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Cadmium and copper inhibit both DNA repair activities of polynucleotide kinase

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ABSTRACT

Human exposure to heavy metals is of increasing concern due to their well-documented toxicological and carcinogenic effects and rising environmental levels through industrial processes and pollution. It has been widely reported that such metals can be genotoxic by several modes of action including generation of reactive oxygen species and inhibition of DNA repair. However, although it has been observed that certain heavy metals can inhibit single strand break (SSB) rejoining, the effects of these metals on SSB end-processing enzymes has not previously been investigated. Accordingly, we have investigated the potential inhibition of polynucleotide kinase (PNK)-dependent single strand break repair by six metals: cadmium, cobalt, copper, nickel, lead and zinc. It was found that micromolar concentrations of cadmium and copper are able to inhibit the phosphatase and kinase activities of PNK in both human cell extracts and purified recombinant protein, while the other metals had no effect at the concentrations tested. The inhibition of PNK by environmentally and physiologically relevant concentrations of cadmium and copper suggests a novel means by which these toxic heavy metals may exert their carcinogenic and neurotoxic effects.

1. Introduction

The biological effects of heavy metal exposure are of growing concern due to the increasing release of such metals into the environment via industrial and agricultural processes, exacerbated by their tendency to accumulate in biotic systems. Many heavy metals are highly toxic, with well-established evidence for several metals having adverse effects on the central nervous and nephrotic systems for example [1]. The International Agency for Research on Cancer has also classified several metals and their compounds as carcinogens [2]. However, the mechanisms by which heavy metals induce their harmful effects are complex and multifactorial, and include the elevation of cellular oxidative stress via either the Fenton-mediated generation of reactive oxygen species (ROS) or inhibition of antioxidant activity; disruption of cell signaling; and inhibition and down-regulation of DNA repair processes (see Beyersmann and Hartwig's recent extensive review on this subject [3]).

The latter of these underlying mechanisms, i.e. disruption of the cell's ability to repair damage to its genetic material, has been the subject of much recent research interest. It is well-established that dysregulation or inhibition of DNA repair processes leads to genetic instability and may ultimately result in cancer. Several heavy metals have been shown to reduce cellular DNA repair capacity by either down-regulation of DNA repair genes or through direct inhibition of the enzymes involved [3]. Metal-induced enzyme inhibition may occur through disruption of the physical structure of the proteins involved. For example, a number of metals, including Cd, Ni, Co and Pb, can displace the Zn of Zn fingers, found in repair enzymes such as XPA and PARP-1 [4]. Alternatively, if a protein requires a metal ion as a cofactor, then another metal with the same charge or ionic radius may compete for binding leading to a change in activity: for example Cd^{2+} can readily compete with Ca^{2+}.

As mentioned above, one possible route for heavy metal-induced carcinogenicity is via elevation of cellular ROS. Oxidative stress is a key determinant of cellular DNA damage, with physiologically derived ROS also playing an important role [5]. One form of DNA damage known to be generated as a result of ROS are single strand breaks (SSBs) in the DNA, which can arise through oxidative attack at sugar residues or as intermediates in the base excision repair (BER) of oxidized bases. Prompt and efficient repair of such damage is essential as strand breaks are both recombinogenic and cytotoxic. Repair of SSBs is carried out via the single strand break repair (SSBR) pathway, which involves a number of enzymes whose combined functions allow the two ends of the break to be rejoined [6]. Interestingly, a number of studies have shown that certain heavy metals are able to delay strand break rejoining in mammalian cells (see for example [7–10]). However, the underlying mechanism for metal-dependent inhibition of single strand break repair is not yet fully understood.

Abbreviations: IPTG, isopropyl β-D-1-thiogalactopyranoside; PNK, polynucleotide kinase; ROS, reactive oxygen species; SCE, sister chromatid exchange; SSB, single strand breaks; SSBR, single strand break repair.

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When SSBs are generated they often carry defective 5′ and 3′ termini, which must be converted into 5′-phosphate and 3′-hydroxyl groups prior to strand resynthesis and ligation. As a result, cells possess a number of enzymes with end-processing activities, such as APE1, aprataxin and Tdp1, that play a critical role in the repair pathway [11]. One enzyme which can repair some forms of damaged termini is the bifunctional enzyme polynucleotide kinase (PNK), which has two semi-independent end-processing activities: 5′-kinase and 3′-phosphatase [12–17]. In addition to its function in SSBR, this enzyme has also been shown to be involved in the repair of double strand breaks via the non-homologous end-joining and alternative end-joining pathways [18,19]. PNK's importance for maintaining genetic stability has been highlighted by RNAi experiments which show that suppression of PNK results in an increased spontaneous mutation rate and elevated sensitivity to carcinogens including ionising radiation, hydrogen peroxide, camptothecin and methyl methanesulfate [20].

The effects of heavy metals on end-processing enzymes have not previously been explored. In the present paper we have therefore employed our previously described system involving fluorescently labeled oligonucleotide substrates [13] to investigate whether heavy metals are able to inhibit PNK-dependent single strand break repair. We find that Cd(II) and Cu(II) are able to inhibit both the kinase and phosphatase activities of PNK, with the other metals investigated, Co(II), Pb(II), Ni(II) and Zn(II), showing no detectable effects at the concentration tested.

2. Materials and methods

2.1. Reagents

Unless stated otherwise, chemicals were from Sigma–Aldrich (Poole, UK) and fluorescent oligonucleotides were supplied by Eurogentec (Belgium). Fluorescently labeled double-stranded substrates were prepared as previously described [13].

2.2. Preparation of recombinant Strep-tagged PNK

A vector encoding N-terminally Strep II epitope-tagged PNK was prepared by site-directed mutagenesis of the bacterial expression vector pETPNK [13], using the following primers (VH Bio, Newcastle, UK) to replace the His-tag with a Strep II-tag: 5′-AGAAGGAGATATACCATGGGCTGGAGCCATCCTCAGTTTGAGAAGAGCAGCGGCCTGGTGCCGCGC-3′ and 5′-GCGCGGCACCAGGCCGCTGCTCTTCTCAAACTGAGGATGGCTCCAGCCCATGGTATATCTCCTTCT-3′. Strep-tagged PNK was then expressed in transformed Rosetta 2 (DE3) pLysS cells (Novagen, Nottingham, UK) following induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. PNK was purified using Strep-Tactin resin (Novagen, Nottingham, UK) according to the manufacturer's conditions and dialyzed into buffer containing 50 mM HEPES pH 7.8, 100 mM KCl, 1 mM DTT and 10% glycerol. Protein concentration was measured by UV absorbance at 280 nm using a calculated extinction coefficient for the tagged protein of 62,730 M^{-1} cm^{-1}.

2.3. Inhibition assays using cell extracts

Whole cell extracts were prepared as described previously [13] from actively dividing early passage MRC5 human lung fibroblasts [21] grown in DMEM (Lonza) supplemented with 10% heat inactivated fetal calf serum (Invitrogen) and 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza) at 37 °C in a humidified incubator with 5% carbon dioxide. Chloride salts of individual heavy metals (200 μM) were pre-incubated with 25 μg of whole cell extract for 10 min at 4 °C in buffer containing 50 mM HEPES–KOH (pH 7.8), 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 2 mM ATP, 0.4 mg/ml BSA, 25 nM phosphocreatine, 2.5 μg creatine phosphokinase, 8.5% glycerol, 20 μM dNTPs, 0.2 mM NAD^+ and 1 μg of single-stranded competitor DNA. The indicated fluorescently labeled nicked substrate was then added to a final concentration of 50 nM and the reaction was incubated for 30 min at 37 °C before quenching by the addition of an equal volume of formamide loading buffer (10 mM EDTA, 98% formamide, 10 mg/ml blue dextran). Samples were heated to 95 °C for 3 min and resolved under denaturing conditions on a 20% denaturing polyacrylamide gel. Gels were visualized using a Typhoon 9410 laser scanner (Amersham). Experiments were carried out in triplicate and a representative gel is shown.

2.4. Metal titrations using recombinant hPNK

Cu or Cd (see figure legends for details) were mixed with the indicated amounts of PNK and incubated for 10 min at 4 °C in buffer containing 50 mM HEPES–KOH (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 0.1 mg/ml BSA. The appropriate substrate (*Substrate 3* for phosphatase and *Substrate 4* for kinase) was added to a final concentration of 50 nM and reactions were incubated for 10 min at 37 °C before quenching by the addition of an equal volume of formamide loading buffer. Samples were then heated to 95 °C for 3 min, resolved in a 20% denaturing polyacrylamide gel and visualised and analysed as described above. All experiments were performed in triplicate and a representative gel is shown in each case. Band intensities were measured using ImageQuant software and the amount of product generated in each case was calculated as the intensity of the product band as a proportion of the total intensities of the product and substrate bands.

3. Results

3.1. Heavy metal inhibition of PNK-dependent SSBR

We tested a panel of divalent heavy metals (Cd, Co, Cu, Ni, Pb and Zn) for the inhibition of cell extract-mediated repair of a dual-labeled oligonucleotide substrate containing a nick flanked by a 5′-hydroxyl and a 3′-phosphate (*Substrate 1*) (see Fig. 1). The use of two different fluorophores, Cy5 and TAMRA, allows both the phosphatase and kinase activities of the enzyme to be investigated simultaneously during SSBR of the substrate [13]. Phosphatase-mediated loss of the 3′-phosphate on the Cy5-labeled strand results

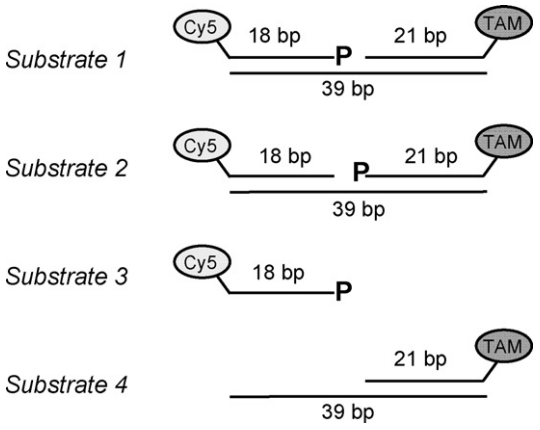

Fig. 1. Schematic of the four fluorescent substrates used to investigate PNK inhibition. Sequences (5′–3′) of the oligonucleotides were (Cy5)-TAGCATCGATCAGTCCTCp (*Substrates 1* and *3*), GAGGTCTAGCATCGTTAGTCA-(TAMRA) (*Substrates 1* and *4*), (Cy5)-TAGCATCGATCAGTCCTC (*Substrate 2*) pGAGGTCTAGCATCGTTAGTCA-(TAMRA) (*Substrate 2*) and unlabeled complementary strand TGACTAACGATGCTAGACCTCTGAGGACTGATCGATGCTA (*Substrates 1*, *2* and *4*).