SUPPRESSION OF XENOBIOTIC-METABOLIZING ENZYME EXPRESSION IN RATS BY ACRIFLAVINE, A PROTEIN KINASE C INHIBITOR

Effects on Epoxide Hydrolase, Glutathione S-Transferases, and Cytochromes P450

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ABSTRACT:

The effects of acriflavine (ACF), a protein kinase C inhibitor, on the expression of hepatic microsomal epoxide hydrolase (mEH), glutathione S-transferases (GSTs), and cytochrome P450 (P450) were assessed in rat hepatic tissue. Northern blot analysis revealed that treatment of rats with thiazole, allyl disulfide (ADS), oltipraz, or clotrimazole at a single dose of 100 mg/kg resulted in 7–18-fold increases in mEH mRNA levels at 24 hr, whereas concomitant ACF treatment (20 mg/kg, im) caused 50–95% inhibition of the chemical-induced increases in hepatic mEH mRNA levels. rGSTA2, rGSTA3, and rGSTM1 mRNA levels were also significantly suppressed at 24 hr in response to a single dose of ACF (20 mg/kg, im). Animals treated with both ACF and ADS showed complete blockage of mEH and GST gene expression as early as 12 hr after treatment. ADS-inducible increases in mEH and rGSTA2 mRNA levels were suppressed at 24 hr after treatment with ACF, in a dose-related manner, with 50% inhibitory dose (ID₅₀) values of 2.0–2.3 mg/kg, whereas gyceraldehyde-3-phosphate dehydrogenase mRNA levels were not altered. Immunoblot analysis revealed that ACF (15 mg/kg/day, im, for 3 days) inhibited induction of mEH or rGSTA2 protein by ADS (100 mg/kg/day, po, for 3 days). The levels of hepatic P450 2B1/2, P450 2C11, and P450 3A1/2 were decreased in rats treated with ACF (15 mg/kg/day, im, for 3 days), whereas P450 1A2 and P450 2E1 expression was not affected. Treatment of rats with ACF in combination with gadolinium chloride, which inhibits mEH and GST expression through calcium channel blocking, shifted the dose-inhibitory response curves for ACF to the left, with 7–15-fold decreases in the ID₅₀ values, indicating that the active site for ACF for suppression of mEH and GST mRNA levels differs from that for gadolinium chloride. Proflavine and safranine O, which are structurally related to ACF, also caused suppression of ADS-induced increases in mRNA levels, in a dose-dependent manner, with ID₅₀ values of 4–9 mg/kg. These results demonstrate that ACF and its related compounds effectively suppress the expression of a battery of hepatic xenobiotic-metabolizing enzymes, including mEH, GSTs, and certain P450 forms.

Expression of mEH, certain GSTs, and P450 forms is altered by xenobiotics and in certain pathophysiological states (e.g., hyperplastic nodules and hepatocellular carcinomas) (Griffin and Noda, 1980). Induction of hepatic xenobiotic-metabolizing enzymes is considered to be part of an adaptive response to chemical and oxidative stress (Hayes and Pulford, 1995). The detoxifying enzymes are transcriptionally activated in response to a variety of xenobiotics and/or reactive oxygen species through the interaction of specific nuclear binding proteins with cis-acting elements present in the upstream regions of the genes. It has been shown that AP-1-like transcription factors are involved in induction of GST by oxidant and prooxidant factors, through interaction of the transcription factors with cis-acting elements in GST genes (Hayes and Pulford, 1995).

ACF neutral, which has been used as a trypanocidal and antibacterial agent, has also been shown to eradicate Friend virus, in combination with other agents (Macadam and Williamson, 1974; Mathé et al., 1994). Our previous studies showed that ACF suppresses the proliferation of tumor cells and that guanosine enhances the antitumor effects of ACF in tumor-implanted animal models (Canellakis and Chen, 1979; Chakraborty et al., 1984; Kim et al., 1997a). ACF has also been studied as a potential anti-infective agent in fish (Yu et al., 1997). ACF binds avidly to plasma and nuclear membranes, and cellular membranes provide sites for binding of ACF in vitro, because of its interaction with polyionic substances (Ferey et al., 1986). Thus, ACF and its related compounds perturb the lipid bilayer and consequently inhibit enzyme activity (Roth et al., 1967). It has been shown that the enzymatic activity of PKC is potently inhibited by the presence of ACF in vitro (Hannun and Bell, 1988).

ACF perturbation of the function of membrane-associated proteins, including PKC, through modulation of cell membrane integrity might
alter the levels of components in signal transduction pathways (including immediate-early gene activation) in vivo and eventually affect a battery of genes activated by the nuclear transcriptional factor complexes. Given the new potential application of ACF as an anticancer agent and the dearth of available information regarding molecular and cellular changes in response to ACF in vivo, the present study was designed to examine the effects of ACF and related acridine derivatives on the expression of hepatic drug-metabolizing enzymes in rats.

Materials and Methods

Materials. ACF (composed of 3,6-diamino-10-methylacridium chloride and 3,6-diaminoacridine, in a ratio of 2:1), proflavine hydrochloride, and safranine O were purchased from Aldrich Chemical Co. (Milwaukee, WI). OZ was a gift from Rhône-Poulenc Rorer (Vitry-sur-Seine, France). [α-32P]dCTP (3000 Ci/mmol) was purchased from New England Nuclear Research Products (Arlington Heights, IL). Form-specific, polyclonal, rabbit anti-rat liver GST antibodies were purchased from Biotrin International (Dublin, Ireland). Polyclonal mouse anti-rat P450 1A1/2, P450 2B1/2, P450 2C11, and P450 2E1 antibodies were obtained from Oxford Biomedical Research (Oxford, MI). Biotinylated goat anti-rabbit IgG, streptavidin-conjugated hors eradish peroxidase, alkaline phosphatase-conjugated goat anti-mouse IgG, and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were supplied by Life Technologies (Gaithersburg, MD). Most reagents for the molecular studies were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal Treatment. Male Sprague-Dawley rats (150–200 g) were obtained from the Korea Food and Drug Administration (Seoul, Korea) and maintained at a temperature of 20–23°C, with a relative humidity of 50%. Animals were caged under a supply of filtered, pathogen-free air. Cheiljedang rodent chow (Seoul, Korea) and water were available ad libitum unless specified. Rats were treated with each of the inducing agents (100 mg/kg/day, for 1 or 3 days) with or without ACF injection (0.5–20 mg/kg/day, im, for 1 or 3 days). To determine the effects of ACF on inducible expression, the compounds (TH, ADS, OZ, and CL) were administered at 100 mg/kg, as studied previously (Kim and Cho, 1996; Kim et al., 1996, 1997b; Kim, 1992). TH was administered ip in an aqueous solution (0.5 ml/200 g rat), whereas ADS was administered by gavage using corn oil as a vehicle (~0.3 ml/200 g rat). OZ and CL were administered by gavage, suspended in a 0.1% carboxymethylcellulose solution (0.5 ml/200 g rat). ACF and related acridine derivatives were injected im at 0.5–20 mg/kg, in a 20 mg/ml aqueous solution, at the same time as the xenobiotics (~0.2 ml/200 g rat). The chemical structures of ACF and related acridine derivatives used in this study are shown in fig. 1. Animals were killed 24 hr after the last treatment, unless otherwise specified, and were fasted for 16 hr before death. Each data point represents the mean ± SD from three independent animal experiments. For most studies, two animals were pooled for each experiment.

Subcellular Fractionation. Male Sprague-Dawley rats were treated with ADS (100 mg/kg/day, po) and/or ACF (15 mg/kg/day, im) for 3 days. Hepatic microsomal and cytosolic fractions were prepared by differential centrifugation. The microsomes and cytosol were prepared from homogenates in 0.1 M Tris acetate buffer (pH 7.4), containing 0.1 M potassium chloride and 1 mM EDTA, by centrifugation at 10,000g for 30 min and then at 100,000g for 90 min. Microsomes were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. Microsomal and cytosolic preparations were stored at −70°C until use.

GST Assay. The activity of cytosolic GST was measured with 1-chloro-2,4-dinitrobenzene as a substrate, as described by Habig et al. (1974).

Immunoblot Analysis. Immunoblot analysis was performed according to previously published procedures (Kim and Cho, 1996; Kim et al., 1996, 1997b). Microsomal and cytosolic proteins were separated by 8% and 11% SDS–polyacrylamide gel electrophoresis, respectively, and electrophoretically transferred to nitrocellulose paper (Laemmli, 1970). The nitrocellulose paper was incubated with either rabbit anti-rat mEH or rabbit anti-rat GST antibodies, followed by incubation with biotinylated secondary antibodies. Immunoreactive proteins were visualized by incubation with streptavidin-horseradish peroxidase, followed by addition of both 4-chloro-1-naphthol and hydrogen peroxide. Replicate nitrocellulose filters were allowed to react with polyclonal mouse anti-rat P450 1A1/2, P450 2B1/2, P450 2C11, P450 2E1, or P450 3A1/2 antibodies, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG as the secondary antibody, and developed using 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium.

cDNA Synthesis and PCR Amplification. Specific cDNA probes for GST genes rGSTA2, rGSTA3, rGSTM1, and rGSTM2 were amplified by reverse transcription-PCR using selective primers for each gene, as described previously (Kim and Cho, 1996; Kim et al., 1996, 1997b). A 510-base pair cDNA probe for GAPDH was amplified by PCR using specific primers based on the sequence of a full-length cDNA (Fort et al., 1985). PCRs were performed for 40 cycles, using the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 3 min.

Northern Blot Analysis. Northern blot analysis was carried out according to the procedures described previously (Kim and Cho, 1996; Kim et al., 1996, 1997b). Total RNA (20 μg) isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and was then transferred to nitrocellulose paper by capillary transfer, followed by hybridization (Chomczynski and Sacchi, 1987; Puissant and Houdéune, 1990). The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 hr. Blots were incubated in hybridization buffer, containing 6× standard saline phosphate/EDTA (1× standard saline phosphate/EDTA contains 0.15 M NaCl, 10 mM NaH2PO4, and 1 mM Na2EDTA, pH 7.4), 200 μg/ml sonicated salmon sperm DNA, 0.1% SDS, and 5× Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin (Pentex fraction V)), at 42°C.

![Chemical structures of ACF neutral, proflavine, and safranine O.](image-url)
for 1 hr without probe. Hybridization was performed at 42°C for 18 hr with a heat-denatured probe, which was random-prime labeled with [\( \alpha ^{32}P \)]dCTP. Filters were washed in 2× SSC (1× SSC contains 0.15M NaCl and 0.015M sodium citrate, pH 7.0)/0.1% SDS for 10 min at room temperature twice and in 0.1× SSC/0.1% SDS for 10 min at room temperature twice. Filters were finally washed in 0.1× SSC/0.1% SDS for 60 min at 60°C. After quantitation of mEH and GST mRNA levels, the membranes were stripped and rehybridized with a cDNA probe for GAPDH, to quantify the amount of RNA loaded onto the membranes. Films were exposed at −70°C for 12–48 hr, using intensifying screens.

Scanning Densitometry. Scanning densitometry was performed with a microcomputer imaging device, model M1 (Imaging Research, St. Catharines, Ontario, Canada). The area of each lane was integrated using MCID software (version 4.20, revision 1.0), followed by background subtraction.

Data Analysis. Data were analyzed using computer programs for pharmacological calculations (Tallarida and Murray, 1987). One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparison of multiple group means. The Student t test was used to determine whether two population means differed significantly. The criterion for statistical significance was set at \( \alpha = 0.05 \) or \( \alpha = 0.01 \).

Results

Effects of ACF on Inducible mEH and GST mRNA Levels. Northern blot analyses were performed to determine the relative changes in mEH and GST mRNA levels in rats. Treatment of rats with a single dose of ACF at a dose of 20 mg/kg (im) caused 20–60% decreases in constitutive mRNA levels for mEH and major GST subunits, including rGSTA2, rGSTA3, and rGSTM1, whereas rGSTM2 mRNA was slightly decreased (fig. 2, table 1). The level of GAPDH mRNA was not altered by ACF. Whereas treatment of animals with a single dose of TH, ADS, OZ, or CL resulted in 18-, 9-, 14-, and 7-fold increases in mEH mRNA levels, respectively, at 24 hr after treatment, concomitant ACF treatment caused 50–95% inhibition of the chemical-inducible increases in hepatic mEH mRNA levels (fig. 2). Inducible expression of rGSTA2, rGSTA3, and rGSTM1 mRNA was also significantly decreased at 24 hr after ACF administration, although the relative level of rGSTM2 was reduced to a lesser extent (table 1).

Time Courses of mEH and GST Gene Expression. The time-dependent changes in mEH and major GST mRNA levels were monitored in rat livers after a single injection of ACF at a dose of 20 mg/kg. Because the effects of ADS on the expression of the enzymes had been studied previously (Kim et al., 1996; Cho and Kim, 1997), ADS was chosen as a model compound for subsequent experiments. Rats treated with a single dose of ADS (100 mg/kg, po) exhibited 6-, 10-, and 9-fold increases in mEH mRNA levels at 6, 12, and 24 hr, respectively (fig. 3). In contrast, animals treated with both ACF and ADS showed complete suppression of mEH gene expression at 12 and 24 hr, although a slight elevation of the mRNA was noted at 6 hr. Comparable time-dependent changes were observed in hepatic rGSTA2 gene expression. The levels of mEH and rGSTA2 mRNA at 24 hr after treatment with ACF appeared to be even less than those in untreated animals (fig. 3). ACF significantly suppressed the ADS-inducible mRNA levels for rGSTA3, rGSTM1, and rGSTM2 at 24 hr.

Dose-Dependent ACF Suppression of Gene Expression. The effects of ACF on hepatic mEH and rGSTA2 gene expression were assessed at 24 hr after treatment, to further determine whether ACF suppresses gene expression in a dose-related manner (fig. 4). ADS-inducible increases in mEH mRNA levels were reduced by ACF in a dose-dependent manner, with the ID\(_{50}\) values (95% confidence limits) being 2.0 (1.6–2.6) mg/kg. Dose-related suppression was also observed for rGSTA2 mRNA levels. The ID\(_{50}\) value (95% confidence limits) for ACF blocking of ADS-inducible increases in rGSTA2 mRNA levels was 2.3 (1.3–4.3) mg/kg.

Immunoblot Analysis of mEH and rGSTA2. mEH and rGSTA2 protein levels were determined by immunoblot analysis 3 days after ADS treatment (100 mg/kg/day, po) of animals, with or without ACF (15 mg/kg/day, im) (fig. 5). Whereas ADS treatment resulted in 4.0 ± 1.2- and 1.2 ± 0.1-fold increases in mEH and rGSTA2 protein levels, respectively, relative to untreated animals, concomitant ACF injection with ADS for 3 days resulted in 2.1 ± 0.3- and 0.9 ± 0.1-fold changes in the protein levels, yielding 47% and 19% decreases, respectively, compared with results without ACF (mean ± SD, significant at \( p < 0.01, N = 3 \)).

The GST catalytic activity was monitored with liver cytosol. The conjugating activity of GST toward 1-chloro-2,4-dinitrobenzene in hepatic cytosol produced from untreated rats or rats exposed to ADS or to both ADS and ACF was 1.11 ± 0.09, 1.63 ± 0.04, and 1.38 ± 0.09 µmol/min/mg protein, respectively. Thus, ACF significantly inhibited ADS-inducible GST activity by 50% (significant at \( p < 0.01, N = 4 \)).

Immunoblot Analysis of P450 Forms. Expression of major P450 forms in response to ACF was examined. The levels of hepatic P450 2B1/2, P450 2C11, and P450 3A1/2 were decreased to 60 ± 10%, 33 ± 12%, and 63 ± 15% of control, respectively, in rats treated with ACF at a dose of 15 mg/kg/day for 3 days (significant at \( p < 0.01, N = 3 \)).
increases in the relative inhibitory potency of ADS-inducible mEH and rGSTA2 mRNA levels, with 15- and 7-fold increases in mEH and GST mRNA levels were suppressed by proflavine in a dose-dependent manner, we were interested in using the previous observation that ACF is composed of 3,6-diamino-10-methyl-acridine and proflavine in a ratio of 2:1, we were interested in using the previous observation that ACF for the suppression of mEH and GST mRNA levels was altered 24 hr after treatment with a single dose of TH (100 mg/kg, ip), ADS (100 mg/kg, po), OZ (100 mg/kg, po), or CL (100 mg/kg, po), with or without a concomitant injection of ACF (20 mg/kg, im). Changes in the mRNA levels, relative to GAPDH, were assessed by scanning densitometry. Each value represents the mean ± SD from three separate animal experiments. Data were analyzed by one-way analysis of variance followed by the Newmann-Keuls test for comparison of multiple group means.

**Gadolinium Chloride Effect.** Given the previous observation that GdCl₃ suppresses mEH and GST mRNA levels through competitive blocking of calcium channels (Kim and Choi, 1997), rats were concomitantly treated with both GdCl₃ (10 mg/kg, iv) and ACF (0.5–10 mg/kg, im), to determine whether the relative potency of ACF for the suppression of mEH and GST mRNA levels was altered 24 hr after a single treatment. Northern blot analysis revealed that ACF in combination with gadolinium chloride reduced the ID₅₀ of ACF for the suppression of P450 2B1/2 (data not shown). Changes in the mRNA levels, relative to GAPDH, were assessed by scanning densitometry. Each value represents the mean ± SD from three separate animal experiments. Data were analyzed by one-way analysis of variance followed by the Newmann-Keuls test for comparison of multiple group means.

### Table 1

<table>
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<tr>
<th></th>
<th>Untreated</th>
<th>TH</th>
<th>ADS</th>
<th>OZ</th>
<th>CL</th>
</tr>
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<tbody>
<tr>
<td>mEH</td>
<td>0.4 ± 0.2</td>
<td>17.9 ± 4.9</td>
<td>9.1 ± 2.5</td>
<td>9.1 ± 3.8</td>
<td>0.3 ± 0.2</td>
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<tr>
<td>rGSTA2</td>
<td>0.8 ± 0.5</td>
<td>14.3 ± 6.8</td>
<td>10.2 ± 3.2</td>
<td>12.0 ± 5.6</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>rGSTA3</td>
<td>0.7 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>2.4 ± 0.5</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>rGSTM1</td>
<td>0.5 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.5</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>rGSTM2</td>
<td>0.6 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>0.4 ± 0.2</td>
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* p < 0.05, in comparison with the respective −ACF control.

### Discussion

In the previous study, we showed that ACF in conjunction with guanosine exhibits enhanced cytotoxicity in animals bearing tumors, including Ehrlich carcinoma, Ehrlich ascites tumor, and P388 leukemia (Kim et al., 1997a). The effect of ACF against tumor cell proliferation was also supported by the intense fluorescent staining of intracellular organelles with ACF, probably as a result of disruption of the plasma membrane (Ferey et al., 1986). Antitumor effects of ACF may be attributable to blockade of the function of proteins bound to the plasma membrane, which would result in perturbation of signaling pathways and impairment of nutrient transport.

ACF binds to the plasma membrane. The ACF binding sites in the plasma membrane include polymeric molecules with phosphate, sulfate, and carboxyl groups (Roth et al., 1967). It is likely that the amino groups present in acridine intercalate, through ionic bonding, with two phosphates present in phospholipids and that the interatomic distance allows the binding of the two amino groups on acridine to the phosphate moieties present in the membrane (Hannum and Bell, 1988).

ACF and its related acridine compounds perturb the lipid bilayer and consequently inhibit enzyme activity *in vitro* (Camellakis and Chen, 1979; Hannum and Bell, 1988). It has been demonstrated that the enzymatic activity of PKC is potently inhibited *in vitro* by the presence of ACF (Hannum and Bell, 1988).

In the present study, we report that ACF suppresses constitutive and inducible hepatic mEH and major GST gene expression. Expression of these hepatic enzymes is mediated by either production of reactive oxygen species and initiation of oxidative stress or activation of xenobiotic-responsive elements present on the upstream regions of the genes. The inducible mRNA levels for GST and mEH in response to typical chemical inducers, including antioxidants, were markedly suppressed by ACF, as shown in this study. In particular, ADS- or OZ-inducible elevations in mEH and rGSTA mRNA levels were decreased >90% by ACF. Maximal blocking of the mRNA increases was observed at 12–24 hr after treatment. This suppression in mEH and major GST mRNA expression could occur through either transcriptional inactivation or rapid turnover of the mRNAs (Daniel, 1993; Eickelmann et al., 1995). ACF suppression of the expression of mEH and major GST genes, as well as certain P450 forms, may be associated with the inhibition of PKC activity. The phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate, a PKC agonist, is known to induce murine rGSTA1/2, whereas 12-O-tetradecanoyl-phorbol-13-acetate, as well as the calcium ionophore A23187, causes a substantial decrease of rGSTA1/2 mRNA levels in HepG2 hepatoma cells through diminished GST α mRNA half-lives (Eickel-
mann et al., 1995). Thus, it is highly likely that the activity of PKC in the plasma membrane is coupled to transcriptional activation of certain GSTs.

mEH and major GST gene expression was inhibited in common by ACF, whereas the constitutive expression of P450 1A2 and P450 2E1 was not altered. ACF, however, caused significant decreases in the expression of P450 2B1/2, 2C11, and 3A1/2 proteins. Thus, ACF differentially affected the expression of P450 forms. Although multiple treatments of tumor-bearing mice with ACF in combination with guanosine produced cytotoxicity of tumor cells, no hepatic injury (i.e., necrosis or degeneration) was observed under the dose regimen used in this study. Thus, suppression of xenobiotic-metabolizing enzymes by ACF was not due to its cytotoxicity, which was supported further by unaltered transcription of the GAPDH gene as well as by the unaffected constitutive expression of P450 1A2 and P450 2E1. ACF showed greater efficacy and potency in blocking the chemical-inducible expression of mEH and GST genes than did proflavine or safra-nine O, which might be associated with the differences in pharmacokinetic profiles and tissue binding affinities.

The extent of inhibition of mEH and GST gene expression by ACF appeared to be greater than that by gadolinium chloride. Previous research in this laboratory showed that GdCl₃ inhibits constitutive and inducible mEH and major GST expression through inhibition of Ca²⁺ influx (Kim and Choi, 1997). ACF appeared to be more effective in suppressing inducible gene expression even when the doses of chemical inducers were elevated 2–4-fold, compared with those used with GdCl₃. Although gadolinium chloride failed to inhibit the inducible expression of mEH and GST caused by ADS at a dose of 100 mg/kg, treatment of rats with GdCl₃ in combination with various doses of ACF caused an increase in the relative inhibitory potency of ACF for
mEH and GST gene expression. These results indicate that the active site for GdCl$_3$ differs from that for ACF.

Inhibition of the function of plasma membrane-associated proteins by ACF may affect activation of the proteins involved in enzyme induction, such as Fos, c-Jun, and Jun B (Volm and Pommerenke, 1995). Diverse chemicals transcriptionally induce the GST genes through AP-1-like complexes (Hayes and Pulford, 1995; Bergelson et al., 1994a,b). It has been shown that increased levels of AP-1-like transcriptional complexes are associated with transcriptional activation of rGSTA2. Another study demonstrated that ACF completely inhibited the activation of nuclear AP-1 complexes by diethylnitrosamine (data not shown), which might contribute to decreases in mEH and GST gene expression.

Several cell surface receptors have been identified that bind LPS. LPS-induced alteration of cell function involves activation of membrane-associated G proteins, which are coupled to intracellular signal transduction systems. Activation of calcium- and phospholipid-dependent PKC has been observed after exposure of cells to LPS (Wightman and Raetz, 1984; Rosoff and Cantley, 1985). NF-κB acts as a messenger to transmit gene induction signals from the cytoplasm to the nucleus, through inactivation of IκB by a phosphorylation event involving PKC (Lenardo and Baltimore, 1989). It has been reported

![Fig. 4. Dose-dependent inhibition by ACF of the ADS-inducible expression of hepatic mEH and GST.](image)

Shown are representative Northern blot analyses carried out with total RNA fractions produced from untreated rats (UN) or rats treated with ADS plus ACF. The mEH, rGSTA2, and GAPDH mRNA levels were determined 24 hr after treatment with ADS (100 mg/kg, po) in combination with ACF at a dose of 0.5, 2, 5, 10, or 20 mg/kg body weight.

![Fig. 5. Immunoblot analyses of rat hepatic microsomal and cytosolic proteins with rabbit anti-rat mEH and rabbit anti-rat rGSTA1/2 antibodies, respectively.](image)

The lanes are associated with hepatic mEH and rGSTA1/2 proteins in untreated rats (UN) or rats treated with ADS (100 mg/kg/day, po) or ADS (100 mg/kg/day, po) plus ACF (15 mg/kg/day, im) for 3 days. Lanes contained 2 μg of microsomal proteins or 1 μg of cytosolic proteins for mEH or rGSTA1/2 blots, respectively. Results were confirmed in multiple immunoblots with different preparations.

![Fig. 6. Effects of ACF on hepatic P450 levels.](image)

Immunoblot analyses of rat hepatic microsomal proteins were carried out with anti-rat P450 1A2, P450 2B1/2, P450 2C11, P450 2E1, and P450 3A1/2 antibodies. Shown are representative immunoblots, with lanes associated with hepatic P450 proteins in rats treated with (+) or without (−) ACF (15 mg/kg/day, im) for 3 days. Each lane contained 10 μg of microsomal proteins. Results were confirmed in multiple immunoblots with different microsomal preparations.

![Fig. 7. Effects of ACF in combination with GdCl$_3$ on mEH and rGSTA2 mRNA levels.](image)

ADS-inducible expression of mEH and rGSTA2 mRNA was assessed 24 hr after treatment of rats with a single dose of ACF, with or without GdCl$_3$ injection. Northern blot analyses were performed with total RNA fractions derived from rats treated with ACF at a dose of 0.5, 1, 2, 5, or 10 mg/kg (im), with or without GdCl$_3$ at a dose of 10 mg/kg (iv). Animals were killed 24 hr after ACF treatment. Percent inhibition of mEH and rGSTA2 mRNA levels, relative to GAPDH, was plotted as a function of ACF dose. Each point represents the mean ± SD from three separate animal experiments. Data were analyzed by one-way analysis of variance followed by the Newmann-Keuls test for comparison of multiple group means. *, p < 0.05, in comparison with the respective control.
that increased phosphorylation of certain proteins occurs after exposure of murine macrophages to LPS, indicating that protein kinases are associated with the signal transduction process (Hewett and Roth, 1993). Gel retardation analysis revealed that ACF suppressed the expression of NF-κB triggered by LPS (data not shown). PKC and other protein kinases (including mitogen-activated protein kinases) functionally associated with the plasma membrane provide signal transduction pathways for external stimuli and are also involved in cellular proliferation. Thus, ACF perturbation of the activities of PKC and other plasma membrane-associated proteins through modulation of cell membrane integrity might lead to alterations in components of the signal transduction pathway involved in transcriptional gene activation. It has been shown that diithiocarbamate and iron chelators potently block the activation of NF-κB (Schreck et al., 1992). Blocking of the LPS-triggered NF-κB activation by antioxidants suggests a role for oxygen radicals in the intracellular signaling by LPS. The possibility that ACF inhibition of NF-κB may also affect a redox-regulated tyrosine kinase or a specific protease cannot be excluded. Expression of certain hepatic detoxification enzymes is dependent on the production of reactive oxygen species, which is strongly elevated in tumor cells. ACF suppression of mEH and major GST gene expression may contribute to the inhibition of tumor cell proliferation. mEH and GST are active in the detoxification of a number of chemicals, including chemotherapeutic agents, by conjugation reaction and drug sequestration. Because the expression of mEH and certain GSTs is highly correlated with tumor cell resistance to chemotherapeutic agents, suppression of detoxifying gene expression in tumor cells by ACF would be of assistance in tumor cell suppression.

References


