

Search for molecular mechanisms in the genotoxicity of nickel

by F William Sunderman, Jr, MD¹

SUNDERMAN FW. Search for molecular mechanisms in the genotoxicity of nickel. *Scand J Work Environ Health* 1993;19 suppl 1:75—80. This paper reviews recent studies done in the author's laboratory on molecular mechanisms of nickel genotoxicity, using as an experimental model the teratogenic effects of bivalent nickel ions (Ni^{2+}) in South African frogs (*Xenopus laevis*). A Ni^{2+} -binding protein, *pNiXa*, was identified in *Xenopus* oocytes and embryos (molecular weight 45 kDa, isoelectric point ~8.5) with a strong homology to human α_1 -antitrypsin, α_1 -antichymotrypsin, and other serine proteinase inhibitors. CNBr peptides of *pNiXa* showed sequence identity to *Ep45*. Nondenatured *pNiXa*, purified by nickel affinity chromatography, inhibits bovine α_1 -chymotrypsin. The possibility that *pNiXa* plays a key role in Ni^{2+} teratogenesis is indicated by (i) the avidity of *pNiXa* for Ni^{2+} , (ii) the presence of *pNiXa* when the embryos are susceptible to Ni^{2+} teratogenesis, and (iii) the potential of the (HX)₂-motif to form Ni^{2+} complexes that could catalyze the formation of oxygen free radicals and thereby damage deoxyribonucleic acid (DNA) and chromosomes.

Key terms: Ni^{2+} -binding proteins, serine proteinase inhibitor, teratogenesis, *Xenopus laevis*.

In previous reviews, I have discussed the carcinogenicity and teratogenicity of nickel compounds and considered possible molecular mechanisms whereby bivalent nickel ions (Ni^{2+}) might produce its genotoxic effects (1—4). In my opinion, the histidyl residues of specific nuclear proteins that bind to deoxyribonucleic acid (DNA) or regulate cell division and gene transcription are likely to be the critical molecular targets, which, through the formation of complexes with Ni^{2+} , could generate oxygen free radicals and thereby cause the genetic damage. This hypothesis rests on the following three pieces of evidence: (i) that certain histidyl-containing peptides and proteins show an exceptionally high affinity for Ni^{2+} , (ii) that certain histidyl-containing peptides and proteins can form Ni^{2+} complexes that react with hydrogen peroxide to generate oxygen free radicals under physiological conditions, and (iii) that DNA strand breaks, DNA-protein cross-links, and chromosome damage, which are biomarkers of nickel-induced genotoxicity, are mediated by oxygen free radical attack on nucleic acids and chromatin-associated proteins.

The evidence pointing to histidyl residues in proteins and peptides as Ni^{2+} ligands of paramount biological importance has been summarized by Martin (5), Andrews et al (6), and Predki et al (7). Although direct binding of Ni^{2+} to DNA has been amply docu-

mented in vitro (8), the affinity of DNA for Ni^{2+} does not rival that of certain proteins, such as urease (from plants) and α -fetoprotein, albumin, and histidine-rich glycoprotein (from animals), all of which have histidyl residues in their metal-binding sites (6, 7, 9, 10). Exceptional selectivity of protein interactions with Ni^{2+} is evident from the high resolution of protein fractionations by chromatography on Ni-iminodiacetate agarose gel (Ni-IDA-Sepharose), which preferentially binds histidyl-containing proteins (10—12).

Histidyl-containing proteins and peptides form Ni^{2+} complexes that catalyze the formation of oxygen free radicals from hydrogen peroxide under physiological conditions and may thereby induce peroxidative damage of nucleic acids, proteins, and lipids (13—18). Several investigations have shown that peroxidation products (eg, malondialdehyde) are formed in vivo as a manifestation of Ni^{2+} toxicity (19—21). The role of oxygen free radicals in the DNA damage induced by carcinogenic nickel compounds has been discussed by Klein et al (22), Lin et al (23), and Higinbotham et al (24).

The marked differences that have been observed in the carcinogenic activities of various nickel compounds probably reflect, in my opinion, the differences in their rates of cellular uptake, intracellular translocation, and eventual dissolution or decomposition to liberate Ni^{2+} (2, 3, 25). The nuclear proteins that hypothetically constitute molecular targets for Ni^{2+} genotoxicity would be expected to bind bivalent zinc ions (Zn^{2+}) or bivalent copper ions (Cu^{2+}) under physiological conditions, but to exchange the native metal ion in the presence of exogenous Ni^{2+} . Histidyl-containing chromatin proteins and transcription factors that bind Ni^{2+} might undergo self-destruc-

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tion by oxygen free radical reactions, in addition to generating destructive free radicals in proximity to chromosomes and double-stranded DNA. Based on recent studies in my laboratory, the zinc-finger motifs found in many transcription factors are unlikely to be molecular targets for Ni^{2+} genotoxicity, since the affinity of one such factor, TFIIIA, is much less for Ni^{2+} than for the native Zn^{2+} (26, 27). On the other hand, the $(\text{HX})_n$ motifs of other transcription factors (eg, *Bicoid*, *ets-74E*, *Chox-1.4*) (28) are more likely targets, since $(\text{HX})_n$ motifs would predictably have equal or greater affinity for Ni^{2+} than for Zn^{2+} or Cu^{2+} . In addition to catalyzing the formation of oxygen free radicals, the substitution of Ni^{2+} in $(\text{HX})_n$ motifs of transcription factors might disturb protein-protein interactions within the transcription apparatus, which could be another molecular mechanism of genotoxicity.

Experimental strategy

As the experimental model for this research program, I decided to investigate the teratogenicity of Ni^{2+} in the South African frog, *Xenopus laevis*. This system has the following advantages: (i) clear-cut morphological end points of teratogenicity and well-standardized assay conditions, using the FETAX procedure (Frog Teratogenesis Assay: *Xenopus*) (29), (ii) an extensive data base for *Xenopus laevis* in reproductive biology and molecular genetics, (iii) mounting evidence that the regulation of oocyte maturation and early embryogenesis in *Xenopus* resembles the regulation of mitosis and differentiation in mammalian cells (30–34), and (iv) the ready availability of *Xenopus* oocytes and embryos at specific stages in adequate quantities for protein isolation, sequencing, identification, and characterization.

My strategy to test the aforestated hypothesis involves several steps, as follows: (i) to demonstrate that Ni^{2+} is teratogenic for *Xenopus* embryos and delineate the time course of their susceptibility to Ni^{2+} -induced malformations, (ii) to devise a Western blotting technique to identify the proteins in *Xenopus* oocytes and embryos that bind Ni^{2+} with the highest affinity, (iii) to predict from their biochemical and physiological characteristics, and the time course of their expression, which of the Ni^{2+} -binding proteins are the most likely to be involved in teratogenesis, (iv) to purify the putative target proteins by metal-chelate affinity chromatography on Ni-IDA-Sepharose, (v) to determine the amino acid sequences of the proteins, establish their physiological functions, and deduce the probable Ni^{2+} -binding sites, and (vi) to test whether the proteins show equal or greater affinity for Ni^{2+} than for Zn^{2+} and Cu^{2+} . The first three steps have already been completed; the next three steps have been largely accomplished in regard to *pNiXa*, one of the major Ni^{2+} -binding proteins of *Xenopus* embryos. In steps seven to nine, which are underway, our research group plans to verify the

metal-binding site of *pNiXa* by testing its CNBr peptides for complexation of $^{63}\text{Ni}^{2+}$, determine whether the Ni^{2+} -*pNiXa* complex catalyzes oxygen free radical reactions in vitro, and see if the Ni^{2+} -*pNiXa* complex induces malformations after microinjection into *Xenopus* embryos during early cleavage stages. This article reviews our progress, summarizing the efforts of numerous collaborators, who are co-authors of the cited papers from our laboratory.

Teratogenic effects of nickel in *Xenopus* embryos

On the basis of radiotracer experiments with $^{63}\text{Ni}^{2+}$, the uptake of Ni^{2+} by *Xenopus* embryos at the two- to four-cell stage averaged 12 to 17 times the uptake by unfertilized eggs (35). Evidently, the structural changes of the vitelline envelope that occur at fertilization enhance the permeability of *Xenopus* embryos to Ni^{2+} . The Ni^{2+} uptake by *Xenopus* embryos during the early cleavage stages was proportional to the ambient Ni^{2+} concentration. These findings suggested that *Xenopus* would be well suited for studies of the embryotoxicity and teratogenicity of Ni^{2+} (35). Experiments in the FETAX (frog embryo teratogenesis assay: *xenopus*) assay confirmed that Ni^{2+} is a potent teratogen for *Xenopus* (36). The most frequent malformations were ocular, skeletal, and intestinal deformities; less common were facial, cardiac, and integumentary malformations. Experiments in which Ni^{2+} exposures were limited to specific postfertilization periods showed that *Xenopus* embryos are the most susceptible to Ni^{2+} -induced anomalies during organogenesis from stages 18 to 36 (ie, approximately one to two days old) (36).

Western blotting technique to identify nickel-binding proteins

A Western blotting technique was devised to identify cellular proteins that bind Ni^{2+} with the highest affinity (26, 37). After separation by polyacrylamide gel electrophoresis (PAGE), the proteins are electroblotted onto a nitrocellulose membrane, renatured in situ in the presence of a reducing agent, probed with $^{63}\text{Ni}^{2+}$ at pH 7.3, washed thoroughly, and visualized by autoradiography. This technique was used to characterize the Ni^{2+} -binding proteins in *Xenopus* oocytes and embryos at various developmental stages (26, 37–39). In previtellogenic oocytes, the autoradiograms showed $^{63}\text{Ni}^{2+}$ binding to (i) transcription factor IIIA, (ii) p43, a protein constituent of 42S ribonucleoprotein particles, and (iii) the 68- and 74-kDa isoforms of *Xenopus* albumin (26). In mature oocytes and in embryos during early cleavage stages, the autoradiograms showed $^{63}\text{Ni}^{2+}$ -binding to two proteins with approximate molecular weights of 45 and 31 kDa (39). The 45-kDa protein, designated *pNiXa* (protein-Ni-*Xenopus*-a), was the most visible in blas-

tula stage embryos (stage 7), became less intense at stage 26, and was barely evident by stage 36. The 31-kDa protein, designated *pNiXb*, persisted to stage 42. A faint 40-kDa $^{63}\text{Ni}^{2+}$ -binding protein, designated *pNiXc*, was seen from stages 7 to 48. In addition, $^{63}\text{Ni}^{2+}$ binding to the isoforms of *Xenopus* albumin appeared at stage 36 and became more intense at stages 42 to 48 (39). *pNiXa* and *pNiXb* bound Ni^{2+} more avidly than Zn^{2+} , Cu^{2+} , or Cd^{2+} (bivalent cadmium ions) whereas TFIIIA bound Zn^{2+} , Cu^{2+} , or Cd^{2+} much more avidly than Ni^{2+} (26, 27, 39). Our attention has been focused initially on *pNiXa*, since it is a soluble protein that is abundant in *Xenopus* embryos during their period of susceptibility to Ni^{2+} -induced teratogenesis.

Purification and characterization of *pNiXa*

pNiXa was isolated from pooled samples of mature oocytes or dejellied eggs obtained from groups of 8 to 10 *Xenopus* females (39, 40). After the samples were homogenized and centrifuged, most proteins in the 40 000 g supernatant were removed by batch adsorption on DEAE (diethylaminoethyl) cellulose; the remaining proteins, including *pNiXa* and *pNiXc*, were adsorbed on phosphocellulose and eluted in a step-gradient of sodium chloride (NaCl). The 0.5 M NaCl eluate, which contained *pNiXa* and *pNiXc*, was further purified by reversed phase high-pressure liquid chromatography (HPLC) on a C-4 column, using an

acetonitrile gradient in dilute trifluoroacetic acid. The *pNiXa*-containing fraction, identified by PAGE and probing Western blots with $^{63}\text{Ni}^{2+}$, yielded a single 45-kDa band on PAGE gels stained with Coomassie blue; the isoelectric point of *pNiXa* was 8.5, according to the mobility in agarose isoelectric focusing gels (39). Sequence analysis of *pNiXa* showed that its N-terminus was blocked. Therefore the protein was cleaved with CNBr, and the peptides were separated by reversed phase HPLC. CNBr-peptide fragments were sequenced by automated Edman degradation, yielding sequence information for 120 residues. Comparisons of amino-acid sequences of the peptide fragments versus the National Biomedical Research Foundation's protein sequence data bank showed significant sequence homology (>5 SD from random) to 38 members of the superfamily of "serpin" proteins (serine proteinase inhibitors) including human α_1 -antitrypsin, human α_1 -antichymotrypsin precursor, rat serine proteinase inhibitor-1, human antithrombin-III precursor, human corticosteroid binding globulin precursor, and human thyroxine binding globulin precursor (39).

Soon after the computer search for sequence homology had been completed, the amino-acid sequence of *Ep45*, an estrogen-regulated serpin of *Xenopus* liver, was reported by Holland et al (41) on the basis of the nucleotide sequence of its nearly full length cDNA. The determined sequences of *pNiXa* were identical to the corresponding sections of *Ep45*;

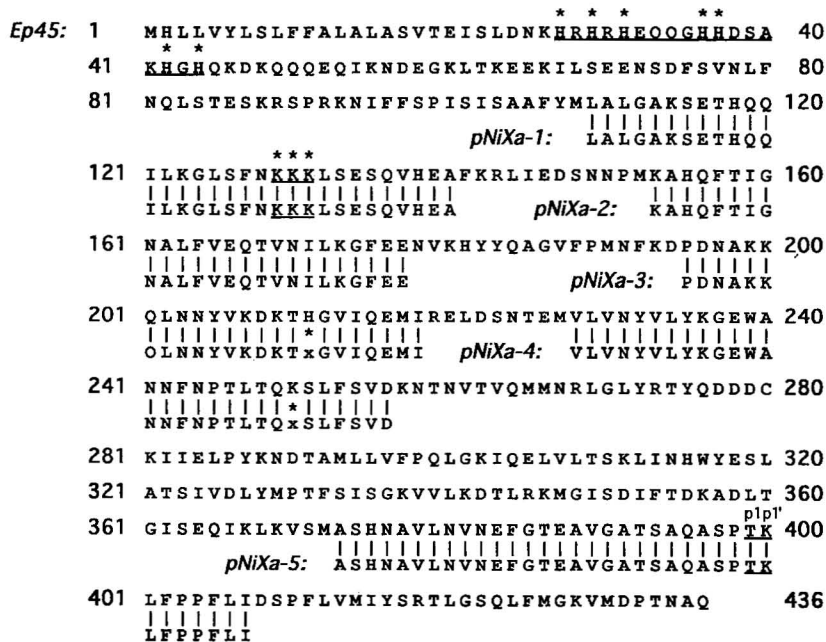


Figure 1. Amino-acid sequences of five peptides of *pNiXa*, as determined by Beck et al (39), correspond exactly to the amino acid sequence of an estrogen-regulated serpin of *Xenopus* liver, *Ep45*, as deduced by Holland et al (41) from the nucleotide sequence of its cDNA. Underlined segments of *Ep45/pNiXa* show the following molecular features: (i) (HX)₃ motifs in the segment from positions 27–44 (His residues identified by asterisks), (ii) DNA-binding KKK-triplet at positions 129–131 (Lys residues identified by asterisks), and (iii) the putative active site for serine proteinase inhibition at positions 399 (Thr = p1) and 400 (Lys = p1').

therefore *pNiXa* of *Xenopus* oocytes and *Ep45* of *Xenopus* liver appear to be the same protein (figure 1). The Ni^{2+} -binding site of *pNiXa* has not been established, but the deduced amino-acid sequence of *Ep45* has a putative metal-binding domain — seven histidines clustered in $(\text{HX})_n$ repeats near the N-terminus. $(\text{HX})_n$ repeats present linear arrays of histidine side chains that can associate in pairs by coordinating metal ions (eg, Zn^{2+} , Cu^{2+}) in a structure termed a “histidine-metal-zipper” (28). Such $(\text{HX})_n$ repeats, which may play a role in protein-protein interactions, have been identified in several *Drosophila* transcription factors (ie, *Paired*, *Bicoid*, *Deformed*, *Odd-Skipped*, *Daughterless*, *Cfla*, *E75A*, and *ets*-oncoprotein-related *74E* gene product), in a chicken transcription factor (*Chox-1.4*), and in a bovine inhibitor of protein kinase C (28, 42). The amino-acid sequence of *pNiXa/Ep45* also contains a DNA-binding lysine-triplet motif (KKK) at positions 129–131; a similar triplet of lysines has been reported in human α_1 -antichymotrypsin (43, 44).

Nondenatured *pNiXa* was purified by nickel-affinity chromatography to test its activity as a serine proteinase inhibitor (39). When *pNiXa*-enriched phosphocellulose eluate was fractionated by chromatography on a Ni^{2+} -IDA-Sepharose column with an imidazole gradient, *pNiXa* was isolated in a narrow peak, free of detectable protein contaminants. After the imidazole was removed by gel filtration, the activity of *pNiXa* as a serine proteinase inhibitor was assayed with bovine pancreatic α -chymotrypsin as the enzyme and N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the substrate. The addition of *pNiXa* to the reaction mixture significantly inhibited α -chymotrypsin activity; under similar conditions, *pNiXa* did not inhibit bovine pancreatic trypsin when N- α -benzoyl-DL-Arg-p-nitroanilide was used as the substrate (39). These assays show that *pNiXa* can function as an inhibitor of α -chymotrypsin activity. The physiological role of *pNiXa* remains to be established, but trials are underway to see if *pNiXa* inhibits the chymotrypsin-like proteinase activity of *Xenopus* oocytes, modulating the germinal vesicle breakdown that occurs during oocyte maturation. The possible role of the Ni^{2+} -*pNiXa* complex as a mediator of teratogenesis in *Xenopus* will be tested by the experiments outlined as steps seven to nine in the section entitled Experimental Strategy.

Discussion

Serine proteinase inhibitors (eg, Bowman-Birk inhibitor, antipain, leupeptin) prevent radiation- and carcinogen-induced cell transformation and suppress the overexpression of certain oncogenes (eg, *c-myc*, *c-fos*) in vivo and in vitro (45–48). The signal pathway whereby *ras*-oncogene-encoded p21 protein induces malignant transformation of NIH 3T3 murine fibroblasts closely resembles the pathway whereby

the oncogenic p21 promotes maturation of *Xenopus* oocytes (30–34). Cleavage of membrane-anchored growth factors [eg, transforming growth factor- α (TGF- α) and stem cell growth factors (Kit ligands KL-1 and KL-2)] to their soluble, active forms depends on multiple serine proteinases, regulated by a signaling mechanism that probably involves serine proteinase inhibitors (49). In view of the anticarcinogenic activity of certain serine proteinase inhibitors, the similar pathways of cell transformation and oocyte maturation, and the evolutionary conservation of proteins of the serpin superfamily, the discovery of a Ni^{2+} -binding serpin with antichymotrypsin activity in *Xenopus* oocytes may be relevant to the genotoxic effects of Ni^{2+} in higher organisms. In any case, I predict that the novel methods that were devised for this investigation will be helpful in efforts to delineate the molecular mechanisms of Ni^{2+} genotoxicity and carcinogenesis in mammalian cells.

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