# Nuclear 3,5,3′-triiodothyronine receptors and malic enzyme activity in liver of rats fed fish oil or cocoa butter

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*The role of different fat supplements (fish oil and cocoa butter), applied intragastrically for 3 weeks, on specific binding of $T_3$ by rat liver nuclear receptors was examined. Fish oil (rich in n-3 polyunsaturated fatty acids) supplementation resulted in a significant ($P < 0.05$) enhancement of the maximal binding capacity (MBC) for $T_3$ with a diminution of the apparent association constant ($K_a$). No changes in nuclear receptor MBC or $K_a$ were observed after cocoa butter administration. Moreover, the rate of fatty acid synthesis in the liver was evaluated by measuring malic enzyme (EC. 1.1.1.40.) activity in rat liver cytosol, which was found to be decreased in both the cocoa butter- ($P < 0.002$) and the fish oil- ($P < 0.001$) treated rats. Interestingly, in fish oil-supplemented animals, not even a higher MBC value for the $T_3$ receptors in liver nuclei and specific binding of $T_3$ to its receptor were able to restore the activity of malic enzyme in liver cytosol. The findings of the same level of thyroid hormones—thyroxine ($T_4$) and triiodothyronine ($T_3$)—in blood of all groups showed that there was apparently no effect of fat supplementation on the thyroid function. However, it may be hypothesized that the decrease of malic enzyme activity in both fat-treated groups may have resulted from a decreased $T_4$ to $T_3$ conversion in liver cells. The data suggest the following: (a) increased dietary fat intake is accompanied by lower lipogenesis in the liver in relation to the degree of fat unsaturation; (b) although polyunsaturated dietary fat increases $T_3$ receptor binding to liver nuclei; (c) thus, an uncoupling between $T_3$ binding and action on malic enzyme activity during raised fish oil intake cannot be ruled out.*

**Keywords:** liver triiodothyronine receptors; fat diet; malic enzyme

## Introduction

It is generally accepted that the lipid composition of subcellular fractions of the liver and other tissues in the rat may be influenced by fatty acid composition of the diet.[1–3] For instance, increased dietary intake of n-3 polyunsaturated fatty acids (PUFA) is known to be accompanied by their increased accumulation in isolated hepatocyte plasma membranes of the rat.[4] Diets rich in n-3 PUFA (30 wt%) result in an increase of n-3 PUFA in membrane lipids. Such changes in cell membrane saturation are followed by changes in cell membrane ordering.[4,5] This is, in turn, connected with changes in hormone receptor binding and/or activities of cell membrane associated enzymes.[5,6] It could be supposed that an increased dietary intake of either unsaturated, or saturated fatty acids may be followed by their enhanced incorporation into intracellular membranes. In fact, mice fed a diet containing a high proportion of linoleic acid had higher contents of n-6 PUFA in liver nuclear membranes than those fed a diet with a low linoleate content.[7] Thus, changes of nuclear membrane-related functions, following dietary manipulations, are predictable.

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Specific receptors for thyroid hormones were originally reported to be localized exclusively in non-histone proteins of liver nuclei and not in the nuclear membrane.[8] However, the presence of nuclear membrane binding sites for steroid and thyroid hormones has recently been documented.[7] These authors also found that mice fed a diet with a high polyunsaturated:saturated (P:S) ratio for 4 weeks had an unsaturated liver nuclear envelope (they exhibited higher levels of C 18:2 n-6), and showed higher specific binding of 3,5,3′ L-triiodothyronine ($T_3$) to nuclear membranes as compared with rats fed a diet with a low P:S ratio. The effect of diets containing corn oil or fish oil on PUFA content of rat liver nuclei, nuclear membrane, and nuclear matrix was also investigated by Iritani et al.[9] In their study, the fatty acid composition of phospholipids in all subcellular fractions investigated was markedly influenced by both oils.

In this regard there are no data on binding of $T_3$ to rat liver nuclear receptors with the exception of that published by Van der Klis et al.[10] These authors have shown that unsaturated fatty acids such as oleic acid, when added in vitro, inhibit nuclear binding of $T_3$ by a competitive mechanism.

Therefore, our recent experiments have been designed to evaluate the effect of PUFA on $T_3$ binding to rat liver nuclear receptors, e.g., in liver nuclei obtained from rats being fed a diet with raised n-3 PUFA content. Special regard was devoted to a potential influence of these diets on the $T_3$-inducible activity of malic enzyme. The data were compared to those obtained in rats fed a diet supplemented with cocoa butter as a source of saturated fatty acids. The significant amount of stearic acid in the cocoa butter was found rather advantageous, as the commercially available laboratory chow for rats in our country also contains a substantial amount of this acid.

## Materials and methods

### *Animals and diets*

Male Wistar rats purchased from Velaz (Prague, Czechoslovakia), weighing approximately 400 g at the beginning of the experiment, were randomized into three groups of 10 animals each. The animals were housed in stainless-steel cages, in a well-ventilated room with temperature kept at 22° C, and a 12hr light/dark cycle. Each group of animals was fed the same commercially available (Velaz, Prague, Czechoslovakia) basal laboratory chow (protein:lipid:carbohydrates = 25:22:53 cal%; originating from wheat scrap 58 wt%, casein 15 wt%, dry milk 13 wt%, vitamins and biofactors 5 wt%, wheat bran 9 wt%). Animals were fed ad libitum the above basal diet, but with different fat supplements: 1 mL of cocoa butter or fish oil (EPA, Martens, Norway, 20.5 wt% n-3 PUFA) applied intragastrically shortly before the end of the daily light period, i.e., at 5:30 p.m. Control animals received 1 mL of physiological saline. Fatty acid composition of the various fats used for supplementation is shown in *Table 1*. After 3 weeks of dietary treatment, animals were sacrificed at 7:30 a.m. by cervical dislocation after an overnight fast. Blood was collected for determination of total serum triglycerides,[11] cholesterol,[12] and fatty acid (FA) composition of the serum. The livers were rapidly removed, weighed, and used for nuclei and cytosol isolation.

**Table 1** Fatty acid composition of dietary fat supplements and of the standard laboratory chow (basal diet)

| Fatty acid (% wt/wt) | Diatary fat | | |
|---|---|---|---|
| | Cocoa butter | Fish oil | Basal diet |
| 14:0 | 3.6 | 4.2 | 1.3 |
| 16:0 | 26.3 | 7.1 | 19.7 |
| 16:1 | 3.0 | 7.4 | 2.9 |
| 18:0 | 19.2 | 0.7 | 6.3 |
| 18:1 (9) | 31.9 | 6.5 | 23.0 |
| 18:1 (7) | 2.6 | 2.7 | 1.8 |
| 18:2 (6) | 10.3 | 1.4 | 32.6 |
| 18:3 (3) | 0.6 | 1.3 | 4.5 |
| 20:4 (6) | — | 1.1 | 0.3 |
| 20:5 (3) | — | 34.0 | 1.8 |
| 22:5 (3) | — | 3.7 | 0.3 |
| 22:6 (3) | — | 19.0 | 1.7 |
| Others | — | 9.9 | 3.8 |
| Total: | | | |
| Saturated | 50.4 | 17.9 | 27.3 |
| Monounsaturated | 37.5 | 20.0 | 27.7 |
| n-6 | 10.3 | 2.5 | 32.9 |
| n-3 | 0.6 | 58.0 | 8.3 |
| P/S | 0.2 | 3.5 | 1.5 |

P/S, polyunsaturated/saturated.

### *Isolation and extraction of nuclei*

Purified rat liver nuclei from non-pooled tissue were freshly prepared by the procedure described by DeGroot and Torresani,[13] with all subsequent steps being carried out at 0–4° C. Tissue was minced and homogenized in 0.32 mol/L sucrose, 1 mmol/L $MgCl_2$, 0.1 mmol/L PMSF, 1 mmol/L DL-dithiothreitol. The liver homogenate was centrifuged at 1,000*g*, the crude pellet washed with the same medium and centrifuged repeatedly at 1,000*g*. The pellet was mixed with 2.3 mol/L sucrose containing 1 mmol/L $MgCl_2$, 0.1 mmol/L PMSF, 1 mmol/L DL-dithiothreitol and then treated by ultracentrifugation at 220,000*g* for 30 min in the swing-out SW 41 rotor in a Beckman L5-50 model (Beckman Instruments, Fullerton, CA USA) ultracentrifuge. Liver nuclei were then washed once in ice-cold SMCT buffer (0.32 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L PMSF, 1 mmol/L DL-dithiothreitol) in the presence of 0.25% Triton X-100 and once in the absence of Triton X-100. This method for isolation of liver nuclei gives a highly purified nuclear fraction as proven by fluorescence microscopy and a protein:DNA ratio of 3.01 ± 0.26, and RNA:DNA ratio of 0.081 ± 0.011.

The nuclear non-histone proteins containing the $T_3$ receptor fraction were obtained directly by extracting purified liver nuclei in NaMPTD buffer (0.3 mol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, 25 mmol/L $NaH_2PO_4$, pH 8.0 and 1 mmol/L dithiothreitol at 0° C, for 1 hr, and separated from the fraction of disrupted nuclei by ultracentrifugation at 135,000*g* (Ti 50.3 rotor, Beckman model L5-50 ultracentrifuge).

### *Binding of $^{125}I$-$T_3$ to nuclear receptors*

The assays on the specific binding of $^{125}I$-$T_3$ (specific activity 44.4 TBq $g^{-1}$, commercially available from the Institute of

Isotopes, Hungarian Academy of Sciences, Budapest, Hungary) were performed at 22° C in 0.5 mL NaMPTD buffer (pH 8.0). Samples containing approximately 200 μg $mL^{-1}$ nuclear protein were incubated for 2 hr with both 7.4 × $10^{-11}$ mol/L $^{125}I$-$T_3$ and increasing concentrations (from 1.5 × $10^{-10}$ to 3.0 × $10^{-9}$ mol/L) of $T_3$. Nonspecific binding of the labeled ligand was distinguished by 3.0 × $10^{-7}$ mol/L $T_3$ per sample. The radioactivity of 0.5 mL aliquots of the resin (Dowex 1-X8, 80 mg $mL^{-1}$) treated supernatant was quantified in a gamma spectrometer (model 4000, Beckman, Fullerton, CA, USA).

### *Thyroid hormones analyses*

The concentration of thyroxine, 3,5,3'-L-triiodothyronine, and reverse triiodothyronine was estimated with the aid of radioimmunoassay using specific antisera prepared at this institute.[14]

### *Enzyme assay*

A spectrophotometric assay described by Freedland et al.[15] was used for the estimation of NADP malate dehydrogenase (E.C.1.1.1.40). The final concentrations of the reagents at 37° C were: Tris (adjusted to pH 7.4) 30 mmol/L, $MnCl_2$ 0.05 mmol/L, sodium malate 0.85 mmol/L, 0.1 mL of the supernatant cytoplasmic (105,000*g*) fraction representing approximately 1 mg of liver protein. The measurements of absorbance were performed at 340 nm. After 5 min of equilibration, the absorbance changes were recorded for several minutes in 30 sec intervals. The results were expressed as rate increment of NADP reduction after the addition of malate following blank subtraction.

### *Lipid analyses*

Lipids were extracted from the diet and/or serum by the method of Folch et al.[16] The methyl esters of FA were prepared (after alkaline hydrolysis of lipids) by esterification with diazomethane and analyzed by gas-liquid chromatography (GLC) using an HRGC 4160 apparatus (Carlo Erba, Italy) equipped with a flame detector. A glass capillary column with stationary phase SP 2340 was used. The details of the GLC analysis were reported elsewhere.[17] Results were quantified with an SP 4000 integrator (Spectra Physics, Heidelburg, Germany).

### *Protein determination*

Protein concentration was determined according to Lowry et al.[18] using bovine serum albumin as standard.

### *Statistical analysis*

All data are presented as mean ± SEM. Two-way analysis of variance (ANOVA) was used to analyze the data, and the Duncan test was used to check the significance of differences ($P < 0.05$) between the means.[19]

## Results

Average body and liver weights, and the amount of food consumed per day by the animals fed the fat-supplemented diets for 3 weeks are shown in *Table 2*. In this study, rats consumed similar amounts of food irrespectively of the dietary regimen. The dietary lipid supplementation had no significant effect on either body or liver weights.

To prove the effectiveness of chosen dietary manipulations, the fatty acid composition of rat serum was determined (*Figure 1*). Fish oil-supplemented rats had increased levels of total omega-3 [mainly due to high levels of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid], and decreased total n-6 fatty acids content of serum total lipids. Supplementation of animals with cocoa butter did not have any effect on total n-6 and n-3 PUFA serum levels in comparison to the control and/or the fish oil-supplemented group. Cocoa butter did not change the total amount of saturated fatty acids in serum total lipids. However, serum DHA levels were significantly reduced in rats supplemented with cocoa butter when compared with control animals (data not shown).

To make sure that the raised serum concentrations of polyunsaturated fatty acids are high enough to have some metabolic and/or regulatory action, serum levels of triglycerides and cholesterol were also measured (*Table 3*). As expected, raised dietary n-3 PUFA intake led to a suppression of both parameters mentioned above. Dietary supplementation of rats with cocoa butter changed neither triglyceride nor cholesterol serum levels.

Binding of L-triiodothyronine to rat liver nuclear receptors in control rats and animals supplemented with fish oil and/or cocoa butter was calculated using the Scatchard plot (*Figure 2*). Fish oil markedly increased the number of $T_3$ nuclear receptors, whereas cocoa butter did not. Parameters evaluated by Scatchard plot analysis, the maximal binding capacity

**Table 2** Food consumption, body weight change, and liver weight in animals fed various diets

| | Basal diet (BD) | BD + cocoa butter | BD + fish oil |
|---|---|---|---|
| Food consumption (g/day) | 21 ± 0.3[a] | 20.5 ± 2.0[a] | 21.1 ± 1.4[a] |
| Body weight (g) Day 0 | 415 ± 10.0[a] | 398 ± 9.3[a] | 411 ± 10.1[a] |
| Day 20 | 438 ± 10.5[a] | 423 ± 10.4[a] | 431 ± 10.6[a] |
| Body weight increment (g) | 22.2 ± 4.0[a] | 25.0 ± 3.7[a] | 21.5 ± 2.1[a] |
| Liver weight (g) | 11.1 ± 0.8[a] | 10.2 ± 0.5[a] | 10.6 ± 0.9[a] |
| Liver/body weight | 0.025 ± .08[a] | 0.024 ± .05[a] | 0.025 ± .09[a] |

The results are expressed as means ± SEM for 10 animals/group. Values in the same row without a common superscript are significantly different based on ANOVA and the Duncan test ($P < 0.05$).

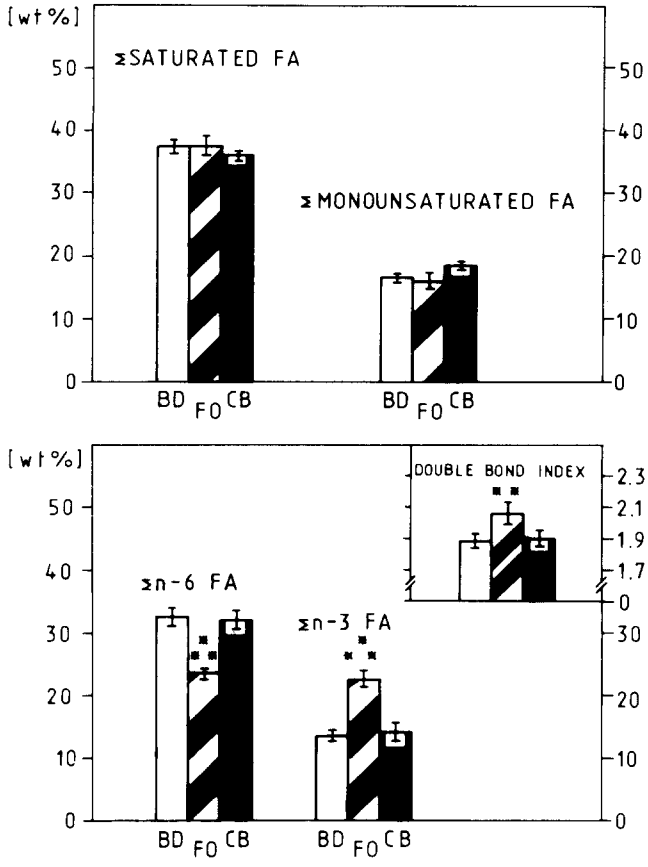


**Figure 1** Fatty acid composition of serum total lipids in rats fed various diets. BD = basal diet; FO = fish oil; and CB = cocoa butter–supplemented basal diet.

(MBC) and the apparent association constants ($K_a$), revealed different effects of fat supplementation on $T_3$-specific binding. MBC of $T_3$ in rat liver nuclear receptors was significantly increased in the fish oil group, as compared to basal diet and cocoa butter fed rats (*Figure 3*). In contrast, $K_a$ for liver nuclear receptors was decreased in rats supplemented with fish oil, and no changes were observed after cocoa butter supplementation, in comparison with data obtained for the control group (*Figure 4*).

To see whether the changes in nuclear binding sites could reflect those in iodothyronine levels in the circulation, thyroxine, triiodothyronine, and reverse triiodothyronine levels in serum were measured (*Table 4*). However, no significant differences in the level of $T_4$ and $T_3$ were observed, while that of metabolically inactive $rT_3$ was increased ($P < 0.05$) in the BD plus

**Table 3** Effect of fish oil and/or cocoa butter supplementation on serum triglycerides and cholesterol levels

| | Basal diet (BD) | BD + cocoa butter | BD + fish oil |
|---|---|---|---|
| Triglycerides (mmol/L) | 1.35 ± 0.20[a] | 1.21 ± 0.20[a] | 0.56 ± 0.10[b] |
| Cholesterol (mmol/L) | 1.40 ± 0.07[a] | 1.45 ± 0.05[a] | 0.95 ± 0.07[b] |

The results are expressed as mean ± SEM for 8 animals/group (due to loss of some samples during processing). Values in the same row without a common superscript are significantly different based on ANOVA and the Duncan test ($P < 0.05$).

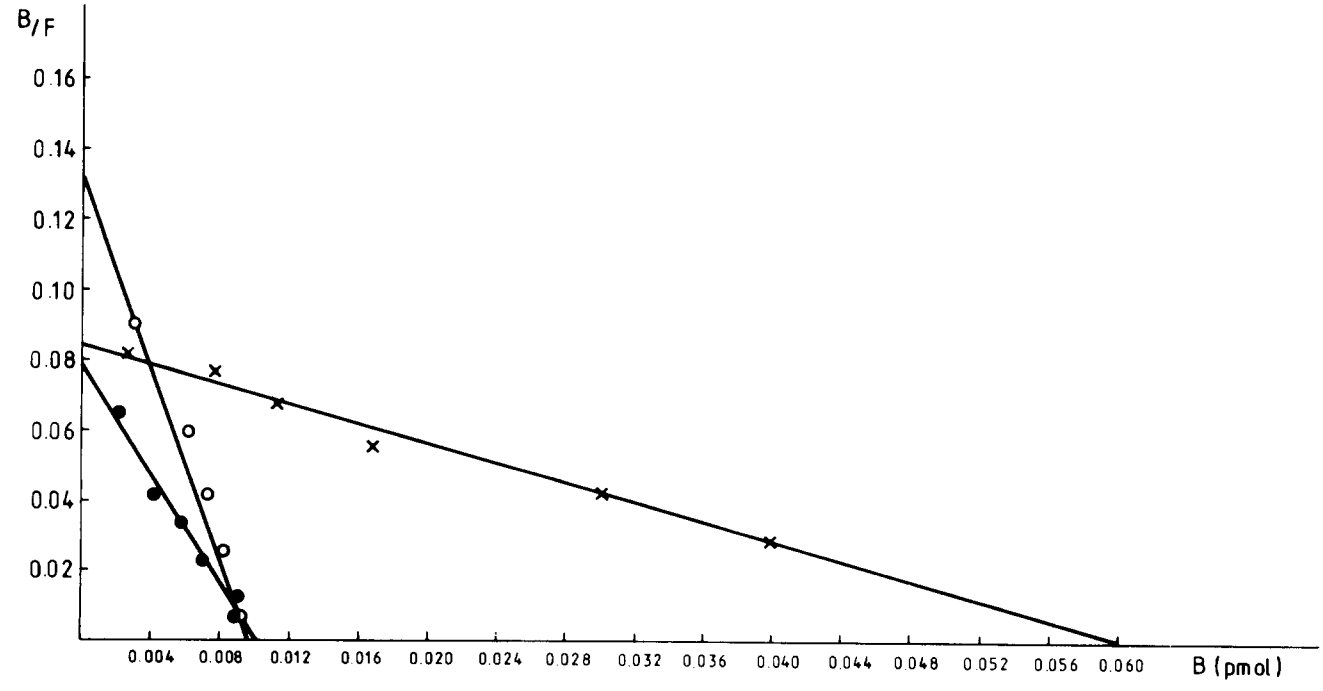


**Figure 2** Representative Scatchard plot of $T_3$ binding to liver nuclear receptor of rats fed basal diet; BD = ○, BD + cocoa butter = ●, or BD + fish oil = x (always one rat per group).

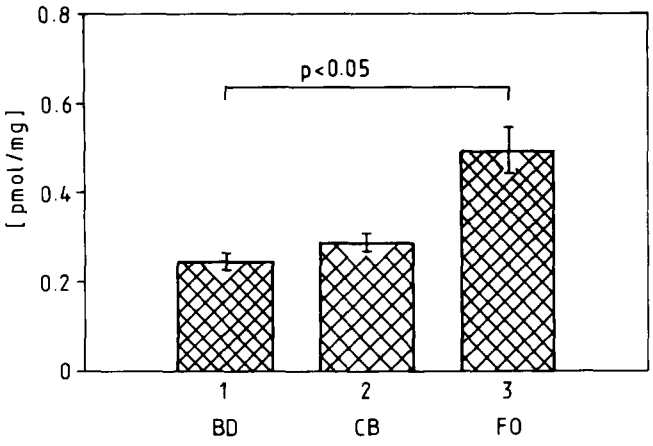


**Figure 3** Effects of various diets on maximal binding capacity (MBC) of $T_3$ as evaluated from the Scatchard plots ($n$ = 10). Rat liver nuclear proteins containing $T_3$ receptors (100 $\mu$g/tube) were incubated with $7.4 \times 10^{-11}$ mol/L $^{125}$I-$T_3$ in the presence or absence of increasing concentrations (from $1.5 \times 10^{-10}$ to $3.0 \times 10^{-7}$ mol/L) of $T_3$ at specified temperature for 2 hr. Results are expressed as pmol $T_3$ bound per mg protein. BD = basal diet; CB = cocoa butter–supplemented diet; FO = fish oil supplemented diet.

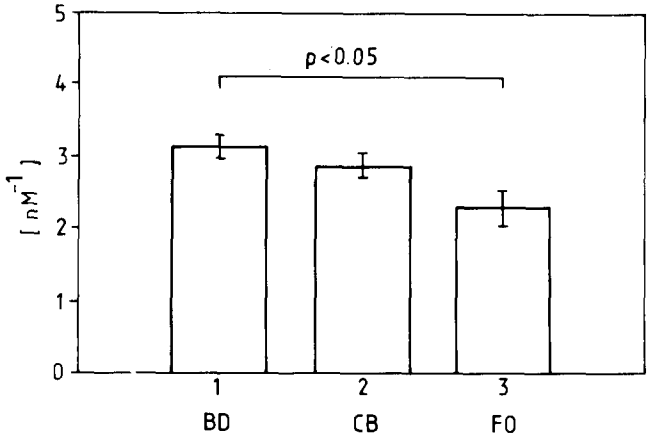


**Figure 4** Effects of various diets on the relative association constant $K_a$ ($nM^{-1}$), calculated from the Scatchard plots ($n$ = 10) under conditions as described in legend to *Figure 2*.

**Table 4** Effect of fish oil and/or cocoa butter supplementation on serum thyroid hormone levels

| | Basal diet (BD) | BD + cocoa butter | BD + fish oil |
|---|---|---|---|
| Thyroxine (nmol/L) | 51.1 ± 2.8[a] | 56.2 ± 7.8[a] | 55.7 ± 5.3[a] |
| Triiodothyronine (nmol/L) | 1.19 ± 0.10[a] | 1.10 ± 0.06[a] | 1.32 ± 0.15[a] |
| Reverse triiodothyronine (nmol/L) | 0.57 ± 0.06[ab] | 0.51 ± 0.03[b] | 0.68 ± 0.08[ac] |

Results are expressed as mean ± SEM for 8–10 animals/group (due to loss of some samples during processing). Values in the same row without a common superscript are significantly different based on ANOVA and the Duncan test ($P < 0.05$).

fish oil group as compared with the animals fed BD plus cocoa butter.

The rate of fatty acid synthesis was evaluated by measuring the malic enzyme activity in rat liver cytosol. Animals of both experimental groups showed a decreased activity of the lipogenic enzyme. In the fish oil group, this effect on malic enzyme activity was more pronounced when compared with the cocoa butter-supplemented animals (*Table 5*).

## Discussion

Present studies indicate that a raised dietary intake of omega-3 PUFA can modulate specific binding of $T_3$ to rat liver nuclear receptors. It is also suggested that the $T_3$-specific binding to both rat liver nuclear receptors and/or $T_3$ binding sites of nuclear membranes might be a result of different phospholipid fatty acid composition.

In our experiments, substantial changes in serum fatty acid composition of rats supplemented intragastrically with fish oil were observed. The intragastric route was chosen with the aim to mimic the human nutrition situation in which marine fish oil concentrates are taken most often in addition to a regular meal. We, and other groups as well, have shown that changes of serum fatty acid composition obtained by in vivo dietary supplementation of omega-3 PUFA are followed by equivalent changes of liver plasma and/or intracellular membrane lipids.[4,9] The lack of effect of dietary cocoa butter on rat liver nuclear specific binding of $T_3$ supports the idea of a stimulatory action of raised omega-3 PUFA liver nuclear content on number of nuclear $T_3$ receptors. Although the transport mode of the thyroid hormone across the nuclear membrane is still unknown, we assume that a higher binding of $T_3$ to nuclear membrane in rats fed an n-3 PUFA rich diet, could affect the number of hormone molecules bound to the nuclear receptors. Such a mechanism however, was not observed in our experiments. The inhibition of nuclear $T_3$ binding with oleic acid in vitro was recently elucidated.[10] As a direct interaction between oleic acid and $T_3$ was excluded, the effect seems to be competitive. It is intriguing to consider a similar mechanism of action for n-3 PUFA on $T_3$ nuclear binding in our experiments.

**Table 5** Effect of dietary fat on the malic enzyme activity in rat liver cytosol

| | Malic enzyme activity (nkat) |
|---|---|
| Basal diet | 185 ± 8.1[a] |
| Cocoa butter | 83 ± 5.2[b] |
| Fish oil | 37 ± 3.3[c] |

Results are expressed as mean ± SEM for 8 animals/group (due to loss of some samples during processing). Values without a common superscript are significantly different based on ANOVA and the Duncan test ($P < 0.002$).

The malic enzyme activity in rat liver cytosol provides a suitable parameter for studying its regulation by both the thyroid hormone and nutritional factors. In particular, potential interactions between them may be responsible for an increase in the activity of this enzyme.[20] The latter authors found that a high carbohydrate-low fat diet raised the activity of the liver malic enzyme. In contrast, feeding rats a high fat diet led to low transcriptional activity, and to a decrease in the mRNA level of the malic enzyme. It was suggested that the enzyme induction was regulated at a pretranslation step. Recently, it was observed that $T_3$ regulates malic enzyme activity on both the transcriptional and the post-transcriptional levels. Moreover, the effect of insulin on malic enzyme synthesis appears to be different from that of carbohydrates.[21] These authors also claim that lipids in the rat liver nuclei are involved in the activity of malic enzyme in a way that is different from that of the enzyme induction by $T_3$.[9]

Our recent data show that in spite of a high MBC for $T_3$ receptors in rat liver nuclei obtained from fish oil-treated rats, the malic enzyme activity was low, and higher binding of $T_3$ to its receptors did not result in restoration of enzyme activity. Studies on the mechanism of action of triiodothyronine on malic enzyme activity brought evidence that $T_3$ induces this enzyme by binding of the $T_3$-receptor complex to the malic enzyme gene responder, and/or $T_3$ induces or activates some essential transcription factors that bind to the promoter.[22] Thus, considering that at least two different regulatory steps participate in regulation of the malic enzyme gene transcription, the exact mechanism responsible for an uncoupling between hormone binding and its action on malic enzyme activity in our experiments remains to be further elucidated.

Because no differences in serum levels of $T_4$ and $T_3$ between groups were found, it may be concluded that at least the amount of hormones available to the liver cells was about the same in each group. However, no conclusion may be drawn from these findings about the transmembrane transport of $T_4$ and the rate of intracellular $T_4$ to $T_3$ conversion, both of which may be influenced by metabolic factors.[23,24] The increased serum level of $rT_3$, which is a metabolically inactive degradation product of $T_4$, may result from a preferential conversion of $T_4$ to $Rt_3$ due to its metabolic changes in some tissues in the fish oil-fed group. This may be supported by our previous finding of increased biliary excretion of $rT_3$ during the infusion of linoleic acid.[25] Such findings might be related to the decreased activity of malic enzyme (*Table 5*) and decreased $T_3$ binding to the liver nuclear receptors (*Figures 2 and 4*).

Our data on decreased malic enzyme activity in the liver suggest that dietary fatty acid composition might be an important factor in the regulation of this lipogenetic enzyme in the liver. However, recent data by Awad et al.[26] obtained in rat epididymal adipose tissue demonstrated that dietary fatty acid composition did not have any effect on in vivo lipolysis or in vitro lipogenesis. That we found a decrease of lipogenesis, and in the other group either no change or a tendency for increased lipogenesis after fish oil supplementation, is not surprising. We have experienced that each main organ in the body (i.e., liver, adipose and muscle tissue) does not always respond to fat (saturated or unsaturated) feeding in the same direction.[4,27] Moreover, it is a well-accepted fact that fish oil suppresses fatty acid synthesis and triglyceride production in the liver.

In summary, this is the first report that demonstrates an increase of $T_3$ binding to rat liver nuclear receptors obtained from rats being fed a diet with a raised n-3 PUFA content. Moreover, not even higher MBC value for the $T_3$ receptors in liver nuclei was able to abolish the fish oil-induced suppression of fatty acid synthesis in the liver, as judged by measuring the malic enzyme activity in liver cytosol. Thus, other regulatory factors independent of $T_3$ action seem to operate in the conditions of our experimental setting.

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