Drug-induced and postnatal hypothyroidism impairs the accumulation of diacylglycerol in liver and liver cell plasma membranes

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Abstract

**Background:** Thyroid hormones are well known modulators of signal transduction. The effect of hyper- and hypo-thyroidism on diacylglycerol/protein kinase C (DAG/PKC) signaling in cardiomiocytes has been determined. Triiodothyronine (T₃) has been shown to prevent the α₁-adrenoreceptor-mediated activation of PKC but does not alter the stimulation of enzyme and hepatic metabolism by phorbol ethers. It has been suggested that the elevation of endogenous DAG in senescent or hypothyroid cells changes the PKC-dependent response of cells to phorbol esters and hormones. In the present study, was examined the formation of DAG and activation of PKC in liver cells from rats of different thyroid status.

**Results:** The results obtained provide the first demonstration of DAG accumulation in liver and cell plasma membranes at age- and drug-dependent thyroid gland malfunction. The experiments were performed in either the [14C]CH₃COOH-labeled rat liver, liver slices or hepatocytes labeled by [14C] oleic acid and [3H]arachidonic acid or [14C]palmitic acid as well as in the isolated liver cell plasma membranes of 90- and 720-day-old rats of different thyroid status. The decrease of T₄ and T₃ levels in blood serum of 720-day-old rats and mercazolil-treated animals was associated with increases of both the DAG mass in liver and liver cell plasma membranes and newly synthesized [14C]DAG level in liver and isolated hepatocytes. Hypothyroidism decreased PKC activity in both membrane and cytosol as well as phospholipid and triacylglycerol synthesis in liver. These hypothyroidism effects were restored in liver by injection of T₄. T₄ administration to the intact animals of different ages decreased the DAG level in liver and isolated plasma membranes and the content of newly synthesized DAG in liver. The reduction of DAG level in liver was not associated with increasing free fatty acid level. DAG labeling ratio [14C]/[3H] in liver slices of rats of different thyroid state sharply differed from PL. DAG was relatively enriched in [14C]oleic acid whereas PL were enriched in [3H]arachidonic acid.

**Conclusions:** The above data have indicated that thyroid hormones are important physiological modulators of DAG level in rat liver and cell plasma membranes. Age- and drug-induced malfunction of thyroid gland resulted in a prominent decrease of glycerolipid synthesis which may promote DAG accumulation in liver.
Background

sn-1,2-Diacylglycerol plays a key role in lipid biosynthesis and signal transduction in a variety of mammalian cells. Thus, to maintain cellular homeostasis, intracellular DAG levels must be tightly regulated. This is illustrated by evidence that inappropriate accumulation of DAG contributes to cellular transformation. For example, cell lines that overexpress PLCγ have a malignant phenotype [1]. Also, cells transformed with one of several oncogenes have elevated DAG levels [2]. Most of the evidences for this pathological effect centers on excessive and/or prolonged activation of PKC, which is a common feature of the transformed state, both in tumors and in cell cultures. Accumulation of DAG with increased membrane-associated PKC is the mechanism of spontaneous hepatocarcinogenesis in choline-deficient rats [3,4]. Aortic endothelial cells have been shown to contain particularly high basal levels of polyunsaturated DAG together with a very high degree of membrane-associated PKC, which is largely insensitive to further activation [5]. Glucose-induced de novo synthesis of DAG and sustained isozyme-selective PKC activation (especially PKC-β) contribute to the pathogenesis of diabetic micro- and macroangiopathy [6]. The fed state was associated with increased DAG level and PKC activity in muscle tissue of insulin-resistant obese Zucker rats [7]. These changes in PKC are likely to exacerbate the hyperglycaemia and hypertriglyceridaemia at obesity-induced diabetes. Hypothyroidism is frequently known to coexist with diabetes [8]. Decreased thyroid hormone levels are often observed in the experimentally diabetic animals [9]. However, little has been known about the effect of hypothyroidism on DAG content in cells. It has been demonstrated that activity of PLC and PLD was depressed [10], and DAG level decreased [11] in thyroxine-induced cardiac hypertrophy. In contrast, experimental hyperthyroidism caused a significant increase in inositol trisphosphate formation and PLC activation in the perfused hearts while hypothyroidism was associated with a decrease in this activity [12]. Hypothyroidism increased the basal PLC-linked inositol phospholipid hydrolysis in rat hypothalamus, whereas L-T₄ supplementation to hypothyroid rats resulted in a complete restoration of hypothalamic inositol phosphate formation to the value of euthyroid control [13].

Numerous investigations demonstrated a novel role of thyroid hormones as modulators of signal transduction. PKC is critical to the mechanism by which thyroid hormones rapidly induce phosphorylation and nuclear translocation of mitogen-activated protein kinase and subsequently potentiate both the antiviral and immunomodulatory actions of IFNγ in cultured cells [14] and regulate the exchange of signaling phospholipids (PL) in hepatocytes [15,16]. It was found that L-T₄ rapidly induced the biphasic DAG accumulation and PKC activation in liver slices and isolated hepatocytes [17]. The effect of L-T₄ on PLC, -D, PKC, and DAG accumulation was too rapid: from seconds to a few minutes. Perfusion of the liver of hypothyroid rats with L-T₄-containing solution during 10 min increased the content of DAG in the liver [18]. However, the effect of hypothyroidism on basal level of DAG in liver has not been determined.

In the present study, we have examined DAG level and PKC activity in liver and isolated membranes during sustained thyroid hormone stimulation and at different thyroid state of organism. Degradation of thyroid gland function with aging or under the mercazolil action decreased PL and TAG synthesis in liver and increased the DAG level in liver slices and isolated liver cell plasma membranes. Hypothyroidism markedly decreased PKC activation. L-T₄ administration to intact or mercazolil-treated rats decreased DAG level and increased PL and TAG synthesis in liver. These results provide the evidence that thyroxine is crucial in lowering DAG by converting them to PL and TAG during sustained hormone action.

Results and Discussion

The present work is aimed at the elucidation of the influence of thyroid functional status on the DAG level in liver and plasma membranes. To determine the role of thyroid hormones in the regulation of DAG level in liver, a comparison of euthyroid, mercazolil- and mercazolil+L-T₄-treated rats or intact animals after injection of L-T₄ has been made.

The results of investigation of thyroid hormones content in blood serum of rats are given in Table 1. The thyroxine and triiodothyronine levels are shown to be markedly lower in blood serum of 720-day-old intact rats and mercazolil-treated rats as compared to appropriate controls. The data obtained are consistent with previous observations of age- and drug-induced thyroid gland malfunction [19–21].

Aging promotes decline in signal transduction in activated cells as evidenced by reductions in DAG and inositol phosphate generation [22,23]. There was also a pronounced age-related increase of PLC activity, inositol 1,4,5-trisphosphate and intracellular free calcium levels in human resting granulocytes [24] and in rat brain cerebellum and striatum [25,26]. We have determined the DAG (Fig. 1A,1B) and [¹⁴C]DAG (Fig. 2A, 3) accumulation in both resting liver and cell plasma membranes of aged rats. Sesnic isolated [¹⁴C]oleic acid prelabeled hepatocytes were found to contain higher basal levels of DAG: 7359 ± 746 as contrasted with 1171 ± 228 cpm/10⁷ cells (P ≤ 0.05, n = 5) in cells of adult animals. Mercazolil leads to a further increase of DAG mass in liver (Fig. 1A) and isolated plasma membranes (Fig. 1B) of animals of different...
L-T4-treated rats showed a marked decrease in the DAG level in liver (Fig. 1A) and isolated membranes (Fig. 1B) and [14C]DAG in liver (Fig. 2B) of rats of different age. However, T4 does not increase free 14C-fatty acid (FFA) accumulation in liver (Fig. 2C). Thus, the impact of DAG lipase in hormone-related decrease of DAG level in liver can be ruled out. Age- and mercazolil-related increase of newly synthesized [14C]DAG in liver slices was observed (Fig. 3). The administration of L-T4 to mercazolil-treated rats reduced [14C]DAG accumulation in liver slices. Mercazolil and L-T4 diminished the age peculiarities of [14C]DAG level in liver. The latter observation is an indication that the observed thyroid status changes of organism at old age may contribute to the age-related changes of DAG level in liver.

Multiple sources of DAGs in mammalian cells have been determined [for review see [27]]. The DAG is generally derived from phosphatidic acid by the action of phosphatidic acid phosphatase (PAP). This reaction is the commitment reaction for the synthesis of TAG and the nitrogen-containing PLs – PC, PE and phosphatidylserine. There are different potential pathways of DAG generation which utilize PIP2 and PC in resting cells. Some reports of PE and phosphatidylinositol (PI) hydrolysis by PLC/PLD and DAG production were published [28,29]. To assess the possibility of PL conversion to DAG in liver slices, we labeled DAG and PL with two different fatty acids, [14C]oleic acid and [3H]arachidonic acid (Table 2). DAG was relatively enriched in [14C]oleic acid, whereas PL were enriched in [3H]arachidonic acid. The results obtained suggest that in liver of hypothyroid animals the accumulated DAG are unlikely to be products of phospholipase-catalised PL hydrolysis. However, radiolabelling will miss some DAG since the fatty acid species chosen will not necessarily label all groups of DAG that may be relevant. Therefore, it is possible that some other molecular species of PL could be the source of DAG in liver of animals of different thyroid state.

It has been demonstrated that DAG accumulates and PC synthesis decreases in the livers of rats fed with choline-deficient diet [3,4,30]. Diet treatment induced the PKC activation in the hepatic plasma membrane. These changes were reversible when choline was returned back to the diet of the deficient animals. Thyroid hormone stimulates lipogenesis by the induction of enzymes in the lipogenic pathway. Acyl-CoA-glycero-3-phosphate acyltransferase, which is known to catalyze a rate-limiting step for the synthesis of phosphatidic acid in rat liver [31], as well as liver diacylglycerol acyltransferase [32] are dependent on thyroid gland function. The PL synthesis by phosphotransferase reaction of DAG with CDPcholine or CDPetanolamine which are generated by the actions of specific cytidytransferases is under thyroid hormone control, too [33–35]. Results of this study provide further evidence that hepatic PC and TAG synthesis is dependent on thyroid status of organism (Fig. 4). It was found that the amount of newly synthesized PC and TAG decreased at old age and in liver homogenates of mercazolil-treated animals. The reduction of 14C-glycerolipid level was corrected by L-T4-treatment of the hypothyroid rat. L-T4 administration to the intact (Fig. 2A,2B) or hypothyroid (Fig. 3) rats reduced the amount of de novo synthesized DAG in liver and increased the synthesis of PLs de novo in isolated hepatocytes [16]. All these experimental observations taken together indicate that drug- and age-depend-

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**Table 1: Blood serum thyroxine and triiodothyronine content in rats of different age and thyroid status**

| Animals          | Age     | T4       | T3        | T4       | T3        |
|------------------|---------|----------|-----------|----------|-----------|
|                   | 90-day-old | 720-day-old |
| Control          | 88.0 ± 6.9 | 1.5 ± 0.07 | 58.2 ± 3.2* | 0.82 ± 0.0* |
| Mercazolil-treated | 48.6 ± 0.9*** | 0.3 ± 0.08*** | 20.6 ± 4.26** | 0         |
| Thyroxine-treated | 410 ± 51.0*** | 5.86 ± 0.48*** | 270 ± 10.0*** | 3.9 ± 0.09*** |

The T4 and T3 content in blood serum was determined by radioimmunoassay kits. The amount of thyroid hormones in serum was represented as nmol per liter. Treatment of the rats by mercazolil was performed as described in "Materials and Methods". T4 (200 µg/100 g body weight) was injected to the normal rats three times a week. The last hormone injection was made 48 h prior to killing. Results are mean ± S.E. of 8 individual experiments. Here and in Table 2 and Figures 1,2,3,4,5 one experiment is equivalent to measurement of the parameters studied in a sample of serum or liver of a single animal. *p < 0.05 vs. 90-day-old rats, **p < 0.05 and *** p < 0.001 vs. control.
Figure 1
Changes in total DAG mass in liver and liver cell plasma membranes following treatment of rats with mercazolil or thyroxine. A, liver homogenates. B, isolated liver cell plasma membranes. T4 (200 µg/100 g weight) was injected to the normal rats three times a week. The last hormone injection was made 48 h prior to killing. Control rats received the same volume of 0.9% NaCl. Mercazolil was injected to the animals and lipids were extracted and separated as described in "Materials and Methods". The intensity of lipid spots was estimated by densitometry using a LKB Ultrascan laser densitometer. Results are mean ± S.E. of four individual experiments. * P < 0.05 vs. 90-day-old rats, ** P < 0.001 vs. control rats, *** P < 0.001 vs. mercazolil-treated rats.
Figure 2
Age- and thyroxine-dependent fluctuations of newly synthesized [\(^{14}C\)]-DAG and [\(^{14}C\)]-FFA level in liver. The liver lipids were labeled by [\(^{14}C\)]CH\(_3\)COOH as described in "Materials and Methods". T\(_4\) (200 µg/100 g weight) was injected to the normal rats 15, 30 and 60 min prior to killing. Control rats received the same volume of 0.9% NaCl. Lipids were extracted and separated as described in "Materials and Methods" and the radioactivity was determined by liquid scintillation counter. Results are mean ± S.E. of six individual experiments. * P < 0.001 vs. 90-day-old rats, ** P < 0.05, *** P < 0.001 vs. control rats.
Figure 3

\( ^{14}\text{C}-\text{DAG} \) accumulation in liver slices of rats of different age and thyroid status. Mercazolil was injected to the animals as described in "Materials and Methods". \( T_4 \) (10 \( \mu \)g/100 g weight) was injected to the mercazolil-treated rats 48 h prior to killing. Control rats received the same volume of 0.9% NaCl. The liver slices were labeled by incubation in Eagle medium containing 10% fetal calf serum, 100 units/liter streptomycin, 100 units/liter penicillin, 13 mg/ml gentamycin and 2.5 \( \mu \)Ci/ml of \( ^{14}\text{C}\)oleic acid for 1 h in 95% O2/5% CO\textsubscript{2} atmosphere at 37\( ^\circ \)C. The lipids were extracted and analyzed as described in "Materials and Methods". Results are mean \( \pm \) S.E. of six individual experiments. * \( P < 0.05 \) vs. 90-day-old rats, ** \( P < 0.05 \) vs. control rats, *** \( P < 0.05 \) vs. mercazolil-treated rats.
ent DAG accumulation is primarily due to reduced hepatic lipogenesis.

In contrast with diabetes[6], dietary-induced insulin resistance [7] and choline-deficiency [3,4], there were no changes in DAG/PKC signalling in skeletal muscle and liver due to normal aging [36]. Hepatic PKC was not activated despite high intracellular DAG in obese Zucker rats [37]. The data on PKC activity at different thyroid status are controversial, perhaps, due to various models of hypothyroidism used [38,39]. In the present study, to determine if DAG accumulation in liver plasma membranes and liver cells could be the reason of sustained PKC activation, the PKC activity in liver of euthyroid and hypothyroid mercazolil-treated rats has been investigated (Fig. 5). There were only slight differences in subcellular distribution of PKC in liver cells of the two groups of rats. However, the total PKC activity in the liver of mercazolil-treated animals was less than in control rats. Addition of exogenous DAG completely abolished the differences between PKC activity in liver cells of eu- hypo- and hyperthyroid rats [39]. It is known that prolonged incubation with

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**Figure 4**

Newly synthesized [14C]-PC and [14C]-TAG in liver slices of rats of different age and thyroid status. Mercazolil was injected to the animals as described in "Materials and Methods". T4 (10 µg/100 g weight) was injected to the mercazolil-treated rats 48 h prior to killing. Control rats received the same volume of 0.9% NaCl. The liver slices were labeled by incubation in Eagle medium containing 10% fetal calf serum, 100 units/liter streptomycin, 100 units/liter penicillin, 13 mg/ml gentamycin and 2.5 µCi/ml of [14C]palmitic acid for 1 h in 95% O2/5% CO2 atmosphere at 37°C. The lipids were extracted and analyzed as described in "Materials and Methods". Results are mean ± S.E. of six individual experiments. * P < 0.05 vs. 90-day-old rats, ** P < 0.05 vs. control rats, *** P < 0.05 vs. mercazolil-treated rats.
phorbol esters depletes cells of PKC α and β [for review see [40]]. In addition, evidence has been obtained for increased degradation of PKC after prolonged cell incubation with phorbol ethers and DAG. Results of the present study suggest that inappropriate DAG accumulation in liver cells of hypothyroid animals contributes to PKC depletion. However, the reduction of PKC isozyme expression can not be ruled out. Further work is required to elucidate this point.

Overnight 12-O-tetradecanoylphorbol 13-acetate (TPA) pretreatment appeared to down-regulate predominantly the alfa-isoform of PKC in rat hepatocytes and to suppress cell signalling in response to vasopressin, TPA and Ca^{2+} ionophore A23187 [41]. Previous data indicated that just the phorbol 12-myristate 13-acetate but not its inactive analog, phorbol 12-myristate 13-acetate 4-O-methyl ether, activated linoleic acid incorporation into PL [15] and phosphoinositide synthesis de novo in liver slices and isolated hepatocytes [16]. PKC-dependent signalling has been indicated in plasma membranes of young intact 90-day-old rats. No effect has been found in plasma membranes isolated from liver of 720-day-old mercazolil-treated rats. These findings suggest that the desensitization of the response to agonist in cell of hypothyroid rats is dependent on PKC down regulation at such pathological state of organism.

Conclusions
In conclusion, results of the present study indicate thyroid hormone as a potentially important physiological modulator of DAG level in rat liver. Unlike our previous work [17] were thyroxine (in physiological concentration) was shown to induce PLC/PLD activation and short-lived DAG accumulation in liver cells, we detected the prominent DAG level decrease under unphysiological doses of

Figure 5
Effect of mercazolil on PKC activity in liver cells. Mercazolil was injected to the animals and PKC activity was determined as described in "Materials and Methods". Results are mean ± S.E. of four individual experiments. * P < 0.05 vs. control rats.
Liver slices of 90-day-old rats were pre-labeled by [14C]oleic and [3H]arachidonic acids and lipids extracted and separated as described in "Materials and Methods". The data are mean ± S.E. of three individual experiments.

hormone administration to rats. Age- and mercazolil-dependent decline of thyroid hormone level in blood serum coincides with elevation of DAG content in liver and liver cell plasma membranes. In contrast, under hypothyroid status of organism synthesis of PL and TAG decreases in liver. The reduction of glycerolipid synthesis and DAG level elevation can be corrected by thyroxine treatment of hypothyroid rats. Thus, thyroid hormone-dependent changes in DAG content are likely associated with glycerolipid synthesis. Extremely high basal DAG level in hypothyroid liver coincides with a declined PKC activity in both cytosol and membrane of liver cells. Stable alterations in DAG metabolism in hypothyroid liver are suggested to lead to disturbances in agonist responsiveness of liver cells.

Materials and Methods

Materials

[14C]oleic acid (58 mCi/mmol) and [3H]arachidonic acid (60 Ci/mmol) – Amersham Corp., [14C]palmitic acid (58 mCi/mmol), [γ-32P]ATP (1000 Ci/mmol) and [14C]CH3COOH (25 mCi/mmol) – BPO Isotop (Russia); DEAE-52-cellulose from Whatman (England); silica gel from Woelm (Germany). Phosphatidylserine was isolated from ox brain; other lipid standards and histone H1 were obtained from Sigma (USA). T4 and mercazolil (1-methyl,2-mercaptoimidazol) were from Zdorov’e Trudyashchikhsya (Kharkov, Ukraine). T4 and T3 radioimmunoassay kits were from Minsk (Belarussia). Other chemicals used were of chemically pure grade.

Animals

Adult 90- and 720-day-old male Wistar rats which had a free access to food and water and were kept at 24°C on a cycle of 12 h light/12 h darkness were used for experiments. Mercazolil was injected intraperitoneally (1 mg/100 g weight) in 0.9% NaCl to the experimental animals every day during 16 days-experiment. In some cases, the mercazolil-treated rats were injected intraperitoneally by T4 (10 µg/100 g weight) 48 h prior to killing. Besides, T4 (200 µg/100 g weight) was injected to the normal rats 15, 30 and 60 min prior to killing or three times a week in which case the last hormone injection was made 48 h prior to killing. Control rats received 0.9% NaCl of the same volume. The animals were starved overnight prior to experiment. The thyroid state of rats was monitored by radioimmunological determination of the T4 and T3 in blood serum.

Experiments with liver slices

The 1 mCi of [14C]CH3COOH was intraperitoneally injected to rats four times every 30 minutes for 2 hours [42]. The liver was perfused with 0.9% NaCl, then removed and washed in Krebs-Henseleit buffer, pH 7.4, containing 2 mM CaCl2 and 0.2% BSA. Pre-labeled slices of liver were used for [14C]-DAG and [14C]-FFA analysis. Besides, the liver slices were labeled by incubation in Eagle medium containing 10% fetal calf serum, 100 units/litre streptomycin, 100 units/litre penicillin, 13 mg/ml gentamycin and 2.5 µCi/ml of [14C]oleic acid and 2.5 µCi/ml [3H]arachidonic acid or 2.5 µCi/ml [14C]palmitic acid alone for 1 h in 95% O2/5% CO2 atmosphere at 37°C. The lipids were extracted and analyzed as described below.

Cell suspension experiments

Hepatocytes were isolated from the 90- and 720-day-old male Wistar rats which had a free access to food and water and were kept at 24°C on a cycle of 12 h light/12 h darkness by the method described in [41]. Preparation of hepatocytes was started between 9:00 and 10:00 a.m. Cells (107/ml) were labeled by incubation in Eagle medium containing 10% fetal calf serum, 100 units/liter streptomycin, 100 units/liter penicillin, 13 mg/ml gentamycin and 2.5 µCi/ml of [14C]oleic acid for 3 h in 95% O2/5% CO2 atmosphere at 37°C. Before lipid extraction, cells were washed twice with a Krebs-Henseleit buffer pH 7.4, containing 2 mM CaCl2, 25 mM HEPES, 0.1% BSA. The lipids were extracted and analyzed as described below.

Isolation of liver cell plasma membranes

Plasma membranes were prepared using differential centrifugation through various concentration of sucrose and characterized by their specific marker enzymes as described in [43]. The lipids were extracted and analyzed as described below.

Extraction and separation of lipids

Lipids were extracted according to Folch et al. [44] and phosphoinositides as described in [45]. The chloroform
phase was collected and dried under N$_2$ at 37°C. The lipids were redissolved in chloroform/methanol (1:2, v/v) and applied on TLC plates. For DAG and FFA isolation the solvent system: hexane/diethyl ether/acetic acid (80:20:2, v/v) was used; for PC and PE separation – chloroform/methanol/acetic acid/water (25:15:4:2, v/v) and for PI, PIP and PIP$_2$ – chloroform/methanol/NH$_4$OH (50:40:10, v/v). Appropriate standards were applied on each plate for quantification. The gel spots containing $[^{14}C]/[^{3}H]$lipids were scraped and transferred to scintillation vials. Radioactivity was measured by scintillation counter.

**Protein kinase C enzyme assay**

Activities of protein kinase C in the cytosol and in the crude membrane fraction of liver cells were determined after partial enzyme purification by chromatography on DEAE-cellulose [46]. The activity of protein kinase C was determined by the transfer of $^{32}$P from [γ-$^{32}$P]ATP into H1 histone in the presence of phosphatidylserine and Ca$^{2+}$ (0.2 mM). Since histone is a poor substrate for calcium-insensitive isoforms of PKC, the predominant isoforms of PKC detected were cPKC and aPKC. The enzyme activity was expressed as pmoles of phosphate transferred from [γ-$^{32}$P]ATP into H1 histone per minute. The protein content was determined by Bradford method [47].

**Abbreviations**

T$_4$ – thyroxine, T$_3$ – triiodothyronine, PKC – protein kinase C, P1 – phosphatidylinositolphosphate, PIP – phosphatidylinositol 4-phosphate, PIP$_2$ – phosphatidylinositol 4,5-bisphosphate, PLC – phospholipase C, DAG – diacylglycerol, FFA – free fatty acids, TAG – triacylglycerol, PL – phospholipids, PAP – phosphatidic acid phosphatase, PC – phosphatidylcholine, PE – phosphatidylethanolamine, TPA – 12-O-tetradecanoylphorbol 13-acetate.

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