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REVIEW

Protein Adduct Formation as a Molecular Mechanism in Neurotoxicity

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Chemicals that cause nerve injury and neurological deficits are a structurally diverse group. For the majority, the corresponding molecular mechanisms of neurotoxicity are poorly understood. Many toxicants (e.g., hepatotoxicants) of other organ systems and/or their oxidative metabolites have been identified as electrophiles and will react with cellular proteins by covalently binding nucleophilic amino acid residues. Cellular toxicity occurs when adduct formation disrupts protein structure and/or function, which secondarily causes damage to submembrane organelles, metabolic pathways, or cytological processes. Since many neurotoxicants are also electrophiles, the corresponding pathophysiological mechanism might involve protein adduction. In this review, we will summarize the principles of covalent bond formation that govern reactions between xenobiotic electrophiles and biological nucleophiles. Because a neurotoxicant can form adducts with multiple nucleophilic residues on proteins, the challenge is to identify the mechanistically important adduct. In this regard, it is now recognized that despite widespread chemical adduction of tissue proteins, neurotoxicity can be mediated through binding of specific target nucleophiles in key neuronal proteins. Acrylamide and 2,5-hexanedione are prototypical neurotoxicants that presumably act through the formation of protein adducts. To illustrate both the promise and the difficulty of adduct research, these electrophilic chemicals will be discussed with respect to covalent bond formation, suspected protein sites of adduction, and proposed mechanisms of neurotoxicity. The goals of future investigations are to identify and quantify specific protein adducts that play a causal role in the generation of neurotoxicity induced by electrophilic neurotoxicants. This is a challenging but critical objective that will be facilitated by recent advances in proteomic methodologies.

Key Words: **toxic neuropathy; axonopathy; protein adduct; electrophilic chemicals; acrylamide; 2,5-hexanedione.**

Exposure to numerous structurally diverse chemicals can cause significant morphological and functional damage to nerve cells in the CNS and PNS. In pursuing the corresponding mechanisms of this neurotoxicity, the majority of previous studies have focused on the impact of these chemicals on neuronal structure (e.g., axons), organelles (e.g., mitochondria), or metabolic processes (e.g., fast anterograde transport, ion regulation). Despite significant research, the pathophysiological and molecular mechanisms for many of these neurotoxicants remains poorly defined. However, a common mechanistic theme in the toxicological sciences has been formation of chemical adducts with macromolecules. It is now recognized that many organ system toxicants (e.g., acetaminophen, chloramphenicol, bromobenzene) and/or their active metabolites are electrophiles (electron deficient) that form irreversible covalent bonds (adducts) with nucleophilic centers (unshared electrons) on proteins, DNA, and RNA. Presumably cell toxicity occurs when adduct formation disrupts the structure and/or function of macromolecules (Coles, 1985; Hinson and Roberts, 1992; Nelson and Pearson, 1990). Since many neurotoxicants and their metabolites are electrophiles (Table 1), it is possible that adduct formation is also a critical step in the neuropathogenic processes initiated by exposure to these chemicals. Indeed, protein adduction has been considered as a possible mechanism of neurotoxic action for the organophosphate insecticides, carbon disulfide, and the hexacarbon solvents (reviewed in Graham *et al.*, 1995; LoPachin and DeCaprio, 2004; Lotti, 2000). Nonetheless, despite some promising research activity, adduct formation has not been adequately explored as a more global consequence of neurotoxicant exposure. Therefore, the purpose of this review is to generate further interest in protein adduction as the initiating pathophysiological process in many types of chemical-induced nerve cell injury. Since electrophile/nucleophile theory has received limited attention in the neurotoxicology literature, we will start with a discussion of the physiochemical principles that govern covalent interactions between biological macromolecules and reactive neurotoxicants. This presentation will be limited to protein adduction since research currently suggests that proteins, and not nucleic acids, are the most likely sites of adduction involved in the pathogenesis of toxic nerve cell injury. We will then discuss

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TABLE 1
Examples of Electrophilic Neurotoxicants (Metabolites)

Acrylamide (glycidamide)	Acrylonitrile (cyanoethylene)
Dithiobiuret	Sodium pyridinethione
p-Bromophenylacetylurea	Carbon disulfide
Isoniazide	Acrolein
2,5-Hexanedione	1,2-Diacetylbenzene
Styrene (styrene oxide)	Methylbromide
Acetaldehyde	Nitrogen mustard
4-Hydroxy nonenal	

how adduction might cause protein dysfunction and how such an effect could mediate toxicity at the cellular level. To provide neurotoxicological context, the general principles and concepts of adduct formation will be related to 2,5-hexanedione (HD) neuropathy, for which the results from two decades of research support a mechanism involving adduction of neuronal proteins. In addition, we will present new evidence that acrylamide (ACR) neurotoxicity is mediated by adduct formation with nerve terminal proteins.

Physiochemical Principles of Protein Adduct Formation

Numerous neurotoxic chemicals and/or their active metabolites are electrophiles that will react with nucleophilic centers that have high electron density. The reaction of an electrophilic species with a nucleophilic molecule generally occurs via a substitution or addition mechanism and involves donation of an electron pair by the nucleophile and subsequent formation of a covalent bond. Many atomic and molecular determinants of electrophilicity and nucleophilicity have been postulated and investigated (see reviews by Chermette, 1999; Parthasarathi *et al.*, 2004). Currently, the most accepted concept classifies electrophiles and nucleophiles according to a ''hard acid/soft base'' (HSAB) model (Loechler, 1994; Pearson and Songstad, 1967; Swain and Scott, 1953). Thus, hard electrophiles have either a high positive charge density or a formal positive charge at the electrophilic center. Conversely, soft electrophiles have a lower positive charge density. Because of their high charge density, hard electrophiles have valence electron shells that are not easily polarized (i.e., deformed), whereas the opposite is true for soft electrophiles. It is the relative tendency of these outer shells to polarize that is a major determinant of the selective reactivity of electrophiles with their nucleophilic targets (see ahead). Hard nucleophiles have high electronegativity and low polarization of valence electrons, whereas soft nucleophiles have low electronegativity and are more polarizable. The softest biological nucleophilic sites are cysteine thiol groups on proteins and glutathione (GSH; Table 2). Of moderate hardness are primary and secondary amino groups (lysine and histidine, respectively) on proteins, whereas the hardest nucleophiles are the oxygen atoms of purines and pyrimidines (Table 2).

TABLE 2
List of Hard and Soft Electrophiles and Hard and Soft Nucleophiles

Hard electrophiles
Alkyl carbonium ions
Benzylic carbonium ions
Soft electrophiles
Acrylamide
Acrolein
Acrylonitrile
Quinones
Hard nucleophiles
Oxygen atoms of purine/pyrimidine bases in DNA
Endocyclic nitrogens of purine bases in DNA
Oxygen atoms of protein serine and threonine residues
Soft nucleophiles
Protein thiol groups
Sulfhydryl groups of glutathione
Primary/secondary amino groups of protein lysine and histidine residues

Based on the HSAB theory, the reaction rates and selectivities of electrophiles and nucleophiles are dependent upon comparable states of ''hardness.'' Specifically, a soft electrophile such as acrylamide will react predominantly with a soft nucleophile such as the thiol group of cysteine. A hard electrophile such as the methyl carbonium ion formed from dimethyl nitrosamine will react with hard nucleophiles such as the oxygen atoms of DNA. This preferential reactivity is due primarily to the high-energy transition state that acts as a barrier to the reaction of, for example, a hard electrophile with a soft nucleophile (Coles, 1985; Pearson and Songstad, 1967). Electrophilic xenobiotics adduct biological nucleophilic sites through a number of covalent reaction mechanisms. For example, the polarized double bonds of acrylamide, acrolein, and other α,β-unsaturated chemicals react by Michael addition of the nucleophile across the vinyl group (Fig. 3). Nucleophilic attack by electrophilic epoxide chemicals (e.g., glycidamide, the oxidized metabolite of acrylamide) occurs through opening of the strained ring (for a more detailed discussion of covalent binding during adduct formation see Coles, 1985; Friedman, 1973; Harding, 1985; Kemp and Vellaccio, 1980).

Relative hardness and softness are clearly important characteristics for covalent bond formation in biological systems. However, protein adduction is dependent not only upon the physiochemical nature of the electrophile but also upon the microenvironment of the nucleophilic center, which can vary significantly even among centers of the same elemental type (e.g., sulfur or amino groups). Thus, nucleophilic reactivity among free sulfhydryl groups on polythiol proteins can be diverse and, consequently, soft electrophilic chemicals will adduct the more reactive thiol groups on a given protein (Vogel and Nivard, 1994). This diversity in nucleophilic reactivity is a function of both steric and electronic factors mediated primarily by protein tertiary structure. For example, it is now

recognized that vicinal acidic and basic amino acids significantly influence reactivity of the target nucleophilic group. In hemoglobin, Cysβ93 is involved in nitric oxide (NO) signaling and is arranged in a three-dimensional motif with the basic amino acid histidine (Hisβ146) and the acidic residue aspartate (Aspβ94; Stamler *et al.*, 1997). In the oxygenated state (relaxed conformation), the sulfur of Cysβ93 is adjacent to the imidazolium group (pKa = 6.0) of histidine, which facilitates deprotonation of the sulfur residue. When compared to other cysteine residues that do not exist in a comparable motif, deprotonation significantly increases the relative nucleophilicity of Cysβ93 (by lowering the pKa) and promotes nitrosylation of the sulfhydryl group. In the deoxygenated state (tense conformation), the Cysβ93 of hemoglobin is in proximity to the carboxyl group of aspartate. This results in protonation of the sulfur and a reduction in both the nucleophilicity and the rate of nitrosylation of this residue. This example demonstrates how reactivity among common nucleophilic sites can vary within a protein and provides a basis for understanding the selectivity and toxicological specificity of protein adduct formation. The example also shows how acid-base catalysis can alternatively regulate the nitrosylation and denitrosylation of a protein nucleophilic center. In turn, this illustrates how the redox-state of a single but functionally critical nucleophile can modulate an important physiological process such as NO signaling (see Hess *et al.*, 2001; Jaffrey *et al.*, 2001; Stamler *et al.*, 1997).

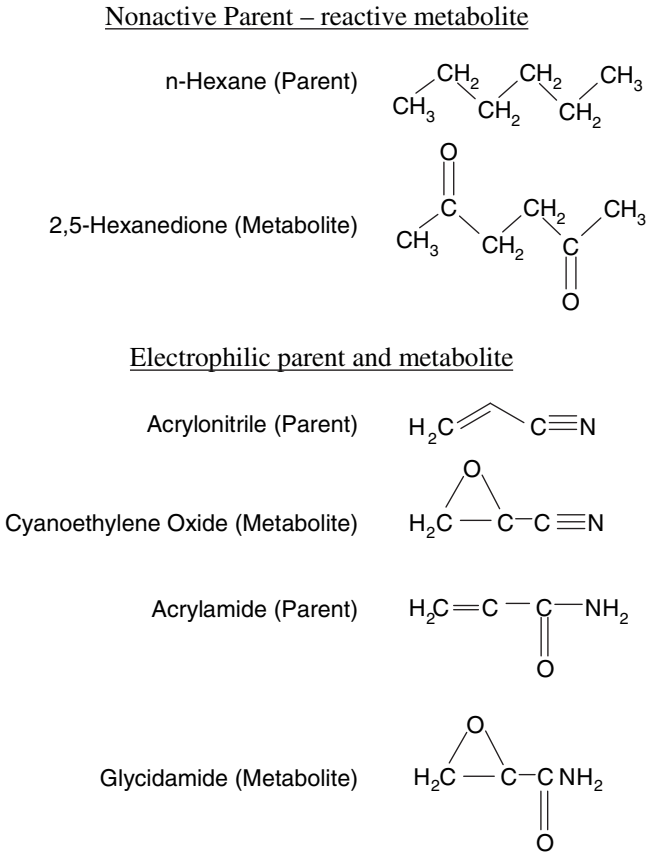

FIG. 1. This figure shows examples of an unreactive parent chemical (n-hexane) and its neurotoxic reactive metabolite (2,5-hexanedione). Also illustrated are several examples of neurotoxicants for which both the parent molecule and metabolite exhibit electrophilic reactivity.

The Influence of Xenobiotic Biotransformation on Sites of Adduct Formation

The biotransformation of a chemical to an active electrophilic derivative appears to be a common mechanism in the toxicological sciences (Fig. 1; Ketterer, 1980; Miller and Miller, 1981). As discussed above, depending upon the physiochemical nature of the electrophile, the resulting metabolite can produce toxicity by reacting with: (1) soft nucleophilic sites on proteins and free thiols such as GSH or (2) harder nucleophilic centers on DNA and RNA. Alternatively, both the parent and metabolite can produce adduct-based toxicity (Fig. 1). Frequently, the parent chemical is a soft electrophile that is metabolically converted to a harder toxic metabolite. For example, acrylonitrile (ACN; cyanoethylene) has been shown to have both carcinogenic and neurotoxic actions (Willhite, 1982; Woutersen, 1998). The vinyl group of ACN is a soft electrophilic center that reacts with free sulfhydryl groups on GSH and protein cysteines (Fig. 1). However, metabolic epoxidation of the double bond produces the relatively hard electrophilic metabolite, cyanoethylene oxide (Fig. 1; Sumner *et al.*, 1999). This epoxide will form adducts primarily with nucleophilic sites on DNA (Guengerich *et al.*, 1981; Oesch *et al.*, 1971). Similarly, acrylamide (ACR) is an α,β-unsaturated carbonyl and is a well-recognized human neurotoxicant and possible carcinogen (Fig. 1; reviewed in LoPachin *et al.*, 2003a; LoPachin, 2004; Dearfield *et al.*, 1995). As a soft electrophile, ACR will adduct thiol groups on proteins and GSH (LoPachin *et al.*, 2003b, 2004a; Tong *et al.*, 2004). Cytochrome P450 2E1-mediated metabolism of ACR yields the epoxide derivative, glycidamide (Fig. 1; Calleman *et al.*, 1990; Sumner *et al.*, 1992). This harder electrophilic metabolite will react with nucleophilic centers on adenine and guanine of DNA (Gamboa da Costa *et al.*, 2003). These examples show that both the parent chemical and oxidative metabolite can exhibit distinct electrophilic characteristics. This difference in electrophilicity predicts correspondingly different nucleophilic targets for the parent (i.e., protein thiols) and harder metabolite (i.e., nucleic acids). That these divergent macromolecular targets mediate different adduct-based toxicities is suggested by the observation that most genotoxic chemicals are hard electrophiles that adduct hard nucleophilic sites on DNA. In contrast, chemicals that produce noncarcinogenic cytotoxicity (e.g., neurotoxicity, hepatotoxicity) are soft electrophiles such as the α,β-unsaturated aldehydes, ketones or related derivatives that bind cysteine sulfhydryl groups on proteins (reviewed in Hinson and Roberts, 1992; Coles, 1985).

The Toxicity Spectrum of Soft Electrophiles

In general, the different preferential toxicities of hard electrophiles (i.e., genotoxicity) and their soft counterparts (i.e., cytotoxicity) are understandable based upon their presumed macromolecular targets; i.e., DNA vs. proteins, respectively. However, soft electrophiles can cause a wide spectrum of cytotoxicity, which is counterintuitive since these chemicals theoretically adduct common nucleophilic sites on proteins. For example, α,β-unsaturated carbonyl compounds are a chemically broad group of soft electrophiles that form cysteine adducts on proteins via Michael additions (Esterbauer *et al.*, 1991; see also Fig. 3). This chemical class includes compounds that produce a variety of organ-specific toxicities; e.g., acrolein and acrylamide (neurotoxicity), allylamine (cardiotoxicity), 2-methylfuran (renal toxicity), and the reactive metabolite of acetaminophen, N-acetyl-p-benzoquinone imine (hepatotoxicity). Because these electrophilic toxicants interact with similar nucleophilic residues that have a wide distribution among diverse tissue proteins, a common, more generalized toxicity might be anticipated. Yet, the observed selective toxicities suggest that individual chemicals of this group differ with respect to their tendency to form protein adducts in specific target organs (Cohen *et al.*, 1997; Tornqvist *et al.*, 2002). The mechanism underlying this apparent selectivity is complex and not completely understood. However, the selective toxicities exhibited by this class of chemicals could be, at least in part, due to characteristic toxicokinetic parameters that determine the tissue distribution and target concentrations of a given electrophile. Also likely involved are the respective physiochemical attributes of both the electrophile (hard vs. soft) and its nucleophilic site (steric and electronic factors) that ultimately determine the probability of adduct formation in different tissues and individual proteins. Thus, although soft electrophilic toxicants react with nucleophilic sites that are common to many proteins across tissues, selective toxicity is possible based on inherent toxicokinetic and physiochemical characteristics that can limit the reaction of an electrophile to a specific set of nucleophiles in a target tissue. Clearly, the selectivity of macromolecular adduct formation is an area of adduct toxicity that requires continued research.

Putative Mechanisms of Adduct-Based Neurotoxicity

In this section, we will discuss mechanisms by which protein adduction might induce neurotoxicity at the molecular and cellular levels. The principles of adduct-based mechanisms are presented schematically in Figure 2. Although substantial evidence supports a role for adduct formation in toxic cell injury, for many chemicals a direct causal relationship has not been established. This is not an indictment of adduct-based mechanisms, but rather reflects the *in vivo* complexity of chemical-protein adduction and the limited analytical methodologies previously available. Recent advances in proteomic techniques should accelerate progress in the detection and quantification of low *in vivo* adduct levels (reviewed in Harder *et al.*, 2003; Liebler, 2002; LoPachin *et al.*, 2003c; Tornqvist *et al.*, 2002). It should also be noted that mechanisms indirectly involving adduct formation, most notably oxidative stress, can also play a role in the production of toxicity. Some electrophilic chemicals lead either directly or indirectly to generation of reactive oxygen species (e.g., superoxide anion, hydroxyl radicals) that subsequently cause oxidative damage. In addition, soft electrophiles that adduct thiol groups can significantly decrease cellular reducing equivalents (e.g., NADPH, glutathione, vitamin E) and thereby shift the redox balance of the cell toward oxidation. Regardless of the oxidative mechanism, lipid peroxidation, protein thiol oxidation and other oxidative changes can, in addition to direct covalent protein modifications, lead to cytotoxicity.

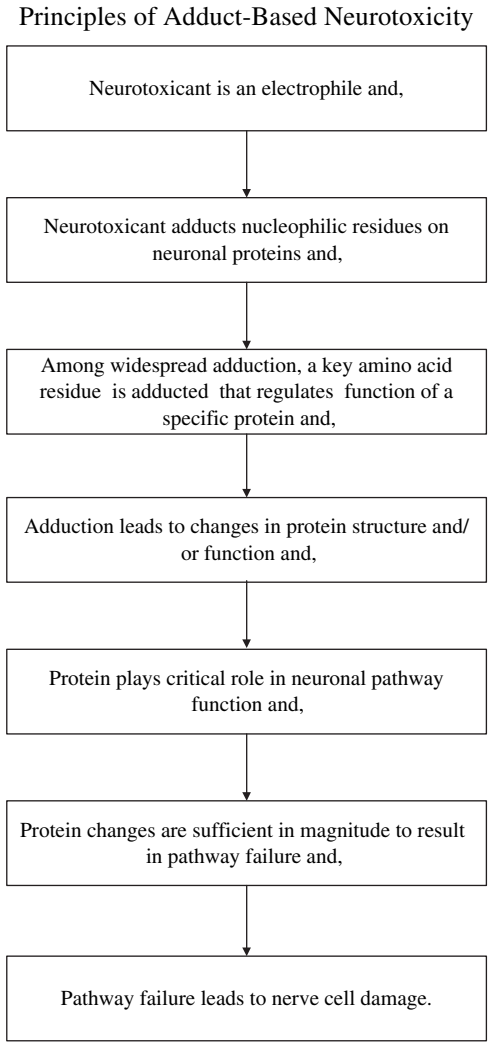

FIG. 2. This figure shows, in schematic form, the salient features of adduct-based neurotoxicity.

We have proposed several possible reasons why, despite adduction of common nucleophilic sites on proteins, soft electrophilic chemicals could produce tissue-specific. Even within the target tissue (e.g., PNS or liver), however, these nucleophilic centers are ubiquitous and diverse cellular pathways and processes exist that might be affected by adduction of constituent proteins. Given this lack of site specificity within the target tissue it is not clear how adduct formation could produce selective toxicity. Accumulating evidence suggests that electrophilic chemicals act by adducting specific amino acids that are critical to tertiary structure and/or protein functions such as enzymatic activity, ion translocation, or protein-protein interactions (reviewed in Friedman, 1973; Harding, 1985; Hinson and Roberts, 1992; Nelson and Pearson, 1990). For example, the role of the skeletal muscle Ca^{2+} release channel/ryanodine receptor (RyR1) in excitation-contraction coupling is highly sensitive to disruption by the sulfhydryl alkylating agent, *N*-ethylmaleimide (NEM; Moore *et al.*, 1999). There are approximately 400 cysteines within the tetrameric RYR1 channel complex that represent potential sites of alkylation (Eu *et al.*, 2000). Nonetheless, several lines of evidence indicate that NEM inhibition is mediated by selective adduction of the sulfhydryl group on Cys 3635 (Eu *et al.*, 2000; Sun *et al.*, 2001; Yamaguchi *et al.*, 2001). Thus, although other cysteine residues are adducted, NEM alkylation of a specific cysteine can lead to impaired RyR1 channel function and thereby promote muscle toxicity. It can also be deduced from this example that a large proportion of the adducts formed on this and other proteins are toxicologically irrelevant since the corresponding amino acids do not play a direct role in either protein structure or function.

Even when adduction of a critical amino acid residue and subsequent changes in protein structure and/or function can be demonstrated, it cannot be assumed a priori that this effect necessarily produces cellular injury. This caveat is particularly germane to the chemical adduction of proteins that are members of metabolic pathways (e.g., glycolysis) or are constituents of complexes (e.g., SNARE core complexes) that mediate neuronal processes (e.g., neurotransmitter release, energy production). Here, the function of the pathway or process is more likely to be affected if the adduct-inhibited protein plays a key regulatory role or is otherwise operationally critical. This concept is best illustrated by the research surrounding the ''glycolysis'' hypothesis of toxic axonopathies. It was proposed that acrylamide inhibited the activities of glycolytic enzymes resulting in an axonal energy deficit and subsequent degeneration of distal fibers (Spencer *et al.*, 1979). Supporting evidence suggested that acrylamide intoxication of rats decreased the activities of neuron specific enolase (NSE) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in brain homogenates (Howland, 1981; Howland *et al.*, 1980). Additional results implied that this effect was due to adduction of cysteine residues in the active sites of these enzymes (Orstan and Gafni, 1990). However, other research showed that acrylamide did not affect lactate production or other indices of brain glycolytic pathway function (Brimijoin and Hammond, 1985; LoPachin *et al.*, 1984; Matsuoka and Igisu, 1992). The absence of an effect on glycolytic flux is consistent with additional data showing that acrylamide did not inhibit the activity of phosphofructokinase (PFK), the rate-limiting glycolytic enzyme (Howland, 1981; Sakamoto and Hashimoto, 1985). Thus, despite adduction of functionally important amino acid residues (cysteines) and subsequent glycolytic enzyme inhibition (e.g., GAPDH and NSE activities), acrylamide did not decrease overall glucose flux. Protein adduct formation is, therefore, not necessarily a prelude to toxicity. Instead, toxicity occurs only when the activities of key regulatory proteins (e.g., PFK) are inhibited by adduct formation.

In the preceding sections, we have defined the physiochemical attributes of electrophilic chemicals and their protein nucleophilic sites of adduction. In addition, we have discussed the molecular mechanisms that might mediate the production of selective cellular toxicity by electrophilic xenobiotics. To place these concepts within a neurotoxicological perspective, in the following section we will provide a brief overview of the substantial evidence supporting a role for protein adduct formation in γ-diketone neuropathy. Also presented will be results from recent studies, which suggest that ACR produces neurotoxicity through adduction of cysteine residues on certain nerve terminal proteins that regulate membrane fusion processes such as neurotransmission.

The Role of Adduct Formation in 2,5-Hexanedione Neurotoxicity

HD is the active γ-diketone metabolite of the neurotoxic hexacarbon solvents *n*-hexane and methyl *n*-butyl ketone (Couri and Milks, 1982; Krasavage *et al.*, 1980). HD intoxication of laboratory animals produces loss of body weight and changes in several neurological parameters, including gait abnormalities (ataxia) and reductions in hindlimb skeletal muscle strength (LoPachin *et al.*, 2002; Spencer and Schaumburg, 1977a). Quantitative morphometric and electrophysiologic studies conducted over the past 20 years have shown that axon atrophy in the PNS and CNS is the morphological hallmark of γ-diketone neuropathy (reviewed in LoPachin and Lehning, 1997; LoPachin *et al.*, 2000; LoPachin and DeCaprio, 2004). Previous investigations have revealed that, regardless of exposure rate (100–400 mg/kg/day), axon atrophy was an early consequence of HD neurotoxicity. HD-induced atrophy developed in conjunction with the onsets of neurological deficits and decreases in nerve conduction velocity (Lehning *et al.*, 2000; Yagi, 1994). Since it is well documented that loss of caliber produces changes in axonal cable properties and nerve conduction (Sakaguchi *et al.*, 1993), the atrophy induced by γ-diketone intoxication could be causally related to the observed nerve dysfunction. Together, these attributes suggest

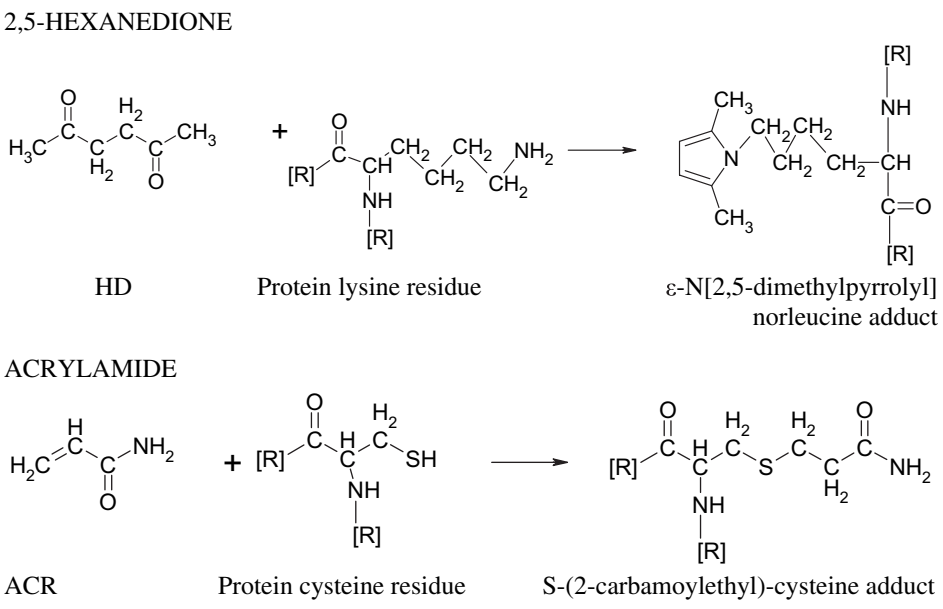

FIG. 3. This figure illustrates the formation of protein adducts by HD and ACR. As a γ-diketone electrophile, HD reacts covalently with nucleophilic lysine ε-amine groups to form 2,5-dimethylpyrrole adducts on proteins (A). ACR is an α,β-unsaturated aldehyde that forms adducts with cysteine sulfhydryl groups via a Michael carbonyl condensation reaction (B).

that the development of axon atrophy is a necessary event in the pathophysiological process that leads to γ-diketone neurological toxicity.

The mechanism by which HD produces axon atrophy is not known, but likely involves either direct or indirect disruption of neurophysiological processes that maintain axon caliber. Based on this premise, the neurofilament (NF) subunits, NF-light (NF-L), NF-medium (NF-M) and NF-heavy (NF-H) are primary target candidates (DeCaprio and O'Neill, 1985), since these proteins play an important role in determining the size of mature axons (reviewed in Muma and Hoffman, 1993). Indeed, we have found that the axon atrophy induced by HD is correlated with a loss of these cytoskeletal proteins in PNS and CNS tissues (Chiu *et al.*, 2000; LoPachin *et al.*, 2004b, 2005). Whereas the reason for this protein loss is not understood, we have hypothesized that HD adduction of NF proteins interferes with the turnover and maintenance of the axonal cytoskeleton (see below). HD is a diketone electrophile that reacts covalently with nucleophilic lysine ε-amine groups (Fig. 3) to form 2,5-dimethylpyrrole adducts on NFs and other proteins (DeCaprio *et al.*, 1982, 1983; DeCaprio and O'Neil, 1985; Graham *et al.*, 1982). Pyrrole formation is unique to γ-diketones, which have a two carbon spacing between the carbonyl functions and is the basis of the structure-activity relationships that have been established for γ-diketone neuropathy (see Anthony *et al.*, 1983; DeCaprio *et al.*, 1988; Genter *et al.*, 1988; Monaco *et al.*, 1985). Graham and colleagues (1991, 1995) suggested that once formed, pyrrole adducts undergo oxidative reactions that yield crosslinked NF proteins. However, the pathophysiological relevance of this secondary step has not been established (reviewed in DeCaprio, 2000; LoPachin and DeCaprio, 2004; LoPachin and Lehning, 1997).

Although evidence has shown that the formation of pyrrole adducts on lysine residues is a critical and required event in the production of HD neuropathy, the low levels of NF adducts formed during γ-diketone exposure and the lack of protein specificity appear to argue against a causative role. Specifically, during *in vivo* γ-diketone exposure non-neuronal proteins (e.g., hemoglobin, serum albumin) that are unlikely to be involved in neurotoxic mechanisms exhibit pyrrole adduct levels that are quantitatively similar to those of cytoskeletal proteins (DeCaprio and O'Neill, 1985; Genter St. Clair *et al.*, 1988; Pyle *et al.*, 1992). This observation suggests a wide range of targets and is consistent with the fact that virtually all proteins contain one or more lysine ε-amine sidechains that are potential sites of adduction for HD. Moreover, *in vivo* and *in vitro* studies showed that only a small fraction (e.g., <5%) of total available lysyl ε-amine groups on NF proteins were converted to pyrrole adducts by HD exposure (DeCaprio and O'Neill, 1985; DeCaprio and Fowke, 1992). If NFs are the primary neuropathogenic target, then one might expect that substantial adduct formation in NF proteins should occur. However, in support of a specific involvement of pyrrole formation, *in vitro* experiments demonstrated that the adducted lysine residues were primarily located within KSP repeats on the C-terminal (''tail'') regions of NF-M and NF-H subunit proteins (DeCaprio and Fowke, 1992; DeCaprio *et al.*, 1997). These regions are critically involved in the interactions of NF subunits with the cytoskeletal polymer (see below). Based on this selectivity, it has been proposed that, despite

a broad range of adducted proteins, γ-diketone neuropathy is mediated by adduction of a limited number of critical lysine amine groups on NF subunits (DeCaprio and Fowke, 1992; DeCaprio *et al.*, 1997).

How does pyrrole formation cause axonal NF protein loss and subsequent atrophy? NF interactions with the cytoskeletal polymer could be modified directly by HD adduction of critical C-terminal lysine groups. Conversion of lysine amine groups to pyrrole adducts will cause a loss of net positive charge and formation of a hydrophobic moiety at the reaction site. This is likely to be followed by changes in NF physiological characteristics, which might include solubility, electrostatic potential, and three-dimensional structure (reviewed in DeCaprio, 1985, 1987; Sayre *et al.*, 1985). If these chemically modified NF proteins cannot interact appropriately with the cytoskeletal network, axon atrophy could develop as accelerated transport and degradation of adducted, unincorporated triplet protein deplete regional subunit levels (for details see LoPachin and DeCaprio, 2004). The elegant proteomic studies involving HD illustrate the important concept that although neurotoxicant exposure might be associated with seemingly indiscriminant adduct formation, adduction of certain key nucleophilic protein residues (i.e., lysine residues within KSP repeats on NF tail regions) might mediate selective compromise of a critical neuronal character (i.e., axon caliber).

The Role of Adduct Formation in Acrylamide Neurotoxicity

Acrylamide (ACR) is a water-soluble, vinyl monomer (Figs. 1 and 3) that has multiple applications in the chemical and manufacturing industries; e.g., ore processing, soil grouting and dye synthesis. In addition, ACR is used extensively in molecular laboratories for separation of macromolecules by gel chromatography and is present in certain foods that have been prepared at very high temperatures (Tareke *et al.*, 2000). Long-term, low-level exposure to monomeric ACR produces ataxia and skeletal muscle weakness in humans and experimental laboratory animal models (LeQuesne, 1985; LoPachin *et al.*, 2002; Spencer and Schaumburg, 1974a). Early morphological studies suggested that the neurological defects associated with ACR intoxication were mediated by degeneration of distal axon regions in the PNS and CNS (reviewed in Spencer and Schaumburg, 1974b, 1977a,b, 1980a). However, substantial evidence from other morphological, electrophysiological, and neurochemical studies now implicate nerve terminals and cerebellar Purkinje neurons as neurotoxicologically relevant sites of ACR action (reviewed in LoPachin *et al.*, 2002, 2003; LoPachin, 2004). Whereas ACR is a soft electrophile, the parent chemical can be metabolized to the reactive epoxide, glycidamide (Calleman *et al.*, 1990). Therefore, at issue is the role of this metabolite in the production of neurotoxicity. A qualitative morphological study has suggested that glycidamide is involved in the mechanism of distal axon degeneration and accompanying neurological deficits in ACR-exposed rats (Abou-Donia *et al.*, 1993). However, other research failed to find evidence for the induction of neurotoxicity by this metabolite (Barber *et al.*, 2001; Brat and Brimijion, 1993; Costa *et al.*, 1992, 1995). Thus, although glycidamide adduction of nucleic acid residues might mediate the genotoxicity associated with ACR intoxication of rodents (see above), neurotoxicity appears to be a product of the parent compound.

The molecular mechanism by which ACR produces nerve terminal and Purkinje cell damage has not been fully delineated. Nonetheless, understanding the structure and chemical properties of ACR can provide insight into the molecular mechanism of neurotoxicity. As indicated previously, ACR is an α,β-unsaturated aldehyde with electrophilic reactivity at the carbonyl carbon atom. As a soft electrophile, ACR could conceivably adduct amines, imidazoles and sulfhydryl groups on proteins via the Michael carbonyl condensation reaction (Fig. 3; Friedman, 1973; Kemp and Vellaccio, 1980). However, the reactivity of free thiols is greater than that of other soft nucleophilic centers and, consequently, the preferential *in vivo* target of ACR is sulfhydryl groups on protein cysteine residues and glutathione (reviewed in Calleman, 1996). Early chemical measurements (Bergmark *et al.*, 1991; Cavins and Friedman, 1968; Dixit *et al.*, 1986; Kemplay and Cavanagh, 1984a,b; Sega *et al.*, 1989) and more recent mass spectroscopy studies (Barber and LoPachin, 2004; Bordini *et al.*, 1999, 2000; Hall *et al.*, 1993) have shown that ACR selectively reacts with cysteine residues of proteins to form S-(2-carbamoylethyl)-cysteine adducts. That cysteine adduct formation in nervous tissue might have mechanistic significance is suggested by the finding that *in vitro* exposure of brain synaptosomes to graded concentrations of ACR (0.001–1.0 M) produced decreases in evoked neurotransmitter release and parallel increases in adduct levels (Barber and LoPachin, 2004). Furthermore, in synaptosomes isolated from brains of ACR-intoxicated rats, cysteine adduct levels increased in concert with the temporal development of neurological deficits (Barber and LoPachin, 2004). These data indicate that the molecular mechanism of ACR neurotoxicity likely involves the formation of thiol adducts on neuronal proteins. Nonetheless, results from other studies do not support this conclusion (Hashimoto and Aldridge, 1970; Lapin *et al.*, 1982; Martenson *et al.*, 1995a,b). For example, Martenson *et al.* (1995a,b) showed that relatively high *in vitro* ACR concentrations (10–100 mM) caused morphological disruptions of dorsal root ganglion growth cones that were similar to changes induced by much lower concentrations of other sulfhydryl reagents; e.g., iodoacetic acid (IAA; 6.75–27 μM) or ethacrynic acid (ECA; 33.5–100 nM). The authors concluded that, since it is unlikely that mM concentrations of ACR are achieved during whole animal intoxication, the mechanism of *in vivo* nerve damage could not involve sulfhydryl alkylation. However, these findings are not necessarily contrary to an involvement of thiol adduction. It is

important to recognize that all three chemicals produced similar *in vitro* neurotoxicity, although the potency of ACR for this effect was significantly lower than the relative potencies of either IAA or ECA. In a recent study of *in vitro* synaptosomal neurotransmitter release, LoPachin *et al.* (2004a) reported a similar dispersion of potencies among ACR, IAA and *N*-ethylmaleimide (NEM) with respect to inhibition of exocytosis. Because ACR is a relatively weak electrophile (Barber and LoPachin, 2004; Cavins and Freidman, 1968), high *in vitro* concentrations are likely required to generate, on an acute basis, intracellular cysteine adduct levels that exceed toxic thresholds. Furthermore, the relatively low potency for adduct formation is consistent with the cumulative *in vivo* neurotoxicity caused by ACR, where relatively long exposure durations or high daily dose-rates are necessary for the induction of neurological deficits (see detailed discussion in Barber and LoPachin, 2004; LoPachin *et al.*, 2004a).

If adduction of protein cysteine residues is the basis of ACR neurotoxicity, it is not clear how such a generalized reaction leads to a specific effect on neurotransmitter release. One possibility is that ACR produces a relatively selective neurotoxic response by reacting with cysteine groups on proteins that regulate membrane fusion processes in nerve terminals and cell bodies. Our most recent research has focused on presynaptic mechanisms and has shown that ACR can form cysteine adducts with many nerve terminal proteins (Barber and LoPachin, 2004; LoPachin *et al.*, 2004a). Among the adducted proteins that have been identified, several play a critical role in synaptic vesicle-membrane fusion and neurotransmitter release; i.e., *N*-ethylmaleimide-sensitive fusion (NSF) protein and synaptosomal associated protein of 25 kDa (SNAP-25; Barber and LoPachin, 2004). In particular, ACR adduction of NSF has significant mechanistic relevance. The fusion of synaptic vesicles with the presynaptic membrane, which is necessary for transmitter release, is mediated by the formation of 7S *cis*-SNARE complexes. NSF is an ATPase that dissociates the SNARE complexes into the corresponding *trans*-protein components; i.e., SNAP-25, synaptobrevin, syntaxin 1. Disassembly of presynaptic SNARE complexes is accomplished by ATP hydrolysis and allows continuous cycling of vesicle-membrane fusion (reviewed in Whiteheart *et al.*, 2001). Recent tandem mass spectrometric analyses of NSF revealed that ACR formed adducts with Cys 264 located within domain I (#255-266) of the nucleotide-binding consensus sequence (Barber and LoPachin, 2004). This residue is critically involved in ATP hydrolysis and, therefore, determines the function of NSF (Matsushita *et al.*, 2003; Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994). Previous studies have shown that sulfhydryl alkylation by NEM inhibits NSF activity, reduces synaptosomal neurotransmitter release, and increases the levels of 7S SNARE complex in exposed synaptosomes (Lonart and Sudhof, 2000; LoPachin *et al.*, 2004a; Nedvetsky *et al.*, 2000; Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994). Similarly, *in vitro* exposure to ACR also produced concentration-dependent decreases in synaptosomal release (LoPachin *et al.*, 2004a) and increased the content of 7S SNARE complexes (Barber and LoPachin, 2004). These findings suggest that ACR does not affect the protein-protein interactions that mediate SNARE core assembly. Rather, the observed accumulation of synaptosomal 7S complexes is consistent with ACR inhibition of NSF ATPase activity, presumably through adduction of Cys 264.

Growing evidence now indicates that the neurological deficits associated with ACR intoxication are mediated by disruption of membrane fusion processes. Whereas the molecular mechanism of this disruption is not known, recent experiments suggest that ACR forms adducts with functionally important cysteine residues (Cys 264) on proteins (NSF) that play a regulatory role in membrane fusion. Whereas our proteomic studies have dealt primarily with presynaptic events, it is possible that Purkinje cell injury in the cerebellum also involves inhibition of critical membrane fusion processes (reviewed in LoPachin *et al.*, 2003b; LoPachin, 2004). In general, it is becoming increasingly obvious that many cellular pathways and processes are regulated by cysteine sulfhydryl groups that act as redox sites for posttranslational modification of protein function by nitric oxide and other presumed signal mechanisms (Broillet, 1999; Forman *et al.*, 2002; Jaffrey *et al.*, 2001; Stamler *et al.*, 2001). This suggests that the diverse toxicities induced by other soft electrophiles (see above) involve adduction of regulatory sulfhydryl groups that are subject to redox-based modulation.

Conclusions and Future Research Directions

In this review we have considered the possible mechanistic roles of protein adduct formation in toxic neuropathies. For other organ system toxicants and/or their active metabolites, covalent interaction with proteins has been a frequently proposed mechanism of cellular toxicity. However, although the formation of adducts by certain neurotoxicants has been exploited as a biomarker of exposure (DeCaprio, 1997; Tornqvist *et al.*, 2002), protein adducts as a neuropathogenic hallmark have received limited attention. Nonetheless, many neurotoxic chemicals have electrophilic centers that could react with nucleophilic amino acid residues to form aducts on neuronal proteins (Table 1). Adduct formation could negatively impact the tertiary structure and/or function of these proteins and thereby interfere with, for example, energy metabolism, axonal transport or presynaptic neurotransmitter release. In contrast, the lack of specificity and low levels of adducts formed with key proteins seems counterintuitive to an involvement in primary pathophysiological processes. Regardless of this apparent enigma, it is possible for electrophilic neurotoxicants to produce specific effects by reacting with functionally critical nucleophilic centers on proteins that regulate cellular pathways or processes. Whereas the molecular mechanism of many neurotoxicants remains poorly defined, for others a greater understanding of neuropathogenesis has been

achieved by investigating the role of protein adduct formation. Thus, for neurotoxicants such as carbon disulfide, isoniazid, dithiobiuret, sodium pyridinethione or p-bromophenylacetylurea the electrophilic nature and nucleophilic targets should continue to be explored as potential mechanistic components. This adduct-based approach could implement new research directions and lead to a better understanding of neurotoxic processes. Finally, understanding the chemistry and pathophysiological consequences of adduct formation could not only benefit neurotoxicology, but might also provide insight into mechanisms of human neurodegenerative diseases. For example, the neurodegeneration associated with Alzheimer's disease (AD) appears to involve oxidative damage characterized by protein oxidation (Hensley *et al.*, 1995), increased expression of antioxidant enzymes (Pappolla *et al.*, 1998), and elevated lipid peroxidation (Sayre *et al.*, 1997). Lipid peroxidation produces several α,β-unsaturated aldehydes (e.g., acrolein, malondialdehyde, 4-hydroxy-2-nonenal) among which, acrolein has the highest nucleophilic reactivity (Picklo *et al.*, 2002; Uchida *et al.*, 1998). Acrolein-protein adducts have been detected in brains of AD patients (Calingasan *et al.*, 1999) and are considered to be a biomarker of the accompanying oxidative stress (Uchida *et al.*, 1998). In addition, it has been hypothesized that acrolein adduct formation with lysine residues on tau proteins might play a role in the development of neurofibrillary tangles that are a pathological hallmark of AD (Calingasan *et al.*, 1999; Picklo *et al.*, 2002). It is interesting to note that acrolein is a ubiquitous environmental pollutant (Ghilarducci and Tjeerdema, 1995). Thus, the pathophysiology of AD could involve protein adduct formation mediated by both endogenous and exogenous neurotoxic components.

The goals for future research should be to identify and quantitate neurotoxicologically important protein adducts and to establish their causal role in the production of nerve cell injury. This latter requirement is perhaps the most challenging, although recent advances in proteomic technology will facilitate such investigations. Current neurotoxicant classification schemes are based on presumed neuronal sites of action (e.g., axon, neuron, nerve terminal) as suggested by previous morphological characterizations (e.g., Spencer and Schaumburg, 1980a). However, morphological changes are often terminal endpoint events and, as such, are insensitive indices of neurotoxicity and of limited value (see LoPachin *et al.*, 2000; LoPachin, 2004; LoPachin and DeCaprio, 2004). As we have discussed, the chemical structure of a neurotoxicant, the resulting toxicokinetics and strength of the corresponding electrophilic center are likely to determine target nucleophilic residues on specific proteins. This in turn could dictate the site of neuronal action and possible neuropathogenic mechanism. Therefore, development of future nosological schemes should incorporate the chemical nature of the toxicant and corresponding potential for protein adduct formation.

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