The opposite correlation between calcium ion and cyclic-AMP regarding the activation of microsomal triglyceride transfer protein in rat liver

Hyun-Jeong Cho¹, Hyeong-Soo Kim², Young-Bin Yu³, Hyo-Chan Kang⁴, Dong-Ha Lee⁵, Man-Hee Rhee⁶, Jae-Youl Cho⁶ & Hwa-Jin Park⁶

¹Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, Daejeon 302-718, ²Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, and Regional Research Center, Inje University, Gimhae 621-749, ³Department of Central Medical Research Institute, Kosin University Medical Center, Busan 602-030, ⁴Department of Medical Laboratory Science, Dong-Eui Institute of Technology, Busan 614-175, ⁵Department of Veterinary Physiology, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, ⁶School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea

INTRODUCTION

TG endogenously synthesized in the liver are secreted into the blood as part of TG-rich very low-density lipoprotein (VLDL)-containing lipids as well as other lipoproteins such as phospholipids, cholesterol ester and apolipoprotein B (apoB). It is well known that TG-rich VLDL in the blood stream is a major cause of atherosclerosis. Specifically, in the liver catalyzes and assembles TG and apoB into VLDL, followed by the secretion of TG-rich VLDL into the blood stream (1-3). Increased hepatic synthesis and production of TG are frequently associated with insulin resistance. These metabolic disturbances often lead to TG accumulation in the liver, contributing to hypertriglyceridemia (4). It is known that fructose or glucose treatment increases the level of TG in rat liver (5, 6). Glucose treatment also increased the level of insulin in serum, an event directly involved in the occurrence of hypertriglyceridemia (7). Recently, we have reported that MTP activity, which promotes hypertriglyceridemia, is stimulated by increased Ca²⁺ levels (7) in the liver as well as increased hepatocytes in rats under insulin resistance. On the contrary, Ca²⁺-induced MTP activity was inhibited in response to the intracellular Ca²⁺ chelator BAPTA-AM, the extracellular Ca²⁺ chelator EDTA, as well as the calmodulin antagonist W-7 (7, 8), all of which suggest Ca²⁺ directly increases MTP activity. On the other hand, the activation of hepatocellular adenylate cyclase, which produces cAMP from ATP, has been reported to suppress the secretion of TG-rich VLDL (9). These previous reports imply that Ca²⁺ and cAMP might possess a particular relationship regarding the release of TG-rich VLDL from liver/hepatocytes into the blood stream. Therefore, we investigated what correlation exists, if any, between cAMP and Ca²⁺ regarding their effect on MTP activity. In this study, new information on the possible regulatory effect of liver cAMP on MTP activity is shown.

RESULTS

Identification of TG accumulation by glucose-feeding

Lipid droplets were increased in the livers of rats administered 10% glucose (GR) compared with those of tap water-treated normal rats (NR) (Fig. 1). Glucose intake in GR was about 10 g/d (data not shown, 7), an event inferred as providing resources for TG synthesis in the liver. This is in agreement with reports that administration of glucose stimulates TG synthesis
Regulation of MTP activity by calcium or cAMP
Hyun-Jeong Cho, et al.

Changes of Ca\(^{2+}\) and cAMP in the liver
The concentration of Ca\(^{2+}\) in the livers (2.5 ± 0.01 nM/liver-g) of GR was approximately 39% higher than that (1.8 ± 0.30 nM/liver-g) of NR (Fig. 2A). In contrast with the Ca\(^{2+}\) level, the level of cAMP (9.9 ± 2.4 pmol/protein-mg) was 30.8% lower compared to that (14.3 ± 3.2 pmol/protein-mg) of NR (Fig. 2B).

Effect of glucose administration on MTP activity
The MTP activity (983 ± 30 nM/protein-mg) of GR was increased by 71% compared to that (576 ± 11 nM/protein-mg) of NR (Fig. 3A). In conclusion, this suggests that MTP activity is increased by high levels of Ca\(^{2+}\) (Fig. 2A) as well as low levels of cAMP (Fig. 2B). In the same manner, it is also shown that MTP activity is decreased by low levels of Ca\(^{2+}\) (Fig. 2A) and high levels of cAMP (Fig. 2B), as shown in NR in Fig. 3A. These results suggest that Ca\(^{2+}\) and cAMP might be involved in the regulation of MTP activity. Therefore, we investigated the relationship between Ca\(^{2+}\) and cAMP in regulating MTP activity as follows (Fig. 3B, C).

Effect of thapsigargin on MTP activity
As shown in Fig. 2A and 3A, MTP activity is increased in GR with high levels of Ca\(^{2+}\). Therefore, one could speculate that MTP activity can be increased by converting the Ca\(^{2+}\) level from low to high. Accordingly, we converted the level of Ca\(^{2+}\) from low to high Ca\(^{2+}\) using thapsigargin, which increases intracellular Ca\(^{2+}\) by inhibiting Ca\(^{2+}\)-ATPase in the endoplasmic reticulum (ER). When various concentrations of thapsigargin were added to NR homogenates having low Ca\(^{2+}\) levels, as shown in Fig. 3B, the MTP activity was 1,141.05 ± 42.21 nM/protein-mg (at 100 nM of thapsigargin), 1,200 ± 42.04 nM/protein-mg (at 300 nM of thapsigargin) and 1,237.1 ± 75.48 nM/protein-mg (at 500 nM of thapsigargin). These figures show that thapsigargin (100, 300 and 500 nM) increased MTP activity by 98.4%, 108.7% and 115.1%, respectively, compared to the MTP activity (575.6 ± 10.3 nM/protein-mg) of NR without thapsigargin treatment. Ethanol (2 × 10\(^{-4}\)%), the vehicle for thapsigargin, had no affect on MTP activity. These results are obvious evidence (Fig. 3B) that intracellular Ca\(^{2+}\) elevation increases MTP activity in the liver.

Effect of db-cAMP on MTP activity
As shown in Fig. 2A, B and 3A, MTP activity is increased in GR having high levels of cAMP and high levels of Ca\(^{2+}\). Therefore, one could speculate that MTP activity can be reduced by converting the level of cAMP from low to high, which is known to reduce intracellular Ca\(^{2+}\) in various cells (10-12). Therefore, a cAMP analogue, db-cAMP, was used to
Regulation of MTP activity by calcium or cAMP
Hyun-Jeong Cho, et al.

Fig. 3. Effect of glucose-administration, thapsigargin and dibutyryl-cAMP on MTP activity. MTP activity was determined as described in Materials and Methods. (A) Effect of glucose-administration on MTP activity. (B) Effect of thapsigargin on MTP activity. Thapsigargin was added to and incubated in liver homogenates from normal rats with low levels of total Ca²⁺ and high levels of cAMP. (C) Effect of dibutyryl-cAMP on MTP activity. Dibutyryl-cAMP was added to and incubated in liver homogenates from 10% glucose-administered rats with high levels of total Ca²⁺ and low levels of cAMP. Data are given as means ± S.D., n = 5. *P < 0.05.

Ca²⁺ levels were increased in livers from GR compared to those of NR (Fig. 2A). When a 10% glucose solution was administered, insulin increased in GR serum (7). Furthermore, insulin increases the level of liver Ca²⁺ (13, 14). Therefore, the increase in liver Ca²⁺ in GR seems to result from elevated insulin caused by glucose administration (7) (Fig. 2). In the present study, although we did not investigate the mechanism of Ca²⁺ mobilization, it is known that hormone-mediated Ca²⁺-signaling is initiated by the agonist-induced activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into two intracellular messengers, inositol-1,4,5-trisphosphate (IP₃), and diacylglyceride (15). The ensuing increase in cytosolic free-Ca²⁺ results in the IP₃-mediated release of Ca²⁺ from internal stores as well as an influx of Ca²⁺ from the external space (15, 16).

Lipid droplets (Fig. 1) containing TG were increased in the livers of GR containing high levels of Ca²⁺ (Fig. 2A). This is in accordance with a report that found Ca²⁺ activates synthesis of the hepatic TG (17). It is known that exogenous glucose decreases the amount of MTP large subunit (97 kDa) mRNA, but increases MTP activity in HepG2 cells (18). This means that MTP mRNA is not related with MTP activity, but other factors like glucose-mediated metabolites regulation may be involved in MTP activation. As described in our previous reports, hepatocellular Ca²⁺ acts as a stimulatory factor of MTP activity (7, 8). This study found that MTP activity was increased in the livers of GR containing high levels of Ca²⁺ (Fig. 2A). This is in agreement with our previous report that glucose-elevated hepatocellular Ca²⁺ level is obvious as the Ca²⁺ inducer thapsigargin increased MTP activity (Fig. 3B), the intracellular Ca²⁺ chelator BAPTA, the extracellular Ca²⁺ chelator EDTA, while inhibiting CaCl₂-elevated MTP activity (7, 8). Additional evidence that Ca²⁺ is involved in the upregulation of MTP activity is that the calmodulin antagonist W-7 inhibits Ca²⁺/calmodulin-dependent protein kinase (CaMPK), play roles in the activation of MTP. Besides CaMPK, the downstream regulatory pathway Ca²⁺/diacylglyceride convert the level of CAMP from low to high as well as reduce Ca²⁺. When various concentrations (100, 300 and 500 μM) of db-cAMP were added to homogenates of livers taken from GR, the MTP activities gradually decreased from 982 ± 29 nM/protein-mg to 874 ± 111 nM/protein-mg at 100 μM of db-cAMP, 838 ± 73 nM/protein-mg at 300 μM of db-cAMP and 684 ± 137 nM/protein-mg at 500 μM of db-cAMP in a dose-dependent manner (Fig. 3C). Furthermore, their activities were inhibited by db-cAMP (500 μM) up to 30.4% (Fig. 3C). Therefore, these results strongly suggest that cAMP decreases MTP activity in the liver.

DISCUSSION

Ca²⁺ levels were increased in livers from GR compared to those of NR (Fig. 2A)
(DG)-dependent protein kinase (C-kinase) also plays a role. In another experiment, we found that H-7 (Km = 6 μM), an inhibitor of C-kinase, inhibited MTP activity in BLK6 mouse liver homogenates by up to 71.5% compared to that (2,812 ± 29 cpn/protein-mg) of control (data not shown). This suggests that MTP activity is increased by C-kinase, which is supported by a report that found probol-12-myristate-13-acetate, an analogue of DG, increased transcription of MTP (19). Hepatic C-kinase is also known to stimulate the release of haptoglobin from liver (20). As such, C-kinase seems to stimulate the release of many materials such as TG-rich VLDL and haptoglobin. Although the effect of hepatocellular Ca2+ on MTP activation could be connected to downstream pathway such as CaMPK and C-kinase, we suggest that the elevation of Ca2+ is a prerequisite to increased MTP activity (7, 8). cAMP and db-cAMP are known to reduce intracellular Ca2+ by promoting the bidirectional uptake of Ca2+ into the ER (11, 12). Therefore, it is obvious that exogenous db-cAMP (Fig. 3C) could inhibit MTP activity by decreasing the level of Ca2+ in GR. We found direct evidence that cAMP might decrease MTP activity using exogenous cAMP in another experiment. When exogenous cAMP was added to liver homogenates followed by incubation, MTP activity was decreased in a dose-dependent manner. Control MTP activity (3,139 ± 57 cpn/mg) was reduced down to 15% of the control by the exogenous provision of 100 μM cAMP and to 22% by 300 μM of exogenous cAMP provision (data not shown). Therefore, the above results seem to naturally propose a working model of the effect of Ca2+ and cAMP on liver MTP activity. First, we suggest that glucose-elevated Ca2+ stimulates the synthesis of TG in order to increase MTP activity (7, 8) (Fig. 1, 2A and 3A). Secondly, lowered Ca2+ levels caused by cAMP (11, 12) might be characterized with respect to decreases in MTP activity (Fig. 3C).

Thirdly, as cAMP and Ca2+ have opposite effects on MTP activity, it can be inferred that Ca2+/calmodulin-activated phosphodiesterase (PDE)-1A (21), an isozyme of PDE, is involved in MTP activation by stimulating the degradation of cAMP. This could be due to the inhibition of Ca2+-or Ca2+/calmodulin-elevated MTP activity by the intracellular Ca2+ chelator BAPTA-AM, the extracellular Ca2+ chelator EDTA, or the calmodulin antagonist W-7 (7, 8). On the contrary, elevated MTP activity in GR containing high levels of Ca2+ was inhibited by db-cAMP (Fig. 3C). Following the increase of liver TG and insulin resistance by glucose (7) (Fig. 1), MTP activity is likewise increased, which promotes hyperlipidemia (7). If hypertriglyceridemia is the most common lipid abnormality associated with insulin resistance (4), Ca2+-increased liver MTP activity caused by glucose might promote type 2 diabetes-associated hypertriglyceridemia since such a disorder is due to an increase in TG-rich VLDL production (7) (Fig. 2A, 3A). On the other hand, db-cAMP reduced glucose-elevated MTP activity (Fig. 3C). This means that cAMP might promote the inhibition of type 2 diabetes-associated hypertriglyceridemia. In conclusion, our results provide new information on the possible regulatory effect of Ca2+ and cAMP on MTP activity.

MATERIALS AND METHODS

Materials

MTP assay kits were obtained from Calbiochem (an Affiliate of Merck KgaA, Darmstadt, Germany). [3H-cyclic AMP] radioimmunoassay kits were obtained from Amersham Bioscience (Buckinghamshire, U.K.). Ca2+ assay kits were obtained from Latron Laboratories (Tokyo, Japan). The other chemical reagents were obtained from Sigma (St. Louis, MO, U.S.A).

Animals and administration

Since MTP assembles TG into VLDL, and its activity increases under TG accumulation in the liver or hepatocytes (7, 8), it was necessary to increase the level of TG in the liver. Therefore, as described in our previous report (7), animals were treated with 10% glucose (6). Sprague Dawley rats (male, 200 g) were divided into two groups and fed ad lib on a standard pellet diet (Sam Yang Oil & Fat Feed Co. Ltd., Korea). Group A, as the normal rat group, had ad lib access to tap water, and group B was given 10% glucose to drink ad lib in place of tap water. The experiment was conducted for 15 days to promote endogenous synthesis of TG in the liver (6, 7). Animals of both groups were not fasted until post-mortem was reached in order to avoid any changes in TG concentration and MTP activity. Animals were anesthetized with 20% urethane before post-mortem.

Preparation of liver homogenates

Liver tissues were homogenized with Tris buffer (150 mM NaCl, 0.5 mM phenylmethylsulfonylfluoride, 20 μg/ml leupeptin, 1 mM mercaptoethanol, 5 mM MgCl2, and 10 mM Tris, pH 7.4) in a glass potter homogenizer (600-1,000 rpm, 5 times). Homogenates were filtered through cotton cheesecloth to remove tissue debris. All procedures were performed in a cold room set at 4°C. The homogenates were stored at −70°C immediately after preparation and were thawed at 4°C prior to experimentation. Protein content was determined by the Lowry method (22).

Observation of liver ultrastructures by transmission electron microscopy (TEM)

Liver tissues were sliced by sharp knife, which were then cut into cubes measuring less than 2 mm. Specimens were subjected to double fixation. Samples were first fixed with Karnovsky fixative (23) for 2 hr at 4°C, washed in phosphate buffer and then post-fixed in 1% osmium tetroxide (Buckinghamshire, U.K.). The other chemical reagents were obtained from Sigma (St. Louis, MO, U.S.A).
scope (Olympus BH-2, Japan). Selected areas of the Epon-embedded tissue were thin (70 nm) sectioned on Reichert Ultratome (LKB Co., Sweden) stained with uranyl acetate and lead citrate (24) and then observed by a Hitachi-600 (Hitachi, Japan) electron microscope at 75 kV.

**Determination of liver cAMP**

cAMP was extracted with 80% ice-cold ethanol from NR and GR liver homogenates. The amount of cAMP was measured using [3H] cAMP-radioimmunoassay kits (Amersham Life Science, Buckinghamshire, U.K.).

**Determination of liver Ca²⁺**

The amount of Ca²⁺ in the liver homogenates was determined by the Moore method (25). Homogenates were dried overnight at 95°C and then ashed for 72 hours at 600°C. The ashed samples were collected in 0.1 N HCl solution containing 0.1% NaCl. Ca²⁺ levels were determined with Ca²⁺ assay kits (Latron Laboratories, Tokyo, Japan).

**MTP assay in liver**

Liver homogenates were sonicated on ice by 2 bursts for 10 seconds using an ultrasonic homogenizer (Bandelin, HD2070, Germany) at 30% power. MTP activity was determined using a commercial kit based on the MTP-mediated transfer of a self-quenched fluorescent neutral lipid from the core of a donor molecule to an acceptor molecule (Calbiochem®, Germany). To determine MTP activity, liver homogenates (200 protein μg/ml) were incubated for 20 hours at 37°C with or without various concentrations of thapsigargin or db-cAMP in the presence of 10 μl of donor molecular containing a fluorescent neutral lipid (TG) and 10 μl of acceptor molecule. To investigate the effect of thapsigargin and db-cAMP, thapsigargin was dissolved in ethanol (2 × 10⁻⁴%) while db-cAMP was dissolved in distilled water, which was then added to the liver homogenates. MTP activity was measured by a spectrofluorometer (SFM 25, Bio-TEK Instrument inc., Vermont, UK) at a fluorescence wavelength of 535 nm and excitation wavelength of 465 nm. The MTP activity was calculated by subtracting observed value of the blank from those of the liver homogenates containing 10 μl of donor molecule and acceptor molecule, and then the value was extrapolated into a standard curve.

**Statistics**

The statistical analysis for each parameter was performed using Student’s t-test where appropriate. At least 95% of probability was accepted as a significant treatment effect.

**Acknowledgements**

This study was supported in part by grants (R01-2007-000-206695-0 to C.J.Y., R.M.H., and P.H.J.) from the Basic Research Program of the Korean Science and Engineering Foundation (KOSEF), Korea, Science & Engineering Foundations, the Ministry of Commerce, Industry and Energy (MOCIE) and the Korean Institute of Industrial Technology Evaluation & Planning (ITEP), through the Biohealth Products Research Center (BPRC) of Inje University, Korea.

**REFERENCES**


Regulation of MTP activity by calcium or cAMP
Hyun-Jeong Cho, et al.