3,5,3’ TRIIODO-L-THYRONINE PREVENTS PANCREATIC β CELL DEATH VIA NONGENOMIC ACTIVATION OF PI3K SIGNALING PATHWAY

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Introduction

Thyroid hormone action

Thyroid hormones, that is, 3,5,3'-triiodothyronine (T_3) and 3,5,3',5'-tetraiodothyronine (T_4), influence a variety of physiological processes, including cell growth, differentiation and metabolism in mammals, metamorphosis in amphibia and development of the vertebrate nervous system (Zhang and Lazar, 2000; Shi et al, 1998; Koibuchi and Chin, 2000). Thyroid hormones are essential for normal development, growth and metabolism (Yen, 2001). The pro-hormone thyroxine (T4) is synthesized in the thyroid gland together with a small amount of the active hormone 3,5,3′-L-triiodothyronine (T3). However, the majority of circulating T3 is generated by pre-receptor ligand metabolism resulting from activity of the iodothyronine deiodinase enzymes D1 and D2 which convert T4 to T3 by 5′ monodeiodination. In contrast, the D3 enzyme inactivates T4 and T3 by 5′ monodeiodination. Thus, the intracellular level of T3 is dependent on the relative activities of these three deiodinases (Bianco et al., 2002).

The growth-stimulatory effect of T_3 has long been recognized in various cellular systems *in vitro*: in rat cerebellar astrocytes and glia cells (Trentin et al, 2001), in rat pituitary cells (Barrera-Hernandez et al, 1999), in hepatocyte proliferation in rats (Francavilla et al, 1994; Pibiri et al, 2001), and in experiments on gene therapy and re-population of hepatocytes (Ledda-Columbano et al, 2000). Recent studies indicate that all these effects are mediated by the interaction of T_3 with the thyroid hormone nuclear receptors (TRs) (Yen, 2001), ligand-dependent transcription factors and members of the steroid hormone/retinoic acid receptor superfamily (Ribeiro et al, 1998).

The actions of thyroid hormone occur through its binding to the thyroid hormone receptor (TR). TR is a nuclear hormone receptor, which heterodimerizes with Retinoid X receptor, or in some cases, with itself. The dimers bind to the thyroid response elements (TREs) in

the absence of ligand and act as transcriptional repressors. The active form of thyroid hormone, 3,5,3’, triiodo-L-thyronine (T_3), binds to TR with much greater affinity than the more abundant 3,5,3’,5’ tetraiodo-L-thyronine (T_4). Binding of T_3 to TR depresses TRE-dependent genes and induces the expression of target genes. This way of action of TRs and of the other steroid receptors, as ligand-dependent transcription factors, has been traditionally involved in the genomic action of T3, which have a considerable latency with response times in hours to days

Thyroid hormone nongenomic effects

However, a number of steroid hormone mediated actions occur within a far more rapid time frame of only a few minutes and are thus incompatible with the classical genomic model of action (Losel and Wehling, 2003). Such rapid non-genomic responses include the progesterone mediated acrosomal reaction (Osman et al., 1989), vasoregulation by estrogens, mineralocorticoids and glucocorticoids (Shaul, 1999; Hafezi-Moghadam et al., 2002 and Losel et al., 2002), and calcium signalling, thermogenesis and the lipolytic activites of catecholamines by thyroid hormone (Davis et al., 1989; Lynch et al., 1985; Segal, 1989 and Wrutniak and Cabello, 1986). Non-genomic or non-classical steroid hormone effects encompass any action that does not directly effect nuclear gene expression. Such non-genomic actions frequently have a short latency, are unaffected by inhibitors of transcription and translation, have agonist and antagonist affinity and kinetics divergent from the classical nuclear receptor and persist in genetically modified mice that lack the classical nuclear receptors. These non-genomic responses are frequently associated with secondary messenger signalling pathways including the phospholipase C (PLC), inositol triphosphate (IP3), diacyl glycerol (DAG), protein kinase C (PKC) and Ca^{2+}_{I} pathway, the adenylyl cyclase, protein kinase A (PKA) and the cyclic AMP-response element binding protein (CREB) pathway and the Ras, Raf1 serine/threonine kinase,

mitogen activated protein kinase kinase (MEK) and the mitogen activated protein kinase (MAPK) pathway (Losel and Wehling, 2003). It has been suggested that such non-genomic actions might be mediated by either membrane associated isoforms of the classical nuclear receptors or by novel membrane receptors with significantly different agonist/antagonist affinities (Losel and Wehling, 2003). The recent identification of such receptors for progestins and estrogens has highlighted the non-genomic actions of steroid hormone (Li et al., 2003; Zhu et al., 2003 and Zhu et al., 2003).

Estrogens are associated with rapid increases in intracellular calcium in endometrial cells (Pietras and Szego, 1975), induction of the phosphatidylinositol 3-kinase (PI3Kinase), Ser/Thr kinase Akt, endothelial NO synthase (eNOS) pathway in endothelium (Haynes et al., 2000), with Giα activation (Russell et al., 2000 and Wyckoff et al., 2001) and with ERK/MAPK signalling (Watters et al., 1997). It has been uncertain whether these actions are mediated by the classical estrogen nuclear receptor (ER) localized in the cell membrane or by a novel membrane receptor (Pietras and Szego, 1977 and Razandi et al., 2002). Even for thyroid hormones, in addition to the genomic or TRE-mediated effects of T_3, non-nuclear or TRE-independent actions of T_3 have recently been described. Although T3 is known to exert many of its actions through the classical genomic regulation of gene transcription, a number of T3 effects occur rapidly and are unaffected by inhibitors of transcription and protein synthesis (Davis et al., 1989 and Davis et al., 2000; Wrutniak-Cabello et al., 2001). However, the levels of circulating thyroid hormones are tightly regulated and stable and thus rapidly mediated responses must involve regulation of pre-receptor ligand metabolism, ligand membrane transport or receptor availability leading to local cell type specific variation in thyroid hormone signalling.

Non-genomic actions of thyroid hormones have been described at the plasma membrane, in the cytoplasm and in cellular organelles (Henneman et al, 2001).

As steroid hormone action, thyroid hormone non genomic action has been related to various second messenger signaling pathways. They have included the modulation of Na^+, K^+, Ca^{2+} and glucose transport, activation of PKC, PKA and ERK/MAPK and regulation of phospholipid metabolism by activation of PLC and PLD (Kavok et al., 2001) In vitro, independent of protein synthesis, T4 induces IP3 and calcium signalling and augments the effects of IFNγ via PKC and PKA (Davis et al., 1989; Lakatos and Stern, 1991 and Lin et al., 1997). In addition, T4-linked to agarose, which does not cross the plasma membrane, has been shown to activate MAPK by a pertussis toxin sensitive mechanism suggesting the actions of a G protein-coupled thyroid hormone membrane receptor (Lin et al., 1999). In vivo, T4 regulates thermogenesis and the lipolytic activites of catecholamines within 30 min (Lynch et al., 1985 and Wrutniak and Cabello, 1989). Non-genomic effects of thyroid hormone have also been reported in the myocardium and vasculature. T3 enhances cardiac output and reduced systemic vascular resistance in normal adult males within 3 min (Schmidt et al., 2002) and cell culture studies suggest that thyroid hormones rapidly, and non-genomically, regulate the Ca^{2+}ATPase enzyme, the Na^+ channel (I_{Na}) via PKC, the K^+ channel (I_K) via PI3-kinase, the Na^+/H^+ anti-porter via PKC and MAPK and the inward rectifying potassium channel (I_{K1}) (Davis and Davis, 2002). T3 also increases sarcoplasmic reticulum Ca^{2+}, cell shortening, contractility and calcium mediated arrhythmic activity suggesting that T3 has a non-genomic, positive ionotropic and arrhythmagenic effect (Wang et al., 2003).

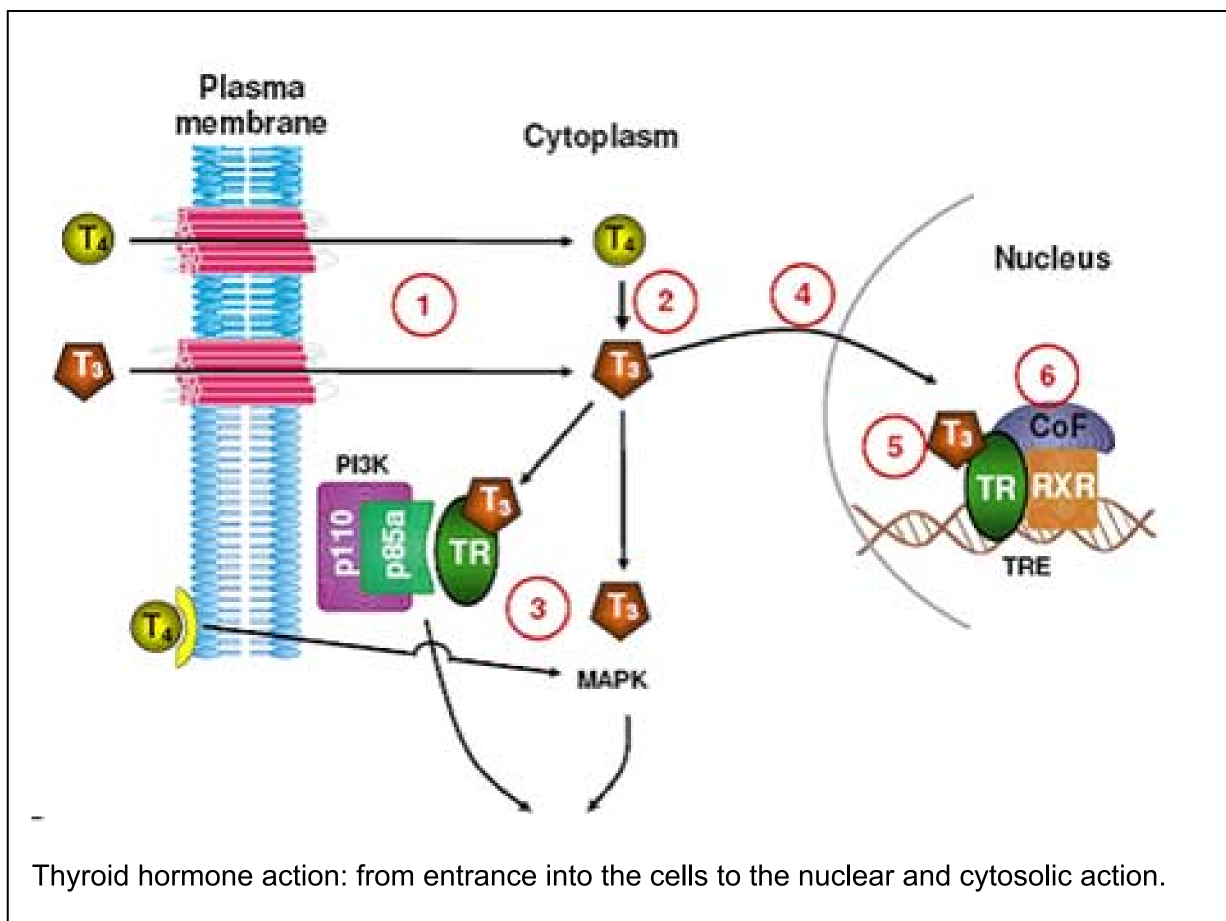
In pituitary cells, T3 has been shown to non-genomically stimulate the ether-a-go-go related gene potassium channel (ERG/KCNH2), which reduces endocrine neuronal excitability, via PI3 kinase and the Rac GTPase, whereas TRH (thyroid releasing hormone) inhibits ERG activity via PKC and the Rho GTPase to increase neuronal excitability (Storey et al., 2002). These observations suggest a possible role for non-genomic T3 signalling in the hypothalamic/pituitary feedback loop..

Although 10% of TRs are cytoplasmic in the absence of T3 (Baumann et al., 2001) no specific membrane associated TR isoform has been identified. Nevertheless, it has been postulated that T4 may interact with a novel G-protein coupled membrane receptor (GPCR) (Lin et al., 1999) leading to rapid serine phosphorylation of nuclear TRs (Davis et al., 2000). This proposed model of Davis and others for the cell surface mediated non-genomic actions of thyroid hormone has been suggested to involve the MAPK and PI3-kinase signalling cascades (Lin et al., 1999 and Storey et al., 2002). This model suggests that, over a time period of 10–30 min, T4 binds a cell surface GPCR (Lin et al., 1999), activates PKC, Ras, Raf1 and MEK resulting in tyrosine phosphorylation, activation and nuclear translocation of MAPK, which in turn phosphorylates a serine residue in the second zinc finger of TR (Davis et al., 2000 and Shih et al., 2001). This phosphorylation results in dissociation of TR from the co-repressors SMRT and NCoR, decreased protease degradation, increased the trascriptional activity (Jones et al., 1994; Leitman et al., 1996 and Ting et al., 1997) and may also regulate RXR heterodimerization (Katz et al., 1995). The nuclear MAPK/TR complex has also been shown to bind and phosphorylate p53 to regulate its transcriptional activity (Shih et al., 2001). In a parallel pathway, T4-activated MEK phosphorylates tyrosine residues in STAT1α and STAT3 (signal transducers and activators of transcription) resulting in their nuclear translocation, further serine phosphoylation by MAPK and activation of gene transcription (Lin et al., 1999). The non genomic actions of thyroid hormone may therefore influence gene transcription by at least three distinct pathways STATs, p53 and TRs. T4 is also thought to initiate DAG formation by phospholipase C resulting in the subsequent PKC activation of phospholipase D (Kavok et al., 2001).

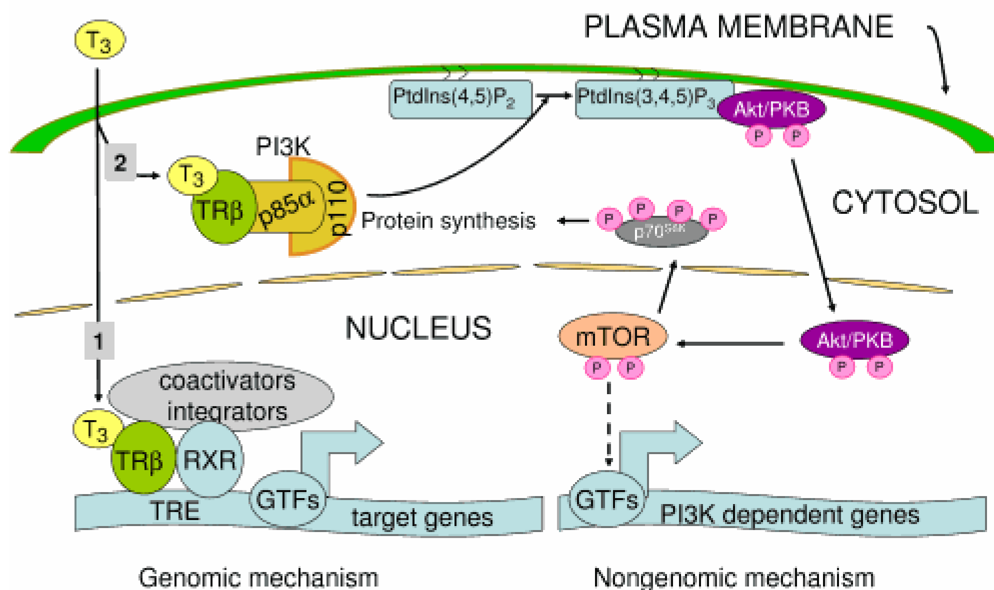
Activation of MAPK by thyroid hormone T3 and rapid modulation of specific cascades imply the existence of membrane receptors for the hormone that may be linked to signal transduction pathways. Membrane binding sites for thyroid hormones were identified years

ago in cell membranes isolated from human and rat hepatocytes (Pliam and Goldfine, 1997, Gharbi J and Torresani J, 1979). It has been recently demonstrated (Bergh et al. 2005) that the extracellular domain of a structural membrane protein, integrin αVβ3, is capable of binding thyroid hormone T4 thus activating the MAPK pathway.

These many observations suggest that the rapid non-genomic effects of thyroid hormone are widespread and may be involved in multiple physiological processes in many different cell types. However, no specific membrane associated TR isoform or thyroid hormone binding G protein-coupled receptors have been identified or cloned and thus the area remains controversial.



Thyroid hormone action: from entrance into the cells to the nuclear and cytosolic action.



(from: Moeller et al, 2006)

Genomic (1) and non-genomic (2) actions of TH are illustrated. Genomic action requires thyroid hormone responsive elements (TREs) for the recognition of genes for direct transcriptional regulation. Non-genomic action is initiated by the TH-dependent activation of PI3K as illustrated in Figure 2. Activation of PI3K leads to sequential activation of Akt/PKB-mTOR-$p70^{S6K}$. Although not well defined, this cascade leads to transcriptional upregulation of some genes such as ZAKI-4α and HIF-1α. GTF: general transcription factors.

Thyroid hormone receptors

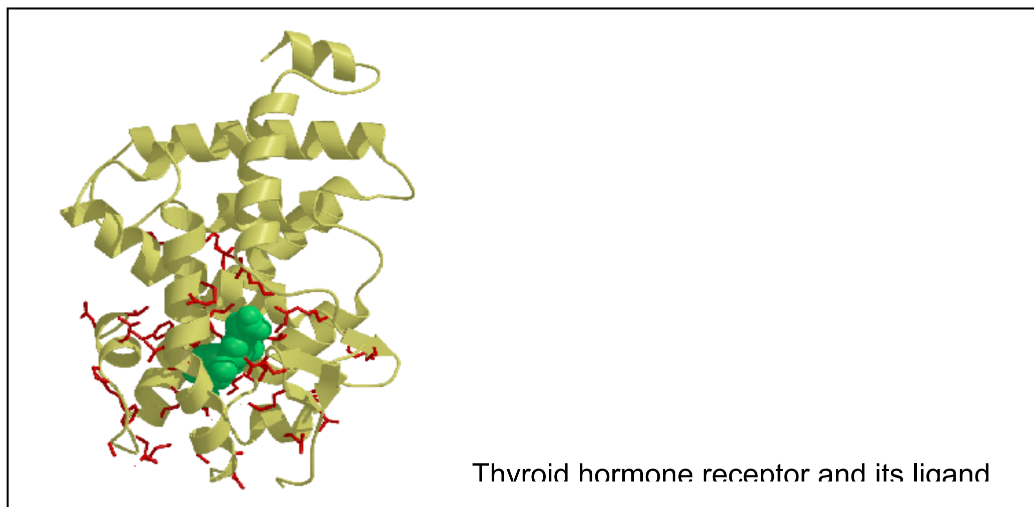
Thyroid hormone receptors (TRs) play a major role in animal physiology. TRs are important and very interesting regulators of diverse aspects, including brain development, hearing, bone growth, morphogenesis, metabolism, intestine, and heart rate in vertebrates. Aberrant functions of TRs induce tremendous defects in these pathways. For example, the human disease of Resistance to Thyroid Hormone (RTH) is a genetically autosomal

dominant inherited syndrome that is caused by mutations in the gene encoding the TRβ. The role of the ligand of TRs, the thyroid hormone, is to modulate the activity and functionality of TRs. T_3 receptors are members of a family of hormone-responsive nuclear transcription factors that are similar in structure and mechanism of action. The functional domains of the receptor include a carboxy-terminal portion, which is important for ligand binding and interactions between receptors, a DNA-binding domain, and an amino-terminal domain, which does not have an identified functional role . The domain that interacts with DNA includes two sequences of amino acids with cysteine residues that form loop structures and chelate a zinc atom, termed "zinc fingers". Several amino acids within this structure serve to determine the specific DNA sequence to which the monomeric receptor binds; others are important for receptor dimerization, the usual functional form for positive regulation. The nature of the DNA sequences that bind T_3 receptors is central to determine which genes are stimulated or inhibited by T_3. In vitro, T_3 receptors bind to DNA as homodimers or as heterodimers with other nuclear proteins, such as the retinoid X receptor (whose ligand is 9-cis-retinoic acid). T_3 disrupts the binding of T_3-receptor homodimers to DNA, but not that of T_3-receptor heterodimers. The ligand-binding domain confers specificity for T_3, and mutations in this domain can result in tissue resistance to thyroid hormone.

There are two TR two genes, THRA and THRB, located on human chromosomes 17 and 3, respectively (Yen, 2001, Harvey and Williams, 2002 and Zhang and Lazar, 2000). The THRA gene generates two mature mRNAs by alternative splicing that encode two proteins, TRα-1 and c-erbAα-2, that differ in their carboxy-termini. TRα-1 is a *bona fide* receptor whereas c-erbAα-2 is unable to bind TH and blocks TR-mediated transcription. The THRB gene encodes two TR isoforms, TRβ-1 and TRβ-2, which most likely are generated by alternative promoter choice (Williams and Brent, 1995) and have differing amino-terminal regions. The major TR isoforms (TRα-1, β-1, β-2) bind T_3 with high affinity

and mediate thyroid hormone-regulated transcription. They also have highly conserved DNA-binding and ligand-binding domains.

Recent findings have elucidated the role of nuclear TRβ1 in mediating some T3 action on cell proliferation (Porlan et al. 2004) and in regulation of specific cell survival cascades (Cao et al. 2005), therefore TRβ1 has been cited as a good candidate for mediating thyroid hormones (TH) nongenomic action.



Thyroid hormone receptor and its ligand

β cell failure in diabetes

Compelling evidences suggest that apoptosis is the principle mode of β-cell death during the development of type 1 and type 2 diabetes (Mauricio and Mandrup-Poulsen, 1998; Chandra et al, 2001). Type 1 diabetes (T1D) is an autoimmune disorder resulting from the destruction of insulin-producing β-cells of pancreatic islets principally by cytotoxic cytokines secreted by infiltrating leukocytes (Mathis et al, 2001). Type 2 diabetes (T2D) is associated with reduced insulin secretion and glucose toxicity that may contribute to β-cell death (Cerasi et al, 2000; Mandrup-Poulsen, 2001).

Type I insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that results from the destruction of insulin-secreting pancreatic islet beta-cells by autoreactive cells and their mediators, such as cytokines (Sjoholm, 1998; Kreuwel and Sherman, 2001). Several recent studies have investigated the role of cell apoptosis in the events leading to the immune-mediated loss of beta-cells either in vitro or ex vivo. Based on studies of pancreatic biopsies taken from diabetes-prone and diabetes-resistant BB/S rats, Lally et al. (2001) showed that the appearance of beta-cell apoptosis in the diabetes-prone group was detectable as early as 68 days of life (long before the onset of diabetes). This was followed by an acceleration of apoptosis rate around 85 days of age in the diabetes-prone group, which co-incided with the onset of hyperglycemia. Similarly, Augstein et al. (1998), using NOD (non-obese diabetic) mice, demonstrated that the frequency of apoptosis of islet cells correlated with the progression of beta-cell destruction and the decline of glycemic control.

Type II diabetes mellitus is characterized by an asymptomatic insulin resistance phase preceding the onset of diabetes, while hyperglycemia occurs when a relative insulin secretory deficiency is manifest (Cerasi et al., 2000; Mandrup-Poulsen, 2001). Studies from autopsies of subjects affected by Type II diabetes demonstrated that in some, although not all individuals with diabetes, there is a marked decrease in beta-cells mass compared to control normoglycemic subjects (Yagihashi, 1996). While a smaller beta-cells mass could result from either an insufficient proliferative capacity or from an excessive rate of cell death, there is evidence of other attributable factors. Several studies have demonstrated that in vitro, free fatty acids, glucose, sulfonylurea, and the islet cell hormone termed amylin can cause beta-cell apoptosis. This suggests that programmed cell death may also be involved in the pathogenesis of Type II diabetes (Schwingshackl et al., 1998; Shimabukuro et al., 1998; Garratt et al., 1999; Federici et al., 2001). Cell apoptosis has also been shown to be the mechanism leading to beta-cells loss in the

Zucker diabetic fatty (ZDF) rat, another model of Type II diabetes. In ZDF rats, the rate of beta-cell proliferation or neogenesis is not reduced and hyperglycemia is detected when the expansion of beta-cells mass is no longer capable of compensating for the degree of insulin resistance and beta-cells apoptosis (Pick et al., 1998; Farilla et al., 2002).

The Akt pathway and its crucial role in β cell function

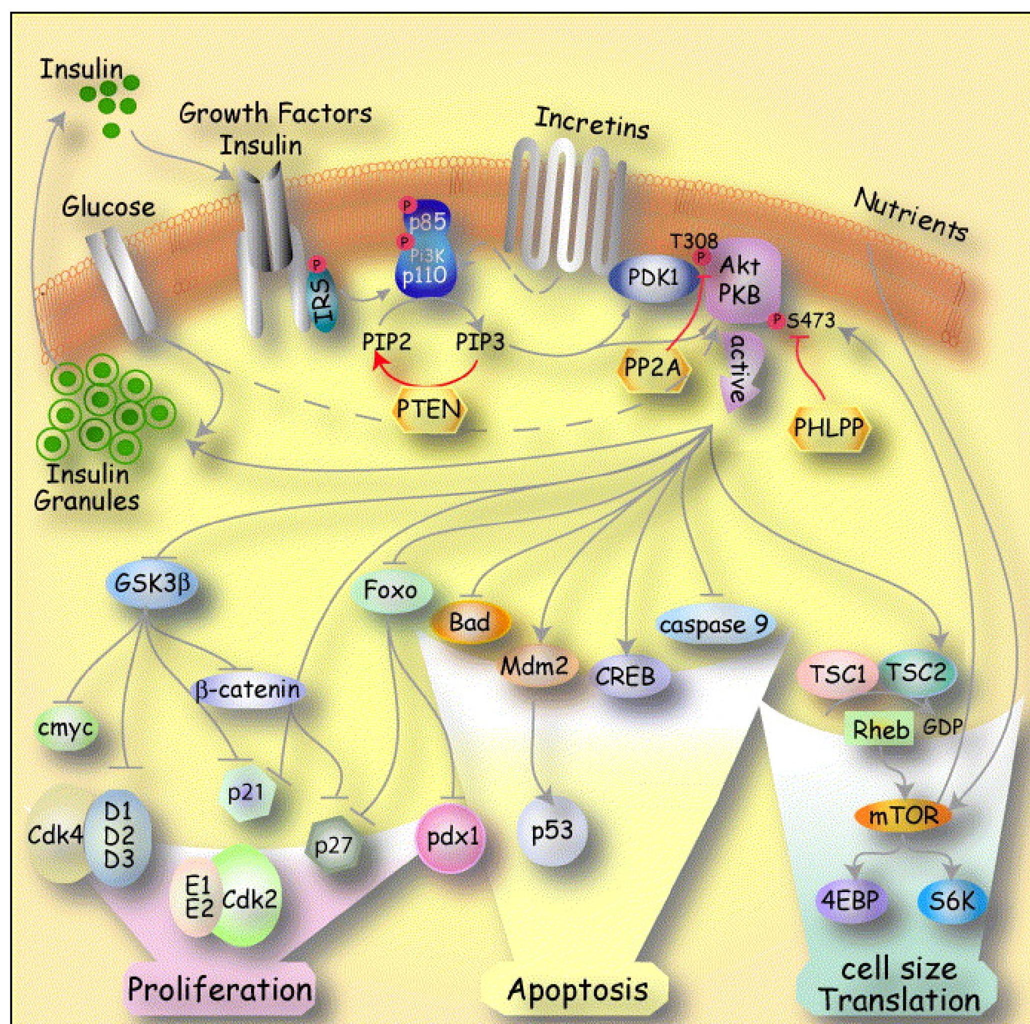
It has recently become evident that PKB/Akt activation plays a pivotal role in β-cell survival (Lingor et al, 2003). In fact, the expression of a constitutively active variant of Akt in β-cells prevents free fatty acid-induced apoptosis (Wrede et al, 2002) and the transgenic β-cell specific expression of the same active Akt is protective against streptozocin-induced diabetes in mice (Tuttle et al, 2001). In pancreatic β-cells, PKB/Akt can be activated by different factors, as IGF-1 and GLP-1 (Buteau et al, 2001; Giannoukakis et al, 2000). When activated, via a cAMP-dependent or independent mechanism, Akt plays a central role in mediating a number of cellular processes, including mitogenesis, survival and differentiation. Most of the downstream proteins of Akt activity are likely expressed in β-cells and might be considered as molecular targets in preventing β-cell death.

Thyroid hormone T3 is also involved in the PI3K pathway: it has recently been reported to regulate the Na,K-ATPase activity via PI3K in alveolar epithelial cells (Lei et al. 2004) and to activate the protein kinase B via PI3K, in human fibroblasts (Cao et al. 2005). Estrogen and retinoic acid have also been recognized to activate PI3K rapidly through the nontranscriptional action of their receptors (Simoncini et al. 2000; Sun et al. 2001; Haynes et al. 2003; Lopez-Carballo et al. 2002). Furthermore, estrogen receptor-α was demonstrated to activate PI3K through binding with p85α either in a ligand-dependent manner in endothelial cells (Simoncini et al. 2000) or in a ligand-independent manner in epithelial cells (Sun et al. 2001).

Cellular processes such as proliferation, survival and glucose metabolism induced by different hormones and growth factors are dependent on the activation of PI-3 Kinase generating D3-phosphorylated phosphoinositides that are capable of binding PKB/Akt . (Ueki K et al. 2002; Liang et Slingerland, 2003). Akt has been implicated as a critical mediator of insulin-stimulated glucose uptake, suppression of apoptosis, stimulation of glycolysis and activation of glycogen and protein synthesys in various cell-culture systems (Coffer PJ et al. 1998). It has recently become evident that PKB/Akt activation plays a pivotal role in β-cell survival (Lingor et al. 2003) and growth.; moreover recent evidences reviewed in Elghazi et al. (2006) underscores the importance of Akt for regulation of β cell mass and function. In pancreatic β-cells, PKB/Akt can be activated by different factors, such as IGF-1 and GLP-1 (Buteau et al. 2001; Giannoukakis et al. 2000), and it is directly activated by glucose (Dickson and Rhodes, 2004). When activated, via a cAMP-dependent or independent mechanism, Akt mediates a large number of cellular processes, including mitogenesis, survival and differentiation.

Activation of Akt/PKB results in phosphorylation of many substrates that control various biological signaling cascades including insulin-mediated glucose transport, protein and glycogen synthesis, proliferation, cell growth, differentiation, and survival (Ahlgren et al., 1996 and Woodgett, 2005). The loss of Akt1/PKBα resulted in normal carbohydrate metabolism and both fetal and postnatal growth defect (Chen et al., 2001 and Cho et al., 2001b). In contrast, Akt2/PKBβ-deficient mice exhibited impaired glucose disposal and diabetes due to a reduction in insulin-stimulated glucose uptake in peripheral tissues and β-cell failure (Cho et al., 2001a). These results suggest that Akt/PKB could play a role in β-cell adaptation to insulin resistance states. Akt1/Akt2 double-knockout mice showed severe growth deficiency, impaired skin development, and skeletal muscle atrophy, and died shortly after birth. The generalized defect in conventional knockouts and expression of different Akt/PKB isoforms with similar biochemical characteristics makes the alterations

in β-cell function in these animal models difficult to interpret. Moreover, overexpression of constitutively active Akt/PKB in β-cells in transgenic mice resulted in augmented β-cell mass by increase in proliferation and cell size and these mice were resistant to streptozotocin-induced diabetes (Bernal-Mizrachi, Wen, Stahlhut, Welling, & Permutt, 2001; Tuttle et al., 2001). Transgenic mice with reduction of Akt/PKB activity in β-cells exhibited normal β-cell mass and defective insulin secretion (Bernal-Mizrachi et al., 2004). Based on these results and experiments in cell lines, it is likely that Akt/PKB is a major mediator of the responses to insulin, insulin-like growth factor (IGFI), incretins, and glucose. The molecules and the mechanisms involved in the regulation of β-cell mass and function by Akt/PKB in β-cells are ill defined, but a specific role for this kinase in regulating pancreatic beta cell proliferation, neogenesis, apoptosis and size together with insulin secretion can be discussed.



In vivo and in vitro experiments suggest that Akt/PKB could mediate the proliferative signals induced by activation of Irs2 signaling (Kitamura et al., 2002 and White, 2002). While the regulation of cell cycle by Akt/PKB has been studied in other model systems, there is little information about its role in cell cycle progression in β-cells (Chang et al., 2003). Recent experiments show that activation of Cdk4 is indispensable for β-cell proliferation induced by Akt/PKB (Bernal-Mizrachi, unpublished observations). One of the mechanisms used for cell cycle regulation by Akt is the phosphorylation and inhibition of glycogen synthase kinase 3β (GSK3β), which results in direct regulation of some main cell cycle regulators such as cyclin D1, c-Myc, β-catenin (Chang et al., 2003) and p21.

Akt/PKB also regulates cell cycle by phosphorylation and inactivation of the Foxo family of forkhead transcription factors (AFX/Foxo4, FKHR/Foxo1, and FKHR-L1/Foxo3a) (Martinez-Gac et al., 2004), even in this case leading to the regulation of a specific cell cycle player: $p27^{kip1}$ (Chang et al., 2003). In particular the PI3K-Akt/PKB pathway elicits events that reduce the abundance of $p27^{Kip1}$ by regulation of transcription, translocation and protein levels, indicating that this cell cycle inhibitor can contribute to β-cell failure during the development of type 2 diabetes in insulin resistant models (Uchida et al., 2005). Similar to $p27^{Kip1}$, Akt/PKB phosphorylates $p21^{CIP}$, thereby inducing cytoplasmic translocation and stabilization (Chang et al., 2003).

Neogenesis of β-cells occurs from progenitor cells arising from pancreatic ducts, but the mechanism behind this observation remains unknown (Bonner-Weir, 2000). Although recent observations suggest that neogenesis is not a major contributor in the maintenance of adult β-cell mass (Dor, Brown, Martinez, & Melton, 2004),the role of neogenesis in regenerative conditions is still a matter of debate. Even in this mechanism induction of Irs2 and Akt/PKB signaling could play a crucial role, since its activation in ductal cells of pancreatectomized mice has been associated to neogenesis (Jetton et al., 2001).

The Akt/PKB signaling is one of the critical pathways regulating cell survival, and its importance in β-cells has been suggested by increased apoptosis observed in *Irs2*$^{-/-}$ mice. In β-cells, Akt/PKB signaling mediates anti-apoptotic effects induced by diverse agents such as glucose, GLP-1, IGF-1, and insulin (Brubaker & Drucker, 2004; Dickson et al., 2001, Srinivasan et al., 2002 and Wang et al., 2004); moreover the activation of Akt/PKB signaling protects β-cells against fatty acid-induced apoptosis and modulates survival to endoplasmic reticulum stress (Srinivasan et al., 2005 and Wrede et al., 2002). Glucose activation of Akt/PKB results in part by a paracrine/autocrine stimulation of the insulin receptor suggesting that glucose can be an important modulator of β-cell survival (Ohsugi et al., 2005). Akt/PKB affects survival by directly regulating members of the Bcl-2 family. Phosphorylation of BAD inhibits the pro-apoptotic activity by releasing it from the Bcl-2/Bcl-X complex and binding to 14-3-3 proteins. Increased apoptosis was associated with decreased Bcl-X_L and Bcl-2 expression in *pdx1*$^{+/-}$ islets, suggesting a potential link between Akt, pdx1, and survival (Johnson et al., 2003).

The regulation of cell size by Akt/PKB is mediated by activation of the mammalian target of rapamycin (mTOR), which targets ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1), key regulators of protein translation and cell size. Mice deficient in S6k1 exhibited glucose intolerance and hypoinsulinemia associated with a 15% reduction in β-cell size, similar to the phenotype in dS6K-null *Drosophila* (Pende et al., 2000). Moreover, overexpression of a constitutively active form of Akt/PKB resulted in increased cell size suggesting that S6K1 relates some of the growth signals induced by Akt/PKB. The potential role of mTOR/S6K signaling in proliferation remains controversial. There is substantial evidence for a role of insulin/IGF signaling in insulin secretion. Experiments in insulinoma cells suggest that the regulation of insulin secretion by PI3K/Akt/PKB is complex and no consensus has yet been achieved, however reduction in Akt/PKB activity in β-cells by overexpression of a kinase-dead Akt/PKB mutant results in

an insulin secretory defect (Bernal-Mizrachi et al., 2004). This secretory dysfunction is similar to that observed in type 2 diabetes but the mechanisms are still unclear.

Preventing diabetes: new strategies

It has become apparent that a number of strategies for preventing the apoptosis of islets and preserving β-cell functions may have therapeutic relevance in preventing the pathogenesis of diabetes (Hui et al, 2004, Giannoukakis et al, 2002). To this aim, various molecules have been studied for their ability to regulate the cellular changes leading to apoptosis in β-cells. Recently, the IGF I / IGF BP3 complex has been recognized to enhance β-cell replication and β-cell survival after exposure to proapototic agents, which indicates this complex as a survival factor for β-cells undergoing apoptosis (Chen et al, 2004). Glucagon-like peptide-1 (GLP-1) is a growth factor for β-cells, but it also has a powerful antiapoptotic action (Hui et al, 2003). The role of glucose is not still clear; however, a pathway activated by glucose and capable of enhancing β-cell survival against apoptosis has been studied (Srinivasan et al, 2002). Some other molecules, as cysteine (Rasilainen et al, 2002), carbon monoxide (Gunther et al, 2002) and the nuclear factor kappa B (Chang et al, 2003) have shown similar protective properties during the apoptotic process in β-cells *in vitro*.

The characterization of the specific apoptotic pathways involved in this disease has led to the individuation of various drugs able to mimic cell death occurring in diabetes. In our study we used streptozocin, S-Nitroso-N-Acetylpenicylamine (SNAP) and Hydrogen peroxide (H_2O_2) as models to study the putative antiapoptotic action of T_3. As demonstrated by several studies, indeed, these agents are able to activate different apoptotic and necrotic pathways in β-cells *in vitro* (Chang et al, 2003; Oyadomari et al, 2001; Rasilainen et al, 2002).

In recent years, numerous studies focused on promoting β-cell survival to counteract the β-cell mass decrease occurring in diabetes. Recent advances have indicated the signal transduction via insulin receptor substrate-2 (IRS-2) and downstream protein kinase B (PKB, also known as Akt) as critical to the control of β-cell survival.

Might T3 play a role in promoting β cell survival ?

Several factors (IGF 1, IGF 2, GLP-1, etc.), known for their proliferative properties and/or their action on cell differentiation, have also been shown to be capable of interfering with the sequence of events leading to cell apoptosis and of promoting cell survival (Giannoukakis et al, 2000; Jill et al, 2000; Perfetti et al, 2000). In this study we hypothesized that thyroid hormone T_3 ─ whose effects on cellular proliferation are well known ─ might affect the regulation of specific apoptotic pathways in the insulin secreting cells rRINm5F and hCM. Our evidences suggest the involvement of the PI3K pathway in mediating this novel survival action of T3; in particular we have shown that T3 is able to induce the phosphorylation of Akt in the islet cells hCM and rRINm5F (Verga Falzacappa et al. 2006). Additionally Cao et al. (2005) have demonstrated that T3 is specifically able to activate Akt in a nongenomic manner. Interestingly, this activation implies the recruitment of the thyroid receptor β1 by the PI3K, suggesting a mechanism similar to those identified for steroids. The PI3K expression is mainly cytoplasmic, and it might be possible that cytoplasmic or cell membrane-bound TRs are responsible for the recruitment of PI3K. Extranuclear TR expression, indeed, has been described long since (Davis et al. 2000; Heery et al. 1997; Zhu et al. 1998).

To examine the specific pathway via which T3 can activate Akt in pancreatic beta cells and to understand further the possible implication of a nongenomic action of T3 in these cellular systems, we suggest a specific role for a “cytoplasmic” thyroid hormone receptor

β1 implicated in the PI3K pathway and able to mediate nongenomic actions of T3 in pancreatic beta cells.

Aim of the study

A major contributing factor in the pathogenesis of both type 1 and type 2 diabetes is an acquired inadequateβ-cell mass that no longer is able to compensate for insulin resistance and/or insulin-secretory demand. Reduced β-cell mass in those cases is predominantly caused by an increased rate of β-cell apoptosis. Therefore, an anti-apoptotic means of promoting β-cell survival is conceivably a viable therapeutic approach to treat or even prevent the onset of type 1 and type 2 diabetes. To this aim several factors (IGF 1, IGF 2, GLP-1, etc.), known for their proliferative properties and/or their action on cell differentiation, have been shown to be capable of interfering with the sequence of events leading to cell apoptosis and of promoting cell survival (Giannoukakis et al, 2000; Jill et al, 2000; Perfetti et al, 2000).Given the well known action of thyroid hormones on cellular processes such as mitogenesis and differentiation, and since we previously demonstrated (Misiti et al, 2005) a differentiative role for thyroid hormone T_3 in pancreatic ductal cells, as β cell precursors; in this study we hypothesized that T_3 might influence some cellular processes in pancreatic β cells. In particular, considering a possible mitogenic role for the hormone in these cells, we intended to verify whether an hormone treatment could counteract an apoptotic process ongoing in the insulin secreting cells rRINm5F and hCM.

The Protein Kinase B (Akt) in pancreatic β-cells plays a critical role in controlling β-cell growth and survival. Indeed, inhibition of PKB activation in β-cells is evidently linked to increased β-cell apoptosis. As such, a therapeutic strategy to alleviate insulin resistance by preventing inhibition of IRS/PI3K/PKB signaling should also have the added bonus of promoting β-cell survival. To this aim we decided to investigate the involvement of this crucial factor in the T_3 survival action on β cells, and further to analyze the mechanisms underlying the relationship between thyroid hormones and Akt in pancreatic β cells.

Aim of this study was to investigate the effect of T3 treatment on pancreatic β cells, in particular to analyze the ability of T_3 to affect β cell proliferation and survival when a diabetes-like apoptosis is induced and to characterize the specific molecular targets and pathways involved.

Materials and Methods

3,5,3'-triiodothyronine (T_3) was obtained from Sigma-Aldrich (St. Louis, MO, USA). BrdU, Hoechst, streptozocin, S-Nitroso-N-Acetylpenicylamine (SNAP), H_2O_2, LY-294,002 hydrochloride, PD098059, Bisphenol-A dimethacrylate (BPA) and cycloheximide (CHX) were obtained from Sigma-Aldrich; Rp-cAMP was purchased from Calbiochem (La Jolla, CA).

Cell Culture

Human insulinoma cell line CM was obtained from Dr. MG Cavallo (Baroni et al, 1999; Cavallo et al, 1996); rat insulinoma cell line RINm5F (Cat. No. CRL-11605) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA).

rRINm5F cells were cultured in RPMI 1640 (ICN, Eschwege, Germany) complemented with 10% Fetal Bovine Serum (ICN); hCM cells were cultured in RPMI 1640 (ICN) containing 5% FBS; all cell culture media were supplemented with L-Glutamine 2 mM according to manufacturer's instructions, and Penicillin 100 μg/ml-Streptomycin 50 μg/ml. Cells were maintained at 37°C under humidified condition of 95% air and 5% CO_2.

To determine the effects of T_3 on cell proliferation, cells were cultured to 60% confluence and exposed to the hormone T_3 or to vehicle alone after 12h. Every 24 hours, fresh aliquots of T_3 (10^{-3}M) were added to culture medium in all the experiments.

In the apoptosis studies, cells were plated in a 96 well plate, and after 12h the hormone T_3 or vehicle alone were added to culture medium; the cells were then exposed to streptozocin (15mM) for 2 hours, SNAP (200μM) for 24h and H_2O_2 (50 μM) for 30 min. in the presence or the absence of the thyroid hormone T_3 10^{-7}M.

To investigate the specific pathways via which T_3 exerts its antiapoptotic and proliferative effect, cells were exposed singly to each inhibitor, i.e., LY-294,002 hydrochloride 50 μM

(PI-3 K inhibitor), PD098059 50 µM (MAPK inhibitor), Rp-cAMP 50µM (PKA inhibitor) and Bisphenol-A 10 µM (T_3 analogue), added only once at the beginning of the individual experiments. Each inhibitor has been utilized as previously described by Hui et al, 2003 and Moriyama et al, 2002.

Streptozocin and H_2O_2 were solubilized and diluted in water immediately before performing each experiment. T_3, SNAP, LY-294,002, PD098059, Rp-cAMP and Bisphenol-A were resuspended in stock solution, according to manufacturer's instructions, and stored at -20°C. Control cultures were grown under the same culture condition as treated cells, but in the absence of drugs. The final concentrations of $NaCl_2$ 0.95%, methanol, ethanol and dymethylsulfoxide were identical in every culture, irrespective of the particular treatment group.

To determine the effects of T_3 (10^{-7}M) on Akt activation in the cell lines, cells were cultured to 60% confluence and after 12h exposed to the hormone T_3, LY-294,002 hydrochloride 10 µM, Bisphenol-A 100 nM, 1µM, 10 µm, TRIAC 100 nM, 1µM, 10 µM, CHX 3 mM added only once, at the beginning of the individual experiments. Each inhibitor has been utilized as previously described by Hui et al, 2003, Moriyama et al, 2002 and LY-294,002, Bisphenol-A and TRIAC were resuspended in stock solution, according to manufacturer's instruction, and stored –20°C. Control cultures were grown under the same culture condition as treated cells but in the absence of drugs. The final concentration of methanol , ethanol and DMSO were identical, in every culture, irrespective of the particular treatment group.

RNA Isolation and RT-PCR Analysis

Total cellular RNA was isolated from rRINm5F cells by using Total RNA Isolation kit (Promega, Madison, WI), according to the manufacturer's protocol. RNA (2 µg) was then subjected to reverse transcription (RT) by using a cDNA synthesis kit OmniScript

(QIAGEN, Chatsworth, CA). Amplification was performed, after a first denaturing step at 94°C for 5 min., for 30 cycles for the cell-cycle-related molecules (cyclins A and D1, p27) and 23 cycles for β–Actin, at a denaturing temperature of 94° for 1 min., at annealing temperature (Table 1) for 1 min. and at an extension temperature of 72° for 30". PCR products were electrophoresed onto a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV light. The relative intensity of the bands was quantitated by densitometric analysis (Total Lab, Nonlinear dynamics, New Castle, UK) and normalized to the co-amplified β–Actin cDNA fragments. Oligonucleotide primers used in these experiments are presented in Table 1.

Immunofluorescence Microscopy

Cells were plated onto multichamber slides (BD Falcon) and culture for 72 hours for Insulin staining or for 12 hours and then exposed to the hormone treatment for the indicated times f or the other protein staining. Slides were washed in PBS 1x, (CAMBREX) air-dried, fixed and permeabilized with acetone/methanol (1:1 vol/vol stored at -20°) for 10 min., and then rehidrated in PBS 1x. Slides were then incubated with 20% fetal bovine serum in PBS 1x for 20 min. and washed three times in PBS 1x. Thereafter slides were stained with primary antibodies (rabbit-anti-PI3K Sta. Cruz, mouse anti TRβ1 Sta. Cruz, mouse anti Akt Sta. Cruz, rabbit anti Phospho Akt 1/2/3- Ser 473 Sta. Cruz) at a dilution of 1:100, for 45 min at RT in a humid chamber. After three washes in PBS 1x, slides were incubated with secondary antibodies (FITC conjugated rabbit anti-mouse IgG, TRITC conjugated rabbit anti mouse IgG, TRITC conjugated swine anti-rabbit IgG, DAKO,Denmark) for 45 min at room temperature in dark, at a dilution of 1:40. Immunofluorescence analyses of cell slides was carried out using an inverted fluorescence microscope equipped for confocal microscopy (Olympus Flowview FV500). Images were processed by the Adobe Photoshop

software.Negative controls including omission of the primary antibody were also performed.

Cell Growth

Cell growth was analyzed by determining Trypan Blue negative cell number in a Thomas's hematocytometer. Rat RINm5F and human CM cells were plated as a monolayer at a density of 4×10^4 and 2.5×10^4 , respectively, in 6 multiwells. After 12h, they were exposed to different doses of T_3 (10^{-9},10^{-7},10^{-11} M) or to vehicle alone. At 24, 48 and 72 hours of continuous exposure, viable cells were harvested and counted. Cell number was determined, and data presented as means ± SD.

The effect of T_3 on cell growth was quantified by assuming that the considered populations were showing exponential growth. The increase in cell numbers was analyzed by fitting to the exponential growth equation:

$$\ln(N) = \ln (N_0) + kt$$

where N = cell number at time t, N_0 = cell number at t_0, K = growth constant

The doubling time td, is given by:

$$Td = \ln (k)/0.963$$

MTT Assay

Cell proliferation was studied by MTT assay (Promega). Cells were plated in 96 multiwells at a 70% confluence. After 12h, cells were exposed to drugs and T_3 for the indicated times. A solution of a tetrazolium salt was added to the culture medium and, after 3h, the metabolic formazan product was solubilized in an organic solution. After 1h of solubilization, the absorbances at 570 and 630 nm were recorded by using a 96 well plate reader.

BrdU Staining

Cell proliferation was determined additionally by BrdU staining. Cells were plated at a 70% confluence onto a multichamber slides (BD Falcon) and then cultured in the presence of T_3 or vehicle alone; during the last 30 min. of the hormone treatment, cells were incubated with BrdU 10μM (Sigma). The slides were then air-dried and cells were fixed in ethanol 70% for 30 min. at 4°C. Slides were then washed in PBS 1x (ICN) and incubated with HCl 3N for 25 min. at RT; the reaction was then neutralized with borax-borate buffer (pH 9.1), and slides were washed in PBS 1x. Slides were incubated sequentially with PBS 1x, FBS 20%, Tween 20 0.5% for 15 min. at RT, and then with mouse monoclonal antibody anti-BrdU (Roche Diagnostic) 1:200, for 1 hour at room temperature. After three washes in PBS 1x, slides were incubated with the secondary antibody anti-mouse TRITC conjugated (Dako), 1:40, for 1h at room temperature in dark. Slides were then washed in PBS 1x twice and stained with Hoechst 1 μg/ml for nuclear detection. Localization and intensity of fluorescence were observed with a fluorescent microscope (Leika). Images were taken by a Canon Digital Camera and processed by the Adobe Photoshop software. Negative controls including omission of the primary antibody were also performed.

TUNEL Assay

Tunel assay was performed by using the In situ Cell Death detection kit (Roche, Basel, CH).

Cells were plated at a 70% confluence onto multichamber slides (BD Falcon) and then exposed to specific drugs and to the hormone. After the removal of the culture medium, the slides were washed in PBS 1x (ICN) and fixed in paraphormaldeide 4% in PBS 1x for 30 min. at Room Temperature. The slides were then washed in PBS 1x and incubated in triton 0.1 % in Sodium Citrate 0.1 % for 2 min. on ice; the slides were then washed in PBS 1x and air-dried. The slides were incubated with the Tunel mixture according to

manufacturer's instructions, for 1h, at 37°C in dark; then samples were washed and counter-stained with Hoechst (1 µg/ml). Tunel positivity was visualized with a Leika (Germany) fluorescence microscope and the images were taken by a Canon digital camera.

Western Blot Analysis

Approximately $3x10^6$ cells were lysed for 30' at 4°C in buffer containing 1% Tween 20, 10% glycerol, 150 mM NaCl, 50 mM Hepes pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM $NaVO_3$, 1 mM Phenylmethylsulfonyl fluoride, protease inhibitors. The lysates were centrifuged at 12,000 rpm for 30' and the total cellular protein content was measured by using the Bradford method (Bio-Rad, Richmond, CA). Cytosol-nuclear protein separation was obtained by nuclear/cytosol fractionation kit (MBL, International Corporation Woburn, MA, US) following manufacturer's instruction. 40 ÷ 60 µg of total extracts and 30 µg of nuclear or cytosol proteins per sample were loaded onto a 10-12% SDS-polyacrylamide gel, electrophoresed, and then blotted onto PVDF membranes (Bio-Rad). As a loading control, proteins were stained with Ponceau. Filters were blocked for non-specific reactivity by incubation for 1h at RT in 5% non-fat dry milk dissolved in PBS 1x, Tween 20 0.1 %, and then incubated for 16h at 4°C, with: TRα1 (S.ta Cruz Biotechnology, Inc., San Diego, CA., 1:500), TRβ1 (S.ta Cruz 1:500), Cyc A (S.ta Cruz, 1:200), Cyc D1 (BD Pharmingen, Franklin Lakes, NY, USA, 1:250), Cyc E (BD Pharmingen, 1:250), p27 (BD Pharmingen, 1:500), Bax (BD Pharmingen, 1:500), Bad (BD Pharmingen, 1:500), Caspase 3 (S.ta Cruz, 1:500), Bcl-2 (S.ta Cruz, 1:500), Bcl-X_L (BD Pharmingen, 1:500), Akt (Sta. Cruz, 1:500), Phospho Akt 1/2/3-Thr 308 (S.ta Cruz, 1:200), Phospho Akt 1/2/3-Ser 473 (Sta. Cruz, 1:500), GSK3α (S.ta Cruz, 1:500), pGSK3-α-Ser 9 (S.ta Cruz, 1:500), and Histone H1 (Sta Cruz, 1:1000), and 1h at RT with β-Actin (Sigma, St. Louis, MO, USA 1:1000) and α-tubulin (Sta. Cruz, 1:1000) ─ diluted in 5% milk, PBS

1x, Tween 20 0.1% . After three washes in PBS 1x, Tween 20 0.1%, the membranes were incubated for 45 min. with the secondary HRP antibodies (anti-goat, anti-mouse, anti-rabbit - Sigma) 1:4000 in milk 5%, PBS 1x, Tween 20 0.1% for 45' at RT. Immunoreactivity was visualized by the ECL immuno-detection system (Amersham Corp, Arlington Heights, IL), following manufacturer's instructions.

The relative band intensity was evaluated by densitometric analysis (Totallab, Nonlinear dynamics) and normalized to β-actin.

Immunoprecipitation

Cells were lysed in 1% NP40, PMSF 0,2mM, NaF 10mM, pepstatin 0,7μg/ml, aprotinin 25μg/ml in PBS1X. After 10' on ice, samples were sonicated and centrifuged at 12000 xg for 15'. The total cellular protein content was measured using Bradford method (Bio-Rad, Richmond, CA, USA). 400 μg of cell lysate were incubated for 2h with 30μl G-protein (Roche Diagnostics, Basel, CH).

After preclearing, the extracts were incubated overnight at 4 ^{o}C with mouse anti TRβ-1 antibody (Sta. Cruz, 1 μg) and 30μl freshly prepared G-protein. The immunoprecipitates were electrophoresed onto an 8% SDS-Polyacrilamide gel, and blotted onto PVDF membrane (Biorad). The membranes were blocked with 5% non-fat dry milk in PBS1X-Tween 20 0,1% for 1h at room temperature and probed with rabbit anti PI3K-p85α (1:500) or anti PI3K-p110 (1:500), and afterwards with mouse anti-TRβ1 antibody (Sta. Cruz, 1:500) diluted in 5% non-fat dry milk in PBS1X-Tween 20 0,1% overnight at 4°C under gentle rocking. After washing in PBS 1X-Tween 20 0,1%, the membranes were incubated with the secondary antibody HRP conjugated (anti-mouse Sigma, anti-rabbit Sigma) 1:4000 in 5% milk, PBS1X, Tween 20 0,1%, for 1h at RT. Immunoreactivity was visualized by the ECL immuno-detection system (Amersham Corp.) following manufacturer's instructions.

PI3K kinase assay

Cells were grown on 100 mm dishes and exposed to T_3 10^{-7}M, LY 10 μM or vehicles alone for 30 min. At the end of the treatment cells were lysed and PI3-K activity was estimated using a commercially available PI3-K ELISA kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions. Protein A beads were then utilized for the PI3-kinase ELISA (Echelon Biosciences Inc, Salt Lake City, UT) following manufacturer's instruction. Briefly, cells were lysed with a low stringency buffer and 100 μg of lysates were incubated with 1 μg of TRβ1 antibody for 1h at 4°C . Then protein G-agarose beads were added to equal amounts of total protein, and the samples were rocked (4°C) for 16 h. The immunoprecipitated TRβ1 were incubated with phosphatidylinositol 4,5-bisphosphate substrate and reaction buffer for 1 h 30 min. The amount of PIP_3 formed from phosphatidylinositol-4,5-bisphosphate by PI3-K activity was detected using a competitive ELISA. The obtained OD values were inversely proportional to the amount of PI(3,4,5)P3 produced by PI3-K activity . Enzyme activity was estimated by comparing the values from the samples to those in the standard curve.

RNA interference

Cells were plated onto 6 multiwells and grown in complete medium, after 24h cells were transfected with siRNA SmartPool THRB (Dharmacon, Lafayette, CO, US). Transfection was performed by incubating cells with 200 pmol of siRNAs in 2 ml of serum free transfection medium using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). After 3h of incubation, normal medium supplemented with serum replaced transfection medium and cells were grown for 30h before starting T3 treatment. Subsequently cells were lysed for Western Blot analysis and samples were analyzed as described

Statistical Analysis

The data were presented as means ± SD. A comparison of the individual treatment was conducted by using Student's *t* test or, if there were more than two groups, by one-way ANOVA , followed by Dunnett or Tukey post-hoc analyses. A P value <0.05 was considered significant.

Results

T_3 induces cell growth and proliferation in islet β-cells and provokes a shortening of the cellular doubling time

The presence of the thyroid receptor isoforms TRα1, TRα2 and TRβ1 and the ability to synthesize insulin have been verified in the insulinoma cell lines rRINm5F and hCM cells.

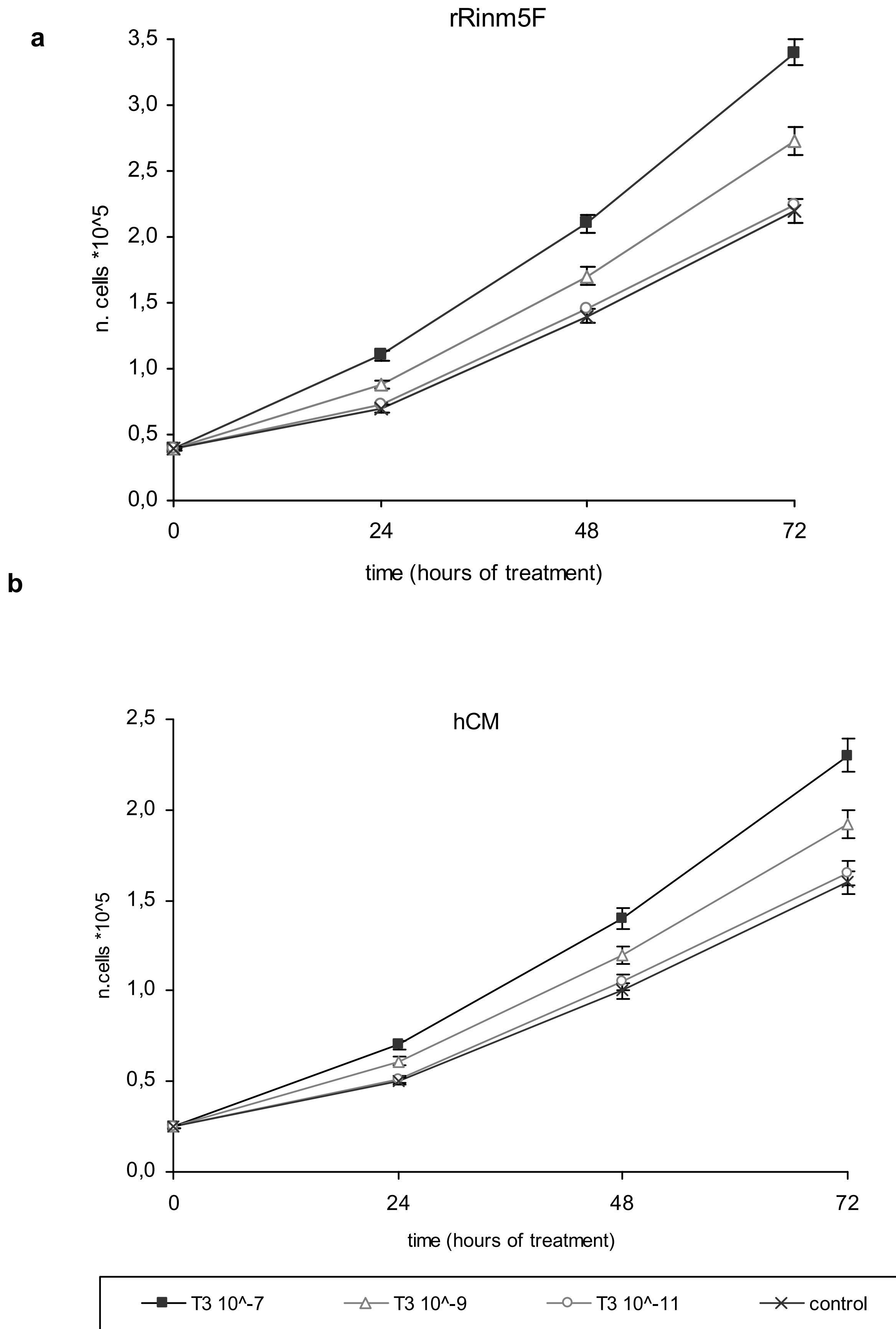
As a first step towards the understanding of the effect of T_3 on insulin secreting cells, we examined the effect of T_3 on the growth of rRINm5F and hCM cells by culturing the cells in the presence or the absence of the hormone treatment.

Cells were treated for three days with different concentrations (10^{-7}, 10^{-9} and 10^{-11} M) of T_3 added every 24h. The number of harvested viable cells was determined, as shown in Figure 1, panels a (rRINm5F) and b (hCM), by counting in a Thoma's hematocytometer. While the dose 10^{-11} M did not affect the cell growth neither in rRINm5F or in hCM cells, the doses 10^{-9} and 10^{-7} M promoted cell growth in both the cell lines. The dose 10^{-7} M showed the highest effect and was chosen for the following experiments. In the Figure 1, panel a, rRINm5F is shown to grow with a doubling time of approximately 30h (calculated as described in Materials and Methods) in the absence of T_3. However, the addition of T_3 in the culture medium led to the stimulation of the cell growth with a doubling time of approximately 20h. The hCM cells showed the same behaviour after treatment with T_3, (Figure 1, panel b). Even in this case, the doubling time of the cell line seemed to be affected by the T_3 (10^{-7}M) treatment after the first 24h. All the treated cells showed a doubling time of approximately 18h, while the doubling time of the cells cultured in the absence of T_3 was 24h. As shown in the Figure 1, panel c, the stimulatory effect of T_3 (10^{-7}M) on cell growth reached a maximum on the 3rd day (72h) of treatment (55% on RINm5F, and 45% on CM cells).

To further analyze the effects of T_3 observed by cell growth analysis, MTT assay was performed in the first 2 days (48h) of treatment to investigate the putative proliferative action of T_3. In the Figure 2, panels a (rRINm5F), and c (hCM), cell proliferation data are expressed as cell viabilities. As shown, the T_3 treatment causes an increment of 10 % (rRINm5F) and of 15% (hCM) in the OD value already after a 6-h treatment. Such increase reached a maximum of 30% (RINm5F) and 35% (CM) after 24h. These data ─ expressed as the increase on the control absorbance value (taken as 100%) ─ were consistent with the cell counting data, which confirms that thyroid hormone T_3 had a proliferative effect on both the cell lines.

This effect was further confirmed by BrdU staining experiments. As shown in the Figure 2, when cells from both rRINm5F (panel b) and hCM (panel d) were cultured in the presence of $T_{3,}$ the majority of nuclei were positive for BrdU. On the contrast, a little number of positive nuclei was visualized in the cells cultured in the presence of vehicle alone, which demonstrates that T_3 induced the DNA sinthesis in RINm5F and CM cells after 24 and 48h of treatment.

Figure 1



c

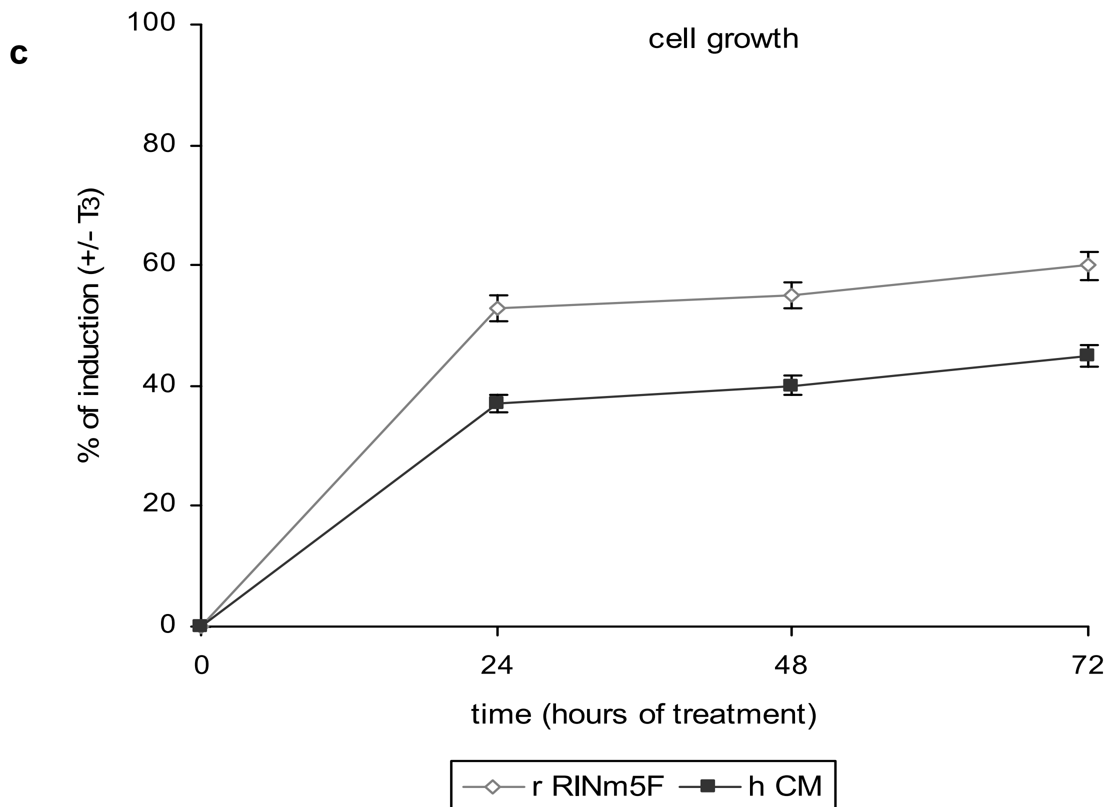
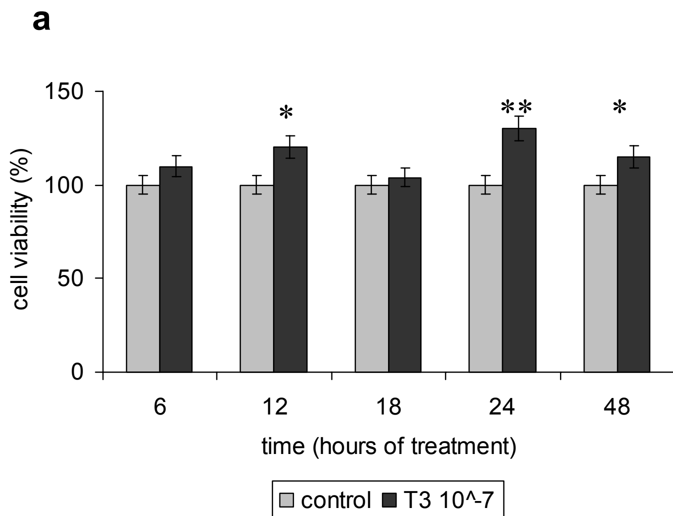
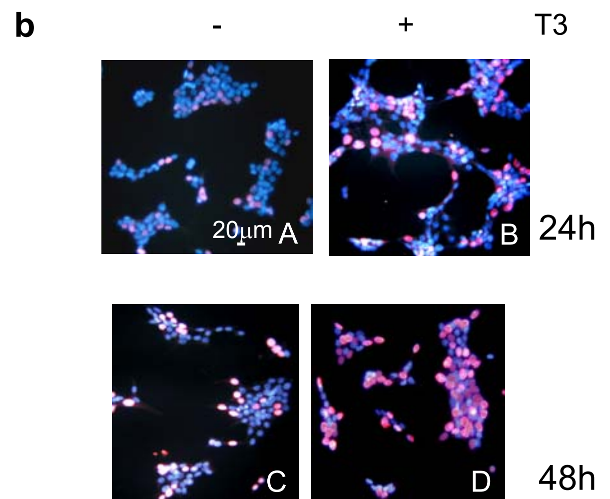


Figure 1 *T3 causes an increment in the growth rate of RINm5F and CM cell lines*
RINm5F (**a**) and CM (**b**) were grown as a monolayer and exposed to different thyroid hormone concentrations (10-7, 10-9 and 10-11M). The graphic shows the effect of the T3 treatment on the cell growth determined by counting Trypan Blue negative cells. y axis: cell number, x axis: hours of T3 treatment. **c**. The percentage of induction (+T3/-T310-7M) on cell growth is shown. The effect on RINm5F cells is shown in grey ◊, while the effect on CM cells is shown in black ■.
All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Tukey post-hoc test.

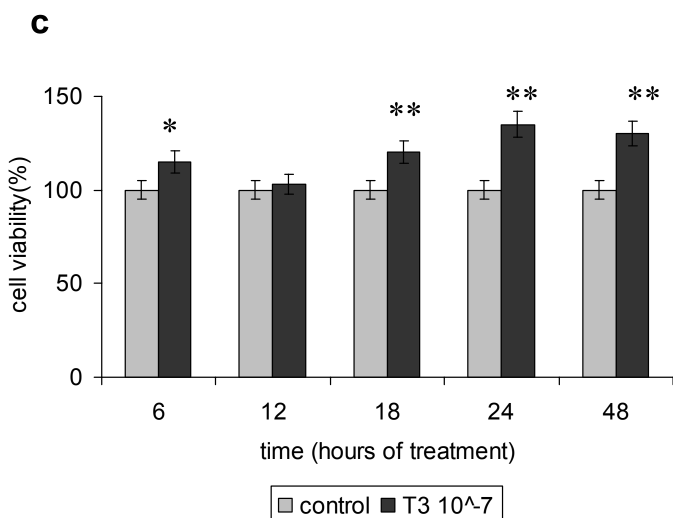
Figure 2

rRINm5F





hCM



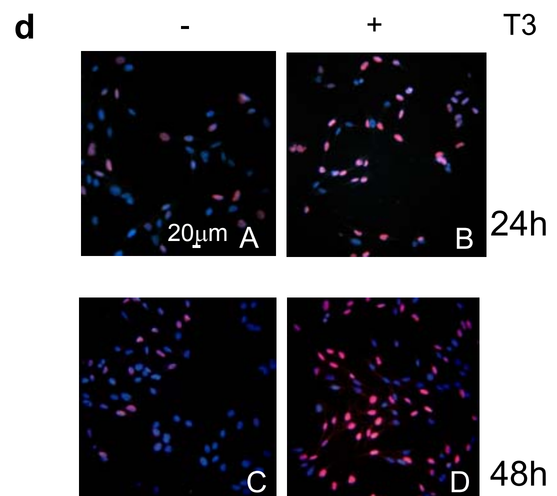


Figure 2 *T3 has a proliferative effect on RINm5F and CM cells*
MTT assay and BrdU staining on RINm5F (**a,b**) and CM (**c,d**) cells are shown.
MTT assays: The cells were grown in 96 multiwells and exposed or not to T3 10-7M for the indicated times (x axis). Cell viabilities are expressed (y axis) as % of the OD (570 nm) control value (taken as 100%). * = $P < 0.05$; ** = $P < 0.01$. A P value <0.05 was considered significant.
Black: T3 treated cells, grey: control cells
All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using Student's *t* test.
BrdU staining: Cells were grown onto multichamber slides and exposed or not to T 3 10-7M for 24 and 48h. Cells were incubated with BrdU for the last 30 min. of theT3 treatment and then immunostained for BrdU (in red). Nuclei were counter-stained with Hoechst (in blue).

T_3 increases the gene and protein expression of cyclins A, D1 and E and decreases the expression of the CDKI $p27^{Kip1}$

We measured the protein levels of the key regulators of the G1-to-S-phase progression, such as cyclins A, D1 and E after treatment with T_3. The kinase activity of a cyclin-cdk dimer is regulated mainly by changes in the protein levels of the cyclin component during the progression through the cell cycle. Cyclin A, D1 and E protein levels increase at the G1- to-S-transition and are important for the cell cycle progression from the G1-to-S-phase (Morgan, 1997). As shown by Western Blot analyses (Figure 3, panel a), the hormone treatment (T_3 10^{-7} M) caused an increase in the cyclins A and D1 protein levels, for the indicated times, leading to a maximum of 50% (Cyc A) and 40% increases (Cyc D1) after 24h in rRINm5F cells. The cyclin E protein levels (Figure 2, panel a) were upregulated (20-30 %) by 24- and 48-h T_3 treatments, while the 6-h hormone treatment didn't seem to affect the protein expression.

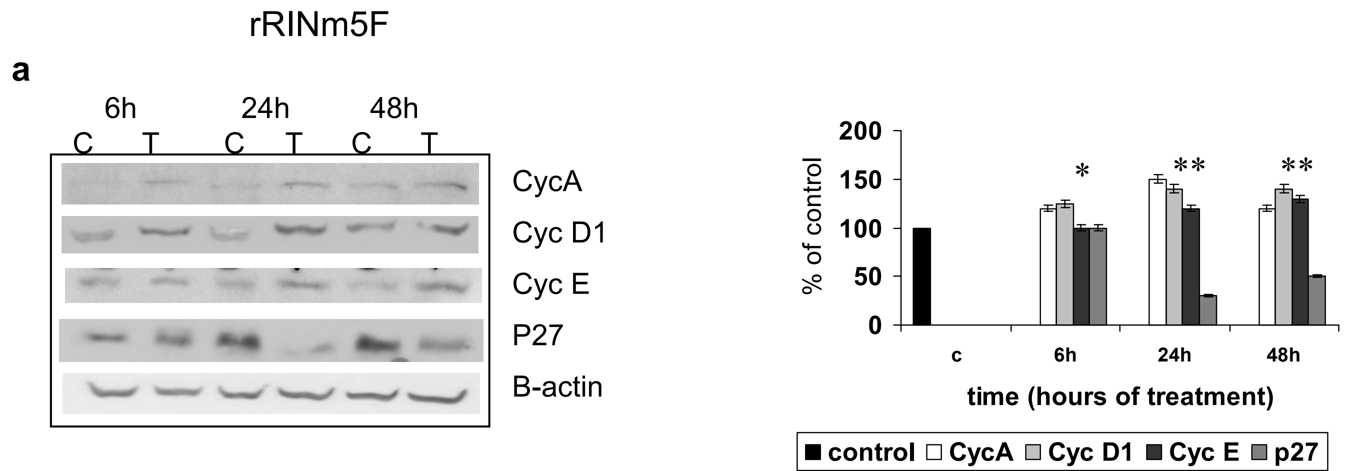
Moreover, we examined the effect of the T_3 treatment on the expression of the CDK Inhibitor $p27^{Kip1}$, which controls the progression through the cell cycle via inhibition of the cdk subunit. As shown in the Figure 3, panel a, the 24h and 48h hormone treatments caused a 70 % and a 50 % decrease of p27 in the protein expression, respectively. These data suggested that the proliferative effect of the thyroid hormone T_3 could depend on a direct regulation of the cyclin and p27 protein levels.

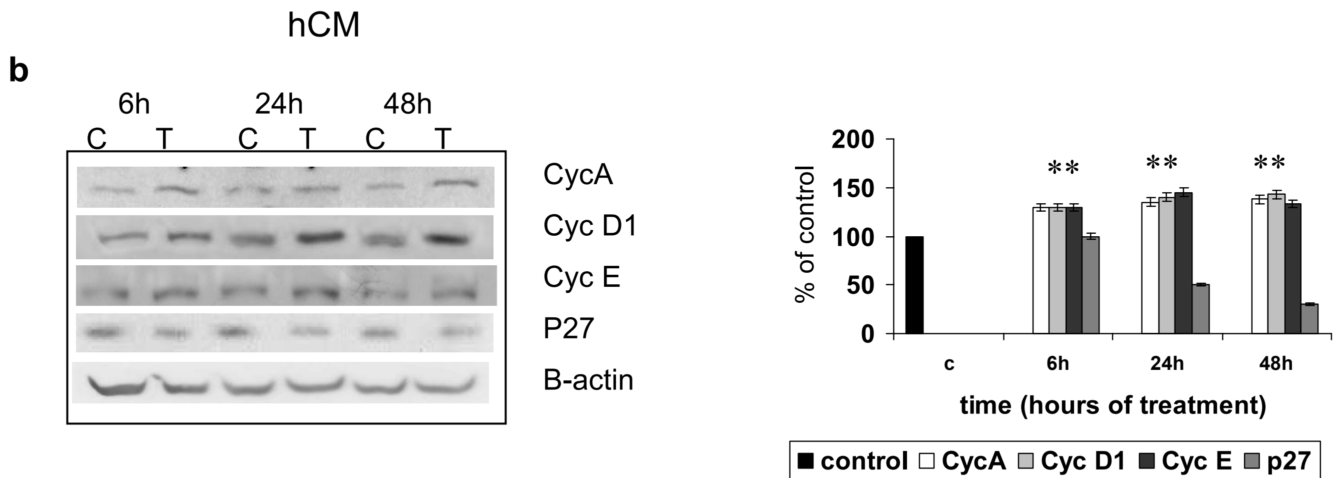
Western Blot analyses (Figure 3, panel b) revealed also that in the human insulinoma cell line hCM the T_3 treatment prompted an upregulation in the protein levels of cyclins A, D1 and E, while the protein levels of p27 resulted downregulated by $T_{3,}$ for the indicated times (6, 24 and 48h).

Since the T_3/TR complex is able to regulate the gene expression (Yen, 2001), we investigated whether the cycle-related molecules were regulated even at mRNA level. RT-PCR experiments have been performed only in rRINm5F, which have been widely used as

a β-cell model *in vitro a*nd largely investigated in their cytological and molecular characteristics. The Figure 4, panel c, shows that all the cycle-related molecules we have analyzed (Cyclins D1, E and p27) were regulated by T_3, as measured by densitometric analyses of RNA levels. Particularly, the hormone treatment led to maximum 50% (Cyc E) and 100% (Cyc D1) increases after 48h, compared to the untreated samples. The p27 gene expression was downregulated by 6h, 24h and 48h hormone treatment, reaching a 50% maximum decrease after 24h.

Figure 3





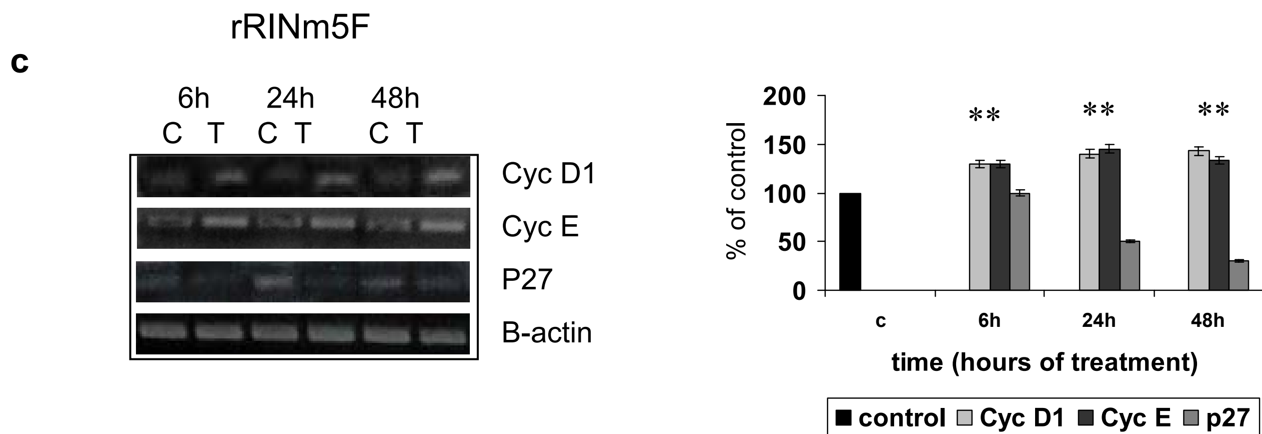


Figure 4 *T3 affects mRNA and protein expression of the main cycle-related molecules in RINm5F and CM cells*
Western Blot analyses for cyclins A, D1 and E, p27Kip1 and β-actin on RINm5F (**a)** and CM (**b**) cells are shown. Cells were grown in the presence of T3 10-7M or of vehicle alone for the indicated times. Western Blot analyses were performed as described in Materials and Methods and a specific band was detected for each analyzed molecule. **c.** RTPCR for cyclins D1 and E, and p27 were performed on RINm5F cultured in the presence or the absence of T3 10-7M for the indicated times, as described in Materials and Methods. Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β-actin for equal loading. Data are presented in the hystogram as percentages (y axis), using control as baseline (100%). x axis: hours of T3 treatment. All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using Student's *t* test. * = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant (control: black; Cyc A: white; Cyc D1: light grey, Cyc E: black; p27: dark grey).

The proliferative effect of T_3 is thyroid hormone receptor-mediated

Given the results obtained for the cell cycle molecules mRNA, and since the transcription regulation happens through the T3/TR action, we decided to investigate whether a T3 antagonist could affect the T3 proliferative effect. The thyroid hormone receptor antagonist Bisphenol A ─ that is, a thyroid hormone analogue capable of disrupting the action of the said hormone through the thyroid hormone receptor (Moriyama et al, 2002) ─ was utilized in MTT experiments to study the specificity of the T_3 proliferative effect. The cells were plated in 96 multiwells and exposed respectively to T_3 (10^{-7}M), BPA (10μM), vehicles alone and concurrent to T_3 and BPA, for 24h. As shown in the Figure 4 the addition of BPA (10 μM) to the culture medium led to a blockade of the proliferative effect of T_3 on both the cell lines. The viability values of the cells exposed to T_3 and BPA for 24h were comparable with the values of the control cells. The adding of BPA alone to the cell cultures did not affect the cell viability (data not shown). These data suggested that the proliferative effect of T_3 might involve the binding of the hormone to its receptors.

Taken together these results suggested that the regulation of the cell cycle-related molecules observed by us might be due to a direct effect of the T_3/TR complex .

Figure 4

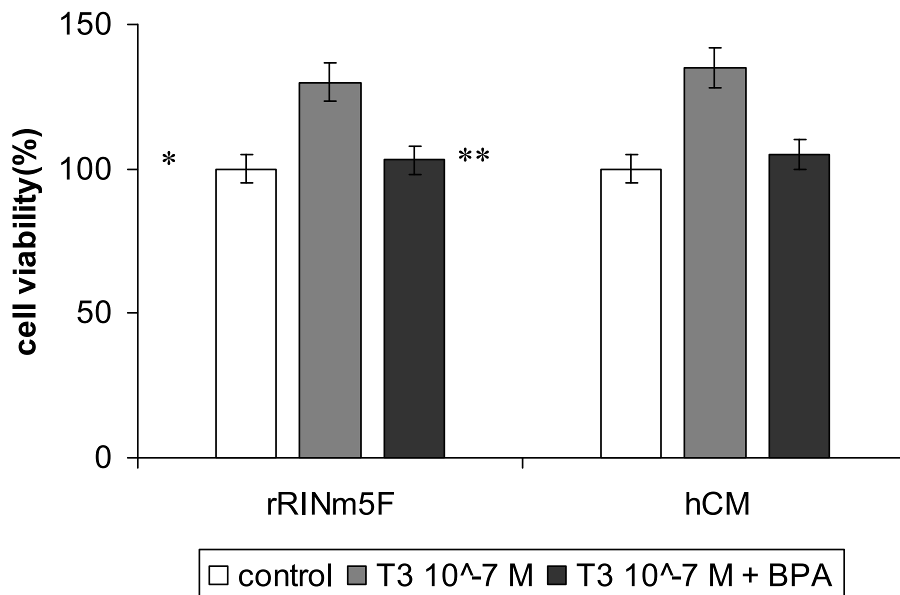


Figure 3 *The T3 proliferative effect is TR-mediated*

RINm5F (**a**) and CM (**b**) cells were immunostained for Insulin.

Cells (both RINm5F and CM) were grown in the presence of T3 10-7M or of vehicle alone for the indicated times. Western Blot analyses for TR α1 and β1 were performed as described in Materials and Methods and a specific band was detected for each analyzed isoform. Densitometric absorbance values from three separate experiments were averaged (± SD) after they had been normalized to β-actin for equal loading. Data are presented in the hystogram as percentages (y axis), using control as baseline (100%). x axis: hours of T3 treatment (control: black; TRα1: white; TRβ1: grey). * = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant.

c. RINm5F and CM cells were cultured in the presence or the absence of T3 (10-7M) and concurrently exposed or not to Bisphenol A (10µM) for 24h. MTT assay results are presented as % of the OD (570 nm) control value (taken as 100%). T3 + BPA (black); T3 (grey); control (white). All the data are presented as means ± SD, and are the results of three independent experiments at least. A comparison of the individual treatment was conducted by using Student's *t* test. * = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant.

T_3 protects insulinoma cells from pharmacological induced apoptosis

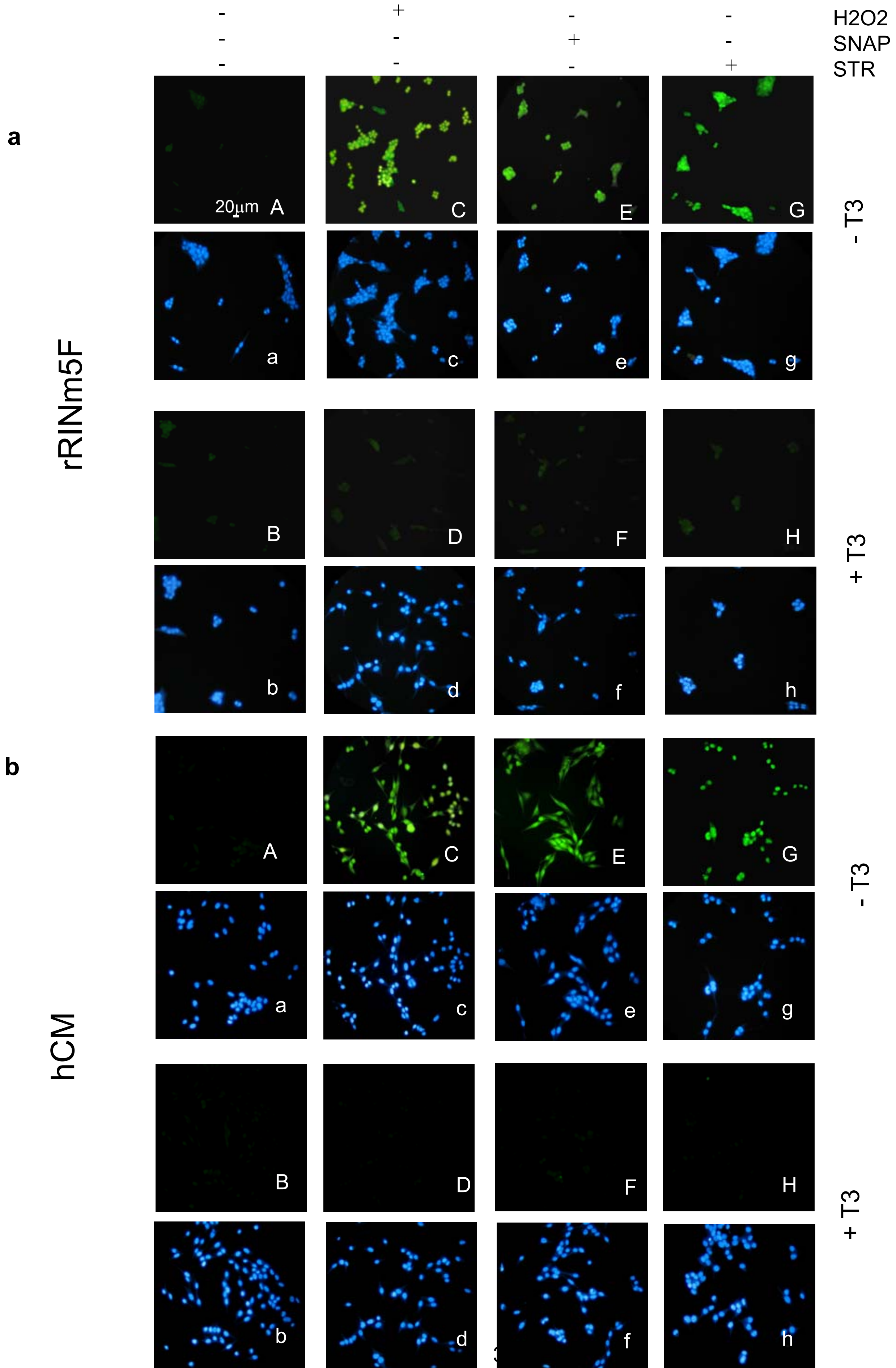
Since many growth factor with well charachterized mitogenic actions have been related to specific survival effect, and given the existence of common player in proliferation and survival pathways, we decided to verify if T3 could also affect the survival of the islet β-cells exposed to proapoptotic agents. To this aim apoptosis was induced by selected proapoptotic drugs in the cells exposed to T_3 or to vehicle alone. The rRINm5F and hCM cells were treated with H_2O_2 50μM for 30 min., SNAP 200μM for 24h and streptozocin 15mM for 2h. As shown in the Figure 5, panels a-b, the TUNEL assay was positive in the majority of the cells treated only with drugs (rRINm5F panels a: C,E,G; hCM, b: C,E,G), which demonstrates the presence of apoptosis, while in the cells treated with T_3 (rRINm5F panel a: D,F,H; hCM, panel b: D,F,H) a minimally positive TUNEL results in a much weaker fluorescence, which suggests that the hormone T_3 is able to counteract the proapoptotic action of the drugs.

For both the analyzed cell lines, control cells cultured without drugs (rRINm5F, panel a: A,B; hCM, panel b: A,B) showed the absence of fluorescence positive nuclei.

The effect on cell metabolism, which is highly affected in the presence of apoptosis, was confirmed by MTT assays (Figure 5, panel c). Cell viability was highly affected by the pharmacological treatment, causing a decrease of up to 40% (streptozocin) compared to the control OD value. In particular, the H_2O_2 treatment caused a decrease of 25% in both the cell lines, while SNAP caused a reduction of 40% (rRINm5F) and of 37% (hCM) in the cell viability values. The treatment with the thyroid hormone T_3 10^{-7}M added before the exposure to H_2O_2 and streptozocin as well as during the exposure to SNAP prevented cell mortality and promoted cell survival. As shown in the Figure 5, panel c, the OD value in the cells pre-treated with the hormone and exposed to proapoptotic drugs was 90-100% of controls.

Interestingly, the antiapoptotic effect of T_3 on cells exposed to H_2O_2 and streptozocin was not present if the cells were exposed to the thyroid hormone immediately after the treatment with the drugs (data not shown), which suggests that the hormone treatment can activate a survival response when it is administered concomitantly to proapoptotic drugs. We can speculate that this action might be due to the capability of the hormone to regulate some intrinsic factors of the cells, as demonstrated hereafter, thus contrasting an apoptotic cascade on-going.

Figure 5



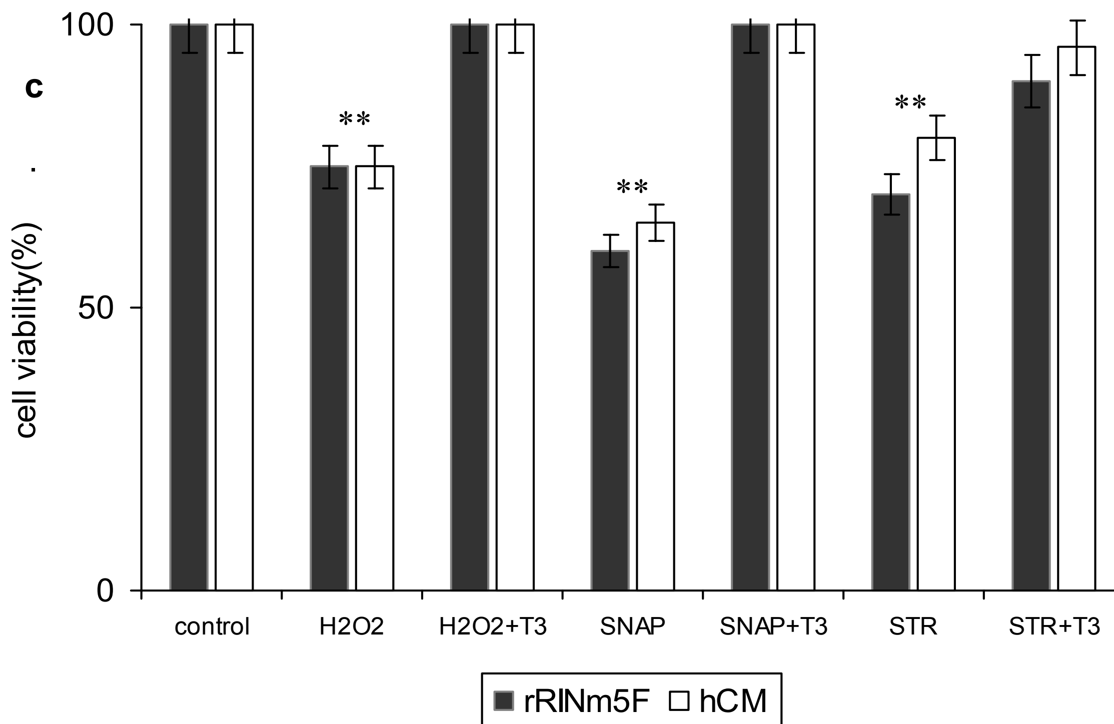


Figure 5 *T3 protects RINm5F and CM cells from pharmacological apoptosis*
Tunel assay on RINm5F cells (**a** uppercase-letter panel) and CM cells (**b** uppercase-letter panel**.**). Cells were cultured in the presence (b,d,f,h) or the absence (a,c,e,g) of T3 10-7M and exposed to different drugs (H2O2 c,d; SNAP e,f; streptozocin g,h). Apoptotic nuclei were detected as Tunel-positive only in the cells exposed to drugs alone (panels c,e,g). Nuclei were counter-stained with Hoechst (lowercase-letter panel). At least ten fields *per* chamber and three independent cultures were examined. **c.** MTT assays were performed on RINm5F (black) and CM (white) cells cultured in the presence or in the absence of T3 (10-7M), and exposed to different drugs (indicated on the x axis). Data are presented as % of the OD (570 nm) control value (taken as 100%) on the y axis, as means ± SD, and are the results of at least five independent experiments. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Dunnett post-hoc test.
* = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant.

T_3 prevention of apoptosis is associated with an increase of cellular antiapoptotic proteins and with a decrease of proapototic proteins

An additional set of experiments was carried out to confirm the ability of T_3 to counteract pharmacologically induced apoptotic cell death in rRINm5F and hCM cells. The observation that T_3 was able to promote cell survival only when it was administered before the drug treatment supported the hypothesis that the hormone might act by enhancing the expression of endogenous antiapoptotic factors or by decreasing the expression of proapoptotic proteins.

Western Blot analyses on rRINm5F (Figure 6, panel a) and hCM cells (Figure 6, panel b) revealed that the T_3 treatment induced an upregulation of the antiapoptotic proteins Bcl-X_L (in the cells exposed to H_2O_2 and streptozocin) and Bcl-2 (when cells were treated with H_2O_2 and SNAP), while the proapoptotic factors Bax and Bad were downregulated by T_3 in the cells exposed to streptozocin and SNAP (Bax) and to streptozocin, SNAP and H_2O_2 (Bad) in both the examined cell lines. All the analyzed proteins were regulated by the T_3 treatment even when the cells were not exposed to drugs, which suggests that the blockade of apoptosis we have observed (Figure 5) was due to a modulation of endogenous pro- and anti- apoptotic factors and occurred according to the inability of the hormone treatment to counteract apoptosis when it was administered immediately after the drugs (data not shown).

The protein expression of the active proapoptotic Caspase 3, which is an upstream regulator of PARP cleavage, was downregulated by the hormone treatment when the cells had been exposed to H_2O_2, SNAP and streptozocin. These data suggest that the thyroid hormone T_3 is able to counteract cell death by regulating the different molecular targets involved in the apoptotic cascade activated by different proapoptotic stimuli.

Figure 6

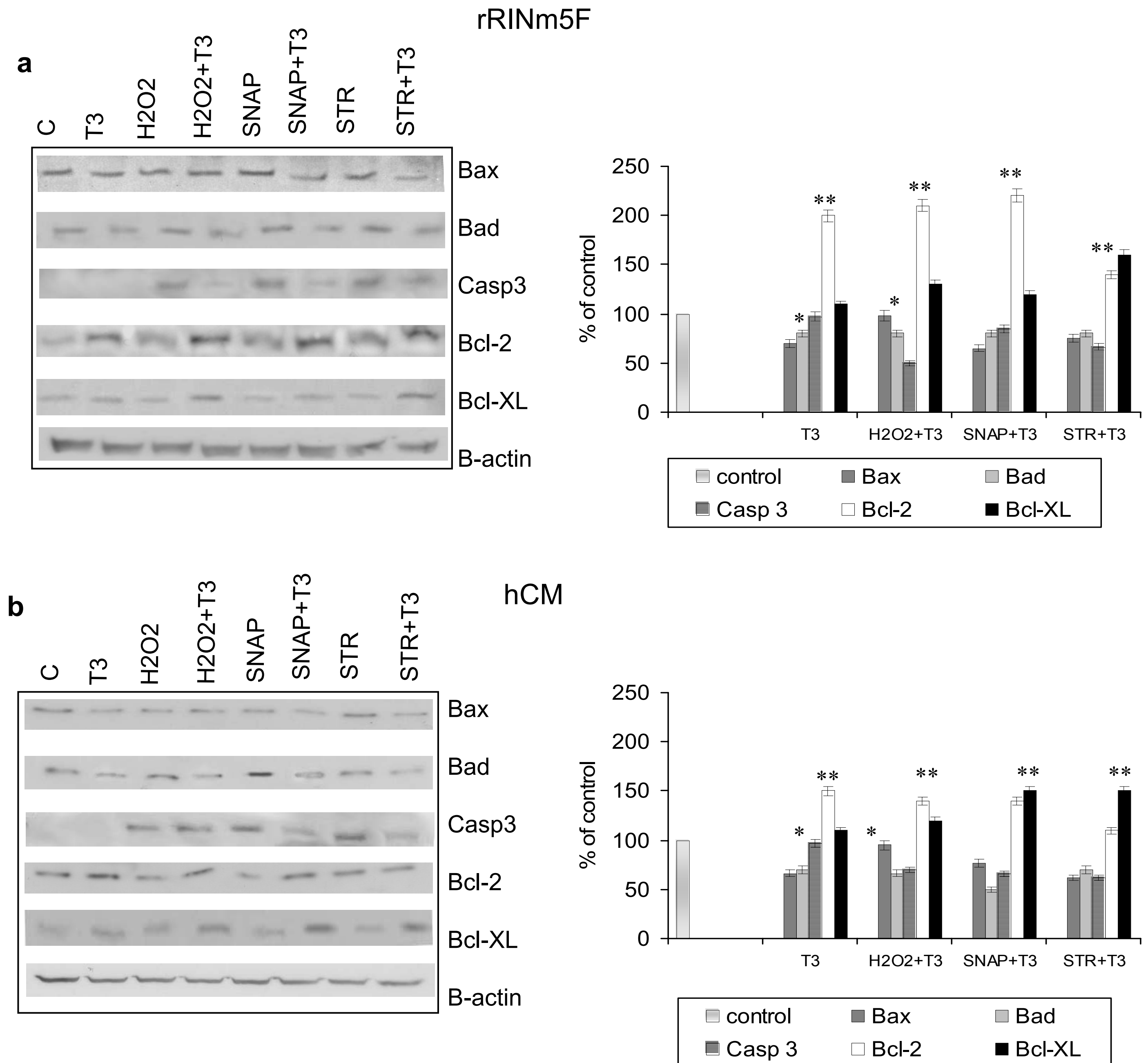


Figure 6 *T3 regulates the protein expression of the major pro- and anti-apoptotic factors in RINm5F (a) and in CM (b) cells.*
Cells were grown in the presence or the absence of T3 10-7M for 24h and concurrently exposed to H2O2 (50 µM, 30min) or SNAP (200 µM 24h), or to streptozocin (15 mM 24h). Western Blot analyses were performed as described in Materials and Methods and a specific band was detected for each analyzed factor. Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to β-actin for equal loading. Data are presented in the hystogram as percentages (y axis), using control as baseline (100%). The different experimental groups are indicated on the x axis. All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Dunnett post-hoc test. * = $P< 0.05$; ** = $P < 0.01$. A P value <0.05 was considered significant.
(control: light grey ; Bax: dark grey, Bad: light grey, Casp3: horizontal stripes, Bcl-2: white, Bcl-XL: black).

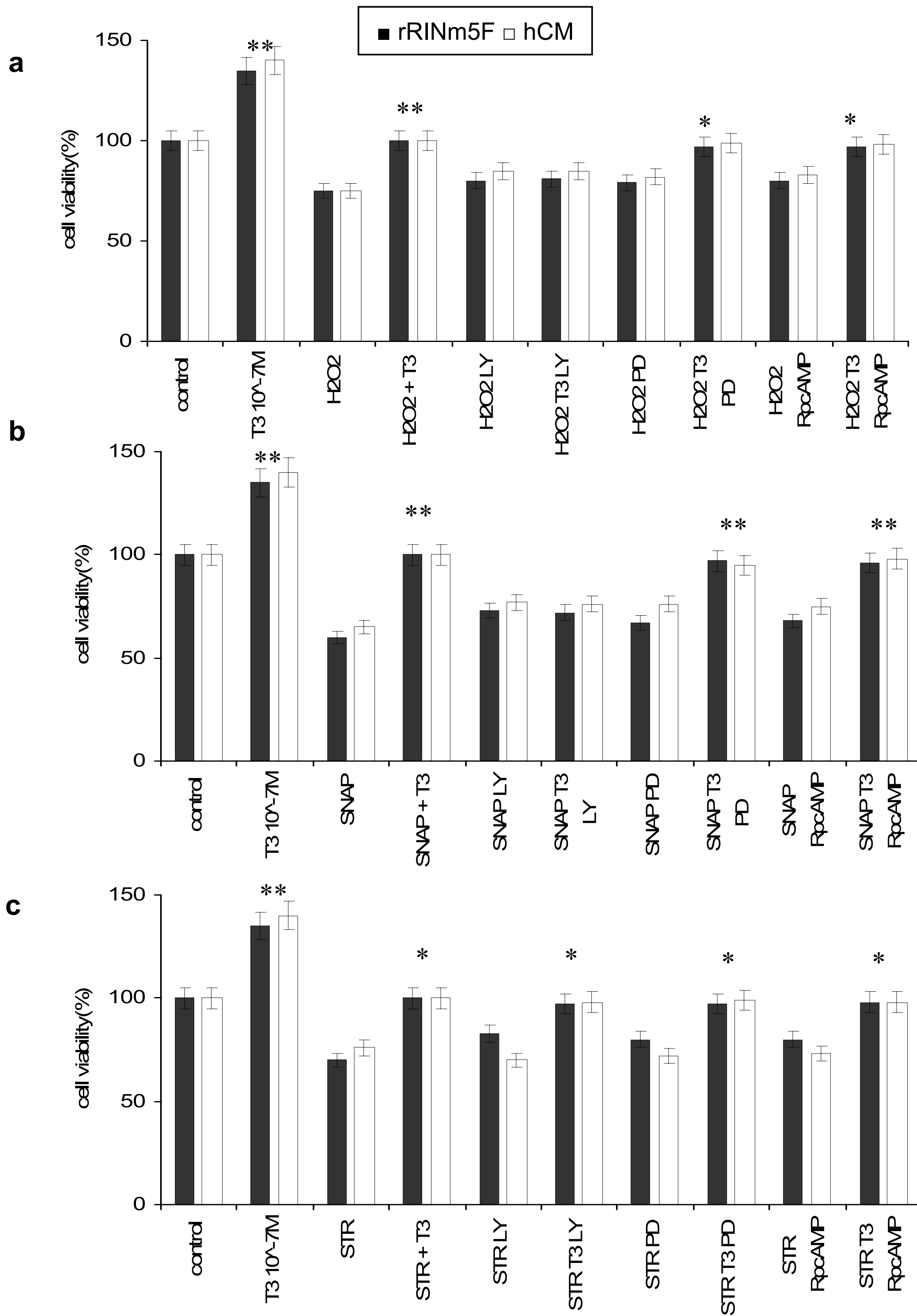
T_3 promotes β-cell survival through the PI-3 kinase/Akt signalling pathway

Once T_3 was determined as capable to promote cell survival in rRINm5F and hCM cells, the signal transduction pathways involved in this process were investigated. In islet β-cells the activation of several serine-threonine kinases ─ including MAP Kinases and PI-3 Kinase ─ have been shown to be involved in cell survival (Franke et al, 2003). We investigated the role of the protein kinases PI-3 K and MAPK in the prevention of apoptosis. The cells were incubated in the presence or the absence of the MAPK inhibitor PD-98059 (50μM), or of the PI-3 kinase inhibitor LY-394002 (50μM), and exposed to the proapoptotic agents. As shown in the Figure 7, panels a (cells exposed to H_2O_2), b (SNAP) and c (streptozocin), MTT assays demonstrated that the MAPK inhibitor did not alter the protective effect of T_3 in the presence of any drugs, while the incubation with the PI-3K-inhibitor abolished the protective effect of T_3 on the cells exposed to H_2O_2 and SNAP, which indicates this effect as PI-3 kinase-dependent. Furthermore, we investigated whether the inhibition of PKA by using Rp-cAMP (50 μM) was able to interfere with the protective effect of the hormone; as shown by MTT assay, the presence of Rp-cAMP did not affect the cell survival induced by the hormone treatment in any case.

A major downstream target of the activated PI-3K is the serine-threonine kinase Akt, and the activation of the enzyme by phosphorylation on Thr^{308} (by PI-3K) and by autophosphorylation on Ser^{473} has been associated with the inhibition of apoptosis (Dickson and Rhodes, 2004). To determine whether T_3-regulated cell survival was associated with the activation of Akt, Western Blot analysis with an antibody specific for the phosphorylated Thr^{308} Akt was performed, too. As shown in the Figure 7, panel d, a specific band for pAkt was detected only in the cells treated with drugs (H_2O_2 and SNAP) and concurrently with T_3 10^{-7}M in both the cell lines, while it was absent in the cells treated with drugs alone, according to MTT results. It is interesting to mention that the phosphorylation of Akt on the Thr residue, as shown in the Figure 7, panel d, was

upregulated also by the hormone treatment alone, in accordance with the very recent observation that T_3 is able to regulate Akt phosphorylation via PI-3 K (Cao et al, 2005). These data suggest that the T_3 treatment is able to promote cell survival via the specific activation of PI-3 K and the phosphorylation of the protein kinase B on Thr 308.

Figure 7



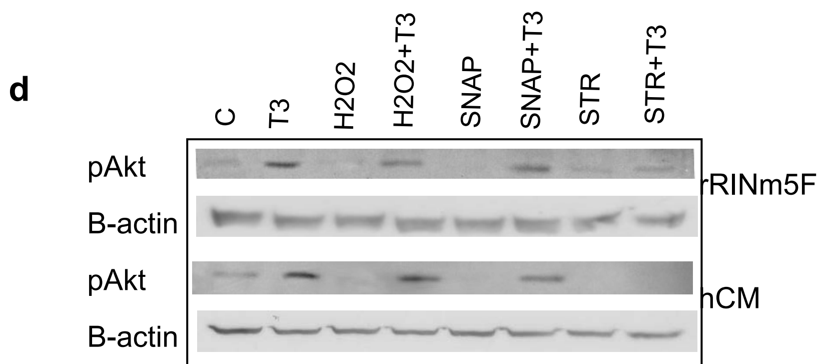


Figure 7 *T3 protects RINm5F and CM cells from apoptosis induced by H2O2 and SNAP via PI-3 K activation and Akt phosphorylation*
RINm5F (black) and CM (white) cells were cultured in the presence or the absence of T3 10-7M, in the presence of LY-294002 hydrochloride (50 μM), PD098059 (50 μM) or Rp-cAMP (50 μM), as indicated on x axis, and concurrently exposed to H2O2 (50 μM, 30min) (**a**), SNAP (200 μM 24h) (**b**) and streptozocin (15mM 24h) (**c**). Cell viabilities were measured by MTT assay and presented as % of the control OD value (y axis). All the data are presented as means ± SD and are the results of five different experiments at least. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Tukey post-hoc test. * = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant.
d. RINm5F (at the edge) and CM (at the bottom) cells were cultured in the presence or the absence of T3 10-7M and concurrently exposed to H2O2, SNAP and streptozocin. Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Thr 308) (77kDa) was detected. The expression of β-actin (44 kDa) was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is showed here.

T3 induces rapid activation of Akt in a CHX-insensitive manner

As we previously demonstrated (Verga Falzacappa et al. 2006), the ability of T_3 to counteract apoptosis in hcm and rRINm5F cells is mediated by PI3K and involves an Akt activation by T3 after 24h of hormone treatment. To examine the mechanisms underlying this new survival effect of the thyroid hormone $T_{3,}$ we decided to examine firstly the earlier time course of Akt activation by T3 treatment. hCM and rRINm5F cells were cultured in the presence or the absence of the hormone treatment (T3 10^{-7}M) for 10 and 30 min., and then were T3 deprived for the same times. In addition cells, in the presence of T3 10^{-7}, were incubated with the thyroid hormone receptor antagonists Bisphenol A and TRIAC (10 μM). The 10μM concentration of each inhibitor was chosen as the more efficacious after preliminary experiments performed with three different concentrations (100 nm, 1μm AND 10 μm) (data not shown). As discussed above BPA can disrupt the action of T3 through the thyroid hormone receptor (Moriyama et al. 2002), differently TRIAC is a thyroid hormone analogue which is specifically able to potentiate T3 effect on transcription through TRβ isoforms (Messier and Langlois, 2000). A negative control for Akt phosphorylation was obtained by incubating the cells with the PI3K inhibitor LY294,002 hydrochloride 10 μM. As shown in figure 8 Ser 473 phosphorylation of Akt was detected as early as 10 min after T3 addition and persisted up to 30 min in both hCM and rRINm5F cells. This effect appeared to be T3 dependent, since the deprivation of the hormone from cell culture media led to a decrease in the phosphorylation level of Akt after 30 min up to the basal level; moreover the effect resulted T3 dependent as suggested by the ability of BPA to block the phosphorylation, and seemed to be independent by gene transcription since the presence of TRIAC could not augment pAkt expression. In particular Western Blot analyses revealed that the activation of Akt by T3 was affected by BPA in a dose-dependent manner, so that we chose the 1μM concentration for the other experiments.

The T3 action we observed did not require de novo protein synthesis; the addiction of the protein synthesis inhibitor CHX to the culture medium indeed did not affect the Ser 473 phosphorylation of Akt even after 30 min of exposure to T3. These data support our hypothesis that the T3 activation of Akt is mediated by a non-genomic mechanism.

Figure 8

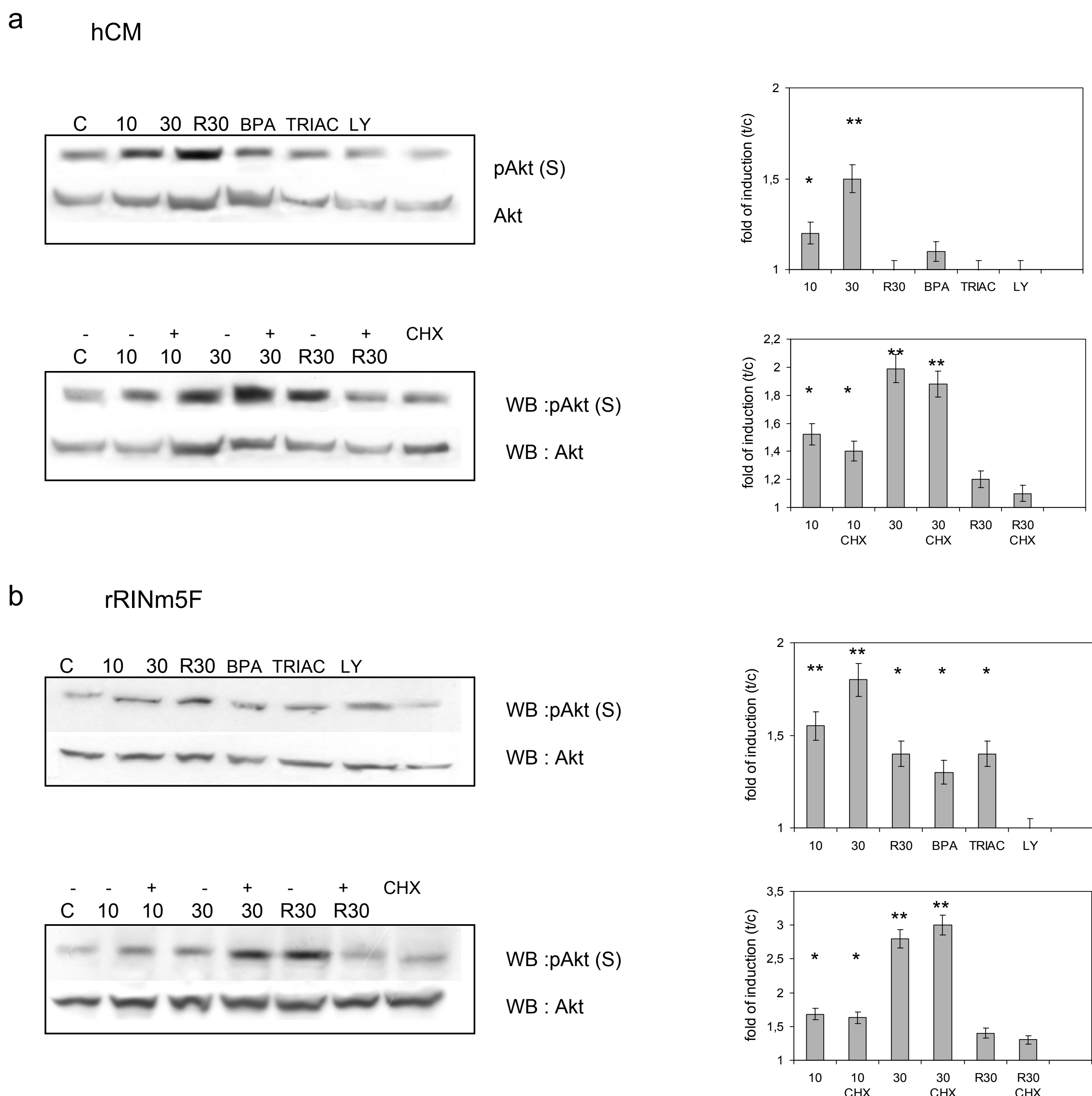


Figure8 T3 induces rapid activation of pAkt in a CHX-insensitive manner
Cells were exposed to T3 (10-7M), and then deprived of the hormone treatment for the indicated times. Cells treated with T3 (30 min) were also exposed to LY (10µM), BPA (100 nM, 1µM and 10µM) or TRIAC (100 nM, 1µM and 10µM) (upper panel), or to BPA 10 µM, TRIAC 10 µM (middle and lower panels). Additionally samples were hormone treated and concurrently exposed to cycloheximide 3 mM for 30' (lower panel). Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Thr 308 or Ser 473) was detected. The expression of unphosphorylated Akt was analyzed as a control for gel loading. Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to Akt for equal loading. Data are presented in the hystogram as percentages (y axis), using control as baseline (100%). The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. * = P< 0.05; ** = P < 0.01. A P value <0.05 was considered significant. At least three different experiments were performed, and a representative one is shown here.

TRβ1 exists in the cytoplasmic region and is able to colocalize with PI3Kp85α

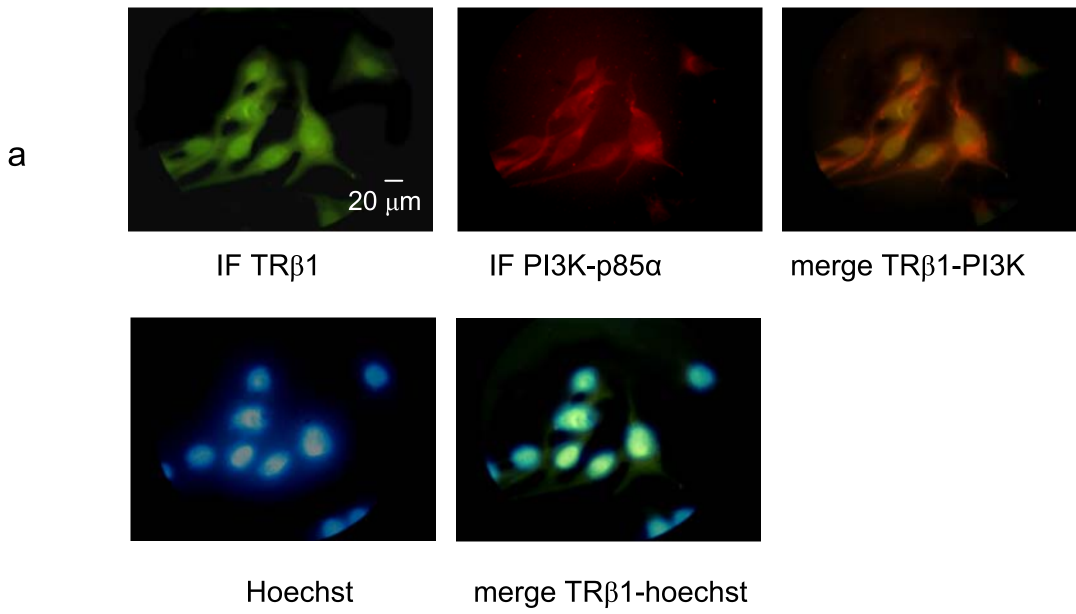
As we observed, the effect of T3 on Akt phosphorylation was rapid and CHX independent. Nongenomic actions of thyroid hormones have recently begun to be elucidated, in particular different hypotheses on the linkage between binding sites and hormone actions have been made. Our results evidenced that BPA but not TRIAC can influence the T3 effect on the activation of Akt, moreover the activation was not influenced by CHX, thus suggesting a nongenomic mechanism for this T3 action, presumably taking place out of the nucleus. The Akt phosphorylation is specifically due to PI3K, which resides at the plasma membrane; since a cytoplasmic location for thyroid receptor has been shown (Davis PJ et al. 2000; Zhu XG, 1998), we decided to investigate whether a thyroid receptor was detectable in a subcellular localization of the examined cells. As shown in figure 3, panels a and d, both hCM and rRINm5F cells immunostained for TRβ1 showed a positivity at the cytoplasmic level, showing the presence of the thyroid receptor in this cell compartment of hCM and rRINm5F cells. Once the cells have been counterstained for the p85α subunit of PI3K images by fluorescence microscopy were merged to evaluate the localization of both the protein analyzed. Interestingly, the TRβ1 and the PI3Kp85α were able to colocalize at a cytoplasmic level. This ability and the expression level of each single protein was not affected by the hormone treatment (data not shown), indicating this to be a ligand independent effect. The cytosol localization of TRβ1 was moreover confirmed by the detection of a specific band in the Western Blot analyses, panels b (hCM) and e (RINm5F), performed on the cytosolic fractions of the cells. As shown, TRβ1 was clearly detectable at the nuclear level in both immunofluorescence and Western Blot analyses, confirming its typical localization is not altered in the analyzed cells.

TRβ1 complexes with PI3Kp85α but not with PI3Kp110 in a ligand-independent manner

Thyroid receptors have recently been demonstrated to act nongenomically similarly to other steroid receptors, in particular Cao et al. (2005) and Storey et al. (2006) have shown an interaction between the subunit p85α of PI3K and the thyroid receptor β1, similar to that observed for the estrogen receptor α (Simoncini et al. 2000). Given the results obtained by the immunofluorescence experiments, we decided to analyze the ability of TRβ1 and PI3K-p85α to form a complex. As shown in figure 9, panels c and f, immunoprecipitation with anti- TRβ1 antibody resulted in pulling down of the catalytic subunit of PI3K p85α in the presence or the absence of the hormone T3 and of its analogues BPA and TRIAC. In the same experimental condition the presence of the regulatory subunit of PI3K p110 was not detectable (data not shown), indicating that only the p85α subunit can be pulled down by TRβ1 .These data indicate that TRβ1 and PI3Kp85α can form a complex in a ligand-independent manner. Together with the evidences obtained by fluorescence microscopy and cytosol Western Blot, these data suggest this complex to be located at the cytosol or at the plasmamembrane level.

Figure 9

a



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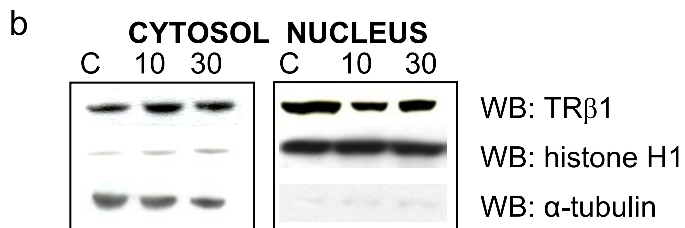

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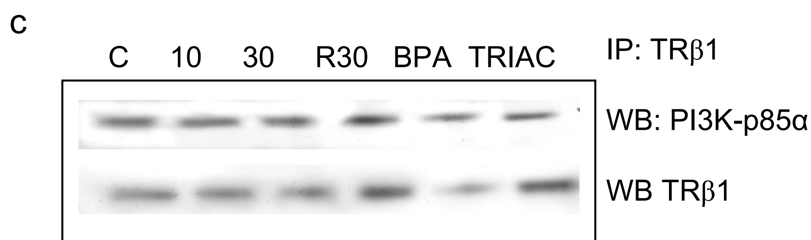

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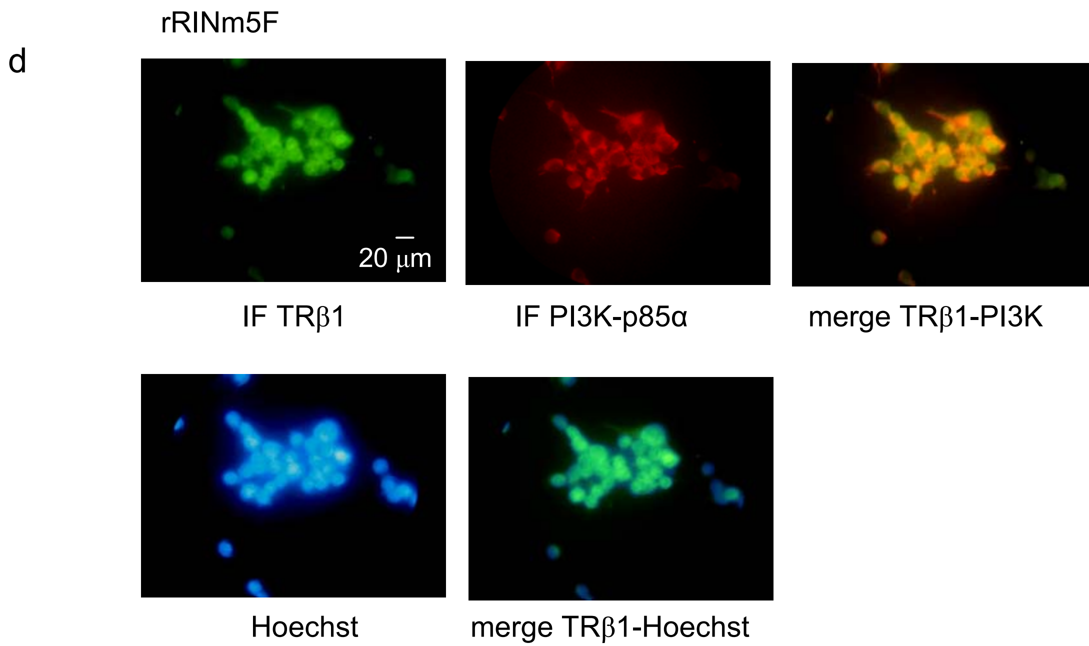

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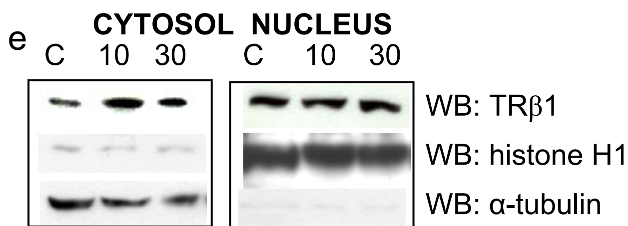

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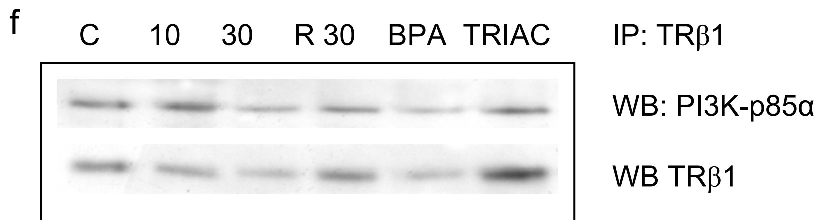

Figure 9 TRβ1 is present at a cytoplasmic level and complexes with PI3Kp85α Immunofluorescence analysis (hCM panel a; rRINm5F panel d). Cells were cultured and immunostained for TRβ1 (green fluorescence), and subsequently for PI3Kp85α (red fluorescence); nuclei were counterstained with Hoechst. The images were then merged to evidence the colocalization of the two signals. The images were acquired and analysed through fluorescence mycroscopy as described in Materials and Methods. Western Blot analyses performed specifically on the cytosol and nuclear fractions of hCM (b) and rRINm5F (e) cell extracts were analysed for the presence of TRβ1, as described in Materials and Methods. Coimmunoprecipitation experiments (hCM panel c; rRINm5F panel f). Cells were exposed to T3 and then deprived of the hormone treatment for the indicated times, and the same cells were also exposed to BPA (10μM), or TRIAC (10μM); immunoprecipitation experiments for TRβ1 were performed on total extracts. Western Blot analyses for PI3Kp85α and TRβ1 were performed as described in Materials and Methods. All the data shown are representative of at least three independent experiments.

T3 induces TRβ1 associated PI3K activity

To investigate whether T3 was able to affect the PI3K activity, a competitive ELISA assay was performed on TRB1 pulled down samples. Samples were collected from cells (hCM and RINm5F) exposed or not to the hormone treatment for 30 mins and exposure to LY 10 μM was used as a negative control. Since the two cell lines showed a similar trend, we decided to show only the results obtained for the human cell line hCM. As shown in figure 10, panel a, the presence of T3 provoked an increase in the kinase activity of about 2 folds; the PI3K activity, infact, was about 90 pmol/mg protein/hour in the control samples of hCM cells, while it reached 200 pmol/mg protein/hour in the hormone treated samples. These data indicate that, although the hormone treatment does not influence the formation of the complex between p85α and TRβ1, it is able to induce the kinase activity in the TRβ1 associated PI3K.

T3 induces Akt activity

To evaluate if the increase in Akt phosphorylation levels was accompanied by an increase in the Akt kinase activity, activation of specific Akt substrates was analyzed by Western Blot analyses. One of the main target of Akt activity are the Glucogen Sinthetase Kinases α/β, which are specifically phosphorylated on the Ser 9 and Ser 21 residues by Akt (Cross et al, 1995); as shown in figure 10, panel b, Western Blot analyses performed on the same samples utilized for Akt activation evaluation, were performed for the analysis of GSK 3 α phosphorylation levels. Interestingly the phosphorylation of GSK3 α was increased by the hormone treatment already after 10 mins and returned to basal level when the hormone treatment was deprived from the cell culture media. Moreover the addition of neither BPA nor TRIAC was able to affect the T3 effect on this phosphorylation. These data suggest that the thyroid hormone T3 is able to fully activate Akt, thus promoting its kinase activity, in both hCM and rRINm5F cells.

Figure 10

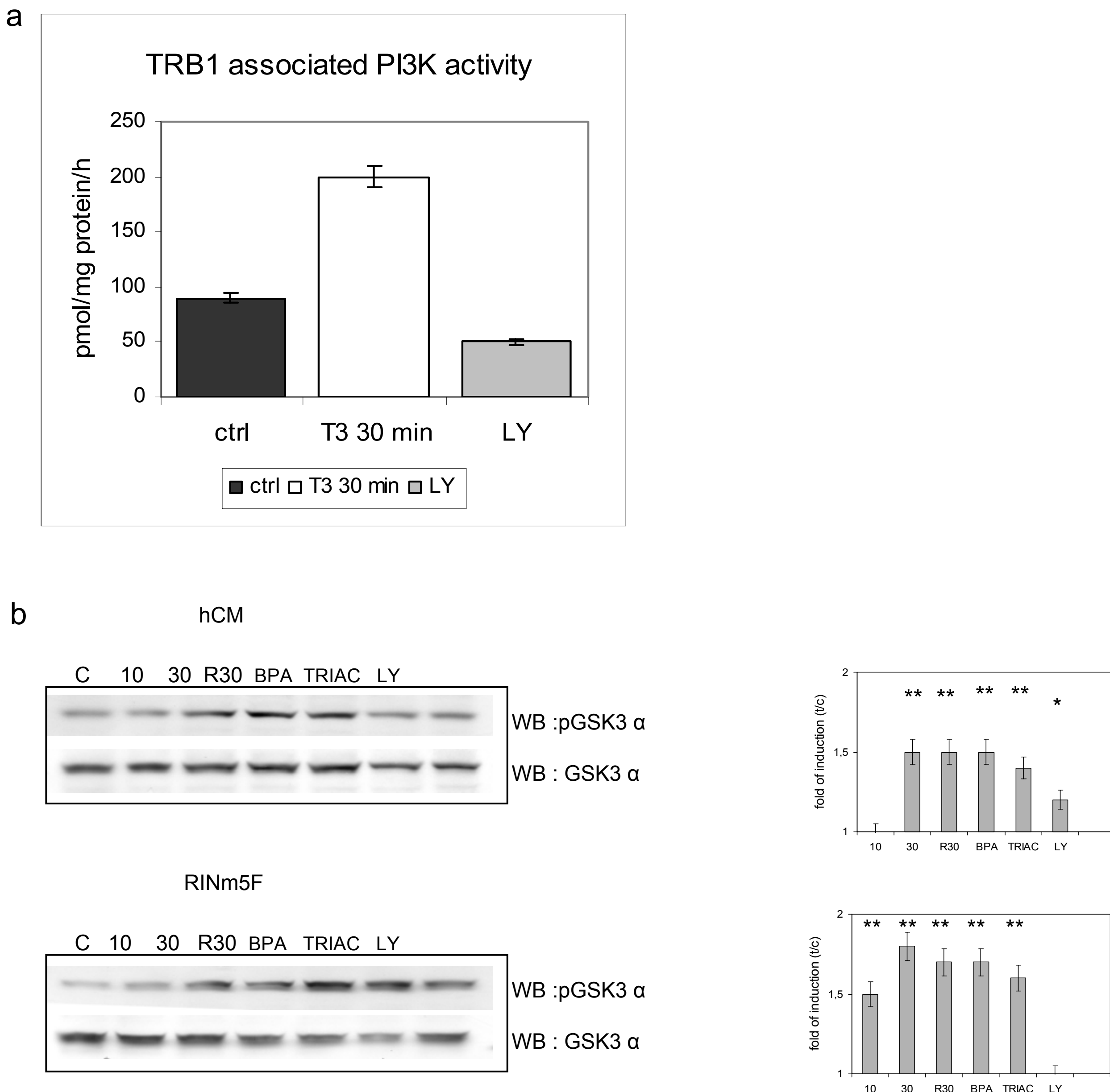

Figure 10 T3 induces TRB1 associated PI3K activity and Akt activity
PI3K activity: hCM and rRINm5f cells were exposed to T3 (10-7M) alone or concurrently to T3 and LY (10 µM) and to vehicle alone for 30 min. Then total extracts were immunoprecipitated for TRB1 and analyzed for PI3K activity as described in Materials and Methods. The kinase activity was estimated by comparing the values from the samples to those in the standard curve. All the data are presented as means ± SD and are the results of five different experiments at least in which every sample was run in triplicate.
Akt activity: hCM and RINm5F cells were cultured in the presence or not of T3 (10-7M) and inhibitors for the indicated times. Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated GSK3α (Ser 9) was detected. The expression of GSK3α was analyzed as a control for gel loading. Densitometric absorbance values from three separate experiments were averaged (± SD), after they had been normalized to GSK3α for equal loading. Data are presented in the hystogram as fold of induction (y axis), calculated as treated sample/control. The different experimental groups are indicated on the x axis. A comparison of the individual treatment was conducted by using Student's *t* test. * = $P< 0.05$; ** = $P < 0.01$. A P value <0.05 was considered significant. At least three different experiments were performed, and a representative one is shown here.

T3 induces the nuclear translocation of activated Akt

To better understand the dinamics of the activation observed, the cells exposed to the hormone treatment (T3) were immunostained for pAkt-Ser 473 and analyzed with a fluorescence microscope equipped for confocal microscopy. After stimulation through PI3K, Akt is recruited to the plasma membrane where it is activated by phosphorylation. Once activated, pAkt is translocated to the different cell compartments, including the nucleus, where its target proteins are.

We have analyzed the T3 effect on the localization of pAkt (Ser 473) in hCM and rRINm5F cells as shown in figure 11, panels a (hCM) and c (rRINm5F). As shown, activated pAkt-Ser 473 was rapidly translocated to the nucleus, leading to a stronger fluorescence in this cell compartment. Interestingly, this effect was promptly increased by the presence of the hormone treatment (30 min), suggesting that T_3 can firstly promote the activation of Akt and later its subsequent nuclear relocalization. To confirm the observed phenomena, Western Blot analyses were performed on the separate cytosol and nuclear fractions of the cells exposed to the hormone treatment for 10 and 30 min. As shown in panels b (hCM) and d (RINm5F), the phosphorilated Akt levels were remarkably augmented in the nuclear fraction of treated cells, suggesting that the increase observed in pAkt levels in the whole proteins is mainly due to the nuclear fraction. The quality and the loading of the extracts were normalized by histone H1 (nucleus) and α-tubulin (cytosol) blotting as shown. These data suggest that in our cellular systems T3 is able to increase Akt activation by specific phosphorylation on Ser 473 and promotes its nuclear localization as resulted in the cells exposed to the hormone.

Figure 11

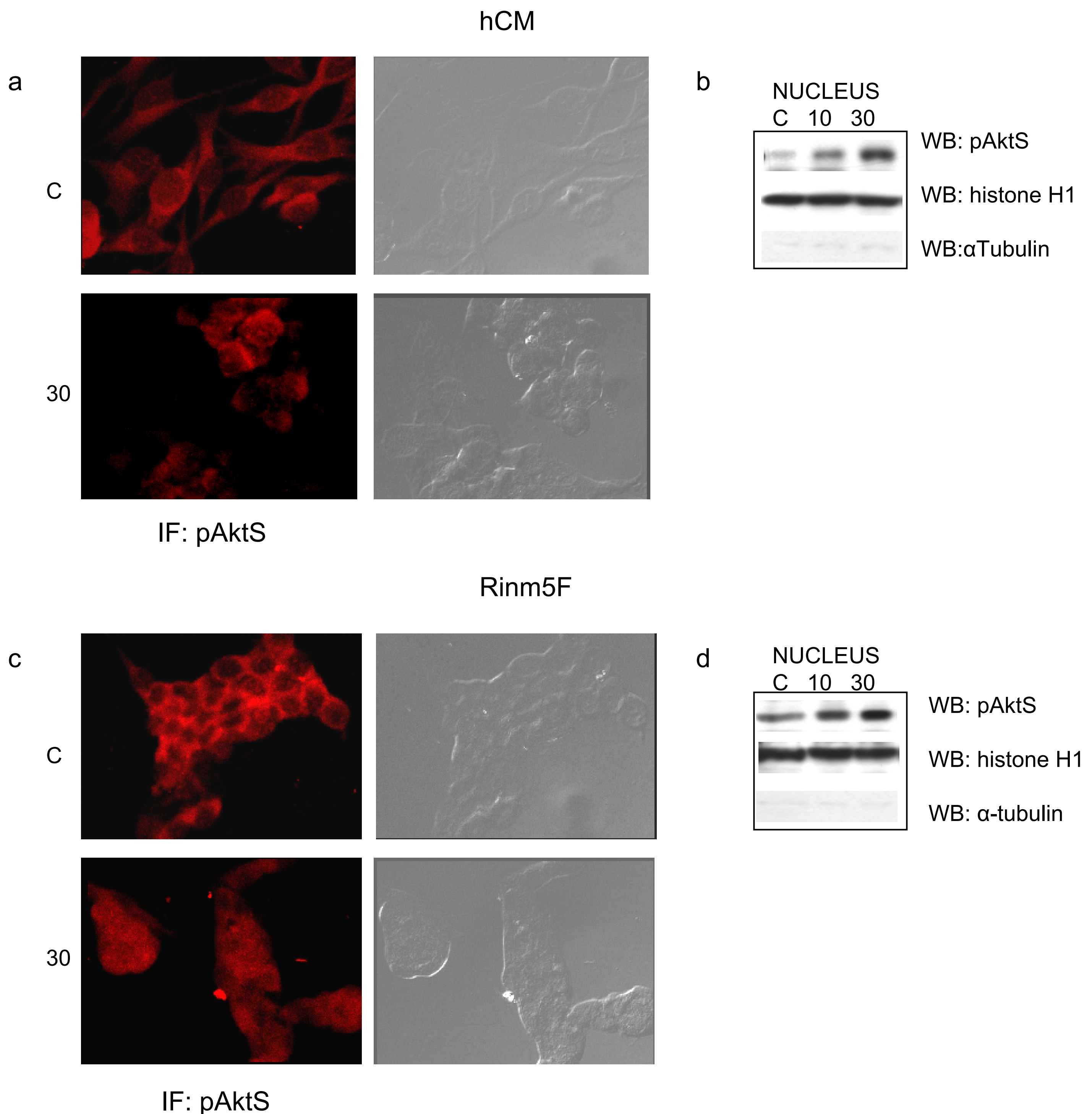

Figure 11 T3 induces nuclear translocation of pAkt
hCM (panel a) and rRINm5F (panel c) cells were exposed to T3 (10-7M) or vehicle alone for 30 min and then immunostained for pAktS as described in Materials and Methods, and images were analyzed by confocal microscopy. Additionally Western Blot analyses on the cytoplasmic and nuclear fractions of proteins extracts from hCM (b) and rRINm5F cells (d) exposed to T3 treatment for 30 min. and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of histone H1 (nuclear) and α-tubulin (cytosolic) were analyzed as a control for gel loading and to exclude the contamination of the cytosol with the nuclear components and *viceversa.* At least three different experiments were performed, and a representative one is shown here.

The T3 activation of Akt in β cells depends on TRβ1

To confirm the crucial role of the thyroid receptor β1 for the T3 activation of Akt via PI3K pathway in hCM and rRINm5F cells, we analyzed if the specific silencing of TRβ1 could alter the T3 ability to promote Akt phosphorylation. TRβ1 was “knocked down” in rRINm5F and hCM by siRNA experiments. hCM and RINm5F cells were transfected with TRβ1 siRNAs and exposed or not to T3 treatment; total extracts were then immunoblotted for TRβ1 and pAkt. As shown in figure 12, panels a and b, when TRβ1 was silenced, as demonstrated by Western Blot analyses, the phosphorylation of Akt induced by T3 was completely abolished, on the other hand the TRβ1 silencing per se was not able to influence the abasal activation of Akt in the β cells analyzed. These data strongly indicated that the ability of T3 to induce Akt activation in the islet cells utilized, is specifically mediated by the thyroid receptor β1.

Figure 12

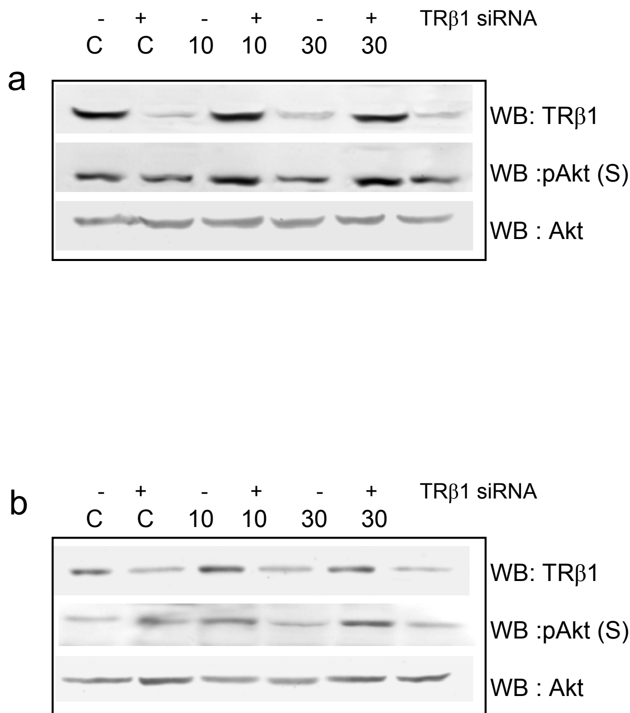

Figure 12 TRβ1 interference abolishes T3 effect on Akt activation
RNA interference experiments to silence TRβ1 expression were performed as described in Materials and Methods on hCM (a) and rRINm5F (b) cells exposed or not to T3 (10-7M) for the indicated times. Western Blot analyses were performed as described in Materials and Methods and a specific band corresponding to the phosphorylated Akt (Ser 473) and TRβ1was detected. The expression of Akt was analyzed as a control for gel loading. t least three different experiments were performed, and a representative one is shown here.

Discussion

In this study we demonstrated a novel T3 action which specifically target pancreatic β cells. At first we analyzed the physiological aspect of this action, secondly we investigate the molecular mechanisms underlting the T3 survival action.

T3 as a survival factor for pancreatic β cells

Throughout the lifetime of a mammal, low levels of β-cell replication and apoptosis are balanced and result in a slowly increasing mass (Bonner Weir, 2000). The physiological control of the β-cell mass in normal and pathological conditions is based on the balance of proliferation, differentiation, and death of the insulin secreting cells. Apoptosis plays an essential role in diabetic pathology, but, as it is involved in the β-cell death usually occurring in islet transplantation, it is also critical for the diabetes treatment (Butler et al, 2003). Factors that can augment the β-cell mass are of particular interest in the field of the diabetes treatment, and to date many growth factors have been already investigated as potential agents to increase or preserve the islet β-cell mass (Nielsen et al, 2001).

In this study we used the rat RINm5F cell line (Bargsten, 2004; Sjohol, 1995), commonly used to study both physiological and pathophysiolological mechanisms of the β-cell damage. Furthermore, in order to investigate our hypothesis in man, too, we decided to use the CM cell line. Human insulinoma cell lines are rarely used because of the difficulties existing in obtaining and culturing them for long periods (Adcock et al, 1975). CM cells represent one of the very few human long-term culture β-cell lines (Baroni et al, 1999; Cavallo et al, 1996). Although the behaviour of none of the most widely used insulin-secreting cell lines perfectly mimics primary β-cell physiology, they are extremely valuable tools for the study of molecular events underlying β-cell function and dysfunction (Poitout et al, 1996).

Thyroid Hormones and Cell Cycle

In agreement with other studies showing a high expression of TRs in the endocrine pancreas (Zinke et al, 2003; Shahrara et al, 1999), we have shown that thyroid receptors are highly expressed in both the selected cell lines, in agreement with the evidences in the pancreatic tissue by Lee et al, 1990.

The thyroid hormone has been suggested to be a paradoxical hormone inducing either growth stimulatory or inhibitory effects, depending on the tissue involved and the treatment regimen (Yen, 2001). We were able to demonstrate that the hormone treatment (T_3 10^{-7}M) led to an induction of up to 55% -rRINm5F- and 45% -hCM- in cell number after 72 hours of treatment and that such induction was accompanied by a shortening of the doubling time of the cell lines we have analyzed, moreover the utilization of the T_3 analogue BPA suggested this induction to be due specifically to the thyroid hormone receptors involvement.

T_3 has been reported to be able to regulate the expression of several genes playing a key role in cell-cycle control via stimulation of the G1-cyclins (Barrera-Hernandez et al, 1999; Pibiri et al, 2001). We have shown that the expression of cyclins A, D1 and E was stimulated by T_3 suggesting that one of the mechanisms by which these proteins were increased was due to the action of T_3 at transcriptional and/or post-transcriptional level. In this study, the indication given by BPA experiments and the increase induced by the T_3 treatment in the TR levels could suggest a relation between TRs and proliferative effect of T_3; however, we cannot rule out that the modulation of the cycle-related molecules may depend on both a direct and an indirect effect of T_3. So, further studies should be addressed to this topic.

β-cell Apoptosis

We have investigated the effects of T_3 on islet β-cells undergoing apoptosis; some cell-cycle regulators can actually influence both cell division and programmed cell death (Vermeulen et al, 2003; King and Cidlowski, 1998). In this study we utilized H_2O_2, SNAP and streptozocin, which have widely been utilized in β-cell apoptosis studies, to induce apoptosis in the RINm5F and CM cells.The selected drugs were able to induce apoptosis, and the administration of T_3 to the islet cells, before (H_2O_2, streptozocin) or during (SNAP) the exposure to the drugs, had a significant protective effect against cell apoptosis. To investigate which factors could be implied in such a protective role of T_3, we examined the expression of some of the main regulators of cell apoptosis, that is, the prosurvival Bcl-2 and Bcl-X_L, and the proapoptotic Bax and Bad. Recent studies (Thomas et al, 2002) demonstrated that isolated human islets express Bax at higher level than Bcl-2, and suggest the balance between pro-survival and pro-apoptotic molecules to be one of the main mechanisms underlying islet cell death by apoptosis. Thus, it could be possible that a reduced expression of Bax might help preserving the β-cell mass. Our data show that T_3 causes an increase in the expression of prosurvival factors in cells treated with drugs, and that this effect is accompanied by a decrease in proapoptotic proteins, suggesting that this might be one of the mechanism involved in the survival activity of the hormone. Moreover, we analyzed the effect of T_3 on the cleavage of proCaspase 3 and we demonstrated that the expression of the active caspase 3 was decreased by the hormone treatment when the cells had been exposed either to streptozocin or to H_2O_2. We also observed a downregulation of the cleaved p85 PARP in the same conditions (data not shown).

All the analyzed proteins were regulated even by the hormonal treatment alone independently from the activation of an apoptotic cascade, suggesting that T_3 acts via a regulation of cellular endogenous factors and in accordance with the observation that the

T_3 was not able to counteract apoptosis, when it was added immediately after the exposure to H_2O_2 and streptozocin.

T_3 As a Survival Factor

PI-3 K and MAPK are two well-known signalling molecules mediating cell proliferation, differentiation and apoptosis. The PI-3 K inhibitor LY294002 was able to abolish the antiapoptotic action of the thyroid hormone ─ which suggests the involvement of a partial regulation via a PI-3 K-dependent mechanism. The activated PI-3 K can inhibit programmed cell death, presumably via Akt (Franke et al, 2003). We evidenced that the phosphorylation of Akt (Thr^{308}), that is specifically due to PI-3 K, occurred only in those cells exposed to H_2O_2 and SNAP concurrently to the thyroid hormone. Such phosphorylation is also enhanced by T_3 alone; this demonstrates that T_3 is able to activate the PI-3 K pathway. Our data are in accordance with the very recent findings by Cao et al (2005), that ascertain a novel role for the thyroid hormone T_3 in activating the PI-3 K pathway in a non-genomic manner. In fact, the authors define the ability of T_3 to activate PI-3 K via the cytoplasmic action of TRβ1, and to regulate directly the phosphorylation of Akt.

It has been demonstrated (Webster and Anwer, 1998) that the involvement of cAMP in the cytoprotection against bile acid-induced apoptosis includes PI-3 K, PKA and MAPK. However, it has also been shown that ─ in the protection from apoptosis ─ signalling through PI-3 K and Akt/PKB may occur independently from cAMP (Ikeuchi et al, 1998). To analyze the role of cAMP in the protective effect of T_3 in our systems *in vitro*, we used the PKA inhibitor Rp-cAMP and demonstrated that this inhibitor did not alter the protective effect of thyroid hormone, which suggests that T_3 is capable of activating a specific survival pathway in islet β-cells undergoing apoptosis. This pathway involves PI-3 K in a cAMP-independent manner.

The MAPK pathways transduce a variety of external signals leading to cell growth, differentiation and apoptosis. Since the use of PD098059 did not affect the ability of T_3 to inhibit apoptosis, we suggest that this pathway is not involved either in the protective action of the thyroid hormone or in the phosphorylation of Akt. However, we did not investigate the other MAPK pathways, thus we do not rule out that these may be involved in the antiapoptotic effect of the hormone, therefore, to this aim, further studies should be conducted .

In conclusion, in the first part of our study we demonstrated that not only the thyroid hormone T_3 should be considered a proliferative factor for islet β-cells *in vitro*, but it also is able to promote survival when an apoptotic cascade is activated. We have shown that this protective effect is due to a regulation of different cellular apoptotic proteins and that the thyroid hormone (T_3) is specifically able to activate the PI-3 K pathway, so regulating the phosphorylation of Akt. These observations may have important clinical and therapeutic implications for the current strategies designed for the diabetes treatment, such as islet transplantation and β-cell protection.

TRβ 1 and the PI3K pathway

The thyroid hormone T3 is usually known to access to the cell interior and to the cell nucleus, where it binds to its nuclear receptors and transactivates thyroid-hormone regulated genes. This action, classically referred to as genomic, happens in hours to days, which is consistent with the typical hormone action, including regulation of cell growth, development and metabolism. On the other hand, a different mechanism has been observed, which induces very fast responses in cells, happening within minutes or even seconds, and it is called nongenomic or extranuclear action. Such effects have actually been known since many years, but their mechanisms still remain unclear. Since the 10% of TRs are cytoplasmic in the absence of T3 (Baumann et al. 2001), one of the main question to be answered is what kind of factors mediate this thyroid hormone action.

In particular it is not yet been clarified whether a new thyroid hormone receptor is involved in the nongenomic action, although no specific membrane associated TR isoform has been identified yet, or if the well known one has novel additional functions.

In this study we evidenced in pancreatic β cells that the PI3K pathway can be considered a target of the nongenomic T3 action and that the thyroid receptor β1 is essential in mediating this T3 action.

Akt and β cell function

The factors that drive β cell proliferation and function under normal or pathological condition are still unknown. Available data indicate that there exists a plethora of β cell growth factors acting in genetically heterogeneous and presumably oligogenic fashion (Nielsen et al. 1999). Cellular processes such as proliferation, survival and glucose metabolism induced by different hormones and growth factors are dependent on the activation of PI3-kinase; moreover the involvement of Akt in the regulation of replication and survival of pancreatic β cells has largely been demonstrated (Lingor et al. 2003; Tuttle

et al. 2001). Multiple Akt substrates are involved in regulating various aspects of β cell function, in this view augmented protein synthesis represents an important component of the growth response, but not the sole effector. Nevertheless the activation of protein kinase B can occurr within minutes and be related to nongenomic action of specific activators of the PI3K pathway.

Akt activation

The thyroid hormones have recently been demonstrated to induce Akt activation (Cao et al. 2005; Kuzman et al. 2005), and this effect happening in minutes or hours. In this study we demonstrated that T3 (10^{-7}M), whose role in β cell growth and survival we previously evidenced (Verga Falzacappa et al. 2006), provokes Akt phosphorylation in minutes, this effect being independent on *de novo* protein synthesis.

Activation of Akt entails a complex series of events involving additional proteins. First the PI3-K generated lipid products PI(3,4,5)P3 and PI(3,4)P2 recruit Akt to the plasma membrane through their affinity for the PH domain of Akt (Burgering et al. 1995; Franke et al. 1997). Once membrane proximal, at least two residues of Akt are rapidly phosphorylated (Thr 308 and Ser 473) by PI3K activity. Active Akt can rapidly translocate to specific intracellular compartments. This ordered series of events is necessary to generate fully activated Akt and has been demonstrated even in *in vivo* models (Scheid et al. 2002), moreover a strict control on the location and activation of Akt probably reflects the direct consequences of chronically activated Akt in disease. In this study we demonstrated how the T3 treatment is able to influence these events, in particular inducing the translocation of active Akt to the nucleus.

TRβ1 and PI3K: T_3 nongenomic action

Optimal signaling through the PI3K pathway depends on a critical molecular balance between the regulatory and catalitic subunits; in particular the p85α subunit is thought to be the major response pathway for most stimuli (Shepherd et al. 1998). The relation between Akt activation and T3 action remains to be elucidated, Cao et al. (2005) and Storey et al. (2006) have demonstrated that the thyroid receptor β1 is able to interact with the regulatory subunit p85α of the PI3K. This ability of a nuclear receptor of complexing with the regulatory subunit of PI3K was already observed for the estrogen receptor ERα (Simoncini et al. 2000), for which controversy exists concerning whether or not it has a role outside the nucleus (Pietras et al. 1977). It has been suggested that such non-genomic actions might be mediated by membrane associated isoforms of the classical nuclear receptors with significantly different agonist/antagonist affinities (Losel 2003). We demonstrated that the β1 isoform of thyroid receptor, not only is able to form a complex with the p85α subunit, but also is clearly detectable in the cytoplasmic area of the cells examined. Interestingly we show that although the interaction between PI3K and a TRβ1 is not influenced by the presence of the hormone, T3 treatment can enhance the TRβ1 associated PI3K activity. In fact it has been demonstrated that thyroid receptors can locate outside the nucleus in the absence of T3 (Baumann et al, 2001), thus expalining that the TRβ1-PI3K binding does not require the T3 presence. Otherwise, the presence of the hormone does stimulate the kinase activity, when it is complexed to TRβ1. These data suggest that the hormone is somehow able to activate the kinase and that this action is TR-mediated. In addition we demonstrated that this T3 action can trigger a cascade of events PI3K dependent; in fact not only Akt is activated by T3, but also its activity is influenced by the presence of the hormone. Our findings evidenced that phosphorylation of GSK3-α, one of the main Akt targets, is enhanced by the hormone treatment and that even this activation shows a trend similar to those observed for Akt phosphorylation itself. These

data suggest that the full activation of Akt induced by T3 via PI3K activity stimulation, leads to an increment even in Akt kinase activity.Moreover, given the evidences of the cytoplasmic localization of TRβ1 our data support the hypothesis that nuclear thyroid receptors might exist also outside the nucleus (Davis PJ et al. 2000; Zhu et al. 1998) playing an important role in non-genomic actions of thyroid hormone.

We also evidenced that the "knock down" of the gene encoding TRβ1, through experiments of RNA interference, led to an abolishment of the Akt activation induced by T3, and once again the role of TRβ1 seems to be essential.

IRS2 role

Phosphoinoisitide 3-kinase plays a pivotal role in the metabolic and mitogenic actions of insulin, following insulin stimulation the IRS proteins bind to class 1a PI3K, thereby increasing its activity (Shepherd et al. 1998, Bonni et al. 1999). In pancreatic β cells the direct binding between IRS-2 and PI3K leads to Akt activation, modulating the cell survival, the proliferation and the cell size (Accili et al. 2001). Moreover, other studies on the knock out mice for IRS-2 evidenced an augmented β cell apoptosis, indicating IRS-2 as crucial in regulating β cell mass and survival (Dickson and Rhodes, 2004). We also analyzed IRS-2 expression after a long-term (24h) treatment with T3; the protein expression of IRS-2 resulted upregulated by the hormone treatment (data not shown) suggesting that T3 interact with the PI3K pathway even at this level. The effect observed could be due to the classical genomic T3 action acting synergistically with nongenomic mechanisms. If all the effects of IRS-2 are mediated by Akt, there must be additional players, which are likely to include effectors of the apoptotic cascade, transcriptional factors and additional kinases. In this context we could speculate that TRβ1 plays a specific role directly entering the PI3K pathway.

Our data showed that the thyroid receptor β1 complexes with the p85α subunit of PI3K and it is well known that even the binding between IRS-2 and PI3K involves the same subunit through the SH2 domain of p85α (Cantley LC. 2002) and the phosphorylated (Tyr) IRS. TR has been shown to be a phosphoprotein, although a tyrosine phosphorylation has not yet been reported (Jones et al. 1994; Davis et al. 2000). Thus it is unlikely that the binding between TRβ1 and p85α we reported involves the SH2 domain. On the other hand, it has been shown that p85α binds some proteins through its Rho-GAP domain, which includes three repeats of LXXLL motifs (Swiss prot accession no. Q63787). This sequence is considered a good candidate to interact with nuclear receptors and it has been identified in various nuclear receptor associating proteins. Given these observations we can speculate that the interaction between TRβ1 and PI3K might take place at the level of the Rho-GAP domain of p85α, without compromising the ability of PI3K to interact with IRS-2. Our data, according to the observation of Cao et al. (2005) support the hypothesis that the interaction between TRβ1 and PI3K takes place in the cytosol or near the plasma membrane. In conclusion we suggest that a "cytoplasmic" thyroid receptor exists in the pancreatic β cells RINm5F and CM, this receptor should be similar to the nuclear TRβ1 form, but with some peculiar characteristics that led its location.

T_3 nongenomic action

Baumann et al. (2001) have shown that TRβ rapidly shuttles between the nucleus and the cytoplasm. The possible modes of interaction between non genomic and genomic thyroid hormone signalling are complex but they appear to act synergistically. The postulated non genomic signalling pathway may complement such actions by generating second messengers and by activating multiple signalling cascades. For example the genomic and non genomic effects of thyroid hormones, in the mitochondria, also appear to be strikingly synergistic. (Bassett et al. 2003). These findings reveal the complexity of the TR signaling

and suggest that a thyroid receptor with high omology to the nuclear one at least, if not the same itself, might be the centre of the effects we characterized in pancreatic beta cells. Short interfering RNAs, designed to specifically act on nuclear TRβ1 mRNA, can silence both cytoplasmic and nuclear TRβ1 without distinction (data not shown); in addition the commercial monoclonal antibody against the nuclear thyroid hormone receptor recognizes something that is responsible for the T3 effect observed and that is located in the cytoplasm, strenghtening our hypothesis that a thyroid hormone receptor with high similarities to the nuclear one but with a peculiar localization is involved. However given the complexity of the TR shuttling mechanisms and since the precise events underlying this phenomenon are not still known , additional studies are needed to clarify the roles of TRβ1 in these non genomic effects of thyroid hormones.

In conclusion we suggest that a “cytoplasmic” thyroid receptor exists in the pancreatic β cells RINm5F and CM that is able to mediate the T3 action on the PI3K signaling pathway; this receptor should be similar to the nuclear TRβ1 form, but with some peculiar characteristics that led its location.

References

Accili D 2001 A kinase in the life of the β cell Journal of Clinical Investigation 108:1575-1576

Adcock R, Austin M, Duckworth WC, Solomon SS, Murrell LR 1975 Human islets cell adenoma: metabolic analysis of the patients and of tumor cells in monolayer Diabetologia 11: 527-535

Bargsten G 2004 Cytological and immunocytochemical characterization of the insulin secreting insulinoma cell line RINm5F Arch Histol Cytol 67(1): 79-94

Baroni MG, Cavallo MG, Mark M, Monetini L and Stoherer B 1999 Beta-cell gene expression and functional characterization of the human insulinoma cell line CM Journal of Endocrinology 161: 59-68

Barrera-Hernandez G, Soo Park K, Dace A, Zhan Q and Cheng SY 1999 Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity Endocrinology 11 (140) :5267-5274

Bassett JHD, Harvey CB and Williams GR 2003 Mechanisms of thyroiod hormone receptor-specific nuclear and extra nuclear actions Molecular and Cellular Endocrinology 213: 1-11

Baumann CT, Maruvada P, Hager GL and Yen PM, 2001 Nuclear cytoplasmic shuttling by thyroid hormone receptors. multiple protein interactions are required for nuclear retention. Journal of Biological Chemistry. Apr 6;276(14):11237-45..

Bergh JJ, Lin H-Y, Lansing L, Mohamed SN, Mousa S, Davis FB, Mousa S and Davis PJ 2005 Integrin αvβ3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis Endocrinology 146:2864-2871

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J. and Larsen, P.R., 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr. Rev. **23**, 38–89.

Bonner-Weir S 2000 Islet growth and development in the adult. J Mol Endocrinol 24:1-6

Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentiki M. 2001 Protein kinase C zeta activation mediates glucagon-like peptide-1 induced pancreatic beta-cell proliferation Diabetes 50:2237-2243

Bonni A, Brunet A, West E, Datta SR, Takasu MA and Greenberg ME 1999 Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. Science, 286: 1358-1362

Burgering BM and Coffer PJ 1995 Protein Kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature, 376: 599-602

Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ and Prentki M 2001 Protein kinase czeta activation mediated glucagon-luke peptide-1 induced pancreatic beta cell proliferation Diabetes 50: 2237-2243

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC 2003 Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes Diabetes 52:102-110

Cantley LC 2002The phosphoinositide 3-kinase pathway; Science 296: 1655-1657

Cao X, Fukushi K, Moeller LC, Refetoff S, Seo H. 2005 Thyroid hormone induces rapid activation of Akt/PKB-m TOR-p70^{S6K} cascade through PI3K in human fibroblasts. Mol Endo Jan 19(1): 102-12

Cavallo MG, Dotta F, Monetini L, Dionisi S, Previti M, Valente L, Toto A, Di Mario U and Pozzilli P 1996 Beta-cell markers and autoantigen expression by a human insulinoma cell line: similarities to native beta cells. Journal of Endocrinology. 150(1): 113-120

Cerasi E, Kaiser N, Leibowitz G 2000 Type 2 diabetes and beta-cells apoptosis. Diabetes Metab 26 (Suppl 3): 13-16

Chandra J, Zhivotovsky B, Zaitsev S, Juntti-Berggren L, Berggren PO, Orrenius S 2001 Role of apoptosis in pancreatic β-cell death in diabetes Diabetes 50 (S1) S44-S47

Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS 2003 Nuclear factor kappa B protects pancreatic beta-cells from tumor necrosis factor-alpha-mediated apoptosis Diabetes 52(5): 1169-1175

Chang I, Cho N, Koh JY, Lee MS 2003 Pyruvate inhibits zinc-mediated pancreatic islet cell death and diabetes Diabetologia 46:1220-1227

Chen W, Salojin KV, Mi QS, Grattan M, Meagher TC, Zucker P, Delovitch TL 2004 Insulin-like growth factor (IGF)-I-IGF-Binding Protein-3 complex: therapeutic efficacy and mechanism of protection against type 1 diabetes Endocrinology 145 (2) :627-638

Coffer PJ, Jin J and Woodgett JR 1998 Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochemical Journal. Oct 1;335 (Pt 1):1-13.

Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA 1995 Inhibition of Glycogen Synthase Kinase-3 by insulin is mediated by Protein Kinase B. Nature 378:785-789

D'Arezzo S, Incerpi S, Davis FB, Acconcia F, Marino M, Farias RN and Davis PJ, 2004 Rapid nongenomic effects of 3,5,3'-triiodo-L-thyronine on the intracellular pH of L-6-myoblasts are mediated by intracellular calcium mobilization and kinase pathways. Endocrinology 145: 1660-1668

Davis, P.J., Davis, F.B. and Lawrence, W.D., 1989. Thyroid hormone regulation of membrane $Ca^{2(+)}$-ATPase activity. Endocr. Res. 15, 651–682.

Davis, P.J. and Davis, F.B., 2002. Nongenomic actions of thyroid hormone on the heart. Thyroid 12, 459–466.

Davis PJ, Shih A, Lin HY, Martino LJ and Davis FB 2000. Thyroxine promotes association of mitogen-activated protein kinase and nuclear thyroid hormone receptor (TR) and causes serine phosphorylation of TR. Journal of Biological Chemistry 275:38032-38039

Davis PJ and Davis FB 2003 Nongenomic action of thyroid hormone. In: Braverman LE, ed. Diseases of the thyroid, 2nd ed. Totowa, NJ: Humana Press, 19-37

Dickson LM and Rhodes CJ 2004 Pancreatic β cell growth and survival is the onset of type 2 diabetes: a role for protein kinase B in the Akt? American Journal of Physiology : Endocrinology and Metabolism 287: E192-E198

Elghazi L, Balcazar N and Bernal-Mizrachi E 2006 Emerging role of protein kinase B/Akt signaling in pancreatic β-cell mass and function The International Journal of Biochemistry and Cell Biology 38 (2006) 157-163

Francavilla A, Carr BI, Azzarone A, Polimeno L, Wang Z, Van Diehl DH, Subbotin V, Prelich JG, Starzl TE 1994 Hepatocyte proliferation and gene expression induced by triiodothyronine in vivo and in vitro. Hepatology 20, 1237-1241

Franke TF, Kaplan DR, Cantley LC and Toker A 1997. Direct regulation of the Akt proto-oncogene product by phosphatidiylinositol-3,4-bisphosphate. Science, 275: 665-668

Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C 2003 PI3-K/Akt and apoptosis: size matters Oncogene 22: 8983-8998

Gharbi J and Torresani J 1979 High affinity thyroxine binding to purified rat liver plasma membranes. Biochemical and Biophysical Research Communication. May 14;88(1):170-7.

Giannoukakis N, Mi Z, Rudert WA, Gambotto A, Trucco M, Robbins P 2000 Prevention of beta-cells dysfunction and apoptosis activation in human islets by adenoviral gene transfer of the insulin-like growth factor I Gene Ther. 7: 2015-2022

Giannoukakis N, Pietropaolo M, Trucco M 2002 Therapeutic strategies for Type 1 and Type 2 diabetes mellitus Diabetes Nutr Metab 15(3) : 173-203

Gunther L, Berbera PO, Haga M, Brouard S, Smith RN, Soares MP, Bach FH, Tobiasch E 2002 Carbon monoxide protects pancreatic beta-cells from apoptosis and improves islet function/survival after transplantation Diabetes 51(4): 994-999

Hafezi-Moghadam, A., Simoncini, T., Yang, E., Limbourg, F.P., Plumier, J.C., Rebsamen, M.C., Hsieh, C.M., Chui, D.S., Thomas, K.L., Prorock, A.J., Laubach, V.E., Moskowitz, M.A., French, B.A., Ley, K. and Liao, J.K., 2002. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat. Med. 8, 473–479.

Haynes, M.P., Sinha, D., Russell, K.S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W.C. and Bender, J.R., 2000. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. Circ. Res. 87, 677–682.

Haynes MP, Li L, Sinha D, Russell KS, Hisamoto K, Baron R, Collinge M, Sessa WC and Bender JR 2003. Src kinase mediates phosphatidylinoditol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen. Journal of Biological Chemistry 278:2118-2123

Heery DM, Kalkhoven E, Hoare S and Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733-736

Hennemann G, Docter RE, Friesema CH, de Jong M, Krenning EP and Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability Endocrine Reviews 22:451-476

Hui H, Nourparvar A, Zhao X and Perfetti R 2003 Glucagon-Like Peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent

Protein kinase A- and a Phosphatidylinositol 3-kinase-dependent pathway Endocrinology 144 (4): 1444-1455

Hui H, Dotta F, Di Mario U, Perfetti R 2004 Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. Journ Cell Physiol 200:177-200

Ikeuchi T, Shimoke K, Kubo T, Yamada M, Hatanaka H 1998 Apoptosis-inducing and –preventing signal transduction pathways in cultured cerebellar granule neurons Hum Cell 11:125-140

Jill DJ, Strutt B, Arany E, Zaina S, Coukell S, Graham CF 2000 Increased and persistent circulating insulin-like growth factor II in neonatal transgenic mice suppresses developmental apoptosis in the pancreatic islets Endocrinology 141: 1151-1157

Jones KE, Brubaker JH and Chin WW 1994 Evidence that phosphorylation events partecipate in thyroid hormone action. Endocrinology 134: 543-548

Katz, D., Reginato, M.J. and Lazar, M.A., 1995. Functional regulation of thyroid hormone receptor variant TR alpha 2 by phosphorylation. Mol. Cell. Biol. 15, 2341–2348.

Kavok, N.S., Krasilnikova, O.A. and Babenko, N.A., 2001. Thyroxine signal transduction in liver cells involves phospholipase C and phospholipase D activation. genomic independent action of thyroid hormone. BMC Cell. Biol. 2, p. 5.

King KL, Cidlowski JA 1998 Cell cycle regulation and apoptosis Annu Rev Physiol 60:601-617

Koibuchi N And Chin W. 2000 Aberrant postnatal cerebellar development in transgenic mouse expressing a dominant negative thyroid hormone receptor. Proc. Annu. Meet. Endocr. Soc. 81d Toronto Canada, p. 374

Kuzman JA, Gerdes AM, Kobayashi S and Liang Q, 2005 Thyroid hormone activates Akt and prevents serum starvation-induced cell death in neonatal rat cardiomyocytes Journal of Molecular and Cellular Cardiology 39(5):841-4.

Lakatos, P. and Stern, P.H., 1991. Evidence for direct non-genomic effects of triiodothyronine on bone rudiments in rats: stimulation of the inositol phosphate second messenger system. Acta Endocrinol. (Copenhagen) 125, 603–608.

Ledda-Columbano GM, Perra A, Loi R, Shinozuka H, Columbano A 2000 Cell proliferation induced by triiodothyronine in rat liver is associated with nodule regression and reduction of hepatocellular carcinoma Cancer Res 60:603-609

Lee JT, Lebenthal E, Lee PC 1990 Thyroidal regulation of rat pancreatic nuclear triiodothyronine receptor during postnatal development Endocrinology 126(1): 209-215

Lei J, Mariash CN and Ingbar DH 2004 3,3',5-triiodo-L-thyronine up-regulation of Na,K-Atpase activity and cell surface expression in alveolar epithelial cells in Src kinase- and phosphoinositide 3-kinase-depenent Journal of Biological Chemistry 279:47589-47600

Leitman, D.C., Costa, C.H., Graf, H., Baxter, J.D. and Ribeiro, R.C., 1996. Thyroid hormone activation of transcription is potentiated by activators of cAMP-dependent protein kinase. J. Biol. Chem. 271, 21950–21955.

Li, L., Haynes, M.P. and Bender, J.R., 2003. Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc. Natl. Acad. Sci. U.S.A. 100, 4807–4812.

Liang J and Slingerland JM 2003 Multiple roles of PI3K/PKB (Akt) pathway in cell cycle progression, Cell Cycle 2(4): 339-345

Lin, H.Y., Yen, P.M., Davis, F.B. and Davis, P.J., 1997. Protein synthesis-dependent potentiation by thyroxine of antiviral activity of interferon-gamma. Am. J. Physiol. 273, C1225–C1232.

Lin HY, Davis FB, Gordinier JK, Martino LJ and Davis PJ. 1999 Thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells. American Journal of Physiology 276:C1014–C1024.

Lingor MK, Dickinson LM, Wrede CE, Briaud I, McCuaig JF, Myers MG Jr and Rhodes CJ 2003 Decreasing IRS-2 expression in pancreatic β-cells (INS-1) promotes apoptosis, which can be compensated for by introduction of IRS-4 expression. Molecular and Cellular Endocrinology 209: 17-31

Losel, R., Feuring, M. and Wehling, M., 2002. Non-genomic aldosterone action: from the cell membrane to human physiology. J. Steroid Biochem. Mol. Biol. 83, 167–171.

Losel R and Wehling M 2003 Nongenomic actions of steroid hormones. Nature Reviews Molecular and Cellular Biology Jan;4(1):46-56.

Lopez-Carballo G, Moreno L, Masia S, Perez P and Barettino D 2002. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. Journal of Biological Chemistry 277:25297-25304

Lynch, M.A., Andrews, J.F. and Moore, R.E., 1985. Low doses of T3 induce a rapid metabolic response in young lambs. Horm. Metab. Res. 17, 63–66.

Mandrup-Poulsen T 2001 Beta-cell apoptosis: stimuli and signalling Diabetes 50(S1) :S 58-63

Mathis D, Vence L and Benoist C 2001 β-cell death during progression to diabetes Nature 414: 792-798

Mauricio D, Mandrup-Poulsen T 1998 Apoptosis and the patogenesis of IDDM: a question of life and death. Diabetes 47: 1537-1543

Messier N and Langlois MF 2000 Triac regulation of transcription is T(3) receptor isoform- and response element-specific. Molecular and Cellular Endocrinology Jul 25;165(1-2):57-66.

Misiti S., Anastasi E., Sciacchitano S., Verga Falzacappa C., Panacchia L., Bucci B., Khouri D., D'Acquarica I., Brunetti E., Di Mario U., Toscano V. and Perfetti R. 3,5,3'-

triiodo-L-thyronine enhances the differentiation of a human pancreatic duct cell line into endocrine cells. Journal of Cellular Physiology, 2005 Jul; 204 (1) :286-96,

Moeller LC, Cao X, Dumitrescu AM, Seo H, Refetoff S. Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor beta through the phosphatidylinositol 3-kinase pathway. Nucl Recept Signal. 2006;4:e020. Epub 2006 Jul 7

Morgan DO 1997 Cyclin-dependent kinases: engines, clocks and microprocessors Annu Rev Cell Dev Biol 13:261-291

Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu A, Kuzuya H, Nakao K. 2002 Thyroid hormone action is disrupted by Bisphenol A as an antagonist J Clin Endocr & Metabol 87(11): 5185-5190

Nielsen JH, Svensson C, Galsgaard ED, Moldrup A and Billestrup N 1999. Beta cell proliferation and growth factors. Journal of. Molecular. Medicine. 77: 62-66

Nielsen JH, Galsgaard ED, Moldrup A, Friedrichsen BN, Billestrup N, Hansen JA, Lee YC, Carlsson C. 2001 Regulation of beta-cell mass by hormones and growth factors. Diabetes; 50 Suppl 1:S25-9.

Osman, R.A., Andria, M.L., Jones, A.D. and Meizel, S., 1989. Steroid induced exocytosis: the human sperm acrosome reaction. Biochem. Biophys. Res. Commun. 160, 828–833

Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M 2001 Nitric oxide-induced apoptosis in pancreatic β cells is mediated by the endoplasmic reticulum stress pathway PNAS vol. 98,19: 10845-10850

Perfetti R, Zhou J, Doyle ME, Egan JM 2000 GLP-1 induces cell proliferation, PDX-1 expression and increases endocrine cell mass in pancreas of old, glucose intolerant rats. Endocrinology 141:4600-4605

Pibiri M, Ledda-Columbano GM, Cossu C, Simula G, Menegazzi M, Shinozuka H and Columbano A 2001 Cyclin D1 is an early target in hepatocyte proliferation induced by thyroid hormone (T3) The FASEB J 15:1006-1013

Pietras, R.J. and Szego, C.M., 1975. Endometrial cell calcium and oestrogen action. Nature 253, 357–359.

Pietras RJ and Szego CM 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265,69-72

Pliam MB and Goldfine ID 1997 High affinity thyroid hormone binding sites on purified rat liver plasma membranes Biochemical and Biophysical Research Communication 79: 166-172

Poitout V, Olson LK, Robertson RP 1996 Insulin-secreting cell lines: classification, characteristics and potential applications Diabetes Metab 22(1): 7-14

Porlan E, Vega S, Iglesias T and Rodriguez-Poena A 2004 Unliganded thyroid hormone receptor beta1 inhibits proliferation of murine fibroblasts by delaying the onset of the G1 cell-cycle signals. Oncogene. Nov 18;23(54):8756-65.

Rasilainen S, Nieminem JM, Levonen AL, Otonkoski T, Lapatto R 2002 Dose-dependent cysteine-mediated protection of insulin-producing cells from damage by hydrogen peroxide Biochem Pharmacol 63(7): 1297-1304

Razandi, M., Oh, P., Pedram, A., Schnitzer, J. and Levin, E.R., 2002. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol. Endocrinol. 16, 100–115.

Ribeiro Rc, Apriletti Jw, Wagner Rl, West Bl, Feng W, Huber R, Kushner Pj, Nilsson S, Scanlan T, Fletterick Rj, Schaufele F, and Baxter Jd. 1998 Mechanisms of thyroid hormone action: insights from X-ray crystallographic and functional studies. Rec. Prog. Horm. Res. 53: 351–394

Russell, K.S., Haynes, M.P., Sinha, D., Clerisme, E. and Bender, J.R., 2000. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc. Natl. Acad. Sci. U.S.A. 97, 5930–5935.

Segal, J., 1989. Action of the thyroid hormone at the level of the plasma membrane. Endocr. Res. 15, 619–649.

Shahrara S, Drvota V, Sylven C 1999 Organ specific expression of thyroid hormone receptor mRNA and protein in different human tissues. Biol Pharma Bul 22(10): 1027-1033

Shaul, P.W., 1999. Rapid activation of endothelial nitric oxide synthase by estrogen. Steroids 64, 28–34.

Shi YB, Su Y, li Q, Damjanovski S 1998 Autoregulation of thyroid hormone receptor genes during metamorphosis: roles in apoptosis and cell proliferation. Int. J. Dev. Biol. 42 : 107-116

Scheid MP and Woodgett JR 2001 PKB/AKT: functional insights from genetic models, Nature, Oct 2001, vol.2 : 760-768

Scheid MP, Marignani PA and Woodgett JR 2002. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Molecular and Cellular Biology, Vol. 22,17: 6247-6259

Schmidt, B.M., Martin, N., Georgens, A.C., Tillmann, H.C., Feuring, M., Christ, M. and Wehling, M., 2002. Nongenomic cardiovascular effects of triiodothyronine in euthyroid male volunteers. J. Clin. Endocrinol. Metab. 87, 1681–1686.

Shepherd PR, Withers DJ and Siddle K 1998 Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochemical Journal: 333: 471-490

Shih A, Lin H-Y, Davis FB and Davis PJ 2001 Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. Biochemistry 40: 2870-2878

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW and Liao JK 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature, 407: 538-541

Sjoholm A. 1995 Regulation of insulinoma cell proliferation and insulin accumulation by peptides and second messengers Ups J Med Sci 100(3): 201-216

Srinivasan S, Bernal-Mizrachi E, Ohsugi M and Permutt MA 2002 Glucose promotes pancreatic islet β-cell survival through a PI 3-kinase/Akt signalling pathway Am J Physiol Endocrin Metabol 283: E748-E793

Storey, N.M., O'Bryan, J.P. and Armstrong, D.L., 2002. Rac and Rho mediate opposing hormonal regulation of the ether-a-go-go-related potassium channel. Curr. Biol. 12, 27–33.

Storey NM, Gentile S, Ullah H, Russo A, Muessel M, Erxleben C and Armstrong DL 2006 Rapid signaling at the plasma membrane by a nuclear receptor for thyroid hormone Proceedings of the National Academy of Sciences, vol 103, no 13, 5197-5201

Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV and Cheng JQ 2001. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between Erα and PI3K. Cancer Research 61:5985-5991

Tang HY, Lin H-Y, Zhang S, Davis FB and Davis PJ 2004 Thyroid hormone causes mitogen-activated protein kinase-dependent phosphorylation of the nuclear estrogen receptor. Endocrinology 145: 3265-3272

Thomas D, Yang H, Boffa DJ, Ding R, Sharma VK, Lagman M, Li B, Hering B, Mohanakumar T, Lakey J, Kapur S, Hancock WW, Suthanthiran M 2002 Proapoptotic Bax is hyperexpressed in isolated human islets compared with antiapoptotic Bcl-2. Transplantation 74:1489-1496

Ting, Y.T., Bhat, M.K., Wong, R. and Cheng, S., 1997. Tissue-specific stabilization of the thyroid hormone beta1 nuclear receptor by phosphorylation. J. Biol. Chem. 272, 4129–4134.

Trentin AG, Alvarez-Silva M and Moura Neto V 2001 Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors Am J Physiol Endocrinol Metab 281:E1088-E1094

Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furrth EE, Polonsky NS, Naji A, Birnbaum MJ 2001 Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt/PKB alpha. Nat. Med. 7:1133-1137

Ueki K, Fruman DA, Brachmann SM, Tseng Y-H, Cantley LC and Kahn CR 2002 Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival Molecular and Cellular Biology Vol 22, No 3:965-977

Verga Falzacappa C, Panacchia L, Bucci B, Stigliano A, Cavallo MG, Brunetti E, Toscano V and Misiti S 2006 3,5,3'-triiodothyronine (T3) is a survival factor for pancreatic beta-cells undergoing apoptosis. Journal of Cellular Physiology 206(2):309-21.

Vermeulen K, Berneman ZN, Van Bockstaele DR 2003 Cell cycle and apoptosis Cell. Prolif. 36: 165-175

Wang, Y.G., Dedkova, E.N., Fiening, J.P., Ojamaa, K., Blatter, L.A. and Lipsius, S.L., 2003. Acute exposure to thyroid hormone increases Na^+ current and intracellular Ca^{2+} in cat atrial myocytes. J. Physiol. 546, 491–499.

Watters, J.J., Campbell, J.S., Cunningham, M.J., Krebs, E.G. and Dorsa, D.M., 1997. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology 138, 4030–4033.

Webster CR, Anwer MS 1998 Cyclic adenosine monophosphate-mediated protection against bile acid-induced apoptosis in cultured rat hepatocytes Hepatology 27: 324-1331

Wyckoff, M.H., Chambliss, K.L., Mineo, C., Yuhanna, I.S., Mendelsohn, M.E., Mumby, S.M. and Shaul, P.W., 2001. Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Galpha(i). J. Biol. Chem. 276, 27071–27076.

Wrede CE, Dickson LM, Lingohr MK, Briaud I, McCuaig JF, Rhodes CJ. 2002 Protein kinase B/Akt prevents fatty acid induced apoptosis in pancreatic β cells (INS-1) J. Biol. Chem. 277: 49676-49684

Wrutniak, C. and Cabello, G., 1989. Influence of tri-iodothyronine or lipid administration on the response of the pituitary-thyroid axis to exposure to cold in the newborn lamb. J. Endocrinol. 121, 361–365.

Yen P.M. 2001 Physiological and molecular basis of thyroid hormone action. Physiological Reviews 81: 1097-1142

Zhang J. and Lazar M.A. 2000 . The mechanism of action of thyroid hormones . Annu. Rev. Physiol. 62 : 439-66.

Zhu XG, Hanover JA, Hager GLand Cheng SY 1998 Hormone-induced translocation of thyroid hormone receptors in living cells visualized using a receptor green fluorescent protein chimera Journal of Biological Chemistry 273: 27058-27063

Zhu, Y., Bond, J. and Thomas, P., 2003. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. U.S.A. 100, 2237–2242.

Zhu, Y., Rice, C.D., Pang, Y., Pace, M. and Thomas, P., 2003. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. U.S.A. 100, 2231–2236.

Zinke A, Schmoll D, Zachmann M, Schmoll J, Junker H, Grempler R, Kirsch G, Walther R 2003 Expression of thyroid hormone receptor isoform alpha 1 in pancreatic islets Exp Clin Endocrinol Diabetes 111(4): 198-202

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