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Antioxidant perturbations in the olfactory mucosa of alachlor-treated rats☆

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Abstract

The chloracetanilide herbicide alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide) induces olfactory mucosal tumors in rats following chronic dietary exposure. Previous reports demonstrated that alachlor exposure was associated with depletion of glutathione (GSH) in liver *in vivo* and *in vitro*, but did not address this issue in the target tissue for the carcinogenic response. In this study we investigated a potential oxidative stress pathway in olfactory tissue by examining perturbations in olfactory mucosal antioxidants. Male Long–Evans rats were fed alachlor for up to 10 days (10–126 mg/kg per day), and intracellular reduced GSH and ascorbate levels were measured in olfactory mucosa. Both GSH and ascorbate rapidly decreased in olfactory mucosa following alachlor exposure, with a subsequent increase in both antioxidants to ~160% of control levels in the high dose group, and recovery of GSH to control levels in all groups by 10 days. Using Western blot analysis, we found that the modifier subunit of the rate-limiting enzyme in GSH synthesis, glutamate-cysteine ligase, increased in olfactory mucosa and remained elevated (126 mg/kg per day group). Two ascorbate transporters were detected by RT-PCR in olfactory mucosa, but neither appeared to be upregulated by alachlor exposure, and ascorbate synthesis was not stimulated in olfactory mucosa by alachlor treatment. Dietary exposure to alachlor depletes olfactory mucosa antioxidants, which may contribute to DNA damage and tissue-specific tumor formation.

Keywords: Olfactory mucosa; Alachlor; Glutathione; Ascorbate; Oxidative stress; Chemical carcinogenesis

1. Introduction

Alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide; alachlor] is a pre-emergent herbicide commonly used in the production of corn, soybeans, rice, and peanuts. Alachlor has been classified by the United States Environmental Protection Agency (USEPA) as a probable human carcinogen based on dose-dependant nasal tumor formation in Long–Evans rats chronically fed alachlor at 2.5–126 mg/kg per day [1]. Alachlor exposure is also associated with tumor formation in the stomach and thyroid of rats and in mouse lungs [2]. A recent epidemiological study found no increase in nasal cancer in humans exposed to high levels of alachlor; however, this study suggested an increased incidence of colorectal cancer in individuals involved in the manufacture of alachlor [3].

Alachlor is metabolized extensively, with the formation of 2,6-diethylaniline as an intermediate metabolite in rodents [4,5]. GSH plays a prominent role in alachlor metabolism, through the displacement of chloride, as well as reaction with a quinoneimine metabolite [4,6]. We have shown that alachlor-induced olfactory mucosal tumors (polypoid adenomas and adenocarcinomas) occur with a relatively short latency (i.e. following 5 months of exposure at 126 mg/kg per day in the diet) and high multiplicity, in that rats treated continuously at this level

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Abbreviations: GSH, reduced glutathione; RT-PCR, reverse transcription-polymerase chain reaction; GSSG, oxidized glutathione; GCL, glutamine-cysteine ligase; GCLC, catalytic subunit of GCL; GCLM, modifier subunit of GCL; SVCT, sodium-dependent vitamin C transporter; TTBS, Tris Tween buffer solution; MMPs, matrix metalloproteinases.

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for 12–18 months often exhibited 10–20 tumors per nasal cavity [7–9].

The complete mechanism of alachlor-related nasal tumor formation in rats has not been elucidated, but we have evidence that metabolic enzymes present in the olfactory mucosa, but not in the liver, bioactivate alachlor to one or more mutagenic species [7]. The sites of alachlor-induced tumor formation in the olfactory mucosa correspond with the distribution of cytochrome P450 2A3 [7], suggesting a role for this enzyme in the formation of a mutagenic/carcinogenic metabolite. The basis for the apparent resistance of mice to the development of alachlor-induced olfactory mucosal tumors [1] is also unclear.

Because several steps in alachlor metabolism involve GSH conjugation [4], we hypothesized that alachlor exposure may produce a sustained decrease in olfactory mucosal antioxidants. Antioxidant depletion could be an important component of alachlor carcinogenesis by inducing oxidative stress that may result in DNA damage [10]. Oxidative DNA damage causes point mutations and is associated with mutagenesis and cell transformation in Syrian hamster embryo cells [11] and tumor formation [12–15]. Glutathione depletion occurred in cultured rat hepatocytes with exposure to alachlor or the alachlor metabolite 2-chloro-*N*-(2,6-diethylphenyl)acetamide [16], and DNA damage resulting from *in vitro* exposure to alachlor has been observed in several model systems. DNA single-strand breaks occurred after alachlor exposure in rat hepatocytes [17], and alachlor was clastogenic in Chinese hamster ovary cells [18]. Although alachlor induced sister chromatid exchange and a clastogenic response in cultured human lymphocytes [19], cytogenetic damage proved to be a relatively insensitive means of detecting alachlor exposure in rats exposed *in vivo* to alachlor [20]. Alachlor caused a weakly positive response in both an Ames-type assay and mouse lymphoma assay with olfactory mucosal metabolic activation [21].

These experiments were designed to extend prior studies to examine the target tissue for alachlor carcinogenesis, the olfactory mucosa, for evidence of antioxidant perturbation in response to acute alachlor exposure. GSH, glutathione disulfide (GSSG), and ascorbate levels were measured in the olfactory mucosa following administration of alachlor in the diet at multiple doses above the threshold dose for tumor formation in this tissue. Based on the treatment-related changes observed in both GSH and ascorbate, levels of GCL and L-gulono-γ-lactone oxidase (ascorbate synthetase), the rate-limiting enzymes for synthesis of GSH and ascorbate, respectively, were measured in control and alachlor-treated rat olfactory mucosa and liver. In addition, olfactory mucosa was examined for the presence of ascorbate transporters, since it was found that this tissue lacks the enzymatic activity required to synthesize ascorbate. The results of these studies are discussed in the context of possible mechanistic implications for alachlor-induced olfactory mucosal carcinogenesis.

2. Materials and methods

2.1. Animals

This work was conducted in accordance with federal animal care guidelines and was pre-approved by the University of Cincinnati Institutional Animal Care and Use Committee. Male Long–Evans rats (4–5 weeks of age) were obtained (Harlan) and were acclimated to the housing conditions and powdered diet for 1–2 weeks before alachlor administration. All rats were between 5 and 7 weeks of age at the inception of treatment. Rats were assigned to treatment groups using a random number table and were housed individually in 18.5 in. × 9.5 in. × 6.5 in. cages containing sterilized corncob bedding (Anderson's Bedding). Age-matched controls were used for each treatment duration. Control rats were housed in the same room as the treated animals and randomly dispersed among the racks. Rats were weighed and 24-hr food consumption per body weight was recorded and used to calculate the concentration of alachlor necessary in the diet to achieve the desired doses. Alachlor, mixed into LM-485 sterilizable mouse/rat powdered diet (Harlan/Tekld), was administered at doses of 0, 10, 31.5, 63, or 126 mg/kg per day for 1, 2, 4, or 10 days (N = 4 rats per treatment per time point). Food was administered in glass cups with stainless-steel lids. Control rats received powdered diet only (N = 4 per time point). Cages and feed cups were sanitized weekly. The environment was maintained at 22 ± 1°, with a relative humidity of 50 ± 10%. Lighting was maintained on a 12-hr dark/light cycle, and animals were allowed tap water *ad libitum*. All rats were sacrificed between 9:00 and 11:00 a.m. to control for possible diurnal variations in GSH or ascorbate levels. Two rats were used per group for Western blot analysis of glutamate-cysteine ligase.

2.2. Reagents

Alachlor (98–99+% pure, Chem Service) was stored, desiccated, at −20° until use. Dr. Terrance J. Kavanagh (University of Washington) provided primary antibodies for detecting GCLC and GCLM subunits, as well as protocols for their use. All other chemicals and reagents were of the highest available grades unless otherwise noted (Sigma–Aldrich).

2.3. Preparation of tissue homogenates for antioxidant assessment

Tissue preparation and glutathione (GSH and GSSG) measurement methods were adapted from previously described methods [22]. Olfactory mucosa was prepared

by removing the ethmoid turbinates (including bone) using straight microscissors. (See [21] for diagram.) Analysis was also made of control liver, as well as livers from a subset of the treated rats. Briefly, 50–100 mg of tissue was homogenized with a steel tissue grinder in 750 μL of modified redox quenching buffer (20 mM HCl, 5 mM diethylenetriaminepentaacetic acid in water) prepared without ascorbic acid. From each homogenate, a 20 μL aliquot was removed for protein assay (Bradford assay, BioRad). Immediately thereafter, 750 μL redox quenching buffer with 10% trichloroacetic acid was added to each sample to precipitate protein. All steps were performed on ice. Samples were centrifuged at 10,000 *g* for 10 min at 4°. Supernatants were removed and centrifuged in filter tubes (Ultrafree Millipore tubes, 0.45 μm cutoff, Millipore Corp.) at 100 *g* for 2 min at 4°. Samples were stored at −70° until they were assayed for glutathione and ascorbate (within 2 weeks).

2.4. Glutathione assay

Approximately half of each tissue homogenate prepared above was used for glutathione measurements according to a previously described fluorometric method [22]. The concentration of GSH and GSSG in each sample was interpolated from known GSH or GSSG standards. Duplicate samples were averaged to get one measurement per tissue per rat.

2.5. Ascorbate assay

The remaining tissue homogenates prepared above were used to measure ascorbate levels using an assay adapted from previously described methods [23]. Ascorbate standards were prepared in 5% trichloroacetic acid, and aliquots of each sample (500 μL sample added to 100 μL water) were processed with known standards (600 μL of standard, ranging from 0 to 100 nmol ascorbate). After the sequential addition of 40 μL 85% H_3PO_4, 300 μL 1% 2,2′-bipyridine, and 40 μL 3% ferric ammonium sulfate, samples were mixed and incubated at room temperature for 30 min. Absorbance was determined at 525 nm. The concentration of ascorbate in each sample was interpolated from the known standards. Single ascorbate measurements were made for each rat tissue sample (due to the limited amount of olfactory tissue that could be harvested from each rat).

2.6. Ascorbate synthesis assays

Microsomes were prepared from the olfactory mucosa and liver of untreated Long–Evans rats as previously described [24]. Microsomes were similarly prepared from the control lungs (in order to evaluate another portal of entry tissue), olfactory mucosa of rats treated for 1 day with alachlor, as well as the livers of rats treated for 2 or 4 days (126 mg/kg per day). These microsomal preparations were used to measure L-gulono-γ-lactone oxidase (ascorbate synthetase) activity in the respective tissues as follows: microsomal proteins (0.5 mg/mL in 100 mM potassium phosphate, pH 7.4) were combined with NAD (4 mM), EDTA (1 mM), and nicotinamide (75 mM); L-gulono-γ-lactone (10 mM), was added to initiate the reaction, in a final volume of 1.4 mL (method designed by our laboratory based on [23]). Measurement of ascorbate concentrations (representing the amount synthesized) in each microsomal preparation was made at several time points between 0 and 20 min of incubation (as described above).

2.7. Western blot analysis for GCL

GCL (catalytic (C) and modifier (M) subunits) levels in olfactory mucosa of untreated rats were compared to GCL levels in rats treated with alachlor (126 mg/kg per day for 1, 2, 4, or 10 days). Western blot analysis was adapted from previously described methods [25]. Ethmoid turbinate tissues were homogenized on ice in homogenizing buffer (20 mM Tris, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 10 μg/mL aprotinin (pH 7.4)), and the homogenates centrifuged for 15 min at 21,000 *g* at 4°. Supernatant proteins (20 μg per lane) were separated by gel electrophoresis under denaturing conditions (12% acrylamide minigels) and transferred to nitrocellulose membrane (BioRad). Equivalent loading and effectiveness of transfer were confirmed by staining the membrane with Ponceau red. Membranes were blocked for 3–4 hr at 4° in 3% bovine serum albumin and 5% powdered milk in TTBS (TTBS = 137 mM NaCl, 0.1% Tween-20, 20 mM Tris, pH 7.6). All antibodies were diluted in TTBS containing 3% bovine serum albumin and 1% ovalbumin. Membranes were incubated with anti-GCLC (1:20,000) overnight (12–16 hr) at 4°. After rinsing four times in TTBS for 10 min at room temperature, membranes were incubated with secondary antibody (horseradish peroxidase-conjugated swine anti-rabbit, 1:1000, Dako Corp.) for 1 hr at 4°. Membranes were rinsed with TTBS four times for 10 min at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) followed by exposure to X-ray film (Kodak X-OMAT LS Scientific Imaging film). After rinsing and repeating the blocking step (above), membranes were then incubated with primary anti-GCLM (1:10,000) overnight at 4°. Membranes were not stripped between primary antibody applications because the differences in molecular mass of the two subunits (72.8 for GCLC and 30.8 for GCLM [26–28]) allowed them to be well resolved on the gels. Rinses, incubation with secondary antibody (swine anti-rabbit, 1:1000), and protein band visualization were repeated as above. Western blot results (densitometry measurements of bands of interest on X-ray films) were expressed as percent control based on quantitation performed using MetaMorph, version 4.6 (Universal Imaging Corp.).

2.8. Detection of ascorbate transporter mRNA by RT-PCR

Primers to detect the ascorbate transporters (SVCT) SVCT-1 and -2 were designed based on their respective cDNA sequences and across introns such that genomic DNA was not detected. Primers for rat SVCT-1 (Genbank accession #AF080452) were designed in the translated region of SVCT-1 in an area with low homology to SVCT-2 based on a BLAST2 alignment search. The sense primer was [AGCTGCGGCCGCCTACTGATCGTCCTG-TTCTCC], encompassing nucleotides 681–701 of the cDNA sequence. The antisense primer was [AGCTAAGC-TTAACATTCCCAGAACTGCAGCC] and encompassed nucleotides 1000–980 of the cDNA sequence. Primers for rat SVCT-2 (Genbank accession #AF080453) were designed in the 3′ untranslated region of the cDNA sequence. The sense primer was [AGCTGGATCCCA-CAGTACGGCATGCATCTG] and encompassed nucleotides 2180–2199 of the sequence. The antisense primer was [AGCTAAGCTTCTAGACCTCGAAGCAGCTTG], encompassing nucleotides 2487–2468 of the cDNA sequence. Transporter expression was evaluated in control rat olfactory mucosa, liver, and lung. For examination of transporter expression during the periods of ascorbate depletion and rebound, olfactory mucosal RNA was also prepared from two rats per time point (126 mg/kg per day dose). RNA was isolated using TriReagent (MRC) per the manufacturer's protocol. The final pellet was resuspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically (A_{260}).

Total RNA (200 ng) was combined with 5 mM $MgCl_2$, 1× reaction buffer (50 mM Tris–HCl, pH 7.5, 20 mM NaCl, 20 μM EDTA, 0.002% NP-40 v/v, and 10% glycerol v/v), 1 mM dNTPs, 1 U/μL RNase inhibitor (50 kDa recombinant enzyme; Roche/Applied Biosystems), 2.5 U/μL reverse transcriptase, and 50 μM oligo d(T) primer was reverse transcribed using a GenAmp RT-PCR kit (Applied Biosystems). For the PCR reaction, 4 μL of the reverse transcription reaction was combined with $MgCl_2$ to a final concentration of 3 mM, 1× reaction buffer, 0.8 mM dNTPs, 0.02 U/μL Taq polymerase and 0.2 μM primers. Thermal cycler conditions were as follows: [95° × 1, 62° × 1, 72° × 1 min] × 30 cycles, followed by an extension step of 5 min at 72°. PCR products were separated on a 1.0% agarose gel in 1× TAE buffer and stained with ethidium bromide.

2.9. Statistics

Statistical significance was determined by one-way ANOVA followed by pairwise multiple comparisons (Tukey test) performed by SigmaStat statistical software, version 2.0. Control GSH and ascorbate values were pooled from all treatment time points. Outliers, determined by the *Q*-test and *t*-value test, were removed from the analysis. Unless otherwise noted, significant differences are those with $P < 0.05$.

3. Results

3.1. Glutathione levels

Glutathione disulfide (GSSG) was uniformly low (<3% of GSH concentration) in all olfactory mucosal samples and did not change with alachlor administration. Consequently, only GSH values were reported for all samples (Fig. 1). Olfactory mucosa and liver from untreated rats contained 27 and 35 nmol GSH/mg protein, respectively, and alachlor treatment did not significantly affect GSH or GSSG levels in liver (data not shown). In contrast, a

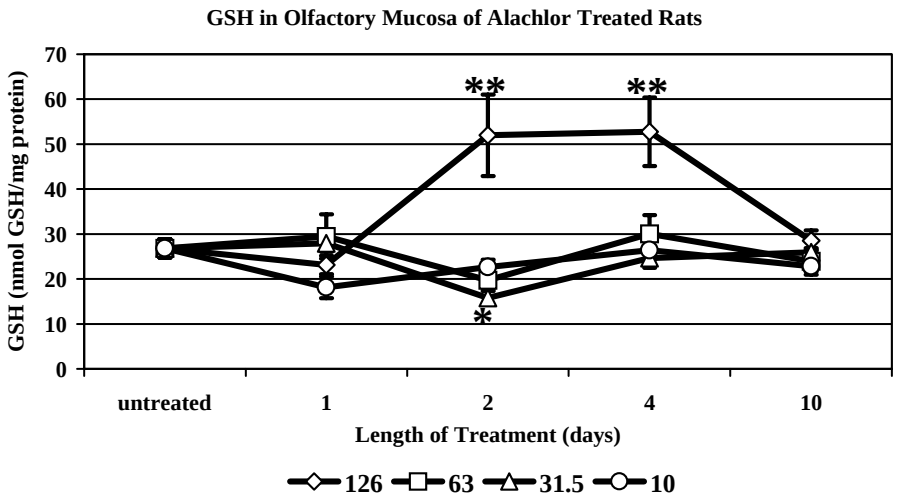

Fig. 1. Reduced glutathione (GSH) levels in olfactory mucosa homogenates from rats treated with alachlor at 10, 31.5, 63, or 126 mg/kg day for 0, 1, 2, 4, or 10 days. Values are nmol GSH/mg protein ± standard error. A significant decrease in olfactory mucosal GSH (∗) was observed after 2 days of treatment at 31.5 mg/kg per day ($P = 0.024$). GSH levels were elevated in olfactory mucosa (∗∗) after 2 and 4 days treatment at 126 mg/kg per day ($P < 0.001$).

significant decrease in olfactory mucosal GSH was observed after 2 days of treatment at 31.5 mg/kg per day ($P = 0.024$). GSH levels were elevated in olfactory mucosa after 2 and 4 days treatment at 126 mg/kg per day ($P < 0.001$). Rats receiving lower doses of alachlor showed a similar pattern of initial decrease in GSH levels after 1 or 2 days of treatment, with return to control levels by 10 days of treatment. The lower alachlor doses did not result in an increase in GSH relative to control levels, as was observed with alachlor treatment at 126 mg/kg per day.

3.2. Ascorbate levels

The concentration of ascorbate in olfactory mucosa from untreated rats was approximately 4-fold higher than liver (olfactory mucosa 90.0 ± 10.5 vs. liver 25.2 ± 2.0 (mean $\pm$ SE, N = 15)). Significant decreases in ascorbate were observed after 1 day at 10 or 126 mg/kg per day, 2 days at 10 or 31.5 mg/kg per day, 4 days at 10 mg/kg per day, and 10 days at 10, 31.5, or 63 mg/kg per day ($P < 0.03$). Increased olfactory mucosal ascorbate was observed after 2 and 4 days at 126 mg/kg per day ($P < 0.02$) (Fig. 2). The increase in ascorbate was similar to the response observed with GSH in the olfactory mucosa, and was not seen in the lower dose groups. After 10 days of treatment, ascorbate had not returned to control concentration in the olfactory mucosa of treated rats. Similar to results with GSH measurements, liver ascorbate levels did not vary significantly with treatment (data not shown).

3.3. GCL in olfactory mucosa

GCLC levels showed a modest increase in olfactory mucosa after 1 day of exposure to alachlor (126 mg/kg per day). After 10 days of treatment, GCLC levels in olfactory mucosa of treated animals were similar to those in control animals. GCLM increased markedly in the olfactory mucosa of treated animals (126 mg/kg per day) as compared to controls. The increase in GCLM was still apparent after 10 days of treatment (Table 1).

3.4. L-Gulono-γ-lactone oxidase (ascorbate synthetase) activity

Ascorbate synthetase activity was measured in membrane preparations from olfactory mucosa, liver, and lung of control and alachlor-treated rats. Ascorbate synthesis was not detected in control rat olfactory mucosa or lung, whereas liver has measurable ascorbate synthetase activity

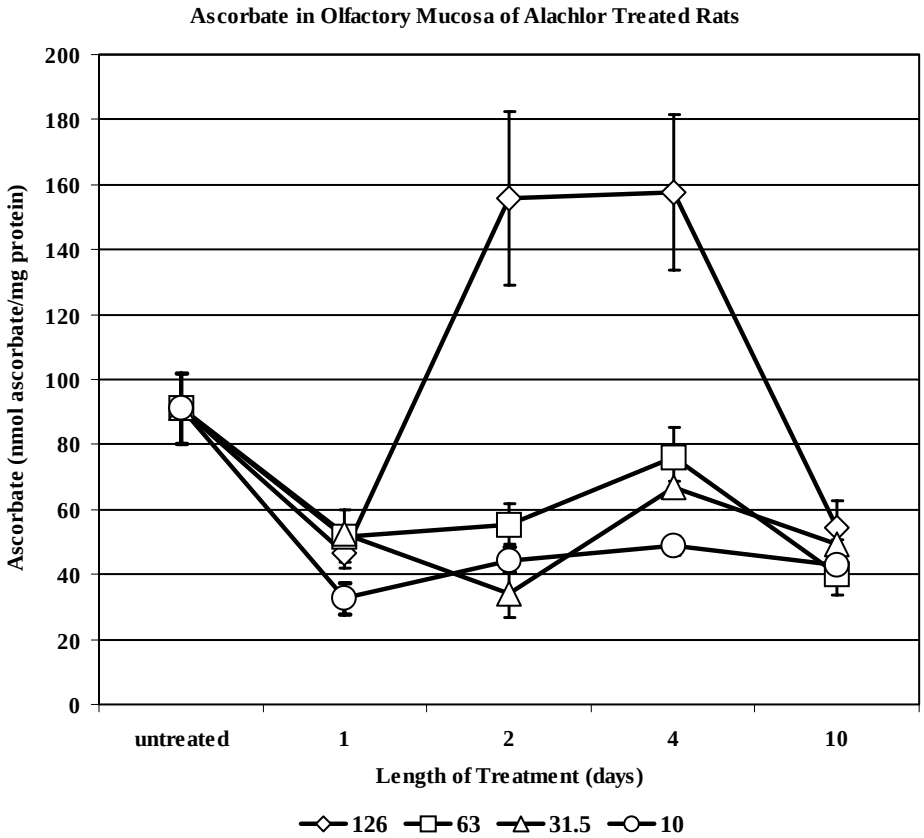

Fig. 2. Ascorbate levels in olfactory mucosa homogenates from rats treated with alachlor at 10, 31.5, 63, or 126 mg/kg per day for 0, 1, 2, 4, or 10 days. Values are nmol ascorbate/mg protein $\pm$ standard error. Significant decreases in ascorbate were observed after 1 day at 10 or 126 mg/kg per day, 2 days at 10 or 31.5 mg/kg per day, 4 days at 10 mg/kg per day, and 10 days at 10, 31.5, or 63 mg/kg per day ($P < 0.03$ for all). Increased olfactory mucosal ascorbate was observed after 2 and 4 days at 126 mg/kg per day ($P < 0.02$ for both time points).

Table 1
GCLC and GCLM in olfactory mucosa

Duration	GCLC %C (±SE)	GCLM %C (±SE)
Untreated	100 (6.3)	100 (17.8)
1 day	137 (11.8)	155.1 (2.7)
2 days	125.3 (20.1)	144.4 (9.2)
4 days	130 (31.6)	142.4 (10.8)
10 days	107.7 (7.3)	147.3 (5.3)

Densitometry measurements showing relative levels of glutamate-cysteine ligase catalytic (GCLC) or modifying (GCLM) subunit following Western blot analysis in olfactory mucosa from alachlor treated rats (126 mg/kg per day). Values are expressed as percent control (standard error).

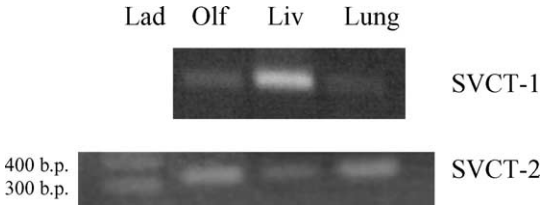

Fig. 4. RT-PCR results, showing expression of both ascorbate transporters in rat olfactory mucosa. SVCT-1 mRNA is enriched in liver (Liv), relative to lung and olfactory mucosa (Olf), whereas SVCT-2 appears to be more highly expressed in olfactory mucosa and lung than in liver. Lad = DNA ladder.

(Fig. 3). Ascorbate synthase activity was not induced in any of these tissues by alachlor exposure (not shown).

3.5. Olfactory mucosal ascorbate transporters

Because ascorbate synthetic activity was not detected in olfactory mucosa, ascorbate must be transported into the cells of this tissue. Since expression of the two known ascorbate transporters (SVCT-1 and -2) has not been described in olfactory mucosa, we initially attempted to characterize ascorbate transporters present in olfactory mucosa by immunohistochemistry and Western blot analysis, but commercially-available antibodies (D-19 and S-19; from Santa Cruz Biotechnology) detected multiple protein bands in liver and olfactory mucosal membrane preparations (data not shown), indicating that they were unsuitable for these purposes. Therefore, RT-PCR for SVCT-1 and -2 mRNA was performed. Both SVCT-1 and -2 were expressed in olfactory mucosa, liver, and lung (Fig. 4). SVCT-1 mRNA was high in liver relative to olfactory mucosa and lung, and SVCT-2 mRNA was high in olfactory mucosa and lung compared to liver. No alachlor-induced changes in the mRNA levels of either SVCT-1 or -2 were detected in olfactory mucosa over the time course of these studies (data not shown).

4. Discussion

Although liver is presumably the initial site of alachlor metabolism [4,5], antioxidant perturbations occurred in the olfactory mucosa, but not in liver, after alachlor administration in the diet. This observation represents the first examination of antioxidant levels in the target tissue for alachlor exposure following *in vivo* alachlor administration, and supports previous findings that extraordinarily high (1000 mg/kg) *in vivo* alachlor doses are required to cause GSH depletion in liver [6]. As evidenced most dramatically at a dose 126 mg/kg per day, alachlor resulted in an initial decline in olfactory mucosal GSH followed by a recovery during which GSH increased above control levels before returning to normal. Increased GSH levels are normally attributed to several factors, including (1) an increase in cellular cysteine levels or, more commonly,

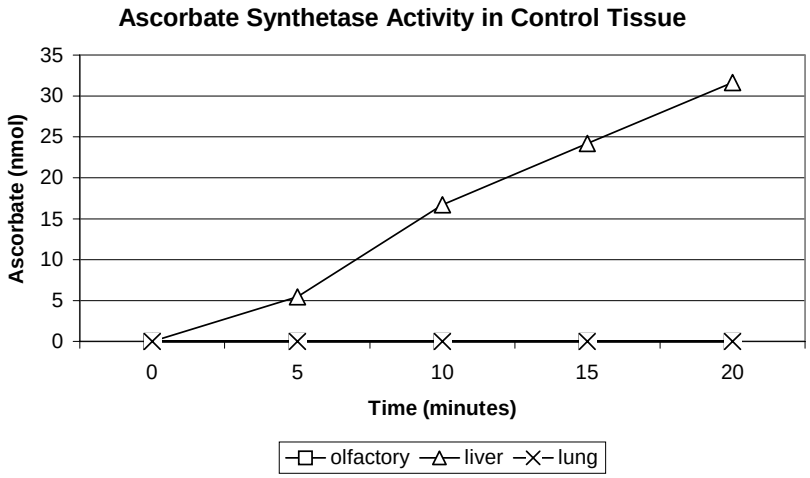

Fig. 3. Ascorbate synthesis following 20 min of incubation of L-gulono-γ-lactone with microsomes from control rat olfactory mucosa, liver, and lung. Reactions contained 0.5 mg/mL microsomal protein, EDTA (1 mM), L-gulono-γ-lactone (10 mM), NAD (4 mM), and nicotinamide (75 mM) in 100 mM potassium phosphate, pH 7.4. L-Gulono-γ-lactone oxidase activity was detected only in liver. No ascorbate synthesis was detected in olfactory or lung microsomes (symbols are superimposed in figure). Alachlor treatment (126 mg/kg per day) did not induce L-gulono-γ-lactone oxidase activity in olfactory mucosa or liver (data not shown).

(2) an increase in the activity of GCL, the rate-limiting enzyme in GSH biosynthesis. Although we did not measure the former, our analyses demonstrated an alachlor-dependent increase in GCL. The most catalytically active form of GCL is a heterodimer comprised of a catalytic (GCLC) and modifier (GCLM) subunit. GCLC is responsible for catalysis, which is less efficient in the absence of GCLM. Indeed, mice lacking GLCM accumulated only approximately 10% of normal GSH levels in all tissues surveyed [29]. This is believed to occur because GCLM reduces non-allosteric feedback inhibition of GCLC by GSH [30–33]. Thus, alachlor treatment initially resulted in a decrease in GSH in olfactory mucosa, which was followed by the up regulation of both GCLC and GCLM, thus increasing GSH biosynthesis to normalize GSH levels. Increases in transcription of both GCLC and GCLM are documented to occur under pro-oxidant conditions, and both genes are positively regulated by electrophile response elements in their 5′-flanking sequences [34–38]. Although regulation of GCLC and GCLM in response to electrophiles has not previously been described in olfactory mucosa, these data support the hypothesis that an electrophilic alachlor metabolite depleted GSH and thereby increased GCLC and GCLM gene expression. Also in agreement with this, we have documented the accumulation of heme oxygenase 1 (HO1) mRNA in olfactory mucosa from alachlor-treated rats [8]. HO1 was transcriptionally induced following oxidant insult of a multitude of cell and tissue types [39].

Following alachlor-induced GSH depletion, we observed a sustained increase in GCLM, but not GCLC, in the olfactory mucosa. Increased levels of GCLM were observed after 1 day of treatment, concomitant with GSH depletion, and remained elevated through 10 days of treatment, as GSH returned to control levels. The prolonged modification in olfactory mucosal GCLM expression associated with alachlor treatment suggests that increased levels of GSH are necessary to eliminate alachlor or metabolites of alachlor formed in this tissue. This contention is supported by the observation that GSH levels return to control levels, while GCLM levels remain elevated. Sustained use of olfactory mucosal GSH for conjugation and elimination of alachlor, as well as metabolites formed in this tissue, may explain the observation that GSH levels decrease without an associated increase in GSSG.

While a significant decrease in GSH was not observed at all doses of alachlor used in these studies, significant diminution of ascorbate was observed at all doses used. Further, ascorbate levels did not return to control levels within the 10 days time course of these studies in all dose groups. It should be noted, however, that levels of GSH are not independent of levels of ascorbate. For example, it has been demonstrated that ascorbate supplementation of rats which have been depleted of GSH using a GCL inhibitor is protective against oxidative pathologies and actually leads to increased GSH levels [40]. Thus, it is not possible to exclude the involvement of GSH in alachlor detoxification even at low alachlor concentration, but in our studies, ascorbate depletion was a more sensitive indirect measure of alachlor-induced olfactory mucosal oxidative stress.

Most vertebrates phylogenetically higher than fish, including rats, express L-gulono-γ-lactone oxidase in the liver and kidney, while primates, guinea pigs, some bats, and passeriform birds do not possess this enzyme, making these latter species dependent on dietary sources of this antioxidant [41]. The enhanced level of ascorbate in olfactory mucosa of alachlor-treated rats following the initial depletion was not due to enhanced olfactory mucosal synthesis of ascorbate, as L-gulono-γ-lactone oxidase (ascorbate synthetase) activity was not detectable in olfactory mucosa of control or alachlor-treated rats. L-Gulono-γ-lactone oxidase activity was detected in control rat liver, as previously reported [42]. Although lung has been previously demonstrated to have ascorbate at a concentration comparable to olfactory mucosa [43], this tissue too lacks ascorbate synthetase activity. Our results extend previous findings to show that toxicant-induced ascorbate perturbation does not stimulate ascorbate synthesis in olfactory mucosa or in liver. We, therefore, hypothesized that one or more ascorbate transporters must be expressed in the olfactory mucosa. Ascorbate transporters, namely ‘sodium vitamin C transporters’ (SVCT)-1 and -2 are expressed in many tissues in multiple species [44]. SVCT-1 is most often found in epithelial cells, whereas SVCT-2 is most often found in metabolically active cells and specialized tissues in the brain, eye, and other organs [44]. Using RT-PCR, we detected expression of both SVCT-1 and -2 in olfactory mucosa. Given that the olfactory mucosa has both epithelial and neuronal elements, our results are consistent with those of other investigators. We investigated upregulation of SVCT-1 and -2 as a possible basis for the increased concentration of ascorbate in olfactory mucosa subsequent to depletion, but no detectable change in message could be detected.

In addition to protection against oxidative stress, ascorbate plays an important role in regulation of extracellular matrix proteins. Ascorbate supplementation has been shown to increase type IV collagen synthesis [45–47], whereas ascorbate depletion decreases type IV collagen synthesis [46–48]. Collagen IV may be important in stabilizing the orientation of cell layers in the olfactory system [49]. Previously published data from our laboratory demonstrates that alachlor exposure leads to disrupted basal cell orientation in the olfactory mucosa [7]. This may be partially due to loss of collagen IV after ascorbate depletion and subsequent instability in cell layers in olfactory epithelium.

We originally expected that the depletion of antioxidants in the olfactory mucosa of alachlor-treated rats would be prolonged. However, GSH levels recovered to control levels within 10 days, concomitant with the upregulation of GCLM. In contrast, although ascorbate levels did not

recover in all dose groups over this time course, ascorbate transporter mRNA levels and ascorbate synthetase activity were unchanged. Despite the fact that GSH levels recovered, acute antioxidant perturbations may have been sufficient to trigger other steps in the carcinogenic process. We have previously demonstrated that genes encoding MMPs 2 and 9 were upregulated following 1 or 4 days of alachlor exposure [8]. Upregulation/activation of MMP-2 and/or MMP-9 and other enzymes involved in extracellular matrix degradation is highly correlated with oxidative stress *in vivo* and *in vitro*, and is associated with angiogenesis and tumor progression in human and murine breast cancers, as well as poor prognosis in human renal cell carcinomas [50–56]. Therefore, acute depletion of GSH and ascorbate may trigger more sustained events involved in both the initiation and promotion of the carcinogenic process.

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