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Impact of cadmium, cobalt and nickel on sequence-specific DNA binding of p63 and p73 *in vitro* and in cells

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ABSTRACT

Site-specific DNA recognition and binding activity belong to common attributes of all three members of tumor suppressor p53 family proteins: p53, p63 and p73. It was previously shown that heavy metals can affect p53 conformation, sequence-specific binding and suppress p53 response to DNA damage. Here we report for the first time that cadmium, nickel and cobalt, which have already been shown to disturb various DNA repair mechanisms, can also influence p63 and p73 sequence-specific DNA binding activity and transactivation of p53 family target genes. Based on results of electrophoretic mobility shift assay and luciferase reporter assay, we conclude that cadmium inhibits sequence-specific binding of all three core domains to p53 consensus sequences and abolishes transactivation of several promoters (e.g. BAX and MDM2) by 50 μM concentrations. In the presence of specific DNA, all p53 family core domains were partially protected against loss of DNA binding activity due to cadmium treatment. Effective cadmium concentration to abolish DNA–protein interactions was about two times higher for p63 and p73 proteins than for p53. Furthermore, we detected partial reversibility of cadmium inhibition for all p53 family members by EDTA. DTT was able to reverse cadmium inhibition only for p53 and p73. Nickel and cobalt abolished DNA–p53 interaction at sub-millimolar concentrations while inhibition of p63 and p73 DNA binding was observed at millimolar concentrations. In summary, cadmium strongly inhibits p53, p63 and p73 DNA binding *in vitro* and in cells in comparison to nickel and cobalt. The role of cadmium inhibition of p53 tumor suppressor family in carcinogenesis is discussed.

1. Introduction

Members of p53 protein family (p53, p63 and p73) control transcription of many genes involved in cell cycle control, DNA repair, cellular senescence or apoptosis in response to DNA damage [1,2]. Evolutionarily older p63 and p73 display strong homology to p53 in three major domains: N-terminal transactivation domain, central DNA binding domain (DBD) and C-terminal oligomerisation domain. Even though subtle structural differences were observed, a generally conserved conformation of DBDs and DNA–protein contact sites were revealed from structure analysis of co-crystals of p53, p63 and p73 DBDs with DNA target sequences. Their central DBDs are highly homologous and share an immunoglobulin-like folded structure [3–5] responsible for binding to sequence-specific response elements (REs), whose overall consensus sequence is similar to that of the canonical p53 consensus sequence (p53CON, containing two half-site decamers 5′PuPuPuC(A/T)(T/A)GPyPyPy3′ in direct orientation, [3]). Mediated by DBD, sequence-specific interactions with REs in regulatory regions of p53 family target genes are crucial for their function as transcription factors.

p53 family belongs to metalloproteins, a zinc ion coordination in DBD by three cysteines and one histidine is necessary for DNA binding of all family members [3–7]. These and other cysteines are involved in already reported p53 redox sensitivity [8]. Recently, we described that p63 and p73 DBDs share redox sensitivity to diamide and hydrogen peroxide with p53 [8,9]. We have shown that oxidation of p63 and p73 DBDs abolished p53CON recognition

Abbreviations: p53CON, p53 consensus sequence; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; p53CD, GSTp53CD (aa 94–312); p63CD, GSTp63CD (aa 114–349); p73CD, GSTp73CD (aa 104–339); p53, full length p53; p53DBD, p53CD (aa 94–312); DBD, central DNA binding domain.

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and that reversibility of DBD oxidation was dependent on zinc accessibility [9]. Similarly, binding of transition metals like cadmium, cobalt, nickel, mercury and copper to the p53 protein abolishes p53 conformation and DNA binding [10–14], likely through very similar set of cysteines and histidines respective to metal ion preferences [10]. Metal sensitivity was currently established only for p53.

Metals can influence p53 on several levels, apart from indirect effects (ROS and NOS induced alterations to DNA bases, altered lipid metabolism and calcium homeostasis) [15–17], p53 is sensitive to local perturbations induced by metal ions themselves [10,12]. In this regard, redox inactive metals, such as cadmium and nickel exhibit their toxicity via binding to sulfhydryl groups of proteins and depletion of glutathione (reviewed in [17]). On the other hand, redox active metals like cobalt are as well potent inducers of oxidative stress, causing generation of free radicals. As environmental pollutants, selected metals pose ever-growing carcinogenic threat. Cadmium (Cd) and nickel (Ni) were classified by IARC as carcinogenic for humans while cobalt (Co) belongs to the group of "possibly carcinogenic to humans" [17].

In this study we used divalent ions of transition metals cadmium, cobalt and nickel to study their effect on sequence-specific binding of p53 family core domains *in vitro* and in cells. Our data show that the sequence-specific DNA binding properties of all three core domains were inhibited at micromolar $CdCl_2$ range and millimolar concentrations of $NiCl_2$ or $CoCl_2$. Also transactivation of MDM2, BAX or p53CON promoter in H1299 cells transfected with p53 family members was inhibited by micromolar concentration of $CdCl_2$ but not yet by $NiCl_2$ or $CoCl_2$. We also found that binding to DNA response element strongly protects core domains from a loss of DNA binding as a response to heavy metal treatment *in vitro*. Additionally, the inhibitory effect of cadmium on p53 family binding *in vitro* was reverted by sub-millimolar concentrations of chelating agent EDTA and only partially by redox agent DTT.

2. Materials and methods

2.1. DNA samples

Fragment p53CON (474 bp) was prepared by *Pvu*II (TAKARA) digestion of supercoiled plasmid pPGM1 (pBSK containing a p53CON sequence AGACATGCCTAGACATGCCT, [18]), second fragment long 2513 bp was used as nonspecific competitor (NON). Plasmids encoding human full length wild-type p53 (p53, aa 1–393, pT7-7p53 [19]), p53 core domain (p53DBD, aa 94–312, pET3dp53, [18]) and GST fusion core domains of p53, p63 (p53CD, aa 94–312; p63CD, aa 114–349; pGEX-4T [6]) and p73 (p73CD, aa 104–339, pGEX-4T [9]) were used for protein expression and purification.

2.2. Protein expression and purification

Full length protein p53 (p53), p53 core domain (p53DBD), and GST fusion p53 family core domains (p53CD, p63CD, p73CD) were purified by protocol described in [9,20]. Proteins after final purification on Superdex 200 HiLoad 26/60 column (Amersham Pharmacia Biotech) in 25 mM Hepes (pH 7.6), 200 mM KCl, 10% glycerol, 1 mM DTT and 1 mM benzamidine were dialyzed against the same buffer with 1 mM TCEP instead of DTT.

2.3. DNA binding assays

Interaction of p53 family proteins with 474 bp long fragment DNA was studied by EMSA in agarose gels. Usually 50–100 ng of proteins were incubated in binding buffer (5 mM Tris-HCl, pH 7.6, 0.01% Triton X-100 and 50 mM KCl with 1 mM TCEP) and mixed with pPGM1/*Pvu*II fragments (200 ng; 474 bp with p53CON, 2513 bp fragment as nonspecific competitor) at molar ratio 3–5/1 (protein tetramer/DNA) for 20 min on ice to reach equilibrium. Before or after addition of DNA the proteins were treated with metal ions ($CdCl_2$, $NiCl_2$ or $CoCl_2$) for 20 min on ice. Samples were loaded onto a 1% agarose gel containing 0.33× TBE buffer, DNA was stained with ethidium bromide.

2.4. Influence of EDTA and DTT on protein-DNA binding inhibited by heavy metals

To study influence of EDTA and DTT on cadmium inhibition the proteins were firstly incubated with cadmium (for 20 min on ice), then 200 ng of pPGM1/*Pvu*II were added and the incubation continued with DTT or EDTA at molar excess (2–200) for 20 min on ice.

2.5. Human cell lines, transfections and luciferase assays

Human non-small cell lung carcinoma line H1299 (p53 null cell line, NCI-H1299, ATCC) was grown in DMEM medium supplemented with 5% FBS (Gibco). The luciferase reporter constructs containing different p53 recognition sites (pGL3-BAX, pGL3-MDM2-APP, pGL3-PGM1 (p53CON sequences were cloned in *Sma*I site in pGL3 promoter vector) [21]), pGL3-promoter (Promega) and pRL-SV40 (Renilla control) were used. H1299 cells seeded in 24-well plates were transfected using Lipofectamine (Invitrogene) according to the manufacturer's instructions. When appropriate, 50–100 ng of the p53 family expression vectors based on pCDNA3.1: pcDNA3-HA-TAp73β, pcDNA3-HA-TAp73γ [22], pcDNA3.1-p53 [21], pcDNA3.1-TAp63α-FLAG (Addgene plasmid 27008, [23]), pCDNA3.1-TAp63γ-(3X)HA (Addgene plasmid 26977, [23]) or empty vector pCDNA3.1 was co-transfected with 200 ng of reporter constructs. About 16 h after transfection cadmium treatment was started, after 12 h treatment extracts were prepared using the Dual Luciferase Assay System kit (Promega) following the manufacturer's protocol (for more details see in [21]). For each construct, relative luciferase activity was defined as the mean value of the Firefly luciferase/Renilla luciferase ratios obtained from at least three independent experiments.

3. Results

3.1. Cadmium inhibits sequence-specific binding of p53 family members

Previously it was shown that cadmium induces modifications of p53 conformation, inhibits p53 sequence-specific binding to DNA and suppresses p53 response to DNA damage [10–14,24]. To characterize the effect of cadmium on sequence-specific binding of other p53 family members, p63 and p73 core domains (p63CD, aa 114–349; p73CD, aa 104–339) were isolated. Proteins p63CD and p73CD recognize p53CON sequences with affinities comparable to p53 core domain also due to fusion of DBD with GST which facilitates dimer formation in solution as was described in [6,9]. We tested the influence of cadmium at the concentration range 10–100 μM on p63CD, p73CD, full length p53 and p53DBD binding to p53CON (inserted in 474 bp DNA fragment, Fig. 1A and B) on agarose gel. Molar ratios of protein to DNA was 3–5/1; at this condition the majority of p53CON fragment was bound by the p53 family proteins (Fig. 1A and B, lanes 2, 6, 10, 14). At first, protein samples were exposed to increasing amount of $CdCl_2$ (10–50 μM) before DNA addition in the presence of 1 mM TCEP in binding buffer (Fig. 1A). Partial inhibition of p63CD and p73CD binding was

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