Different activation patterns of rat xenobiotic metabolism genes by two constituents of garlic

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Diallyl sulfide (DAS) and diallyl disulfide (DADS) are natural components that could account for the anticarcinogenic properties of garlic, at least in part, through the activation of xenobiotic detoxifying metabolism. The aim of this work was to describe the effect of DAS and DADS on xenobiotic-related gene expressions and to study molecular mechanisms relaying DAS effect. We describe the different effects of DAS and DADS on hepatic CYP2B1/2, CYP3A and epoxide hydrolase (EpH) mRNAs in rats, in terms of activation profile, doses and kinetics. The activation profile varied with the mode of chemical administration, i.e. gastric infusion or intraperitoneal (i.p.) injection. Using gastric infusion, DAS and DADS proved different efficiencies at enhancing the mRNA level of the three drug-metabolizing enzymes. After an i.p. administration, we observed a specific activation of CYP2B1/2 gene by DAS. The DAS-mediated CYP2B1/2 activation occurred at transcriptional level and through an okadaic acid-sensitive pathway. In rat livers, a short sequence (NR1) derived from the CYP2B1/2 promoter was stimulated by DAS and we observed a nuclear accumulation of a DNA–protein complex binding NR1. Because constitutively activated receptor (CAR) is a major transcription factor driving the xenobiotic-induced stimulation of CYP2B1/2 through NR1, the role of CAR as a preferential mediator of DAS effect is discussed.

Introduction

Garlic (Allium sativum) is reputed worldwide as having beneficial effects for improving health, but the biological and molecular effects of its main compounds remain poorly understood. Garlic is particularly rich in organosulfur compounds that have been reported in animals or in cell lines to have anticarcinogenic properties by involving diverse cellular events, as diverse as proliferation, drug metabolism, apoptosis, gene expression, redox status or inter-cellular communication (1–6). Of sulfur compounds naturally occurring in garlic, diallyl sulfide (DAS) and diallyl disulfide (DADS) are the most abundant (7). DADS and DAS are formed from the natural precursor allicin when a garlic glove is crushed. The concentration of allicin in garlic is 5 mg/g and it has been estimated that a 70 kg individual eating 1.4 g of garlic per day would receive 0.1 mg/kg body wt of sulfides (8).

Until now, it has been established that DAS and DADS are potent modulators of drug metabolism enzymes (9–12). The ability of DAS and DADS to modulate phase I and II drug metabolizing–enzymes could explain their anti-promoting and anti-initiating effects in chemically induced cancer in rodents (9,13). By modulating xenobiotic enzyme activity, DAS and DADS could also influence drugs pharmacokinetics, as it has been studied in the case of garlic supplement consumption and saquinavir (14) or ritonavir (15). DAS and DADS modulate xenobiotic detoxifying metabolism through transcriptional or post-transcriptional effect (12). Although the transcriptional effects of botanicals tend to be clarified (16), mechanisms underlying the transcriptional effects of DAS and DADS remain poorly understood. The involvement of constitutively activated receptor (CAR) as a DAS-inducible factor has been suggested previously (17). CAR and its homologue pregnane X receptor (PXR) have both been considered archetypes of xenosensor transcription factors. Under the influence of various chemicals, they modulate the transcription of genes involved in the metabolism of xenobiotics (18). Although they have overlapping functions with similar DNA recognition motifs, PXR and CAR have some specific features relative to cellular regulation (19). CAR displays a constitutive activity that is either repressed by androstane metabolites (20) or stimulated by agonists such as TCPOBOP or phenobarbital (PB) (21).

Here, we describe in rats, the different effects of DAS and DADS on cytochromes P450 (CYP2B1/2, CYP3A) and epoxide hydrolase (EpH) expression, in terms of activation profile, doses and kinetics. DAS and DADS, directly injected in the stomach, were both strong inducers of CYP2B1/2 and EpH. After an intraperitoneal administration (i.p.), CYP2B1/2 gene was stimulated by DAS but remained unchanged in the presence of DADS. Then, we focused our work on mechanisms involved in the activation of CYP2B1/2 gene expression by DAS. Results of gene profile inductions, in vivo transfections, gel shift assays, and drug sensitivity assays show that DAS-induced CYP2B1/2 transcription at least in part through a short DNA recognition sequence (NR1) and thus suggest a preferential involvement of CAR as DAS-activated transcription factor. Given its effect on the CAR receptor and on CYP2B1/2 gene stimulation, DAS could be considered as a PB-like food constituent.

Materials and methods

Reagents and plasmids

DADS (CH₂ = CHCH₂SSCH₂CH = CH₃) and DAS (CH₂ = CHCH₂SCH₂CH = CH₃) (Figure 1), actinomycin D and okadaic acid were purchased from Sigma-Aldrich (L’Isle d’Abeau Chesnes, France). Two independent lots of DADS (purity 80%) and DAS (purity 97%) were used without further

Abbreviations: EpH, epoxide hydrolase; DADS, diallyl disulfide; DAS, diallyl sulfide; PB, phenobarbital.

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 insurers: €36,000-€45,000 (approx. US$40,000-€50,000) for a 6-month period.
purification as the impure part was made up only of other allyl sulfides (9). PB was provided by Dr M-H Siess (INRA, Dijon, France). The plasmid (NR1) tk-luciferase was a kind gift from Dr Negishi (Laboratory of reproductive and developmental toxicology, NC, USA) (22) and pSV-β-gal was purchased from Promega (Madison, USA).

Animals and drug injections

All aspects of the protocol conformed to the International Guiding Principles for Biomedical Research Involving Animals.

Wistar male rats weighing 300–320 g were housed individually and were fed M25 pellets (Dietex, Saint-Gratien, France). DAS and DADS were administered either by using a gastric catheter as described previously (3) or by i.p. injection. The livers were removed under isoflurane anaesthesia (Abbott; Rungis, France).

In the case of gastric administration, DAS and DADS diluted in 13 ml of rapeseed oil were infused in the stomach for 4 h (3.25 ml/h/300 g) as described previously (23,24). DAS and DADS were both injected at 80 mg/100 g and 8 mg/100 g of body wt. Control rats received 13 ml of rapeseed oil alone with the same rate of infusion (3.25 ml/h/300 g). Rat livers were removed as soon as the infusion ended (S0), at 18 h after the end of infusion (S18), or at 24 h (S24). In a few cases, animals receiving DAS at high doses died. This situation was never observed with DAS, suggesting that DAS is less harmful than DADS, as shown previously (25).

In the case of IP injection, PB, diluted in 0.9% NaCl, was injected at a dose of 10 mg/100 g of body wt. DAS was IP-injected as a pure solution at a dose of 80 mg/100 g (i.e. 0.09 ml/100 g). Control rats received 0.1 ml/100 g of NaCl (0.9% w/v) alone. Either actinomycin D (150 μg/100 g of body wt) or okadaic acid (1.5 μg, 3 μg or 6 μg/100 g of body wt) was IP administered with DAS or PB. Six hours after IP injection, the livers were removed to prepare RNA and nuclear proteins.

Northern blot

Total RNAs were extracted from livers with Qiagen affinity columns (Rneasy midi kit, Qiagen). Fifteen RNAs were separated on 1% agarose-denaturing gel and transferred onto nylon membrane (Hybond-N, Pharmacia Biotech). The preparation of nuclear extracts was carried out at 4°C and was assayed for luciferase activity by chemiluminescence (Roche Molecular Biochemicals, Mannheim, Germany) or the β-gal reporter gene assay by chemiluminescence (Roche Molecular Biochemicals, Mannheim, Germany). As recommended by the supplier, tissue lysates were incubated 60 min at 50°C, to reduce background from eukaryotic β-galactosidase. Data are expressed as arbitrary values of chemiluminescent activity and are means of 10 measurements per rat. Data are expressed as means ± standard deviation and the significance was assayed by t-test with Sigmastat software.

Nuclear extracts and gel shift assay

The preparation of nuclear extracts was carried out at 4°C following the procedure described previously (27). The livers were from rats IP-injected with DAS (80 mg/100 g), PB (10 mg/100 g) or NaCl (0.9%). For gel shift assay, double strand oligonucleotides were designed with the following upper strand: 5’-TCAGTCTGACCTTCTGACACTT-3’ (NR1), 5’-TCAGTCTCT-GTGTCTCTGACCTT-3’ (NR1m) (21,28); 5’-ATCCATTGAGATTAA- CAAGTTCAAAGT-3’ (ER6/DR4) (29). Probes were labelled by kination with [γ-32P]ATP to obtain 5000 c.p.m.1 ng. Approximately 0.3 mg of radioactive probe was added in 20 μl reaction buffer containing 100 ng poly(dI·dC), 10 mM HEPES (pH 8), 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 2 mM DTT, 17% (v/v) glycerol, 100 μg/ml albumin, 4 mM spermidine. For competition assays, 5 or 10 molar excess of unlabelled oligonucleotide (NR1 or NR1m) was mixed with the radioactive probe. Liver nuclear extracts (10 μg) were added just after the reaction reaction for 10 min at 4°C. The samples were loaded and migrates in a non-denaturing 5% acrylamide gel.

Statistical analysis

Data are means ± standard deviation and the significance was assayed by t-test (Figure 5) or by an Anova test and a Student–Newman–Keul’s test (Figures 2 A,B, C,D) with Sigmastat software (P < 0.05).

Results

The effects of DAS and DADS on drug-metabolizing enzymes were studied at mRNA levels in rats receiving compounds by a gastric catheter and sampled at the end of the infusion (S0), at 18 h after the end of the infusion (S18) or 24 h (S24). As illustrated in Figure 2A, DAS, when injected at 80 mg/100 g of body wt, markedly increased mRNA levels of CYP2B1/2, CYP3A and EpH. The DAS-induced stimulation of the three mRNAs was observed at S18 and maintained until S24. At S24, CYP 2B1/2 mRNA level was the most enhanced by DAS, with a 21 ± 3 (n = 3) stimulation factor (Figure 2B). In contrast to DAS, DADS had no statistical significant effect on CYP3A mRNA (Figure 2B). DADS was less efficient than DAS to stimulate CYP2B1/2 (Figure 2B) and this effect was detectable only at S24 (Figure 2A). In the case of EpH, DAS and DADS displayed a similar range of stimulation at S18 and S24 (Figure 2B).

With a 10-fold lower amount of DAS (8 mg/100 g), the induction of CYP2B1/2 and EpH was not observed. In contrast, 8 mg/100 g of DADS did stimulate mRNAs (Figure 2C). In turn, when considering the range of induction and the dose-dependent activation, DAS was more efficient than DADS in stimulating these drug-metabolizing genes.

After an i.p. of DAS (80 mg/100 g), a 3.6 ±0.4, n = 6) stimulation of CYP2B1/2 mRNA was observed (Figure 3A and B). Interestingly, the effect of DAS became specific of CYP2B1/2 after an IP injection, as no significant stimulation of CYP3A was observed. A slight effect of DAS was detected on EpH but it was not statistically different in comparison with controls. DADS, yet efficient after a gastric infusion (Figure 2), did not enhance CYP2B1/2 after an IP injection (no significant induction in comparison with controls). EpH remained activated by DADS whatever the mode of
administration (compare Figures 2 and 3). To investigate whether the amount of mRNAs reflected a regulation at a transcriptional level, the effects of DAS were studied in the presence of actinomycin D (ac.D), a transcriptional inhibitor (Figure 4). Values represented stimulation factors of CYP2B1/2, CYP3A and EpH mRNAs. Each signal was normalized by the 18S amount. Data are means of values obtained with three rats for each treatment (except for ac.D, n = 2). *Significantly different from controls.

As the induction of CYP2B1/2 gene transcription by strong activator such as PB is impaired in the presence of okadaic acid (OA), we studied the stimulation of CYP2B1/2 in the presence of increasing amounts of OA and 80 mg/100 g DAS. Even with the weakest doses (1.5 μg), OA inhibited the DAS-induced stimulation of CYP2B1/2 (Figure 4). In accordance with previous results, PB induction of the CYP2B1/2 gene was also abolished in the presence of 3 μg OA (30). The DAS-mediated activation of CYP2B1/2 occurred through an OA-sensitive pathway, as it is the case for PB.

The PB-induced activation of CYP2B2 is driven by a PB-responsive enhancer module, composed by two direct repeat sequences (NR1 and NR2) (22,28). We therefore tested whether the transcription of a reporter gene (luciferase) enhance a housekeeping promoter activity, as 

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and two rats received PB + 80 mg/100 g, and one rat received PB (10 mg/100 g) or a mixture of drugs with okadaic acid (OA). Three rats were treated with DAS, PB or serum, one rat received DAS + OA (6 µg/100 g) or DAS + OA (3 µg/100 g) or DAS + OA (1.5 µg/100 g) and two rats received PB + OA (3 µg/100 g). Livers were removed 6 h after the injection.

![Fig. 4](https://academic.oup.com/carcin/article-abstract/27/10/2090/2392212)

**Fig. 4.** Okadaic acid impaired DAS-induced CYP2B1/2 expression. Typical northern blot autoradiography obtained with 15 µg total liver RNA. Indicated probes were sequentially hybridized with the same membrane. Rats were IP-injected with physiological serum (−), DAS (80 mg/100 g), PB (10 mg/100 g) or a mixture of drugs with okadaic acid (OA). Three rats were treated with DAS, PB or serum, one rat received DAS + OA (6 µg/100 g) or DAS + OA (3 µg/100 g) or DAS + OA (1.5 µg/100 g) and two rats received PB + OA (3 µg/100 g). Livers were removed 6 h after the injection.

at a lower, yet reproducible, extent by DAS. This complex was progressively displaced when non-labelled NR1 was added in 5 or 10 molar excesses. NR1m bearing mutations in the recognition site was not efficient to compete the complex, thus confirming the specific DNA–protein interaction. We checked that the nuclear proteins of each sample (control or treated) had a comparable capacity to bind DNA by using yet another recognition site (Sp1) (data not shown). It has been shown that NR1 binds both CAR and PXR, but with a clear preference for CAR (31,32). Interestingly, the nuclear extracts from DAS-treated rats did not form any complex with ER6/DR4 sequence (Figure 6B), which has a higher affinity for PXR than for CAR (33). Our data suggest that CAR is involved in the NR1 binding complex after a DAS treatment.

**Fig. 5.** In vivo activation of (NR1)3-tk-luci construct by IP DAS treatment. 80 µg (NR1)3-tk-luci and pSV-βgal was coated with jetPEI™ and transfected into livers of rats. 18 h after transfection, rats were IP-injected with NaCl (control), DAS (80 mg/100 g). Six hours after IP treatment, a small area of liver that had been shot with plasmids was removed, crushed and 10 dosages per rat were assayed for reporter gene activity. Diagrams represented means of arbitrary units obtained by chemiluminescent assays of luciferase and β-galactosidase activity. *Significantly different (P < 0.05) in comparison with controls.

### Discussion

Our work dealt with DAS and DADS-mediated activation of CYP2B1/2 and CYP3A genes that are both responsible for the metabolism of a large spectrum of drugs. We also studied EpH as a surrogate of second phase enzymes inducible by organosulfur compounds (34). We show here that DAS and DADS stimulated CYP2B1/2, CYP3A and EpH mRNA differently in terms of activation profile and range of stimulation factors. DAS was a more potent activator of CYP2B than DADS [(11) and Figure 2] and CYP3A was less induced than CYP2B1/2 and EpH (Figure 2). This result could be related to observations that supplementation with garlic oil did not significantly alter the levels of CYP3A4 in healthy volunteers (35). The profile of stimulation with DAS and DADS depended on the mode of chemical administration, i.e. gastric infusion or i.p. injection. DAS induced a stimulation of CYP3A after a gastric infusion whereas it did not stimulate CYP3A after an IP injection (compare Figure 2, Figure 3 and Figure 4). The different effects observed between DAS and DADS as well as the different effects observed when they were administered by IP or by gastric infusion could be triggered by the production of distinct metabolites and as a consequence by different signalling pathways involved. The main metabolites produced from DAS are allyl mercaptan and allyl methyl sulfide, which are detected both in primary rat hepatocytes (36) and in the liver of rats after an oral administration of DADS (37). DAS has been shown to be converted in diallyl sulfoxide and diallyl sulfone in vivo (38) and in allyl methyl sulfide in primary rat hepatocytes (36).

In the case of an IP injection, our results suggest that CAR is preferentially involved in DAS-mediated activation of CYP2B. The possible role of CAR in the effect of DAS is supported (i) by the DAS-sensitive gene panel such as CYP2B1/2 (Figures 3 and 4), (ii) by the inhibitory effect of ac.d (39) and Figure 3) and okadaic acid (Figure 4), (iii) by the capacity of NR1 at relaying the effect of DAS (Figure 5), (iv) by the presence of a NR1/binding complex (Figure 6), (v) by a previous study (17). CAR and PXR are both nuclear receptors with overlapping functions and with similar DNA recognition motifs, so it is difficult to discriminate between these two factors. Nevertheless, our data suggested that PXR is not implicated in DAS effect when administered by IP injection. Indeed, CYP3A, which is the most sensitive target of PXR (40), was not stimulated by DAS after an IP injection (Figures 3 and 4). Moreover, no binding complex was observed after DAS treatment by using an ER6/DR4 element, yet being a preferential site of recognition for PXR (29). At last, the PXR intracellular localization is not impaired by OA treatment (41), while, in our study, DAS-modulated CYP2B activation was abolished by OA (Figure 4). We postulate that DAS, by being a weak activator (in comparison with PB), led to the activation of the highest sensitive targets of CAR like CYP2B and NR1. After a gastric infusion or by extension in the case of classical nutrition using garlic, it is possible that both CAR and PXR are involved.

As it has been shown previously (17,42), DAS and PB i.p. injection led to a similar drug-sensitive induction profile of CYP2B1/2 mRNA (Figures 3 and 4), PB being stronger than DAS. Yet another common feature between PB and DAS is their ability to induce (NR1)3-tk-luci in vivo (Figure 5), but not in cell lines (like CV1 or HepG2) [data not shown and (28)]. None of the cell lines, even those transiently transfected...
with an expression vector for CAR, responds to DAS by inducing (NR1) 3-tk-luci (data not shown). Like in the case of PB, it can be postulated that DAS activates CAR through an indirect mechanism and that the transport of CAR into the nucleus is an essential step leading to DAS-induced CYP2B activation (28,43,44). Even though a direct binding of DAS with CAR seems to be unlikely, supplementary data are necessary to determine how DAS (or its metabolites) activates CAR.

Garlic is commonly appreciated for its flavour in traditional meals but is also consumed as a food supplement in the form of odourless and tasteless pills (45). These diet supplements are used because garlic is known for decreasing cardiovascular diseases and cancers risks. However, no study has established any clear protective effect of garlic, let alone of the supplements (46–48). An increasing web of studies indicates that the consumption of dietary supplements could have deleterious effects per se and could also modify the efficiency of conventional medicine (49). A long-term consumption of high doses of garlic oil leads to a modification of xenobiotic metabolism enzymes in human serum (50). Therefore, consistent with the suspicion of interference between garlic consumption and drug metabolism, we here strengthen molecular evidence for these interactions.

References
Activation patterns of rat metabolism genes

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