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Inhibition of gluconeogenesis by vanadium and metformin in kidney-cortex tubules isolated from control and diabetic rabbits

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Abstract

Effect of vanadyl acetylacetonate (VAc) and metformin on gluconeogenesis has been studied in isolated hepatocytes and kidney-cortex tubules of rabbit. Glucose formation from alanine + glycerol + octanoate, pyruvate or dihydroxyacetone was inhibited by 50–80% by 100 μM VAc or 500 μM metformin in renal tubules of control and alloxan-diabetic animals, while the inhibitory action of these compounds in hepatocytes was less pronounced (by about 20–30%). In contrast to VAc, metformin increased the rate of lactate formation by about 2-fold in renal tubules incubated with alanine + glycerol + octanoate. In view of VAc-induced changes in intracellular gluconeogenic intermediates and gluconeogenic enzyme activities, it is likely that this compound may decrease fluxes through pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase. In contrast to VAc, metformin-induced decrease in renal gluconeogenesis may result from a decline of cytosolic oxaloacetate level and consequently PEPCK activity. Following 6 days of VAc administration (1.275 mg V kg^{-1} body weight daily) the blood glucose level in alloxan-diabetic rabbits was normalised while blood glucose changes in control animals were not observed. On the contrary, in diabetic animals treated for 6 days with metformin (200 mg kg^{-1} body weight day^{-1}) a high blood glucose level was maintained. Unfortunately, VAc-treated control and diabetic rabbits exhibited elevated serum urea and creatinine levels. In VAc-treated animals vanadium was accumulated in kidney-cortex up to 7.6 ± 0.6 μg V g^{-1} dry weight. In view of a potential vanadium nephrotoxicity a therapeutic application of vanadium compounds needs a critical re-evaluation.

Keywords: Gluconeogenesis; Vanadium; Metformin; Diabetes; Renal tubules; Hepatocytes

1. Introduction

Both metformin and vanadium compounds are hypoglycaemic agents which do not stimulate insulin secretion [1,2]. Metformin (*N*,*N*-dimethylbiguanide) is one of the oral drugs used for more than 40 years to treat patients with type 2 diabetes mellitus without causing overt hypoglycaemia [2]. Pharmacological studies have indicated that metformin acts by (a) improving peripheral sensitivity to insulin [3], (b) inhibiting gastrointestinal absorption of glucose [3] and (c) decreasing hepatic glucose production [4,5]. However, the action of this drug on glucose formation is still controversial because metformin has also been reported as the agent with no effect on hepatic gluconeogenesis [6,7].

Vanadium compounds have been demonstrated to improve hyperglycaemia and aberrant glucose homeostasis in animal models of type 1 and type 2 diabetes mellitus as well as in clinical studies on a limited number of diabetic subjects [8,9]. From several years there has been a great interest in both understanding the mechanism of vanadium action and also in developing new vanadium compounds as potential oral insulin alternatives in diabetes treatment. Vanadyl acetylacetonate (VAc), organically chelated vanadium, is more potent than free vanadium in correcting the hyperglycaemia [10,11]. Moreover, it is of a greater hydrolytic and redox stability in comparison with other vanadium compounds [12]. Vanadyl derivative was also coupled with metformin to form potentially synergistic compound for diabetes treatment but no positive associative effects were apparent [13]. Despite an impressive

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Abbreviations: VAc, vanadyl acetylacetonate; PEPCK, phosphoenolpyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase.

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anti-diabetic action, vanadium compounds have been associated with several toxic effects (reviewed in [14]). Some other studies have, however, failed to detect changes in the level of urea, creatinine and transaminases, indicating no toxic action on kidney and liver functions [15,16]. It is commonly accepted that liver is the main gluconeogenic organ [17], while the role of the kidney in the post-absorptive state is not significant [18]. However, according to other studies renal glucose release accounts for 25% of systemic glucose production [17] and increases to 50% during prolonged starvation or diabetes mellitus [19] suggesting that renal gluconeogenesis is of physiological importance for the maintenance of blood glucose homeostasis. In view of observations that: (i) the influence of vanadium and metformin on glucose formation in kidney is unknown and (ii) these two compounds accumulate in the kidney [20,21], the aim of the present study was to investigate their action on gluconeogenesis in kidney-cortex tubules isolated from control and diabetic rabbits, which exhibit similar to human intracellular localisation of gluconeogenic enzymes [22].

2. Material and methods

2.1. Animals and isolation of kidney-cortex tubules, hepatocytes, cytosol and mitochondria

The experiments were performed with male white Termond rabbits weighing approximately 1–1.2 kg. Animals were maintained on the standard rabbit chow with free access to water and food and starved for 40 hr before experiments. Alloxan-diabetes was induced by the single injection of alloxan (150 mg kg^{-1} body weight) dissolved in 10 mM citrate buffer (pH 4.5) into the marginal vein of the ear of a rabbit starved for 48 hr [23]. After injection animals were allowed a standard diet and water *ad libitum*. To avoid hypoglycaemic shock, animals were given 1% glucose solution to drink during 24 hr after the alloxan treatment. Only those alloxan–treated animals which exhibited decreased or stabilised body weight and blood glucose concentration in excess of 300 mg 100 mL^{-1} 3 days after treatment were considered diabetic and used for experiments. Rabbit kidney-cortex tubules and hepatocytes were obtained as described previously by Jarzyna *et al.* [24]. Approximately 95% of both hepatocytes and renal tubules of control and diabetic rabbits excluded Trypan blue.

Mitochondria were isolated from the kidney-cortex using a solution containing 225 mM mannitol, 75 mM sucrose, 5 mM MOPS, 0.1 mM EDTA and 1% BSA (pH adjusted to 7.2 with Tris) as reported by Harris *et al.* [25], but the final wash was made with 0.3 M mannitol.

Cytosol for measurement of PEPCK (diphosphate:oxaloacetate carboxy-lyase; EC 4.1.1.38) activity was obtained according to MacDonald *et al.* [26]. For measurement of FBPase (D-fructose-1,6-bisphosphate phosphohydrolase; EC 3.1.3.11) activity the enzyme was purified according to Ozaki *et al.* [27], omitting ultrafiltration step. Cytosolic fraction for determination of G6Pase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) activity was obtained as described in [28].

2.2. Incubation of renal tubules and hepatocytes

Both isolated kidney-cortex tubules and hepatocytes were incubated at 37° in 25 mL Erlenmeyer flask sealed with rubber stoppers under atmosphere of O_2: CO_2 (95:5) in Krebs–Ringer bicarbonate buffer, pH 7.4, in the presence of substrates indicated in legends to figures and tables. The rates of gluconeogenesis under all conditions studied were linear at least for 90 min of incubation. Reactions were stopped following 60 min of incubation by either the addition of 1 mL sample to 0.1 mL of 35% perchloric acid or centrifugation of kidney-cortex tubules suspension through the silicon oil layer to 1 mL of 10% perchloric acid.

2.3. Analytical methods

Pyruvate carboxylase (pyruvate:carbon-dioxide ligase; EC 6.4.1.1) activity in kidney-cortex mitochondria was measured as described by Michalik and Bryl|a [29]. PEPCK activity in mitochondria and cytosol was measured according to Bryl|a and Dzik [30] and MacDonald *et al.* [26], respectively, using GTP instead of ITP. FBPase and G6Pase activities were assayed spectrophotometrically according to Ozaki *et al.* [27] and Gierow and Jergil [31], respectively. Oxygen uptake was measured using Clark oxygen electrode.

Glucose, lactate, pyruvate, malate, phosphoenolpyruvate, triose phosphates, 3-phosphoglycerate + 1,3-bisphosphoglycerate, fructose-1,6-bisphosphate, fructose-6-phosphate, glucose-6-phosphate, 3-hydroxybutyrate and acetoacetate were estimated either spectrophotometrically or fluorimetrically according to Bergmeyer [32]. Amino acids were determined by HPLC (Beckman Instruments) as their DABS-derivatives according to Chang *et al.* [33]. Blood glucose was analysed with a glucose analyser (Accutrend GCT, Boehringer Mannheim). Urea was measured in blood plasma as ammonium in agreement with da Fonseca-Wollheim and Heinze [34], following treatment of neutralised samples with urease, whereas creatinine was determined by the reaction of Jaffe as described by Michalik *et al.* [35].

Metformin was determined according to Teerlink *et al.* [36], while vanadium level in renal tubules as well as in kidney-cortex was measured using graphite-furnace atomic absorption spectrometry (Solaar M6, Waters) fitted with a 6F 95 graphite furnace atomiser and a programmable sample dispenser. For determination of vanadium concentration 0.5 g of dry weight of kidney-cortex was dissolved in 10 mL of concentrated nitric acid (Suprapur,

Merck). Kidney mineralisation was made for 15 min at 180° in microwave labstation (Ethos plus, Milestone). Before measurement samples were diluted to 50 mL with ultra-pure deionized water. The vanadium concentration was determined by comparison with a calibration curve obtained with the use of vanadium standard solutions. Detection limit was 4.5 μg L^{-1} (~0.5 μg g^{-1} dry weight).

Protein content in both mitochondrial and cytosolic fractions was determined according to Layne [37], while that in partially purified FBPase preparation was measured according to Bradford [38].

The cytosolic oxalacetate concentration was calculated from the intracellular malate concentration using lactate dehydrogenase and malate dehydrogenase equilibrium constants and assuming near-equilibrium for both reactions [39].

2.4. Chemicals

Enzymes, coenzymes and nucleotides for metabolites determination were from Boehringer. Aqua Scynt for ^{14}C labelled CO_2 incorporation was from Biocare. VAc was from Aldrich Chemicals Company, Inc, while metformin was obtained from Polfa. All others chemicals were from Sigma Chemicals Co. Octanoate-albumin emulsion was prepared according to Williamson *et al.* [40].

2.5. Expression of results

Data shown are means ± SD for at least three separate experiments. The statistical significance of differences was calculated by ANOVA two-factors test.

3. Results

3.1. Glucose formation and substrate utilisation

With no substrate added to the incubation medium the rates of glucose synthesis in hepatocytes and kidney-cortex tubules isolated from both control and diabetic rabbits were negligible (3.0 ± 1.1 and 4.4 ± 1.1 in hepatocytes of control and diabetic rabbits, respectively, $N = 5$ and 2.1 ± 0.9 and 3.1 ± 1.0 in renal tubules of control and diabetic animals, respectively, $N = 5$). Although amino acids, pyruvate, lactate and glycerol are commonly accepted as main gluconeogenic substrates ([41], for review) rabbit kidney-cortex tubules produce efficiently glucose from amino acids only in the presence of glycerol and either fatty acids [42] or ketone bodies [43]. Therefore, in all experiments with the use of amino acids (at 2 mM concentration) rabbit renal tubules were incubated with both 2 mM glycerol and 0.5 mM octanoate. 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 [42]. Other substrates were added at 5 mM concentrations.

The data of Fig. 1 show that increasing concentrations of VAc and metformin resulted in an augmented decrease of glucose formation in kidney-cortex tubules regardless of the gluconeogenic substrate applied. At 100 μM concentration VAc inhibited this process from alanine + glycerol+ octanoate and dihydroxyacetone by about 60–80%, while metformin achieved 70–80% of inhibition at 500 μM concentration. Although in the presence of pyruvate the addition of glycerol + octanoate increased glucose formation from 60.0±5.4 to 98.5 ± 6.0 nmol hr^{-1} mg^{-1} dry weight ($N = 6$, $P < 0.01$), however, the inhibitory effects of both VAc and metformin on gluconeogenesis were similar (17.0 ± 1.6 and 20.1 ± 3.1 nmol hr^{-1} mg^{-1} dry weight, respectively, $N = 6$, $P < 0.005$). In following experiments VAc and metformin were used at 100 and 500 μM concentrations, respectively.

As shown in Fig. 2, the inhibition of gluconeogenesis by VAc was much less significant in hepatocytes (by about 20–30%) than in renal tubules (by about 60–80%), while metformin did not practically affect this process in hepatocytes, indicating a different sensitivity of these two organs to the action of both compounds. In agreement with Kida *et al.* [44] glucose formation in diabetic rabbit renal tubules was increased for about 30–40% in comparison with values determined for control animals. However, the inhibitory effect of VAc on glucose formation was similar in tubules of both control and diabetic rabbits, whereas metformin was less effective in alloxan-treated animals (about 50–60% inhibition of glucose production). Moreover, in contrast to VAc, metformin caused a significant increase in lactate production in renal tubules incubated with alanine + glycerol + octanoate (from 60.0 ± 1.9 to 108.0 ± 10.8 nmol mg^{-1} dry weight hr^{-1}, $N = 7$, $P < 0.005$).

In spite of increased glucose formation in kidney-cortex tubules of diabetic rabbits utilisation of glycerol was lower than that determined in renal tubules of control animals, while both alanine and pyruvate consumptions were not altered (Table 1) probably due to a smaller substrate utilisation for lactate production in kidney-cortex tubules of diabetic animals (65.5 ± 5.6 and 47.8 ± 5.3 nmol hr^{-1} mg^{-1} dry weight with pyruvate and alanine + glycerol+ octanoate, respectively, $N = 5$, $P < 0.05$) in comparison with control rabbits (94.0 ± 7.5 and 74.8 ± 8.9 nmol hr^{-1} mg^{-1} dry weight with pyruvate and alanine+ glycerol + octanoate, respectively, $N = 5$).

Inhibitory effect of vanadium on glucose formation might be due to a decrease in alanine, glycerol and pyruvate utilisations (by 40, 35 and 20%, respectively) in both control and diabetic rabbits. However, a decline in alanine utilisation does not seem to result from inhibition of alanine uptake as the rate of alanine transport into kidney-cortex tubules was not altered in the presence of VAc (data not shown). Metformin lowered alanine, pyruvate and glycerol consumptions in control animals (by about 35, 20, 20%, respectively) while it was less effective in diabetic animals.

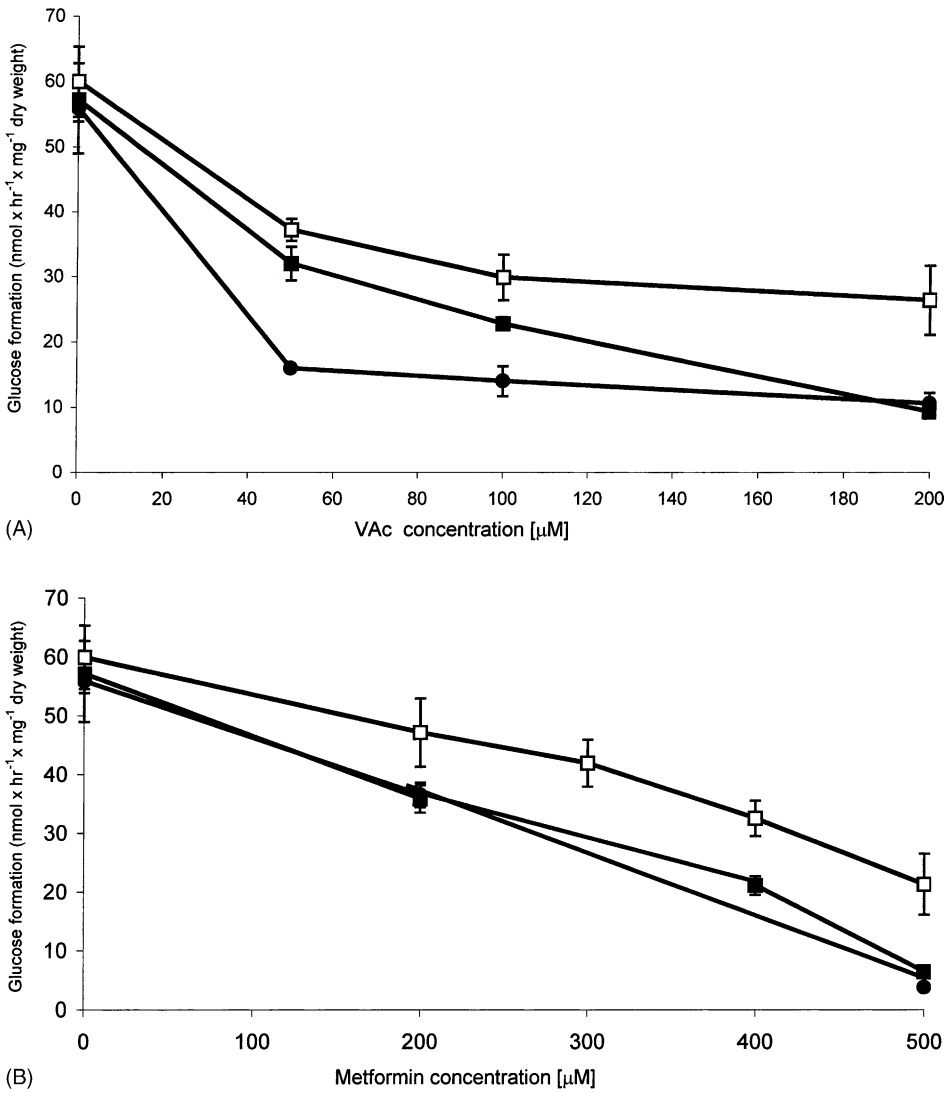

Fig. 1. Effect of various concentrations of VAc (A) and metformin (B) on glucose formation in isolated kidney-cortex tubules incubated with: 2 mM alanine + 2 mM glycerol + 0.5 mM octanoate (■), 5 mM pyruvate (□) or 5 mM dihydroxyacetone (●). Results are means ± SD of 8–10 separate experiments.

3.2. Vanadium and metformin accumulations in renal tubules

Following the rise of VAc concentration in the reaction medium from 50 to 100 μM the intracellular vanadium level increased by about 100-fold (from 0.7 ± 0.1 to 81.7 ± 6.7 μg V g^{-1} dry weight, $N = 6$, $P < 0.005$), indicating a marked accumulation of vanadium in the presence of 100 μM VAc. On the contrary, the increase in metformin concentration from 200 to 500 μM in the renal tubule suspension resulted in the 2.5-fold rise of the intracellular drug content following 60 min of incubation (from

Table 1
Effect of VAc and metformin on substrate utilisation in kidney-cortex tubules of control and diabetic rabbits

Rabbits	Additions	Alanine (nmol hr^{-1} mg^{-1} dry weight)	Glycerol (nmol hr^{-1} mg^{-1} dry weight)	Pyruvate (nmol hr^{-1} mg^{-1} dry weight)
Control	None	152 ± 10	165 ± 3	395 ± 28
	Vac	94 ± 9**	107 ± 8***	302 ± 22***
	Metformin	99 ± 7*	137 ± 10**	319 ± 19***
Diabetic	None	159 ± 16	92 ± 4	364 ± 23
	Vac	94 ± 8***	61 ± 5*	298 ± 27***
	Metformin	124 ± 10*	87 ± 9	342 ± 15*

Kidney-cortex tubules were incubated for 1 hr in the absence and presence of either 100 μM VAc or 500 μM metformin, as indicated. Results are means ± SD of 5–6 separate experiments.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

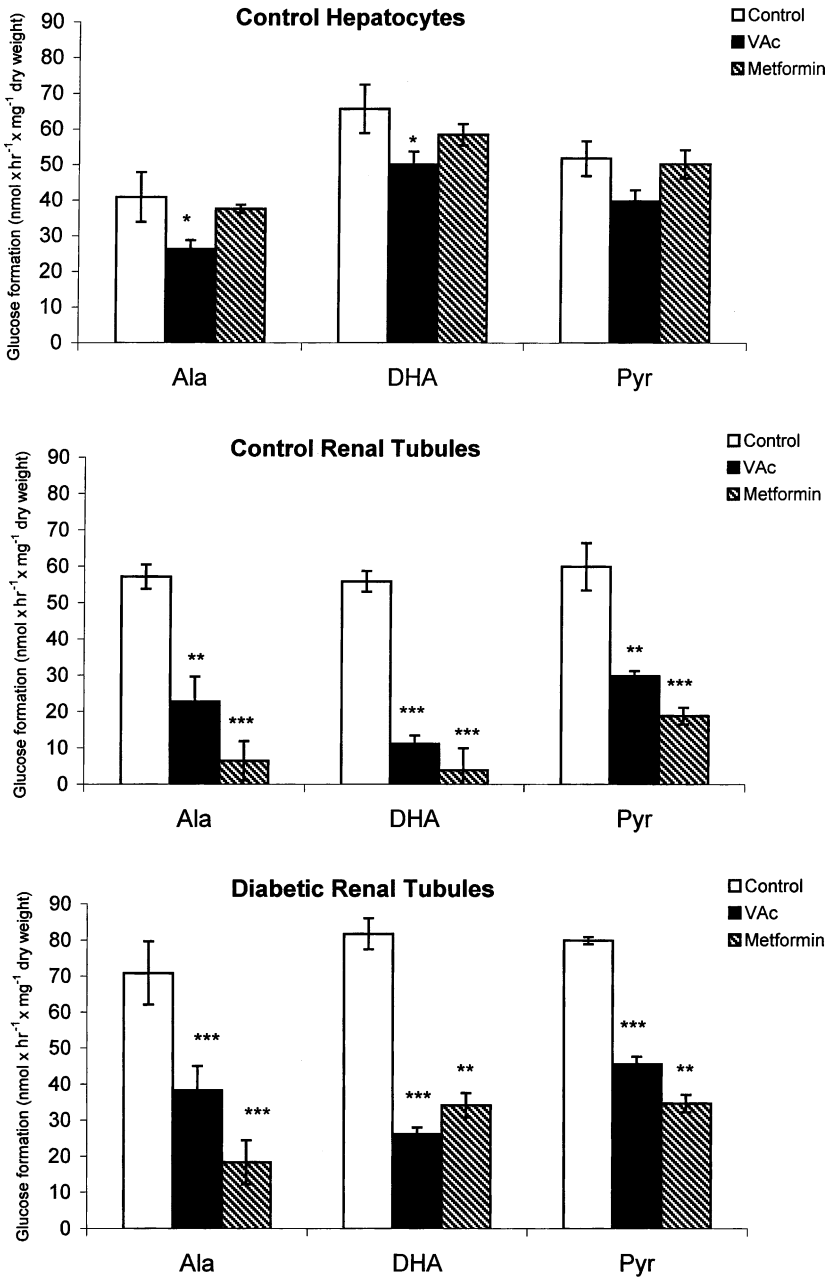

Fig. 2. Effect of VAc (100 μM) and metformin (500 μM) on glucose formation in hepatocytes and kidney-cortex tubules isolated either from control or diabetic rabbits. Kidney-cortex tubules were incubated with 2 mM alanine + 2 mM glycerol + 0.5 mM octanoate while pyruvate and dihydroxyacetone were added at 5 mM concentrations. Hepatocytes were incubated with substrates at 5 mM concentrations. Results are means ± SD of 5–9 separate experiments. (*) indicates $P < 0.05$, (**) indicates $P < 0.01$, (***) indicates $P < 0.005$.

209.8 ± 9.9 to 447.8 ± 14.3 μg g^{-1} dry weight, $N = 6$, $P < 0.005$).

3.3. Gluconeogenic intermediates and enzyme activities

In order to identify the steps responsible for inhibition of glucose synthesis pathway we have measured both the VAc- and metformin-induced changes in gluconeogenic intermediates levels in renal tubules incubated with alanine + glycerol + octanoate. As VAc at 100 μM decreased the intracellular ATP level from 4.65 ± 0.10 to 2.95 ± 0.10 nmol hr^{-1} mg^{-1} dry weight ($N = 5$, $P < 0.005$), while 50 μM concentration of this compound did not affect the intracellular ATP content (4.6 ± 0.1 and 4.5 ± 0.1 nmol hr^{-1} mg^{-1} dry weight without and with VAc, respectively, $N = 5$). We have compared the influence of vanadium on intracellular content of metabolites at both 100 and 50 μM VAc concentrations. A decline of ATP level in the presence of 100 μM VAc may be due to inhibition of respiration as the oxygen uptake by kidney-cortex mitochondria incubated with glutamate+ malate was decreased by 100 μM VAc by about 20% (from 24.3 ± 1.6 to 19.2 ± 1.5 nmol O_2 min^{-1} mg^{-1} protein, $N = 4$, $P < 0.005$). No changes in ATP level have been

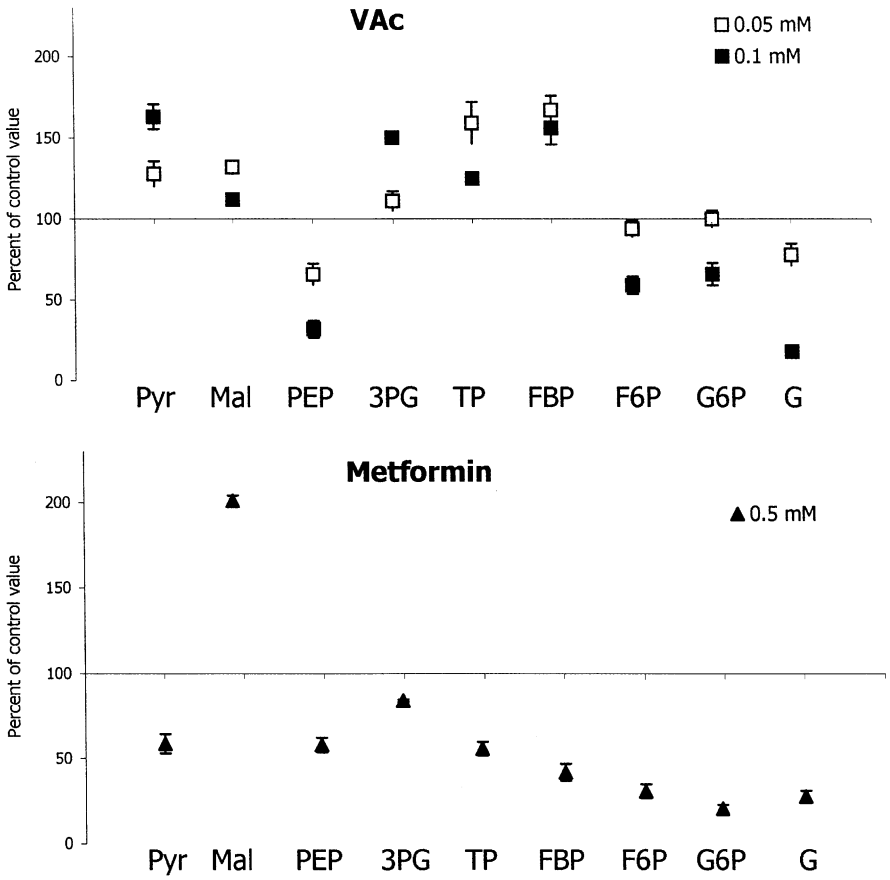

Fig. 3. The influence of VAc and metformin on changes in intracellular levels of gluconeogenic intermediates in renal tubules incubated with alanine + glycerol + octanoate. Experimental conditions as well as control values of metabolites are presented in Table 2. The concentrations of intracellular metabolites in the presence of 50 and 100 μM VAc or 500 μM metformin are expressed as percentage of control values measured without VAc or metformin. Metabolites listed from left to right are: pyruvate (Pyr), malate (Mal), phosphoenolpyruvate (PEP), 3-phosphoglycerate plus 1,3-diphosphoglycerate (3PG), glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate (TP), fructose-1,6-bisphosphate (FBP), fructose-6-phosphate (F6P), glucose-6-phosphate (G6P), glucose (Glc). Results are means ± SD of 6–8 separate experiments.

observed in the presence of 500 μM metformin in kidney-cortex tubules (4.6 ± 0.1 and 4.8 ± 0.1 nmol hr^{-1} mg^{-1} dry weight, without and with metformin, respectively, $N = 6$). In addition, we have observed no effect of this drug on glutamate + malate oxidation following as long as 4 hr incubation of kidney-cortex mitochondria with metformin (15.6 ± 1.4 and 14.8 ± 0.9 nmol O_2 min^{-1} mg^{-1} protein, without and with metformin, respectively, $N = 4$). At both concentrations applied vanadium induced a marked accumulation of pyruvate, malate, 3-phosphoglycerate + 1,3-bisphospho-glycerate, triosephosphates and fructose-1,6-bisphosphate accompanied by a significant decline of phosphoenolpyruvate, fructose-6-phosphate, glucose-6-phosphate and glucose levels (Fig. 3), indicating a decrease in pyruvate carboxylase, PEPCK, FBPase and G6Pase activities. Metformin induced elevation of the intracellular malate content accompanied by a decreased phosphoenolpyruvate concentration indicating an inhibition of PEPCK activity (Table 2).

As in the presence of both pyruvate and alanine + 2-oxoglutarate 50 and 100 μM VAc resulted in a similar decline of incorporation of $[^{14}C]CO_2$ into isolated kidney-cortex mitochondrial metabolites from 27.9 ± 1.9 and 49.3 ± 3.5 to 23.1 ± 1.4 and 35.5 ± 1.7 nmol $10\ min^{-1}$ mg^{-1} of protein, respectively ($N = 5$, $P < 0.01$) an inhibition of pyruvate carboxylation by VAc could be responsible for

Table 2
Intracellular content of gluconeogenic intermediates in renal tubules incubated in the presence of alanine + glycerol + octanoate

Metabolite	Alanine + glycerol + octanoate
Pyruvate	0.16 ± 0.01
Malate	0.47 ± 0.03
Phosphoenolpyruvate	0.17 ± 0.01
3-phosphoglycerate + bisphosphoglycerate	0.52 ± 0.05
Triosephosphate	0.13 ± 0.01
Fructose-1,6-bisphosphate	0.08 ± 0.01
Fructose-6-phosphate	0.06 ± 0.01
Glucose-6-phosphate	0.26 ± 0.01
Glucose	4.07 ± 0.30

Kidney-cortex tubules were incubated with 2 mM alanine in the presence of 2 mM glycerol and 0.5 mM octanoate. Metabolite contents (nmol mg^{-1} dry weight) were determined following 1 hr incubation and centrifugation through a silicon oil layer, as described in Section 2. Results are means ± SD of 5–7 separate experiments.

Table 3
Effect of VAc and metformin on PEPCK activites in cytosolic and mitochondrial fractions of kidney-cortex

Additions	Inhibitor concentrations (μM)	Mitochondria		Cytosol	
		$-MnCl_2$	$+MnCl_2$	$-MnCl_2$	$+MnCl_2$
None	–	23.5 ± 1.9	22.5 ± 1.3	21.9 ± 0.8	30.7 ± 2.7
VAc	50	22.3 ± 1.0	22.1 ± 1.2	13.4 ± 1.0**	34.4 ± 1.7
	100	18.8 ± 1.5*	24.3 ± 1.5	7.4 ± 0.7***	31.5 ± 2.8
Metformin	500	23.5 ± 1.5	22.7 ± 0.9	22.8 ± 1.2	33.5 ± 2.2

Enzyme activities are expressed as nmol min^{-1} mg^{-1} protein. $MnCl_2$ was added at 0.1 mM concentration. Data shown are means ± SE of 5–7 separate experiments.
* $P < 0.05$;** $P < 0.01$; *** $P < 0.005$.

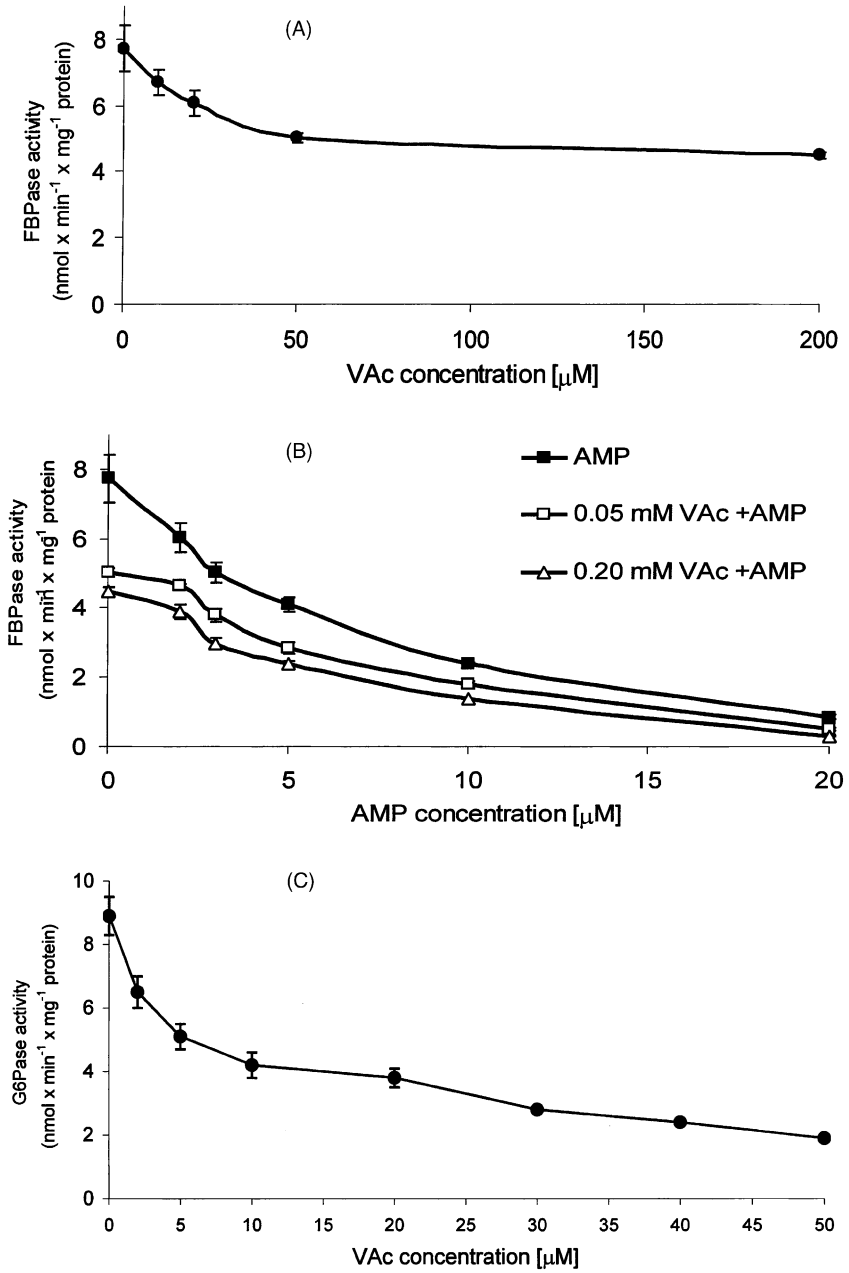

Fig. 4. Inhibition by VAc of rabbit kidney-cortex fructose-1,6-bisphosphatase (A and B) and glucose-6-phosphatase (C). FBPase activity was measured in the presence of either VAc alone (A) or both AMP and VAc (B). Fructose-1,6-bisphosphate and glucose-6-phosphate were added at 20 μM and 5 mM concentrations, respectively. Values are means ± SE of 5–7 separate experiments performed in duplicate.

the vanadium-induced decrease of both glucose formation (cf Fig. 1) and substrate utilisation (cf Table 1). In contrast to VAc, metformin did not affect pyruvate carboxylation (data not shown).

At 50 and 100 μM concentrations VAc significantly decreased (by about 40 and 60%, respectively) the cytosolic PEPCK activity (Table 3). Moreover, the addition of 100 μM $MnCl_2$, a PEPCK activator [45], abolished the VAc-induced enzyme inhibition. The inhibitory effect of VAc on the mitochondrial PEPCK was considerably smaller than that shown on the cytosolic one and it was also abolished on the addition of $MnCl_2$. Surprisingly, metformin at 500 μM concentration did not affect the PEPCK activity in both cytosolic and mitochondrial fractions. However, as both lactate/pyruvate (22.7 ± 1.8 and 63.8 ± 5.8 without and with metformin, respectively, $N = 5$, $P < 0.01$) and 3-hydroxybutyrate/acetoacetate ratios (0.53 ± 0.02 and 0.84 ± 0.03 without and with metformin, respectively, $N = 5$, $P < 0.025$) were significantly increased following the addition of metformin to renal tubule suspension incubated with alanine + glycerol+ octanoate as a substrate, this drug may cause an elevation of $NADH/NAD^+$ ratio in both cytosol (from $2.5 \times 10^{-3} \pm 0.2 \times 10^{-3}$ to $7.1 \times 10^{-3} \pm 0.3 \times 10^{-3}$, $N = 5$, $P < 0.01$) and mitochondria (from $26 \times 10^{-3} \pm 2 \times 10^{-3}$ to $41 \times 10^{-3} \pm 3 \times 10^{-3}$, $N = 5$, $P < 0.025$) as calculated from changes in lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios, respectively. Following the addition of 500 μM metformin the cytosolic oxalacetate concentration (calculated in view of changes in $NADH/NAD^+$ ratios) decreased from 1.10 ± 0.10 to 0.56 ± 0.06 μM ($N = 5$, $P < 0.01$), whereas level of this metabolite in the mitochondrial fraction was very low ($0.09 \pm 6.1 \times 10^{-3}$ μM, $N = 5$). This may indicate an importance of the cytosolic enzyme for the inhibitory action of metformin on renal gluconeogenesis.

In addition to pyruvate carboxylase and PEPCK, VAc also decreased FBPase activity in the kidney-cortex cytosolic fraction (Fig. 4A) and augmented the inhibitory action of AMP on the enzyme activity (Fig. 4B). Moreover, VAc was also a potent inhibitor of G6Pase activity in cytosolic fraction of both kidney-cortex (Fig. 4C) and liver (18.6 ± 1.3 and 10.1 ± 1.3 nmol min^{-1} mg^{-1} protein, without and with 5 μM VAc, respectively; $N = 5$, $P < 0.01$).

3.4. Body weights and blood glucose in vanadium- and metformin-treated rabbits

The insulin-mimetic effect of VAc was investigated in alloxan-treated rabbits in comparison with that exhibited in

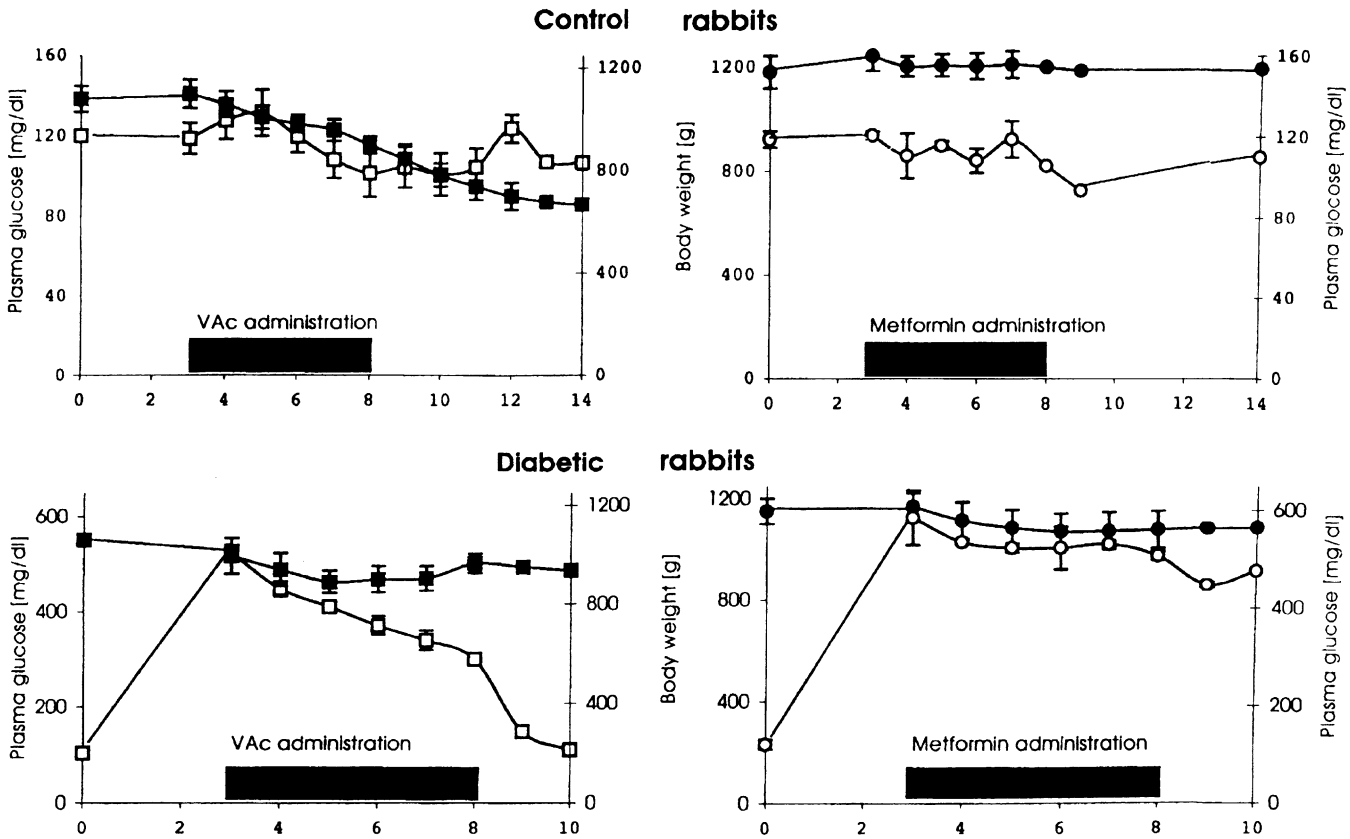

Fig. 5. Effect of VAc (□) and metformin (○) treatment on blood glucose (empty symbols) and the body weight (filled symbols) of control and diabetic rabbits. VAc was administrated i.p. once a day for 6 days at the dose of 1.275 mg V kg^{-1} body weight [10], while metformin was injected twice a day at the dose of 100 mg kg^{-1} body weight [75]. Values are means ± SD of 4–6 rabbits in each group.

control animals. At the dose of 1.275 mg V kg^{-1} body weight VAc was administrated intraperitoneally (i.p.) to rabbits for 6 days to bypass gastrointestinal tract. As shown in Fig. 5 during vanadium treatment the blood glucose content in control animals did not change despite 30% decrease in the rabbit body weight. On the contrary, blood glucose levels of diabetic animals were progressively falling down to achieve control values on 1–2 days following vanadium withdrawal regardless of no differences in the body weight. In contrast to VAc, the treatment of control and diabetic rabbits with metformin (200 mg kg^{-1} body weight day^{-1}) affected neither body weight nor blood glucose levels. It is necessary, however, to point out that plasma urea and creatinine levels were markedly elevated following 6 days of VAc treatment of control animals (from 18.7 ± 1.7 and 1.27 ± 0.2 mg dL^{-1} up to 132.8 ± 9.7 and 2.2 ± 0.3 mg dL^{-1}, respectively, $N = 5$, $P < 0.05$). In diabetic rabbits plasma urea and creatinine concentrations were also higher than in control rabbits (122.0 ± 2.7 and 2.8 ± 0.3 mg dL^{-1}, respectively, $N = 5$) and maintained high during vanadium treatment (104.0 ± 10.3 and 2.6 ± 0.2 mg dL^{-1}, respectively, $N = 5$). Following six i.p. injections of VAc, vanadium content in kidney-cortex increased up to 7.6 ± 0.6 μg V g^{-1}dry weight of tissue in both diabetic ($N = 5$) and control animals ($N = 5$). In view of (i) an extensive accumulation of vanadium in kidneys of VAc-treated animals and (ii) increased urea and creatinine levels in vanadium-treated control and diabetic animals, it seems that vanadium should not be recommended as an insulin-mimetic therapeutic agent.

4. Discussion

4.1. Vanadium action

In agreement with earlier reports for hepatocytes [1] VAc also diminished gluconeogenesis in kidney-cortex tubules (cf Fig. 2). However, vanadium is more potent inhibitor of gluconeogenesis in renal tubules than in hepatocytes. Differences in the VAc effects on glucose synthesis in liver and kidney may be caused by the different isoforms of regulatory gluconeogenic enzymes. For example, the activity of FBPase, a key enzyme of this process, is not affected by ribose-1,5-bisphosphate in rabbit liver [46], whereas it is inhibited in rat kidney-cortex [27].

Vanadium compounds produce their insulin-mimetic effects through an inhibition of tyrosine phosphatases acting on the insulin receptor and/or at points distal to the receptor in the insulin signalling pathway [47]. Besides, vanadium has also been shown to exert direct inhibitory effects on a number of various cellular enzymes [1,48]. An inhibitory action of vanadium has also been reported with respect to the liver PEPCK activity as well as mRNA level of this enzyme [49–51]. Thus, it may be suggested that (i) PEPCK is a target enzyme for vanadium action in both liver and kidney and (ii) vanadium at least in liver can act in short-term (metabolic) as well as in long-term (genic) manners. Inhibition of FBPase activity in isolated hepatocytes was explained by the vanadium-induced increase of fructose-2,6-bisphosphate level, an inhibitor of FBPase [52]. However, in our hands, vanadium directly affected this enzyme activity and in addition it potentiated the inhibitory action of AMP (cf. Fig. 4A and B).

Inhibitory action of VAc on G6Pase in kidney-cortex is in agreement with that reported for hepatic enzyme [50]. However, a smaller effect of VAc on hepatic gluconeogenesis than on renal one (cf Fig. 2) may be due to an increased vanadium accumulation in kidney-cortex (7.6 ± 0.6 μg V g^{-1} dry weight, $N = 10$) in comparison with that in liver (1.9 ± 0.3 μg V g^{-1} dry weight, $N = 8$) as observed in rabbits following 6 days of VAc treatment.

Commonly described signs of vanadium toxicity in experimental animals include a diminished food consumption, weight loss and even death [20]. In the most of these studies vanadium compounds were typically administrated in the drinking water. In order to overcome poor absorption from the gastrointestinal tract and gastrointestinal toxicity i.e. diarrhoea, in the present study rabbits were treated with vanadium by i.p. injection of VAc at dose equal to 1.275 mg kg^{-1} day [10] i.e. much lower than that administered orally [53]. In contrast to Reul *et al.* [10] who have observed blood glucose decrease by about 25% after one i.p. injection of VAc to diabetic rats, six injections to diabetic rabbits were required to normalise blood glucose level. Despite a similar vanadium-induced inhibition of gluconeogenesis in kidney-cortex tubules isolated from both control and diabetic animals (cf Table 1) VAc did not alter blood glucose level in control rabbits (cf Fig. 5). This may be due to both an inhibition of gluconeogenesis and an activation of liver glycogenolysis [54,55] and glycogenesis in rat adipocytes and muscle [56,57]. Thus, the net balance of blood glucose level may not change significantly in control organisms, in agreement with blood glucose measurements in athletes treated for 3 months with vanadyl sulphate [58]. On the contrary, VAc-induced decline in blood glucose level in diabetic rabbits may result from an inhibition of gluconeogenesis as the liver glycogen content in diabetes is reduced [59]. Unfortunately, while in diabetic rabbits increased blood glucose levels were reduced following vanadium treatment (cf. Fig. 5), elevated serum urea and creatinine concentrations were measured in vanadium-treated control rabbits in agreement with those reported for vanadium-treated rats (reviewed in [20]). Other authors failed to detect changes in levels of latter compounds [60,61]. Moreover, vanadium was detected to accumulate in rat kidney [20] and rabbit kidney-cortex, implying an additional risk of vanadium toxicity. In our opinion it should be used neither to improve glucose uptake into skeletal muscles [58] nor to treat diabetes [8,9].

4.2. Metformin action

Although metformin has been used worldwide to treat type 2 diabetes for several decades, its mechanism of action on glucose homeostasis remains controversial. It either does not affect [6] or reduces endogenous glucose production both independently and in interaction with insulin [62]. In view of *in vitro* studies an inhibition of hepatic gluconeogenesis [63] may result from: (i) a decrease in the uptake of gluconeogenic precursors by liver cells [64], (ii) a diminished flux through pyruvate carboxylase and PEPCK [65] as well as glucose-6-phosphatase [63], (iii) an enhanced flux through pyruvate kinase (EC 2.7.1.40 ATP-pyruvate 2-O-phosphotransferase) [66,67] and (iv) a decreased expression of genes encoding glucose-6-phosphatase and PEPCK [5]. Moreover, changes in fluidity of cell membrane have also been suggested as factors contributing to the metformin action [68].

It is necessary to point out that in studies reporting a decline of hepatic glucose formation metformin was used at very high (1–50 mM) concentrations [5,66]. The concentration of metformin in human serum vary typically between 10–100 μM [69]. However, in patients with lactic acidosis and other pathological conditions such as renal impairment, a significant accumulation of this drug may occur in plasma (over 50 mg L^{-1} i.e. above 300 μM) [70]. Moreover, in kidney and liver metformin concentration may exceed twice those measured in serum [21]. In our hands, 500 μM metformin considered as the high therapeutic level [64], did not practically affect hepatic gluconeogenesis (cf Fig. 2), while it resulted in a significant decrease of glucose production in kidney-cortex tubules (cf Figs. 1 and 2). In contrast to Kuhnle *et al.* [71], it is not, however, due to a direct action of metformin on the mitochondrial pyruvate carboxylase and PEPCK (cf Table 3). In agreement with observations for rat hepatocytes [5] the metformin-induced decline of flux through the cytosolic PEPCK may result from a decreased concentration of cytosolic oxaloacetate, the substrate of this enzyme, probably due to an elevation of NADH/NAD^+ ratios, observed also by Owen *et al.* [72] and by EL-Mir *et al.* [73] following the addition of metformin into isolated hepatocytes at 2 and 10 mM concentration, respectively. Moreover, the possibility of a metformin-induced increase in flux through pyruvate kinase activity cannot be excluded. However, in agreement with others [62,65] no changes in ATP level have been observed in kidney-cortex tubules, so postulated in hepatocytes [66] an increased flux through pyruvate kinase activity due to diminished ATP content cannot explain a metformin-induced decrease in renal gluconeogenesis.

Although metformin has been reported to permeate very slowly across the mitochondrial inner membrane and decrease the complex I activity of the respiratory chain at 10 mM concentration [72], the inhibitory effect of the drug on renal gluconeogenesis does not seem to be due to a progressive inhibition of complex I as we have observed no effect of 500 μM metformin on oxidative phosphorylation. Moreover, it is difficult to believe that clinically relevant concentration of a relatively safe drug, prescribed to 25% of type 2 diabetic patients [74], would reduce the level of such a crucial cofactor. In view of these observations metformin-induced decrease in gluconeogenesis in isolated renal tubules may result mainly from an elevation of NADH/NAD^+ ratio, probably due to an increase in lactate production, affecting the cytosolic oxaloacetate level and consequently PEPCK activity. However, it is necessary to keep in mind that isolated kidney-cortex tubules represent an experimental model used to elucidate metformin action.

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