The Effects of Inhibition of Gluconeogenesis in Suckling Newborn Rats

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Inhibition of gluconeogenesis with 3-mercaptopycolinate in suckling newborn rats caused a fall in blood [glucose], but no change in their high plasma [free fatty acid] and blood [ketone bodies]. Active gluconeogenesis seems to be more important than sparing of glucose by high concentrations of fat-derived substrates for the maintenance of normal blood [glucose] in suckling newborn rats.

Immediately after birth, the rat is fed with milk, a low-carbohydrate and high-fat diet (Luckey *et al.*, 1954). Nevertheless, the blood [glucose] is maintained at normoglycaemic values throughout the suckling period (Page *et al.*, 1971). The low carbohydrate intake of the suckling rat could be partly counter-balanced by the decreased glucose utilization (Vernon & Walker, 1972; Snell & Walker, 1973a). Under these conditions, the role of gluconeogenesis is relevant in the regulation of blood [glucose]. Studies *in vitro* with tissue slices have indicated that the maximal rate of gluconeogenesis in the liver and kidney of the suckling rat is greater than that of the normally fed adult [see Snell & Walker (1973b) for a review]. The importance of this process during suckling is also supported by the considerable increase in activity of key regulatory enzymes of gluconeogenesis (Ballard & Hanson, 1967; Vernon & Walker, 1968). However, the extent to which this occurs *in vivo* and the relative contribution of various gluconeogenic substrates (lactate, amino acids and glycerol) are uncertain. The finding that 3-mercaptopycolinate is an effective inhibitor of gluconeogenesis at the phosphoenolpyruvate carboxykinase level (Di Tullio *et al.*, 1974) prompted us to study the effect of this compound in suckling newborn rats.

Experimental

Animals

All newborn rats of an albino Wistar strain were born between 15:00 and 18:00 h on day 22 of gestation. They were left with their mother for 16 h after delivery in a room in which the external temperature was 22°C. The newborn rats began to suckle between 1 and 2 h after birth.

Experimental design

Each litter of 16 h-old newborn rats was divided into two groups. The newborn rats in the first group were injected subcutaneously with sodium 3-mercaptopycolinate (pH 7.4) at a dose of 75 mg/kg body wt.

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and were immediately placed in a Humidicrib (Jouan SA, Paris, France) in which the temperature was maintained at 37°C and the relative humidity at 70%. The newborn rats in the second group were injected with the same volume (50 μ l) of 0.9% NaCl. In the experiments in which blood and hepatic metabolites and plasma hormones were determined, the newborn animals were killed 1 h after injections of 3-mercaptopycolinate or NaCl. In the gluconeogenesis studies, the newborn rats were injected intraperitoneally with labelled precursor (1 μ Ci of [U-¹⁴C]lactate or 1 μ Ci of [U-¹⁴C]alanine or [1-¹⁴C]-glycerol) 30 min after 3-mercaptopycolinate or NaCl injections, placed again in the Humidicrib and were killed 30 min after injection of labelled precursor.

Blood and liver sampling

Blood samples for metabolite, amino acid and hormone assays were collected as described previously (Girard *et al.*, 1973, 1975). The liver was removed rapidly from unanaesthetized newborn rats and freeze-clamped between metal blocks previously cooled in liquid N₂ (Wollenberger *et al.*, 1960). The average time elapsing between sampling and deep-freezing was 2 s. The frozen liver was weighed, pulverized in a mortar, extracted with HClO₄ and neutralized, as described by Williamson *et al.* (1967).

Measurement of gluconeogenesis rates *in vivo* from ¹⁴C-labelled substrates

The technique used and the method for calculation of gluconeogenesis rate *in vivo* were as described previously (Girard *et al.*, 1975, 1976), by using lactate and glycerol spaces equal to the total body water, i.e. 80 ml/100 g body wt., and glucose and alanine spaces equal to extracellular body water, i.e. 60 ml/100 g body wt. The separation of labelled glucose, lactate, alanine or charged compounds was performed by ion-exchange chromatography (Kreisberg *et al.*, 1972). Labelled glucose was separated from labelled glycerol by descending paper chromatography (Vernon & Walker, 1970).

Assays

Enzymic assays were performed for glucose, pyruvate, lactate, glycerol, alanine, acetoacetate and 3-hydroxybutyrate as previously described (Girard *et al.*, 1973). Plasma unesterified fatty acids were measured by the radioactive nickel method of Ho (1970). Enzymic assays were also used for ATP (Lamprecht & Trautschold, 1963), ADP and AMP (Adam, 1963), malate (Hohorst, 1963) and phosphoenolpyruvate (Czok & Eckert, 1963). The liver [oxaloacetate] and the liver cytosolic and mitochondrial free [NAD⁺]/[NADH] were calculated as described by Williamson *et al.* (1967).

Amino acid analysis

The analysis of free amino acids was performed on blood de-proteinized with sulphosalicylic acid by using a Beckman 120C amino acid analyser as described previously (Girard *et al.*, 1975, 1976).

Hormones

Plasma insulin and glucagon were determined by radioimmunoassay as described previously (Girard *et al.*, 1973, 1975). Glucagon was assayed by using 30K antiserum (Faloona & Unger, 1974), which is considered to be specific for pancreatic glucagon. Results are expressed as means $\pm$ S.E.M.; significant differences were determined by using Student's *t* test for non-paired populations.

Special chemicals

Enzymes and coenzymes were from Boehringer Corp. (Paris), 75006 Paris, France. 3-Mercaptopicolinic acid was a gift from Dr. H. L. Saunders, Smith, Kline and French Laboratories, Philadelphia, PA 19101, U.S.A.

Results

Blood metabolites

The injection of 3-mercaptopicolinate (75 mg/kg, subcutaneously) in 16 h-old suckling newborn rats resulted in a dramatic fall in blood [glucose] after 1 h; the total decrease in concentration was 3.9 mm (Table 1). It also resulted in increases in blood [lactate] and [pyruvate] of 0.72 and 0.044 mm respectively at 1 h (Table 1). The blood [alanine] did not change significantly. Similarly, the blood concentration of 20 amino acids measured by an amino acid analyser was not changed by the injection of 3-mercaptopicolinate (results not shown).

The injection of 3-mercaptopicolinate caused a decrease in blood [acetoacetate] of 0.38 mm after 1 h, but the decrease in blood [3-hydroxybutyrate] of 0.33 mm was not statistically significant. There was a 6-fold increase in the blood [3-hydroxybutyrate]/[acetoacetate] ratio. Plasma [unesterified fatty acids] increased by 0.12 mm 1 h after 3-mercaptopicolinate injection, but blood [glycerol] did not change.

Liver metabolites

The effect of 3-mercaptopicolinate on the pattern of liver metabolites is shown in Fig. 1, and indicates a point of inhibition between oxaloacetate and phosphoenolpyruvate, i.e. at the level of phosphoenolpyruvate carboxykinase. The injection of 3-mercaptopicolinate also caused a decrease in hepatic [3-hydroxybutyrate] and [acetoacetate] from 4.04 ± 0.34 and 0.83 ± 0.05 μ mol/g for NaCl controls ($n=9$) to 2.52 ± 0.14 ($P < 0.001$) and 0.22 ± 0.02 ($P < 0.01$) μ mol/g ($n=10$) respectively. The cytosol [NAD⁺]/[NADH] and the [ATP], [ADP], [AMP] and [alanine] in the liver remained unchanged after 3-mercaptopicolinate injection (results not shown).

Table 1. Blood and plasma metabolite concentrations after injection of NaCl or 3-mercaptopicolinate in suckling newborn rats. Suckling newborn rats (16 h-old) were injected subcutaneously with NaCl or 3-mercaptopicolinate. The blood was collected 1 h later. Significant differences were determined by using Student's *t* test. * $P < 0.05$, ** $P < 0.001$, when comparing animals injected with 3-mercaptopicolinate with animals injected with NaCl. The number of animals in each group is shown in parentheses. Values are means $\pm$ S.E.M. For other details see the text.

	Metabolite concn. (μ mol/ml)	
	NaCl (8)	3-Mercaptopicolinate (9)
Blood [glucose]	5.5 ± 0.2	$1.6 \pm 0.2^{**}$
Blood [pyruvate]	0.11 ± 0.01	$0.15 \pm 0.01^{**}$
Blood [lactate]	1.51 ± 0.12	$2.23 \pm 0.10^{**}$
Blood [glycerol]	0.23 ± 0.01	0.24 ± 0.02
Plasma [unesterified fatty acid]	0.84 ± 0.03	$0.96 \pm 0.06^*$
Blood [acetoacetate]	0.45 ± 0.03	$0.06 \pm 0.01^{**}$
Blood [3-hydroxybutyrate]	2.47 ± 0.35	2.14 ± 0.23
Total [ketone bodies]	2.62	2.20
Blood [alanine]	0.36 ± 0.03	0.40 ± 0.02
[Lactate]/[pyruvate]	14.2	14.9
[3-Hydroxybutyrate]/[acetoacetate]	5.5	33

Fig. 1. Cross-over plot showing the hepatic metabolite concentrations after injection of 3-mercaptopicolinate in suckling newborn rats

Suckling newborn rats (16 h-old) were injected subcutaneously with NaCl (●) or 3-mercaptopicolinate (○). The liver was removed 1 h later and immediately freeze-clamped. Values shown are the means $\pm$ S.E.M. for nine or ten determinations. The concentrations of each metabolite in the liver of control rats were: lactate, $2 \mu\text{mol/g}$; pyruvate, $0.06 \mu\text{mol/g}$; malate, $0.51 \mu\text{mol/g}$; calculated oxaloacetate, 3.6 nmol/g ; phosphoenolpyruvate, $0.21 \mu\text{mol/g}$; glucose, $2.5 \mu\text{mol/g}$. Although the method used for calculation of oxaloacetate is not entirely satisfactory since [malate] is not the same in all the cell compartments, it seems to be acceptable in the present study, because of the large changes observed.

By contrast, the injection of 3-mercaptopicolinate caused a decrease in hepatic mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ from 4.2 ± 0.4 for NaCl controls ($n = 9$) to 1.6 ± 0.2 ($P < 0.001$) ($n = 10$).

Rate of gluconeogenesis *in vivo*

As expected, a marked inhibition (88%) of gluconeogenesis from labelled lactate and alanine was observed in newborn rats injected with 3-mercaptopicolinate. Surprisingly, gluconeogenesis from glycerol was also inhibited by 40%. The rate of conversion of ^{14}C -labelled precursors into labelled glucose

(in $\mu\text{mol/h}$ per 100 g body wt.) in NaCl-injected newborn rats was: 22 ± 1 from lactate ($n = 15$); 1.8 ± 0.2 from alanine ($n = 10$); 6.5 ± 0.4 from glycerol ($n = 8$). In newborn rats injected with 3-mercaptopicolinate these rates were: 2.5 ± 0.2 from lactate ($n = 10$); 0.25 ± 0.03 from alanine ($n = 8$); 3.9 ± 0.1 from glycerol ($n = 8$). All these differences were statistically significant ($P < 0.001$). The specific radioactivity of lactate, alanine or glycerol in the blood at the time of sampling was always higher in newborn rats injected with 3-mercaptopicolinate than in controls. This ruled out a possible decrease of gluconeogenesis secondary to an increased pool size of substrate and a decreased specific radioactivity of the glucose precursor.

Plasma insulin and glucagon

Plasma insulin decreased dramatically, from $1.85 \pm 0.16 \text{ ng/ml}$ for the NaCl controls to $0.46 \pm 0.04 \text{ ng/ml}$, in association with hypoglycaemia, in the newborn rats injected with 3-mercaptopicolinate. No significant variations in plasma glucagon were observed ($1275 \pm 133 \text{ pg/ml}$ for the NaCl controls versus $1375 \pm 60 \text{ pg/ml}$ for the newborn rats injected with 3-mercaptopicolinate). Each value was the mean of six determinations. The insulin/glucagon molar ratio fell dramatically, from 0.80 for NaCl controls to 0.19, and reflected a strongly catabolic state in the newborn rats injected with 3-mercaptopicolinate.

Discussion

In the present study 3-mercaptopicolinate was used to examine the metabolic effects of a blockage of gluconeogenesis in the suckling newborn rats. As previously reported for starved or diabetic adult rats (Di Tullio *et al.*, 1974; Blackshear *et al.*, 1975), 3-mercaptopicolinate inhibited gluconeogenesis at the level of phosphoenolpyruvate carboxykinase and caused hypoglycaemia in 16 h-old suckling rats. The development of a profound hypoglycaemia in suckling newborn rats occurred in spite of the high plasma [unesterified fatty acids] and blood [ketone bodies]. This suggests that, in the newborn rat, a potential sparing of glucose utilization by the high plasma [unesterified fatty acids] and [ketone bodies] is not sufficient to maintain a normal blood glucose concentration when gluconeogenesis is inhibited. A high rate of gluconeogenesis seems to be a prerequisite to maintain blood glucose homeostasis in these animals fed with milk, which is a high-fat, high-protein and low-carbohydrate diet (Luckey *et al.*, 1954). The 50% rise in blood [lactate] when gluconeogenesis is inhibited strongly suggests that lactate is the most important substrate for gluconeogenesis in newborn rats. By contrast, no increase in blood [alanine] or other gluconeogenic amino acids is observed. This

differs markedly from the results obtained in 10-day-old and adult rats, where an increase in [alanine] was observed in animals infused with 3-mercaptopicolinate (Blackshear *et al.*, 1975; Snell, 1976). This discrepancy could be explained by a difference in the quantitative importance of alanine as gluconeogenic precursor during development. Glycerol does not appear to make a major contribution to blood glucose regulation in suckling newborn rats, since, when gluconeogenesis is inhibited by only 40% by 3-mercaptopicolinate, hypoglycaemia nevertheless arose. From the data available, it is not possible to give an explanation for the effect of 3-mercaptopicolinate on gluconeogenesis from glycerol, but a similar observation has also been made in isolated perfused adult rat liver (Jomain-Baum *et al.*, 1976).

The present results show that inhibition of gluconeogenesis induced by 3-mercaptopicolinate is accompanied by a decrease in ketogenesis which is not secondary to a decrease in plasma [unesterified fatty acid], since it rises rather than falls. The injection of 3-mercaptopicolinate to newborn rats caused an accumulation of reducing equivalents in mitochondria, but not in the cytosol, as indicated by the changes in liver $[NAD^+]/[NADH]$. This early accumulation of reducing equivalents in mitochondria could result from an inhibition of mitochondrial transport of dicarboxylic acid, as has been demonstrated previously after quinolinic acid administration in adult rats (Spydervold *et al.*, 1973, 1974). These results are in agreement with the previous observations by Blackshear *et al.* (1975), who suggested that inhibition of gluconeogenesis by 3-mercaptopicolinate may decrease ketogenesis through the more reduced mitochondrial redox state. The inhibitory effect of 3-mercaptopicolinate on ketogenesis is already apparent after 1 h in the liver, whereas the decrease in blood [ketone bodies] is not yet obvious. A likely explanation for this finding is that the liver is the organ producing ketone bodies. Similarly, in severely ketotic rats, 3-mercaptopicolinate caused only a 30% decrease of blood [ketone bodies] 1 h after the injection, but an 80% decrease 3 h later (Blackshear *et al.*, 1975).

A decrease in plasma insulin, but no change in plasma glucagon, occurred during hypoglycaemia induced by the injection of 3-mercaptopicolinate. This result is in agreement with previous observations showing that pancreatic A cells of neonatal rats were insensitive to acute variations of blood glucose concentration, but that B cells were fully responsive to changes in blood glucose (Girard *et al.*, 1974).

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