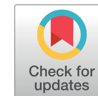

Verapamil Prevents Decline of IGF-I in Subjects With Type 1 Diabetes and Promotes β-Cell IGF-I Signaling

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Diabetes 2023;72:1460–1469 | https://doi.org/10.2337/db23-0256

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ISLET STUDIES

Verapamil promotes functional β-cell mass and improves glucose homeostasis in diabetic mice and humans with type 1 diabetes (T1D). Now, our global proteomics analysis of serum from subjects with T1D at baseline and after 1 year of receiving verapamil or placebo revealed IGF-I as a protein with significantly changed abundance over time. IGF-I, which promotes β-cell survival and insulin secretion, decreased during disease progression, and this decline was blunted by verapamil. In addition, we found that verapamil reduces β-cell expression of IGF-binding protein 3 (IGFBP3), whereas IGFBP3 was increased in human islets exposed to T1D-associated cytokines and in diabetic NOD mouse islets. IGFBP3 binds IGF-I and blocks its downstream signaling, which has been associated with increased β-cell apoptosis and impaired glucose homeostasis. Consistent with the downregulation of IGFBP3, we have now discovered that verapamil increases β-cell IGF-I signaling and phosphorylation/activation of the IGF-I receptor (IGF1R). Moreover, we found that thioredoxin-interacting protein (TXNIP), a proapoptotic factor downregulated by verapamil, promotes IGFBP3 expression and inhibits the phosphorylation/activation of IGF1R. Thus, our results reveal IGF-I signaling as yet another previously unappreciated pathway affected by verapamil and TXNIP that may contribute to the beneficial verapamil effects in the context of T1D.

ARTICLE HIGHLIGHTS

- Verapamil prevents the decline of IGF-I in subjects with type 1 diabetes (T1D).
- Verapamil decreases the expression of β-cell IGF-binding protein 3 (IGFBP3), whereas IGFBP3 is increased in human and mouse islets under T1D conditions.
- Verapamil promotes β-cell IGF-I signaling by increasing phosphorylation of IGF-I receptor and its downstream effector AKT.
- Thioredoxin-interacting protein (TXNIP) increases IGFBP3 expression and inhibits the phosphorylation/activation of IGF1R in β-cells.
- Regulation of IGFBP3 and IGF-I signaling by verapamil and TXNIP may contribute to the beneficial verapamil effects in the context of T1D.

IGF-I has been shown to play an important role in β-cell biology and diabetes. As an effector hormone of growth hormone, IGF-I is primarily produced in liver, plays an important role in childhood growth, and has anabolic effects in adults (1–3). As an endocrine hormone, IGF-I acts on many tissues, including pancreatic islets (1–3). IGF-I binding to its specific and ubiquitously expressed IGF-I receptor (IGF1R) leads to phosphorylation and activation of the receptor tyrosine kinase and initiation of the downstream signaling cascade. This signaling most notably includes phosphorylation and activation of protein kinase B/AKT, promoting pathways involved in cell growth, differentiation, and glucose metabolism. The majority of circulating IGF-I is bound to IGF-I binding proteins (IGFBPs), which can prolong its half-life and maintain its stability (4). On the other hand, at the cellular level, the binding of IGFBPs to IGF-I prevents IGF-I from interacting with its receptors and thereby inhibits the activation of the IGF-I signaling cascade (4). In β-cells, IGF-I signaling has been reported to control apoptosis and insulin secretion (5–8).

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Received 1 April 2023 and accepted 23 July 2023
This article contains supplementary material online at https://doi.org/10.2337/figshare.23739180.

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In addition, IGFBPs have been shown to play an important role in β-cell survival and function (9–12). So far, six protein members (IGFBP1–IGFBP6) have been identified in the IGFBP family, of which IGFBP3 is the most abundant (4). Interestingly, IGFBP3 has been suggested to promote apoptosis of insulin-secreting cells (12), and IGFBP3-overexpressing transgenic mice exhibited impaired glucose homeostasis because of inhibited insulin secretion (10,13). In addition, IGFBP3 has been shown to be increased in subjects with diabetes (11). Moreover, a recent study reported that IGFBP3 inhibition preserved β-cells and delayed diabetes onset in the short term and promoted β-cell expansion in the long term (11). Mice with triple knockout of IGFBP3, 4, and 5 have also been found to have higher numbers of β-cells and increased insulin secretion (9).

IGF-I and its signaling have also been extensively studied in human type 1 diabetes (T1D). Circulating IGF-I levels were found to be significantly lower in individuals with T1D compared with age- and sex-matched healthy subjects (14,15). Furthermore, IGF-I levels were decreased in individuals with undetectable C-peptide compared with subjects with T1D who had retained C-peptide production, suggesting that serum IGF-I correlates with residual β-cell function (16). In fact, the reduction in serum IGF-I levels has been found to occur even prior to T1D diagnosis in subjects positive for autoantibodies and to progress with disease duration, suggesting that serum IGF-I might serve as a novel biomarker in T1D progression (17).

Verapamil, an approved antihypertension drug, has recently been found to also have antidiabetic effects. We initially demonstrated that verapamil protects against β-cell death and diabetes in different mouse models of diabetes (18), and our randomized controlled clinical trial of verapamil revealed that it promotes endogenous β-cell function and lowers insulin requirements in adults with recent-onset T1D (19). This preservation of β-cell function has now been further confirmed in a new, independent verapamil trial in children with T1D (20,21). Recently, we further found that verapamil has immunological effects and reverses T1D-associated elevation in circulating T-follicular helper cell markers and in the T1D autoantigen chromogranin A (CHGA) (22). In addition, verapamil promoted an immunomodulatory, antioxidative, and antiapoptotic gene expression profile and decreased detrimental thioredoxin-interacting protein (TXNIP) in human islets (22). Intriguingly, pharmacological or genetic inhibition of TXNIP has previously been shown to have antidiabetic effects in a variety of diabetes models (18,23–25), and we have demonstrated that verapamil effectively downregulates TXNIP in INS-I β-like rat cells and primary islets of mice and humans (18).

Interestingly, on the basis of our serum proteomics and islet transcriptomics data, we now have discovered that verapamil also modulates changes in circulating IGF-I levels occurring in subjects with T1D and in the expression of IGFBPs in human islets, suggesting that verapamil may regulate IGF-I signaling. The current study was therefore aimed at defining the effects of verapamil on IGFBPs and IGF-I signaling and exploring the potential novel link with TXNIP signaling. Using serum samples of individuals with T1D before and after 1 year of treatment with verapamil or placebo and primary human and mouse islets as well as INS-1 cells, we found that verapamil indeed preserves circulating IGF-I levels in the context of T1D, downregulates islet IGFBPs, and promotes IGF-I signaling.

RESEARCH DESIGN AND METHODS

Human Serum Samples

All human studies were approved by the University of Alabama at Birmingham institutional review board, and written informed consent was obtained from all subjects in accordance with the criteria set by the Declaration of Helsinki. Subjects with T1D had been diagnosed within 3 months and were positive for at least one T1D-associated autoantibody. All continued on their standard insulin regimen but were taking no other diabetes medication during the study. They were taking randomly assigned verapamil (360 mg sustained release daily) or placebo in a double-blinded fashion for 1 year. C-peptide area under the curve (AUC) at the end of the trial was assessed during a mixed-meal tolerance test as described previously (26,27). The characteristics of the study subjects who provided the proteomics serum samples are listed in Supplementary Table 1.

Animal Studies

All mouse studies were approved by the University of Alabama at Birmingham animal care and use committee. NOD mice (RRID:IMSR_JAX:001976) and NOD-resistant (NOR) mice (RRID:IMSR_JAX:002050) were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been used extensively as a mouse model of spontaneous autoimmune T1D. To follow the development of diabetes, blood glucose was measured using a glucometer (LifeScan, Malvern, PA), and mice were considered diabetic if their blood glucose was >250 mg/dL on 2 consecutive days. Txnip-deficient and wild-type control mice have been described previously (23,28–30). Mouse islets were isolated by collagenase digestion as described previously (31,32).

Human Islets and Tissue Culture

Human islets were obtained from the Integrated Islet Distribution Program (Supplementary Table 2) and allowed to recover overnight in RPMI medium (Thermo Fisher Scientific, Waltham, MA) with 5 mmol/L glucose, 10% FBS, 1% penicillin/streptomycin, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 10 mmol/L HEPES. On the following day, 250 islets were handpicked per sample and incubated in 5 mmol/L glucose RPMI medium with or without cytokine cocktail (50 units/mL human interleukin 1β [IL-1β], 1,000 units/mL human tumor necrosis factor-α [TNF-α], and 1,000 units/mL human interferon-γ [INF-γ]; R&D Systems, Minneapolis, MN) for 24 h. INS-1 cells were grown

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in RPMI medium with 11.1 mmol/L glucose, 10% FBS, 1% penicillin/streptomycin, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 0.05 mmol/L 2-mercaptoethanol (31,32). To test the effect of verapamil on gene expression, INS-1 cells were treated with or without 100 μmol/L verapamil (Sigma-Aldrich, St. Louis, MO) for 24 h. To test the effect of verapamil on the IGF-I signaling, INS-1 cells were treated with or without 100 μmol/L verapamil for 24 h, followed by cotreatment with 5 nmol/L recombinant rat IGF-I (R&D Systems) for 30 min. INS-LacZ and INS-TXNIP cells have been described previously (32).

Proteomics/Liquid Chromatography-Tandem Mass Spectrometry

Ten subjects, five treated with placebo and 5 with verapamil, had sufficient usable serum for liquid chromatography-tandem mass spectrometry (LC-MS/MS) at baseline and after 1 year of receiving verapamil or placebo; therefore, their samples were used for proteomics analysis. A total of 20 serum samples, collected for each subject at both time points, was analyzed using a standardized workflow as previously described (22). Sample processing, LC-MS/MS analysis, data processing, and statistical analyses were performed as described in detail previously (22). The only difference was that for the current study, the focus was on protein abundance changes over time regardless of the drug treatment. Subjects were modeled as random effects, and the full model was formulated as protein $\sim$ (1|subject) + time point. Significance was tested using a nested model approach. The test was two-tailed and performed using the lme4 package (33) in R (https://www.R-project.org).

ELISA

Serum IGF-I levels were assessed using the human IGF-I Quantikine ELISA Kit (R&D Systems). Serum CHGA levels were measured using the Human Chromogranin A ELISA Kit (Epitope Diagnostics, San Diego, CA).

RNA Sequencing

Human islets from three different donors were placed overnight at 5 mmol/L glucose, and then 250 islets were handpicked per sample and incubated in 25 mmol/L glucose RPMI medium with or without 100 μmol/L verapamil for 24 h prior to RNA extraction using an miRNeasy Mini Kit (QIAGEN, Germantown, MD). RNA sequencing was performed by Exiqon/QIAGEN as described previously (22) and included preparation of libraries using TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA) and single-end sequencing with >40 million reads obtained per sample. For data processing and analysis, RNA sequencing reads were aligned to the *Homo sapiens* reference genome (GRCh38.p7) using STAR (version 2.4.2a) with an average of ~90% reads uniquely mapped. Alignments were quantified using Salmon (version 0.8.2), and differential expression analysis was performed using DESeq2. The DESeq2 model accounted for the experimental design of paired treated and untreated samples from each individual. DESeq2 was used to determine the significance of the differential expression.

Quantitative Real-Time PCR

Total RNA was extracted using an miRNeasy Mini Kit. RNA was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 System (Roche) using SYBR Green (Thermo Fisher Scientific). All primers used are listed in Supplementary Table 3. All samples were corrected for the 18S ribosomal subunit run as an internal standard.

Western Blotting

Protein extracts were prepared using lysis buffer containing HEPES (50 mmol/L), Nonidet P-40 (10%), sodium fluoride (100 mmol/L), sodium pyrophosphate (10 mmol/L), EDTA (4 mmol/L), phenylmethanesulphonyl fluoride (1 mmol/L), leupeptin (2 μmol/L), activated sodium orthovanadate (2 mmol/L), and okadaic acid (100 nmol/L). Protein concentrations were measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Hudson, NH), and equal amounts of protein were loaded. Bands were visualized using ECL Plus (GE Healthcare) and quantified using ImageJ software (https://imagej.nih.gov/ij/index.html). The following antibodies were used: rabbit anti-IGFBP3 IgG (RRID:AB_2123233), rabbit anti-IGFBP5 IgG (RRID:AB_2554033) (Thermo Fisher Scientific); rabbit anti-phosphorylated IGF1R (pIGF1R) IgG (RRID:AB_10548764), rabbit anti-pAKT IgG (RRID:AB_2315049), rabbit anti-AKT IgG (RRID:AB_915783), mouse anti-β-actin IgG (RRID:AB_2242334), horse anti-mouse IgG (RRID:AB_330924), and goat anti-rabbit IgG (RRID:AB_2099233) (Cell Signaling Technology); rabbit anti-IGF1R IgG (RRID:AB_11011902) (Novus Biologicals); and mouse anti-TXNIP IgG (RRID:AB_592934) (MBL Life Science).

Statistical Analysis

Student *t* tests were used to calculate the significance of a difference between two groups. Data sets containing multiple groups were analyzed using one-way ANOVA, and the significance of a correlation was determined by Pearson product moment correlation. All tests were two-tailed, and $P < 0.05$ was considered significant.

Data and Resource Availability

The proteomics and RNA sequencing data analyzed during the current study are available in the ProteomeXchange repository (https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD026601) and the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181328), respectively.

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RESULTS

Verapamil Prevents Decline of Serum IGF-I in Subjects With T1D

To assess potential systemic changes in circulating factors over time, we performed a global proteomics analysis using LC-MS/MS of serum samples from subjects with T1D at baseline and after 1 year of receiving verapamil or placebo (ProteomeXchange data set PXD026601) (22). Statistical analysis using a linear regression model identified 59 proteins in which relative abundance over time was significantly altered ($P < 0.05$) (Supplementary Table 4). We next conducted a pathway analysis using Enrichr and found that IGF-I–related terms came up repeatedly (Supplementary Table 5). Indeed, we discovered that IGF-I was one of the top five differentially changed proteins after 1 year in the analysis of changes in protein abundance over time. In addition, two-tailed t test indicated that after 1 year, IGF-I (unlike most other proteins) was also significantly more abundant in the verapamil group compared with the placebo group (P = 0.016). Specifically, in subjects with T1D receiving placebo, IGF-I abundance decreased significantly during year 1 after diagnosis (Fig. 1*A*), consistent with the lower IGF-I levels reported previously in T1D (14,15). Of note, this decline was blunted in subjects receiving verapamil (Fig. 1*B*). To further confirm the LC-MS/MS results, we also measured serum levels of IGF-I by ELISA. The results were very much in alignment and again revealed significantly decreased IGF-I levels in the placebo group (Fig. 1*C*) but not in subjects receiving verapamil treatment (Fig. 1*D*). These results suggest that verapamil prevents the decline of IGF-I in subjects with T1D.

Serum IGF-I has been reported to correlate with residual β-cell function (16), and indeed, we found in our study that even 1 year after T1D diagnosis, serum IGF-I was positively correlated with stimulated C-peptide AUC, the gold standard for measuring remaining β-cell function (Fig. 1*E*). In addition, we recently identified serum CHGA as a biomarker that is increased in T1D and normalized in response to treatment with verapamil (22). Our analysis now reveals a highly significant inverse correlation between IGF-I and CHGA after 1 year of verapamil treatment, irrespective of whether serum levels were assessed by LC-MS/MS or ELISA (Fig. 1*F* and *G*). On the basis of this finding, we also calculated the IGF-I/CHGA ratio and discovered that it shows a very strong and highly significant correlation with the C-peptide AUC (R = 0.87, P = 0.001) (Fig. 1*H*), which was more robust than that with IGF-I alone.

Verapamil Decreases IGFBP Expression in Human Islets

To further explore the effects of verapamil on β-cell function and survival, we performed RNA sequencing of human

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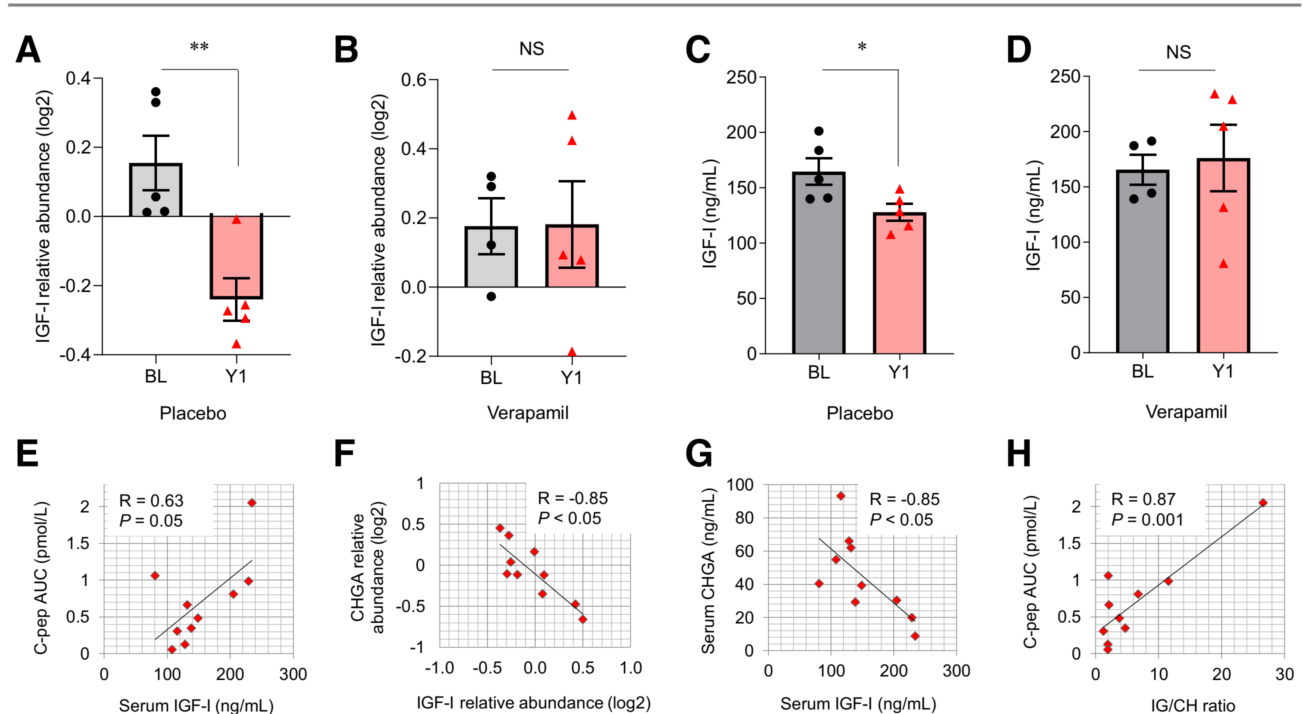

Figure 1—Verapamil prevents decline of serum IGF-I in subjects with T1D. *A* and *B*: IGF-I as assessed by LC-MS/MS (y-axis represents relative abundance levels in zero-centered $\log_2$ form) in serum at baseline (BL) or after 1 year (Y1) of subjects with T1D receiving placebo or verapamil. *C* and *D*: Serum IGF-I levels at BL or Y1 as assessed by ELISA in subjects with T1D receiving placebo or verapamil. *E*–*H*: Correlation of serum IGF-I and mixed-meal–stimulated C-peptide (C-pep) AUC (*E*) or CHGA assessed by LC-MS/MS (*F*) or ELISA (*G*) or of the IGF-I/CHGA ratio (IG/CH) and C-pep AUC (*H*) at Y1. Subject characteristics are described in Supplementary Table 1. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$.

islets from three different donors treated with or without verapamil, each serving as its own control. Of the genes identified to be differentially regulated, 907 were upregulated and 619 downregulated (Gene Expression Omnibus data set GSE181328) (22). Interestingly, we now discovered that several genes from the IGFBP family are among the top altered genes. As shown in Fig. 2*A*, IGFBP2, 3, 4, and 5 (four of the six members of the IGFBP family) were decreased in response to verapamil treatment, whereas expression of IGF1R remained unaltered. To confirm the result of the RNA sequencing, we performed qRT-PCR and found that the three most highly expressed IGFBPs (IGFBP3, 4, and 5) were significantly decreased by verapamil in human islets (Fig. 2*B*–*D*). These results suggest that verapamil decreases the expression of IGFBPs in human islets, which would be expected to enhance IGF-I interaction with its receptor and thereby promote IGF-I signaling (4).

IGFBP3 Expression Is Increased in Human Islets Exposed to T1D-Associated Inflammatory Cytokines and in Diabetic NOD Islets

To test the expression of IGFBP3 under T1D conditions, we treated human islets with T1D-associated cytokines (cocktail of IL-1β, TNF-α, and IFN-γ). We found that IGFBP3 was significantly induced by T1D-associated cytokines (Fig. 3*A*). In addition, we measured the expression of Igfbp3 in islets of diabetic NOD mice, a mouse model of autoimmune-induced T1D, and again found that Igfbp3 was significantly increased in islets of diabetic NOD mice compared with nondiabetic NOR control mice (Fig. 3*B*). These results suggest that islet expression of IGFBP3 is upregulated under T1D conditions.

Inflammatory Cytokine-Induced IGFBP3 Is Associated With Increased TXNIP and Decreased MAFA in Human Islets

Next, we assessed the expression of proapoptotic TXNIP in the same human islets exposed to T1D-associated inflammatory cytokines where we had observed the increase in IGFBP3. The results revealed a significant increase also in TXNIP (Fig. 3*C*). In addition, we measured changes in MAFA, a key factor in insulin transcription and β-cell function, and found that it was dramatically decreased in response to inflammatory cytokines (Fig. 3*D*). Intriguingly, these changes were strongly correlated with the elevation in IGFBP3 (Fig. 3*E* and *F*). Together, these results support the notion that this IGFBP3 signaling pathway may also modulate β-cell function and survival.

Verapamil Decreases Igfbp mRNA and Protein Expression in INS-1 Cells

We have found that verapamil prevents the decline of IGF-I in subjects with T1D and that verapamil decreases the expression of IGFBPs in human islets. IGFBPs bind to IGF-I and inhibit IGF-I signaling by preventing IGF-I from interacting with its receptor (4). Hence, we hypothesized that verapamil may increase IGF-I signaling in β-cells. To test our hypothesis, we first tested whether verapamil could also inhibit the expression of Igfbps in INS-1 β-like cells as it did in human islets. In fact, verapamil significantly downregulated the mRNA and protein expression of IGFBPs in INS-1 cells (Fig. 4), suggesting that this effect is not restricted to the mRNA level, one particular isoform, or primary human or mouse islets but also occurs in this rat cell line and that INS-1 cells, therefore, provide a viable model for further testing our hypothesis.

Verapamil Promotes IGF-I Signaling

To test the effects of verapamil on IGF-I signaling, we treated INS-1 cells with IGF-I and verapamil. As expected, we found that IGF-I significantly induced pIGF1R, while it had no effect on total IGF1R (Fig. 5*A*). Interestingly, verapamil treatment led to a significant further increase in IGF-I–induced pIGF1R (Fig. 5*A* and *B*). In addition, we found that verapamil also significantly increased IGF-I–induced pAKT (Fig. 5*A* and *C*), which is a downstream effector of IGF1R signaling. Moreover, verapamil decreased TXNIP protein levels (Fig. 5*A* and *D*) consistent with its

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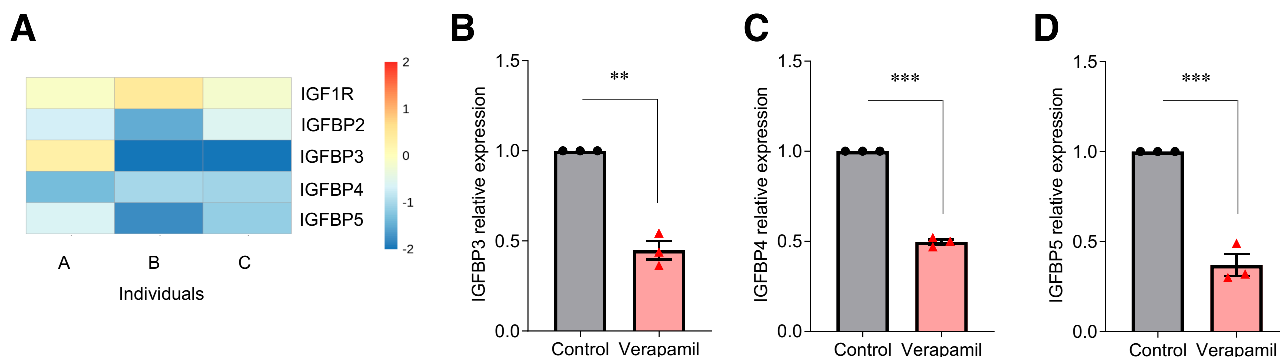

Figure 2—Verapamil decreases IGFBP expression in human islets. *A*: Heat map showing the change of IGFBPs in response to verapamil treatment (color scale represents $\log_2$ fold change). *B*–*D*: Expression levels of IGFBP3, IGFBP4, and IGFBP5 were assessed by qRT-PCR in human islets treated with or without 100 μmol/L verapamil for 24 h. Data are mean ± SEM (*n* = 3 human islet donors). $^{**}P < 0.01$, $^{***}P < 0.001$.

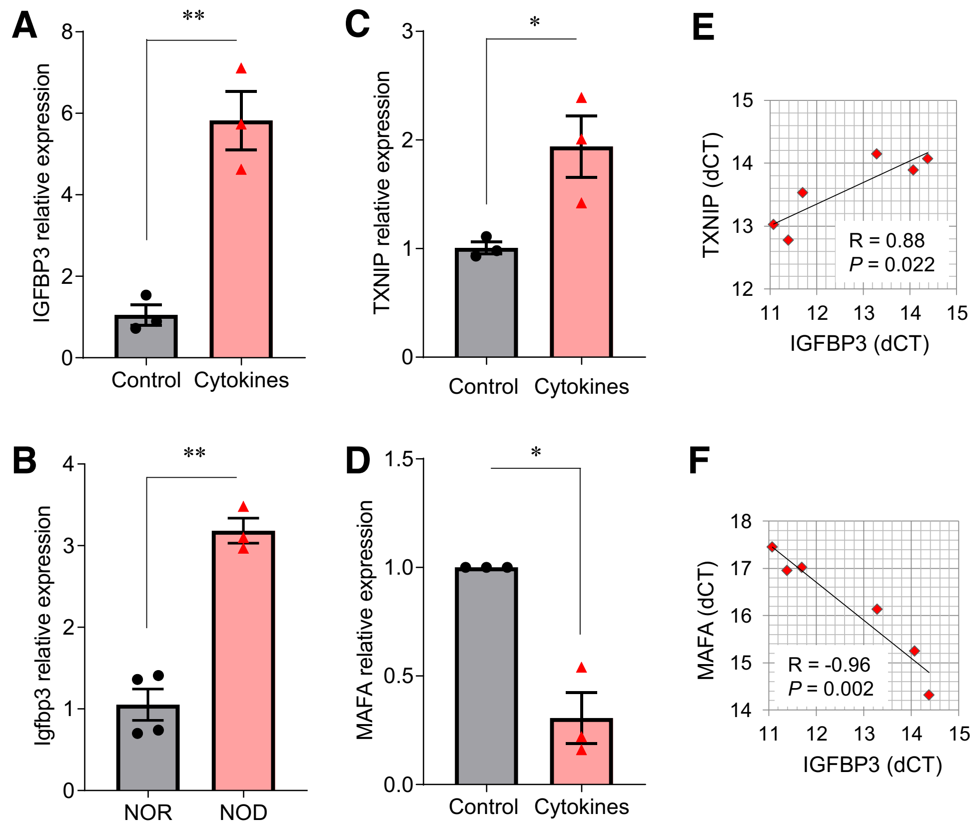

Figure 3—IGFBP3 expression is increased in human islets exposed to T1D-associated inflammatory cytokines and in diabetic NOD islets. *A*: IGFBP3 mRNA expression levels were assessed by qRT-PCR in human islets exposed to T1D-associated cytokine cocktail (50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ) or PBS control for 24 h. *B*: Igfbp3 mRNA expression levels were assessed by qRT-PCR in mouse islets of female diabetic NOD mice and female nondiabetic NOR control mice. *C* and *D*: TXNIP and MAFA expression as assessed by qRT-PCR in the same human islets exposed to inflammatory cytokines as shown in *A*. *E* and *F*: Correlation of IGFBP3 expression and TXNIP or MAFA. Data are mean ± SEM (*n* = 3 human islet donors; *n* = 3–4 mice per group). *$P < 0.05$, **$P < 0.01$. dCT, ΔΔCt.

role as a general TXNIP inhibitor. These results suggest that verapamil indeed increases β-cell IGF-I signaling.

TXNIP Regulates Igfbp3 Expression and IGF-I Signaling

Our previous studies have shown that TXNIP is increased in β-cells in the context of diabetes and promotes β-cell apoptosis (23,24) and dysfunction (32) and that verapamil inhibits β-cell expression of TXNIP, resulting in the beneficial antidiabetic effects of verapamil (18). We have now found that the TXNIP inhibitor verapamil also regulates β-cell IGFBPs and IGF-I signaling, suggesting that TXNIP may also be involved in this regulation. Indeed, we found that the expression of Igfbp3 was significantly increased in INS-1 cells overexpressing human TXNIP (Fig. 6*A*), whereas Igfbp3 was decreased in islets of Txnip-deficient mice compared with wild-type control mice (Fig. 6*B*). Moreover, pIGF1R was significantly decreased in the context of TXNIP overexpression (Fig. 6*C* and *D*). Taken together, these results suggest that TXNIP induces β-cell Igfbp3 expression and inhibits IGF1R phosphorylation/activation.

DISCUSSION

In summary, our results demonstrate for the first time that verapamil prevents the decline of circulating IGF-I in subjects with T1D and inhibits IGFBPs in human islets and β-cells, leading to increased β-cell IGF-I signaling. In addition, we discovered that the proapoptotic protein TXNIP is involved in regulating IGF-I signaling in β-cells.

IGF-I is a key hormone that plays an important role in childhood growth and has anabolic effects in adults (1–3). The serum level of IGF-I increases during childhood, reaching its highest value during puberty, followed by a gradual decline during adulthood (34,35). In subjects with T1D, serum IGF-I levels have been found to be significantly lower than in healthy subjects (14,15), and with disease duration, the reduction in serum IGF-I level progresses (17). Our present findings of decreased IGF-I levels at 1 year compared with at the time of T1D diagnosis in the placebo arm align with this observation. Interestingly, subjects receiving verapamil were able to maintain their IGF-I levels, and no significant decline was found. This likely reflects the simultaneous preservation of β-cell function observed in the verapamil group (19,22), as endogenous insulin secretion has also been shown to stimulate IGF-I production (36). In fact, we found

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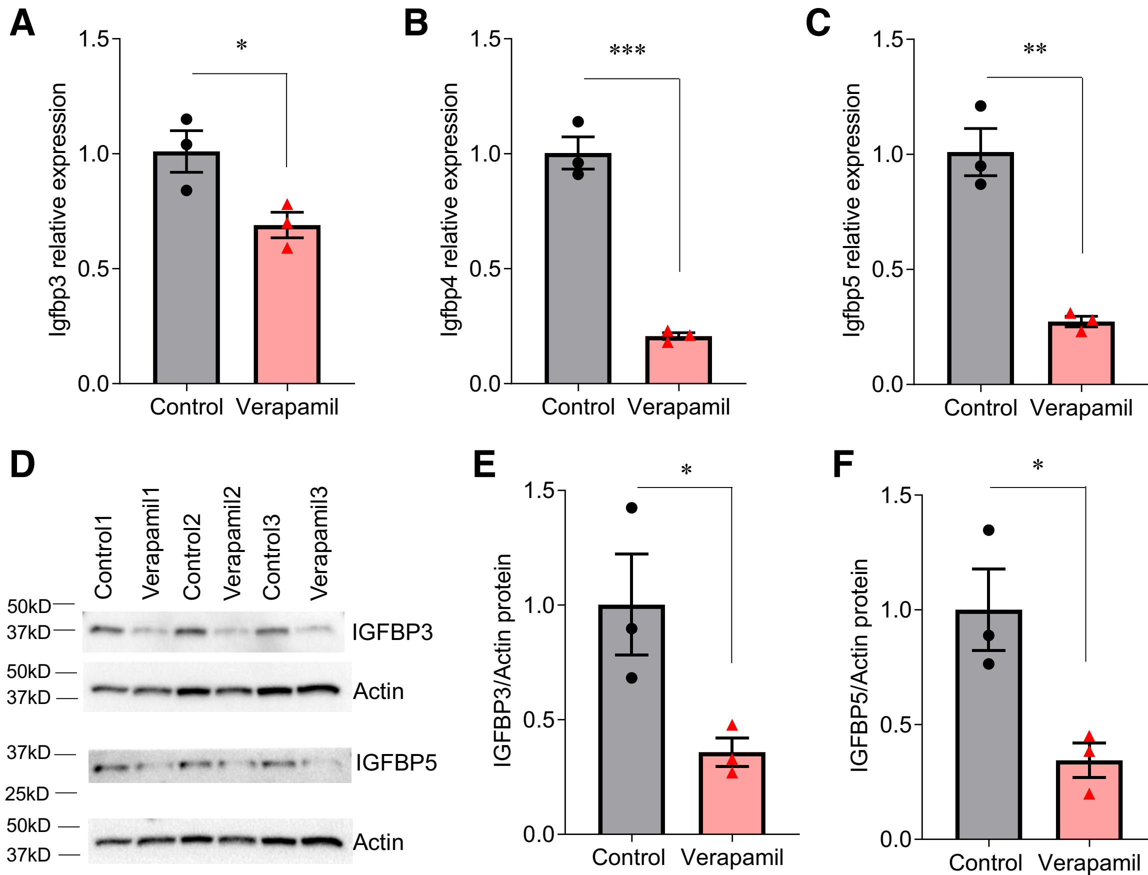

Figure 4—Verapamil decreases Igfbp mRNA and protein expression in INS-1 cells. *A–C*: Igfbp3, Igfbp4, and Igfbp5 mRNA expression levels were assessed by qRT-PCR in INS-1 cells incubated at 11.1 mmol/L glucose and treated with or without 100 μmol/L verapamil for 24 h. *D–F*: IGFBP3 and IGFBP5 protein levels were assessed by Western blotting in INS-1 cells treated with or without 100 μmol/L verapamil for 24 h and the relative change in IGFBP3 and IGFBP5 protein corrected for actin was quantified. Data are mean ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

that IGF-I, and especially the IGF-I/CHGA ratio, positively correlated with stimulated C-peptide AUC. This aligns with reports of serum IGF-I correlating with residual β-cell function (16) and serving as a biomarker of T1D progression (17). Of note, serum IGF-I and CHGA can be obtained from a single routine blood test, and our results therefore suggest that IGF-I, CHGA, and/or the IGF-I/CHGA ratio might become helpful markers to monitor T1D therapies targeting

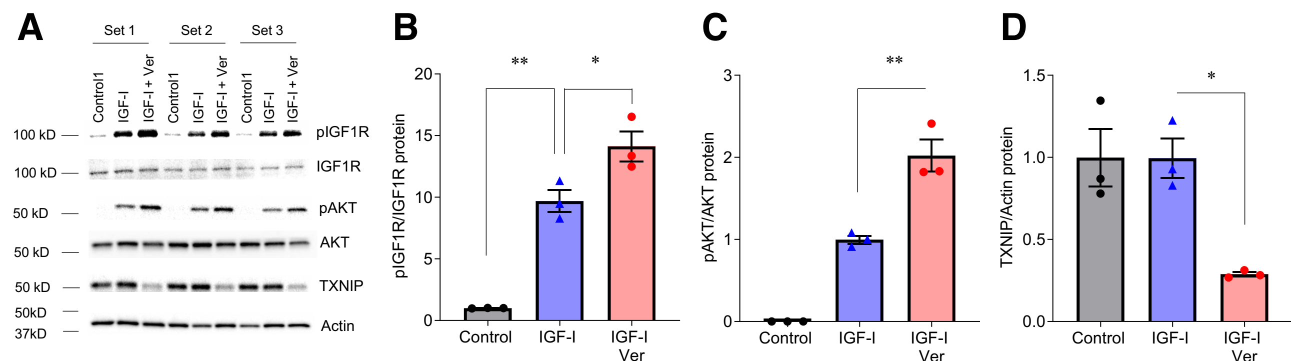

Figure 5—Verapamil promotes IGF-I signaling. *A*: INS-1 cells were treated with or without 100 μmol/L verapamil for 24 h followed by 30 min exposure to 5 nmol/L IGF-I, and pIGF1R, total IGF1R, pAKT, total AKT, TXIP, and actin protein levels were assessed by Western blotting. *B* and *C*: Effects on IGF1R phosphorylation/activation were assessed by quantifying band intensities of pIGF1R corrected for total IGF1R and of pAKT corrected for total AKT. *D*: Effects on TXNIP expression were assessed by quantifying band intensities of TXNIP normalized to actin. Data are mean ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$. Ver, verapamil.

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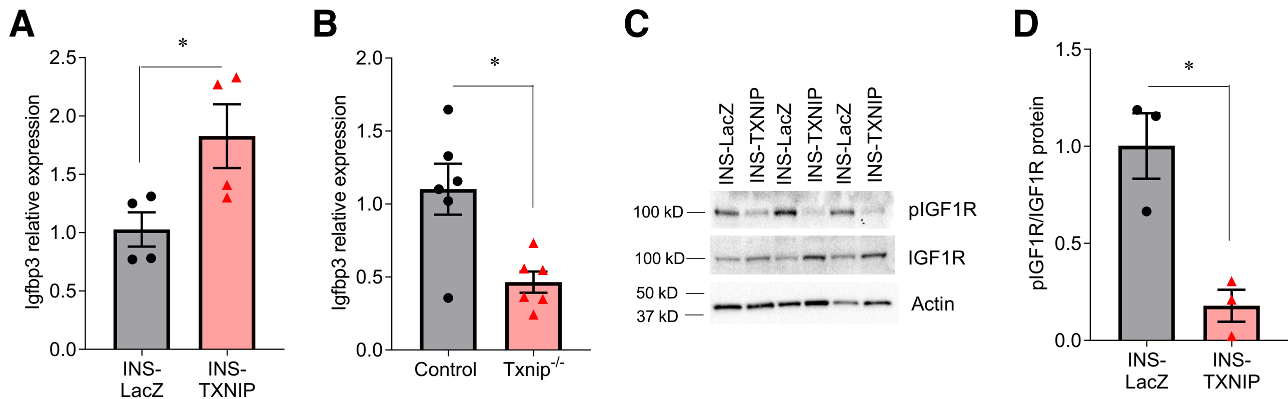

Figure 6—TXNIP regulates Igfbp3 expression and IGF-I signaling. *A* and *B*: Igfbp3 mRNA expression levels were assessed by qRT-PCR in INS-1 cells stably transfected to overexpress human TXNIP (INS-TXNIP) or LacZ control (INS-LacZ) and in islets of Txnip-deficient (Txnip$^{-/-}$) or control mice. *C*: pIGF1R and IGF1R protein levels were assessed by Western blotting in INS-TXNIP or INS-LacZ. *D*: Effects on IGF1R phosphorylation/activation were assessed by quantifying band intensities of pIGF1R corrected for total IGF1R. Data are mean ± SEM (*n* = 3–4 independent experiments and 6 mice/group). *$P < 0.05$.

β-cell preservation. In addition, IGF-I has been shown to promote β-cell mass (8), β-cell function (6,7), and insulin sensitivity (37,38). Hence, the verapamil-mediated increase of IGF-I may also contribute to the beneficial effects of verapamil observed in subjects with T1D (19).

Interestingly, we found that verapamil also significantly decreased IGFBP3 in human islets and INS-1 cells. Of note, RNA sequencing of β-cell–enriched human islet fractions confirmed that IGFBP3 is indeed expressed in β-cells (39,40). Also, we observed that IGFBP3 was increased in human islets exposed to proinflammatory cytokines as well as in islets of diabetic NOD mice. These findings are consistent with the negative effects attributed to elevated β-cell IGFBP3, as IGFBP3 has been shown to inhibit β-cell function and induce apoptosis (10,11,13). Since increased IGFBP binding of IGF-I prevents IGF-I from interacting with its receptor and thereby inhibits IGF-I signaling (4), we hypothesized that verapamil-induced downregulation of IGFBP3, the main IGFBP isoform, may increase IGF-I signaling. Indeed, we found that verapamil induced activation/phosphorylation of the IGF1R and of its main downstream signaling target AKT. However, since we have found that verapamil not only downregulates IGFBP3 but also modulates IGFBP4 and 5 expression, we cannot exclude the possibility that the downregulation of IGFBP4 and 5 might also contribute to the observed verapamil effects. Thus, verapamil seems to promote IGF-I signaling by upregulating its circulating levels, as well as by enhancing its ability to act at the cellular level (9).

IGFBP3 has been reported to modulate IGF-I signaling globally (in circulation) as well as locally (4), but the exact mechanisms, especially of the latter, are still not fully understood. Since IGFBP3 is thought to act by sequestering IGF-I and preventing it from binding to its receptor, it is tempting to speculate that at the cellular level, some paracrine/autocrine processes might be involved as described previously (41) and may mediate some of the verapamil effects observed. In addition, IGFBP3 is known to also have IGF-I–independent effects that include decreased insulin secretion and cell proliferation and increased apoptosis (4,13,42). As such, it is conceivable that IGFBP3 is also involved in some of the previously reported β-cell effects of TXNIP (24,43).

The β-cell–protective, antidiabetic effects of verapamil are thought to be mediated by downregulation of β-cell TXNIP (18,19,22), raising the question about whether TXNIP might also be involved in the verapamil-induced regulation of IGF-I signaling in the current studies. Indeed, TXNIP was downregulated by verapamil in the presence of IGF-I, and gain- and loss-of-function experiments revealed that TXNIP in turn regulates Igfbp3 expression. Specifically, TXNIP not only induced Igfbp3 expression but also dramatically inhibited IGF1R phosphorylation/activation. This represents the first time that TXNIP has been found to inhibit IGF-I signaling. Thus, our current studies have also uncovered a novel link between TXNIP and IGF-I signaling that might contribute to the detrimental effects of increased β-cell TXNIP, as well as to the beneficial effects of verapamil-mediated TXNIP inhibition. In addition, there appears to be a feedback mechanism, as a previous study has shown that IGF-I in turn negatively regulates TXNIP and identified TXNIP as a downstream target for IGF-I signaling (44). This is again consistent with the beneficial β-cell effects attributed to IGF-I signaling and the detrimental ones associated with increased TXNIP.

The idea of using repurposed verapamil for the treatment of T1D continues to garner support. On the basis of promising verapamil studies in diabetes mouse models (18), we conducted the first randomized, double-blind, placebo-controlled verapamil trial in adults with recent-onset T1D (19). The results revealed that verapamil promoted remaining β-cell function and reduced insulin requirements and hypoglycemic events for 1 year. Our more recent follow-up study

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demonstrated that these beneficial effects were sustained for at least 2 years after T1D diagnosis with continuous use of verapamil but were lost upon discontinuation, providing proof of the causality of the effects (22). This outcome is consistent with the protective effects on β-cell survival and function associated with genetic β-cell deletion of TXNIP in mouse models (23) and, as such, can be attributed to verapamil-induced TXNIP inhibition. However, given the known involvement of IGF-I signaling in β-cell function and survival (5–8), our current findings raise the possibility that verapamil-induced downregulation of IGFBP3 and promotion of IGF-I signaling may also play a role in these antidiabetic effects.

Surprisingly, we discovered that verapamil also had immunomodulatory effects, including normalization of T-follicular helper cell markers and downregulation of MHC class I and II molecules (22). Interestingly, IGFBP3 and IGF-I have also been suggested to play a role in β-cell autoimmunity in children (45). Most recently, the beneficial effects of verapamil on β-cell function have been independently confirmed in children with new-onset T1D in the Hybrid Closed-Loop Therapy and Verapamil for β-Cell Preservation in New-Onset Type 1 Diabetes (CLVer) trial (20). This further underscores the relevance and importance of understanding the underlying mechanisms and processes responsible for the verapamil effects in the context of T1D.

One limitation of the current study is the small number of samples from subjects with T1D. Therefore, the verapamil-mediated preservation of circulating IGF-I levels, as well as the usefulness of IGF-I or the IGF-I/CHGA ratio as biomarkers, will need to be confirmed in serum samples from a larger verapamil trial, such as the ongoing Verapamil SR in Adults With Type 1 Diabetes (Ver-A-T1D) (46) or the just published CLVer trial (20,21).

Funding. This work was supported by National Institutes of Health grants R01DK078752 (to A.S.), R01DK122160 (to W.-J.Q.), and University of Alabama at Birmingham Diabetes Research Center Pilot and Feasibility Award P30DK079626 (to G.X.). Human pancreatic islets were provided by the National Institute of Diabetes and Digestive and Kidney Diseases–funded Integrated Islet Distribution Program at City of Hope (2UC4DK098085).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. G.X. conceived the project; designed, performed, and analyzed the experiments; and wrote the manuscript. J.C. isolated the mouse islets and helped with the human islet processing. B.L. contributed to the visualization of the RNA sequencing analysis. P.S. was responsible for the RNA sequencing analysis. W.-J.Q. conducted the LC-MS/MS studies and analyses. A.S. initiated the project, supported the work and the analysis, and revised the manuscript. G.X. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 83rd Scientific Sessions of the American Diabetes Association, San Diego, CA, 23–26 June 2023.

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