

Anti-apoptotic effects of 3,5,3'-tri-iodothyronine in mouse hepatocytes

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Abstract

The present study demonstrates that 3,5,3'-tri-iodothyronine (T₃) in physiological dose range inhibits tumor necrosis factor α (TNF α)/Fas-induced apoptosis in mouse hepatocytes. T₃ pretreatment prevented Fas-induced early stage of apoptosis signs assessed by flow cytometry analysis of the annexin V positive cell population. T₃ attenuated TNF α /Fas-induced cleavage of caspase-8 and DNA fragmentation. We found that T₃ exerted its anti-apoptotic effects by mobilization of several non-genomic mechanisms independent of transcriptional activity. Inhibition of protein kinase A (PKA), extracellular signal-regulated kinase (ERK), and Na⁺/H⁺ exchanger blocked T₃-dependent anti-apoptotic effects indicating an involvement of these intracellular targets into T₃-induced signaling cascade. Furthermore, physiological concentrations of T₃, but not reverse T₃, caused increases in intracellular

cAMP content and activated PKA. T₃ markedly induced phosphorylation of ERK. We also detected T₃-dependent intracellular alkalinization that abolished TNF α -induced acidification. PKA inhibitor KT-5720 blocked T₃-induced activation of ERK and intracellular alkalinization confirming the upstream position of PKA signaling. We further detected that hepatocytes from hypothyroid mice are more sensitive to TNF α /Fas-induced apoptosis than euthyroid animals *in vivo*. Together, these findings imply that T₃ triggers PKA- and ERK-regulated intracellular pathways capable of driving and ensuring hepatocytes survival in the presence of death receptor ligand-induced damage under chronic inflammatory conditions.

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Introduction

Thyroid hormones (THs) are widely known regulators of growth, metabolism, and development (Feng *et al.* 2000, Yen 2001, Davis & Davis 2002). Multiple studies demonstrated that liver is an important target organ for THs. THs exerted mitogenic effects on hepatic metabolism, on the level of gene expression (Francavilla *et al.* 1994, Feng *et al.* 2000, Yen 2001) and on cell-cycle progression (Alisi *et al.* 2005, Furumoto *et al.* 2005). Majority of the functions of THs were mediated by the *c-erbA* proto-oncogenes – THs nuclear receptors, members of the steroid/thyroid hormone nuclear receptor subfamily that also function as ligand-modulated transcription factors (Yen 2001).

Beyond the abundant genomic effects, THs have been shown to initiate fast non-genomic signaling (Yen 2001, Davis & Davis 2002, Incerpi 2005). It is important that most of plasma membrane and cytosol targets of THs have been strongly circumstantially linked to mitogenic and anti-apoptotic signaling pathways (Qi *et al.* 1999, Laoag-Fernandez *et al.* 2004, Verga Falzacappa *et al.* 2006). For instance, THs were able to activate the Na⁺/H⁺ exchanger (NHE) and reduce intracellular acidity produced by activated sodium

currents in different cells (Wang *et al.* 2003, D'Arezzo *et al.* 2004). THs induced activation of protein kinase C (PKC), protein kinase A (PKA), and mitogen activated protein kinase (MAPK) via putative G-protein-coupled receptor (GPCR; Lin *et al.* 1999, Yen 2001, D'Arezzo *et al.* 2004). Activation of the MAPK cascade by physiological concentrations of THs caused translocation of phosphorylated extracellular signal-regulated kinase (ERK) to the cell nucleus resulting in serine phosphorylation of the nuclear THs receptor (TR) and transcriptional activity (Yen 2001, Davis & Davis 2002, Shih *et al.* 2004).

THs may also exert a fast permissive action on the target cells' responsiveness to other active peptide and hormones. The status of THs modulated the steady-state levels of specific G-proteins induced by β -adrenergic agonists (Daza *et al.* 1997). It has also been shown that THs positively regulated epidermal growth factor receptor signaling pathway (Lin *et al.* 1999, Di Fulvio *et al.* 2000, Shih *et al.* 2004) – established survival regulator of Fas-induced apoptosis (Gibson *et al.* 1999). However, the effects of THs on Fas-induced apoptosis in hepatocytes have not yet been addressed.

Death receptor ligands (DRLs) have been demonstrated to impact hepatocellular damage (Zang *et al.* 2000, Ding & Yin

2004). Hepatocyte apoptosis initiated by DRLs like tumor necrosis factor- α (TNF α) or Fas contributed to a variety of liver diseases, including acute liver failure, viral and inflammatory hepatitis, alcoholic hepatitis, and ischemia/reperfusion injury (Luedde *et al.* 2002, Ding & Yin 2004). Several intriguing clues from clinical reports alluded to possible correlation between thyroid dysfunction and inflammation-related liver disorders. It was found that hepatitis sensitizes hepatocytes to TNF α apoptosis (Ding & Yin 2004) and, on the other hand, is often accompanied by autoimmune hypothyroidism via impaired THs secretion (Antonelli *et al.* 2004). However, the possibility that hypothyroidism impacts the DRLs-dependent liver damage has not yet been explored given the demonstrated importance of TH status for liver regeneration (Alisi *et al.* 2005, Malik *et al.* 2005). Supporting the protective role of THs during liver injury, it has been shown that THs regulated the expression and activity of the proteins involved in the control of cell cycle, apoptosis, and growth arrest in hepatocytes after partial hepatectomy (Malik *et al.* 2005). The analysis of the phosphorylated forms of MAPK, as survival and growth markers, revealed that they are induced during hepatic regeneration in euthyroid and hyperthyroid rats whereas they are negatively regulated in hypothyroid rats (Alisi *et al.* 2004). Therefore, considering *in vivo* and *in vitro* data, we questioned the ability of THs to influence hepatocyte survival signaling pathways.

The results of our experiments demonstrate that T₃ serves as an important anti-apoptotic factor in hepatocyte survival after DRLs-initiated apoptosis. T₃ mobilizes several fast-signaling mechanisms and abrogates Fas/TNF-induced nuclear fragmentation and activation of caspase-8 via increases in intracellular cAMP, induction of PKA and ERK, and maintenance of intracellular pH (pHin) in homeostasis.

Materials and Methods

Chemicals

3,5,3'-Tri'-iodothyronine (T₃), reverse T₃ (rT₃), and 8-bromoadenosine 3',5'-cyclic monophosphate (Br-cAMP) were purchased from Calbiochem (San Diego, CA, USA). MEK-1 inhibitor, U0126, was purchased from Cell Signaling Technologies (Beverly, MA, USA). bis carboxyethyl-5-(6)-carboxyfluorescein (BCECF/AM), 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), actinomycin D (ActD), forskolin, amiodarone, KT-5720 (PKA inhibitor), cycloheximide, staurosporine (StSp), and digitonin were purchased from Sigma. Mouse TNF α and anti-Fas antibodies (clone Jo2) were purchased from Pharmingen (San Diego, CA, USA). Fas receptor, phospho-ERK (pERK), and ERK total antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Fas-associated death domain-like interleukin-1 β -connecting enzyme-inhibitory protein (FLIP) CT polyclonal antibodies were from StressGen Biotechnology

(Victoria, BC, Canada). The anti-caspase-8 antibody that recognizes the 18 kDa active fragment of caspase-8 (p18) was from Oncogene Research Products (Boston, MA, USA). Unless otherwise specified, the rest of the chemicals used in this study were obtained from Sigma.

Animals and hepatocyte culture experiments

Mouse liver and primary hepatocyte cultures have been widely used for Fas-induced apoptosis studies (Fladmark *et al.* 1997, Ding & Yin 2004). Wild-type male mice 8-weeks of age were assigned to one of the following groups: (a) euthyroid (sham operated); (b) hypothyroid that had been thyroidectomized surgically, allowed to stabilize, and used only if they had gained no weight for a minimum of 1 week prior to hepatocyte preparation or further treatment. The Fas-injury model group received a single i.p. injection of 0.15 μ g/g body weight Jo2 anti-Fas antibody (PharMingen, Franklin Lakes, NJ, USA) or TNF α (0.1 μ g/g body weight) 6 or 10 h before liver extraction. Animals were placed under inhaled isoflurane anesthesia for isolation of blood and liver tissue. Plasma was prepared from whole blood by centrifugation at 4000 g for 30 min at 4 °C. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by routine clinical chemical methods using enzyme assay kits as described previously (Zang *et al.* 2000). The concentrations of THs (T₄ and T₃) in mouse blood were measured as previously described (Escobar-Morreale *et al.* 1996). Experiments with mice were conducted according to the National Research Council's guidelines for the care and use of laboratory animals.

For *in vitro* experiments, hepatocytes were isolated from male wild-type mice by the collagenase perfusion method (Francavilla *et al.* 1994). Briefly, livers were first perfused *in situ* with oxygenated 0.5 mM EGTA in a calcium-free salt solution (8 ml/min, 37 °C for 5 min), and then with a 0.04% collagenase solution (Wako Pure Chemical Industries Co., Osaka, Japan) for 10 min. Livers were then gently minced and filtered through a polyamide mesh. Hepatocyte viability was >90%, as determined by Trypan blue dye exclusion. Hepatocytes were diluted to a concentration of 2×10^5 cells/ml and plated in 12-well plates (1 ml/well). The plates were previously coated with mouse collagen type I in Dulbecco's modified Eagle's medium (DMEM; GIBCO). The cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES (pH 7.4), 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 5% CO₂ incubator at 37 °C for 16 h before various treatments. Hepatocytes were pretreated with vehicle or different concentrations of T₃ for various time periods. For apoptosis induction, cells were treated with ActD (0.2 μ g/ml) and Jo2 (0.5 μ g/ml) or recombinant mouse TNF α (10 ng/ml) as described previously (Ogasawara *et al.* 1993). We used a serum-free culture medium in *in vitro* experiments with hormones/inhibitors treatments so as to avoid the possible influences of various hormones contained in serum.

cAMP RIA

Nearly confluent plates of cells, plated at a density 2×10^6 cells per 160 mm dish, were treated with various agents and then harvested in 500 μ l TNE media (40 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 1.5 mM EDTA). Cells, collected into microcentrifuge tubes kept on ice, were pelleted (100 g for 5 min). The cell pellets were resuspended in 200 μ l ice-cold cAMP extraction buffer (50 mM Tris-HCl (pH 7.5) and 4 mM EDTA). Extract aliquots were boiled for 10 min, and cell debris was pelleted. The [³H]cAMP RIA kit (Amersham) was used to measure the cAMP content of samples. Incubations were carried out at 4 °C for 3 h, and charcoal-dextran-treated samples were analyzed for radioactivity.

PKA assay

The cytosolic fractions that had been separated from cells after various treatments were used for protein kinase assays (Shih *et al.* 2004). The PKA activity was determined using a PKA Assay Kit, according to the manufacturer's directions (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, cells were lysed, sonicated, centrifuged, and total protein was determined. A mixture of the cytosolic fraction (5 μ g protein), assay dilution buffer (pH 7.2), cAMP, PKC/CaMK inhibitor cocktail, Mg/ATP [γ -³²P]ATP (5 μ Ci or 3000 Ci/mmol), and Kemptide (a synthetic peptide containing the consensus PKA phosphorylation sequence) as substrate was incubated at 30 °C for 10 min, in a total volume of 60 μ l. A 25 μ l aliquot of the reaction mixture was applied to phosphocellulose filter paper (Whatman p81) and washed repeatedly in 75 mM phosphoric acid, and dried. The radioactivity was quantitated by scintillation. The results were expressed in terms of nanomoles phosphate/minute per milligram protein. The PKA assay was also performed in the presence of the protein kinase inhibitor peptide of PKA. These values were similar to background counts obtained in the absence of Kemptide substrate, indicating the specificity of the assay (data not shown).

DNA fragmentation

DNA fragmentation was demonstrated by harvesting total cellular DNA and 1.2% agarose gel electrophoresis stained with ethidium bromide. Briefly, after the indicated treatment, both adherent and detached cells were harvested, washed with PBS, and lysed in 50 mM Tris-EDTA (pH 7.4), 1% SDS, and 0.5 mg/ml proteinase K for 3 h at 50 °C. Samples were then extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in 20 mM Tris-EDTA (pH 8.0). After digesting RNA with RNase (0.1 mg/ml) at 37 °C for 1 h, DNA was separated by electrophoresis.

Immunoblot analysis

Cells were harvested and lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na₃VO₄, 10 mM NaF, 1 mM EDTA, and protease inhibitors (Roche Molecular Biochemicals). Protein concentrations in cell homogenates were determined with DCA protein assay (Bio-Rad) using BSA as standard. Proteins were normalized prior to gel loading. Aliquots of cell lysates were resolved by 8–12% SDS-PAGE and transferred to Hybond-P membranes (Amersham). The membranes were then probed with appropriate antibodies according to the manufacturer's standard method. The immunocomplexes were detected with an enhanced chemiluminescence PLUS kit (Amersham Pharmacia Biotech). Densitometry was performed on a Typhoon 9410 Mode Imager using the ImageQuant program (Amersham).

Annexin V staining and analysis

Annexin V-FITC binding to externalized phosphatidylserine has been used for apoptosis detection. Cells were plated in culture plates and exposed to various pretreatments. After apoptosis induction by Fas/TNF α /ActD, the cells were harvested, washed with ice-cold PBS, and subsequently resuspended and incubated (in the dark) with 100 μ l annexin V incubation reagent that includes FITC-conjugated annexin V (Trevigen, Gaithersburg, MD, USA) and propidium iodide (PI) for 30 min at room temperature. Flow cytometry analyses were conducted using Epics XL-MCL flow cytometer (Coulter, Hialeah, FL, USA) at 488 nm wavelength. The percentage of apoptotic cells was determined as a percentage of annexin V-FITC-positive cells.

Measurement of intracellular pH

Intracellular pH (pH_{in}) was determined by fluorescence intensity of BCECF/AM (Sigma) using both flow cytometry and fluorimetry as described previously (Musgrove *et al.* 1986, D'Arezzo *et al.* 2004).

For fluorimetric determination, cells (10^8 cells/ml) were washed twice and then loaded with 1 μ M BCECF (in dimethylsulfoxide) for 30 min at 37 °C in the dark with gentle shaking in sodium HEPES buffer (D'Arezzo *et al.* 2004). Then the cells were washed twice and resuspended in the same buffer at 10^6 cells/ml, and introduced in a thermostatic cuvette at 37 °C with constant stirring for the continuous recording of the pH-sensitive fluorescence on a Perkin-Elmer LS50B luminescence spectrometer (Perkin-Elmer Corp., Norwalk, CT, USA). pH_{in} was estimated from the ratio of the 530 nm fluorescence signals obtained at 500 nm (pH sensitive) and 450 nm (isosbestic) excitation wavelengths. Routinely at the end of each experiment, the calibration of fluorescence was carried out by established methods (Musgrove *et al.* 1986, D'Arezzo *et al.* 2004) in high potassium HEPES buffer containing the H⁺/K⁺ ionophore

nigericin (10 μ g/ml). The calculations of ion concentration were carried out as described in Musgrove *et al.* (1986).

For cytofluorimetric pHin determination, cells were treated according to the designed protocol with or without hormones or inhibitors. The cells were then washed with media containing RPMI 1640, containing 10 mM HEPES and 2.5% serum, loaded with BCECF (as mentioned above), and analyzed using Epics XL-MCL flow cytometer as described previously (Musgrove *et al.* 1986). Excitation of the probe was performed at 488 nm with an argon laser. Emission fluorescence was filtered through a 525 band pass (green) and a 610 long pass (red) fluorescence filters.

Statistical analysis

All data are expressed as means \pm s.d. Unpaired Student's *t*-tests were used for comparison between two groups. For multiple comparisons, results were analyzed by ANOVA followed by the Dunnett's test. *P* values <0.05 were considered statistically significant.

Results

Thyroid hormones suppress TNF α /Fas-induced apoptosis in mouse hepatocytes

In our experiments, mouse primary hepatocyte culture has been treated for 4 h with death receptor ligands (DRLs) – Fas (0.5 μ g/ml) or TNF α (10 ng/ml) plus ActD (0.2 μ g/ml; Fladmark *et al.* 1997, Ding & Yin 2004). Apoptosis was assessed by flow cytometry detecting annexin V labeling of phosphatidylserine. DRLs-treated hepatocytes exhibited significantly increased annexin V binding (Figs 1 and 2).

The major form of THs secreted from thyroid gland is L-thyroxine (T₄); however, most of THs actions were induced by 3,5,3'-tri-iodothyronine (T₃) – the product of T₄ deiodination (Yen 2001). We have assayed the effect of both T₄ and T₃ on Fas-induced apoptosis in hepatocytes. To detect the effect of T₃/T₄ on DRLs-induced apoptosis hepatocytes were pretreated with one of the hormones (150 nM) or vehicle for 0.25–6 h before stimulation with Fas/or TNF α /ActD. THs exposure decreased Fas and TNF α /ActD-induced

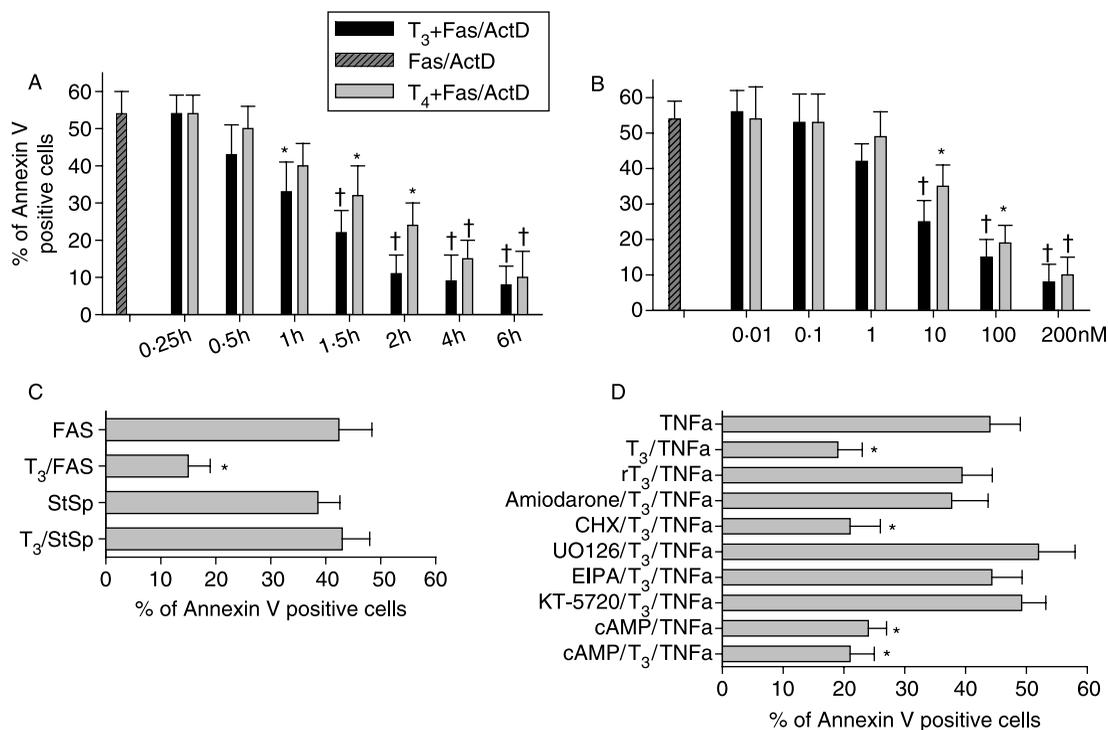


Figure 1 THs (T₃ and T₄) inhibit Fas-induced apoptosis in mouse hepatocytes. (A) T₃/T₄ (150 nM) time-dependent and (B) T₃/T₄ (2 h exposure) dose-dependent anti-apoptotic effects. Apoptosis has been detected by annexin V flow cytometry after 4 h stimulation with anti-Fas ligands (0.5 μ g/ml) and ActD (0.2 μ g/ml) as described in Materials and Methods. Values are expressed as means \pm s.d. (*n*=4 for Fas-treated controls, while *n*=8 for T₃/T₄-pretreated groups) from four separate experiments. Statistically different data are marked as **P*<0.05; †*P*<0.01 (Fas-treated versus T₃/T₄-pretreated cells). (C and D) T₃-induced preventive effects on Fas/TNF α -induced apoptosis are independent of transcriptional and translational activities, but conditioned by PKA, ERK, and intracellular pH. The percentage of annexin V-positive cells is indicated. Used concentrations and time of treatment: 150 nM T₃ or rT₃ for 2 h, TNF α (10 ng/ml) or Fas (0.5 μ g/ml) + ActD (0.2 μ g/ml) for 4 h; 1 μ M amiodarone; StSp (1 μ M) for 4 h; 60 μ g/ml CHX (60 min), 500 nM KT-5720, 10 μ M UO126, and 60 μ M EIPA. All experiments have been repeated at least thrice and one representative experiment is shown. **P*<0.05; †*P*<0.01.

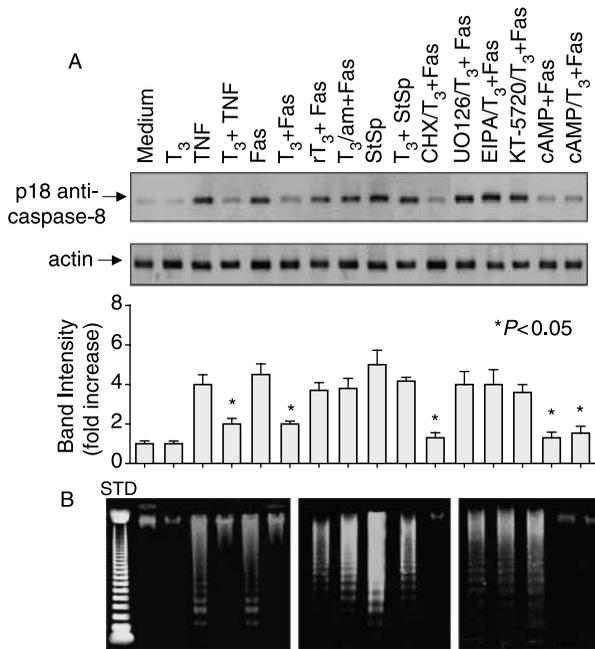


Figure 2 T₃ prevented Fas/TNF α -induced activation of caspase-8 and DNA degradation in mouse hepatocytes. (A) Hepatocytes were pretreated with the following agents separately or in combinations: 150 nM T₃ or rT₃ for 2 h, TNF α (10 ng/ml) or Fas (0.5 μ g/ml)+ActD (0.2 μ g/ml) for 4 h; 1 μ M amiodarone; StSp (1 μ M) for 4 h; 60 μ g/ml CHX (60 min), 500 nM KT-5720, 10 μ M UO126, and 60 μ M EIPA. Cell lysates were subjected to SDS-PAGE and caspase-8 activation was detected by immunoblotting using anti-caspase-8 antibody that recognizes the 18 kDa active fragment of caspase-8 (p18). Equal protein loading was confirmed by actin staining of the blot. The bar graphs represent band intensities obtained from three individual experiments. Results were normalized by actin and expressed as means \pm s.d. **P*<0.05 versus medium (control). (B) Fragmented DNA was obtained from 5 \times 10⁵ cells as described in Materials and Methods and loaded in each lane. DNA ladder of 123 bp is marked as STD (standard). Experiment has been repeated four times. One representative experiment is shown.

apoptosis (Fig. 1). The preventive effect was also dose dependent and has been detected as early as 30-min T₃ pretreatment. T₄ demonstrated effects similar to but lower than those induced by T₃. No protective effect was observed when T₃ was simultaneously applied or post-applied after Fas/TNF α /ActD treatments. Application of reverse T₃ (rT₃) did not prevent DRL-induced apoptosis (Figs 1D and 2).

Structurally similar to biologically active THs, the potent anti-arrhythmic drug amiodarone (1 μ M) used as a TR inhibitor (Van Beeren *et al.* 1996), prevented the T₃ effects (Figs 1D and 2). However, T₃ did not protect hepatocytes from 1 μ M StSp-induced apoptosis (Fig. 1C) that activated the mitochondrial death pathway independently from death receptors. Our results indicate that in hepatocytes, T₃ selectively inhibits DRL-mediated apoptosis, and the observed protection does not result from a general inhibitory effect on cell death.

Next, we investigated whether new protein synthesis is required for the anti-apoptotic effect of T₃. As shown in Figs 1D and 2, T₃-mediated protection from TNF α -induced apoptosis occurred equally well in the presence or in the absence of cycloheximide (CHX; 60 μ g/ml), indicating that the T₃ protective effect was independent of new protein synthesis. It was also independent of activation of transcription because we have added ActD in all experiments along with application of TNF α /Fas to sensitize hepatocytes to apoptosis (Ding & Yin 2004). Therefore, the involvement of cytoplasmic, non-genomic signaling pathways has to be considered. Indeed, anti-apoptotic and mitogenic effects of THs were associated with the activation of several non-genomic mechanisms, including activation of PKA, ERK, and NHE in different cells (Yen 2001, Davis & Davis 2002, D'Arezzo *et al.* 2004, Furumoto *et al.* 2005, Incerpi 2005). In our experiments, the THs-induced anti-Fas protective effect was reversed by pretreatment (30 min) and co-incubation with PKA inhibitor KT-5720 (500 nM), ERK activation inhibitor UO126 (10 μ M), and NHE inhibitor EIPA (60 μ M; Figs 1 and 2). KT-5720, UO126, and EIPA have been tested separately and did not affect cell viability or DNA integrity during overnight treatment (data not shown).

Successful inhibition of T₃ effects by KT-5720 indicated an involvement of cAMP/PKA into T₃-induced anti-apoptotic signaling. To elucidate whether this anti-apoptotic effect of THs is causally linked to the increases in cAMP induced by T₃, we examined the effect of 100 μ M 8-bromoadenosine 3',5'-cyclic monophosphate (Br-cAMP) on Fas-induced apoptosis. Pretreatment with Br-cAMP significantly reduced the number of apoptotic cells compared with TNF α /Fas-treated controls. However, we have not observed an additive effect of Br-cAMP and T₃ (Fig. 1D).

Hepatocyte apoptosis initiated by DRLs-like TNF α or Fas elicits the recruitment of several cellular pathways resulting in the activation of caspase-8 and caspase-3, translocation of Bid into mitochondria with subsequent cytochrome *c* release (Van Beeren *et al.* 1996). The cytosolic cytochrome *c* accumulation results in the formation of the apoptosome and DNA degradation (Ding & Yin 2004). Therefore, DNA fragmentation and caspase activation have been described as apoptosis markers in hepatocytes challenged with Fas/TNF α /ActD (Ding & Yin 2004). In our experiments, T₃ prevented Fas-induced caspase-8 activation (Fig. 2). The protective effect of T₃ was inhibited in the presence of KT-5720, UO126, and EIPA indicating the necessity of PKA, ERK, and NHE activation to overbalance Fas-induced apoptosis and prevent caspase-8 activation. Protective T₃ effects have also been confirmed using the DNA fragmentation assay (Fig. 2B).

T₃-induced increases in cAMP and PKA activation are important for prevention of Fas-induced apoptosis

In hepatocytes, cAMP protected against apoptosis induced by TNF and Fas ligands (Van Beeren *et al.* 1996, Fladmark *et al.*

1997, Li *et al.* 2000). To confirm the cAMP participation in T_3 -mediated effects, we next examined how T_3 affects the cellular cAMP content. Dose-response studies revealed that low, physiological concentrations of T_3 , but not rT_3 caused a sixfold increase in the cellular cAMP content (Fig. 3). The effect was blocked by pretreatment with amiodarone. Used as a positive control, forskolin (5 μ M) increased cAMP nine times above the basal level. Intracellular cAMP levels returned to the control level by 6 h despite continued exposure to hormonal ligands.

To assess whether protein and RNA synthesis were required for the stimulatory effect of T_3 on intracellular cAMP concentration, cells were pretreated with CHX and ActD for 60 min prior to the addition of the hormone. CHX/ActD-treated cells showed no inhibition of T_3 -induced stimulation of cAMP, although the inhibitors prevented protein and RNA synthesis by >95% (data not shown). This implies that T_3 -induced stimulation of cAMP accumulation does not require RNA or protein synthesis. The early time course of cAMP

increase is also consistent with the apparent absence of a requirement for RNA synthesis.

In classic signaling cascades, cAMP activates PKA, a cAMP-dependent serine threonine kinase, which in turn regulates intracellular pathways via phosphorylation of signaling intermediates (Kopperud *et al.* 2003). Since T_3 increased intracellular cAMP, we asked whether the changes in cAMP were significant enough to affect the activation of PKA. We have measured PKA activity stimulated by T_3 . As shown in Fig. 3, T_3 induced activation of PKA that decreased to baseline levels by 24 h. The specificity of this response was confirmed by the finding that amiodarone blocked T_3 effects and rT_3 did not stimulate PKA activity (Fig. 3C). The T_3 -induced increase in PKA activity was not affected by TNF α (Fig. 3D). These data suggest that T_3 signaling is a potential source of PKA activation that might be required for inhibition of DRL-dependent apoptosis in hepatocytes.

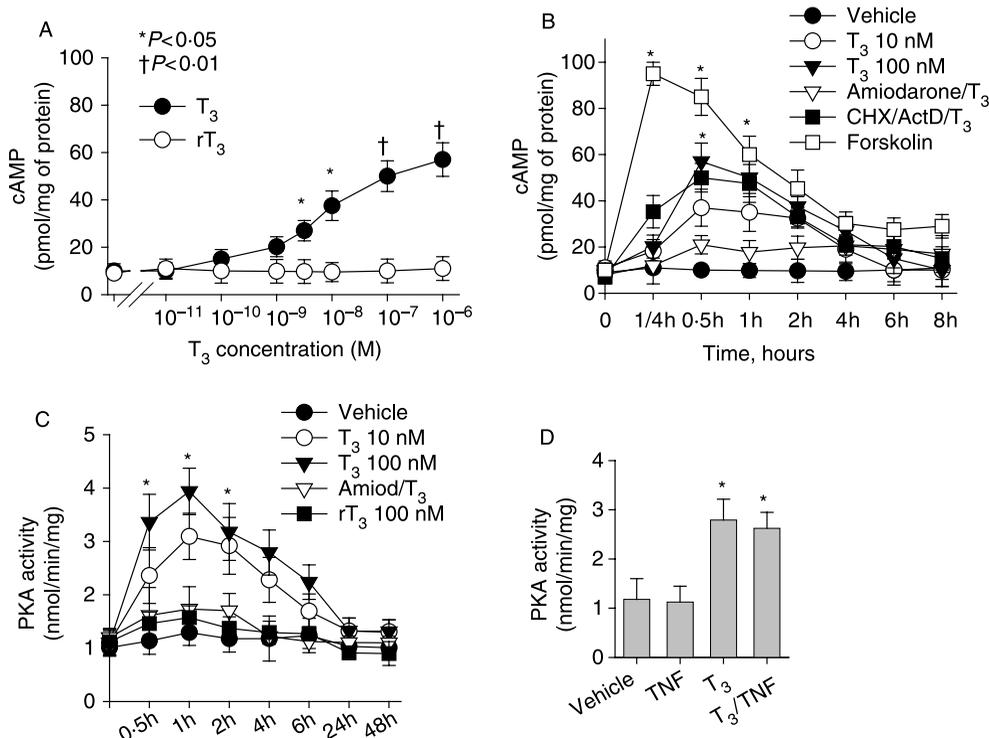


Figure 3 T_3 stimulates increases in the cellular cAMP content and PKA activity in mouse hepatocytes. (A) T_3 or rT_3 was applied dose dependently for 60 min. Intracellular cAMP content was determined by RIA. * P <0.05 and † P <0.01 indicate statistically significant effects of T_3 treatment compared with rT_3 -treated cells. (B) Time-dependent changes in intracellular cAMP have been measured after treatment with or without 10 or 100 nM T_3 , 1 μ M amiodarone (30-min pretreatment), ActD (0.2 μ g/ml), 60 μ g/ml CHX (60-min pretreatment), or with 5 μ M forskolin. Inhibitors were added before treatment with 100 nM T_3 . * P <0.05 T_3 treatments versus vehicle-treated controls. (C) PKA activity was measured in hepatocytes at the indicated time points using 10 or 100 nM T_3 , 100 nM rT_3 , and 1 μ M amiodarone (Amiod). PKA activity was determined by measuring incorporation of radiolabeled phosphate into a PKA-substrate peptide (Kemptide) and subtracting background counts obtained in the absence of substrate. (D) Bar graphs represent data obtained from four individual PKA assay experiments after 1-h stimulation with the marked reagents as follows: 10 nM T_3 , 10 ng/ml TNF α \pm ActD (0.2 μ g/ml), separately and combined. Results are expressed as means \pm s.d. * P <0.05 versus vehicle-treated controls.

T₃ activates ERK to suppress Fas-mediated apoptosis

ERK is a member of the MAPK family that participates in mitogenic signaling through complex phosphorylation cascades converting cell surface signals into nuclear transcription programs. Induction of ERK signaling by cAMP/PKA-dependent pathway has been shown (Kopperud *et al.* 2003, Rudolph *et al.* 2004). Importantly, ERK is also known to attenuate Fas-mediated apoptosis in hepatocytes (Sautin *et al.* 2001). We have detected that the inhibition of ERK activation by U0126 suppressed the T₃-dependent protection against Fas-induced apoptosis (Figs 1 and 2).

To confirm the ERK-dependent mechanisms of T₃-mediated prevention of Fas-induced apoptosis, we addressed the activation of MAPK as a potential target for T₃ action. In our experiments, T₃ markedly induced the phosphorylation of ERK in hepatocytes, starting at 30 min and lasting up to 10 h (Fig. 4A). The stimulation of ERK phosphorylation by T₃ also occurred in the presence of the apoptotic trigger (Fas/TNF α ; Fig. 4B). We have not detected any significant increases in ERK activation after time-course treatment (15 min to 4 h) with TNF α or Fas/ActD in primary mouse hepatocytes (data not shown). U0126, used as a positive control, effectively inhibited T₃-dependent ERK activation (Fig. 4B). PKA inhibition by KT-5720 also prevented T₃-induced ERK activation suggesting PKA activation is upstream of ERK signaling. Together, these data supported our findings that T₃ inhibits Fas-mediated apoptosis in hepatocytes through activation of the ERK signaling pathway by a PKA-dependent mechanism.

T₃ prevents TNF α -induced intracellular acidification

Low pH-dependent (acidic) nucleases are often involved in intranucleosomal genome degradation. Fas-ligands have been shown to induce not only DNA degradation, but also cytosole acidification via inhibition of NHE (Gottlieb *et al.* 1996). In our experiment, NHE inhibitor EIPA abrogated T₃-dependent anti-apoptotic effects indicating the importance of pHin regulation for cell survival (Figs 1 and 2). Our data suggested that T₃ effects could be mediated by activation of the NHE and subsequent intracellular alkalization.

To assess whether T₃ regulates intracellular pH in hepatocytes, we performed conventional BCECF fluorescence measurements. We analyzed the distribution of TNF α /Fas- and vehicle-treated cells according to their pHin using flow cytometry and fluorimetry. Treatment of hepatocytes with TNF α or Fas led to the appearance of a subpopulation of cells with a more acidic pHin than vehicle-treated cells (Fig. 5) as it was noted previously that exposure to DRL-stimulated decreases in pHin (Gottlieb *et al.* 1996, D'Arezzo *et al.* 2004). In contrast, shift of the main cell population towards alkalized pH was observed after the addition of physiological concentrations of T₃ indicating significant rise in the pHin (Fig. 5). Using a fluorimetric assay, we confirmed these data and found that T₃ induced gradual increases of hepatocyte pHin. EIPA caused suppression of the T₃-induced increases in pHin. Treatment with rT₃ had no effect on hepatocyte pHin. Our experiments were carried out in HCO₃-free media, which renders the NHE as the main mechanism for regulation of pHin (Gottlieb *et al.* 1996).

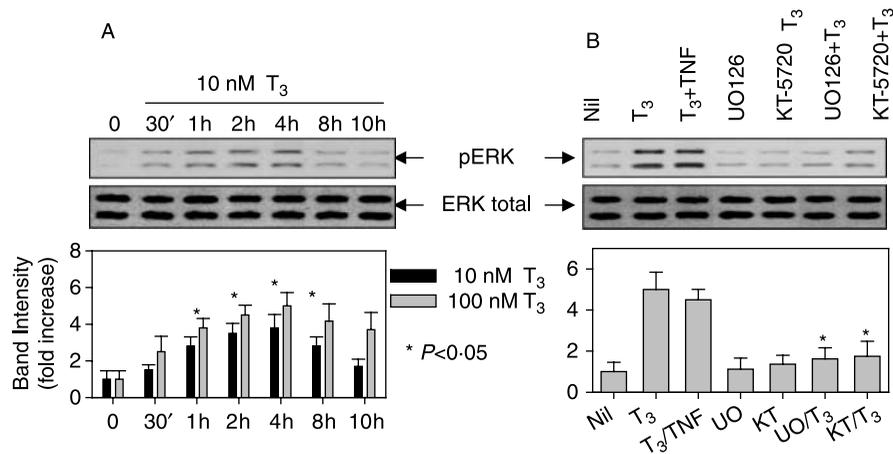


Figure 4 T₃ induces ERK activation in mouse hepatocytes. (A) Hepatocytes were treated with 10 nM T₃ for various periods of time. Bar graphs represent normalized band intensities obtained from four individual western blotting experiments with 10 and 100 nM T₃. (B) Hepatocytes were treated with 100 nM T₃ (1 h), 10 ng/ml TNF α (4 h), 500 nM KT-5720 (30 min), or 10 μ M U0126 (30 min), as marked. Cell lysates were subjected to 12% SDS-PAGE and probed with anti-phosphorylated ERK (pERK; the upper panels). The filter was stripped and reprobed using anti-ERK total (the lower panels) antibodies. Representative blots are shown, and the results were verified in three additional independent experiments. Bar graphs represent band intensities obtained from three individual experiments. Results were normalized by total ERK and expressed as means \pm s.d. **P* < 0.05 versus zero time treatment or nil.

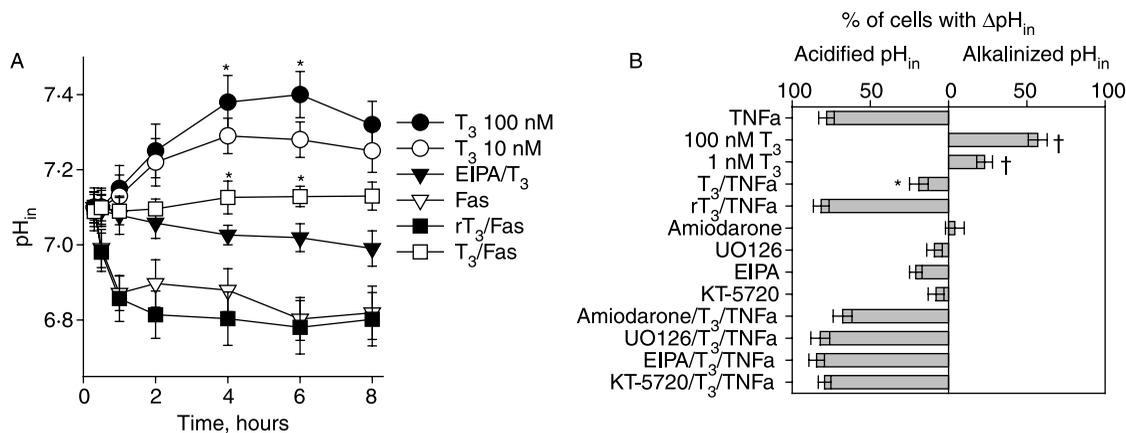


Figure 5 T_3 changes pH_{in} in mouse hepatocytes. (A) Time course of pH_{in} changes was specific for T_3 and blocked by EIPA that was determined by fluorimetric method as described in Materials and Methods. T_3 , but not rT_3 pretreatment inhibited Fas-induced acidification. Used reagents and concentrations: 10 and 100 nM T_3/rT_3 , 0.5 μ g/ml Fas, 0.2 μ g/ml ActD, 60 μ M EIPA separately and combined. (B) T_3 induces intracellular alkalinization and prevents Fas-dependent acidification detected by flow cytometry analysis using BCECF-loaded mouse hepatocytes. The bar graphs represent statistically analyzed data obtained from four individual experiments after 4-h stimulation. Used concentrations and time of treatment as described in Fig. 1D. All inhibitors were combined with 100 nM T_3 . Results are expressed as means \pm s.d. * $P < 0.05$ (Fas- or T_3 -treated cells versus nil), † $P < 0.01$ (Fas-treated versus T_3 -pretreated cells).

Control application of EIPA alone induced slow acidification over 3-h treatment (data not shown).

As evident from flow cytometry analysis of mouse hepatocytes, T_3 induced cytosolic alkalinization contrary to Fas stimulation that was marked by cytosolic acidification. T_3 pretreatment, but not rT_3 , prevented Fas effects and markedly increased pH_{in} when compared with TNF α /Fas-treatment alone (Fig. 5B). EIPA abrogated T_3 -induced pH effects. These results suggest that T_3 can compensate the DRLs-dependent inhibition of the NHE and prevent acute cytosolic acidification in mouse hepatocytes.

Hypothyroidism enhances Fas-induced apoptosis *in vivo*

The liver of mice is particularly sensitive *in vivo* to anti-Fas antibodies, which cause liver apoptosis with a phenotype that resembles fulminant hepatitis marked by activation of caspases (Ogasawara *et al.* 1993). We addressed the activation of caspase-8 in livers extracted from hypothyroid (thyroidectomized) and euthyroid mice. The thyroidectomized animals injected with placebo were severely hypothyroid with decreased T_4/T_3 levels (5 ± 2.4 and 0.2 ± 0.38 ng/ml plasma). T_4 and T_3 blood concentrations in euthyroid mice were 60 ± 14 and 1.5 ± 0.4 ng/ml plasma respectively. Anti-Fas antibodies (0.15 μ g/g body weight) or TNF α (0.1 μ g/g body weight) at non-lethal concentrations were injected 6 or 10 h before liver extraction. At 6 h after injection of Fas *in vivo*, as shown in Fig. 6, we could detect $20 \pm 7\%$ increases in activation of caspase-8 in hepatocytes from euthyroid animals. TNF α did not induce detectable caspase-8 activation under these experimental conditions. However, Fas/TNF α effect was accelerated during 10-h exposure. Nevertheless,

the group of animals survived well under the experimental conditions and was relatively resistant to Fas-mediated apoptosis. Hypothyroid animals demonstrated significant adverse response to TNF α and anti-Fas treatment. We detected an increased sensitivity to Fas-mediated death by increased caspase-8 cleavage in hypothyroid when compared with euthyroid mice. The increased sensitivity to Fas antibodies suggested an intrinsic change in the Fas cell death pathway. The increased damage of hepatocytes in hypothyroid mice after anti-Fas treatment *in vivo* was further confirmed by the significantly elevated serum AST and ALT levels when compared with control euthyroid mice (Table 1). However, as the time passed, AST and ALT levels increased slightly further in the euthyroid mice as well, but to a significantly lesser degree than in hypothyroid animals. These data implied that livers from hypothyroid mice are more sensitive to an otherwise non-lethal dose of anti-Fas antibodies.

These findings were not related to an enhanced cell surface expression of Fas (Fig. 6). Neither did we find a difference in the hepatic expression of cFLIP (Fig. 6B), which interferes with the autoproteolytic maturation of Fas-FADD-bound caspase-8 proximal in the Fas apoptotic pathway (Irmeler *et al.* 1997).

Discussion

THs have been shown to modulate fundamental processes associated with development, including cell proliferation, differentiation, and apoptosis in nearly all tissues (Feng *et al.* 2000, Yen 2001, Furumoto *et al.* 2005). Regarding hepatic homeostasis, it has recently been detected that thyroid status strongly impacted liver regeneration altering the expression

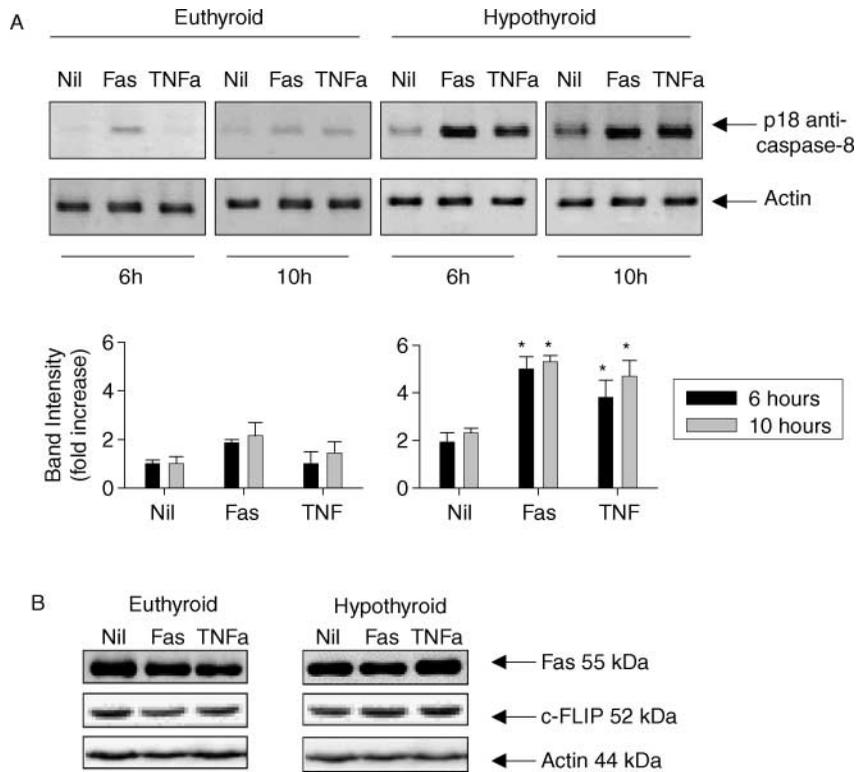


Figure 6 *In vivo* DRLs-induced apoptosis is enhanced in hypothyroid mice. (A) Hypothyroid mice demonstrated higher Fas/TNF α -induced activation of caspase-8 after exposure to Fas/TNF α for 6 and 10 h. The bar graphs represent p18 caspase-8 band intensities from three individual experiments. Results were normalized by actin and expressed as means \pm s.d. * P <0.05 (DRLs-treated versus nil). (B) Fas and cFLIP expressions were determined by immunoblotting of hepatocyte lysates from in hypo- and euthyroid mice. All experiments have been repeated at least thrice and one representative experiment is shown.

and activity of proteins involved in the cell-cycle control (Francavilla *et al.* 1994, Feng *et al.* 2000, Alisi *et al.* 2005). Although THs are known to exert many of their effects via classical regulation of gene transcription, a large number of their actions occur rapidly and are unaffected by inhibitors of transcription and protein synthesis (Yen 2001, Davis & Davis 2002, Incerpi 2005). Analyzing available data indicated that thyroid status influences sensitivity to apoptosis (Lin *et al.* 1999, Qi *et al.* 1999, Antonelli *et al.* 2004, Laoag-Fernandez *et al.* 2004, Verga Falzacappa *et al.* 2006), we questioned whether T₃-activated signaling could suppress DRLs-induced apoptosis. In this study, we found that THs counteract apoptotic effects of Fas/TNF and prevent hepatocyte apoptosis in a rapid non-genomic manner independent of transcriptional activity (Figs 1 and 2). T₃ effects resulted in inhibition of DRLs-induced annexin V labeling, caspase-8 activation, and DNA degradation. As T₃-mediated protection is independent of new protein synthesis, it is probable that the observed inhibition of apoptosis upstream of caspase-8 was due to post-translational mechanisms. We further analyzed the intracellular signaling by which THs modulated DRLs-induced apoptosis in hepatocytes. We found

that T₃ affected several intracellular targets, such as production of cAMP, PKA, ERK, and pHin.

We detected that T₃ dose- and time-dependently increased cellular content of cAMP and activated PKA in hepatocytes

Table 1 Effect of a single i.p. injection of anti-Fas antibodies on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in hypothyroid and euthyroid mice. Values are expressed as means \pm s.d. (n =6 for the saline-treated controls, while n =10 for the anti-Fas-treated groups).

	Treatment	ALT (U/ml)	AST (U/ml)
Mice Euthyroid	Saline	87 \pm 43	172 \pm 84
	Anti-Fas 6 h	2178 \pm 1023*	1567 \pm 927*
	Anti-Fas 10 h	4108 \pm 2256	3212 \pm 1986
Hypothyroid	Saline	116 \pm 41	398 \pm 276
	Anti-Fas 6 h	12 049 \pm 5698	8696 \pm 3955
	Anti-Fas 10 h	15 634 \pm 5994	10 226 \pm 4899

Note: All mice were treated intraperitoneally with either saline or anti-Fas antibodies (0.15 μ g/g body weight). *Statistically different from the anti-Fas-treated hypothyroid mice as determined by non-paired *t*-test (P <0.05).

(Fig. 3). Our observations are consistent with previous reports regarding enhanced intracellular cAMP content in different cells after THs application (Yen 2001, Shih *et al.* 2004). THs modulated the activity of phosphodiesterase (Morgan *et al.* 1982) and mimicked the effects of cAMP-inducing hormones (Muller & Seitz 1983, Daza *et al.* 1997). Furthermore, cAMP-induced PKA activation has been shown to mediate THs effects (Lin *et al.* 1999), such as potentiation of growth hormone receptor signaling in cancer cells (Shih *et al.* 2004). Consistently, we found that the activation of PKA is necessary for T_3 -initiated apoptosis prevention in hepatocytes. In our experiments (Fig. 2), the treatment of hepatocytes with Fas resulted in apoptosis induction and generation of the p18 active form of caspase-8, which are strongly inhibited in the presence of T_3 or cAMP. Pretreatment of the cells with the PKA inhibitor KT-5720 abolished the ability of T_3 to reduce caspase-8 activation and inhibit apoptosis. These results have been confirmed in annexin V and DNA fragmentation assays (Figs 1 and 2).

PKA and its activator cAMP are established mediators of differential sensitivity to apoptosis (Van Beeren *et al.* 1996, Fladmark *et al.* 1997, Kopperud *et al.* 2003, Li *et al.* 2000). Increases in intracellular cAMP have been shown to prevent Fas-induced apoptosis in hepatocytes (Van Beeren *et al.* 1996, Fladmark *et al.* 1997). Moreover, activated PKA induced a prolonged stimulation of hepatocyte proliferation (Kopperud *et al.* 2003). Beyond PKA signaling, an increase in intracellular cAMP induced distinct mechanisms of anti-apoptosis, such as phosphorylation of Bad (Harada *et al.* 1999), glycogen synthase kinase-3 (Feng *et al.* 2000), and Akt pathway (Kopperud *et al.* 2003). Therefore, we cannot exclude the possibility that cAMP/PKA might also regulate additional steps in the T_3 signaling cascade.

There are many possible targets for a modulation of the DRL signaling, although only a few protein synthesis-independent signal-based mechanisms that regulate DRL-dependent apoptosis have been reported. In this case, MAPK/ERK signaling was shown to phosphorylate DRL receptors directly, thereby causing inhibition of its cytotoxic ability (Cottin *et al.* 1999). We detected ERK activation in response to different concentrations of T_3 (Fig. 4). Inhibition of ERK activation by MEK inhibitor U0126 significantly prevented T_3 anti-apoptotic effects. Our results are consistent with the recently published *in vivo* data on the analysis of the phosphorylated forms of ERK. It has been revealed that ERK phosphorylation is induced during hepatic regeneration in euthyroid and hyperthyroid rats, whereas it is negatively regulated in hypothyroid animals (Alisi *et al.* 2004, 2005). Supportingly, several groups found that THs initiated non-genomic induction of ERK via putative membrane GPCR (Lin *et al.* 1999, Shih *et al.* 2004). Whether T_3 prevents DRLs-induced apoptosis through GPCR remains to be determined.

ERK activity might be sufficient to trigger the THs protective effect as was shown for other ERK-activating agents (Sautin *et al.* 2001). However, T_3 -induced activation of

ERK was blocked by PKA inhibition (Fig. 4B). It appears that PKA activation is required for THs-promoted ERK activation and apoptosis prevention. Therefore, we suggest that the increases in intracellular cAMP and the activation of PKA are possible upstream mediators of ERK activation in response to T_3 . Indeed, ERK activation has been linked to cAMP production (Rudolph *et al.* 2004) and PKA activation (Saxena *et al.* 1999). Furthermore, ERK was proposed as a downstream effector of cAMP signaling linked to hepatocyte survival via activation of the small GTPases, Rap1/2 (Caron 2003).

One of the downstream targets of PKA and ERK activation is the regulation of intracellular pHin. It is well known that plasma membrane of hepatocytes has a system of membrane transporters that supports neutral pHin (D'Arezzo *et al.* 2004). Fas-induced apoptosis has been reported to induce cytosolic acidification via inhibition of NHE (Gottlieb *et al.* 1996). Acidic pHin stimulates endonuclease activity and DNA fragmentation (Gottlieb *et al.* 1996). Our presented results confirmed that Fas/TNF are able to induce cytosolic acidification. In contrast, T_3 -treated cells respond with a significant increase in pHin responsible for the recovery and/or maintenance of normal pHin (Fig. 5). We have observed that Fas-induced caspase-8 activation and DNA degradation can be prevented by T_3 that is also able to block Fas-induced intracellular acidification. The T_3 effect on the intracellular pH was completely blocked by EIPA – the specific NHE antiport inhibitor.

Cytosolic alkalization and activation of NHE are known as non-genomic anti-apoptotic mechanisms. For instance, cAMP and PKA have been shown to activate NHE (Borgese *et al.* 1992, Caron 2003). Our results are in agreement with these studies and indicate that THs signaling is an important modulator of pHin that impacted hepatocyte survival. The raise in pHin induced by T_3 should be sufficient to stop DNA degradation and/or prevent further activation of the endonuclease. We also found that T_3 -induced activation of PKA and ERK signaling are necessary for regulation of intracellular pHin in hepatocytes because the PKA and ERK inhibitors, KT-5720 and U0126, blocked T_3 -initiated pHin effects (Fig. 5).

Our results are in agreement with findings that NHE might be stimulated via the mechanism of direct phosphorylation of NHE either by ERK itself or by p90rsk, a newly described ERK-activated kinase (Moor & Fliegel 1999). Furthermore, THs were shown to activate the NHE by a rapid, non-genomic mechanism in rat skeletal muscle (D'Arezzo *et al.* 2004), L-6 myoblasts (D'Arezzo *et al.* 2004), and cat atrial myocytes (Wang *et al.* 2003). This T_3 effect is thought to be mediated non-genomically via activation of ERK (Wang *et al.* 2003, D'Arezzo *et al.* 2004).

DRL-induced hepatic apoptosis is considered as an important cause for a variety of liver diseases, such as viral hepatitis, cholestasis, and ischemic or septic liver injury (Luedde *et al.* 2002). For instance, playing a primary role in inflammation TNF α mediates severe hepatocellular damage

(Zang *et al.* 2000, Ding & Yin 2004). Therefore, the suppression of TNF α -induced hepatocyte apoptosis in clinical aspects is critical. Our *in vivo* data demonstrated that euthyroid mice are more resistant to TNF α /Fas-induced apoptosis when compared with hypothyroid animals (Fig. 6). The level of caspase-8 activation was increased in hypothyroid compared with euthyroid mice after Fas/TNF α injections. The significantly elevated serum AST and ALT levels in hypothyroid mice confirmed the increased hepatocyte sensitivity to Fas-induced apoptosis (Table 1). Taken together, all our results demonstrate that the THs-initiated inhibition of the DRL apoptotic signal occurs at the very proximal stages of apoptotic signaling because T₃-induced signaling seems to suppress apoptosis before activation of caspase-8. Taken together, our *in vitro* and *in vivo* data indicate that T₃-activated signaling pathway mediated by activation of PKA, ERK, and balanced pHin strongly enable survival of hepatocytes. Therefore, THs could be an important target in the therapeutic intervention that can block unwanted hepatocellular apoptotic cell death during inflammation and/or septic liver failure.

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