Thyroid hormone receptor TR β 1 mediates Akt activation by T₃ in pancreatic β cells

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Abstract

It has recently been recognized that thyroid hormones may rapidly generate biological responses by non-genomic mechanisms that are unaffected by inhibitors of transcription and translation. The signal transduction pathways underlying these effects are just beginning to be defined. We demonstrated that thyroid hormone T₃ rapidly induces Akt activation in pancreatic β cells rRINm5F and hCM via thyroid hormone receptor (TR) β 1. The phosphorylation of Akt was T₃ specific and dependent. Coimmunoprecipitation and colocalization experiments revealed that the phosphatidylinositol 3 kinase (PI3K) p85 α subunit and the thyroid receptor β 1 were able to form a complex at the cytoplasmic level in both the cell lines, suggesting that a 'cytoplasmic TR β 1' was implicated. Moreover, we evidenced that T₃ treatment was able to induce kinase activity of the TR β 1-associated PI3K. The silencing of TR β 1 expression through RNAi confirmed this receptor to be crucial for the T₃-induced activation of Akt. This action involved a T₃-induced nuclear translocation of activated Akt, as demonstrated by confocal immunofluorescence. In summary, T₃ is able to specifically activate Akt in the islet β cells rRINm5F and hCM through the interaction between TR β 1 and PI3K p85 α , demonstrating the involvement of TR β 1 in this novel T₃ non-genomic action in islet β cells.

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Introduction

Thyroid hormone, T₃, is classically known for its ability to regulate gene expression via binding with thyroid hormone receptors (TRs) on specific promoter regions of target genes. TRs act as ligand-dependent transcription factors and have been traditionally involved in the genomic action of T₃, which have a considerable latency with response times in hours to days. Among TR isoforms, two major functional TRs with specific physiological functions have widely been recognized, TR α 1 and TR β 1 (Ribeiro *et al.* 1998, Yen 2001). Recent findings have elucidated the role of nuclear TR β 1 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, TR β 1 could be considered a good candidate for mediating thyroid hormones (THs) non-genomic action.

Non-genomic actions of thyroid hormones have been described at the level of the plasma membrane, cytoskeleton, cytoplasm, and organelles of mammalian cells (Hennemann *et al.* 2001). Some of these actions rapidly lead to post-translational modification of nucleoproteins, including thyroid and estrogen nuclear receptors, and are mediated by MAPK ERK 1/2; (Davis

et al. 2000, Shih *et al.* 2001, D'Arezzo *et al.* 2004, Tang *et al.* 2004). Upstream mitogen activated protein kinase (MAPK), protein kinase C (PKC), and the phosphatidylinositol pathways may be activated by iodothyronines; these effects have also been shown in cells that lack functional nuclear thyroid hormone receptors (Lin *et al.* 1999, Shih *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 (Gharbi & Torresani 1979, Pliam & Goldfine 1997). It has recently been demonstrated (Bergh *et al.* 2005) that the extracellular domain of a structural membrane protein, integrin α V β 3, is capable of binding thyroid hormone T₄, thus activating the MAPK pathway.

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 non-transcriptional action of their receptors (Simoncini *et al.* 2000, Sun *et al.* 2001, Lopez-Carballo *et al.* 2002, Haynes *et al.* 2003). Furthermore, estrogen receptor α was demonstrated to activate PI3K through binding with p85 α 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 PI3K generating D3-phosphorylated phosphoinositides that are capable of binding PKB/Akt (Ueki *et al.* 2002, Liang & 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 synthesis in various cell culture systems (Coffer *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 evidence reviewed in Elghazi *et al.* (2006) underscore 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-I and GLP-1 (Giannoukakis *et al.* 2000, Buteau *et al.* 2001), and it is directly activated by glucose (Dickson & Rhodes 2004). When activated, via a cAMP-dependent or -independent mechanism, Akt mediates a large number of cellular processes, including mitogenesis, survival, and differentiation.

We have previously demonstrated that thyroid hormone T_3 (10^{-7} M) protects pancreatic β cells from a pharmacologically induced apoptosis. Our evidence suggested the involvement of the PI3K pathway in mediating this novel survival action of T_3 ; in particular, we have shown that T_3 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 T_3 is specifically able to activate Akt in a non-genomic manner. Interestingly, this activation implies the recruitment of the thyroid receptor β 1 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 (Heery *et al.* 1997, Zhu *et al.* 1998, Davis *et al.* 2000).

To examine the specific pathway via which T_3 can activate Akt in pancreatic β cells and to understand further the possible implication of a non-genomic action of T_3 in these cellular systems, we suggest a specific role for a 'cytoplasmic' thyroid hormone receptor β 1 involved in the PI3K pathway and able to mediate non-genomic actions of T_3 in pancreatic β cells.

Materials and methods

3,5,3'-Tri-iodothyronine (T_3), LY-294 002 hydrochloride, 3,5,3'-tri-iodoacetic acid (TRIAC), bisphenol A dimethacrylate (BPA), and cycloheximide (CHX) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

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

rRINm5F cells were cultured in RPMI 1640 (Cambrex Corp., East Rutherford, NJ, USA) complemented with 10% fetal bovine serum (Cambrex Corp.); hCM cells were cultured in RPMI 1640 (Cambrex Corp.) containing 5% FBS; all cell culture media were supplemented with L-glutamine 2 mM, according to manufacturer's instruction, and penicillin 100 μ g/ml and streptomycin 50 μ g/ml. Cells were maintained at 37 °C under humidified conditions of 95% air and 5% CO₂.

To determine the effects of T_3 (10^{-7} M) on Akt activation in the cell lines, cells were cultured to 60% confluence and exposed to the hormone T_3 , 10 μ M LY-294 002 hydrochloride, 10 μ M bisphenol A, 10 μ M TRIAC, and 3 mM CHX added only once, at the beginning of the individual experiments. Each inhibitor has been utilized as previously described by Moriyama *et al.* (2002) and Hui *et al.* (2003); LY-294 002, bisphenol A, and TRIAC were resuspended in stock solution, according to the manufacturer's instruction, and stored at -20 °C. Control cultures were grown under the same culture conditions 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.

Western blot analyses

Approximately, 3×10^6 cells were lysed for 30 min at 4 °C in buffer containing 1% Tween 20, 10% glycerol, 150 mM NaCl, 50 mM Hepes (pH 7.0), 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaF, 10 mM Na₄P₂O₇, 2 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors. The samples were then centrifuged at 12 000 r.p.m. for 30 min and the total cellular protein content was measured using the Bradford method (Bio-Rad). Cytosol-nuclear protein separation was obtained by nuclear/cytosol fractionation kit (MBL, International Corporation, Woburn, MA, USA) following the manufacturer's instruction. Forty micrograms total extracts and 30 μ g nuclear or cytosol proteins per sample were loaded onto a 10%

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 1 h at RT in 5% non-fat dry milk dissolved in PBS 1×, Tween 20 0.1% and then incubated for 16 h at 4 °C, with: TRβ1 (Santa Cruz Biotechnology, Inc., San Diego, CA, USA; 1:500), Akt (Santa Cruz, 1:500), phospho Akt 1/2/3-Ser 473 (Santa Cruz, 1:500), GSK3α (Santa Cruz, 1:500), pGSK3α-Ser 21 (Santa Cruz, 1:500), and histone H1 (Santa Cruz, 1:1000), and 1 h at RT with β-actin (Sigma, 1:1000), and α-tubulin (Santa Cruz, 1:1000), diluted in 5% milk, and PBS 1×, Tween 20 0.1%. After three washes in PBS 1× (Cambrex Corp.), Tween 20 0.1%, the membranes were incubated with the secondary HRP antibodies (anti-mouse, anti-rabbit; Sigma) 1:4000 in milk 5%, PBS 1×, Tween 20 0.1% for 45 min at RT. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp.) following the manufacturer's instruction.

Immunofluorescence microscopy

Cells were plated onto multichamber slides, (BD Falcon, Franklin Lakes, NJ, USA) cultured to 60% confluence and then exposed to the hormone treatment for the indicated times. Slides were washed in PBS 1×, air-dried, fixed, and permeabilized with acetone/methanol (1:1 (v/v) stored at −20 °) for 10 min, and then rehydrated in PBS 1×. Slides were then incubated with 20% fetal bovine serum in PBS 1× for 20 min and washed thrice in PBS 1×. Thereafter, slides were stained with primary antibodies (rabbit anti-PI3K, Santa Cruz; mouse anti-TRβ1, Santa Cruz; rabbit anti-phospho Akt 1/2/3-Ser 473, Santa Cruz) at a dilution of 1:100, for 45 min at RT in a humid chamber. After three washes in PBS 1×, slides were incubated with secondary antibodies (FITC-conjugated rabbit anti-mouse IgG, TRITC-conjugated swine anti-rabbit IgG, DAKO, Glostrup, Denmark) for 45 min at room temperature in the dark, at a dilution of 1:40. Immunofluorescence analysis of cell slides was carried out using an inverted fluorescence microscope equipped for confocal microscopy (Olympus Flowview FV500, Olympus Mikroskopie, Hamburg, Germany). Negative controls including omission of the primary antibody were also performed.

Immunoprecipitation

Cells were lysed in 1% NP40, 0.2 mM PMSF, 10 mM NaF, 0.7 µg/ml pepstatin, and 25 µg/ml aprotinin in PBS 1×. After 10 min on ice, samples were sonicated and centrifuged at 12 000 *g* for 15 min. The total cellular protein content was measured using the Bradford method (Bio-Rad). Cell lysate (400 µg) was

incubated for 2 h with 30 µl G-protein (Roche Diagnostics). After preclearing, the extracts were incubated overnight at 4 °C with mouse anti-TRβ1 antibody (Santa Cruz, 1 µg) and 30 µl freshly prepared G-protein. The immunoprecipitates were electrophoresed onto an 8% SDS-polyacrylamide gel, and blotted onto PVDF membrane (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in PBS 1×–Tween 20 0.1% for 1 h at room temperature and probed with rabbit anti-PI3K p85α (1:500), and afterwards with mouse anti-TRβ1 antibody (Santa Cruz, 1:500) diluted in 5% non-fat dry milk in PBS 1×–Tween 20 0.1% overnight at 4 °C under gentle rocking. After washing in PBS 1×–Tween 20 0.1%, the membranes were incubated with the secondary antibody HRP conjugated (anti-mouse, anti-rabbit; Sigma) 1:4000 in 5% milk, PBS 1×, Tween 20 0.1%, for 1 h at RT. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp.) following the manufacturer's instructions.

PI3K kinase assay

Cells were cultured in 100 mm dishes to 60% confluence and exposed to T₃ 10^{−7} M, LY 10 µM or vehicles alone for 30 min. At the end of the treatment, cells were lysed and PI3K activity was estimated using a commercially available PI3K ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA) according to manufacturer's instructions. Protein G beads were then utilized for the PI3K ELISA (Echelon Biosciences, Inc.) following manufacturer's instruction. Briefly, cells were lysed with a low stringency buffer and 100 µg lysates were incubated with 1 µg TRβ1 antibody for 1 h 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 samples were incubated with phosphatidylinositol 4,5-bisphosphate substrate and reaction buffer for 1 h 30 min. The amount of PIP₃ formed from phosphatidylinositol 4,5-bisphosphate by PI3K activity was detected using a competitive ELISA. The optical density (OD) values obtained were inversely proportional to the amount of PI(3,4,5)P₃ produced by PI3K activity. Enzyme activity was estimated by comparing the values from the samples with those in the standard curve.

RNA interference

Cells were plated onto six multiwells and grown in complete medium after 24 h cells were transfected with siRNA SmartPool THRB (Dharmacon, Lafayette, CO, USA). Transfection was performed by incubating cells with 200 pmol siRNAs in 2 ml serum-free transfection medium using lipofectamine reagent (Invitrogen). After 3 h of incubation, normal medium supplemented

with serum replaced transfection medium and cells were grown for 30 h before starting T₃ treatment. Subsequently, cells were lysed for western blot analysis and samples were analyzed as described earlier.

Results

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

As we previously demonstrated (Verga Falzacappa *et al.* 2006), hCM and rRINm5F cells exposed to T₃ (10⁻⁷ M) treatment are able to counteract an apoptotic cascade ongoing. This survival effect is mediated by PI3K and involves an Akt activation by T₃ after 24 h of hormone treatment. At first, we decided to examine an 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 30 min. In addition, cells in the presence of T₃ 10⁻⁷ M were incubated with the thyroid hormone receptor antagonists, bisphenol A and TRIAC (10 μM). The 10 μM concentration of each inhibitor was selected as the more efficacious after experiments of dose-response performed with different concentrations (100 nM, 1, and 10 μM; data not shown). BPA is a thyroid hormone analog capable of disrupting the action of the said hormone through the thyroid hormone receptor (Moriyama *et al.* 2002), while TRIAC is a thyroid hormone analog which is specifically able to potentiate T₃ effect on transcription through TRβ isoforms (Messier & Langlois 2000). A negative control for Akt phosphorylation was obtained by incubating the cells with the PI3K inhibitor LY-294 002 hydrochloride 10 μM. As shown in Fig. 1, a significant increase in Ser 473 phosphorylation of Akt was detected as early as 10 min after T₃ addition and persisted for 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 which appeared to be T₃ dependent as suggested by the ability of BPA to block the phosphorylation, seemed to be independent of gene transcription, since the presence of TRIAC could not augment pAkt levels.

The T₃ action we observed did not require *de novo* protein synthesis; the addition 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.

TRβ1 exists in the cytoplasmic region and is able to colocalize with PI3K p85α

As we observed, the effect of T₃ on Akt phosphorylation was rapid and CHX independent. Our results showed 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 non-genomic mechanism for this T₃ action, presumably taking place outside 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 (Zhu 1998, Davis *et al.* 2000), we decided to investigate whether a thyroid receptor was detectable in a subcellular localization of the examined cells. As shown in Fig. 2a 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 proteins analyzed. Interestingly, the TRβ1 and the PI3K p85α were able to colocalize at a cytoplasmic level. This ability and the expression level of each single protein were 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, Fig. 2b (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.

TRB1 complexes with PI3K p85α in a ligand-independent manner

Thyroid receptors have recently been demonstrated to act non-genomically 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 Fig. 2c 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 analogs BPA and TRIAC. In the same experimental conditions, 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. TRβ1 and PI3K p85α can

Figure 1 T_3 induces rapid activation of pAkt in a CHX-insensitive manner. Cells (a, hCM; b, rRINm5F) were exposed to T_3 (10^{-7} M), and then deprived of the hormone treatment for the indicated times. Cells treated with T_3 (30 min) were also exposed to LY (10 μ M), BPA (10 μ M), and TRIAC (10 μ M; upper panels). Additionally, samples were hormone treated and concurrently exposed to 3 mM CHX for 30 min (lower panels). Western blot analyses (WB) were performed as described in Materials and methods and a specific band corresponding to the phosphorylated Akt (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 ($\pm$ s.d.), after they had been normalized to Akt for equal loading. Data are presented in the histogram 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. *P* < 0.05 was considered significant. At least three different experiments were performed, and a representative one is shown here.

form a complex in a ligand-independent manner, and together with the evidence obtained by fluorescence microscopy and cytosol western blot, these data suggest this complex that is located at the cytosol or at the plasma membrane level.

T₃ induces TRβ1-associated PI3K activity

To investigate whether T₃ was able to affect the PI3K activity, a competitive ELISA assay was performed on TRβ1 pulled down samples. Samples were collected from cells (hCM and RINm5F) exposed or not to the hormone treatment for 30 min and exposure to LY 10 μM was used as a negative control. As shown in Fig. 3a, the presence of T₃ provoked an increase in the kinase activity of about twofold; the PI3K activity was indeed about 90 pmol/mg protein per hour in the control samples of hCM cells, while it reached 200 pmol/mg protein per hour in the hormone-treated samples. The same experiments performed on rRINm5F showed a similar trend (data not shown). 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 a specific Akt substrate was analyzed by western blot analyses. The main targets of Akt activity are the glucocorticoid synthetase kinases α and β, which are specifically phosphorylated on the Ser 21 and Ser 9 residues respectively by Akt (Cross *et al.* 1995); as shown in Fig. 3b, western blot analyses performed on the same samples utilized for Akt activation evaluation, were performed for the analysis of GSK3α phosphorylation levels. Interestingly, the phosphorylation of GSK3α on Ser 21 was increased by the hormone treatment already after 10 min; moreover, the addition of neither BPA nor TRIAC was able to affect the T₃ effect on this phosphorylation suggesting that the thyroid hormone T₃ is able to fully activate Akt, thus promoting its kinase activity, in both hCM and rRINm5F cells.

T₃ induces the nuclear translocation of activated Akt

To better understand the dynamics 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 in rRINm5F cells as shown in Fig. 4a (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 T₃ can promote the activation of Akt and 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 Fig. 4b (hCM) and d (rRINm5F), the phosphorylated 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 examined by histone H1 (nucleus) and α-tubulin (cytosol) blotting as shown. These data suggest that in our cellular models, T₃ is able to increase Akt activation by specific phosphorylation on Ser 473 and promotes its nuclear localization as shown in the cells exposed to the hormone.

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 T₃ ability of the to promote Akt phosphorylation. TRβ1 was 'knocked down' in hCM and rRINm5F cells by RNAi experiments. The cells were transfected with TRβ1 siRNAs and exposed or not to T₃ treatment; total extracts were then immunoblotted for TRβ1 and pAkt-Ser 473. As shown in Fig. 5a and b, when

Figure 2 TRβ1 is present at a cytoplasmic level and complexes with PI3K p85α. Immunofluorescence (IF) analysis (hCM, a; rRINm5F, d). Cells were cultured and immunostained for TRβ1 (green fluorescence), and subsequently for PI3K p85α (red fluorescence); nuclei were counterstained with Hoechst. The images were then merged to show the colocalization of the two signals. The images were acquired and analyzed through fluorescence microscopy as described in Materials and methods. Western blot (WB) analyses performed specifically on the cytosol and nuclear fractions of hCM (b) and rRINm5F (e). Cell extracts were analyzed for the presence of TRβ1 as described in Materials and methods. Coimmunoprecipitation (IP) experiments (hCM, c; rRINm5F, f). Cells were exposed to T₃ and then deprived of the hormone treatment for the indicated times (min), and the same cells were also exposed to BPA (10 μM) or TRIAC (10 μM); immunoprecipitation experiments for TRβ1 were performed on total extracts. Western blot analyses for PI3K p85α and TRβ1 were performed as described in Materials and methods. All the data shown are representative of at least three independent experiments.

(a) TRB1 associated PI3K activity

(b) hCM

RINm5F

Figure 3 (a) T_3 induces TRβ1-associated PI3K and Akt activities. PI3K activity: hCM and rRINm5f cells were exposed to T_3 (10^{-7} M) alone or concurrently with T_3 and LY (10 μ M) and to vehicle alone for 30 min. Then, total extracts were immunoprecipitated for TRβ1 and analyzed for PI3K activity as described in Materials and methods. The kinase activity was estimated by comparing the values from the samples with those in the standard curve obtained following manufacturer's instruction. All the data are presented as means $\pm$ s.d. and are the results of five different experiments at least in which every sample was run in triplicate. (b) Akt activity: hCM and RINm5F cells were cultured in the presence or not of T_3 (10^{-7} M) and inhibitors for the indicated times (min). Western blot (WB) analyses were performed as described in Materials and methods and a specific band corresponding to the phosphorylated GSK3α (Ser 21) was detected. The expression of GSK3α was analyzed as a control for gel loading. Densitometric absorbance values from three separate experiments were averaged ($\pm$ s.d.), after they had been normalized to GSK3α for equal loading. Data are presented in the histogram 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 treatments was conducted by using Student's *t*-test. * $P < 0.05$; † $P < 0.01$. $P < 0.05$ was considered significant. At least three different experiments were performed, and a representative one is shown here. ctrl, control.

TR β 1 was silenced, as demonstrated by western blot analyses, the phosphorylation of Akt induced by T₃ was completely abolished; on the other hand, the TR β 1 silencing *per se* was not able to influence the basal

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 β 1.

Figure 4 T₃ induces nuclear translocation of pAkt. hCM (a) and rRINm5F (c) cells were exposed to T₃ (10⁻⁷ M) or vehicle alone for 30 min and then immunostained for pAkt-Ser 473 as described in Materials and methods, and images were analyzed by confocal microscopy. Additionally, western blot (WB) analyses on the cytoplasmic and nuclear fractions of proteins extracts from hCM (b) and rRINm5F cells (d) exposed to T₃ treatment for 30 min were performed and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of histone H1 (nuclear) and α -tubulin (cytosolic) was analyzed as a control for gel loading and to exclude the contamination of the cytosol with the nuclear components and vice versa. At least three different experiments were performed, and a representative one is shown here.

Figure 5 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 (10⁻⁷ M) 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 TRβ1 was detected. The expression of Akt was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here.

To exclude the possibility that T₃ effects on Akt depend on the amount of TRβ1, we performed a western blot to analyze TRβ1 expression in treated cells (data not shown). Neither hCM nor rRINm5F TRβ1 protein levels were affected by T₃ treatment, strongly indicating that the effect we observed was completely independent of the receptor amount.

Discussion

The thyroid hormone T₃ is known to access the cell interior and 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 effects, 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 non-genomic or extranuclear action. Such effects have actually been known for many years, but their mechanisms still remain unclear. One of the main questions to be answered is which are the mediators of this thyroid hormone action; in particular it is not yet clear whether a new thyroid hormone receptor is involved in the non-genomic action, although no

specific membrane-associated TR isoform has been identified yet, or if the well known receptor has novel additional functions, since 10% of TRs are cytoplasmic in the absence of T₃ (Baumann *et al.* 2001).

In this study, we have shown in pancreatic β cells that a non-genomic T₃ action involving the PI3K pathway exists and that the thyroid receptor β1 is essential in mediating this T₃ action.

The factors that drive β cell proliferation and function under normal or pathological conditions 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 PI3K; moreover, the involvement of Akt in the regulation of replication and survival of pancreatic β cells has been demonstrated (Tuttle *et al.* 2001, Lingor *et al.* 2003). 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 occur within minutes, and is related to non-genomic action of specific activators of the PI3K pathway. The thyroid hormones have recently been demonstrated to induce Akt activation (Cao *et al.* 2005, Kuzman *et al.* 2005), and this effect occurs 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 of *de novo* protein synthesis.

Activation of Akt entails a complex series of events involving additional proteins. First, the PI3K-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 & Coffey 1995, Franke *et al.* 1997). Once membrane proximal, Akt is phosphorylated on two residues (Thr 308 and Ser 473), then 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). In this study, we demonstrated how T₃ treatment is able to influence these events, in particular inducing the translocation of active Akt to the nucleus.

Optimal signaling through the PI3K pathway depends on a critical molecular balance between the regulatory and the catalytic subunits; in particular, the p85α subunit is thought to be the major response pathway for most stimuli (Shepherd *et al.* 1998). The relationship 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 has already been 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 & Szego 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 & Wehling 2003). We demonstrated that the β1 isoform of the 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 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 confirming that the TRβ1-PI3K binding does not require the presence of T₃. 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 that are 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 showed that phosphorylation of GSK3α, one of the main Akt targets, is enhanced by the hormone treatment and that even this activation shows a trend similar to the one 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 evidence of the cytoplasmic localization of TRβ1, our data support the hypothesis that nuclear thyroid receptors might exist also outside the nucleus (Zhu *et al.* 1998, Davis *et al.* 2000) playing an important role in non-genomic actions of thyroid hormones.

We also showed that the 'knockdown' 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.

Phosphoinositide 3-kinase plays a pivotal role in the metabolic and mitogenic actions of insulin, in particular the direct binding between IRS-2 and PI3K leads to Akt activation in pancreatic β cells, modulating the cell survival, the proliferation and the cell size (Accili 2001). Given this crucial role of IRS-2 in PI3K action in pancreatic β cells, we analyzed its expression after

long-term (24 h) treatment with T₃; interestingly the protein levels of IRS-2 were upregulated by the hormone treatment (data not shown), suggesting that T₃ can interact with the PI3K pathway even at this level. In fact, if all the effects of IRS-2 are mediated by Akt, there must be additional players, which are likely to include also transcriptional factors and additional kinases. In this context, we could speculate that TRβ1 plays a specific role directly entering the PI3K pathway.

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 signaling are complex, but they appear to act synergistically. The postulated non-genomic signaling pathway may complement such actions by generating second messengers and by activating multiple signaling 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 homology to the nuclear one at least, if not the same itself, might be implicated in the effects we characterized in pancreatic β 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, strengthening our hypothesis that a thyroid hormone receptor with high similarities to the nuclear one but with a peculiar localization is involved.

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 seems to be similar to the nuclear TRβ1 form, but with some peculiar characteristics that led its location.

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