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The garlic ingredient diallyl sulfide inhibits cytochrome P450 2E1 dependent bioactivation of acrylamide to glycidamide

Dirk Taubert [a,*], Reinhild Glöckner [b], Dieter Müller [b], Edgar Schömig [a]

[a] *Department of Pharmacology, University Hospital of Cologne, Gleueler Str. 24, D-50931 Cologne, Germany*
[b] *Institute of Pharmacology and Toxicology, Friedrich Schiller University Jena, Nonnenplan 4, D-07743 Jena, Germany*

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Abstract

Genotoxic effects of acrylamide are supposed to result from oxidative biotransformation to glycidamide. After incubation of rat liver slices with acrylamide we detected free glycidamide using a liquid chromatography tandem mass spectrometric method. Glycidamide formation was diminished in the presence of the cytochrome P450 2E1 inhibitor diallyl sulfide (DAS), which is a specific ingredient of garlic. This may be relevant to human health since the suggested carcinogenic risk of dietary acrylamide may be reduced by concomitant intake of garlic.

Keywords: Acrylamide; Glycidamide; Diallyl sulfide; Garlic; Rat liver slices; Cytochrome P450 2E1

1. Introduction

Based upon findings of carcinogenicity studies in rats (Johnson et al., 1986; Friedman et al., 1995), genotoxic effects in cultured mammalian cells and in somatic cells of treated animals (Besaratinia and Pfeifer, 2003), acrylamide is considered to be a probable human carcinogen (IARC Monographs, 1993). The potential carcinogenicity attained considerable interest with the discovery of high concentrations of acrylamide in common heated starch-rich foodstuffs (e.g. French fries, potato chips, cakes, bread) (Tareke et al., 2002) formed by Maillard reaction from reducing sugars and asparagine at processing temperatures above 120 °C (Mottram et al., 2002; Taubert et al., 2004).

Experimental studies (Besaratinia and Pfeifer, 2003) and investigations of in vivo genotoxicity (Manjanatha et al., 2005) have demonstrated a dose dependent increase of mutation frequency after exposure to acrylamide. There is evidence that the genotoxicity of acrylamide predominantly results from metabolic conversion to its epoxide derivative glycidamide (Twaddle et al., 2004; Doerge et al., 2005a) and subsequent formation of glycidamide–DNA adducts (Gamboa da Costa et al., 2003; Doerge et al., 2005b; Ghanayem et al., 2005a). Paulsson et al. (2003) reported that in mice the induction of micronuclei per unit of glycidamide in blood, a measure of genotoxicity, was identical when glycidamide was directly administered or when it arose as a metabolite from acrylamide administration. Recently, glycidamide was shown to be an inducer of genotoxicity or mutagenicity in V79 cells and human lymphocytes, while acrylamide was inactive in these models (Baum et al., 2005). Sumner et al. (1999) found cytochrome P450 2E1 (CYP2E1) to be the specific liver enzyme

* Corresponding author. Tel.: +49 221 478 4196; fax: +49 221 478 5022.
E-mail address: dirk.taubert@medizin.uni-koeln.de (D. Taubert).

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involved in this reaction in mice. Furthermore, in mice pretreated with 1-aminobenzotriazole, an inhibitor of CYP2E1, acrylamide induced mutations in spermatids were substantially reduced (Adler et al., 2000).

Two major metabolic pathways for acrylamide have been reported (Calleman, 1996; Dybing et al., 2005). One pathway is conjugation with glutathione to form the urinary metabolites *N*-acetyl-*S*-(3-amino-3-oxypropyl) cysteine and *N*-acetyl-*S*-(2-carbamoylethyl) cysteine. The second pathway is epoxidation to glycidamide. Most of glycidamide is metabolized by conjugation with glutathione to form mercapturic acids or metabolized by epoxide hydrolase (Friedman and Chemistry, 2003; Boettcher et al., 2005). However, only free unchanged glycidamide is supposed to account for the genotoxicity of acrylamide by formation of promutagenic DNA adducts (Gamboa da Costa et al., 2003; Doerge et al., 2005b; Segerback et al., 1995).

Administration of garlic (Allium sativum) has been shown to reduce the incidence of various chemically induced tumors in animal models (Milner, 1996). Epidemiologic studies indicate that frequent consumption of garlic or garlic extracts is associated with reduced cancer risk (Fleischauer and Arab, 2001). One of the primary constituents of garlic suggested to be responsible for this anticarcinogenic action is allyl sulfides that are arising from decomposition of the native cysteine sulfoxide alliin (Amagase et al., 2001). Allyl sulfides are thought to exert their protective effects in part by inhibition of CYP2E1, thereby preventing the formation of genotoxic oxidative metabolites from xenobiotics (Milner, 2001; Yang et al., 2001). A potent inhibition of CYP2E1-mediated bioactivation of procarcinogenes was reported for diallyl sulfide (DAS) as well as its metabolites diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) (Brady et al., 1991).

Using an in vitro model (rat liver slices), we wanted to test the hypothesis that biotransformation of acrylamide leads to free glycidamide and that the glycidamide formation is inhibited by DAS.

2. Experimental

2.1. Reagents

Glycidamide and D3-glycidamide (purity > 98% w/w) were synthesized from acrylamide and D3-acrylamide, respectively by H_2O_2 oxidation of acrylonitrile, as described (Payne and Williams, 1961). Deuterated acrylamide ([2,3,3-$^2H_3$]-acrylamide, 98% purity w/w) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Acrylamide (purity > 99% w/w) and all other chemicals were from Sigma.

2.2. Incubation of rat liver slices

Metabolism experiments were performed using precision-cut rat liver slices. The liver was dissected out of ether sacrificed male Wistar rats weighing 200–300 g and placed into ice-cold Krebs–Henseleit–HEPES buffer. Preparation of liver slices (thickness about 250 μm) was performed directly out of the liver lobes with a Krumdieck tissue slicer, as described previously (Lupp et al., 2001).

Four slices each were placed in bidirectionally shaking Erlenmeyer flasks, filled with 5 ml carbogen aerated William's Medium E (pH 7.4, 37 °C) supplemented with insulin (1 μmol/l), L-glutamine (2 mmol/l) and ampicillin (10 mg/l). Slices were incubated for 0.5, 1, 2, 4, and 24 h, respectively with 1.4 μmol/l acrylamide in the presence of either 100 μmol/l or 1000 μmol/l diallyl sulfide or DAS free solvent (dimethylsulfoxide, 0.2% final concentration). All experiments were performed in duplicate. Glycidamide was determined in the medium. Incubation of glycidamide with DAS for 24 h in William's Medium E had no effect on glycidamide concentration, which excludes a direct interaction between glycidamide and DAS.

2.3. LC–MS/MS detection of glycidamide

Analysis was performed on a triple-quadrupole tandem mass spectrometer (TSQ Quantum Ultra, Thermo Electron, Dreieich, Germany) equipped with a thermostated (5 °C) Surveyor autosampler and a thermostated (30 °C) Surveyor HPLC system (Thermo Electron) operating in positive electrospray ionization (ESI^+) mode. Spray voltage was set at 4000 V and capillary temperature was kept at 350 °C. Nitrogen sheath gas and auxiliary gas pressure were 40 and 4 psi, respectively. Argon collision gas pressure was 1.0 Torr. The multiplier gain was tuned to 6 Mio to achieve maximal sensitivity. Fifteen microlitre aliquots of William's Medium E samples were injected onto a 5 μm Hypersil BDS C_{18} column (50 mm × 2.1 mm; Thermo Electron), and eluted isocratically at a flow rate of 0.25 ml/min (run time 5.0 min). The mobile phase consisted of 5% (v/v) acetonitrile/0.1% formic acid and 95% (v/v) deionized water/0.1% formic acid.

Precursor ion $[M+H]^+ \rightarrow$ product ion transition (single reaction monitoring (SRM)) used for quantification of glycidamide was m/z 88 → 44 (collision energy 20 eV). Detection of the internal standard (IS) D3-glycidamide (500 ng/ml) was performed by monitoring the m/z 91 → 44 transition (collision energy 20 eV). Response ratios of glycidamide versus IS were linear over a concentration range of 0.1–75 ng/ml (0.00115–0.86128 μmol/l) glycidamide ($r^2 > 0.990$). The limit of detection (LOD) was 0.1 ng/ml (0.00115 μmol/l) glycidamide.

3. Results and discussion

Incubation of rat liver slices with acrylamide resulted in a time dependent formation of free gly-

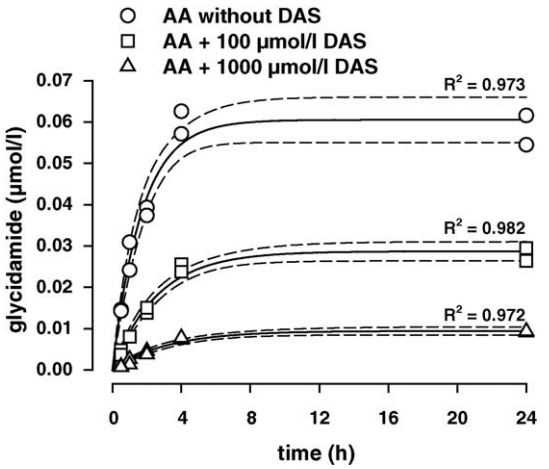

Fig. 1. Time dependence of free glycidamide formation following incubation of rat liver slices with 1.4 μmol/l acrylamide (AA) in the presence or absence of diallyl sulfide (DAS). Individual concentration vs. time data are fitted with an exponential model ($c(t) = c_{max}(1-e^{-kt})$). Dashed lines represent 95% confidence intervals. R^2 denotes the coefficient of determination.

cidamide (Fig. 1). Fitting the glycidamide concentration versus time data with different non-linear equations using Table Curve 2D v5.01 (SYSTAT Software Inc.) revealed that the kinetics of glycidamide formation were best explained by the exponential equation $c(t) = c_{max}(1-e^{-kt})$ with a coefficient of determination of $R^2 = 0.973$ ($c(t)$: glycidamide concentration at time t, c_{max}: extrapolated maximal glycidamide concentration, k: reaction rate constant of glycidamide formation). This equation agrees with the mathematical description of cumulative drug elimination after applying a single dose assuming linear pharmacokinetics in a one-compartment model. The maximal glycidamide concentration c_{max} was 0.0609 μmol/l, i.e. 5.3% of the acrylamide dose was metabolized to free glycidamide (Table 1). The half maximal concentration of glycidamide was achieved after $t_{1/2} = 71$ min of incubation, so that a near maximal level (90% of c_{max}) was already observed after 4 h.

Coincubation of the liver slices with acrylamide and DAS resulted in a dose dependent inhibition of glycidamide formation (Fig. 1). In the presence of 100 μmol/l DAS, the maximal glycidamide level ($c_{max} = 0.0287$ μmol/l) achieved just one half of the value in DAS free incubation medium and in the presence of 1000 μmol/l DAS the respective level of inhibition was 85% (Table 1). DAS and its metabolites DASO and $DASO_2$ are competitive inhibitors of CYP2E1 (with $DASO_2$ additionally operating as a suicide inhibitor) (Brady et al., 1991); representative K_i values obtained for inhibition of rat microsomal CYP2E1 by DAS were 27 μmol/l for the demethylation of *N*-nitrosodimethylamine (Brady et al., 1988) and 188 μmol/l for the hydroxylation of *p*-nitrophenol (Brady et al., 1991) which fall in the range of the DAS concentration (<100 μmol/l) for half maximal inhibition of glycidamide formation. Previous work also demonstrated that DAS, DASO and $DASO_2$ are selective inhibitors of CYP2E1 (Brady et al., 1991, 1988; Kwak et al., 1994), some other CYP isoforms such as CYP1A1/2 and CYP1B1/2 were induced (Guyonnet et al., 2000). Hence, the strong suppression of glycidamide formation by DAS indicates that CYP2E1 is the major enzyme responsible for epoxidation of acrylamide. This is further supported by recent studies in CYP2E1 knockout mice revealing an almost complete (95%) inhibition of acrylamide conversion to glycidamide compared to wild-type mice (Ghanayem et al., 2005a) and the absence of acrylamide induced genotoxicity (Ghanayem et al., 2005b).

The lowest K_m values reported for the metabolism by microsomal CYP2E1 were in the range of 14–22 μmol/l (Brady et al., 1991; Yoo et al., 1990). Thus, a substrate concentration of 1.4 μmol/l acrylamide ($\ll K_m$) is likely to correspond to non-saturating conditions, implying that the observed velocity and proportion of conversation of acrylamide to glycidamide occurred at maximal levels. This situation also applies to the dietary exposure to acrylamide with an estimated median daily intake of

Table 1
Kinetic parameters of glycidamide formation following incubation of rat liver slices with 1.4 μmol/l acrylamide (AA) in the presence or absence of diallyl sulfide (DAS)

Compound	c_{max} glycidamide (μmol/l) [95% CI]	Formation rate constant k (1/h) [95% CI]	P value for difference
AA without DAS	0.0609 [0.0551–0.0655]	0.585 [0.438–0.731]	
AA + 100 μmol/l DAS	0.0287 [0.0264–0.0308]	0.383 [0.306–0.460]	0.0022
AA + 1000 μmol/l DAS	0.0092 [0.0080–0.0103]	0.316 [0.236–0.396]	0.000016

c_{max} and k were obtained from non-linear regression of the data with an exponential function ($c(t) = c_{max}(1-e^{-kt})$). Significance of the differences between the curves in the presence of DAS compared to the absence of DAS was assessed by pairwise multiple comparison procedure (Dunn's Method). $P < 0.05$ was considered statistically significant. CI denotes confidence interval.

0.5 μg/kg (0.007 μmol/kg) body weight (i.e. a total of about 0.5 μmol per day) (Boon et al., 2005). Marked anticancerogenic effects of garlic or garlic extracts have consistently been observed at a daily intake of more than 2 g. In humans a mean inhibition of CYP2E1 activity by 31% has been reported after administration of a single oral dose of 0.2 mg/kg (1.75 μmol/kg) body weight (i.e. a total of about 125 μmol) of DAS (Loizou and Cocker, 2001). Since DAS is found in processed garlic at an approximate concentration of 0.3% (w/w) (Voigt and Wolf, 1986), this corresponds to the ingestion of about 4.5 g or 2–3 cloves of garlic. Hence, in individuals with high intake of garlic the DAS concentrations may achieve pharmacologically active levels that inhibit transformation of dietary acrylamide to glycidamide.

Evaluation of pharmacokinetics of acrylamide in rodents indicates a lower proportion of acrylamide transformation to glycidamide in rats than in mice (Calleman, 1996). The few available data from studies on urinary pharmacokinetics of acrylamide in humans (Sorgel et al., 2002; Fennell et al., 2005) suggest that metabolism of acrylamide in humans shows more similarities with rats strengthening the possible relevance of the here employed model for human toxicokinetics. Moreover, liver slices exhibit advantages over other more artificial in vitro systems, such as cultured hepatocytes, because the normal tissue architecture, the cell heterogeneity and cell–cell interactions are maintained (Gebhardt et al., 2003). Models of isolated perfused liver, although principally closer to in vivo conditions, are not superior to liver slices for assessing metabolism: slices combine the advantages of completely preserved metabolic and transport capacities with an easy handling and the saving of animals.

Based on the lack of associations between dietary acrylamide ingestion and cancer risk in epidemiological studies, the causality of nutritional acrylamide for development of cancer has recently been questioned (Mucci et al., 2003; Pelucchi et al., 2006). However, assuming a very low individual acrylamide dependent lifetime cancer risk in the range of 0.7–4.5/100,000 as considered by regulatory authorities from animal toxicity studies (World Health Organization, 1985; Environmental Protection Agency, 1985), the statistical power of these observational studies appears too low to prove the hypothesis. In contrast, growing and consistent evidence of the genotoxic potency of acrylamide or its metabolite glycidamide from in vitro and animal studies further supports the hypothesis that dietary acrylamide is carcinogenic in humans.

In summary, we have demonstrated that the proposed ultimate carcinogen glycidamide is formed from acrylamide in the liver by epoxidation with the cytochrome P450 enzyme CYP2E1. The biotoxification of acrylamide is diminished by applying the CYP2E1 inhibitor DAS which is exclusively formed in garlic and may account for the anticarcinogenic effects observed in individuals with regular intake of high amounts of garlic.

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