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Vahidnia, A.

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Chapter 6: Arsenic neurotoxicity IV

# Mechanism of Arsenic-Induced Neurotoxicity May Be Explained Through Cleavage of p35 to p25 by Calpain

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# Mechanism of Arsenic-Induced Neurotoxicity May Be Explained Through Cleavage of p35 to p25 by Calpain

**A Vahidnia [a,*], RJHM van der Straaten [a], F Romijn [b], J van Pelt [b], GB van der Voet [c], FA de Wolff [d]**

[a] Department of Clinical Pharmacy and Toxicology, [b] Department of Clinical Chemistry, Leiden University Medical Center, Leiden, the Netherlands, [c] Department of Environmental and Toxicologic Pathology, Armed Forces Institute of Pathology, Washington, United States, [d] Chair of Clinical & Forensic Toxicology (emeritus), Leiden University Medical Center, Leiden, the Netherlands.

**Abstract**

In recent studies we have demonstrated that arsenic (As) metabolites change the composition of neuronal cytoskeletal proteins *in vivo* and *in vitro*. To further examine the mechanism of arsenic-induced neurotoxicity with various arsenate metabolites ($iAs^{V}$, $MMA^{V}$ and $DMA^{V}$) and arsenite metabolites ($iAs^{III}$, $MMA^{III}$ and $DMA^{III}$) we investigated the role of the proteolytic enzyme calpain and its involvement in the cleavage of p35 protein to p25, and also mRNA expression levels of calpain, cyclin-dependant kinase 5 (*cdk5*) and glycogen synthase kinase 3 beta (*gsk3ß*). A HeLa cell line transfected with a p35 construct (HeLa-p35) was used as a model, since all other proteins such as calpain, CDK5 and GSK3ß are already present in HeLa cells as they are in neuronal cells. HeLa-p35 cells were incubated with various As metabolites and concentrations of 0, 10 and 30 µM for duration of 4 hours. Subsequently the cells were either lysed to study their relative quantification levels of these genes or to be examined on their p35-protein expression. P35-RNA expression levels were significantly ($p<0.01$) increased by arsenite metabolites, while p35 protein was cleaved to p25 (and p10) after incubation with these metabolites. The cleavage of p35 is caused by calcium ($Ca^{2+}$) induced activation of calpain. Inhibition of calpain activity by calpeptin prevents cleavage of p35 to p25. These results suggest that cleavage of p35 to p25 by calpain, probably As-induced $Ca^{2+}$-influx may explain the mechanism by which arsenic induces its neurotoxic effects.

**Keywords:** Arsenate and arsenite metabolites; Calpain; HeLa cells; Neurotoxicity; p35.

## 1. Introduction

Long-term exposure to As causes serious effects to various organs such as bladder, kidney, liver, lung and skin cancer, cardiovascular disease and neurological effects.

Neurological effects of As may develop within a few hours after ingestion but are usually seen 2-8 weeks after exposure (Kishi et al. 2001; Jha et al. 2002). It is usually a symmetrical sensori-motor polyneuropathy, often resembling Guillain-Barré syndrome (Perriol et al. 2006). The predominant clinical features of neuropathy are paresthesias, numbness, and pain, particularly of the soles of the feet. Electrophysiological studies performed on patients with As neuropathy have revealed a reduced nerve conducting velocity (NCV), typical of those seen in axonal degeneration, which was confirmed by sural nerve biopsy (Hilmy et al. 1991). However, the mechanism of action of As-induced neurotoxicity is poorly understood.

Neurological disorders have been correlated with disorganization and changes in the expression levels of neuronal cytoskeleton proteins such as neurofilament-high (NF-H), neurofilament-middle (NF-M), neurofilament Low (NF-L) and microtubule associated protein tau (MAP-tau) (Trojanowski et al. 1993).

In specific neurodegenerative diseases such as Alzheimer's Disease (AD), the accumulation of amyloid beta peptide is believed to be an early and critical event leading to synapse and neuronal cell loss (Smith et al., 2006). Subsequently, neurofibrillary tangles are formed by tau hyperphosphorylation (Alonso et al. 1996). This hyperphosphorylation is caused by proteolytic cleavage of p35 to p25 by calpain at a specific site post-translationally, whereby p25 acts as hyperactivator of CDK5. P35 and also p25 are the neuron specific activator of CDK5. CDK5 is involved in terminal differentiation of neurons and muscle cells. The p35/CDK5 protein complex is required for proper development of the central nervous system and is this complex is required for neurite outgrowth (Nikolic et al. 1996). Calpain is a $Ca^{2+}$ dependant kinase enzyme, which is activated by the internal increase of $Ca^{2+}$ in the cells. Activated calpain is responsible for the cleavage of p35 to p25 (Lee et al. 2000). Hyperphosphorylation of cytoskeletal proteins is a potential mechanism of neurodegenerative disease, which is result of p25 overexpression, such as in AD-patients (Monaco 2004; Ahlijanian et al. 2000). A p25-mediated increase in CDK5 activity results in hyperphosphorylation of tau and neurofilaments (NFs). As a result microtubule (MT) network will destabilize leading to disruption of cytoskeleton (Trojanowski et al. 1993; Alonso et al. 1996). Glycogen synthase kinase 3 is involved in energy metabolism, neuronal cell development, and body pattern formation. GSK3ß also regulates the microtubule cytoskeleton during axon outgrowth (Goold & Gordon-Weeks 2001).

In our previous studies, we have shown that $iAs^{III}$ causes compositional changes in sciatic nerve proteins, namely reduction in NF-L expression (Vahidnia et al. 2006). Furthermore, *in vitro* studies with various arsenic metabolites have shown that $MMA^{V}$ and $DMA^{V}$ affect expression of neurofilaments and tau genes, but not $iAs^{III}$ (Vahidnia et al. 2007). Neurofilament-L decrease may play an important role in the pathological changes of nervous system, since NF-L is the only NF protein capable of organizing and co-assembling of filaments on its own *in vivo*. Both NF-H and NF-M need a NF-L protein to form a heteropolymer in the cytoskeletal framework (Carpenter & Ip 1996). Arsenite is also known to phosphorylate tau proteins (Giasson et al. 2002) and we have also seen similar results in sciatic nerves of rats exposed to $iAs^{III}$, whereby tau proteins were hyperphosphorylated (as yet unpublished). Hyperphosphorylated tau proteins have a reduced ability to bind to microtubules. Decrease in NF-L and hyperphosphorylation of tau proteins under As-induced toxicity.

Here we investigated a possible mechanism of As-induced neurotoxicity by which arsenite metabolites may be responsible for hyperphosphorylation and degradation of NF and tau proteins caused by cleavage of p35 to the p25 fragment. Transfected HeLa-p35 cells were

incubated with various As metabolites. Gene expression levels were monitored for p35, calpain, CDK5 and GSK3ß. Furthemore, the cleavage of p35 to p25 after incubation with arsenite metabolites was studied by western blot experiments.

## 2. Materials

2.1. Chemicals.

The following arsenic metabolites and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): (catalogue no.: PS-281), Sodium meta-arsenite ($As^{III}$) (product no. 228699-100G), Arsenic acid sodium ($As^{V}$) (product ref. A6756-50G), Trypsin-EDTA solution 1x (catalogue no. T3924). Dimethyl arsenic acid ($DMA^{V}$) (catalogue no. PS-51) and Disodium methyl arsenate ($MMA^{V}$) were obtained from Chem Service (West Chester, USA). Methylarsonous diiodide ($MMA^{III}$) and Dimethylarsinous iodide ($DMA^{III}$) were purchased from Dr. Cullen of the Department of Chemistry at the University of British Columbia, Vancouver, Canada. AlamarBlue dye was purchased from BioSource International, Inc. (catalogue no. DAL1100. Camarillo, CA). RNeasy Mini Kit 250 was supplied by Qiagen Inc (catalogue no.74106, Valencia, CA, USA). Deoxyribonucleotide triphosphate (dNTPs) 5 mM, 0.1 M dithiothreitol (DTT), 5x buffers, reverse transcriptase (RT) and RNA inhibitor 40 units/µl (RNA sin) were purchased from Promega (Benelux BV, Netherlands). T-25 flasks with air-filter cap for cell growth were obtained from Greiner bio-one GmbH (catalogue no. 690175. Frickenhausen, Germany). Monoclonal antibody to GAPDH (catalog no. Ab8245) and rabbit polyclonal anti-p35 (catalog no. Ab10570) were supplied by Abcam (Cambridge, United Kingdom). Rabbit polyclonal anti-Calpain (H-60) (catalog no. sc-30065), goat anti-rabbit IgG-HRP (catalog no. sc-2004) and goat anti-mouse IgG-HRP (catalog no. sc-2055) were purchased from Santa Cruz biotechnology, Inc. (Califorina, USA). Cyclin-dependant kinase 5, regulatory subunit 1 (CDK5R1, p35) construct (catalog no. IOH11747-pDEST26) was purchased from RZPD (Berlin, Germany) and Lipofectamine™ 2000 (catalog no. 11668-019) from Invitrogen (Breda, The Netherlands). Calpeptin (catalog no. 03-34-0051) was purchased from Calbiochem (Darmstadt, Germany). Kodak Biomax XAR film (catalog no. 165 1454) was obtained from Kodak (Shelton, CT, USA). ECL plus™ western blotting detection reagent was bought from Amersham Biosciences (Piscataway, NJ, USA). SYBRGreen PCR-mastermix 2x (P/N: 4309155, Foster City, CA, USA) was purchased from Applied Biosystems. Phosphate-buffered saline (PBS, NaCl 145 mmol/l, phosphate 1.4 mmol/l and pH 7.5) was prepared from analytical grade reagents by the Department of Pharmacy. The used primers were all obtained from Isogen Bioscience B.V (IJsselstein, the Netherlands) with a final concentration of 10 pmol/µl in Tris/EDTA-buffer (Table 1).

Table 1. Primer sequences used for the RT-PCR.

| Name | Sequence (5’-->3’) | Gene |
|---|---|---|
| ß2-Microglobuline-Sense | GATGCTGCTTACATGTCTCG | Household |
| ß2-Microglobuline-Anti-sense | CCAGCAGAGAATGGAAAGTC | Household |
| Cdk5-Sense | GCACAAGAACATCGTCAGGCTTCA | Target |
| Cdk5-Anti-sense | GTGTAGCACATTGCGGCTATGACA | Target |
| Gsk3β-Sense | TGATTCAGGAGAACTGGTCGCCAT | Target |
| Gsk3β-Anti-sense | TTTCCGGAACATAGTCCAGCACCA | Target |
| µ-Calpain-Sense | ACGAGAACTTCAAGGCCCTCTTCA | Target |
| µ-Calpain-Anti-sense | TGCCATCACGATCCATGAGGTTCA | Target |
| m-Calpain-Sense | ACCGAGAAATCGACGTTGACAGGT | Target |
| m-Calpain-Anti-sense | CAGCCGAACCAAACACCGAACAAA | Target |
| p35-Sense | TGACATGCCTGTACCTCTCCTACT | Target |
| p35-Anti-sense | CGGAGAAGACCTGTGTGAAGTAGTGT | Target |

2.2 Cell cultures.

Most of the materials for cell culture were purchased from Invitrogen (Breda, The Netherlands); otherwise more specific details are followed in brackets. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, catalog no. ) and were supplemented with 10% heat-inactivated foetal bovine serum obtained from Greiner bio-one GmbH (catalogue no. 758093) (Frickenhausen, Germany) and supplemented with 100 IU/ml penicillin/streptomycin (catalogue no. 15140-122), and maintained at 37 °C, 5% $CO_2$ in a humidified incubator.

2.3. Cell Transfection.

HeLa cells were seeded in T25 flasks for a transient transfection and grown to 70-80% confluence for 24 h. HeLa cells were exposed to a mixture of 30 µl of Lipofectamine Reagent$^{TM}$ (Invitrogen) and 8 µg of plasmid DNA for 5 h in serum-free medium, after which the medium was refreshed by a full culture medium. After 24 h, the transfected cells were trypsinized and seeded in a 6-wells plate and grown to a 80-90% confluence, ready for treatment.

2.4. Protein expression.

Calpain, CDK5, GAPDH, GSK3β and p35 expression was analyzed by the western blot technique in triplicate. Transfected HeLa cells were grown in 6-wells plates. After trypsinization, the cells were centrifuged at 350xg and washed twice with PBS for 6 minutes at 4ºC. Afterwards, the cells were collected in 100µl of lysis buffer containing 25mM Tris-base, 2.5% SDS (w/v) and 2.5 mM DTT at pH 6.8. After addition of lysis buffer, cells were incubated at 100ºC for 5 minutes before and after lysing by freeze-thawing in liquid nitrogen (-80ºC) and a water bath (37ºC), 3 to 4 times for 3 minutes each. Protein concentrations of the homogenates were measured by the BCA$^{TM}$ Protein Assay Kit. To obtain the same protein concentrations prior to analysis by SDS-PAGE, the protein samples were standardized with lysis buffer. The protein samples were analyzed on an 8 or 12% acrylamide separation gel. The western blot analysis of the same samples was performed in triplicate.

2.5. Experimental design.

2.5.1. Relative Quantification of various genes.

For RT-PCR, cells were cultivated in triplicate for each As metabolite and concentration. From each culture, PCR was performed in duplicate, so each PCR was performed six times in total (n=6). For the study of gene expressions, cells were grown in 6-wells plates, from Corning Incorporated Costar (catalogue no. 3506) (Frickenhausen, Germany) till 80-90% confluency was reached. Various As metabolites ($iAs^{III}$, $iAs^{V}$, $MMA^{III}$, $MMA^{V}$, $DMA^{III}$ and $DMA^{V}$) in concentrations of 0, 10 and 30 µM dissolved in cell culture medium were incubated for 4-hours. Afterwards, cells in 6-wells plate were washed twice with PBS and RNA was isolated and purified from the cells by using Qiagen RNeasy mini columns (for more details, see RNeasy mini protocol for isolation of total RNA from animal cells). Ultimately cDNAs with a final concentration of 20 ng/µl were synthesized from the isolated RNAs and the samples were analyzed with the Applied Biosystems 7300/7500 Real Time PCR System (Nieuwerkerk a/d IJssel, the Netherlands). The used software, Relative Quantification (RQ), determines the change in expression of a nucleic acid sequence (target)

in a test sample relative to the same sequence in a calibrator sample, after all the measurements are standardized through a chosen household gene (Livak et al. 2001). The relative expressions of each gene for the various concentrations are compared to their control.

2.5.2. Cleavage of p35 to p25 by calcium and arsenite metabolites.

The conversion of p35 to p25 was analyzed by western blot. The transfected HeLa-p35 cells were seeded and grown in 6-wells plates and incubated until 80-90% confluence was reached. To investigate cleavage of p35 to p25, cultures were stimulated with either $CaCl_2$ in a range of 0 to 2 mM or arsenite metabolites at end concentrations of 10 or 30 µM (without addition of any $CaCl_2$) for 4 hours in the incubator at 37˚C in a 5 % $CO_2$ atmosphere. For inhibition experiments, calpeptin, a cell-permeable calpain inhibitor, was used with an end concentration of 50 µM and incubated an hour prior to challenge with various calcium concentrations or arsenite metabolites. After treatment, cells were collected for further analysis with SDS-PAGE and western blot.

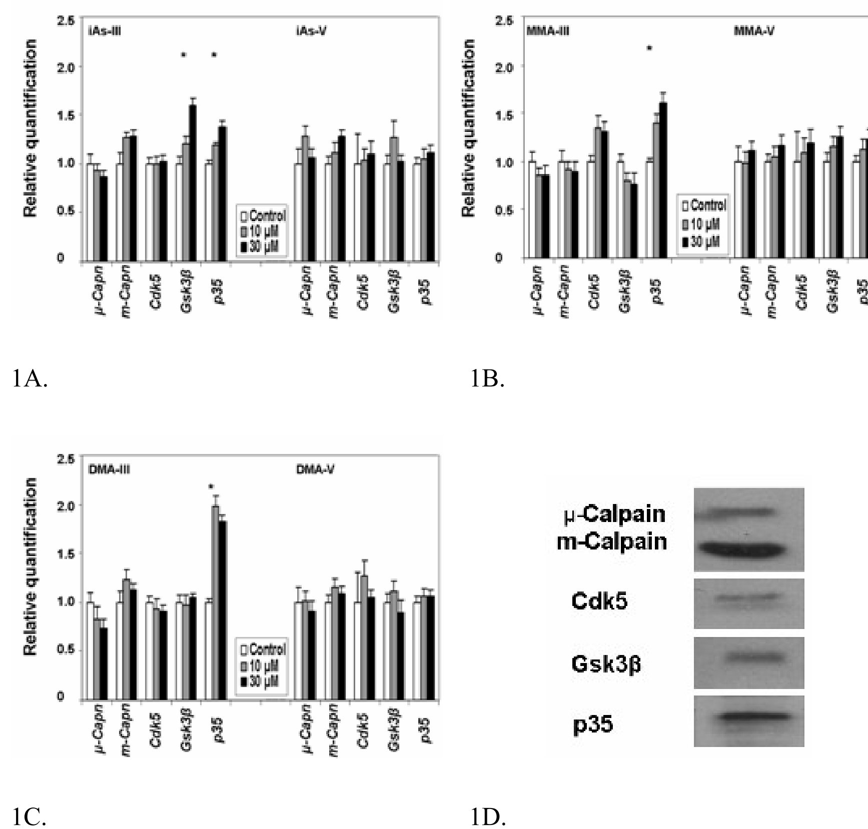
2.6. Statistical analysis.

The data for each gene were statistically evaluated for each concentration using SPSS 14.0 for Windows. Chi-Squared for non-parametric tests (Kruskal-Wallis test) was performed to evaluate the data for concentration and As metabolites as obtained for each gene. A level of $p<0.05$ was accepted as statistically significant.

## 3. Results

3.1. Relative Quantification of various genes.

After treament of the transfected cells with various arsenic metabolites, relative quantification indicated that the significant changes on RNA levels were caused by arsenite metabolites on the increase of p35 gene expression ($p<0.05$) (Fig. 1A-C). *Gsk3ß* was significantly increased in its incubation only after incubation with $iAs^{III}$ ($p<0.05$). Arsenate metabolites did not cause significant changes in the expression levels of any of the genes. The presence of the genes coding for calpain (76 and 80 kDa), CDK5, GSK3ß and the transfected p35 proteins was confirmed using the appropriate antibodies (Fig. 1D).



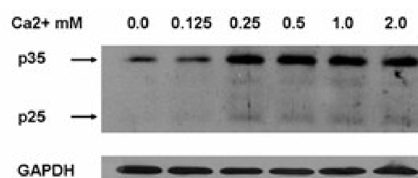

1A. 1B.

1C. 1D.

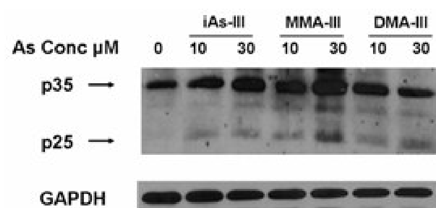
Fig. 1. Effect of various arsenic metabolites, iAs (1A), MMA (1B) and DMA (1C) and concentrations on the various genes involved in phosphorylation of cytoskeletal proteins (*, $p<0.05$). Relative mean values ± SD; n=6. Fig. 1D shows the presence of the μ- and m-calpain, CDK5, GSK3β and the transfected p35 proteins in HeLa cell line.

3.2. Cleavage of p35 to p25 by calcium and arsenite metabolites.

Calcium induced proteolytic cleavage of p35 from 0.25 mM upwards (Fig. 2A). The arsenite metabolites caused a cleavage of p35 to p25 at concentrations of 10 and 30 µM of each metabolite.




2A.

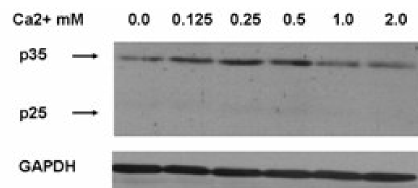



2B.

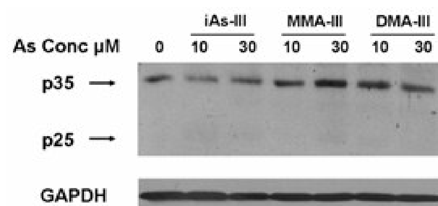
Fig 2. Cleavage of p35 to p25. A titration series of $Ca^{2+}$ ranging from 0 to 2 mM shows that a minimum concentration of 0.25 mM of $Ca^{2+}$ is needed for the cleavage (2A). All three arsenite metabolites (without addition of any $CaCl_2$) cleave p35 to p25 at concentrations of 10 and 30 µM (2B).

3.3. Inhibition of p35 cleavage to p25 by calpeptin.

Pretreatment of the transfected cells with 50 µM calpeptin inhibited the cleavage of p35 to p25 after incubation of calpeptin with various $Ca^{2+}$ concentrations (Fig. 3A) and 10 and 30 µM concentrations of arsenite metabolites (Fig. 3B).




3A.




3B.

Fig 3. Cleavage of p35 to p25 is inhibited by pretreatment with 50 µM of calpeptin. Titration series of $Ca^{2+}$ ranging from 0 to 2 mM shows no cleavage to p25 is visible after $Ca^{2+}$ treatment (3A). The arsenite metabolites (without addition of any $CaCl_2$) are not able to cleave p35 to p25 at concentrations of 10 and 30 µM after pre-incubation with calpeptin (3B).

## 4. Discussion

A number of metal compounds, such as lead and mercury are known to induce neurotoxicity. The organic mercury compound methylmercury has been shown to induce the cleavage of p35 to p25 after treatment of neuronal cells through calpain activation (Sakaue et al.; 2005).
In this *in vitro* study, we first evaluated RNA expression profiles of μ-calpain (calpain-I), m-calpain (calpain-II), *cdk5*, *gsk*3β and *p35* after treatment with various arsenic metabolites. These genes are involved in hyperphosphorylation and degradation of some of cytoskeletal proteins in the nerves. The p35 RNA expression levels were significantly ($p<0.05$) increased after incubation with all the arsenite metabolites at 10 and 30 μM, while GSK3β expression was only significantly ($p<0.05$) induced by $iAs^{III}$ (Fig. 1A-C). However, the arsenate metabolites had no significant effects on any of the gene expression levels. The transfected HeLa cells were also used to characterize their protein expression of the used genes as basis of our model (Fig. 1D).
In our previous study in rats, we showed that NF-L protein expression was decreased after treatment with $iAs^{III}$ (Vahidnia et al. 2006), while its expression on RNA level was unchanged after treatment with $iAs^{III}$ *in vitro* (Vahidnia et al. 2007). This suggests that the reduced expression is a post-translational activity. Calpain could 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). Furthermore, cleavage of p35 to p25 is a $Ca^{2+}$-induced conversion by calpain, which is seen in AD patients (Lee et al. 2000). Here we have shown that $Ca^{2+}$ induces cleavage of p35 to p25 (Fig. 2A) and p25 overexpression after incubation with various arsenite metabolites and 10 and 30 μM concentrations (Fig. 2B). This is in agreement with other studies that show the breakdown of cdk5/p35 into cdk5/p25 increases its kinase activity and neurotoxicity (Camins et al., 2006)
Other studies with PC12 cells under oxidative stress circumstances have shown an increase of calcium in the cells and up-regulation of calpain leading to degradation of NF-L protein (Ray et al. 2000). Furthermore, 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 activation of calpain, through influx of $Ca^{2+}$, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process. Here we have shown that inhibition of calpain by 50 μM calpeptin prevented both $Ca^{2+}$- and arsenite-induced cleavage of p35 to p25 (Fig. 3A and 3B).
From these results can be concluded that arsenite treated cells show cleavage of p35 to p25, which is probably mediated by an increase of $Ca^{2+}$ in the cells. An increase in internal $Ca^{2+}$ levels and subsequent calpain activation may be the primary step in the mechanism of arsenite-induced their neurotoxicity. In addition, calpain splits p35 into p25, which in turn is a hyper-activator of CDK5. This p25/CDK5 complex is responsible for the hyperphosphorylation of MAP-tau and NF proteins. Although RNA expression levels of *cdk5* are unaffected with the various arsenic metabolites (Fig. 1A-C), a complex of p25/CDK5 has shown to be responsible for hyperphosphorylayion of tau proteins (Lee et al. 2000; Town et al. 2002). Hyperphosphorylation of tau reduces its ability to associate with microtubuli proteins as their organizer as it has been seen in AD patients (Kesavapany et al. 2003).
Degradation and hyperphosphorylation of neuronal proteins may lead to disruption of neurofilament organization, resulting in pathophysiological changes in the axon. In conclusion, we have proposed a mechanism of $As^{III}$-induced neurotoxicity. Future studies will show how $Ca^{2+}$-influx is affected by arsenite metabolites.

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