Phosphatidylinositol 3-kinase/nuclear factor- κ B signaling pathway is involved in the regulation of IGF-I on Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein expression in cultured FRTL thyroid cells

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Abstract

It is known that decreased apoptosis of thyrocytes may be involved in the formation of goiters in patients with Graves' disease, and growth factors are involved in regulating the size of the thyroid gland. The purpose of our study was to investigate mRNA and protein levels of an antiapoptotic protein, namely, Fas-associated death domain-like interleukin-1-converting enzyme (FLICE)-inhibitory protein (FLIP). The results showed that in FRTL thyroid cells, treatment with IGF-I upregulated mRNA and protein levels of FLIP in a dose-dependant manner. While a specific nuclear factor- κ B (NF- κ B) inhibitor, BAY11-7082, blocked this effect. Further study demonstrated that IGF-I induced the DNA-binding activity of NF- κ B in association with decreased expression of the NF- κ B inhibitory protein I κ B α . These findings implied that IGF-I increased FLIP expression by enhancing the activation of NF- κ B in FRTL thyroid cells. Using a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, we also found that PI3K was involved in the pathway by which IGF-I activated NF- κ B and increased FLIP expression. When treated with IGF-I and LY294002, decreased NF- κ B DNA binding activity and increased expression of I κ B α protein were detected in cultured thyroid cells, which further confirmed that NF- κ B was under the control of the PI3K pathway. Taken together, our results suggest that IGF-I regulates the expression of FLIP in FRTL cells by activating the PI3K/NF- κ B cascade.

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Introduction

Thyroid goiter is a main feature of Graves' disease. There are several consequences of goiter, such as compression of airways and so on, which influence the life quality of patients. An imbalance between the proliferation and the apoptosis of thyroid cells may be crucial for goiter formation (Irmeler *et al.* 1997, Ashkenazi & Dixit 1998). However, the precise mechanism of goitrogenesis in terms of thyrocyte growth regulation is yet to be understood. It has been known that cellular Fas-associating protein with death domain-like interleukin-1-converting enzyme (FLICE)-inhibitory protein (FLIP) blocks Fas-induced apoptosis. In Graves' disease, upregulation of FLIP leads to an absence of thyrocyte apoptosis and thyroid goiter (Mitsiades *et al.* 2001).

Insulin-like growth factor-I (IGF-I) is an important hypertrophic and antiapoptotic factor for a number cell types (Kofidis *et al.* 2004, Radcliff *et al.* 2005). It produces its biologic effects by means of the IGF-I receptor, which

mediates many of these effects by activating phosphatidylinositol 3-kinases (PI3Ks). In the thyroid, IGF-I collaborates with thyrotropin and stimulates the growth of thyroid cells (Kimura *et al.* 2001, Saito *et al.* 2001). In the study of thyroid goiter formation, interest in the role of IGF-I has increased. IGF-I may be important in the pathogenesis of Graves' disease, and it is involved in the formation of thyroid goiter (Maiorano *et al.* 1998, Miyakawa *et al.* 2003). Our previous study showed that serum IGF-I levels were positively correlated with thyroid volumes observed in Graves' disease. In thyroid tissues, IGF-I was locally produced and more strongly expressed than in normal thyroid tissue (Zhong *et al.* 2006). These findings indicated that IGF-I has autocrine and paracrine roles in the formation of thyroid goiters. However, whether IGF-I is correlated with FLIP expression and what the signaling pathways are remain uncertain.

A growing body of evidence demonstrates that nuclear factor- κ B (NF- κ B) is a key regulator of the transcription of genes that control cellular proliferation

and antiapoptosis (Ghosh *et al.* 1998, Barkett & Gilmore 1999, Karin & Ben-Neriah 2000). When bound to an inhibitory protein, I κ B α , NF- κ B is present in latent form in the cytoplasm. On activation, degradation of I κ B α frees NF- κ B to enter the nucleus and induce the transcription of target genes (Pahl 1999). During cell apoptosis and proliferation, NF- κ B may have an important role beyond IGF-I-mediated thyroid tissues and cell lines. IGF-I protects neurons (Heck *et al.* 1999) and lymphocytes (Jimenez Del Rio & Velez-Pardo 2006) from apoptosis by activating NF- κ B. However, the relationship between IGF-I and NF- κ B is not well characterized in normal thyroid lines.

All these observations prompted us to determine if IGF-I influences FLIP expression in thyroid cells by affecting the activity of NF- κ B as a downstream target. We found that IGF-I upregulated FLIP expression in FRTL thyroid cells by means of PI3K-dependent pathway that finally led to the activation of NF- κ B.

Materials and methods

Cell culture and treatment

FRTL thyroid cells (ATCC catalog number CRTL-1486) were grown in 6H medium consisting of Coon's modified F12 medium supplemented with 5% fetal bovine serum and a mixture of six hormones: bovine thyrotropin (1 milliunit/ml), insulin (10 μ g/ml), hydrocortisol (0.4 ng/ml), transferrin (5 μ g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was added to all cells every 2 or 3 days and cell passaging was done every 7–10 days. As appropriate for the experiments to be performed, the cells were shifted to 4H medium containing no thyrotropin, no insulin, and only 0.2% calf serum 2 days before they were used. Then cells were washed twice with PBS. For some experiments, the PI3K inhibitor LY294002 (10 μ M) or the NF- κ B inhibitor BAY11-7082 (50 μ M) was added 1 h before IGF-I treatment. Then, IGF-I was added for 24 h.

Real-time PCR

Total RNA was extracted from cells using a standard Trizol RNA isolation method. Reverse transcription of 1 μ g RNA was carried out according to the instructions of a commercial Takara RT kit (Takara, Japan). Quality of the RNA and cDNA was checked using DU640 nucleic-acid analyzer (Beckman Coulter, Fullerton, CA, USA). Primers against rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. Following sense and antisense primers were used, respectively: for FLIP, 5'-GCG GTT TGA CCT GCT CAA GA-3' and 5'-AGC AGC ACC CTA TAA TCG

GAA A-3', and for GAPDH, 5'-TGG TGG ACC TCA TGG CCT AC-3' and 5'-CAG CAA CTG AGG GCC TCT CT-3'. Real-time PCR was performed with a kit (Quantitect SYBR Green; Qiagen Inc.) by following the manufacturer's instructions. The total reaction volume was 25 μ l, and 100 ng cDNA was used as the template. Fluorescence was detected using a detection system (Prism 7700; ABI, Foster city, CA, USA). PCR products were visualized with gel electrophoresis to confirm a single product of the correct size. Ratios of the target gene to GAPDH were calculated and compared.

Western blotting

FRTL cells were harvested, centrifuged, and washed with PBS. They were lysed for 20 min on ice in RIPA buffer containing 1 $\times$ PBS, 1% NP-40, 0.1% SDS, 5 mM ethylenediaminetetraacetic acid, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Cells were then centrifuged at 12 000 *g* for 10 min at 4 °C. The resulting supernatants were collected and frozen at –80 °C or used immediately.

Protein concentrations were determined using a protein assay (bicinchoninic acid, BCA; Pierce, Rockford, IL, USA). We heated 40 μ g of each sample for 30 min at 60 °C, then analyzed them by performing 10% SDS-PAGE and by electroblotting them onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk for 1 h and then incubated with the specific primary antibody overnight at 4 °C. Afterward, they were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using the enhanced chemiluminescent method, and immunoreactive bands were quantified using an imaging system (AlphaImager 2200; Alpha Innotech Corporation, San Leandro, CA, USA). Values were corrected with the absorbency of the internal control (actin).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts for EMSA were prepared with nuclear and cytoplasmic extraction reagents (NE-PER; Pierce). The supernatant fraction containing the cytoplasm and the nuclear extract were prepared separately. Protein concentrations were determined using a protein assay (BCA; Pierce). The amount of protein in each lane was constant in each experiment. EMSA was performed using a commercial chemiluminescent kit (Lightshift; Pierce). The NF- κ B consensus oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled with biotin (Sangon, Shanghai, China). Nuclear extracts (2–3 μ l) were incubated for 20 min at room temperature with 50 ng/ μ l poly (dI.dC; Pierce), 2.5% glycerol, 5 mM

MgCl₂, 0.05% NP-40, and 50 fmol biotin-labeled NF- κ B oligonucleotide probe. Then, 5 μ l of 5 $\times$ binding buffer was added to each 20 μ l binding reaction.

The reaction mixture was subjected to nondenaturing 5% PAGE in 0.5 $\times$ tris-borate-EDTA (TBE) buffer and electroblotted onto a positively charged nylon membrane. After cross-linking with u.v. light, the membrane was blocked, washed, and incubated with a substrate working solution (Lightshift; Pierce). Finally, the membrane was exposed to X-ray film for 2–5 min. Optical density was assessed with the use of a densitometer analysis software (AlphaImager 2200; Alpha Innotech Corporation). Binding reactions with a 200-fold excess of unlabeled double-stranded oligonucleotides (NF- κ B or Sp1) were processed as described above and used as controls for binding specificity.

Data analysis

All experiments were repeated at least thrice. Values are given as the mean $\pm$ s.d. Data were analyzed using SPSS 10.0 software (SPSS, Chicago, IL, USA). Statistical significance was assessed by performing unpaired Student's *t* test. $P < 0.05$ indicated a statistically significant difference.

Results

IGF-I upregulates gene and protein expression of FLIP in a dose-dependent manner

We first determined whether IGF-I regulated FLIP gene expression in FRTL cells. Results of the reverse-transcription PCR showed that IGF-I increased FLIP mRNA levels compared with levels in control cells not treated with IGF-I (Fig. 1A). When we validated this change with real-time PCR, a quantitative method with high-sensitivity, FLIP mRNA levels increased by 30% with 10 ng/ml IGF-I and 50% with 50 ng/ml compared with controls (Fig. 1B). This indicated IGF-I upregulated FLIP gene expression in a dose-dependent manner. Western blots also showed that IGF-I stimulated FLIP protein expression in a dose-dependent manner (Fig. 1C and D). All indicated IGF-I enhanced FLIP mRNA and protein expression in FRTL cells.

PI3K is involved in the IGF-I-induced increase in FLIP expression

When we used LY294002, a widespread pharmacological inhibitor of PI3K, to ascertain whether PI3K was involved in the regulation of IGF-I on FLIP,

Figure 1 IGF-I stimulates FLIP mRNA and protein expression in a dose-dependent manner. Starved FRTL cells were treated with various concentrations of IGF-I for 24 h and harvested for analysis. (A) Representative reverse-transcription PCR results. (B) Histogram of real-time PCR results. (C) Cells were treated with 10 or 50 ng/ml IGF-I for 24 h, washed with phosphate-buffered saline, and lysed. For each sample, 40 μ g protein was used for western blotting. β -Actin served as a control to determine equal protein loading. (D) Densitometric results from three experiments of Western blotting. * $P < 0.05$ versus untreated group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

reverse-transcription and real-time PCR showed that LY294002 pretreatment decreased the IGF-I-induced FLIP mRNA content to 70% of that observed in cells treated with IGF-I alone ($P<0.05$; Fig. 2A and B). This reduction was paralleled by a significant decrease in FLIP protein level ($P<0.05$; Fig. 2C and D), which suggested that IGF-I induced FLIP expression depending on PI3K activity in FRTL cells.

IGF-I decreases expression of inhibitory I κ B α protein

Activation of NF- κ B depends on the degradation of its inhibitory protein I κ B α . When we measured I κ B α protein levels in the cytoplasm using specific anti-I κ B α antibody, results suggested that I κ B α protein expression decreased by about 80% in FRTL cells treated with IGF-I for 24 h compared with untreated cells ($P<0.05$; Fig. 3). Pretreatment with PI3K inhibitor LY294002 diminished IGF-I-induced degradation of I κ B α , indicating IGF-I induced I κ B α degradation in the cytoplasmic fraction by activating PI3K.

IGF-I activates binding activity of NF- κ B DNA through PI3K pathway

The action of NF- κ B in regulating target genes is based on its DNA-binding activity. To assess whether IGF-I has any effect on NF- κ B DNA-binding activity, EMSA was performed. Results showed that treatment with IGF-I resulted in strong NF- κ B-specific gel-retarded bands in nuclear samples extracted from FRTL cells relative to untreated cells (Fig. 4, lane 2).

To investigate whether PI3K is also involved in increased NF- κ B-DNA-binding activity stimulated by IGF-I, PI3K inhibitor were added, and faint gel-retarded bands were found in the nuclear extracts (Fig. 4, lane 3), indicating involvement of PI3K in the effect of IGF-I on NF- κ B. NF- κ B-DNA binding activity was specific because the band disappeared with an excess of unlabeled NF- κ B oligonucleotides (Fig. 4, lane 4) or with only free labeled probe (Fig. 4, lane 6) but not with Sp1 oligonucleotides (Fig. 4, lane 5). Taken together, these above results indicated that IGF-I/PI3K-mediated degradation of I κ B α resulted in the translocation of activated NF- κ B into the nucleus to facilitate the binding of NF- κ B with the target gene.

FLIP expression depends on a signaling pathway involving IGF-I, PI3K, and NF- κ B

To further confirm the effect of NF- κ B on FLIP expression, FRTL cells were exposed to NF- κ B specific inhibitor, BAY11-7082. Treatment of FRTL cells with BAY11-7082 markedly decreased IGF-I-induced FLIP expression (Fig. 5). Inhibition of NF- κ B by BAY11-7082 combined with inactivation of PI3K by LY294002 decreased IGF-I-induced FLIP protein expression (Fig. 5A, lane 5) more than the decrease caused by inhibiting NF- κ B or PI3K alone (Fig. 5A, lanes 3 and 4). Semiquantitative analysis showed that FLIP protein levels decreased 30% with PI3K inhibitor, 40% with NF- κ B inhibitor, and 80% with both inhibitors, compared with cells treated with only IGF-I (Fig. 5B).

Figure 2 Effect of PI3K inhibitor LY294002 on IGF-I-induced FLIP mRNA and protein expression. Starved cells were preincubated with 50 ng/ml IGF-I for 24 h with or without LY294002 (10 μ M). Reverse transcription (A) and real-time (B) PCR were performed with RNA from FRTL cells. Results from three independent experiments are shown. (C) Equal amounts of total protein were loaded and western blotting was performed to measure FLIP protein expression. A representative blot is shown. (D) Densitometric results from three experiments. * $P<0.05$ versus untreated group. † $P<0.05$ versus IGF-I-treated group.

Figure 3 IGF-I decreases the level of inhibitory I κ B α protein by means of PI3K. Starved cells were pretreated with 10 μ M LY294002 for 1 h then 50 mg/ml IGF-I for 24 h. Their cytoplasmic portions were extracted, and protein levels of I κ B α were measured on western blots. Equal amounts of protein were loaded to allow direct comparisons. β -Actin was the loading control. (A) Representative western blot. (B) Densitometric results from three independent experiments. * $P < 0.05$ versus untreated group. † $P < 0.05$ versus IGF-I-treated group.

Discussion

In the present study, we identified NF- κ B, a new target of IGF-I-related actions in FRTL cells. IGF-I stimulated the activation of PI3K, increasing the expression of antiapoptotic protein FLIP, at least partly by enhancing NF- κ B activity.

Our previous study and others have shown that the size of the thyroid in Graves' disease is closely correlated with the serum content of IGF-I (Lakatos *et al.* 2000), and with the localized expression of IGF-I in thyroid tissues (Maiorano *et al.* 1998, Vella *et al.* 2001). Animal models of thyroid goiter demonstrated that increased expression of IGF-I may contribute to early goiter formation (Phillips *et al.* 1994). On the other hand, upregulated antiapoptotic moleculars, such as FLIP, were also proved to be involved in thyroid goiter formation through preventing apoptotic destruction of thyroid. Our present observation showed that IGF-I induced a marked increase in both FLIP mRNA and protein content of normal thyroid line, which was consistent with studies of thyroid carcinoma cells that IGF-I induced FLIP expression (Poulaki *et al.* 2002). Induction of FLIP by IGF-I suggests a new mechanism

Figure 4 NF- κ B DNA-binding activity in FRTL cells. Equal amounts of nuclear protein extract were prepared from untreated cells (lane 1) and from cells treated with 50 ng/ml IGF-I without (lane 2) or with (lane 3) 10 μ M LY294002. Biotin-labeled NF- κ B oligonucleotide was used as a probe. Solid and open arrows indicate bands of NF- κ B/DNA complexes and free probe respectively. Dark bands reflect increased NF- κ B DNA binding activity. Specificity of the NF- κ B complex was confirmed in competition experiments performed with the same nuclear extracts incubated with an excess of unlabeled NF- κ B (lane 4) or Sp1 (lane 5) oligonucleotides; these oligonucleotides eliminated binding and had no effect on binding respectively. Lane 6 is the negative control with only labeled free probe. Similar results were obtained from several experiments.

by which IGF-I and FLIP may contribute to thyroid goiter. Although some attention has focused on the effect of IGF-I on FLIP expression, little information is available about its signaling pathway in normal thyroid tissue and in cell lines.

Evidence from several cell lines indicates that specific tyrosine kinase receptors, such as PI3K, mediate the actions of IGF-I (Withers & White 2000, Lessmann *et al.* 2006). Although a previous study showed that PI3K did not affect FLIP expression (Osaki *et al.* 2004), some antiapoptotic proteins (such as FLIP, survivin, and X-linked mammalian inhibitor of apoptosis protein (XIAP)) were recently identified as substrates for PI3K (Kim *et al.* 2004, Alladina *et al.* 2005). Peruzzi *et al.* (1999) reported that IGF-I-mediated, mitogen-activated protein kinase-dependent survival signals may predominate only when the PI3K pathway is disabled, implicating PI3K as a key regulator in the actions of IGF-I. Our results showed that under the stimulation of IGF-I, inhibition of PI3K activity resulted in reduced mRNA and protein expression for FLIP, which suggests that PI3K is involved in the IGF-I/FLIP signaling pathway.

Figure 5 Effect of NF- κ B inhibitor BAY11-7082 on protein levels of FLIP. Cells were starved for 48 h. LY294002 (10 μ M) and/or BAY11-7082 (50 μ M) were added 1 h before treatment with 50 ng/ml IGF-I for 24 h. Western blotting was performed to measure protein levels of FLIP. Equal amounts of protein were loaded to allow direct comparisons. β -Actin was the loading control. (A) Representative blot. (B) Densitometric results from three experiments. * P <0.05 versus untreated group. † P <0.05 versus IGF-I-treated group. ‡ P <0.05 versus group treated with IGF-I and one inhibitor.

NF- κ B is one of the most critical transcription factors involved in the control of cellular proliferation and apoptosis. The effect of IGF-I on NF- κ B has recently garnered interest but results still remained controversial. Our confirming experiment demonstrated that the amount of I κ B α decreased when FRTL cells were stimulated with IGF-I, with a corresponding enhancement of NF- κ B DNA-binding activity in the nuclei. In addition, concomitant upregulation of FLIP gene transcription occurred. Further results showed that inhibition of NF- κ B activity eliminated the IGF-I-induced increase in FLIP gene transcription and protein levels, to a certain extent. These suggest that IGF-I-induced FLIP expression is at least partly due to the increased NF- κ B-mediated transcription of the gene for FLIP.

Since both PI3K and NF- κ B were involved in the mediation of FLIP expression induced by IGF-I, we examined whether they played roles through a common pathway. The concept that PI3K activation promoted NF- κ B activity (Heck *et al.* 1999) was recently

challenged because studies showed that inhibition of PI3K increased DNA binding of NF- κ B in monocyte-derived dendritic cells (Aksoy *et al.* 2005). In addition, insulin-related activation of NF- κ B in mammalian cells did not involve PI3K (Bertrand *et al.* 1998). Our present results showed that the PI3K inhibitor, LY294002, blocked the IGF-I-induced increase in NF- κ B-mediated DNA-binding activity, with an increase in I κ B α levels, which indicates NF- κ B is a downstream molecular of PI3K pathway. Further confirmation was performed by adding LY294002 and BAY11-7082 together to the cultured cells before treated with IGF-I. More obvious decline in FLIP expression was detected as compared with cells treated with BAY11-7082 only, indicating that these two signal molecules were tightly involved in the course of IGF-I regulating FLIP. Therefore, all these suggest that the effect of IGF-I on FLIP is most likely to involve a PI3K/NF- κ B-dependent mechanism.

In conclusion, our results have important implications on understanding the mechanism of IGF-I-mediated upregulation of FLIP in FRTL cells. To our knowledge, we are the first to demonstrate that the PI3K/NF- κ B signaling pathway is involved in this mechanism. This involvement may explain why IGF-I plays an important role in the formation of thyroid goiters by upregulating antiapoptotic proteins. Our observation provides valuable therapeutic information. However, potential involvement of other signaling pathways and the precise underlying mechanisms still need further investigation.

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