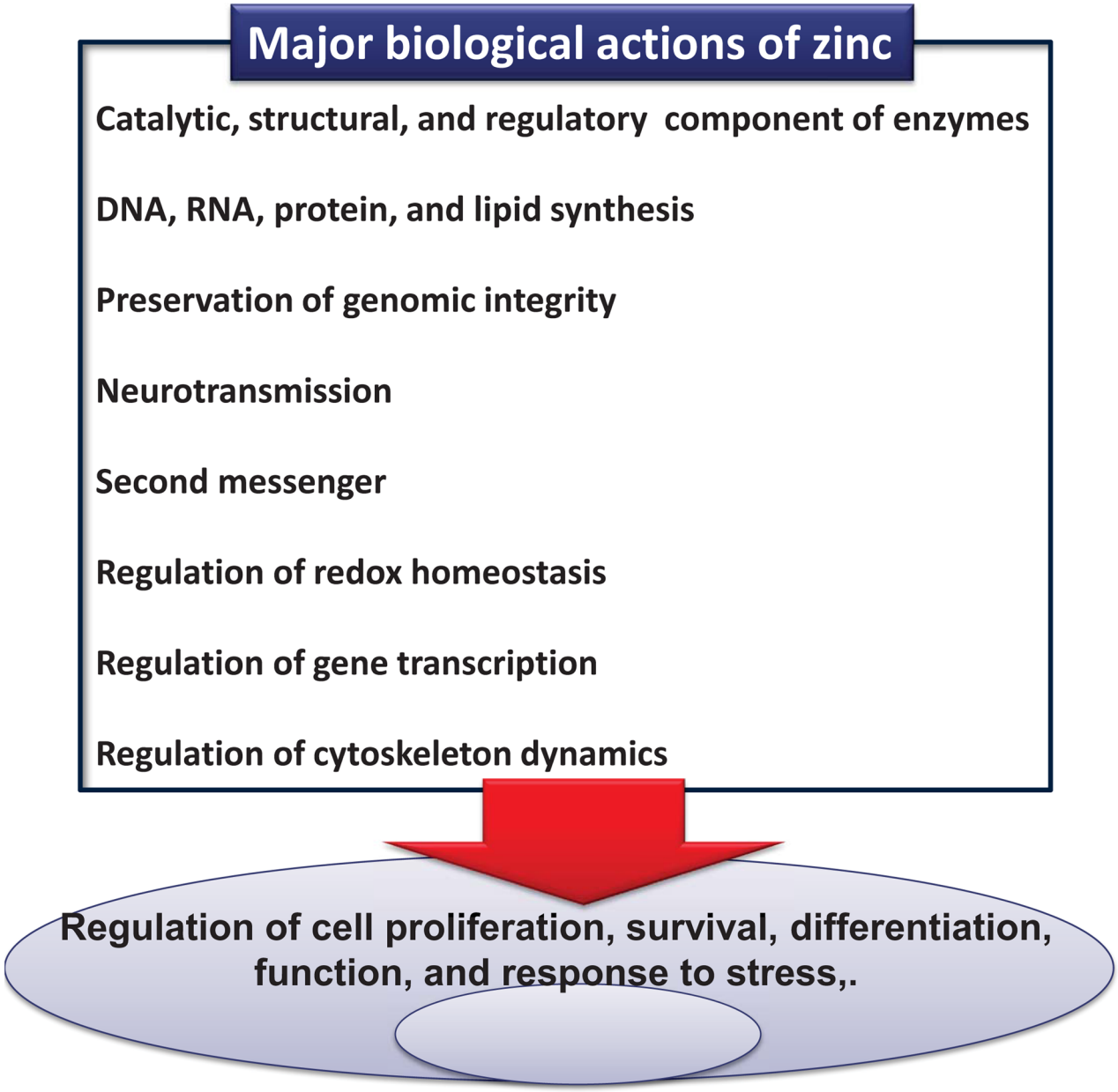

Figure 1.
Biological actions of zinc.

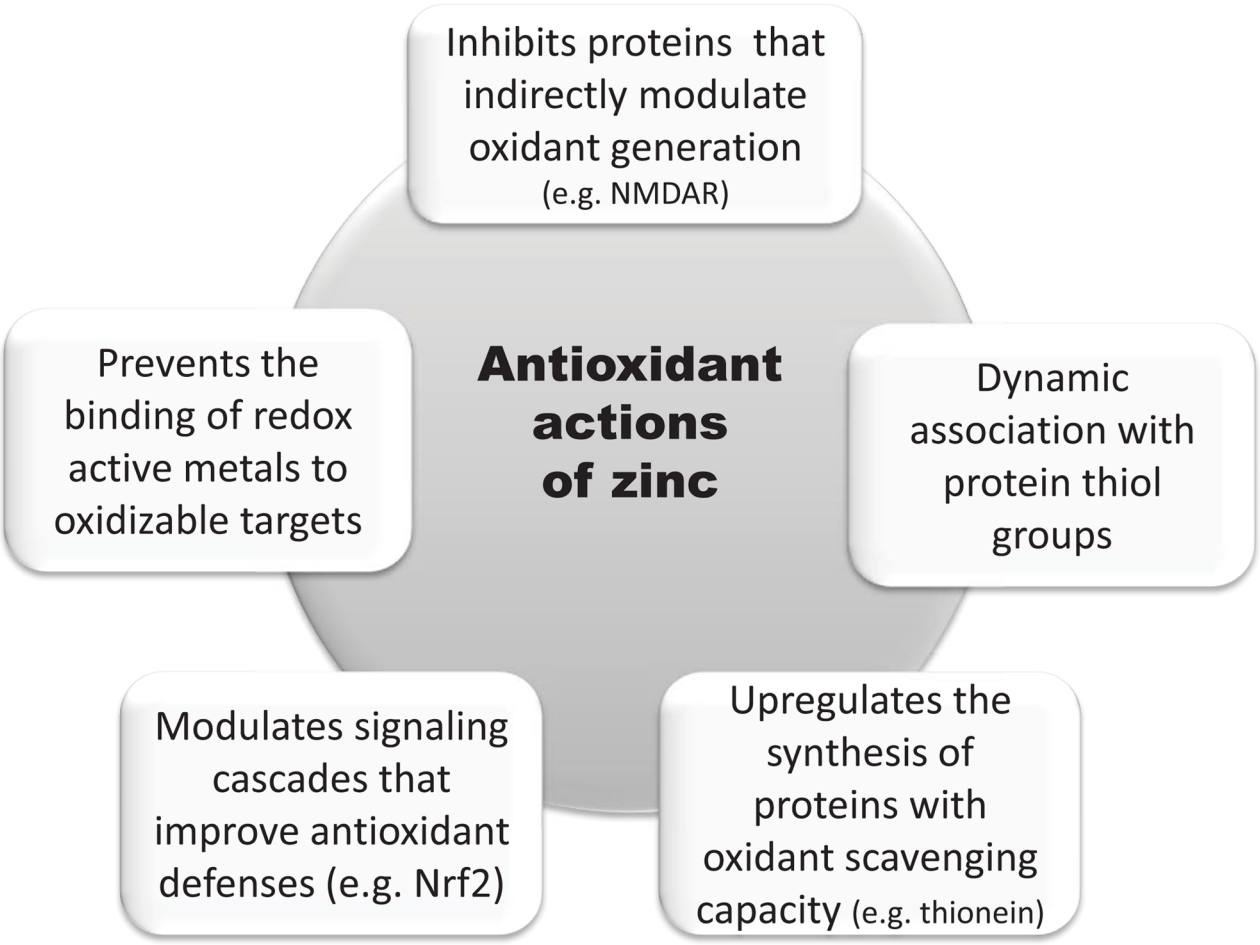

Figure 2.
Mechanisms involved in the “antioxidant” actions of zinc.

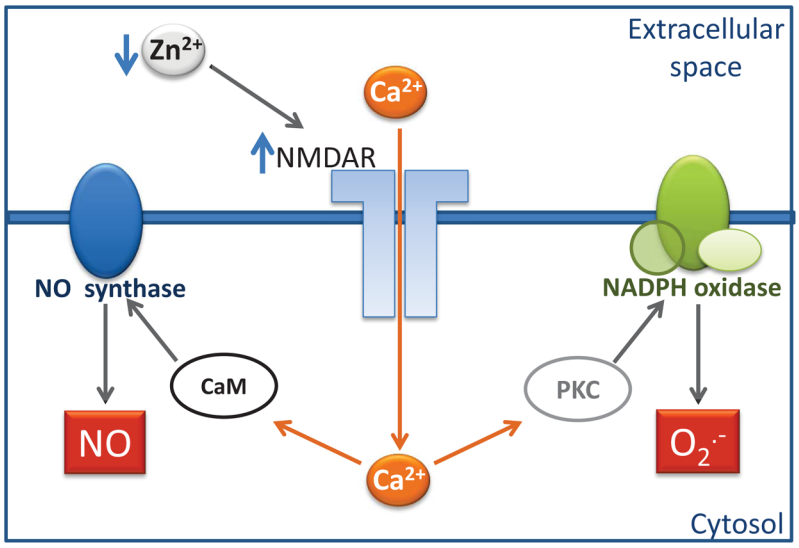

Figure 3. Zinc availability regulates NO and superoxide anion production in neuronal cells through the modulation of the NMDAR

The neuronal NMDAR is blocked by zinc (Zn^{2+}) at two different and independent sites. A decrease in extracellular zinc levels leads to the release of that inhibition, and to the influx of calcium (Ca^{+}) through the NMDAR channel. The increase in cellular calcium activates protein kinase C (PKC) which activates NADPH oxidase. NADPH oxidase catalyzes the formation of superoxide anion which can be subsequently converted into other reactive species. On the other hand, calcium can bind to caldmodulin (CaM) and activate NO synthase leading to an increased generation of NO. This figure summarizes findings from Aimo et al. [6].

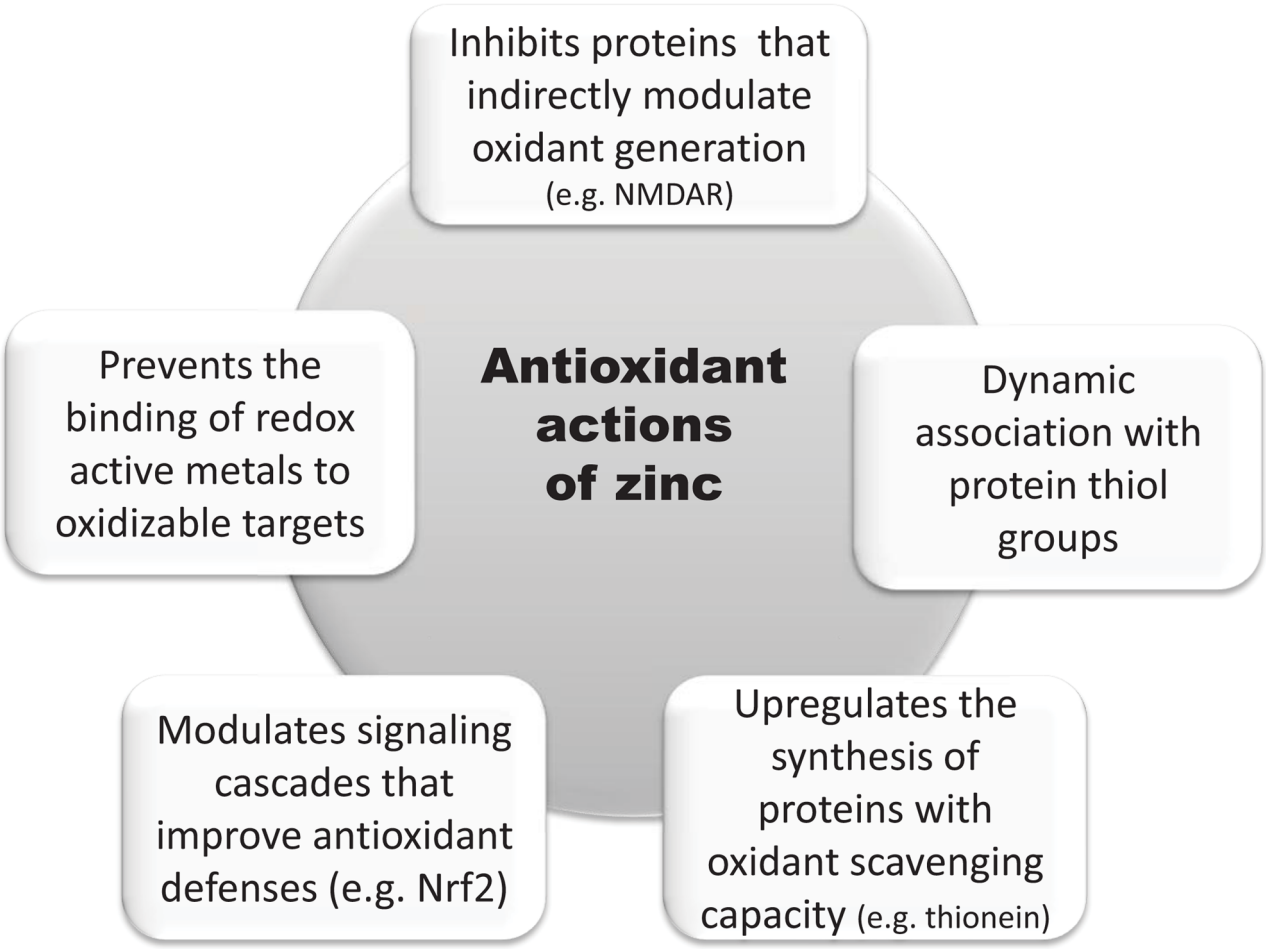

Figure 4. Zinc inhibits Fe^{2+} binding to liposomes and lipid oxidation, acting in conjunction with hydrophilic and lipofilic antioxidants

A- Correlation between the capacity of zinc (Zn) (15–250 μM) to inhibit Fe^{2+} (25 μM) binding to liposomes (phosphatidylcholine:phosphatidylserine 60:40 mol%) and to inhibit lipid oxidation (measured as 2-thiobarbituric acid-reactive substances). **B-** Zinc (Zn) (25 μM), (−)-epicatechin (EC) (0.5 μM) and 3-tocopherol (AT) (0.01 mol%) jointly act inhibiting Fe^{2+} (25 μM)-initiated lipid oxidation in liposomes. Adapted from [43].

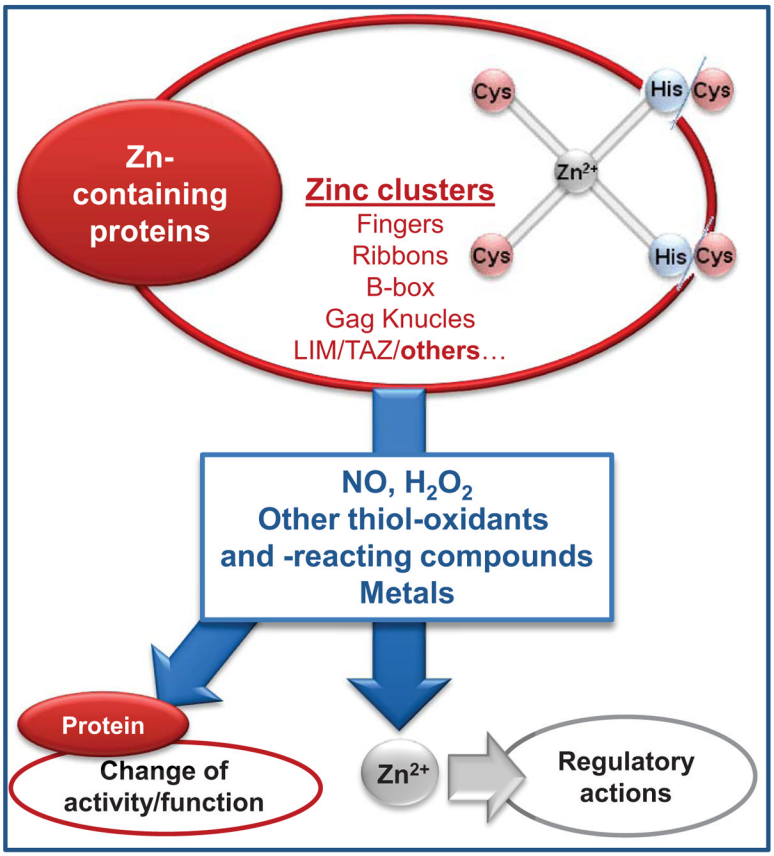

Figure 5. Release of zinc from zinc-sulfur binding domains

Zinc can bind tetrahedrally to binding domains in proteins through Cys4 or Cys2His2 interactions. Different molecules including NO, H_2O_2, oxidized GSH, metals, and molecules that react with thiols or have the capacity to oxidize them, cause the release of free zinc. The released zinc can have different regulatory actions, while the modified protein may or may not undergo changes in activity/function.

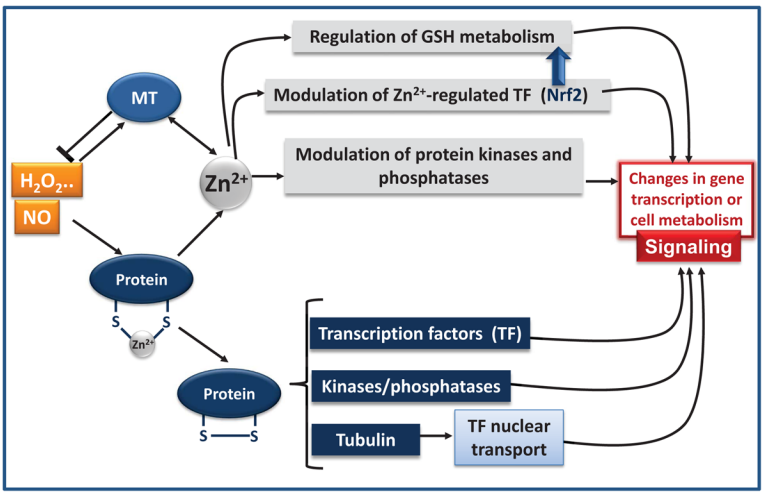

Figure 6. Zinc in the regulation of redox signaling

NO, H_2O_2, oxidized GSH, and other oxidant species can modify zinc (Zn^{2+}) thiolates leading to zinc release from MT and other proteins. Those species can exert reversible or irreversible chemical modifications of thiol residues (the formation of a disulfide is exemplified in this figure). If the protein is involved in signaling (e.g. transcription factor (TF), kinase, phosphatase, tubulin) a change in activity would lead to changes in signaling modulation. On the other hand, the released zinc can per se regulate transcription factors and the activity of phosphatases and kinases involved in signaling. A direct modulation of transcription factor Nrf2 by zinc will affect GSH synthesis, which would indirectly affect cell redox homeostasis and redox signaling. Zinc is part of the antioxidant network protecting biological systems from oxidative stress. Protein thiol-bound zinc participates in redox sensing. Zinc regulates proteins directly or indirectly involved in redox homeostasis. Zinc can modulate redox signaling through multiple mechanisms. NO and oxidants release zinc bound to sulfur clusters.