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Inhibition of Thymidylate Synthase and Thymidine Kinase by Okadaic Acid in Regenerating Rat Liver after Partial Hepatectomy

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ABSTRACT. The effects of okadaic acid, a potent and specific inhibitor of protein phosphatases 1 and 2A, on liver regeneration after partial (70%) hepatectomy were investigated. The injection of okadaic acid (25 μg/kg body weight) inhibited the increases in the activities of thymidylate synthase and thymidine kinase in regenerating rat liver at 24 hr after partial hepatectomy, with a concomitant reduction in DNA content. Northern blot analysis showed that the suppression of thymidylate synthase and thymidine kinase activities was caused by comparable decreases in their mRNA levels. The protein levels of thymidylate synthase and thymidine kinase were confirmed by immunoblotting assay to be proportional to the activity and mRNA levels. These findings suggest that okadaic acid-sensitive protein phosphatases are involved in transcriptional control of the dTMP-synthesizing enzymes during liver regeneration. BIOCHEM PHARMACOL 52;6:879–884, 1996.

KEY WORDS. okadaic acid; thymidylate synthase; thymidine kinase; DNA synthesis; liver regeneration

Protein phosphorylation and dephosphorylation play a pivotal role in the regulation of gene expression and cell proliferation. While the importance of protein kinases is well understood, the physiological role of protein phosphatases has not been fully elucidated. Okadaic acid, a cell permeant polyether derivative of fatty acid, is known to be a potent, highly specific inhibitor of PP1§ and PP2A [1], two of the four major serine/threonine phosphatases in the cytosol of mammalian cells [2–4]. Okadaic acid has, therefore, provided a good means to study the function of these protein phosphatases. However, work with this drug has generated apparently opposing results. Okadaic acid has been shown to be a tumor promoter in skin carcinogenesis [5] and to stimulate expression of certain proto-oncogenes [6, 7]. On the other hand, recent experiments indicate that okadaic acid can be a growth inhibitor in that it blocks proliferation [8–10] and induces apoptosis [11] and mitotic arrest [12, 13]. However, very little is known about the role of protein phosphatases in *in vivo* cellular proliferation.

The regenerative response of liver following removal of 70% of its mass provides a suitable *in vivo* model in which to study the role of protein phosphatase in the regulation of cell proliferation. The early stages of the regenerative response in rats consist of a hypertrophic, prereplicative phase, lasting approximately 12–16 hr, during which the rate of protein synthesis increases, and a subsequent hyperplastic phase that is characterized by the onset of DNA synthesis at about 18 hr, peaking at about 24 hr, followed by mitosis [14, 15]. Thus, a well-defined, and to an extent synchronous, cellular proliferation marks the first 24 hr after partial hepatectomy [16]. Recently, the expression and activity of PP1 were reported to increase at 12 hr after PH [17]. Okadaic acid represses the expression of cdc2 and cyclin A in mouse NIH 3T3 fibroblasts [10]. These findings suggest the involvement of protein phosphatases in G_1/S transition and S phase of cell proliferation.

To study the possible role of protein phosphatases in DNA synthesis during liver regeneration, we investigated the effects of okadaic acid on the activities, the protein, and the mRNA levels of TS (EC 2.1.1.45) and TK (EC 2.7.1.21). TS and TK catalyze the formation of thymidylate via the *de novo* and salvage pathways, respectively. We previously described that these are the rate-determining enzymes of DNA synthesis and that the capacity of liver regeneration is evaluated by the induction of these enzymes [18–25]. We show here that okadaic acid inhibited DNA synthesis in regenerating liver by the repression of the mRNA levels of TS and TK.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following sources: [*methyl*-^{3}H]thymidine (2.9 TBq/mmol), ICN Biomedicals, Inc.

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§ *Abbreviations:* PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; TS, thymidylate synthase; TK, thymidine kinase; and PH, partial (70%) hepatectomy.

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(Irvine, CA, U.S.A.); [5-^{3}H]deoxyuridine monophosphate (403 GBq/mmol), Amersham International (Buckinghamshire, England); okadaic acid, Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Durapore membrane filter, Nihon Millipore Ltd. (Yonezawa, Japan); goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase, Cappel Research Laboratories (Durham, NC, U.S.A.); DIG RNA labeling kit and DIG luminescent detection kit, Boehringer Mannheim GmbH (Mannheim, Germany); oligo(dT)cellulose (type 3), Collaborative Research Inc. (Bedford, MA, U.S.A.); GeneScreen nylon membrane, NEN Research Products (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Animals

Male Wistar rats weighing 170–190 g were used for all experiments. The animals were kept in a temperature-controlled room with 12-hr alternating light and dark cycles and given commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water *ad lib.* at all times. A two-thirds partial hepatectomy (PH) was performed under diethyl ether anesthesia by the procedure of Higgins and Anderson [26]. Okadaic acid (25, 10 and 5 μg/kg body wt), dissolved in 50% propylene glycol, was injected intraperitoneally immediately or at 8 hr after PH. Control rats received the same quantity of 50% propylene glycol as the experimental animals. The rats were killed and their livers were excised at 24 hr after PH. All liver samples were dissected in half: one part was stored immediately at −80° and used for RNA isolation; the other half was used for the determination of TS and TK activities, the content of DNA, RNA, and protein, and the content of TS and TK proteins by an immunoblotting assay. Animal protocols were performed in accordance with international criteria for the use and care of experimental animals as outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Determination of Enzymatic Activity and Content of DNA, RNA, and Protein

The excised liver was homogenized with 5 vol. of 50 mM Tris–HCl buffer, pH 7.3, containing 0.25 M sucrose, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The DNA and RNA contents of the liver homogenate were measured by the diphenylamine [27] and orcin [28] reactions, respectively, after extraction with trichloroacetic acid by the procedure of Schneider [28]. Protein was determined by the method of Lowry *et al.* [29] using bovine serum albumin as standard. After a 20% liver homogenate was centrifuged at 36,000 *g* for 30 min at 4°, the supernatant fraction was used as the enzyme preparation. The activities of TS and TK were determined as described previously [18] and shown as picomoles product formed per minute per milligram protein at 37°.

Isolation of RNA

Total RNA was extracted from liver in 4 M guanidinium isothiocyanate [30] and fractionated by affinity chromatography on an oligo(dT)-cellulose column to obtain poly(A)$^+$-rich RNA. The concentrations of RNA samples were measured by absorbance at 260 nm. The purities of RNA samples were determined by the ratio of $A_{260\,nm}/A_{280\,nm}$ (>1.8) and by electrophoresis in formaldehyde agarose gel stained with ethidium bromide.

Preparation of RNA Probe

The *Pst*I fragment (0.7 kb) from the mouse TS cDNA clone pMTS3 or the *Bam*HI fragment (1.1 kb) from the mouse TK cDNA clone pMTK4 (ATCC 37556) was subcloned into plasmid pGEM 3Z (Promega). After linearization of the plasmid, T7 RNA-polymerase was employed to obtain run-off transcripts of the antisense strands. Transcription and labeling were performed utilizing a commercial DIG RNA labeling system according to the manufacturer's instructions.

Northern Blot Analysis

The RNA preparations were denatured and electrophoresed on 1.4% agarose/2.2 M formaldehyde gels. After blotting the RNA samples on the gel to nylon membrane, the blotting efficiency was examined by the intensity of ethidium bromide fluorescence signals of rRNA on the filter. Hybridization and quantitative analysis of the blots were carried out as described previously [31]. The comparable levels of glyceraldehyde-3-phosphate dehydrogenase mRNA were also confirmed in RNA samples used for the blotting.

Antibodies and Immunoblot Analysis

Anti-polyclonal rat TS antibodies were prepared with the purified TS from regenerating rat liver as we described previously [32, 33]. Anti-polyclonal rat TK antibodies were prepared against the bacterial expressed and purified rat TK polypeptide. The rat TK was expressed using host bacteria *Escherichia coli* BL21 (DE32) after cloning a polymerase chain reaction product of the rat TK cDNA clone pRtk-1 into pAR 2156 expression vector [34]. The resulting polypeptide had a molecular weight of 26,000 and was purified by affinity chromatography as described previously [35]. Immunoblots were performed using a 1:70 dilution of anti-TS antisera or 1:500 dilution of anti-TK antisera. The immunoblot was developed with the goat anti-(rabbit IgG) conjugated to horseradish peroxidase and assayed quantitatively as described previously [33].

RESULTS

Effects of Okadaic Acid on the Activities of TS and TK, the Content of DNA, RNA, and Protein, and the Liver Weight of Regenerating Liver

The activities of TS and TK at 24 hr after PH are shown in Table 1. TS activity was increased by about four times

compared with activity of the normal rat (resting in G_0 state; just after PH). The injection of okadaic acid immediately after PH at a dose of 25 μg/kg body weight inhibited the activity of TS to 52% of the control value. TK activity was also depressed by the injection of okadaic acid to 11% of the control, which was comparable to the normal level. At a dose of 10 μg of okadaic acid/kg body weight, the level of TS activity was similar to that of the control. However, TK activity was still inhibited significantly compared with the control. When the dose of okadaic acid was reduced to 5 μg/kg body weight, the decrease in TK activity was not observed. These results indicated that okadaic acid inhibited the increases in TS and TK activities of 24 hr-regenerating liver after PH in a dose-dependent manner. Liver DNA content was also reduced significantly accompanied by decreases of TS and TK activities by injection of 25 μg of okadaic acid/kg body weight. At this dose level, RNA content and gross parameters such as protein content and liver weight were also reduced significantly. The DNA, RNA, and protein contents and liver weight in rats injected with 10 or 5 μg of okadaic acid were similar to those of the control.

When okadaic acid was administered 8 hr after PH at a dose of 25 μg, in contrast to the immediate injection, RNA and protein contents and liver weight were not influenced significantly. However, the inhibition of the increases in the activities of the enzymes and the concomitant decrease in DNA content in 24-hr regenerating liver were observed to be similar to those in animals injected immediately after PH.

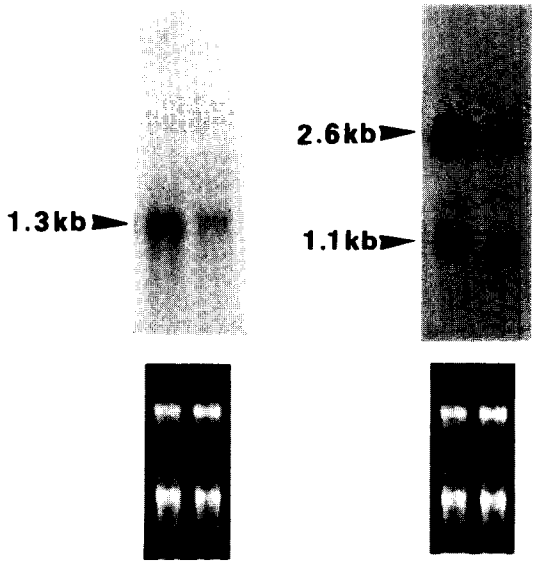

FIG. 1. Effects of okadaic acid on mRNA levels of TS and TK in regenerating liver. Shown are northern blots of TS mRNA (left) and TK mRNA (right) in regenerating liver. Poly(A)⁺ RNA (7 μg) from regenerating liver at 24 hr after PH was fractionated in agarose-formaldehyde gel, blotted to GeneScreen, and hybridized with the DIG-labeled TS or TK probe as described in Materials and Methods. Lanes contained regenerating liver samples from the control (left lane) and okadaic acid-treated [25 μg/kg body wt, administered at 8 h after PH) (right lane) rats. The photograph of the gel exhibiting ethidium bromide fluorescence is shown in the lower panels, demonstrating equal loading of RNA samples.

Effect of Okadaic Acid on mRNA Levels of TS and TK

TS mRNA was just detectable in normal quiescent liver, started to elevate at 12 hr, and reached a maximum at 24 hr after PH, as previously reported [31]. The injection of okadaic acid at 8 hr reduced 1.3-kb TS mRNA induction by PH, as shown in Fig. 1. Scanning densitometry showed that the level of TS mRNA in okadaic acid-treated rats decreased to 37% of the control (Table 2). This reduction in TS mRNA level closely correlated with the decrease in TS activity. TK mRNA levels were also found to be decreased, in proportion to the reduction in TK activity, by the injection of okadaic acid (Fig. 1 and Table 2). These results clearly demonstrated that okadaic acid repressed the gene expression of TS and TK in the prereplicative phase of regenerating liver after PH.

TABLE 1. Effects of okadaic acid on liver regeneration after partial hepatectomy

Treatment	injection time after PH (hr)	dose (μg/kg)	Enzymatic activity (pmol/min/mg protein)		Total liver content (mg)			Liver weight (g)
			TS	TK	DNA	RNA	Protein	
Control			65.1 ± 3.1	357.7 ± 31.1	6.63 ± 0.16	41.3 ± 1.2	587.7 ± 24.0	3.2 ± 0.06
Okadaic acid	0	25	34.0 ± 2.8*	40.5 ± 8.4*	4.48 ± 0.16*	31.4 ± 1.3*	471.4 ± 17.8*	2.7 ± 0.08*
	0	10	78.1 ± 6.2	228.4 ± 29.7*	6.26 ± 0.26	40.3 ± 1.9	622.5 ± 44.7	3.2 ± 0.11
	0	5	77.2 ± 6.3	341.9 ± 22.8	6.77 ± 0.16	42.0 ± 0.7	608.6 ± 11.8	3.3 ± 0.10
	8	25	23.2 ± 2.7*	50.4 ± 10.5*	5.29 ± 0.14*	40.2 ± 2.4	522.9 ± 14.4	3.0 ± 0.04
	8	10	62.3 ± 4.2	159.1 ± 32.4*	6.70 ± 0.19	41.0 ± 1.4	589.8 ± 10.8	3.3 ± 0.04
	8	5	70.2 ± 10.5	263.4 ± 22.0	6.60 ± 0.15	43.6 ± 2.4	585.8 ± 21.4	3.2 ± 0.04
Normal (G_0, just after PH)			14.8 ± 2.3*	31.3 ± 8.2*	4.97 ± 0.09*	27.6 ± 0.5*	491.7 ± 14.9*	2.6 ± 0.09*

Okadaic acid (25, 10, or 5 μg/kg body weight) was injected intraperitoneally immediately (0 hr) or at 8 hr after PH. At 24 hr after PH, the activities of TS and TK, the liver content of DNA, RNA, and protein, and liver weight were determined as described in Materials and Methods. Values are means ± SEM of 5–10 rats.

* Significantly different from control, $P < 0.05$.

TABLE 2. Effects of okadaic acid on the mRNA and protein levels of TS and TK in 24 hr-regenerating liver

		TS		TK
Enzymatic activity (%)		35.5 ± 7.3		15.5 ± 3.1
mRNA level	(1.3-kb)	37.3 ± 6.1	(2.6-kb)	18.5 ± 7.3
			(1.1-kb)	22.2 ± 2.4
Protein content		43.5 ± 8.7		10.5 ± 1.7

Okadaic acid (25 μg/kg body wt) was injected at 8 hr after PH. The liver was excised and dissected into two portions at 24 hr after PH. One portion was used for the determination of the enzymatic activity and of the contents of TS and TK protein by immunoblotting and the other was for RNA isolation and northern blotting as described in Materials and Methods. TS and TK activities are expressed as a percentage of the control value, 59.3 ± 12.8 and 264.6 ± 50.3 pmol/min/mg protein, respectively. The mRNA and protein levels of TS and TK were relative to 100 arbitrary units of the control. Values are means ± SD of 4 rats.

Effects of Okadaic Acid on the Protein Levels of TS and TK

To investigate the TS and TK protein levels, immunoblot analyses using the specific antisera were carried out. TS protein immuno-reacted as a 35 kDa protein band in 24-hr regenerating liver of the control or okadaic acid-injected rat on the immunoblots, as shown in Fig. 2. The relative amounts of TS protein in the rats injected with okadaic acid at 8 hr were reduced to 44% of the control (Fig. 2 and Table 2). This decrease correlated with the levels of the enzymatic activity and its mRNA.

On immunoblots using the anti-rat TK sera that were prepared with rat TK protein expressed in *E. coli* BL21 (DE3), a single band with a molecular mass of 26 kDa was detected in induced bacteria lysate and the supernatant fraction of 24-hr regenerating rat liver, but not in uninduced bacteria and normal rat liver, as shown in Fig. 2. When okadaic acid was injected at 8 hr after PH, the TK protein in 24-hr regenerating liver was barely detectable (Fig. 2). The scanning densitometry showed that the TK protein level of the treated rat was reduced to 10% of the control (Table 2). The reduction in TK protein level also corresponded to the decreases in the activity and the mRNA observed in TS.

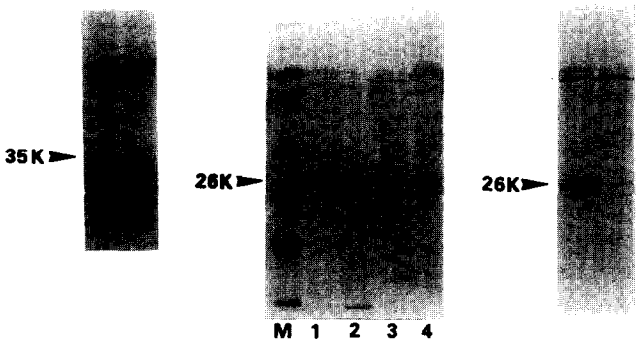

FIG. 2. Effects of okadaic acid on protein levels of TS and TK in regenerating liver. Left panel: Immunoblot analysis of TS protein. The supernatant fractions (30 μg of protein) of 24 hr-regenerating liver of the control (left lane) and okadaic acid-treated (25 μg/kg injected at 8 hr) (right lane) rats were separated on SDS/10% polyacrylamide gel, transferred, and developed as described in Materials and Methods. Middle panel: Immunoblot of uninduced bacteria lysates (lane 1), induced bacteria lysates (lane 2), and the supernatant fractions of 24-hr regenerating rat liver (lane 3) and normal rat liver (lane 4) with anti-rat TK antisera. Lane M was prestained protein molecular-mass markers (175-, 83-, 62-, 47.5-, 32.5-, 25-, and 16.5-kDa). Right panel: Immunoblot analysis of TK protein. The supernatant fractions (200 μg protein) of 24-hr-regenerating liver from the control (left lane) and okadaic acid-treated (25 μg/kg injected at 8 hr) (right lane) rats were separated on SDS/12.5% polyacrylamide gel and submitted to immunoblotting assay as described in Materials and Methods.

DISCUSSION

The results presented here show that okadaic acid inhibited DNA synthesis by the repression of TS and TK during liver regeneration after PH. This was consistent with the *in vitro* study which demonstrated that DNA replication of the epidermal growth factor-stimulated hepatocytes is abolished by okadaic acid in primary culture [9]. The growth inhibition was also observed in human leukemia K562 [12] and NIH fibroblasts [10]. These effects of okadaic acid on cell cycle progression are believed to be mediated through the inhibition of PP1 and PP2A. In this experiment, TS and TK activities were decreased by the injection of okadaic acid at a dose level as low as 25 μg (30 nmol) per kg body weight. The increase in DNA content of liver was also suppressed (Table 1). However, the negative effect on RNA and protein contents and on liver weight was also observed when okadaic acid was injected immediately after PH. The rate of ribosomal RNA and mRNA synthesis increases during the first 6 hr after PH, in the early phase of liver regeneration [36]. To eliminate the inhibitory effects on the overall rate of cellular transcription, okadaic acid was injected at 8 hr after PH, at which time the second stage of liver regeneration, regulated by catecholamines through α_1-adrenoceptor and calcium ions, starts [18, 21]. The injection of okadaic acid at 8 hr repressed the increases in TS and TK activities and DNA content without the decreases in RNA and protein contents and liver weight.

Northern blot analysis clearly showed that these repressions were caused by the reductions in TS and TK mRNA (Fig. 1 and Table 2). The reduction of TS or TK mRNA by okadaic acid correlated closely with the decrease in its activity (Table 2). Therefore, in this case, translational regulation is not apparent. This is further supported by the immunoblot analysis, which showed the close correlation between immunoreactive protein, mRNA, and TS and TK activities as shown in Table 2. These results indicate that okadaic acid suppresses the gene expression of dTMP-synthesizing enzymes, TS and TK, in regenerating liver after PH. This is the first evidence suggesting that okadaic acid-sensitive protein phosphatases are involved in the regulation of DNA synthesis at the mRNA level of DNA synthesizing enzymes in an *in vivo* model system, the regenerating liver.

It is known that okadaic acid blocks cells in metaphase [12, 37, 38]. Recently, okadaic acid was reported to cause an abrupt and complete cessation of primary rat hepatocyte cell cycle progression at the restriction point in late G_1 [39]. The growth inhibition by okadaic acid in NIH3T3 fibroblasts was also due to its effect on later events in G_1 with the concomitant repression of cdc2 and cyclin A expression [10]. In regenerating liver after PH, cyclin A and cdk2 were reported to increase during late G_1 peaking S phase [40]. Loyer *et al.* [41] also reported that the activation of cdc2 and cdk2 took place during the S phase, and these two kinase proteins would participate in the regulation of DNA synthesis during liver regeneration. The PP1 activity in nuclei was found to increase in G_1 to S transition in the cell cycle of regenerating liver [17]. Okadaic acid would, therefore, prevent these late G_1 events required to stimulate the expression of TS and TK which occurs at the G_1 to S transition during liver regeneration, although a direct effect of okadaic acid on the regulation of transcription of TS and TK is not excluded.

In conclusion, our paper has provided evidence for the inhibitory effects of okadaic acid on DNA synthesis accompanied by the repression of TS and TK in regenerating rat liver. Further study remains, however, to elucidate the molecular mechanism of the actions of okadaic acid on the expression of these particular genes during liver regeneration.

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