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Perturbation of glycerol metabolism in hepatocytes from *n*3-PUFA-depleted rats

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Abstract. Second generation *n*3-PUFA-depleted rats represent a good animal model of metabolic syndrome as they display several features of the disease such as liver steatosis, visceral obesity and insulin resistance. The goal of our study was to investigate the influence of *n*3-PUFA deficiency on hepatic glycerol metabolism. Aquaglyceroporin 9 (AQP9) allows hepatic glycerol transport and consequently contributes to neoglucogenesis. AQP9 knockout mice display hypertriacylglycerolemia, one of the hallmarks of the metabolic syndrome. Our data show reduced AQP9 expression at the protein level in *n*3-PUFA-depleted rats, without any changes at the mRNA levels. [U-^{14}C]glycerol uptake was increased in hepatocytes from *n*3-PUFA-depleted animal cells. The apparent discrepancy between decreased AQP9 protein expression, and increased [U-^{14}C]glycerol uptake could be explained by an observed increase in glycerol kinase activity.

Introduction

Metabolic syndrome is a common disease associated with an increased risk of type 2 diabetes, atherosclerosis and cardiovascular disease (1,2). Its major characteristics include insulin resistance, dyslipidemia, abdominal obesity and hypertension (1,2). Nutritional pattern plays an important role in the development and progression of the metabolic syndrome (3,4). ω3 polyunsaturated fatty acids (*n*3-PUFA) consumption may improve dyslipidemia and insulin resistance, and reduce adiposity, triacylglycerolemia, and retard the development of type 2 diabetes (5-7). Over the last 40-50 years, increased intake of ω6 polyunsaturated fatty acids (*n*6-PUFA) has counterbalanced the decreased intake in *n*3-PUFA in Western populations (8,9).

Second generation *n*3-PUFA-depleted rats display several features of the metabolic syndrome (10-14), making them a valuable model for metabolic syndrome.

Transmembrane glycerol transport is ensured by aquaglyceroporins belonging to the aquaporins (AQPs) family of water-permeable channel proteins accounting for transcellular water permeability in many organisms (15,16). The aquaglyceroporins (AQP3, AQP7, AQP9, AQP10) are permeable to glycerol and urea in addition to water (15,17,18). Regulation of AQP9 expression in hepatocytes participates in the control of glycerol metabolism during fasting and refeeding (19-22). AQP9-knockout animals are characterized by increased plasma glycerol and triacylglycerol levels (23).

The purpose of our study was to investigate the consequences of metabolic syndrome on glycerol metabolism using both control and second generation *n*3-PUFA-depleted rats.

Materials and methods

Animals. Female control and second generation *n*3-PUFA-depleted rats were 22 weeks of age. The *n*3-PUFA-depleted rats were prepared as previously described (12,13). Rats were given access to water and food *ad libitum* (either control or *n*3-PUFA-depleted). The fatty acid pattern of lipids in the diets offered to control animals and *n*3-PUFA-depleted rats was previously reported (12). Liver tissue was removed following euthanasia in a CO_2 chamber. All animal experiments were conducted in compliance with the Institutional Animal Care and Use Committee.

ω3 fatty acids pattern in liver tissue. The methods used to measure the fatty acid content and pattern in total lipids from liver tissue has been previously described (24). ω3 fatty acid content was determined on liver triglycerides and phospholipids from both control and *n*3-PUFA-depleted rats and expressed as μg/mg of tissue.

Real-time RT-PCR. Real-time RT-PCR analyses were performed as previously described (25). Primer sequences are shown in

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Table I. qRT-PCR primer sequences and efficiency.

Gene	Sequence of primers	Amplicons (bp)	Efficiency
AQP9	Sense: CGGAAAACTGCTCGTCGTAG Antisense: CACCCAGGTTTCTGGAGTCA	161	1.00±0.02
Arbp	Sense: GAGGTGCTGGACATCACAGA Antisense: AGACAAAGCCAGGACCCTTT	153	0.96±0.06
PpiA	Sense: GGGTTCCTCCTTTCACAGAA Antisense: TCCCAGGACCTGTATGCTTC	143	1.00±0.02
β-actin	Sense: GGGTTACGCGCTCCCTCATG Antisense: CCACGCTCGGTCAGGATCTTC	90	1.01±0.03
RPL	Sense: AGGCACCAGTCGGACCGATA Antisense: GAAGCCGCTAGGCAGCATGT	159	1.00±0.04

Efficiencies are expressed as mean ± SD (n=3).

Table II. ω3 fatty acids pattern in liver triglycerides and phospholipids.

	Triglycerides		Phospholipids	
Rats	Control	*n3*-PUFA-d	Control	*n3*-PUFA-d
C18:3ω3	144.60±9.20	ND[a]	42.84±2.86	ND[a]
C18:4ω3	ND	ND	ND	ND
C20:5ω3	57.54±3.91	ND[a]	134.10±11.66	ND[a]
C22:3ω3	ND	ND	ND	ND
C22:5ω3	125.60±10.76	ND[a]	381.00±16.66	40.94±1.47[a]
C22:6ω3	346.40±36.62	ND[a]	4162.00±156.50	368.10±11.63[a]

Omega3 fatty acid contents from liver triglycerides and phospholipids were determined as described in Material and methods in control (n=12) and *n3*-PUFA-depleted (n=6) rats. ND, not detected, levels under the limit of detection. The results are expressed as the mean ± SEM in μg of fatty acid/mg of tissue. [a]$p<0.0001$ for the difference between control and *n3*-PUFA-depleted rats, unpaired Student t-test.

Table I. Out of seven housekeeping genes tested, the most stable were PpiA, Arbp, RPL and β-actin.

Western blot analysis. Crude plasma membrane protein preparation and Western blot analysis were performed as previously described (25). Proteins were immunolabeled using an affinity-purified antibody to AQP9 at a 1:500 dilution (Alomone, Jerusalem, Israel).

Hepatocyte isolation. Hepatocytes were isolated from overnight-starved control and *n*3-PUFA-depleted rats according to the collagenase perfusion technique (26) slightly modified (27). Rats were anesthetized by intraperitoneal injection of Nembutal (1.6 ml/kg). Following abdominal incision, a catheter was introduced into the portal vein and ligated. The liver was successively perfused for 5 min with Hank's solution containing 0.5 mM EDTA and 0.2% phenol red and then for 15 min with Hank's solution containing 5.0 mM $CaCl_2$, 0.2% phenol red and 0.5 mg/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO, USA). The liver was placed in a Petri dish containing Hank's solution prior to removal of the liver capsule and subsequent hepatocytes release. The obtained cellular suspension was filtered and the hepatocytes were washed three times by sedimentation-suspension in buffered bicarbonate and HEPES medium (28) containing 1.0 mg/ml bovine serum albumin. Cell viability was estimated by cell count following trypan blue staining.

Glycerol uptake in hepatocytes. Glycerol uptake (29) was performed in a 1.5 ml microfuge tube using ~$60x10^3$ isolated hepatocytes that were incubated at 37˚C for 1-20 min in 0.12 ml Krebs-Ringer bicarbonate buffer containing 0.2 mM [U-^{14}C]glycerol 0.7 μCi/ml. At the end of incubation, the hepatocytes were separated by centrifugation at 5000 x g for 3 min following the addition of dibutylphthalate/di-isononylphthalate (10:3) oils (Sigma-Aldrich, Steinheim, Germany). Cells were then mixed with 5 ml scintillation liquid and the radioactivity was counted using a β-counter. Total glycerol uptake was calculated after correction for the blank value found under the same experimental conditions in the absence of hepatocytes. The extracellular space and total cellular water space were determined for each experiment using 2.0 mM L-[1-^{14}C]glucose (0.38 μCi/ml) and 2.5 μCi/ml ^{3}HOH, respectively.

Table III. Relative expression of AQP9 mRNA in liver tissue from *n*3-PUFA-depleted rats and control rats.

	Control rats	*n*3-PUFA-depleted rats
Relative AQP9 expression (%)	100±12	115±8

RNA extraction and qRT-PCR were performed as described in Materials and methods. AQP9 mRNAs was detected by qRT-PCR in liver tissue from *n*3-PUFA-depleted rats (n=4) and control rats (n=4). The mRNA levels were first normalized using the normalization factor (NF) determined by GeneNorm analysis of 4 housekeeping genes and then compared to the control samples set at 100%. These normalized values (mean ± SEM) are shown in the right column. Mann-Whitney tests were used to evaluate the statistical differences between the groups (p>0.05).

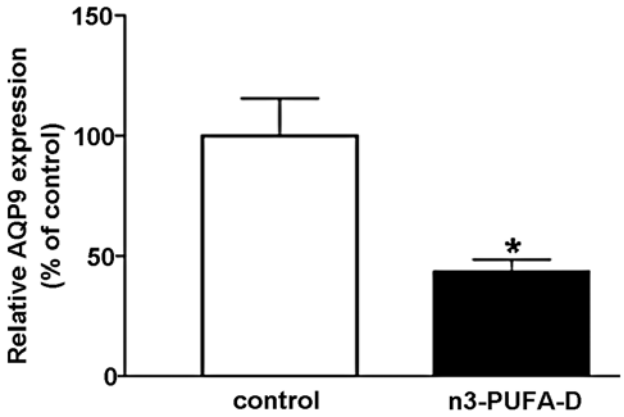

Figure 1. Relative hepatic AQP9 protein expression levels. Relative protein expression levels were determined as described in Material and methods. The results are expressed as the mean ± SEM in percentage of control (n=4 in both animal groups). *p<0.05 compared to control rats, Mann-Whitney test.

Determination of glycerol kinase activity. Glycerol kinase activity was measured by mixing 50 µl of tissue homogenate (10 mg wet weight/ml) with 50 µl of a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 6 mM $MgCl_2$, 60 mM KCl, 10 mM KH_2PO_4, 5 mM ATP, 0.5 mM or 25 mM glycerol and [U-^{14}C]glycerol (0.25 µCi/ml). After 30 min incubation at 37˚C, reaction was stopped by the addition of 1 ml cold water, then phosphorylated product was separated by anion-exchange chromatography on AG1X8.

Statistical analysis. Data are expressed as the mean ± SEM together with the number of separate determination (n) or degree of freedom (d.f.). Mann-Whitney U-test or Student t-test was used to evaluate the statistical differences between the groups. Covariance analysis was performed to determine regression coefficient. All statistical analyses were carried out using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA).

Results

ω3 fatty acid patterns in liver triglycerides and phospholipids. Determination of n3 fatty acid patterns showed significant decrease of C18:3ω3, C20:5ω3, C22:5ω3 and C22:6ω3 in both liver triglycerides and phospholipids from *n*3-PUFA-depleted rats as compared to control animals (p<0.0001, Table II). The levels of both C18:4ω3 and C22:3ω3 in liver triglycerides and phospholipids from both control and *n*3-PUFA-depleted rats were below the limit of detection (Table II).

Table IV. Distribution spaces in hepatocytes.

	Control rats	n3-PUFA-D rats
^{3}HOH	4.01±0.59 (15)	3.97±0.59 (15)
L-[1-^{14}C]glucose	1.40±0.51 (15)	2.27±0.30 (15)
[U-^{14}C]glycerol	28.95±2.76 (19)	54.26±7.26 (19)
Intracellular ^{3}HOH	2.62±0.29 (15)	1.70±0.37 (15)
Intracellular [U-^{14}C]glycerol	27.55±3.14 (32)[a]	52.00±8.20 (32)[a]

The distribution spaces are the mean ± SEM and are expressed as nl/10^3 cells after 20 min of incubation. [a]Degree of freedom.

Hepatic AQP9 expression. AQP9 mRNA levels in liver tissue from *n*3-PUFA-depleted rats (115±8%, n=4) were not significantly different (p>0.05) from those of control rats (100±12%, n=4) (Table III). However, the relative liver protein level of AQP9 was significantly decreased (p<0.05) in *n*3-PUFA-depleted rats (43.82±4.71%, n=4) compared to that of control rats (100.00±15.68%, n=4) (Fig. 1).

Hepatic [U-^{14}C]glycerol uptake. After 20 min incubation, the mean value for the apparent distribution space of ^{3}HOH exceeded that of L-[1-^{14}C]glucose in hepatocytes from both control animals and *n*3-PUFA-depleted rats (Table IV). As judged from the paired differences between these two measurements, the mean value of the intracellular ^{3}HOH space, which failed to differ significantly in hepatocytes from control animal and *n*3-PUFA-depleted rats, yielded an overall value of 2.16±0.25 nl/10^3 cells (n=30, p<0.001 versus zero).

After 20 min incubation, the apparent distribution space of [U-^{14}C]glycerol (28.95±2.76 nl/10^3 cells; n=19) largely exceeded (p<0.001) that of L-[1-^{14}C]glucose (1.40±0.51 nl/10^3 cells; n=15) in hepatocytes from control rats (Table IV), yielding an intracellular glycerol apparent content of 5.51±0.63 pmol/10^3 cells (d.f.=32). A comparable situation prevailed in the hepatocytes from *n*3-PUFA-depleted animals, except that the apparent glycerol content was almost twice higher (10.40±1.64 pmol/10^3 cells, d.f.=32, p<0.008) than in control animals.

The apparent glycerol content of hepatocytes was converted to an intracellular concentration, taking into account the intracellular ^{3}HOH space. The intracellular glycerol concentration

Table V. Hepatic glycerol kinase activity.

Glycerol concentration (mM)	Glycerol kinase activity (nmol/mg wet weight tissue per min)	
	Control rats	*n*3-PUFA-D rats
0.5	0.061±0.003	0.072±0.001[a]
25.0	58.283±3.022	66.117±1.253[a]

Glycerol kinase activity was determined in liver homogenates from n3-PUFA-depleted rats (n=6) and control rats (n=6) at two glycerol substrate concentrations (0.5 and 25 mM). Glycerol kinase activity is the mean ± SEM and is expressed as nmol/mg wet weight tissue per min. Student t-tests were used to evaluate the statistical differences between the groups. [a]p<0.05, compared to control rats.

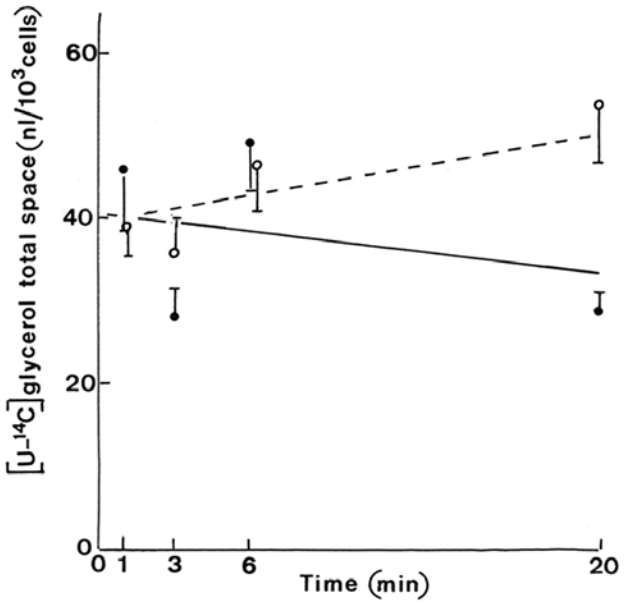

Figure 2. Time course of changes in the total apparent intracellular distribution space of [U-^{14}C]glycerol in hepatocytes. Values for control animals (closed circles and solid line) or *n*3-PUFA-depleted rats (open circle and dashed line) are displayed. The lines were obtained by regression analysis. Mean values ± SEM are derived from 19-20 separate measurements collected in experiments using 4 rats from each group in each case.

of hepatocytes from *n*3-PUFA-depleted rats (6.13±1.69 mM, d.f.=46) was almost 3-fold higher than that found in control animals (2.10±0.39 mM, d.f.=46, p<0.025). Even the latter value was about 10-fold higher (p<0.001) than the extracellular concentration of glycerol (0.20 mM).

Net [U-^{14}C]glycerol uptake time course was investigated by incubating the hepatocytes for 1, 3, 6 and 20 min in the presence of [U-^{14}C]glycerol. In the hepatocytes from control animals, no significant correlation was found between the individual values for the total apparent distribution space of [U-^{14}C]glycerol and the length of incubation (r= -0.2046; n=78) (Fig. 2). After short incubation periods (1, 3 and 6 min), the values recorded in the hepatocytes from *n*3-PUFA-depleted rats were not significantly different (p>0.8) from those measured in control animals. They averaged in *n*3-PUFA-depleted rats 102.5±7.4% (n=59) of the mean corresponding values recorded at the time of the incubation in control rats (100.0±7.4%, n=59). However, in the hepatocytes of *n*3-PUFA-depleted rats, a significant positive correlation (r= +0.2699; n=78; p<0.05) was observed between the individual values for the total apparent distribution space of [U-^{14}C]glycerol and the length of incubation. Covariance analysis, confirmed that the regression coefficient for the time-related changes in the apparent distribution space of [U-^{14}C]glycerol, was vastly different (p<0.001) in the hepatocytes from *n*3-PUFA-depleted rats (+0.8866) compared to control animals (-0.5984). The mean values eventually reached after 20 min incubation were significantly higher (p<0.009) in *n*3-PUFA-depleted rats than in control animals. Even the overall mean value found in the hepatocytes from control animals (38.01±2.45 nl/10^3 cells, n=78) remained significantly lower (p<0.01) than that found after 20 min incubation in the hepatocytes from *n*3-PUFA-depleted rats (54.26±7.26 nl/10^3 cells, n=19) (Table IV).

Hepatic glycerol kinase activity. Hepatic glycerol kinase is known to display a double Km for glycerol: one of 25 μM and one of 2.5 mM (30-33). Therefore, glycerol kinase activity was measured using 0.5 and 25 mM glycerol. At both glycerol concentrations, glycerol kinase activity was significantly higher in liver homogenates from *n*3-PUFA-depleted rats than in control rats (Table V).

Discussion

The effect of *n*3-PUFA depletion was clearly observed in both liver triglycerides and phospholipids. Indeed, liver triglycerides and phospholipids from *n*3-PUFA-depleted rats exhibited C18:3ω3, C20:5ω3, C22:5ω3 and C22:6ω3 levels much lower to those observed in control rats, while C18:4ω3 and C22:3ω3 levels were undetectable in liver triglycerides and phospholipids from both control and *n*3-PUFA-depleted rats.

Aquaglyceroporins account for cellular glycerol transport. Our data indicate reduced hepatic AQP9 protein expression in *n*3-PUFA-depleted rats compared to control rats. However, no difference was detected between the relative AQP9 mRNA levels in liver from *n*3-PUFA-depleted compared to control animals. This was somehow unexpected as insulin is known to induce a down-regulation of AQP9 expression at the transcriptional level (34); and as PUFA-depleted rats furthermore suffer from chronic hyperinsulinemia (13). However, the reduced AQP9 protein expression could result from the insulin resistance concomitantly displayed by the *n*3-PUFA-depleted rats, together with other features of metabolic syndrome.

Finally, the levels of most proteins do not necessarily correlate with the levels of their transcripts (35). Therefore, the observed reduced AQP9 protein expression appears to result from a translational effect and/or modification of protein turnover, rather than from a transcriptional effect. Further studies will however be required to clarify this point.

Another non-insulin-dependent regulation of AQP9 could be related to nutritional cues. Indeed, *n*3-PUFA bind to peroxisome proliferator-activated receptors (PPARs) (36,37) and some aquaglyceroporin expression can be enhanced in response to PPARγ activation (38,39). Consequently, the lack of effects of PPARγ activation, due to *n*3-PUFA deficiency, might account for the absence of effect on AQP9 mRNA levels. Even thought the effects of *n*3-PUFA on post-translational mechanisms are unknown, such mechanisms could be responsible for the reduction in AQP9 protein expression. Additional studies will be required to further clarify these possibilities.

Measurements of hepatic [U-^{14}C]glycerol uptake also showed some differences between *n*3-PUFA-depleted and control rats. The faster [U-^{14}C]glycerol uptake by hepatocytes from *n*3-PUFA-depleted rats resulted in a two-fold higher value in *n*3-PUFA-depleted versus control rats after 20 min incubation. Such differences may reflect a higher rate of gluconeogenesis in *n*3-PUFA-depleted rats versus normal animals, paralleling glucose intolerance and insulin resistance.

The apparent discrepancies between the decreased AQP9 protein level in *n*3-PUFA-depleted rats and increased [U-^{14}C]-glycerol uptake could be explained by intracellular glycerol metabolism. Indeed, in hepatocytes, insulin increases glucose uptake despite no change in GLUT-2 expression but merely because of increased glucose phosphorylation and incorporation into glycogen. GLUT-2 expression can be directly assessed by measuring the permeability using non-metabolized analogues, such as 3-O-methylglucose or deoxyglucose; unfortunately no such analogues exist for glycerol. Considering the large amount of glycerokinase activity in hepatocytes (40), glycerol metabolites may be generated in liver. Therefore, it is conceivable that the measured glycerol uptake reflects differences in intracellular metabolism rather than a modest modification of aquaglyceroporins expression at the cell membrane. This indeed seems to be the case as glycerol kinase activity, measured at substrate saturation for both Km of the enzyme, is significantly increased in *n*3-PUFA-depleted rats compared to control rats.

In conclusion, *n*3-PUFA-depleted rats exhibit decreased hepatic AQP9 protein levels, increased [U-^{14}C]glycerol uptake and increase glycerol kinase activity. Additional studies will be required to refine our understanding with respect to both the post-translational mechanisms regulating hepatic AQP9 protein, its cellular localisation, as well as the mechanisms controlling glycerol metabolism.

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