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1,2-*sn*-Diacylglycerol accumulates in choline-deficient liver

A possible mechanism of hepatic carcinogenesis via alteration in protein kinase C activity?

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Choline deficiency is associated with triacylglycerol accumulation in the liver, and is the only nutritional state known to trigger hepatic cancer spontaneously. In two different experiments, rats were pair-fed for 6 weeks with control (0.2% choline), or choline-deficient (CD) (0.002% choline) diets. Hepatic choline and phosphocholine declined in CD animals to 54% and 16% of control levels, respectively. In control livers, 1,2-*sn*-diacylglycerol (1,2-*sn*-DAG) concentration was (in nmol/g wet wt) 144 (±25; mean±SE); while in CD livers it was 792 (±140) in the first experiment. In the second experiment the values were 375 (±26) and 1147 (±74), respectively. 1,2-*sn*-DAG, a precursor of triacylglycerol, is an endogenous activator of protein kinase C (PKC). PKC is the presumed site of action of the tumor-promoting phorbol esters. We suggest that the 1,2-*sn*-DAG accumulating in CD liver could bind PKC, altering its activity, and thus contribute to the carcinogenic effect of CD diets.

Choline deficiency; Diacylglycerol; Protein kinase C; Tumor promoter; Carcinogenesis; (Rat liver)

1. INTRODUCTION

Choline deficiency is the only nutritional state which causes hepatic cancer by itself (i.e. in the absence of known carcinogenic agents). During choline deficiency, extremely large amounts of lipids (mainly triacylglycerol) accumulate in liver cells, eventually filling the entire hepatocyte [1]. Normally, triglycerides are secreted in very low density lipoproteins (VLDL), an essential component of which is phosphatidylcholine (PtdCho) [2–4]. In choline deficiency synthesis of PtdCho is decreased and, in its absence, VLDL formation is compromised causing lipids to accumulate. Hepatocytes, prepared from choline-deficient rats (CD) were unable to secrete VLDL until choline or methionine was made available [4]. Ultrastructural abnormalities of the endoplasmic reticulum and Golgi system, consistent with diminished VLDL transport, have been observed in electron microscopic studies of hepatocytes from rats fed CD diet [5]. Triacylglycerol and glycerophospholipids (including PtdCho) are synthesized using 1,2-*sn*-diacylglycerol (1,2-*sn*-DAG) as a precursor. In association with massive accumulation of triacylglycerol and with decreased synthesis of PtdCho, it is possible that 1,2-*sn*-DAG might accumulate in CD liver. Indeed, total concentrations of diglycerides (i.e. the sum of the 1,2- and the 2,3-*sn*-diacylglycerols) have been previously found to increase 6-fold in the livers from rats fed CD, low-methionine diets for one week [6].

1,2-*sn*-DAG is also a second messenger, formed when plasma membrane receptors for certain hormones, neurotransmitters or growth factors are coupled to phospholipase C. The 1,2-*sn*-DAG molecule remains within the membrane after hydrolysis of phospholipids, and can activate a regulatory enzyme, protein kinase C (PKC) [7]. During activation, which requires the presence of

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calcium and phosphatidylserine, PKC is translocated from the cytosol to the plasma membrane [7,8]. 1,2-*sn*-DAG markedly increases the affinity of PKC for calcium, thereby activating the enzyme without a net increase in intracellular calcium concentration [7]. 1,2-*sn*-DAG containing unsaturated fatty acids are the most potent in this respect, while 1,3- or 2,3-*sn*-DAG neither activates nor inhibits the enzyme [7]. This means that the 2,3-*sn*-DAG liberated from triglyceride by the action of lipoprotein lipase, and heparin-released hepatic lipase will not activate PKC [7]. The appearance of 1,2-*sn*-DAG in membranes is usually transient, and therefore PKC is activated only for a short time after a receptor has been stimulated. Tumor-promoting phorbol esters have higher affinity than 1,2-*sn*-DAG for the same site on PKC; they cause translocation to membranes and longer-lasting activation [7]. Prolonged activation of PKC by these compounds leads to down-regulation of the enzyme (i.e. proteolysis to a form which is not bound to the membrane [7]). It is believed that the carcinogenic effects of phorbol esters may be explained by their interactions with PKC (though it is not clear whether activation or down-regulation is more important in this regard). If this is true then – in situations with prolonged and/or abnormally high concentrations – 1,2-*sn*-DAG might act as an endogenous tumor promoter. We report that large amounts of 1,2-*sn*-DAG accumulate in CD livers. We propose that this 1,2-*sn*-DAG may cause a long-term alteration of hepatic PKC activity and thus act as an endogenous tumor promoter contributing to the carcinogenic effect of choline deficiency upon liver.

2. MATERIALS AND METHODS

In two experiments, Sprague-Dawley rats (150 g males), were pair-fed for 6 weeks with a semisynthetic diet (ICN Nutritional Biochemicals custom control diet: 10% casein, 10% a-soy protein, 20% lard, 56% sucrose, 4% salt mix W, ICN vitamin mix; the control diet contained 0.2% choline bitartrate). The CD diet omitted choline bitartrate and contained 0.002% choline by our assay. Animals were anesthetized with nembutal and the livers were collected by freeze-clamping between tongs cooled in liquid nitrogen. Tissue samples were pulverized under liquid nitrogen using a mortar and pestle, forming a homogeneous powder. The powders were extracted according to the method of Blight and Dyer [9], and 1,2-*sn*-DAG content in the organic portion of the extract was determined using the method of Priess et al. [10]. This procedure utilizes 1,2-*sn*-DAG kinase, purified from *E. coli* (Lipidex Inc., Westfield, NJ), and [γ-^{32}P]ATP to form radiolabelled phosphatidic acid, which is isolated by thin-layer chromatography and its radioactivity determined by liquid scintillation spectrometry. This assay is specific for 1,2-*sn*-DAG and does not detect other isomers. Since large amounts of triglycerides are present in the extracts of CD livers, we added several control samples containing triolein to our 1,2-*sn*-DAG assay. No detectable [^{32}P]phosphatidic acid was formed during the assay of those samples. Choline and phosphocholine, were measured in an aliquot of the aqueous portion of the extracts. Approximately 4000 dpm of ^{14}C-labeled standards ([*methyl*-^{14}C]choline chloride and [*methyl*-^{14}C]phosphocholine from New England Nuclear, Boston, MA) were added to permit detection of eluted peaks using an on-line radioactivity monitor after separation by high-pressure liquid chromatography (HPLC). In addition, internal standards labeled with stable isotopes were added to permit calculation of and correction for recovery during the assay [[^{15}N]choline chloride, phosphocholine-d9 chloride (*N,N,N-trimethyl*-d9; barium salt; MSD Isotopes, Merck Chemical Division, St. Louis, MO), the amount for each was equivalent to approximately 10% of the amount normally found in that tissue]. Hepatic choline and phosphocholine were purified from the aqueous phase of the extract by HPLC. The chromatographic conditions were based on the method described [11]. We used a normal phase column (Pecosphere-3C Si, 4.6 × 83 mm; Perkin-Elmer, Norwalk, CT) and the elution was with a binary nonlinear gradient of acetonitrile/ethanol/acetic acid/1.0 M ammonium acetate/water/0.1 M sodium phosphate (weak mobile phase: 800:68:2:3:127:10; strong mobile phase: 400:68:44:88:400:10) at a flow rate of 1.5 ml/min. Choline and phosphocholine, were collected and then the latter was hydrolyzed with 6 M HCl at 80°C for 24 h to form free choline. The chloroform phase of the extracted tissue was used to assay the organic metabolites of choline. PtdCho and lysoPtdCho were purified by thin-layer chromatography and then hydrolyzed in 6 M methanolic-HCl at 80°C for 60 min, liberating choline. A volatile derivative of choline (demethylation with benzenethiolate and formation of propionyl ester) was then purified using gas chromatography (6 ft × 2 mm i.d. glass column packed with 3% OV-17 on GC22 precoated with 1% 4-dodecyldiethylenetriamine succinamide) and measured with a mass selective detector (MSD; Hewlett Packard 5970 GC/MSD) [12]. Use of internal standards allowed correction for recovery of losses during the isolation procedures. Protein was determined using the method of Bradford [13].

3. RESULTS AND DISCUSSION

Choline-deficient rats had fatty infiltration of the liver, with obvious hepatic abnormalities visible at the light microscopic level. The amount of protein in the livers of control animals was higher (142.8 ± 0.01 mg/g) than in livers of CD (138.8 ± 0.004 mg/g). Choline and phosphocholine levels declined in CD livers to 54% and 16% of those of controls, respectively (table 1). There were no significant changes in hepatic PtdCho or lysoPtd-

Table 1

Effect of choline deficiency on choline and phosphocholine in rat liver

	Choline (nmol/g wet wt)	Phosphocholine (nmol/g wet wt)
Control	81 ± 9.9	1417 ± 356
Deficient	44 ± 4.2	230 ± 58
	$p = 0.03$	$p = 0.01$

Rats were pair-fed control or choline-deficient diets for 6 weeks. Hepatic choline and phosphocholine were measured by gas-chromatography mass-spectrometry after purification by high-performance liquid chromatography. Results are expressed as means ± SE ($n = 3$/group). Statistical significance of differences between groups was determined by t-test

Cho concentrations. 1,2-*sn*-DAG concentrations increased 3–5-fold in CD liver compared to control tissues in two separate experiments (table 2). 1,2-*sn*-DAG levels in control livers were in agreement with those observed in normal isolated hepatocytes [10]. It has been reported that the sum of 1,2-*sn*-DAG and 2,3-*sn*-DAG increased from 300 to 1800 nmol/g in rat liver after one week of choline deficiency [6]. Unfortunately, at the time of these investigations, no convenient technique existed to measure 1,2-*sn*-DAG specifically. Recent studies indicate that the physiological state of the cell determines the ratio of 1,2-*sn*-DAG to 2,3-*sn*-DAG. For example in the parotid, 2,3-*sn*-DAG constituted approximately 8% of DAG at rest, and stimulation of β-receptors specifically increased the formation of 2,3-*sn*-DAG, such that it constituted over 30% of the total DAG [14]. This 2,3-*sn*-DAG was ineffective in stimulating PKC activity [14]. Thus, it is important that we observe specific increases in 1,2-*sn*-DAG levels in CD liver (table 2) because only this stereoisomer can activate PKC.

Table 2

Effect of choline deficiency on 1,2-*sn*-diacylglycerol in rat liver

	1,2-*sn*-Diacylglycerol (nmol/g wet wt)	
	Expt I	Expt II
Control	144 ± 25	375 ± 26
Deficient	792 ± 140	1174 ± 74
	$p = 0.02$	$p = 0.0001$

Rats were pair-fed control or choline-deficient diets for 6 weeks in two separate experiments. Hepatic 1,2-*sn*-diacylglycerol was measured by a radioenzymatic assay. Results are expressed as means ± SE ($n = 3$/group in expt I; and $n = 9$–10/group in expt II). Statistical significance of differences between groups was determined by t-test

Accumulation of lipids was not homogeneous among liver cells. When, in preliminary experiments, we isolated hepatocytes from CD livers, we observed three populations of cells which differed according to their sedimentation behavior in Krebs-Ringer buffer: one population (similar to that present in control livers) sedimented readily; a second population sedimented slowly, and a third population (absent from control livers) floated. It is possible that 1,2-*sn*-DAG concentrations in the floating cells were even higher than those observed in total homogenate. We conclude that choline deficiency was associated with a remarkable increase in hepatic 1,2-*sn*-DAG concentrations, reaching values higher than those occurring after stimulation of a receptor linked to phospholipase C activation (e.g. vasopressin receptor [10,15]) and of the order of magnitude needed to activate PKC in vitro [16]. The concentrations of 1,2-*sn*-DAG achieved in CD liver (table 2) were several-fold higher than the concentrations of exogenous 1,2-*sn*-DAG used to activate PKC in platelets [17,18] or to modify responses to α_1-receptors in the liver [19]. We suggest that such elevations in 1,2-*sn*-DAG concentrations could chronically stimulate PKC in vivo.

Many studies have demonstrated that feeding a choline-devoid diet to experimental animals is carcinogenic [20–30]. There are several mechanisms which have been suggested to explain this effect: increased cell death, increased cell proliferation, decreased DNA methylation, and increased lipid peroxidation. In the CD liver there is a progressive increase in cell proliferation, related to regeneration after parenchymal cell death [29,31]. Cell proliferation, with associated increased rate of DNA synthesis, could be the cause of greater sensitivity to chemical carcinogens [29]. Other stimuli for increased DNA synthesis are associated with carcinogenesis: hepatectomy and necrogenic chemicals are examples [29]. However, Shinozuka et al. [30] found that the rate of cell proliferation could be dissociated from the rate at which preneoplastic lesions formed during choline deficiency, suggesting that cell proliferation was not the sole condition acting as a promoter of liver cancer. In-

terestingly, partial hepatectomy activates PKC in the liver [32,33] prior to DNA synthesis [33]. Methylation of DNA is important for the regulation of gene expression [34]. It has been suggested that the under-methylation of DNA (decreased 5-methylcytosine content in nuclear DNA), observed during choline deficiency, is responsible for carcinogenesis [26]. Another proposed mechanism is based upon the observation of increased lipid peroxidation in hepatic nuclei isolated from CD animals [35]. Lipid peroxides in the nucleus could be a source of free radicals which could modify DNA, and initiate tumor formation.

The carcinogenic effects of tumor-promoting phorbol esters, as well as of several oncogenes have been linked to the activation of PKC [36]. For example, NIH 3T3 cells transformed with Ha-*ras* or Ki-*ras*, v-*src*, and v-*fms* oncogenes have elevated 1,2-*sn*-DAG levels [37,38] as well as tonic activation and partial down-regulation of PKC [37,38]. Activated PKC, in turn, may participate in mechanisms leading to the induction of expression of the *c-myc* oncogene [39,40]. We propose that the accumulation of 1,2-*sn*-DAG in CD liver may cause prolonged activation of PKC, thus acting as an endogenous tumor promoter.

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