

Effects of Ranolazine on Carbohydrate Metabolism in the Isolated Perfused Rat Liver

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Abstract

The action mechanism of ranolazine, an antiangina drug, could be at least partly metabolic, including inhibition of fatty acid oxidation and stimulation of glucose utilization in the heart. The purpose of the present work was to investigate if ranolazine affects hepatic carbohydrate metabolism. For this purpose, the hemoglobin-free isolated perfused rat liver was used as the experimental system. Ranolazine increased glycolysis and glycogenolysis and decreased gluconeogenesis. These effects were accompanied by an inhibition of oxygen consumption. The drug also changed the redox state of the NAD⁺-NADH couple. For the cytosol, increased NADH/NAD⁺ ratios were observed both under glycolytic conditions as well as under gluconeogenic conditions. For the mitochondria, increased NADH/NAD⁺ ratios were found in the present work in the absence of exogenous fatty acids in contrast with the previous observation of a decreasing effect when the liver was actively oxidizing exogenous oleate. It seems likely that ranolazine inhibits gluconeogenesis and increases glycolysis in consequence of its inhibitory actions on energy metabolism and fatty acid oxidation and by deviating reducing equivalents in favour of its own biotransformation. This is in line with the earlier postulates that ranolazine diminishes fatty acid oxidation, shifting the energy source from fatty acids to glucose.

Keywords

Glycogenolysis, Glycolysis, NAD⁺-NADH Redox Potentials, Gluconeogenesis, Ketogenesis

1. Introduction

Ranolazine is an agent for angina medication, commercialized under the trade name of Ranexa™. Its action mechanism is not clear and there are at least two different hypotheses. The first one postulates that ranolazine

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diminishes fatty acid oxidation, shifting the energy source from fatty acids to glucose [1]-[5]. An alternative interpretation with increasing experimental support postulates that ranolazine could exert its effects by improving the deficient functioning of sodium channels [6]-[8].

Irrespective of its clinical importance, alterations in fatty acid metabolism by ranolazine were in fact observed. A concomitant reduction in fatty acid oxidation and increase in glucose oxidation, for example, were found in experiments with the perfused rat heart [4]. These effects were accompanied by a reduction in the levels of acetyl-CoA in the heart tissue [4]. In the liver, which is the site of ranolazine biotransformation [9], it has been shown that ranolazine inhibits oleate net uptake (40% at 200 μ M ranolazine) by diminishing the transfer of this fatty acid from the extracellular albumin site to the intracellular space [10]. No effect on the coefficient for intracellular sequestration of oleate was found. Inhibition of net uptake is thus not the consequence of an acyl-CoA synthetase inhibition. Consistently, ranolazine also inhibits the extra oxygen consumption caused by oleate, as well as the extra ketogenesis induced by this substrate. It seems thus that in the liver ranolazine acts on fatty acid metabolism by at least two mechanisms: inhibition of cell membrane permeation and inhibition of the mitochondrial electron transfer via pyridine nucleotides [10]. The latter involves possibly the NADH dehydrogenase, but a direct effect on specific enzymes, especially β -hydroxybutyrate dehydrogenase, cannot be excluded.

Fatty acid and carbohydrate metabolism are always interconnected in mammalian cells. It is well known, for example, that fatty acid oxidation increases gluconeogenesis [11] [12], that the availability of carbohydrates enhances fatty acid synthesis and also that ketogenesis, even from endogenous sources, is strongly influenced by the presence of gluconeogenic substrates such as lactate [13] [14]. These interrelationships are caused by many factors which include activation/deactivation of key enzymes, alterations in the redox status of the cytosolic and mitochondrial NAD^+ -NADH systems and alterations in energy metabolism. Considering, thus, the action of ranolazine on fatty acid metabolism, it seems also worth to investigate its possible actions on carbohydrate metabolism. In the present work the actions of ranolazine on both glycolysis from endogenous sources and gluconeogenesis driven by exogenous substrates were measured in addition to the adenine nucleotide levels, in the hope of expanding knowledge about the actions of this drug in the liver.

2. Materials and Methods

2.1. Materials and Animals

First, the liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the assay procedures and fatty acid-free bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of at least 98% - 99% purity.

Male albino rats (Wistar strain; 220 - 250 g) were used. They received a standard laboratory diet and water ad libitum prior to the surgical removal of the liver. Animal handling and experiments were done in accordance with the world-wide accepted ethical guidelines for animal experimentation.

2.2. Liver Perfusion

For the surgical procedure the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. Hemoglobin-free, non-recirculating perfusion was done according to the technique described elsewhere [15] [16]. After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The hepatic artery was closed (monovascular perfusion) and the bile duct was left open. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted to between 30 and 35 $\text{mL}\cdot\text{min}^{-1}$, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs/Henseleit-bicarbonate buffer is [15] [16]: 115 mM NaCl, 25 mM NaHCO_3 , 5.8 mM KCl, 1.2 mM Na_2SO_4 , 1.18 mM MgCl_2 , 1.2 mM NaH_2PO_4 and 2.5 mM CaCl_2 . Substrates and ranolazine were dissolved in the perfusion fluid according to necessity. Samples of the effluent perfusion fluid were collected at 4-minute intervals and analyzed for their metabolite content.

2.3. Analytics

The following compounds in the outflowing perfusate were assayed by means of standard enzymatic procedures

[17]: lactate, pyruvate, glucose, β -hydroxybutyrate and acetoacetate. The oxygen concentration in the outflowing perfusate was monitored polarographically employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [16] [18].

2.4. Determination of the Hepatic Contents of Adenine Mononucleotides

The perfused liver was rapidly frozen in liquid nitrogen and a portion weighing between 2 and 4 g triturated and used for extraction. The adenine mononucleotides (AMP, ADP, ATP) were extracted with a 0.6 M perchloric acid solution. After mixing the liver powder with 6 to 12 mL of the perchloric acid solution the suspension was homogenized in a van-Potter homogenizer. The homogenate was centrifuged at 2°C (10 minutes at 3000 g) and the supernatant was neutralized with potassium carbonate. The neutralized extract was kept in ice bath until HPLC analysis.

The adenine mononucleotides in the samples were separated and quantified by HPLC. The apparatus (Shimadzu, Japan) consisted in a system controller SCL-10AVP, two pumps model LC10ADVP, a column oven model CTO-10AVP, and a UV-VIS detector model SPD-10AVP. A reversed-phase column C18 HRC-ODS (5 μ m; 150 \times 6 mm I.D.; Shimadzu, Japan), protected with a pre-column GHRC-ODS (5 μ m; 10 \times 4 mm I.D.; Shimadzu, Japan), was used with a gradient from reversed-phase 0.044 M phosphate buffer solution pH 6.0 to 0.044 M phosphate buffer solution plus methanol (1.1) pH 7.0 at 0.8 mL \cdot min⁻¹. The gradient was (in % of methanol): 0 min, 0%; 2.5 min, 0.5%; 5 min, 3%; 7 min, 5%; 8 min, 12%; 10 min, 15%; 12 min, 20%; 20 min, 30%. Temperature was kept at 35°C and the injection volume was always 20 μ L. The UV-absorbance detector was auto-zeroed at the start of each chromatogram and the absorbance was measured at 254 nm.

The identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained injecting standards in the same conditions, as well as by spiking liver samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. The calibration curves were constructed by separating chromatographically standard solutions of the compounds. Linear relationships were obtained between the concentrations and the areas under the absorbance curves.

2.5. Statistical Analysis

The error parameters presented in the text and tables are standard errors of the means. Differences between pairs of means were analyzed by means of Student's t test or Student's paired t-test according to the context. The 5% level ($p < 0.05$) was adopted as a criterion of significance.

3. Results

3.1. Glycogen Catabolism and Glycolysis

In order to investigate the action of ranolazine on glycogen catabolism and glycolysis, livers from fed rats were perfused with substrate-free perfusion fluid, in an open system. Under these conditions, the livers release glucose, lactate and pyruvate as a result of glycogen degradation [15] [19]. Lactate plus pyruvate production are a good estimate for the glycolytic activity because, under these conditions, the processing of pyruvate by the pyruvate dehydrogenase complex and pyruvate carboxylase proceeds at very low rates [20] [21]. **Figure 1** shows the time course of the experiments in which 100 μ M ranolazine was infused. Oxygen uptake was diminished ($p < 0.05$, paired t-test) and remained so during the entire ranolazine infusion period. Lactate and glucose productions were both increased shortly after initiation of ranolazine infusion ($p < 0.05$). Both variables also remained elevated during the ranolazine infusion. Pyruvate production was not affected. Due to the elevated lactate production, the lactate to pyruvate ratio was increased, meaning also an elevated cytosolic NADH/NAD⁺ ratio, due to the lactate dehydrogenase equilibrium [13] [22]. Upon cessation of the infusion all variables tended to return to values close to those before initiation of ranolazine infusion (basal rates).

3.2. Lactate Gluconeogenesis and Ketogenesis from Endogenous Sources

For investigating the action of ranolazine on glucose synthesis livers from 24 hours fasted rats were used in order to minimize the interference by glycogen catabolism [13]. The liver cells under these conditions can survive

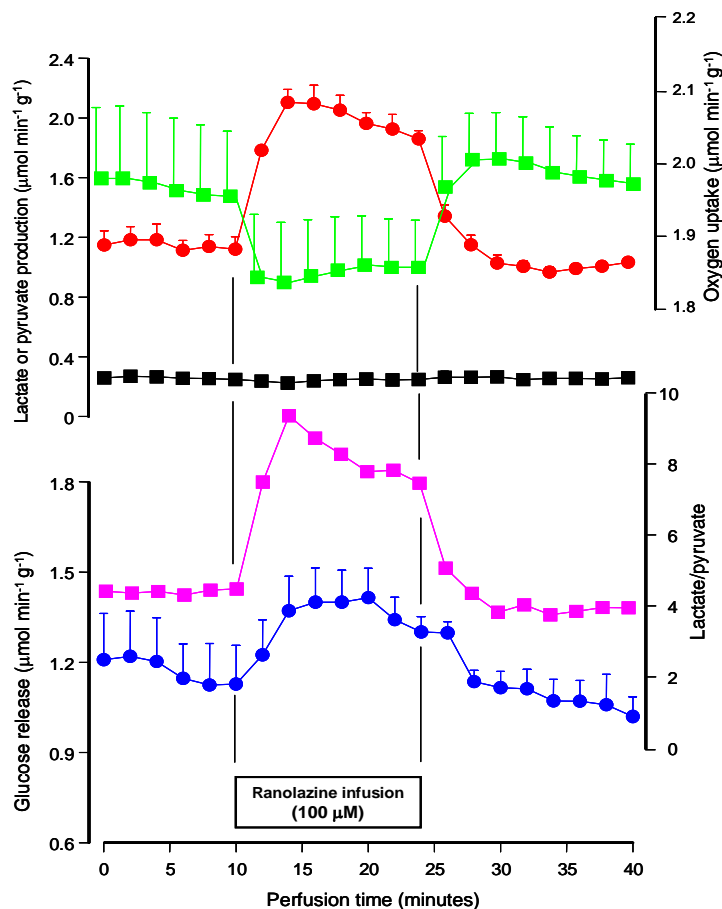


Figure 1. Time course of the effects of 100 μM ranolazine on metabolic fluxes derived from glycogen catabolism and on oxygen uptake in livers from fed rats. Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Samples of the effluent perfusate were collected for glucose, lactate and pyruvate assay. Oxygen in the outflowing perfusate was monitored polarographically. The data represent the means (\pm SEM) of three liver perfusion experiments.

mainly at the expense of the oxidation of endogenous fatty acids and amino acids. The intense β -oxidation increases the acetyl-CoA levels, which in turn leads to a significant ketogenesis (β -hydroxybutyrate + acetoacetate production), as revealed by panels A and B of **Figure 2**. The introduction of ranolazine under these conditions produced a rapid decrease in ketogenesis and oxygen uptake ($p < 0.05$; **Figure 2(A)**). The β -hydroxybutyrate to acetoacetate ratio, on the other hand, was increased by ranolazine. This effect differs from that observed when ketogenesis was mainly due to exogenous oleate oxidation, where the introduction of ranolazine decreased the β -hydroxybutyrate/acetoacetate ratio [10]. The introduction of 5 mM lactate further decreased ketogenesis and increased the β -hydroxybutyrate/acetoacetate ratio. It also produced increments in oxygen uptake and glucose production. The latter effect, however, was much less pronounced than that found when lactate was infused in the absence of ranolazine, as shown in **Figure 2(B)**. This means that ranolazine inhibited gluconeogenesis, with an inhibition degree of 85% ($p < 0.05$). It should be remarked that lactate alone also decreased ketogenesis ($p < 0.05$; **Figure 2(B)**) to the same low levels as those found when ranolazine and lactate were both present (**Figure 2(A)**). Lactate also increased the β -hydroxybutyrate/acetoacetate ratio, either alone (**Figure 2(B)**) or when ranolazine was also present (**Figure 2(A)**). This observation, in addition to the increment in oxygen uptake caused by lactate in the presence of ranolazine, indicates that the reducing equivalents derived from lactate oxidation were transferred to the mitochondria. It should be noted that the β -hydroxybutyrate to acetoacetate ratio reflects the mitochondrial NADH to NAD^+ ratio [13] [22].

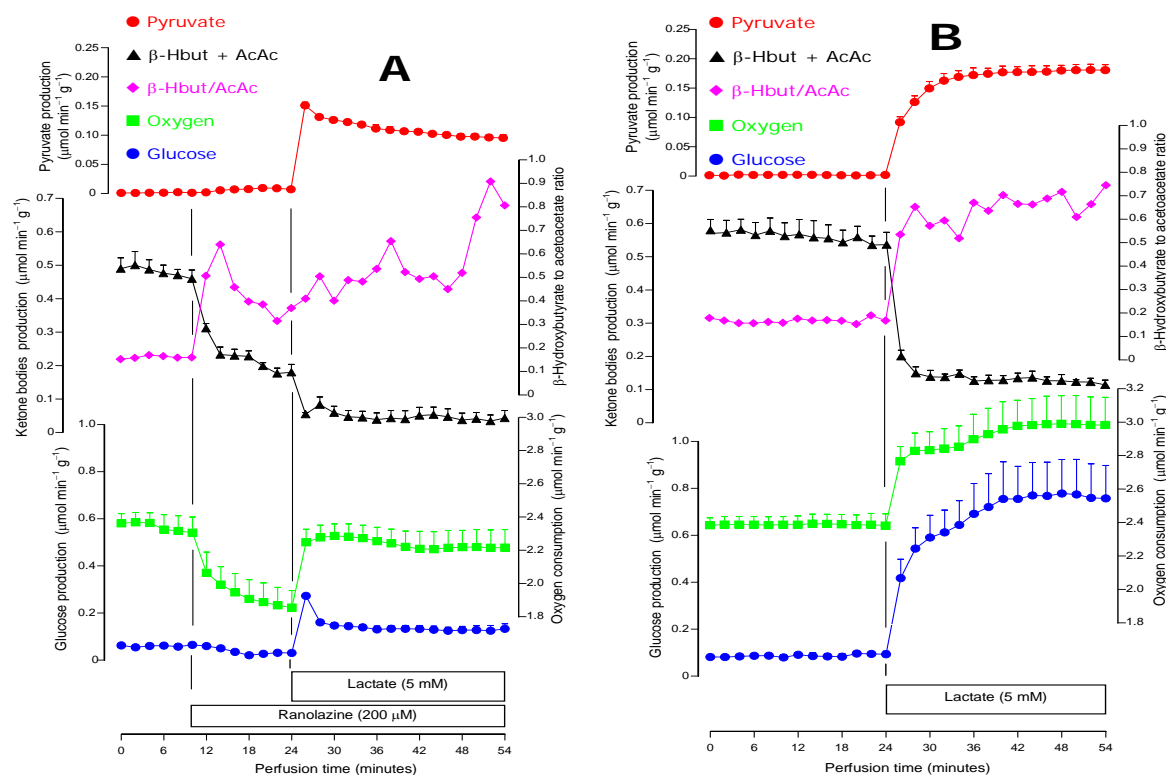


Figure 2. Effects of ranolazine on lactate gluconeogenesis and ketogenesis from endogenous sources. Livers from 24 hours fasted rats were perfused with substrate-free medium initially and with 5 mM lactate containing medium at the times indicated in the bars near to the time scale. In the experiments of panel A 200 μM ranolazine was infused as indicated. Perfusate samples were collected for metabolite measurements by means of enzymatic procedures. Oxygen uptake was measured polarographically. Data are means plus mean standard errors of 4 (panel A) and 5 (panel B) liver perfusion experiments. Legends: $\beta\text{-Hbut}$, $\beta\text{-hydroxybutyrate}$; AcAc, acetoacetate.

3.3. Pyruvate Gluconeogenesis and Ketogenesis from Endogenous Sources

The same kind of experiments that were done with 5 mM lactate as gluconeogenic substrate were also done with 5 mM pyruvate. The infusion of lactate induces a highly reducing state in the cell whereas the infusion of pyruvate induces the opposite, *i.e.*, a highly oxidizing condition [22]. Concerning ketogenesis, the actions of pyruvate were similar to those of lactate: an additional inhibition in the presence of ranolazine ($p < 0.05$; **Figure 3(A)**) and a strong inhibition in the absence of the drug ($p < 0.05$; **Figure 3(B)**). The $\beta\text{-hydroxybutyrate/acetate}$ ratio was considerably increased by pyruvate in the presence of ranolazine, even though strong fluctuations were apparent (**Figure 3(A)**). In the absence of ranolazine, unlike to what happened with lactate, the increase in the $\beta\text{-hydroxybutyrate/acetate}$ ratio caused by pyruvate was relatively modest (**Figure 3(B)**), an expected phenomenon if one considers that the transformation of pyruvate generates much less reducing equivalents than that of lactate. The response of gluconeogenesis to pyruvate in the presence of ranolazine differed from that of lactate: it was slower (**Figure 3(A)**) when compared to the control condition (**Figure 3(B)**) with a progressive increase so that at the end of the experiment the inhibition of gluconeogenesis was relatively small, only 17% ($p < 0.05$). Lactate production from pyruvate, finally, was 25% higher in the presence of ranolazine ($p < 0.05$).

3.4. Adenine Mononucleotide Levels

Table 1 lists the hepatic contents of AMP, ADP and ATP in livers from fed rats perfused with substrate-free medium in the absence (control) and presence of ranolazine. The levels of AMP, ADP and ATP are very close to those found under exactly the same conditions (substrate-free perfused rat liver of fed rats) using enzymatic assays [23]-[25]. As revealed by **Table 1**, 200 μM ranolazine did not produce significant changes in the cellular

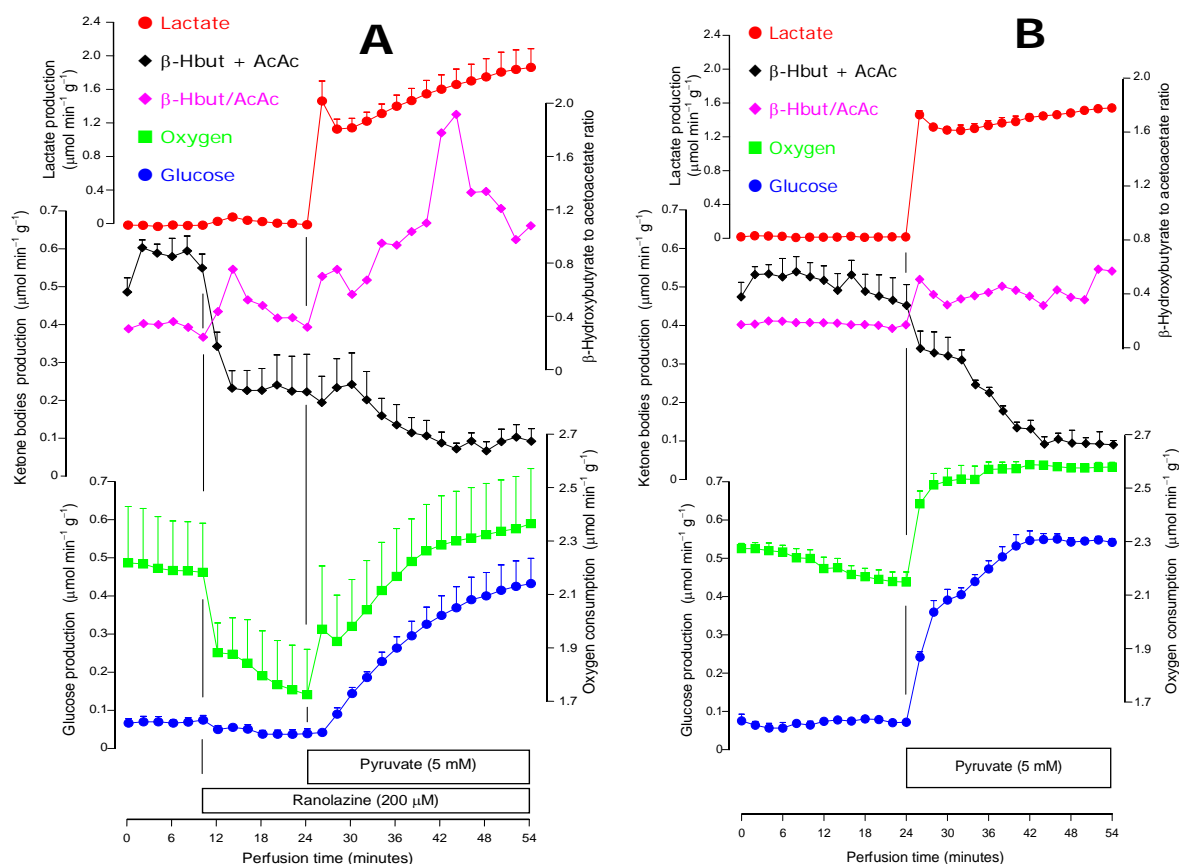


Figure 3. Effects of ranolazine on pyruvate gluconeogenesis and ketogenesis from endogenous sources. Livers from 24-hours fasted rats were perfused with substrate-free medium initially and with 5 mM pyruvate containing medium at the times indicated in the bars near to the time scale. In the experiments of panel A 200 μM ranolazine was infused as indicated. Perfusate samples were collected for metabolite measurements by means of enzymatic procedures. Oxygen uptake was measured polarographically. Data are means plus mean standard errors of 4 (panel A) and 5 (panel B) liver perfusion experiments. Legends: $\beta\text{-Hbut}$, β -hydroxybutyrate; AcAc, acetoacetate.

Table 1. Contents of adenine mononucleotides of livers from fed rats in the presence and absence of 200 μM ranolazine. The extraction and assay procedures are described in the Materials and Methods section. Error parameters are mean standard errors. The p values refer to student's t test.

	Control	Ranolazine (200 μM)	p
	$\mu\text{mol (gram liver wet weight)}^{-1}$		
ATP	2.08 ± 0.16 (n = 6)	2.20 ± 0.25 (n = 3)	>0.05
ADP	0.88 ± 0.07 (n = 6)	0.76 ± 0.07 (n = 3)	>0.05
AMP	0.36 ± 0.07 (n = 6)	0.12 ± 0.01 (n = 3)	<0.05

ATP and ADP contents, although a tendency toward diminished ADP levels is apparent. The AMP levels, however, were significantly diminished. This means also increased ATP/AMP ratios in the presence of ranolazine.

Table 2 shows the hepatic contents of AMP, ADP and ATP in livers from 24-hours fasted rats perfused with lactate as the gluconeogenic substrate. Under these conditions the ATP content was diminished by 200 μM ranolazine (–21%). The AMP and ADP contents, under these conditions, were not affected by ranolazine.

4. Discussion

The results obtained in the present work confirm the general hypothesis stated in the Introduction, namely, that

Table 2. Contents of adenine mononucleotides of livers from fasted rats in the presence of 5 mM lactate and 5 mM lactate + 200 μ M ranolazine. The extraction and assay procedures are described in the materials and methods section. Error parameters are mean standard errors. The p values refer to Student's t test.

	5 mM lactate	5 mM lactate + 200 μ M ranolazine	p
	μ mol (gram liver wet weight) ⁻¹		
ATP	1.986 \pm 0.083 (n = 5)	1.566 \pm 0.031 (n = 3)	<0.05
ADP	0.999 \pm 0.111 (n = 5)	1.091 \pm 0.063 (n = 3)	>0.05
AMP	0.313 \pm 0.018 (n=5)	0.354 \pm 0.060 (n = 3)	>0.05

ranolazine could be active on carbohydrate metabolism in the liver. In principle one is authorized to say that the drug increases glycolysis and decreases gluconeogenesis. Additionally it tends to increase the NADH/NAD⁺ ratio in both the cytosolic and in the mitochondrial compartment, as indicated by the lactate/pyruvate and β -hydroxybutyrate/acetoacetate ratios [12] [13]. For the cytosolic NADH/NAD⁺ ratio, increases were observed both under glycolytic conditions as well as under gluconeogenic conditions when pyruvate was the substrate (increased lactate production). For the mitochondrial NADH/NAD⁺ ratio, increases were observed in the present work in the absence of exogenous fatty acids, but it should be remarked that the opposite was found when the liver was actively oxidizing an exogenous fatty acid [10].

An important cause for the increased glycolysis and diminished gluconeogenesis is probably the inhibition of oxygen uptake caused by ranolazine and the consequently diminished rate of oxidative phosphorylation. It is well established by studies with other inhibitors that such an action usually causes stimulation of glycolysis and inhibition of gluconeogenesis [13] [26]. Inhibition of mitochondrial oxygen uptake, in turn, may have two causes. The first one is a direct inhibition of electron transfer at complex I [27]; the second cause is a direct inhibition of fatty acid oxidation, as suggested by experiments in which the oxidation of palmitoyl-CoA and oleoyl-CoA by isolated mitochondria was measured [10]. The fact that ranolazine inhibited the ketone body production from endogenous sources strongly corroborates the view of a direct inhibition of fatty acid oxidation. The latter may be even more important than complex I inhibition as suggested by the observation that the infusion of lactate or pyruvate in the presence of ranolazine still increased oxygen uptake to levels close to those found before the infusion of ranolazine suggesting that the reducing equivalents generated by the oxidation of lactate or pyruvate were still partly able to reach the cytochrome c oxidase system, a phenomenon that requires the participation of complex I. Even so, it is likely that complex I is inhibited to a certain extent, because the increased β -hydroxybutyrate to acetoacetate ratios in the presence of ranolazine indicate a diminished capacity of oxidizing the mitochondrial NADH [22].

Since ranolazine inhibits oxygen uptake, it seems reasonable to assume that this also represents diminished mitochondrial ATP production. Corroborating this, a diminution of the ATP content was found in the perfused liver of fasted rats and under gluconeogenic conditions. However, no such diminution was found in the liver of fed rats under glycogenolytic and glycolytic conditions. The fact that the ATP content was not affected by ranolazine in the fed state is most probably an event caused by the compensatory ATP production in the glycolytic pathway, which is stimulated by ranolazine (Figure 1). Actually, in the case of the experiments shown in Figure 1, in which 100 μ M ranolazine was infused, the excess lactate production was approximately equal to 0.7 μ mol min⁻¹.g⁻¹. On stoichiometric grounds, this corresponds also to a net ATP production rate of 0.7 μ mol·min⁻¹.g⁻¹. The diminution of oxygen uptake, on the other hand, was around 0.1 μ mol O₂ min⁻¹.g⁻¹ (or 0.2 μ g-atom oxygen min⁻¹.g⁻¹), which corresponds to a decrease of 0.5 μ mol·min⁻¹.g⁻¹ in mitochondrial ATP production if one assumes a P/O ratio of 2.5 [21]. The diminished phosphorylation is, thus, fully compensated, or even exceeded, by the increased glycolysis, what explains the absence of effects of ranolazine on the cellular ATP contents.

Another phenomenon that can be contributing for gluconeogenesis inhibition is the deviation of reducing equivalents and intermediates of the gluconeogenic pathway because of the extra NADPH consumption in the microsomal electron transport chain due to ranolazine transformation [9]. The strong influence of this phenomenon on gluconeogenesis has been unequivocally demonstrated by experiments with aminopyrine [28]. That this may be occurring with ranolazine is corroborated by the previous observations that the drug was able to increase hepatic oxygen consumption in the presence of 2 mM cyanide, a condition where changes in oxygen consumption no longer reflect the mitochondrial respiratory chain [10]. This increase was equal to 0.14 \pm 0.02

$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ with 200 μM ranolazine and it requires a constant supply of NADPH which must come from the reactions catalyzed by the NADPH-supplying dehydrogenases [28]. Two of them, the malic enzyme and the glucose 6-phosphate dehydrogenase interfere with gluconeogenesis because their action drains away or recycles intermediates of the gluconeogenic pathway [28] [29].

5. Conclusion

In conclusion, it seems likely that ranolazine inhibits gluconeogenesis and increases glycolysis in consequence of its inhibitory actions on energy metabolism and fatty acid oxidation and by deviating reducing equivalents in favour of its own biotransformation. Lactate gluconeogenesis, which is more strongly inhibited, is particularly important because of the predominance of high lactate to pyruvate ratios under *in vivo* conditions. All these observations are in line with the earlier postulates that ranolazine diminishes fatty acid oxidation, shifting the energy source from fatty acids to glucose [1]-[5]. Nevertheless, there were also indications that the action of ranolazine on metabolism may not be restricted to the sites of action that were already identified in the present and previous work [10] [27]. The unusual response of pyruvate gluconeogenesis in the presence of ranolazine, for example, suggests a more complex mechanism of action. Identification of other sites of action depends no doubt on additional experimental work.

Acknowledgements

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