EFFECT OF 12-O-TETRADECANOYLPHORBOL 13-ACETATE ON DNA SYNTHESIS IN PROLIFERATING RAT HEPATOCYTES IN PRIMARY CULTURE

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Summary : DNA synthesis in primary culture of rat hepatocytes was initiated over 16h after the beginning of culture and reached a peak between 32h and 40h under the conditions of this study. The addition of 12-O-tetradecanoylphorbol 13-acetate (TPA) (100ng/ml) during the initial 24h of culture inhibited DNA synthesis, while addition between 16h or 20h and 24h stimulated DNA synthesis. Chelator-extractable and calcium-dependent protein kinase activity in the soluble fraction of hepatocytes with TPA addition during the initial 24h of culture was apparently reduced at 8, 12, 16 and 24h. It is suggested that TPA addition just prior to the initiation of DNA synthesis activates proten kinase C (PKC) and stimulates DNA synthesis, whereas TPA addition at an early stage of culture inhibits DNA synthesis, possibly because of PKC depletion.

Index Terms
12-O-tetradecanoylphorbol 13-acetate (TPA), DNA synthesis, primary culture of rat hepatocytes

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INTRODUCTION

Tumor-promoting phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA)[1] are closely related to cell proliferation and carcinogenesis in a variety of tissues[2-5]. TPA has a specific binding activity to protein kinase C (PKC), and is known to directly activate it[6,7]. This enzyme is usually activated by 1,2-diacyl glycerol, which is released during phosphoinositide breakdown induced by the receptor-mediated stimulation of several types of hormones and neurotransmitters[8,9]. The authors have previously reported that PKC activity in the soluble fraction of hepatectomized rat liver showed a biphasic decrease prior to the initiation of DNA synthesis, and that administration of TPA at the time of the operation resulted in inhibition of DNA synthesis[10]. TPA-induced activation of PKC appears to have an inhibitory effect on liver regeneration. On the other hand, it is known that continuous TPA treatment causes a total loss of PKC[11-13]. To elucidate the relationship between this TPA effect and liver regeneration, we investigated the effects of TPA on DNA synthesis in primary cultures of rat hepatocytes. Primary cultures of rat hepatocytes have previously been used in studies on factors associated with replicative DNA synthesis in the liver[14-18].

MATERIALS AND METHODS

Materials and animals Collagenase Type I, insulin, dexamethasone and H1 histone (Sigma Type III-S) were obtained from Sigma Chemical Company, St.Louis,MO. 1- (5-Isoquinolinesulfonyl) -2-methylpiperazine (H-7) was obtained from Seikagaku Kogyo Co., LTD., Tokyo, Japan[19]. Williams' E medium[20], fetal bovine serum (FBS) , Dulbecco's PBS without Ca^{++} and Mg^{++}, and Hanks' balanced salt solution (HBSS) were purchased from Flow Laboratories Inc., USA. [6-^{3}H] Thymidine (>15Ci/mmol) and [γ-^{32}P] ATP (>10Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA. TPA, dimethylsulfoxide (DMSO) , N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) , trypsin, ethylene bis (β-aminoethylether) -N, N, N', N'-tetraacetic acid (EGTA) , the scintillation fluid (Univer-Gel II) and other chemicals were obtained from Nakarai Chemicals, LTD., Kyoto, Japan. Male Sprague-Dawley rats, each weighing about 200g, were obtained from Japan SLC, Inc.

Primary culture of hepatocytes Hepatocytes were isolated from rats which had fasted overnight according to the method described by Tanaka et al.[21] with some modifications. The liver was perfused with 100ml $Ca^{++}Mg^{++}$-free Dulbecco's PBS containing 0.5mM EGTA followed by 200ml of HBSS containing 5mM $CaCl_2$ and collagenase (100mg) . The hepatocytes were dispersed, washed and resuspended at a concentration of 2.5×10^{5}cells/ml in Williams' E medium containing 10mM HEPES buffer (pH 7.4) , insulin (1μM) , dexamethasone (1μM) and 10% FBS. The cells were plated on Falcon plastic dishes at a density of 5×10^{4}cells/cm^2 and maintained at 37℃ under 5% CO_2 in air. Ninety-six-well plates and 35-mm dishes were used for [6-^{3}H] Thymidine incorporation and DNA determination, respectively. Following a 24h culture period, the medium was replaced with fresh Williams' E medium containing no serum or hormones.

Estimation of DNA synthesis DNA synthesis was estimeted by the incorporation of [6-^{3}H] Thymidine into the DNA of hepatocytes. [6-^{3}H] Thymidine (0.1μCi/well) was exposed to cultured hepatocytes for 8h or 24h prior to harvest. The cells were washed with $Ca^{++}Mg^{++}$- free Dulbecco's PBS, trypsinized and filtered through a glassfiber filter in ice-cold 10% trichloroacetic acid (TCA) with a cell harvester (Labo Science Co.) . The filter was placed in scintillation fluid to measure radioactivity. DNA was determined by the fluorometric method of Setaro and Morley[22].

Assay for calcium-dependent protein kinase activity in the soluble fraction of hepatocytes Cultured hepatocytes (3.75×10^{6}cells) were scraped with a rubber policeman, washed twice with Ca^{++} Mg^{++}-free Dulbecco's PBS, placed in 4ml of homogenization buffer (20mM Tris, 10mM EDTA, 2mM EGTA, 2mM phenylmethanesulfonyl fluoride, pH 7.4) , homogenized with a Potter-Elvejhem homogenizer and precipitated by centrifugation at $100{,}000\times g$ for 1h. The soluble fraction containing EGTA- and EDTA-extractable (chelator-extractable) protein kinases was saved and protein was determined by the method of Lowry et al.[23]. All procedures were performed at 4℃. Calcium-dependent protein kinase activity was assayed by measurement of the enzymatic transfer of ^{32}P from [γ-^{32}P]ATP to H1 histone, in a total volume of 200μl containing 50mM Tris-HCl (pH 7.0) , 10mM $MgCl_2$, 100μg of H1 histone, 10μg of phosphatidyl serine, 10mM 2-mercaptoethanol, 0.5mM $CaCl_2$, 2 nmol [γ-^{32}P] ATP and 60μl of sample (50-200μg of protein). Basal activity was measured in the presence of 1mM EGTA instead of

$CaCl_2$. After incubation at 30℃ for 6 min, the reaction was terminated by adding 1ml of ice-cold 20% TCA and followed by adding 3ml of 5% TCA and 0.5mg of bovine serum albumin. The mixture was centrifuged at 3,000 rpm for 15 min. The precipitate was washed three times with 5ml of 10% TCA and dissolved in 1ml of 1N NaOH to measure radioactivity. Calcium-dependent protein kinase activity was determined by subtracing the ^{32}P incorporation into

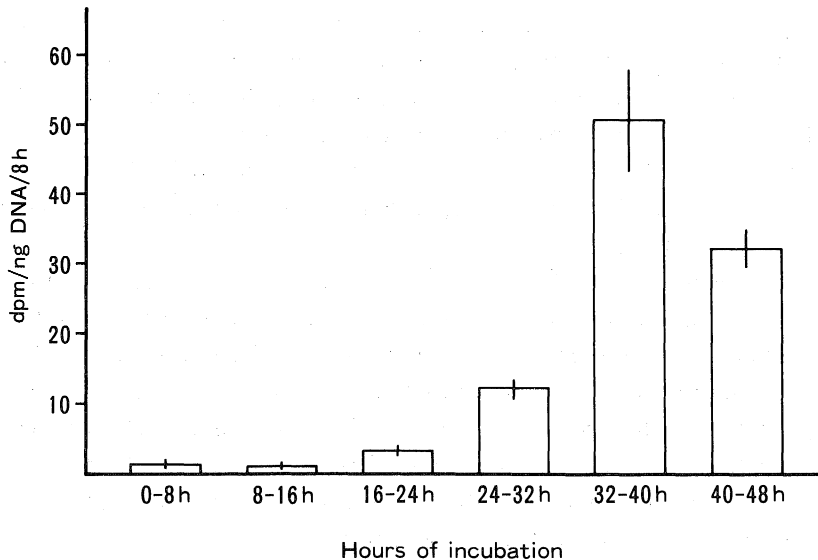

Fig. 1. Time course of DNA synthesis in primary culture of rat hepatocytes. DNA synthesis was determined by the incorporation of [6-^{3}H] Thymidine into DNA of hepatocytes. [6-^{3}H] Thymidine (0.1 μCi/well) was added to cultured hepatocytes at 8-h intervals after inoculation of cells. After exposure. the cells were havested. Data are the mean±SD of 5 wells.

Table 1. The effect of the addition of TPA on DNA synthesis in primary culture of rat hepatocytes

DNA synthesis (dpm/ng DNA/24 h)		
	TPA addition during	
	0 h-24 h	24 h-48 h
Control	32.23±4.41	
TAP 1 ng/ml	31.78±4.38	34.86±6.43
10 ng/ml	28.14±5.55	30.94±2.36
100 ng/ml	22.31±2.57(*)	34.71±6.95

	TPA addition during			
	12 h—24 h	16 h—24 h	20 h—24 h	22 h—24 h
TAP 100 ng/ml	26.89±9.65	50.61±9.41*	48.95±7.07*	37.38±6.22

DNA synthesis was determined by the incorporation of [6-^{3}H] Thymidine into DNA in cultured hepatocytes. [6-^{3}H] Thymidine (0.1 μCi/well) was exposed to cultured hepatocytes from 24 h to 48 h. After exposure, the cells were harvested.
Data are means ±SD of 5 wells
*$p<0.01$, significantly high compared with control.
(*)$p<0.01$, significantly low compared with control.

histone noted in the presence of EGTA from that noted in the presence of $CaCl_2$.

Measurements and statistical analysis of data Radioactivity was measured in a liquid scintillation counter (Model LS-7500, Beckmann Instruments Inc.) . Data in all fiqures are expressed as the mean±SD of 5 wells. Statistical analysis was performed using the unpaired t-test.

RESULTS

The time course of DNA synthesis in primary cultures of hepatocytes is shown in Fig. 1. DNA synthesis was determined by the incorporation of [6-^{3}H] Thymidine into DNA of hepatocytes at 8-h intervals after inoculation of cells. A significant increase in DNA synthesis following a latent period (16h) was found during the third 8-h interval (2.89±0.28 dpm/ng DNA/8h) and the fourth 8-h interval (14.01±1.78 dpm/ng DNA/8h) . DNA synthesis reached a peak in the fifth 8-h interval (50.44±7.42 dpm/ng DNA/8h) and declined during the following interval (32.28±2.85 dpm/ng DNA/8h) .

The effect of the addition of TPA on DNA synthesis in primary culture of hepatocytes is shown in Table 1. DNA synthesis was determined by the incorporation of [6-^{3}H] Thymidine into DNA in hepatocytes between 24h and 48h of culture. TPA was dissolved in DMSO, of which the final concentration was adjusted to 0.02%. TPA addition at a concentration of 100 ng/ml during the initial 24h significantly inhibited DNA synthesis (69% of control) , while addition between 24h and 48h had no significant effect. In contrast, TPA addition between 16h or 20h and 24h significantly stimulated DNA synthesis (stimulation by TPA addition during 16h-24h and 20h-24h was 157% and 152%, respectively) .

Chelator-extractable and calcium-dependent protein kinase activity in the soluble fraction of hepatocytes with or without TPA addition during the initial 24h of culture was assayed at 0, 8, 12, 16 and 24h (Table 2) . Protein kinase activity in the TPA-treated hepatocytes was apparently reduced at 8, 12, 16 and 24h, while that in control appeared to decrease between 16h and 24h.

Addition of H-7 during the initial 24h of culture inhibited DNA synthesis in a concentration-dependent manner (0.5-32μM) (Fig.2) . Using H-7, 50% inhibition of DNA

Table 2. Calcium-dependent protein kinase activity in the soluble fraction of hepatocytes with and without TPA addition during the initial 24 h of culture

Hours	Protein kinase activity (pmol^{32}P/min/mg protein)	
	TPA (−)	TPA (+)
0 h	0.1291	
8 h	0.1152	0
12 h	0.1128	0.0244
16 h	0.0934	0.0007
24 h	0.0491	0

Data are means of duplicate assay.
Protein kinase activity is expressed as pmol of ^{32}P transferred from [γ-^{32}P] ATP to H1 histone in 1 min (mg protein) $^{-1}$.

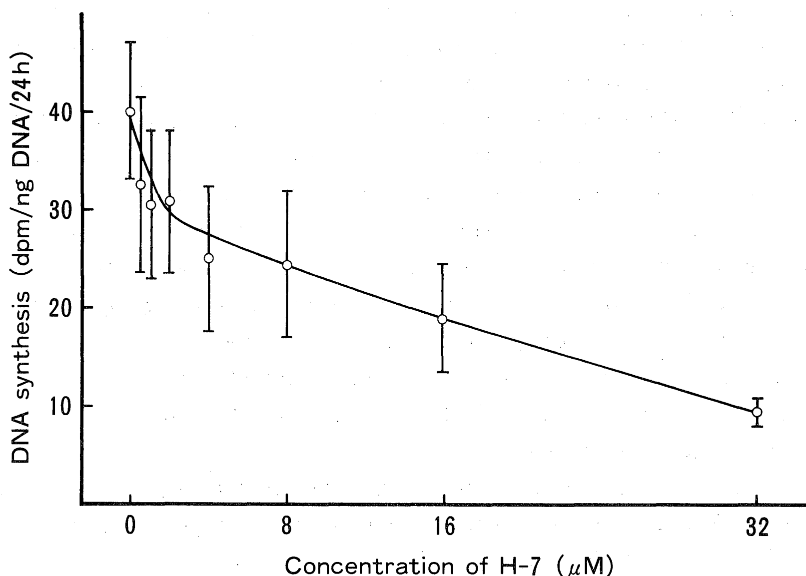

Fig. 2. Effect of H-7 on DNA synthesis in primary cultures of rat hepatocytes, H-7 (0.5-32 μM) was added during the initial 24 h of culturing. DNA synthesis was determined by the incorporation of [6-^{3}H] Thymidine into DNA in cultured hepatocytes. [6.-^{3}H] Thymidine (0.1 μCi/well) was exposed to cultured hepatocytes from 24h to 48h. After exposure,the cells were harvested. Data are the mean$\pm$SD of 5 wells.

synthesis was achieved at a dose of 16μM.

DISCUSSION

The proliferation of hepatocytes in primary culture is known to be affected by a variety of culture conditions such as cell density[15], serum supplementation[15,18], growth factors[14,18], hormones[14] and nutrients[16,17]. Under the conditions of this study, DNA synthesis of hepatocytes in primary culture was initiated over 16h after the initiation of culture and reached a peak between 32h and 40h. The peak of DNA synthesis in primary cultures of hepatocytes occurred later than that observed in partially hepatectomized liver, less than 24h after partial hepatectomy[10].

TPA addition during the initial 24h significantly inhibited DNA synthesis, but addition between 24h and 48h had no effect. On the other hand, TPA addition between 16h or 20h and 24h significantly stimulated DNA synthesis. Thus, the effect of TPA on DNA synthesis in hepatocytes varied with the timing of its addition.

TPA is known to activate PKC directly and induce rapid intracellular translocation of PKC[6,7]. It has been reported that translocated PKC progressively decreases and is lost after long exposure to TPA in various cell lines such as Swiss 3T3 mouse fibroblasts[11], HEL-37 mouse epidermal cells[12] and MCF-7 human breast cancer cells[13]. On the other hand, It is

known that PKC phosphorylates epidermal growth factor (EGF) receptors and inhibits their tyrosine kinase activity[24], which is believed to play an important role in liver regeneration[18].

We have reported that soluble PKC in regenerating rat liver shows a biphasic decrease prior to the initiation of DNA synthesis,and concluded that this decrease in soluble PKC implies translocation of PKC to the plasma membrane[10]. The translocation of PKC to the plasma membrane is thought to be associated with the activation of PKC[25]. In this study, we found that soluble calcium-dependent protein kinase activity (which could include the activity of non-translocated PKC) in hepatocytes in primary cultures decreased from 16h to 24h, similar to the second decrease in soluble PKC activity observed in regenerating liver. It is conceivable that PKC is activated not only in the process of in vivo liver regeneration but also in primary culture of hepatocytes. On the other hand, early and apparent reduction of soluble calcium-dependent protein kinase activity in TPA-treated hepatocytes is inferred to be caused by TPA-induced down-regulation of PKC.

Taken together, these observations suggest that addition of TPA just prior to the initiation of DNA synthesis (at late G_1 phase) enhances the translocation of PKC and stimulates DNA synthesis, whereas addition of TPA at an early stage of culture (more than 24h before the initiation of DNA synthesis) inhibits DNA synthesis, possibly because of PKC depletion as a result of early activation of PKC.

The mechanism of PKC action on liver regeneration remains unknown. The decrease of soluble calcium-dependent protein kinase in primary culture of hepatocytes may induce DNA synthesis in a manner similar to the elevation of cyclic AMP levels in regenerating rat liver reported by MacManus et al.[26]. These phenomena may be associated with the induction of ornithine decarboxylase, which is reported to increase in cells at the G_1/S phase of the cell cycle[27,28].

The effect of H-7 addition on DNA synthesis in primary culture of hepatocytes was also studied. The addition of H-7 during the initial 24h culture period inhibited DNA synthesis in a dose-dependent manner. The inhibition of DNA synthesis was achieved more efficiently with low doses of H-7 than with TPA. H-7 is the most potent known inhibitor of PKC among isoquinolinesulfonamides, though it also inhibits cyclic AMP-dependent or calmodulin-dependent protein kinase[29]. Thus, PKC and/or other protein kinase seem to be essential for the proliferation of cultured hepatocytes.

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