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Potential toxins of acute liver failure and their effects on blood-brain barrier permeability

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Summary. The effects of potential toxins of hepatic coma on the blood-brain barrier (BBB) permeability of the rat have been examined using the Oldendorf technique. Classical toxins of hepatic failure such as ammonia, methyl octanoate, mercaptans, and phenol caused significant increases in BBB permeability. A slight increase in permeability occcurred following infusion of peroxidized linoleic acid and unconjugated bilirubin but no increase after infusion of bile acids. *E. coli* endotoxin infused into rats following partial hepatectomy also increased the BBB permeability.

Livingstone et al.[13] in 1977 demonstrated an increase in BBB permeability in hepatectomized rats as they passed into a coma which was followed by cerebral oedema and death. No single toxin is likely to account for all the features of hepatic encephalopathy but raised plasma levels of water soluble toxins, such as ammonia and amino acids, as well as lipophilic toxins such as phenols[2], fatty acids[11], mercaptans, bile acids[1] and bilirubin have all been reported in liver failure. Recently there has been great interest in the disturbed balance of cerebral neurotransmitters secondary to changes in the brain and plasma amino acid profiles. Systemic endotoxemia may play a contributory role during liver failure since the function of Kupffer cells, which normally remove endotoxin, appears to be impaired in these patients[3]. The effects of toxins on BBB permeability have received little attention. In this paper we report changes in permeability of the BBB demonstrated using the Oldendorf technique[16] in normal rats following administration of a number of these toxins.

Method

Male Wistar rats weighing 250–300 g (King's College Hospital Medical School) were used for all studies. The rats were anesthetized with ether and i.p. sodium pentobarbitone (30 mg/kg) prior to administration of the toxins.

Administration of toxins. Phenol, endotoxin (*E. coli* 0111), unconjugated bilirubin, glycocholic, taurocholic, arachidonic and peroxidized linoleic acid were each infused into the femoral vein for 20 min prior to the measurement of the brain uptake index. Ammonium acetate and methyloctanoate were given as single i.p. doses. Ethanethiol was administered by inhalation to fully conscious rats in a fume chamber over a period of 5 min. The quantity of each toxin injected was based on the blood volume of the rat and was calculated to give a plasma concentration which would approximate to the pathologically raised levels seen in patients with fulminant hepatic failure. The bile acids were dissolved in 3 ml buffered albumin while the remaining toxins were dissolved in 3 ml of phosphate buffered saline prior to infusion by syringe pump over 20 min (Scientifica and Cook Electronics Ltd, London, UK). Infusion of phosphate buffered saline alone did not alter the brain uptake index in control rats. The estimated plasma concentrations of potential toxins, their routes of administration and the resultant brain uptake indices are shown in table 1.

Table 1. Routes of administration, estimated plasma concentrations and brain uptake indices of ^{14}C inulin following administration of potential toxins in fulminant hepatic failure

Substances	Route of administration	No. of rats	Injected concentration	Estimated plasma concentration	Brain uptake index (%)	Significance*
Ammonium acetate	i.p.	7	9.6 mmoles/kg	800 µmoles/l	8.2±0.7	$p<0.001$
Methyl octanoate	i.p.	7	3.47 mmoles/kg	2400 µmoles/l	6.3±0.5	$p<0.001$
Ethanethiol	Inhalation	3	1.5 mmoles/l	250 µmoles/l	4.9±0.7	$p<0.005$
Phenol	i.v.	8	1.3 mmoles/kg	200 µmoles/l	5.8±0.7	$p<0.001$
Bilirubin	i.v.	6	8.0 mmoles/kg	80 µmoles/l	3.2±0.6	NS
Glycocholic acid	i.v.	5	8.0 mmoles/kg	80.0 µmoles/l	2.5±0.3	NS
Taurocholic acid	i.v.	7	8.0 mmoles/kg	80.0 µmoles/l	2.5±0.5	NS
Arachidonic acid	i.v.	6	3.6 mmoles/kg	30 µmoles/l	2.7±0.3	NS
Oxidized linoleic	i.v.	6	3.6 mmoles/kg	30 µmoles/l	3.2±0.4	$p<0.02$
Normal rats		16			2.6±0.3	

The number of rats studied and route of administration of each toxin is shown, as well as mean brain uptake index±SD for each toxin.
* Significance of the brain uptake index compared with normal rats determined by Student's t-test.

The brain uptake indices following i.v. infusion of low (6 mg/kg) and high (12 mg/kg) doses of *E. coli* endotoxin were measured in normal rats and in rats subjected to a partial hepatectomy (70%) 48 h earlier as described by Higgins and Anderson[9]. Toxins were obtained from Sigma Chemical, London, Hopkins and Williams, Essex, and B.D.H. Chemicals, Dorset.

Measurement of brain uptake index. Following administration of toxin the brain uptake index was determined by the Oldendorf technique. The methodology has been described in detail elsewhere[16] and only a brief description will be given here. The right common carotid artery was surgically exposed and cannulated with a 27-gauge needle. 0.2 ml of a mixture of ^{3}HOH and ^{14}C labeled inulin was rapidly injected and exactly 15 sec later the rat was decapitated, the skull opened and the brain removed and transected at the level of the superior and inferior colliculi. That part of the forebrain rostral to the transection was then divided along the midline into 2 halves. The half forebrain ipsilateral to the injection was divided into equal portions. Each portion was put in a glass scintillation vial containing 1 ml Soluene 350 (Packard Instr., USA) and incubated at 37 °C for at least 30 h, until fully dissolved. Following the addition of 15 ml of scintillation fluid each vial was counted on a Packard 3320 automatic liquid scintillation spectrophotometer.

During the course of one capillary passage most of the ^{3}HOH in the injected mixture leaves the capillaries and distributes to the rapidly exchangeable 'water space'. The amount of test substance lost to the brain on a single passage through the microcirculation can be calculated from the ratio of ^{14}C to ^{3}H in brain tissue relative to that in the injectate:

$$\text{brain uptake index} = \frac{\text{tissue } ^{14}C/\text{tissue } ^{3}H}{\text{injectate } ^{14}C/\text{injectate } ^{3}H}$$

Results

The brain uptake index for ^{14}C inulin in normal rats was 2.6±0.3%. A highly significant increase in the blood-brain barrier permeability to inulin ($p < 0.01$) was observed following injection of ammonium acetate, methyl octanoate, phenol and inhalation of the mercaptan, ethanethiol (table 1). Unconjugated bilirubin given i.v. in phosphate buffered saline did not cause a significant increase in blood-brain permeability, although there was some increase in uptake of inulin in 3 of the 6 rats.

No significant increase in brain uptake index was seen with either arachidonic acid or buffered bile acids. Linoleic acid that was 5% oxidized, as assessed by thio-barbituric acid assay, caused a slight increase in brain uptake index to 3.2% ($p < 0.02$).

Neither the 6 mg/kg nor the 12 mg/kg dose of endotoxin caused an increase in the brain uptake of inulin. A significant increase in BUI was seen with a dose of 6 mg/kg in partially hepatectomized rats (table 2).

Discussion

Of all the toxins tested ammonia, the free fatty acid methyloctanoate, unconjugated phenol and the mercaptan ethanethiol, had the most profound effect on blood-brain barrier permeability. The synergistic effect of these toxins, which when given together result in the production of encephalopathy is well documented[22].

The increase in brain uptake index of inulin was greatest following i.p. ammonium acetate. One of the

Table 2. Effect of femoral vein infusion of *E. coli* endotoxin on blood brain barrier permeability in normal and partially hepatectomized rats

Category	Dose of endotoxin	No. of rats	Brain uptake index (%), mean±SD	Significance*
Normal control rats	-	16	2.6±0.3	
Normal rats	2 mg	5	2.6±0.3	NS
Normal rats	4 mg	5	2.8±0.2	NS
Partial hepatectomy	2 mg	7	3.2±0.3	$p<0.05$

* Denotes level of significance of ^{14}C inulin BUI compared with normal control rats (determined by Student's t-test).

most important toxic effects of ammonia is the inhibition of cerebral energy metabolism[15]. James et al.[10] proposed that hyperammonemia stimulated brain glutamine synthesis, resulting in rapid exchange of brain glutamine for plasma neutral amino acids. Hyperammonemia may therefore contribute to encephalopathy indirectly by raising the brain concentration of neutral amino acids and thus altering neurotransmitter metabolism. Other possible mechanisms of ammonia toxicity have recently been discussed by Flannery et al.[6].

A potential role for short chain fatty acids in the etiology of hepatic encephalopathy was shown by Chen et al.[4], who demonstrated increased levels of butyrate, valerate and octanoate in the blood and CSF of patients with hepatic encephalopathy. We have demonstrated a significant increase in blood-brain barrier permeability following IP injection of octanoate ($p < 0.001$) but not of the other short chain fatty acids. However, a damaging effect of oxidised linoleic acid was demonstrable. In liver cell necrosis the phospholipids of the liver become oxidized by free radical mediated reactions and it is possible that the fatty acids of the plasma might become peroxidized. Such circulating oxidized fatty acids could affect the blood-brain barrier as we have demonstrated although there is no evidence of damaging peroxidation within the brain[8].

Extremely small doses of mercaptans can cause reversible coma in rats. In fulminant hepatic failure methanethiol derived from methionine is incompletely metabolized by the liver. The brain is thus exposed to increased concentrations of methanethiol and dimethyl sulphide (CH_3SCH_3) which have been shown to cause confusion, disorientation, lethargy and eventually coma. Zieve et al.[21] suggested that the role of mercaptan in the pathogenesis of hepatic coma was as an augmenter of the toxic effects of ammonia and free fatty acids, but our data has shown that the mercaptan ethanethiol increases the blood-brain barrier permeability in normal rats.

The 6-fold elevation in free phenols found in patients with fulminant hepatic failure is derived from the gut in addition to the increased blood levels of tyrosine and phenylalanine. Phenols are known to be capable of causing convulsions and coma as they are lipophilic and can complex magnesium ions that normally bind to ATP within neurones[14]. It has also been shown that phenols inhibit many hepatic and cerebral enzymes and in common with ammonia, fatty acids and mercaptans inhibit Na^+/K^+ ATPase[19].

The effects of unconjugated bilirubin are of interest in relation to the causation of kernicterus in the new born. Animal experiments do not support the traditional view that kernicterus is due to an increase in 'free' bilirubin[18] and it is now apparent that the primary defect is an increased permeability of the blood-brain barrier[12]. Only 3 of the 6 rats studied, following i.v. injection of unconjugated bilirubin, showed changes in blood-brain barrier permeability. The reason for this variable response has yet to be established.

Endotoxin has been shown to damage endothelial cells of cerebral capillaries[17] but in this study did not increase the blood-brain barrier permeability in normal rats presumably because of removal by the Kupffer cells in the liver. However, following partial hepatectomy infusion of the lower dose of endotoxin resulted in a significant increase in permeability which was associated with a deterioration in clinical condition. Although the effect of partial hepatectomy itself on BBB permeability was not investigated, it is unlikely that the permeability is increased since the biochemical abnormalities associated with a two-thirds partial hepatectomy have virtually resolved by 48 h postoperatively[20].

The exact mechanisms by which these toxins cause an increase in the permeability of the blood brain barrier are not yet established although inhibition of Na^+/K^+ ATPase demonstrated in other studies[5,7,19] is likely to be involved. Widening of the interendothelial tight junctions is a further mechanism whereby the permeability of the barrier might be increased.

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Short Communications

Ferric chloride oxidation of isoeugenol[1]

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Summary. Ferric chloride oxidation of isoeugenol gave 5 products. The elucidation of the structures of those products and the mechanism of their formation are discussed.

Material and methods. The oxidation of isoeugenol **1** has been studied previously as a model for the observation of the formation of lignin-related dimers during ferric chloride oxidation[2] and enzymatic oxidation[3]. Photolysis[4] and free radical oxidation[5] of isoeugenol also give similar products. Further studies showed that free radical oxidation of isoeugenol[6] produced 4 trilignols. In all other experiments reported[7], the ferric chloride oxidation of isoeugenol yielded only product dehydrodiisoeugenol **2**. In the experiments described here, the oxidation of isoeugenol (10 g) acetone solution with aqueous ferric chloride in order to prepare **2** actually yielded 4 crystalline products, **3** (14 mg), **4a** (120 mg), **5** (16 mg) and **6a** (58 mg) in addition to **2** (5.3 g). The products are dilignols with a new C_β-C_β linkage except that **2** is dilignol with a C_α-O linkage. The structures were elucidated as follows.

Result and discussion. **4a** (m.p. 219–220 °C) shows IR-absorption bands at ν_{max} 3400, 1600 and 1500 cm^{-1} and NMR signals at δ_{CD_3OD} 2.11 and 2.42 (each 3H, s), 3.84 and 3.98 (each of 3H, s), 6.66, 6.70 and 7.00 (each 1H, ABX system), 7.04, 7.30 and 7.47 (each 1H, s). Its acetate **4b** (m.p. 176–177 °C), prepared from **4a** with Ac_2O-pyridine, expressed 2 acetyl groups [ν_{max} 1760 cm^{-1}; δ_{CDCl_3} 2.16 and 2.28 (each 3H, s)] instead of 2 hydroxy groups. With diazomethane the tetramethyl ether **4c** (m.p. 178–179 °C) was obtained. It was identical with dehydroguaiaretic acid dimethyl ether[8] isolated from *Guaiacum officinale.* **6a** (m.p. 247–249 °C) is a tetraol (ν_{max} 3485, 3420, and 3250 cm^{-1}) whose structure was elucidated by analytic and spectral data of its triacetate **6b**. **6b** (m.p. 216–218 °C) exhibits bands at 3520, 1760, 1715, 1605 and 1505 cm^{-1} in the IR and signals at δ_{CDCl_3} 0.93 (3H, d, J = 7Hz), 1.34 (3H, s), 1.92 (1H, s, −OH), 2.05–2.25 (1H, m, C(2)-H), 2.12, 2.21 and 2.31 (each 3H, s), 3.70 (1H, d, J = 9Hz, C(1)-H), 3.81 (6H, s), 5.87 (1H, s, C(4)-H), 6.65 and 7.11 (each 1H, s), and 6.83, 6.82 and 7.08 (each 1H, ABX system). **6a** gave **4a** on refluxing in acetone solution with p-TsOH. According to the spectral data, 2 hydroxyl groups must be located on C(3) and C(4). **6a** was not hydrogenolyzed with 10% Pd-C in methanol indicating that the C(1)-hydroxyl group has an α-equatorial orientation. The 1,2-glycol was shown to be cis because **6a** gave an amorphous acetonid **7** [ν_{max} 3400, 1580, 1110, and 1010; δ_{CDCl_3} 2.16 (6H, s)]. The large coupling constant (J = 9Hz) of C(1)-H indicates that both aryl and methyl groups have a diequatorial orientation.

5 (m.p. 210–211 °C) is also a tetraol (ν_{max} 3400 and 3225 cm^{-1}); its structure was elucidated by analytic and spectral data. **4a** was also obtained from **5** by treatment with acid. Both aryl group and methyl group in **5** have a diequatorial orientation due to C(1)-H with large coupling constant δ_{CD_3OD} 3.48 (1 H, d, J = 9 Hz). The C(4)-H signal appears at δ 4.19 (s). Therefore that 2 hydroxyl groups are located at C(3) and C(4) position. **5** gave no reaction with 2,2-dimethoxypropane in the presence of acid. The result indicates that the 1,2-glycol is a diaxial orientation. **3** (m.p. 158–159 °C, λ_{max} 281 nm; log 4.50) exhibits bands at 3390, 1600 and 1510 cm^{-1} in the IR and signals at δ_{CDCl_3} 1.06 (3H, d, J = 7Hz), 1.77 (3H, d, J = 1.2Hz), 2.36 (1H, m, C(2)-H), 3.63 (1H, d, J = 3Hz, C(1)-H), 3.78 and 3.80 (each 3H, s), 5.43 and 5.45 (each 1H, s, 2-OH), 6.09 (1H, br s, C(4)-H), and 6.50–6.80 (5H, m). Treatment with OsO_4 in pyridine yielded **6a**. The hydrogenation product of **3** was identical with quaiacin **8** (m.p. 197–199 °C).[9] In the tetrahydronaphthalene derivatives **5**, **6**, **7**, and **8** the aryl groups prefer to be in quasi-equatorial orientation. But in the dihydronaphthalene derivative (as **3**), the aryl group prefers the quasi-axial orientation. Therefore C(1)-H of **3** shows a small diequatorial coupling constant (J = 3Hz)[10].