

## Feeding of a deep-fried fat causes PPAR $\alpha$ activation in the liver of pigs as a non-proliferating species

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Recent studies have shown that dietary oxidised fats influence the lipid metabolism in rats by activation of PPAR $\alpha$ . In this study, we investigated whether a mildly oxidised fat causes activation of PPAR $\alpha$  in pigs which are non-proliferators like man. Eighteen pigs were assigned to two groups and received either a diet containing 90 g/kg of a fresh fat or the same diet with 90 g/kg of an oxidised fat prepared by heating for 24 h at 180°C in a deep fryer. Pigs fed the oxidised fat had a higher peroxisome count, a higher activity of catalase and a higher mRNA concentration of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in the liver and a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat ( $P < 0.05$ ). Hepatic mRNA concentrations of acyl-CoA oxidase and carnitine palmitoyltransferase-1 tended to be increased in pigs fed the oxidised fat compared to pigs fed the fresh fat ( $P < 0.10$ ). Pigs fed the oxidised fat, moreover, had higher mRNA concentrations of sterol regulatory element-binding protein (SREBP)-1 and its target genes acetyl-CoA carboxylase and stearoyl-CoA desaturase in the liver and higher mRNA concentrations of SREBP-2 and its target genes 3-hydroxy-3-methylglutaryl-CoA reductase and LDL receptor in liver and small intestine. In conclusion, this study shows that even a mildly oxidised fat causes activation of PPAR $\alpha$  in the liver of pigs. Up-regulation of SREBP and its target genes in liver and small intestine suggests that the oxidised fat could stimulate synthesis of cholesterol and TAG in these tissues.

### Oxidised fat: Pig: PPAR $\alpha$ : Cholesterol: Triacylglycerols

The typical western diet contains large quantities of PUFA that are heated or processed to varying degrees. In fast-food restaurants fat is heated in fryers for up to 18 h daily, at temperatures close to 180°C (Frankel *et al.* 1984). Several studies with animals have been performed to investigate the effects of oxidised fats on the metabolism (reviewed in Cohn, 2002). Recently, it has been shown in rats that oxidised fats are able to influence the lipid metabolism by activation of PPAR $\alpha$  (Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004), a transcription factor belonging to the nuclear hormone receptor superfamily (Schoonjans *et al.* 1996). This is probably due to the occurrence of hydroxy- and hydroperoxy fatty acids such as hydroxy octadecadienoic acid and hydroperoxy octadecadienoic acid which are potent activators of PPAR $\alpha$  (Delerive *et al.* 2000; Mishra *et al.* 2004; König & Eder, 2006). Activation of PPAR $\alpha$  leads to an increase in the transcription of genes related to fatty acid transport across the cell membrane, intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake, and both mitochondrial and peroxisomal fatty acid  $\beta$ -oxidation, gluconeogenesis and ketogenesis

(Mandard *et al.* 2004). Recently, it has been shown that PPAR $\alpha$  activation influences also the expression or the proteolytic activation of sterol regulatory element-binding proteins (SREBP), transcription factors which control fatty acid synthesis and cholesterol homeostasis (Patel *et al.* 2001; Guo *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). Therefore, PPAR $\alpha$  activation stimulates not only the degradation of fatty acids by enhancing  $\beta$ -oxidation but affects also the synthesis of cholesterol and TAG. Reduced liver and plasma concentrations of TAG and cholesterol are typical effects observed in animals treated with PPAR $\alpha$  agonists, and such effects have been also observed in rats administered oxidised fats (Huang *et al.* 1988; Eder & Kirchgessner, 1998; Eder, 1999; Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004).

Regarding the expression of PPAR $\alpha$  in tissues and the effects of PPAR $\alpha$  activation on transcription of its target genes, there are great differences between various species. In rodents, PPAR $\alpha$  is highly expressed, and activation of PPAR $\alpha$  not only induces many genes involved in various metabolic pathways such as  $\beta$ -oxidation, ketogenesis and

**Abbreviations:** ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7 $\alpha$ -hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TBARS, thiobarbituric acid-reactive substances.

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gluconeogenesis but also causes severe peroxisome proliferation in the liver (Peters *et al.* 2005). In contrast to rodents, PPAR $\alpha$  agonists do not induce peroxisome proliferation in the liver of many other species, such as guinea pigs, swine, monkeys and man (Holden & Tugwood, 1999). These non-proliferating species have a lower expression of PPAR $\alpha$  in the liver and the response of many genes to PPAR $\alpha$  activation is much weaker than in proliferating species. For that reason, effects related to PPAR $\alpha$  activation observed in rodents cannot be directly applied for non-proliferating species such as man. Therefore, it remains unknown whether oxidised fats are able to cause PPAR $\alpha$  activation also in non-proliferating species.

The aim of the present study was to investigate whether a dietary oxidised fat, prepared by heating sunflower oil under usual deep-frying conditions (180°C) for 24 h in a deep fryer, is able to activate PPAR $\alpha$  and to cause peroxisome proliferation in pigs. Pigs have been chosen as a model since they belong – like man – to the non-proliferating species (Yu *et al.* 2001; Peffer *et al.* 2005) and since pig liver cells show a similarity to human liver cells in the gene response to PPAR $\alpha$  agonists (Cheon *et al.* 2005). We focused our analyses on liver and small intestine as both tissues exhibit a high expression of PPAR $\alpha$  (Braissant *et al.* 1996; Lemberger *et al.* 1996). Moreover, both tissues play an important role in whole body lipid homeostasis, i.e. synthesis and secretion of lipoproteins rich in TAG and cholesterol (Lindsay & Wilson, 1965; Dietschy *et al.* 1993). We examined the expression of various genes involved in lipid metabolism which have been already shown to be influenced by PPAR $\alpha$  activation. Furthermore, in both tissues we determined gene expression of SREBP and important SREBP target genes involved in fatty acid synthesis and cholesterol uptake and synthesis.

## Materials and methods

### Animals

For the experiment, eighteen male 8-week-old crossbred pigs ((German Landrace  $\times$  Large White)  $\times$  Pietrain) were kept in a room under controlled temperature at  $23 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$  relative humidity with light from 06.00 to 18.00 hours. One day before the beginning of the experimental feeding period, the pigs were weighed and randomly allocated to two groups with body weights of 12.0 (SD 1.1) kg in the control group and 12.2 (SD 0.9) kg in the treatment group. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

### Diets and feeding

Both groups of pigs received a nutritionally adequate diet for growing pigs containing (in g/kg) wheat (400), soyabean meal (230), wheat bran (150), barley (100), sunflower oil or test oil (90), and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14.4 MJ metabolisable energy and 185 g crude protein/kg. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. During the feeding period, the amount of diet offered each day was increased

continuously from 400 to 1200 g. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 d.

### Preparation of the test fats

To prepare the oxidised fat, sunflower oil obtained from a local supermarket was heated at a temperature of 180°C for 24 h in a deep fryer. This treatment caused a loss of PUFA and tocopherols. The major fatty acids in the fresh and the oxidised fat, respectively, were (g/100 g total fatty acids): palmitic acid (16:0), 6.30 v. 6.70; stearic acid (18:0), 4.0 v. 4.2; oleic acid (18:1*n*-9), 22.8 v. 23.8; linoleic acid (18:2*n*-6), 63.6 v. 59.9. Other fatty acids were present only in small amounts (<0.5 g/100 g fatty acids). To equalise the fatty acid composition of the fresh and the oxidised fat, the fresh fat was composed of a mixture of sunflower oil and palm oil (93:7, w/w). To adjust dietary vitamin E concentrations, we analysed the native concentrations of tocopherols in the fresh fat and in the oxidised fats after the thermal treatment. With consideration of the native tocopherol concentrations of the dietary fats, the diets were supplemented individually with all-*rac*- $\alpha$ -tocopheryl acetate (the biopotency of all-*rac*- $\alpha$ -tocopheryl acetate is considered to be 67% of that of  $\alpha$ -tocopherol). The final vitamin E concentration was 620 mg  $\alpha$ -tocopherol equivalents/kg in both fats. Concentrations of lipid peroxidation products were determined after the fats have been already included into the diets. Therefore, lipids of the diets were extracted by *n*-hexane and isopropanol (3:2, v/v; Hara & Radin, 1978). Concentration of thiobarbituric acid-reactive substances (TBARS; Sidwell *et al.* 1954), conjugated dienes (Recknagel & Glende, 1984), peroxide value (Deutsche Gesellschaft für Fettwissenschaft, 1994), acid value (Deutsche Gesellschaft für Fettwissenschaft, 1994) and concentration of total carbonyls (Endo *et al.* 2001) were determined in the extracted fat.

### Sample collection

After completion of the feeding period the animals were killed under light anaesthesia. Each pig was fed its respective diet 4 h before being killed. After killing, blood was collected into heparinised polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 g, 10 min, 4°C). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900 000 g at 4°C for 1.5 h. Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions  $\delta < 1.006$  kg/l VLDL plus chylomicrons,  $1.006 < \delta < 1.063$  kg/l LDL and  $\delta > 1.063$  kg/l HDL were removed by suction. The liver was dissected and weighted and samples were stored at  $-80^\circ\text{C}$  until analysis. For preparation of liver homogenate, 1 g liver tissue was homogenised in PBS by TissueLyser (Qiagen, Haan, Germany), centrifuged at 600 g for 10 min at 4°C and the supernatant was stored at  $-20^\circ\text{C}$  until analysis. For isolation of intestinal epithelial cells, the abdomen was immediately opened after killing, and a 35 cm intestinal segment was dissected starting at 30 cm distal to the pyloric sphincter, and flushed twice with ice-cold wash buffer (PBS containing 0.2 mM-phenylmethylsulphonyl fluoride and 0.5 mM-dithiothreitol, pH 7.4).

The isolation of porcine intestinal epithelial cells was performed by the modified distended intestinal sac technique according to Fan *et al.* (2004). In brief, the intestinal segments were filled with 100 ml preincubation buffer (PBS containing 27 mM-sodium citrate, 0.2 mM-phenylmethylsulphonyl fluoride and 0.5 mM-dithiothreitol, pH 7.4), sealed with strings and filled intestinal segments were incubated in a saline bath (154 mM-NaCl) for 15 min at 37°C. Afterwards, the pre-incubation buffer was discarded, and the intestinal segments were filled with isolation buffer (PBS containing 1.5 mM- $\text{Na}_2\text{EDTA}$ , 0.2 mM-phenylmethylsulphonyl fluoride, 0.5 mM-dithiothreitol and 2 mM-D-glucose, pH 7.4). Two major cell fractions, consisting of the upper and the crypt cell fraction, were sequentially isolated from intestinal segments through two consecutive incubations with isolation buffer at 37°C for 40 (upper cell fraction) and 60 min (crypt cell fraction), respectively. Each cell fraction was collected separately, and washed twice with ice-cold PBS. Afterwards, cells were retained by centrifugation (400g, 4 min, 4°C) and immediately frozen at -80°C. For further analysis, we used the crypt cell fraction as it has been shown that these cells have a 6–8-fold higher capacity of lipid synthesis than villus cells (Shakir *et al.* 1978).

#### Lipid analysis

Lipids from liver were extracted with a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids were dissolved using Triton X-100 (De Hoff *et al.* 1978). Concentrations of TAG and cholesterol in plasma and lipoproteins and those of liver were determined using enzymatic reagent kits (cat. no. 113009990314 for cholesterol and cat. no. 157609990314 for TAG; Ecoline S<sup>+</sup>, DiaSys, Holzheim, Germany).

#### Preparation of liver microsomal and cytosolic fractions

Liver (1 g) was homogenised in 10 ml 0.1 M-phosphate buffer, pH 7.4, containing 0.25 M-sucrose using a Potter-Elvehjem homogeniser. Homogenates were centrifuged at 1000g for 10 min at 4°C, and the supernatant was centrifuged at 15 000g for a further 15 min. The microsomal pellet was obtained by centrifugation of the 15 000g supernatant at 105 000g for 60 min. The resulting cytosolic fraction in the supernatant was separated, microsomal pellets were resuspended in the homogenisation buffer and all samples were stored at -20°C for further analysis. The protein concentrations of cytosolic and microsomal fractions were determined with the BCA reagent according to the protocol of the supplier (Interchim, Montelucon, France) using bovine serum albumin as standard.

#### RT-PCR analysis

Total RNA from liver tissue and enterocytes, respectively, was isolated by the TissueLyser (Qiagen) using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm (SpectraFluor Plus; Tecan, Crailsheim, Germany). The quality of all RNA samples was furthermore assessed by agarose gel electrophoresis.

Total RNA (1.2 µg) was used for cDNA synthesis as described previously (König & Eder, 2006). The mRNA concentration of genes was measured by real-time detection PCR using SYBR<sup>®</sup> Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1.25 U Taq DNA polymerase, 500 µM-dNTP and 26.7 pmol of the specific primers. For determination of mRNA concentration a threshold cycle ( $C_t$ ) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the  $\Delta\Delta C_t$  method as previously described (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalisation. The PCR primers used for real-time RT-PCR were obtained from Operon (Köln, Germany) and Roth (Karlsruhe, Germany), respectively, and are listed in Table 1.

#### Enzyme assays

Superoxide dismutase (SOD) activity in liver cytosol was determined according to the method of Marklund & Marklund (1974) with pyrogallol as the substrate. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. The activity of glutathione peroxidase in liver cytosol was determined with *t*-butyl hydroperoxide as substrate according to the method of Paglia & Valentine (1967). One unit of glutathione peroxidase activity is defined as 1 µmol reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate oxidised/min. The activity of glutathione *S*-transferase was determined using 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* (1974). One unit of glutathione *S*-transferase is defined as one nmol substrate consumed/min. Catalase activity in liver homogenate was determined using  $\text{H}_2\text{O}_2$  as substrate according to the method of Aebi (1986). One unit of catalase activity is defined as the amount consuming 1 mmol  $\text{H}_2\text{O}_2$ /min.

#### Determination of conjugated dienes, thiobarbituric acid-reactive substances and $\alpha$ -tocopherol

Lipids from liver were extracted using a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). After drying the lipid extracts, 1 mg extract was dissolved in 1 ml *n*-hexane. The concentrations of conjugated dienes were calculated by using the molar extinction coefficient for conjugated dienes at 234 nm ( $\epsilon = 29\,500$  mol/cm). The concentrations of TBARS were measured in liver homogenates as described (Brandsch *et al.* 2002). The concentration of  $\alpha$ -tocopherol in liver tissue was determined by HPLC (Brandsch *et al.* 2002).

#### Determination of $\text{H}_2\text{O}_2$

To determine the  $\text{H}_2\text{O}_2$  content in liver homogenates, the method for cell culture systems described by Royall & Ischiropoulos (1993) was modified, using dihydrorhodamine 123 as substrate. Homogenates were incubated with 27.5 µM-dihydrorhodamine 123 for 1 h at 37°C in a final volume of 400 µl. After incubation, the fluorescence of rhodamine 123, the oxidation product of dihydrorhodamine 123, was measured (excitation wavelength 485 nm, emission wavelength 538 nm).

**Table 1.** Characteristics of the specific primers used for RT–PCR analysis

Gene	Forward primer (from 5' to 3')	Reverse primer (from 5' to 3')	bp	Annealing temp. (°C)	NCBI GenBank
ACC	CTCCAGGACAGCACAGATCA	GCCGAAACATCTCTGGGATA	170	60	AF175308
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218	60	AF185048
apo CIII	GACACCTCCCTTCTGGACAA	TCCCAGAAGTCGGTGAACCT	185	60	NM_001002801
CPT-1	GCATTTGTCCCATCTTTCGT	GCACTGGTCCCTTCTGGGATA	198	60	AF288789
CYP7	TATAGGGCACGATGCACAGA	ACCTGACCAGTCCGAGATG	200	60	NM_001005352
FAS	GAACACGGCCTAGAAGTGG	ATCTGGATCCTGCAGATGG	199	62	NM_213839
FATP	GGTTCAGCCTGTTGAATGT	AACAAAACCTTGGTGCTTGG	275	60	DQ192231
FDPS	GAAAGGCAGGATTTTCATCCA	AGAAGGCTTGGAGCAGTTCA	259	60	AY609787
GAPDH	AGGGGCTCTCCAGAATCATCC	TCGCGTGCTCTTGTGGGGTTGG	446	60	AF017079
Glutathione peroxidase	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCCGGTCA	190	60	NM_214201
Glutathione S-transferase	TTTTTGCCAACCCAGAAGAC	GGGGTGTCAAATACGCAATC	246	60	NM_214300
HMG-CoA-R	GGTCAGGATGCGGCACAGAACG	GCCCCACGGTCCCAGTCTCTATG	127	65	S79678
I-FABP	TACAGCCTCGCAGACGGAACG	TGCTTGATGAGGAGAGGAAAACAG	276	59	AY960624
Insig-1	AGAGGGAGTGGGCCAGTGTGATGC	ACGGGAGCCAGGAGCGGATGTAG	276	65	AY336601
Insig-2	AAATCACGCCAGCGCTAAAGTG	TCCTACTCCAAGGCCAAAACCAC	127	60	AY585269
LDL receptor	AGAACTGGCGGCTGAAGAGCATC	GAGGGGTAGGTGTAGCCGTCCTG	115	60	AF118147
L-FABP	TTCGGTGCATGTCTAAGCTG	TGAGAGGGAGAGGATGAGGA	200	60	DQ182323
mAAT	TATGTCACCGTGCAGACCAT	CTCCTTCCACTGCTCAGGAC	309	60	M11732
mHMG-CoA-S	GGACCAAACAGACCTGGAGA	ATGGTCTCAGTGCCCACTTC	198	62	U90884
MTP	CAGGACGGCAAAGAAAGAAGG	ATGGGAAGCAAACCACAAGG	199	60	AY217034
NPC1	ACGCGGTATCTTTGGTCAAC	AGTGGCTCCCAGCAAGACTA	266	60	AF169635
NPC2	GGAGGGGAGGAGAAATCAAG	ATTCGGGTCTTGTCTGGTTG	267	60	NM_214206
PPAR $\alpha$	CAGCCTCCAGCCCTCGTC	GCGGTCTCGGCATCTTCTAGG	382	58	DQ437887
SCD	ACGTTGTGCCAGTGAAGTCAAG	GTCTTGGCCTCTTGTGCTTC	206	62	NM_213781
SOD	TCCATGTCCATCAGTTTGGGA	CTGCCAAAGTCATCTGGTTT	250	60	AF396674
SREBP-1	CCTCTGTCTCTCCTGCACC	ACAAAGAGAAGCGCCAAGAA	213	62	NM_214157
SREBP-2	CGCTCGGAATCCTGCTGTG	GGTGCGGGTCCGTGCTGTG	103	65	DQ020476

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7 $\alpha$ -hydroxylase; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; NPC, Niemann-Pick type C; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein.

As previously shown by Walrand *et al.* (2003), dihydrorhodamine 123 is specifically oxidised by H<sub>2</sub>O<sub>2</sub>.

#### Determination of 3-hydroxybutyrate

Concentration of 3-hydroxybutyrate in plasma was determined using an enzymatic assay (cat. no. 10907979035; R-Biopharm AG, Darmstadt, Germany).

#### Transmission electron microscopy

Liver tissues were fixed in 3% sodium cacodylate-buffered glutaraldehyde (pH 7.2) and post-fixed with 1% osmium tetroxide. After washing three times, probes were dehydrated in an ethanol series and embedded in Spurr's epoxy resin. For observations with an EM 900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany), ultrathin sections (80 nm) were mounted on copper grids. Catalase is known to be located in peroxisomes specifically and was marked for a better visualisation of peroxisomes. For immunohistochemistry, ultrathin sections were blocked for 30 min with 1% bovine serum albumin and 0.1% Tween in PBS and incubated overnight with sheep polyclonal anti-catalase serum (1:50; Biotrend, Köln, Germany). For detection of primary antibody, sections were incubated for 1 h with a gold-marked donkey-anti-sheep antibody (1:25; Biotrend) and finally stained with uranyl acetate/lead citrate. Peroxisomes were counted in 1000 different prints per liver sample for each animal with a magnification of 12 000 $\times$ .

#### Statistics

The results were analysed using Minitab (State College, PA, USA) statistical software (release 13). Statistical significance of differences of the mean values of the two groups of pigs was evaluated using Student's *t* test. Mean values were considered significantly different for  $P < 0.05$ .

## Results

#### Fatty acid composition and concentration of lipid peroxidation products in the dietary fats

Palmitic, stearic, oleic and linoleic acid were the major fatty acids in the dietary fats. The sum of these fatty acids accounted for about 95 g/100 g total fatty acids in the fats (Table 2). Amounts of stearic, oleic and linoleic acid were nearly identical in both fats; the amount of stearic acid was slightly higher in the fresh fat than in the oxidised fat. Peroxide value, acid value and concentration of conjugated dienes were 4–5-fold higher in the oxidised than in the fresh fat included in the diet (Table 2). The concentration of total carbonyls was 10-fold higher and that of TBARS was 30-fold higher in the oxidised than in the fresh fat (Table 2).

#### Body weights, antioxidant status and concentrations of lipid peroxidation products in the liver

Body weights of the pigs at the end of the experiment on day 28 did not differ between the two groups (25.6 (SD 1.4) v. 26.0 (SD 1.5) kg in pigs fed the oxidised fat v. pigs fed the fresh fat;

**Table 2.** Major fatty acids and concentrations of some lipid peroxidation products in the fresh and the oxidised fat after inclusion into the diet

	Fresh fat	Oxidised fat
Major fatty acids (g/100 g fatty acids)		
16:0	9.0	6.7
18:0	4.1	4.2
18:1 <i>n</i> -9	23.7	23.8
18:2 <i>n</i> -6	59.8	59.9
Peroxidation products		
Conjugated dienes (mmol/kg)	22.7	89.1
TBARS ( $\mu$ mol/kg)	9	271
Peroxide value (mEq O <sub>2</sub> /kg)	2.5	10.0
Acid value (g KOH/kg)	1.6	8.0
Total carbonyls (mmol/kg)	2.5	24.5

TBARS, thiobarbituric acid-reactive substances.

nine pigs per group). Pigs fed the oxidised fat had a higher mRNA concentration and a higher activity of SOD and a lower activity of microsomal glutathione *S*-transferase in the liver than pigs fed the fresh fat ( $P < 0.05$ ; Table 3). Activities of glutathione peroxidase and cytosolic glutathione *S*-transferase as well as mRNA concentrations of these enzymes in the liver did not differ between both groups of pigs (Table 3). Concentrations of total, reduced and oxidised glutathione in the liver also did not differ between the two groups of pigs whereas the concentration of  $\alpha$ -tocopherol was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ( $P < 0.05$ ; Table 3). Concentration of TBARS in the liver did not differ between the two groups of pigs whereas the concentration of conjugated dienes was slightly but significantly higher in pigs fed the oxidised fat than in pigs fed the fresh fat ( $P < 0.05$ ; Table 3).

#### Indices of peroxisome proliferation

Liver weights of the pigs were not different between the two groups but pigs fed the oxidised fat had a higher peroxisome count and a higher activity of catalase in the liver than pigs fed the fresh fat ( $P < 0.05$ ; Table 4). Relative mRNA concentration of acyl-CoA oxidase (ACO), a peroxisomal enzyme, in the liver, was 34% higher in pigs fed the oxidised fat than in control animals ( $P = 0.062$ ; Table 4). The concentration of H<sub>2</sub>O<sub>2</sub> which is mainly released from peroxisomal oxidases was not different between the two groups of pigs (Table 4).

#### mRNA concentrations of genes in liver and intestine

In liver, mRNA concentrations of PPAR $\alpha$  and genes involved in fatty acid transport and oxidation [liver fatty acid binding protein (L-FABP), carnitine palmitoyltransferase-1 (CPT-1)], fatty acid and cholesterol synthesis [SREBP-1 and -2, insulin-induced gene-1 and -2, fatty acid synthase, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R)], cholesterol uptake (LDL receptor), bile acid synthesis [cholesterol 7 $\alpha$ -hydroxylase (CYP7)], lipoprotein assembly and secretion [microsomal TAG transfer protein (MTP)], inhibition of lipoprotein lipase (apo CIII) and ketogenesis [mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA-S)]

**Table 3.** mRNA concentrations and activities of antioxidant enzymes and concentrations of antioxidants and lipid peroxidation products in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (n 9)		Oxidised fat (n 9)	
	Mean	SD	Mean	SD
Superoxide dismutase				
mRNA concentration (relative)	1.00	0.22	1.24*	0.14
Activity (U/mg protein)	42.7	8.4	58.8*	6.0
Glutathione S-transferase	202	42	144*	13
mRNA concentration (relative)	1.00	0.31	1.27	0.23
Activity in microsomes (U/mg protein)	202	42	144*	13
Activity in cytosol (U/mg protein)	760	262	761	186
Glutathione peroxidase				
mRNA concentration (relative)	1.00	0.13	1.14	0.22
Activity (U/mg protein)	4.72	0.77	5.14	0.75
Glutathione, total (nmol/mg)	2.13	0.51	2.17	0.37
Glutathione, reduced (nmol/mg)	1.70	0.50	1.84	0.55
Glutathione, oxidised (nmol/mg)	0.21	0.12	0.17	0.10
$\alpha$ -Tocopherol (nmol/g)	14.5	2.5	11.9*	1.8
Conjugated dienes ( $\mu$ mol/mg protein)	16	1	18*	3
TBARS (mmol/g)	7.2	1.6	7.3	2.8

TBARS, thiobarbituric acid-reactive substances.

Mean values were significantly different from those of the fresh fat group: \* $P < 0.05$ .

were determined (Fig. 1). Pigs fed the oxidised fat had significantly higher mRNA concentrations of mHMG-CoA-S, a classical PPAR $\alpha$  target gene, SREBP-1 and its target genes ACC and SCD, and SREBP-2 and its target genes HMG-CoA-R and LDL receptor than control pigs fed the fresh fat ( $P < 0.05$ ). mRNA concentration of CYP7 was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ( $P < 0.05$ ). mRNA concentrations of CPT-1 and MTP, two other PPAR $\alpha$  target genes, tended to be increased in pigs fed the oxidised fat ( $P = 0.074$  and  $P = 0.065$ , respectively) compared to pigs fed the fresh fat whereas mRNA concentrations of PPAR $\alpha$ , L-FABP, apo CIII, insulin-induced gene-1 and -2, and fatty acid synthase were not different between pigs fed the fresh fat and those fed the oxidised fat (Fig. 1).

In enterocytes, relative mRNA concentrations of PPAR $\alpha$  and of proteins involved in fatty acid uptake (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein, mitochondrial aspartate aminotransferase), fatty acid oxidation (ACO, CPT-1), intracellular trafficking of cholesterol (Niemann-Pick type C1 and 2) and fatty acid synthesis (SREBP-1, fatty acid synthase) were not different between pigs fed the oxidised fat and those fed the fresh fat (Fig. 2).

However, mRNA concentration of SREBP-2 and its target genes HMG-CoA-R and LDL receptor, involved in cholesterol synthesis and uptake, were higher in pigs fed the oxidised fat than in pigs fed the fresh fat ( $P < 0.05$ ; Fig. 2). mRNA concentration of farnesyl diphosphate synthase did not differ between the two groups of pigs (Fig. 2).

#### Concentrations of TAG and cholesterol in liver, plasma and lipoproteins

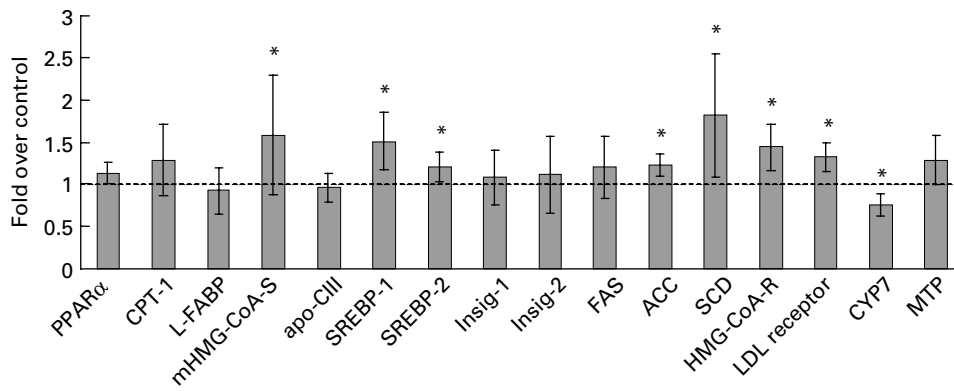
Concentrations of TAG in liver, plasma and TAG-rich lipoproteins did not differ between pigs fed the fresh fat and those fed the oxidised fat. Concentrations in pigs fed the oxidised fat *v.* pigs fed the fresh were (nine pigs per group): liver, 88 (SD 20) *v.* 91 (SD 19)  $\mu$ mol/g; plasma, 0.96 (SD 0.26) *v.* 1.09 (SD 0.17) mmol/l; chylomicrons + VLDL, 0.80 (SD 0.25) *v.* 0.93 (SD 0.16) mmol/l. Concentrations of cholesterol in liver, plasma, LDL and HDL were also not different between the two groups of pigs. Concentrations in pigs fed the oxidised fat *v.* pigs fed the fresh were: liver, 73 (SD 14) *v.* 69 (SD 10)  $\mu$ mol/g; plasma, 2.63 (SD 0.32)

**Table 4.** Indices of peroxisome proliferation in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (n 9)		Oxidised fat (n 9)	
	Mean	SD	Mean	SD
Liver weight (g)	673	63	700	64
Peroxisome count (number/print)	366	67	515*	91
Acyl-CoA oxidase mRNA concentration (relative)	1.00	0.33	1.34†	0.37
Catalase (U/mg protein)	0.75	0.14	0.89*	0.13
H <sub>2</sub> O <sub>2</sub> (fluorescence/g liver)	29 372	12 343	29 437	8361

Mean values were significantly different from those of the fresh fat group: † $P < 0.1$ ; \* $P < 0.05$ .



**Fig. 1.** Relative mRNA concentrations (--- represents 1.00) of various genes involved in hepatic lipid metabolism in enterocytes of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT-PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation. Values are means with their standard deviations depicted by vertical bars ( $n$  9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7 $\alpha$ -hydroxylase; FAS, fatty acid synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: \* $P$  < 0.05.

v. 2.83 (SD 0.22) mmol/l; LDL, 0.96 (SD 0.16) v. 0.97 (SD 0.15) mmol/l; HDL, 1.02 (SD 0.18) v. 1.13 (SD 0.11) mmol/l.

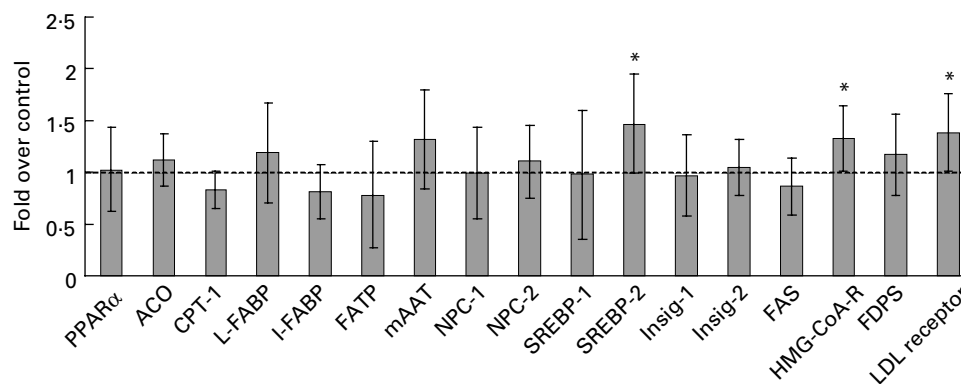
#### Concentration of 3-hydroxybutyrate in plasma

Pigs fed the oxidised fat had a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat (1.23 (SD 0.58) v. 0.52 (SD 0.27) mmol/l;  $P$  < 0.05).

#### Discussion

In the present study, pigs were fed a diet containing an oxidised fat prepared under usual deep-frying conditions. The relatively low concentrations of lipid peroxidation products (conjugated dienes, TBARS, peroxides and carbonyls) in the oxidised fat indicate that this fat was mildly oxidised. Concentrations of peroxidation products in this fat were indeed even

lower than in soyabean oil or hydrogenated animal-vegetable oil blends used for frying of potatoes at 190°C over a period of 24 h (Frankel, 1998). The reason for the relatively low degree of oxidation is that we did not add foodstuffs to be fried during the preparation of the oil as we wanted to avoid contamination of the oil with food ingredients. It is well known that ingredients of foodstuffs, i.e. metal ions, enhance the lipid peroxidation process during frying of fats (Kubow, 1992). The concentration of conjugated dienes which include the potent PPAR $\alpha$  activators hydroxy- and hydroperoxy fatty acids (Delerive *et al.* 2000; König & Eder, 2006) was approximately four times higher in the oxidised fat than in the fresh fat. The finding of an increased activity of SOD and a slightly elevated concentration of conjugated dienes, together with the observation of a slightly reduced concentration of  $\alpha$ -tocopherol, indicates that the oxidised fat produced oxidative stress in the liver of the pigs. It has been demonstrated that under



**Fig. 2.** Relative mRNA concentrations (--- represents 1.00) of various genes involved in intestinal lipid metabolism in livers of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT-PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation. Values are means with their standard deviations depicted by vertical bars ( $n$  9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; NPC, Niemann-Pick type C; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: \* $P$  < 0.05.

oxidative stress, expression of SOD is stimulated and concentration of  $\alpha$ -tocopherol is reduced due to an enhanced consumption (Liu & Huang, 1995; Ruiz-Gutierrez *et al.* 1999; Atalay *et al.* 2000). However, the oxidative stress produced by the oxidised fat was very moderate as concentrations of TBARS and glutathione remained completely unchanged. In rodents treated with PPAR $\alpha$  agonists such as fibrates or WY-14,643, production of H<sub>2</sub>O<sub>2</sub> is largely increased due to a strong up-regulation of peroxisomal oxidases, and this causes oxidative stress and contributes to hepatocarcinogenesis in these species (Peters *et al.* 2005). In the present study, feeding the oxidised fat did not increase the concentration of H<sub>2</sub>O<sub>2</sub> in the liver. This was probably due to two reasons: first, there was only a slight increase in the mRNA concentration of ACO, one of the enzymes producing H<sub>2</sub>O<sub>2</sub>; second, activity of catalase, the key enzyme of decomposition of H<sub>2</sub>O<sub>2</sub> in peroxisomes was increased. Therefore, generation of H<sub>2</sub>O<sub>2</sub> did not contribute to oxidative stress in animals treated with oxidised fat. The reason for the moderate oxidative stress may be that a part of the dietary lipid peroxidation products is absorbed in the intestine and reaches the liver via lipoproteins (Staprans *et al.* 2005). Production of oxidative stress by intake of strongly oxidised fats has been shown several times in rodents (Yoshida & Kajimoto, 1989; Liu & Huang, 1996; Liu & Lee, 1998; Ammouche *et al.* 2002; Eder *et al.* 2004; Keller *et al.* 2004a, b). The present study shows for the first time that even a mildly oxidised fat, as used in human nutrition, can induce moderate oxidative stress in pigs as a non-proliferating species.

To find out whether the mildly oxidised fat caused activation of PPAR $\alpha$  in the liver of pigs, we determined mRNA concentrations of the classical PPAR $\alpha$  target genes ACO, CPT-1 and mHMG-CoA-S as well as peroxisome count, activity of catalase and plasma concentration of 3-hydroxybutyrate. Recent studies in pigs have shown that activation of PPAR $\alpha$  in pigs, by either treatment with clofibrate or by fasting, leads to an increased expression of these PPAR $\alpha$  target genes, and in turn stimulates mitochondrial and peroxisomal  $\beta$ -oxidation and ketogenesis (Yu *et al.* 2001; Peffer *et al.* 2005; Cheon *et al.* 2005). The finding of an increased peroxisome count together with increased activity of catalase, a peroxisomal enzyme, a significantly increased mRNA concentration of mHMG-CoA-S and an increased plasma concentration of 3-hydroxybutyrate strongly indicate that the oxidised fat caused PPAR $\alpha$  activation in the liver of the pigs. The finding that mRNA concentrations of ACO and CPT-1, two other classical PPAR $\alpha$  target genes were also increased by 34 and 29 %, although not significantly different to control, supports the assumption that the oxidised fat induced hepatic PPAR $\alpha$  activation in the pigs. It has been shown that these two enzymes are only moderately up-regulated in pig liver by PPAR $\alpha$  agonists. For instance, in pigs treated with clofibrate, a strong PPAR $\alpha$  agonist, hepatic gene expression of CPT-1 and ACO was only 1.89- and 1.42-fold, respectively, increased over control while gene expression of mHMG-CoA-S was increased 3.32-fold (Cheon *et al.* 2005). This presents an explanation for the observations that mHMG-CoA-S was significantly increased in pigs treated with oxidised fat and that ACO and CPT-1 were only slightly increased. The finding that mRNA concentration of MTP, a gene recently shown to be up-regulated by PPAR $\alpha$  activation (Ameen *et al.* 2005), tended to be increased in the liver of pigs fed the oxidised fat

also indicates that the oxidised fat caused PPAR $\alpha$  activation in the liver. Recently, studies in rats have already shown that oxidised fats are able to activate PPAR $\alpha$  in the liver (Huang *et al.* 1988; Chao *et al.* 2001; Stülzle *et al.* 2004). In these rat studies, up-regulation of PPAR $\alpha$  target genes in the liver was much stronger than in pigs of the present study. This may have two different reasons: first, most PPAR $\alpha$  target genes respond stronger to PPAR $\alpha$  activation in rats than in non-proliferating species such as pigs or man; second, fats used in the rat studies were more strongly oxidised than the mildly oxidised fat used in the present study. The present study shows for the first time that even a mildly oxidised fat causes activation of PPAR $\alpha$  in pigs which are, as man, less sensitive to PPAR $\alpha$  agonists than rodents.

To study whether the oxidised fat caused PPAR $\alpha$  activation in small intestine, we considered in addition to the classical PPAR $\alpha$  target genes ACO and CPT-1, several genes involved in fatty acid transport (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein and mitochondrial aspartate aminotransferase) and cholesterol trafficking (Niemann-Pick type C1 and 2) in intestinal tissue. All these genes have been shown to be up-regulated by PPAR $\alpha$  activation (Darimont *et al.* 1998; Motojima *et al.* 1998; Mochizuki *et al.* 2001; Chinetti-Gbaguidi *et al.* 2005). The finding that none of these genes was up-regulated in cells of small intestine indicates that oxidised fat caused no or even weak PPAR $\alpha$  activation and does not influence intestinal fatty acid transport and cholesterol trafficking.

Synthesis of lipids in mammalian cells is controlled by a network involving the action of insulin-induced genes and SREBP, and it has been recently shown in several studies that this network is influenced by PPAR $\alpha$  activation (Guo *et al.* 2001; Patel *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). The present study shows that feeding a mildly oxidised fat increased the mRNA concentration of SREBP-1 and its target genes ACC and SCD, two key enzymes of *de novo* fatty acid synthesis, in the liver. These alterations may be caused by activation of PPAR $\alpha$  in the liver. Knight *et al.* (2005) found that treatment with WY 14,643, a synthetic PPAR $\alpha$  agonist, causes a strong up-regulation of enzymes involved in hepatic fatty acid synthesis and stimulates fatty acid synthesis in wild-type mice but not in PPAR $\alpha$  null mice. Knight *et al.* (2005) suggest that up-regulation of hepatic fatty acid synthesis is a compensatory response on the increased fatty acid oxidation to maintain a constant cellular TAG level. The finding that TAG levels in liver and plasma were not reduced in pigs fed the oxidised fat compared to control pigs indeed suggests that an increased  $\beta$ -oxidation of fatty acids was compensated by an increased fatty acid synthesis. As there is no evidence for a direct action of PPAR $\alpha$  on the promoter regions of SREBP-1 and ACC genes, it is likely that the increased mRNA concentrations of these genes are an indirect result of PPAR $\alpha$  activation. In contrast, SCD is not only dependent on SREBP-1 but has also a PPAR response element in its promoter (Miller & Ntambi, 1996). Therefore, its transcription may have been in part directly stimulated by PPAR $\alpha$  activation. An up-regulation of SCD which catalyses the formation of MUFA from SFA has also been observed in pigs treated with clofibrate (Cheon *et al.* 2005). These findings of the effects of the oxidised fat on gene expression of lipogenic enzymes observed in pigs are opposite to those observed in rats in which a dietary oxidised fat causes a down-regulation of lipogenic enzymes and a strong reduction of liver and plasma TAG (Eder & Kirchgessner, 1998; Eder *et al.* 2003).



It is moreover shown that feeding the mildly oxidised fat led to a moderate but significant up-regulation of SREBP-2, and its target genes HMG-CoA-R and LDL receptor, in both liver and small intestine. The present findings suggest that the oxidised fat could have stimulated synthesis and uptake of cholesterol in these tissues. As this effect occurs not only in the liver but also in the small intestine where no PPAR $\alpha$  activation was found in pigs fed the oxidised fat, it is questionable whether these effects are linked to PPAR $\alpha$  activation. The finding that hepatic genes involved in cholesterol synthesis were not altered in pigs treated with clofibrate indeed suggests that PPAR $\alpha$  activation does not influence SREBP-2 controlled transcription of genes involved in cholesterol homeostasis (Cheon *et al.* 2005). On the other hand, treatment with the PPAR $\alpha$  agonist WY 14,643 caused an up-regulation of genes involved in hepatic cholesterol synthesis in wild-type mice but not in PPAR $\alpha$  null mice, indicating that PPAR $\alpha$  activation indeed could directly stimulate cholesterol synthesis (Knight *et al.* 2005). It should be noted, however, that there is also another study that found a suppression of gene expression and proteolytic activation of SREBP-2, and a strong down-regulation of its target genes accompanied by reduced cholesterol synthesis in rats (König *et al.* 2006). The effect of PPAR $\alpha$  activation on SREBP-2-dependent cholesterol synthesis is not yet clear and may also be different between various species. Besides an up-regulation of genes involved in synthesis and uptake of cholesterol, we found a down-regulation of CYP7, the key enzyme of bile acid formation, in the liver. It has been shown in human and rat liver cells that PPAR $\alpha$  agonists lower CYP7 expression probably by reducing the availability of hepatic nuclear factor 4 $\alpha$  which is required for binding to a DR-1 in CYP7 promoter (Marrapodi & Chiang, 2000; Patel *et al.* 2000). Therefore, we assume that down-regulation of CYP7 in the liver of pigs fed the oxidised fat was caused by PPAR $\alpha$  activation induced by the oxidised fat. Increased hepatic cholesterol synthesis and uptake of cholesterol into the liver, together with a decreased bile acid synthesis, is expected to increase hepatic cholesterol concentration. In contradiction to this, liver and plasma cholesterol concentrations were unchanged in pigs fed the oxidised fat compared to pigs fed the fresh fat. We assume that the changes in gene expression were too small to induce phenotypical alterations of cholesterol concentrations.

In conclusion, the present study shows that a mildly oxidised fat causes PPAR $\alpha$  activation in the liver of pigs as indicated by an increased peroxisome count, a moderate up-regulation of PPAR $\alpha$  target genes and a stimulation of ketogenesis. Moreover, the oxidised fat led to an up-regulation of the expression of SREBP-1 and SREBP-2 and their target genes involved in TAG and cholesterol synthesis, suggesting a stimulation of lipid synthesis. As the fat used in the present study was even less oxidised than fats used for deep-frying of foods, and as there exists a similarity in the gene response to PPAR $\alpha$  agonists between pig and human liver cells, deep-fried fats could exert similar effects in man.

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Sebastian Luci and Bettina König contributed equally to this work.

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