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The Regulation of Ribonucleoside Diphosphate Reductase by the Tumor Promoter Orotic Acid in Normal Rat Liver In Vivo

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Our earlier studies have shown that in normal hepatocytes, orotic acid (OA) inhibits DNA synthesis induced by several growth factors in vitro and after two-thirds partial hepatectomy (PH) in vivo. As in the normal liver OA induces an imbalance in nucleotide pools (specifically, an increase in uridine nucleotides, including deoxyuridine nucleotides, and a decrease in adenosine nucleotides, including ATP) and creation of this imbalance is crucial for the mitoinhibitory effects of OA, we hypothesized that ribonucleoside diphosphate reductase (RNR), a key enzyme in DNA synthesis that is regulated by nucleotide/deoxynucleotide levels, might be one of the targets for the inhibition of DNA synthesis by OA. To test this hypothesis, we subjected male Fischer 344 rats (130–150 g) to two-thirds PH in the absence or in the presence of OA (a 300-mg tablet of OA methyl ester implanted intraperitoneally at the time of two-thirds PH). The rats were killed at different times later, and their livers were processed for analysis of levels of RNR enzyme activity, protein, and mRNA transcripts. The results obtained indicated that treatment with OA resulted in a near-100% inhibition of RNR induced by two-thirds PH in rat liver, as monitored by enzyme activity and protein level. Furthermore, this inhibition was paralleled by a decrease in the mRNA transcripts for both the M1 and M2 subunits of *RNR*. Nuclear run-off assays indicated that this decrease in the levels of mRNA transcripts could not be attributed to an effect on transcription. However, administration of OA 20 h after two-thirds PH, when *RNR* mRNA transcripts were maximally induced, resulted in increased degradation of the RNR M1 and M2 subunits. Taken together, these results indicate that OA treatment decreases RNR levels induced by two-thirds PH, at the levels of enzyme activity, protein, and mRNA transcripts, and the decreased levels of mRNA transcripts appeared to be due to increased degradation of the transcripts. *Mol. Carcinog. 24:188–196, 1999.*

Key words: ribonucleoside diphosphate reductase; orotic acid; mitoinhibition; liver tumor promotion

INTRODUCTION

Previous studies by us and subsequently by others have shown that orotic acid (OA), a precursor of uridine nucleotides, is an excellent tumor promoter for liver [1–3] and other organs [4–6]. Furthermore, it was observed that both in vitro and in vivo, OA inhibits DNA synthesis in normal rat hepatocytes [7–9] but not in hepatocytes from hepatic nodules [10,11]. We therefore hypothesized that OA might exert its liver tumor–promoting effect by selectively inhibiting DNA synthesis in normal hepatocytes while having little or no effect on initiated hepatocytes. This would give the latter a selective advantage in responding to growth stimuli and developing into foci, nodules, and finally hepatocellular carcinoma. An extension of these studies to determine the sites at which OA might exert its mitoinhibitory effects in normal hepatocytes led us to postulate that ribonucleoside diphosphate reductase (RNR) may be one of the target sites for the mitoinhibitory effects of OA. The rationale for this is based on the following considerations: (i) dietary OA leads to an imbalance in nucleotide pools characterized by an increase in uridine and deoxyuridine nucleotides and a decrease in adenosine nucleotides, including ATP [12,13], and this imbalance is crucial for OA to exert its mitoinhibitory effects in the liver, and (ii) RNR is one of the enzymes involved in DNA synthesis that is sensitive to the fluctuations of nucleotide/deoxynucleotide pools. For example, while ATP is a positive effector required for the reduction of CDP and UDP [14], their reduction has been shown to be inhibited by increased levels of dUTP, dGTP, and TTP in many mammalian systems, including the rat liver [14,15].

The RNR enzyme protein is made up of two subunits. M1 is a 170-kDa dimer that contains the binding sites for the substrates and for allosteric effectors

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Abbreviations: OA, orotic acid; RNR, ribonucleoside diphosphate reductase; PH, partial hepatectomy; i.p., intraperitoneally; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHFR, dihydrofolate reductase; TS, thymidylate synthetase; Gal-Tase, β-1,4-galactosyltransferase; Act D, actinomycin D.

[16]. M2 is also a dimer, of 88 kDa, and contains stoichiometric amounts of nonheme iron and a unique tyrosyl radical essential for enzyme activity [17]. Several studies have shown that the levels of RNR activity correlate with the level of DNA synthesis [18–22]. In adult resting rat liver, RNR enzyme activity is barely detectable. However, administration of a proliferative stimulus, such as two-thirds partial hepatectomy (PH), increases RNR activity between 12 and 24 h later and is followed by a decline to basal levels by 72 h after PH [20,22–24].

The study reported here was designed to determine whether OA treatment inhibits the RNR expression induced by two-thirds PH and, if it does, how. The results obtained indicated that OA treatment decreased RNR induced by two-thirds PH, at the levels of enzyme activity, protein, and mRNA transcripts for the M1 and M2 subunits. Furthermore, the decrease in mRNA transcripts was not a reflection of impaired transcription but appeared to be due to increased degradation of mRNA transcripts of both the M1 and M2 subunits of *RNR*.

MATERIALS AND METHODS

Rats

In this study, we used male Fischer rats weighing 130–150 g (Charles River, St. Constant, Quebec). They were acclimatized for 1 wk before use and were maintained on a semisynthetic basal diet (Diet 101; Dyets Inc., Bethlehem, PA) and water ad libitum.

Preparation of Cytosol and Measurement of RNR Enzyme Activity

The rats were subjected to a two-thirds PH with or without a 300-mg tablet of OA (OA methyl ester; Sigma Chemical Co., St. Louis, MO) implanted intraperitoneally (i.p.) at the time of PH and were killed 24 h later. All further operations were performed at 4°C. Freshly excised liver from animals in each group was minced and gently homogenized in homogenization buffer (0.05 M HEPES, pH 7.5; 0.25 M sucrose; and 1 mM dithioerythreitol (Sigma Chemical Co.)). The crude homogenate was centrifuged at 100 000 $\times g$ for 1 h. The resulting clear lipid-free cytosol was treated with streptomycin sulfate (final concentration, 0.7%) for 20 min and centrifuged at 10 000 rpm. The supernatant was treated with ammonium sulfate (80% saturation), stirred for 30 min, and centrifuged at 10 000 rpm for 10 min. The pelleted protein precipitate was dissolved in 1/10 the original volume of cold HEPES buffer (homogenization buffer without sucrose) and desalted on a G-25 Sephadex column equilibrated with approximately 100 mL of HEPES buffer. The column was eluted with the same buffer, and the first 1.5 mL of the eluate was collected. This clear cytosol was aliquoted, frozen at –70°C, and used as the source for the RNR enzyme. Protein concentration was estimated by using the BCA assay (Pierce, Rockford, IL).

RNR enzyme activity was measured by a modification of the methods described by Steeper and Steuart [25], Takeda and Weber [26], and Youdale and MacManus [27]. Briefly, the assay mixture (final volume, 150 μL) contained 400 nmol of ATP and 1500 nmol of dithioerythreitol (both in sodium phosphate buffer, pH 7.5); 800 nmol of magnesium acetate and 8 nmol of ferric chloride (both in water); 4950 nmol of HEPES buffer, pH 7.5; 8 nmol of [^{14}C]CDP (sp. act., 477 mCi/mmol); and 1 mg of the enzyme protein. After incubation at 37°C for 30 min, the reaction was stopped by boiling for 4 min, and the samples were centrifuged at 10 000 rpm in an Eppendorf centrifuge. The dCDP formed was separated from the unreacted CDP by boronate affinity chromatography [28] with Affigel 601 (BioRad Labs., Richmond, CA). For this purpose, the supernatant was treated with 1 mg of snake venom phosphodiesterase (Boehringer Mannheim, Indianapolis, IN) in 6 mM Tris and 2 mM magnesium acetate, pH 9.2, at 37°C for 90 min. The reaction was stopped by boiling for 4 min and centrifuged. An aliquot of the supernatant was applied to the boronate column equilibrated with 1 M ammonium acetate and 0.1 M magnesium acetate, pH 8.8, and the unbound dCDP was eluted with the same buffer in 10 1.5-mL fractions. The unreacted CDP bound to the column was similarly eluted with 0.1 M formic acid. The radioactivity in each fraction was measured with an LKB scintillation counter (Pharmacia LKB Nuclear, Gaithersburg, MD) after addition of 10 mL of Aquasol (Universol; ICN Biochemicals). An aliquot of the sample applied to the column was similarly counted to determine the total radioactivity applied to the column. The enzyme activity was expressed as nmol of dCDP formed/mg of protein/30 min.

Western Blot Analysis

Cytosolic fractions used for the measurement of RNR enzyme activity were analyzed on vertical 12.75% sodium dodecyl sulfate–polyacrylamide gels essentially as described by Laemmli [29]. Five microliters of a prestained low-range molecular weight marker (19–107 kDa; BioRad Labs.) run along with the samples served as a standard. The samples were electrophoresed in a BioRad Mini-Protean II apparatus against tank buffer (0.025 M Tris, pH 8.3; 0.195 M glycine; and 0.1% sodium dodecyl sulfate) at 20 mA for 90 min. After electrophoresis, the gel was equilibrated in ice-cold transfer buffer (0.025 M Tris, pH 8.3; 0.195 M glycine; and 20% (v:v) methanol) for 20 min. The proteins were then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) with a BioRad Mini Trans Blot Cell at 100 V at 4°C for 1 h. For the detection of M1 protein, the blot was probed with a mouse monoclonal antibody to the M1 subunit of RNR (AD203; InRo Biomedtedk, Umea Sweden) at a concentration of 5 μg/mL followed by goat anti–mouse immunoglobulin G (Sigma Immuno-

chemicals, St. Louis, MO) conjugated with alkaline phosphatase at a dilution of 1:3000. The alkaline phosphatase reaction was developed with a BioRad alkaline phosphatase conjugate substrate kit, and the blot was then destained in distilled deionized water, air dried, and photographed.

Isolation of RNA from Rat Liver

Total RNA was isolated from the liver essentially by the method of Chirgwin et al. [30]. Poly(A^+) RNA was isolated from the total RNA by oligo dT chromatography as described by Maniatis et al. [31].

Electrophoresis of RNA and Northern Hybridization

Electrophoresis of RNA in formaldehyde-agarose gels, transfer to nitrocellulose filters, and northern hybridization were performed as described earlier [32]. The probes for the M1 and M2 subunits of *RNR* were kindly supplied by Dr. L. Thelander (University of Umea, Sweden); probes for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, ATCC 57090) and mouse dihydrofolate reductase (*DHFR*, ATCC 37295) were from the American Tissue Type Collection (Manassas, VA). The cDNAs for mouse thymidylate synthetase (*TS*) and bovine β-1,4-galactosyl-transferase (*Gal-Tase*) were gifts from Dr. L. F. Johnson (Department of Genetics, Ohio State University, OH) and Dr. J. Shaper (Johns Hopkins Oncology Center, Baltimore, MD), respectively. All the probes were radiolabeled with [^{32}P]dCTP (sp. act., >6000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by using the Multiprime labeling kit (Amersham Corp.).

Nuclear Run-Off Assay

Rat liver nuclei for the nuclear run-off assays were isolated essentially as described by Schibler et al. [33]. The livers from two rats per group were pooled, and 2 g of freshly excised liver was used for the preparation of nuclei. The purified nuclei were resuspended in glycerol storage buffer, and the integrity and number of nuclei were determined with a hemocytometer under phase-contrast microscopy. In vitro elongation of nascent transcripts was performed by using the method of McKnight and Palmiter [34] with minor modifications. Routinely, 2×10^7 nuclei prepared as described above were used per assay. The reaction was performed at 30°C for 30 min in a reaction mixture containing 100 μCi of [^{32}P]-labeled UTP (sp. act., >650 Ci/mmol; ICN, Montreal, Canada), the other three unlabeled nucleotides, and 50 μL of RNAse inhibitor (vanadyl ribonuclease inhibitor; BRL, Burlington, Canada). The reaction was terminated by adding RNAse-free DNAse I (Pharmacia Biotech, Piscataway, NJ). Two negative control reactions were performed: one was incubated with α-amanitin (1 mg/mL; Boehringer Mannheim) and the other without one of the unlabeled nucleotides. At the end of the reaction, the labeled RNA was isolated as follows. The nuclei were first lysed with an equal volume of 2× lysis buffer (20 mM Tris, pH 7.4; 1% sodium dodecyl sulfate; 50 mM EDTA; and 0.2 M NaCl). Then, proteinase K (Boehringer Mannheim) was added to a final concentration of 200 μg/mL, and the mixture was incubated at 37°C for 15 min. The samples were then extracted with phenol–chloroform–isoamyl alcohol (25:24:1 v:v:v). The RNA was precipitated with 0.3 M potassium acetate and 2.5 vol of absolute ethanol at –20°C overnight. The precipitated RNA was washed in 70% ethanol, air dried, and dissolved in DNAse buffer (10 mM Tris, pH 8.0; 5 mM $MgCl_2$; 150 mM KCl; and 2.5 mM dithioerythreitol). The samples were then subjected to DNAse I (10 U) digestion at 37°C for 1 h and reextracted with phenol–chloroform–isoamyl alcohol. The final RNA pellet was resuspended in 100 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and purified through Sephadex G-50 NICK columns (Pharmacia Biotech) as per the manufacturer's instructions. An aliquot was removed from each sample and the incorporation of labeled UTP determined.

Binding of Target Plasmids to Nitrocellulose and Hybridization

The plasmids containing the M1 and M2 subunits of *RNR* and *Gal-Tase* (1 and 3 μg) were linearized with the appropriate restriction enzymes and, after denaturation and neutralization, were slot blotted onto nitrocellulose membranes [35]. The blots were baked in a vacuum oven for 2 h at 80°C and prehybridized at 42°C overnight as described previously [32]. For hybridization, the radioactive RNA samples obtained above were denatured by boiling for 10 min and quenched on ice. One sample per blot was then injected into a bag containing a blot, such that each bag had the same amount of radioactivity (usually 5×10^6 cpm/mL). Hybridization was performed at 42°C for 72 h. The blots were then washed and subjected to autoradiography at –70°C as described earlier [32].

Estimation of DNA Content and Nucleotide Pools

The livers were homogenized in 0.5 N perchloric acid. The amount of DNA in the perchloric acid–precipitable fraction of the liver homogenate was estimated by Burton's method [36]. The perchloric acid–soluble supernatant was used for the estimation of nucleotide pools as described previously [10].

RESULTS

In this study, we studied only the cytosolic RNR enzyme as a representive of all cellular RNR activity. Although RNR activity has been reported to be present in the outer nuclear membrane, most RNR activity (75%) is present in the cytosol. Furthermore, the changes in the activity in the two fractions are similar during the DNA synthesis phase [37]. To determine the effect of OA on the induction of RNR activity, rats were subjected to two-thirds PH, at which time, in some animals, a 300-mg tablet of OA

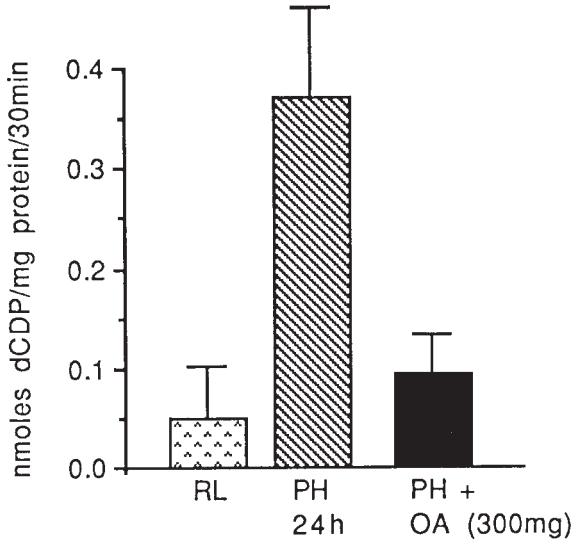

Figure 1. Effect of OA on hepatic RNR enzyme activity induced by two-thirds PH. Rats were subjected to two-thirds PH either in the presence of OA (a 300-mg tablet of OA implanted at the time of PH (PH+OA)) or in its absence (PH) and killed 24 h after PH. Liver removed at the time of PH served as resting liver (RL). Other details are given in the text. The values are the means ± SD from four rats, each assayed in triplicate.

was implanted i.p. All animals were killed 24 h later. This time point was chosen because maximum RNR enzyme activity has been reported in rat liver 24 h after two-thirds PH [23]. The results presented in Figure 1 indicated that compared with resting liver, there was an increase in the RNR enzyme activity 24 h after two-thirds PH. OA inhibited the induction of enzyme activity at 24 h after PH, suggesting that RNR may be a target for the mitoinhibitory effects of OA.

To determine whether the inhibition of enzyme activity reflected a decrease in the amount of protein, samples from the experiment described above were analyzed by western blotting with an antibody against the M1 subunit of RNR. The results presented in Figure 2 suggested that M1 (approximately 80 kDa) was present in low amounts in resting liver. However, there was an increase in protein levels 24 h after PH. Interestingly, OA inhibited this increase in the M1 subunit induced by two-thirds PH. Unfortunately, antibodies for the M2 subunit were not available. Because both subunits are required for enzyme activity, the results for one of the subunits should be adequate to assess total enzyme activity.

In the light of these results, it was of interest to determine whether the inhibition of RNR activity by OA at the protein level was also reflected at the level of mRNA transcripts for both the M1 and M2 subunits of *RNR* induced in response to two-thirds PH. Accordingly, animals were subjected to two-thirds PH with or without OA and were killed 24 and 48 h after PH. Poly(A+) RNA was isolated from the livers of each animal. Northern blot analysis with the probes for the M1 and M2 subunits showed that M1 and M2 transcripts were barely detectable in resting liver (Figure 3). However, there was an increase in the expression of both the M1 and M2 subunits 24 and 48 h after PH. Surprisingly, in the OA-treated livers this increase in the levels of mRNA transcripts for both the M1 and M2 subunits of *RNR* in response to two-thirds PH was not seen at 24 h. However, as the OA tablet completely dissolved and the effects of OA wore off, transcripts of both M1 and M2 subunits could be seen in the OA-treated livers at 48 h after PH (Figure 3). The increase in the levels of transcripts coincided with the decrease in the imbalance in nucleotide pool levels and an increase in DNA synthesis in these animals (data not shown). Further, coadministration of adenine, an agent that competes with OA for 5-phosphoribosyl-1-pyrophosphate and inhibits the OA-induced accumulation of uridine nucleotides, counteracted the inhibitory effects of OA at the level of *RNR* mRNA transcripts (data not shown). These findings support the conclusion that the inhibitory effect of OA on RNR is mediated through an imbalance in nucleotide pools created by OA.

To determine whether the inhibitory effect of OA was specific for *RNR* transcripts, the expression of other cell cycle–related genes and a housekeeping gene was examined by sequential hybridization of the blots in Figure 3 with radiolabeled probes for the c-*myc* and Ha-*ras* genes. In conformity with our ear-

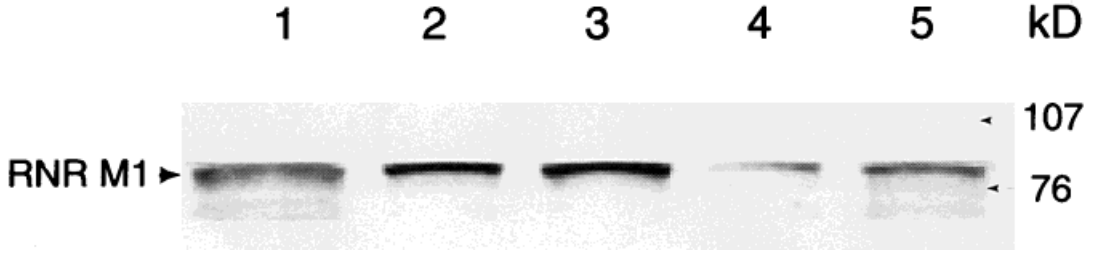

Figure 2. Effect of OA on hepatic RNR M1 protein levels induced by two-thirds PH. Cytosolic preparations obtained from the livers of rats killed in the experiment described in Figure 1 were used for western analysis. Each lane contains protein from two pooled liver samples. Lane 1, resting liver, 40 μg of protein; lanes 2 and 3, 20 and 40 μg of protein, respectively, from livers after two-thirds PH; lanes 4 and 5, 20 and 40 μg of protein, respectively, from livers after two-thirds PH plus OA. The arrowheads on the right indicate the positions of the molecular weight markers, whose sizes are given. Other details are given in Materials and Methods.

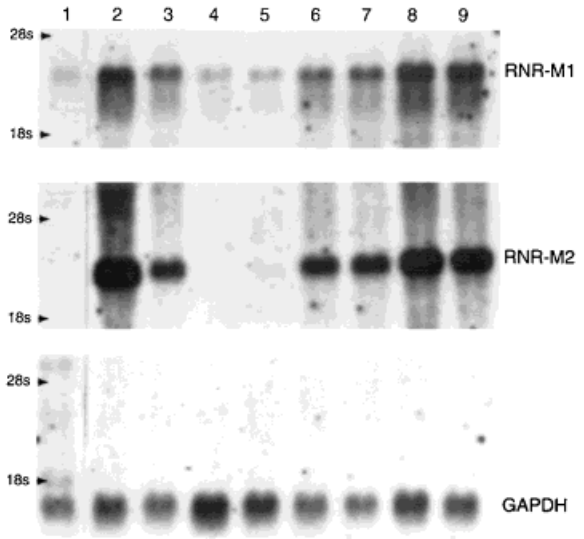

Figure 3. Effect of OA on the levels of mRNA transcripts for the M1 and M2 subunits of *RNR* induced by two-thirds PH. Rats were subjected to two-thirds PH in the presence or absence of OA, and liver removed at the time of PH served as a resting control. Rats were killed 24 and 48 h after PH. Ten micrograms of poly(A+) RNA was electrophoresed on formaldehyde-agarose gels, and northern hybridization was performed with ^{32}P-labeled cDNA probes for the *RNR* M1 and *RNR* M2 subunits and *GAPDH.* Each lane contains poly(A+) RNA from one rat. Lane 1, resting liver; lanes 2 and 3, liver 24 h after two-thirds PH in the absence of OA; lanes 4 and 5, liver 24 h after PH in the presence of OA; lanes 6 and 7, liver 48 h after PH in the absence of OA; lanes 8 and 9, liver 48 h after PH in the presence of OA. The arrowheads indicate the positions of the 28s and 18s RNA markers.

lier results from hepatocytes in primary culture [32], the in vivo expression of c-*myc* and Ha-*ras* was not inhibited by OA treatment (data not shown). These results suggested that the inhibitory effect of OA may not be a general one on cell cycle–related genes but may be more closely related to the expression of those genes whose products are required for DNA synthesis. To test this possibility, we investigated the expression of two other genes whose products are required for DNA synthesis, *DHFR* and *TS*. The results presented in Figure 4 show that while the induction of expression of these genes was not significantly affected at 8 h after PH, their levels at 16 and 24 h decreased more rapidly in the OA-treated livers. This may reflect increased degradation of their transcripts in the presence of OA.

That the steady-state levels of the mRNA for both the M1 and M2 subunits of *RNR* induced by two-thirds PH at 24 h were decreased in OA-treated livers raised two possibilities: that OA treatment exerted this effect at the transcriptional level or at the post-transcriptional level. To examine whether the effect was at the transcriptional level, nuclear run-off assays were performed with isolated purified nuclei from the livers of rats killed 24 h after two-thirds PH with or without a 300-mg tablet of OA implanted i.p. at the time of PH. The results of the nuclear run-off assays shown in Figure 5 indicated that compared with resting liver, two-thirds PH induced the transcription of both the M1 and M2 subunits of *RNR* and *Gal-Tase*, which was used as a control. Interestingly, OA treatment did not significantly inhibit the transcription of either the M1 or M2 subunits of *RNR* or of *Gal-Tase*. Densitometric analysis of the autoradiograms obtained from three different experiments further confirmed these conclusions. Taken together, these results suggest that OA could exert its effect at any point beyond initiation of *RNR* mRNA transcripts, including processing, transport of the mRNA to the cytoplasm, and its subsequent degradation.

The next series of experiments were therefore designed to determine the effect of OA on the stability of the mRNA for the two subunits of RNR. Initial experiments were performed with actinomycin D (Act D), a compound conventionally used to study the stability of mRNAs. However, Act D prevents the

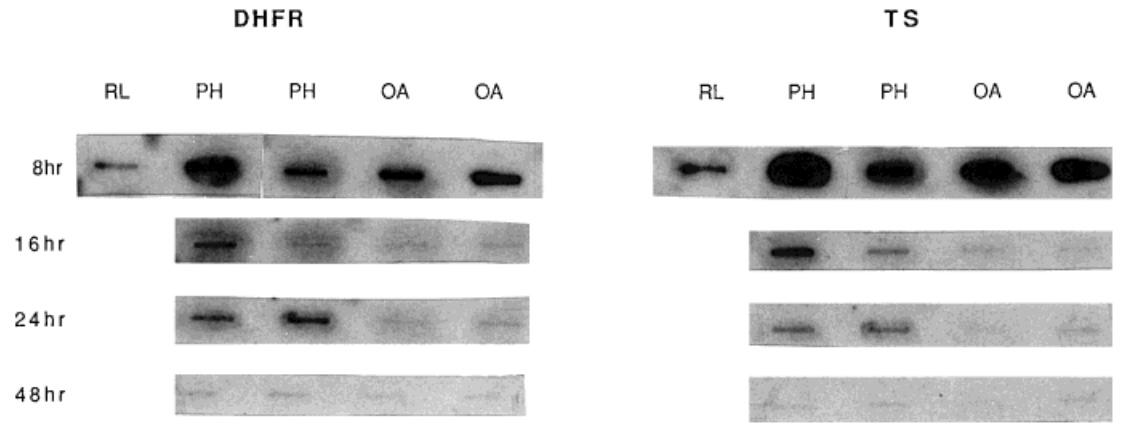

Figure 4. Effect of OA on the levels of mRNA transcripts for *DHFR* and *TS.* Slot blots were made with 10 μg of poly(A+) RNA from livers of rats killed 8, 16, 24, and 48 h after PH in the absence (PH) or presence (OA) of OA and sequentially hybridized with ^{32}P-labeled probes for *DHFR* and *TS.* RL, control liver removed at the time of PH. Each lane contains poly(A+) RNA from two pooled liver samples.

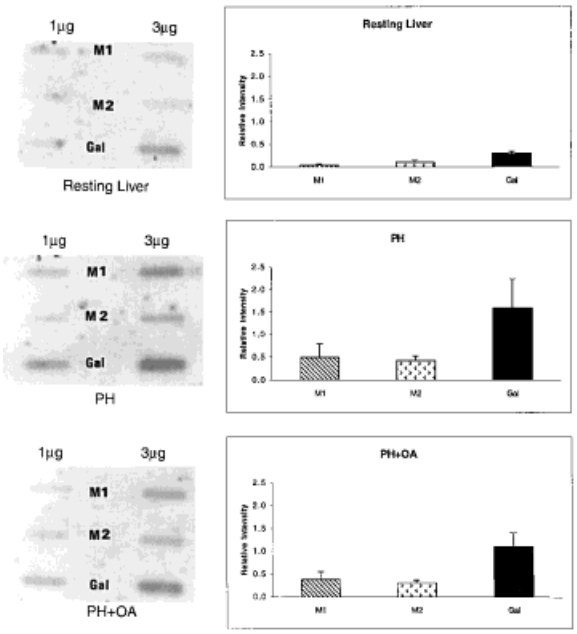

Figure 5. Nuclear run-off analysis of RNA from rat liver nuclei isolated from animals 24 h after two-thirds PH in the presence or absence of OA. The rats were subjected to two-thirds PH in the absence (PH) or presence (PH+OA) of a 300-mg tablet of OA implanted i.p. at the time of PH. Another group of rats did not receive any treatment and served as a resting control. All the rats were killed 24 h later, and nuclei were prepared from the freshly excised livers. Four rats per group were used, and livers from two rats were pooled for the preparation of nuclei. Details of the assay are given in Materials and Methods. Gal, Gal-Tase. The lefthand panels are autoradiograms. On the right, densitometric analysis of the 3-µg samples is presented. Relative intensity refers to the intensity of the band relative to the intensity of an equal area of background. The values are the means ± SD from three experiments with four rats per group.

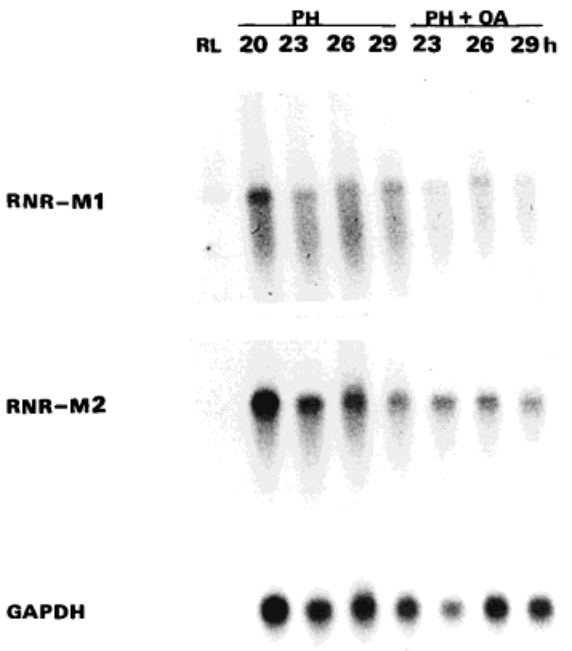

Figure 6. Effect of OA on the stability of the mRNA transcripts for the M1 and M2 subunits of *RNR* induced by two-thirds PH. Rats were subjected to two-thirds PH, and 20 h later, some received OA (a 300-mg tablet i.p.; PH+OA), whereas the others served as untreated controls (PH). Rats were then killed every 3 h up to 29 h after PH. Ten micrograms of poly(A+) RNA (from the pooled livers of three rats per time point) was electrophoresed on formaldehyde-agarose gels, and the blots were hybridized with ^{32}P-labeled cDNA probes for the *RNR* M1 and M2 subunits and *GAPDH.* Other details are given in the text. RL, resting liver.

accumulation of uridine nucleotides by OA [38]. Because the accumulation of uridine nucleotides is a prerequisite for the mitoinhibitory effect of OA, an alternate approach was developed that did not involve the use of Act D. The experimental protocol used consisted of first inducing the expression of RNR for up to 20 h by two-thirds PH. At this time, some animals received an intraperitoneal implant of a 300-mg tablet of OA. Rats were then killed every 3 h up to 29 h after PH to determine the effect of OA on the stability of the *RNR* mRNA levels that were induced at 20 h. The rationale was that if OA influenced the degradation of the *RNR* transcripts, then the mRNA levels induced by PH should decrease more rapidly in the OA-treated animals than in the controls. As shown in Figure 6, both the M1 and M2 subunits were induced in response to PH at 20 h. Thereafter, the levels declined slowly up to 29 h in both the controls and the OA-treated animals. Interestingly, in the OA-treated rats, the levels of both the M1 and M2 subunits of RNR declined faster. However, OA did not seem to have any significant effect on the levels of mRNA transcripts for *GAPDH.*

Because OA could influence the stability of preformed mRNA, it was of interest to determine whether OA would also inhibit DNA synthesis when implanted after *RNR* expression was induced. Accordingly, rats were subjected to two-thirds PH, and 16 h later, a 300-mg tablet of OA was implanted i.p. in some rats. Subsequently, rats were killed every 6 h up to 24 h after OA administration. The inhibitory effect of OA on the DNA content (Figure 7) was evident 12 h after OA treatment and increased further at 24 h. This inhibitory effect was coincident with the imbalance in nucleotide pools, as shown by a sixfold increase in the ratio of uridine to adenosine plus inosine by 6 h and an 11-fold increase at 24 h after OA tablet implantation (Figure 8). These data suggest that OA can exert its effect even after cells have started expressing *RNR*, perhaps by the degradation of the transcripts.

DISCUSSION

The success of DNA replication depends on the expression of several genes in a predetermined temporal sequence. In this complex sequence, the enzyme RNR plays a crucial role by providing a continuous

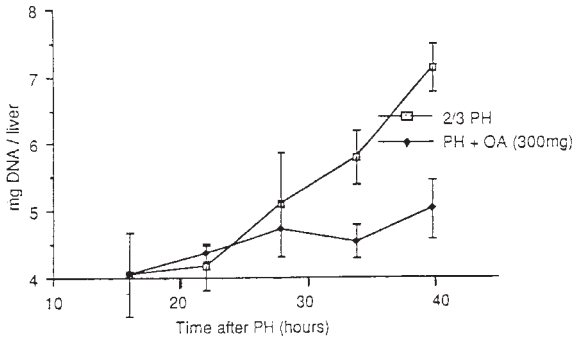

Figure 7. Effect on liver DNA content of a 300-mg OA tablet implanted 16 h after PH. Rats were subjected to two-thirds PH, and 16 h later, some received a 300-mg tablet of OA i.p. (PH+OA), whereas the rest served as untreated controls (PH). Rats (three per group) were killed every 6 h up to 24 h after OA administration. Their livers were quickly frozen in liquid nitrogen and processed for DNA content and nucleotide pool analysis (Figure 8). The values shown are the means ± SD from three rats per group.

and balanced supply of the deoxyribonucleotides required for DNA synthesis, and it is not surprising that RNR is regulated at multiple sites. As RNR activity is regulated by nucleotide pools and OA exerts its effects through an imbalance in nucleotide pools, it seemed reasonable to hypothesize that RNR may be one of the targets for the mitoinhibitory effects of OA. The in vivo studies undertaken to examine the validity of this hypothesis indicated that OA inhibited RNR enzyme activity measured 24 h after two-thirds PH (Figure 1), suggesting that RNR may indeed be a target for mitoinhibition by OA. These results are in agreement with earlier ones that had suggested that the reduction in CDP and UDP can be inhibited by dUTP, dGTP, and dTTP [14,15] and that dUTP levels increase after OA treatment [13]. Because deoxynucleotides have been shown to inhibit RNR enzyme activity in vitro [17], and OA treatment does induce an imbalance in nucleotide pools, it may be argued that this imbalance may be responsible for the observed inhibition of the in vitro enzyme activity. However, because the protein fraction used in the in vitro assay for RNR was partially purified and free of in vivo–generated nucleotide pools, and because the inhibition of RNR enzyme activity by OA was also paralleled by a decrease in the protein levels (Figure 2, western blot) and mRNA transcript levels for both the M1 and M2 subunits of *RNR* (Figure 3, northern analysis), it may be concluded that the decreased enzyme activity is indeed a true reflection of the decreased levels of transcripts and protein.

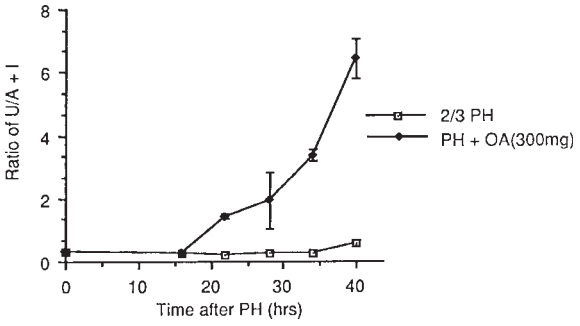

Figure 8. Effect of a 300-mg OA tablet implanted 16 h after two-thirds PH on hepatic uridine to adenosine plus inosine nucleotide ratio. Livers from the experiment described in Figure 7 were used for nucleotide pool analysis. The details are given in the legend to Figure 7 and in the text.

The observation that the creation of an imbalance in nucleotide pools by OA can influence the levels of *RNR* mRNA transcripts raises interesting possibilities. Nuclear run-off assays indicated that the transcripts were initiated normally in vivo in OA-treated liver. Therefore, the effect of OA could have been at the level of processing, transport of mRNA to the cytoplasm, or subsequent degradation. Experiments designed to determine the stability of mRNA in OA-treated rat livers indicated that OA treatment seemed to decrease the stability of mRNA transcripts of both subunits of RNR. The degradation of RNR transcripts may not reflect general degradation of mRNAs, because OA treatment did not appear to influence the stability of mRNA transcripts of *GAPDH* (a housekeeping gene) or *Gal-Tase* (an induced gene). Interestingly, while the expression of some cell cycle–related genes like c-*myc,* c-*fos*, and Ha-*ras* is not significantly affected by OA in vitro [32] or in vivo (this study, data not shown), the stability of the transcripts for *DHFR* and *TS* appeared to be decreased by OA treatment (Figure 4).

Therefore, the question that arises from these studies is how nucleotide pools affect the stability or degradation of *RNR* mRNA transcripts. The regulation of mRNA stability has emerged as an important control mechanism for gene expression. Several different mechanisms with unique features for each gene have been reported [39–42]. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate has been shown to increase the expression of RNR by increasing the half-life of *RNR* mRNA transcripts. This is achieved by downregulation of the cis-trans interaction between specific cis elements in the 3′ untranslated region of the mRNAs for the M1 and M2 subunits of *RNR* and protein factors in the cytoplasm [43,44]. It is important to determine whether similar or other mechanisms are involved in the decreased stability of the *RNR* mRNAs in the presence of OA-induced nucleotides pool imbalance.

It has been hypothesized that OA promotes liver carcinogenesis by the differential mitoinhibitory mode. If RNR is one of the targets by which OA inhibits DNA synthesis in normal hepatocytes, then it may be speculated that RNR in initiated hepatocytes (and, by extrapolation, in hepatic foci or nodules) could be relatively resistant to the effects of OA. This relative resistance can be achieved by not accumulating OA-induced uridine nucleotides and/or by gen-

erating a modified RNR that is relatively less sensitive to fluctuations in nucleotides pools. Mutant forms of the M1 subunit of RNR that have alterations in the allosteric sites that disrupt the binding of nucleotides effectors have been reported [45]. One of these mutant forms is in fact resistant to feedback inhibition by dATP and exhibits a mutator phenotype in cells [46]. Differences in the sensitivity of RNR from different sources to nucleotides pool changes has been documented [47,48]. For example, RNR from herpes virus is relatively insensitive to fluctuations in nucleotides pools [49,50]. In hydoxyurea-resistant cells, the stability of *RNR* messages is increased by altering the cis-trans interactions between the 3′ untranslated sequences in the mRNAs and their binding proteins [51]. Furthermore, overexpression of the M2 subunit of RNR has been reported to be a malignancy determinant that cooperates with activated oncogenes in enhancing transformation and malignancy potential [52]. The inability of nodule hepatocytes to accumulate uridine nucleotides, which leads to nucleotide imbalance [11] and the hepatocytes' resistance to the mitoinhibitory effects of OA [53], suggests that RNR may not be a target for OA's effects on the nodules. In this context, it is interesting that hepatic nodules promoted by the resistant-hepatocyte model also do not accumulate uridine nucleotides when exposed to OA and are resistant to mitoinhibition by OA [11,54]. Further, 2-acetylaminofluorene, the mitoinhibitor used in the resistant-hepatocyte model of liver tumor promotion, also inhibits RNR [55]. The observation that two liver tumor promoters inhibit DNA synthesis by targeting RNR makes us speculate that the RNR in the nodule hepatocytes and the one present in the normal hepatocytes may behave differently toward liver tumor promoters that promote by the differential mitoinhibitory mode. It will be interesting to determine how the stability of *RNR* mRNA transcripts is regulated in normal and nodule hepatocytes. Such information will be helpful in understanding the mechanism of liver tumor promotion, especially by agents that promote by a differential mitoinhibitory mode and inhibit DNA synthesis by targeting RNR in normal hepatocytes.

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