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Fatty acids and glycerol or lactate are required to induce gluconeogenesis from alanine in isolated rabbit renal cortical tubules

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Summary. In isolated rabbit renal cortical tubules, glucose synthesis from 1 mM alanine is negligible, while the amino acid is metabolized to glutamine and glutamate. The addition of 0.5 mM octanoate plus 2 mM glycerol induces incorporation of [U-^{14}C]alanine into glucose and decreases glutamine synthesis, whereas oleate and palmitate in the presence of glycerol are less potent than octanoate. Gluconeogenesis is also significantly accelerated when glycerol is substituted by lactate. In view of an increase in $^{14}CO_2$ fixation and elevation of both cytosolic and mitochondrial NADH/NAD$^+$ ratios, the activation of glucose formation from alanine upon the addition of glycerol and octanoate is likely due to (i) stimulation of pyruvate carboxylation, (ii) increased availability of NADH for glyceraldehyde-3-phosphate dehydrogenase and (iii) elevation of mitochondrial redox state causing a diminished provision of ammonium for glutamine synthesis. The induction of gluconeogenesis in the presence of alanine, glycerol and octanoate is not related to cell volume changes. The results presented in this paper show the importance of free fatty acids and glycerol for regulation of renal gluconeogenesis from alanine. The possible physiological significance of the data is discussed.

Keywords: Amino acids – Alanine – Fatty acids – Glutamine and glutamate synthesis – Glycerol and lactate metabolism – Malate-aspartate shuttle – Rabbit kidney-cortex tubules – Renal gluconeogenesis

Introduction

Alanine is considered to be a major gluconeogenic substrate in liver (Jungas et al., 1992). In contrast, despite the presence of alanine aminotransferase in proximal nephron, this amino acid is not converted into glucose in rat and dog (Lemieux et al., 1988) or guinea pig kidney-cortex preparations when used as the sole substrate (Michoudet et al., 1988), while low glucose formation in the

presence of alanine has been measured in isolated human renal cortical tubules (Fouque et al., 1996). Although it has been proposed that the major role of alanine in renal metabolism is its synthesis rather than utilization (Lemieux et al., 1988), this amino acid is effectively converted into glutamine in isolated guinea pig renal cortical tubules with the use of pyruvate carboxylase which plays an essential role in generation of 2-oxoglutarate needed for alanine transamination (Michoudet et al., 1988). Moreover, in isolated rabbit kidney-cortex tubules metabolizing 2-oxoglutarate, alanine is converted into glucose, indicating that generation of 2-oxoglutarate in renal tubules may limit alanine utilization as glucose precursor (Zabłocki and Bryła, 1989).

Glycerol, which is not used as gluconeogenic substrate in rabbit renal tubules (Wirthenson et al., 1981; Zabłocki and Bryła, 1988), has been shown to accelerate glucose formation from pyruvate and glutamate (Zabłocki and Bryła, 1988), while both lactate and glycerol stimulate glucose synthesis from aspartate in renal tubules isolated from rabbit (Lietz and Bryła, 1995). Moreover, fatty acids have been reported to enhance glucose production from lactate in rat renal cortical tubules (Guder and Wieland, 1972). In view of these data, studies were undertaken on the effect of glycerol, lactate and fatty acids on gluconeogenesis from alanine in isolated rabbit kidney-cortex tubules.

Material and methods

Preparation and incubation of kidney-cortex tubules

Fed rabbits (male, Termond strain, 2–3 kg body weight) were anesthetized with pentobarbital injected into ear marginal vein (30 mg per kg body weight). Renal cortex tubules were isolated as described previously (Zabłocki et al., 1983) and incubated at 37°C in 2 ml of Krebs-Ringer bicarbonate buffer (about 10 mg dry weight) in 25 ml plastic Erlenmeyer flasks sealed with rubber stoppers under the atmosphere of 95% O_2 + 5% CO_2. Substrates were added at concentrations given in legends to Tables and Figures. Fatty acids were included into reaction medium as albumin emulsions. For the measurement of total production of metabolites in renal tubule suspension, the reaction was stopped after 60 min of incubation by the addition of 35% perchloric acid (0.1 vol of tubule suspension). No significant differences could be seen in the ratios of lactate dehydrogenase to glutamate dehydrogenase under various conditions of incubation, indicating negligible leakage of cytosolic enzymes. Moreover, fatty acids did not decrease the intracellular ATP content following 60 min of incubation of tubules with alanine + glycerol (5.04 ± 0.44 and 4.86 ± 0.42 μmol per g dry wt in the presence and absence of octanoate in the incubation mixture, respectively).

Analytical methods

Both utilization of alanine, glycerol, lactate and production of amino acids, lactate, glycerol-3-phosphate, ammonium and glucose were estimated from measurements of metabolites in samples withdrawn from the reaction medium. The intracellular content of metabolites in isolated kidney tubules was measured in samples following centrifugation of tubule suspension through the silicone oil into a perchloric acid solution as described previously (Zabłocki et al., 1983). The isotopic studies used for investigation of the contribution of different substrates to glucose formation were performed according to Exton and Park (1967) and Pilkis et al. (1976). Fixation of $^{14}CO_2$ by renal tubules was determined in the incubation mixture containing a trace amount of sodium [^{14}C]-

bicarbonate (Michoudet et al., 1988). Oxygen consumption determinations were performed as described by Harris et al. (1981) in magnetically stirred thermostated chambers using polarographic techniques. Intracellular water was determined as described by Weinberg et al. (1990) for suspension of isolated renal proximal tubules. Glucose, ammonium, lactate, malate, pyruvate, 3-hydroxybutyrate, acetoacetate, glycerol, glycerol-3-phosphate, dihydroxyacetone phosphate were estimated either spectrophotometrically or fluorimetrically by standard techniques described by Bergmeyer (1965). Amino acids were determined by HPLC after derivatization of samples with 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) as described by Chang et al. (1983).

Enzymes and chemicals

Collagenase (EC 3.4.24.3), type IV, DABS-Cl, lactic acid, L-alanine, octanoic, oleic and palmitoic acids, aminooxyacetate (AOA) and L-methionine sulphoximine (MSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enzymes for metabolite determination were from Boehringer (Mannheim, Germany), Reanal (Budapest, Hungary) and P.O.Ch. (Gliwice, Poland). [U-^{14}C]lactate, [U-^{14}C]glycerol and [U-^{14}C]L-alanine were from Amersham Searle Corporation (Amersham, U.K.), while [^{14}C]sodium bicarbonate was from Swierk (Poland). Other reagents were from P.O.Ch. (Gliwice, Poland).

Results

Alanine utilization, glucose formation, oxygen consumption, and amino acid production

The utilization of 1 mM alanine by isolated rabbit renal cortical tubules was linear over 60 min of incubation and was equal to 148.5 ± 8.7 μmol/h per g dry weight. It was not accompanied by accumulation of ammonium, aspartate, lactate or pyruvate in the incubation mixture. Amino acid consumption was not changed on the addition of glycerol, octanoate nor glycerol plus octanoate (157.2 ± 8.4, 154.2 ± 12.4 and 140.6 ± 15.0 μmol/h per g dry weight, respectively).

As presented in Table 1, the rate of glucose formation in rabbit renal tubules incubated with 1 mM alanine was negligible. Moreover, the addition of either 2 mM glycerol, which was not utilized for gluconeogenesis, or 0.5 mM octanoate did not affect glucose synthesis. In contrast, 2 mM lactate significantly increased the rate of glucose formation in comparison with that determined with either alanine or lactate alone. Interestingly, the addition of 0.5 mM octanoate to renal tubules incubated with alanine plus glycerol resulted in a marked enhancement of gluconeogenesis. When octanoate was substituted by either 0.5 mM oleate or 0.5 mM palmitate, the rates of glucose synthesis were lower than those measured with octanoate by about 50 and 80%, respectively (36.7 ± 6.3 and 15.2 ± 2.6 μmol/h per g dry wt, respectively). This might be due to their rather low utilization as concluded from the rates of oxygen consumption (58.1 ± 4.4, 83.7 ± 5.8, 68.1 ± 1.1 and 65.4 ± 2.2 μmol/min per g dry wt, without and with octanoate, oleate or palmitate, respectively, $P < 0.001$ vs control value measured without fatty acid for 3 experiments). The maximal rate of gluconeogenesis in the presence of octanoate was observed with 1 mM alanine and either 2 mM glycerol or lactate. It should be

Table 1. Glucose, glutamine and glutamate synthesis in renal tubules incubated with various substrates

Substrates	Alanine	Glucose	Glutamine	Glutamate
None	−	2.1 ± 1.1	32.5 ± 2.3	19.0 ± 1.0
None	+	4.5 ± 1.0	85.3 ± 14.7	37.0 ± 5.0
Glycerol	−	6.8 ± 2.5	32.2 ± 3.7	19.3 ± 0.9
Glycerol	+	5.4 ± 3.4	87.6 ± 15.7	29.6 ± 6.1
Octanoate	−	2.5 ± 1.5	33.3 ± 2.0	37.0 ± 5.7[a]
Octanoate	+	4.7 ± 0.9	57.4 ± 9.4[f]	91.2 ± 12[f]
Glycerol + octanoate	−	6.5 ± 1.8	30.5 ± 4.0	39.0 ± 3.7[a]
Glycerol + octanoate	+	66.2 ± 6.7[c]	57.1 ± 9.9[c]	93.8 ± 10.6[c]
Lactate	−	11.6 ± 4.4[a]	35.3 ± 3.2	41.7 ± 5.7[a]
Lactate	+	31.5 ± 8.7[d]	44.5 ± 4.5[f]	66.5 ± 9.6[f]
Lactate + octanoate	−	23.0 ± 3.4[d]	23.2 ± 2.4[b]	50.0 ± 4.4[a]
Lactate + octanoate	+	62.3 ± 6.3[e]	39.3 ± 2.9[e]	92.7 ± 8.7[e]

Renal tubules were incubated with various substrates for 60 min. Alanine and fatty acids were added at 1 and 0.5 mM concentrations, respectively, while glycerol and lactate were 2 mM. Values expressed as μmol/h per g dry wt are means ± SD for 5–10 experiments. [a] $P < 0.001$, [b] $P < 0.05$ vs control with no alanine and other substrate added; [c] $P < 0.001$ vs control with alanine + glycerol; [d] $P < 0.001$ vs control with lactate alone; [e] $P < 0.001$ vs control with alanine + lactate; [f] $P < 0.001$ vs control with alanine alone.

mentioned, however, that in the presence of 1 mM alanine, octanoate induced a significant stimulation of glucose synthesis at as low as 0.2 mM and 0.5 mM glycerol concentration (17.1 ± 2.9 and 40.5 ± 1.5 μmol of glucose/h per g dry wt, respectively, $P < 0.001$ vs control with alanine + octanoate, for 3 experiments), while increasing glycerol concentration above 2 mM did not further accelerate glucose formation (data not shown).

In the absence of fatty acid and glycerol, glutamate and glutamine were the main products of alanine metabolism in rabbit renal tubules. The addition of glycerol did not significantly change the rates of glutamine and glutamate formation, while octanoate resulted in about 40% inhibition of glutamine synthesis, accompanied by a marked accumulation of glutamate. In contrast to glycerol, the presence of lactate in renal tubules incubated with alanine resulted in an inhibition of glutamine formation by about 50% accompanied by about 2-fold increase in glutamate accumulation. Octanoate augmented the lactate-induced action on both glutamine and glutamate synthesis. The decrease in glutamine formation was not due to inhibition of endogenous glutamine synthesis since glycerol, lactate, octanoate and glycerol + octanoate did not affect glutamine formation when alanine was absent from the reaction medium. However, slight reciprocal changes in glutamate and glutamine production were observed on the addition of lactate + octanoate to the reaction medium not containing alanine.

The levels of ammonium in the incubation mixture were undetectable spectrophotometrically under all conditions studied. However, a massive release of ammonium was observed in renal tubules incubated with alanine in

the presence of 1 mM MSO, an inhibitor of glutamine synthetase (Meister, 1984). Ammonium production did not change on the addition of glycerol (88.4 ± 11.5 and 85.7 ± 10.2 μmol/hour per g dry wt, with alanine and alanine + glycerol, respectively, for 3 experiments). In contrast, it was markedly decreased in the presence of octanoate (to 56.5 ± 6.52 μmol/hour per g dry wt, $P < 0.001$ vs control with alanine alone), indicating that the fatty acid could reduce the activity of glutamate dehydrogenase generating ammonium for glutamine formation. Since lactate-induced inhibition of glutamine production from alanine was not accompanied by a decrease in alanine utilization (140.1 ± 10.6 μmol/hour per g wt.), the depressed glutamine formation was probably due to inhibition of ammonium generation via glutamate dehydrogenase as confirmed by a decline of ammonium release from alanine (to 50.7 ± 2.5 μmol/hour per g dry wt, $P < 0.001$ vs control with alanine alone) in tubules incubated in the presence of MSO.

The contribution of alanine, glycerol and lactate to gluconeogenesis

In isolated rabbit kidney-cortex tubules incubated with alanine, glycerol and octanoate, glucose production displayed a pronounced lag phase of about 20 min before the rate of glucose synthesis became maximal (Fig. 1A). Therefore, in order to establish the mechanisms responsible for the induction of glucose synthesis in the presence of alanine, glycerol and octanoate, the incorporation of [^{14}C]labeled substrates into glucose was analyzed at 20, 40 and 60 min of incubation. In renal tubules incubated with alanine and glycerol, octanoate stimulated incorporation of both [U-^{14}C]alanine (Fig. 1B) and [U-^{14}C]glycerol (Fig. 1C) into glucose by about 5- and 10-fold, respectively. ^{14}C-glucose formation from [U-^{14}C]alanine under these conditions was linear with time over 60 min of incubation, while both [U-^{14}C]glycerol (Fig. 1C) and [U-^{14}C]lactate (Fig. 1D) incorporation into glucose in the presence of alanine and octanoate displayed a lag phase of about 20 min. This might indicate that the delay in glycerol incorporation into glucose could be responsible for the low rate of glucose synthesis observed during initial 20 min of incubation of tubules with alanine, glycerol and octanoate (cf. Fig. 1A). In contrast to glycerol, the addition of lactate to tubules incubated with alanine resulted in an enhancement of [U-^{14}C]alanine incorporation into glucose by about 4-fold (Fig. 1B), while [U-^{14}C]lactate contribution to gluconeogenesis under these conditions was increased only by about 40% (Fig. 1D). However, the addition of octanoate in the presence of alanine plus lactate caused twofold stimulation of incorporation of both [U-^{14}C]alanine and [U-^{14}C]lactate into ^{14}C-glucose in comparison with values measured in the absence of fatty acid.

Glycerol and lactate utilization as well as glycerol-3-phosphate and lactate synthesis

As presented in Fig. 2, without alanine and fatty acid added, glycerol removal as well as glycerol-3-phosphate (glycerol-3P) and lactate synthesis were

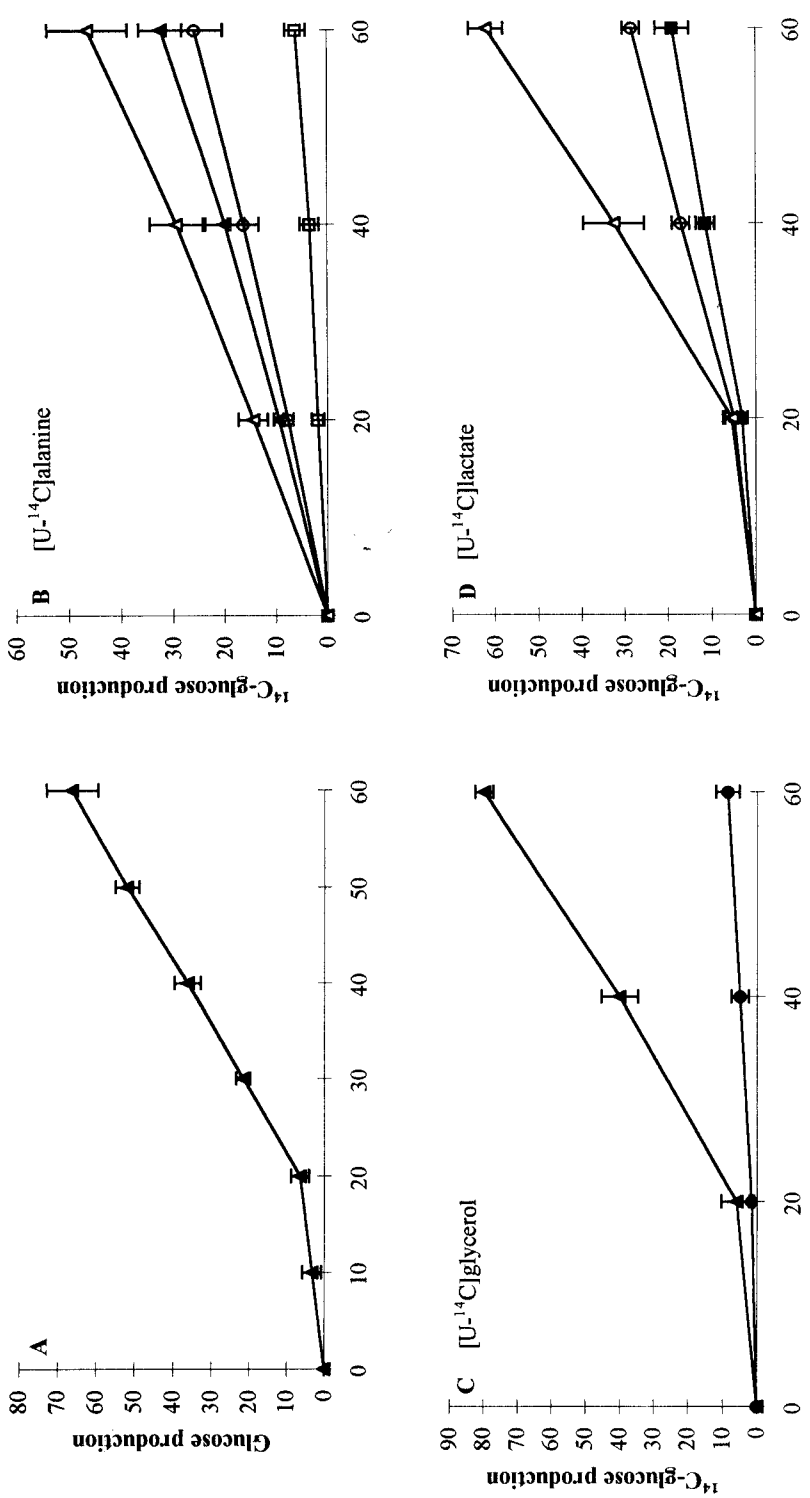

Fig. 1. The time course of glucose synthesis in renal tubules incubated with alanine, glycerol and octanoate (**A**) and ^{14}C-glucose synthesis from [U-^{14}C]alanine (**B**), [U-^{14}C]glycerol (**C**) and [U-^{14}C]lactate (**D**) under various experimental conditions. Additions: alanine (□), lactate (■), alanine + glycerol (●), alanine + lactate (○), alanine + glycerol + octanoate (▲) and alanine + lactate + octanoate (△). The experimental conditions were as described in the legend to Table 1 and under Materials and methods. Glucose production is expressed in μmol per g dry wt (**A**) or in μmol of 3^{14}C-units incorporated into glucose per g dry wt (**B**,**C**,**D**)

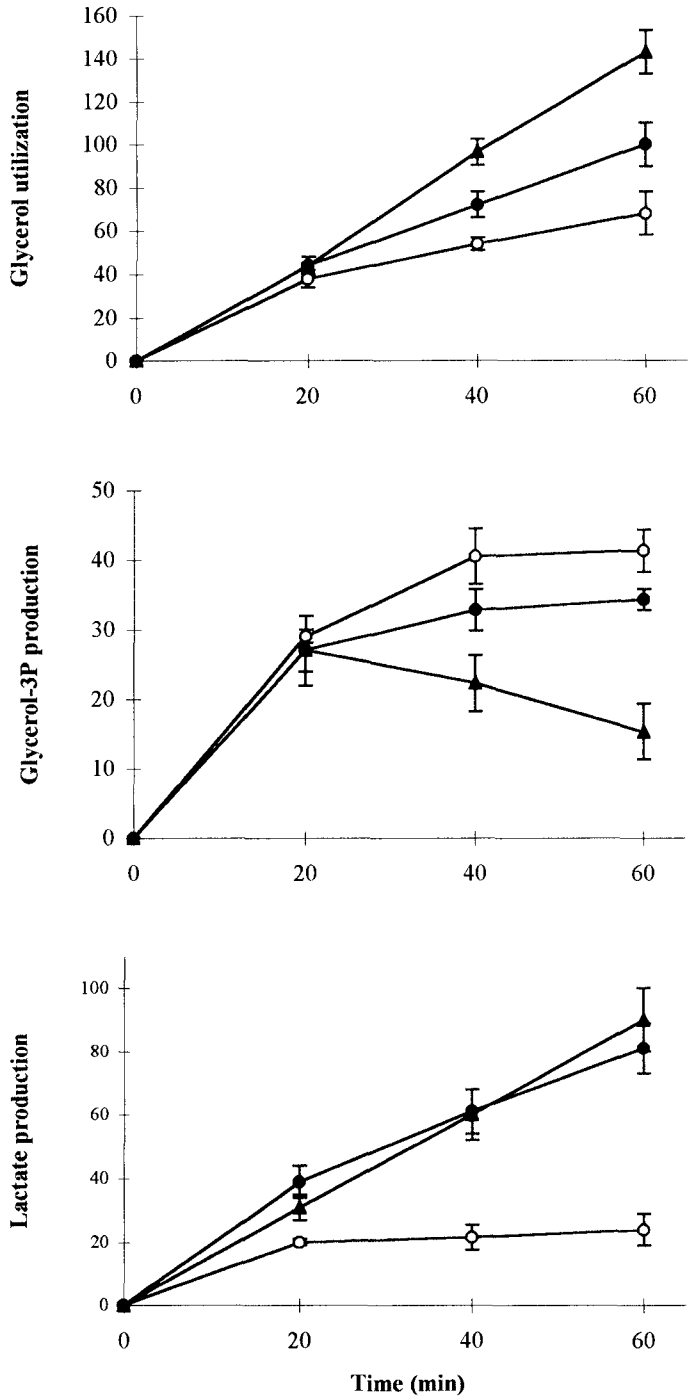

Fig. 2. Glycerol utilization and both glycerol-3P and lactate production in renal tubules incubated with glycerol (○), glycerol + alanine (●) and glycerol + alanine + octanoate (▲). The experimental conditions were as described in the legend to Table 1. Glycerol utilization as well as glycerol-3P and lactate formation are expressed in μmol per g dry wt

significantly reduced between 20 and 60 min of incubation. The addition of alanine in the presence of glycerol resulted in about 50% enhancement of glycerol utilization, accompanied by about 30% decrease in glycerol-3P accumulation. The production of lactate under these conditions was increased by about 2-fold and was linear over 60 min of incubation (89.1 ± 10.5 μmol/hour per g dry wt). Surprisingly, the addition of octanoate to renal tubules incubated with alanine plus glycerol resulted in a further stimulation of glycerol utilization (up to 140.2 ± 10.4 μmol/hour per g dry wt) accompanied by a marked reduction of glycerol-3P accumulation, while it did not affect lactate production. Similarly, an enhancement of glucose synthesis in the presence of alanine and lactate (cf. Table 1) was accompanied by a significant acceleration of lactate utilization in comparison with that determined with lactate alone (from 57.7 ± 8.6 to 135.3 ± 10.5 μmol/h per g dry wt, with lactate and lactate + alanine, respectively, $P < 0.001$ for four experiments).

Cellular NADH/NAD⁺ ratios

Since the addition of octanoate to renal tubules incubated with alanine plus glycerol resulted in glutamate accumulation (cf. Table 1) accompanied by an increase in glycerol utilization and decrease in glycerol-3P production (cf. Fig. 2) it is likely that fatty acid-induced activation of gluconeogenesis might be due to changes in the cellular redox state. Therefore, we have estimated [lactate]/[pyruvate] and [glycerol-3P]/[dihydroxyacetone phosphate] ([glycerol-3P]/[DHAP]) ratios, indicating the cytosolic NADH/NAD^+ ratio as well as [3-hydroxybutyrate]/[acetoacetate] ([3-HB]/[AcAc]) ratio reflecting the mitochondrial redox state. As shown in Table 2, on the addition of octanoate plus glycerol to renal tubules incubated with alanine, the [lactate]/[pyruvate] and [3-HB]/[AcAc] ratios were almost twice higher in comparison with those observed in the presence of alanine alone, while [glycerol-3P]/[DHAP] ratio was increased by about 36-fold. Since the 2-oxoglutarate content remained unchanged under conditions studied (data not shown), the increase in the mitochondrial NADH/NAD^+ ratio might be responsible for inhibition of glutamate dehydrogenase resulting in glutamate accumulation and a decline of glutamine synthesis (cf. Table 1). Moreover, induction of gluconeogenesis observed on the inclusion of octanoate to renal tubules incubated with alanine + glycerol was accompanied by about 25% stimulation of $^{14}CO_2$ fixation (43.0 ± 1.63 and 55.1 ± 1.65 μmol of $H^{14}CO_3^+$/hour per g dry wt, for alanine + glycerol and alanine + glycerol + octanoate, respectively, $P < 0.001$ for 3 experiments) and an increase of malate content (from 0.16 ± 0.04 to 0.52 ± 0.08 μmol per g dry wt), indicating acceleration of pyruvate carboxylation.

Since the addition of alanine plus octanoate increased both glycerol utilization for gluconeogenesis (cf. Fig. 1C) and its consumption by renal tubules (Fig. 2), it seemed interesting to estimate redox changes induced by these substrates in rabbit renal tubules incubated with glycerol. Surprisingly, on the addition of octanoate in the presence of alanine + glycerol, both [lactate]/

Table 2. The intracellular levels of lactate, pyruvate, glycerol-3P, DAHP, 3-HB and AcAc as well as cytosolic and mitochondrial NADH/NAD^+ ratios in renal tubules incubated with alanine, glycerol, alanine + octanoate, alanine + glycerol and alanine + glycerol + octanoate

Metabolites and ratios	Metabolite levels and ratios				
	+ Alanine	+ Glycerol	+ Alanine + octanoate	+ Alanine + glycerol	+ Alanine + glycerol + octanoate
Lactate	1.11 ± 0.13	2.53 ± 0.17	2.36 ± 0.32[d]	3.98 ± 0.10[d]	2.39 ± 0.15[b,d]
Pyruvate	0.32 ± 0.04	0.29 ± 0.01	0.30 ± 0.05	0.30 ± 0.04	0.31 ± 0.06
Glycerol-3P	0.17 ± 0.02	40.3 ± 3.61	0.32 ± 0.04[d]	35.3 ± 1.22[c,h]	14.7 ± 2.51[a,c,e,g]
DHAP	0.02 ± 0.01	0.10 ± 0.02	0.02 ± 0.01	0.11 ± 0.02[d]	0.07 ± 0.01[b,c,d,e,f]
3-HB	0.04 ± 0.01	0.05 ± 0.01	0.21 ± 0.05[d]	0.04 ± 0.01	0.14 ± 0.02[b,d]
AcAc	0.31 ± 0.03	0.27 ± 0.01	0.72 ± 0.05[d]	0.28 ± 0.05	0.45 ± 0.05[b,d,e,f,h]
$\frac{[\text{Lactate}]}{[\text{Pyruvate}]}$	3.47 ± 0.41	8.72 ± 0.58	7.87 ± 1.07[d]	13.3 ± 1.33[h]	7.71 ± 0.48[a,c]
$\frac{[\text{Glycerol-3P}]}{[\text{DHAP}]}$	5.86 ± 0.79	420.0 ± 37.1	11.1 ± 1.39[d]	334. 0 ± 20.3[c,h]	219.0 ± 37.2[a,c,h]
$\frac{[\text{3-HB}]}{[\text{AcAc}]}$	0.16 ± 0.3	0.18 ± 0.04	0.28 ± 0.03[d]	0.18 ± 0.03	0.31 ± 0.02[a,d,h]

Renal tubules were incubated with substrate concentrations shown in the note to Table 1 and separated from the reaction medium as described under Materials and methods. The intracellular levels of metabolites expressed as μmol/g dry wt are means ± SD for four separate experiments and were measured following 60 min of incubation.

[a] $P < 0.001$, [b] $P < 0.05$ vs control with alanine + glycerol, [c] $P < 0.001$, [d] $P < 0.05$ vs control with alanine, [e] $P < 0.001$, [f] $P < 0.05$ vs control with alanine + octanoate, [g] $P < 0.001$, [h] $P < 0.05$ vs control with glycerol.

[pyruvate] and [glycerol-3P]/[DHAP] ratios were smaller by about 1.7- and 1.5-fold in comparison with values measured with glycerol plus alanine, suggesting a decrease in cytosolic NADH/NAD^+ ratio. Furthermore, octanoate induced a marked increase in intracellular glutamate, aspartate and malate levels (28.9 ± 3.34, 4.35 ± 0.24 and 0.52 ± 0.08 μmol per g dry wt, respectively) in comparison with values measured without fatty acid (9.67 ± 2.56, 2.25 ± 0.14 and 0.13 ± 0.02 μmol per g dry wt, respectively, $P < 0.001$ for 4 experiments), suggesting an enhanced operation of malate-aspartate shuttle followed by a decrease in cytosolic redox state and activation of glycerol utilization (cf. Fig. 2). In agreement with this hypothesis, 0.07 mM methylene blue which was reported to decrease the cytosolic NADH/NAD^+ ratio (Niwa et al., 1986) caused stimulation of gluconeogenesis by about 30% (from 65.1 ± 7.5 to 88.5 ± 8.7 μmol/h per g dry wt, $P < 0.001$ for 3 experiments) accompanied by about 50% decrease in intracellular glycerol-3P level (from 15.2 ± 3.3 to 7.5 ± 0.17 μmol per g dry wt, $P < 0.001$) when added to renal tubules incubated with alanine, glycerol and octanoate.

Comparison of glycerol- and lactate-induced changes in intracellular redox state suggests that NAD(H) poor might not be equilibrated between lactate and glycerol-3P dehydrogenases. Firstly, in contrast to glycerol, lactate added to renal tubules incubated with alanine resulted in an elevation of intracellular [3-HB]/[AcAc] ratio in comparison with the value calculated with alanine as the sole substrate (from 0.17 ± 0.03 to 0.28 ± 0.03, $P < 0.001$ for 3 experiments). Secondly, in contrast to glycerol, in renal tubules incubated with lactate alanine caused a decrease in [lactate]/[pyruvate] ratio (from 12.51 ± 1.23 to 7.01 ± 0.05, $P < 0.05$ for 3 experiments) enhancing lactate incorporation into glucose (cf. Fig. 1D). Thirdly, in renal tubules incubated with alanine the addition of glycerol plus octanoate produced 36-fold increase in [glycerol-3P]/[DHAP] ratio, whereas [lactate]/[pyruvate] ratio was only twofold higher than that with amino acid as the sole substrate, it seems possible that NAD(H) pool might not be equilibrated between lactate and glycerol-3P dehydrogenases.

The effect of low malate concentration on glucose synthesis induced by octanoate in the presence of alanine and glycerol

As the addition of octanoate to renal tubules incubated with alanine and glycerol resulted in an increase in intracellular levels of aspartate, glutamate and malate, the main components of malate-aspartate shuttle, we have investigated the effect of low concentrations of both aspartate and malate on the rate of glucose synthesis. The addition of 0.2 mM aspartate to renal tubules incubated with alanine, glycerol and octanoate neither abolished the lag phase nor stimulated glucose synthesis probably due to its inability to penetrate the mitochondria, since as shown by La Noue et al. (1973), the intramitochondrial rather than the cytosolic aspartate is required for malate-aspartate shuttle operation. In contrast, in the presence of 0.2 mM malate gluconeogenesis became linear and enhanced by about 70% the value obtained in the presence

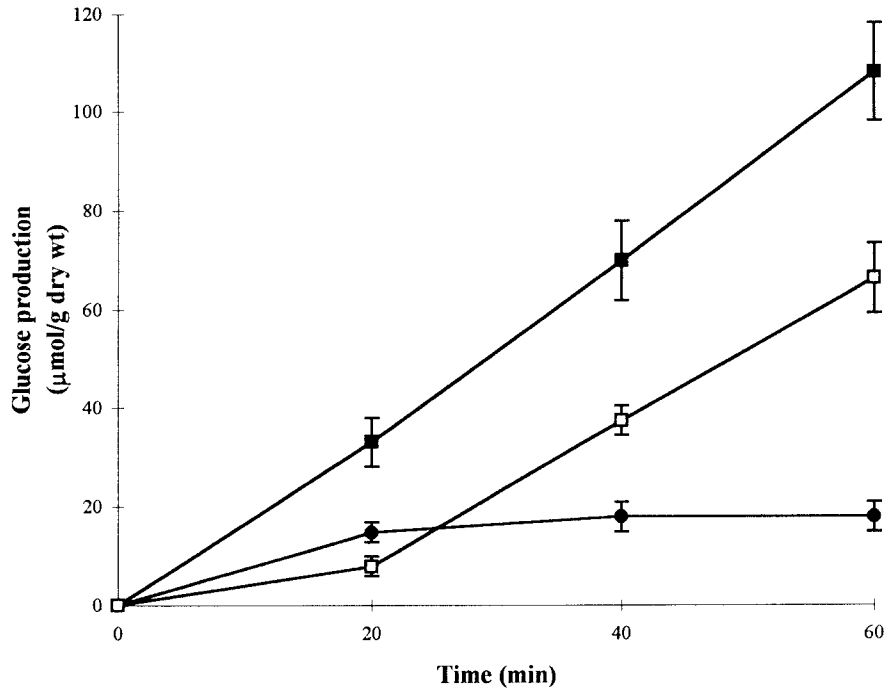

Fig. 3. The effect of 0.2 mM malate on glucose synthesis in renal tubules incubated with alanine + glycerol in the absence or presence of octanoate. (□) alanine + glycerol + octanoate, (●) alanine + glycerol + malate, (■) alanine + glycerol + octanoate + malate. The experimental conditions were as described in the legend to Table 1

of alanine, glycerol and octanoate (Fig. 3). Moreover, this was accompanied by a marked increase in intracellular levels of aspartate following 20 min of incubation (2.54 ± 0.15 and 4.77 ± 0.38 μmol per g dry weight, without and with malate, respectively, $P < 0.001$ for 4 experiments) as well as a significant decrease in intracellular levels of glycerol-3P at both 20 min (26.7 ± 5.1 and 17.41 ± 3.3 μmol per g dry wt, in the absence and presence of malate, respectively, $P < 0.05$) and 60 min of incubation (16.8 ± 4.2 and 7.35 ± 1.3 μmol per g dry wt, in the absence and presence of malate, respectively, $P < 0.05$). Thus, it seems likely that the delay in glucose production in the absence of exogenous malate was caused by a limited generation of aspartate required for operation of the malate-aspartate shuttle and oxidation of cytosolic NADH.

Cell volume measurements

Alanine has been reported to induce rapid (i.e. occurring within minutes after exposure to amino acid) cell swelling in both hepatocytes (Wettstein et al., 1990) and renal proximal tubules (Beck and Potts, 1990). However, the addition of 1 mM alanine to rabbit renal tubules incubated with glycerol and octanoate did not affect the intracellular water content over 20 min of incuba-

tion (1.80 ± 0.04, 1.80 ± 0.07 and 1.74 ± 0.07 μliters per mg dry wt determined at 0, 10 and 20 min of incubation, respectively). In contrast, the presence of both alanine and 2 mM aminooxyacetate (AOA), an inhibitor of aminotransferases, resulted in cell swelling (1.96 ± 0.08 and 2.10 ± 0.09 μliters of intracellular water per mg dry wt at 10 and 20 min of incubation, respectively, $P < 0.05$ vs corresponding control value measured without AOA). In view of the observation that the addition of AOA to renal tubules incubated with alanine, glycerol and octanoate produced a complete inhibition of gluconeogenesis (data not shown), it seems likely that activation of glucose synthesis in renal tubules incubated with alanine, glycerol and octanoate (cf. Table 1) is not related to intracellular water changes.

Discussion

In agreement with data for guinea pig renal cortex (Michoudet et al., 1988), in rabbit kidney-cortex alanine is converted mainly into glutamine and glutamate (cf. Table 1), while glucose, aspartate, ammonium, lactate and pyruvate production is negligible. However, we have shown that, on a decrease of glutamine synthesis in the presence of octanoate and either glycerol or lactate, both glucose formation and alanine incorporation into glucose are markedly activated (cf. Table 1 and Fig. 1). Similarly, a substantial induction of glucose synthesis from alanine was also observed in rabbit renal tubules incubate glycerol and ketone bodies (Lietz et al., 1997). Oleate and palmitate are less potent for stimulation of gluconeogenesis in the presence of alanine and glycerol probably due to their lower utilization as concluded from oxygen consumption measurements. Octanoate has also been shown to exert much more pronounced inhibitory effect on glycolytic flux in rat hepatocytes incubated with lactate in comparison with that caused by oleate (Morand et al., 1994).

The possible pathways for glucose and glutamine synthesis in rabbit renal tubules incubated with alanine, fatty acids and either glycerol or lactate are summarized in Fig. 4. The lack of ammonium production from alanine in rabbit renal tubules is probably due to a high activity of glutamine synthetase which effectively consumes ammonium ions for glutamine synthesis in both rabbit (Dugelay and Baverel, 1991) and guinea pig kidney cortex preparations (Baverel et al., 1990). However, on the addition of MSO, an inhibitor of glutamine synthetase, to rabbit renal tubules incubated with alanine, a massive release of ammonium into incubation media occurs, indicating that glutamate dehydrogenase activity is masked by a very efficient operation of glutamine synthetase. Similar phenomenon has also been observed in rabbit renal cortical tubules matabolizing glutamine (Dugelay and Baverel, 1991) or aspartate (Lietz and Bryła, 1995), as well as in guinea pig renal tubules incubated with alanine (Michoudet et al., 1988) and aspartate (Baverel et al., 1990). In contrast, in human renal cortical tubules which are deficient of glutamine synthetase activity, alanine is utilized for ammoniagenesis (Fouque et al., 1996).

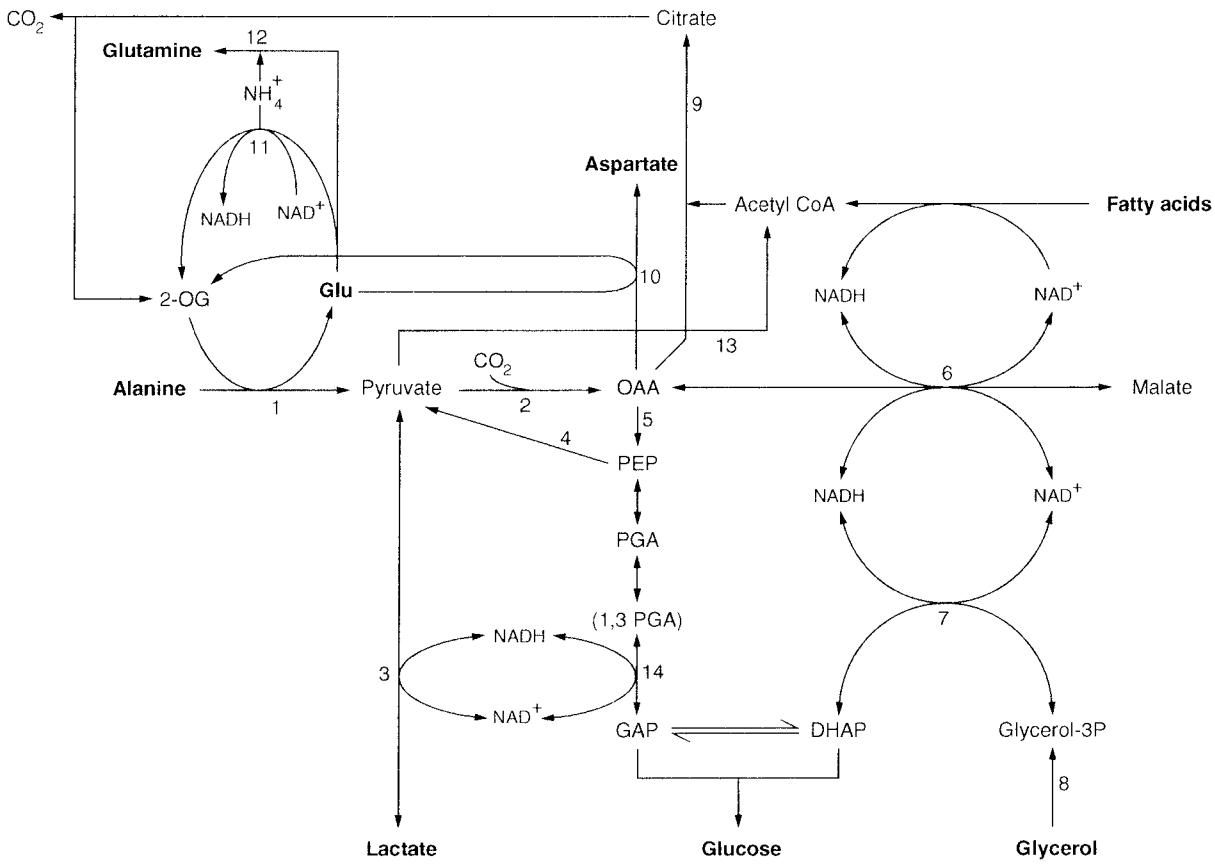

Fig. 4. The possible pathways for glucose and glutamine synthesis in rabbit renal tubules incubated with alanine, fatty acids and either glycerol or lactate. The numbers indicate the following enzymes: *1.* alanine aminotransferase; *2.* pyruvate carboxylase; *3.* lactate dehydrogenase; *4.* pyruvate kinase; *5.* phosphoenolpyruvate carboxykinase; *6.* malate dehydrogenase; *7.* glycerol-3-phosphate dehydrogenase; *8.* glycerol kinase; *9.* citrate synthase; *10.* aspartate aminotransferase; *11.* glutamate dehydrogenase; *12.* glutamine synthetase; *13.* pyruvate dehydrogenase; *14.* glyceraldehyde-3-phosphate dehydrogenase; *PGA*, 3-phosphoglycerate; *DHAP*, dihydroxyacetone phosphate; *glycerol-3P*, glycerol-3-phosphate; *1,3 PGA*, 1,3-bisphosphoglycerate; *GAP*, glyceraldehyde-3-phosphate; *PEP*, phosphoenolpyruvate; *OAA* oxalacetate; *2-OG*, 2-oxoglutarate

Activation of glucose formation from alanine in the presence of glycerol and fatty acids (cf. Fig. 1) can be due to elevation of the cytosolic NADH/NAD^+ (cf. Table 2) ratio resulting in an increase in glyceraldehyde-3-phosphate dehydrogenase activity. Moreover, the elevation of the mitochondrial NADH/NAD^+ ratio observed on the addition of glycerol + octanoate to renal tubules incubated with alanine (cf. Table 2), may cause stimulation of pyruvate carboxylase activity due to (i) an increased sensitivity of pyruvate carboxylase to its activator acetylCoA (Wałajtys-Rode et al., 1992), (ii) a depression of intramitochondrial content of oxalacetate, an inhibitor of pyruvate carboxylase (Scrutton and Young, 1972) or (iii) an inhibi-

tion of pyruvate dehydrogenase activity (Scholz et al., 1978). This might lead to an enhanced provision of alanine-derived pyruvate for the carboxylation reaction. Thus, the increased glucose formation from alanine in the presence of glycerol and octanoate might be due to a "switch" in the metabolism of pyruvate within mitochondria from oxidation to carboxylation. Additionally, diminished glutamine production observed upon the addition of octanoate in the presence of alanine and glycerol (cf. Table 1) may cause an enhanced provision of alanine carbon skeletons for gluconeogenesis.

In view of different degrees of change for [lactate]/[pyruvate] and [glycerol-3P]/[DHAP] ratios on the addition of both glycerol and octanoate to renal tubules incubated with alanine (cf. Table 2), it seems likely that NAD(H) pool might not be equilibrated between lactate and glycerol-3P dehydrogenases (cf. Table 2). This is in agreement with our previous observation concerning aspartate metabolism in rabbit renal tubules (Lietz and Bryła, 1995) as well as with data of Berry et al. (1992) for hepatocytes incubated with lactate. To explain this phenomenon, the latter authors postulate the possibility of intracellular compartmentation ("metabolite channeling') whereby enzymes of glycolysis are segregated from those of gluconeogenesis and under certain metabolic conditions reducing equivalents cooperating with one pool may not be accessible to another.

Both the increased utilization, as well as accelerated incorporation of glycerol into glucose on the addition of alanine and octanoate (cf. Figs. 1 and 2), are due to a decrease in the cytosolic $NADH/NAD^+$ ratio in comparison with that determined with glycerol alone (cf. Table 2), resulting in the stimulation of glycerol-3P dehydrogenase. This is in agreement with data for rat liver (Berry et al., 1973; Werner and Berry, 1974) indicating that transport of reducing equivalents from cytosol into mitochondria limits the rate of glucose formation from glycerol as the sole substrate. According to Friedrichs (1975), compounds that can form glutamate like ammonium and several amino acids stimulate gluconeogenesis from lactate in isolated rat renal tubules through an increased provision of glutamate and, thereby, aspartate, resulting in an activation of malate-aspartate shuttle. Similarly, it has been recently shown that asparagine stimulates glucose formation from lactate in isolated hepatocytes (Efthivoulou et al., 1975). Moreover, both alanine and asparagine have been reported to exert stimulatory action on gluconeogenesis in rat hepatocytes metabolizing sorbitol (Sugano et al., 1988), while aspartate has been shown to accelerate glucose formation from glycerol and lactate in rabbit renal cortical tubules (Lietz and Bryła, 1995).

Since stimulation of gluconeogenesis from glycerol observed on the addition of octanoate and alanine (cf. Fig. 1C) is associated with an increased intracellular content of glutamate, aspartate and malate, it seems likely that a decrease in the cytosolic $NADH/NAD^+$ ratio accompanied by stimulation of glycerol utilization (cf. Table 2 and Fig. 2) is due to activation of malate-aspartate shuttle by alanine and fatty acid. The presence of the amino acid and octanoate results in an acceleration of pyruvate carboxylation and elevation of aspartate level (cf. Fig. 4). A decrease in cytosolic $NADH/NAD^+$ ratio observed on the addition of octanoate to tubules incubated with alanine

and glycerol is accompanied by a reciprocal change in the mitochondrial redox state (cf. Table 2). This is in agreement with Scholz et al. (1995) who have reported that in hearts isolated from rabbits, cytosolic and mitochondrial redox states do not necessarily have to change in parallel fashion.

In view of recent studies, amino acid-induced cell volume alterations may affect a variety of metabolic pathways not primarily serving cell volume regulation, representing a new principle of metabolic control (Häussinger and Lang, 1991; Lang et al., 1993). Although Beck and Potts (1990) have measured the rapid 20% increase of cell volume in perfused rabbit renal proximal tubules in the presence of 6.6 mM alanine, we have not observed cellular water changes on the addition of 1 mM alanine to rabbit renal cortical tubules incubated with glycerol and octanoate. However, the addition of 2 mM AOA to renal tubules incubated with alanine, glycerol and octanoate produced an increase in intracellular water content by about 15%. According to Häussinger and Lang (1991), the aminooxyacetate-induced increase in cell volume in the presence of alanine might be due to inhibition of alanine metabolism resulting in an increase of amino acid accumulation leading to an enhanced osmotic gradient of alanine across plasma membrane. Since the extent of amino acid-induced swelling of rat hepatocytes depends on the amino acid concentration (Häussinger and Lang, 1991), the inability of 1 mM alanine to increase cellular water content in the presence of glycerol and octanoate in rabbit renal tubules may also be due to a low intracellular level of this amino acid, which according to our calculations does not exceed 3 mM concentration.

Physiological implications

Although in the early stages of starvation the liver is the main gluconeogenic organ, in prolonged starvation renal gluconeogenesis contributes to approximately 50% of the total body glucose synthesis. Moreover, renal glucose formation has been shown to be markedly increased in diabetic and acidotic animals (Klahr and Hammerman, 1985), suggesting that renal gluconeogenesis is of physiological importance for the maintenance of blood glucose homeostasis. Concentration of alanine in rabbit serum is equal to approximately 0.5 mM (Silbernagl, 1985); while in humans fed a low protein diet, concentration of this amino acid in serum rises up to 0.7 mM (Prior et al., 1996). The changes of free fatty acid levels in blood reach an amplitude of about 15-fold (Randle, 1995), whereas concentrations of glycerol and lactate in serum have been reported to alter between 0.04–0.4 mM and 1–2 mM, respectively, responding to the physiological state of organisms (Lin, 1977; Emmet and Seldin, 1985). Circulatory levels of glycerol are known to rise following 24 h starvation of rabbits (Lin, 1977). Moreover, infusion of insulin in men (Fanelli et al., 1993) as well as administration of either isoproterenol or adrenaline to fed, fasted or diabetic rabbits (Lin, 1977) have also been shown to elevate the glycerol level in serum.

In view of the present data, it is likely that alanine plays an important regulatory role in renal gluconeogenesis. In the presence of physiological levels of metabolites elevating both cytosolic and mitochondrial redox state (i.e. glycerol, lactate and fatty acids), this amino acid may significantly contribute to renal glucose synthesis *in vivo*. In addition, both alanine and fatty acids are required to activate utilization of glycerol and lactate carbon skeletons for gluconeogenesis. Thus, alanine and fatty acids serve as potent modulators of renal metabolism of these substrates.

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References

Baverel G, Martin G, Michoudet Ch (1990) Glutamine synthesis from aspartate in guinea-pig renal cortex. Biochem J 268: 437–442

Beck JS, Potts DJ (1990) Cell swelling, co-transport activation and potassium conductance in isolated perfused rabbit kidney proximal tubules. J Physiol 425: 369–378

Bergmeyer HU (1965) Methods of enzymatic analysis, 2nd edn. Verlag Chemie GmbH, Weinheim; Academic Press, New York London

Berry MN, Kun E, Werner HV (1973) Regulatory role of reducing-equivalent transport from substrate to oxygen in the hepatic metabolism of glycerol and sorbitol. Eur J Biochem 33: 407–417

Berry MN, Phillips JW, Grivell AR (1992) Interactions between mitochondria and cytoplasm in isolated hepatocytes. Curr Top Cell Reg 33: 309–328

Chang J, Knecht R, Braun DG (1983) Amino acid analysis in the picomole range by precolumn derivatization and high-performance liquid chromatography. Methods Enzymol 91: 41–48

Dugelay S, Baverel G (1991) Concomitant synthesis and degradation of glutamine in isolated rabbit kidney tubules. Biochim Biophys Acta 1075: 191–194

Efthivoulou M-A, Philips JW, Berry MN (1995) Abolition of the inhibitory effect of ethanol oxidation on gluconeogenesis from lactate by asparagine or low concentrations of ammonia. Biochim Biophys Acta 1244: 303–310

Emmet M, Seldin W (1985) Metabolic acidosis and alcalosis. In: Seldin DW, Giebish G (eds) The kidney. Physiology and physiopathology. Raven Press, New York, pp 1567–1639

Exton JH, Park CR (1967) Control of gluconeogenesis in liver. J Biol Chem 242: 2622–2636

Fanelli C, Calderone S, Epifano L, De Vincenzo A, Modarelli F, Pampanelli S, Perriello G, De Feo P, Brunetti P, Gerich JE, Bolli GB (1993) Demonstration of a critical role for free fatty acids in mediating counterregulatory stimulation of gluconeogenesis and suppression of glucose utilization in humans. J Clin Invest 92: 1617–1622

Fouque D, Dugelay Y, Martin G, Combet J, Baverel G (1996) Alanine metabolism in isolated human kidney tubules. Use of a mathematical model. Eur J Biochem 236: 128–137

Friedrichs D (1975) On the stimulation of gluconeogenesis by L-lysine in isolated rat kidney cortex tubules. Biochim Biophys Acta 392: 255–270

Guder WG, Wieland OH (1972) Metabolism of isolated kidney tubules. Additive effect of parathyroid hormone and free fatty acids on renal gluconeogenesis. Eur J Biochem 31: 69–79

Harris SJ, Balaban RS, Barret L, Mandel LJ (1981) Mitochondrial respiratory capacity and Na^+ – and K^+ – dependent adenosine triphosphatase-mediated ion transport in the intact renal cell. J Biol Chem 256: 10319–10328

Häussinger D, Lang F (1991) Cell volume in the regulation of hepatic function: a mechanism for metabolic control. Biochim Biophys Acta 1071: 331–350

Jungas RL, Mitchell L, Brosnan JT (1992) Quantative analysis of amino acid oxidation and related gluconeogenesis in humans. Physiol Rev 72: 419–448

Klahr S, Hammerman M (1985) Renal metabolism. In: Seldin DW, Giebish G (eds) The kidney. Physiology and physiopathology. Raven Press, New York, pp 699–718

Lang F, Ritter M, Völkl H, Häussinger D (1993) The biological significance of cell volume. Renal Physiol Biochem 16: 48–65

LaNoue KF, Wałajtys E, Williamson JR (1973) Regulation of glutamate metabolism in interactions with citric acid cycle in rat heart mitochondria. J Biol Chem 248: 7171–7183

Lemieux G, Berkofsky J, Lemieux C, Quenneville A, Marsolais M (1988) Real importance of alanine in renal metabolism: in vitro studies in dog. Am J Physiol 255: R42–R45

Lietz T, Bryła J (1995) Glycerol and lactate induce reciprocal changes in glucose formation and glutamine production in isolated rabbit kidney-cortex tubules incubated with aspartate. Arch Biochem Biophys 321: 501–509

Lietz T, Winiarska K, Bryła J (1997) Ketone bodies activate gluconeogenesis in isolated rabbit renal cortical tubules incubated with glycerol and amino acids. Acta Biochim Polon 44: 323–332

Lin ECC (1977) Glycerol utilization and its regulation in mammals. Annu Rev Biochem 46: 765–795

Meister A (1984) Enzymology of glutamine. In: Häussinger D, Sies H (eds) Glutamine metabolism in mammalian tissues. Springer, Berlin Heidelberg New York Tokyo, pp 3–15

Michoudet Ch, Martin G, Baverel G (1988) Pyruvate carboxylation in glutamine synthesis from alanine by isolated guinea-pig renal cortical tubules. Pflugers Arch 412: 7–11

Morrand Ch, Besson C, Demigne Ch, Remesy Ch (1994) Importance of the modulation of glycolysis in the control of lactate metabolism by fatty acids in isolated hepatocytes from fed rats. Arch Biochem Biophys 309: 254–260

Niwa H, Yamano T, Sugano T (1986) Hormonal effects and control of gluconeogenesis from sorbitol, xylitol and glycerol in perfused chicken liver. Comp Biochem Physiol 85B: 739–745

Pilkis SJ, Rion JP, Claus TH (1976) Hormonal control of [^{14}C]glucose synthesis from [^{14}C]dihydroxyacetone and glycerol in isolated rat hepatocytes. J Biol Chem 251: 7841–7852

Prior RL, Crim MC, Castaneda C, Lammi-Keefe C, Dawson-Hughes B, Rosen CJ, Spindler AA (1996) Conditions altering plasma concentrations of urea cycle and other amino acids in erderly human subjects. J Am Coll Nutr 15: 237–247

Randle PJ (1995) Metabolic fuel selection: general integration at the whole-body level. Proc Nutr Soc 54: 317–327

Scholz R, Olson MS, Schwab AJ (1978) The effect of fatty acids on the regulation of pyruvate dehydrogenase in perfused rat liver. Eur J Biochem 86: 519–530

Scholz TD, Laughlin MR, Balaban RS, Kupriyanov VV, Heineman FW (1995) Effect of substrate on mitochondrial NADH, cytosolic redox state and phosphorylated compounds in isolated hearts. Am J Physiol 268: H82–H91

Scrutton MC, Young MR (1972) Pyruvate carboxylase. In: Boyer PD (ed) The enzymes. Academic Press, New York London, pp 1–35

Silbernagl S (1985) Amino acids and oligopeptides. In: Seldin DW, Giebish G (eds) The kidney. Physiology and physiopathology. Raven Press, New York, pp 1677–1701

Sugano T, Nishimura K, Sogabe N, Shiota M, Oyama N, Noda S, Ohta M (1988) Ca-dependent activation of the malate-aspartate shuttle by norepinephrine and vasopressin in perfused rat liver. Arch Biochem Biophys 264: 144–154

Wałajtys-Rode E, Zapareto J, Moehren G, Hoek JB (1992) The role of matrix calcium in the enhancement of mitochondrial pyruvate carboxylation by glucagon pretreatment. J Biol Chem 267: 370–379

Weinberg JM, Davis JA, Abarzua M, Smith RK, Kunkel R (1990) Ouabain-induced lethal proximal tubule cell injury is prevented by glycine. Am J Physiol 258: F346–F355

Werner HV, Berry MN (1974) Stimulatory effect of thyroxine administration on reducing-equivalent transport from substrate to oxygen during hepatic metabolism of sorbitol and glycerol. Eur J Biochem 42: 315–324

Wettstein M, vom Dahl S, Lang F, Gerok W, Häussinger D (1990) Cell volume regulatory responses of isolated perfused rat liver. The effect of amino acids. Biol Chem Hoppe-Seyler 37: 493–501

Wirthenson G, Vandewalle A, Guder WG (1981) Renal glycerol metabolism and the distribution of glycerol kinase in rabbit nephron. Biochem J 198: 543–549

Zabłocki K, Bryła J (1988) Effect of glycerol on gluconeogenesis in isolated rabbit kidney-cortex tubules. Biochim Biophys Acta 970: 231–240

Zabłocki K, Bryła J (1989) Utilization of alanine for glucose formation in isolated rabbit kidney-cortex tubules. FEBS Lett 259: 144–148

Zabłocki K, Gemel J, Bryła J (1983) The inhibitory effect of octanoate, palmitate and oleate on glucose formation in rabbit kidney tubules. Biochim Biophys Acta 757: 111–118

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