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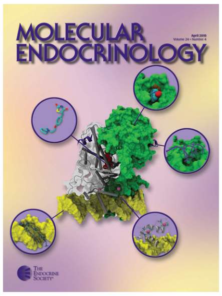

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Thyroid Hormone-Mediated Activation of the ERK/Dual Specificity Phosphatase 1 Pathway Augments the Apoptosis of GH4C1 Cells by Down-Regulating Nuclear Factor-κB Activity

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Thyroid hormone (T_3) plays a crucial role in processes such as cell proliferation and differentiation, whereas its implication on cellular apoptosis has not been well documented. Here we examined the effect of T_3 on the apoptosis of GH4C1 pituitary cells and the mechanisms underlying this effect. We show that T_3 produced a significant increase in apoptosis in serum-depleted conditions. This effect was accompanied by a decrease in nuclear factor-κB (NF-κB)-dependent transcription, IκBα phosphorylation, translocation of p65/NF-κB to the nucleus, phosphorylation, and transactivation. Moreover, these effects were correlated with a T3-induced decrease in the expression of antiapoptotic gene products, such as members of the inhibitor of apoptosis protein and Bcl-2 families. On the other hand, ERK but not c-Jun N-terminal kinase or MAPK p38, was activated upon exposure to T_3, and inhibition of ERK alone abrogated T_3-mediated apoptosis. In addition, T_3 increased the expression of the MAPK phosphatase, dual specificity phosphatase 1 (DUSP1), in an ERK-dependent manner. Interestingly, the suppression of DUSP1 expression abrogated T_3-induced inhibition of NF-κB-dependent transcription and p65/NF-κB translocation to the nucleus, as well as T_3-mediated apoptosis. Overall, our results indicate that T_3 induces apoptosis in rat pituitary tumor cells by down-regulating NF-κB activity through a mechanism dependent on the ERK/DUSP1 pathway. (*Molecular Endocrinology* 22: 2466–2480, 2008)

THE THYROID HORMONE T_3 influences a variety of physiological processes in mammals, including cell growth and metabolism (1). Most, if not all, of these actions are mediated by nuclear T_3 receptors, which are widely expressed in mammalian tissues. Pituitary somatolactotrope cells are a well-established target of T_3 and require trophic support for survival in culture (2). GH4C1 cells, which are derived from a rat pituitary tumor, have been widely used as an *in vitro* model to understand the mechanisms of T_3 action (3). Nevertheless, there is still certain controversy regarding the effect of T_3 and its receptors on cell proliferation. In fact, the cell type, as well as the developmental or pathophysiological state, appears to determine whether T_3 activates (4–6) or blocks (7–10) the proliferation of many cultured cells through mechanisms, involving either growth factors, MAPK pathways, cyclins, or the tumor suppressor p53. In particular, T_3 has been shown to modulate the proliferation of different rat pituitary cell lines and it is known to promote the division of GH4C1 cells by stimulating the secretion of an autocrine growth factor (11). Indeed, T_3 stimulates growth of GC cells by shortening the G_1 phase of the cell cycle (12). Moreover, it has been shown that T_3-dependent cell growth of GH1 cells requires a serum-derived mediator for T_3 responsiveness (13). In contrast, exposing the GH3 pituitary cell line to T_3 was recently shown to increase the expression of mitochondrial proapoptotic molecules Bax and Bak, while decreasing that of the antiapoptotic Bcl-2, potentially initiating an apoptotic response (14).

The inducible nuclear factor-κB (NF-κB) is an important transcription factor involved in the regulation of immune, inflammatory, apoptotic, and carcinogenic processes in response to a wide variety of stimuli (15, 16). Under resting conditions, NF-κB exists in the cytoplasm as a heterotrimer of p50, p65, and IκBα. The phosphorylation, ubiquitination, and degradation of

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* A.C. and A.S.-P. contributed equally to this work.

Abbreviations: DTT, Dithiothreitol; DUSP1; dual specificity phosphatase 1; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; HS, horse serum; IAP, inhibitor of apoptosis protein; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; NF-κB, nuclear factor-κB; PARP, polyadenosine ribose polymerase; PI, propidium iodide; QRT-PCR, quantitative RT-PCR; r-IAP, rat inhibitor of apoptosis protein; SD, serum deprived; siRNA, small interfering RNA; TK, thymidine kinase.

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IκBα lead to the translocation of NF-κB complexes to the nucleus, where they bind to specific DNA response elements (17). In addition, several studies have shown that posttranslational modification of NF-κB proteins, including phosphorylation, can influence its transcriptional activity (18). NF-κB regulates the expression of a large number of genes the products of which are involved in apoptosis, including several members of the inhibitor of apoptosis proteins (IAP) and the Bcl-2 families (19).

The MAPK family comprises related serine/threonine protein kinases that direct cellular responses to proliferative cues or stressful stimuli, integrating different signals (20). Conventional members of the MAPK family include the ERKs, the c-Jun N-terminal kinases (JNKs), and MAPK p38. These MAPKs regulate diverse cell activities such as gene expression, proliferation, differentiation, and apoptosis (21–23). In general, it is accepted that JNK and MAPK p38 mainly promote apoptosis, whereas ERK activation is typically associated with cell survival, proliferation, and differentiation, as reflected by its activation by mitogens and some cell survival factors (23). However, activation of ERK by different stimuli has recently been found to contribute to cell death in certain cell types. In this regard, a persistent activation of ERK has been shown to induce cell death in primary neuron cultures (24), rat hepatocytes (25), and TtT-97 mouse thyrotrope tumor cells (26, 27). Interestingly, a correlation between ERK activation and cell death has also been shown in the pituitary, and sustained ERK activation in GH3 pituitary cells increases cell death (28). Similarly, epidermal growth factor triggers programmed cell death in pituitary GH4C1 cells by a mechanism involving ERK activation and the down-regulation of Bcl-2 (29).

MAPK phosphatases dephosphorylate MAPK at Thr/Tyr residues critical for activation, thereby contributing to the down-regulation of MAPK activity. Dual specificity phosphatase 1 (DUSP1), also known as MAPK phosphatase 1, is the founder member of this family, and it is induced by a variety of stimuli including growth factors, nuclear receptors, and stress stimuli (30). The expression of DUSP1 can be regulated through multiple pathways involving both transcriptional and posttranscriptional mechanisms, and inhibition of ERK substantially impairs the induction of DUSP1 by different stimuli (31, 32). DUSP1 has recently been identified in several cells as a critical regulator of many activities, including control of the homeostatic balance (33), immune challenge (34), inflammation (35), and proliferation or apoptosis (36, 37). In particular, the role of DUSP1 in apoptosis is controversial although it has been shown to be fundamental to prevent the cell death induced by chemotherapy agents in diverse tumor cells (37–39). Moreover, oxidative stress-mediated cell death is enhanced in DUSP1$^{-/-}$ mouse embryonic fibroblasts (40). By contrast, a proapoptotic role for DUSP1 has been described in other systems, and DUSP1 can mediate the ERK-dependent cell death induced by diverse stimuli in primary neuron cultures (41, 42) and in NIH3T3 cells (43). Interestingly, a link between DUSP1 induction and the antiproliferative effects of glucocorticoids in osteoblasts has also been demonstrated recently (44).

In this study, for the first time we demonstrate cross talk between T_3 signaling and both the NF-κB and the ERK/DUSP1 pathways in GH4C1 pituitary cells. We show that T_3 induces apoptosis in serum-starved GH4C1 cells, which is an established model for analyzing apoptotic pathways (45). This effect is controlled by a complex mechanism that involves the inhibition of NF-κB activity by induction of DUSP1, which in turn is dependent on ERK activation. These results could contribute to better understanding the molecular mechanism of action of T_3 in different physiopathological situations in pituitary cells.

RESULTS

Thyroid Hormone Induces Cell Death in the Serum-Starved Cells

T_3 plays a crucial role in cell proliferation and differentiation in different cell contexts. To evaluate the effects of T_3 on the apoptosis of GH4C1 cells, we first analyzed the sub-G_1 hypodiploid cell population by flow cytometry after propidium iodide (PI) staining. Cells were cultured for 48 h in growth medium supplemented with 10% charcoal-stripped-fetal bovine serum (FBS) (control) or 0.1% charcoal-stripped-FBS [serum-deprived (SD)], in the absence or presence of T_3 (Fig. 1A). As expected, T_3 did not induce apoptosis when added in the presence of 10% serum. However, exposure to T_3, in combination with the proapoptotic stimulus serum deprivation, induced apoptosis that affected about 25% of the cell population after 48 h. Apoptosis was induced by 10% by exposure to T_3 for 24 h when added in serum-depleted conditions, whereas T_3 appeared to be cytotoxic at 72 h (data not shown). For this reason the 48-h incubation time was chosen in subsequent experiments for analyzing the apoptotic mechanism employed by T_3.

Apart from PI staining, annexin V can be used to mark the redistribution of phosphatidylserine to the outer leaflet of the plasma membrane, a hallmark of programmed cell death. Cells were treated in the same conditions as in Fig. 1A, stained with both annexin V and PI, and analyzed by flow cytometry (Fig. 1B). The data show that in high serum conditions 93.49% of cells were viable (annexin V and PI double negative), and that the percentage of early apoptotic cells (annexin V single positive) or late apoptotic cells (annexin V and PI double positive) was low (3.89% and 2.89%, respectively). The PI-positive cells were labeled as necrotic and accounted for 1.80% of the cell population. Treatment of the cells with T_3 did not significantly

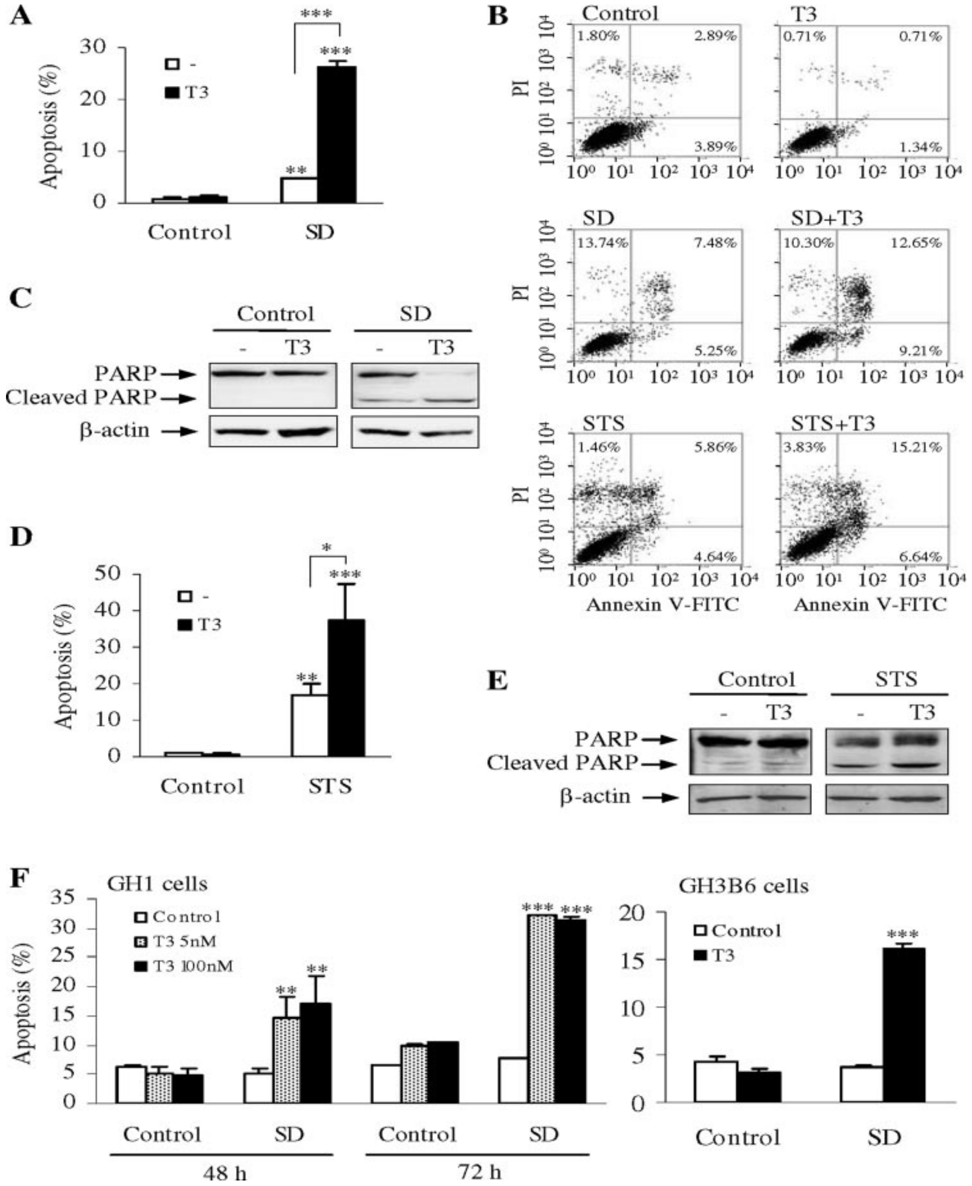

Fig. 1. Thyroid Hormone Induces Cell Death

A, GH4C1 cells were incubated for 48 h in medium supplemented with 10% (control) or 0.1% (SD) charcoal-stripped-FBS in the presence or absence of T_3 (5 nM). Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. The values represent the mean ± SD of three independent experiments performed in duplicate. B, GH4C1 cells were incubated as in panel A or panel D, and apoptosis was examined by the Annexin V/PI dual-staining assay, as detected by flow cytometry. A representative experiment is shown. C, Cells were incubated as in panel A, and Western blotting was performed to determine the expression of PARP, ensuring equal protein loading with β-actin. A representative experiment is shown. D and E, Cells were incubated for 48 h in medium supplemented with 10% charcoal-stripped-FBS in the presence or absence of T_3 (5 nM), and staurosporine (STS, 500 nM) was added for the last 24 h. Apoptosis levels shown in panels D and E were measured as in panels A and C, respectively. F, GH1 or GH3B6 cells were incubated for 48 h or 72 h, respectively, in medium supplemented with 10% (control) or 0.1% (SD) charcoal-stripped-HS in the presence or absence of 5 nM T_3 (GH1 cells) or 100 nM (GH1 and GH3B6 cells). Apoptosis levels were measured as in panel A. FITC, Fluorescein isothiocyanate.

modify these percentages. However, when cells were incubated in the presence of 0.1% serum (SD), viability decreased to 73.53%, and the percentages of cell population that displayed early or late apoptosis were increased up to 5.25% and 7.48%, respectively. The treatment with T_3 in low serum further reduced the viable cells to about 67.84%, but augmented the percentages of the cells in both the early and the late stage of apoptosis up to 9.21% and 12.65%, respectively (SD + T_3). Thus, dual staining with annexin V and PI allowed clear discrimination between unaffected cells, early apoptotic cells, and late apoptotic cells.

To further verify that T_3 did indeed mediate apoptosis, we examined the effects of T_3 treatment on the caspase-induced cleavage of polyadenosine ribose polymerase (PARP, Fig. 1C). The results obtained indicate that although low levels of PARP cleavage were detected in GH4C1 cells incubated in low serum, cleavage increased in the presence of T_3. In contrast, PARP was not cleaved in cells incubated with T_3 plus 10% serum. These results indicate that the enhanced cytotoxicity induced by T_3 in low serum was indeed due to apoptosis. The above results, together with additional data showing that T_3 reduces the tetramethyl rhodamine methyl ester fluorescence and induces mitochondrial depolarization in GH4C1 cells (data not shown) confirmed the proapoptotic effect of T_3 when cells were SD.

To evaluate whether the effects of T_3 on apoptosis were dependent on the apoptotic trigger employed, the cells were incubated under high serum conditions in the presence or absence of staurosporine, a known proapoptotic stimulus in GH4C1 cells (29), and apoptosis was analyzed by flow cytometry after PI staining (Fig. 1D). The results showed that about 20% of cells undergo apoptosis upon incubation with staurosporine, and that T_3 increased apoptosis up to about 40% (Fig. 1D). Apoptosis was also assessed by dual Annexin V and PI staining (Fig. 1B), and the results showed that staurosporine caused both early and late apoptosis in about 4.64% and 5.86% cell population, respectively, *vs.* 3.89% and 2.89%, respectively, in control cells. Incubation of the cells in the presence of staurosporine plus T_3 did raise the percentage of both early and late apoptotic cells up to 6.64% and 15.21%, respectively (Fig. 1B). These results were confirmed by the measurement of PARP cleavage, which was detected after incubation with staurosporine, and was enhanced by treatment of the cells with staurosporine in combination with T_3 (Fig. 1E).

To rule out cell type-specific effects of T_3 on pituitary cell apoptosis, we examined the effects of T_3 on apoptosis of the other rat pituitary cell lines. To that purpose, GH1 or GH3B6 cells were incubated in the same conditions as in Fig. 1A for 48 and 72 h (GH1 cells) or 72 h (GH3B6 cells), and apoptosis was measured by flow cytometry after PI staining (Fig. 1F). As expected, T_3 did not induce apoptosis when added in the presence of 10% serum. However, exposure to T_3 when cells were SD significantly induced apoptosis in GH1 or GH3B6 cells. These results show that different pituitary cell lines are susceptible to the proapoptotic effect of T_3 in low serum condition, revealing a potential physiological relevance of T_3 on pituitary apoptosis.

NF-κB Activity Is Inhibited by Thyroid Hormone in Cells Grown in Low Serum

Because NF-κB is a transcription factor known to be involved in antiapoptotic processes, we determined whether T_3 might induce apoptosis by suppressing NF-κB activity. NF-κB-dependent transcription was measured in GH4C1 cells transfected with a luciferase reporter (3x NF-κB-Luc) after exposure for 48 h to T_3 (Fig. 2A). T_3 did not affect NF-κB activity in cells grown in 10% serum; however, in SD cells there was a 1.5-fold increase in NF-κB-dependent transcription, although this activity was reduced by 60% by exposure to T_3 (Fig. 2A). By contrast, the thymidine kinase (TK)-Luc control reporter showed a very low activity, which was not modified in the presence of T_3 (data not shown). As mentioned previously, NF-κB is regulated, in part, by a cellular process that involves phosphorylation and degradation of its inhibitory subunit IκBα, permitting active NF-κB complexes to translocate to the nucleus and activate transcription. Thus, we measured the kinetics of IκBα degradation upon exposure to T_3 for 48 h and found that T_3 did not significantly modify the total IκBα levels in cytoplasmic extracts from cells incubated under all conditions (Fig. 2B). We next analyzed the effect of T_3 on IκBα phosphorylation and found that phosphorylated IκBα levels increased after serum depletion, whereas T_3 abrogated this effect (Fig. 2B). We then examined the effect of T_3 on p65/NF-κB levels in cytosolic extracts, which remained quite high and unchanged under all conditions. In contrast, nuclear p65/NF-κB levels increased after serum depletion, although they were almost completely suppressed after 48 h exposure to T_3 (Fig. 2B). Because NF-κB activity is enhanced by p65/NF-κB phosphorylation, we tested whether exposure to T_3 affected the phosphorylation of p65. A strong reduction in p65/NF-κB phosphorylation at Ser276 (pp65) was observed in cells cultured in low serum and exposed to T_3, whereas there was little phosphorylated nuclear p65/NF-κB in cells grown in high serum both in the presence or absence of T_3 (Fig. 2B). The levels of phosphorylated p65/NF-κB were almost undetectable in cytosolic extracts from the same cells and remained unchanged in any condition (Fig. 2B). The levels of tubulin and histone H3 were used as controls to validate the integrity of the cytoplasmic and nuclear extracts, respectively (Fig. 2B).

Because we detected differences in the levels of nuclear p65/NF-κB after exposure to T_3, we determined the NF-κB binding activity in nuclear extracts from cells that were incubated with T_3 for 48 h. No specific NF-κB-DNA binding complexes were observed when cells were incubated in the presence of 10% serum, whereas complex formation was induced in low serum. Moreover, there was a decrease in the NF-κB-DNA binding complexes when these cells were exposed to T_3 (Fig. 2C).

NF-κB may be activated by stimulation of the transactivation domain in the p65/NF-κB subunit. Therefore, we studied whether the differences observed after T_3 treatment were also dependent on the transcriptional activation of p65/NF-κB. We used a plasmid encoding the Gal4-p65 fusion protein to address this question, where the sequences encoding the DNA binding domain of Gal4 have been fused with se-

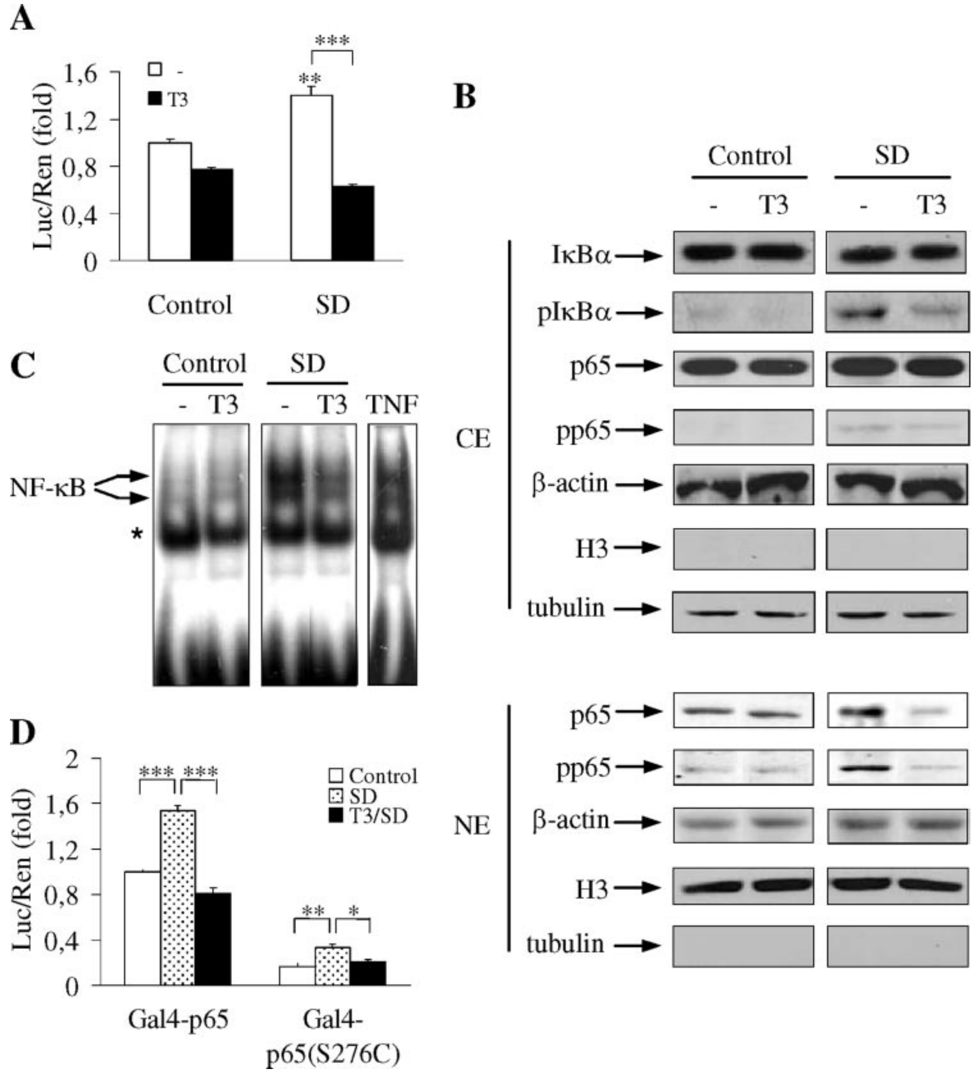

Fig. 2. NF-κB Activity Is Inhibited by Thyroid Hormone

A, GH4C1 cells were transiently transfected with a NF-κB-driven luciferase reporter plasmid (3× NF-κB-TK-Luc) and pRL-TK-*Renilla* and then treated with T_3 (5 nM) for 48 h in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS. Cell extracts were prepared and assayed for luciferase and *Renilla* activities. The luciferase levels were normalized to those of *Renilla* and expressed as the induction over the controls. The data shown represent the mean ± SD of three independent experiments performed in duplicate. B, Cells were incubated with T_3 (5 nM) in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS for 48 h. The cytosolic extracts (CE) and the nuclear extracts (NE) were analyzed by Western blotting using antibodies against IκBα, the phosphorylated form of IκBα (pIκBα), p65, and the phosphorylated form of p65 (pp65). Equal protein loading was evaluated by β-actin. The levels of tubulin and histone H3 were used as controls to validate the integrity of the cytoplasmic and nuclear extracts, respectively. The blots shown are from one of three representative experiments. C, Cells were treated as in panel B, and nuclear extracts were prepared and tested for NF-κB binding activity by EMSA. TNFα (10 ng/ml) was used as a positive control. The *asterisk* indicates a nonspecific band. A representative experiment of three replicates is shown. D, Cells were transiently transfected with the pGal4-Luc reporter and the pRL-TK-*Renilla*, together with either Gal4–p65 or the mutant Gal4–p65(S276). Cells were then treated with T_3 (5 nM) for 48 h in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS. Luciferase levels in the cell extracts were assayed, normalized with *Renilla*, and expressed as the induction over controls. The data shown represent the mean ± SD of three independent experiments performed in duplicate.

quences encoding p65/NF-κB. Its cotransfection with a Gal4-Luc reporter plasmid allowed us to determine whether cellular signals triggered by T_3 regulate gene expression by specifically targeting the p65/NF-κB protein. Like NF-κB-dependent transcription, low serum increased the basal activity of Gal4-p65 about 1.5-fold (Fig. 2D), and incubation of the cells with T_3 decreased it by about 50%. By contrast, the Gal4 control reporter showed very low activity that was not modified in the presence of T_3 (data not shown). These results indicate that T_3 inhibits NF-κB transcriptional activity by reducing the potential p65/NF-κB transactivation. Because T_3 affects p65/NF-κB phosphorylation at Ser276 (Fig. 2B), we tested the effect of T_3 on the transactivation potential of a Gal4-p65 mutant construct in which the Ser276 was replaced by cysteine [Gal4-p65(S276C)]. As expected, the basal activity of this mutant was very low (Fig. 2D), and T_3 further decreased the activity of this mutant by 39%, albeit to a lesser extent than the Gal4-p65 activity, which was reduced in T_3-treated cells by 55.4%. Hence, p65/NF-κB phosphorylation at the Ser276 contributes to, but is not necessary for, the regulation of p65/NF-κB transactivation by T_3.

Thyroid Hormone Alters the Levels of NF-κB-Dependent Antiapoptotic Genes

The results above suggest that T_3 induces cell apoptosis in low serum by a mechanism involving NF-κB inhibition. NF-κB up-regulates the expression of several genes involved in cell survival, including IAP and members of the Bcl-2 family. To test whether T_3 affected the expression of these NF-κB-responsive genes, GH4C1 cells were incubated for 48 h in the presence or absence of T_3, and the expression of IAP and Bcl-2 family members was determined by quantitative RT-PCR (QRT-PCR). Incubation of cells in low serum induced the expression of the antiapoptotic genes, rat inhibitor of apoptosis protein 1 (r-IAP1) and 3 (r-IAP3) (Fig. 3, A and B), and Bcl-x_L (Fig. 3C), and exposure to T_3 provoked a marked decrease in the expression of these genes. However, T_3 did not greatly modify the expression of r-IAP3 or Bcl-x_L when added to cells in 10% serum, although a mild reduction in r-IAP1 expression was observed albeit less than in low serum (Fig. 3A). The effects of T_3 were not general, because other members of these families, including survivin and r-IAP2, were neither expressed under basal conditions nor affected by T_3 treatment (data not shown). These perturbations in the expression of the antiapoptotic genes might contribute to the potentiation of the cell death caused by T_3 in low serum.

The Apoptosis Induced By Thyroid Hormone Is Specifically Mediated by ERK

JNK and MAPK p38 have been well characterized as proapoptotic kinases that transduce cell death signaling in many cell types (23). To further analyze the mechanisms by which T_3 induced apoptosis, we first examined the effects of T_3 on the activity of these kinases by measuring their phosphorylation (Fig. 4A). JNK phosphorylation was dramatically augmented by serum depletion, although this modification was not altered by T_3 (Fig. 4A, *upper panel*). MAPK p38 phosphorylation was not detected in GH4C1 cells at either concentration of serum or by the presence or absence of T_3 (Fig. 4A, *middle panel*). ERK is the other member of the MAPK family involved in apoptosis in different cell types. To analyze whether ERK was involved in T_3-mediated apoptosis, we examined the effects of T_3 on ERK activity (Fig. 4A, *lower panel*). Like JNK, incubation of the cells in serum-depleted conditions stimulated ERK, and T_3 caused a further increase in this activation. A discrete increase in ERK phosphorylation was also observed in the presence of T_3 and 10% serum.

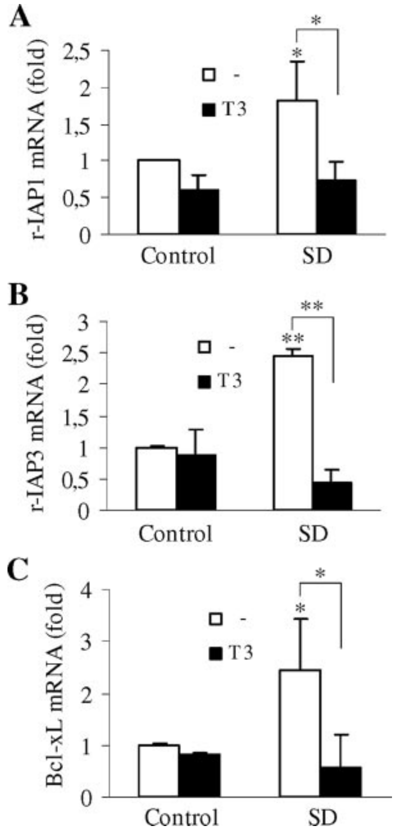

Fig. 3. Thyroid Hormone Alters the Levels of NF-κB-Dependent Antiapoptotic Genes

GH4C1 cells were maintained in the presence or absence of 5 nM T_3 in 10% (control) or 0.1% (SD) charcoal-stripped-FBS for 48 h. Total RNA was prepared, and QRT-PCR was carried out using primers against r-IAP1 (A), r-IAP3 (B), Bcl-x_L (C), and GAPDH. The mRNA levels were normalized by GAPDH, and the results were expressed as the changes in mRNA expression. The data shown represent the mean ± SD of three independent experiments.

To analyze the possible role of these proteins on T_3-induced apoptosis in GH4C1 cells, we next pretreated the cells for 30 min with specific inhibitors of MAPK pathways, followed by incubation with T_3 for 48 h. Exposing cells to the inhibitor of MAPK p38 (SB203580) or JNK (SP600125) did not significantly alter T_3-induced apoptosis, whereas the phenomenon was completely abrogated by incubation of the cells in the presence of the MAPK/ERK kinase (MEK) inhibitor U0126 (Fig. 4B). These results indicate that T_3-induced apoptosis in

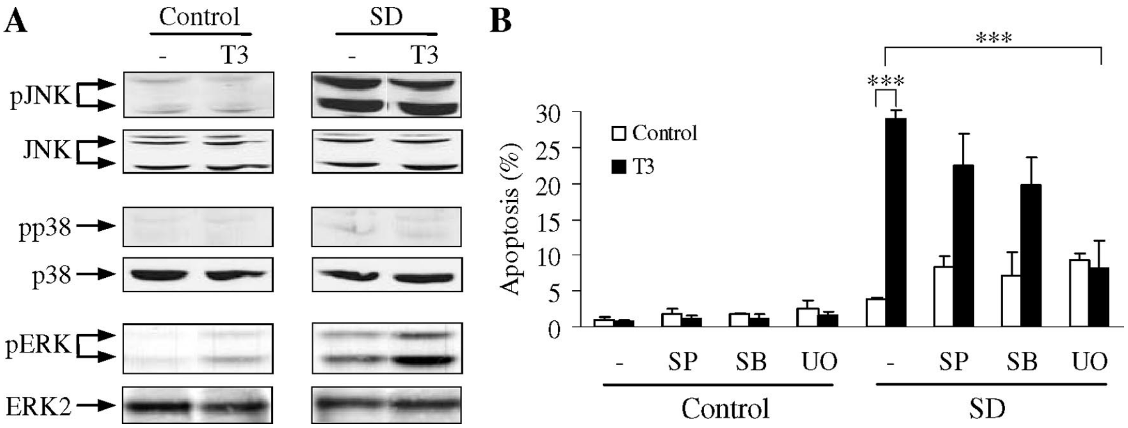

Fig. 4. The Apoptosis Induced by Thyroid Hormone Is Specifically Mediated by ERK

A, GH4C1 cells were maintained in the presence or absence of T_3 (5 nM) in 10% (control) or 0.1% (SD) charcoal-stripped-FBS for 48 h. Total cell lysates were prepared and analyzed by Western blotting with antibodies against phospho-JNK, total JNK, phospho-p38, total p38, phospho-ERK, or ERK2. The *blots* shown are from a representative experiment performed twice with similar results. B, Cells were incubated for 48 h in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS, in the presence or absence of T_3 (5 nM) plus vehicle or the inhibitors SP600125 (5 μM), SB203580 (5 μM), or U0126 (10 μM). Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. The values represent the mean ± SD of three independent experiments performed in duplicate. SB, SB203580; SP, SP600125; U0, U0126.

low serum is specifically mediated by the MEK/ERK pathway.

Thyroid Hormone Induces DUSP1 at a Posttranslational Level

To further examine the mechanism by which T_3 induces ERK-dependent apoptosis in GH4C1 cells, we measured the levels of DUSP1, a dual specificity phosphatase that dephosphorylates and deactivates members of the MAPK family, and that has been shown to be involved in apoptosis in many cell types. Cells were first incubated for 48 h with T_3 in normal or low serum, and the levels of DUSP1 were then measured in total cell extracts by Western blotting. In the presence of 10% serum, DUSP1 levels were almost undetectable, and they were not significantly affected by T_3 (Fig. 5A). In contrast, serum depletion produced a slight increase in DUSP1 expression by cells that was further exacerbated by exposure to T_3. Because the induction of DUSP1 is dependent on MAPK pathways, we analyzed DUSP1 expression after exposure to T_3 in combination with specific MAPK inhibitors. Strikingly, incubation of the cells in the presence of the MEK inhibitor U0126 completely abolished the T_3-mediated induction of DUSP1 (Fig. 5B). By contrast, the specific inhibitors of JNK or MAPK p38 did not alter the levels of this phosphatase (data not shown). This result indicates that T_3 induces DUSP1 levels by a mechanism dependent on the MEK/ERK pathway.

It has been previously established that activation of ERK is sufficient to induce DUSP1 protein by a mechanism that implies an increase in its half-life (46). Because we have shown that T_3 induces DUSP1 protein in an ERK-dependent manner, we then investigated whether T_3 affected DUSP1 protein stability. First, mRNA from T_3-treated or control cells was subjected to Northern blotting using a probe against DUSP1. As shown in Fig. 5C, DUSP1 mRNA was expressed at similar levels in control and T_3-treated cells, confirming that T_3 does not regulate DUSP1 expression at a transcriptional level. We next examined the stability of DUSP1 protein in GH4C1 cells. To that purpose, cycloheximide was added to the cells after 48 h of T_3 treatment, and DUSP1 protein levels were measured by Western blotting at different time points (Fig. 5D). As expected, DUSP1 levels decayed very rapidly in control cells, showing a half-life of 12 ± 2.4 min. However, incubation with T_3 resulted in a significant increase of DUSP1 protein levels, which decayed at a lower rate in the presence of cycloheximide (half-life of 24.23 ± 5.21 min). To confirm that T_3 was indeed decreasing the degradation of the DUSP1 protein by the ubiquitin-proteasome system, cells were treated in the presence or absence of the inhibitor MG132, and DUSP1 levels were monitored by Western blotting. Figure 5E shows that DUSP1 protein was, in fact, stabilized in the presence of the proteasome inhibitor, both in control and T_3-treated cells.

All these data confirm that T_3 induces DUSP1 protein at a posttranslational level by a mechanism dependent on ERK.

The Effects of Thyroid Hormone on the NF-κB Pathway and Apoptosis in GH4C1 Cells Are Mediated by DUSP1

We next investigated the role of DUSP1 on both T_3-mediated inhibition of the NF-κB pathway and apoptosis in GH4C1 cells. To address this question, cells were transiently transfected with either a control small interfering RNA (siRNA) (siControl) or with two specific siRNAs to knock down DUSP1 expression (siDUSP1).

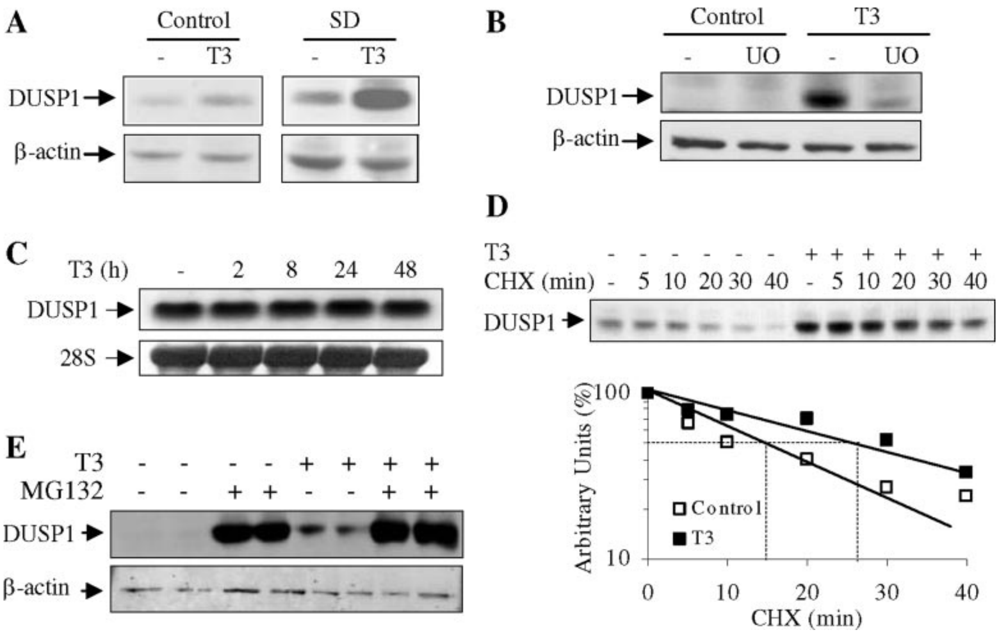

Fig. 5. Thyroid Hormone Induces DUSP1 at a Posttranslational Level

A, GH4C1 cells were incubated for 48 h with T_3 (5 nM) in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS, and the total cell extracts were analyzed by Western blotting using antibodies against DUSP1. Equal protein loading was evaluated by assessing β-actin. B, Cells were incubated for 48 h in 0.1% charcoal-stripped-FBS with T_3 (5 nM) plus vehicle or U0126 (10 μM), and DUSP1 expression was analyzed by Western blotting. Equal protein loading was evaluated by assessing β-actin. C, Cells were incubated for the indicated times in 0.1% charcoal-stripped-FBS with T_3 (5 nM). Total RNA was prepared and subjected to Northern blotting using a specific probe against DUSP1. D, Cells were incubated for 48 h in 0.1% charcoal-stripped-FBS with T_3 (5 nM), cycloheximide (10 μg/ml) was then added, and DUSP1 protein levels were detected by Western blotting. The *blot* and the *graph* shown are from one representative experiment performed three times with similar results. E, Cells were incubated in 0.1% charcoal-stripped-FBS with MG132 (10 μM) for 4 h before the addition of T_3 (5 nM). After 48 h incubation, total cell extracts were analyzed by Western blotting using antibodies against DUSP1. U0, U0126.

When the levels of DUSP1 mRNA were monitored by QRT-PCR, the DUSP1 siRNAs attenuated DUSP1 mRNA expression, reducing the transcript levels by about 80% of that in control cells (Fig. 6A). Hence, the siRNAs were capable of reducing DUSP1 expression.

To assess the role of DUSP1 on the T_3-mediated inhibition of NF-κB transcription, cells were cotransfected with the 3×NF-κB-Luc reporter together with the control siRNA or the DUSP1 siRNAs. As expected, when the cells were incubated for 48 h with T_3 in low serum, T_3 reduced NF-κB-mediated transcription to about 40% of control cells (Fig. 6B, *left panel*). By contrast, the inhibition by T_3 was almost completely abolished in DUSP1 siRNA-transfected cells (Fig. 6B, *left panel*). Because we have shown that T_3 induces DUSP1 in an ERK-dependent manner (Fig. 5B), we analyzed the effect of the MEK inhibitor U0126 on the T_3-mediated inhibition of NF-κB-dependent transcription. As expected, incubation of the cells with U0126 partially impaired the inhibition of NF-κB-dependent transcription caused by T_3, reaching a T_3-mediated inhibition of 46% in cells treated with U0126 *vs.* a 70% inhibition in cells incubated in the absence of the inhibitor (Fig. 6B, *right panel*). To examine whether the effect of DUSP1 on NF-κB-mediated transcription involved modification of p65/NF-κB nuclear translocation, cells were transfected with either the control or the DUSP1 siRNAs and incubated for 48 h with or without T_3 in low serum. T_3 almost completely diminished p65/NF-κB translocation to the nucleus in control cells, whereas it did not affect the level of p65/NF-κB in the nucleus of cells transfected with the DUSP1 siRNAs (Fig. 6C). These data indicate that DUSP1 mediates the inhibition of the NF-κB pathway caused by T_3 in GH4C1 cells.

Because we have shown that the MEK/ERK pathway is involved in T_3-induced apoptosis (Fig. 4B) and DUSP1 induction (Fig. 5B) in GH4C1 cells, we explored the possible involvement of DUSP1 in T_3-induced apoptosis. GH4C1 cells were transfected with either the control siRNA or the specific DUSP1 siRNAs and then incubated with T_3 for 48 h. Subsequently, apoptosis was measured in these cells by flow cytometry (Fig. 6D). Although the basal level of apoptosis in these cells was high, probably due to the transfection procedure itself, nevertheless T_3 enhanced the apoptosis caused by serum depletion in cells transfected with the control siRNA. Interestingly, cells transfected with the DUSP1 siRNA showed less apoptosis than control cells when incubated in low serum, and T_3 did not augment apoptosis under these conditions (Fig. 6D). To confirm the involvement of DUSP1 in T_3-me-

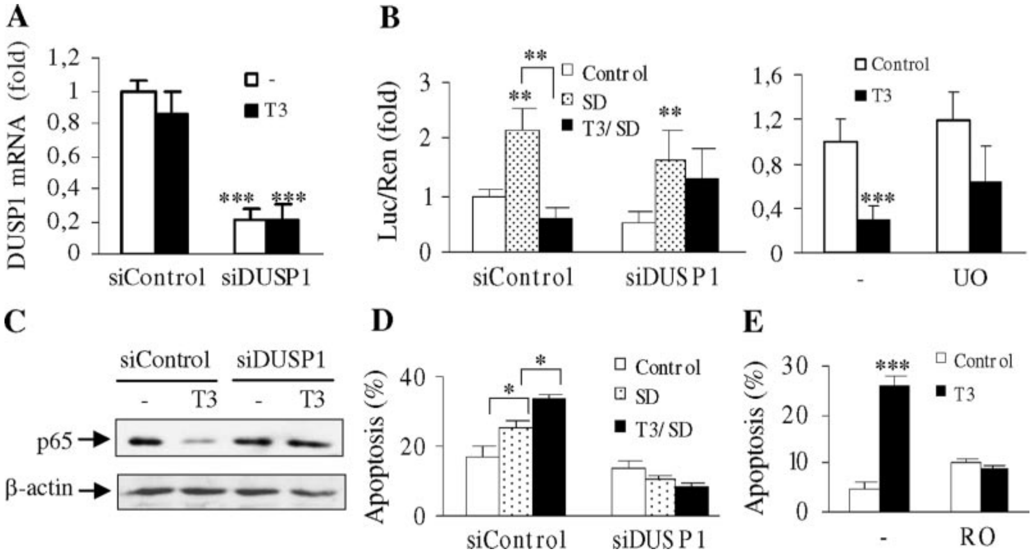

Fig. 6. The Effects of Thyroid Hormone on the NF-κB Pathway and Apoptosis in GH4C1 Cells Are Mediated by DUSP1

A, Cells were transiently transfected with either a control siRNA (siControl) or with two different siRNAs to knock down DUSP1 (siDUSP1), and they were treated for 48 h with T_3 (5 nM) in 0.1% charcoal-stripped-FBS. Total RNA was extracted, and the levels of DUSP1 mRNA were monitored by QRT-PCR. DUSP1 mRNA levels were normalized by GAPDH, and the results are expressed as the change in mRNA expression. The values represent the mean ± SD of a representative experiment performed in duplicate and repeated twice with similar results. B (*left*), Cells were cotransfected with the 3×NF-κB-Luc reporter and the control siRNA or the DUSP1 siRNAs and then incubated for 48 h with T_3 in the presence of 10% (control) or 0.1% (SD) charcoal-stripped-FBS. Cell extracts were prepared and assayed for luciferase and *Renilla* activities. The luciferase levels were normalized with those of *Renilla* and expressed as the induction over the controls. The data shown represent the mean ± SD of two independent experiments performed in duplicate. B (*right*), Cells were transiently transfected with a NF-κB-driven luciferase reporter plasmid (3×NF-κB-TK-Luc) and pRL-TK-*Renilla* and then treated for 48 h in 0.1% charcoal-stripped-FBS with T_3 (5 nM) plus vehicle or U0126 (10 μM). Cell extracts and luciferase activity were measured as above. C, Cells were transfected with either the control siRNA or the DUSP1 siRNAs and incubated for 48 h with or without T_3 in 0.1% charcoal-stripped-FBS. The nuclear extracts were analyzed by Western blotting using antibodies against p65. Equal protein loading was evaluated by assessing β-actin. The *blots* shown are from one representative experiment performed twice with similar results. D, Cells were transfected with either the control siRNA or the DUSP1 siRNAs and incubated for 48 h in the presence or absence of T_3 in 10% (control) or 0.1% (SD) charcoal-stripped-FBS. Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. Values represent the mean ± SD of two independent experiments performed in duplicate. E, Cells were incubated for 48 h in 0.1% charcoal-stripped-FBS with T_3 (5 nM) plus vehicle or RO-31-8220 (5 μM). Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. The values represent the mean ± SD of two independent experiments performed in duplicate. RO, RO-31-8220; U0, U0126.

diated apoptosis, we next incubated the cells for 48 h with T_3 in the presence or absence of the DUSP1 inhibitor, RO-31-8220. Incubation of the cells with the inhibitor completely prevented the induction of apoptosis by T_3 (Fig. 6E).

Together, these data demonstrated that both T_3-mediated inhibition of NF-κB and T_3-induced apoptosis in GH4C1 cells are mediated by ERK-induced DUSP1 expression.

DISCUSSION

The data presented here demonstrate that T_3 induces apoptosis in GH4C1 pituitary cells through a mechanism dependent on ERK, DUSP1, and NF-κB. Indeed, we show that T_3 induces DUSP1 via ERK, leading to a down-regulation of NF-κB activity and the induction of apoptosis.

Programmed cell death and apoptosis have a fundamental role in tissue homeostasis. Growth factors and serum provide both mitogenic and antiapoptotic signals to cells and therefore play an important role in maintaining the homeostatic balance between cell proliferation and cell death. The mitogenic effects of these factors have been well described, and a growing body of literature has demonstrated that their withdrawal can induce programmed cell death in several cell systems. In particular, it has been shown that primary pituitary cells require trophic support for survival in culture, and serum deprivation is an established model for analyzing apoptotic pathways in these cells (2). Our data support the idea that low serum conditions are necessary for T_3 to induce apoptosis in diverse pituitary cell lines, including GH4C1, GH1, and GH3B6 cells. The proposal that T_3 modulates apoptosis in pituitary cells is in agreement with previous findings that T_3 augments the mitochondrial proapoptotic molecules Bax and Bak, diminishing the antiapoptotic protein Bcl-2 in the closely related pituitary cell line, GH3 (14). However, our results seem to contrast with those indicating that T_3 mediates the proliferation of GC and GH4C1 cells (11, 12). It is probable that the different cell context explains this apparent contradiction and indeed, in these earlier studies the cells were maintained in medium supplemented with 10% charcoal-stripped fetal calf serum, conditions in which T_3 does not induce apoptosis of GH4C1 cells. In contrast, the induction of apoptosis by T_3 in cells maintained in low serum is in accordance with the suggestion that T_3 stimulates the division of GH4C1 cells through the secretion of a growth factor, which might promote proliferation in conjunction with other activities present in serum (11). Together, these data show that T_3 can exert opposing effects depending on the cell context, promoting either cell proliferation or cell death.

Our results also show that T_3 causes sustained activation of ERK and that T_3-mediated apoptosis of GH4C1 cells in low serum is dependent on this kinase. T_3 can activate ERK to a lesser extent in cells incubated in high serum conditions, although this is not sufficient to induce apoptosis. These findings suggest that a threshold of ERK activation is necessary to promote apoptosis in our cells. The role of T_3 in ERK signaling has been previously described in TtT-97 mouse thyrotrope tumor cells, in which T_3 activates the ERK pathway and induces the arrest of S-phase progression (26, 27). In general, ERK activation is associated with cell survival and proliferation (47), although activation of ERK by different stimuli has recently been found to contribute to cell death in diverse primary cultured cells (24, 25). More interestingly, sustained activation of ERK was shown to be crucial in the apoptosis caused by different stimuli in GH3 (28) and in GH4C1 cells (29). Our results support this idea because T_3 can act as a proapoptotic molecule in GH4C1 cells through a mechanism that involves ERK activation.

To our knowledge, this is the first time T_3 has been shown to induce DUSP1 expression, although this is in agreement with a growing number of reports where other nuclear receptors regulate DUSP1. For instance, it was previously shown that glucocorticoid receptors induce DUSP1 expression in different cell types (39, 48–50), whereas other nuclear receptors have also been shown to regulate DUSP1 expression, albeit to a lesser extent. For example, retinoid acid up-regulates DUSP1 expression in different cells (51–53), and estrogen receptors increase basal levels of DUSP1 in mesangial cells (54). It is also significant that T_3-mediated induction of DUSP1 is dependent on the MEK/ERK pathway. Although different mechanisms have been shown to be involved in DUSP1 expression, the main regulator seems to be ERK, which induces, phosphorylates, and stabilizes DUSP1 by impeding its proteolytic degradation (46, 55). Our data show that T_3 induces DUSP1 protein by a posttranslational mechanism, increasing the half-life of the protein and decreasing its degradation via the ubiquitin-proteasome pathway. Although the DUSP1 phosphorylation level remains to be examined, our results suggest that T_3 might induce DUSP1 phosphorylation via ERK in GH4C1 cells.

DUSP1 seems to be crucial for T_3-mediated apoptosis of GH4C1 cells because silencing DUSP1 expression abrogates the induction of apoptosis caused by T_3. Interestingly, this effect is comparable to that produced by blocking MEK and the downstream ERK pathway, illustrating the link between the effects of T_3, ERK signaling, and DUSP1 induction. In contrast, specific inhibition of JNK or MAPK p38 signaling has no effect on T_3-mediated apoptosis, demonstrating that these kinases do not participate in this process. However, JNK and MAPK p38 are likely to fulfill other important functions in pituitary cells, which may be regulated by DUSP1 and therefore, affected by T_3. In this regard, we observed that T_3 can down-regulate the increase of MAPK p38 phosphorylation in response to TNFα in GH4C1 cells (data not shown), and this could influence processes other than apoptosis.

This study also provides evidence that T_3 can induce apoptosis by down-regulating NF-κB activity. The function of NF-κB in anterior pituitary cells has, to date, received little attention; however, our results show that NF-κB can be regulated by T_3. Previous studies have shown that inhibition and interruption of the NF-κB pathway can modulate the expression of Bcl-2 and IAP family members in different cell types. We found that T_3 exerts an inhibition of Bcl-x_L mRNA expression, a member of the Bcl-2 family recently shown to play an important role in the programmed cell death of pituitary GH4C1 cells triggered by epidermal growth factor (29). Moreover, T_3 inhibits r-IAP1 and r-IAP3 mRNA expression, two members of the IAP family. These findings strongly suggest that the T_3-induced apoptosis in GH4C1 cells is achieved, in part, by the regulation of NF-κB-responsive genes known to be important modulators of apoptotic processes. The specific role of each of these proapoptotic genes, as well as other still unidentified genes, in T_3-mediated apoptosis in GH4C1 cells remains to be established.

Although NF-κB can regulate T_3-mediated transcription (56, 57), for the first time our data show a negative regulation of the NF-κB signaling pathway by T_3. Functional cross talk between nuclear receptors and NF-κB has been reported for various classes of receptors. Activated NF-κB impairs the function of receptors for glucocorticoids, progesterone, androgens, and cholesterol, as shown in several *in vitro* studies (58–61). In accordance with our data, these receptors inhibit the action of NF-κB in the presence of their cognate ligands. For example, glucocorticoids exert their antiinflammatory effects, at least partially, through the inhibition of NF-κB. Moreover, glucocorticoids can inhibit NF-κB in a variety of ways in different cell contexts, including inhibition of DNA binding, IκB kinase (IKK) activity, and p65/NF-κB transactivation (58). Similarly, estrogens can act through the estrogen receptor to inhibit NF-κB by a variety of mechanisms (62, 63). We show here that T_3 inhibits NF-κB-dependent transcription in GH4C1 cells by affecting the phosphorylation of IκBα and translocation of active NF-κB complexes from the cytosol to the nucleus, by binding to κB sequences in DNA, and by p65/NF-κB transactivation through a mechanism involving the ERK activation and DUSP1 induction.

We also demonstrate that T_3-mediated inhibition of p65/NF-κB translocation to the nucleus depends on DUSP1 expression, because impairing DUSP1 gene expression abrogates the effect of T_3. Regulation of the NF-κB pathway by DUSP1 is not surprising, considering that this protein has been identified as one of the components of the IKK signalsome. Indeed, DUSP1 coassociates with IKKα, and it was speculated that DUSP1 (or the DUSP1-reactive protein identified in the IKK signalsome) might be the phosphatase responsible for down-regulating IKKα/β activity (64). This down-regulation may occur by direct dephosphorylation of IKK at the crucial 176/177 serine residue or, perhaps, by modulating the activity of an upstream kinase. This hypothesis could explain our data showing that T_3 induced inhibition of NF-κB through a mechanism dependent on DUSP1 expression, although this remains to be demonstrated.

Although nuclear translocation of NF-κB has been regarded as the principal method to activate NF-κB-dependent gene expression, alternate mechanisms of NF-κB activation are emerging, such as the phosphorylation of the p65/NF-κB subunit (18, 65). The phosphorylation of p65/NF-κB in the cytoplasm or nucleus is specific to both stimuli and cell type. So far, a great number of p65/NF-κB protein kinases have been identified, such as the IKK complex, protein kinase A, glycogen synthase kinase 3, protein kinase C, CK2, p38 MAPK, or AKT, and their role in NF-κB activation has been extensively described (18). In most cases, this phosphorylation enhances the transactivation potential of p65. Our data also show that T_3 decreases both the phosphorylation of nuclear p65/NF-κB and its transactivation potential, which correlates with the decrease in NF-κB-dependent transcription observed. These results are in agreement with earlier data showing that p65/NF-κB phosphorylation enhances p65/NF-κB transactivation potential. It has been shown previously that p65/NF-κB and T_3 nuclear receptors do not physically interact in yeast two-hybrid systems (56), suggesting that the repressive effect of T_3 on p65/NF-κB transactivation is not due to a direct interaction between these transcription factors. However, we cannot exclude a possible interaction in the context of native promoters. An alternative mechanism for the transrepression of NF-κB activity by T_3 could involve the sequestering of common cofactors shared between NF-κB and TR, such as cAMP response element-binding protein-binding protein/p300 or histone deacetylases (HDACs). p65/NF-κB has been shown to interact with HDAC1 and HDAC2, inhibiting the transactivation function of NF-κB in both basal and stimulated situations (66). Because T_3 attenuates p65/NF-κB-dependent transcription, we examined whether treatment with the HDAC inhibitor, trichostatin A, had any effect on T_3-NF-κB-dependent transcription. Trichostatin A did counteract the inhibitory action of T_3 on NF-κB-dependent transcription (data not shown), and these data indicate that HDAC activity is involved in the control of NF-κB transcription by T_3 in GH4C1 cells, although the underlying mechanism requires further investigation.

In conclusion, our data show a novel proapoptotic function of T_3 in GH4C1 cells that is produced through ERK activation, DUSP1 induction, and NF-κB down-regulation. These results could have important implications for the understanding of pituitary cell homeostasis in different physiopathological situations, including the apoptotic process that occurs naturally in the pituitary gland during postnatal life.

MATERIALS AND METHODS

Materials and Plasmids

Tissue culture media and sera were obtained from Life Technologies, Inc. (Gaithersburg, MD). The antibodies used were: antidual phosphorylated ERK, antiphosphorylated p38 MAPK, anti-pSer^{276}-NF-κB, and antiphospho-IκBα (Cell Signaling Technology, Danvers, MA); antiphosphorylated JNK (Promega Corp., Madison, WI); anti-ERK2, anti-p38 MAPK, anti-JNK, anti-MAPK phosphatase 1, anti-NF-κB/p65, anti-IκBα, and anti-PARP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-β-actin and antitubulin (Sigma Chemical Co., St. Louis, MO); antihistone H3 (Abcam, Inc., Cambridge, MA); and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the DUSP1 inhibitor RO-31-8220 were all obtained from Calbiochem (La Jolla, CA). The MEK inhibitor U0126 was purchased from Promega. T_3 and MG-132 were from Sigma. The 3×NF-κB-TK-Luc reporter plasmid, which contains a three-tandem repeat of the NF-κB-binding motif of the *H-2k* gene upstream of the thymidine kinase minimal promoter (67) was kindly provided by Dr. M. Fresno (Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Cientificas-UAM, Madrid, Spain). The plasmid containing the Gal4-DNA-binding domain fused to the full-length human p65/NF-κB coding sequence (pGal4-p65) (68) and the Gal4-p65 mutant construct [pGal4-p65(S276C)] were obtained from the BCCM/LMBP Plasmid Collection (Ghent, Belgium). The plasmid pGal4-Luc and the probe for EMSAs (69) were kindly provided by Dr. R. Perona (Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Cientificas-Universidad Autónoma de Madrid, Madrid, Spain).

Cell Culture

GH4C1 cells were cultured in DMEM supplemented with 10% FBS. GH3B6 or GH1 cells were cultured in Ham's F10 or RPMI medium supplemented with 10% horse serum (HS) and 2.5% FBS, respectively. In the experiments, the cell medium was replaced by fresh medium containing either 10% or 0.1% AG1x8 resin and charcoal-stripped FBS (for GH4C1 cells) or HS (for GH1 and GH3B6 cells), and cells were incubated for the indicated time periods in the presence or absence of T_3. Control cells were incubated with the same volume of the vehicle used to dissolve the different compounds.

Cell Transfection

GH4C1 cells were transfected by electroporation, as described previously (70). Briefly, 2–3 × 10^6 cells were mixed with the reporter plasmids and exposed to a high-voltage pulse (170–200 V, 960 μF) in a Bio-Rad electroporator with a capacitor extender (Bio-Rad Laboratories, Richmond, CA). The cells from each electroporation were plated in different dishes with hormone-depleted media and incubated with the different treatments as necessary.

siRNA Oligos and Transfections

siRNA oligos for DUSP1 were obtained from Ambion, Inc. (Austin, TX): siRNA (identification no. 55543), forward (5′-GGG UCA CUA CCA GUA CAA Gtt-3′), reverse (5′-CUU GUA CUG GUA GUG ACC Ctc-3′); siRNA (identification no. 55635), forward (5′-GGC AGA CAU UAG CUC CUG Gtt-3′), reverse (5′-CCA GGA GCU AAU GUC UGC Ctt-3′). Transfections were carried out by electroporation as described above using the siPORT siRNA Electroporation Buffer (Ambion), and 2 μM of each siRNA or the scrambled siRNA (silencer negative control 1 siRNA, Ambion). Cells were plated on 60-mm plates and left for 24 h in DMEM supplemented with 10% FBS. The media were replaced with DMEM plus 0.1% or 10% resin and charcoal-stripped FBS, and the cells were then incubated in the presence or absence of T_3 for 48 h.

NF-κB Reporter Assays

The NF-κB reporter assay was performed as described previously (67) with minor modifications. Cells were transfected as described above with 2 μg of the 3×NF-κB-TK-Luc or TK-Luc reporter plasmids and 0.6 μg of the common internal transfection standard *Renilla* in the pRL-TK plasmid, which was used to normalize transfection efficiency. The cells were harvested in 100 μl reporter lysis buffer (Promega) 48 h after transfection, and dual luciferase and *Renilla* reporter assays were performed following the protocol provided by the manufacturer. To measure the transactivating potential of p65/NF-κB, cells were transfected with the fusion construct pGal4-p65, pGal4-p65(S276C), or pGal4 alone (2 μg) and the pGal4-Luc reporter plasmid (4 μg). Luminescence was measured in 10 μl of the cell extract, and the protein concentration was determined by the Bradford protein assay. Each treatment was performed in duplicate cultures that normally showed less than a 5% variation in luciferase activity. Each experiment was repeated at least three times with similar differences in regulated expression, and all data are expressed as the mean ± SD.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was performed on the Real-Time PCR System Mx3005P (Stratagene), monitoring the increase of fluorescence due to the binding of SYBR Green to double-stranded DNA. Dissociation analysis was performed at the end of each PCR to ensure that only the specific product was amplified. The first-strand cDNA template was synthesized from 5 μg of total RNA using oligodeoxythymidine in 20 μl of water, following the instructions of the SuperScript First Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). For a 25-μl PCR, 2 μl of cDNA template was mixed with forward and reverse primers (each primer at final concentration150 nM) and 2× Brilliant SYBR Green QPCR Master Mix (Stratagene). The gene-specific primers and the conditions of each reaction were as follows: *r-IAP1*: forward (5′-GAT TTG CAC ACT CGC TAC CT-3′), reverse (5′-CCA GTT GCT CAG TTT CCC AC-3′), 95 C, 30 sec, 54 C, 1 min, 72 C, 1 min, 40 cycles; *r-IAP3*: forward (5′-CGG CAG TAG ATA GAT GGC AG-3′), reverse (5′-CTC TCT GGG GCT TAA ATG GG-3′), 95 C, 30 sec, 54 C, 1 min, 72 C, 1 min, 40 cycles; *Bcl-x*$_L$: forward (5′-TAT TGG TGA GTC GGA TTG CA-3′), reverse (5′-GAG ATC CAC AAA AGT GTC CCA-3′), 95 C, 30 sec, 52 C, 1 min, 72 C, 1 min, 40 cycles; *DUSP1:* forward (5′-GAT CAA CGT CTC GGC CAA TT-3′), reverse (5′-GCA CAA ACA CCC TTC CTC CA-3′), 95 C, 30 sec, 50 C, 30 sec, 72 C, 1 min, 40 cycles; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward (5′-ACA CTG CAT GCC ATC ACT GCC-3′), reverse (5′-GCC TGC TTC ACC ACC TTC TTG-3′), 95 C, 30 sec, 55 C, 1 min, 72 C, 1 min, 40 cycles. To quantify changes in gene expression, the ΔΔCt method was used to calculate the relative changes normalized against the GAPDH gene.

Northern Blot Analysis

Northern blot was performed as previously described (48). Briefly, total RNA was isolated using the TRI Reagent (Sigma), and 10 μg RNA samples were electrophoresed on denaturing formaldehyde-agarose gels. RNA was transferred to Hybond N membrane, and DUSP1 mRNA was detected with a specific cDNA probe labeled with the Ready-to-go kit (Amersham Pharmacia Biotech, Piscataway, NJ). Signals were visualized by exposure to Kodak Biomax film (Eastman Kodak, Rochester, NY).

Cell Extracts

For total cell extracts, cells were harvested after treatment in 100 μl lysis buffer [20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol, 100 mM KCl, 1% Triton X-100, 0.3% 2-mercaptoethanol, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$] supplemented with protease inhibitors, and the lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4 C. Nuclear extracts were prepared as described (71). Briefly, cells were washed and recovered in a hypotonic buffer containing: 10 mM HEPES (pH 7.4), 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 1% Triton X-100, and 0.2% sucrose supplemented with protease inhibitors. After a 10-min incubation at 4 C, the cell nuclei were collected by centrifugation at 30,000 × *g* at 4 C for 10 min. The pellet containing the nuclei was resuspended in elution buffer (20 mM HEPES, pH 8; 25% glycerol; 0.42 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT) supplemented with protease inhibitors, and after a 45 min extraction at 4 C the nuclear membranes were sedimented at 30,000 × *g* for 30 min at 4 C. The nuclear proteins were finally collected in the supernatant and stored in aliquots at −70 C.

Western Blot Analysis

Cells were incubated as described in the figure legends and then harvested in lysis buffer as described above. The protein content of the cell or nuclear extracts was normalized, the samples were separated by SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were probed with the primary antibodies indicated and that in turn were detected with a peroxidase-coupled secondary antibody. Antibody binding was visualized using the enhanced chemiluminescence system (Amersham).

EMSA

DNA binding was assessed by EMSA according to standard protocols. As such, 2 μg of the nuclear extracts was incubated in 40 mM HEPES (pH 7), 12.5 mM $MgCl_2$, 140 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.01% Nonidet P-40, 4% Ficoll, and 0.2 μg of polydeoxyinosinic deoxycytidylic acid for 15 min at 4 C. Subsequently 10,000 cpm of a ^{32}P-labeled probe containing the NF-κB binding site (69) was added to the samples and left for 20 min at room temperature to permit DNA-protein binding. The protein-DNA complexes formed were separated in 5% nondenaturing polyacrylamide gels in 0.5% Tris-borate-EDTA buffer, and the gels were then dried and the bands visualized by exposure to Kodak Biomax film.

Flow Cytometry Analysis of Apoptosis

Apoptosis was identified and quantified by flow cytometry after PI staining. Adherent and floating cells were collected after treatment, washed with ice-cold PBS, and fixed with 70% ice-cold ethanol (30 min, 4 C). The fixed cells were washed twice with PBS and treated with ribonuclease (1 mg/ml for 30 min at 37 C). Cellular DNA was stained PI (5 μg/ml in PBS), and the cells were analyzed on a FACScan flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ). The percentages of cells in different phases of the cell cycle were calculated from the DNA histograms. Cells with sub-G_1 DNA content were considered apoptotic.

Apoptosis was also examined by the Annexin V/PI dual staining assay. Cells were collected after treatment and were mixed with 10 μl of fluorescein isothiocyanate-conjugated annexin V reagent (R&D Systems, Abingdon, UK) and 10 μl of 3 mM PI. After incubation, samples were analyzed by flow cytometry. The percentage of early apoptotic cells, late apoptotic cells, and dead cells is shown at the *top* of each panel.

Statistical Analysis

All data are expressed as means ± SD. In statistical analysis, Student's *t* test was performed using the SSC-Stat software (version 2.18, University of Reading, Reading, UK). The statistical significance of difference between groups was expressed by asterisks (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$).

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