

# Induction of pancreatic acinar cell proliferation by thyroid hormone

G M Ledda-Columbano, A Perra, M Pibiri, F Molotzu  
and A Columbano

Department of Toxicology, Oncology and Molecular Pathology Unit, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy  
(Requests for offprints should be addressed to G M Ledda-Columbano; Email: gmledda@unica.it)

## Abstract

Thyroid hormone is known to elicit diverse cellular and metabolic effects in various organs, including mitogenesis in the rat liver. In the present study, experiments were carried out to determine whether thyroid hormone is able to stimulate cell proliferation in another quiescent organ such as the pancreas. 3,5,3'-L-tri-iodothyronine (T3) added to the diet at a concentration of 4 mg/kg caused a striking increase in nuclear bromodeoxyuridine (BrdU) incorporation of rat acinar cells 7 days after treatment (the labeling index was 46.7% in T3-treated rats vs 7.1% in controls). BrdU incorporation was limited to the acinar cells, with duct cells and islet cells being essentially negative. The increase in DNA synthesis was accompanied by the presence of several mitotic figures. Histological examination of the pancreas did not exhibit any sign of T3-induced toxicity. Determination of the apoptotic index, measurement of the serum levels of  $\alpha$ -amylase and lipase, and glycemia determination did not show any increase over control values, suggesting that the enhanced proliferation of acinar cells was a direct effect induced by

T3 and not a regenerative response consequent to acinar or  $\beta$ -cell injury. Additional experiments showed that DNA synthesis was induced as early as 2 days after T3 treatment (the labeling index was 9.4 vs 1.9% in controls) and was associated with increased protein levels of cyclin D1, cyclin A and proliferating cell nuclear antigen, with no substantial differences in the expression of the cyclin-dependent kinase inhibitor p27. The mitogenic effect of T3 on the pancreas was not limited to the rat, since extensive acinar cell proliferation was also observed in the pancreas of mice treated with T3 for 1 week (the labeling index was 28% in T3-treated mice vs 1.8% in controls). Treatment with three other ligands of nuclear receptors, ciprofibrate, all-*trans* retinoic acid and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, induced little or no pancreatic cell proliferation. These results demonstrated that T3 is a powerful inducer of cell proliferation in the pancreas and suggested that pancreatic acinar cell proliferation by selected agents may have potential for therapeutic use.

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## Introduction

The thyroid hormones influence a variety of physiological processes, including cell growth and metabolism in mammals, metamorphosis in amphibians and development of the vertebrate nervous system (Samuels *et al.* 1988). Most of these actions are mediated by thyroid hormone (L-tri-iodothyronine; T3) nuclear receptors (TRs) that are encoded by two genes ( $\alpha$  and  $\beta$ ) and are expressed as several isoforms (Cheng 1995, Lazar 1993, Hoppenheimer *et al.* 1996, Yen 2001). The TRs are ligand-regulated transcription factors that are members of the steroid hormone/retinoic acid receptor family of nuclear receptors, which includes the retinoic acid receptors (RARs), the retinoid X receptors, the vitamin D receptor, the peroxisome proliferator-activated receptors (PPARs) and some orphan receptors (Mangelsdorf *et al.* 1984). In the past few years it has become evident that several ligands of the steroid/thyroid hormone receptor superfamily possess mitogenic activity for rodent liver (Columbano *et al.* 1997,

Pibiri *et al.* 2001), and that lack of receptors results in a blunted proliferative response following treatment with their respective ligands (Lee *et al.* 1995, Wei *et al.* 2000). We have recently shown that the hepatocyte response to T3 is associated with a rapid induction of cyclin D1 and an accelerated entry into the S phase (Pibiri *et al.* 2001). Based on the finding that an early induction of cyclin D1 was found following treatment with other ligands with mitogenic activity (Ledda-Columbano *et al.* 2000a), it has been proposed that cyclin D1 is a direct target gene of the transcriptional activity of these receptors (Ledda-Columbano & Columbano 2003).

The pancreas of adult organisms, like liver, is a quiescent organ that, similar to adult hepatic tissue, has a potential for regeneration after partial pancreatectomy (Brochenbrough *et al.* 1988, Pap *et al.* 1991, Bonner-Weir *et al.* 1993) and acinar cell necrosis (Slater *et al.* 1998). The mechanisms involved in the regulation of normal pancreas growth and its regeneration have not yet been fully elucidated, although activation of p42/p44

mitogen-activated protein kinase increased expression of the immediate early genes, *c-fos*, *c-jun* and *c-myc*, and cyclins D and E, and increased activity of cyclin-dependent kinase-2, hyperphosphorylation of retinoblastoma (pRb) and reduction of the expression of the cyclin kinase inhibitors p15, p21 and p27 have been found to be associated with pancreas regeneration after 90% pancreatectomy (Lu & Logsdon 1992, Duan & Williams 1994, Morriset *et al.* 1999, Charland *et al.* 2001).

However, much less is known about the capacity of pancreatic cells to respond to direct mitogenic stimuli such as those elicited by primary mitogens, such as ligands of nuclear receptors, namely agents that, in other organs, induce cell proliferation in the absence of cell death.

In the current study, we have analyzed the effect of four different ligands of the nuclear receptor of the steroid/thyroid hormone receptor superfamily: T3, ciprofibrate, all-*trans* retinoic acid (tRA) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), ligands of TRs, PPARs, RARs and constitutive androstane receptor (CAR) respectively on pancreas cell proliferation. Results showed that T3 treatment for 1 week was able to induce massive acinar cell proliferation in both rats and mice, in the absence of any sign of cellular damage. The entry of acinar cells into the cell cycle was associated with increased expression of cyclin D1, proliferating cell nuclear antigen (PCNA) and cyclin A. Little or no mitogenic effect on pancreatic cells was observed with other ligands of nuclear receptors.

## Materials and Methods

### Animals

Eight- to ten-week-old male F-344 rats and CD-1 female mice purchased from Charles River (Milan, Italy) were used in these experiments. The animals were fed a laboratory chow diet provided by Ditta Mucedola (Settimo Milanese, Italy) and had free access to food and water. All procedures were performed in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals and the guidelines of the animal ethics committee of this University. All experiments were performed in a temperature-controlled room with 12 h darkness:12 h light cycles. T3 and tRA (Sigma Chemical Co., St Louis, MO, USA) were added to a basal diet at a final concentration of 4 mg/kg and 150 mg/kg diet respectively. Ciprofibrate, a gift from Sanofi Winthrop (Guildford, Surrey, UK) was added to the basal diet at a final concentration of 0.025%. TCPOBOP was a gift from Dr B A Diwan, National Cancer Institute, Frederick, MD, USA). Hematoxylin and eosin were from Carlo Erba Reagenti (Milan, Italy).

Rats and mice were fed a diet supplemented with T3, tRA or ciprofibrate for 2 days or 1 week or were given a single intragastric dose of TCPOBOP (3 mg/kg body

weight). For the determination of cell proliferation, animals were given bromodeoxyuridine (BrdU; Sigma Chemical Co.), dissolved in the drinking water (1 mg/ml), throughout the experimental period (Ledda-Columbano *et al.* 1998).

### Histology and immunohistochemistry

Pancreas sections were fixed in 10% buffered formalin and processed for staining with hematoxylin-eosin or immunohistochemistry immediately after death. The remaining pancreas was snap-frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until use.

For the determination of hepatocyte proliferation, mouse monoclonal anti-BrdU antibody was obtained from Becton Dickinson (San Jose, CA, USA) and the peroxidase method was used to stain BrdU-positive hepatocytes. Peroxidase goat anti-mouse immunoglobulin (Dako EnVision+TM Peroxidase Mouse; Dako Corporation, Carpinteria, CA, USA) was used. Four micron thick sections were deparaffinized, treated with 2 M HCl for 1 h, then with 0.1% trypsin type II (crude from porcine pancreas; Sigma, Milan, Italy) for 20 min, and treated sequentially with normal goat serum (1:10) (Dako Corporation), mouse anti-BrdU (1:100) and Dako EnVision+TM Peroxidase Mouse (ready to use). The sites of peroxidase binding were detected by 3,3'-diaminobenzidine. The labeling index (L.I.) was expressed as the number of BrdU-positive nuclei/100 nuclei. Results are expressed as the means  $\pm$  S.E. of four to five animals per group. At least 2000 acinar cell nuclei per pancreas were scored. The incidence of apoptotic bodies was determined by scoring 3000–4000 acinar cells per pancreas. Only apoptotic bodies containing hematoxylin-positive nuclear fragments were recorded. The apoptotic index (A.I.) was expressed as the number of apoptotic bodies/100 acinar cells.

### Serum $\alpha$ -amylase, lipase and glucose determination

Immediately after death, blood was collected from the inferior vena cava and analysed for blood chemistry. Briefly, the blood samples were centrifuged at 1500 r.p.m. for 20 min and the serum was tested for  $\alpha$ -amylase, lipase and glucose using commercially available kits from Boehringer (Mannheim, Germany).

### Western blot analysis

Total cell extracts were prepared from frozen pancreas powdered in a liquid nitrogen-cold mortar. Equal amounts of powder from different animals were resuspended in 1 ml lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 135 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.1% mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 10 mM iodoacetic acid and

20 µg/ml each of aprotinin, pepstatin and leupeptin). Several protease inhibitors were added to the isolation buffer to minimize protein degradation during the isolation protocol. Extracts were incubated for 30 min on ice, centrifuged at 12 000 r.p.m. at 4 °C and the supernatants recovered. All inhibitors used were purchased from Boehringer with the following exception: PMSF, NaF and DTT were purchased from Sigma Chemical Co. and iodoacetic acid from ICN Biomedicals (Irvine, CA, USA). The protein concentration of the resulting total extracts was determined according to Bradford (1976) using bovine serum albumin as standard (DC Protein Assay; BioRad). For immunoblot analysis, equal amounts (from 100 to 150 µg/lane) of proteins were electrophoresed on SDS 12% or 8% polyacrylamide gels. Acrylamide and bis-acrylamide were purchased from ICN Biomedicals (Irvine, CA, USA). After gel electrotransfer onto nitrocellulose membranes to ensure equivalent protein loading and transfer in all lanes, the membranes and the gels were stained with 0.5% (w/v) Ponceau S red (ICN Biomedicals) in 1% acetic acid and with Coomassie blue (ICN Biomedicals) in 10% acetic acid respectively. Before staining, gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Sigma Chemical Co.). After blocking in Tris-buffered saline containing 0.05% Tween 20 (Sigma Chemical Co.) and 5% non-fat dry milk (TBS-T), membranes were washed in TBS-T and then incubated with the appropriate primary antibodies diluted in blocking buffer. Whenever possible the same membrane was used for detection of the expression of different proteins. Depending on the origin of primary antibody, filters were incubated at room temperature with anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were identified with a chemiluminescence detection system, as described by the manufacturer (Supersignal Substrate; Pierce, Rockford, IL, USA). When necessary, antibodies were removed from filters by 30-min incubation at 60 °C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 7.6) and the membranes reblotted as above.

#### Antibodies

For immunoblotting experiments, mouse monoclonal antibody directed against cyclin D1 (72-13 G; Santa Cruz Biotechnology), p27 (Kip1-p27; Transduction Laboratories, Lexington, KY, USA), actin (clone AC40; Sigma Chemical Co.) and PCNA (PC-10; Santa Cruz Biotechnology) were used; rabbit polyclonal antibodies against cyclin A (C-19) was from Santa Cruz Biotechnology.

#### Statistical analysis

Comparison between treated and control groups was performed by Student's *t*-test.

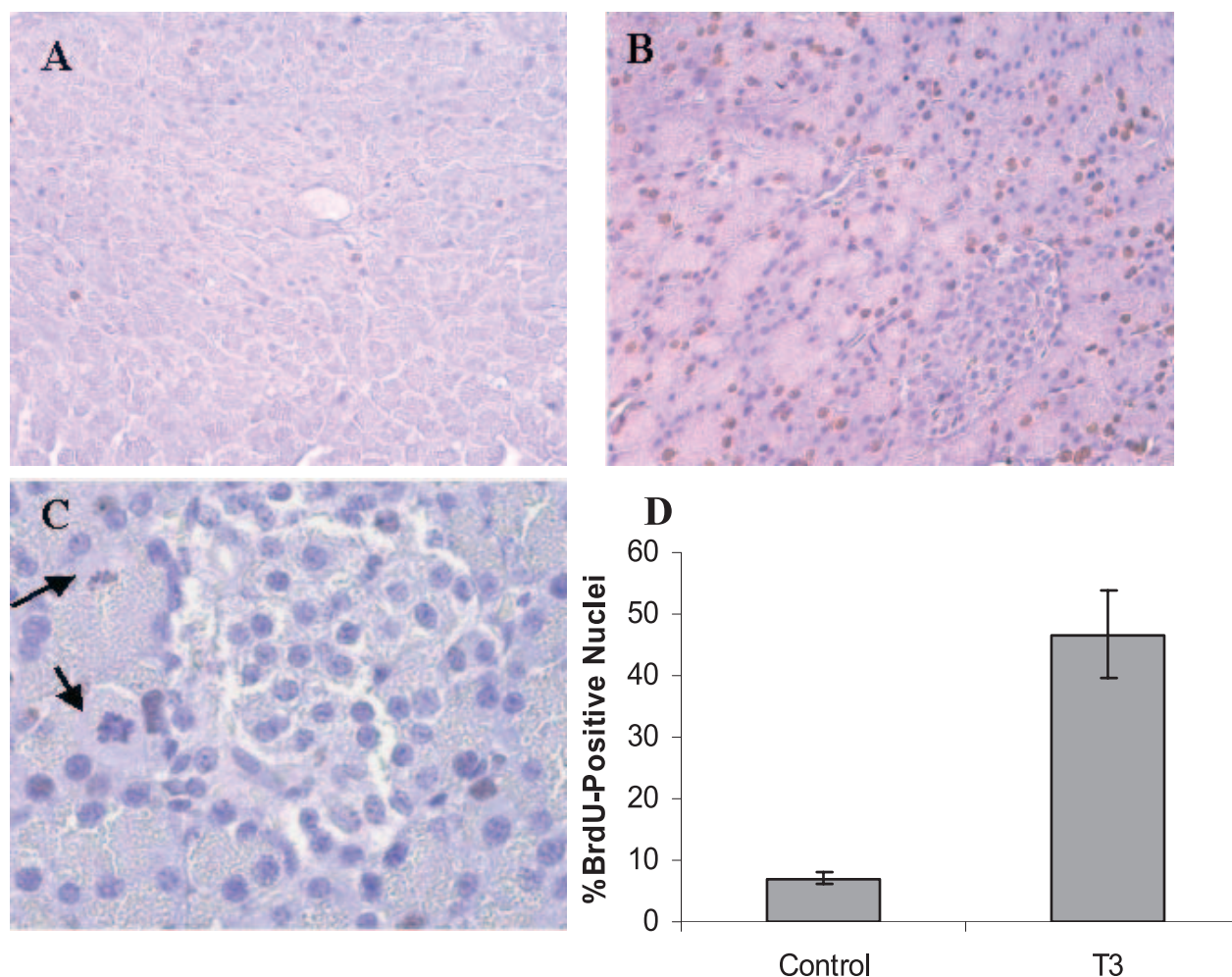
## Results

Previous work showed that T3 feeding for 7 days stimulated cell proliferation in rat liver (Ledda-Columbano *et al.* 2000b). To evaluate the effect of T3 on normal pancreatic cell turnover, experiments were performed wherein F-334 rats were fed a basal diet supplemented with 4 mg T3/kg diet for 1 week. To determine the extent of cells entering into the S phase, T3-fed rats and controls were given BrdU (1 mg/ml, dissolved in the drinking water) throughout the experimental period. The results showed that while pancreatic cells from control rats were essentially BrdU-negative (Fig. 1A), T3 treatment resulted in increased pancreatic cell proliferation (Fig. 1B). At this time-point, most of the labeled cells were acinar cells with islet cells and ductular cells being almost completely unaffected. The L.I. was dramatically increased over the control values (the L.I. of T3-treated rats was 46.7 vs 7.1% of controls) (Fig. 1D); the increased DNA synthesis of acinar cells was associated with the presence of mitotic figures (Fig. 1C). No difference in the distribution of BrdU-positive cells was observed between peri-insular and tele-insular acinar cells. The increased proliferative activity of acinar cells was accompanied by an increase, although not statistically significant, in pancreas weight ( $0.96 \pm 0.13$  vs  $0.73 \pm 0.01$  g in controls). No evidence of a change in cell volume or other clear morphological alterations were found in pancreatic acinar cells following T3 treatment.

To establish whether the proliferative effect induced by T3 could be the consequence of T3-induced pancreatic cell damage and compensatory regeneration or, rather, a direct mitogenic effect, the serum values of  $\alpha$ -amylase and lipase, two secretory enzymes known to be released in the serum during pancreatitis, were determined. As shown in Table 1, no increase in the activity of either of the enzymes was observed in T3-treated rats, the values being 184 U/l and 18 U/l for  $\alpha$ -amylase and lipase respectively vs 236 U/l and 17 U/l in controls. Since apoptotic cell death might have occurred in the absence of changes in the serum levels of amylase and lipase, histological analysis was performed to determine the presence of apoptotic bodies in pancreas sections of T3-treated rats. Results did not reveal any significant change in the number of apoptotic bodies compared with controls (the A.I. was  $0.033 \pm 0.020\%$  in T3-treated rats vs  $0.021 \pm 0.010\%$  in controls).

Lack of toxicity of T3 in  $\beta$  cells was also demonstrated biochemically by determination of the serum values of glucose that were not only not increased over control values but, on the contrary, showed a decrease (Table 1), most probably as a consequence of the increased peripheral uptake of glucose caused by T3.

Additional experiments performed to determine the onset of DNA synthesis in T3-treated rats showed that DNA synthesis began in the first 48 h, as demonstrated by the increased BrdU incorporation (the L.I. was 9.43% in T3-treated rats vs 1.91% in controls) (Fig. 2A).



**Figure 1** Representative photomicrographs demonstrating immunohistochemical staining for BrdU in the pancreas of (A) untreated or (B) T3-treated rats. Rats were fed a T3-supplemented diet (4 mg/kg diet) or a basal diet for 7 days. Immediately after the administration of T3 in the diet, rats were given BrdU (1 mg/ml) in the drinking water for 7 days. A BrdU-negative pancreatic islet is evident ( $\times 200$ , sections counterstained with hematoxylin). (C) Acinar cells in mitosis in the proximity of a pancreatic islet are indicated by the arrows ( $\times 200$  stained with hematoxylin). (D) The LI. of rat pancreatic acinar cells. The LI. was expressed as number of BrdU-positive acinar cell nuclei/100 nuclei. At least 2000 acinar cells per pancreas were scored. Results are expressed as means  $\pm$  s.e. of four to five rats per group.

A key role in the control of the cell cycle is played by a complex formation between cyclins and cyclin-dependent kinases (CDKs). Activation through phosphorylation of

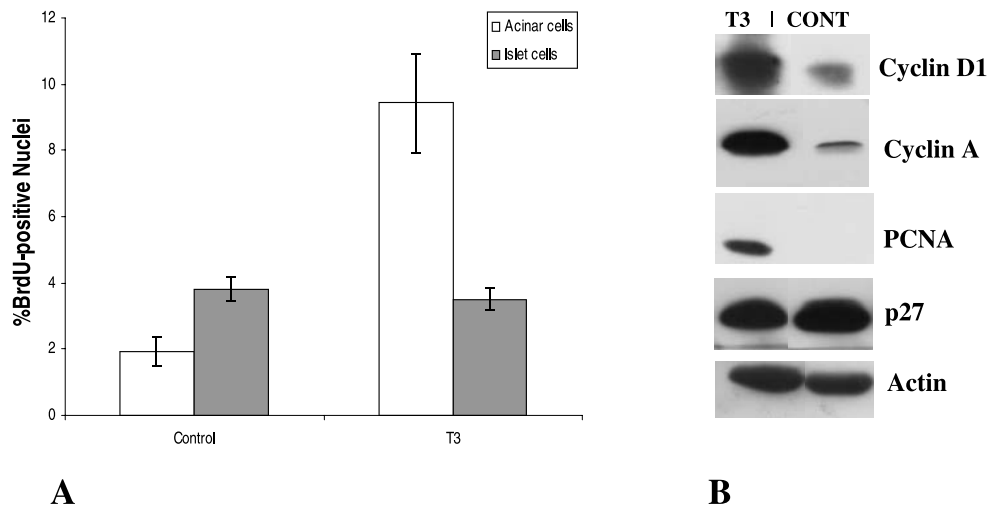
**Table 1** Effect of T3 feeding for 7 days on rat  $\alpha$ -amylase, lipase and glucose serum levels. Values are expressed as means  $\pm$  s.e. of four to six animals per group

	$\alpha$ -amylase (U/l)	Lipase (U/l)	Glucose (mg/dl)
Control	236 $\pm$ 90	17 $\pm$ 4	145 $\pm$ 4
T3	184 $\pm$ 77*	18 $\pm$ 4	95 $\pm$ 5*

\* $P < 0.005$  compared with controls.

cyclin-CDK complexes leads to progression into the cell cycle (Weinberg 1995, Sherr 1996). We therefore determined the expression of cyclin D1, cyclin A, a cell cycle-regulatory protein specifically expressed during the S phase and PCNA after T3 treatment. As shown in Fig. 2B, while pancreatic levels of cyclin D1, cyclin A and PCNA were very low or undetectable in untreated rats, they were induced at a significantly higher level 2 days after T3, concomitantly with the increased BrdU incorporation (Fig. 2A). No significant difference in the protein content of the CDK inhibitor p27 was observed between untreated and T3-fed rats, indicating that entry





**Figure 2** (A) The L.I. of rat acinar cells 2 days after T3 feeding. Rats fed a T3-supplemented diet were given BrdU (1 mg/ml) in the drinking water for 48 h. At least 2000 acinar cell nuclei per pancreas were scored. The L.I. was expressed as number of BrdU-positive acinar cell nuclei/100 nuclei. Results are expressed as means  $\pm$  s.e. of four to five rats per group. (B) Western blot analysis of cyclin D1, cyclin A and PCNA. Protein extracts were prepared from the pancreas of rats killed 2 days after T3 feeding, as described in Materials and Methods. Appropriate loading was confirmed by staining the gel with Coomassie blue. Each lane represents a pool of three samples. CONT, control.

into the S phase of acinar cells was due to an increased expression of cyclins rather than to inhibition of the CDK inhibitors.

We next asked the question as to whether the mitogenic effect of T3 is restricted to the rat pancreas or could also be exerted in other species. To answer this question, CD-1 mice were fed T3 for 7 days, at the same concentration as above. Results showed that T3 was able to induce a strong proliferative response in mouse acinar cells. As shown in Table 2, the L.I. was 28 vs 1.8% in controls. Similarly to rats, islet cells and ductular cells were largely unaffected by the mitogenic action of T3. These results thus demonstrated that the mitogenic effect of T3 on pancreatic acinar cells is species independent.

**Table 2** Effect of treatment with T3, ciprofibrate, tRA and TCPOBOP on pancreatic acinar cell proliferation in mice and rats. Values are expressed as means  $\pm$  s.e. of four to six animals per group

Treatment	Days of treatment	Species	BrdU-positive acinar cell nuclei (%)
Control	7	Mice	1.81 $\pm$ 0.86
T3	7	Mice	28.30 $\pm$ 3.94*
Control	7	Mice	0.02 $\pm$ 0.01
tRA	7	Mice	0.54 $\pm$ 0.24
Control	4	Mice	0.26 $\pm$ 0.14
TCPOBOP	4	Mice	0.72 $\pm$ 0.15
Control	7	F-344 rats	3.30 $\pm$ 0.13
Ciprofibrate	7	F-344 rats	9.20 $\pm$ 0.17*

\* $P < 0.005$  compared with the respective controls.

It has been shown that several ligands of nuclear receptors of the superfamily of steroid/thyroid hormone receptors, including T3, share the capacity to induce hepatocyte proliferation (Ledda-Columbano & Columbano 2003). We therefore asked the question as to whether other ligands of the same nuclear receptor superfamily could mimic T3 in stimulating acinar cell proliferation in the pancreas. To answer this question, ciprofibrate, a ligand of PPAR- $\alpha$  and a powerful rat liver mitogen, and two known inducers of mouse hepatocyte proliferation, tRA and TCPOBOP (Ledda-Columbano *et al.* 2000a, 2003), ligands of RAR and CAR respectively were used.

As shown in Table 2, although ciprofibrate was able to induce some degree of proliferation, the number of acinar cells undergoing DNA synthesis after 7 days of treatment was much lower than that seen after T3 (the L.I. was 9.2% in ciprofibrate-treated rats vs 3.3% in controls). Moreover, two other ligands of nuclear receptors that induce mouse liver cell proliferation, tRA and TCPOBOP, did not exert any significant effect on mouse acinar cells proliferation (the L.I. was 0.54 and 0.72% vs an L.I. less than 0.3% in the respective controls).

### Discussion

Several studies have shown that thyroid hormones may modulate the function of pancreatic islets, including a decrease in the expression of thyrotropin-releasing hormone and insulin secretion in islets (Hinata *et al.* 1994,

Fragner *et al.* 1999). In addition, proinsulin mRNA levels are suppressed in the pancreatic  $\beta$ -cell line RIN-m5F cells by T3 (Fernandez-Mejia & Davidson 1992). On the other hand, much less is known about the effect of T3 on acinar cells. The present study has demonstrated that T3 exerts a strong proliferative effect on the acinar cells of rat and mouse pancreas. The mitogenic effect exerted by T3 is not associated with pancreatic injury but is a primary event. There are three main points rising from this study: (i) the finding that T3 is also a mitogen for rodent pancreas demonstrated that this hormone is, among several other biological effects, strongly involved in activating signal transducing pathways leading to cell cycle entry in different organs; (ii) this mitogenic effect in extra-hepatic tissues appears to be unique to T3 and not shared by other nuclear receptor ligands; and (iii) the mitogenic activity of T3 may be potentially useful for allowing repopulation of acinar cells in damaged pancreas.

As to the first point, it is interesting to note that while T3 is a powerful mitogen for rat liver it has a much weaker effect on mice (A Columbano 2002, unpublished observations). On the contrary, in the present study we have found that the proliferative response of pancreatic acinar cells to T3 is very strong in both species. In the light of the present findings, and based on our preliminary observations showing that T3 induces re-entry into the cell cycle of rat cardiomyocytes, associated with increase in cyclin D1 expression (A Columbano 2004, unpublished observations), it appears that the mitogenic effect elicited by T3 is quite ubiquitous, thus making this hormone unique among other mitogens. The present study demonstrating that T3 is a powerful mitogen for pancreatic acinar cells may also provide a powerful and simple model for studying the molecular mechanisms associated with cell cycle activation in various organs and cellular types. As to the other ligands of nuclear receptors investigated in this work, ciprofibrate at a concentration that induces a high extent of proliferation in rat liver showed a much weaker, although significant, mitogenic effect in the pancreas, with tRA and TCPOBOP being completely ineffective. Whether the strong proliferative response of acinar cells observed after T3, unlike other ligands of nuclear receptors used in this study, depends upon the availability of the different receptors (TR, PPAR, RAR and CAR) in this organ is, at present, unclear.

The mitogenic effect of T3 observed under the present condition might potentially be useful for experimental studies addressed to pancreas repopulation or to clarify aspects related to the process of transdifferentiation or dedifferentiation. Indeed, in spite of several studies addressing the clarification of the latter points, it is still uncertain whether acinar cells have the potential to differentiate into ductular-like cells and if these newly formed acinoductular cells may act as (facultative) stem cells. The transition of acinar cells to ductular cells has been shown in cultured pancreatic cells using appropriate

markers for these cells (Bockman 1995, Reid & Walker 1999). It was recently reported (Rooman *et al.* 2001, 2002) that cultured rat pancreatic cell aggregates, initially highly enriched in acinar cells, lost their acinar phenotype and acquired a phenotype, similar to precursor cells, which are characterized by the expression of pancreatic exocrine transcription factor and the pancreatic duodenal homeobox-1. Furthermore, in transgenic mice expressing the interferon- $\gamma$  gene, islet neogenesis has been shown to continue from the intermediate (acinoductular) cells that express a duct cell antigen (carbonic anhydrase II) and an exocrine enzyme (amylase) (Gu *et al.* 1994). Finally, recent studies have suggested dedifferentiation of acinar cells into acinoductular cells in regenerating rat pancreas (Tokoro *et al.* 2003).

Although no attempts were made in the present study to study the fate of newly dividing acinar cells, the data reported here may provide a simple, reproducible and very efficient experimental system to study the mechanisms underlying pancreatic cell proliferation and the process of transdifferentiation. Furthermore, in view of the powerful and quite rapid effect of T3 on acinar cell DNA synthesis, compared with other models such as regeneration after pancreatectomy, the present model can be utilized to achieve successful gene transfer in acinar cells.

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