Enzyme activities of energy metabolism in muscle and brown fat of germ-free rats

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INTRODUCTION

The growth and body weight of adult germ-free (GF) rats are similar to those of microbially-associated (MA) (*Snyder & Wostmann* 1987), while the sizes of several body organs are different (*Gordon & Wostmann* 1960, *Gordon et al.* 1966, *Wostmann et al.* 1982). The resting oxygen consumption of the intact GF rat is 20–30 % lower than that of their MA counterparts (*Desplaces et al.* 1963, *Levenson et al.* 1968, *Wostmann et al.* 1968, 1983). The slight differences in body chemical composition (*Levenson* 1978) are insufficient to explain the differences in oxygen consumption. Also the thyroid gland shows normal function in adult GF rats and mice (*Levenson* 1978, *Sewell & Wostmann* 1975).
Oxygen consumption of muscle tissue is about 20 % of the total oxygen consumption of the body at rest. The brown adipose tissue (BAT) has a high metabolic activity, and is an important component of facultative thermogenesis (*Himms-Hagen* 1985). The metabolic activity of BAT is under sympathetic control. This resembles shivering thermogenesis in skeletal muscle, which is also an oxidative process but controlled by somatic motor nerves. Although GF rats have lower oxygen consumption and smaller heart size than MA animals, there are no reports on enzyme activities of energy metabolism either in the heart, skeletal muscles or BAT of these animals.
In this study enzyme activities of energy metabolism in skeletal and myocardial muscles and BAT were measured in adult GF and MA littermate rats of both sexes. Additionally, the animals were used to reevaluate whether some differences given in the literature on organ sizes between inbred GF and MA male rats (*Gordon et al.* 1966, *Wostmann* 1984) are consistent also when the comparisons are made in animals from an outbred colony, using littermates reared under strictly similar conditions, with the exception of the microbiological status.

MATERIALS AND METHODS

Animals

Outbreed Han:WIST rats of both sexes were used. All the animals were born from GF parents. They were housed in transparent Trexler-type flexible PVC-film isolators (Kleinfeld GmbH, Hannover, FRG) in groups of 2–4 animals in polycarbonate shoe-box type cages (type III; 420×260×150 (h) mm) on autoclave-sterilised softwood beddings changed once a week. All the isolators were kept in the same room with a light-dark cycle 12:12 hours (6 a.m. – 6 p.m.) in room temperature (for details, see Annual Report 19). The rats had free access to autoclave-sterilised rodent diet (MR5 of Central Institute for Laboratory Animal Breeding, Hannover, FRG) and autoclave-sterilised tap water from bottles. The microbiological status of the GF animals was controlled weekly (for details, see Annual Report 19). Within two days after the birth the litters were divided. In order to be associated by a normal complete gastrointestinal flora part of the offspring was transferred into isolators under MA foster dams taken from the specified pathogen-free barrier unit, and having a normal pathogen-free microbial flora. The other part of the litter remained with the original mother until weaning. All the animals were reared in isolators until sampling.

Procedure

The rats were sacrificed at the age of 78–107 days by cervical dislocation within two hours

after leaving the isolator. The timing of the sacrifice was arranged so that every GF animal had a MA counterpart of the same sex killed at the same time of the day. The heart, caecum, BAT and muscle sample (median part of m. gastrocnemius) were immediately cut free and weighed. All preparations were done by the same person. The relative organ sizes were calculated as mg/g of body weight, corrected by subtraction of the weight of the caecal content. All tissue samples for enzyme assays were immediately transferred into and prepared in ice-cold 0.9 % NaCl, subsequently smoothly dried and frozen in liquid nitrogen, and stored at –20°C until assayed. For histological examination, a piece of the posterior lobe of the BAT was fixed in 10 % aqueous formaldehyde. After dehydration and embedding in paraffin, 7 µm sections were cut and stained with hematoxylin-eosin. The density of fat cell nuclei in the sections were calculated from photomicrographs.

For dry weight determination, pieces of BAT were dried at 100°C for 48 h. The fat content of BAT tissue was measured using a commercial kit for neutral fat (Boehringer Mannheim, FRG).

For enzyme assays the tissue samples were homogenised with a Potter-Elvehjem all-glass homogeniser (20 mg of tissue/1 ml ice-cold 0.1-M Tris-Hcl buffer, pH 7.6) and centrifuged for 10 min (1000 × g, at 4°C). The activities of citrate synthase (CS) (E.C. 4.1.3.7) (*Srere* 1969), lactate dehydrogenase (LHD) (E.C. 1.1.1.27) (Boehringer Mannheim, commercial kit), malate dehydrogenase (MDH) (E.C. 1.1 1.37) (*Englard & Siegel* 1969), phosphofructokinase (PFK) (E.C. 2.7.11.) (*Boström et al.* 1974) and succinate dehydrogenase (SDH) (E.C. 1.3.99.1) (*Earl & Korner* 1965) were determined from supernatant with methods referred. The enzyme activities were measured at 37°C in quartz cuvettes with 1 cm light path in a Cary 118 C-type spectrophotometer. The assay for a given parameter for all animals was always performed in a single series. Glycogen content of muscle tissue was measured after acid hydrolysis (1 N HCl, 2 h, 100°C) as glucose by the o-toluidine method (*Hultman* 1967). Protein content of the supernatants was measured by the method of *Lowry et al.* (1951) with bovine serum albumin as standard.

Two-tailed Student's t-test was used for evaluation of statistical significance of differences between means.

RESULTS

The body weights of the animals, and the relative weights (mg/g of body weight corrected for caecal content) of heart, liver, kidneys, spleen, adrenals, testes, caecal content, and BAT are given in Table I. The weights of the GF and MA animals were similar. The weights of the hearts in the GF females were lower than those in the MA females. In males the tendency of a smaller heart was also seen, but no statistically significant difference oc-

Table I. Body weights (g), relative organ weights (mg/g b.w.) of heart, liver, kidneys, spleen, adrenals, testes, caecal content and interscapular brown adipose tissue (BAT) of adult germ-free (GF) and microbially-associated (MA) Han:WIST rats (x ± SE, n= 9-14).

	GF females	GF males	MA females	MA males
Body (g)	211 ± 4.9	352 ± 11.0	205 ± 3.5	346 ± 8.4
Heart	3.20 ± 0.075**	2.95 ± 0.076	3.51 ± 0.080	3.09 ± 0.062
Liver	31.2 ± 0.370**	33.4 ± 0.510***	34.3 ± 0.730	36.8 ± 0.420
Kidney	7.22 ± 0.156*	6.82 ± 0.192	6.76 ± 0.104	6.40 ± 0.084
Spleen	2.57 ± 0.083	2.24 ± 0.083	2.74 ± 0.059	2.25 ± 0.061
Adrenal	0.29 ± 0.007	0.018 ± 0.005***	0.28 ± 0.008	0.015 ± 0.005
Testes		9.67 ± 0.283		9.87 ± 0.178
Caecal content	88.5 ± 5.13***	60.4 ± 3.61***	14.9 ± 0.81	15.0 ± 0.79
BAT	0.95 ± 0.056*	0.87 ± 0.050	1.17 ± 0.067	0.89 ± 0.024

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to MA rats of the same sex.

Table II. Glycogen content (mg/g) and activities (U/g) of citrate synthase (CS), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), phosphofructokinase (PFK) and succinate dehydrogenase (SDH) in the myocardium and in m. gastrocnemius (medial par) of adult germ-free (GF) and microbially-associated (MA) Han:WIST rats (x±SE, n=10-11).

	Myocardial muscle		M. gastrocnemius	
	GF females	MA females	GF females	MA females
Glycogen	10.7±0.62	11.8±0.84	6.7±0.51	7.6±0.37
CS	137.5±3.22	148.2±4.59	23.7±1.57	21.1±1.82
LDH	696.6±34.36	703.3±37.70	886.3±32.49	825.5±37.01
MDH	626.9±11.29	639.2±16.61	172.7±19.72	151.0±10.92
PFK			10.1±0.79	9.3±1.20
SDH	36.2±3.21	42.6±1.00	4.2±0.37	4.6±0.45

curred. In females the weights of the BAT were also lower in the GF than in MA, while in males there was no difference. Both sexes of GF rats had smaller livers than their MA counterparts. In GF females the kidneys were heavier than in the MA females, but in males the difference was statistically insignificant. The GF males had larger adrenals than the MA males. The caecal contents were 4–6 times heavier in the GF than in the MA rats.

The glycogen content and the activities of CS, LDH, MDH, PFK and SDH in myocardial and skeletal muscles from female rats are given in Table II. In these parameters there were no significant differences between the GF and MA rats.

Cell densities, fat, water and protein contents, and the activities of CS, MDH and SDH in BAT are given in Table III. There were no differences in cell densities between GF and MA females. Fat content in BAT was lower and conversely water content was higher in GF than in MA females. In BAT of GF females the activities of CS and MDH were higher than in their MA counterparts. In males similar tendencies were observed, but the differences were not statistically significant.

DISCUSSION

The differences in enzyme activities as well as in fat and water contents in BAT between GF and MA rats is a new finding. The metabolic activity of BAT is controlled by adrenergic nervous system. In female GF rats the weight and fat content of BAT was lower than in MA females, but the activities of CS and MDH were higher in the tissue homogenate. These differences between GF and MA rats are similar to the changes occurring in rats

Table III. Cell density (nuclei/0.01 mm^2 in 7 μm slides), activity (U/g) of citrate synthase (CS), malate dehydrogenase (MDH), and succinate dehydrogenase (SDH), fat water (% of wet weight) and protein content (mg in 1000xg tissue homogenate supernatants from 1 g) of interscapular brown adipose tissue (BAT) of adult germ-free (GF) and microbially-associated (MA) Han:WIST rats (x±SE, for males n=12, for females n=9).

	GF females	GF males	MA females	MA males
Cell density	46.2±4.85		42.6±3.08	
CS	182.7±9.98**	141.5±10.58	133.5±7.52	122.1±14.36
MDH	358.7±11.27***	251.3±14.37	255.4±17.63	214.9±18.49
SDH	12.8±1.48	6.9±1.36	11.3±3.28	6.3±2.27
Tissue fat	31.9±1.64*	43.1±1.93	38.3±2.39	42.7±1.12
Tissue water	54.5±1.81**	41.8±2.03	45.5±1.28	39.8±1.15
Protein content	76.1±1.68	54.3±0.56	65.6±2.54	49.2±2.29

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to MA rats of the same sex.

during prolonged beta-adrenergic blockade by alprenolol. After three weeks treatment with alprenolol the BAT weight is decreased and the activities of the oxidative enzymes CS and MDH are increased (*Harri* 1977, 1978). It has been speculated whether the adrenergic inhibitory substance, found in the intestinal content and especially in the caecum of GF rats, might enter into circulation and be related to the lower metabolic rate in GF rats (*Gordon & Bruckner* 1984).

The similar body weights of the GF and MA animals are in agreement with earlier reports (*Ratcliffe* 1987, *Wostmann* 1984). Whenever differences between GF and MA animals were seen, they were generally larger in females than in males. The most pronounced difference between the GF and MA rats was the 4–6-fold larger caeum in the GF animals, as expected. This, as well as the finding of the smaller hearts in GF rats is in accordance with previous results obtained from male rats (*Gordon et al.* 1966, *Snyder & Wostmann* 1987). In this study, however, the difference in heart size of males was smaller than found earlier (*Gordon et al.* 1966) and too small to reach a statistical significance. The rearing conditions of the GF and MA rats in the study of *Gordon et al.* (1966) were not strictly similar for the two groups as was the case in this study, which may have influenced the difference in heart sizes in the GF and MA groups in their study.

Enzyme activities from the myocardial muscle were measured from females because the difference in heart weights between GF and MA rats was larger in them. In spite of the smaller hearts, the GF females had practically the same CS, LDH, MDH and SDH activities as MA females. Also the glycogen content of the myocardium was similar in GF and MA females. In striated muscle from m. gastrocnemius, representing a muscle with mixed type (both fast and slow) muscle fibers, these enzyme activities were also similar in GF and MA female rats. Since GF and MA rats are reported to have similar water contents in striated muscle (*Gordon et al.* 1966, psoas), there is probably no difference in the actual *in vivo* activities of these enzymes in GF and MA rats.

In the present study the activities of several enzymes of energy metabolism of the heart and skeletal muscle in GF rats were quite comparable to those in MA rats, in spite of the smaller mass of the myocardial muscle especially in GF female rats. Thus the decreased metabolic rate and oxygen consumption in GF rats is likely to be due to factors other than muscular enzymes for energy metabolism.

In conclusion, the present results confirm earlier findings that the heart and liver of the GF rats are somewhat smaller than in the MA ones. The activities of CS and MDH were higher in BAT of the GF than in the MA rats, but not in myocardial or skeletal muscle. Moreover, the differences between the GF and MA rat groups seem generally greater in females than in males.

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Summary

The heart is smaller in germ-free (GF) rats and the resting oxygen consumption is 20–30 % lower than that in their microbially-associated (MA) counterparts. In this study organ weights and enzyme activities of energy metabolism were measured from myocardial and skeletal muscles and interscapular brown adipose tissue (BAT) of GF and MA rats. GF and MA rats had similar body weights. Caecum was 4–6 times heavier in GF than in MA rats. Especially in females the heart, liver and BAT were lighter, and kidneys heavier in GF than in MA rats. The adrenals of GF males were heavier than in MA males. There were no differences between GF and MA females in glycogen content and activities of citrate synthase, lactate dehydrogenase, malate dehydrogenase and phosphofructokinase from myocardial and skeletal muscles. BAT was lighter in GF than in MA females and it contained less fat. The activities of

citrate synthase and malate dehydrogenase were higher in the BAT of GF females and the same tendency was seen in the males. The results suggest that the decreased oxygen consumption in GF rats is likely to be due to factors other than muscular energy metabolism.

Sammendrag

Hos kimfri (GF) rotter er hjertet mindre og hvile iltforbruget 20–30 % lavere end hos mikrobe-associerede (MA) kontroldyr. I dette studie måltes organvægte og energimetaboliske enzymaktiviteter i hjerte- og skeletmuskulatur, samt interscapulært brunt fedtvæv (BAT) hos GF og MA rotter. GF og MA rotter havde ensartet kropsvægt. Caecum var 4–6 gange tungere hos de kimfri rotter, og især hos hunnerne var hjerte, lever og BAT lettere og nyrerne tungere end hos MA rotter. GF hannernes binyrer var tungere end MA hannernes. Der var ingen forskel mellem GF og MA hunnerne i glycogenindhold og aktivitet af citratsynthase, lactatdehydrogenase, malatdehydrogenase og fosfofructokinase fra hjerte- og skeletmuskulatur. BAT var lettere i GF end i MA hunrotter og det indeholdt mindre fedt. Aktiviteten af citratsynthase og malatdehydrogenase var højere i BAT hos GF hunner og en lignende tendens sås hos hannerne. Resultaterne indicerer, at det mindre iltforbrug hos GF rotter sandsynligvis skyldes andre faktorer end muskelenergistofskiftet.

Yhteenveto

Rotilla, joilta puuttuvat kaikki mikrobit (germfree, GF) on pienempi sydän ja hapenkulutus kuin rotilla, joilla on normaali mikrobikasvusto (microbially-associated, MA). Työssä punnittiin elinten painoja sekä mitattiin sydän- ja luustolihaksesta ja ruskeasta rasvasta energia-aineenvaihduntaan liittyvien entsyymien aktiivisuuksia. GF- ja MA-eläimet olivat samanpainoisia. Umpisuolen paino oli GF-eläimillä 4–6-kertainen MA-rottiin verrattuna. Erityisesti GF-naaraissa sydän, maksa ja lapaluiden välinen ruskea rasvakudos olivat kevyemmät ja munuaiset painavammat kuin MA rotilla. GF-urosten munuaiset olivat painavammat kuin MA-urosten. Naaraista mitattiin sydän- ja luustolihaksen glykogeenipitoisuus sekä sistrattisyntaasin, laktaattidehydrogenaasin, malaattidehydrogenaasin ja fosfofruktokinaasin aktiivisuudet. GF- ja MA-eläinten välillä ei näissä ollut eroa. GF-naaraiden ruskea rasvakudos oli kevyempi ja sen rasvapitoisuus pienempi kuin MA-naaraiden. GF-naaraiden ruskeassa rasvakudoksessa sitraattisyntasiin ja malaattidehydrogenaasin aktiivisuudet olivat korkeammat kuin MA-naaraissa. Uroksissa näkyi samanlainen suuntaus. Näiden tulosten mukaan pienempi hapenkulutus GF-rotissa ei selity lihaskudoksen aineenvaihduntaentsyymien avulla.

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