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Acrylamide: A Dietary Carcinogen Formed in Vivo?

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Acrylamide, a chemical formed during heating of human foods, reacts with N-terminal valine in hemoglobin (Hb) and forms stable reaction products (adducts). These adducts to N-terminal valine in Hb have been used to estimate daily intake of acrylamide. Daily intake of acrylamide estimated from Hb adduct levels was higher than daily intake estimated from dietary questionnaires, possibly indicating other sources of exposures. Therefore, in this study the possible endogenous formation of acrylamide was investigated by treating mice with $FeSO_4$, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine−hydrochloric acid (MPTP), or methamphetamine (METH). Acrylamide Hb adducts were determined, and a significant increase ($p < 0.05$) in acrylamide Hb adduct levels was observed 24 h following treatment with $FeSO_4$ and 72 h following treatment with MPTP or METH. The results of this study show that acrylamide Hb adduct levels are increased in mice treated with compounds known to induce free radicals, thus suggesting the endogenous production of acrylamide.

KEYWORDS: Acrylamide; endogenous formation; oxidative stress; MPTP; METH

INTRODUCTION

Acrylamide is a chemical that is classified *as a probable human carcinogen* by the U.S. EPA and the IARC (*1, 2*) on the basis of studies in rats (*3*) and mice (*4, 5*). Acrylamide is extensively used in the industrial manufacture of polyacrylamide. Accidents, environmental and in occupational settings, led to human exposures making it evident that acrylamide is neurotoxic to humans (*6, 7*). Acrylamide is easily absorbed following all routes of exposure. In vivo acrylamide is metabolized to glycidamide (*8–10*) by CYP 2E1 (*11*).

Both acrylamide and glycidamide react with nucleophilic sites in macromolecules, such as the blood protein hemoglobin (Hb), and form stable reaction products, adducts (*12, 13*). Adducts from acrylamide and glycidamide to the N-terminal valine in Hb can be quantified using a modified *N*-alkyl Edman method (*13*) and can be used to indicate and estimate exposure levels of reactive electrophiles (*14*).

Acrylamide has been observed as background Hb adducts in blood samples from "unexposed" humans (*10*). The origin for these background adduct levels was unknown until the discovery of the formation of acrylamide in heated human foods that are rich in starch (*15, 16*). This finding showed that almost the entire population is exposed to acrylamide on a daily basis and that the major cause for the observed background adducts was the ingestion of heated starchy food. Background Hb adduct levels in adult humans range from 12 to 50 fmol/mg of globin (*17, 18*); dietary intake estimated from these adduct levels corresponds to a daily intake that is about 3-fold higher than acrylamide intake calculated from dietary intake (*17*). The discrepancy in estimated dietary intake calculated from in vivo dose surrogate or from information obtained by combining dietary intake data and published acrylamide levels in foods points to the potential presence of additional source(s) for acrylamide exposure.

α,β-Unsaturated carbonyl chemicals, such as acrolein and crotonaldehyde, are known to be formed as a result of oxidative stress (*19, 20*). The aim of this study is to investigate whether acrylamide, an α,β-unsaturated carbonyl, can also be formed endogenously by oxidative stress. To investigate the possible endogenous formation of acrylamide, mice were exposed to $FeSO_4$, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or methamphetamine (METH). These chemicals exert their damage through oxidative stress. $FeSO_4$ is a chemical known to generate hydroxyl radicals (*21, 22*), and MPTP and METH are chemicals known to produce neurotoxicity by generating oxidative stress and free radicals such as superoxide and nitric oxide (*23–25*).

MATERIALS AND METHODS

Reagents. $FeSO_4 \cdot 7H_2O$ (99.7%) was obtained from J. T. Baker (Phillipsburg, NJ), and METH was purchased from Sigma (St. Louis, MO). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine−hydrochloric acid (MPTP) was purchased from Research Biochemical International (Natick, MA). Pentafluorophenyl isothiocyanate (PFPITC) (>97%) and formamide (>99.5%) were purchased from Fluka (Buchs, Switzerland). All other solvents were of analytical grade, and Milli-Q water was used throughout.

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Animal Handling Procedures. Procedures involving the care and handling of mice were reviewed and approved by the NCTR Laboratory Animal Care and Use Committee. Male mice (C57BL/6N) weighing 25−30 g were obtained from the NCTR colony. NIH 31 standard diet and tap water were provided ad libitum throughout the experiment. The initial experiment consisted of pooled blood sample (from four mice) from controls and from mice treated with MPTP. This study was followed with an experiment in which mice were treated with MPTP (four controls and four MPTP treated) and individual samples analyzed. To investigate if the observed effect is due to oxidative stress, mice were treated with 3.2 mg/kg of body weight (bw) of iron in $FeSO_4 \cdot 7H_2O \times 2$, 20 mg/kg of bw MPTP × 2, and 10 mg/kg of bw METH × 4 ($n = 4$ in each group). To investigate the effect of time on acrylamide Hb adduct levels in blood, animals were treated with 3.2 mg/kg of bw of iron in $FeSO_4 \cdot 7H_2O \times 2$, $FeSO_4$, and blood collected at different time intervals following treatment. Control mice treated with saline solution ($n = 4$) were included in each experiment group and were kept under the same conditions. In all experiments animals were sacrificed 24, 48, or 72 h following treatment and erythrocytes collected for analysis.

Isolation of Globin and Derivatization. Red blood cells were separated from the whole blood, and globin was immediately precipitated according to the method of Mowrer et al. (*26*), centrifuged, washed twice with ethyl acetate, and dried. The adducted N-terminal valine residues from 10 mg of globin samples were derivatized according to the *N*-alkyl Edman method (*27*). PFPTHs of acrylamide and glycidamide adducted N-terminal valines, AA-Val PFPTH and GA-Val PFPTH, respectively, were then purified and analyzed using LC-MS/MS.

Mass Spectrometry. A Quattro Micro triple-quadruple mass spectrometer (Waters Corp., Milford, MA) equipped with an electrospray interface with a source block temperature of 100 °C and a desolvation temperature of 400 °C was used. Nitrogen gas was used as desolvation gas (750 L/h), and argon was used as collision gas, at a collision cell pressure of 4.5×10^{-3} mBar. Positive ions were acquired in multiple-reaction monitoring mode for the following transitions according to Tareke et al. (*28*):

AA-VAL-PFPTH: m/z 395.9 → m/z 378.9

GA-VAL-PFPTH: m/z 411.9 → m/z 394.9

AA-VAL-PFPTH-$^{13}C_5$: m/z 400.9 → m/z 383.9

GA-VAL-PFPTH-$^{13}C_5$: m/z 416.9 → m/z 399.9

A cone voltage of 15 V and a collision energy of 22 eV were used for all transitions.

RESULTS

Acrylamide and glycidamide adduct levels from all control mice (four experiments, $n = 4$ each; and one experiment, $n = 1$ pooled sample), mice treated with MPTP (two experiments, $n = 4$ each; and one experiment, $n = 1$ pooled sample), and mice treated with $FeSO_4$ for 24 h (two experiments, $n = 4$ each) were analyzed and grouped on the basis of treatment.

The mean values of acrylamide hemoglobin (Hb) adducts (in mice 24, 48, and 72 h following treatment with $FeSO_4$) and glycidamide Hb adducts (in mice 48 and 72 h following treatment with $FeSO_4$) increased significantly by 10−22 and 30−40%, respectively ($P = 0.002$ and $P = 0.001$, respectively; determined by one-way analysis of variance) (**Figure 1**). The mean values of acrylamide adduct levels in mice 72 h after treatment with MPTP or METH were also significantly increased by 20% ($P < 0.001$ determined by one-way analysis of variance) (**Figure 2**). Effects of individual treatments on acrylamide and glycidamide Hb adduct levels compared to levels in Hb from control mice were also analyzed using Student's t test (**Figures 1** and **2**). In $FeSO_4$-treated mice, the increase in acrylamide Hb adduct levels was observed 24 h after treatment, and glycidamide levels was observed starting 48 h after treatment (**Figure 1**), whereas in mice treated with MPTP or METH the increase in acrylamide Hb adduct levels was observed 72 h post-treatments

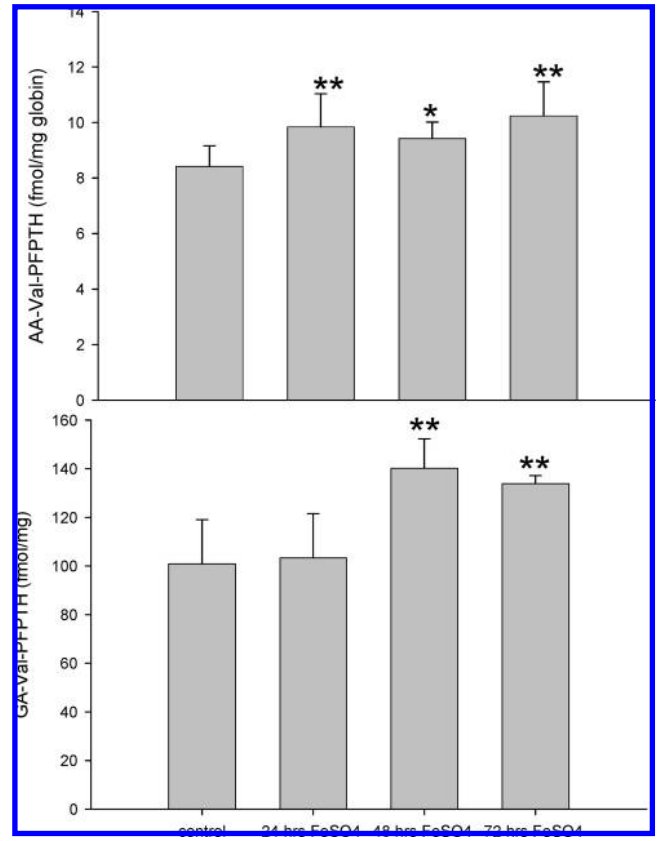

Figure 1. Acrylamide and glycidamide adducts to N-terminal valine in hemoglobin from mice treated with $FeSO_4$. Blood samples were collected from control ($n = 15$) mice and mice treated for 24 ($n = 8$), 48 ($n = 3$), or 72 ($n = 3$) h following treatment. ∗, $P < 0.05$; ∗∗, $P < 0.005$, using Student's t test.

(**Figure 2**). No increase in glycidamide Hb adducts was observed in mice treated with MPTP or METH 24, 48, or 72 h following treatment. Not enough blood was collected from two controls and two $FeSO_4$-treated mice (one 48 h and one 72 h after treatment).

DISCUSSION

The formation of acrylamide Hb adducts in humans is proportional to exposure levels. This was demonstrated in a study in which human subjects were orally exposed to 0.5, 1, and 3 mg/kg of bw of $^{13}C_3$ acrylamide. Hemoglobin adduct levels in the study demonstrated a clear linear increase in a dose-dependent manner (*29*). However, epidemiological studies revealed that daily intake of acrylamide estimated from measured Hb adduct levels is higher than intake levels estimated using dietary questionnaires (*17, 18*). This observed gap in estimations of daily intake could be due to several underlying factors including the following: (1) the estimation of dietary intake of acrylamide from measured acrylamide Hb adduct levels uses several assumptions; (2) the dietary questionnaires used for the correlation studies were not designed specifically for the investigation of acrylamide intake; (3) the present knowledge on dietary sources for acrylamide exposure is inconclusive; (4) the presence of other sources and the possible endogenous formation of acrylamide were not considered.

The present study examined the possibility of endogenous formation of acrylamide as a result of oxidative stress. To illustrate this, mice were exposed to iron, which is known to generate hydroxyl radicals through Fenton's reaction (*21*), and

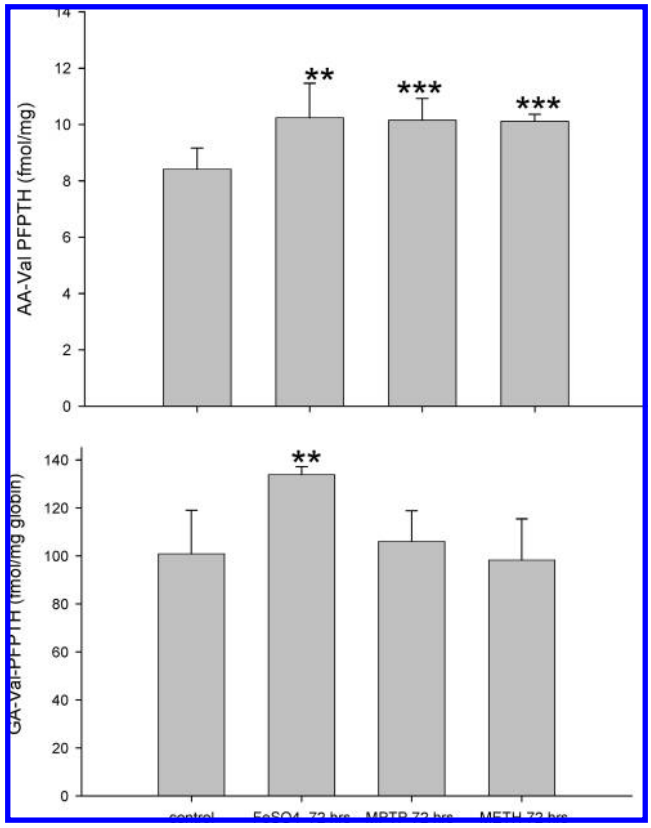

Figure 2. Acrylamide adducts to N-terminal valine in hemoglobin from mice treated with $FeSO_4$ ($n = 3$), MPTP ($n = 9$), or METH ($n = 4$). Blood samples were collected 72 h following treatment. **, $P < 0.005$; ***, $P < 0.001$, using Student's t test.

MPTP and METH, which are selective dopaminergic neurotoxicants that have been shown to produce oxidative stress and free radicals such as superoxide and nitric oxide (*23, 25*).

The results from this study demonstrated a small, but significant, increase in acrylamide Hb adduct levels following treatment with $FeSO_4$, MPTP, or METH.

Because the common underlying mechanism for the toxicity of $FeSO_4$, MPTP, or METH is through free radical formation and because there is no plausible metabolic pathway that may lead to direct formation of acrylamide from these chemicals, we hypothesize that acrylamide was formed endogenously as a result of oxidative stress. The increase in glycidamide Hb adduct levels in mice treated with $FeSO_4$ indicates that the metabolism of acrylamide to glycidamide is not affected. However, it is possible that inhibition of phase 2 enzymes as a result of oxidative stress may lead to decreased clearance of acrylamide and glycidamide and, therefore, increased acrylamide and glycidamide Hb adduct levels. In this case a parallel change in acrylamide and glycidamide adduct levels should be expected, because both acrylamide and glycidamide are presumably detoxified with the same enzymes. In the study we observed a selective increase in acrylamide Hb adduct levels in mice treated with $FeSO_4$ after 24 h and with MPTP and METH after 72 h. However, analysis of mercapturic acids of acrylamide [*N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine, AAMA] and glycidamide [*N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine, GAMA] would clarify the influence of the treatment on phase 2 enzymes and consequently on the clearance of both acrylamide and glycidamide.

So far there is no known mechanism for the endogenous formation of acrylamide. A possible pathway for its formation is that acrolein, a chemical known to be formed during lipid peroxidation, may be a precursor. Acrolein is formed as a result of oxidative stress both endogenously (*20*), in experimental settings (*30*) and during heating of lipids (*31*). Spraying acrolein gas over heated asparagine was shown to produce acrylamide (*30*). Therefore, a plausible mechanism is that acrolein, which is endogenously formed by oxidative stress, reacts with amino groups that may be formed as byproducts of protein oxidation, leading to the formation of acrylamide.

The observed increment in Hb adduct levels is subtle compared to the 3-fold discrepancy between the intake of acrylamide estimated from adduct levels and that estimated from dietary intake. However, more studies in humans with physiological conditions, such as diabetes, and neuron degenerative diseases, such as Parkinson's disease, Alzheimer's, and ALS, that induce or are mediated through chronic oxidative stress are warranted to clarify the effect of long-term chronic oxidative stress on the internal dose of acrylamide and glycidamide. Results from this study clearly demonstrate an increased internal dose of acrylamide that, irrespective of whether acrylamide is formed endogenously or increased due to inhibited detoxification, needs to be considered in risk assessment and in the estimation of the dietary intake of acrylamide.

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