Measuring Glycerol Turnover, Gluconeogenesis From Glycerol, and Total Gluconeogenesis With [2-^{13}C]glycerol: Role of the Infusion-Sampling Mode

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Mass isotopomer distribution analysis (MIDA) of glucose during infusion of [2-^{13}C]glycerol is a new method for measuring total gluconeogenesis (GNG). Since this method relies on calculation of the isotopic enrichment (IE) of hepatic triose phosphates (TP), the results should be independent of the sites of tracer infusion and blood sampling. Postabsorptive and starved rats were infused with [2-^{13}C]glycerol and sampled either in the arterial-venous (A-V) or venous-arterial (V-A) modes. Blood was also sampled from the portal vein. In both postabsorptive and starved rats, glycerol turnover rate (Rt) and the percent contribution of glycerol to total glucose production were higher in the A-V mode than in the V-A mode ($P < .05$). Glycerol IE in portal venous blood was intermediate between IE values observed in peripheral arterial and venous blood. Its use for calculating the contribution of glycerol to glucose production reconciled the results obtained with the two infusion-sampling modes in both postabsorptive and starved rats; this contribution was increased by starvation ($P < .01$). In postabsorptive rats, total GNG calculated from MIDA of glucose accounted for approximately 50% of glucose production whatever the infusion-sampling mode (A-V, 48.8% ± 4.7%; V-A, 52.2% ± 3.9%). This contribution increased to 90% in starved rats, again, with no difference between A-V (95.2% ± 1.8%) and V-A (89.2% ± 1.3%) modes. In conclusion, during infusion of [2-^{13}C]glycerol, total GNG measured from MIDA of glucose is independent of the infusion-sampling mode, contrary to calculations of Rt and GNG from glycerol. Measurement of glycerol IE in portal venous blood reconciles the results obtained with the two modes with respect to the contribution of glycerol to GNG.

ABNORMALITIES of gluconeogenesis (GNG) are considered to play an important role in the elevated hepatic glucose production (HGP) observed in diabetes[1] and other pathological conditions such as stress[2] or hyperthyroidism.[3] However, precise determination of the in vivo contribution of GNG to total HGP with isotopes is difficult. Approaches using the incorporation into glucose of label from lactate, pyruvate, or alanine are complicated by isotopic exchanges at the oxaloacetate crossroad, resulting in dilution of the label and underestimation of GNG rates.[4-5] Various solutions to this problem have been proposed. Methods using labeled acetate infusion to calculate a correction factor[6-8] are of limited value in vivo, mainly because extrahepatic metabolism of acetate produces labeled metabolites interfering with the direct labeling by acetate of liver Krebs cycle intermediates and glucose.[9-10] More recently, the combination of [3-^{13}C] or [3-^{14}C]lactate infusion with the noninvasive sampling of liver glutamine by phenylacetate and a mathematical model of Krebs cycle and GNG has been used to calculate relative flux rates through the main enzymes of Krebs cycle and of GNG and a dilution factor between hepatic lactate and phosphoenolpyruvate isotopic enrichments (IEs).[11] This approach appears valid for in vivo studies[9] and provides important insight into hepatic metabolism in vivo. However, this approach still has some limitations: (1) GNG from glycerol is not taken into account, and this metabolic pathway could play a significant role in some pathological situations.[12] (2) During in vivo studies in humans, lactate IE is measured in peripheral blood, and a possible dilution between peripheral blood and liver is not corrected. Actually, we have shown in rats in vivo that such a dilution of IE is present.[13] (3) Lastly, lactate IE in peripheral blood, and thus calculated lactate turnover rates and gluconeogenic rates, are dependent on the infusion-sampling mode.[13-15] These last two limitations could be less important in humans than in small animals such as rats.[13] However, a fully satisfactory technique for measuring GNG in vivo should include GNG from glycerol and be independent of both the infusion-sampling mode and any dilution of IE between peripheral blood and liver.

Mass isotopomer distribution analysis (MIDA) is a new method[16-18] for measuring with stable isotope–labeled tracers and gas chromatography–mass spectrometry techniques the synthesis of a molecule synthesized by the addition of several identical precursors. Glucose is formed by the addition of two triose phosphates (TP). Indeed, we[19] and Neese et al[20] provided evidence that infusion of [2-^{13}C]glycerol and MIDA of glucose is a valid method for measuring GNG. This method allows calculation of the IE of hepatic TP. The contribution of GNG to HGP is determined by the comparison of the IEs of plasma glucose and hepatic TP. Thus, in addition to including GNG from glycerol, this method is theoretically independent of the infusion-sampling mode and of any dilution between peripheral blood and liver. To test these assumptions, we infused rats with [2-^{13}C]glycerol in the arterial-venous (A-V) and venous-arterial (V-A) modes to measure glycerol turnover rate ([Rt] from circulating glycerol IE), GNG from glycerol (by comparing glycerol and glucose IE), and total GNG (by MIDA of glucose).

MATERIALS AND METHODS

Materials

Chemicals were acquired from Sigma Chemicals (St Louis, MO) or Merck (Darmstadt, Germany), and enzymes were from Boehringer (Mannheim, Germany). [2-^{13}C]glycerol (99% atoms ^{13}C)

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was obtained from Isotec (St Quentin, France). [6,6-$^2H_2$]glucose (99% atoms ^2H) and [1-^{13}C], [2-^{13}C], [4-^{13}C], and [4-5-$^{13}C_2$]glucose (99% atoms ^{13}C) were from Eurisotop (Gif-sur-Yvette, France), and [6-^{13}C]glucose (99% atoms ^{13}C) was from Sigma Chemical. [3-^3H]glucose was from Isotopochim (Ganogobie-Peyrins, France).

In Vivo Studies

Sprague-Dawley rats (IFFA-Credo, L'Arbresle, France) were studied in the postabsorptive state (6 hours after food withdrawal, ie, food was withdrawn at 8 AM and tracer infusion started at 2 PM; n = 14, 250 to 280 g body weight) or after 48 hours of starvation (n = 14, 210 to 240 g body weight). After anesthesia with pentobarbital sodium (6 mg/100 g body weight), catheters were inserted in the right atrium through the right jugular vein and in the aorta through the left carotid artery. [2-^{13}C]glycerol and [3-^3H]glucose (0.12 μCi/min for starved rats and 0.24 μCi/min for postabsorptive rats) were infused either through the jugular vein (and blood sampled through the arterial catheter, mode V-A, n = 6 for postabsorptive and starved rats) or through the arterial catheter (and blood sampled through the venous catheter, mode A-V, n = 8 for postabsorptive and starved rats). The tracer was infused for 3 hours after a priming dose (infusion rate × 10 for 1 minute). The labeled-glycerol infusion rates were 8 to 9 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ in starved rats and 10 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ in the postabsorptive group. Blood was sampled before tracer infusion and at 150, 165, and 180 minutes. After the last peripheral blood sampling, the abdomen was quickly opened and blood collected from the portal vein.

Analytical Procedures

Glucose and glycerol levels were measured by enzymatic assays.[19] Glucose and glycerol were purified from neutralized perchloric acid extracts of blood by sequential anion-cation–exchange chromatography.[21] One part of the neutralized extracts was evaporated before chromatography to measure glucose radioactivity. The neutral eluate from the ion-exchange chromatography was split into two parts. One was used to measure glycerol IE by gas chromatography–mass spectrometry with the use of the triacetate derivative, as previously described.[19-21]

The other part was used to prepare the aldonitrile pentaacetate[22] and the methyloxime trimethylsilyl[22] derivatives of glucose. These derivatives were used to measure the mass isotopomer distribution of glucose, its total enrichment, symmetry or lack of symmetry of the labeling, and distribution of labeling on the carbon atoms of glucose; these procedures have been described in detail previously.[19] All these analyses were performed on a gas chromatograph (HP 5891; Hewlett Packard, Palo Alto, CA) equipped with a 25-m fused silica capillary column (model OV 1701; Chrompack, Bridgewater, NJ) and interfaced with a mass spectrometer (model HP 5971A; Hewlett Packard) operating in the electronic impact-ionization mode. Standard curves were run with each series of biological samples.

Calculations

Glucose Rt was calculated from glucose specific radioactivity and tracer infusion rate using equations for steady state. Glycerol Rt was calculated from the tracer infusion rate, I, and glycerol IE measured in arterial or peripheral venous blood and expressed as mole percent excess (MPE). We calculated total glycerol Rt as Rt = I/IE (MPE) and endogenous glycerol Rt as end Rt = (I/IE) − I. GNG from glycerol was calculated from the ^{13}C enrichment of glycerol and glucose with the equation, % glucose formed from glycerol = (^{13}C glucose IE/[2 × ^{13}C glycerol IE]) × 100, using ^{13}C glycerol IE measured either in peripheral (arterial or venous) blood or in portal venous blood. ^{13}C glucose IE was calculated from the fractional abundance of the isotopomers of glucose, g_0 (no excess ^{13}C), g_1 (one excess ^{13}C), and g_2 (two excess ^{13}C; $g_0 + g_1 + g_2 = 1$), as ^{13}C glucose IE = $g_1 + (2 \times g_2)$.

Calculation of total GNG from MIDA of glucose has been described in detail previously.[19] In short, from the ratio $r = g_1/g_2$ (with g_1 being the fractional abundance of glucose labeled with one excess ^{13}C and g_2 the fractional abundance of glucose labeled with two excess ^{13}C), one can calculate the enrichment, p, of hepatic TP as $p = 2/(r + 2)$. This holds theoretically only if the labeling of glucose is symmetrical, ie, if the two TP have the same enrichments; however, we[19] and others[20] have shown that significant errors occur only when large disequilibriums are observed, a situation not encountered in this study (see the Results). Comparison of the calculated IE of TP to the IE of glucose yields the contribution (as percentage) of GNG to glucose production. Lastly, the appearance rate (Ra) of hepatic TP was calculated as proposed by Neese et al[20] as the ratio of labeled glycerol infusion rate over TP enrichment.

Values are presented as the mean ± SE. Comparisons were performed using Student's *t* test for paired data (within-group comparison) or nonpaired data (between-group comparison).

RESULTS

Table 1 shows the total ^{13}C IE of glucose in peripheral blood and of glycerol in peripheral and portal venous blood in the four groups of rats studied, as well as the calculated values for glycerol Rt. In both postabsorptive and starved rats, glycerol IE was lower in the A-V than in the V-A mode, and therefore, both total and endogenously produced glycerol Rt were markedly higher in the A-V mode. Glycerol IE in portal venous blood was intermediate, ie, higher than in peripheral venous blood but lower than in

Table 1. Glucose and Glycerol IE, Glycerol Rt, and Contribution to HGP in Postabsorptive and Starved Rats Studied Using the A-V or V-A Infusion-Sampling Modes (mean ± SE)

	Postabsorptive				Starved			
	A-V (n = 8)		V-A (n = 6)		A-V (n = 8)		V-A (n = 6)	
Parameter	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal
Glucose IE	10.2 ± 1.2		10.7 ± 0.9		18.4 ± 0.7		14.8 ± 1.2	
Glycerol IE	33.1 ± 0.9	43.6 ± 2.0*	45.2 ± 1.7†	37.6 ± 1.6*†	27.5 ± 1.5	40.7 ± 1.4*	40.7 ± 1.3†	31.3 ± 3.0*†
Total glycerol Rt	30.5 ± 0.9	—	22.2 ± 0.8†		29.5 ± 1.5	—	19.7 ± 0.7†	—
Endogenous glycerol Rt	20.4 ± 0.9	—	12.2 ± 0.8†	—	21.7 ± 1.6	—	11.8 ± 0.8†	—
% HGP from glycerol	15.7 ± 2.2	12.0 ± 1.7*	11.8 ± 1.0†	14.4 ± 1.8*	34.2 ± 2.4‡	23.5 ± 1.6*‡	18.3 ± 1.8†‡	25.2 ± 4.0*‡

*$P < .05$ *v* peripheral values of the same group.

†$P < .05$ *v* A-V mode in postabsorptive (or starved) rats.

‡$P < .01$ *v* corresponding postabsorptive group.

arterial blood, showing that there was some dilution by glycerol released by splanchnic tissues. Glycerol concentrations could be measured in peripheral blood and portal venous blood for most rats in the postabsorptive groups, and suggest glycerol release also by splanchnic tissues, since portal concentrations, slightly lower than peripheral venous levels (180 ± 10 *v* 216 ± 22 μmol · L^{-1}, $P > .20$), in rats studied in the A-V model were slightly higher than in arterial blood (186 ± 23 *v* 165 ± 10 μmol · L^{-1}, $P > .20$) in rats studied in the V-A mode. This comparison could not be performed in starved rats, since we did not have enough portal venous blood for measuring glycerol level. In addition, we measured peripheral blood glycerol concentration before (time zero) and at the end of tracer infusions (180 minutes). In postabsorptive rats, this concentration increased in both the A-V (122 ± 15 to 216 ± 22 μmol · L^{-1}) and V-A (75 ± 5 to 165 ± 10 μmol · L^{-1}) modes ($P < .05$). There was no significant increase in starved rats. The percent contribution of glycerol to total HGP calculated using glycerol IE in peripheral blood was also dependent on the infusion-sampling mode: this contribution was higher in the A-V than in the V-A mode. Use of portal venous glycerol IE yielded intermediate values that were no longer different between the two modes. Lastly, whatever the glycerol IE value used, the contribution of glycerol to glucose was increased by starvation.

Table 2 shows the mass isotopomer distribution of glucose and the corresponding r, p, and calculated total contribution of GNG to HGP. As in a previous report,[19] greater than 95% of excess ^{13}C in glucose was found as expected on carbons 2 and 5 (data not shown). There was some isotopic disequilibrium, with a labeling ratio between the upper and lower parts of glucose of 1.09 to 1.43. Taken in account or not, this disequilibrium did not modify the results, in agreement with previous studies,[19,20] and the results shown are those calculated assuming equilibrium. In postabsorptive rats, GNG accounted for approximately 50% of HGP, and results obtained with the A-V or V-A mode were identical. This contribution increased, as expected, to approximately 90% in starved rats, again with no differences between the two modes. The contribution of gluconeogenic substrates other than glycerol was calculated by subtracting from total GNG the contribution of glycerol calculated using portal venous glycerol IE. In postabsorptive and starved rats, glycerol accounted for about 25% of GNG and the other substrates for the remaining 75%.

Table 2. MIDA of Glucose and Calculation of the Contribution of GNG to HGP in Postabsorptive and Starved Rats Studied in the A-V or V-A Infusion-Sampling Mode (mean ± SE)

	Postabsorptive		Starved	
Parameter	A-V (n = 8)	V-A (n = 6)	A-V (n = 8)	V-A (n = 6)
g_1	9.12 ± 1.03	9.61 ± 1.21	16.63 ± 0.54	13.51 ± 0.95
g_2	0.54 ± 0.08	0.54 ± 0.08	0.87 ± 0.10	0.62 ± 0.09
r	16.95 ± 0.92	17.80 ± 1.70	19.10 ± 1.27	21.90 ± 1.50
p	10.55 ± 0.49	10.10 ± 0.75	9.47 ± 0.57	8.31 ± 0.51
% HGP from GNG	48.8 ± 4.7	52.2 ± 3.9	95.2 ± 1.8*	89.8 ± 1.3*

Abbreviations: r, ratio of g_1 to g_2; p, calculated IE of hepatic TP.
*$P < .01$ *v* corresponding postabsorptive group.

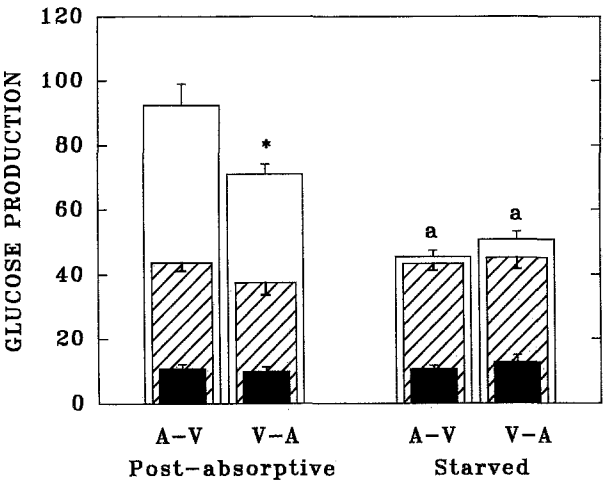

Fig 1. Glucose production and GNG in postabsorptive and starved rats studied in the A-V and V-A mode. (□) Total glucose production; (▨) total GNG; (■) GNG from glycerol (calculated using portal venous glycerol IE). [a]$P < .01$ *v* postabsorptive groups; *$P < .05$ *v* corresponding group in the A-V mode. Values are μmol · kg^{-1} · min^{-1}.

Since glucose Rts were measured, all these fluxes calculated as percentages could be converted to absolute values, and Fig 1 shows these results. In starved rats, glucose Ra was approximately 50 μmol · kg^{-1} · min^{-1} and all absolute fluxes (total GNG, GNG from glycerol, and GNG from other substrates) were comparable in the A-V and V-A modes. The flux of glycerol to glucose was also identical (21.2 ± 1.8 μmol · kg^{-1} · min^{-1} in the A-V mode *v* 25.6 ± 4.0 in the V-A mode). In postabsorptive rats, glucose Ra was slightly lower in the V-A mode (71 ± 3 *v* 93 ± 7 μmol · kg^{-1} · min^{-1}, $P < .05$). Liver glycogen (18.8 ± 3.1 *v* 24.1 ± 1.7 mg/g · liver) and glucose (8.6 ± 0.4 *v* 10.4 ± 0.8 mmol/L) were also lower in rats studied in the V-A mode. Therefore, although food was removed at the same time in all rats, it seems that rats studied in the V-A mode were in a slightly more advanced transition from the fed to the fasted state. Despite this slight difference in glucose Ra, there were no differences in any gluconeogenic fluxes between the two modes (Fig 1). The flux of glycerol to glucose was also comparable (21.9 ± 2.4 in the A-V mode *v* 19.8 ± 2.6 μmol · kg^{-1} · min^{-1} in the V-A mode).

The contribution of glycerol to hepatic TP was estimated by comparing the calculated IE of TP with portal venous glycerol IE. This percent contribution was nearly identical to the percent contribution of glycerol to GNG in all groups of rats (Table 3). When using the IE of glycerol in peripheral blood, for this calculation, there was a mild discrepancy in the V-A mode and a large one in the A-V mode. Lastly, Ra of TP was calculated according to Neese et al.[20] The comparison of Ra of TP with the flux of TP going to glucose (calculated as twice the absolute GNG rate) shows that in starved rats, 95% to 100% of TP Ra was directed to GNG; in postabsorptive rats, this percentage was 95% in the A-V mode and 77% in the V-A mode.

Table 3. Contribution of Glycerol (%) to GNG and to TP Synthesis (calculated from either portal or peripheral glycerol IE) and Estimated Ra of Hepatic TP (mean ± SE)

Mode	GNG From Glycerol (%)	TP Synthesized From Glycerol (%)		Ra TP ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)
		With Portal IE	With Peripheral IE	
Postabsorptive				
A-V (n = 8)	24.8 ± 2.7	24.3 ± 2.5	32.1 ± 2.4*	94 ± 4
V-A (n = 6)	27.8 ± 2.5	27.0 ± 2.9	22.9 ± 2.8*	99 ± 3
Starved				
A-V (n = 8)	24.7 ± 1.9	24.6 ± 1.7	35.5 ± 3.2*	85 ± 5
V-A (n = 6)	28.4 ± 4.8	28.3 ± 4.0	21.0 ± 1.6*	94 ± 5

*$P < .05$ *v* corresponding value in the first 2 columns.

DISCUSSION

Our results confirm that during isotopic studies of in vivo glycerol metabolism, glycerol IE and thus calculated Rt are dependent on the infusion-sampling mode used, with higher IE and lower Rt with the V-A mode.[15] This problem is observed with other compounds[13-15] characterized by high fractional turnover rates. The difference in IE between the two modes is attributed to the fact that infusion of the tracer results in an abrupt increase of tracer concentration downstream of the infusion site, and that when the sampling site is localized between the infusion site and the tissues metabolizing the compound studied, the newly infused tracer does not mix with the interstitial and tissue pool and is not diluted by unlabeled molecules released by tissues.[13-14] The A-V mode is considered to yield the more correct Rt value, and when this mode cannot be used, as in human subjects, a correction procedure for the data obtained in the commonly used V-A mode can be used.[23] As expected, these differences in glycerol IE also bear on the calculation of GNG from glycerol, with a higher calculated contribution in the A-V mode in both postabsorptive and starved rats. Moreover, neither arterial nor venous glycerol IE yielded a correct estimation of portal venous IE that was intermediate between the other two values. The decrease between arterial and portal venous IE shows that substantial lipolysis occurs within splanchnic tissues. Use of portal venous glycerol IE reconciles the results obtained with the two infusion-sampling modes in both postabsorptive and starved rats, showing that it is the most appropriate way of calculating GNG from glycerol. As expected, the percent contribution of glycerol to total HGP was increased by starvation.

In contrast, calculation of the total contribution of GNG to HGP using MIDA of glucose should be independent of the infusion-sampling mode, since it relies on the determination of intrahepatic TP enrichment. Indeed, in both starved and postabsorptive rats, values obtained with the A-V and V-A mode were identical. Starvation increased the contribution of GNG to HGP from 50% to approximately 90%. As in previous reports,[19,20] this increase in the percentage of HGP from GNG was due to a decrease with starvation of the absolute flux of glucose production from nongluconeogenic pathways, while the absolute gluconeogenic flux was unchanged. In addition, the relative contribution of glycerol and of other gluconeogenic substrates to GNG was not modified by starvation. Lastly, the good agreement between the percent contribution of glycerol to GNG and the percent of TP synthesized from glycerol calculated from the MIDA of glucose (Table 3) supports the validity of this method.

Neese et al[20] proposed the use of MIDA of glucose to calculate, in addition to GNG, the Ra of hepatic TP as the ratio of labeled-glycerol infusion rate to hepatic TP enrichment. This calculation gives further insight into intrahepatic metabolism. However, its validity depends on the assumption that all the infused ^{13}C-labeled glycerol enters the hepatic TP pool sampled, ie, that all glycerol is taken up by the liver. Neese et al[20] tested this assumption by calculating the recovery of ^{13}C in the end products of hepatic TP metabolism, and found in starved rats a 92% to 96% recovery. In the present report, the ^{13}C recovery in glucose (calculated from labeled-glycerol infusion rate and ^{13}C Ra in glucose, ie, Ra glucose × ^{13}C glucose IE), 95% to 100% in starved rats and 76% to 95% in postabsorptive rats, was also compatible with this assumption. On the other hand, evidence has been presented that significant quantities of glycerol are taken up and metabolized by other tissues.[24] Labeled carbon from glycerol could thus be incorporated during extrahepatic metabolism into products such as lactate or glutamate, sent to the liver, and finally incorporated in end products of TP metabolism without crossing the glycerol-3-phosphate pool. However, this indirect pathway of incorporation into hepatic TP and next into glucose results in incorporation of label into positions other than carbons 2 and 5 of glucose. Since we found greater than 95% of the ^{13}C on carbons 2 and 5, this pathway seems quantitatively minor in the conditions of our study.

The present results, as well as our previous report[19] and that of Neese et al,[20] support the usefulness of ^{13}C-labeled glycerol infusion and MIDA of glucose to calculate GNG, since GNG was found to account for almost the totality of glucose production in starved rats. Results in postabsorptive rats also support the validity of this method, although its accuracy cannot be fully demonstrated in these conditions, in the absence of another independent direct measure of either glycogenolysis or GNG. However, Landau et al[25] and Previs et al[26] recently reported discordant results, respectively, in humans and in rats. Landau et al found that in subjects fasted 60 hours, GNG calculated from [U-^{13}C]glycerol infusion and MIDA of glucose accounted only for 50% to 60% of glucose production. Previs et al found in rats fasted for 2 days and also infused with [U-^{13}C]glycerol that the contribution of GNG was only 75%. These investigators ascribed these underestimates of GNG to a heterogeneity of hepatic TP labeling, itself related to a periportal to pericentral decrease of glycerol concentration and enrichment. We have no definite explanations for this discrepancy between our results and those of Neese et al on one hand and Landau et al and Previs et al on the other. It may be due to differences in the choice of tracer ([2-^{13}C] versus [U-^{13}C]glycerol) or in the methods

used for MIDA of glucose. It could also be related to differences in the level of glycerol IE attained. Landau et al enriched the glycerol pool only 8% to 12%, whereas we and Neese et al[20] enriched it approximately 30% to 40%. This large enrichment, besides allowing higher glucose-labeling, resulted in an increase in glycerol concentration[19] and thus in hepatic glycerol load. We found previously[19] that in these conditions, hepatic glycerol-3-phosphate concentrations increase. This suggests that the high labeled-glycerol infusion rate used in these rats allowed homogenous labeling of the hepatic TP pool and thus a correct estimation of GNG.

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