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Acrolein Modifies and Inhibits Cytosolic Aspartate Aminotransferase

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Acrolein is a reactive lipid peroxidation byproduct, which is found in ischemic tissue. We examined the effects of acrolein on cytosolic aspartate aminotransferase (cAAT), which is an enzyme that was previously shown to be inhibited by glycating agents. cAAT is thought to protect against ischemic injury. We observed that acrolein cross-linked cAAT subunits as evidenced by the presence of high molecular weight bands following SDS-PAGE. Acrolein-modified cAAT resisted thermal denaturation when compared with native cAAT. We also observed a decrease in intrinsic fluorescence (290 nm, ex; 380 nm, em). These observations are consistent with an acrolein-induced change in conformation that is more rigid and compact than native cAAT, suggesting that intramolecular cross-links occurred. Acrolein also inhibited activity, and the inhibition of enzyme activity correlated with the acrolein-induced formation of cAAT cross-links.

Keywords: Acrolein; Cytosolic aspartate aminotransferase; Lipid peroxidation; Ischemia

Abbreviations: cAAT, cytosolic aspartate aminotransferase; AMPs, acrolein-modified proteins; RFUs, relative fluorescence units; HMW, high molecular weight; M_r, relative molecular mass; PLP, pyridoxal 5-phosphate

INTRODUCTION

Cytosolic aspartate aminotransferase (cAAT) (EC 2.6.1.1) is a key enzyme of the malate–aspartate shuttle, which is necessary for optimal fuel utilization and energy production in many diverse cell types. Various experimental models demonstrated that cAAT plays a pivotal role in ischemia.[1–6] cAAT catalyzes the reversible reaction: L-glutamate + oxaloacetate (or pyruvate) ↔ α-ketoglutarate + L-aspartate (or L-alanine), which requires pyridoxal 5-phosphate (PLP). The literature supports the hypothesis that cAAT and the mitochondrial isoform promote glycolysis and ATP production in ischemic tissues. The observed increase in glutamate utilization in anoxic rat cardiac myocytes[1] is consistent with cAAT having a protective effect during ischemia. Perfusion of isolated rat hearts with cAAT substrates prevents injury to the myocardium[2] and improves mechanical recovery[3,4] following ischemia. Additionally, inhibitors of cAAT cause left ventricular failure in the isolated guinea pig heart model[6] and reduce the rate of oxygen consumption in rat cerebral cortex slices.[5]

cAAT activity may be in part modulated post-transcriptionally. Nutrition presumably plays a role in regulating cAAT activity.[7] Decreased cAAT activity in the diabetic rat model corresponds to increased levels of glycated cAAT,[8] supporting a post-translational mechanism of control. Reactive sugars inhibit cAAT when isolated from rat liver[9] or pig heart.[10] cAAT is remarkably thermostable[11] making this enzyme an effective model for studying mechanisms of post-translational modification.[8–10,12–14]

This study examined the inhibitory effects of acrolein ($CH_2 = CH–CHO$), which is derived from the peroxidation of lipids[15] and is a product of myeloperoxidase activity.[16] Acrolein impairs cell function[17–19] and may be atherogenic.[20,21] It reacts with DNA polymerase,[22] proteinase inhibitor[23] and

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carbonic anhydrase.[24] Acrolein-modified proteins (AMPs) can be seen immunohistochemically in several disease models[25–27] suggesting that AMPs may be useful diagnostic markers. Acrolein reacts with amino acid residues forming diverse adducts as well as Schiff base cross-links.[28] We present data here indicating that acrolein formed cross-links between cAAT subunits and inhibited activity.

MATERIALS AND METHODS

Materials

Acrolein was obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of acrolein were freshly prepared prior to each experiment. Porcine cardiac cAAT was purchased either from Sigma or Roche Molecular Biochemicals (Indianapolis, IN) with an activity of $243.8 \pm 11.03\,\mu\text{mole} \times \text{min}^{-1} \times \text{mg prot}^{-1}$. No further purification procedures were performed with the exception of dialysis to remove salts. All other chemicals were of reagent grade or better.

Preparation and Examination of Acrolein-modified cAAT

Unless otherwise indicated cAAT (0.2–2 mg/ml) was incubated in acrolein (0–10 mM) in a 100 mM sodium phosphate buffer (pH = 7.4) at various temperatures (23–50°C) for various times. Sodium azide (0.5 mM) was added to longer incubations. Following incubation, samples were dialyzed to remove unreacted acrolein. Samples were dialyzed (1:2000, Spectra/Por®CE DispoDialyzer, MWCO 10000, Spectrum Laboratories, Inc.) against a 100 mM sodium phosphate buffer (pH = 7.4) containing sodium azide (0.5 mM) for 5 h. After preparing acrolein-modified cAAT, the following analyses were performed.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using Tris–glycine (12% polyacrylamide) pre-cast mini-gels (Sigma Chemical Co.) in a mini-Protean II apparatus (BioRad Laboratories, Hercules, CA) with a Tris (25 mM)–glycine (192 mM, pH = 8.3)–SDS (0.1%, w/v) buffer. Samples were heated (4 min at 95°C) in a Tris buffer containing SDS (0.1%) and β-mercaptoethanol (5%, v/v) prior to electrophoresis. Coomassie-stained protein bands were quantified using the SigmaGel software program (SPSS, Inc., Chicago, IL). Digitized photographs of gels were converted to gray-scale bitmap files before equivalent areas were integrated up from a lower threshold set at 50.

Intrinsic Protein Fluorescence

Intrinsic protein fluorescence was determined[29] using an LS50B luminescence spectrometer (Perkin–Elmer Corp., Shelton, CT). Relative fluorescence units (RFUs) were measured following preparation of acrolein-modified cAAT and compared with unmodified cAAT controls. The excitation and emission wavelengths were 290 nm (slit width = 5 nm) and 380 nm (slit width = 5 nm), respectively, setting the integration time to 10 s.

Thermal Denaturation

Susceptibility to thermal denaturation was measured as a temperature-dependent increase in light scattering using nonspecific absorbance at 450 nm.[30] Quartz cuvettes were used in a programmable Peltier-heated cell holder in an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) with a heating rate set at 0.5°C/min (initial and final temperatures were 40°C and 88°C, respectively).

cAAT Activity

Maximal cAAT activity was measured spectrophotometrically using a coupled-enzyme assay.[10] Incubated samples were diluted in a 100 mM sodium phosphate buffer (pH = 7.4) containing 0.1 mM pyridoxal 5-phosphate (PLP) (final assay PLP concentration was 1 μM). Aliquots (0.2 μg cAAT) were assayed at 37°C in an 80 mM sodium phosphate buffer (pH = 7.4) containing 200 mM L-aspartate, 12 mM α-ketoglutarate, 0.2 mM NADH and 1.1 I.U. malate dehydrogenase. Absorbance (340 nm) changes due to NADH oxidation were followed during the reaction. The extinction coefficient for NADH (6.22 absorbance of one μmole NADH per ml at 340 nm in a 1 cm light path) was used to calculate activity.

Statistical Analyses

Data are expressed as means ± SD. Statistical differences were assessed using either Student's *t*-tests for independent means or one-way ANOVAs followed by two-tailed protected *t*-tests. Where indicated linear regression analyses were performed using SigmaPlot 4.0 (SPSS, Inc.), and Pearson coefficients were evaluated for significance.

RESULTS

Acrolein-induced Cross-linking of cAAT

SDS-PAGE of acrolein-modified cAAT (incubation conditions: 0–10 mM acrolein for 2.5 h at 37°C)

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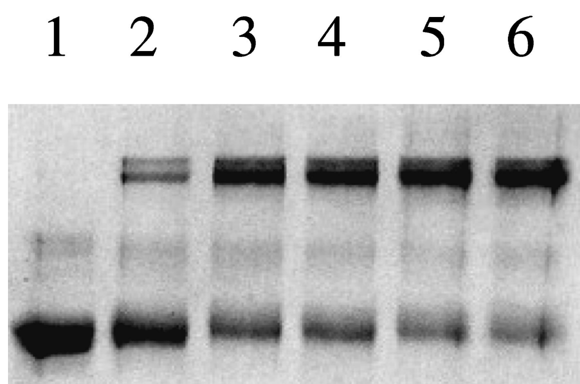

FIGURE 1 Acrolein-induced formation of HMW proteins. SDS-PAGE was performed following cAAT incubation in the presence of acrolein (0–10 mM) for 2.5 h at 37°C. Lane 1: unmodified cAAT; Lanes 2–6: acrolein-modified cAAT prepared by incubation with 2, 4, 6, 8 and 10 mM acrolein, respectively.

revealed a high molecular weight (HMW) doublet (Fig. 1). The relative molecular masses (M_r) of the acrolein-modified cAAT were $M_r = 95000$ and 104000 (unmodified cAAT subunit, $M_r = 41000$). There was an approximate 2:1 stoichiometry in M_r values between the acrolein-modified cAAT and the unmodified cAAT, suggesting that cross-linked products were formed. The remaining mass may be attributed to acrolein molecules attached to the protein, although protein modification may have contributed to changes in electrophoretic migration.

The protein bands were quantified and then plotted as a function of acrolein concentration (Fig. 2). The intensities of the 95000-M_r and 104000-M_r bands, which represent cross-linked cAAT, were combined in the analysis. As acrolein concentration increased, we observed an increase in cross-linking and a loss of native cAAT. The increase in cross-linked protein correlated with the decrease in native

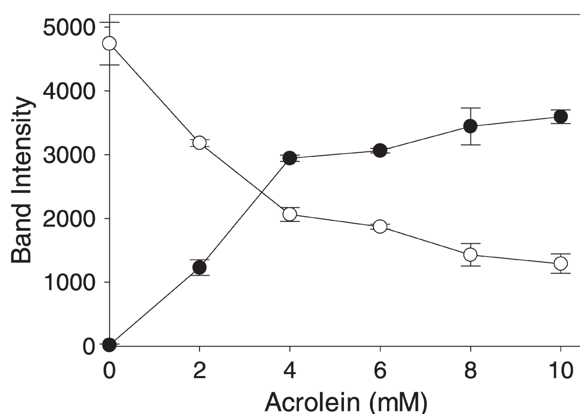

FIGURE 2 Quantity of HMW protein increases with increased acrolein. Protein bands from SDS-PAGE gel presented in Figure 1 were quantitated and values presented as a function of acrolein concentration. HMW protein bands (closed circles); native cAAT protein band (open circles). Data is given as averages ± SD of triplicate readings from a representative experiment.

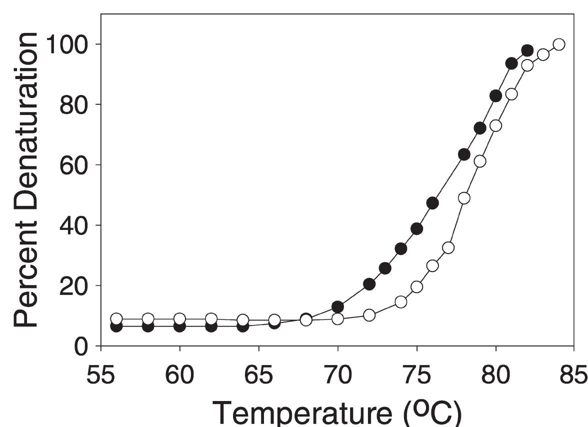

FIGURE 3 Effects of acrolein on thermal denaturation of cAAT. Thermal denaturation was measured as described in Materials and Methods following incubation of cAAT in the presence and absence of acrolein (2 mM) for 17.5 h at 37°C. Native cAAT (closed circles); acrolein-treated cAAT (open circles). Data are from a representative experiment.

cAAT as assessed by linear regression analysis (Pearson coefficient, $r = 0.99$, $P < 0.005$).

Thermal Denaturation

Acrolein decreased the susceptibility of cAAT to thermal denaturation above 70°C, which was determined by light scattering (Fig. 3). Following cAAT incubation with acrolein (2 mM for 17.5 h at 37°C), a higher temperature was required for helix to coil transition. The Tm (temperature at which half of the cAAT molecules were denatured) of unmodified cAAT was 77.3 ± 0.64°C (M ± SD). The Tm for acrolein-modified cAAT was 79.1 ± 0.99°C. This observation suggests that acrolein-modification rigidifies the protein.

Intrinsic Fluorescence of Acrolein-modified cAAT

Intrinsic protein fluorescence decreased following incubation of cAAT with acrolein (2.5 mM for 19 h at 37°C) (Fig. 4). Fluorescence intensity ($\lambda_{ex} = 290$ nm; $\lambda_{em} = 380$ nm) is attributed to the number and location of tryptophan residues as well as the quenching effects of PLP.[29,31] The RFUs of the acrolein-modified cAAT was approximately one half that of unmodified cAAT. This observation further suggests that acrolein caused irreversible conformational changes.

Effects of Acrolein on cAAT Activity

Acrolein (0–10 mM for 2.5 h at 37°C and 50°C) irreversibly inhibited cAAT activity (Fig. 5). This inhibition was concentration and temperature-dependent. When cAAT activity was plotted versus formation of cross-linked product (Fig. 6), linear regression analysis revealed a significant correlation

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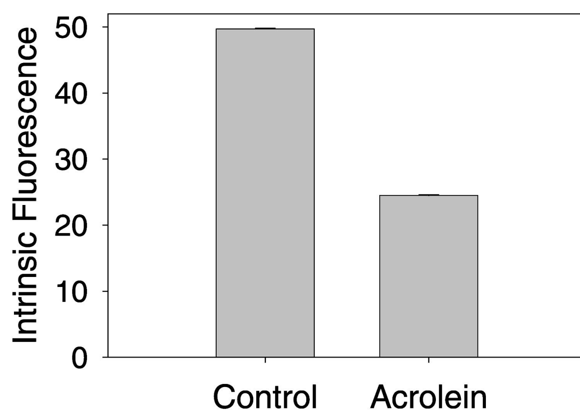

FIGURE 4 Effects of acrolein on intrinsic fluorescence of cAAT. Relative fluorescence units (RFUs) were measured for cAAT following incubation in the presence and absence of acrolein (2.5 mM for 19 h at 37°C). Samples were dialyzed prior to analysis to remove free acrolein. Measurements were at $\lambda_{ex} = 290$ nm (slit = 5 nm); $\lambda_{em} = 380$ nm (slit = 5 nm), 10 s integrations. Data is presented as averages of multiple readings ± SD from two experiments.

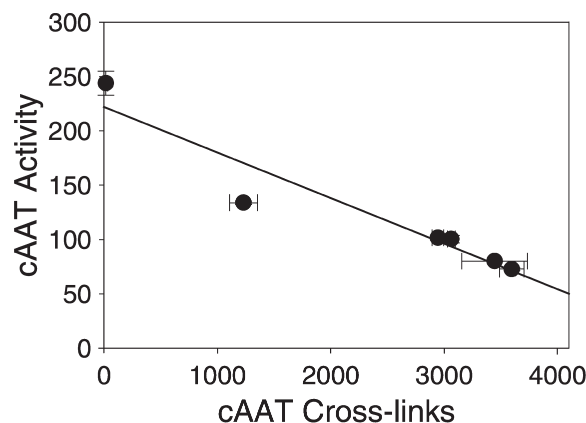

FIGURE 6 Correlation of cAAT inhibition and formation of cross-links. cAAT was incubated in the presence of acrolein (0–10 mM) for 2.5 h at 37°C prior to determination of cAAT activity and cross-linked product. The values representing activity measurements are derived from Figure 5. Activity is presented as μmole × min^{-1} × mg $prot^{-1}$. Cross-linked product is given as intensity values from densitometric analysis of SDS-PAGE gels. Regression line was drawn using SigmaPlot 4.0 (SPSS, Inc.).

(Pearson coefficient, r = 0.95, $P < 0.01$). Loss of enzyme function was directly related to the rate of cAAT cross-link formation.

DISCUSSION

We report on the properties of acrolein-modified cAAT demonstrating that acrolein alters protein structure and adversely affects enzyme activity. Acrolein, an inflammatory byproduct, exhibits toxicity[17–19] and atherogenicity.[20,21] The toxicity of acrolein is due to its reactivity to macromolecules[22–24] forming AMPs, which may be useful markers for the diagnosis of atherosclerosis,[25] diabetic

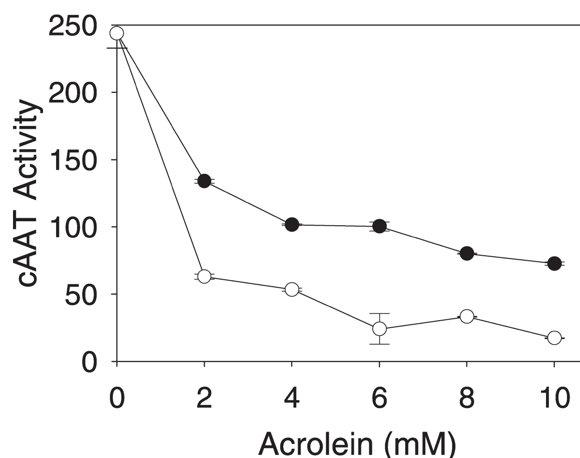

FIGURE 5 Inhibition of cAAT activity. cAAT activity was measured following incubation in the presence of acrolein (0–10 mM) for 2.5 h at 37°C (closed circles) and 50°C (open circles). Activity is presented as μmole × min^{-1} × mg $prot^{-1}$. Data is given as averages ± SD of triplicate assays from two experiments.

nephropathy[26] and Alzheimer's disease.[27] The major acrolein-derived adduct is FDP-lysine (N′[3-formyl-3,4-dehydropiperidino]), although other adducts form including the β-substituted propanal adduct ($R-NH-CH_2-CH_2-CHO$) and the Schiff base cross-link ($R-NH-CH_2-CH_2-CH=N-R$), where R represents a lysyl, histidinyl or cysteinyl residue.[28]

Acrolein chemically modified cAAT affecting protein structure. Subunit cross-links were formed (Figs. 1 and 2). The HMW doublet seen in SDS-PAGE is presumably acrolein-dependent cross-linked cAAT subunits, which may be due to the single Michael addition to one subunit and the Schiff base attachment to another subunit ($cAAT-NH-CH_2-CH_2-CH=N-cAAT$). An FDP-lysine on one subunit may form Schiff base cross-links because FDP contains a free aldehyde group. After subtracting the cross-linked subunit molecular mass from the HMW bands, the remaining relative molecular mass may be attributed to attached acrolein molecules (acrolein = 56 Da). Additionally, protein modification may have contributed to changes in electrophoretic migration. We observed that acrolein increased conformational rigidity (Fig. 3). This observation is consistent with other studies involving similar chemical modifications of proteins where conformational flexibility decreases.[12,32] We also observed that acrolein decreased intrinsic protein fluorescence (Fig. 4) suggesting that there may be increased quenching by bound PLP in the modified cAAT due to rigidification of the protein. A more compact structure would decrease the proximity of the tryptophans to PLP promoting energy transfer and thereby decrease fluorescence emission. This

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interpretation is consistent with previous studies[29,31] that examined the unfolding dynamics of PLP-dependent enzymes. The acrolein-induced increase in protein rigidity and the formation of a more compact structure may be due to intramolecular cross-linking.

Acrolein irreversibly inhibited enzyme activity (Fig. 5) in a concentration and temperature-dependent manner, providing further evidence that a chemical reaction is involved. In fact, acrolein-induced formation of cAAT cross-links directly correlated with inhibition of enzyme activity (Fig. 6). Additionally, the loss in activity may be attributed to the acrolein-induced decrease in flexibility and increase in compaction as illustrated in Figs. 3 and 4. These observations are consistent with the acrolein-dependent decrease in biological activity of other proteins[22–24] that exhibit very different structural motifs.

cAAT plays a pivotal role in ischemia by promoting glycolysis and ATP production.[1–6] In our *in vitro* study, we observed that an inflammatory byproduct (acrolein) modified and inhibited cAAT. It is plausible that the acrolein in ischemic tissue[33–34] may contribute to cell injury by modifying and inhibiting cAAT *in vivo*. The decreased cAAT activity in erythrocyte aging[35] and diabetes[8] is presumably due to chemical modification of cAAT. Interestingly, a dietary deficiency in essential fatty acids also decreases cAAT activity.[7] Because essential fatty acids down-regulate cytokine-induced lipid peroxidation,[36,37] deficiencies in essential fatty acids may promote acrolein-induced cAAT modification and inhibition. Further research is required to determine if acrolein modifies cAAT *in vivo*.

In summary, we report that acrolein chemically modified cAAT, which resulted in protein cross-links. Acrolein-modified cAAT exhibited a change in conformation that was more rigid and compact relative to native cAAT. We observed that acrolein irreversibly inhibited cAAT activity in a concentration and temperature-dependent manner. Furthermore, enzyme inhibition correlated with acrolein-induced protein cross-linking.

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