Benzoate stimulates glutamate release from perfused rat liver

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In isolated perfused rat liver, benzoate addition to the influent perfusate led to a dose-dependent, rapid and reversible stimulation of glutamate output from the liver. This was accompanied by a decrease in glutamate and 2-oxoglutarate tissue levels and a net K^+ release from the liver; withdrawal of benzoate was followed by re-uptake of K^+ . Benzoate-induced glutamate efflux from the liver was not dependent on the concentration (0–1 mM) of ammonia ($NH_3 + NH_4^+$) in the influent perfusate, but was significantly increased after inhibition of glutamine synthetase by methionine sulphoximine or during the metabolism of added glutamine (5 mM). Maximal rates of benzoate-stimulated glutamate efflux were 0.8–0.9 $\mu\text{mol}/\text{min per g}$, and the effect of benzoate was half-maximal ($K_{0.5}$) at 0.8 mM. Similar V_{max} values of glutamate efflux were obtained with 4-methyl-2-oxopentanoate, ketomethionine (4-methylthio-2-oxobutyrate) and phenylpyruvate; their respective $K_{0.5}$ values were 1.2 mM, 3.0 mM and 3.8 mM. Benzoate decreased hepatic net ammonia uptake and synthesis of both urea and glutamine from added $NH_4\text{Cl}$. Accordingly, the benzoate-induced shift of detoxication from urea and glutamine synthesis to glutamate formation and release was accompanied by a decreased hepatic ammonia uptake. The data show that benzoate exerts profound effects on hepatic glutamate and ammonia metabolism, providing a new insight into benzoate action in the treatment of hyperammonaemic syndromes.

INTRODUCTION

Sodium benzoate has been used successfully for treatment of a variety of hyperammonaemic states, such as inborn errors of urea synthesis or liver cirrhosis (Brusilow *et al.*, 1979; Batshaw *et al.*, 1982; Kodama *et al.*, 1983; Mendenhall *et al.*, 1986; Batshaw & Monahan, 1987). Thereby blood ammonia ($NH_3 + NH_4^+$) is decreased, and the patients improve clinically. However, the mechanism of action of benzoate is incompletely understood. In the liver, glycine as a vehicle for waste nitrogen combines with benzoate, yielding hippurate, which is then rapidly excreted into the urine. This may represent an alternative pathway for the elimination of waste nitrogen. However, studies in intact rats and mice showed that benzoate was also able to potentiate ammonia toxicity (O'Connor *et al.*, 1982; Masmoswe *et al.*, 1986; Palekar *et al.*, 1989), which could be prevented by addition of L-carnitine (O'Connor *et al.*, 1987). Overdosage of benzoate produced symptoms similar to those found in hyperammonaemia (Batshaw *et al.*, 1982). These symptoms may partly be attributed to neurotransmitter changes in the brain (Colombo, 1987). On the other hand, the deleterious effect of benzoate was attributed to the accumulation of benzoyl-CoA, which could deplete the acetyl-CoA pool, leading to an impaired formation of N-acetylglutamate and consequently an inhibition of urea synthesis (O'Connor *et al.*, 1987; Wendler & Tremblay, 1982). No direct inhibition was found of mitochondrial urea-cycle enzymes in hyperammonaemic rats treated with sodium benzoate (Colombo *et al.*, 1988).

In view of this discrepancy, we studied the effect of benzoate on nitrogen metabolism in perfused rat liver.

There is a remarkable structural and functional organization of ammonia metabolism in the liver acinus. In the direction of hepatic blood flow, the two major pathways of hepatic ammonia detoxication are anatomically switched behind each other: urea synthesis and glutaminase are found in periportal hepatocytes, whereas glutamine synthetase is exclusively localized in a small hepatocyte population at the perivenous boundary of the liver acinus [for reviews see Häussinger *et al.* (1988) and Traber *et al.* (1988)]. We found that benzoate inhibits both periportal urea synthesis and perivenous glutamine synthesis from ammonia and leads to a marked stimulation of glutamate efflux from the liver.

MATERIALS AND METHODS

Liver perfusion

Livers of male Wistar rats of 120–250 g body wt., fed *ad libitum* on stock diet (Altromin), were perfused as described previously (Sies, 1978), without recirculation of the perfusate, with bicarbonate-buffered Krebs–Henseleit saline plus L-lactate (2.1 mM) and pyruvate (0.3 mM), if not stated otherwise. Perfusion fluid was equilibrated with O_2/CO_2 (19:1). Perfusate flow rate was 3.2–4.5 ml per min/g and was kept constant throughout the individual perfusion experiment. The temperature was 37 °C.

Glutamine synthetase in perfused rat liver was inhibited by intraperitoneal injection of methionine sulphoximine (5 mg/100 g body wt.) 2–3 h before the perfusion experiment and by addition of methionine

Abbreviation used: SITS, 4-acetamido-4'-isothiocyantostilbene-2,2'-disulphonic acid. The term 'ammonia' refers to the sum of both NH_3 and NH_4^+ .

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sulphoximine to the perfusion fluid at a concentration of 0.2 mM (Häussinger & Gerok, 1983).

Assays

The concentrations of oxoglutarate, glutamate, glutamine, ammonia, urea, lactate, pyruvate and glucose in the perfusate or in the tissue extracts were determined by enzymic methods based on the procedures described in Bergmeyer (1974). When 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid (SITS) was present, glutamate was measured with an automatic amino acid analyser (LC 700; Biotronic, Munich, Germany), because the background absorbance at NADH-specific wavelengths in the presence of SITS did not allow enzymic determination of glutamate. Benzoate was measured by reversed-phase h.p.l.c. as described by Bachmann (1988).

The K^+ and H^+ concentrations in the perfusate were continuously monitored with a selective K^+ electrode (Radiometer, Munich, Germany) and a glass electrode, respectively.

Tissue samples (500–1000 mg) of perfused rat liver were taken by the freeze-clamp technique and, neutralized $HClO_4$ extracts were prepared as described by Häussinger *et al.* (1975). In the experiments to determine the effect of benzoate (1 mM) on 2-oxoglutarate and glutamate tissue levels, a sample of liver tissue was taken by the freeze-

clamp technique after a 40 min perfusion. These perfusion experiments were carried out either without addition of benzoate or in the presence of benzoate (1 mM), with the benzoate addition to the influent perfusion-medium starting at 20 min of perfusion.

Data are given as means $\pm$ S.E.M.

Chemicals

All enzymes, pyruvate, 2-oxoglutarate, NAD^+ and NADH were from Boehringer (Mannheim, Germany). Quinidine and SITS were from Sigma (Munich, Germany). Lactic acid was from Roth (Karlsruhe, Germany). All other chemicals were from Merck (Darmstadt, Germany).

RESULTS

Stimulation of glutamate release from perfused rat liver by benzoate

In isolated perfused rat liver, glutamate is released into effluent perfusate at a rate of 40–60 nmol/min per g, corresponding to an effluent glutamate concentration of about 10–15 μM , as shown by Häussinger & Gerok (1984) and also in the present work. These low rates of glutamate release were observed for more than 100 min of perfusion, and were not significantly affected by addition of

Fig. 1. Effect of benzoate on the concentrations of glutamate (a) and K^+ (b) in effluent perfusate

Results are representative of a series of five similar perfusion experiments.

Fig. 2. Effect of NH_4Cl (1 mM) and glutamine (5 mM) (a) and methionine sulfoximine (b) on benzoate (1 mM)-induced glutamate release from perfused rat liver

In experiments with glutamine (5 mM), dibutyryl cyclic AMP (20 μM) was present, to maintain a high glutaminase flux. Data are given as means $\pm$ S.E.M. ($n = 4-6$ different experiments).

ammonia or glucagon (Häussinger & Gerok, 1984). Simultaneously, hepatic glutamate levels remained high, indicating the maintenance of a several-hundredfold intra-/extra-cellular glutamate concentration ratio in perfused rat liver, as also described previously (Häussinger & Gerok, 1984). Addition of benzoate to the perfusion medium, however, led to a rapid and marked stimulation of glutamate release from the liver, which was fully reversible. The low basal rates of glutamate efflux were reached again within about 1 min (i.e. the first time point of glutamate assay) after stopping the benzoate infusion (Fig. 1a). Benzoate concentrations as low as 15 μM were effective to stimulate glutamate release significantly from the liver. Simultaneously, benzoate infusion led to a net K^+ release from the liver and a net K^+ re-uptake after benzoate withdrawal (Fig. 1b). With an influent benzoate concentration of 1 mM, the stimulation of glutamate efflux from the liver was maximal within 1 min, and thereafter declined to reach a steady state which was maintained for more than 10 min (Fig. 2a). Since benzoate is used in the treatment of hyperammonaemia, NH_4Cl was added in concentrations up to 1 mM, corresponding to plasma levels found in hepatic encephalopathy. The benzoate-induced glutamate output from the liver was not affected by NH_4Cl (Fig. 2a). However, it was significantly increased after inhibition of glutamine synthetase by methionine sulfoximine

Table 1. Kinetics of glutamate efflux induced by organic acids in perfused rat liver

Data were obtained by double-reciprocal-plot analysis of the maximal increase of glutamate efflux from livers after addition of different benzoate concentrations. The influent perfusate contained glutamine (5 mM), to guarantee a continuous intracellular glutamate replenishment. V_{max} = maximal rate of glutamate efflux induced by the organic acids. $K_{0.5}$ = concentration of organic acids required in influent perfusate to achieve a half-maximal glutamate efflux. r = linear correlation coefficient; n = number of determinations in double-reciprocal-plot analysis. Data for 4-methyl-2-oxopentanoate, phenylpyruvate and ketomethionine were calculated from data given in a previous study (Häussinger & Gerok, 1984).

Effector	V_{max} ($\mu\text{mol/min per g}$)	$K_{0.5}$ (mM)	r	n
Benzoate	0.90	0.8	0.990	8
Benzoate*	0.83	0.75	0.970	16
4-Methyl-2-oxopentanoate	0.92	1.2	0.997	10
Phenylpyruvate	0.74	3.8	0.990	8
Ketomethionine	1.08	3.0	0.994	8

* No glutamine present in the perfusate.

Fig. 3. Effect of benzoate (1 mM) on the effluent concentrations of urea and ammonia (a) and glutamine and glutamate (b) in livers perfused with NH_4Cl (0.3 mM)

Results shown are representative of a series of four perfusion experiments.

(Fig. 2b). With 0.3 mM- NH_4Cl in the influent, the maximal glutamate release after benzoate addition was 381 ± 18 nmol/min per g ($n = 4$) and 259 ± 22 nmol/min per g ($n = 5$) in the presence and absence of methionine sulfoximine, respectively. During the subsequent metabolic steady state, which was reached after about 10–15 min, the rates of extra glutamate release were higher in the presence of methionine sulfoximine than without it (167 ± 9 and 89 ± 2 nmol/min per g respectively; both $n = 4$). Also, in the presence of glutamine (5 mM) and dibutyl cyclic AMP (20 μM) [in order to maintain a high glutaminase activity (Joseph & McGivan, 1978; Häussinger *et al.*, 1983)], benzoate-induced glutamate efflux from the liver was markedly enhanced (Fig. 2a). It should be noted that benzoate, apart from its stimulatory effect on glutamate release, did not lead to a measurable increase in efflux of other amino acids, such as alanine, glycine, serine, threonine, histidine, branched-chain or aromatic amino acids (results not shown).

The benzoate-stimulated glutamate output from the liver was dependent on the portal benzoate concentration (Fig. 1a). Double reciprocal plot analysis (maximal rate of benzoate-induced glutamate efflux versus benzoate concentration in the influent) yielded maximal rates of glutamate release from the liver of about 0.9 μmol/min per g (Table 1), with half-maximal effects ($K_{0.5}$) being obtained at influent benzoate concentrations of about 0.8 mM. 4-Methyl-2-oxopentanoate ('ketoisocaproate'), phenylpyruvate and 4-methylthio-2-oxobutyrate (keto-

methionine) were shown also to be effective in stimulating glutamate efflux from the liver (Häussinger & Gerok, 1984). This was not the case for leucine, phenylalanine, methionine, pyruvate, oxaloacetate, oxoglutarate or 3-methyl-2-oxobutanoate (oxoisovalerate) (Häussinger & Gerok, 1984). As also shown in Table 1, the maximal rates of glutamate efflux from the liver induced by 4-methyl-2-oxopentanoate, phenylpyruvate, ketomethionine and benzoate were similar, i.e. in the range of 0.74–1.08 μmol/min per g. However, benzoate and 4-methyl-2-oxopentanoate were the most effective organic acids, exhibiting the lowest $K_{0.5}$ values.

The increased output of glutamate from the liver under the influence of benzoate was accompanied by a 40–50 % decrease in the tissue contents of glutamate and 2-oxoglutarate. In the absence and presence of benzoate (1 mM), the glutamate tissue contents were 2.47 ± 0.26 ($n = 7$) and 1.39 ± 0.07 ($n = 5$) μmol/g wet wt. of liver respectively, and those of 2-oxoglutarate were 0.345 ± 0.018 ($n = 7$) and 0.163 ± 0.023 ($n = 5$) μmol/g wet wt. of liver respectively. During infusion of benzoate at a concentration of 1 mM, the tissue benzoate level was 0.71 ± 0.02 μmol/g liver wet wt. ($n = 5$). Taking into account the extracellular and intracellular water spaces in perfused rat liver, as described by Soboll *et al.* (1976), an intracellular benzoate concentration of about 0.8 mM can be estimated. This value is higher than that expected by assuming benzoate equilibration by non-ionic diffusion across the hepatocyte plasma membrane according to the

Fig. 4. Effect of benzoate (1 mM) on the metabolism of NH_4Cl (0.3 mM) during inhibition of glutamine synthetase by methionine sulfoximine

Results shown are representative of a series of four perfusion experiments.

pH gradient (about 0.3 pH unit more acidic inside the cell) (Sies *et al.*, 1973, 1974; Soboll *et al.*, 1988).

Effect of benzoate on hepatic urea and glutamine synthesis

When livers were perfused with a near-physiological ammonia concentration, benzoate (1 mM) decreased urea and glutamine production by $12.7 \pm 2.0\%$ ($n = 4$) (from 0.77 ± 0.05 to 0.68 ± 0.06 $\mu\text{mol}/\text{min}$ per g) and $34 \pm 2\%$ ($n = 4$) (from 303 ± 34 to 200 ± 26 nmol/min per g) respectively. Simultaneously, ammonia release into the effluent perfusate increased from 0.10 ± 0.02 to 0.33 ± 0.02 $\mu\text{mol}/\text{min}$ per g ($n = 4$), indicating that hepatic ammonia uptake was inhibited by benzoate and was not compensated for by increased glutamate formation and release from the liver (Figs. 3a and b). It should be noted that during metabolic steady states the benzoate-induced decrease in hepatic glutamine synthesis of 103 ± 10 nmol/min per g ($n = 4$) was roughly matched by an increased net glutamate release (89 ± 2 nmol per g; $n = 4$). In fact, the release of total nitrogen (urea- plus glutamine- plus glutamate-nitrogen plus ammonia) was not affected by benzoate, and was 1.54 ± 0.11 and 1.55 ± 0.11 $\mu\text{mol}/\text{min}$ per g ($n = 4$) in the absence and presence of benzoate respectively. These data show that in the liver-perfusion model benzoate did not significantly affect the formation of nitrogenous products other than glutamate, glutamine or urea, and further suggest that benzoate did not interfere with the basal rate of breakdown of endogenous amino acids or proteins in the liver. Inhibition of urea and glutamine synthesis from NH_4Cl by benzoate was observed at all ammonia concentrations studied (0–1 mM). Also, when glutamine synthetase was inhibited by methionine sulfoximine,

benzoate (1 mM) inhibited urea synthesis from NH_4Cl (0.3 mM) and decreased ammonia uptake by the liver (Fig. 4), although benzoate-induced glutamate efflux from the liver was significantly higher after inhibition of glutamine synthetase (Fig. 2b). During metabolic steady states, which were reached after about 15 min of benzoate infusion, and glutamine synthetase being inhibited, ammonia release into the effluent was increased by benzoate by about 60% (from 0.43 ± 0.06 to 0.67 ± 0.08 $\mu\text{mol}/\text{min}$ per g; $n = 4$). Simultaneously, urea-nitrogen synthesis decreased by about 45% (from 0.99 ± 0.04 to 0.58 ± 0.07 $\mu\text{mol}/\text{min}$ per g; $n = 4$), whereas glutamate release rose by about 310% (from 58 ± 6 to 225 ± 13 nmol/min per g; $n = 4$) in the plateau phase. Again, total nitrogen release from the liver was not affected by benzoate addition (1.48 ± 0.10 and 1.47 ± 0.05 $\mu\text{mol}/\text{min}$ per g in the absence and the presence of benzoate respectively).

DISCUSSION

Effect of benzoate on hepatic nitrogen metabolism

The data in this paper show that benzoate inhibits urea and glutamine synthesis, diminishes hepatic ammonia uptake and induces a marked stimulation of glutamate output from the liver. The last observation resembles that obtained with the keto-analogues of methionine, leucine, isoleucine, phenylalanine or tyrosine (Häussinger & Gerok, 1984). It is likely that stimulation of glutamate output by benzoate and these keto acids involves a similar mechanism, as suggested by the similarities of the maximal rates of glutamate efflux (Table 1). Interestingly, benzoate and 4-methyl-2-oxopentanoate, which are used

for the treatment of hyperammonaemic syndromes, were the most effective agents promoting glutamate release from the liver. Maximal glutamate efflux from the liver, i.e. about $0.9 \mu\text{mol/min per g}$ (Table 1), is remarkably high; for comparison the rates of urea and glutamine formation from NH_4Cl (1–2 mM) in perfused rat liver were reported to be about $1.1 \mu\text{mol/min per g}$ (Häussinger *et al.*, 1975) and $0.4\text{--}0.6 \mu\text{mol/min per g}$ (Häussinger *et al.*, 1983; Stoll & Häussinger, 1989) respectively. This suggests that another important site of control of hepatic nitrogen metabolism is a modulation of glutamate export from the liver by benzoate or keto-analogues of some branched-chain or aromatic amino acids. The data also show that the view held previously on the impermeability of the liver plasma membrane to glutamate (Ross *et al.*, 1967; Hems *et al.*, 1968) requires revision. The inhibition of urea and glutamine synthesis by benzoate, however, cannot be explained exclusively by a shift of ammonia from these pathways into the glutamate-efflux pathway, because hepatic ammonia uptake was decreased by benzoate. It is likely that the inhibition of urea synthesis is also due to an impaired formation of *N*-acetylglutamate, the activator of carbamoyl-phosphate synthetase, as suggested by O'Connor *et al.* (1987). Whereas in our study benzoate impairs urea and glutamine synthesis from NH_4Cl (0.3 mM) in perfused rat liver by 11% and 35% respectively, it is conceivable that *in vivo* a simultaneous benzoate-induced stimulation of glutamate output from the liver can be beneficial under conditions when urea and/or glutamine synthesis is already largely impaired [for example in liver cirrhosis (Kaiser *et al.*, 1988) or in inborn defects of urea-cycle enzymes]. Benzoate-stimulated release of glutamate from the liver may then represent an alternative pathway for detoxication of waste ammonia: glutamate is formed and released from the liver, instead of urea and glutamine. However, at least in our experimental system of isolated perfused rat liver, no condition was found in which benzoate improved hepatic ammonia clearance.

In perfused rat liver, we have shown that vascular glutamate is taken up almost exclusively into perivenous glutamine synthetase-containing hepatocytes, whereas the much larger periportal compartment seems not to contribute significantly to total hepatic glutamate uptake (Häussinger & Gerok, 1983; Häussinger *et al.*, 1989). Whether such a strict compartmentation also holds for the benzoate-induced glutamate efflux from the liver is an interesting, but as yet unanswered, question.

Potential mechanisms of benzoate-induced glutamate efflux from the liver

Considering the chemical structure of the organic acids capable of stimulating glutamate efflux from the liver, it is now apparent that, besides the carboxylic group, not an α -oxo group but the α -double bond seems to be an important structural feature in the molecule. However, it remains unclear why the keto-analogues of leucine and isoleucine as well as α -oxobutyrate are effective in stimulating glutamate efflux, whereas the keto-analogue of valine is not (Häussinger & Gerok, 1984).

The mechanism of benzoate-induced glutamate efflux from the liver remains unclear. Two possibilities must be considered: an efflux of glutamate driven by the electrochemical gradient through an anion channel, which is opened under the influence of benzoate, and a glutamate/

Fig. 5. Effect of benzoate (1 mM) on effluent H^+ and K^+ concentrations

benzoate exchange mechanism. In line with the first suggestion ('electrogenic efflux') would be the observation that glutamate release is followed by a K^+ efflux from the liver and that the benzoate-induced glutamate efflux is completely inhibited by quinidine (1 mM), with glutamate release rates being $8 \pm 4 \text{ nmol/min per g}$ ($n = 3$) in the absence and presence of benzoate (1 mM). A simultaneous net K^+ release from the liver (Fig. 1b) may then represent a secondary mechanism for charge compensation. However, the time courses of benzoate-induced K^+ and glutamate efflux were different (cf. Figs. 1a and b); in particular, a net K^+ release was observed only during the first 6 min of benzoate addition (Fig. 5), whereas the stimulation of glutamate release was much more prolonged (Figs. 3 and 4). The alternative suggestion of a glutamate/benzoate exchange implies that such an electroneutral exchanger is driven by intra-/extra-cellular glutamate and benzoate concentration gradients. During metabolic steady states, i.e. after equilibration of benzoate between the extra- and intra-cellular space, net glutamate release via this glutamate/benzoate exchanger must therefore involve a cycling of benzoate across the plasma membrane (Scheme 1).

Although hypothetical, such a mechanism, combining benzoate/glutamate exchange with a non-ionic benzoate diffusion out of the cell as depicted in Scheme 1, would be compatible with a variety of experimental findings in our study. It would predict a steady-state acidification of the extracellular space, as indeed is shown in Fig. 5. It would also predict a steady-state benzoate concentration in the intracellular space which is greater than the concentration expected from a simple non-ionic diffusion equilibrium with an intra-/extra-cellular pH difference of 0.3 (see the Results section). An intracellular alkalization, resulting from the operation of Scheme 1, could explain the net K^+ release after benzoate addition, because this condition is known to open pH-sensitive K^+ channels (Lang *et al.*, 1988). A benzoate/glutamate exchange could also explain why benzoate-induced glutamate efflux is maximal during the first 1–2 min of

Scheme 1. Hypothetical mechanism of benzoate-stimulated glutamate efflux from the liver.

For details see the text. Glu⁻, glutamate anion; B⁻, benzoate anion; BH, undissociated benzoic acid.

benzoate addition. Its subsequent decline may result from a dissipation of the intra-/extra-cellular benzoate and glutamate gradients. SITS (1 mM), an inhibitor of anion exchange, decreased the benzoate-induced stimulation of glutamate efflux [from 15 ± 4 to 65 ± 4 nmol/min per g ($n = 3$) in the presence of SITS; from 56 ± 5 to 306 ± 30 nmol/min per g ($n = 6$) in the absence of SITS].

Interestingly, evidence has been found for a glutamate/glutamate exchange in perfused rat liver (Häussinger *et al.*, 1989) and a glutamate/cysteine exchange in fibroblasts (Bannai, 1986). It is conceivable that such mechanisms also mediate a benzoate/glutamate exchange in our experiments. A benzoate- and keto-acid-stimulated glutamate export system in the liver plasma membrane certainly adds another site of control for hepatic nitrogen metabolism in general, which might be relevant to an understanding of the therapeutic effects of benzoate.

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