Molecular and Cellular Biochemistry **178:** 213–217, 1998.

Steady state changes in mitochondrial electrical potential and proton gradient in perfused liver from rats fed a high fat diet

Maria P. Mollica,[1] Susanna Iossa,[1] Giovanna Liverini[1] and Sibylle Soboll[2]

[1]*Department of General and Environmental Physiology, University of Naples 'FEDERICO II, Italy;* [2]*Institute of Physiological Chemistry I, University of Düsseldorf, Germany*

Received 27 February 1997; accepted 15 July 1997

Abstract

In this work the protonmotive force (Δp), as well as the subcellular distribution of malate, ATP, and ADP were determined in perfused liver from rats fed a low fat or high fat diet, using density gradient fractionation in non acqueous solvents.

Rats fed a high fat diet, despite an enhanced hepatic oxygen consumption, exhibit similar Δp to that found in rats fed a low fat diet, but when we consider the two components of Δp, we find a significant decrease in mitochondrial/cytosolic pH difference (ΔpH_m) and a significant increase in mitochondrial membrane potential ($\Delta\psi_m$) in rats fed a high fat diet compared to rats fed a low fat diet, which tend to compensate each other. In rats fed a high fat diet the concentration ratio of malate and ATP/ADP does not reflect the changes in ΔpH_m and $\Delta\psi_m$, which represent the respective driving force for their transport.

The findings are in line with an increase in substrate supply to the respiratory chain which is, however, accompanied by a higher energy turnover in livers from HFD rats. By this way the liver could contribute to the lack of weight gain from the high caloric intake in HFD rats. (Mol Cell Biochem **178:** 213–217, 1998)

Key words: mitochondria, respiration, metabolism, adenosine triphosphate, calories, diet

Abbreviations: ME intake – metabolizable energy intake; Δp – protonmotive force; Δψ – membrane potential; ΔpH – mitochondrial proton gradient; c – cytosolic; m – mitochondrial; pm – plasma membrane; DMO – 5,5-dimethyloxazolidinedione; TPMP – triphenylmethyl-phosphonium bromide

Introduction

We have previously shown that rats fed a high fat diet become hyperphagic, but fail to exhibit excess weight gain through a facultative increase in energy expenditure [1, 2]. A contribution to the increase in energy expenditure may be given by the liver. In fact hepatocytes from rats fed a high fat diet showed increased respiration rates following addition of NAD-linked and FAD-linked substrates [3]. In addition, we have shown that a large part of this increase is due to an increased mitochondrial oxygen consumption [4]. We have also suggested that the increased mitochondrial oxygen consumption can be due to an increase in both substrate supply to the electron transport chain and ATP turnover. Since the above parameters influence protonmotive force (Δp) [5], which in turn is an important controlling factor of the mitochondrial respiration [6], it appeared of interest to verify whether the increase in oxygen consumption found in rats fed a high fat diet is accompanied by variations in Δp. To this purpose, in rats fed a low fat or high fat diet, we have determined Δp, by measuring both the electrical ($\Delta\Psi_m$) and the chemical (ΔpH_m) component. It is well known that $\Delta\Psi_m$ is the driving force for electrogenic transport systems, whereas ΔpH_m influences the transport of metabolites where

Address for offprints: G. Liverini, Dipartimento di Fisiologia Generale ed Ambientale, Via Mezocannone 8, I-80134 Napoli, Italy

electroneutrality is maintained via proton symport or hydroxyl ion antiport [7]. Therefore, changes in ΔpH_m and $\Delta\Psi_m$ could also influence substrate supply to the cell and to the mitochondrial compartment. In the light of the above observations, we have also measured the mitochondrial/cytosolic distribution of malate, ATP and ADP, since the distribution of malate is influenced by ΔpH_m, whereas the distribution of ATP and ADP is influenced by $\Delta\Psi_m$.

Materials and methods

Sixteen male Wistar rats of about 30 days of age (bred in the animal house of the Department of Physiological Chemistry I, University of Dusseldorf) were divided into two groups of eight rats with the same mean body weight (about 80 g). A control group (CD rats) was fed *ad libitum* a standard stock diet (percentage of total metabolizable energy: 29 protein, 10.6 lipid, and 60.4 carbohydrate, J/J; 15.88 kJ gross energy/g). The second group (HFD rats) had free access to a high fat diet (28% control diet, 39.5% lyophilized meat, 17.8% butter, 12% alphacel, 0.7% AIN vitamin mix, 2% AIN mineral mix, g/g; percentage of total metabolizable energy: 29 protein, 50 lipid, 21 carbohydrate, J/J; 19.85 kJ gross energy/g) [8, 9]. This diet is characterized by a high fat content and by the presence of a meat component which is among the flavours most preferred by rats [10, 11]. The experiment lasted 15 days. All rats had free access to water and were housed individually in grid-bottomed cages at 24°C under an artificial circadian 12 h light/dark cycle.

At the end of the experimental period, four CD and HFD rats were starved for 16 h and thereafter they were used for energy balance measurements. Other four CD and HFD rats were starved for 16 h and thereafter they were used for oxygen consumption measurement in perfused liver and determination of ΔpH_m, $\Delta\Psi_m$ and metabolite content. We used 16 h-fasted rats to avoid variations in liver substrate supply of metabolic fuels coming from food, caused by differences in both diet composition and energy intake. The energy balance, however, was the same as in fed CD and HFD rats.

Measurement of oxygen consumption in perfused liver

Rats were anaesthetized with pentobarbital (12 mg/100 g b.w.) and livers were perfused with a medium containing 120 mM NaCl, 5 mM KCl, 50 mM Hepes, pH 7.4, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$ in a non-recirculating system [12]. The fluid was pumped through a temperature regulated (37°C) membrane oxygenator supplemented with 100% O_2 to obtain about 90% O_2 saturation prior to entering the liver via a cannula inserted in the portal vein. The arterial oxygen concentration, maintained constant by the oxygenator, was measured before and after each experiment. The effluent perfusate was collected via a cannula placed in the vena cava and it passed by a platinum electrode for continuous monitoring of the venous oxygen concentration before being discarded. The flow rate was adjusted to the metabolic activity of the liver as judged from the venous oxygen concentration. It varied from experiment to experiment between 5–6 $ml \times min^{-1} \times g^{-1}$, but it was constant in each individual experiment. Oxygen consumption was calculated from arterio-venous oxygen concentration differences and the constant flow rate, and was referred to the wet weight of the liver.

Determination of subcellular ΔpH gradients, membrane potentials and metabolite contents

After oxygen consumption measurement, rat livers were perfused in a closed system with 200 ml of perfusate medium supplemented with 15 µCi of [2–^{14}C]5,5-dimethyl-2,4-oxazolidinedione ([^{14}C]DMO) and 15 µCi of [^{3}H] triphenylmethyl-phosphonium bromide ([^{3}H]TPMP) for 30 min, and perfusate samples were taken for determination of specific radioactivities of DMO and TPMP in the extracellular space. Then, the perfusion was stopped by freeze-clamping of the liver, the frozen tissue was ground in liquid nitrogen, freeze-dried and sonicated in a mixture of heptane/CCl_4 at –10°C and then fractionated by density-gradient centrifugation in a gradient obtained from heptane/CCl_4 mixture (1.28–1.33Kg/l) [13]. The gradient yielded eight fractions, each containing different proportions of mitochondrial and cytosolic protein. Specific activities of marker enzymes for mitochondria (citrate synthase) and cytosol (phosphoglycerate kinase), as well as metabolite contents were measured enzymically [14] in each fraction, and specific radioactivities were determined by liquid-scintillation counting. Protein contents were measured as described by Lowry *et al*. [15]. Metabolite contents and specific radioactivities of mitochondrial (m) and cytosolic (c) compartments were extrapolated from the activities of marker enzymes and the contents of metabolites in each fraction of the density gradient [13]. Corrections were made for extracellular specific radioactivities of [^{14}C]DMO and [^{3}H]TPMP in each fraction [16]. Concentrations were calculated on the basis of mitochondrial and cytosolic water contents of 0.8 and 3.8 µl/mg of compartmental protein, respectively [17].

Subcellular ΔpH and ΔΨ were calculated from the specific activities of the labelled indicators in the respective compartments according to equations (1) and (2):

$$\Delta pH = log\ ([^{14}C]DMO)_{in}/([^{14}C]DMO)_{out} \quad (1)$$

$$\Delta\Psi = 2.303\frac{RT}{nF} \times log([^3H]TPMP)_{in}/([^3H]TPMP)_{out} \quad (2)$$

where n is number of electrons and F is Faraday constant.

Δp was calculated as $\Delta\Psi_m + 61\ \Delta pH_m$.

Energy balance measurements

Body weight and food intake were measured daily to allow calculations of body weight gain and gross energy intake. The faeces were also collected daily for energy content measurements. The collected faeces were dried and ground to a powder before determining their energy content with a bomb calorimeter (Parr adiabatic calorimeter calibrated with dry benzoic acid standard). The gross energy content of low-fat and high-fat diets was also determined by the bomb calorimeter. Digestible energy intake (taking into account the food spillage) was obtained by subtracting the energy measured in the faeces from the gross energy intake as measured from daily food consumption. Metabolizable energy (ME) intake was expressed as digestible energy intake × 0.96 [18]. The gain in energy was obtained by subtracting the energy content of an initial group (four rats killed at the beginning of the study) from that of each of the two experimental groups. The carcasses were autoclaved, chopped into small pieces, thoroughly mixed, and homogenized in water (final volumes equal to twice the carcass weight) with a Polytron. Samples of homogenates were dessicated into a dry powder from which small pellets (about 200 mg) were made. The energy content was measured with the bomb calorimeter. Energy expenditure was calculated from the difference between ME intake and energy gain; gross efficiency was calculated as the percent of ME intake stored as body energy.

Other four CD and HFD rats were starved for 16 h and thereafter they were used for the determination of serum free fatty acid (FFA) levels. Rats were anesthetized with ethyl ether and blood was collected via the abdominal aorta. Serum samples were stored at –20°C until the time of analysis. FFA serum levels were measured using the acyl-CoA synthetase/acyl-CoA oxidase method using the enzymic kit obtained from Boehringer-Mannheim Biochemia, Milano, Italy.

Statistical analysis

The data are summarized using means and standard errors of four different rats. Statistical significance between the means was examined by two-tailed Student's *t*-test. Probability values less than 0.05 were considered to indicate a significant difference.

Materials

All enzymes and coenzymes were from Boehringer (Mannheim, Germany) or Sigma (Munich, Germany). Chemicals were from Merck (Darmstadt, Germany). Radiochemicals were from NEN (Dreieichen, Germany).

Results

Table 1 shows the results of energy balance measurements in CD and HFD rats. Mean initial and final body weights were not significantly different between the two groups, yielding a daily body weight gain of about 7 g in both groups. Plasma FFA were significantly enhanced in 16 h fasted HFD rats compared to CD rats. Also a significant increase in ME intake and energy expenditure and a significant decrease in gross efficiency were found in HFD rats, compared to CD rats.

Oxygen consumption measured in perfused livers was significantly higher (+22% $p < 0.05$) in HFD rats compared to CD rats and was calculated to be 122.3 ± 2.2 and 149.6 ± 4.3 μmol O_2/h × g wet weight in CD and HFD rats, respectively.

Table 2 shows the results of $\Delta\psi_m$, ΔpH_m and Δp in CD and HFD rats. The subcellular pH differences were determined from the distribution of DMO. The distribution of the labelled indicator between perfusate, cytosol and mitochondria of perfused liver revealed that the mitochondrial matrix has a more alkaline milieu than the cytosol in both CD and HFD rats. Moreover, the cytosolic pH significantly increased and the mitochondrial decreased in HFD rats, compared to CD rats. Therefore ΔpH_m was significantly lower in HFD rats. On the other hand, HFD rats showed a significant increase in $\Delta\psi_m$, so that Δp was not different in CD and HFD rats. No variation was found in $\Delta\psi_{pm}$.

The subcellular distribution of metabolites in livers from CD and HFD rats is shown in Table 3. The mitochondrial/cytosolic concentration ratio of malate and ATP/ADP ratios were not different in HFD rats compared to CD rats. In both

Table 1. Energy balance and serum free fatty acid (FFA) levels in CD and HFD rats

	CD rats	HFD rats
Initial body weight, g	80 ± 1	80 ± 1
Final body weight, g	180 ± 1	179 ± 1
Body weight gain, g	100 ± 1	99 ± 1
ME intake, kJ	2850 ± 23	3870 ± 16*
Body energy gain, kJ	620 ± 33	658 ± 20
Energy expenditure, kJ	2230 ± 38	3212 ± 28*
Gross efficiency†, %	22 ± 1	17 ± 1*
Serum FFA levels, mM	1.0 ± 0.1	1.2 ± 0.1*

Mean values with their standard errors for 4 rats referred to 15 day period. *$p < 0.05$ compared to CD rats (Two-tailed Student's *t*-test); ME – metabolizable energy intake; †(Body energy gain/ME intake) × 100.

Table 2. Subcellular pH and electrical potential difference in perfused livers from CD and HFD rats

	CD rats	HFD rats
ΔpH (cytosol-perfusate)	–0.02 ± 0.05	0.29 ± 0.03*
ΔpH (mitochondria-cytosol)	0.89 ± 0.03	0.17 ± 0.01*
pH perfusate†	7.4	7.4
pH cytosol†	7.37 ± 0.05	7.70 ± 0.03*
pH mitochondria†	8.25 ± 0.06	7.86 ± 0.02*
$\Delta\psi_m$ (mV)	118 ± 10	143 ± 3*
Δp (mV)	172 ± 12	154 ± 3
$\Delta\psi_{pm}$ (mV)	76 ± 8	59 ± 3

Mean values with their standard errors for 4 rats. †Calculated from the individual data; *p < 0.05 compared to CD rats (Two-tailed Student's *t*-test); $\Delta\psi_m$ – Mitochondrial membrane potential; Δp-proton motive force; $\Delta\psi_{pm}$ – Plasma membrane potential.

Table 3. Subcellular concentration ratios in liver from CD and HFD rats

	CD rats	HFD rats
$(Malate)_m/(Malate)_c$†	5.1 ± 0.5	7.1 ± 1.4
$(ATP/ADP)_m$	0.6 + 0.1	0.6 ± 0.1
$(ATP/ADP)_c$	4.1 ± 1.0	4.2 ± 0.6
$\Delta pH^{\dagger}_{malate}$	0.34 ± 0.02	0.41 ± 0.04
$\Delta\psi_{m\ (ATP/ADP)}^{\dagger}$ (mV)	48 ± 8	52 ± 5

Mean values with their standard errors for 4 rats. *p < 0.05 compared to CD rats (Two-tailed Student's *t*-test); ΔpH_{malate} – 1/2log $(malate)_m/(malate)_c$; $\Delta\psi_m$(ATP/ADP ratio) – RT/F log $(ATP/ADP)_c(ATP/ADP)_m$. †Calculated from the individual data.

groups of rats, the $(ATP/ADP)_c$ ratio was higher than $(ATP/ADP)_m$. Consequently, no variation was found in both ΔpH_m calculated from the distribution of malate and $\Delta\psi_m$ calculated from the ATP/ADP ratio.

Discussion

The results obtained in this work on energy balance measurements (Table 1) and liver respiration are in agreement with our previous ones [3] indicating the presence in HFD rats of some regulatory mechanisms which are useful to limit fat gain. One of these mechanisms can involve hepatic metabolic adaptations [2, 3, 19], which may in turn affect the relationship between respiration, Δp and phosphorylation. In fact, an increase in oxygen consumption in perfused liver could be achieved by increasing the supply of reducing equivalents to the electron transport chain (which will raise Δp), and/or it could be due to an increased cytosolic ATP turnover (which will lower Δp) [20]. The results of the present study show that perfused livers from HFD rats, despite an enhanced oxygen consumption, exhibit similar Δp to that found in CD rats (Table 2). Accordingly, no variation in mitochondrial and cytosolic ATP/ADP ratios are found in HFD rats compared to CD rats (Table 3). These findings are in agreement with our previous suggestion, based on experiments in isolated hepatocytes [3], of a concomitant increase in both, substrate supply and ATP turnover, in liver cells from HFD rats.

Interestingly, when we consider the two components of Δp, we find a significant decrease in ΔpH_m and a significant increase in $\Delta\psi_m$ in HFD rats compared to CD rats (table 2), which tend to compensate each other. The lowering of ΔpH_m could be the effect of a decoupling action of FFA [21–23]. Decoupling can be accompanied by a lowered efficiency of proton extrusion by respiratory chain proton pumps or an increase in proton backflow, thus lowering the mitochondrial/cytosolic proton gradient. This possibility appears reasonable in view of the fact that HFD rats show a significant increase in serum FFA levels after 16 h fasting (Table 1), even though the FFA level also increased to some extent in CD rats after this time. We can exclude that the break down of the mitochondrial proton gradient is produced by a change in proton backflow through the mitochondrial inner membrane due to high fat diet. In fact, we have previously found no variation in mitochondrial proton leak measured in both isolated mitochondria [8] and isolated hepatocytes [4] from HFD rats.

The significant increase in $\Delta\psi_m$ found in HFD rats (Table 2) follows the decrease in ΔpH_m, so that a new steady state is set up where $\Delta\psi_m$ is compensatory increased by movement of other ions than protons, to keep Δp constant. Similar observations have been made in isolated mitochondria [24].

Δp consisting of a chemical (ΔpH_m) and an electrical ($\Delta\psi_m$) component, not only provides the driving force for ATP synthesis, but also influences the distribution across the mitochondrial membrane of several ions and metabolites that are critical for mitochondrial function [7]. We have measured the subcellular concentrations of three metabolites, whose distribution between mitochondria and cytosol is influenced by either $\Delta\psi_m$ (ATP/ADP), or ΔpH_m (malate). The results show that neither $[(ATP/ADP)_c/(ATP/ADP)_m]$ reflects $\Delta\psi_m$ nor the distribution ratio of malate ΔpH_m. Apparently in both control and HFD rats the kinetics of metabolism and transport are such that metabolism dominates transport rates in determining the concentrations of these metabolises in the different compartments [16]. In addition, the concentration ratio of malate does not reflect the change in ΔpH_m in HFD rats. In fact, the decrease in ΔpH_m found in HFD rats is not followed by a decrease in the malate concentration ratio. This result is in agreement with our previous hypothesis of a shift of the reaction catalyzed by malate dehydrogenase in favour of malate production at the expense of oxalacetate [2]. The decreased ΔpH_m would allow the export of malate to the cytosol, where it can be converted to pyruvate or oxalacetate, with the formation of NADPH or NADH. The reducing equivalents can be finally retransported into the mitochondria through the α-glycerophosphate shuttle, whose activity

increases in HFD rats [2], and oxidized through the respiratory chain from complex II onwards. This mechanism would thus lead to a fall in hepatic metabolic efficiency, with a following wasteful increase in the oxidation of energetic substrates, such as fatty acids and could contribute to the increased energy expenditure of HFD rats.

In conclusion, our present results show that the increased hepatic respiration in HFD rats is associated with reciprocal changes in $\Delta\psi_m$ and ΔpH_m at a practically unchanged protonmotive force. These findings are in line with the notion of an increase in substrate supply to the respiratory chain on one hand and a lowering of efficiency of cytosolic ATP-supply due to decoupling and/or higher cytosolic ATP-turnover in HFD rats, on the other hand. Both effects would compensate each other so that protonmotive force remains unchanged.

Acknowledgements

The authors wish to thank Professor Antonio Barletta for helpful discussion and Sonja Grundel and Myung-Hee Oh for technical assistance. This work was supported by a grant from Ministero dell'Università e del la Ricerca Scientifica e Tecnologica.

References

1. Liverini G, Iossa S, Barletta A: Hepatic mitochondrial respiratory capacity in hyperphagic rats. Nutr Res 11: 1671–1682, 1984
2. Iossa S, Mollica, MP, Lionetti L, Barletta A, Liverini G: Hepatic mitochondrial respiration and transport of reducing equivalents in rats fed an energy dense diet. Int J Obes 19: 539–543, 1995
3. Iossa S, Mollica MP, Lionetti L, Barletta A, Liverini G.: Energy balance and liver respiratory activity in rats fed on an energy-dense diet. Brit J of Nutr 77: 99–105, 1997
4. Lionetti L, lossa S, Brand MD, Liverini G: The mechanism of stimulation of respiration in isolated hepatocytes from rats fed an energy-dense diet. J Nutr Biochem 7: 571–576, 1996
5. Brown GC, Lakin-Thomas PL, Brand MD: Control of respiration and oxidative phosphorylation in isolated rat liver cells. Eur J Biochem 192: 355–362, 1990
6. Horrum MA, Tobin RB, Ecklund RE: Thyroid hormone effects on the State IV proton motive force in rat liver mitochondria. Mol Cell Biochem 103: 9–13, 1991
7. La Noue KF, Schoolwerth HC: Metabolite transport in mitochondria. Ann Rev Biochem 48: 871–921, 1979
8. Lionetti L, lossa S, Brand MD, Liverini G: Relationship between membrane potential and respiration rate in isolated liver mitochondria from rats fed an energy dense diet. Mol Cell Biochem 158: 133–138, 1996
9. Iossa S, Lionetti L, Mollica MP, Barletta A, Liverini G. Thermic effect of food in hypothyroid rats. J Endocrinol 148: 167–174, 1996
10. Naim M, Brand JG, Kare MR, Carpenter RG: Energy intake, weight gain, and fat deposition in rats fed nutritionally controlled diets in a multichoice ('cafeteria') design. J Nutr 115: 1447–1458, 1995
11. Allard M, LeBlanc J: Effects of cold acclimation, cold exposure and palatability on postprandial thermogenesis in rats. Int J Obes 12: 169–178, 1988
12. Scholz R, Hansen W, Thurman RG: Interaction of mixed function oxidation with biosynthetic processes. Eur J Biochem 38: 64–72, 1973
13. Elbers R, Heldt H W, Schmucker P, Soboll S,. Wiese H. Measurement of the ATP/ADP ratio in mitochondria and in the extramitochondrial compartment by fractionation of freeze-stopped liver tissue in non-acqueous media. Hoppe-Seyler's Z Physiol Chem 355: 378–393, 1974
14. Bergmeyer HU: Methoden der Enzymatischen Analyse. 2nd edn., Verlag Chemie, Weinheim (ed), 1974
15. Lowry OH, Rosebrough NJ, Farr AL, Randall R: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275, 1951
16. Soboll S, Elbers R, Scholz R, Heldt HW: Subcellular distribution of di- and tricarboxylates and pH gradients in perfused rat liver. Hoppe-Seyler's Z Physiol Chem 361: 69–76, 1980
17. Soboll S, Scholz R, Freisl M, Elbers R, Heldt HW: In: JM Tager, HD Soling, JR Williamson (eds). Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies. North-Holland, Amsterdam and Oxford, pp 29–40, 1976
18. Barr HG, McCracken KJ: High efficiency of energy utilization in "cafeteria" and forcefed rats kept at 29°C. Br J Nutr 51: 379–387, 1984
19. Liverini G, Iossa S, Mollica MP, Lionetti L, Barletta A: Hepatic fatty acid-supported respiration in rats fed an energy dense diet. Cell Biochem Funct 14: 283–289, 1996
20. Nobes CD, Hay WW, Brand MD: The mechanism of stimulation of respiration by fatty acid in isolated hepatocytes. J Biol Chem 265: 12910–12915, 1990
21. Soboll S, Grundel S, Schwabe U, Scholz R: Influence of fatty acids on energy metabolism. Eur J Biochem 141: 231–236, 1984
22. Rottenberg, H, Hashimoto, K: Fatty acid uncoupling of oxidative phosphorylation in rat river mitochondria. Biochemistry 25: 1747–1755, 1986
23. Wrigglesworth JM, Sharpe MA, Cooper CE: Regulation of electron flux through cytochrome c oxidase: pH, ΔpH and fatty acids. Biochem Soc Trans 21: 781–784, 1993
24. Mitchell P, Moyle J: estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. Eur J Biochem 7: 471–484, 1969