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Inhibition by interferon α-2b of rat liver regeneration: effect on ornithine decarboxylase and total protein synthesis☆

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Abstract

Polyamines are key factors in macromolecule synthesis during liver regeneration. It has been postulated that interferon-α (IFNα) decreases putrescine levels in regenerating liver by inhibiting ornithine decarboxylase (ODC) activity, the main enzyme in polyamine biosynthesis. In the present study, we analysed the effects of a pharmacological dose of IFNα on polyamine and ODC levels during the regenerative process following partial hepatectomy in rats. Synthesis of ODC by isolated hepatocytes from IFN-treated rats with regenerating livers was also assessed. Furthermore, we investigated the effect of IFNα-2b on DNA and total protein synthesis in 24-hr regenerating livers. No effect on DNA synthesis was observed at the dose of IFNα-2b used, but total protein synthesis decreased significantly in IFNα-2b-treated rats undergoing liver regeneration (7.0 ± 2.0 and $12.1 \pm 1.7\% \cdot min^{-1}$ in hepatectomized rats treated with IFNα-2b and saline, respectively). ODC levels were also reduced significantly (by 50%) in hepatectomized rats treated with IFNα-2b versus saline. In parallel with the ODC decrease, the concentrations of putrescine and spermidine (63 ± 25 vs 101 ± 15 nmol/g liver and 1.08 ± 0.35 vs 2.14 ± 0.22 μmol/g liver, respectively, in IFNα-2b- and saline-treated hepatectomized rats) showed similar, significant diminutions. Moreover, the incorporation of [^{35}S]methionine into ODC was decreased dramatically in isolated hepatocytes from IFNα-2b-treated hepatectomized rats 12 hr after surgery. In conclusion, the protein synthesis rate in regenerating liver was impaired by therapeutic doses of IFNα-2b. In addition, the results presented in this study suggest that IFNα-2b negatively regulates ODC synthesis, causing a reduction in polyamine levels during liver regeneration.

Keywords: IFNα-2b; Partial hepatectomy; ODC regulation; Polyamines; Putrescine; Spermidine; DNA synthesis

1. Introduction

Non-parenchymal cytokines, such as IL-1, IL-6, and TNFα, normally regulate many liver metabolic functions [1]. The participation of cytokines during liver regeneration is also well described. In this connection, the role of TNFα and IL-6 in the initiation, and the inhibitory effect of TGFβ and activin on the shutdown, of hepatic regeneration have been investigated intensively [2,3].

IFNα, classified as a type I interferon, represents a family of more than 20 different proteins that are produced mainly by leukocytes and exhibit diverse antiviral, immunomodulatory, and antiproliferative effects [4]. Many of its actions are exerted by the interaction of the IFNα receptor–kinase complex with STAT transcription factors, which, once activated, form multimers that bind to the regulatory elements of cytokine-inducible genes [5, 6]. In regenerating liver, there is a puzzling overlap of the downstream pathways induced by different proinflammatory cytokines and hepatocyte mitogens [3]. Type I IFNs decrease the level of putrescine in regenerating murine liver. In fact, Nishiguchi *et al.* [7] have demonstrated that IFN$\alpha+\beta$ inhibit ODC, the enzyme that catalyses putrescine production during liver regeneration. Later studies with different subclasses of IFNα also showed significant reductions of ODC activity after partial hepatectomy [8, 9]. Partial hepatectomy in rodents constitutes an appropriate model for studying liver regeneration. In this model, hepatocytes rapidly enter into

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Abbreviations: IL, interleukin; TNFα, tumor necrosis factor-α; TGFβ, transforming growth factor-β; IFNα, interferon-α; and ODC, ornithine decarboxylase.

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the G1 phase of the cell cycle and, after the induction of a specific pattern of genes, progress to the S phase [10]. During liver regeneration, the *odc* gene is activated early [11]. ODC protein expression is regulated at different levels [12–14], and the final rate of ODC synthesis results from the balance among *odc* transcriptional activity, mRNA stability, and translation efficiency. The effect of IFNα-2b on ODC protein expression *in vivo* has never been studied, even though understanding this effect is of great help in explaining the changes in ODC activity and putrescine levels during regeneration.

Putrescine is essential for DNA and protein synthesis during hepatic regeneration [15, 16]. It has been postulated that high doses of IFNα reduce DNA synthesis after partial hepatectomy in rats by decreasing the concentration of putrescine [8, 9]. In this respect, Wong *et al.* [8] demonstrated that this diminution depends on the IFNα subtype used. In fact, IFNα-2b, commonly employed in HCV patients [17], when administered as a single, therapeutic dose prior to partial hepatectomy, seemed not to modify the rate of DNA synthesis in regenerating livers of rats [8]. However, no studies examining its putative effect on protein synthesis were carried out.

In the current study, we analyzed total protein synthesis, 24 hr following partial hepatectomy, in the livers of rats treated with a pharmacologic dose of recombinant IFNα-2b. We also investigated whether the described reductions of ODC activity and polyamine levels were due to a diminution in ODC expression. Since ODC is mainly down-regulated by its rapid turnover [14], we evaluated labelled amino acid incorporation into ODC in isolated hepatocytes from hepatectomized animals treated with IFNα-2b.

2. Materials and methods

2.1. Chemicals

Recombinant IFNα-2b (BIOFERON) was a gift from BioSidus. [^{3}H]Thymidine, [^{3}H]phenylalanine, L-tyrosine decarboxylase, Protein A-Sepharose, mouse IgG anti-rat ODC, putrescine, spermidine, and spermine were from the Sigma Chemical Co. [^{35}S]Methionine was from NEN Life Science Products. All solvents and reagents for HPLC assays were from Merck. Reagents for SDS–PAGE and western blotting were from Sigma. The Enhanced Chemiluminescence (ECL) western blotting kit was from Amersham Pharmacia Biotech Inc. Kodak AR X-Omat radiographic film was purchased from Sigma. Horseradish peroxidase-conjugated anti-mouse antibody was obtained from Amersham.

2.2. Animals and treatments

Adult, male Wistar rats (330–380 g) were used throughout our studies. Rats were housed two per cage and maintained in a room at constant temperature with a 12-hr light–dark cycle. The animals had free access to pelleted rat food and water. All the experimental protocols were according to the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, Publication No. 86–23, revised 1985). For the surgical procedures, rats were anesthetized with a single dose of sodium pentobarbital (50 mg/kg body wt, i.p.). Surgeries were carried out between 9:00 and 11:00 a.m. to minimize the influence of circadian variations.

Rats were randomized into two groups: sham-operated (S, i.e. laparotomy and gentle manipulation of the liver) and partially hepatectomized (PH, i.e. removal of median and left lateral lobes) [18]. Half of each group was treated with saline or with IFNα-2b (6.5×10^5 U/kg body wt, i.p.) administered twice, 16 hr before and at the moment of surgery. The dose used was comparable to that used for therapeutic purposes [8, 17].

Rats were killed and bled by cardiac puncture 24 or 12 hr after the sham operation or hepatectomy. Livers were removed and washed *ex situ* with ice-cold saline.

To evaluate the possible effect of IFNα-2b on food intake, body weight was controlled throughout the treatments, and no significant differences were found between the hepatectomized groups treated with either saline or IFNα-2b.

2.3. DNA synthesis

Liver samples (1 g) were used for the measurement of [^{3}H]thymidine incorporation into DNA 24 hr after surgery. The radioactive compound (10 μCi/200 g body wt, i.p) was administered 1 hr before killing the rats. Liver tissues were processed as described previously [19], and radioactivity in the acid-insoluble fraction was measured in a liquid scintillation counter (1214 Rack Beta, Pharmacia).

2.4. Protein synthesis

In vivo protein synthesis was measured as described by Garlick *et al.* [20] with slight modifications. Briefly, [^{3}H]phenylalanine was administered as a single injection diluted with an excess of the unlabelled amino acid (50 μCi/100 g body wt, 150 μmol/100 g body wt) into the femoral vein, 10 min before killing the rats. Aliquots of liver tissue were homogenized with cold 2% $HClO_4$ and centrifuged at 3000 *g* for 15 min at 4°. The supernatant was used for the measurement of free [^{3}H]phenylalanine. The pellet was washed and solubilized for 1 hr at 37° with 0.3 M NaOH. This solution was reprecipitated with 70% $HClO_4$ and centrifuged at 3000 *g* for 15 min at 4°. The precipitate was washed with 2% $HClO_4$, resuspended in 6 M HCl, and hydrolyzed for 24 hr at 110°. The hydrolysate was dissolved in 0.3 M citric acid and used for assessing protein-linked [^{3}H]phenylalanine. To discount the labelled tyrosine formed from [^{3}H]phenylalanine, phenylalanine was converted into β-phenethylamine by enzymatic reaction with l-tyrosine

decarboxylase (0.7 U, 0.5 mg pyridoxal phosphate/mL) for 6 hr at 50°. β-Phenethylamine was extracted by adding 8 mL heptane and 2 mL of 0.01 M H_2SO_4, the organic layer was removed, and radioactivity was detected in the aqueous phase by liquid scintillation counting. The rate of protein synthesis was calculated as follows:

$$K_S = \frac{S_B \times 100}{S_A \times t}$$

where S_A is dpm in the supernatant · g liver^{-1}, i.e. free radioactivity; S_B is dpm in the hydrolysate · g liver^{-1}, i.e. protein-linked radioactivity; and t is 10 min.

2.5. Polyamine levels

Samples from each liver were obtained and processed as described [21]. Dansyl derivatives of putrescine, spermidine, and spermine were detected by HPLC equipped with a spectrofluorometer detector (Waters 600 Pump, Waters 474 Scanning Fluorescence Detector). Aqueous solutions of polyamines were processed and used as standards [21].

2.6. ODC protein

Liver lysates were used for detecting total ODC protein. One gram of liver tissue was homogenized in 3 mL of lysating RIPA buffer containing PBS, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors. After 30 min of incubation at 0° and three freeze–thaw cycles, protein concentration was determined [22], and equal amounts (150 μg) were immunoprecipitated by incubation with anti-ODC for 1.5 hr at room temperature with gentle agitation. Another incubation for 1 hr at 0° was carried out after the addition of 4 mg/mL of Protein A-Sepharose. The precipitated material was washed, resuspended in sample buffer for western blotting, and boiled for 3 min. The resulting samples were loaded, and proteins were separated by SDS–PAGE (10% acrylamide) [23]. Proteins were then transferred onto nitrocellulose membranes [24]. Membranes were incubated for 2 hr with the appropriate dilution (1:200) of monoclonal antibody against rat ODC. A horseradish peroxidase-conjugated anti-mouse antibody was added, and ODC was revealed by enhanced chemiluminescence (Amersham) following the recommendations of the manufacturer. Membranes were exposed to radiographic films, and the bands were assessed by densitometry (Shimadzu CS 9000).

2.7. ODC synthesis

Twelve and twenty-four hours after surgery, isolated hepatocytes from all livers were obtained by enzymatic digestion [25]. Cell viability, assessed by trypan blue exclusion, was greater than 85% in all samples. Hepatocytes were resuspended in plastic vials with Dulbecco's modified Eagle's methionine-free medium and preincubated at 37° for 30 min under a carbogen stream. Labelled methionine (100 μCi) was added to 2×10^7 cells and incubated for 30 min at 37°. The following procedures were done as described previously [26] with slight modifications. Briefly, synthesis was stopped by adding cold 5 mM methionine and collecting the cells by centrifugation (500 *g*, 4°, 5 min). Cells were washed three times with PBS and resuspended in 1 mL of ice-cold RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin and 1 μg/mL of aprotinin. After three freeze–thaw cycles, hepatocytes were centrifuged (30,000 *g*, 4°, 20 min), the pellet was discarded, and the supernatant was used for protein determination [27]. Equal amounts of protein (3 mg) were incubated with anti-ODC (20 μL stock antibody/mL lysate) at room temperature for 1 hr 30 min after which 4 mg of Protein A-Sepharose was added and the mixture was reincubated for 1 hr at 4° with gentle shaking. The immunoprecipitate was washed exhaustively, dissolved in 40 μL of SDS–PAGE sample buffer, and boiled for 3 min. Equal volumes of the samples were loaded and, after SDS–PAGE (10% acrylamide) [23], gels were fixed and impregnated with 1 M sodium salicylate, 1% glycerol solution for 30 min, dried, and exposed to radiographic film at −70°.

2.8. Statistical analysis

Values are expressed as means ± SEM. The non-parametric Mann-Whitney test was performed to evaluate all the data. The level of significance was set at a limit of $P < 0.05$.

3. Results

3.1. [^{3}h]thymidine incorporation into DNA

DNA synthesis was measured 24 hr after surgery in all the experimental groups (N = 5) (Fig. 1). DNA synthesis rates were significantly higher in regenerating rat livers, as compared with the sham groups. IFNα-2b treatment did not affect the magnitude of this augmentation.

3.2. Decrease of protein synthesis

Figure 2 summarizes the fractional rate of protein synthesis 24 hr after surgery in sham-operated and partial-hepatectomized rats treated with saline or IFNα-2b (N = 5). Protein synthesis increased significantly in the partially hepatectomized group, as compared with the sham-operated groups (12.1 ± 1.7 and 6.2 ± 1.3%· min^{-1}, respectively), and IFNα-2b prevented this augmentation (7.0 ± 2.0% · min^{-1}).

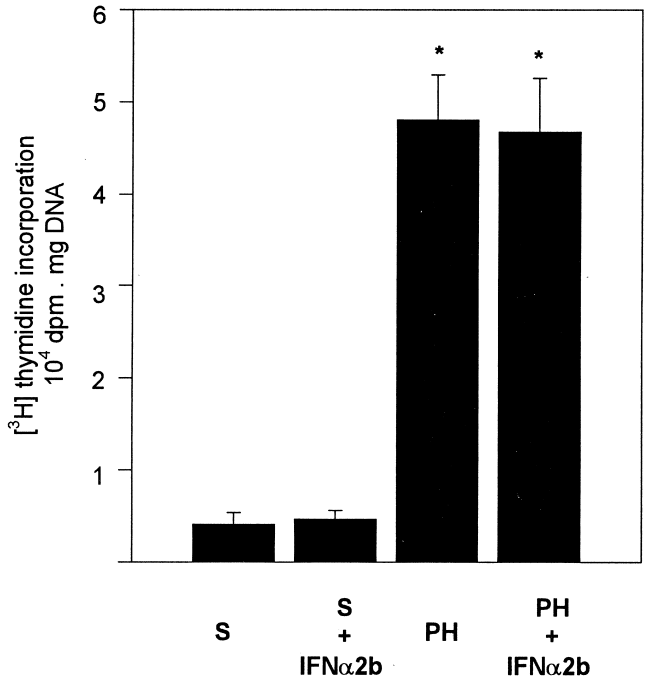

Fig. 1. DNA synthesis. Results represent means ± SEM of five animals. Experimental conditions: S, saline-treated sham rats; S+IFNα-2b, IFNα-2b-treated sham rats; PH, saline-treated partial-hepatectomized rats; and PH+IFNα-2b, IFNα-2b-treated partial-hepatectomized rats. Key: (*) significant difference vs S ($P < 0.01$).

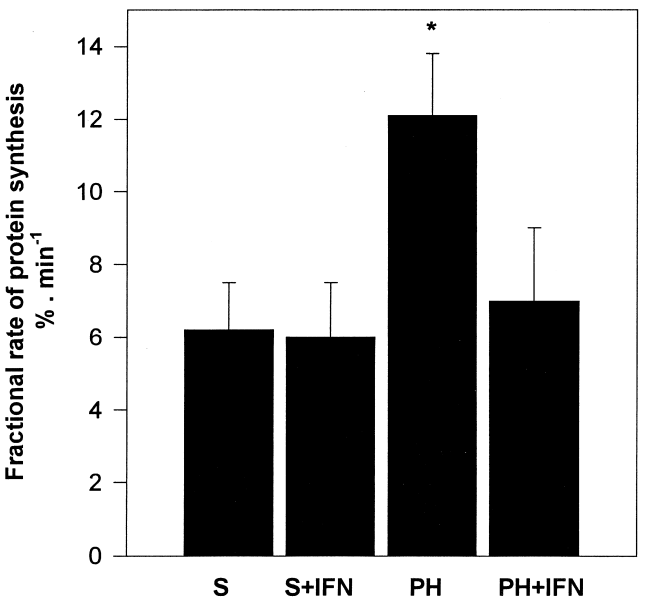

Fig. 2. Fractional rate of total protein synthesis. The incorporation of [^{3}H]phenylalanine into protein was assessed, and the values were calculated as described in "Materials and methods." Results represent means ± SEM of five animals. Experimental conditions: S, saline-treated sham rats; S+IFN, IFNα-2b-treated sham rats; PH, saline-treated partial-hepatectomized rats; and PH+IFN, IFNα-2b-treated partial-hepatectomized rats. Key: (*) significant difference vs S ($P < 0.01$).

3.3. Polyamine concentrations and total ODC protein in hepatic lysates

Figure 3 illustrates putrescine, spermidine, and spermine levels at 24 hr in all the groups (N = 5). As can be seen, putrescine content was reduced significantly in hepatectomized rats treated with IFNα-2b (63 ± 25 vs 101 ± 15 nmol/g liver in IFNα-2b- and saline-treated PH rats, respectively). Spermidine concentration was decreased in a similar way by IFNα-2b treatment (1.08 ± 0.35 vs 2.14 ± 0.22 μmol/g liver in IFNα-2b- and saline-treated PH rats, respectively); the spermine level showed no significant modification (0.26 ± 0.05 vs 0.32 ± 0.03 μmol/g liver in IFNα-2b- and saline-treated PH rats, respectively).

ODC protein was measured by immunoprecipitation and western blotting (Fig. 4). The level of ODC protein increased during liver regeneration (saline-hepatectomized) but significantly decreased in 24 hr in IFNα-2b-treated hepatectomized rats (densities from 5 animals, 401 ± 17 and 192 ± 24 arbitrary units in saline-PH and IFNα-2b-PH, respectively).

3.4. Diminution of ODC synthesis

De novo ODC synthesis by isolated hepatocytes was reduced significantly in the IFNα-2b-treated hepatectomized group 12 hr after surgery, the mean percentage of this diminution being 79% (Fig. 5). Twenty-four hours after the operation, ODC synthesis was undetectable.

4. Discussion

In the present study, we analyzed the effect of IFNα-2b on total protein synthesis during liver regeneration. We also studied its effect on ODC expression. The most important findings can be summarized as follows: Therapeutic doses of IFNα-2b produced a significant diminution in the rate of protein synthesis during liver regeneration and a marked reduction of ODC synthesis and polyamine levels.

IFNα inhibition of DNA synthesis after partial hepatectomy in rodents [7–9] or in cultured hepatoma cells [28] has been demonstrated. In these studies, the decrease in the rate of DNA synthesis was associated with a reduction in ODC activity and the resultant decrease in putrescine levels.

DNA and protein syntheses peak around 24 hr after partial hepatectomy [10]. At the doses used, IFNα-2b did not modify DNA synthesis but surprisingly decreased total protein synthesis 24-hr post-hepatectomy (see Fig. 2). Although these results do not allow us to discount a direct effect of IFNα on protein elongation [27], the diminution observed could be attributed to a decrease in putrescine and/or spermidine in hepatectomized animals treated with IFNα-2b. In fact, we showed that IFNα-2b decreased putrescine and spermidine concentrations in regenerating liver by 40–50%. In this connection, a correlation between putrescine levels and the rate of protein synthesis has been reported [29]. Furthermore, changes in putrescine concen-

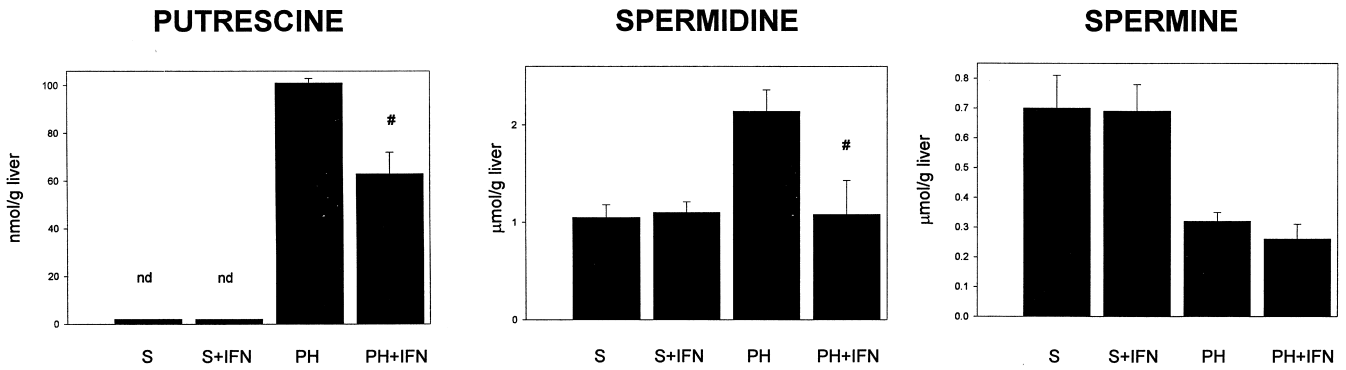

Fig. 3. Polyamine concentrations at 24 hr. Results represent means ±SEM of five animals. Experimental conditions: S, saline-treated sham rats; S+IFN, IFNα-2b-treated sham rats; PH, saline-treated partial-hepatectomized rats; and PH+IFN, IFNα-2b-treated partial-hepatectomized rats. ND, non-detectable. Key: (#) significant difference vs PH ($P < 0.01$).

tration perturb protein synthesis in cultured cells previous to any change in DNA synthesis [30].

Previous reports explain the inhibitory effect of IFNα on putrescine levels, both *in vivo* and in cultured cells [7–9, 28, 31, 32], by the decrease of ODC activity. Wong *et al.* [8] showed no changes in ODC and putrescine levels when IFNα-2b was administered at a pharmacological dose. In this regard, it should be noted that in the same experiments the authors reported that another subtype of IFNα, IFNα-2a, did decrease ODC activity and putrescine concentration. In the present work, IFNα-2b was used at a similar, pharmacological dose, but administered twice, before and at the moment of surgery, instead of once prior to partial hepatectomy, as used by the other investigators [8]. Considering previous and present observations, it can be postulated that IFNα at therapeutic doses is able to reduce ODC and putrescine levels during regeneration. Many of the discordant results can be attributed to differences in the bioavailability of IFNα at the initial regenerative phases following partial hepatectomy.

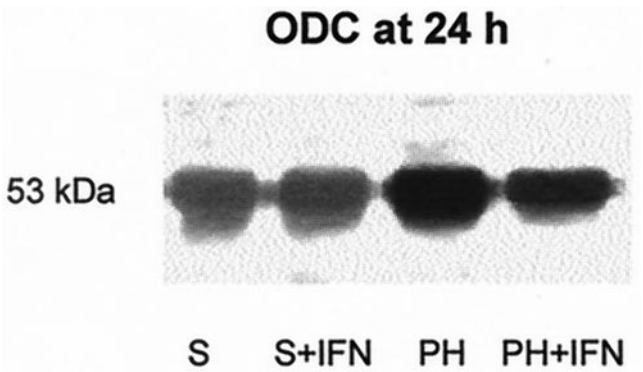

Fig. 4. ODC protein in hepatic lysates at 24 hr. The figure shows a typical ECL-western blot. Samples were prepared as described in "Materials and methods," immunoprecipitated, and immunodetected. Gels were loaded with the immunoprecipitate from approximately 150 μg of protein in the lysate. Experimental conditions: S, saline-treated sham rats; S+IFN, IFNα-2b-treated sham rats; PH, saline-treated partial-hepatectomized rats; and PH+IFN, IFNα-2b-treated partial-hepatectomized rats.

ODC protein status in hepatectomized rats treated with IFNα-2b and the rate of ODC synthesis in isolated hepatocytes were assessed in this study. We showed a dramatic decrease of ODC synthesis at 12 hr in hepatocytes from hepatectomized rats treated with IFNα-2b (see Fig. 5). In a previous study, Nishiguchi *et al.* [7] suggested that inhibition of ODC activity by a combination of type I IFNs, IFN$\alpha+\beta$, was cyclic AMP-mediated. However, the putative regulation of ODC synthesis by IFNα is not understood. Our results strongly support the hypothesis

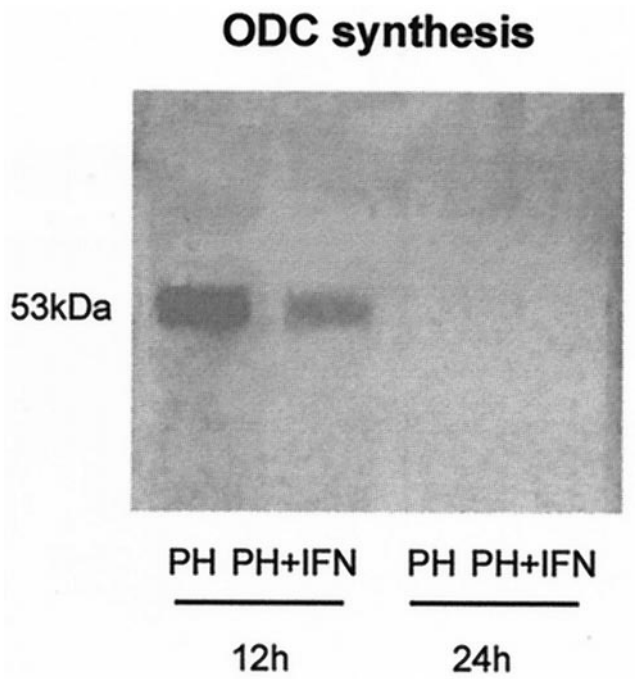

Fig. 5. ODC synthesis at 12 or 24 hr after surgery. Hepatocytes from three rats from each experimental group were pooled, and 2 x 10^7 cells were incubated with [^{35}S]methionine and lysed as described in "Materials and methods." Equal amounts of total protein (3 mg) were subjected to immunoprecipitation with anti-ODC and Protein A-Sepharose. Each lane was loaded with the resultant immunoprecipitate. Experimental conditions: PH, hepatocytes from saline-treated partial-hepatectomized rats; PH+IFN, hepatocytes from IFNα-2b-treated partial-hepatectomized rats.

that the reduction of ODC activity by IFNα-2b is due to a diminished synthesis. Further studies are necessary to explain the decrease of ODC synthesis, mainly at post-transcriptional and post-translational levels, since ODC mRNA levels in cultured 3T3 fibroblasts [31], hepatoma cells [28], or Daudi cells [32] were found alternatively unaltered, increased, or decreased by IFNα, even when ODC activity and putrescine levels were reduced. ODC protein is rapidly turned over by a process involving its binding to ODC antizyme and its degradation by the 26S proteasome [33–35]. It is therefore of interest to study the possible effect of IFNα not only on ODC antizyme regulation but also on polyamine uptake. Also, of note, it was recently proven that IFNγ enhances ODC degradation in HeLa and SW620 cells by inducing its proteasomic proteolysis [36]. On the other hand, the observed changes in spermidine levels could reflect not only the diminution of its precursor, putrescine, but also a putative regulation by IFNα-2b of spermidine/spermine *N*-acetyltransferase (SAT) in the regenerating liver.

Wong *et al.* [8] reported that, of the three IFNα subtypes tested, only one, IFNα-2a, inhibited DNA synthesis when administered at clinical doses before partial hepatectomy in rats. However, at higher doses, all three IFNα subtypes namely, 2a, 2b, and n1, decreased DNA synthesis at 24 hr. In our hands, and according to these results, pharmacological doses of IFNα-2b did not perturb either the first major peak of DNA synthesis at 24 hr, or the second minor peak at 48 hr (data not shown), even though they reduced polyamine concentration. DNA synthesis is a relatively late consequence of the growth response, beginning once multiple pathways have been activated during the immediate-early response [37]. Although an early role for IFNα-2b during the onset of DNA replication (around 10 hr after hepatectomy) cannot be ruled out, the results described point out that therapeutic doses of IFNα-2b are not high enough to decrease either the rate of DNA synthesis at 24 hr or PCNA levels (data not shown), two parameters which indicate that the hyperplasic component of liver regeneration is preserved [29]. Nevertheless, the decreases in hepatic putrescine and spermidine levels found at physiologic doses of IFNα-2b suggest the existence of other possible disturbances in growth response during regeneration. In fact, it has been shown that polyamine levels can mediate different growth-regulation signals involving cellular targets other than nucleic acids [38–43].

In conclusion, we have described two effects produced by pharmacological doses of IFNα-2b on liver regeneration, namely, an important diminution of ODC protein expression with a parallel decrease in polyamine levels, and, also, a reduction in total protein synthesis.

IFNα-2b is widely used in patients undergoing potential liver regeneration, as in the case of transplanted hepatitis C virus (HCV) [44, 45] or compensated cirrhotic HCV patients [46], hepatic repair being crucial for a favorable prognosis. Results presented herein point out a basic mechanism by which pharmacological doses of IFNα-2b could impair the regenerative process.

Acknowledgments

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