Letter to the Editor

The Role of NF mRNA and Calpain in NF Reduction of Acrylamide Neuropathy*

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The purpose of this study was to study the role of neurofilament (NF) mRNA and calpain in NF reduction of acrylamide (ACR) neuropathy. Male Wistar adult rats were injected *i.p.* every other day with ACR (20 mg/kg·bW or 40 mg/kg·bW) for 8 weeks. NF mRNA expression was detected using RT-PCR and the calpain concentration was determined using western blot analysis. The NF mRNA expression significantly decreased while the level of m-calpain and μ-calpain significantly increased in two ACR-treated rats groups regardless of the ACR dose. The light NF (NF-L) protein expression was significantly correlated with NF-L mRNA expression. Combined with previous data, the concentrations of three NF subunits were negatively correlated with the calpain levels. These findings suggest that NF-L mRNA and calpain mediated the reduction in NF of ACR neuropathy.

Acrylamide (ACR) is a water-soluble α, β-unsaturated carbonyl derivative of the type-2 alkene chemical class and is considered to be prototypical toxicant that produce distal axonopathy. ACR has extensive manufacturing applications in paper, textile, and fabric industries and in scientific research for the electrophoretic separation of macromolecules[1]. In addition it is present in certain foods prepared at very high temperatures[1].

ACR polymers have very low toxicity and do not degrade into their monomeric forms. However, unreacted residual monomers present in the polymers or released during production may be harmful to human health. ACR causes central-peripheral distal polyneuropathy, which is characterised by distal swellings and secondary degeneration[2]. Exposure of both humans and laboratory animals to monomeric ACR produces neurotoxic syndromes such as, ataxia, skeletal muscle weakness, and weight loss[3-4].

Neurofilaments (NFs) are neuron-specific in most mature neurons and are most abundant in cytoskeletal elements, especially myelinated axons. NFs are synthesised in the cell body and are assembled as obligate heteropolymers that require light NF (NF-L) with either medium NF (NF-M) or heavy NF (NF-H) for proper polymer formation. Disruption of the triplet protein has complex consequences. NF disorganisation can sometimes produce deleterious effects and even cause neuronal death; and is a hallmark of various neurodegenerative diseases.

The relationship between the NF protein and ACR neuropathy may be complex. Our laboratory previously reported that ACR induces NF reduction in the sciatic nerve accompanied by typical behavioural changes (e.g., ataxia, hind limb weakness, and foot splay) and significant decreases of body weight based on the baseline values[5].

The mechanism of NF alteration in the peripheral nervous system has not been completely elucidated yet. NFs are synthesised in neuronal cell bodies and then move outward along the axon via slow transport along microtubules. NF protein deficiency can be caused by defective perikaryal synthesis, cell body gene expression downregulation, abnormal transport and/or protein degradation by intracellular proteases. However, few researches have detailed whether such NF changes are a marker for ACR intoxication or whether the NF reductions are caused by NF mRNA and/or calpain disorders.

NF-L, NF-M, and NF-H are coded on chromosome 8p21, 8p21, and 22q12.2, and are consist of 543, 916, and 1020 amino acids, respectively. Transcription of these genes yields NF mRNAs, which are translated exclusively in perikarya and proximal dendrites because axons have no ribosomes. However, information on the effects of ACR on NF gene expression in the sciatic nerve is limited.

Calpain is one of the most abundant neutral proteinases in the nervous system and is involved in the pathophysiology of many neurologic disorders,

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including Alzheimer's disease, sciatic nerve injury, diabetes and aluminium-induced neuropathy[6].

In this study, the levels of NF mRNA expression were determined via RT-PCR and the calpain levels were determined via immunoblot in the sciatic nerves of ACR-treated rats in order to investigate the mechanism of ACR-induced NF protein alterations in peripheral nerves and to explore the mechanisms of ACR-induced neuropathy.

27 Male Wistar rats were divided into two experimental groups and one control group. The rats were injected *i.p.* every other day with ACR (40 mg/kg·bW or 20 mg/kg·bW) for 24 treatments in order to induce subchronic neurological deficits. The ACR was dissolved in 0.9% normal saline and was administered at 3 mL/kg. Age-matched, non-treated control rats were administered an equivalent volume of 0.9% normal saline. The onset and development of neurotoxicity were determined and quantified through gait score assessment and weekly weight change. Gait score assessment and sciatic nerves supernatant preparation were conducted according to LoPachin[7].

In order to assess changes in calpain content using SDS-PAGE, the sciatic nerves supernatant preparation, protein quantitation, electronphoresis, and transmem- berane were manipulated according to the method previous reported[5]. For m-calpain and of μ-calpain electrophoresis, 50 μg and 40 μg total protein of sciatic nerve supernatant fraction were placed into respective lanes and GAPDH was used as the loading control. In order to determine whether the decrease in NF proteins was due to corresponding reductions in NF gene expression, the effects of ACR on NF-L, NF-M, and NF-H mRNAs was measured. Total RNA was extracted using chloroform/isoamyl alcohol. The specific primers for NF-L, NF-H, NF-M, and GAPDH gene were designed (Supplemental Table 1, see the website www.besjournal.com). The data from this measurement are expressed as the mean±SD. The gait scores of the ACR-treated groups and the control group were compared using the Mann-Whitney U test. The body weights and integrated optical densities (IOD) of the immunoreactive blots/bands were expressed as means±SD. Statistical analysis of IOD was performed using one-way ANOVA and the body weights was performed using two-way ANOVA, followed by an LSD or Dunnett's (equal variances not assumed) post-hoc tests using SPSS 13.0 statistical software. The correlations were calculated using SAS 9.2 statistical software. The differences were considered statistically significant ($P<0.05$).

Both groups of ACR-treated rats showed abnormal behaviour, such as lethargy, reduced locomotors activity, and mild ataxia. The rats of low-dose group progressively exhibited hind limb abduction, foot splay and hopping gait, whereas those in the high-dose group exhibited foot dragging and hind limb paralysis.

Gait disturbances are a relatively sensitive marker for the onset and progression of ACR-induced neurologic changes in rats. It can be applied in mechanistic studies on neurotoxicity[7]. The initial gait scores increased from 1±0 (normal gait) to 4±0 (severely abnormal gait) under high-dose ACR and relatively slowly increased to 2±0 (slightly abnormal gait) under low-dose ACR. [Supplemental Figure (A), see the website www.besjournal.com]. ACR hindered body weight gain in a dose-dependent manner. Only the high-dose ACR dosage treatment caused significant weekly weight gain retardation. [Supplemental Figure (B), see the website www.besjournal.com]. Based on the data from gait scores and body weights, we observed that the gait scores peaked and the body weight decreased remarkably in the high-dose ACR group by the end of the experiment. These results suggest that the rats developed ACR neurotoxicity under this intoxication method and we presumed that the high-ACR group could develop nerve injury.

NF proteins are synthesised in the cell body and conveyed through the axon via slow transport. NF mRNA levels represent the net outcome between synthesis (transcription) and RNA turnover. Changes in gene expression caused by toxin exposure affect protein levels and their dependent processes, and the diminished NF proteins could have resulted from decreased cell body gene expression[8]. Local de novo NF-L synthesis in the sciatic nerve of rats has been identified[9]. However, the sciatic nerve axon is also a potential synthesis site for NF polypeptides. The result from our present study also showed that NF-L, NF-M, and NF-H mRNA decreased consistently regardless of ACR-dose (Figure 1, $P<0.01$). The NF mRNA downregulation in our study might indicate inhibition of cell function or cell death in the rat peripheral nervous system under ACR exposure.

Proteinases are widespread in neuronal or nonneuronal eukaryotic cells. They play an important role in protein turnover in neuronal perikaryon and axons, and in the digestion of transported cytoskeletal proteins. Findings from this

study showed that both the μ-calpain and the m-calpain protein levels increased significantly (P<0.01) regardless of the ACR dose (Figure 2).

In order to study the effect of NF mRNA and calpain levels to the NF reduction in ACR neuropathy, the correlation of NF protein[5] with its mRNA expression level (Figure 1D) and calpain content (Figure 2) were analyzed using SAS statistical software combined with the data from previous studies. As shown in Supplemental Figure 2 (see the website www.besjournal.com), NF-L protein level was significantly correlated with its mRNA expression level. The correlation coefficient was 0.6753 (P<0.01). On the contrast, NF-M and NF-H level were correlated poorly (P>0.05) with its mRNA expression level. Considering the changes in gene expression partly corresponded with those of the corresponding protein content, the observed reduction in NF gene expression might only be partly responsible for the NF synthesis reduction in the sciatic nerves.

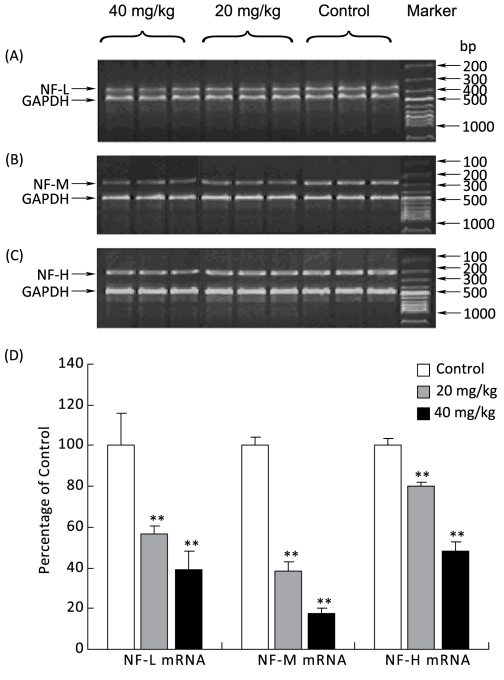

Figure 1. The decrease in NF mRNA levels in sciatic nerves. Representative RT-PCR electrophoresis bands of NF-L, NF-M, and NF-H are shown in (A), (B), and (C), respectively. Graph (D) shows the mRNA band values quantified by image analysis. Specific NF subunit mRNA levels are normalized to GAPDH levels for each lane. The results are presented as mean percentages of the corresponding control ±SD (n=9). The asterisk indicates statistically significant differences ($^{**}P$<0.01).

The western blot analysis demonstrated an overwhelming increase in m-calpain and μ-calpain level in the sciatic nerves of the ACR-treated rats, which might be related to upregulated expression and secretion in sciatic nerves. Both m-calpain and μ-calpain level was negatively correlated with the levels of all three NFs (P<0.01 Supplemental Figure 2). Calpain remains a proenzyme until activated by increased calcium levels. Aside from the calpain protein content, calpain activity should be considered when considering the potential factors that affect NF protein levels during ACR injury. Gupta reported that ACR inhibits m-calpain activity in vitro sciatic nerve model but does not affect μ-calpain activity[10]. Hence, the effect of calpain activity on NF decline needs further evaluation.

In sum, the results from our present study suggest that ACR poisoning significantly reduced the transcription of all three NF genes and increased the calpain content in the sciatic nerves. Combined with data previously reported, finding from this study shows that NF protein is positively correlated with NF-L mRNA level and negatively correlated with both m-calpain and μ-calpain level. These findings suggest that the downregulation in NF-L mRNA and increased calpain expression partly account for the ACR-induced reduction in NF protein in the sciatic nerves.

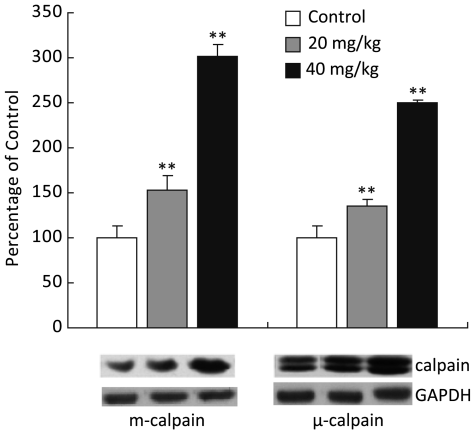

Figure 2. Increased calpain protein content in the sciatic nerves. The graph represents the μ-calpain and m-calpain expression. The representative electrophoresis bands of μ-calpain and m-calpain are also shown below in the corresponding graphs. The results are presented as mean percentages of the corresponding control±SD (n=9). Asterisks indicate statistically significant differences ($^{**}P$<0.01).

The authors declare that they have no conflicts of interest.

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