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PROTEIN TARGETS OF ACRYLAMIDE ADDUCT FORMATION IN CULTURED RAT DOPAMINERGIC CELLS

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Abstract

Acrylamide (ACR) is an electrophilic unsaturated carbonyl derivative that produces neurotoxicity by forming irreversible Michael-type adducts with nucleophilic sulfhydryl thiolate groups on cysteine residues of neuronal proteins. Identifying specific proteins targeted by ACR can lead to a better mechanistic understanding of the corresponding neurotoxicity. Therefore, in the present study, the ACR-adducted proteome in exposed primary immortalized mesencephalic dopaminergic cells (N27) was determined using tandem mass spectrometry (LTQ-Orbitrap). N27 cells were characterized based on the presumed involvement of CNS dopaminergic damage in ACR neurotoxicity. Shotgun proteomics identified a total of 15,243 peptides in N27 cells of which 103 unique peptides exhibited ACR-adducted Cys groups. These peptides were derived from 100 individual proteins and therefore ~0.7% of the N27 cell proteome was adducted. Proteins that contained ACR adducts on multiple peptides included annexin A1 and pleckstrin homology domain-containing family M member 1. Sub-network enrichment analyses indicated that ACR-adducted proteins were involved in processes associated with neuron toxicity, diabetes, inflammation, nerve degeneration and atherosclerosis. These results provide detailed information regarding the ACR-adducted proteome in a dopaminergic cell line. The catalog of affected proteins indicates the molecular sites of ACR action and the respective roles of these proteins in cellular processes can offer insight into the corresponding neurotoxic mechanism.

Keywords

toxic neuropathy; neurotoxicity; nerve terminal; proteomic analysis; protein adduct formation

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Introduction

Monomeric acrylamide (ACR) is an unsaturated carbonyl derivative and is therefore a member of a large class of toxic, structurally-related chemicals known as the type-2 alkenes. ACR is used to manufacture polyacrylamide materials such as glue and plastics and in scientific laboratories for the production of polyacrylamide gels for the electrophoretic separation of macromolecules. ACR is a component of cigarette smoke and is a contaminant in certain potato- or grain-based foods that are prepared at high temperatures. In addition, ACR can be ubiquitous in some occupational environments (LoPachin and Gavin, 2012; Moorman et al., 2012). Long-term ACR exposure causes neurotoxicity characterized by cognitive deficits, gait abnormalities and skeletal muscle weakness in humans and experimental animal models. Consequently, there are significant health concerns regarding ACR exposure (LoPachin et al., 2003). Recent chemical and proteomic studies indicate that ACR and the type-2 alkenes cause toxicity via a common molecular mechanism involving protein inactivation through the formation of irreversible covalent adducts (reviewed in LoPachin and Gavin, 2012; LoPachin et al., 2012).

Whereas protein inactivation is mediated by the chemical interaction between ACR and the cogent nucleophilic amino acid target, the general mechanism of neurotoxicity remains ambiguous since the specific proteins affected and their corresponding pathways or cellular processes have not been adequately identified. For example, we have shown that ACR can form adducts with, and thereby inactivate, presynaptic cysteine (Cys)-directed proteins (e.g., *N*-ethylmaleimide sensitive factor (NSF), synaptosomal-associated protein 25 (SNAP-25), and the dopamine transporter. This subsequently leads to disruption of neurotransmitter uptake, storage, and release in central nervous system (CNS) glutaminergic and dopaminergic nerve terminals (Barber and LoPachin, 2004; LoPachin et al., 2004, 2006). That the nerve terminal proteome was a significant ACR target has been subsequently confirmed by cleavable isotope-coded affinity tagging (ICAT) and tandem mass spectrometric analysis of striatal synaptosomes from ACR-intoxicated rats (Barber et al., 2007). However, based on the generic electrophilic reactivity of ACR, a more global effect on nerve terminal processes might be expected. Indeed, ICAT analysis identified other presynaptic proteins (e.g., complex I NADH-ubiquinone oxidoreductase) and their respective processes (e.g., mitochondrial energy production) as putative ACR targets (Barber et al., 2007). Thus, identifying additional proteins that are susceptible to adduction can increase our mechanistic understanding of ACR neurotoxicity.

In the present study, we used liquid chromatography–mass spectrometry (LC-MS/MS) proteomics to identify ACR adducts on Cys-containing peptides isolated from exposed rat primary immortalized mesencephalic dopaminergic cells (N27). This study builds upon previous ICAT investigations that identified ACR adducted proteins in isolated rat striatal synaptosomes (Barber et al. 2007). In this study, sub-network enrichment analyses were also conducted to assemble interaction networks that can connect specific proteins with neuronal processes potentially affected by ACR adduction.

2. Materials and Methods

2.1 In vitro maintenance of N27 cells and exposure to ACR

Rat primary immortalized mesencephalic dopaminergic cells (N27) were maintained in RPMI-1640 (1X) with L-glutamine media (Gibco, Carlsbad CA USA) in a humidified atmosphere of 5% CO₂ at 37°C which was supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (100x; Invitrogen, Carlsbad CA USA). N27 cells were grown to 50% confluency in 75 cm² Corning CellBIND culture flasks (Corning Incorporated, Corning, NY, USA). Based on data from preliminary experiments, cells were

exposed to either light (C12) or heavy (C13) ACR (100 μ mol) in media for 6 days. The light and heavy ACR isotopes were used to determine, by mass, whether the same proteins were adducted across independent experiments because samples were mixed prior to fractionation and mass spectrometry. The ACR exposure scenario (100 μ mol x 6 days) used in the present study did not cause significant cell loss or overt signs of toxicity and is presumed to model low-level environmental exposures. Regardless, previous animal studies (e.g., Barber and LoPachin, 2004; Barber et al., 2007; LoPachin et al., 2004) indicate that protein adducts appear in advance of neurotoxicity.

Following *in vitro* ACR treatment, cells were harvested using 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (2 ml; Gibco) for 3 min and trypsin activity was quenched using 1 mg/ml soybean trypsin inhibitor (2 ml; Roche Diagnostics, Indianapolis, In, USA). Cells were pelleted by centrifugation (100 g for 6 min at 4°C) and then gently washed (3x) in ice-cold 1X phosphate-buffered saline (PBS). Cells were then resuspended in lysis buffer (20 mM Tris-HCl pH 7.7, 1 mM EDTA, 150 mM NaCl, 0.4% Nonidet P-40, and protease inhibitor) and lysed on ice for 15 minutes in the dark. Lysed cells were centrifuged at 13,000 g for 5 minutes at 4°C and proteins were acetone-precipitated (1 part lysis buffer/6 parts acetone). Proteins were reconstituted in Tris buffer (100 mM; pH 8.8) and treated with 10 μ l of 5 mM DTT for 15 minutes (55°C). This was followed by incubation with 2 mg iodoacetamide for 1 hour at room temperature in the dark. Approximately 400 μ g of protein was trypsin-digested overnight at 37 °C before online desalting and fractionation.

2.2 LC MS/MS and protein / peptide identification

A nano flow liquid chromatograph (U3000, Dionex, Sunnyvale, CA) coupled to an electrospray ion trap mass spectrometer (LTQ-Orbitrap, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide identification experiments. The sample was first loaded onto a pre-column (5mm $\times$ 300 μ m ID packed with C18 reversed-phase resin, 5 μ m, 100Å) and washed for 8 minutes with aqueous 2% acetonitrile with 0.04% trifluoroacetic acid. The trapped peptides were then eluted onto the analytical column, (C18, 75 μ m ID $\times$ 15 cm, Pepmap 100, Dionex, Sunnyvale, CA). The 120-minute gradient was programmed as follows: 95% solvent A (2% acetonitrile + 0.1% formic acid) for 8 minutes, solvent B (90% acetonitrile + 0.1% formic acid) from 5% to 50% in 90 minutes, increasing from 50% to 90% B in 7 minutes, then held at 90% for 5 minutes. Re-equilibration was achieved by decreasing solvent B from 90% to 5% in 1 minute and holding at 5% B for 10 minutes. The flow rate for the analytical column was 300 nl/min. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. The MS scans were acquired in the Orbitrap to obtain accurate peptide mass measurements, and the MS/MS scans were acquired in the linear ion trap using 60 second exclusion for previously sampled peptide peaks. There were 10 fractions that were investigated in total.

Tandem mass spectra were extracted by Xcalibur version 2.0. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot) and Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12). Mascot was set up to search the Sprot_20100810 database (selected for *Rattus*, unknown version, 7552 entries) assuming the digestion enzyme trypsin. Sequest was set up to search the SwissProt_RAT_082510 database (unknown version, 7554 entries) also assuming trypsin digestion. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.2 Da. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 2.5 Da. Oxidation of methionine, iodoacetamide derivative of cysteine, histidine, lysine, acrylamide adduct of cysteine, and acrylamide (i.e. propionamide) on cysteine, histidine and lysine were specified in Sequest as variable modifications. Oxidation of methionine, iodoacetamide derivative of cysteine, acrylamide adduct of cysteine and acrylamide on cysteine were specified in Mascot

as variable modifications. Figure 1 depicts the chemical structure and formation of the carboxamidoethylcysteine group.

Scaffold (version Scaffold_2.1.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 50.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.3 Protein modeling and sub-network enrichment analysis

Three-dimensional (3D) structure predictions were carried out for rat annexin A1 with SWISS-MODEL (Schwede et al., 2003; Arnold et al., 2006) and 3D-JIGSAW (Bates et al., 2001) in order to learn more about Cys adduct formation in this protein. RasMol version 2.7.5.2 was used to visualize the protein space filling models.

To determine the involvement of ACR-adducted proteins in defined cellular networks and whether these proteins were associated with specific neuronal functions, proteins in the ACR proteome were assessed using pathway enrichment and sub-network enrichment analysis (SNEA). All analyses were performed in Pathway Studio 7.1 (Ariadne, Rockville, MD, USA) using ResNet 7.0 (Mammals). Gene Entrez ID was used to map proteins adducted by ACR into interaction networks. SNEA was performed for expression targets and binding partners. This approach has been previously described in vertebrate taxa for genes and proteins (Kotelnikova et al., 2012; Martyniuk et al., 2012). The criteria for a well-supported network were that (1) 5 or more proteins adducted by ACR were localized to a given network and (2) that there was a corresponding enrichment of $P < 0.05$. Disease pathways were built using all significant expression and binding partner networks. Pathogenic processes were mapped onto the protein network to achieve a more refined interpretation of ACR neurotoxicity through the potential relevance of specific protein adducts. Connectivity scores > 15 (strongly supported network) were used to generate the pathogenic network for ACR adducted proteins and the shortest distance between expression and binding relationships among entities was used.

3. Results

3.1. ACR adduct formation on Cys-containing peptides

In the following description of proteins containing ACR adducts, the lower case c in the peptide sequence denotes the adducted cysteine. Using the LTQ Orbitrap, shotgun proteomics detected a total of 15,243 peptides in ACR-exposed N27 cells. There were 103 unique peptides from 100 different proteins that exhibited ACR-adducted Cys residues (Table 1) and, therefore ~0.7% of the proteome was adducted. Based on peptide sequence analyses, 73 proteins were identified with a probability greater than 80% and 40 proteins were identified with better than 90% probability. All additional information on data collected using mass spectrometry and adducted peptides is provided in Appendix 1.

Proteins containing more than one adducted peptide included thrombospondin-4 (THBS4) and pleckstrin homology domain-containing family M member 1 (Appendix 1). Two peptides with overlapping amino acids sequences were detected for annexin A1 (cEDMSVNQDLADTDAR and GDRcEDMSVNQDLADTDAR; Figure 2A and 2B). Thus, annexin contained a thiol group (Cys189) located at C1 of peptide sequence cEDMSVNQDLADTDAR (and C4 for GDRcEDMSVNQDLADTDAR; Table 2) that was

highly sensitive to ACR adduct formation. Based upon the predicted protein structure from SWISS MODEL and 3D-JIGSAW, Cys189 is located in an external loop and, when the residue range of 41 to 344 was considered (Figure 3; amino acid residues modeled 41–344; QMEAN Z-Score = 0.92 and QMEAN score 4 = 0.84), this residue is relatively accessible to interactions with other proteins and compounds. There are 5 other Cys in annexin A1 that appear to be less available to ACR adduct formation relative to Cys189 (e.g. Cys263, Cys270, Cys324, and Cys343 are located in predicted alpha helices, and are predicted to be less accessible due to their location within the interior of the annexin A1 tertiary structure).

Proteins exhibiting ACR adducted Cys groups (> 90% protein identification probability) were involved in broad cytophysiological processes; i.e., receptor and ion channel activity (melanocortin receptor 3, voltage-dependent anion-selective channel protein 3, and inositol 1,4,5-trisphosphate receptor type 2), cell structure (tubulin beta-7 chain, collagen alpha-1(III) chain, intercellular adhesion molecule 1 (ICAM1)), cellular metabolism (aspartyl-tRNA synthetase, ATP-binding cassette sub-family A member 7, triosephosphate isomerase (TPI)) and neuronal function (glial cell line-derived neurotrophic factor, Huntingtin-associated protein 1) (Table 1).

3.2 Pathways and sub-networks represented by ACR adducted proteins

To describe some of the functional relationships among proteins that were adducted by ACR, we constructed protein interaction networks. SNEA revealed that expression targets of the hormone leptin, angiotensinogen, fibroblast growth factor 2 (basic) (FGF2), and proteins that were involved in Ras and Mapk signaling pathways contained ACR adducted proteins (Table 3). There were 19 proteins that contained ACR adducts that also are binding partners for peroxiredoxin (PRDX), a protein with a significant role in oxidative stress responses.

ACR adducts were found on proteins associated with hypertrophy, depression, and neoplasms (Figure 4). Many proteins were associated to each other in the network by expression and/or binding relationships. For example, there was a “hub” of connections for protein binding (purple circle and lines) that include: low-density lipoprotein related protein 2 (LRP2), acid phosphatase 1 (ACP1), sh3 domain, ICAM1, and CAMK1. Moreover, processes related to neurodegeneration were among the disease entities (i.e., nerve degeneration and neurotoxicity) and therefore a second pathway was constructed (Figure 5) to focus on these specific entities. Proteins involved in neurotoxicity included annexin A1, ionotropic kainate glutamate receptor, LRP2, ICAM1, and c-fos induced growth factor (vascular endothelial growth factor D). Protein networks reveal additional information about cell processes that might be associated with ACR neurotoxicity through Cys adduction.

4. Discussion

In this study, proteomic analysis of ACR-exposed dopaminergic cells identified 100 unique proteins that exhibited at least one Cys-adduct. ACR adduct formation was Cys-specific because neither Lys nor His adducts on proteins were detected. Furthermore, although many proteins possess Cys residues, only a small fraction of the N27 proteome was identified as having an ACR adduct. Therefore, the ACR-protein interactions were selective with respect to the amino acid adduct and protein target. This selectivity is consistent with the developing concept that ACR and other type-2 alkenes produce cytotoxicity via a common molecular mechanism involving the rapid formation of irreversible covalent adducts at specific cysteine residues; e.g., ACR specifically targets Cys152 of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) compared to other Cys groups (Cys156 and Cys247) in the molecule (Martyniuk et al., 2011). Thus, the characteristic α , β -unsaturated carbonyl structure of the type-2 alkenes is a soft electrophile and, as such, ACR will form Michael-type adducts with soft nucleophilic sites on proteins. Although cysteine residues are the

demonstrated type-2 alkene targets, at intracellular pH (7.4), corresponding sulfhydryl groups ($pK_a = 8.4$) exist primarily in the non-nucleophilic thiol (0) state. Consequently, sulfhydryl thiol groups are kinetically unfavored sites for adduct formation. Similarly, the nitrogen groups of histidine (imidazole ring) and lysine (ϵ -amino group) residues are also kinetically unfavorable adduct sites because these are harder, relatively weak nucleophiles. However, ionization of cysteine sulfhydryl groups yields the anionic thiolate (-1), which is a soft, highly nucleophilic state that reacts rapidly and preferentially with soft electrophiles such as ACR. Sulfhydryl thiolate groups can be found in pK_a -lowering microenvironments such as cysteine-centered catalytic triads that play a role in regulating protein function as in the example of Cys152 of GAPDH (reviewed in LoPachin and Barber, 2006; LoPachin and Gavin, 2012; LoPachin et al., 2012). Previous kinetic and proteomic studies have shown that type-2 alkene adduction at thiolate-based catalytic triads of numerous enzymes impairs corresponding function (Dalle-Donne et al., 2007; Eliuk et al., 2007; Martyniuk et al., 2011; Seiner et al., 2007). Our data therefore provide additional evidence that, as a soft electrophile, ACR and other type-2 alkenes target sulfhydryl thiolate groups on specific cysteine residues (LoPachin et al., 2007a,b). Although the activities of adducted proteins were not determined, the selectivity of adduct formation identified in the present study suggests that function is impaired through ACR-thiolate interactions. This level of protein dysfunction could translate to impairment of related cell pathways (see ahead).

The diverse spectrum of ACR-adducts reflects targeted proteins that are important components of numerous cell processes and pathways: metabolism (ATP synthase subunit b, TPI), cell structure (annexin A1, collagen α -1(III) chain, tubulin β -7 chain), translation (ribosomal proteins, elongation factor, eukaryotic translation initiation factor 5B) and cell signalling (GTPases, rab GDP dissociation inhibitor beta, Ras GTPase-activating proteins). Similarly, in striatal synaptosomes from ACR intoxicated rats, Barber et al. (2007) identified adducts on Cys residues of proteins that were also involved in cell metabolism (hexokinase, isocitrate dehydrogenase, sodium potassium ATPase subunits) and structure (myosin, actins). Together, these experiments provide corroborating evidence that proteins involved in cell structure and general metabolism are target-rich environments for ACR. However, it cannot be assumed that the adducted proteins detected in this study are dysfunctional, nor can it be assumed that such protein adducts will have toxicological consequences at the pathway or process level. Rather, the present data provide detailed information regarding potentially vulnerable cell components that might be involved in mediating ACR neurotoxicity and therefore warrant investigation. That the present proteomics approach has utility is suggested by the studies of Barber et al. (2007), who also identified adducted presynaptic proteins involved in neurotransmission (*N*-ethylmaleimide sensitive factor, vesicular (v)-ATPase, synaptotagmin, SNAP-25). It is important to note that the present study used shotgun proteomics, whereas Barber et al. (2007) used ICAT analysis. The latter technique is a more targeted approach that enriches for peptides containing Cys residues (> 500 ACR adducted peptides detected by Barber et al., 2007). However, issues related to the incomplete reactivity of ICAT tags with the proteome and the numerous analytical steps needed to identify adducts are significant disadvantages of this proteomic method. Although the shotgun approach has less analytical steps, fewer ACR-adducted peptides are likely to be detected (~100 this study) due to the complexity of proteome analyzed. Thus, the combined use of shotgun proteomics and ICAT analysis has provided complimentary, detailed information regarding the cellular proteome adducted by ACR and possibly other soft electrophiles.

Pathway and sub-network analyses indicated a connection between proteins adducted by ACR and those associated with disease states such as diabetes, inflammation, and atherosclerosis among others. For example, proteins that included thrombospondin 4 (Frolova et al., 2010), Peroxiredoxin-1 (Kisucka et al., 2008), and fibroblast growth factor 2

(Liao et al., 2009) all play a role in the etiology of atherosclerosis. Angiotensinogen is a precursor molecule for angiotensins that are involved in learning, cognition and memory (reviewed in Wright and Harding, 2012). There is evidence that angiotensins such as AngII and AngIV are directly involved in neuroprotection, and perturbations to the renin-angiotensin system have been directly linked to neurodegenerative diseases such as Alzheimer's (Kehoe et al., 2009) and PD (Mertens et al., 2010). However, no studies have associated ACR exposure to changes in the angiotensionogen system.

Our data indicate that proteins related to neurodegeneration and neurotoxicity were significant targets of ACR adduct formation. In particular, Cys189 of annexin located in peptide GDRcEDMSVNQDLADTDAR was especially susceptible to ACR adduction due to its location within the tertiary structure of this protein. Annexins are present in eukaryotic cells and are a multigene family of structurally related hydrophilic proteins. Annexins have diverse and multiple roles within the cell which include inhibition of phospholipase activity, exocytosis and endocytosis, signal transduction, organization of the extracellular matrix, anti-inflammatory properties, resistance to reactive oxygen species and roles in DNA replication (reviewed in Gerke and Moss, 2002). Based on the prediction that Cys189 is located more externally in the protein, we hypothesize that the formation of ACR adducts with annexin A1 can prevent important interactions between cell membranes and other proteins. In support of this, annexins have been shown to interact with cytoskeletal proteins, form complexes with Ca²⁺-binding proteins, and to be involved in Ca²⁺-dependent phospholipid binding and vesicle aggregation (Gerke and Moss, 2002).

It is also plausible that ACR causes nerve damage by inhibiting the neuroprotective effects of annexin A1 and other proteins. This suggestion is based on reports that infusion of annexin A1 into rats following damage to peripheral nerves has been shown to be neuroprotective (Facio and Burnett, 2012). This might also be the case for other proteins that are found within the nerve degeneration network of this study. Thus for example, peroxisome proliferator-activated receptor gamma also has neuroprotective roles in the CNS and has been shown to reduce oxidative stress and inflammation (García-Bueno et al., 2005; Yu et al., 2008). The theory that ACR causes CNS nerve damage by inhibiting the function of neuroprotective proteins warrants further investigation.

The common molecular facet of the pathway connectivity demonstrated in this study might be electrophilicity, since there is reasonable evidence that pathogenic conditions such as atherosclerosis and nerve degeneration involve oxidative stress and the generation of electrophilic mediators such as acrolein and 4-hydroxy-2-nonenal (HNE; LoPachin and Gavin, 2008, 2012). Like ACR, these mediators are soft electrophiles of the type-2 alkene chemical class and would be expected to form adducts with a common protein pool (LoPachin et al., 2007a,b). It is not surprising that ACR might be connected to these and other diseases that potentially involve type-2 alkenes. For example, it has been suggested that early nerve terminal damage in Alzheimer's disease is caused by regional oxidative mitochondrial damage with subsequent evolution of acrolein and other electrophilic unsaturated aldehydes. Subsequent adduction of proteins involved in neurotransmission leads to loss of presynaptic activity and neurodegeneration (LoPachin and Gavin, 2008). Thus, our pathway and sub-network analyses might reflect the possibility that certain disease states are linked by common electrophilic mediators and a similar pool of adducted proteins. The use of network analysis in integrating information on ACR adducted proteins in the context of human diseases generates new hypothesis regarding the mechanisms of ACR neurotoxicity. Future studies should examine the degree to which adducted proteins within a particular cellular pathway result in adverse functional effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Acrylamide (ACR) induces neurotoxicity by forming irreversible adducts with thiolates

The ACR-adducted proteome was characterized in mesencephalic dopaminergic cells (N27)

Less than 1.0% (103 peptides) of the N27 cell proteome contained ACR adducts

ACR-adducted proteins are associated with neurotoxicity and neurodegeneration

The catalog of adducted proteins offers new insight into neurotoxic mechanisms of ACR

Figure 1. Chemical structure of the formation of the carboxamidoethylcysteine group on the peptides. (A) thiol group in peptide sequence in single amino acid (B) formation of carboxamidoethylcysteine after acrylamide adduction (C) molecular showing the R groups to indicate the continuing peptide sequence.

Figure 2.

Spectrum obtained from two peptides derived from ANXA1 that contain the same cysteine group. (A) The peptide was detected with mass-to-charge ratio 928.8691. The tandem mass spectrum matched the following sequence, CEDMSVNQDLADTDAR, indicating that the Cys was modified (+74); the detection of b2 is consistent with this localization. The assignment was made with Sequest with XCorr 3.30 and Δ CN 0.45 (B) The peptide was detected with mass-to-charge ratio 728.6382. The tandem mass spectrum matched the following sequence, GDRCEDMSVNQDLADTDAR, indicating that the Cys was modified (+74); the detection of b5 is consistent with this localization. The assignment was made with Sequest with XCorr 4.62 and Δ CN 0.43.

Figure 3.

Predicted 3D structure for rat annexin A1 using two different protein prediction algorithms (SWISS-MODEL and 3D-JIGSAW). Cys189 is predicted to be more accessible using SWISS-MODEL when compared to prediction of 3D-JIGSAW however both models predicted that Cys189 would be located more externally in the protein when compared to other Cys groups.

Figure 4.

Disease states and processes (i.e. nerve degeneration and inflammation) that were associated with proteins that contain ACR adducts. Abbreviations are as follows; ABCA7, ATP-binding cassette, sub-family A (ABC1), member 7; ACP1, acid phosphatase 1, soluble; AGT, angiotensinogen (serpin peptidase inhibitor, clade A, member 8); ANXA1, annexin A1; CAMK1, calcium/calmodulin-dependent protein kinase I; COL3A1, collagen, type III, alpha 1; FDX1, ferredoxin 1; FGF2, fibroblast growth factor 2 (basic); FIGF, c-fos induced growth factor (vascular endothelial growth factor D); GRIK3, glutamate receptor, ionotropic, kainate 3; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; ICAM1, intercellular adhesion molecule 1; LDL, low density lipoprotein; LEP, leptin; LIMK1, LIM domain kinase 1; LRP2, low density lipoprotein-related protein 2; MAPK1, mitogen-activated protein kinase 1; MAPK3, mitogen-activated protein kinase 3; MAPK14, mitogen-activated protein kinase 14; MC3R, melanocortin 3 receptor; MMP14, matrix metalloproteinase 14 (membrane-inserted); PIK3CB, phosphoinositide-3-kinase, catalytic, beta polypeptide; PPARG, peroxisome proliferator-activated receptor gamma; PRDX1, peroxiredoxin 1; PPP1R12A, protein phosphatase 1, regulatory (inhibitor) subunit 12A; PTPN11, protein tyrosine phosphatase, non-receptor type 11; Ras, RAS oncogene homolog; SPZ1, spermatogenic leucine zipper 1; SYNGAP1, synaptic Ras GTPase activating protein 1 homolog (rat); THBS4, thrombospondin 4;

Figure 5. Proteins that had ACR adducts that were involved directly with the process of nerve degeneration and neuron toxicity. Abbreviations are listed in Figure 3.

Table 1

ACR adducted peptides identified using shotgun proteomics in rat N27 dopaminergic cells. There were 103 unique peptides identified that contained an ACR adduct. Adducted cysteine (Cys) groups are indicated with a small “c” in the peptide sequence. Note that a group of proteins have multiple Cys-containing peptides that are susceptible to ACR adduct formation and that some peptides containing multiple Cys groups are adducted. Complete information on the peptides and localization of adducted Cys groups are reported in Appendix 1.

Major Cell function /Process	Protein name	Protein accession numbers	Protein molecular weight (Da)	Peptide sequence
Cellular Metabolism/Mitochondria	Adrenodoxin, mitochondrial	ADX_RAT	20116.8	RLLVcGTR
	Aspartyl-tRNA synthetase, cytoplasmic	SYDC_RAT	57108.7	QMciCADFEK
	ATP synthase subunit b, mitochondrial	AT5F1_RAT	28851.5	cIGDLK
	ATP-binding cassette sub-family A member 7	ABCA7_RAT	237705	VAMVASGSLcCCGSPLFLRR
	Mitochondrial import inner membrane translocase subunit Tim13	TIM13_MOUSE	10440.1	cIAMCMDR
Cell Signalling	Triosephosphate isomerase	TPIS_RAT	26830.7	cLGELICTLNAAK
	Calcium/calmodulin-dependent protein kinase type I	KCC1A_RAT	41621.6	DCcVEPGSELPPAPPSSR
	COP9 signalosome complex subunit 1	CSN1_RAT	53411.8	HVINMcLNVIK
	GTPase Hras	RASH_RAT	21296.2	LNPPDESGLGcMScK
	GTPase IMAP family member 4	GIMA4_RAT	35805.6	YcLFNNK
	GTPase IMAP family member 8	GIMA8_RAT	77054.9	GRVcAFNNK
	Islet amyloid polypeptide	IAPP_RAT	9996.7	KcNTATCATQR
	Latent-transforming growth factor beta-binding protein 2	LTBP2_RAT	189841	KVTNDVcSQPLR
	Low molecular weight phosphotyrosine protein phosphatase	PPAC_RAT	18133.8	cCKAFLEK
	Mitogen-activated protein kinase 1	MK01_RAT	41259.9	KISPFHQYTYcQRTLRL
	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform	PK3CB_RAT	122595	AKGKEAMHTcLK
	Protein phosphatase 1 regulatory subunit 12A	MYPT1_RAT	115268	ScSFGR
	Protein strawberry notch homolog 1	SBNO1_RAT	140727	FFKYLCIASKVK
	Rab GDP dissociation inhibitor beta	GDIB_MOUSE	50521.1	TDDYLDQPcETINR
	Rab proteins geranylgeranyltransferase component A 1	RAE1_RAT	72462.2	KFLQcLGR
	Ras GTPase-activating protein SynGAP	SYGP1_RAT	144708	GKGGcPAVR
	Serine/threonine-protein kinase TAO3	TAOK3_RAT	105458	GcYLYK
	TANK-binding kinase 1-binding protein 1	TBKB1_RAT	67137.4	SPASPScPSPVPQRR
	Type II inositol-3,4-bisphosphate 4-phosphatase	INP4B_RAT	105230	KLNGIRFTccK
	Vascular endothelial growth factor D	VEGFD_RAT	37093.6	cLPTGPRHPYSIIR
Cell Structure	Alpha-1-acid glycoprotein	A1AG_RAT	23557.8	cSEQKQKQLELEK
	Annexin A1	ANXA1_RAT	38813	GDRcEDMSVNQDLADTDAR

Major Cell function /Process	Protein name	Protein accession numbers	Protein molecular weight (Da)	Peptide sequence
	Annexin A1	ANXA1_RAT	38813	cEDMSVNQDLADTDAR
	Collagen alpha-1(III) chain	CO3A1_RAT	138919	NcRDLKFCHPELK
	Collagen alpha-1(XXVII) chain	CORA1_RAT	187795	VcRDLMDeEQK
	Contactin-associated protein like 5-3	CTP5C_RAT	145704	ANSLGFIGcLSSVQYNHIAPLK
	Cullin-associated NEDD8-dissociated protein 2	CAND2_RAT	139659	TLIQcLGSVGRQAGHR
	Cysteine desulfurase, mitochondrial	NFS1_RAT	49995	cVLDScR
	Echinoderm microtubule-associated protein-like 2	EMAL2_RAT	70690.8	ITQEVLGAHDGGVFALcALR
	Integrin alpha-7	ITA7_RAT	124178	NVTLDcAQGTAK
	Intercellular adhesion molecule 1	ICAM1_RAT	60124.2	cRAFSSR
	Tubulin beta-7 chain	TBB7_CHICK	49652.6	TAVcDIPPR
Ion Channel and Receptors	5-hydroxytryptamine receptor 2A	5HT2A_RAT	52833.4	SLQKEATLcVSDLSTRAK
	Glutamate receptor, ionotropic kainate 3	GRIK3_RAT	104057	NcNLTIQIGGLIDSK
	Inositol 1,4,5-trisphosphate receptor type 2	ITPR2_RAT	307044	cGAFMSKLINHTKK
	Low-density lipoprotein receptor-related protein 2	LRP2_RAT	519241	TCSAGEFScANGRCVR
	Melanocortin receptor 3	MC3R_RAT	35848.8	IAALPPADGVAPQQHScMK
	Nuclear receptor subfamily 2 group C member 2	NR2C2_RAT	65327.8	cLEMGMK
	Potassium voltage-gated channel subfamily H member 6	KCNH6_RAT	105691	QDLDeWHR
	Tyrosine-protein phosphatase non-receptor type 11	PTN11_RAT	68440	cNNSKPK
	Voltage-dependent anion-selective channel protein 3	VDAC3_RAT	30780.8	ScSGVEFSTSGHAYTDTGK
	Voltage-dependent calcium channel subunit alpha-2/delta-3	CA2D3_RAT	122190	cERLKAQK
Neuronal Function	Fragile X mental retardation protein 1 homolog	FMR1_RAT	66762.6	QMCAK
	Glial cell line-derived neurotrophic factor	GNDF_RAT	23601.7	YCSGScEAAETMYDK
	Huntingtin-associated protein 1	HAP1_RAT	70195	KFITDPAYFMERcDTR
	Mesencephalic astrocyte-derived neurotrophic factor	MANF_RAT	20370.9	KDSQIcELKYDK
	N-acetylneuraminate cytidyltransferase	NEUA_RAT	48111.6	RVGLSAVPADAcSR
	Neurogenic locus notch homolog protein 2	NOTC2_RAT	265341	VASFScLcPEGK
Protein Catabolism	E3 ubiquitin-protein ligase RNF123	RN123_RAT	149063	VcATCFDLSVSLLR
	Ubiquilin-like protein	UBQLN_RAT	67775.8	cQMEQLVLVSMGR
Proteins with Domains	FACT complex subunit SSRP1	SSRP1_RAT	80899.7	ELQcLTPR
	F-box/LRR-repeat protein 20	FXL20_RAT	30443.2	LRHLDLAScTSITNMSLK
	FERM domain-containing protein 8	FRMD8_RAT	51763.4	QGSVVcSR
	Homeobox protein unc-4 homolog	UNC4_RAT	53861.9	ATPANTAATAGLDFTPLPcAPR

Major Cell function /Process	Protein name	Protein accession numbers	Protein molecular weight (Da)	Peptide sequence
	LIM domain kinase 1	LIMK1_RAT	72576.2	cLEHPNVLK
	RIMS-binding protein 2	RIMB2_RAT	115598	LNTAAcTYR
	Spermatogenic leucine zipper protein 1	SPZ1_RAT	45797	cPNDCLTK
	WAP four-disulfide core domain protein 1	WFDC1_RAT	23211.9	CPPPPRTLPPGAcQATR
	Zinc finger protein 574	ZN574_RAT	99334.3	cLLcSR
Response to Stress/Xenobiotics	Metallothionein-1; Short=MT-1	MT1_RAT	5987.3	KSCcSCCPVGCSKCAQGCVCCK
	Peroxiredoxin-1	PRDX1_RAT	22092.2	HGEVcPAGWKPGSDTIKPDVVK
Transcription/Translation	40S ribosomal protein S2	RS2_RAT	31214.2	cSKEVATAIR
	60S ribosomal protein L11	RL11_CHILA	20235.2	NEKIAVHcTVRGAK
	60S ribosomal protein L12	RL12_RAT	17828.1	cTGGEVVGATSALAPK
	DNA polymerase delta catalytic subunit	DPOD1_RAT	123586	LALTLRPcAPILGAK
	Elongation factor 1-gamma	EF1G_RAT	50043.4	AAAPAPEEEMDEcEQALAAEPK
	Eukaryotic translation initiation factor 5B	IF2P_RAT	137673	RLAHcEELR
	Threonyl-tRNA synthetase, mitochondrial	SYTM_RAT	81655	LLGVLAEScGGR
	Transcriptional adapter 2-alpha	TAD2A_RAT	51382.7	TKEEcEK
Various Functions	Autophagy-related protein 7	ATG7_RAT	77419.7	cLLLGAAGTLGCNVARTLMGWGVR
	Carbonyl reductase family member 4	CBR4_RAT	25268.8	VcAVFGGSR
	Chromodomain-helicase-DNA-binding	CHD8_RAT	290678	cKKNK
	protein 8			
	DDB1- and CUL4-associated factor 8	DCAF8_RAT	66137	FLPNSGDSTLAMcAR
	Embryonic polyadenylate-binding protein 2	EPAB2_RAT	29843.6	ELLSPETIGcFFPGAPK
	Epithelial splicing regulatory protein 1	ESRP1_RAT	75003.8	FFKGLNIAKGGAALcLNAQGR
	Intestinal mucin-like protein	MUC2L_RAT	91478.1	cTFFSCMK
	Kinesin-like protein KIF27	KIF27_RAT	158864	SIAQLQGVKPKVcR
	Lanosterol synthase	ERG7_RAT	83284	GIRcLLGK
	Lupus La protein homolog	LA_RAT	47761.7	MGcLLK
	Mast cell carboxypeptidase A	CBPA3_RAT	47927.8	KAIFMDcGIHAR
	Max-interacting protein 1	MXI1_RAT	26004.5	RAHLRLcLER
	Pleckstrin homology domain-containing family M member 1	PKHM1_RAT	117492	KGcPRCAR
	Pleckstrin homology domain-containing family M member 1	PKHM1_RAT	117492	GKSWISEDDFcRPPK
	Protein hairless	HAIR_RAT	127289	LCVAcGRIAGAGKNR
	Protein SEC13 homolog	SEC13_RAT	35528.8	KFASGGcDNLK
	Protein YIPF4	YIPF4_RAT	27266.5	IRcVLMPPMPSLGFNRQVVR
	Ribonucleoside-diphosphate reductase subunit M2	RIR2_RAT	45022.1	EYLFNAIETMPcVKK
	SCO-spondin	SSPO_RAT	550599	cANGDcALK
	Selenoprotein P	SEPP1_RAT	43064.7	IAYcEKR

Major Cell function /Process	Protein name	Protein accession numbers	Protein molecular weight (Da)	Peptide sequence
	Solute carrier family 2, facilitated glucose transporter member 5	GTR5_RAT	55527.3	GALLFNNIFSILPAILMGcSK
	Spindle and kinetochore-associated protein 2	SKA2_RAT	16499.3	ScICAILNK
	Taperin	TPRN_RAT	80254.2	EGGcPRPAISDTDK
	Thrombospondin-4	TSP4_RAT	108196	GDAcDDDMDGDGIK
	Thrombospondin-4	TSP4_RAT	108196	cGPKCPGYTGDQTR
	Torsin-2A	TOR2A_RAT	35853.2	SWVQGNLTAcGR
	Transmembrane protein 199	TM199_RAT	23142.6	NVTcQDAQcGGTSLDLGK
	Uncharacterized protein C18orf54 homolog	CR054_RAT	40829	KcGDKIPELLIK
	Vacuolar protein sorting-associated protein 54	VPS54_RAT	108826	cKNicPPK

Table 2

Peptides derived from Annexin A1 (MW = 38.81 kDa, IPI00231615) that are detected sites of ACR adduction. Small case c denotes the thiol group containing an ACR adduct.

Protein name	Protein identification probability	Peptide sequence	Peptide identification probability	Variable modifications identified by spectrum
Annexin A1	100.0%	GDRcEDMSVNQDLADTDAR	95.0%	C4: Propionamide (+71.04)
Annexin A1	100.0%	GDRcEDMSVNQDLADTDAR	95.0%	C4: Propionamide (+71.04)
Annexin A1	100.0%	cEDMSVNQDLADTDAR	95.0%	C1: Acrylamide_heavy (+74.06)
Annexin A1	100.0%	cEDMSVNQDLADTDAR	95.0%	C1: Acrylamide_heavy (+74.06)
Annexin A1	100.0%	GDRcEDMSVNQDLADTDAR	95.0%	C4: Acrylamide_heavy (+74.06)

Table 3

Protein networks involving ACR adducted proteins. The column containing “entities in network” indicates those identified as having ACR adducts in the particular network. For example, there were 7 ACR adducted proteins detected in this study that regulate the expression of angiotensinogen.

Protein Seed	Overlap	Entities in Network	p-value
Low-density lipoprotein	5	ICAM1,HRAS,PPARG, MMP14,FDX1	0.005
Leptin	6	ICAM1,PPARG,MMP14, COL3A1,FDX1,MC3R	0.006
Angiotensinogen	7	ICAM1,PPARG,MMP14,LRP2, COL3A1,THBS4,FDX1	0.013
MAPK3	5	MAPK1,ICAM1,PPARG, MMP14,COL3A1	0.015
MAPK1	7	MAPK1,ICAM1,PPARG, MMP14,LRP2,COL3A1,ABCA7	0.018
FGF2	6	ICAM1,PPARG,MMP14, FIGF,PTPN11,FDX1	0.030
Ras	5	ICAM1,PPARG, MMP14,PRDX1,SPZ1	0.031
MAPK14	5	ICAM1,PPARG, ANXA1,COL3A1,PRDX1	0.043
Peroxiredoxin-1 ^a	19	MAPK1,ICAM1,PPARG,MMP14,PTPN11,LRP2,PPP1R12A,COL3A1,GRIK3, THBS4,LIMK1,PIK3CB,ACPI,CAMK1,PRDX1,SPZ1,SYNGAP1,FDX1,ABCA7	P<0.001
Protein 14-3-3 ^a	6	PTPN11,PPP1R12A,LIMK1,PIK3CB,CAMK1,PRDX1	P<0.001

^a indicates that entities in network are binding partners. All others are involved in expression networks. Abbreviations are as follows: ABCA7, ATP-binding cassette, sub-family A (ABCI), member 7; ACPI, acid phosphatase 1, soluble; ANXA1, annexin A1; CAMK1, calcium/calmodulin-dependent protein kinase I; COL3A1, collagen, type III, alpha 1; FDX1, ferredoxin 1; FIGF, c-fos induced growth factor (vascular endothelial growth factor D); GRIK3, glutamate receptor, ionotropic, kainate 3; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; ICAM1, intercellular adhesion molecule 1; LIMK1, LIM domain kinase 1; LRP2, low density lipoprotein receptor-related protein 2; MAPK1, mitogen-activated protein kinase 1; MC3R, melanocortin 3 receptor; MMP14, matrix metalloproteinase 14 (membrane-inserted); PIK3CB, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta; PPARG, peroxisome proliferator-activated receptor gamma; PPP1R12A, protein phosphatase 1, regulatory subunit 12A; PRDX1, peroxiredoxin 1; PTPN11, protein tyrosine phosphatase, non-receptor type 11; SPZ1, spermatogenic leucine zipper 1; SYNGAP1, synaptic Ras GTPase activating protein 1; THBS4, thrombospondin 4.