Biochemical Pharmacology, Vol. 52, pp. 863–867, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00353-X

Effect of Aniline on Ethanol Oxidation and Carbohydrate Metabolism in Isolated Hepatocytes

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ABSTRACT. The addition of aniline to isolated hepatocytes derived from fasted rats and incubated with ethanol, caused a 30–60% decrease in the rate of ethanol oxidation. The degree of inhibition was dependent on aniline concentration, 5 mM causing near-maximal inhibition. Aniline reduced the activity of alcohol dehydrogenase in a noncompetitive manner, but had no effect on aldehyde dehydrogenase activity nor on reducing-equivalent transfer between the cytoplasm and mitochondria. The inhibition of alcohol dehydrogenase by aniline was associated with a decrease in the inhibitory effects of ethanol on glycolysis. Aniline, added to hepatocytes in the presence or absence of ethanol, inhibited gluconeogenesis from lactate and pyruvate, but not from sorbitol or fructose. BIOCHEM PHARMACOL 52;6:863–867, 1996.

KEY WORDS. hepatocyte; ethanol oxidation; glycolysis; gluconeogenesis; alcohol dehydrogenase; aniline

In contrast to the ready inhibition of drug metabolism by an acute dose of ethanol [1–4], the inhibition of ethanol metabolism by drugs is uncommon. Inhibition of ethanol oxidation may occur as a result of a decrease in the activity of the enzymes responsible for the conversion of ethanol to acetate, namely alcohol dehydrogenase [5, 6] and aldehyde dehydrogenase [7, 8], competition for cofactors, or interference in the process of disposal of cytoplasmic reducing equivalents generated during ethanol oxidation [9–11].

Studies conducted almost 25 years ago [12, 13] reported that various drugs (i.e. acetanilide, aminopyrine, antipyrine, and aniline) depressed fructose- or pyruvate-stimulated ethanol oxidation, but no explanation of their mechanism of action has been reported. The aim of the present work was to investigate the effect of aniline on ethanol oxidation and determine whether or not aniline modulates the influence of ethanol oxidation on other metabolic processes.

MATERIALS AND METHODS

Materials

Collagenase and enzymes for metabolite determination were from Boehringer Mannheim (Sydney, NSW) as was bovine serum albumin (fraction V) that was defatted according to the method of Chen [14]. Aniline was purchased from Sigma (St. Louis, MO, U.S.A.). [U-^{14}C]Ethanol was obtained from Amersham (Sydney) and Ready-Safe scintillation cocktail was from Beckman (Fullerton, CA). Both aniline and [U-^{14}C]ethanol were redistilled before use. [U-^{14}C]Glucose was purchased from DuPont NEN Products (Boston, MA, U.S.A.). Crystallized equine liver alcohol dehydrogenase was from Sigma and yeast alcohol dehydrogenase was from Boehringer Mannheim Australia Pty., Ltd. All other chemicals were of the highest quality commercially available.

Methods

Hepatocytes from male Hooded Wistar rats (280–320 g body mass), fasted for 24 hr to deplete liver glycogen, were prepared by a modification [15] of the method of Berry and Friend [16], in which 1 mM calcium ions were added to the washing medium immediately following perfusion. Liver cells (90–120 mg wet wt) were incubated at 37° in 2 mL of a balanced bicarbonate-buffered medium containing 2.25% albumin under a gas phase of 95% O_2 and 5% CO_2 [17].

The incubation mixtures were deproteinized by the addition of an equal volume of ice-cold 1 M perchloric acid and neutralized with 2 M KOH. Metabolites were measured by standard enzymatic techniques [18], using a COBAS FARA automated analyzer (Roche Diagnostics, Basel, Switzerland), the data being transferred to a microVAX computer (Digital Equipment Corporation, Maynard, MA) for subsequent processing.

To measure the rate of glycolysis, hepatocytes were incubated with glucose in the presence of 0.5 μCi [U-^{14}C]glucose; the $^{14}CO_2$ produced was trapped in phenylethylamine and measured by liquid scintillation counting [19]. Total glycolysis was measured, in three-carbon units, from the sum of lactate + pyruvate + ⅓ CO_2. Flux of reducing

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Received 26 October 1995; accepted 5 February 1996.

TABLE 1. Effect of various substrates on the rate of ethanol oxidation

Additions	$J_{Ethanol}$ (μmol/min/g wet wt)	
	(-) Aniline	(+) Aniline (5 mM)
None	0.58 ± 0.01 (20)	0.36 ± 0.01† (6)
Lactate (10 mM)	1.60 ± 0.05* (20)	0.76 ± 0.01† (15)
Pyruvate (12 mM)	2.09 ± 0.17* (4)	0.89 ± 0.04† (4)
Fructose (10 mM)	1.77 ± 0.09* (5)	0.90 ± 0.06† (4)
Glucose (40 mM)	1.20 ± 0.08* (4)	0.61 ± 0.06† (4)

Isolated hepatocytes from fasted rats were incubated, as described in Materials and Methods, in 2 mL of incubation medium at 37° with the substrates indicated. All vessels contained 13 mM [1-^{14}C]ethanol. The rate of ethanol oxidation ($J_{Ethanol}$) was measured between 10–30 min. Results are the mean ± SEM with the number of individual cell preparations shown in parentheses. * $P < 0.001$ relative to the value for ethanol alone; † $P < 0.001$ relative to the value for incubations without aniline.

equivalents from sorbitol to oxygen was estimated by the sum of glucose + fructose + ½ lactate [20]. Ethanol oxidation was measured by a radioactive technique [21].

The activity of rat hepatocyte alcohol dehydrogenase was determined in extracts that were obtained by centrifugation (12000 *g*, 2 min) of a suspension of cells exposed for 10 min at 4° to an equal volume of 1% Triton X-100. These extracts were particulate-free on microscopy. Alcohol dehydrogenase activity was determined spectrophotometrically in 1 mL of 0.1 M glycine, pH 10, containing 4.5 mM NAD^+ and 25 mM ethanol at 37° [22]. The activities of high and low K_m acetaldehyde dehydrogenase were determined in particulate-free extracts and the activity of the low K_m enzyme was also measured using mitochondria isolated by the method described in [23]. Aldehyde dehydrogenase was assayed spectrophotometrically at 37° in 1 mL of 0.15 M sodium pyrophosphate, pH 8, containing 6 mM NAD^+ and acetaldehyde [22]. Two different concentrations of acetaldehyde (0.2 mM for the low K_m isoenzyme or 20 mM for the total enzyme activity) were used as substrate. All data are expressed as the mean ± SEM; statistical comparisons were based upon the Student's *t*-test.

RESULTS AND DISCUSSION

In the absence of added substrates, the average rate of ethanol oxidation by hepatocytes from fasted rats was 0.58 ± 0.01 μmol/min/g wet wt (N = 20), and was constant over a 30-min incubation period. The addition of 5 mM aniline to hepatocytes metabolizing ethanol alone decreased the rate of ethanol oxidation by approximately 33% (Table 1). In the presence of substrates, such as lactate, pyruvate, fructose, or glucose, the rate of ethanol oxidation by isolated liver cells was stimulated 2- to 4-fold [24–27] (Table 1). The addition of 5 mM aniline caused a 50–60% inhibition of these stimulated rates (Table 1). Aniline inhibited ethanol oxidation by hepatocytes in a dose-dependent manner (Fig. 1), a concentration of 5 mM causing almost maximal inhibition. This inhibition was present from the commencement of the incubation and was constant throughout

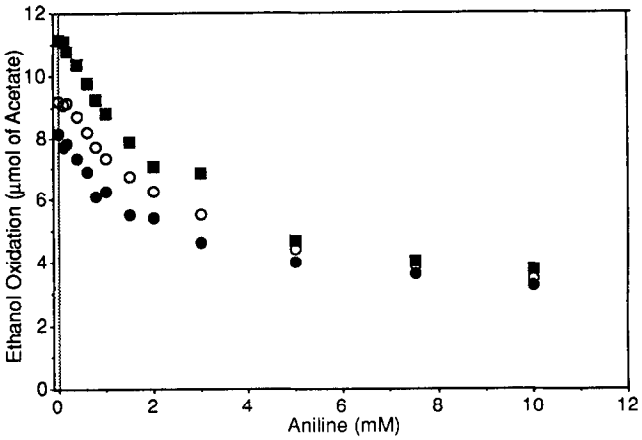

FIG. 1. Effect of a variable concentration of aniline on ethanol oxidation. Isolated hepatocytes from fasted rats were incubated for 40 min at 37°, as described in Materials and Methods, with 13 mM [1-^{14}C]ethanol and 10 mM lactate (●), 10 mM pyruvate (■) or 10 mM fructose (○). The data are from a representative experiment.

the experiment, indicating that aniline itself, rather than one of its metabolic products, was the responsible agent. The rate of ethanol oxidation by hepatocytes isolated from fed rats was inhibited by aniline to the same extent as the rate by cells from fasted rats (data not shown).

It is now widely accepted that the principal enzymatic system involved in the conversion of ethanol to acetaldehyde is alcohol dehydrogenase [28–30]. This has been confirmed in isolated hepatocytes, where alcohol dehydrogenase was shown to account for 90% of ethanol oxidation [31]. The so-called microsomal ethanol-oxidizing system has a relatively high K_m for ethanol [32] and the role of catalase in the metabolism of ethanol has been reported to be minor [33, 34]. Hence, alcohol dehydrogenase normally

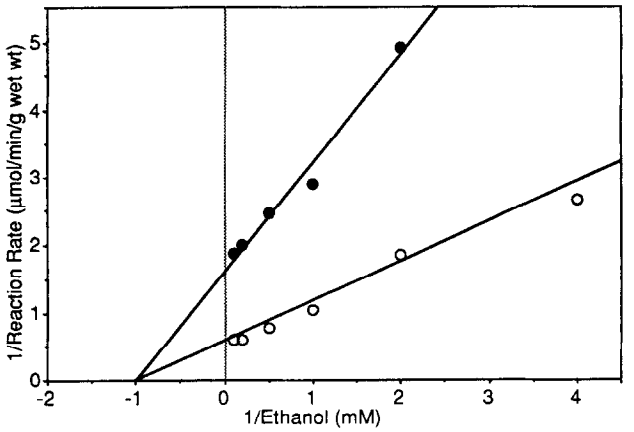

FIG. 2. Lineweaver-Burk plot of alcohol dehydrogenase activity. Particulate-free extracts were prepared from isolated hepatocytes as described in Materials and Methods. The activity of alcohol dehydrogenase was measured in particulate-free extracts in the absence (○) or presence of 5 mM aniline (●) with various concentrations of substrate (ethanol) at 37°. The data are from a representative experiment.

TABLE 2. Effect of sorbitol on the rate of glucose, lactate and fructose accumulation in the presence or absence of aniline

Additions	$J_{Glucose}$ (μmol/min/g wet wt)	$J_{Lactate}$ (μmol/min/g wet wt)	$J_{Fructose}$ (μmol/min/g wet wt)	Flux of reducing equivalents
Sorbitol (10 mM)	1.58 ± 0.08 (10)	0.17 ± 0.03 (8)	0.01 (6)	1.68 ± 0.19 (6)
Sorbitol (10 mM); aniline (5 mM)	1.51 ± 0.06 (5)	0.39 ± 0.12 (5)	0.01 (3)	1.72 ± 0.29 (3)

Isolated hepatocytes from fasted rats were incubated, as described in Materials and Methods, in 2 mL of incubation medium at 37° with the substrates indicated. The rates of glucose accumulation ($J_{Glucose}$), of lactate accumulation ($J_{Lactate}$), and of fructose accumulation ($J_{Fructose}$) were measured between 10–40 min. Flux of reducing equivalent transfer was determined from the sum of glucose + fructose + ½ lactate. Results are the mean ± SEM, with the number of individual cell preparations shown in parentheses.

accounts for the bulk of ethanol oxidation. The activities of the main enzymes responsible for the oxidation of ethanol to acetaldehyde and, thence, to acetate, alcohol dehydrogenase, and aldehyde dehydrogenase, respectively, were measured in particulate-free extracts and isolated mitochondria. The addition of 5 mM aniline significantly inhibited the activity of alcohol dehydrogenase, reducing it from 1.44 ± 0.15 to 0.64 ± 0.10 μmol/min/g wet wt (N = 5) ($P < 0.01$). A Lineweaver-Burk plot (Fig. 2) revealed that alcohol dehydrogenase was inhibited by aniline in a noncompetitive manner. Aniline (5 mM) inhibited crystalline equine liver alcohol dehydrogenase activity by 51 ± 4% (N = 3), but had no effect on crystalline yeast alcohol dehydrogenase. Equine and rat liver alcohol dehydrogenase were inhibited by aniline to a similar degree. This suggests that the action of aniline on dehydrogenases is specific for mammalian alcohol dehydrogenase. The question arose as to whether or not aniline inhibited other zinc-containing dehydrogenases, in addition to alcohol dehydrogenase [35, 36]. The effect of aniline on lactate dehydrogenase, which contains zinc [37], was examined. However, the activity of lactate dehydrogenase was not changed by aniline (data not shown). Aniline did not alter the activity of aldehyde dehydrogenase in particulate-free extracts. But, because the acetaldehyde arising from ethanol oxidation is thought to be oxidized mainly within the mitochondria [38], the activity of the low K_m mitochondrial enzyme was also measured and found to be unaffected by aniline (data not shown).

A potential rate-limiting step in ethanol oxidation from fasted rats is the transfer of reducing equivalents from the cytoplasm to mitochondria [11, 39, 40]. To test the effects of aniline on intercompartmental reducing-equivalent transfer, hepatocytes were incubated with sorbitol. The cytoplasmic reducing equivalents generated during the oxidation of this substrate are transferred to the mitochondria by both the malate/aspartate and α-glycerophosphate shuttles [20, 41]. The rate of reducing-equivalent transfer was of the same order as the activity of alcohol dehydrogenase, measured in particulate-free extracts, and was not affected by aniline (Table 2). Hence, it appears that aniline depressed ethanol oxidation solely by its inhibition of alcohol dehydrogenase. Aniline is not a potent inhibitor of the enzyme compared with 4-methylpyrazole [5, 6] which, at a concentration of 10 μM yielded the same degree of inhibition induced by 5 mM aniline.

Ethanol oxidation elevates the [lactate]/[pyruvate] ratio [42–44] and impairs glycolysis [45, 46] and gluconeogenesis [44, 47], though the latter inhibition can be relieved by asparagine [48, 49]. A not unexpected finding, therefore, was that aniline prevented the large rise in the [lactate]/[pyruvate] ratio brought about by incubation of hepatocytes with lactate and ethanol. The [lactate]/[pyruvate] ratio of cells incubated with lactate and ethanol for 30 min was 74.6 ± 6.9 (N = 20); in the presence of aniline, this value was reduced to 27.2 ± 2.1 (N = 16) ($P < 0.001$). Aniline also decreased the inhibition of glycolysis from ethanol (Table 3). On the other hand, aniline not only failed to

TABLE 3. Effect of ethanol and aniline on the rate of glycolysis

Additions	$J_{Pyruvate}$ (μmol/min/g wet wt)	$J_{Lactate}$ (μmol/min/g wet wt)	J_{CO_2} (μmol/min/g wet wt)	Rate of glycolysis
Glucose (40 mM)	0.35 ± 0.04 (6)	1.56 ± 0.13 (6)	2.02 ± 0.08 (3)	2.58 ± 0.20 (3)
Glucose; aniline (5 mM)	0.25 ± 0.02† (6)	1.80 ± 0.11 (6)	2.19 ± 0.14 (3)	2.78 ± 0.19 (3)
Glucose; [1-^{14}C]ethanol (13 mM)	0.01* (7)	0.18 ± 0.01* (7)	0.29 ± 0.03* (3)	0.29 ± 0.02* (3)
Glucose; [1-^{14}C]ethanol; aniline	0.09 ± 0.01§ (7)	1.22 ± 0.09‡ (7)	1.48 ± 0.22‡ (3)	1.80 ± 0.19§ (3)

Isolated hepatocytes from fasted rats were incubated, as described in Materials and Methods, in 2 mL of incubation medium at 37° with the substrates indicated. The rates of pyruvate accumulation ($J_{Pyruvate}$), of lactate accumulation ($J_{Lactate}$), and of CO_2 accumulation (J_{CO_2}) were measured between 10–30 min. The rate of glycolysis, in three-carbon units, was calculated from the sum of lactate + pyruvate + ⅓ CO_2. Results are the mean ± SEM with the number of individual cell preparations shown in parentheses. *$P < 0.001$ relative to the value for glucose alone; †$P < 0.05$ relative to the value for glucose alone; ‡$P < 0.001$ relative to the value for glucose plus ethanol; §$P < 0.01$ relative to the value for glucose plus ethanol.

TABLE 4. Effect of ethanol and aniline on the rate of glucose accumulation from lactate, pyruvate, or fructose

Additions	$J_{Glucose}$ (μmol/min/g wet wt) (−) Aniline	(+) Aniline (5 mM)
Lactate (10 mM)	0.66 ± 0.01 (20)	0.20 ± 0.02* (12)
Lactate; [1-^{14}C]ethanol (13 mM)	0.61 ± 0.02† (20)	0.20 ± 0.02‡ (16)
Pyruvate (12 mM)	0.70 ± 0.07 (4)	0.42 ± 0.05‖ (4)
Pyruvate; [1-^{14}C]ethanol (13 mM)	1.10 ± 0.03§ (4)	0.53 ± 0.03¶ (4)
Fructose (10 mM)	2.37 ± 0.17 (8)	2.17 ± 0.13 (5)
Fructose; [1-^{14}C]ethanol (13 mM)	2.09 ± 0.19 (5)	2.24 ± 0.10 (3)

Isolated hepatocytes from fasted rats were incubated, as described in Materials and Methods, in 2 ml of incubation medium at 37° with the substrates indicated. The rate of glucose accumulation ($J_{Glucose}$) was measured between 30–40 min for lactate; between 5–30 min for pyruvate, and between 10–40 min for fructose. Results are the mean ± SEM with the number of individual cell preparations shown in parentheses. * $P < 0.001$ relative to the value for lactate alone; † $P < 0.05$ relative to the value for lactate alone; ‡ $P < 0.001$ relative to the value for lactate plus ethanol; § $P < 0.01$ relative to the value for pyruvate alone; ‖ $P < 0.05$ relative to the value for pyruvate alone; ¶ $P < 0.001$ relative to the value for pyruvate plus ethanol.

relieve the inhibition of gluconeogenesis from lactate induced by ethanol, but reduced the rate of glucose synthesis still further (Table 4). This inhibition of gluconeogenesis by aniline was also manifest in the absence of added ethanol (Table 4). A similar inhibition by aniline of glucose synthesis occurred when cells were incubated with pyruvate in the presence or absence of ethanol (Table 4).

Because aniline had no inhibitory effect on glycolysis in the absence of added ethanol (Table 3), nor on intercompartmental reducing-equivalent transfer (Table 2), it can be inferred that its probable mode of action was inhibition of one of the gluconeogenic enzymes, glucose 6-phosphatase, fructose 1,6-diphosphatase, pyruvate carboxylase, or phosphoenolpyruvate carboxykinase. Because glucose synthesis from fructose was not impaired (Table 4), it seems likely that the enzymes near the distal end of the gluconeogenic pathway can be excluded from consideration. Malate and phosphoenolpyruvate were measured to examine whether or not pyruvate carboxylase and/or phosphoenolpyruvate carboxykinase were possible targets for aniline inhibition. The quantity of malate measured in cells incubated with either lactate or pyruvate was not changed by the inclusion of aniline (data not shown); hence, there was no apparent inhibition of pyruvate carboxylation by aniline. The concentration of phosphoenolpyruvate was found to be negligible in isolated hepatocytes, so no conclusion could be reached on the effect of aniline on phosphoenolpyruvate carboxykinase. Inhibition of gluconeogenesis from lactate by mixed-function oxidation drugs, such as aminopyrine and alprenolol, has been previously reported [50–52]. The main theory of how these drugs inhibit glucose synthesis proposes diversion of reducing equivalents for NADPH synthesis [50, 53]. Further studies on the inhibition of glucose synthesis from lactate or pyruvate by aniline are currently in progress.

This work was supported in part by grants from the National Health and Medical Research Council of Australia. M-A. Efthivoulou holds a Postgraduate Scholarship from the Drug and Alcohol Services Council of South Australia.

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