Am J Physiol Cell Physiol 307: C150–C161, 2014.
First published May 7, 2014; doi:10.1152/ajpcell.00308.2013.

Thyroid hormone inhibition in L6 myoblasts of IGF-I-mediated glucose uptake and proliferation: new roles for integrin αvβ3

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Submitted 30 September 2013; accepted in final form 14 April 2014

Incerpi S, Hsieh MT, Lin HY, Cheng GY, De Vito P, Fiore AM, Ahmed RG, Salvia R, Candelotti E, Leone S, Luly P, Pedersen JZ, Davis FB, Davis PJ. Thyroid hormone inhibition in L6 myoblasts of IGF-I-mediated glucose uptake and proliferation: new roles for integrin αvβ3. *Am J Physiol Cell Physiol* 307: C150–C161, 2014. First published May 7, 2014; doi:10.1152/ajpcell.00308.2013.—Thyroid hormones L-thyroxine (T_4) and 3,3′,5-triiodo-L-thyronine (T_3) have been shown to initiate short- and long-term effects via a plasma membrane receptor site located on integrin αvβ3. Also insulin-like growth factor type I (IGF-I) activity is known to be subject to regulation by this integrin. To investigate the possible cross-talk between T_4 and IGF-I in rat L6 myoblasts, we have examined integrin αvβ3-mediated modulatory actions of T_4 on glucose uptake, measured through carrier-mediated 2-deoxy-[^{3}H]-D-glucose uptake, and on cell proliferation stimulated by IGF-I, assessed by cell counting, [^{3}H]-thymidine incorporation, and fluorescence-activated cell sorting analysis. IGF-I stimulated glucose transport and cell proliferation via the cell surface IGF-I receptor (IGFIR) and, downstream of the receptor, by the phosphatidylinositol 3-kinase signal transduction pathway. Addition of 0.1 nM free T_4 caused little or no cell proliferation but prevented both glucose uptake and proliferative actions of IGF-I. These actions of T_4 were mediated by an Arg-Gly-Asp (RGD)-sensitive pathway, suggesting the existence of crosstalk between IGFIR and the T_4 receptor located near the RGD recognition site on the integrin. An RGD-sequence-containing integrin inhibitor, a monoclonal antibody to αvβ3, and the T_4 metabolite tetraiodothyroacetic acid all blocked the inhibition by T_4 of IGF-I-stimulated glucose uptake and cell proliferation. Western blotting confirmed roles for activated phosphatidylinositol 3-kinase and extracellular regulated kinase 1/2 (ERK1/2) in the effects of IGF-I and also showed a role for ERK1/2 in the actions of T_4 that modified the effects of IGF-I. We conclude that thyroid hormone inhibits IGF-I-stimulated glucose uptake and cell proliferation in L6 myoblasts.

glucose transport; insulin-like growth factor type I; fluorescence-activated cell sorting; tetraiodothyroacetic acid; thyroxine; triiodothyronine; mitogen-activated protein kinase; extracellular regulated kinase 1/2; phosphatidylinositol 3-kinase

NONGENOMIC ACTIONS OF THYROID HORMONES are usually rapid in onset, do not primarily involve nuclear thyroid hormone receptors (TRs), and may be initiated at the plasma membrane level, in cytosol or at certain organelles (7, 20). Thyroid hormones also nongenomically affect membrane transport systems, such as glucose transporters, Ca^{2+}-ATPase and Na^+/K^+-ATPase activities, the Na^+/H^+ exchanger, and certain ion channels (1, 7, 11, 14, 16, 17, 32, 33, 40, 41 56–61, 74). The signal transduction mechanisms utilized by thyroid hormones in nongenomic actions in both mammalian and nonmammalian cells appear to involve protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK, specifically ERK1/2) pathway, as well as the phosphoinositide 3-kinase (PI3K) pathway and Akt/PKB activation (19, 42, 43). Some of these hormonal effects were shown a decade or more ago in cells that lacked functional nuclear TRs (42, 45, 64).

Thyroid hormone also modulates the actions of interferon-γ (45) and growth factors, such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α) (64), and vascular growth factors (15, 50), by nongenomic mechanisms. Some of the authors of the present paper have previously shown that both EGF and TGF-α cause ERK1/2 activation and expression of c-*fos* in HeLa cells (64) and that T_4 increases EGF- and TGF-α-induced ERK1/2 activation in these cells. Both effects of the hormone were mimicked by T_4-agarose, a reformulation of thyroid hormone that does not gain access to the cell interior, and were inhibited by 3,3′,5,5′-tetraiodothyroacetic acid (tetrac). This compound is a naturally occurring, deaminated derivative of T_4 and is often an inhibitor of the nongenomic actions of T_4 and T_3 (7, 20); when intracellular, however, tetrac has also been shown to increase or decrease certain differentially regulated genes (26, 68). With regard to EGF and TGF-α, it is known that thyroid hormone can enhance the autocrine/paracrine effects of EGF and also block the actions of TGF-α (64). Thus nongenomic actions of thyroid hormone are not uniformly stimulatory.

One of our laboratories has established that the extracellular domain of a structural plasma membrane protein, integrin αvβ3, bears a receptor for thyroid hormone and hormone analogs (3, 7). Ligand-protein interactions at the receptor activate several signal transduction systems, including the ERK1/2 pathway, by a PKC-dependent mechanism (3), and PI3K. Occlusion of the receptor site prevents activation of ERK1/2 by iodothyronines and inhibits cellular actions of thyroid hormone downstream of ERK1/2. Thyroid hormone-activated ERK1/2 phosphorylates TR-β1 and other transcriptionally active proteins in the course of their translocation from cytoplasm to nucleus (18, 20). Basal transcriptional rates of thyroid hormone-responsive genes are at least in part maintained by this mechanism, and certain of these genes are

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involved in stimulating angiogenesis and tumor cell proliferation (7, 19, 20).

Because integrin αvβ3 contains a cell surface receptor for thyroid hormone but also is a coreceptor for insulin-like growth factor type I (IGF-I) (11), we postulated that thyroid hormone might modulate IGF-I actions. IGF-I is a small protein that supports cellular growth and has high structural homology to proinsulin; it increases tissue insulin sensitivity through autocrine, paracrine, and endocrine mechanisms and contributes to glucose homeostasis. Through interaction with its receptor and consequent stimulation of tyrosine kinase and PI3K activities, IGF-I stimulates the transport of glucose into smooth muscle and skeletal muscle cells. Recent studies on smooth muscle cells have shown that transduction of the IGF-I signal may involve not only its own receptor, but also integrin αvβ3 (12). The integrin is of structural, as well as functional, importance to muscle (65).

In the present studies we determined whether thyroid hormone modulates typical skeletal muscle cell responses to IGF-I, including glucose uptake as a short-term response and cell proliferation as a long term response, consistent with support of muscle mass (8, 10). We demonstrate in L6 myoblasts that T_4 at a subnanomolar free hormone concentration inhibits IGF-I stimulation of glucose uptake and of cell proliferation and that these effects are mediated by communication ("crosstalk") between the αvβ3 integrin and the IGF-I receptor (IGFIR). This is not surprising, given the recently described complex formation that occurs between IGFIR and αvβ3 (23). The involvement of PI3K in IGF-I actions (12) on glucose uptake and cell proliferation and of the activated ERK1/2 signal transduction pathway in the actions of both T_4 (20) and IGF-I (12) caused us to study involvement of these pathways in the crosstalk at the plasma membrane between T_4 and IGF-I.

MATERIALS AND METHODS

Cell culture. L6 rat skeletal muscle cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin, in an atmosphere of 5% CO_2 at 37°C, and were kept in culture as myoblasts by continuous passages at preconfluent stages as previously reported (14).

Measurement of free T_4 concentration. Culture medium free T_4 concentration was directly measured by a chemiluminescent immunoassay (Access Immunoassay System; Beckman Coulter, Brea, CA).

Glucose uptake assay. L6 cells were seeded in multiwell plates (12 wells) at a density of about 3×10^4 cells/well and were confluent after 4–5 days. On days of experiments, the cells were depleted of serum for 4 h by placement in serum-free DMEM. Cells were then washed with HEPES-buffered saline containing 11 mM glucose plus activators and/or inhibitors (5). The glucose concentration of 11 mM was used throughout the experiments to mimic a postprandial situation with increased insulin concentration and activation of the signaling cascade that results in the translocation of glucose transporter 4 (GLUT4) to the plasma membrane (71). Preliminary experiments did not show any significant difference between glucose uptake experiments carried out at 5.5 mM and 11.0 mM glucose, as has also been shown to be the case in adipocytes (55).

Glucose uptake was measured using radioactive 2-deoxyglucose, as described by Blair et al. (5). Total 2-deoxyglucose concentration was 10 μM, and the radiolabeled 2-deoxyglucose concentration was 1 μCi/well (specific activity 8.5–11 Ci/mmol; GE Healthcare, Buckinghamshire, UK). In experiments involving T_4, the cells were preincubated with T_4 for 30 min before addition of 2-deoxyglucose. Measurement of glucose uptake was carried out at 37°C for 10 min, during which time aliquots were taken for control measurements of free radioactivity. After 10 min, the multiwell plate was placed on ice for several minutes to stop the reaction.

Medium containing radiolabeled glucose was discarded, and cells were washed twice on ice in the multiwell plate with cold NaCl (0.9%). After the last wash, 0.6 ml of warmed NaOH (0.1 M at 80°C) was added to each well. The multiwell plate was transferred to 37°C for 15 min for cell detachment, and cell pellets were then collected and added to separate liquid scintillation vials, together with buffer up to a volume of 1.0 ml. An additional 5 ml of scintillation fluid was added to each vial (Optiphase HiSafe 3; Perkin Elmer, Shelton, CT), and the contents were then counted in a scintillation counter (Tricarb, Canberra Packard; Perkin Elmer). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B, and this value was subtracted from all other values. Results are reported as picomoles of 2-deoxyglucose/well × 10 min.

Proliferation studies. Cell proliferation was measured in cells seeded on 60 × 15 mm Petri dishes, $1.0–1.5 \times 10^5$ cells/dish, and grown in DMEM supplemented as reported above, in the presence or absence of thyroid hormone, IGF-I, and activators or inhibitors of signaling pathways. Media were changed every 24 h. For experiments carried out at fixed times, the cells were grown as reported for 48 h and then stimulated with thyroid hormones, IGF-I, and inhibitors or activators for 24 h. Up to 96 h after seeding, and after 24 h of hormone treatment, the cells were harvested with mild trypsinization and counted in a Neubauer chamber.

Analysis of DNA synthesis. [^{3}H]-Thymidine incorporation was used to measure the mitogenic response (49). DNA synthesis experiments were carried out by preparation of cell monolayers. Approximately 2×10^5 cells were seeded in Petri dishes (60 × 15 mm) and allowed to grow for 72 h in the presence or absence of thyroid hormone (T_4). IGF-I and activators and inhibitors were added to the cells 24 h before a [^{3}H]-thymidine pulse of 1 μCi/ml of [^{3}H]-thymidine (specific activity 23.0 Ci/mmol) and incubated for an additional 3 h at 37°C. Petri dishes were then placed on ice and washed twice with cold PBS; 0.6 ml of NaOH 0.1 M (about 80°C) was then added to each sample, and the dishes were placed in a 37°C incubator for 15 min. Detached cell suspensions were then transferred to scintillation vials, and each sample was washed with 0.4 ml of PBS. Washes were also added to the corresponding vials. Scintillation liquid (5 ml) was added and the radioactivity determined in each sample in a Tricarb scintillation counter.

Fluorescence-activated cell sorting analysis. L6 cells were grown in 60 × 15 mm Petri dishes for 4 days and used at 90% confluence. Cells were stimulated with either T_4 or IGF-I or with T_4 + IGF-I for 24 h. Cells were then stained with 500 μl propidium iodide, and the cell cycle phase was determined with a Becton Dickinson cytofluorimeter. The number of cells in each phase was obtained and analyzed, using the embedded cell cycle analysis tool WinMDI 2.8.

Western blotting. L6 cells were seeded in 10-cm dishes at a density of about 1×10^6 cells/dish. The cells were serum starved for two days before plating. Cells were exposed to signaling pathway inhibitors for 30 min, then treated with activators (T_4, IGF-I), individually or in combination for the times indicated in the figures. Cells were collected and lysed in hypotonic buffer. Protein concentration was measured by BCA reagent (Pierce, Rockford, IL). Samples (20 μg) of denatured protein were resolved in each lane of 10% SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad, Hercules, CA). After being blocked in 2% BSA in Tris-buffered saline containing 0.1% Tween 20, membranes were incubated with one of the following antibodies: anti-phospho-Akt (Gene Tex, Irvine, CA), anti-phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA), anti-IGFIR (Cell Signaling Technology), or anti-phospho-IGFIR (Abcam, Cambridge, MA). Anti-GAPDH (Gene Tex) antibody was the loading control.

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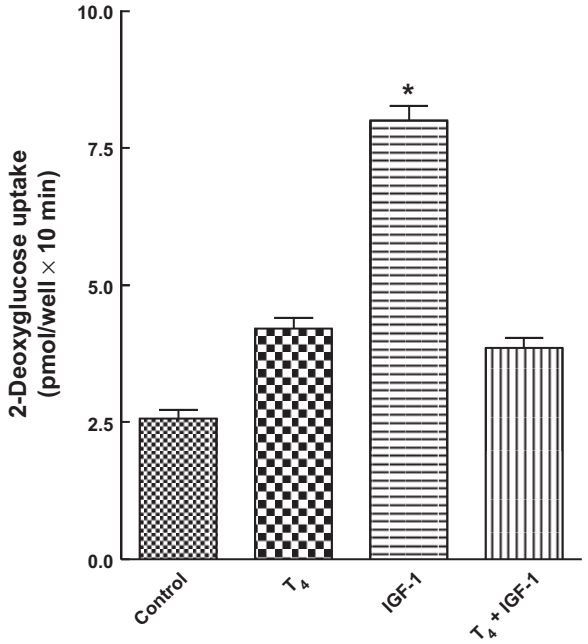

Fig. 1. Effects on glucose uptake by L6 cells of thyroid hormone (T_4) alone, insulin-like growth factor type I (IGF-I) alone, and IGF-I in combination with T_4. Data base is all experiments carried out with T_4 and IGF-I. The data are means ± SD of 36 different experiments carried out in duplicate. *$P < 0.001$ compared with control, T_4, and T_4 + IGF-I.

Secondary antibodies were either goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse IgG (Santa Cruz Biotechnology). Protein bands were detected by chemiluminescence, using the BioSpectrum Imaging System (UVP). Immunoblotting experiments were carried out in duplicate three times.

Materials. DMEM, antibiotics, and fetal bovine serum were obtained from GIBCO (Grand Island, NY). 2-Deoxy-D-[^{3}H]glucose and [^{3}H]-thymidine were from GE Healthcare (Buckinghamshire, UK). HEPES, Tris, 3,3′,5-triiodo-L-thyronine (T_3, sodium salt), 3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-L-alanine (L-thyroxine or T_4, sodium salt), tetrac, human recombinant IGF-I, echistatin, cytochalasin B, picropodophyllotoxin (PPP), ionomycin, Arg-Gly-Asp (RGD), and Arg-Gly-Glu (RGE) peptides were supplied by Sigma (St. Louis, MO). PD 98059 and wortmannin were from Alexis Biochemicals (Laufelfingen, Switzerland). Monoclonal anti-αvβ3 (clone LM609) was obtained from Immunological Sciences/Società Italiana Chimici (Rome, Italy). All other chemicals were of the purest grade available from Merck (Darmstadt, Germany).

Statistical analysis. The results are reported as means ± SD and analyzed by one-way ANOVA, followed by post hoc Bonferroni's multiple comparison test or by the Student's *t*-test; these were carried out using the Prism4 statistics program (GraphPad, San Diego, CA). Differences were considered significant at $P < 0.05$.

RESULTS

Short-term modulation by thyroid hormone of basal and IGF-I-mediated glucose transport in L6 myoblasts; involvement of integrin αvβ3 in thyroid hormone action. T_4 added at a total concentration of 100 nM (0.1 nM free T_4, directly measured in medium) significantly increased 2-deoxyglucose uptake; IGF-I (10 nM) produced a greater uptake of about threefold. However, T_4 inhibited significantly the IGF-I-activated glucose transport in L6 myoblasts (Fig. 1). The results reported in Fig. 1 were obtained by pooling all the samples obtained for control, IGF-I, T_4, and T_4 + IGF-I from the different sets of experiments shown in the subsequent figures. We also tested T_3 and found that this hormone was able only variably to inhibit IGF-I-mediated glucose transport (results not shown). The aim of the current studies thus became the definition of the signal transduction pathway(s) involved in the inhibition of the IGF-I effect by T_4.

Glucose uptake in skeletal muscle cells has been well characterized in the last 20 years by several groups (4, 5, 24, 59, 61, 69). We carried out experiments to confirm our experimental model, measuring glucose uptake in L6 myoblasts induced by 0.1 nM insulin or 10 nM IGF-I (Fig. 2*A*). Uptake studies in the presence of insulin or IGF-I showed a threefold increase in glucose uptake compared with basal levels and appear to be consistent with the literature (24). The involvement of IGFIR in glucose uptake by L6 myoblasts was shown using a phar-

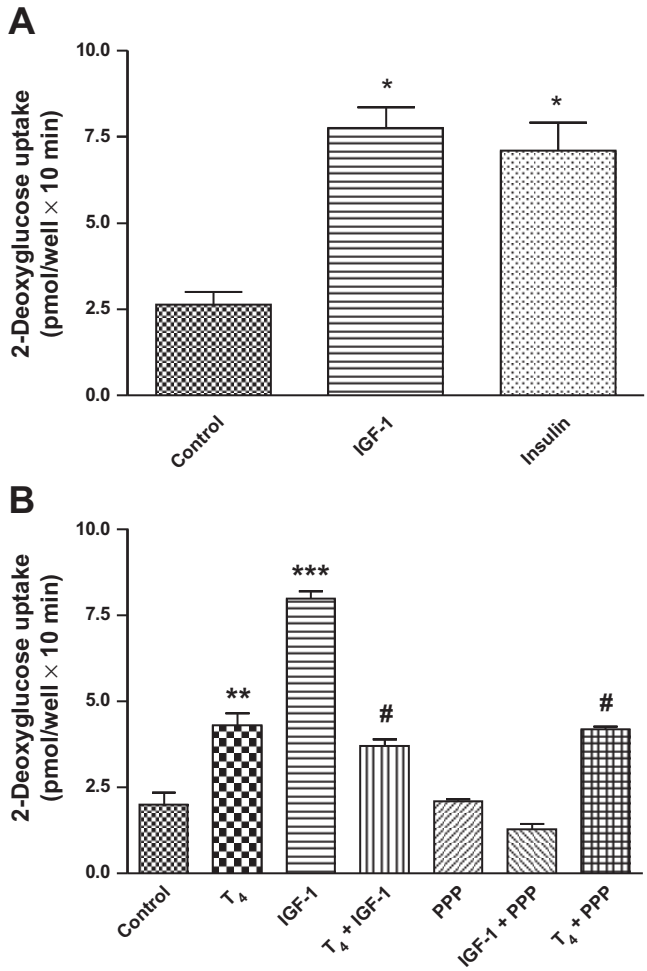

Fig. 2. Glucose uptake in the presence of IGF-I (10 nM) and insulin (0.1 nM) and an inhibitor of IGF-I receptor (IGFIR), picropodophyllotoxin (PPP), on IGF-I-mediated glucose uptake in L6 myoblasts. *A*: effects of insulin (0.1 nM) and IGF-I (10 nM, 30-min preincubation) on 2-deoxyglucose uptake. Data are reported as means ± SD of at least 3 separate experiments carried out in duplicate. Effects of both IGF-I and insulin were significant vs. control at the *$P < 0.001$ level. *B*: effect of 60-min preincubation with PPP (2 μM) on IGF-I-mediated glucose uptake in L6 myoblasts. Data are reported as means ± SD of 2 different experiments carried out in duplicate. **$P < 0.01$ at least, compared with IGF-I, PPP, and IGF-I + PPP; ***$P < 0.001$ compared with all, #$P < 0.05$, at least, compared with PPP and IGF-I + PPP.

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macological inhibitor, the cyclolignan PPP (2 μM), widely used to assess the involvement of IGFIR and its tyrosine kinase activity in the effects mediated by the growth factor. Preincubation of cells for 1 h with this compound, at concentrations not affecting the insulin receptor (25), completely prevented IGF-I activation of glucose uptake, whereas it did not affect the stimulation by thyroid hormone (70; Fig. 2*B*).

Thyroid hormones have been reported by Bergh et al. (3) to initiate their nongenomic, rapid-onset effects through interaction with the integrin αvβ3. At the hormone receptor site on the integrin, there is a domain that binds T_3 exclusively and a second domain that is responsive to T_4 and also binds T_3 but is less responsive to the latter (43). Because the inhibitory effect of T_3 on glucose uptake in myoblasts by IGF-I was variably

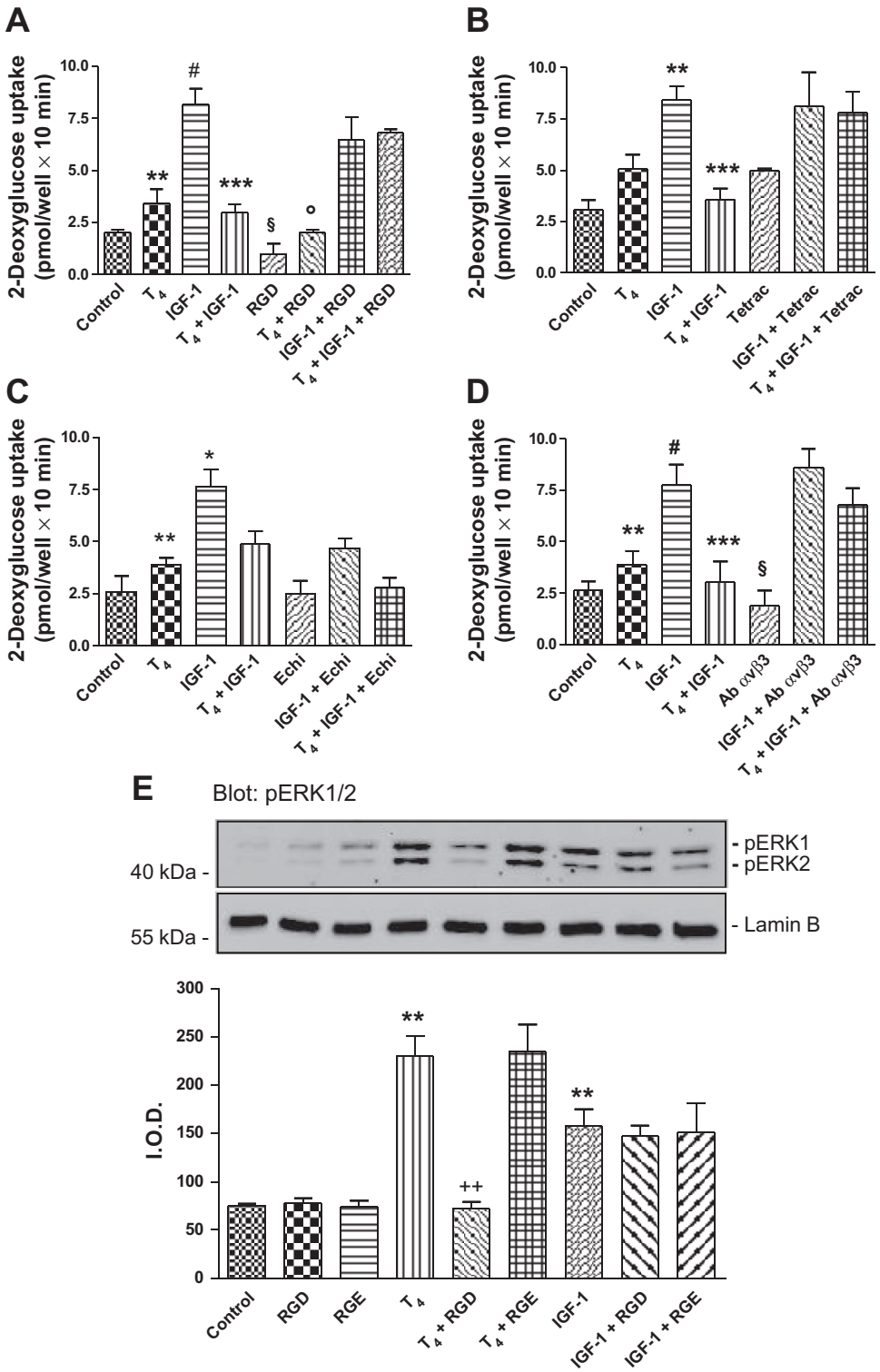

Fig. 3. Effects of T_4 (0.1 nM free) and integrin αvβ3 ligands/inhibitors on basal and IGF-I-mediated glucose uptake in L6 myoblasts and on activation (phosphorylation) of ERK1/2. For the experiments reported in this figure and in the following figures, the cell treatment periods were as follows: thyroid hormone for 30 min, IGF-I for 25 min, Arg-Gly-Asp (RGD) peptide, 3,3′,5,5′-tetraiodothyroacetic acid (tetrac), and echistatin for 15 min before addition of T_4, and anti-αvβ3 integrin for 20 min before the addition of T_4. Results are reported as means ± SD of at least 3 independent experiments, unless otherwise stated, carried out in duplicate. *A*: effects of RGD peptide (10 μM). $^{**}P < 0.05$ at least, compared with IGF-I and IGF-I + T_4 + RGD; $^{\#}P < 0.001$, compared with T_4 + IGF-I, RGD, and T_4 + RGD; $^{***}P < 0.05$, with respect to IGF-I + RGD and IGF-I + T_4 + RGD; §$P < 0.001$, with respect to IGF-I + RGD and IGF-I + T_4 + RGD;°$P < 0.01$ at least, compared with IGF-I + RGD and IGF-I + T_4 + RGD. *B*: effects of tetrac (10 μM). $^{**}P < 0.01$, compared with IGF-I + T_4; $^{***}P < 0.05$, compared with IGF-I, Tetrac + IGF-I, and T_4 + Tetrac + IGF-I. The differences among groups were statistically significant as from 1-way ANOVA ($P = 0.0002$). *C*: effects of echistatin (Echi) (100 nM). Results are reported as means ± SD of 4 different experiments carried out in duplicate. $^{*}P < 0.05$, compared with Echi, Echi + IGF-I, and Echi + T_4 + IGF-I, $^{**}P < 0.01$ compared with IGF-I. The differences among groups were significantly different by 1-way ANOVA ($P < 0.0001$) and post hoc Bonferroni's multiple comparison test. *D*: effects of anti-αvβ3. Results are reported as means ± SD of 4 different experiments carried out in duplicate. $^{**}P < 0.05$ compared with IGF-I + Ab αvβ3; $^{\#}P < 0.05$ at least, compared with T_4 + IGF-I and Ab αvβ3; $^{***}P < 0.01$ compared with Ab αvβ3 + IGF-I; §$P < 0.01$ at least, compared with Ab αvβ3 + IGF-I and Ab αvβ3 + T_4 + IGF-I. *E*: immunoblot analysis of activation (phosphorylation) of L6 cell ERK1/2 by IGF-I and by T_4 in absence and presence of RGD or Arg-Gly-Glu (RGE) peptide. Studies were carried out in duplicate ×3. $^{**}P < 0.01$, compared with control; $^{++}P < 0.01$, compared with T_4 alone. IOD, integrated optical density.

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demonstrated and, when present, less than that of T_4 (results not shown), we proceeded to study only the mechanism of action of T_4 on glucose uptake. To confirm the involvement of αvβ3 integrin in the actions of IGF-I and thyroid hormone in myoblasts, we tested the ability of the RGD peptide, a small ligand that binds to the integrin RGD recognition site (20), to affect the modulatory effects of T_4. It is in fact known that the T_4 site on the integrin is close to the RGD recognition site (20). The RGD peptide completely blocked the inhibition by T_4 of IGF-I-mediated glucose uptake and returned stimulation of glucose uptake to the values obtained with IGF-I alone (Fig. 3*A*). This result demonstrated that the integrin plays a role in the T_4 effect.

Tetrac has been shown to inhibit the short-term effects of thyroid hormone at the plasma membrane level (14, 42) and is considered a probe for the involvement of the integrin αvβ3 in plasma membrane-initiated actions of T_4; the agent also exhibits pharmacological antitumor properties (19, 26, 44, 49). As expected, tetrac (10 μM) prevented the inhibitory effect of T_4 on IGF-I-induced glucose uptake; surprisingly, tetrac itself stimulated basal glucose uptake comparably to T_4 (Fig. 3*B*).

Echistatin is a disintegrin protein of 49 amino acids (5.4 kDa) obtained from the venom of the viper *Echis carinatus*. It is an inhibitor of integrin αvβ3 (2, 47). Disintegrins contain the RGD sequence; by binding to the RGD recognition site on the integrins, they block ligand interaction with certain extracellular matrix (ECM) proteins (27). Echistatin alone did not affect glucose uptake, but it significantly inhibited IGF-I-mediated glucose uptake and to the same extent as T_4. The combination of echistatin, IGF-I, and T_4 returned the glucose uptake to basal level (Fig. 3*C*). An antibody to the αvβ3 integrin, LM609, did not affect the activation of glucose uptake by IGF-I but LM609

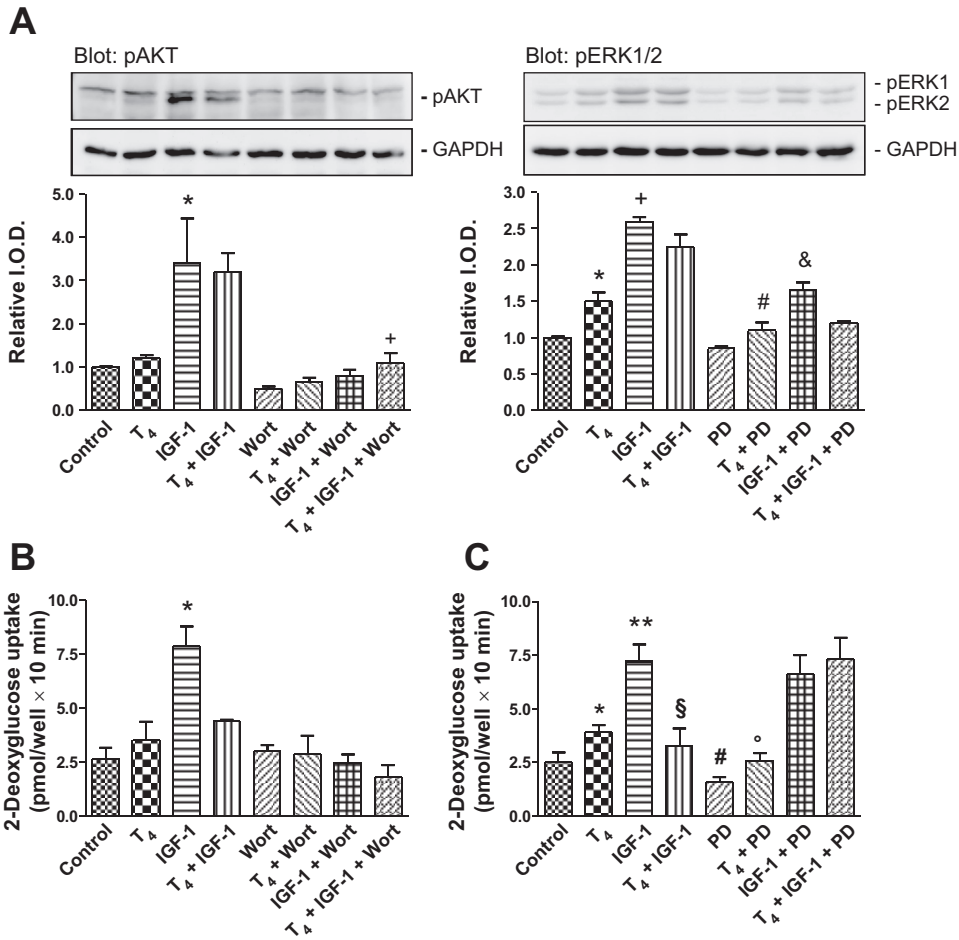

Fig. 4. Roles of phosphoinositide 3-kinase (PI3K) and MAPK/ERK pathways in inhibition by T_4 of IGF-I-stimulated glucose uptake in L6 myoblasts. Wortmannin (Wort), 100 nM (*A*, *left; B*), and PD 98059 (PD), 10 μM (*A*, *right; C*), were applied to cells 10 min before T_4 treatment. *A*, *left*: immunoblots of activated Akt (pAkt) in lysates of L6 cells treated with T_4, IGF-I, wortmannin, and combinations of the agents. Bar graphs are means ± SD of blot densities in duplicate runs of 3 experiments. $^*P < 0.01$ compared with control; $^+P < 0.01$ compared with IGF-I alone. *Right*: immunoblots of activated ERK1/2 (pERK1/2) in lysates of L6 cells treated with T_4, IGF-I, PD 98059, and combinations of the agents. $^*P < 0.05$ vs. control; $^+P < 0.01$ compared with control; $^\#P < 0.01$ compared with IGF-I alone; $^\&P < 0.05$ vs. IGF-I alone. Results of glucose uptake studies in *B* and *C* are reported as means ± SD of at least 3 different experiments carried out in duplicate. *B*: T_4 inhibits the stimulatory effect of IGF-I on 2-deoxyglucose uptake. Wortmannin had no appreciable effect on unstimulated glucose uptake or on the action of T_4 but completely inhibited the uptake stimulated by IGF-I either with or without T_4. Results are reported as means ± SD of 3 different experiments carried out in duplicate. $^*P < 0.05$ compared with all. *C*: MAPK activation inhibitor, PD 98059 (10 μM), suppressed the inhibitory action of T_4 on IGF-I stimulation of 2-deoxyglucose uptake. Results are reported as means ± SD of at least 3 different experiments carried out in duplicate. $^*P < 0.05$ compared with IGF-I + T_4 + PD, $^{**}P < 0.05$ at least compared with IGF-I + T_4, PD, and PD + T_4; $^\#P < 0.01$ at least compared with IGF-I + PD and IGF-I + T_4 + PD; $^\circ P < 0.05$ at least compared with IGF-I + PD and IGF-I + T_4 + PD.

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completely prevented the inhibition by T_4 of IGF-I-mediated glucose uptake (Fig. 3*D*). Echistatin thus appears to affect crosstalk between the integrin and IGFIR more potently than the anti-αvβ3 antibody (Fig. 3, *C* and *D*).

The functional contribution of αvβ3 to the action of T_4 was additionally examined by measurement of αvβ3-dependent activation (phosphorylation) of ERK1/2, a factor essential to the interaction of T_4 and IGF-I on glucose transport. L6 cells were treated with T_4 or IGF-I in the presence and absence of RGD or RGE peptide, and pERK1/2 was measured (Fig. 3*E*). Both T_4 and IGF-I increased pERK1/2, but RGD peptide affected only the thyroid hormone-induced activation of the enzyme. Control RGE peptide was without effect. Thus the mechanisms of ERK1/2 activation by T_4 and IGF-I are discrete, and only the thyroid hormone effect requires the integrin in L6 cells. These results are consistent with glucose uptake studies in Fig. 3*A*.

Short-term modulation of thyroid hormone on IGF-I-mediated glucose transport in L6 myoblasts: signal transduction by MAPK and Akt. The signal transduction pathways involved in the effect of T_4 on IGF-I action were studied by immunoblotting and with pharmacological probes (Fig. 4). Western blotting showed that IGF-I, but not T_4, caused phosphorylation of Akt (Fig. 4*A*, *left*). A PI3K inhibitor, wortmannin (100 nM), blocked the action of IGF-I on pAKT. Consistent with these observations, wortmannin eliminated the action of IGF-I on glucose transport and had no effect on glucose transport in the presence of T_4 (Fig. 4*B*).

Both IGF-I and T_4 increased pERK1/2 (Fig. 4*A*, *right*), and these actions were inhibited by PD 98059 (10 μM), an inhibitor of the MAPK pathway at MEK. The PD compound did not affect stimulation of glucose transport by IGF-I (Fig. 4*C*) but did reduce glucose transport in the presence of T_4 alone. Furthermore, PD 98059 eliminated the inhibitory effect of T_4 on IGF-I-stimulated glucose uptake (Fig. 4*C*).

These results supported a role for the PI3K/Akt pathway as the principal mediator of glucose transport activation by IGF-I and established that pERK1/2 is a critical contributor to the inhibitory action of thyroid hormone (T_4) on IGF-I-enhanced glucose uptake. The T_4 effect was shown above to require αvβ3 and thus to be a nongenomic action of the hormone.

Phosphorylation of IGFIR. We also examined IGFIR phosphorylation in L6 cells in response to IGF-I and T_4, individually and in combination (Fig. 5). Exposure of myoblasts to IGF-I for 30 min expectedly increased pIGFIR, whereas T_4, alone did not promote phosphorylation of IGFIR. Despite the physical interaction of IGFIR and αvβ3 (23), the action of T_4 alone on glucose uptake is unrelated to activation of IGFIR. When added with IGF-I, T_4 caused a small but significant ($P < 0.05$) decrease in the phosphorylation state of IGFIR. Thus the inhibitory effect of T_4 on IGF-I-stimulated glucose transport in myoblasts may in part reflect suboptimal phosphorylation of IGFIR.

Thyroid hormone modulation of IGF-I-stimulated proliferation in L6 myoblasts. Actions of IGF-I include *1*) stimulation of expression and activity of the different glucose transporters, and *2*) action as a growth factor on cell proliferation in different cell systems (8, 9). We determined whether thyroid hormone modulated the stimulatory effect of IGF-I on cell proliferation, as it may of other growth factors (64). We first confirmed that IGF-I increased L6 myoblast number (Figs. 6, *A* and *B*, 7, *A* and *B*). T_4 had a small stimulatory effect on cell proliferation (Fig. 6, *A* and *B*) that was inconsistent (Fig. 7, *A* and *B*), but the hormone significantly inhibited the stimulatory effect of IGF-I on proliferation (Figs. 6, *A* and *B*, and 7, *A* and *B*). Addition of either tetrac or echistatin to the combination of T_4 and IGF-I completely eliminated the inhibition by thyroid hormone of the proliferative IGF-I effect (Fig. 6, *B* and *D*). This again implicates αvβ3 integrin in the inhibitory effect of thyroid hormone on the IGF-I-mediated effects, as shown above (Fig. 3, *B* and *D*). RGD peptide is an αvβ3 ligand but did not produce the same effects as tetrac. For example, it did not in the presence of T_4 restore the effect of IGF-I on cell proliferation (Fig. 6, *B* and *D*). This is not surprising because tetrac and RGD peptide do not have identical effects at the hormone receptor site on the integrin, particularly in the S2 domain of the receptor that is primarily affected by T_4 (43). The decrease in cell proliferation observed with RGD alone (Fig. 6, *B* and *C*) might be due to cell detachment by this compound. This has been described with long incubation times. However, the concentrations of RGD peptide employed in our experiments were lower than those used in previous reports, and this possibility appears less likely (30).

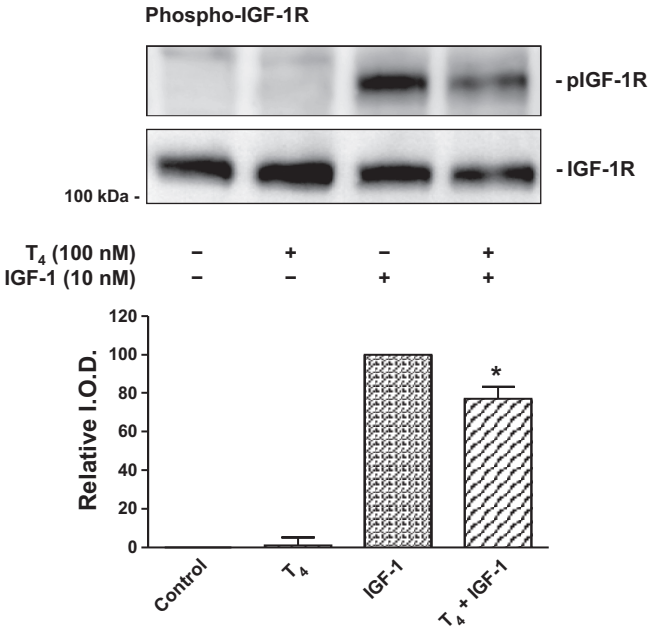

Fig. 5. Phosphorylation (activation) of IGFIR in L6 myoblasts in response to IGF-I, T_4, and IGF-I + T_4. Cells were incubated with IGF-I or thyroid hormone or both for 30 min before harvest and analysis. Blot is representative of 3 experiments involving duplicate cell samples. Reduction in IGFIR phosphorylation obtained with T_4 in the presence of IGF-I was significant at the $^*P < 0.05$ level.

We next examined the participation of Akt and ERK1/2 signal transduction pathways in IGF-I-stimulated cell proliferation and its inhibition by thyroid hormone. PI3K/Akt mediates both short- and long-term cellular responses to IGF-I (10). Figure 7*A* shows that ERK inhibition with PD 98059 (10 μM) had no effect on the action of IGF-I or on cell counts but eliminated the inhibitory effect of T_4 on cell proliferation of IGF-I. In contrast, wortmannin (100 nM) completely prevented induction of cell proliferation by IGF-I so that the cell counts remained at control levels (Fig. 7*B*). This further substantiates

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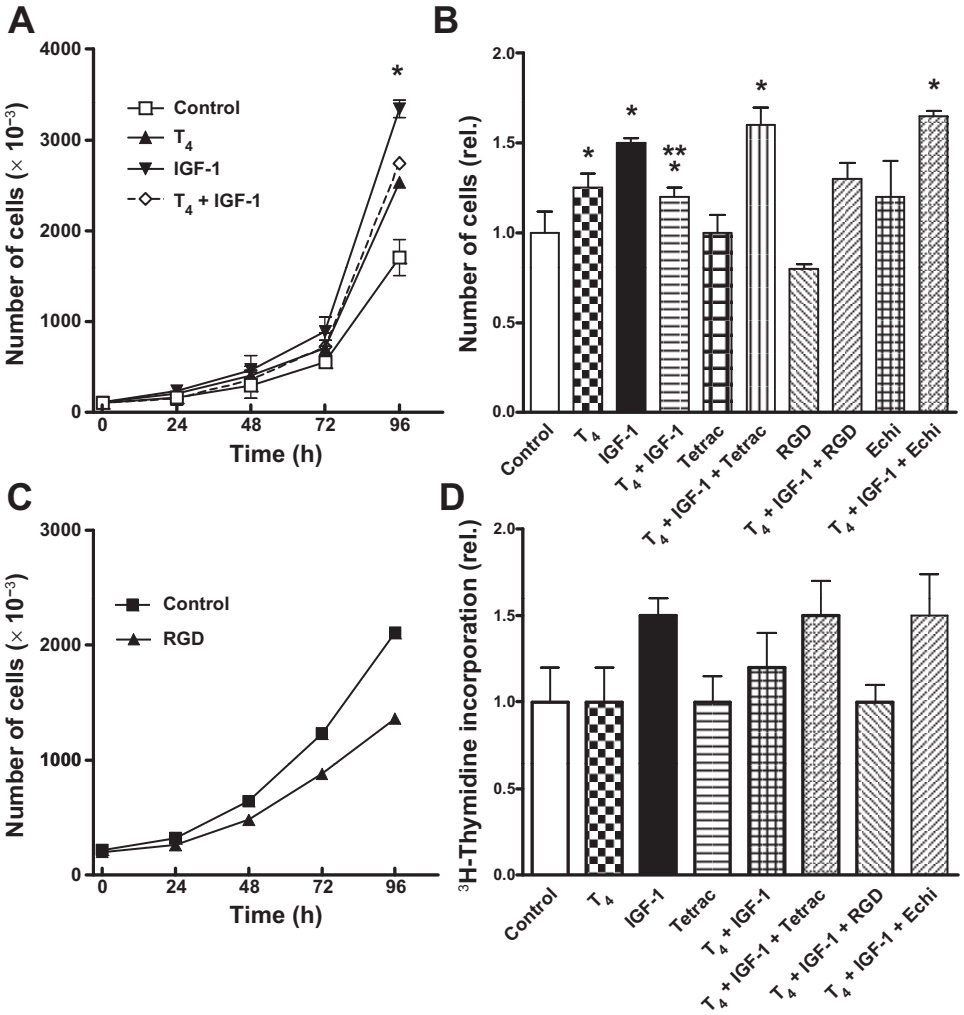

Fig. 6. Effects of T_4 (0.1 nM free), RGD peptide (10 μM), tetrac (10 μM), or echistatin (100 nM) on cell proliferation stimulated by IGF-I (10 nM) in L6 myoblasts. Results are reported as means ± SD of at least 3 different experiments, unless otherwise stated. *A*: effect of T_4, IGF-I, and T_4 + IGF-I on cell proliferation. Cells were seeded on 60 × 15 mm Petri dishes and stimulated and counted every 24 h, as described in MATERIALS AND METHODS. $*P <$ 0.01 vs. all. At 96 h, all differences were significant. *B*: effect of tetrac, RGD peptide, echistatin, and T_4 on cell proliferation activated by IGF-I. $*P < 0.05$ at least, compared with tetrac + T_4 + IGF-I, RGD, and Echi + T_4 + IGF-I; $**P < 0.001$ compared with IGF-I, tetrac + T_4 + IGF-I, and Echi + T_4 + IGF-I. *C*: effect of RGD peptide (10 μM) on cell proliferation assessed by cell counting; the graph shows a representative experiment of 2 similar. *D*: effect of T_4 and IGF-I, in the presence or absence of RGD, tetrac, echistatin, on ^{3}H-thymidine incorporation. Results are reported as means ± SD of 3 different experiments carried out in duplicate. 1-way ANOVA gave significant differences among the groups ($P = 0.0188$).

roles for the PI3K pathway in the mediation of the IGF-I responses that are both short term (glucose uptake; Fig. 4) and long term (cell counts/cell proliferation at 24 h) (Fig. 7*B*).

We also examined the possibility that the effect of thyroid hormone on IGF-I-mediated cell proliferation might be the result of a modification of different phases of cell cycle. Thyroid hormone is considered to be a regulator of the time course of cell differentiation, and this hormone-induced inhibition could represent in L6 myoblasts the starting point of such an effect also for L6 myoblasts. Table 1 presents the percentages of cells in the different phases of the cell cycle after T_4, IGF-I, or T_4 + IGF-I treatment ($n = 3$), and Fig. 8 presents representative scans. T_4 caused an increase in the number of dead cells (Table 1), but the hormone did not significantly affect the cell cycle. IGF-I promoted trends in the cell cycle (decreased cells in G1, increased cells in S and G2/M, compared with control cells). These results are consistent with the proliferative action of IGF-I (39). When thyroid hormone and IGF-I together were incubated with cells, there was a small but significant reduction in the proliferative effect of IGF-I. In agreement with this, there was also, compared with IGF-I alone, a significant increase in cells in G0/G1 and a decrease of cells in G2/M in the presence of T_4 + IGF-I. The S phase was not significantly affected (Table 1), suggesting that T_4 in L6 myoblasts might slow the cell cycle and promote differentiation, as already reported for other cell lines (46).

DISCUSSION

The principal findings here are that thyroid hormone as T_4 inhibits two actions of IGF-I in L6 myoblasts, glucose uptake and proliferation. Glucose uptake is inhibited by T_4 within a short period of time (minutes), whereas the effect on stimulation by IGF-I on cell proliferation persists for a day or more. IGF-I treatment of rat myoblasts increased glucose uptake three- to fourfold compared with basal levels. It has been shown elsewhere that this effect of IGF-I is very fast and independent of protein synthesis and apparently reflects translocation of glucose transporters from intracellular stores to the plasma membrane (4, 38, 72). In the current studies, when T_4 was added to cells together with IGF-I (10 nM), IGF-I-stimulated glucose uptake was significantly inhibited, suggesting the existence of crosstalk between the T_4 receptor on integrin αvβ3 and IGFIR and consistent with the ternary complex formation described between the integrin and IGFIR (23). Acting alone, T_4 variably increased glucose uptake. Studies elsewhere have shown that T_3 consistently stimulated cellular glucose uptake (59, 61), probably attributable to an increase in

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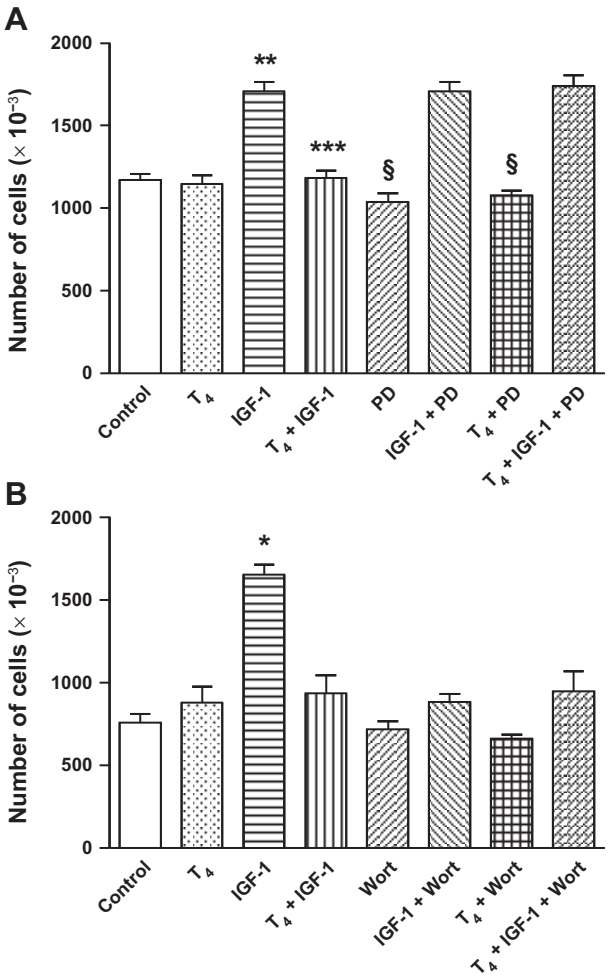

Fig. 7. Effects of inhibitors of MAPK (PD 98059) and PI3K (wortmannin) pathways on cell proliferation in L6 myoblasts. Results are reported as means ± SD of 3 independent experiments carried out in duplicate. *A*: effect of MAPK pathway inhibitor, PD 98059 (10 μM). Results are reported as means ± SD of 3 independent experiments carried out in duplicate. $^{**}P < 0.001$ compared with IGF-I + T_4, PD, and T_4 + PD; $^{***}P < 0.001$ compared with IGF-I, IGF-I + PD, and IGF-I + T_4 + PD; §$P < 0.001$ compared with IGF-I + PD and IGF-I + T_4 + PD. *B*: effect of PI3K pathway inhibitor, wortmannin (100 nM), on cell proliferation. Results are reported as means ± SD of 3 different experiments carried out in duplicate. $^{*}P < 0.001$ compared with all of the others.

activity of the glucose transporter. It may be noted that the T_4 added in the current experiments resulted in a directly measured near-physiological free T_4 concentration of 10^{-10} M in the cell culture medium.

The mechanisms by which T_4 inhibits the effect of IGF-I on glucose transport and may increase glucose uptake are incompletely understood. IGF-I action in the present studies caused phosphorylation of IGFIR (Fig. 5), and results obtained with PPP (Fig. 2*B*) indicated that the activation of IGFIR indeed was required for action of IGF-I on glucose uptake in L6 cells. Downstream of IGFIR, glucose uptake promoted by IGF-I reflected Akt activation (phosphorylation) (Fig. 4*A*, *left*). ERK1/2 was also phosphorylated in response to IGF-I, but this was not linked to glucose uptake (Fig. 4*C*). In contrast, the inhibitory action of T_4 on the stimulation of glucose uptake by IGF-I was ERK1/2 dependent (Fig. 4*A*, *right*, Fig. 4*C*). IGF-I also can activate ERK1/2, but our results indicate that IGF-I and T_4 cause phosphorylation of ERK1/2 by different mechanisms upstream of the enzyme and with discrete consequences downstream of these kinases. As shown here, T_4 does not affect activation of Akt but does have a small but significant negative effect on phosphorylation of IGFIR by IGF-I (Fig. 5), which may contribute to the blockade of IGF-I-induced glucose transport by thyroid hormone. Integrin αvβ3 is expressed on the cell surface of various mammalian cells including skeletal muscle (64).

The thyroid hormone-integrin interaction, particularly that with T_4, activates the MAPK (ERK1/2) signal transduction pathway, promoting complex cellular responses such as angiogenesis (3, 44, 50). The extracellular domain of αvβ3 includes an RGD recognition site, essential for the interaction with polypeptide ECM ligands that contain the RGD sequence (73). The binding of T_4 to the integrin and the consequent activation of the MAPK pathway and downstream physiological responses are inhibited by the RGD peptide. This indicates that the T_4 receptor on the integrin is proximal to the RGD recognition site (13, 21), but the actions of RGD peptide and tetrac at the TR on αvβ3 are not identical (44). Tetrac inhibits the nongenomic effects of thyroid hormone by competing with the hormone for the plasma membrane binding site (3, 14, 22, 43). In the present studies, RGD peptide and tetrac both blocked the effect of thyroid hormone on IGF-I-stimulated glucose uptake in L6 myoblasts, demonstrating the involvement of the integrin αvβ3 in the T_4 effect. The RGD peptide was also effective in preventing the inhibition by T_3 of IGF-I-mediated glucose uptake (not shown), confirming that T_3 also binds to this integrin, as previously shown (13, 44). Very recently, short-term triiodothyronine (10^{-9} M) exposure was shown to normalize glucose transport in thyroid hormone-deprived L6 myotubes; the effect was additive to that of insulin. In these studies, there was no translocation of insulin-sensitive glucose transporters to the plasma membrane (69).

To further establish the role of integrin αvβ3 in the interaction of thyroid hormone and IGF-I effects on myoblast glucose transport, we employed a monoclonal antibody to the αvβ3 integrin (LM 609) and a disintegrin, echistatin. Disintegrins have been considered to be antagonists of the receptor, although recent evidence indicates that they can have agonist effects on integrins (2, 51). Our data suggest that echistatin is not exclusively an antagonist of integrin αvβ3 but that it may have a direct inhibitory effect on glucose uptake stimulated by IGF-I (Fig. 3*C*). In the proliferation assay, echistatin behaved like RGD. In the presence of both IGF-I and T_4, echistatin

Table 1. *Distribution of L6 cells in the different phases of cell cycle after treatment for 24 h with T_4, IGF-I, or T_4 + IGF-I*

	Dead Cells	G0/G1	S	G2/M
Control	10.3 ± 3.5	73.0 ± 3.4	2.2 ± 2.5	14.8 ± 3.5
T_4	18.3 ± 5.0*	59.0 ± 10.0	4.4 ± 2.1	18.2 ± 4.2
IGF-I	8.7 ± 0.2	54.8 ± 3.5	8.7 ± 2.0	27.7 ± 2.5
IGF-I+ T_4	7.4 ± 0.9	62.4 ± 3.6†	7.9 ± 1.9	22.0 ± 2.5†

Results are reported as means ± SD of 3 different experiments. $^{*}P < 0.02$ by Student's *t*-test compared with insulin-like growth factor type I (IGF-I) and IGF-I + T_4, same column. †$P < 0.05$ by Student's *t*-test compared with IGF-I, same column.

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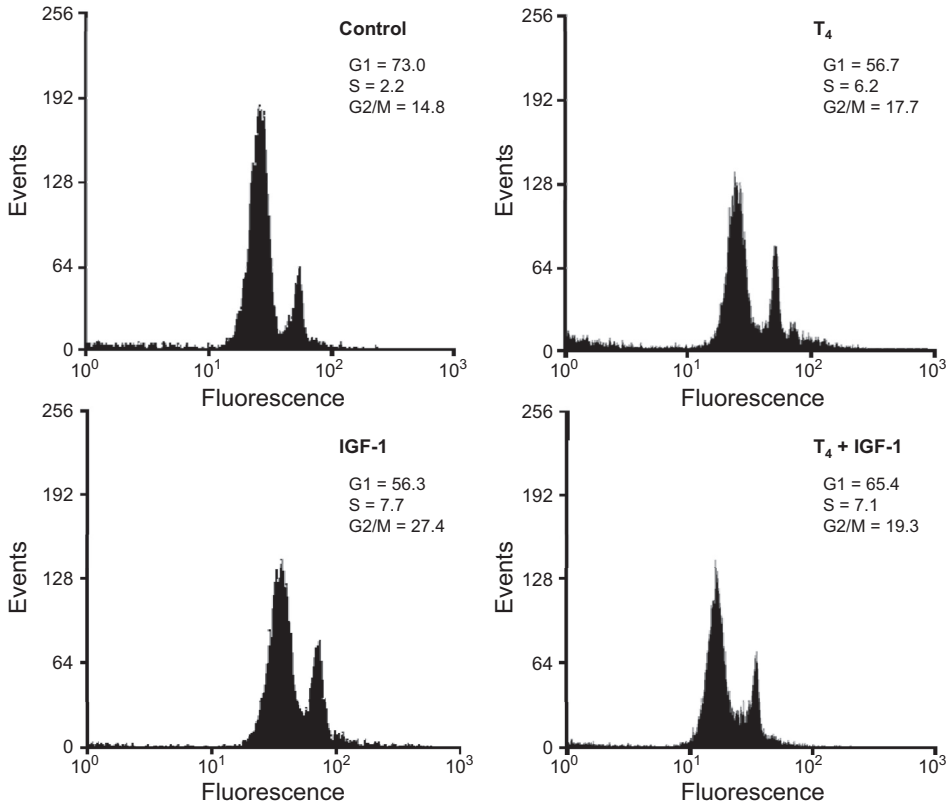

Fig. 8. Fluorescence-activated cell sorting analysis of DNA in L6 myoblasts after incubation for 24 h with T_4 (0.1 nM free), IGF-I (10 nM), and T_4 + IGF-I. The histogram shows a representative experiment and shows the number of events vs. fluorescence emission. G1, S, and G2/M indicate the different cell cycle phases. Data from multiple analyses are presented in Table 1.

blocked the inhibitory effect of T_4 on cell proliferation, mimicking RGD and tetrac. To summarize, results obtained with RGD peptide, tetrac, echistatin, and the antibody for the integrin αvβ3 support the existence of crosstalk between the integrin receptor for T_4 and the IGFIR. Acting via αvβ3, thyroid hormone increases phosphorylation of the IGFIR, consistent with clustering and physical interaction of the proteins on the cell surface and function of the integrin as a coreceptor for IGF-I (9, 11). Experiments carried out by others on vascular smooth muscle cells show that full expression of the activities of IGF-I requires activation of integrin αvβ3 by ECM proteins, such as vitronectin (11, 36, 48, 53). Reported by Segal and Ingbar in 1990 (61), the T_3-induced increase in glucose transport in rat thymocytes was among the first nongenomic effects of thyroid hormone to be identified. The effect was ascribed to activation of transporters, rather than to transporter translocation. Thyroid hormone stimulated the uptake of glucose without affecting transporter number or affinity, as indicated by the binding of [^{3}H]-cytochalasin B (61). In contrast to the observations of Segal and Ingbar involving T_3 and glucose uptake, we find that the effect of T_4 on glucose transport in myoblasts is primarily modulation of IGF-I-stimulated glucose uptake and secondarily or variably on sugar transport in the absence of IGF-I. At the 10^{-10} M free T_4 concentration employed, T_4 opposed IGF-I-enhanced glucose uptake by an integrin αvβ3-mediated mechanism distinct from the classical mechanism of IGF-I action.

Signal transduction studies of thyroid hormone and IGF-I that we conducted on glucose uptake involved inhibitors of PI3K/Akt and MAPK. Wortmannin, an inhibitor of PI3K, inhibited IGF-I stimulation of glucose uptake, as expected (8, 9). MAPK pathway inhibition with PD 98059 did not affect IGF-I action but did block the actions of T_4. We confirmed the pharmacological inhibitor studies involving wortmannin and PD with immunoblots of activated Akt and ERK1/2. These findings are in agreement with previous reports from our laboratories on several nongenomic effects of thyroid hormones that may involve these signal transduction pathways (14, 18, 21, 43). Figure 4, *A* and *B*, indicates that the addition of T_4 does not significantly change the abundance of pAkt or pERK1/2 in IGF-I-stimulated cells. The upstream pathway sources of pERK1/2 in the blot band phosphoprotein are different in IGF-I-treated cells in the presence and absence of T_4, and the downstream consequences may be different ("targeted pools"). Our results, however, do suggest that the small decrease in activated IGFIR obtained with T_4 in IGF-I-treated L6 cells contributes to the decrease in glucose uptake seen with thyroid hormone. We do not yet know whether T_4 is capable of affecting translocation of hormone-sensitive GLUT transporters from the cytoplasm to the plasma membrane or their intrinsic activity (52, 66), an action that T_3 appears to induce (61). Any stimulation of glucose uptake that T_4 may achieve, e.g., that shown here in Fig. 3*C*, could reflect MAPK/ERK1/2-dependent stimulation of intrinsic activity of the glucose transporter (52, 61, 66).

In addition to its effect on IGF-I-stimulated cell glucose uptake, T_4 impaired the action of IGF-I on myoblast proliferation, as measured by cell counting, thymidine incorporation experiments, and fluorescence-activated cell sorting experiments. The inhibitory effect is mediated by the MAPK pathway because PD 98059 reversed the inhibitory effect of T_4 on proliferation. This is similar to the action of the PD compound

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on the T_4-IGF-I interaction on glucose uptake. Thus T_4 is to be added to a group of endogenous small molecules or peptides that may importantly modulate insulin or IGF-I activity in muscle cells (29, 34, 35, 63).

Tetrac completely reversed the inhibitory effects of T_4 on cell proliferation induced by IGF-I, implicating the iodothyronine receptor on αvβ3 in this action of thyroid hormone. The results obtained with echistatin support those obtained with tetrac; that is, the disintegrin completely eliminated the inhibition of IGF-I-induced cell proliferation by T_4. RGD peptide was unable to reverse the inhibition by T_4 of glucose uptake in IGF-I-treated L6 myoblasts. As we have already noted, the effects of RGD peptide may overlap with, but are not identical to, those of tetrac (44).

L6 myoblasts are customarily grown in the high-glucose medium (4.5 g/l) used in the present studies (MATERIALS AND METHODS). Increased ambient glucose may usefully retard myogenesis (28) or confluency and myotube formation. Other cell lines, such as neural stem cells (6), are also known to require high-glucose medium. Apoptosis has been studied in neural stem cells grown in high ambient glucose and has been shown to occur only at glucose concentrations above the 4.5 g/l level used in the current study on myoblasts. Thus we believe it is unlikely that the medium glucose level used here influenced the cell proliferation results obtained.

Glucose intolerance is common in hyperthyroidism. Decreased peripheral insulin sensitivity with impaired insulin secretion are factors contributing to the development of abnormal glucose tolerance in the hyperthyroid state (31, 67). However, the mechanisms involved in this action of thyroid hormone are incompletely understood (54). An insight provided by the current studies is that thyroid hormone impairs glucose uptake in L6 myoblasts that is promoted by IGF-I, functionally giving rise to IGF-I resistance that can be considered as a part of a differentiation process involving L6 cells. One approach to interpretation of these results is that impairment by thyroid hormone of IGF-I-stimulated glucose uptake in L6 myoblasts is antianabolic and might contribute to differentiation of these cells.

ACKNOWLEDGMENTS

We thank Dr. Graziella Medori, Laboconsult s.r.l., Rome, for the assay of free T_4.

GRANTS

We gratefully acknowledge the financial support from the Italian Ministry for University and Research, General Management for Strategies and Development of Internationalization of Scientific and Technological Research; a grant from the Ordway Research Institute, Albany, NY; and a Lab Visit Grant from the Society of Endocrinology to Dr. R. G. Ahmed. Drs. F. B. and P. J. Davis appreciate the financial support of M. Frank and Margaret D. Rudy.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.I., P.D.V., R.G.A., P.L., and J.Z.P. conception and design of research; S.I., M.-T.H., H.-Y.L., G.-Y.C., P.D.V., A.M.F., R.S., E.C., S.L., and P.J.D. analyzed data; S.I., M.-T.H., H.-Y.L., P.D.V., R.G.A., E.C., S.L., P.L., J.Z.P., F.B.D., and P.J.D. interpreted results of experiments; S.I., G.-Y.C., and A.M.F. prepared figures; S.I., J.Z.P., and P.J.D. drafted manuscript; S.I., P.L., J.Z.P., F.B.D., and P.J.D. edited and revised manuscript; S.I., M.-T.H., H.-Y.L., G.-Y.C., P.D.V., A.M.F., R.G.A., R.S., E.C., S.L., P.L., J.Z.P., F.B.D., and P.J.D. approved final version of manuscript; M.-T.H., H.-Y.L., G.-Y.C., P.D.V., A.M.F., R.S., E.C., and S.L. performed experiments.

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