Extracellular glycerol regulates the cardiac energy balance in a working rat heart model

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The fasting (0.33 mM) and fed (3.33 mM) states. Cardiac energy heart perfused in the working mode. Glycerol concentrations modeled glycerol supply, energy demand, and intermediary metabolism lead-

increased palmitate uptake and significantly decreased its oxidation. Over, increasing glycerol supply did not affect glucose oxidation but rather increased glycerol oxidation rate. More-

Increased glycerol supply and energy demand resulted in an increase in glycerol oxidation rate, but few have led to significant success. Carnitine palmitoyl transferase-1 (CPT-1) inhibitors such as etomoxir or oxfenicine were developed to decrease glycemia by shifting myocardium metabolism to glucose oxidation. Etomoxir was shown to improve insulin sensitivity in type 2 diabetic patients (9) and to increase glucose oxidation in normal and diabetic heart (21), but chronic administration resulted in cardiac hypertrophy induced by triacylglycerol (TAG) accumulation due to the inability of the cardiomyocyte to handle the nonoxidized long-chain FAs (20). Trimetazidine is the only molecule with a clinical success and acts by a simultaneous reduction in β-oxidation (3, 11) and an increase in phospholipid (PL) synthesis (7, 22, 23). Increasing PL synthesis resulted in a decrease in TAG synthesis and a significantly enhanced incorporation of long-chain FAs into membranes (24), which reduces the intracellular stores of long-chain FAs available for β-oxidation (7). We have reported earlier that the increase in PL synthesis due to trimetazo-

zidine is associated with a significant increase in cellular glycerol uptake. For this reason, we hypothesized that glycerol could be a key component controlling the metabolic ratio between oxidation and FAs for energy metabolism, between PL/TAG synthesis and between energy metabolism and lipid synthesis.

In cardiomyocytes, glycerol is a key metabolite that controls several steps of lipid metabolism (27). In a previous paper (5), we reported that, in cultured rat cardiac cells, glycerol is phosphorylated to glycerol-3-phosphate and dose dependently contributes to energy production through oxidation, to membrane homeostasis via PL synthesis, and to lipid storage as TAG. The present study was performed with physiological glycerol concentrations mimicking the fasting (0.33 mM) and fed (3.33 mM) states, in a working rat heart model. The energy demand was influenced by pacing the heart at either standard [350 beats/min (bpm)] or high rate (450 bpm). Increasing glycerol supply and energy demand resulted in an increased glycerol uptake, a significantly increased glycerol oxidation, and a parallel decrease in β-oxidation. Dietary

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glycerol could be involved in the regulation of the oxidation balances, and in PL/TAG synthesis balance.

MATERIALS AND METHODS

Animals

The study was performed on male Wistar rats (280–330 g). These investigations were carried out in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, Revised 1996). Animal quarters and all animal experimental procedures were in agreement with national regulations (Agreement No. A92-019-01), under license (No. 92-181, delivered April 2003).

Determination of the Glycerol Doses

To assess the low and high doses of glycerol to be supplied to the working heart, two groups of rats (n = 6, 300 g) received an intragastric administration of glycerol (1.5 and 2 g/kg). Blood was collected every 30 min during a 3-h period, and glycerol concentration was determined in plasma. The two concentrations to be used in the preliminary study were determined from the sum of all radioactive fractions when radiolabeled glycerol was added.

Oxidation Evaluation

The cardiac capacity to use glycerol, palmitate, or glucose as metabolic substrates for energy production, according to glycerol availability, was evaluated using [U-14C]glycerol, [9,10-3H]palmitate, and/or [U-14C]glucose (SA = 3,996 Bq/ml) (Perkin Elmer). The direct measurement of mitochondrial oxidation was made by quantitative collection of [14C]O2 and [3H]2O produced from the working heart, perfused with [14C]- and [3H]-substrates, respectively. The heart perfusion medium was collected every 10 min (over the 30-min perfusion), and 1 ml was introduced in a closed chamber containing a Whatman filter saturated with hyamine hydroxide at 1 M (Perkin Elmer) to trap the [14C]O2. The remaining soluble CO2 was released from the liquid by addition of 1 ml of HCl (1 M). Additionally, the gas mixture (95% O2–5% CO2) outflow containing [14C]O2 was allowed to bubble in 40 ml of a 1 M hyamine hydroxide solution (Perkin Elmer). The radioactivity present on the filter and in the hyamine solution was determined in Insta-Fluor scintillation liquid (Perkin Elmer). FA oxidation was evaluated through the measurement of [3H]2O produced from [9,10-3H]palmitate. [3H]2O was separated from [9,10-3H]palmitate by extraction on a charcoal column (200 μl charcoal-1 ml water) and centrifuged for 10 min at 16,000 rpm (4°C) to remove charcoal. The radioactivity present in the supernatant was evaluated on a β-scintillation counter.

Glycerolipids

At the end of the experiment, the heart was washed to remove the remaining radioactivity and homogenized in 30 ml of chloroform-methanol (2:1, vol/vol) according to Folch et al. (4). The radioactivity of the homogenate was evaluated. A 0.73% NaCl solution was added.

Table 1. Influence of substrate and heart rate on functional parameters

<table>
<thead>
<tr>
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<th>Glucose (5.5 mM)</th>
<th>Palmitate (0.4 mM)</th>
<th>Palmitate (0.4 mM)</th>
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<tbody>
<tr>
<td></td>
<td>[14C]Glycerol (mM)</td>
<td>[14C]Glycerol (mM)</td>
<td>[3H]Palmitate (mM)</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>0.33 3.33 0.33 3.33</td>
<td>0.33 3.33 0.33 3.33</td>
<td>0.33 3.33 0.33 3.33</td>
</tr>
<tr>
<td>Mean pressure, mmHg</td>
<td>65.7±0.46 65.8±0.38 65.7±0.54 65.8±0.28</td>
<td>65.5±0.34 66.0±0.45 65.9±0.36 65.8±0.38</td>
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<tr>
<td>Cardiac flow, ml/min</td>
<td>45.5±2.11 44.8±2.65 45.8±3.23 46.5±3.51</td>
<td>45.3±2.47 46.1±2.86 46.4±2.24 45.6±2.87</td>
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Influence of substrate (glucose, glycerol, palmitate) and heart rate (bpm) on functional parameters (n = 6). Heart weight was similar in all groups. Glycerol (mM) was perfusion radiolabeled.
to cause phase separation. The radioactivity was determined in the aqueous and organic fractions. In the organic fraction, the PLs were separated from nonphosphorus lipids (NPLs) on silica cartridges (Supelco, Sigma), and the radioactivity of each class was evaluated.

Statistical Analysis

All data were expressed as means ± SE and submitted to a two-way analysis of variance (ANOVA) (1). When significantly different, the means were compared by the Newman-Keuls test. Differences were considered significant at $P < 0.05$.

RESULTS

Determination of the Glycerol Range

A preliminary study was made to assess the glycerol range to be used in the perfusion medium. The results are shown in Fig. 1. Whatever the concentration of glycerol administrated, all the rats displayed a roughly similar level of plasma glycerol 3 h after administration. The mean value (0.33 mM) was chosen as the low glycerol concentration. At peak (30 min after administration), the glycerol concentration in plasma was close to 7 and 5 mM for the rats receiving 2 and 1.5 g/kg, respectively. The high glycerol concentration (3.33 mM) was chosen as the mean plasma concentration in the two rat groups 60 min after administration.

Heart Function

The ECG, intraventricular pressure, and heart rate were recorded throughout the experiment and are reported in Table 1. The results show that, whatever the conditions, modifying substrates (glucose, glycerol, palmitate) or heart rates (350 or 450 bpm) had no effect on physiological parameters, suggesting that all hearts were in good physiological condition.

Influence of Glycerol Supply on the Synthesis of Glycerolipids and Energy Production

Glycerolipid synthesis. The influence of glycerol supply on cardiac lipid synthesis was evaluated by following the intracellular fate of [U-14C]glycerol at low energy requirement (350 bpm) and higher energy requirement (450 bpm). The results presented in Fig. 2A show that, in 30 min, glycerol uptake increased when extracellular glycerol supply increased from 0.33 to 3.33 mM. At 350 bpm, the glycerol uptake increased from 1.4 to 9.7 mol/heart ($P < 0.001$). At 450 bpm, the glycerol uptake increased from 3.8 to 34.2 mol/heart ($P < 0.001$). Moreover, these results also showed that increasing the contraction rate from 350 to 450 bpm induced a threefold increase in glycerol uptake (whatever the glycerol supply). The fate of intracellular nonoxidized glycerol was evaluated in these conditions in rat hearts by analyzing the radioactivity incorporated from [U-14C]glycerol into PLs and NPLs. NPLs mainly represent the TAG pool. The incorporation of glycerol in NPL increased 10-fold when glycerol supply increased from 0.33 to 3.33 mM ($P < 0.001$, Fig. 2B). At low glycerol supply, the synthesis of NPLs was not significantly influenced by heart rate. Conversely, at high glycerol supply, the TAG synthesis was sixfold higher at 450 bpm compared with 350 bpm ($P < 0.001$, Fig. 2B). The influence of supply and demand on PL synthesis was also significant (Fig. 2C). At 350 bpm, PL synthesis was increased 8-fold when glycerol supply increased from 0.33 to 3.33 mM, whereas at 450 bpm, it increased 30-fold. As shown in Fig. 2C, the PL synthesis did not increase with heart rate at low glycerol concentration but was significantly raised by increasing the rate at high glycerol supply.

Energy production. The metabolic implication of glycerol in cardiac energy production followed by the mitochondrial pro-
GLYCEROL AND CARDIAC METABOLISM

A 0.33 mM GLYCEROL

B 3.33 mM GLYCEROL

Fig. 3. Influence of glycerol supply (0.33 vs. 3.33 mM) on glycerol (μmol·min⁻¹·heart⁻¹) in isolated, perfused working rat heart at 350 and 450 bpm. A: 0.33 mM glycerol supply. B: 3.33 mM glycerol supply. Values are means ± SE (n = 6). Heart weight was similar in all groups. *P < 0.05. ***P < 0.001.

Influence of Other Substrates on Glycerol Metabolism

Glycerolipid synthesis. The involvement of glycerol in cardiac lipid synthesis was evaluated at 30 min in hearts paced at 350 bpm and perfused with either glucose (5.5 mM) or palmitate (0.4 mM), using [U-14C]glycerol as tracer. As shown in Fig. 4A, glycerol uptake at 30 min was significantly higher in palmitate-perfused hearts and glucose-perfused hearts, despite a significant increase in glycerol uptake (see Fig. 4A). Conversely, at high glycerol supply, the glycerol oxidation was significantly raised in palmitate-perfused hearts compared with glucose-perfused hearts (P < 0.05), which is consistent with the higher uptake.

Influence of Glycerol on FA Metabolism

Glycerolipid synthesis. The influence of glycerol supply was evaluated at 30 min on hearts perfused with a palmitate medium (0.4 mM). Palmitate metabolism was followed by the intracellular palmitate fate of [9,10-3H]palmitate. As shown in Fig. 6A, the palmitate uptake was significantly higher at 3.33 mM glycerol than at 0.33 mM glycerol supply (8.3 and 5.0 μmol/heart, respectively; P < 0.001). The metabolism of complex lipids was similarly affected (Fig. 6B). The turnover of NPLs increased with glycerol supply from 0.3 to 2.5 μmol/heart (P < 0.001), and the synthesis of PLs (Fig. 6B) increased from 1.1 to 3 μmol/heart (P < 0.001).

Energy production. The influence of glycerol concentration (0.33 and 3.33 mM) on palmitate oxidation was evaluated in the same conditions (after 30 min of perfusion). As shown in Fig. 6C, the increase in glycerol supply significantly decreased palmitate oxidation from 3.4 to 2.3 μmol/heart at 30 min (P < 0.01).

DISCUSSION

Glucose and FAs are the two main energy sources in the myocardium. In physiological conditions, the adult heart derives most of its energy from mitochondrial FA β-oxidation but switches between carbohydrate and fat fuel sources to balance ATP production and substrate supplies (7, 10, 19). One of the objectives in cardiovascular disease prevention is to lower the utilization of FAs greedy in oxygen consumption. Glycerol is a backbone for glycerolipid synthesis. In a previous paper, we reported that glycerol is a metabolic substrate for cultured cardiomyocytes (5) and contributes to the regulation of metabolic pathways and energy balance. This study addressed the question of the influence of energy demand on glycerol metabolism in a model of isolated working rat heart. As shown, both glycerol supply and energy demand levels increased cardiac glycerol uptake. Although significant at low glycerol supply, the effect of heart rate was more pronounced at high...
glycerol concentration. The metabolic utilization of glycerol was modified by energy demand. The involvement of glycerol in PL turnover paralleled glycerol supply and uptake. The synthesis of PLs increased with glycerol supply (8-fold from 0.33 to 3.33 mM glycerol at low heart rate, 30-fold at higher heart rate). Increasing the contraction rate increased the synthesis of PLs only when glycerol supply was high. The present results confirm previous data showing that, in isolated cells, glycerol uptake and PL turnover paralleled glycerol supply (5). In addition, the working heart model used in the present study showed that increasing energy demand resulted in an increase in PL turnover. At higher heart rate, membrane homeostasis may require an increased PL synthesis, which may not be fulfilled when glycerol availability is low. The maintenance of membrane integrity is highly compromised in cells repeatedly contracting as cardiac myocytes (18), which may contribute to PL homeostasis requirement. Increased cardiac membrane homeostasis may be primarily associated with periods of high plasma glycerol level and may then not be favored in fasting periods. Moreover, the reconstitution of TAG stores mainly occurs when plasma glycerol is high and when the PL synthesis is overflowed. This study also showed that the incorporation of glycerol in TAGs was also increased at high glycerol supply. Obviously, this could be considered as detrimental to the heart, since accumulation of TAG could be occurring. However, this result was obtained in isolated, perfused rat heart in 30 min, in conditions that allow the assessment of TAG turnover without allowing any increase in TAG total content. Such an accumulation can only be determined in vivo, a condition that is, however, the succession of high and low glycerol supply to the heart.

The cardiac myocytes release lipases in the intercellular space, such as phospholipases and TAG lipases (8), that contribute to the glycerol supply to the heart. Tildon and Roeder (26) reported the capacity of a heart homogenate to oxidize glycerol. The present data confirm that glycerol is readily oxidized to CO₂ in the heart and contributes to energy production, under the control of both glycerol availability and energy demand. This result is consistent with the suggestion by Good-
win et al. (6) that an acute catecholamine-induced increase in heart work augmented carbohydrate oxidation because of the activation of the pyruvate dehydrogenase (PDH) complex. Lloyd et al. (13) outlined the beneficial effects of increasing cardiac carbohydrate metabolism.

In this study, increasing glycerol supply from 0.33 to 3.33 mM resulted in a 10-fold increase in glycerol oxidation. This oxidation was also raised by the increase in heart rate. Since all the hearts were perfused with glucose, these results suggest that increasing cardiac work affects the recruitment of other substrates for energy production. In cardiomyocytes, the glycerol contribution increased the pyruvate availability without lowering glucose oxidation, suggesting an additional recruitment of activated PDH (5). To investigate the competition between substrates, hearts were perfused with either glucose or palmitate. Whatever the second substrate, increasing glycerol supply resulted in a raise in glycerol oxidation, although less pronounced with glucose than with palmitate (3- vs. 8-fold, respectively), probably because glycerol uptake was higher with palmitate-enriched medium than with glucose-enriched medium. At low glycerol supply, glycerol oxidation was lower in palmitate-perfused hearts, whereas PL synthesis was very high (200-fold higher than in glucose perfusion), and the complex lipid turnover ratio was in favor of the PL synthesis (PL/TAG synthesized/min = 4). The weak fulfillment of membrane homeostasis when glycerol supply was low could explain the reduced contribution of glycerol to energy production in the presence of palmitate. Conversely, at high glycerol supply, both glycerol oxidation and glycerolipid synthesis were higher in palmitate perfusion than in glucose perfusion. These conditions favor TAG synthesis more than PL synthesis (PL/TAG synthesized/min = 0.6), suggesting that lipid storage occurs in the heart only when the availability of both FAs and glycerol overflows the membrane homeostasis requirements. The competition of glycerol and FAs as substrates for energy in the working heart is of high interest. Increasing glycerol supply increased palmitate uptake but reduced its β-oxidation and favored its incorporation in complex lipids. The oxidation of glycerol increases the mitochondrial acetyl-CoA content, the excess of which is released in the cytoplasm and converted to malonyl-CoA by acetyl-CoA carboxylase. In turn, this malonyl-CoA inhibits the CPT-1 activity, which lowers β-oxidation. This mechanism was demonstrated in isolated cardiomyocytes (5), and we showed in this study that, in the working heart also, increasing glycerol oxidation lowers β-oxidation. Trimetazidine, an anti-anginal drug, has been shown to directly inhibit FA oxidation in the heart and thus increase glucose oxidation. The FA oxidation inhibition is secondary to the inhibition of long-chain 3-ketoacyl-CoA thiolase (11, 15). This enzyme is inhibited by acetyl-CoA generated after oxidation reactions (28). The inhibition of FA oxidation by glycerol, observed in this study, may also result from the inhibition of long-chain 3-ketoacyl-CoA thiolase by the acetyl-CoA derived from glycerol oxidation, which increases intracellular acetyl-CoA, since glucose oxidation is not affected (5). The cardiac function in ischemic conditions and the functional recovery after reperfusion are known to be improved by decreasing the contribution of FAs to cardiac energy (2, 7, 11, 14).

On the basis of numerous papers referring to the functional benefit of lowering β-oxidation (7) and previous results showing that glycerol oxidation has no effect on glucose oxidation, the effect of glycerol on the glucose/FA oxidation balance appears to be of high interest. The excess in β-oxidation observed in pathological conditions such as ischemia and
diabetes is associated with the long-chain acyl-CoA stimulation of CPT-1 and the acceleration of the production of NADH and acetyl-CoA with consequent inactivation of PDH (2, 7, 14). Conversely, the reduction of β-oxidation by glycerol may favor PDH activation (5), which may, in turn, improve the coupling between glycolysis and glucose oxidation and decrease cellular protons and acidosis and hence calcium overload (12, 17). However, the adverse consequence of CPT-1 inhibition is the accumulation of nonoxidized FAs that lead to cardiomyopathy and cardiac hypertrophy (7, 20, 25). This study shows that increasing intracellular glycerol is associated with the augmentation in PL turnover and long-chain FA incorporation into membranes. The present results suggest that decreasing β-oxidation by increasing glycerol oxidation may prevent the detrimental effects of CPT-1 inhibitors such as etomoxir, since the mechanism of glycerol allows the handling of nonoxidized FAs.

In conclusion, the control of energy production through the decrease of β-oxidation and probably the FA/glucose balance appears as a clear strategy in cardiac cytoprotection and glycerol as a key regulator in balancing lipid metabolic pathways. The results presented here show that 1) glycerol is a significant substrate in cardiac energy production; 2) glycerol cardiac uptake is controlled by energy demand when the circulating glycerol supply is high; 3) at high circulating glycerol concentration, the intracellular available glycerol is preferentially oriented toward the synthesis of PLs; 4) increasing the glycerol supply results in the preferential incorporation of long-chain FAs into the TAG pool; and 5) increased glycerol oxidation decreases β-oxidation, as reported previously in cardiomyocytes, without concurrent glucose oxidation. These results demonstrate that glycerol contributes to the regulation of cardiac metabolism and energy balance.

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