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EFFECT OF ETHANOL ON GLYCEROL METABOLISM IN RAT LIVER DURING DIFFERENT HORMONAL CONDITIONS

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Abstract—The effect of ethanol on the metabolism of glycerol and its regulation was studied in rat liver *in vitro*, under different hormonal conditions. Glycerol utilization by normal rat liver slices is inhibited to about 50 per cent upon addition of ethanol. Ethanol addition to normal animals increases hepatic concentrations of α-glycerophosphate and 5′AMP, which have been observed to inhibit hepatic glycerokinase. Glucagon administration to normal animals also causes an increase in hepatic α-glycerophosphate and 5′AMP content and consequently lowers glycerol utilization. Insulin administration neither effects α-glycerophosphate, AMP content, nor the rate of glycerol utilization. In alloxan diabetes, although α-glycerophosphate concentration is lowered, glycerol utilization is not different from normals as hepatic AMP concentrations are doubled. Blocking of glycolysis by 2-deoxyglucose-6-phosphate lowers ethanol mediated accumulation of α-glycerophosphate in insulin and glucagon treated groups. Ethanol addition causes an increase in glycerol release from all groups, except that treated with insulin. The activities of hepatic glycerokinase and α-glycerophosphate dehydrogenase in the insulin treated or diabetic groups are not different from the control group. This investigation shows that an increase in either of the two inhibitors of glycerokinase, namely α-glycerophosphate, or 5′AMP can result in an inhibition of hepatic glycerol utilization.

GLYCEROL like ethanol is predominantly metabolized by liver[1] in the mammalian organism. Glycerol is rapidly converted to glucose and lipids or oxidized to CO_2. During starvation glycerol has been shown to be a precursor for gluconeogenesis.[2]

The metabolism of glycerol is strongly inhibited by the simultaneous metabolism of ethanol both *in vivo*[3] and *in vitro*.[4] Under these conditions it has been suggested that the glycerokinase mediated phosphorylation of glycerol to α-glycerophosphate is inhibited,[5] possibly through either of two metabolites namely α-glycerophosphate[6] which accumulates during ethanol metabolism *in vivo*[7,8] and *in vitro*,[4] and AMP as an inhibitor of glycerokinase [5,6] or both of them. In previous investigations increased α-glycerophosphate removal, produced by the administration of thyroid hormone, relieved the inhibitory effect of ethanol on glycerol metabolism.[4,9]

In the present study the metabolism of glycerol and its relationship with ethanol metabolism has been investigated after insulin treatment, glucagon administration and under conditions of acute insulin deficiency produced by alloxan diabetes and anti-insulin serum treatment. Attempts to study the regulatory role of α-glycerophosphate

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in the hepatic metabolism of glycerol were made by combining the different hormonal conditions, with the addition of ethanol.

The accumulation of α-glycerophosphate after ethanol treatment has been suggested to be caused by a shift in the α-glycerophosphate dehydrogenase reaction in favour of α-glycerophosphate as a consequence of an increased NADH/NAD ratio. The ratio [α-glycerophosphate]/[dihydroxyacetone phosphate] has been observed to increase 1·5-fold after ethanol administration.[8] In order to study the pathway(s) contributing to α-glycerophosphate accumulation during ethanol metabolism 2-deoxyglucose-6-phosphate, a metabolic inhibitor of the phosphohexose-isomerase step was used. This would block the conversion of α-glycerophosphate to glucose as well as its formation from glucose. Conditions were also produced in the liver in which the concentration of only one of the two alleged inhibitors of glycerokinase was increased. The concentration of glycerol and free fatty acids in the blood is greatly influenced by the hormones which influence the rate of lipolysis. Therefore, the influence of free fatty acids on the activities of glycerokinase and α-glycerophosphate dehydrogenase was also studied, to assess the regulatory role of free fatty acids in the metabolism of glycerol.

MATERIALS AND METHODS

Preparation of animals. Wistar rats were obtained from NOVO industry A/S, Denmark and maintained on Stock diet supplied *ad lib.* Male animals weighing 200–250 g were used.

Hyperinsulinism was produced by subcutaneous injection of 5u/rat of glucagon free Insulin NOVO (ten times recrystallized, D. Sp. no. 138). twice in 48 hr.[10] The control rats received a blank solution of NaCl. Insulin treatment dropped the blood sugar level from a control value of 98 to 55 mg %.

Acute insulin deficiency was produced in two different groups. One group was given 60 mg/kg of alloxan monohydrate[11] though the tail vein. The animals having blood glucose concentrations of 300 mg % or more were used after 48 hr. Blood sugar was determined using arsenomolybdate reagent as described previously.[12] Glucose in the urine was tested by clinistix. The other group was given 1·0 ml of guinea pig anti-insulin serum capable of neutralizing 6u insulin through the femoral vein under nembutal anaesthesia, and animals used after 90 min. The corresponding controls were given normal guinea pig serum for the same time period.

The animals treated with glucagon were anaesthetized by nembutal (intraperitoneal 50 mg/kg). A femoral vein was cannulated and crystalline insulin free glucagon (cysteine treated, D. Sp. no. 2514) dissolved in Krebs–Henseleit bicarbonate buffer (pH 7·4) was administered intravenously at the rate of 50 μg/min for the first minute, followed by 5 μg/min for a total period of 30 min.[13] Controls were treated similarly by infusion of Krebs–Henseleit bicarbonate buffer.

Incubation of tissue. The rats were killed by cervical dislocation. The livers were rapidly chilled in ice, blotted and slices 0·3 mm thick were cut at 4° by a McIlwain Buddle slicer. The slices (about 0·3–0·5 g) were transferred to prepared Erlenmeyer flasks containing 10 ml of cold Krebs–Henseleit bicarbonate buffer (pH 7·4) with the additions indicated in the tables. The flasks were shaken in an incubator at 37° and gassed with $O_2 + CO_2$ (95 : 5) for 5 min at the beginning of the incubation. At 15-min intervals 250 μl of incubation medium was deproteinized by addition of 250 μl of

perchloric acid (6% final conc.). The total incubation period was 60 min. Glycerol was determined enzymatically[14] on the neutralized supernatant whereas α-glycerophosphate was determined enzymatically[15] in liver slices. The effect of ethanol (4 mM) on glycerol release by the liver slices from different groups was studied by measuring the concentration of glycerol in the medium after 60 min of incubation.

Determination of AMP. For the determination of AMP content in liver slices from different groups of animals the following procedure was used. After incubation for 1 hr the slices were removed and rapidly frozen by pressing them between pre-cooled aluminium blocks.

The frozen slices were powdered in a mortar with the addition of liquid nitrogen. The powder was extracted in 6% (v/v) $HClO_3$ containing EDTA (5mM). The $HClO_4$ was removed as $KClO_4$ at 0° by the addition of $KHCO_3$. After removal of $KClO_4$ by centrifugation AMP was measured enzymatically[16] on the supernatant.

Enzyme assays. The hepatic glycerokinase was measured at room temperature (25°) on the supernatant from 20% fresh liver homogenate prepared in a medium containing KCl (0·15 M) and EDTA (1 mM) centrifuged at 32,000 *g* for 15 min. The assay method was essentially as described by Bubbitz and Kennedy.[17]

The activity of cytoplasmic NAD-dependent α-glycerophosphate dehydrogenase was assayed on a 32,000 *g* supernatant from 10% liver homogenate prepared in 0·25 M sucrose solution. The α-glycerophosphate dehydrogenase was measured at pH 7·5 according to Beisenheiz *et al.*[18] In some experiments effect of fatty acids like laurate, palmitate and oleate was studied on the activities of hepatic glycerokinase and α-glycerophosphate dehydrogenase.

Liver supernatant fractions were pre-incubated for 10 min at 37° in a glycine buffer pH 9·0 with different concentrations of fatty acids.

The enzymes and cofactors were purchased from C. F. Boehringer & Soehne (Mannheim, Germany). Short acting, insulin (D. Sp. nr. 138) was glucagon free and crystalline glucagon (D.Sp. nr. 2514) was insulin free. Both hormones were purchased from NOVO Industri A/S., Copenhagen. Alloxan monohydrate, lauric acid, palmitic acid were purchased from British Drug House, Ltd., England.

RESULTS

Glycerol utilization

As shown in Table 1, liver slices from insulin treated, alloxan diabetic and anti-insulin serum treated when incubated in Krebs–Henseleit bicarbonate buffer with the addition of 5 mM glycerol, did not show a significant change in the rate of glycerol utilization as compared to the normal control animals. Glucagon treatment (200 μg for 30 min), however, decreased the glycerol utilization significantly. A 50 per cent inhibition of glycerol utilization in the presence of ethanol (4 mM) observed in the control animals did not change after insulin treatment, alloxan diabetes and anti-insulin serum treatment.

The addition of ethanol caused a significantly higher glycerol release from the normal livers compared to diabetic and glucagon treated (Table 1). In the insulin treated group this increase was not observed.

α-glycerophosphate concentration

The content of α-glycerophosphate in the liver slices was significantly ($P<0·01$)

TABLE 1. EFFECT OF INSULIN ADMINISTRATION, ALLOXAN DIABETES, AIS TREATMENT AND GLUCAGON ADMINISTRATION ON THE UPTAKE OF GLYCEROL AND CONTENT OF GLYCEROPHOSPHATE IN LIVER SLICES

Treatment	Additions	Rate of glycerol utilization (μmoles/g wet wt./hr)	Glycerol release (μmoles/g/hr)	Glycerophosphate content (μmoles/g wet wt./hr)
1. Control (injected with saline)	none		0·24 ± 0·04 (4)	0·50 ± 0·05 (6)
	4 mM ethanol		0·49 ± 0·06	1·24 ± 0·04 (6)
	5 mM glycerol	31·8 ± 2·0 (6)		1·40 ± 0·07 (6)
	glycerol + ethanol	15·7 ± 1·2 (6)		2·80 ± 0·05 (6)
2. Insulin treated (5 u insulin/kg) (B.W. in 48 hr)			0·19 ± 0·05 (4)	0·58 ± 0·04 (6)
	4 mM ethanol		0·21 ± 0·27	1·34 ± 0·07 (6)
	5 mM glycerol	32·3 ± 1·9 (6)		1·30 ± 0·06 (6)
	glycerol + ethanol	18·7 ± 1·7 (6)		2·80 ± 0·05 (6)
3. Alloxan diabetes (60 mg alloxan/kg B.W. for 48 hr)			0·50 ± 0·06 (4)	0·25 ± 0·01 (6)
	4 mM ethanol		0·89 ± 0·08	0·53 ± 0·03 (6)
	5 mM glycerol	30·9 ± 2·6 (6)		0·52 ± 0·02 (6)
	glycerol + ethanol	14·9 ± 1·2 (6)		0·70 ± 0·05 (6)
4. AIS-treated (1·0 ml AIS/rat for 90 min)			0·45 ± 0·04 (6)	0·35 ± 0·07 (6)
	4 mM ethanol		0·57 ± 0·06	0·70 ± 0·02 (6)
	5 mM glycerol	30·3 ± 1·8 (6)		0·71 ± 0·02 (6)
	glycerol + ethanol	15·2 ± 1·6 (6)		1·02 ± 0·06 (6)
5. Glucagon-treated (200 μg glucagon/ rat in 30 min)			0·52 ± 0·03 (4)	0·74 ± 0·03 (4)
	4 mM ethanol		0·70 ± 0·06 (4)	1·40 ± 0·04 (4)
	5 mM glycerol	22·9 ± 2·3 (4)		1·36 ± 0·02 (4)
	glycerol + ethanol	15·0 ± 2·0 (4)		3·30 ± 0·07 (4)

The liver slices from the rats given the treatment indicated in the table were incubated for 60 min at 37° in 10 ml Krebs–Henseleit bicarbonate buffer with the additions indicated. The results are expressed as average ± S.E.M. with the number of animals used in parentheses.

TABLE 2. EFFECT OF 2-DEOXYGLUCOSE 6-PHOSPHATE ADDITION ON THE α-GLYCEROPHOSPHATE CONCENTRATION IN LIVER SLICES FROM CONTROL, INSULIN TREATED, ALLOXAN DIABETIC AND GLUCAGON TREATED ANIMALS

Treatment	Additions	Glycerophosphate content (μmoles/g/hr)
1. Control	(a) Ethanol (4 mM)	1·24 ± 0·04 (6)
	(b) Ethanol + 2-deoxyglucose-6-phosphate	1·20 ± 0·04 (6)
2. Insulin treated	(a) Ethanol (4 mM)	1·30 ± 0·07 (6)
	(b) Ethanol + 2-deoxyglucose-6-phosphate	1·00 ± 0·05 (6)
3. Alloxan-diabetic	(a) Ethanol (4 mM)	0·53 ± 0·02 (6)
	(b) Ethanol + 2-deoxyglucose-6-phosphate	0·51 ± 0·02 (6)
4. Glucagon treated	(a) Ethanol (4 mM)	1·57 ± 0·07 (6)
	(b) Ethanol + 2-deoxyglucose-6-phosphate	1·27 ± 0·06 (6)

Liver slices from rats given different treatments indicated in the table were incubated for 60 min at 37° in 10 ml Krebs–Henseleit bicarbonate buffer with the additions indicated. Results are expressed as average ± S.E.M. with the number of animals given in parentheses.

higher in the glucagon treated animals than in control group. Alloxan diabetes and anti-insulin serum treatment significantly lowered the content of α-glycerophosphate (Table 1). The addition of glycerol (5 mM) to all groups caused an increase in the α-glycerophosphate content. Addition of ethanol (4 mM) caused an increase in α-glycerophosphate in all groups.

Addition of 2-deoxyglucose 6-phosphate to the liver slices from insulin and glucagon treated groups significantly ($P<0.01$) lowered the ethanol mediated increase in the α-glycerophosphate concentration (Table 2). In the case of normal, alloxan diabetic and anti-insulin serum treated animals the ethanol mediated accumulation of α-glycerophosphate remained unchanged.

AMP content

Addition of ethanol to control, insulin treated, alloxan diabetic, AIS treated and glucagon treated animals resulted in an increase in hepatic AMP content (Table 3).

Treatment of control animals with alloxan, anti-insulin serum and glucagon resulted in significant increase in AMP content in liver. However, hepatic AMP content in insulin treated group was in the same range as in controls.

TABLE 3. EFFECT OF ETHANOL, INSULIN, ALLOXAN, ANTI-INSULIN SERUM AND GLUCAGON ON THE CONTENT OF AMP IN RAT LIVER SLICES

Treatment	Additions	AMP content (μmoles/g wet wt.)
1. Control	none	0·138 ± 0·020 (4)
	ethanol (4 mM)	0·200 ± 0·014 (4)
2. Insulin treated	none	0·130 ± 0·030 (4)
	ethanol (4 mM)	0·198 ± 0·016 (4)
3. Alloxan diabetic	none	0·258 ± 0·018 (4)
	ethanol (4 mM)	0·350 ± 0·022 (4)
4. AIS treated	none	0·221 ± 0·016 (4)
	ethanol (4 mM)	0·294 ± 0·020 (4)
5. Glucagon treated	none	0·260 ± 0·018 (4)
	ethanol (4 mM)	0·340 ± 0·020 (4)

Liver slices from rats given the treatment indicated in the table were incubated for 60 min at 37° in 10 ml Krebs–Henseleit bicarbonate buffer with or without ethanol. At the end of incubation slices were pressed between the pre-cooled aluminium blocks extracted in $HCLO_4$ and AMP determined enzymically.

TABLE 4. EFFECT OF INSULIN ADMINISTRATION, ALLOXAN DIABETES AND GLUCAGON ADMINISTRATION ON THE ACTIVITIES OF HEPATIC GLYCEROKINASE AND GLYCEROPHOSPHATE DEHYDROGENASE

Treatment	Hepatic enzyme activity	
	Glycerokinase	Glycerophosphate dehydrogenase (units/g wet liver)
1. Control (saline injected)	0·96 ± 0·08 (6)	32·0 ± 4·2 (4)
2. Insulin treated	1·00 ± 0·06 (6)	34·1 ± 3,8 (4)
3. Alloxan diabetes	0·87 0·04 (6)	30·0 ± 3·6 (4)

The activities of hepatic glycerokinase and glycerophosphate dehydrogenase were determined spectrophotometrically at room temperature (25°). For glycerokinase supernatant from 20% fresh liver homogenate prepared in a medium containing (0·15 M) and EDTA (1 mM) was used. Glycerophosphate dehydrogenase was assayed on the supernatant from 10% liver homogenate.

Glycerokinase and α-glycerophosphate dehydrogenase

Treatment of normal animals with insulin to produce hyperinsulism, or treatment with alloxan to produce acute diabetes, did not result in a significant alteration in the activities of either hepatic glycerokinase or α-glycerophosphate dehydrogenase (Table 4). The activities of these two enzymes also remained unaltered upon pre-incubation with free fatty acids (up to 1 mM). As also the rate of glycerol uptake by liver slices remained unaffected when incubated with palmitate, or oleate (0·4 mM) in the presence of serum albumin. The rate of glycerol uptake in the presence or absence of free fatty acids was 31·8 μmoles . g^{-1} . hr^{-1}.

DISCUSSION

The levels of serum glycerol have been reported to be elevated in uncontrolled diabetes and lowered by insulin or glucose administration or both of them.[20,21] Opinions differ as to whether this lowering effect of glucose and insulin is due to reduced glycerol output from the adipose tissue or to increased glycerol utilization by liver. The unchanged utilization rate by the liver slices after the administration of insulin and acute insulin deficiency produced by alloxan and anti-insulin serum treatment (Table 1) as compared to the control animals supports the former view. The glycerol utilization has also previously been observed to be not significantly different in alloxan diabetic groups as compared to normal animals[22].

As is evident from the result given in Table 1, addition of ethanol to liver slices from the acute diabetic group or normal control group inhibited the rate of glycerol utilization to about the same extent (50 per cent). As the hepatic concentration of α-glycerophosphate in the diabetic group after addition of ethanol was significantly lower than in the normal animals, the inhibition of glycerol utilization produced upon ethanol addition in this group would be expected to change if α-glycerophosphate was the metabolite solely inhibiting the activity of hepatic glycerokinase and consequently the rate of glycerol utilization. However, as mentioned above, this is not the case. Therefore, some other metabolite might be exerting the inhibitory effect on the glycerol metabolism under these conditions. The other possible metabolite exerting regulatory effect was probably AMP, as the hepatic concentrations of AMP after ethanol addition to the diabetic group were much higher than in control animals after ethanol addition.

In the control group, the glycerol utilization in the presence of ethanol might be regulated by an increase in both hepatic α-glycerophosphate and AMP content. In the diabetic group this effect is obtained by an increase in only AMP content, which is greater than in normal animals. In the case of the insulin treated group the extent of glycerol utilization is similiar to that of the normal animals. This is not surprising, since ethanol addition to the insulin treated group produces comparable changes to controls in the content of the two proposed inhibitors of glycerokinase, namely α-glycerophosphate and AMP. In the case of adipose tissue[27] insulin has also been observed not to influence the content of AMP. However, unlike in liver, in adipose tissue α-glycerophosphate content is significantly increased upon insulin administration.[27]

The inhibited rate of glycerol utilization observed in liver slices from glucagon treated animals (without additions) may be explained on the basis of increases in the concentration of both α-glycerophosphate and AMP. The effect of glucagon in this

respect is like the ethanol effect, where both glycerophosphate and AMP concentrations are increased. However, at present it is difficult to assign a physiological significance to this inhibitory effect of glucagon on the uptake of glycerol. This might be of significance in conditions of glucagon mediated lipolysis where glycerol is produced by the hydrolysis of triglycerides and diglycerides, but perhaps not used for gluconeogenesis. Glucagon administration in perfused rat livers has failed to stimulate gluconeogenesis from glycerol.[24] Conversion of glycerol to glucose has also been observed to be inhibited in conditions where high concentrations of AMP[25] are obtained by cyanide administration.

It seems from these studies that hepatic concentrations of both α-glycerophosphate and AMP exert a regulatory effect on the rate of glycerol utilization. The glycerol utilization rate by liver in conditions of starvation also supports this idea, as this rate during starvation is not different from that of fed animals.[26] Under conditions of starvation, although the content of hepatic α-glycerophosphate is greatly lowered as compared to fed animals,[27] which might allow the glycerokinase reaction to proceed at a maximal rate, the hepatic content of AMP is almost doubled, which in turn would inhibit the reaction to the extent that finally the glycerol utilization is not different from fed animals.

Experiments conducted with the metabolic inhibitor 2-deoxyglucose-6-phosphate to assess the pathway(s) contributing to the α-glycerophosphate accumulation during ethanol metabolism indicated that carbon for the α-glycerophosphate formation in a normal animal is supplied through the gluconeogenic route. Addition of ethanol would further enhance this effect as ethanol addition has previously been observed to induce gluconeogenesis in fed animals.[28] On the other hand in a condition like insulin administration where gluconeogenesis is inhibited, addition of 2-deoxyglucose-6-phosphate significantly decreased the accumulation of α-glycerophosphate. In contrast to the insulin treated group in the alloxan treated group which has increased need of gluconeogenesis, addition of 2-deoxyglucose-6-phosphate did not significantly affect the α-glycerophosphate accumulation.

The unchanged activities of two hepatic enzymes directly involved in glycerol metabolism namely glycerokinase and α-glycerophosphate dehydrogenase (Table 4) after insulin administration or alloxan treatment as compared to normal animals, were also consistent with the unchanged glycerol utilization (Table 1). In contrast to acute insulin deficiency, chronic diabetes has been observed to produce a decrease[29] in the activity of hepatic glycerokinase when expressed on the basis of total protein. Subsequent administration of insulin to these diabetic animals resulted in an increased activity of hepatic glycerokinase.

The effect of free fatty acids on the activities of glycerokinase and α-glycerophosphate dehydrogenase was investigated, in view of an interrelation between metabolism of glycerol and ethanol and the fact that enzymes of ethanol metabolism have been observed to be inhibited by free fatty acids.[30,31] The uninfluenced activities of two enzymes of glycerol metabolism by free fatty acids, suggest that in conditions of glucagon induced lipolysis the influence of the hormone on the hepatic glycerol utilization is at least not a result of the changes in the plasma free fatty acids. The plasma free fatty acids level has been observed to remain unaltered on oral glycerol administration to human subjects.[32]

Addition of ethanol to the adipose tissue [33] in normal animals has been observed to

increase the glycerol release. In the present study the unchanged glycerol in the medium (Table 1) with liver slices from insulin treated animals and significantly large glycerol release from acute diabetic animals support the antilipolytic effect of insulin, while higher release of glycerol by glucagon treated animals support lipolytic effects of glucagon.

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