Effects of n–3 and n–6 polyunsaturated fatty acid-enriched diets on lipid metabolism in periportal and pericentral compartments of female rat liver lobules and the consequences for cell proliferation after partial hepatectomy

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Abstract The effects of a low fat diet or diets enriched with either n-6 or n-3 polyunsaturated fatty acids (safflower or fish oil, respectively) on lipid metabolism in periportal and pericentral zones of female rat liver lobules were investigated in relation with cell proliferation after partial hepatectomy. It was found that cell proliferation was localized almost exclusively in periportal and midzonal areas and was significantly reduced by 60% after a fish oil diet only. The fish oil diet caused a strongly increased β-oxidation capacity in peroxisomes and a moderately increased catalase activity. Catalase activity was mainly localized pericentrally, particularly after partial hepatectomy, whereas the capacity of lipid peroxidation product formation was doubled only in periportal zones in rats on a fish oil diet. The capacity of glucose-6-phosphate dehydrogenase activity to produce NADPH was distinctly lower in both zones of liver lobules as a result of the fish oil diet. Localization patterns and activity in liver lobules of NADPH-cytochrome c (P450) reductase were not significantly affected by fish oil diet. Therefore, it is concluded that elevated peroxisomal β-oxidation and increased lipid peroxidation capacity in periportal zones of liver lobules coincide with reduced cell proliferation in hepatectomized rats on fish oil diet. These findings support the hypothesis that lipid peroxidation products are involved in the regulation of cell proliferation.—**Van Noorden, C. J. F.** Effects of n-3 and n-6 polyunsaturated fatty acid-enriched diets on lipid metabolism in periportal and pericentral compartments of female rat liver lobules and the consequences for cell proliferation after partial hepatectomy. *J. Lipid Res.* 1995. **36**: 1708–1720.

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Lipid peroxidation is down-regulated in proliferating hepatocytes in regenerating liver after partial hepatectomy (1), in the Novikoff rat liver tumor (2), the Yoshida rat liver tumor (3), and in slowly growing hepatomas (4). The low rates of lipid peroxidation in proliferating hepatocytes seem to be caused by low levels of polyunsaturated fatty acids (PUFA), NADPH-cytochrome c (P450) reductase and cytochrome P450 and elevated levels of lipid-soluble antioxidants such as α-tocopherol (vitamin E) and reduced glutathione (3, 5, 6). These findings support the hypothesis that lipid peroxidation products are involved in the same mechanisms that regulate cell proliferation (3, 7–9). When DNA synthesis is at a maximum, lipid peroxidation is lowest and vice versa. When DNA is uncovered during the cell cycle, it would be relatively susceptible to damage by lipid peroxidation products (10). Therefore, lipid peroxidation production is decreased and antioxidant levels are increased during the cell cycle as a protective mechanism (6, 9, 11). Moreover, enzymes that are essential for cell proliferation such as DNA polymerases are inhibited by the lipid peroxidation products 4-hydroxynonenal, malondialdehyde, 4-hydroxynonadienal, and 4-hydroxyhexenal (11–14). Furthermore, the regulatory enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase, is also inhibited by lipid peroxidation products (15). This enzyme is one of the main producers of NADPH necessary for the cytochrome P450 system,

Abbreviations: PUFA, polyunsaturated fatty acids; BrdU, bromodeoxyuridine; PGE_2, prostaglandin E_2; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PVA, polyvinyl alcohol; tetranitro BT, tetranitroblue tetrazolium chloride; ED-1, monoclonal antibodies directed against an intracellular antigen of monocytes and macrophages; ED-2, monoclonal antibodies directed against a membrane antigen on resident macrophages such as Kupffer cells.

for cholesterol and fatty acid biosynthesis, and for glutathione reductase (16).

Important sources of lipid peroxidation products in humans are dietary PUFA of the n–3 and n–6 classes. These PUFA are needed in the arachidonic acid metabolism for prostaglandin and leukotriene synthesis (9, 17). Variations in type and quantity of dietary lipids alter rates of fatty acid biosynthesis and oxidation. High levels of PUFA in the diet reduce fatty acid biosynthesis and induce fatty acid oxidation in the liver (18). Animal studies suggest that n–6 PUFA may promote cancer growth (19), possibly by stimulation of prostaglandin synthesis (11, 20). PUFA of the n–3 class inhibit synthesis of prostaglandins of the second series by inhibition of cyclooxygenases and lipoxygenases and therefore, may inhibit cancer growth (21, 22).

The aim of the present study was to investigate whether diets enriched with n–3 or n–6 PUFA would affect hepatocyte proliferation after partial hepatectomy in female rats and if so, whether lipid metabolism plays any direct role. For this purpose, the incorporation of bromodeoxyuridine (BrdU) in hepatocyte nuclei before and at 48 h after partial hepatectomy was studied in relationship with relevant metabolic parameters such as lipid content, formation of lipid peroxidation products, activities of acylCoA oxidase (involved in peroxisomal fatty acid β-oxidation) (23), catalase, glucose-6-phosphate dehydrogenase, and NADPH-cytochrome c (P450) reductase. Because liponeogenesis occurs mainly pericentrally and lipid oxidation is predominant periportally (24), and liver cell proliferation after partial hepatectomy starts in periportal zones (25–27), the study has been performed with quantitative histochemical methods in order to elucidate differences in periportal and pericentral zones of liver lobules. Distribution patterns of Kupffer cells and macrophages before and after partial hepatectomy were investigated as well because prostaglandin E_2 (PGE_2) is released by these cells. PGE_2 is known to induce hepatocyte proliferation and plays an important role in the regenerative response after partial hepatectomy (28, 29). Female rats were used because partial hepatectomy has a more profound effect on carbohydrate and lipid metabolism in female rat liver than in male rat liver (30).

MATERIALS AND METHODS

Twenty-five female Wistar rats (6–7 weeks old; TNO substrain; TNO, Zeist, The Netherlands) were maintained for 2 weeks under constant environmental conditions with food and water freely available. The temperature was kept at 21–22°C and the relative humidity at 60%. The rats were exposed to a 12-h light–dark cycle (light, 07.00–19.00; darkness, 19.00–07.00) throughout the acclimatization and experimental periods. All rats were fasted for 24 h prior to the diet period. Then, the rats were randomly divided into five treatment groups; two groups of five rats were kept on a low fat diet, five on a n–3 PUFA-containing fish oil diet, five on a n–6 PUFA-containing safflower oil diet, and five rats were kept on a restricted low fat diet of 7 g per day that matches the amount of food consumed by the rats on fish oil diet (Fig. 1). Rats in one group on low fat diet and on PUFA-containing diets were housed five per cage and rats in one group on low fat diet and on the restricted low fat diet were housed individually at the Academic Medical Center animal facility in accordance with the guidelines for animal care of the institute.

Treatment

The animals were kept for 3 weeks on the respective diets. Then, the rats underwent partial hepatectomy according to the method of Higgins and Anderson (31). Ligation and excision of the median and left lateral lobes of the livers resulted in two-thirds hepatectomy. Anesthesia was performed with ether (ca. 2 min) followed by Nembutal (40 mg/kg body weight, i.p.). Small pieces of liver tissue up to 5 mm^3 were chilled in small capped vials in liquid nitrogen and stored at –80°C until further use. This tissue served as control material of the animals before operation.

At 48 h after partial hepatectomy, the rats were anesthetized with ether, the abdomen was opened, and the liver remnants were removed immediately and chilled in liquid nitrogen as described above. The survival rate of the animals in all experimental groups was 100%. All animal experiments were carried out in accordance with guidelines for animal care of the institute at the Academic Medical Center.

Diets

Diets were prepared by Hope Farms (Woerden, The Netherlands). Fish oil (Pronova Biocare, Sandefjord, Norge) and safflower oil (Sigma Chemical Co., St. Louis, MO) were added at the Academic Medical Center animal facility. Fish oil and safflower oil were kept under nitrogen at 4°C and were added each day to the food immediately before it was given to the animals. In this way auto-oxidation of the PUFA was kept to a minimum.

Vitamin E levels in the food were kept as low as possible at 35 mg per kg of the low fat diet (5% (v/w) soybean oil) and at 75 mg per kg of the n–3 and n–6 PUFA diets (20% (v/w) fish oil or safflower oil, respectively). Other vitamins, minerals, essential fats, and proteins were present at sufficiently high concentrations in the food to provide adequate growth (32). Complete composition of the diets is presented in **Table 1.** Diets were composed in such a way that each animal in the

TABLE 1. Composition of diets

Nutrients	Diets (% by Weight)		
	Low Fat	n-6 PUFA[a]	n-3 PUFA[b]
Cellulose/Didacel 2 + 4	5.00	6.03	6.03
Cerelose 02001 (CPC)	54.29	30.89	30.89
Pro Melka 11003 (CPC)	10.00	12.10	12.10
Casein, vitamin free	20.00	24.10	24.10
Vitamin mix (-vitamin E)	0.25	0.25	0.25
Vitamin E (50%)	0.01	0.02	0.02
Mineral mix	0.25	0.30	0.30
$CaHPO_4 \cdot 2H_2O$	1.30	1.57	1.57
$CaCO_3$	1.00	1.21	1.21
KH_2PO_4	0.70	0.84	0.84
KCl	0.70	0.84	0.84
NaCl	0.30	0.36	0.36
$MgSO_4 \cdot 7H_2O$	0.40	0.48	0.48
MgO	0.20	0.24	0.24
Methionin	0.20	0.24	0.24
Choline · HCl (50%)	0.40	0.50	0.50
Soybean oil	5.00	-	-
Safflower oil	-	20.00	-
Fish oil	-	-	20.00
Cholesterol	0.10	0.10	0.05
n-6 PUFA	-	14.9	-
n-3 PUFA	-	-	14.2

[a]n-6 PUFA, n-6 polyunsaturated fatty acids (safflower oil).
[b]n-3 PUFA, n-3 polyunsaturated fatty acids (fish oil).

diet group would receive similar amounts of the various nutrients and similar amounts of energy (Dr. G. Smulders, Hope Farms). The rats on fish oil diet appeared to eat significantly less (ca. 7 g per day). Therefore, one group of rats received a restricted low fat diet of 7 g per day to exclude the possibility that the experimental results in the fish oil diet group were due to differences in food intake.

BrdU incorporation

BrdU (15 mg; Sigma) was injected i.p. 1 h before partial hepatectomy and at 23 and 47 h after partial hepatectomy and BrdU incorporation was analyzed immunocytochemically (27). Cryostat sections (thickness 8 μm) were cut on a motor-driven cryostat fitted with a retraction microtome (Bright, Huntingdon, UK) at a cabinet temperature of -25°C. Sections were picked up onto clean glass slides and kept in the cryostat cabinet until used.

The immunocytochemical procedure was started by allowing the sections to dry for at least 10 min at room temperature. Incorporated BrdU was detected by an indirect two-step labeling technique using peroxidase-conjugated IgG. Endogenous peroxidase activity was inhibited by preincubation of the sections in methanol containing 0.3% (w/v) hydrogen peroxide for 10 min at room temperature. Sections were briefly rinsed in phosphate-buffered saline (PBS) and incubated in 0.5 N HCl for 30 min at room temperature and rinsed again briefly in PBS. Sections were then incubated in a solution of monoclonal anti-BrdU antibodies (Eurodiagnostics, Apeldoorn, The Netherlands; 1:10 diluted in PBS) for 1 h at room temperature, followed by two washes in PBS. The sections were finally incubated in a solution of peroxidase-conjugated rabbit anti-mouse IgG (Dako, Santa Barbara, CA; 1:200 diluted in PBS) and 1% (v/v) rat serum for 1 h at room temperature. After two washes in PBS, the antibody binding sites were visualized by incubation for 10 min at room temperature in a solution of 0.05% (w/v) diaminobenzidine-tetrachloride (Sigma) in 50 mM Tris-HCl (pH 7.6) to which 0.01% (v/v) hydrogen peroxide had been added just before use. After a rinsing step in distilled water, the specimens were counterstained with hematoxylin and embedded in glycerin-gelatin. Control incubations were performed with an irrelevant antibody (anti-collagen type II; Sanbio, Uden, The Netherlands) replacing the primary antibody in the same dilution.

The number of labeled nuclei was counted in sections independently and in a blinded fashion by two observers. A light microscope was used with a 40× objective and an 8× eye piece with a quadrangular diaphragm of 1 × 1 cm limiting field of view. The number of nuclei per area was counted in two perpendicular directions in each of two sections of each liver. In this way, 1000 nuclei per section were counted twice. The labeling index was determined as the percentage labeled nuclei of the total number of nuclei per area.

Kupffer cells and macrophages

Cryostat sections (8 μm thick) were air-dried for 30 min at room temperature and fixed in acetone at 4°C for 7 min. The sections were air-dried again for 30-45 min to remove the acetone.

Monoclonal antibodies (Serotec, Oxford, UK) ED-1, directed against an intracellular antigen of monocytes and macrophages, and ED-2, directed against a membrane antigen on resident macrophages such as Kupffer cells, were used (33). Antibodies were diluted 1:500 in 0.01 M PBS, pH 7.4, containing 0.2% (w/v) bovine serum albumin (BSA; Merck, Darmstadt, Germany). Incubations lasted for 60 min at room temperature in a closed moist incubation chamber. Antibody binding was visualized as described above. Controls were performed by incubation with an irrelevant antibody (anti-collagen type II) instead of the primary antibodies in the same dilution.

Lipid content

The lipid content of livers was demonstrated histochemically by incubating unfixed cryostat sections in a saturated solution of Sudan Black B (Merck) in 70%

(v/v) ethanol for 15 min at room temperature as described by Bayliss High (34). The solution was filtered just before use. Afterwards, sections were rinsed thrice in distilled water for 2 min each, dried, and mounted in glycerin–gelatin.

Lipid peroxidation capacity

Lipid peroxidation capacity was analyzed in periportal and pericentral zones of lobules of all livers before and after partial hepatectomy as described by Thomas et al. (35). Unfixed cryostat sections were dried for 5 min at room temperature and then incubated for 10 min at 37°C with a pro-oxidant system containing 0.2 mM NADPH (Boehringer, Mannheim, Germany), 1 mM ADP (Boehringer), and 15 μM $FeCl_2$ dissolved in 50 mM Tris-maleate buffer (pH 7.4). Lipid peroxidation products were detected after rinsing the sections briefly in Tris-maleate buffer containing 0.15 mM KCl and treatment for 1 min with 5% trichloroacetic acid and four rinses for 5 min in 0.9% (w/v) NaCl. Then, sections were incubated for 16 h at room temp in 0.9% NaCl solution containing 0.1% (w/v) 3-hydroxy-2-naphthoic acid hydrazide (Janssen, Beerse, Belgium) dissolved in dimethylsulfoxide (final concentration 10% (v/v)) and 5% (v/v) acetic acid. Incubations were performed in a humidified atmosphere to avoid evaporation, and afterwards, sections were rinsed four times for 5 min in 15% (v/v) dimethylsulfoxide in an aqueous solution of 1 mM HCl. Finally, sections were incubated for 20 min at room temperature in a solution of 0.1% (w/v) Fast Blue B (Serva, Heidelberg, Germany) in 100 mM phosphate buffer (pH 6.5). The slides were rinsed for 5 min in running tap water and for 5 min in distilled water and then mounted in glycerin–gelatin.

Acyl-CoA oxidase activity

Acyl-CoA oxidase (E.C.1.3.3.6) activity was measured in total homogenates using the procedure described by Kvannes and Flatmark (36).

Catalase activity

Catalase (E.C.1.11.1.6) activity was measured using a newly devised method (37) based on the peroxidative action of catalase in the presence of excess ethanol. The assay medium contained the following standard components: 100 mM potassium phosphate (pH 7.4), 1.5 mM NAD, 10 mM pyrazole, 2 M ethanol, 5 mM H_2O_2, 0.05% (w/v) Triton X-100, and 1.5 U/ml aldehyde dehydrogenase (aldehyde: NAD(P) oxidoreductase, E.C.1.2.1.5 from yeast; Boehringer). The increase in absorbance at 340 nm was measured in time at 10-sec intervals using a COBAS-BIO centrifugal analyzer (Hoffmann-LaRoche, Basle, Switzerland).

Localization of catalase activity in liver lobules was performed in cryostat sections (thickness 8 μm) that were fixed in 0.3% (w/v) glutaraldehyde (Merck) in distilled water for 5 min at room temp as described by Van Noorden and Frederiks (38). After rinsing thrice in distilled water, catalase activity was demonstrated with a medium containing 100 mM glycine–NaOH buffer (pH 10.5), 2% (w/v) polyvinyl alcohol (PVA; weight average M_r 70,000–100,000; Sigma), 5 mM diaminobenzidine, and 18 mM hydrogen peroxide. Incubations lasted for 30 min at 37°C. Control incubations were performed either in the absence of hydrogen peroxide or in the presence of hydrogen peroxide and 10 mM 3-amino-1,2,4-triazole (Serva).

Glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) activity was analyzed histochemically in unfixed cryostat sections (thickness 8 μm) as described by Van Noorden and Frederiks (38). The reaction was performed with a medium containing 100 mM phosphate buffer, pH 7.45, and 18% (w/v) PVA, 0.8 mM NADP (Boehringer), 4 mM $MgCl_2$, 10 mM glucose-6-phosphate (Serva), 5 mM sodium azide, 0.32 mM 1-methoxyphenazine methosulfate (Serva), and 5 mM tetranitroblue tetrazolium chloride (Tetranitro BT; Serva). Control media lacked substrate and coenzyme (38). Sections were allowed to dry for 5 min at 37°C and then were incubated for 5 min at 37°C with preheated media.

NADPH-cytochrome c (P450) reductase activity

NADPH-cytochrome c (P450) reductase (E.C.1.6.2.4) activity was analyzed histochemically in unfixed cryostat sections (thickness 8 μm) as described by Van Noorden and Frederiks (38). The reaction was performed with a medium containing 100 mM phosphate buffer, pH 7.45, and 18% (w/v) PVA, 5 mM NADPH (Boehringer) and 5 mM tetranitro BT. Control media lacked substrate or 5 mM NADP was added as competitive inhibitor of the reductase (38). Sections were allowed to dry for 5 min at 37°C and then incubated for 10 min at 37°C with preheated media.

All enzyme incubations were stopped immediately by rinsing thoroughly in 100 mM phosphate buffer (pH 5.3) at 56°C and sections were mounted in glycerin–gelatin.

Photomicrography and cytophotometry

Photomicrographs were taken with an Olympus Vanox-T photomicroscope with an SPlanApo objective (10 ×, N.A. 0.40). In the present study, periportal zones in liver lobules are defined as large areas comprising periportal and midzonal cells, whereas pericentral areas consist of a smaller area around the central veins.

Cytophotometrical analysis of the final reaction products in periportal and pericentral zones of liver lobules was performed as described in detail by Van Noorden and Frederiks (38) with a Vickers M85a scanning and integrating cytophotometer. Measurements were made at 557 nm for tetranitro BT-formazan, at 550 nm for lipid peroxidation products and at 595 nm for the Sudan Black B-stained sections. A 6.3 × planachromatic objective (N.A. 0.20), a bandwidth setting of 65, a scanning spot with an effective diameter of 3.2 μm, and a mask with a diameter of 63 μm were used. The area scanned in each measurement was 3117 μm^2. Per rat and per zone, ten cytophotometric readings were made in each of three sections both for test reactions in the presence of substrate and for control reactions in the absence of substrate (and coenzyme). Ten periportal and ten pericentral zones were selected in each section in a blinded fashion for measurement of the test reactions and the same zones were taken in the control sections for measurement of the control reactions. The relative integrated absorbance was converted into mean integrated absorbance by reference to a calibration curve (38). In the case of enzyme reactions, mean integrated absorbance values were converted into μmoles of substrate converted per min per g wet weight of liver tissue by recourse to the molar extinction coefficient of 19,000 for tetranitro BT-formazan (38). Reaction rates of enzymes were calculated by subtracting from absorbance values obtained for test reactions absorbance values as measured in consecutive sections that were incubated for control reactions. For each rat, the mean ± standard deviation (SD) was calculated both for periportal and pericentral zones.

Statistical analysis

To test for statistical significance of differences of parameters between diet groups and between periportal and pericentral zones in liver lobules, the Wilcoxon two sample test ($2\alpha = 0.05$) was applied.

RESULTS

Body weight

Figure 1 shows the weight of the animals during the experiments. All animals in one diet group showed similar development with respect to body weight. Animals on low fat diet gained on average 26 g of weight, animals on restricted low fat diet lost 7 g of their weight, animals on n-3 PUFA-containing diet showed an average loss of 6 g over 3 weeks, and animals on n-6 PUFA-containing diet gained on average 45 g of weight.

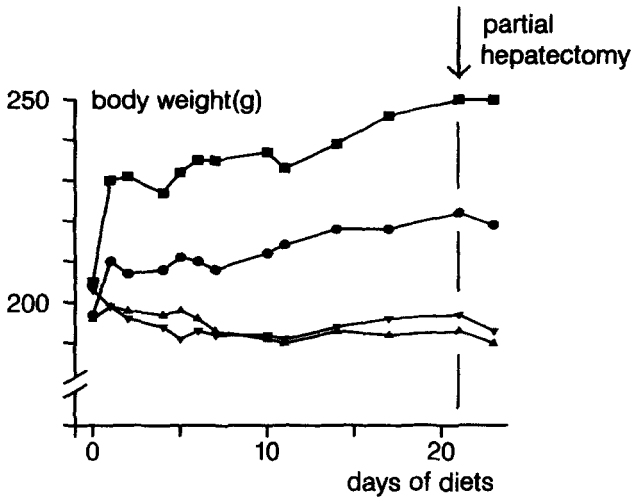

Fig. 1. Body weight of the animals from the day the diet was started (day 0) until the end of the experiment at 48 h after partial hepatectomy (day 23); ●-●, low fat diet; ■-■, n-6 PUFA-enriched diet; ▲-▲, n-3 PUFA-enriched diet; ▼-▼, restricted low fat diet of 7 g per day per animal.

Cell proliferation

Cell proliferation as detected with BrdU incorporation was very low before partial hepatectomy (in all 25 rats < 0.2%). The number of cells that had been or were in S-phase in the 48 h after partial hepatectomy increased dramatically (**Table 2, Fig. 2**). The percentage of positive nuclei was approximately 20% in the low fat diet groups and the restricted low fat diet group, less than 10% in the n-3 PUFAs diet group, and 16% in the n-6 PUFA diet group. The decrease by 60% in the n-3 PUFA diet group versus the low fat diet groups was significant. In all five diet groups the larger part of positive nuclei was localized in periportal zones (Fig. 2).

Kupffer cells and monocytes

Partial hepatectomy induced an increase in the number of Kupffer cells and newly recruited monocytes as demonstrated with ED-1. Resident macrophages as detected with ED-2 were present in low amounts before

TABLE 2. Percentage of bromodeoxyuridine-positive nuclei (% BrdU) of liver cells at 48 h after partial hepatectomy in the different diet groups

Diet	% BrdU ± SD
Low fat (group I)	22.5 ± 5.7
Low fat (group II)	21.1 ± 6.0
Restricted low fat	18.7 ± 5.6
n-6 PUFA	16.3 ± 5.8
n-3 PUFA	9.5 ± 5.7[a]

[a]Significantly different from controls.

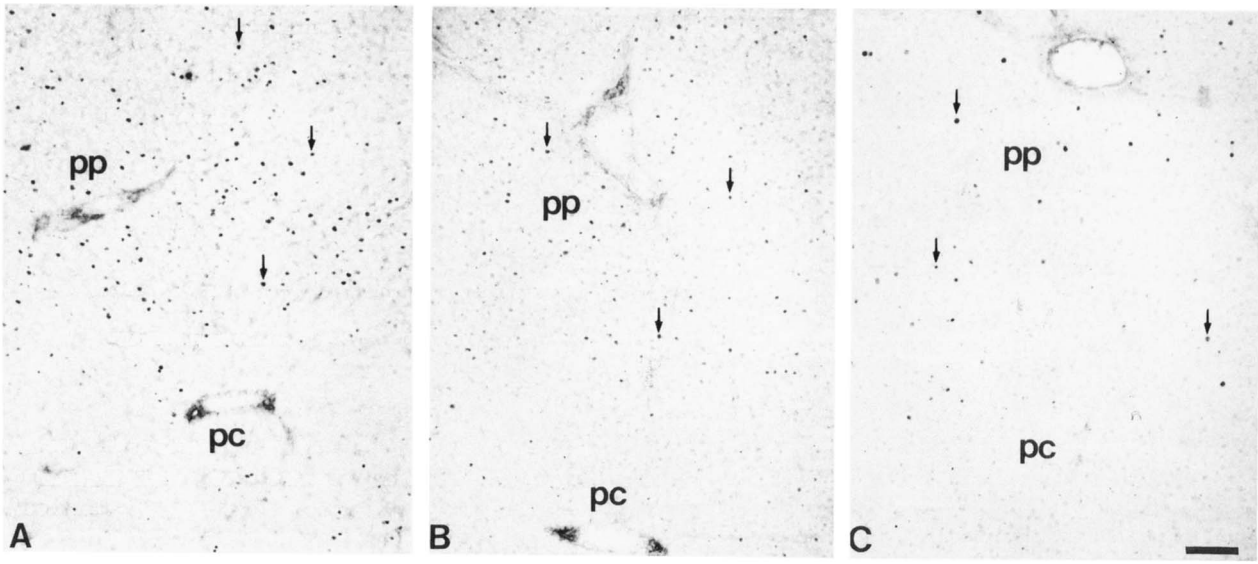

Fig. 2. Incorporation of bromodeoxyuridine (BrdU) in liver cells as detected immunocytochemically at 48 h after partial hepatectomy using cryostat sections of livers of animals on low fat diet (A), n–6 PUFA diet (B), or n–3 PUFA diet (C). Positive nuclei (arrows) are mainly localized in periportal (pp) and midzonal areas but not in pericentral zones (pc). Bar = 100 μm.

operation, but at 48 h after partial hepatectomy the amount of ED-2-positive cells was vastly increased, particularly in periportal zones. These findings were independent of the diet (data not shown).

Lipid content

The lipid content in livers before partial hepatectomy was low and preferentially localized in periportal zones (data not shown). At 48 h after partial hepatectomy, lipid droplets had accumulated in hepatocytes, particularly in rats on low fat and n–6 PUFA diets. Lipid droplet accumulation in hepatocytes of animals on n–3 PUFA diet was decreased and its localization was distinctly different from that in the other groups. Livers of this diet group showed a finer granular distribution pattern of intracellular lipid droplets closely linked with the plasma membrane (**Fig. 3**).

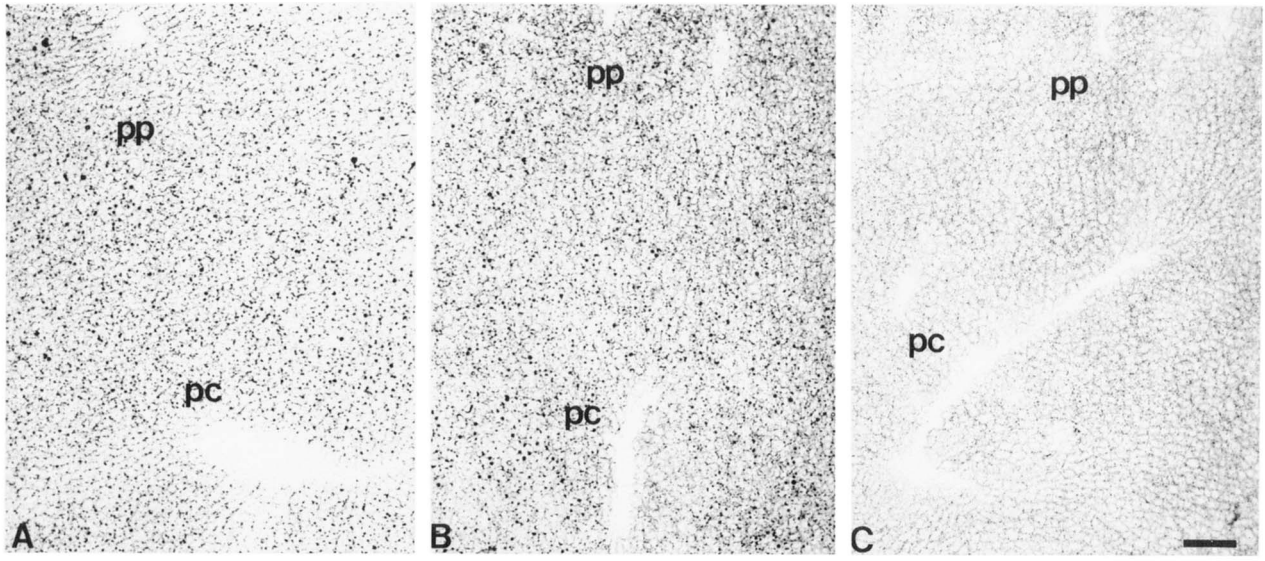

Fig. 3. Distribution patterns of lipids in liver lobules of rats on low fat diet (A), n–6 PUFA diet (B), or n–3 PUFA diet (C); pp, periportal zones; pc, pericentral zones. Bar = 100 μm.

TABLE 3. Capacity of lipid peroxidation product formation in periportal and pericentral zones of rat liver lobules after different diets and before (control) and at 48 h after partial hepatectomy

	Control		After Partial Hepatectomy	
Diet	Periportal	Pericentral	Periportal	Pericentral
Low fat	0.32 ± 0.10[a]	0.38 ± 0.04	0.29 ± 0.06	0.29 ± 0.08
n-6 PUFA	0.05 ± 0.01[b]	0.05 ± 0.01[b]	0.17 ± 0.04[b,c]	0.16 ± 0.08[b,c]
n-3 PUFA	0.52 ± 0.15[b]	0.34 ± 0.10	0.36 ± 0.12[c]	0.32 ± 0.04

[a]Expressed as mean integrated absorbance ± SD at 550 nm.
[b]Statistically significant difference between lipid peroxidation capacity in animals on low fat and PUFA-enriched diets.
[c]Statistically significant difference between lipid peroxidation capacity before and after partial hepatectomy.

Lipid peroxidation capacity

The cytophotometric detection of the capacity to generate lipid peroxidation products revealed that there was no distinct heterogeneity within liver lobules before partial hepatectomy irrespective of treatment or diet with the exception of n-3 PUFA diet. The capacity appeared to be strongly decreased after n-6 PUFA diet and significantly increased in periportal areas after n-3 PUFA diet (**Table 3**). After partial hepatectomy, the capacity to generate lipid peroxidation products was significantly increased in the animals on n-6 PUFA diets and significantly decreased in periportal zones of liver lobules in rats on n-3 PUFA diet (Table 3).

Acyl-CoA oxidase and catalase activity

The activity of acyl-CoA oxidase, the regulatory enzyme of peroxisomal β-oxidation of long chain fatty acids, was significantly increased in livers of rats on n-3 PUFA diet (**Table 4**). The activity was 4- to 5-fold higher than in other diet groups before and after operation. The n-6 PUFA diet caused a small but significant 1.35-fold increase in comparison with low fat diet before partial hepatectomy. This difference disappeared after operation.

Catalase activity in livers of animals on n-6 and n-3 PUFA diets was significantly higher before but not after partial hepatectomy. Catalase activity was strongly reduced after partial hepatectomy, independent of the diet. Before operation, catalase activity was evenly distributed over the zones in liver lobules except for the animals fed on n-3 PUFA. In this group, catalase activity was localized preferentially in pericentral zones (**Fig. 4**). After operation, catalase activity was almost completely lost in periportal compartments, particularly after n-3 PUFA diet (Fig. 4).

The ratio of acyl-CoA oxidase and catalase activity was 3- to 4-fold higher before operation in the n-3 PUFA diet group than in the other diet groups. After partial hepatectomy, this ratio had increased by 8- to 10-fold in all three diet groups, mainly because catalase activity was strongly decreased after operation independent of the diet (Table 4).

Glucose-6-phosphate dehydrogenase activity

Activity of the regulatory enzyme of the pentose phosphate pathway was very high after low fat diet, and even higher after n-6 PUFA diet (**Fig. 5, Table 5**). Moreover, it was distinctly higher in pericentral zones than in periportal zones in lobules of livers of animals on low fat and n-6 PUFA diets before operation. Rats on n-3 PUFA diet did not show such a distinct heterogeneity of the enzyme activity in liver lobules (Fig. 5,

TABLE 4. Acyl-CoA oxidase and catalase activity and ratio of the activities of acyl-CoA oxidase and catalase in livers of rats after different diets and before (control) or at 48 h after partial hepatectomy

	Acyl-CoA Oxidase[a]		Catalase[a]		Ratio[b]	
Diet	Control	Partial Hepatectomy	Control	Partial Hepatectomy	Control	Partial Hepatectomy
Low fat	2.5 ± 0.1	2.9 ± 1.1	1220 ± 82	343 ± 280[d]	2.0 ± 0.1	18.7 ± 19.3[d]
n-6 PUFA	3.3 ± 0.4[c]	2.8 ± 0.3	1396 ± 71[c]	137 ± 24[d]	2.4 ± 0.3[c]	21.1 ± 3.3[d]
n-3 PUFA	14.8 ± 2.5[c]	11.7 ± 1.3[c]	1888 ± 99[c]	224 ± 127[d]	7.9 ± 1.8[c]	63.9 ± 26.7[c,d]

[a]Activity expressed as nmoles H_2O_2 produced or converted per min per mg protein.
[b]Ratio of activities of acyl-CoA oxidase and catalase × 1000.
[c]Statistically significant difference between low fat and PUFA-enriched diets.
[d]Statistically significant difference between activity or ratio before and after partial hepatectomy.

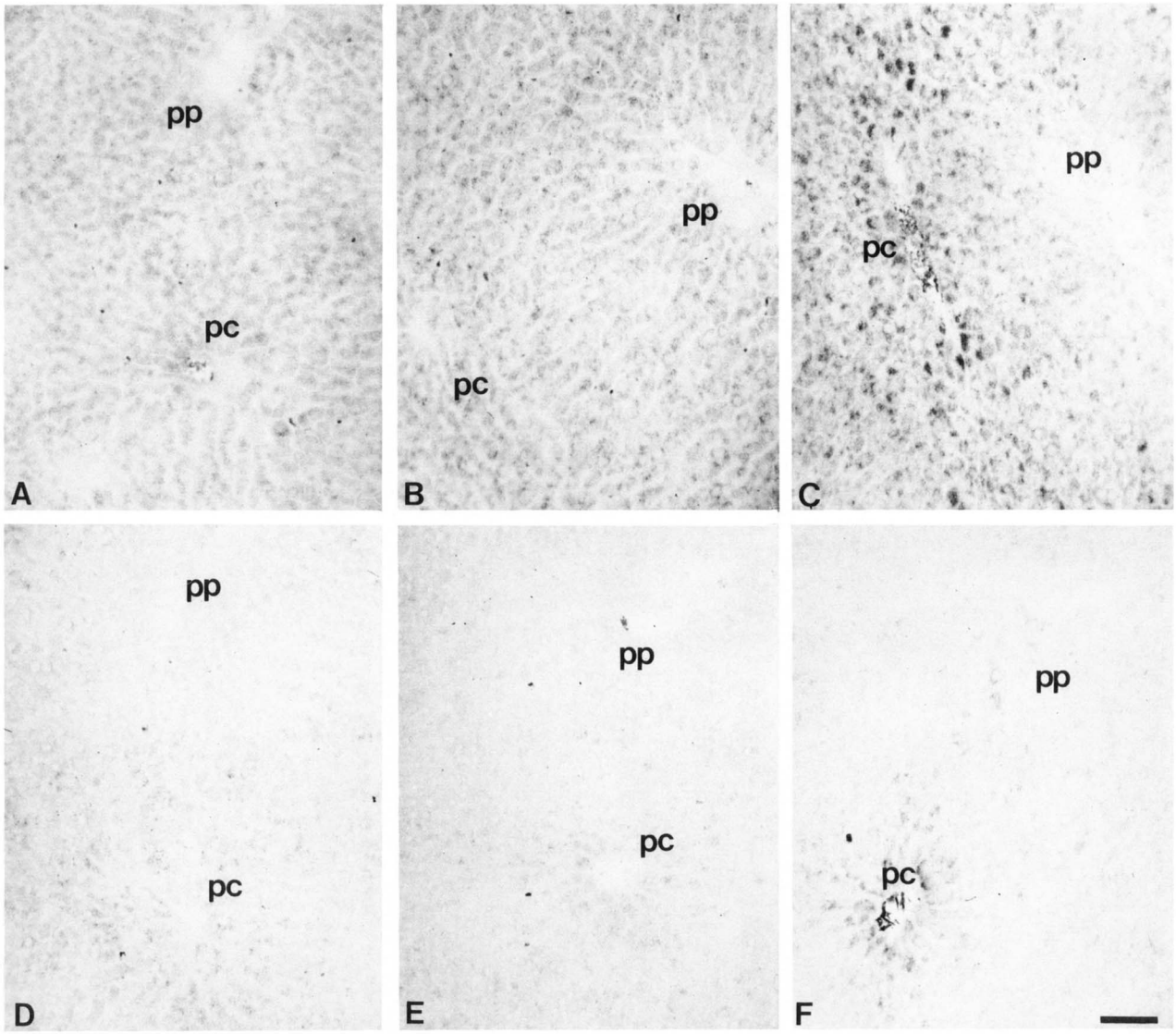

Fig. 4. Distribution pattern of catalase activity in liver lobules of rats on low fat diet (A, D), n–6 PUFA diet (B, E), or n–3 PUFA diet (C, F) before (A, B, C) or at 48 h after partial hepatectomy (D, E, F); pp, periportal zones; pc, pericentral zones. Bar = 100 μm.

Table 5). After partial hepatectomy and low fat or n–6 PUFA diets, activity was decreased significantly in both zones of the lobules. Partial hepatectomy caused a significant increase of glucose-6-phosphate dehydrogenase activity in pericentral zones of liver lobules in rats on n–3 PUFA diet.

NADPH-cytochrome c (P450) reductase activity

Reductase activity was highest in pericentral zones. This distribution pattern was constant irrespective of diet or operation (data not shown).

DISCUSSION

Liver cell proliferation during the first 48 h after partial hepatectomy is reduced by approx. 60% in rats fed on a n–3 PUFA-enriched diet. n–6 PUFA-enriched diet did not significantly affect cell proliferation in comparison with low fat diet. The percentage nuclei positive for BrdU incorporation that we found in rats on low fat diet at 48 h after partial hepatectomy (20%; Table 2) was distinctly lower than the percentage (36%) found by Frederiks et al. (27) at 24 h after operation. This was a surprising but constant finding in our control

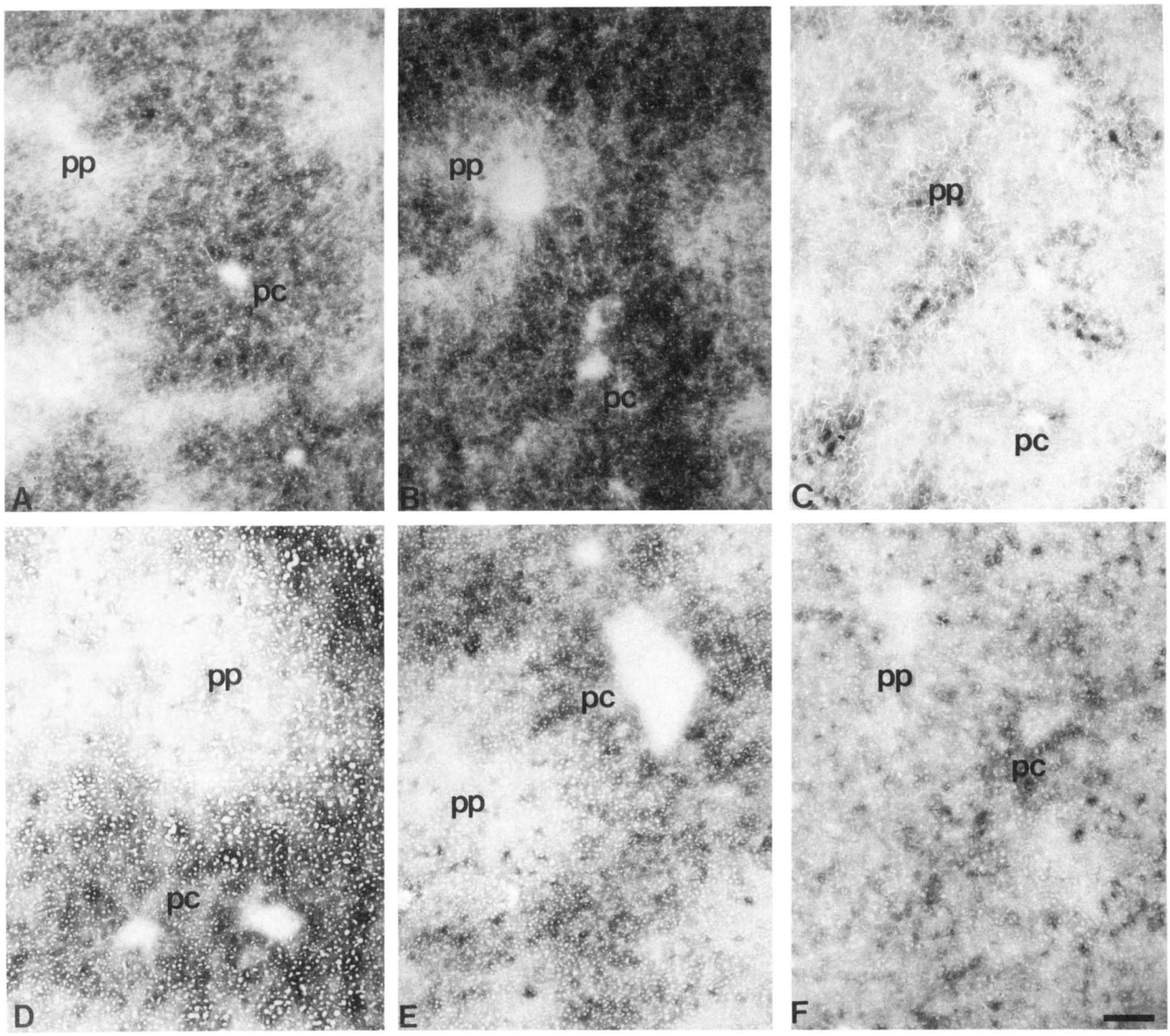

Fig. 5. Distribution pattern of glucose-6-phosphate dehydrogenase activity in liver lobules of rats on low fat diet (A, D), n–6 PUFA diet (B, E), or n–3 PUFA diet (C, F) before (A, B, C) or at 48 h after partial hepatectomy (D, E, F); pp, periportal zones; pc, pericentral zones. Bar = 100 μm.

groups (Table 2). Moreover, BrdU was administered at 0, 23, and 47 h after operation in the present study. The larger part of positive cells was found periportally and midzonally independent of diet. Percentages of proliferating liver cells in periportal, midzonal, and pericentral areas after partial hepatectomy as reported in the literature vary considerably. Straatsburg et al. (I. H. Straatsburg, M. A. Boermeester, A. P. J. Houdijk, W. M. Frederiks, R. I. C. Wesdorp, P. A. M. Van Leeuwen, and C. J. F. Van Noorden, unpublished results) found 12% nuclei positive for proliferating cell nuclear antigen (PCNA) in periportal zones and 4% in pericentral zones at 24 h after operation. With the use of [^{3}H]thymidine incorporation, 5% (25) and 30% (39) in all three zones or 5, 2, and 3% in periportal, midzonal, and pericentral zones, respectively, (26) were found at 48 h after partial hepatectomy. These large variations in percentages of proliferating liver cells demonstrate that several factors influence regeneration after partial hepatectomy. Straatsburg et al. (unpublished results) concluded that spill-over of endotoxins in the circulation is inhibitory to liver cell proliferation and here we demonstrate that n–3 PUFA diet is inhibitory as well (Fig. 2).

All reports mentioned above demonstrate that cell proliferation during the first period after partial hepatectomy occurs mainly periportally and midzonally (Fig. 2) irrespective of the (patho)physiological circumstances. The metabolic zonation within lobules changes

TABLE 5. Glucose-6-phosphate dehydrogenase activity in periportal and pericentral zones of rat liver lobules after different diets and before (control) and at 48 h after partial hepatectomy

Diet	Control		After Partial Hepatectomy	
	Periportal	Pericentral	Periportal	Pericentral
Low fat	12.4 ± 1.7[a]	24.3 ± 4.7[b]	8.1 ± 1.3[d]	16.4 ± 3.1[b,d]
n-6 PUFA	15.1 ± 5.3	40.1 ± 17.4[b]	9.0 ± 1.9[d]	20.3 ± 4.1[b,d]
n-3 PUFA	6.2 ± 1.7[c]	6.1 ± 1.6[c]	6.9 ± 2.4	12.5 ± 5.6[b,d]

[a]Expressed as μmoles glucose-6-phosphate converted per min per g wet weight of tissue.
[b]Statistically significant difference between periportal and pericentral zones.
[c]Statistically significant difference between low fat and PUFA-enriched diets.
[d]Statistically significant difference between activity before and after partial hepatectomy.

after partial hepatectomy because dividing cells become glycolytic. Normally, gluconeogenesis and glycogen synthesis from pyruvate are periportal processes (24). These processes are reallocated from periportal zones to mainly pericentral zones (30, 40) and coincide with at least two other metabolic changes in liver lobules, namely lipid accumulation in droplets causing a fatty liver (Fig. 3) and hydrogen peroxide metabolism in peroxisomes (Table 4). Lipid droplets appear mainly periportally and midzonally (30; Straatsburg et al., unpublished results) where the larger part of cell proliferation takes place and most Kupffer cells and macrophages are found. The lipids are partly recruited from adipocytes in the periphery (41, 42) and/or derived from de novo fatty acid and triglyceride synthesis in hepatocytes (43).

In the present study, the amount and the intracellular localization of lipids were affected by n-3 PUFA in the diet (Fig. 3). This change in lipid accumulation and reduced cell proliferation in the n-3 PUFA diet group could be caused by alterations in the release of cytokines such as tumor necrosis factor α and interleukin-6 or prostaglandins such as PGE_2 by activated Kupffer cells. PGE_2 is a stimulus for hepatocyte proliferation (44, 45). Reduced cell proliferation in livers of the n-3 PUFA group could be due to reduced PGE_2 secretion by Kupffer cells because PGE_2 synthesis is inhibited by n-3 PUFA diet (21, 22). However, n-6 PUFA did not enhance cell proliferation after partial hepatectomy whereas PGE_2 synthesis is stimulated by n-6 PUFA (11, 19). If any effect of n-6 PUFA was noted in the present study it would be inhibition of cell proliferation (16% positive nuclei after n-6 PUFA diet versus 20% after low fat diet). Moreover, the diets did not affect the number of Kupffer cells and newly recruited monocytes before and after operation. These findings indicate that either PGE_2 production by Kupffer cells does not play a main role in the in vivo regulation of cell proliferation after partial hepatectomy or that PGE_2 stimulation is already optimal after partial hepatectomy under normal feeding conditions.

On the other hand, decreased amounts of lipids in livers of rats on n-3 PUFA diet (Fig. 3), the stimulatory effects of n-3 PUFA diet on the β-oxidation capacity in peroxisomes (see also ref. 46), and their vastly reduced catalase-mediated hydrogen peroxide capture capacity in peroxisomes (Table 4) coincide strongly with reduced cell proliferation. Moreover, catalase activity was distributed homogeneously in liver lobules in rats on low fat and n-6 PUFA diets but was highest in pericentral zones in lobules of rats on n-3 PUFA diets (Fig. 4). In these rats, catalase activity was localized almost exclusively in a small pericentral rim after partial hepatectomy. Therefore, it is conceivable that after partial hepatectomy in liver of rats on n-3 PUFA diet a combination of factors causes high levels of lipid peroxidation products that inhibit cell proliferation. First, the capacity for hydrogen peroxide production due to peroxisomal β-oxidation of long chain fatty acids is elevated (Table 4). Second, periportal and midzonal peroxisomal catalase activity is decreased (Fig. 4) and third, the capacity of lipid peroxidation product formation is enhanced (Table 3). These metabolic changes due to n-3 PUFA diet could cause formation of lipid peroxidation products particularly in periportal zones where most of the cell proliferation takes place after partial hepatectomy. Hydrogen peroxide can leak from peroxisomes into the cytoplasm and cause lipid peroxidation as has been described for rats treated with peroxisome proliferators such as clofibrate (47, 48). Treatment with peroxisome proliferators can cause hepatocellular cancer in rats (49). This effect has been ascribed to elevated β-oxidation as monitored by increased activity of acyl-CoA oxidase and a limited increase of catalase activity (48, 50). Increased acyl-CoA oxidase activity could also explain inhibition of cell proliferation in the present study. In fact, two theories on the effects of hydrogen peroxide and lipid peroxidation products are being investigated at present. The first theory proposes that oxidative damage induces carcinogenesis (48, 49), whereas the second theory proposes that lipid peroxidation products down-regulate cell proliferation (1-3, 5, 7-9, 51) and induce differentiation

(52, 53). Our in vivo findings support the latter hypothesis. Distinct peroxisomal subpopulations with different metabolic properties have been described not only in various cell types (54) but also in one and the same cell (55–57). Peroxisomal biogenesis is dynamic and the composition of peroxisomes can be altered, for example due to partial hepatectomy (58), tumor growth (59), or treatment with peroxisome proliferators (60). Therefore, cellular metabolic heterogeneity should be taken into account in the study of the physiological role of lipid peroxidation products.

Differences in calorie intake were avoided as much as possible in the present study (Table 1), but rats on low fat diet and safflower oil-enriched diet ate more than rats on fish oil-enriched diet (Fig. 1). Therefore, a group of rats received a restricted low fat diet of 7 g per day per animal. Reduced food intake did not affect the proliferation rate of liver cells after partial hepatectomy (Table 2). It can be concluded that decreased cell proliferation in livers of rats on fish oil diet was due to n–3 PUFA. Moreover, reduced calorie intake has been reported to stimulate rather than inhibit hepatocellular proliferative burst after partial hepatectomy (61).

Composition of the diets also had a dramatic effect on glucose-6-phosphate dehydrogenase activity. In normal mature female rats, glucose-6-phosphate dehydrogenase activity is equivalent to approx. 4–6 and 8–10 μmol glucose-6-phosphate converted per min per g wet weight of tissue in periportal and pericentral zones, respectively (30, 62). Low fat or n–6 PUFA diets resulted in a 2- to 4-fold increase in activity. Partial hepatectomy reduced the activity 1.5- to 2-times in both metabolic zones (Table 5), whereas in normally fed female rats, the distribution pattern of glucose-6-phosphate dehydrogenase activity becomes more or less homogeneous after partial hepatectomy (30). The homogeneous distribution pattern and the low activity of glucose-6-phosphate dehydrogenase are male-like rather than female-like (30) although estrogen levels are increased and testosterone levels are decreased in the circulation after partial hepatectomy thus causing hepatic feminization after partial hepatectomy (63, 64). Low fat and n–6 PUFA diets resulted in maintenance of the female pattern after operation (Fig. 5, Table 5) whereas n–3 PUFA diet caused a change in the distribution pattern after partial hepatectomy that is more female-like than before operation. Low fat or carbohydrate-rich diets induce glucose-6-phosphate dehydrogenase activity (65, 66). The enzyme is considered to be a member of the family of lipogenic enzymes which also includes fatty acid synthase, acetyl-CoA carboxylase, and NADP-dependent malate dehydrogenase (66). It is remarkable that n–6 PUFA and low fat diets induce glucose-6-phosphate dehydrogenase in a similar way. The more so, because lineoleate and arachidonate, which both belong to the n–6 PUFA class (11, 17), can repress glucose-6-phosphate dehydrogenase expression in vitro (66). This discrepancy underlines the complexity of regulation mechanisms in vivo.

The distribution patterns of glucose-6-phosphate dehydrogenase activity in livers of rats on different diets and before or after partial hepatectomy also cannot be explained by a direct inhibition of its activity by lipid peroxidation products (15). Before operation, elevated lipid peroxidation products generated from the n–3 PUFA diet could be the inhibitory cause but after operation glucose-6-phosphate dehydrogenase activity is significantly elevated in pericentral zones (Table 5) whereas lipid peroxidation product formation in peroxisomes should be highest in livers from rats on n–3 PUFA after operation (Table 4).

We did not find large variations in the distribution pattern or levels of NADPH-cytochrome c (P450) reductase activity due to diet or operation, although loss of one-third of its activity was observed at 48 h after partial hepatectomy in comparison with sham-operated rats using biochemical means (67). Furthermore, NADPH production capacity by means of glucose-6-phosphate dehydrogenase activity was strongly reduced after n–3 PUFA diet (Table 5). Therefore, enhanced lipid peroxidation on the basis of Ω-oxidation via microsomal NADPH-dependent cytochrome P450 activity does not seem to play a main role in the present in vivo study in contrast with the in vitro study of Morisaki et al. (9).

In conclusion, it can be stated that controlled cell proliferation in vivo is inhibited by n–3 PUFA diet but not significantly affected by n–6 PUFA diet. The most likely regulation mechanism behind this effect is elevated peroxisomal hydrogen peroxide production in combination with an elevated capacity of lipid peroxidation production. These in vivo studies on lipid metabolism in relationship with controlled cell proliferation in different compartments of liver lobules are in line with studies on cancer cell growth which is also affected by n–3 PUFA (20–22, 32). Studies on the metabolic backgrounds of cancerous growth in animals on low fat, n–3, and n–6 PUFA diets are in progress.

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Box 2109, N-3202 Sandefjord, Norge, by the supply of n-3 polyunsaturated fatty acid-containing fish oil.

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