

Lipid peroxidation is not a prerequisite for the development of obesity and diabetes in high-fat-fed mice

Florence M. Sohet¹, Audrey M. Neyrinck¹, Evelyne M. Dewulf¹, Laure B. Bindels¹, Laurence Portois², Willy J. Malaisse², Yvon A. Carpentier², Patrice D. Cani¹ and Nathalie M. Delzenne^{1*}

¹Unit of Pharmacokinetics, Metabolism, Nutrition and Toxicology, Louvain Drug Research Institute, Université catholique de Louvain, Av. E. Mounier, 73/69, 1200 Brussels, Belgium

²Laboratory of Experimental Surgery, Université Libre de Bruxelles, Brussels, Belgium

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The mechanism, by which a high-fat (HF) diet could impair glucose metabolism, is not completely understood but could be related to inflammation, lipotoxicity and oxidative stress. Lipid peroxides have been proposed as key mediators of intracellular metabolic response. The purpose of the present study was to analyse, in mice fed with a HF diet, the possible association between obesity and glucose tolerance on the one hand, and between oxidative stress and lipid peroxidation on the other hand. The present results show that a HF diet (70% energy as fat), *v.* a high-carbohydrate chow diet (control), increases body weight and fat mass development, and impairs glycaemia and insulinaemia within 4 weeks. It also promotes the expression of NADPH oxidase in the liver – signing both oxidative and inflammatory stress – but decreases thiobarbituric acid-reactive substances content in the liver as well as in epididymal, subcutaneous and visceral adipose tissues. HF diet, with elevated vitamin E content, induces high concentration of α -tocopherol in liver and adipose tissues, which contributes to the protection against lipid peroxidation. Thus, lipid peroxidation in key organs is not necessarily related to the development of metabolic disorders associated with diabetes and obesity.

High-fat feeding: Lipid peroxidation: Inflammation: Vitamin E: Diabetes

The prevalence of diabetes and obesity is increasing worldwide with about 200 million diabetic and more than a billion overweight individuals in the world^(1,2). Oxidative stress is often considered to play an important role in the development of insulin resistance and complications of diabetes^(3,4). Antioxidants have been for long proposed as potentially useful compounds in the management of diabetes, since they may reduce the level of advanced glycation end products known to cause microvascular alterations (neuropathy, retinopathy and renal dysfunction)^(5,6). Increased content of fatty acids and their metabolites is also suggested to reduce insulin sensitivity in different tissues: muscle; liver; adipose tissue. This lipotoxicity could induce oxidative and inflammatory stress and consequently impair insulin signalling^(3,7). At a cellular level, oxidative stress consists of increased production of reactive oxygen species (ROS) through the intervention of enzymatic, non-enzymatic (hyperglycaemia, glucose oxidation) and/or mitochondrial pathways (mitochondrial respiratory chain, xanthine oxidase, cyclo-oxygenase, lipoxygenase, NADPH oxidase...)^(6,8). NADPH oxidase appears as a key ROS-producing enzyme in the background of the metabolic syndrome^(9,10). ROS interact with PUFA, producing reactive lipid by-products (lipoperoxide radicals, lipid peroxides and degradation products such as aldehydes), prone to interact with macromolecules such as proteins and DNA and leading to cellular dysfunction.

ROS production modulates intracellular kinase activities (Jun N-terminal kinase, NF- κ B...), leading to increased phosphorylation of Ser/Thr IR and insulin receptor substrate, and to a decreased Tyr phosphorylation of insulin receptor substrate, both resulting in impaired insulin action and glucose transport^(3,11). This link could serve as a rationale to assess the interest of antioxidant in the management of early phases of diabetes development (insulin resistance). Increased intake of vitamin E – which counteracts peroxy radical reactivity – have been largely proposed in this context^(6,12). Insulin resistance associated with obesity has been often studied in animal models receiving a high-fat (HF) diet^(13–15). It has been suggested that fat mass accretion promotes oxidative stress in the adipose tissue upon diet induced obesity in mice⁽¹⁶⁾. The present study aimed at assessing the potential relationship between lipid peroxidation, vitamin E status and glucose/insulin response in obese and diabetic mice fed a HF diet.

Experimental methods

Animals and diets

Ten-week-old male C57BL/6J mice (Charles River) were housed in groups of four mice per cage (eight per group) at 22°C with sequential light/dark cycle (lights off 18.00 hours;

Abbreviations: AUC, area under the curve; CT, control; HF, high fat; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance.

* **Corresponding author:** Nathalie M. Delzenne, fax +32 02 764 73 59, email nathalie.delzenne@uclouvain.be

lights on at 06.00 hours) and were given free access to diet and water. They were either fed a control (CT; AO4, SAFE, Villemois-sur-Orge, France) or a HF (SAFE) diet for 28 d. The CT diet contained the following (g/100 g dry diet): protein 19.3; total carbohydrate 70.4 (including starch 38, saccharose 3, cellulose 5 and non-digestible carbohydrates 8); lipid 3; vitamin 1.3; mineral mixture 6. The HF diet contained, in percentage of energy content, 72 % lipids (with proportion of 2.4:1 lard: maize oil), 28 % protein, and <1 % carbohydrates as described^(17,18). Food intake, taking into account spillage, was recorded weekly for a month. The mean value for the weekly assessment was calculated. At the end of experiment, retro-orbital vein blood samples were collected in EDTA tubes and plasma was stored at -80°C . Mice were killed by cervical dislocation after a 6 h period of fasting; fasting started at 07.30 hours and mice were killed from 13.30 hours. All mice experiments were approved by the local committee and the housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230314).

Oral glucose tolerance test

An oral glucose tolerance test was performed 3 weeks after the beginning of the feeding period^(14,15,19). Six-hour-fasted mice received an oral load of glucose of 3 g/kg body weight with a 660 g/l glucose solution; fasting started at 07.30 hours and glucose administration at 13.30 hours. Different parameters related to glucose homeostasis have been measured. Blood glucose was determined with a glucose meter (Roche Diagnostics Belgium, Vilvoorde, Belgium) on 3–5 μl of blood collected from the tip of the tail vein, 30 min before and 0, 15, 30, 60, 90 and 120 min following oral glucose administration. Plasma insulin was measured on 5 μl of plasma collected from tail blood at 0 and 15 min using an ELISA kit (Mercodia, Upssala, Sweden). The insulin resistance index was obtained by the multiplication of the area under the curve (AUC) of glucose between 0 and 15 min after glucose administration and the AUC of insulin between 0 and 15 min after glucose administration; its unit is $\text{mM}\mu\text{M}\text{min}^{2(18,20,21)}$. The AUC was calculated from basal glycaemia (30 min before glucose administration) up to 120 min after the glucose administration, with no correction of the area by the basal value.

Blood samples

TNF- α , IL-6, IL-1 α , IL-1 β , monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 α were determined in 25 μl of plasma using a Multiplex kit (Bio-Rad, Nazareth, Belgium) and measured by using Luminex technology (Bio-Plex SystemTM, Bio-Rad) according to the manufacturer's instructions.

Tissue samples

Fat pads (visceral, epididymal and subcutaneous) and liver were removed and immersed in liquid N_2 and stored at -80°C for further mRNA and/or lipid analysis. Full and empty caecum, liver, spleen and fat tissues were weighed. The caecal contents were collected and maintained at -80°C . Liver was excised: most of it was immediately

clamped in liquid N_2 and kept at -80°C for lipids and mRNA analysis. Total fat mass stands for the sum of epididymal, visceral and subcutaneous adipose tissues.

Tissues analyses

TAG, phospholipids and cholesterol were measured in the liver tissue after an extraction with chloroform–methanol according to Folch *et al.*⁽²²⁾. Tissue lysate was prepared in phosphate buffer by using Ultra-Turrax (IKA, T10 basic, Boutersem, Belgique), until complete tissue lysis. Lipids were extracted by mixing 125 μl lysates with 1 ml of chloroform–methanol (2:1). The chloroform phase was evaporated under nitrogen flux, and the dried residue was solubilised in 100 μl of isopropanol. Liver phospholipids (Wako, Brussels, Belgium), TAG and cholesterol (Elitech diagnostics, Brussels, Belgium) concentrations were measured using kits coupling enzymatic reaction and spectrophotometric detection of reaction end products.

Lipid peroxidation was evaluated by measuring thiobarbituric acid-reactive substances (TBARS). Tissue lysate was prepared in saline by using Ultra-Turrax (IKA), until complete tissue lysis. Aldehydes contained in tissue lysate reacted with thiobarbituric acid forming an aldehyde–thiobarbituric acid complex, which can be spectrophotometrically detected⁽²³⁾.

To analyse the fatty acid pattern of epididymal adipose tissue and diets, lipids were first extracted from epididymal adipose tissue and diets by the method of Folch *et al.*⁽²²⁾. The concentration of individual fatty acids was then analysed by GLC^(24,25). The internal standard used for fatty acids concentrations in total lipid extract was 15:0 at 100 mg/l solved in chloroform–methanol (2:1).

In order to measure liver glutathione, tissue proteins were aggregated using TCA and supernatant was then recuperated. *ortho*-Phthalaldehyde was added to supernatant and formed adducts with the glutathione contained in the supernatant. Those can then be detected by fluorimetry⁽²⁶⁾.

Vitamin E was extracted from tissues or diet with hexane. The concentration of vitamin E was then analysed by HPLC coupled with a Gilson fluorescent detector^(27,28).

Liver phosphorylated form of p-IGF-1R (Y1135/Y1136), p-Akt (S473), p-GSK-3 β (S9) and p-p70S6K (T421/S424) were measured using Luminex technology. Liver tissue were homogenised in lysis buffer (50 mM-2-amino-2-hydroxy-methyl-propane-1,3-diol, 1 % NP40, 5 mM-EDTA, 250 mM-NaCl, 50 mM-NaF, 1 mM- Na_3VO_4 , proteases inhibitors cocktail (Roche Diagnostics Belgium)). After 15 min on ice and centrifugation (13 000 g, 10 min, 4°C), supernatants were snap frozen and aliquots were stored at -80°C . Protein content of supernatants was determined using the Bradford method. The assay was performed by using a Multiplex kit (Akt pathway phospho7plex, BioSource, Nivelles, Belgium) through Luminex technology (Bio-Plex, Bio-Rad) according to the manufacturer's instructions.

Real-time quantitative PCR

Total RNA was isolated from subcutaneous adipose tissue and liver using TriPure isolation reagent kit (Roche Diagnostics Belgium). cDNA was prepared by reverse transcription of

1 µg total RNA using the Kit Reverse transcription System (Promega, Leiden, The Netherlands). Real-time PCR were performed with the StepOnePlus real-time PCR system and software (Applied Biosystems, Den IJssel, The Netherlands) using SYBR Green for detection. RPL19 RNA was chosen as a housekeeping gene. Primers and gene detailed are summarised in Table 1. All tissues were run in duplicate in a single 96-well reaction plate and data were analysed according to the $2^{-\Delta\text{CT}}$ method⁽¹⁵⁾. The identity and purity of the amplified product were checked through the analysis of the melting curve carried out at the end of amplification.

Statistical analysis

Results are presented as means with their standard errors. Statistical significance of differences was analysed using Student's *t* test. Correlations between parameters were assessed by Pearson's correlation test (GraphPad Software, San Diego, CA, USA; www.graphpad.com). $P < 0.05$ was considered as statistically significant.

Results

Effect of high-fat diet on obesity and metabolic alterations

Weekly energy intake was monitored during the 4 weeks of experience. CT and HF mice had the same food intake in g (total food intake during the 4 weeks of experiment – mean for four mice: 262 and 281 g for CT and HF groups, respectively) but HF-fed mice had an increased energy intake (total energy intake during the 4 weeks of experiment – mean for four mice: 208 and 397 kJ for CT and HF groups, respectively). Total body weight gain was significantly increased in HF mice (Table 2). Liver weight relative to the body weight was significantly decreased in HF mice, whereas spleen weight relative to the body weight was increased. Relative total body fat mass was significantly increased in HF mice (total fat mass – means with their standard errors in g: CT 3.5 (SE 0.1); HF 4.8 (SE 0.4); $P < 0.05$), with a significant effect shown for the epididymal tissue. Both caecal tissue and caecal content weights relative to the body weight were significantly reduced in HF animals.

HF mice exhibited a higher fasting glycaemia (Fig. 1(A)) when compared with CT, whereas fasting insulinaemia tend to be higher but such a difference failed to achieve significance (Fig. 1(B)). Fifteen minutes after oral glucose loading, insulinaemic response was strongly increased in HF mice (insulinaemia at 15 min – mean in pm: CT 279.4 (SE 13.22), HF 345.8 (SE 21.37); $P < 0.05$) and their glucose tolerance was significantly impaired (see AUC of glycaemia,

Fig. 1(D)). The insulin resistance index, AUC glucose 0–15 min \times AUC insulin 0–15 min mM pm min^2 – was significantly increased in HF mice (Fig. 1(C)). From those observations, we can confirm that HF diet increases body weight gain, energy intake and adiposity, and promotes glucose intolerance.

Modulations of tissue fatty acids and vitamin E content by high-fat diet

Liver lipid content (TAG, cholesterol and phospholipids) was not significantly modified by the dietary treatment (data not shown). The fatty acid pattern has been measured both in the diet and in the epididymal adipose tissue. Total fatty acid was approximately 20 times higher in HF diet compared with the CT one, all types of fatty acid (saturated, monounsaturated and polyunsaturated) being increased (Table 3). The HF diet was also characterised by a higher 18:0:16:0 ratio (Table 3). In the epididymal adipose tissue, total fatty acid concentration was not significantly modified by the diet. HF feeding significantly increased MUFA, and tended to decrease PUFA. Concerning specific modifications of tissue fatty acids, we observed a significant increase in both stearic (18:0) and oleic (18:1n-9) acids, whereas no modifications of palmitic acid (16:0) occurred (Table 3).

Vitamin E was strongly (more than 10-fold) increased in the HF v. CT diet. This dietary difference strongly modulated tissues' vitamin E concentrations, mostly in liver tissues of HF-fed animals (more than 5-fold the CT value) and to a lesser extent in the epididymal adipose tissue ($\times 1.5$; Table 4).

Effect of high-fat diet on tissue markers of inflammatory and oxidative stress

HF-fed mice showed a significant increase in plasma IL-1 α levels, without significant modifications in IL-1 β and TNF- α plasma concentrations (Table 5). Others peptides associated with inflammation and the metabolic syndrome, and namely secreted by the adipose tissue (monocyte chemoattractant protein-1, macrophage inflammatory protein-1 α and IL-6), reached similar serum concentrations in both groups (data not shown). mRNA levels of TNF- α , IL-1 α and also iNOS were measured in the liver and subcutaneous adipose tissue and showed no difference between groups (Table 5). CD68 mRNA levels were also measured as a marker of macrophages fixed in the liver and in subcutaneous adipose tissue, but no differences was detected between groups (Table 5). Phosphorylated proteins implicated in the insulin signalling were quantified in the liver tissue. As insulin signalling and NF κ B pathways interact, IGF-1R, Akt, GSK-3 β and p70S6K

Table 1. Primers sequences used for real-time quantitative PCR

	Forward primer (5' to 3')	Reverse primer (5' to 3')
CD68	CTTCCCACAGGCAGCACAG	AATGATGAGAGGCAGCAAGAGG
iNOS	AGGTACTIONCAGCGTGCTCCAC	GCACCGAAGATATCTTCATG
TNF α	TGGGACAGTGACCTGGACTGT	TTCGGAAAGCCCATTGAGT
IL1 β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
NADPH oxidase	TTGGGTCAGCACTGGCTCTG	TGGCGGTGTGCAGTGCTATC

iNOS, inducible NO synthase.

Table 2. Body and tissue weight in mice at the end of dietary treatments
(Mean values with their standard errors)

	CT		HF	
	Mean	SE	Mean	SE
Body weight (g)	23.82	0.28	24.63	0.39
Body weight gain (g)	0.98	0.27	1.90*	0.25
Liver (g/100 g body wt)	3.68	0.03	3.39*	0.09
Spleen (g/100 g body wt)	0.24	0.01	0.28*	0.01
Visceral adipose tissue (g/100 g body wt)	0.63	0.04	0.79	0.07
Epididymal adipose tissue (g/100 g body wt)	1.27	0.07	1.73*	0.13
Subcutaneous adipose tissue (g/100 g body wt)	1.65	0.07	2.30	0.31
Caecal tissue (g/100 g body wt)	0.43	0.02	0.27*	0.02
Caecal content (g)	0.21	0.02	0.12*	0.02

CT, control; HF, high fat.

Statistically significant differences between groups were determined by Student's *t* test (CT *n* 7, HF *n* 8): **P*<0.05.

can be seen as markers of inflammation. All were slightly – but not significantly – decreased in HF-fed mice (Table 5). HF diet thus increases plasma IL1- α levels, without modifying the other inflammatory parameters analysed.

Concerning the oxidative stress, HF diet significantly increases liver NADPH oxidase expression as compared with CT (Fig. 2), measured by the expression of Nox2 mRNA. Subcutaneous adipose tissue NADPH oxidase mRNA level was not affected by the treatment (data not shown). Glutathione was measured in the liver (glutathione– means with their

standard errors in nmol/mg prot: CT 7.0 (SE 0.4), HF 7.7 (SE 0.4) but no significant modification was observed. Strikingly, mice fed a HF diet exhibited a significant decrease in TBARS contents in the liver, the epididymal and subcutaneous adipose tissues (Fig. 3). TBARS closely and inversely correlates with vitamin E content in the liver and epididymal adipose tissue (Fig. 4).

Insulin resistance is often related to different markers of both inflammatory and oxidative stress. In the present study, insulin resistance index positively correlates with hepatic

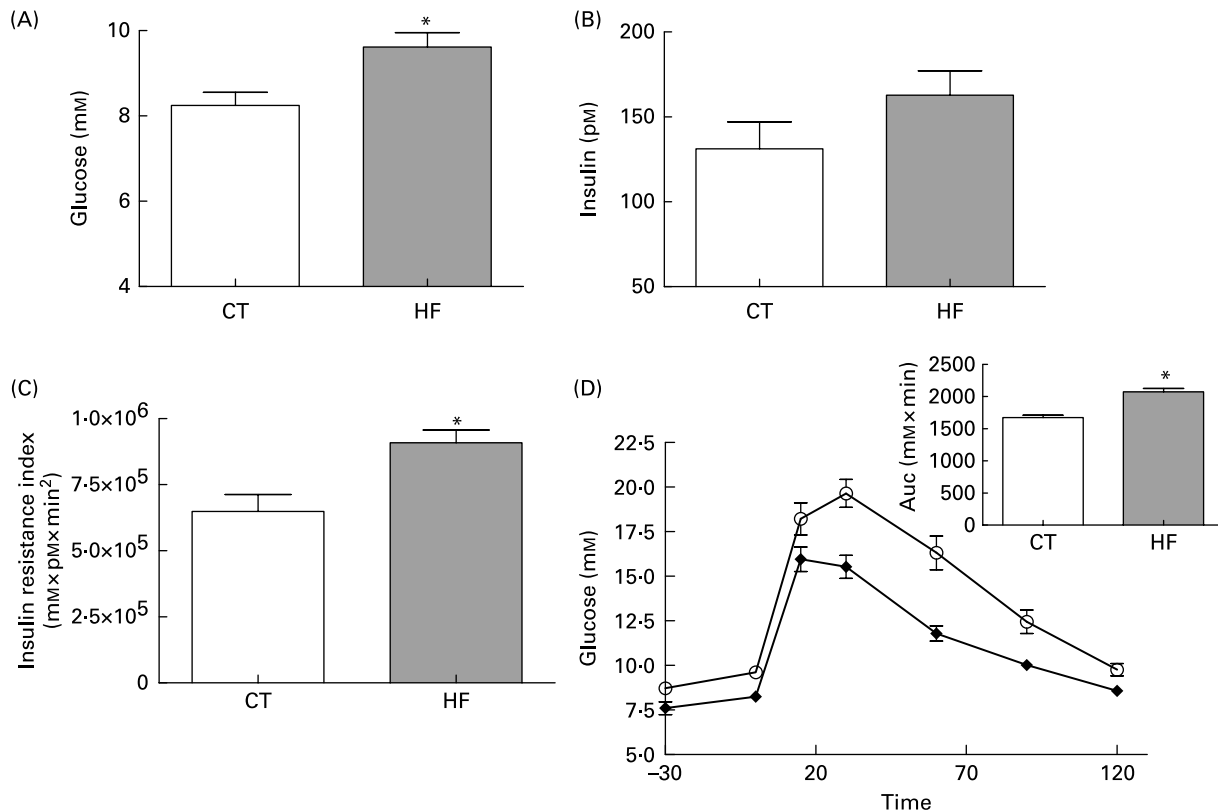


Fig. 1. Glucose homeostasis at the fasting state (before the glucose load) and upon oral glucose tolerance test (OGTT). (A) Fasting plasma glycaemia (mm); (B) fasting plasma insulinaemia (pM); (C) insulin resistance index (mm pM min⁻²); (D) evolution of glycaemia after glucose loading (mm, \blacklozenge control (CT); \circ high fat (HF)) and area under the curve (AUC) of glycaemia (inserts). Data are means with their standard errors (CT *n* 7, HF *n* 8). **P*<0.05.

Table 3. Summary of fatty acid (FA) pattern of control (CT) and high-fat (HF) diets and epididymal adipose tissue of mice at the end of dietary treatments

(Mean values with their standard errors)

	Diet (n 1, % total FA)		Adipose tissue (n 4, % total FA)			
	CT	HF	CT		HF	
			Mean	SE	Mean	SE
SFA	23.08	31.98	26.09	0.65	24.75	0.36
Including C16:0	16.19	21.47	21.79	0.58	19.79*	0.37
C18:0	2.14	8.99	1.78	0.06	3.19*	0.09
MUFA	17.56	39.04	37.81	0.69	44.10*	0.27
Including C18:1n9	16.54	37.00	31.65	0.63	40.07*	0.45
PUFA	59.36	28.98	36.10	0.28	31.15*	0.32
Including C18:2n6	55.70	27.34	35.74	0.28	30.83*	0.32

Statistically significant differences between groups were determined by Student's *t* test: **P*<0.05.**Table 4.** Vitamin E concentrations in diets and tissues of mice at the end of dietary treatments

(Mean values with their standard errors)

	CT		HF	
	Mean	SE	Mean	SE
Diet (µg/g diet, n 1)	14		153	
Liver tissue (µg/g tissue, CT n 7, HF n 8)	7.6	1.0	38.9*	2.1
Epididymal adipose tissue (µg/g tissue, CT n 7, HF n 8)	17.9	0.7	25.0*	2.5

CT, control; HF, high fat.

Statistically significant differences between groups were determined by Student's *t* test: **P*<0.05.**Table 5.** Inflammatory markers in blood, liver and subcutaneous adipose tissue at the end of dietary treatments

(Mean values with their standard errors)

	CT mice		HF mice	
	Mean	SE	Mean	SE
Blood				
IL1α (pg/ml)	63.55	15.01	144.70*	29.76
IL1β (pg/ml)	23.10	4.84	16.10	3.52
TNFα (pg/ml)	18.82	1.69	15.41	1.80
Liver				
IL1β (mRNA level, relative expression)	1.00	0.11	1.88	0.49
TNFα (mRNA level, relative expression)	1.00	0.09	1.41	0.41
CD68 (mRNA level, relative expression)	1.00	0.06	1.17	0.16
iNOS (mRNA level, relative expression)	1.00	0.26	0.79	0.22
p-Akt (units/mg total target protein)	17.53	2.30	15.34	0.91
p-P70S6K (units/mg total target protein)	27.13	2.84	20.46	2.10
p-GSK-3β (units/mg total target protein)	21.35	1.45	18.95	0.97
p-IGF-1R (units/mg total target protein)	8.43	1.01	6.98	0.49
Subcutaneous adipose tissue				
IL1β (mRNA level, relative expression)	1.00	0.22	1.32	0.24
TNFα (mRNA level, relative expression)	1.00	0.17	1.14	0.09
CD68 (mRNA level, relative expression)	1.00	0.18	0.88	0.10
iNOS (mRNA level, relative expression)	1.00	0.35	0.74	0.21

CT, control; HF, high fat.

Statistically significant differences between groups were determined by Student's *t* test (CT n 7, HF n 8): **P*<0.05.

NADPH oxidase mRNA level and negatively correlates with hepatic TBARS (Fig. 5).

Discussion

Mice fed a diet consisting of HF (70 % in energy) and protein exhibit strong alterations of insulin sensitivity⁽¹⁴⁾. In the present study, we clearly see that the HF diet disturbs glucose homeostasis, by increasing glycaemic response upon an oral glucose load, and by increasing fasting glycaemia. Could it be related to fatty acid composition of the diet? The profile of fatty acids seems important when taking into account the effect of a HF diet on metabolic disorders^(29–32). In the present study, the HF diet given to mice was characterised by an increase in all types of fatty acids (SFA, MUFA and PUFA), 18:0 becoming important as a source of saturated fat. In the adipose tissue, we observe a huge increase in 18:0 and 18:1n-9 fatty acids, with minor modifications of other types of major fatty acids. The increase in 18:1n-9 could be related to an increase in dietary intake or due to the desaturation of 18:0, namely through stearyl-CoA desaturase-1 activity. In fact, an increase in stearyl-CoA desaturase-1 activity is associated with insulin resistance⁽²⁹⁾. On the other hand, an increase in MUFA improves the insulin sensitivity in the postprandial state⁽³¹⁾. Therefore, it is rather difficult to clearly state that the modifications of fatty acid profile explain *per se* the metabolic alterations upon a HF diet.

Several data suggest that oxidative and inflammatory stresses are implicated in the alterations of insulin response, and therefore promote diabetes associated with obesity^(3,33,34). This phenomenon is related to endotoxaemia, which promotes fat mass development, insulin resistance by a CD14/TLR-dependent mechanism⁽¹⁵⁾. Here, we show

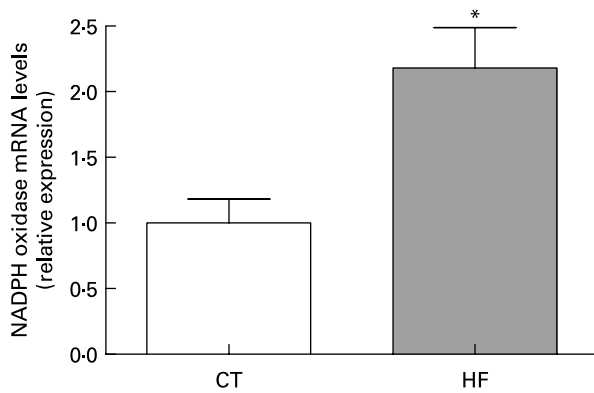


Fig. 2. Liver NADPH oxidase mRNA levels measured at the end of dietary treatments. Data are means with their standard errors (control (CT) n 7, high fat (HF) n 8). * P < 0.05.

that there is only a small increase in specific proinflammatory cytokines – namely IL-1 α – in the serum of HF-fed mice, and there is no modification of all other inflammatory markers measured either in the serum, the adipose tissue or in the liver. The role of IL-1 α , which increases upon metabolic endotoxaemia⁽¹⁷⁾ in the pathogenesis of diabetes, remains poorly studied up to now, but it would merit attention, since it is also correlated with the modulation of the gut microflora, a new target associated with obesity in the HF-fed mice models⁽¹⁷⁾. Anyway, we show here that HF diet does not necessarily lead to a significant inflammation process, despite it is able to disturb glucose homeostasis.

Besides inflammatory burst clearly associated with the disruption of insulin signalling^(33,34), ROS production also appears as an important phenomenon in glucose homeostasis alterations associated with fat mass development. This relation has been largely studied in the adipose tissue, where a key role for NADPH oxidase in oxidative burst has been proposed⁽¹⁶⁾.

Here, we show that NADPH oxidase mRNA is also increased in the liver tissue of HF-fed diabetic animals, a phenomenon correlated with insulin resistance index, and which is not related to lipid accumulation in the liver tissue. The relevance of the increased NADPH oxidase expression in the production of active ROS such as superoxide anion remains to be proven, as well as the mechanism involved in the increase in NADPH oxidase expression in the liver and adipose tissue upon HF feeding. However, the increase in NADPH oxidase expression could not be directly linked to the amount of lipid peroxides in the liver and adipose tissue of HF-fed mice. Strikingly, in the present model, some features of oxidative stress are decreased upon HF feeding since lower liver peroxidation (TBARS) was observed both in the liver and in the adipose tissues. Furthermore, there is no modification of GSH content or endogenous peroxidase activity – assessed by histochemical analysis – data not shown – in the liver tissue upon dietary treatment. Only a slight decrease in adipose tissue PUFA is observed in HF-fed animals, as compared with the huge decrease in lipid peroxides. Even if we may not exclude the fact that it participates to the decrease in lipid peroxidation, we can reasonably assume that the decrease in PUFA may not constitute the sole explanation.

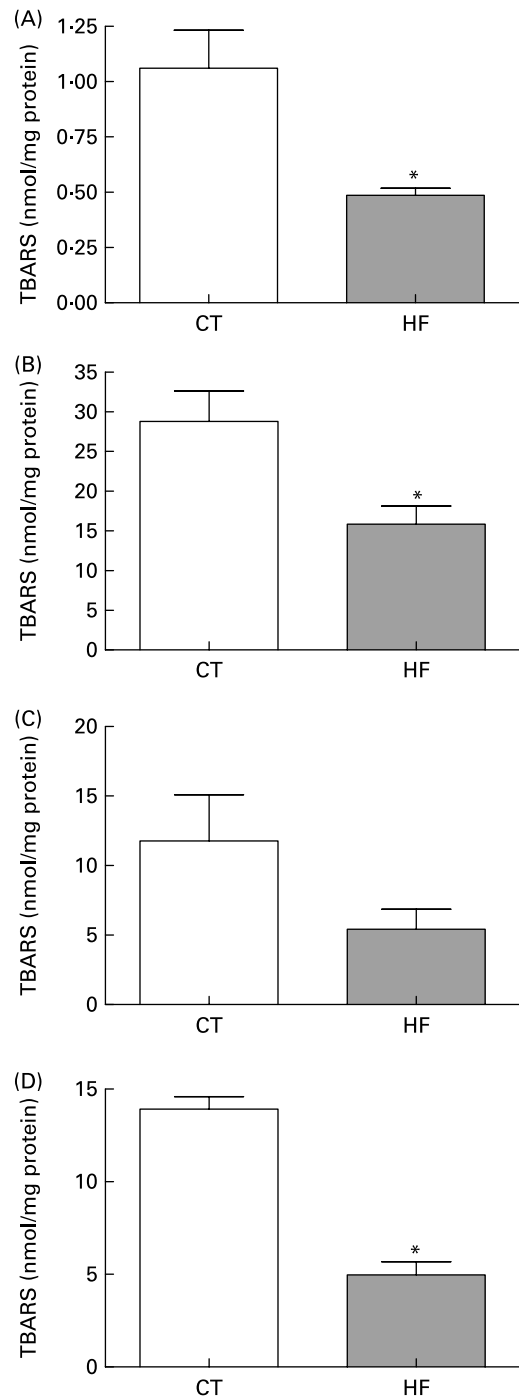


Fig. 3. Thiobarbituric acid-reactive substance (TBARS) levels in different tissues – (A) liver, (B) epididymal, (C) visceral and (D) subcutaneous adipose tissues – in mice at the end of dietary treatments. Data are means with their standard errors (control (CT) n 7, high fat (HF) n 8). * P < 0.05.

Arsov *et al.*⁽³⁵⁾ have also demonstrated that TBARS are lower in a model of HF diet-induced steatosis. Here, we propose that this ‘protection’ against lipid peroxidation is merely due to the high content of vitamin E in the HF diet, leading to a huge increase in vitamin E content in the liver tissue, and in a less extent, in the adipose tissue. It is important to note that vitamin E is the most potent peroxyl radical scavenger among nutrients⁽¹²⁾.

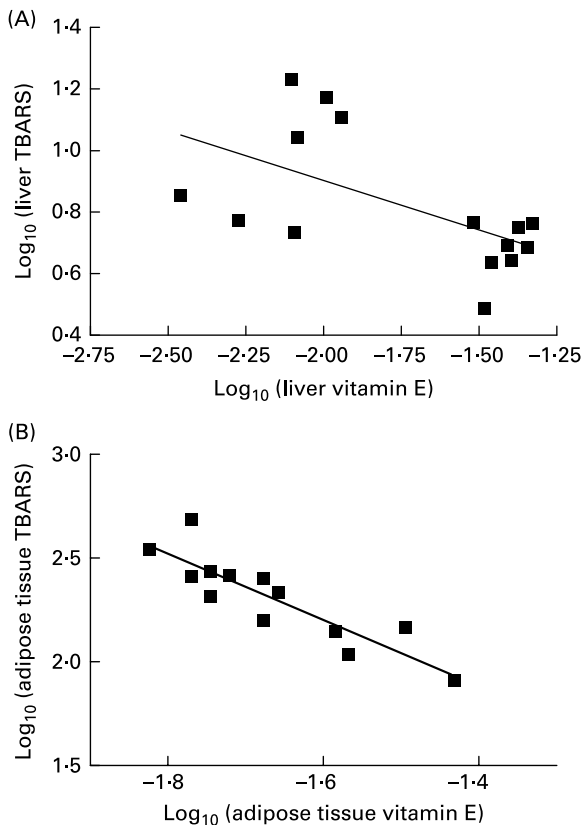


Fig. 4. Correlations between tissue vitamin E and thiobarbituric acid-reactive substances (TBARS): (A) in the liver and (B) in the epididymal adipose tissue. Vitamin E and TBARS levels are expressed as \log_{10} (liver n 15, adipose tissue n 13). (A) $r = -0.5824$, $P = 0.0227$; (B) $r = -0.8805$, $P < 0.0001$.

We may thus conclude that lipid peroxides are not implicated in the metabolic alterations causing insulin resistance and diabetes in this dietary model, but could contribute to the occurrence of other features associated with obesity, namely low tone inflammation. Interestingly, other authors had shown that another marker of lipid peroxidation – namely isoprostanes measured in the urines – is not involved in the development of glucose intolerance in human beings⁽³⁶⁾. The lack of association between lipid peroxidation and metabolic disturbances must be taken into consideration in the nutritional advices devoted to counteract or to prevent diabetes. In fact, the clinical trials conducted to date failed to provide adequate support for the use of vitamin E in diabetes⁽⁶⁾.

The production of reactive species remains, namely through NADPH oxidase, important in the perturbation of insulin signalling in different tissues, including the liver^(3,37,38). In that context, in addition to the NADPH oxidase, mitochondrial and endoplasmic reticulum stresses may be considered as important targets to take into account when assessing the effect of interesting nutrients or nutritional approach to counteract diabetes associated with obesity^(39,40). The present study also supports the fact that the content of vitamin E – or other antioxidants? – in experimental diets could be taken into account when assessing some specific features associated with obesity and metabolic disorders (inflammation and oxidative stress).

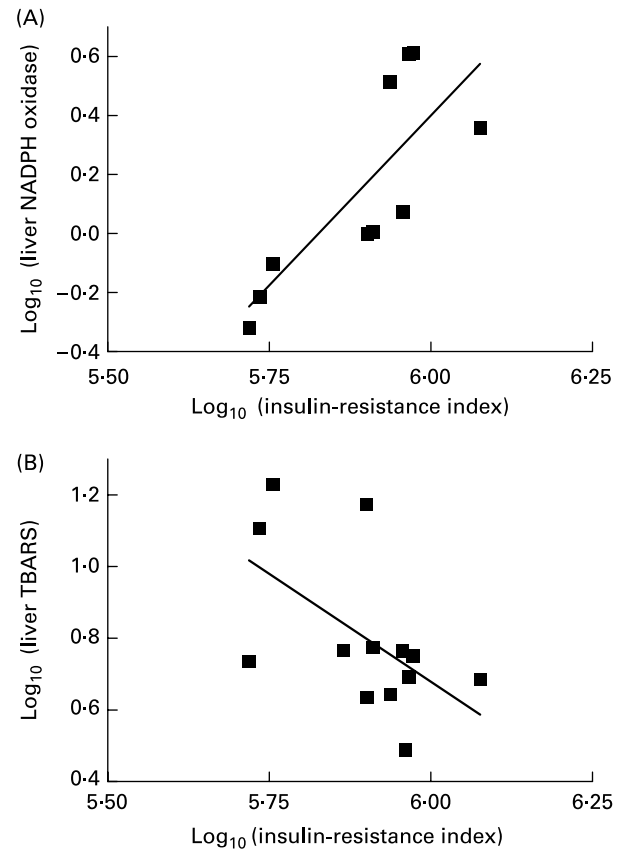


Fig. 5. Correlations between insulin resistance index and: (A) liver NADPH oxidase mRNA, (B) liver thiobarbituric acid-reactive substances (TBARS). Insulin resistance index, NADPH oxidase and TBARS levels are expressed as \log_{10} (A) n 10, (B) n 13. (A) $r = 0.7912$, $P = 0.0064$; (B) $r = -0.5616$, $P = 0.0458$.

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References

1. Kahn SE, Hull RL & Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **444**, 840–846.
2. Yach D, Stuckler D & Brownell KD (2006) Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nat Med* **12**, 62–66.

3. Evans JL, Goldfine ID, Maddux BA, *et al.* (2002) Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* **23**, 599–622.
4. Maritim AC, Sanders RA & Watkins JB III (2003) Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* **17**, 24–38.
5. Grattagliano I, Vendemiale G, Boscia F, *et al.* (1998) Oxidative retinal products and ocular damages in diabetic patients. *Free Radic Biol Med* **25**, 369–372.
6. Fardoun RZ (2007) The use of vitamin E in type 2 diabetes mellitus. *Clin Exp Hypertens* **29**, 135–148.
7. Schrauwen P (2007) High-fat diet, muscular lipotoxicity and insulin resistance. *Proc Nutr Soc* **66**, 33–41.
8. Poli G, Leonarduzzi G, Biasi F, *et al.* (2004) Oxidative stress and cell signalling. *Curr Med Chem* **11**, 1163–1182.
9. Roberts CK, Barnard RJ, Sindhu RK, *et al.* (2006) Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome. *Metabolism* **55**, 928–934.
10. Grattagliano I, Palmieri VO, Portincasa P, *et al.* (2007) Oxidative stress-induced risk factors associated with the metabolic syndrome: a unifying hypothesis. *J Nutr Biochem* **19**, 491–504.
11. Evans JL (2007) Antioxidants: do they have a role in the treatment of insulin resistance? *Indian J Med Res* **125**, 355–372.
12. Traber MG & Atkinson J (2007) Vitamin E, antioxidant and nothing more. *Free Radic Biol Med* **43**, 4–15.
13. Burcelin R, Crivelli V, Dacosta A, *et al.* (2002) Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. *Am J Physiol Endocrinol Metab* **282**, E834–E842.
14. Cani PD, Knauf C, Iglesias MA, *et al.* (2006) Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. *Diabetes* **55**, 1484–1490.
15. Cani PD, Amar J, Iglesias MA, *et al.* (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761–1772.
16. Furukawa S, Fujita T, Shimabukuro M, *et al.* (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* **114**, 1752–1761.
17. Cani PD, Neyrinck AM, Fava F, *et al.* (2007) Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* **50**, 2374–2383.
18. Cani PD, Bibiloni R, Knauf C, *et al.* (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57**, 1470–1481.
19. Knauf C, Cani PD, Perrin C, *et al.* (2005) Brain glucagon-like peptide-1 increases insulin secretion and muscle insulin resistance to favor hepatic glycogen storage. *J Clin Invest* **115**, 3554–3563.
20. Lin HY, Yu IC, Wang RS, *et al.* (2008) Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. *Hepatology* **47**, 1924–1935.
21. Guerre-Millo M, Rouault C, Poulain P, *et al.* (2001) PPAR-alpha-null mice are protected from high-fat diet-induced insulin resistance. *Diabetes* **50**, 2809–2814.
22. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.
23. Esterbauer H & Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* **186**, 407–421.
24. Lepage G & Roy CC (1986) Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res* **27**, 114–120.
25. Dahlan W, Richelle M, Kulapongse S, *et al.* (1992) Effects of essential fatty acid contents of lipid emulsions on erythrocyte polyunsaturated fatty acid composition in patients on long-term parenteral nutrition. *Clin Nutr* **11**, 262–268.
26. Hissin PJ & Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* **74**, 214–226.
27. Hatam LJ & Kayden HJ (1979) A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. *J Lipid Res* **20**, 639–645.
28. Traber MG & Kayden HJ (1989) Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins. *Am J Clin Nutr* **49**, 517–526.
29. Riserus U (2008) Fatty acids and insulin sensitivity. *Curr Opin Clin Nutr Metab Care* **11**, 100–105.
30. Calder PC & Deckelbaum RJ (2008) Omega-3 fatty acids: time to get the messages right! *Curr Opin Clin Nutr Metab Care* **11**, 91–93.
31. Lopez S, Bermudez B, Pacheco YM, *et al.* (2008) Distinctive postprandial modulation of beta cell function and insulin sensitivity by dietary fats: monounsaturated compared with saturated fatty acids. *Am J Clin Nutr* **88**, 638–644.
32. Summers LK, Barnes SC, Fielding BA, *et al.* (2000) Uptake of individual fatty acids into adipose tissue in relation to their presence in the diet. *Am J Clin Nutr* **71**, 1470–1477.
33. Wellen KE & Hotamisligil GS (2005) Inflammation, stress, and diabetes. *J Clin Invest* **115**, 1111–1119.
34. Shoelson SE, Lee J & Goldfine AB (2006) Inflammation and insulin resistance. *J Clin Invest* **116**, 1793–1801.
35. Arsov T, Larter CZ, Nolan CJ, *et al.* (2006) Adaptive failure to high-fat diet characterizes steatohepatitis in Alms1 mutant mice. *Biochem Biophys Res Commun* **342**, 1152–1159.
36. Boyne MS, Sargeant LA, Bennett FI, *et al.* (2007) Isoprostanol, a marker of lipid peroxidation, may not be involved in the development of glucose intolerance. *Diabetes Res Clin Pract* **76**, 149–151.
37. Milagro FI, Campion J & Martinez JA (2006) Weight gain induced by high-fat feeding involves increased liver oxidative stress. *Obesity (Silver Spring)* **14**, 1118–1123.
38. Cave M, Deaciuc I, Mendez C, *et al.* (2007) Nonalcoholic fatty liver disease: predisposing factors and the role of nutrition. *J Nutr Biochem* **18**, 184–195.
39. Hotamisligil GS (2005) Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes. *Diabetes* **54**, Suppl. 2, S73–S78.
40. Rolo AP & Palmeira CM (2006) Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol* **212**, 167–178.