Studies into the mechanism of arsenic-induced neurotoxicity

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Chapter 8

General Discussion and Summary

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Arsenic

Arsenic is a metalloid with the symbol As and atomic number 33. It has an atomic mass of 74.92. It has been known since ancient times, though probably in compound form rather than in its pure state. Arsenic has a long history of medical applications; before penicillin was developed an arsenic compound was used to treat syphilis. Arsenic has also been used in combination with other materials in pigments, poison gases and insecticides (such as Paris Green, calcium arsenate, lead arsenate and lewisite as chemical agent for warfare) and is well known from former use as a rat poison. In forensic science, it is well-known from a great number of homicidal cases over many centuries. Arsenic is known to be used as a preservative in tanning and taxidermy, as well as in Wolman's salt as a wood preservative and even playground materials. Other uses of As compounds are or have been used in ammunition manufacturing. Its use in combination with other metals helps to improve the hardness of bullets.

Arsenic is a notoriously poisonous metalloid with known hazardous effects to human health. The element exists in both organic and inorganic forms and either form can also exist in trivalent (-3, +3 or arsenite, As^{III}) or pentavalent oxidation state (+5 or arsenate, As^{V}). In many animal species arsenic metabolism is characterized by two main types of reactions: (1) reduction reactions of pentavalent to trivalent arsenic, and (2) oxidative methylation reactions in which trivalent forms of As are sequentially methylated to form monomethyl- (MM-) and dimethyl- (DM) products: $iAs^{V} \rightarrow iAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$. Although there may be differences in the potency of different chemical forms, it is generally considered that arsenites tend to be more toxic than arsenates and inorganic is more toxic than the organic form. Intoxication with As may occur in the *acute* (short-term) and *chronic* (long-term) forms, which are separate syndromes. Acute As exposure may cause nausea, vomiting, diarrhea, weakness, loss of appetite, shaking, cough, headache and neuropathy. Chronic exposure may lead to a variety of symptoms including skin pigmentation, numbness, and cardiovascular disease. It is also known to cause various forms of cancer such as skin (non-melanoma type), kidney, bladder, lung, prostate and liver cancer and, again, neuropathy.

The presence and levels of As can be analyzed in urine, blood and hair. Tests may either be devised to measure the total As content in tissues or bodyfluids, or the different types of arsenic species. For total As concentration measurement is carried out with atomic absorption spectrometry (AAS) in various samples. Atomic absorption spectroscopy is based on the absorption of light to measure the concentration of gas-phase atoms. Since samples are usually liquids or solids, the analyte atoms or ions must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption. Applying the Lambert-Beer law directly in AAS is difficult due to variations in atomization efficiency from the sample matrix, and non-uniformity of concentration and path length of analyte atoms (in graphite furnace AAS). Concentration measurements are usually determined from a calibration curve after calibrating the instrument with standards of known concentration. Prior to quantification of the analyte by AAS it is usually necessary to destroy the organic matrix and bring the element into inorganic solution. In many cases, As samples are usually predigested with a digestive acid such as a mixture of perchloric and nitric acid (Sakamoto et al. 2001). The samples for As analysis prior to measurements are added to the

calibration matrix consisting of 1M HCl (reduction acid) in order to convert all the pentavalent to trivalent As.

When studying the hazardous effects of various As metabolites, determination of total arsenic concentration needs to be completed with the analysis of individual arsenic species and compounds. Various techniques such as high pressure liquid chromatography (HPLC) coupled with ICP-MS, ICP-AES or AAS are most suitable for the quantification of arsenic species (Londesborough et al. 1999).

Arsenic and neurotoxicity

Exposure to As may affect both the central and peripheral nervous systems; symptoms include tremors, headaches, numbness, irritability, muscular weakness, convulsions and coma. Although peripheral nervous system (PNS) impairment is common in As-exposed populations, its mechanism is poorly understood. Arsenic effects manifest themselves weeks after first exposure as both central and peripheral neuropathy. The most frequent neurological manifestation by As is peripheral neuropathy that may last for several years, if not life-long. Peripheral neuropathy may lead to rapid severe ascending weakness, similar to the Guillain-Barré syndrome, requiring mechanical ventilation (artificial respiration). From human clinical cases studied by Le Quesne and McLeod (1977) it has become clear that As exposure results in a late reaction of the nervous system, which was established by decrease in their nerve conducting velocity (NCV) measurements (Le Quesne & McLeod 1977). Patients exposed to As show significantly lower Nerve Conduction Velocities (NCVs) in their peripheral nerves in comparison to their referents (Greenberg 1996; Tseng et al. 2006; Otto et al. 2007). Although this reduction in NCV is a hallmark in As-induced neurotoxicity, its mechanism of action on the molecular level is not known. Therefore, the project described in this thesis was aimed at elucidating the probable mechanism of As-induced neurotoxicity in animals *in vivo* and in cell cultures *in vitro*.

The animal studies in this thesis were designed to answer questions about the effect of As on the peripheral nervous system after sub-acute and chronic intoxication of laboratory rats. Axonal integrity is maintained by three categories of cytoskeletal proteins: neurofilaments, which are found in high concentrations along the axons, microtubules and actin filaments (microfilaments). Neurofilaments are specific to neurons and found in high concentrations along the axons of vertebrate neurons. Three types of neurofilament proteins exist, which co-assemble *in vivo*, forming a heteropolymer that contains Neurofilament-Light (NF-L, 68 kDa) plus either Neurofilament-Medium (NF-M, 150 kDa) or Neurofilament-Heavy (NF-H, 200 kDa) proteins. During axonal growth, new neurofilament subunits are incorporated all along the axons. In an intact nerve, the level of neurofilament proteins control how fast electrical signals travel down the axon. Microtubules consist of tubulin subunits. Their stability is regulated by the interaction with microtubule-associated proteins (MAPs). MAP function includes both stabilizing and destabilizing microtubules, guiding microtubules toward specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell. MAP-tau is a microtubule-associated protein, which is primarily expressed in neurons. They are required for tubulin assembly into the microtubules and stabilize the assembled microtubules.

Chapter 3 of this thesis describes the compositional changes in rat sciatic nerves after a single exposure to arsenite i.v. with various doses. As a first step in this study, the short- and long-term effects of arsenite on rat sciatic nerve proteins were studied as a model for peripheral axonopathy. Male Wistar rats were exposed to inorganic arsenite (iAs^{III}) given as a single dose i.v. dissolved in PBS (between 0 and 20 mg/kg) in a tail vein. The doses used for short-term single arsenic exposure were 0-, 15- and 20-mg/kg iAs^{III} (n=3). The long-term single exposures were 0-, 3- and 10-mg/kg iAs^{III} (n=9). In the short-term section of the experiment,

rats were kept in metabolic cages for the intended duration of 3, 6 and 9 hours. In the long-term section of the experiment, rats were kept in metabolic cages 24 hours after injection. Afterward, rats that received the same dose were combined into one group and housed for 2, 3 and 4 weeks in plastic cages on sawdust. The difference between the long- and short-term studies is the result of the variation in dose range and the duration of urine collection. After sacrifice, sciatic nerves were excised and the protein composition was analyzed. Protein analysis of sciatic nerves showed disappearance of neurofilament and fibroblast proteins in rats treated with arsenite doses of 15 and 20 mg/kg in comparison to the control groups. Some fibroblast protein bands with band sizes of 40 and 140 kDa degraded after a high dosage in the 20-mg/kg dose group. The analyzed neurofilament-M and -L proteins decreased dose-dependently over time. Arsenic affects the composition of proteins in the rat sciatic nerve, especially the neurofilaments. The reduction of signals in western blot analysis revealed changes in cytoskeletal composition, which may well lead to neurotoxic effects *in vivo*.
Chapter 4 of this thesis describes studies on the compositional changes in rat sciatic nerves after semi-chronic exposure. In our previous study in rats exposed to As, we observed an effect of As on neurofilaments in the sciatic nerve. This study deals with the effects of iAs^{III} in Wistar rats on the cytoskeletal protein composition of the sciatic nerve after subchronic intoxication. Inorganic arsenite dissolved in phosphate-buffered saline (PBS) was orally administered daily in doses of 0, 3 and 10 mg/kg body weight/day (n = 9 rats/group) by intragastric route for 4, 8 and 12 week periods. The control group received only PBS without added arsenite. Toxicokinetic measurements revealed a saturation of blood As levels in the 3- and 10-mg/kg dose groups at approximately 14 µg/ml, with an increase in renal clearance of As at increasing doses. After exsanguination, sciatic nerves were excised and the protein composition was analyzed. Analysis of the sciatic nerves showed compositional changes in their proteins. Protein expression of NF-H and NF-M remained unchanged. Neurofilament protein Low (NF-L) expression was reduced, while µ- and m-calpain protein expression was increased, both in a dose/time pattern. Furthermore, NF-H protein was hypophosphorylated; while NF-L and microtubule-associated protein tau (MAP-tau) proteins were phosphorylated.

These two *in vivo* studies present direct evidence for the As effect on the neuronal skeleton, as a basis for neurotoxicity. The first real proof of As involvement was the elevated As measurements in the sciatic nerve tissues of the rats in sub-acute and semi-chronic induced-toxicity in these rats. The second evidence was achieved by the decrease in cytoskeletal NF-L protein. Several studies that were performed to elucidate the role of neurofilament proteins have concluded that both NF-H and NF-M each need a NF-L protein to form a heteropolymer (Carpenter & Ip 1996). The third evidence was achieved through the (hyper) phosphorylation of MAP-tau and NF-L. Phosphorylation of the MAP-tau and NF-L proteins leads to conformational changes leading to destabilization and disruption of the cytoskeletal framework. These results suggest that the mechanism of As induced-neurotoxicity lays in the cytoskeletal proteins that have been affected by As in various ways.

In the in vitro studies, effects of other As species were tested in various cell culture models (chapter 5) and the manner of their hyperphosphorylation was further studied (Chapter 6) for a better understanding of the disruption of neuroskeletal integrity by As.
Chapter 5 of this thesis describes the effects of various arsenic metabolites (iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}) on two different cell lines derived from the peripheral (ST-8814) and central (SK-N-SH) nervous system. The effects of As metabolites were examined on the relative quantification levels of the cytoskeletal genes, neurofilament-light (NEFL), neurofilament-medium (NEF3), neurofilament-heavy (NEFH) and microtubule associated protein-tau (MAPT), using Real-Time PCR. Various As metabolites (iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}) and concentrations of 0, 0.3, 1.0 and 3.0 µM dissolved in their appropriate cell culture

medium were incubated for 24 and 48 hours in triplicate. Afterward, cDNAs were synthesized from the isolated RNA to determine their relative quantification (RQ) and follow the changes in expression of these genes under influence of the various As metabolite and concentrations. Our results showed that iAs^{III} and iAs^{V} have no significant effects on either cell lines. Conversely, MMA^{V} and DMA^{V} cause significant changes in expression levels of NEF3 and NEFL genes, while the expression level of the NEFH gene is significantly increased in both cell lines. Increase in NF-H may suggest a compensatory mechanism. However, this needs further study.

In chapters 3 to 5 we have demonstrated that arsenic metabolites change the composition of cytoskeletal proteins *in vivo* and *in vitro*. Furthermore, in chapter 4 we demonstrated that calpain expression is increased with the increase of As dose and exposure time. To further examine the mechanism of arsenic-induced neurotoxicity with various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}), we studied the role of p35 and calpain enzyme (chapter 6) and its involvement in hyperphosphorylation of cytoskeletal proteins. Calpain is a calcium-activated cytoplasmic protease that seems to be involved in some neurodegenerative diseases such as Alzheimer disease (AD) (Lee et al. 2000). Alzheimer patients form neurofibrillary tangles through tau hyperphosphorylation. Calpain has also been shown to be responsible for NF-L degradation, since neuroblastoma cells (SY-5Y) treated with arsenic trioxide (trivalent As) show an increase in intracellular calcium (Florea et al. 2007). Studies in PC12 cells under oxidative stress circumstances have shown an increase in calcium within the cells and up-regulation of calpain leading to degradation of NF-L protein (Ray et al. 2000). Moreover, inactivation of calpain by calpain inhibitor (MDL-28170) prevents NF-L breakdown (Kunz et al. 2004; Lopez-Picon et al. 2006). These results suggest that As-induced destabilization and disruption of the cytoskeletal framework is partly due to increased expression of calpain, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process.

Another approach to destabilization and disruption of the cytoskeletal framework is through phosphorylation of cytoskeletal proteins. In a normal situation, p35 binds to cyclin-dependent kinase 5 (Cdk5), which is responsible for neurite-outgrowth. In diseased patients, p35 is cleaved to p25 by calpain, whereby p25 binds to Cdk5, resulting in hyper-activation of Cdk5 and hyperphosphorylation of tau and neurofilament proteins (Fig. 1)

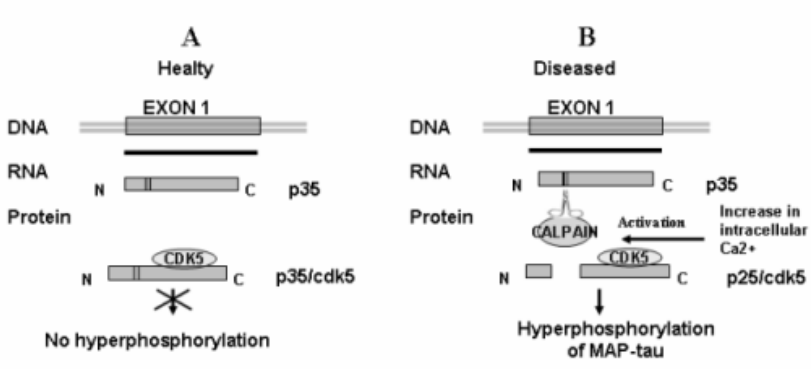

Fig.1. Cleavage of p35 to p25 by calpain. A. Healthy subjects where complex of p35/cdk5 is responsible for the neurite outgrowth. B. Influx of Ca^{2+} in patients with Alzheimer's disease, for example, results in activation of calpain protease. Calpain cleaves p35 to p25, whereby the p25/Cdk5 complex results in hyperphosphorylation of MAP-tau proteins.

In chapter 6 (Arsenic Neurotoxicity IV), we tried to examine whether the cleavage of p35 to p25 can be induced by As. Furthermore, we studied mRNA expression levels of calpain, cyclin-dependant kinase 5 (Cdk5) and glycogen synthase kinase 3 beta (Gsk3ß). A transfected HeLa cell line with a p35 construct (HeLa-p35) was used as a model, since all other necessary proteins such as calpain, Cdk5 and Gsk3ß are already present in HeLa cells. HeLa-p35 cells were incubated with various As metabolites and concentrations of 0, 10 and 30 µM for duration of 5 hours, after which the cells were either lysed to analyze p35 protein expression or examined on the relative quantification levels of the genes.
Calpain activation may contribute to As-induced neurotoxicity in two ways. As shown in chapter 4, incubation with As leads to hyperphosphorylation of MAP-tau and NF proteins, resulting in deregulation and disorganization of the cytoskeletal framework on the one hand. On the other, it causes calpain activation-induced degradation of NF-L protein (Kunz et al. 2004). These authors have shown that calpain inhibition prevents inflammation-induced NF-L breakdown in the spinal cord. This may suggest that inorganic arsenic causes degradation on the protein level *in vivo* by activating proteases such as calpain. In chapters 3 and 4, we showed that NF-L protein expression was decreased after treatment with iAs^{III}. It is reasonable to suggest that since As results in activation of calpain through influx of Ca^{2+} that calpain is responsible for NF-L degradation in a calcium-induced proteolytic process. The results found in our *in vivo* and *in vitro* experiments suggest that As exerts its toxic effects in two different manners depending on the As species. Rats treated with iAs^{III} showed a decrease in NF-L expression on their protein level, while the *in vitro* study with iAs^{III} (chapter 5) showed no changes in expression on the mRNA level after treatment with inorganic As.

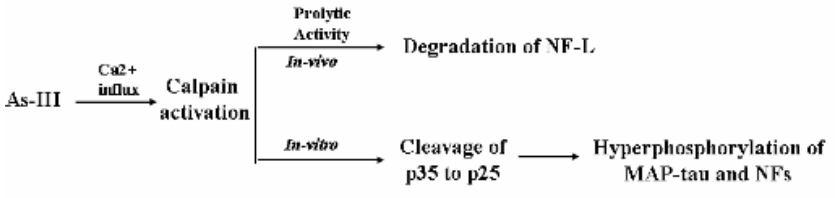

Fig. 2. Two probable mechanism of As^{III} induced neurotoxicity with involvement of calpain activity. As^{III} results in decrease of NF-L protein, which could be the result of a calpain-induced proteolytic process. As^{III} induces hyperphosphorylation of cytoskeletal proteins through calpain-induced activation of the cleavage of p35 to p25.

Effects of As on the DNA level is not reserved to cytoskeletal genes only. Arsenic is also known to be carcinogenic and to be involved in inhibition of DNA repair mechanisms (Andrew et al. 2006). However, the carcinogenic mechanism of As has yet to be fully understood. Many studies have shown the adverse effects of As on chromosomal and DNA level (Colognato et al. 2007; Yedjou & Tchounwou 2007) and to some extent on the effects of nucleotide excision repair and As. Andrew *et al.* (2006) have shown that people exposed to As in drinking water have decreased DNA repair abilities, namely the decrease of excision repair cross-complementing 1 (ERCC1) (Andrew et al. 2003; Andrew et al. 2006). In chapter 7 of this thesis, we describe the effect of various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}) on three Chinese hamster ovary (CHO) cell lines, AA8 (wild type), UV20 (ERCC1 deficient) and UV5 (ERCC2 deficient). Cytotoxicity to the As metabolites was assessed by determining the concentration at which 50% of the cells in culture is killed (LC_{50}). The LC_{50} was only determined for trivalent As metabolites, since

pentavalent As metabolites did not reach complete lethality at the highest concentration. Our results showed that in CHO cells, methylated arsenites are more cytotoxic to AA8 and UV20 cell types than As^{III} in the inorganic form, while absence of *ERCC2* in UV5 cell types contributes to higher resistance to methylated arsenite species.

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