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Mutation Research 533 (2003) 183–200
Fundamental and Molecular Mechanisms of Mutagenesis
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Review

Signaling by carcinogenic metals and metal-induced reactive oxygen species

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Received 20 May 2003; received in revised form 4 August 2003; accepted 4 August 2003

Abstract

Epidemiological data indicate that exposure to metal and metalloid species, including arsenic(III), chromium(VI), and nickel(II), increases the risk of cancer, particularly of the lung and skin. Alterations in normal signal transduction as a result of exposure to carcinogenic metals, and to metal-catalyzed reactive oxygen species (ROS) formation, appear to play an important role in the etiology of metal-induced carcinogenesis. Signaling components affected by metals include growth factor receptors, G-proteins, MAP kinases, and nuclear transcription factors. This article reviews current literature on the effects of carcinogenic metals and metal-induced ROS on cancer-related signaling pathways. In addition, the mechanisms by which those changes occur, and the role of those changes in carcinogenesis are discussed.

Keywords: Metals; Signal transduction; Reactive oxygen species

1. Introduction

Exposure to certain metal and metalloid species, particularly in industrial settings, is associated with an increased risk of cancer. Based on epidemiological data arsenic, chromium, nickel, beryllium, and cadmium are confirmed human carcinogens, while lead, cobalt, antimony, and iron are considered potential carcinogens [1–8].

The most common sites for metal-related cancers are the lung and skin. Increased risks for cancers of the prostate, kidney, bladder, and liver, as well as for lymphoma and leukemia, have also been reported [9]. Excess occupational exposure to metals, particularly in mining, smelting, and metal-plating operations is considered to be a major cause of metal-related cancer [6]. Environmental contamination by lead, arsenic, and cadmium are also associated with increased cancer risk. Arsenic is of particular concern in several countries including Bangladesh, India, and Argentina due to its presence in groundwater at high concentrations [10,11]. The presence of metals including

Abbreviations: AP-1, activator protein-1; ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; BMAPK-1, big MAPK-1; EGF, epidermal growth factor; ERK, extracellular-regulated kinase; GADD, gene arrest and DNA damage; GSH, reduced glutathione; HIF-1, hypoxia inducible factor-1; HO-1, heme-oxygenase-1; IL, interleukin; JNK, c-jun-NH_2-terminal kinase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; NADPH, reduced nicotine adenine dinucleotide phosphate; NFκB, nuclear transcription factor-kappa B; NFAT, nuclear factor of activated T cells; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

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doi:10.1016/j.mrfmmm.2003.08.025

cadmium, arsenic, nickel, and chromium in cigarettes has lead to speculation that these metals contribute to the five-fold increase in lung cancer risk for smokers versus non-smokers [12].

The complex chemistry of metals defies simple categorization with respect to carcinogenesis. Even among metals recognized as carcinogens, there is great variation with respect to carcinogenic potential. These differences are primarily a function of oxidation state. For example, exposure to chromium(VI) increases human cancer risk, whereas chromium(III) exposure does not [13]. Other factors, including the organic/inorganic nature of metal compounds and the methylation state of organic metal compounds also affect carcinogenic potential [14–17].

Numerous studies have examined the effects of metals and metal-induced ROS on DNA damage and the inhibition of DNA repair [18–22]. Although these effects are relevant to carcinogenesis, recent data indicate that metal-induced alterations in signal transduction also play a role in the etiology of cancer either independently of, or in concert with, DNA damage [23–26]. Metal exposure has been shown to activate or inactivate cancer-related genes and their protein products. These include growth factor receptors, ras, c-src, the MAPKs, and nuclear transcription factors such as NFκB, NFAT, AP-1, HIF-1, and p53 [25–32]. In short, metals can affect multiple aspects of cellular function, including proliferation, apoptosis, differentiation, and cell transformation [33,34]. Many of these effects appear to be related to ROS formation, but there is also evidence that metals can affect cell signaling independently of free radical generation [35].

It is far beyond the scope of this review to discuss all of the cancer-related genes/proteins affected by metals. Rather, this review seeks to address several key questions regarding metal-related signaling and cancer:

(a) What effects do metals have on signal transduction?
(b) What are the mechanisms by which metals affect signal transduction?
(c) How are changes in signaling involved in metal-induced carcinogenesis?
(d) How do metal-induced ROS affect signal transduction?

2. Effects of metals on signal transduction

Metals affect the gene transcription, expression and activation of numerous signaling proteins including growth factor receptors, G-proteins such as ras, tyrosine kinases such as c-src, MAPK proteins, and nuclear transcription factors such as NFκB, NFAT, AP-1, HIF-1, and p53. These effects may involve either activation or inactivation. Effects may be direct and through the interaction of metals with proteins, or indirect and through the formation of metal-induced ROS. The effects of metals on signaling pathways may mimic extracellular ligands such as insulin, or physical conditions such as hypoxia, via mechanisms that are poorly understood.

2.1. Growth factor receptors

A number of carcinogenic metals have been shown to affect growth factor receptors, including arsenic, chromium, nickel, beryllium, and cobalt. Growth factor receptors for EGF, VEGF, PDGF and for insulin are receptor tyrosine kinases. They are essential to the normal function of cells and are often initiators of MAPK signaling. The mutation or overexpression of growth factor receptors augments the invasive and metastatic characteristics of cancer. Because of the importance of growth factor receptors in cancer a number of new drugs have been designed to inhibit them, including Iressa, which targets the EGF receptor, and Gleevec (also known as STI571 or Imatinib), which inhibits PDGF receptor phosphorylation [36–38].

The EGF receptor is overexpressed in pre-malignant and cancerous lung tissue, as well as in cancers of the prostate, kidney, and bladder [39,40]. This receptor appears to play a role in cancer invasiveness, partly due to its role in angiogenesis [41]. Arsenic is capable of inducing EGF receptor phosphorylation in BEAS cells, as are the essential nutrients copper and zinc. Downstream effectors of the EGF receptor including ras, MAPKK-1, MAPKK-2, ERK-1, and ERK-2 were activated by all three of these metals [42,43]. Exposure of normal human kidney epithelial cells to nickel(II) in vitro led to their immortalization and to a 260% increase in EGF receptor expression [44].

Disregulation of the VEGF receptor is involved in tumor promotion and angiogenesis. VEGF transcription and expression is induced in response to hypoxia

and to the metals cobalt, nickel, cadmium, and arsenic. Although VEGF may be produced by a number of cell types including macrophages, keratinocytes, smooth muscle cells, tumor cells, and endothelial cells, only endothelial cells have receptors for VEGF [45]. Both cobalt(II) and nickel(II)-induced transcription of VEGF in endothelial cells in a manner similar to that observed under hypoxic conditions. These metals may, therefore, cause autocrine stimulation of VEGF in endothelial cells [45]. Tissue hypoxia induces VEGF expression in tumors in a ROS-dependent manner [46]. Cadmium inhibits a tyrosine kinase downstream of the VEGF receptor [47]. Sodium arsenite-induced transcription of VEGF in OVCAR-3 human ovarian cancer cell line and in mouse fibroblasts is mediated by p38 [48].

Ligand binding of EGF and VEGF receptors causes an increase in cytoplasmic pH by the cellular export of H^+ ions and an increase in cytoplasmic calcium, both of which are essential for proliferation [49]. Exposure to cadmium, nickel, zinc, copper, and beryllium can also increase intracellular calcium, which may partially explain the effects of metals on cell proliferation [50]. Cadmium(II) caused rapid increases in intracellular calcium in human fibroblasts through a mechanism independent of tyrosine kinase activity, and that involved an orphan receptor and a G-protein [51]. Nickel(II) caused intracellular calcium increases in primary rat hepatocytes. The essential nutrients zinc(II) and copper(II) had similar effects. It was proposed that intracellular calcium may have been increased via the interaction of these metals with cell-surface iron receptors [52]. Beryllium exposure increased intracellular calcium through inositol-1,4,5-triphosphate-dependent mechanisms in mouse peritoneal macrophages [30].

The PDGF receptor is a growth factor for mesenchymal cells, endothelial cells and fibroblasts and is overexpressed in lung and prostate cancer [53,54]. The PDGF inhibitor Gleevec has been shown to inhibit the growth of the human lung cancer cell line A549 [38]. The production of PDGF by tumor-associated macrophages may be involved in the promotion of non-small cell lung cancers [55]. Both nickel and cobalt can induce PDGF production by macrophages [56].

Chromium, nickel, and cadmium interfere with insulin signaling by altering phosphorylation patterns of downstream insulin targets or by mimicry of insulin action. Chromium(VI) was shown to interfere with insulin function in H4 hepatoma cells by reducing the phosphorylation of insulin targets in a manner resembling that of the tumor promoter phorbol-12-myristyl-13-acetate [57]. Nickel(II) interfered with the lipogenic effects of insulin in Wistar rats, suggesting an interaction with the insulin receptor and/or downstream targets [58]. Cadmium(II) mimics insulin action causing the movement of glucose transporter proteins to the cell surface of rat adipocytes. This effect was independent of the insulin receptor, however, and was mediated through kinase activity [52].

2.2. Ras

Ras is a membrane-bound G protein family that interacts with tyrosine kinase receptors and activates MAPK signaling as well as other signaling pathways such as PI3K. There are three ras family members: H-ras, K-ras, and N-ras. Of these, metals have been reported to affect only H-ras and K-ras. Ras is normally involved in cell growth and differentiation. Ras is responsive to growth factors, cytokines, and to cellular stress, such as UV radiation and ROS. The ras gene is mutated in approximately 30% of all human cancers although the incidence of mutations in some cancers is as high as 90% [59]. Ras mutations have been linked to cancers of the lung, skin, liver, bladder, and colon [60,61].

The metals arsenic, nickel, iron, and beryllium have been reported to cause ras mutations and affect ras signaling in animal and in vitro studies. Little data is available with regard to the effects of metals on ras in humans, however [62]. Rats given dimethylarsinic acid in drinking water for 2 years demonstrated a low (10%) incidence of H-ras mutations in bladder tumors [63]. Nickel subsulfide alone, or with iron, induced K-ras mutation and kidney sarcomas in F344 rats. G:T transversions were observed in codon 12 of the K-ras gene, possibly due to the formation of 8-hydroxyguanosine. More K-ras mutations were observed in the group treated with nickel plus iron than in the nickel only group [64]. Beryllium fluoride-induced cell proliferation in mouse peritoneal macrophages via p21-ras and NFkB-dependent pathways. Beryllium also caused an increase in MAPKK-1, ERK-1, p38, and JNK [30]. A study of the effects of chromium

exposure on p21-ras protein levels in urine detected no differences between chromium refinery workers and an unexposed cohort. In addition, no mutated p21-ras protein was found in the urine of the chromium workers as had been previously observed in patients with cancers of the urinary tract, bladder, and prostate [65–67].

2.3. Src

The non-receptor tyrosine kinase src is activated by ligand binding of receptor tyrosine kinases and by stressors such as ROS and UV radiation. Once activated, src binds to the cytoplasmic membrane via myristylation and can be involved in signaling pathways for growth factor receptors, MAPKs, NFκB, and PI3K [68]. Overexpression of c-src is associated with cancers of the colon, breast, pancreas, bladder, and of the head and neck [69]. Despite its reported interaction with several signaling pathways affected by carcinogenic metals, few studies have been conducted on the effects of metals on c-src activation. Arsenic(III)-induced c-src activation was found to be essential for the activation of the EGF receptor and ERK in a human uroepithelial cell line [32]. Several organic forms of trivalent chromium (tris-(1,10-phenanthroline)chromium, tris-(2,2′-bipyridyl)chromium, trans-diaqua-1, 2-bis(salicylideneamino)ethanechromium, and trans-diaqua-1,3-bis(salicylideneamino) propanechromium)-induced lymphocyte apoptosis through the induction of the src family members p56lck, p59fyn, and p53/56lyn. Based on the use of dichlorofluorescein dyes the induction of apoptosis appeared to be related to ROS production [14].

2.4. MAPKs

At present there are four known families of MAPK proteins: ERK, JNK, p38, and BMAPK-1. Together, these MAPKs transduce signals for a diverse array of cellular functions, including proliferation, apoptosis, and differentiation [33,34]. Carcinogenic metals have been observed to stimulate MAPKs in a variety of cell lines. Arsenic(III), chromium(III), and chromium(VI) were found to enhance the phosphorylation of ERK-1 and ERK-2, as well as JNK and p38 in the human bronchial epithelial cell line, BEAS. Arsenic(III) was the most potent activator of the MAPKs in BEAS cells [70]. Activation of either p38 or JNK by arsenic(III) activated GADD45 in BEAS-2B cells which induced cell cycle arrest. Activation of GADD45 causes arsenic-induced cell cycle arrest [71]. Arsenic-induced apoptosis is JNK1-dependent, but p53 independent in JB6 cells [72]. Both arsenic(III) and cadmium(II) activate HO-1 in the chicken hepatoma cell line LMH via ERK, p38, and the nuclear transcription factor AP-1 [73]. Chromium(VI) and cadmium(II) strongly activated the expression of JNK and p38 in the human non-small cell lung cancer line CL3. Cadmium(II) was the strongest activator of JNK and p38 and was also the most potent inducer of apoptosis in CL3 cells [74]. Cadmium(II)-induced apoptosis in the human T cell line CCRF-CEM through activation of ERK, p38, and (to a lesser extent) JNK. JNK was activated exclusively through MKK7. ERK was activated to a lesser degree by both metals [75]. Cadmium(II) activated HO-1 in the human breast cancer cell line MCF-7 through p38 [76]. Treatment of murine peritoneal macrophages with beryllium difluoride increased the activation of ERK1, JNK, and p38 [30]. No effects of carcinogenic metals have been reported for BMAPK-1 although it can be activated by hydrogen peroxide, a ROS that can be formed by metal-catalyzed reactions [77]. A number of proteins involved in MAPK signalling contain zinc-finger domains, including Raf-1 and PI3K [78,79]. Substitution of nickel(II), cobalt(II), or cadmium(II) for zinc(II) in zinc-finger proteins may alter MAPK signaling and could represent an epigenetic mechanism of metal-induced carcinogenesis. Zinc-finger regions may be found in the protein products of a number of cancer-associated genes, including src, raf-1, and erbA [80].

2.5. NFκB

NFκB is a general term for heterodimeric nuclear transcription factors of the rel family, typically p50 (NFκB-1) and p65 (relA). NFκB may be activated by a variety of stimuli including cytokines, MAPK signaling, and ROS. The mechanism of NFκB activation is through phosphorylation of IκB by IκB kinase and subsequent degradation of IκB by the 26S proteosome. This allows the freed NFκB to be translocated to the nucleus. NFκB activation is generally associated with the inhibition of apoptosis by transcriptional activation of numerous genes and the induction of proliferation

via interaction with cyclin D1 [81,82]. The activation of NFκB is required for the transformation of cells by ras presumably due to the ability of NFκB to inhibit apoptosis. Ras activates NFκB through MAPK and non-MAPK pathways by facilitating the transactivation of the p65 subunit of NFκB [83]. NFκB regulates the expression of a number of cancer-related genes including GADD45, bcl-2, Cox-2, and c-*myc* [84–87].

Arsenic and chromium have been reported to affect NFκB, in some cases as inducers, in others as inhibitors. Arsenic may stimulate or inhibit NFκB activation and DNA binding depending on concentration, treatment duration, and the cell type in question. In the HRS lymphoma cell line arsenic-induced apoptosis by inhibiting IκB kinase and NFκB activation [88]. In contrast to the results observed in HRS cells, high arsenic concentrations activated NFκB in the JB6 mouse keratinocyte cell line resulting in p53-independent apoptosis. Lower arsenic concentrations-induced proliferation in the same cell line [89]. Arsenic caused dose- and time-dependent transactivation of NFκB in mouse epidermal JB6 cells through ERK and JNK-mediated mechanisms [90]. NFκB DNA binding was increased in rat lung slices treated with arsenic(III) [91]. Arsenic was also found to transiently increase DNA binding by NFκB in cultured lung epithelial cells [92]. In human GM847 fibroblasts, short-term (24 h) treatment with arsenic(III) increased NFκB binding of DNA while longer (10–20 weeks) treatment decreased NFκB binding. Short-term arsenic(III) treatment also increased the expression of thioredoxin and Ref-1, both of which can increase NFκB-DNA binding via chemical reduction of NFκB [93].

Nickel, beryllium, cobalt, cadmium, lead, and iron influence NFκB activation and DNA binding. Nickel enhanced NFκB activity in murine 3T3 fibroblast and BEAS-2B bronchial epithelial cell lines [94]. Both nickel(II) and cobalt(II)-induced NFκB-DNA binding in the human umbilical vein epithelial cell line (HUVEC) [95]. B200 cells, which are resistant to nickel or hydrogen peroxide-induced damage, also displayed low levels of NFκB-DNA binding and high levels of reduced glutathione relative to control (3T3) cells [29]. Treatment of murine peritoneal macrophages cells with beryllium fluoride or cadmium(II) increased NFκB protein levels [30,96]. Lead activated NFκB in CD^{4+} T lymphocytes [97]. Iron-induced NFκB activation as well as the transcription of IL-6 and TNF-α in rat hepatic macrophages [98].

2.6. AP-1

The group of nuclear transcription factors collectively referred to as AP-1 is composed of hetero- and homodimer subunits of proteins from the fos, jun, jun dimerization partner (JDP), and activating transcription factor (ATF) families. C-jun and c-fos appear to be the subunits most closely associated with malignancy. AP-1 is activated by a wide variety of stimuli including growth factors, inflammatory cytokines, UV radiation, and oxidative stress [99]. AP-1 expression is controlled by the ERKs, which activate fos genes, and by JNK and p38, which regulate the activation of jun [100,101]. AP-1 activation by metals generally favors proliferation but may, under conditions of extreme cell stress, activate apoptosis. The ultimate effect of active AP-1 depends upon the cell type involved, the dimeric composition of AP-1, and the other molecular signals being received by the cell [102,103].

Arsenic induces the activation of AP-1 in a variety of cell types including epithelial cells, fibroblasts, type II cells, and alveolar macrophages. Methylated arsenic forms may be more potent activators of AP-1 than inorganic forms. In human bladder cells the methylated trivalent arsenic species methylarsine oxide and iododimethylarsine were found to be more potent inducers of the binding of AP-1 sites on DNA sites by c-jun, jun B, and jun D than an inorganic form of arsenic(III). The activation of AP-1 by methylated forms of trivalent arsenic was mediated by ERK-1 and ERK-2 [15]. Dimethylarsenic and inorganic arsenic(III) were also found to increase AP-1 DNA binding activity in the bladder of C57BL/6 mice [16,104]. As with NFκB, arsenic(III) affects AP-1 activation and DNA binding in a time-dependent manner. Short-term (24 h) exposure of GM847 fibroblast cells to arsenic increased the expression of c-fos and c-jun, and AP-1 binding to DNA, while chronic exposure (10–20 weeks) reduced all of these measures [93]. The level of nuclear c-jun as well as AP-1 DNA binding was increased in rat lung slices treated with arsenic(III). AP-1 proteins were primarily found in type II epithelial cells and alveolar macrophages of the lung slices [91]. Some studies indicate that AP-1 activation by arsenic is dependent on protein kinase C [105,106].

Chromium, nickel, cadmium, lead, cobalt, and iron also increase AP-1 activation. In vitro induction of AP-1 by chromium(VI) was mediated by p38 and inhibited by the antioxidant acetylsalicylic acid [107]. The role of nickel in the activation of AP-1 is unclear. In one study nickel(II) increased transcription of c-jun and c-fos, increased expression and activation of c-jun, and activated AP-1 in BEAS-2B human bronchial epithelial cells. A second study found no activation of AP-1 by nickel(II) in mouse epidermal or fibroblast cell lines or in BEAS-2B cells [94,108]. As with NFkB, lower levels of AP-1 DNA binding were observed in cells resistant to nickel toxicity [29]. The transformation of BALBc/3T3 cells by cadmium(II) involved an increase in c-fos and c-jun that was dependent on superoxide radicals, hydrogen peroxide, and calcium. Once transformed, these cells were capable of forming tumors in nude mice [109]. The activation of AP-1 in JB6 cells by cadmium is primarily through the ERK/MAPK pathway, although p38 and JNK pathways were also activated. Induction of AP-1 by cadmium was enhanced by phorbol ester and by EGF [110]. Both lead(II) and cobalt(II) have been shown to induce AP-1 DNA binding in the PC12 pheochromocytoma cell line. Lead induced the expression of the AP-1 subunits c-fos and c-jun via pathways involving PKC, MAPKK, and JNK [111,112]. The induction of AP-1 by cobalt(II)-induced apoptosis and was inhibited by antioxidants [113]. Coal mined from Pennsylvania, which contains high levels of bioavailable iron, was found to enhance AP-1 in JB6 cells through a mechanism that involved p38 and ERKs [114].

2.7. NFAT

Members of the NFAT family of nuclear transcription factors were first observed as effectors of cytokine signaling in T cells. Of the five NFAT members identified thus far, four (NFAT1–NFAT4) are calcium-dependent. These four NFATs affect the production of cytokines including interleukins, TNF-α, interferon-γ, and granulocyte macrophage-colony-stimulating factor in multiple organ sites and cell types [115]. NFAT proteins have been reported to be involved in a wide variety of developmental processes, including the regulation of skeletal muscle growth and differentiation, as well as angiogenesis, chondrogenesis and adipogenesis [116–121]. Like NFκB, inactive NFAT components are present in the cytoplasm and translocate to the nucleus upon activation. Unlike NFκB, NFAT is activated by dephosphorylation. This dephosphorylation occurs when the phosphatase calcineurin is activated by high intracellular calcium levels [122].

Calcium-dependent forms of NFAT can activate gene transcription in conjunction with AP-1. Due to the close proximity of their DNA binding sites, NFAT and AP-1 can form ternary complexes and can synergistically activate one another. Through these interactions, NFAT and AP-1 co-regulate expression of interleukins, TNF-α, interferon-γ, granulocyte macrophage-colony-stimulating factor, Fas ligand, CD25, and Cox-2 [122]. In addition to AP-1, NFAT proteins can also interact with NFκB [123].

Several studies by Huang et al. indicate that metals affect the expression of NFAT through ROS-related mechanisms. Both vanadium(IV) and (V) were found to induce the expression of NFAT in JB6 mouse epidermal and PW mouse embryo fibroblast cells. Vanadium(V) consistently induced the highest expression of NFAT. This expression was found to be dependent both on calcium and H_2O_2. It was proposed that elevated intracellular H_2O_2 may cause increases in cytoplasmic calcium levels, thus activating NFAT [124]. Hydrogen peroxide was also found to be a causative agent of NFAT induction by nickel(II) chloride or nickel(III) subsulfide in PW cells. Nickel subsulfide-induced NFAT to a greater degree than did nickel chloride [125]. In a third study, both coal containing high levels of bioavailable iron and ferrous sulfate (a major form of iron found in coal) alone induced both NFAT and AP-1 in JB6 cells [114].

2.8. HIF-1

HIF-1α is a heterodimeric transcription factor that is strongly induced by hypoxia and is overexpressed in many cancers. It is composed of HIF-1α and HIF-1β. HIF-1α is strongly induced by low cellular oxygen while HIF-1β is expressed constitutively. HIF-1 activity increases as cells undergo transformation to a carcinogenic phenotype [126,127]. HIF-1 regulates the expression of erythropoeitin, HO-1, aldolase, enolase, and lactate dehydrogenase A, all of which have important implications for carcinogenesis. Ras and c-src induce HIF-1 [128,129]. Chromium(VI)-induced HIF-1α through a p38 and hydrogen peroxide-dependent

mechanism in the DU145 human prostate cancer cell line [130]. Costa et al. [131,132] used a variety of human and rodent cell lines to demonstrate that HIF-1 is induced by both soluble and insoluble nickel. Although insoluble nickel is generally considered to be more carcinogenic than soluble forms, microarray expression analysis of the effects of nickel revealed that acute exposure to either soluble or insoluble nickel induce HIF-1 to a similar degree [126].

2.9. p53

It has been estimated that the p53 gene is mutated in greater than 50% of all human cancers [133]. The p53 protein functions as a transcription factor and is involved in the activation of apoptosis-inducing genes including (non-ras) p21 and GADD45 [134,135]. The binding of p53 to DNA requires zinc. This may make the p53 protein especially susceptible to inactivation by transition metals since they are known to substitute for zinc [136]. In addition, p53 is sensitive to and can be activated by changes in oxidative conditions within the cell, due its content of to labile cysteine residues [26]. A number of metals including, arsenic, chromium, nickel, beryllium, cadmium, cobalt, and iron can affect p53 expression.

The effects of arsenic on p53 are dependent on the cell type in question and the chemical form of arsenic. The organic/inorganic, oxidation and methylation states of arsenic were observed to influence p53 protein levels in U2OS osteosarcoma cells. Two inorganic forms of arsenic(III), arsenic trioxide and sodium arsenite, induced p53 in a dose- and time-dependent manner. The dimethylated forms of arsenic(III) or arsenic(V) used in the study (dimethylarsinic acid and iododimethylarsenic, respectively) also induced p53 in a dose- and time-dependent manner. In contrast, monomethylated forms had no effect on p53 activation. Overall, arsenic(III) trioxide was the strongest inducer of p53 [17].

Arsenic(III) is unique among metals in that it is both a carcinogen and a chemotherapy agent. As arsenic(III) trioxide, it has been used successfully as a treatment for acute promyelocytic leukemia [137,138]. Arsenic trioxide induces apoptosis in 90% of myeloma cells possessing mutated p53 versus 40% of cells with wild-type p53. Caspase-3 was induced in both cell types. In contrast, caspase-8 was also induced only in mutant cells while caspase-9 was induced only in cells possessing wild-type p53 [139]. Arsenic trioxide-induced apoptosis in the small cell lung cancer line NeI-H and the gastric cancer cell lines AGS and MKN-28 by the induction of p53. In NeI-H cells, bcl-2 was also induced, whereas caspase-3 was activated in the gastric cancer cell lines [140,141].

A frequent target of arsenic-induced cancer is the skin, but the role of p53 in these cancers remains unclear. Overexpression of p53 was found in 44% of squamous cell carcinomas but only 14% of basal cell carcinomas of patients in an area of Taiwan where arsenic intoxication is endemic [142]. p53 protein accumulation was found in 78% of the pre-malignant skin lesions of patients treated with arsenic-containing medications but mutations of the p53 gene were found in only 30% [143]. Basal cell carcinomas induced by arsenic exposure were found to overexpress p53 less frequently than sporadic basal cell carcinomas [144]. The human keratinocyte cells line HaCat, exposed to arsenite for 14 days, exhibited dose- and time-dependent decreases in p53 in conjunction with dose- and time-dependent increases in mdm2 expression [145]. Arsenic may affect p53 gene expression by hypermethylation. Hypermethylation of the p53 promoter was observed when A549 cells were treated with either sodium arsenite or arsenate [146].

Chromium(VI) can indirectly induce p53-mediated apoptosis in multiple ways: by causing DNA damage, via DNA binding by chromium(VI) reduction products, by activation of MAPKs upstream of p53, through the oxidative activation of p53 itself, and by enhancing the activity of other carcinogens. Reduced forms of chromium and the ROS generated during reduction reactions have the capacity to damage DNA. DNA strand breaks and other forms of damage activate upstream kinases including DNA protein kinase, ATM, and ATR, all of which are capable of activating p53 [147]. Despite the capacity of chromium and its reduction products to damage DNA, the p53 gene may not be a major target for chromium-induced mutation in humans. A study of former chromium workers with lung cancer indicated that p53 mutations are uncommon [148]. In another study comparing chromium workers with the general population, elevated blood levels of p53 were found in 19% of chromium workers [149].

The binding of chromium(III) and chromium(V) to DNA can result in the activation of the MAPKs, JNK and p38. This, in turn, can activate p53. Chromium(VI)-enhanced p53 activity in a dose-dependent manner in A549 cells by increasing protein expression, by enhancing p53 transactivation, and by causing mdm2 dissociation from p53. The dissociation of mdm2 resulted from phosphorylation of serine 15 of the p53 protein, and was ERK/MAPK-dependent [150]. p53 was shown to be essential to chromium(VI)-induced apoptosis in human lung fibroblasts. Treatment with chromium(VI)-induced p53 protein expression and transactivation. Cells with reduced p53 levels were resistant to apoptosis induced by chromium(VI) [151]. Chromium(VI) enhances the binding of the carcinogen benzopyrene-diol-epoxide to the p53 gene in normal human lung fibroblast cells. Interestingly, the binding sites (exons 7 and 8, codons 248, 273, and 282) are "hot spots" associated with lung cancer causation [152].

Chromium(VI)-induced p53 activation and apoptosis in a dose-dependent manner in A549 cells [153]. Using a variety of enzymes, cofactors and antioxidants, it was determined that the activation of p53 was hydrogen peroxide and hydroxyl radical-dependent as well. The addition of SOD, which catalyzes the formation of the hydroxyl radical precursor, hydrogen peroxide, increased p53 activity in chromium(VI)-treated A549 cells. NADPH, which enhances hydroxyl radical formation through chromium(VI) reduction, also increased p53 activity. Catalase, which catalyzes the conversion of hydrogen peroxide to water and oxygen, decreased p53 activity. The general antioxidant acetylsalicylic acid and the hydroxyl radical scavenger sodium formate reduced p53 activity by preventing the generation of hydroxyl radical from hydrogen peroxide. Finally, the use of the chelator deferoxamine, which prevented chromium's ability to catalyze hydroxyl radical formation, reduced p53 activity. Based on this data, it was concluded that hydrogen peroxide (and the hydroxyl radical derived from it)-induced p53 in A549 cells [154]. The apoptotic effects of chromium(VI) are time-dependent. Early apoptotic events (0–3 h treatment) induced by chromium(VI) are ROS-dependent, whereas later events (3–24 h treatment) are both ROS- and p53-dependent in A549 cells [155].

Nickel induces p53 expression and p53 gene mutations in vitro, but its effects on human p53 expression are not well-documented. Nickel(II) acetate-induced p53 activation, cell cycle arrest, and apoptosis in Chinese hamster ovary cells [156]. Nickel(II) chloride induces the accumulation of wild-type, but not mutant p53 in MCF7 and A549 cells [157]. A T:C transition mutation was observed at codon 238 of the p53 genes in human kidney epithelial cells immortalized with nickel(II) [158]. No increase in nasal p53 levels were observed in nickel refinery workers as compared with workers in non-nickel exposed occupations [159].

The effects of beryllium on p53 in animals are equivocal. An increase in lung neoplasms was observed for p53 ± knockout mice versus mice homozygous for wild-type p53 after inhalation exposure to beryllium [160]. F344/N rats exposed to beryllium by inhalation showed no evidence of p53 mutations in the lung, although 64% of the animals developed tumors [161].

Cadmium-induced p53 in a variety of model systems via MAPK-dependent and MAPK-independent mechanisms. TNF-α enhanced cadmium-induced apoptosis in NIH3T3 and BALBc/3T3 fibroblast cell lines by a p53-dependent mechanism [162]. When Clara cells and type II cells from rat lung were exposed to cadmium acetate, both displayed p53 and Bax-dependent apoptosis which was independent of ROS generation. Type II cells were more cadmium-sensitive, however [163]. Cadmium treatment induced the transcription of p53, c-*myc* and c-jun in human prostate epithelial cell line RWPE-1 prior to apoptosis. A large percentage (35%) of the cells did not undergo apoptosis, however. The authors suggested that cadmium treatment may select for apoptosis-resistant cell sub-populations [164]. Cadmium chloride treatment-induced apoptosis in the prostate of Wistar rats in a dose and time-dependent manner. Transcription of p53 also increased in a dose- and time-dependent manner, indicating that the observed apoptosis may have been p53-dependent [165]. Cadmium chloride induced the phosphorylation of p53 at serine 15 in the MCF7 breast cancer cell line by a PI3K-dependent, MAPK-independent mechanism [166]. In a second study, cadmium inhibited the binding of wild-type p53 to DNA in MCF7 cells via alteration of p53 protein structure. In the same study, cadmium inhibited the expression of p53 in response to DNA-damaging agents such as hydrogen peroxide [136].

Cobalt and iron affect p53 transcription and expression, while iron alone induces p53 mutations. Cobalt(II) chloride inhibited the transcription of p53 in HeLa cells in a manner similar to hypoxia or HIF-1α protein. Site-directed mutagenesis data indicated that the E-box of the p53 promoter was responsible for cobalt-induced transcriptional inhibition [167]. Individuals afflicted with the iron storage disease hemachromatosis possess extremely high iron levels and are 200 times more likely to develop liver cancer than the general public. A study of hemachromatosis patients found elevated levels of p53 mutations in the liver, specifically G:C to T:A transversions in codon 249 and C:G to A:T and C:G transversions in codon 250. In addition, p53 protein expression was increased [168]. Interestingly, iron deprivation as a result of deferoxamine treatment has been shown to inhibit p53 expression in ML-1 and Raji cells which carry wild-type and mutant p53 genes, respectively [169].

3. ROS and metal-induced signal transduction

Carcinogenic metals alter normal signal transduction in a number of ways: through DNA damage, DNA binding, the inhibition of DNA repair, gene silencing, and through the production of ROS [19,21,26,131,155,170,171]. ROS have been observed to affect several aspects of signal transduction, including MAPK signaling, calcium signaling, and the activation of transcription factors including NFκB, AP-1, NFAT, and HIF-1 [105,124,172–177]. Here, we discuss the effects of hydroxyl radicals, thiyl radicals, superoxide radicals, and hydrogen peroxide, all of which can be formed through metal-catalyzed reactions, on signal transduction pathways associated with carcinogenesis.

3.1. Hydroxyl radical formation

Transition metals (metals that can exist in multiple oxidation states) catalyze the formation of ROS, by donating or accepting single electrons. ROS, including hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$) interact to form the hydroxyl radical ($^{\bullet}OH$) by Fenton-type and Haber Weiss-type reactions [178].

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + {}^{-}OH + {}^{\bullet}OH \quad (1)$$

$$M^{(n+1)} + O_2^{\bullet -} \rightarrow M^{n+} + O_2 \quad (2)$$

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{M^{n+}/M^{(n+1)+}} O_2 + {}^{-}OH + {}^{\bullet}OH \quad (3)$$

Eqs. (1)–(3) demonstrate the effects of transition metals on the formation of the hydroxyl radical. In Fenton-type reactions (Eq. (1)), metal oxidation ($M^{n+} \rightarrow M^{(n+1)+}$) is coupled to hydrogen peroxide disproportionation to form the hydroxyl ion and the hydroxyl radical. Eq. (2) demonstrates the ability of superoxide to reduce an oxidized metal through the donation of a single electron. By combining Eqs. (1) and (2), we arrive at Eq. (3), the Haber–Weiss reaction. It is the reduction of metal by superoxide in Eq. (2) that catalyzes the heterolytic cleavage of hydrogen peroxide to form the hydroxyl radical and hydroxyl anion in Eq. (3). The Haber–Weiss reaction will not proceed unless it is coupled to metal reduction [179]. The reactions described in Eqs. (1)–(3) can function in a cycle wherein metals are repeatedly oxidized and reduced (thus the term redox cycling).

Due to metal catalysis, this oxidation/reduction cycle results in the rapid formation large quantities of the highly toxic hydroxyl radical. Chromium(VI), nickel(II), cobalt(II), and iron(II) are capable of producing hydroxyl radicals and inducing DNA damage via Fenton and Haber–Weiss-type reactions under physiological conditions. Two carcinogenic metals (cadmium and lead) are incapable of directly inducing free radical formation through Fenton-like reactions. Instead, they deplete cells of gluthathione and other sulfhydryls. This dramatically lowers the reducing capacity of the cell and allows free radicals to be produced at a higher than normal rate [180,181].

Metal chelation may enhance or inhibit the Fenton reaction depending on the metal and the chelator in question. Chelation of iron(II) by EDTA enhances the formation of hydroxyl radical while deferoxamine, a chelator that prevents reaction of iron with hydrogen peroxide, reduces its formation. The generation of hydroxyl radicals by nickel(II) and cobalt(II) is enhanced by peptide and protein chelation, which may have important implications in vivo. In addition to the Fenton and Haber–Weiss reactions, metals can also catalyze the formation of the hydroxyl radical by reacting with hyperchlorite (HOCl), which is produced by phagocytes. Hyperchlorite can also react with myeloperoxidase to form singlet oxygen ($^1O_2$) [18].

The hydroxyl radical has been implicated in MAPK signaling and gene expression in a variety of cell types. Furthermore, hydroxyl radicals affect cellular processes with important implication for carcinogenesis, including apoptosis and angiogenesis. The increased p38 expression observed in the human small cell lung cancer line CL3 treated with Cr(VI) is hydroxyl radical-dependent [182].

In oncogenically-transformed rat fibroblast cells depleted of glutathione, inhibitors of Haber–Weiss reactions (antioxidants and metal chelators) also inhibited apoptosis. In contrast, non-transformed rat fibroblasts underwent apoptosis as a result of hydrogen peroxide, but not hydroxyl radical formation [183]. Another study has indicated that decreased hydroxyl radical formation, which occurs under hypoxic conditions, resulted in the expression of erythropoietin, a gene associated with angiogenesis. Treatment of HepG2 liver cells with cobalt or nickel(II) chloride resulted in a reduction in hydroxyl radical formation and an increased expression of erythropoeitin [184].

3.2. Thiyl radicals

As indicated earlier, ROS formed by Fenton and Haber–Weiss reactions, such as the hydroxyl radical, can damage macromolecules, including DNA, RNA, and protein [18,185]. The hydroxyl radical can react with cysteine-containing proteins to form thiyl radicals. Eq. (4) shows the reaction of a sulfhydryl-containing protein with the hydroxyl radical to form a thiyl (protein-S$^{\bullet}$) radical. Thiyl radicals may form additional radicals or may react with reducing agents in the cells, such as glutathione. In Eq. (5), the thiyl radical reacts with glutathione to form an intermediate that can react with molecular oxygen to form a glutathionylated protein and superoxide (Eq. (6)). Note that the superoxide formed can react with hydrogen peroxide from Haber–Weiss-type reactions (Eq. (3)) to form additional hydroxyl radicals. This series of reactions is important because many proteins involved in signaling (EGF receptor, c-src, and phosphatases) possess sulfhydryl groups, which are available to react with ROS [31].

$$\text{protein-SH} + \text{OH}^{\bullet} \rightarrow \text{protein-S}^{\bullet} + \text{H}_2\text{O} \quad (4)$$

$$\text{protein-S}^{\bullet} + \text{GSH} \rightarrow \text{protein-SS}^{\bullet -} + \text{G} \quad (5)$$

$$\text{protein-S-S}^{\bullet -}\text{G} + \text{O}_2 \rightarrow \text{protein-S-S-G} + \text{O}_2^{\bullet -} \quad (6)$$

3.3. Superoxide and hydrogen peroxide

Neutrophils, eosinophils, and macrophages are capable of superoxide formation as part of a “respiratory burst”. NADPH oxidase, a flavoenzyme formed within the cell membrane in response to immune challenge and other stressors, catalyzes the formation of superoxide. Eq. (7) shows the formation of superoxide by NADPH oxidase.

$$\text{NADPH} + 2\text{O}_2 \xrightarrow{\text{NADPH oxidase}} \text{NADP}^{+} + \text{H}^{+} + 2\text{O}_2^{\bullet -} \quad (7)$$

In addition to reactions catalyzed by NADPH oxidase, superoxide may also be formed as a by product of cox-2, lipoxygenase, and xanthine oxidase-catalyzed reactions [186]. Exposure to the carcinogenic metals arsenic and chromium has been shown to increase superoxide production in vitro and in vivo [187–189]. In addition to effects on superoxide production, metals including chromium, cadmium, copper and lead have been reported to inhibit SOD in vivo [190–192]. In this way, metals may increase superoxide formation and inhibit its removal from biological systems.

A number of tumors have been found to possess low levels of superoxide dismutase, indicating that superoxide may play a role in carcinogenesis. Indeed, overexpression of superoxide dismutase has been shown to suppress tumor growth in a two-stage skin cancer model [193]. Superoxide may play a role in cancer through its ability to affect ras signaling and through its ability to liberate sequestered iron. The use of the antioxidants GSH and melatonin inhibited ras signaling in NIH/3T3 fibroblast cells, whereas transformation with Ha-ras caused an increase in superoxide production [194]. Superoxide is capable of liberating iron from ferritin and hemosiderin, thus allowing for additional ROS formation [195]. This is of particular interest because iron has been proposed to be the ultimate carcinogen in metal-induced carcinogenesis [18].

The superoxide formed by the reaction in Eq. (7) can be quickly converted to hydrogen peroxide by the action of SOD, or by glutathione peroxidase. Alternatively, the superoxide formed by this reaction may spontaneously dismutate to form hydrogen

peroxide. In either case, the resulting hydrogen peroxide can participate in Fenton and Haber–Weiss-type reactions. Hydrogen peroxide may represent a link between metal-induced cancer and signal transduction via its effects on MAPK signaling. Hydrogen peroxide is believed to activate MAPK signaling by deactivation of protein tyrosine phosphatases [196]. Hydrogen peroxide can reversibly oxidize thiolate anions ($-S^-$), which are found only in proteins where cysteines are surrounded by positive charges. Protein tyrosine phosphatases, which serve to "turn off" activated kinases contain thiolate anions. Transient oxidation of critical cysteines can inactivate phosphatases in vivo, allowing kinase signaling to continue until phosphatases are reactivated by reduction. In a normal cell, ROS are rapidly eliminated by enzymatic and chemical reduction. It is possible that the rapid formation and disappearance of ROS also serve as "on and off" switches for cellular signaling in adjacent cells [197].

Interference with intercellular signaling by hydrogen peroxide may change gene expression in such a way that uncontrolled growth and resistance to apoptosis is encouraged. Hydrogen peroxide is the major ROS inducer of HIF-1 and VEGF [130]. Hydrogen peroxide formation is also associated with the ras/MAPK signaling pathways. Transformation of NIH/3T3 fibroblasts with H-ras resulted in increased production of hydrogen peroxide [194]. Hydrogen peroxide is essential for the activation of p38 and ERKs in UV-treated JB6 cells [198]. Transient increases in intracellular calcium and expression of the inflammatory cytokine interleukin-6 in human lung fibroblasts were induced by hydrogen peroxide in an ERK-dependent manner [199]. Hydrogen peroxide also stimulated ERK 1/2 expression in rat pleural epithelial cells in a dose- and time-dependent manner [200].

4. Future perspectives

Studies of the effects of metals and metal-induced ROS on cell signaling have previously focused on individual genes or proteins, due to the technological and temporal limitations of examining multiple interactions. With the advent of genomics, and now proteomics, a paradigm shift is occurring wherein thousands of genes or proteins can examined at once. The effects of several of the carcinogenic metals including arsenic, chromium, nickel, and cadmium on gene expression have been studied using a genomic method, the microarray [126,201–203]. This work has linked metals to the expression of genes previously unassociated with metal treatment, thus more clearly defining their effects on signaling. The new field of metabonomics, combined with information from proteomics and genomics will provide a clearer picture of the effects of metals on the 3-dimensional network that is signal transduction, replacing the 2-dimensional, linear understanding that we have today.

Acknowledgements

Many thanks to Steve Leonard and Fei Chen for their help during the preparation of this manuscript.

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