Integrated Stress Response Signaling Pathways Induced by Supraphysiological Concentrations of Thyroid Hormone Inhibit Viral Replication

Mohammad Ishaq and Ven Natarajan

Laboratory of Molecular Cell Biology, Applied and Developmental Research Directorate, Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research, Frederick, MD, USA.

ABSTRACT: Supraphysiological concentrations (SPCs) of triiodo-l-thyronine (T3) have been used in the treatment of a number of nonviral diseases. However, the signaling mechanisms that regulate the function of T3 at these concentrations and their role in modulating cellular stress pathways and antiviral responses are unknown. Here, we have investigated the effects of SPCs of T3 on integrated stress response (ISR) signaling pathways and the replication of vesicular stomatitis virus (VSV). T3 amplified Poly IC-induced activation of RNA-dependent protein kinase, induced phosphorylation of eIF2α, stress granule (SG) formation, IRE1α phosphorylation, XBP1 splicing, and the expression of stress markers. T3 inhibited VSV replication by modulating SG formation and the expression of stress response markers. ISR activator guanabenz also inhibited VSV replication and amplified T3-induced anti-VSV response. To summarize, we have uncovered novel functions of T3 at SPCs as an activator of ISR signaling pathways and an inhibitor of VSV replication. This study offers a proof of principle of the concept that ISR activating agents like SPC of T3 and guanabenz can be potential antiviral agents.

KEYWORDS: thyroid hormone, integrated stress response, endoplasmic reticulum stress, vesicular stomatitis virus, guanabenz

Introduction

Following viral infection, cellular innate immune response is induced, which triggers the activation of interferon regulatory factors and NF-kB transcription factors and facilitates the production of antiviral type 1 interferons (IFNs) and pro-inflammatory cytokines. IFNs induce a wide range of gene products called interferon-stimulated genes (ISGs) that function as effectors of the type 1 IFN response. A number of studies have linked the transcriptional programs activated by innate immune responses with integrated stress response (ISR) that includes endoplasmic reticulum (ER) stress and unfolded protein response (UPR). In addition to the induction of IFNs, viral infections also trigger ER stress response. A best-studied stress-responsive signaling pathway is the UPR. The UPR induces transmembrane stress sensors, IRE1, ATF6, and PERK, that are localized in the ER. Activation of IRE1 induces an unconventional cytoplasmic splicing event that generates spliced form of XBP1 called XBP1S. The latter is an active transcription factor necessary to maintain homeostasis during the adaptation phase of the stress response. Activation of PERK induces the selective phosphorylation of a subunit of eukaryotic initiation factor 2 (eIF2α), which results in attenuation of protein translation, thereby decreasing the load on the ER proteostasis network. The translational stress is associated with cytoplasmic translocation of mRNAs that encode interferon-regulated proteins and consolidation of these proteins with transcripts to form RNA protein complexes that are termed stress granules (SGs). Phosphorylated eIF2α selectively promotes translation of the transcription factor ATF4, which targets stress-responsive genes, including CHOP, the transcription factor. CHOP induces transcription of the protein phosphatase 1 regulatory subunit GADD34 that binds to the catalytic subunit of protein phosphatase 1 and dephosphorylates eIF2α. This negative feedback loop turns off signaling and facilitates restoration of ribosomal translation following ER stress.

The double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is an ISG that is induced and activated during viral infection and plays a key role in type 1 IFN production. Like PERK, activated PKR also phosphorylates the subunit of translation initiation factor eIF2, resulting in translational shutdown and inhibition of viral replication. Thus, PKR plays a central role as an ISG that links IFN response pathways to ISR during viral infections.

Nuclear hormone receptors play important roles in the development and homeostasis of both adaptive and innate immune responses. A number of nuclear receptor ligands...
have been reported to have anti-inflammatory activities. An earlier report has indicated that thyroid hormone receptor (THR) agonist triiodo-L-thyronine (T3) at physiological concentrations can potentiate the inhibition of vesicular stomatitis virus (VSV) replication by IFN-γ; however, these authors did not observe any inhibition of VSV replication by T3 alone. Although supraphysiological concentrations (SPCs) of T3 have been used in the treatment of chronic fatigue syndrome, prophylaxis-resistant psychiatric illnesses, and obesity, the mechanism of T3 function at SPCs and their role in modulating cellular stress pathways and stress-mediated antiviral responses are unknown. In this paper, we report that T3 hormone at SPCs activates ISR pathways that include PKR activation, SG formation, and activation of ER-mediated stress pathways. We also show that SPCs of T3 inhibit VSV replication by modulating stress response and guanabenz (GBZ), an inhibitor of eIF2α dephosphorylation, induces SG formation and together with T3 induces strong antiviral response.

**Methods**

**Cells and reagents.** HeLa cells (ATCC) were maintained in DMEM medium containing 10% fetal bovine serum. Polynosinic–polycytidylic acid (Poly IC), 3′,5′-triiodo-L-thyronine sodium salt (T3), thapsigargin (TG), and GBZ acetate were purchased from Sigma-Aldrich. VSV-GFP was kindly provided by Dr. S. Balachandran (Fox Chase Cancer Center, Philadelphia, PA). Antibodies to T3 and VSV-G were obtained from Santa Cruz Biotechnology, Inc. Antibodies to total PKR, phospho-PKR, PERK, IRE1, GAPDH, TIAR, and GBZ were from Novus Biologicals. IRE1 inhibitor 4μC was obtained from EMD Millipore.

**Poly IC transfections.** HeLa cells were transfected with 5.0 μg Poly IC in 6-well plates using FuGENE HD (Promega Corporation) either in the presence or absence of DMSO or T3. Cells were harvested after indicated time for RNA isolation or protein extraction.

**Western blot analysis.** Protein extracts were electrophoresed in a 10% or 12% NuPAGE Bis Tris Gel using NuPAGE MES-SDS running buffer (Life Technologies) and transferred to a PVDF membrane using XCell Blot Module (Life Technologies). After treatment with primary antibodies, protein was detected using fluorophore–labeled secondary antibodies and the Odyssey Infrared Imaging System (LI-COR Biotechnology).

**Fluorescence microscopy.** Cells were grown in chamber slides, transfected with 2.5 μg Poly IC using FuGENE HD, and incubated for 24 hours. Cells were fixed with 4% formaldehyde for 30 minutes, quenched with 0.25 M glycine for 45 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were blocked with 5% goat serum/0.2% Triton X-100, treated with primary antibodies for overnight at 4°C or 1–2 hours at room temperature, and labeled with Alexa Fluor-conjugated (Life Technologies) secondary antibodies (1:2,000). Cells were mounted in ProLong Gold Antifade Reagent with DAPI (Life Technologies). Immunofluorescence images were obtained using an Axioimager D1 Zeiss microscope. The densitometric mean intensity of nuclear p-eIF2α was quantified using AxioVision software from Zeiss Imager D1 fluorescent microscope.

**Real-time RT-PCR.** Total RNA was isolated using Qiagen RNeasy kit by following manufacturer’s protocol. For real-time RT-PCR, total RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Life Technologies) in the presence of 2.5 μM random hexamers. The products of reverse transcription were subject to real-time PCR with gene–specific primers using 7500 Fast Real-Time PCR System and Fast SYBR Green Master Mix from Applied Biosystems. The comparative threshold cycle method was used to calculate the relative gene expression. The data were normalized to GAPDH mRNA levels.

**VSV infection.** HeLa cells (2 × 10^4) in duplicate were seeded and grown in chamber slides for 16 hours, pretreated with T3, GBZ, or DMSO for 4 hours, infected with one TCID<sub>50</sub> of VSV-GFP for 4 hours either in the presence or absence of T3, DMSO, or GBZ as indicated. The cells were washed with PBS and incubated in the medium containing T3, GBZ, or DMSO for 16 hours. Cells were fixed in 4% formaldehyde for 20 minutes at 37°C, washed with PBS, and quenched with 0.25 M glycine for 40 minutes at 4°C, and GFP-positive cells were studied under a fluorescent microscope. For quantitation of VSV replication, cells were seeded in 12-well plates, pretreated with T3, GBZ, or DMSO for four hours, infected with one TCID<sub>50</sub> of VSV-GFP for four hours. The cells were washed with PBS and incubated in the medium containing T3, GBZ, or DMSO for 16 hours. For the quantitation of secreted VSV, the supernatants were spiked with Rous sarcoma virus (RSV) for normalization. RNA was isolated from the supernatants using Qiagen RNeasy kit following manufacturer’s protocol. For the quantitation of VSV and RSV RNAs, samples were reverse transcribed using random primers and the products of reverse transcription were subject to real-time PCR with VSV-G, VSV-M, and RSV-specific primers using 7500 Fast Real-Time PCR System and Fast SYBR Green Master Mix from Applied Biosystems. The comparative threshold cycle method was used to calculate the relative gene expression. To quantify the endogenous viral proteins, cell pellets from these experiments were used for the isolation of proteins. The endogenous expression of VSV-G protein was studied by Western blot analysis using VSV-G–specific antibodies.

**Cell proliferation assay.** HeLa cells (2 × 10^4) were seeded in 96-well plates in 100 μL medium for 24 hours and treated with either DMSO or T3 for 24 hours. Cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–based CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega Corporation), as per the manufacturer’s instructions.
Statistical analysis. The results shown are representative of three independent experiments each carried out in duplicate and expressed as mean ± SD. Statistical analysis was performed using Student’s t-test. A value of \( P < 0.05 \) was considered significant. Statistical significance are indicated by *, **, and *** for \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \), respectively. NS, not significant.

Results

T3 at SPCs amplifies Poly IC-mediated expression and activation of PKR. We studied the effect of T3 hormone on dsRNA mimic Poly IC-induced expression of PKR in HeLa cells. HeLa cells were either mock transfected or transfected with Poly IC in the presence of DMSO or different concentrations of T3 ranging from 1 to 75 μM. We found that T3 enhanced the Poly IC-induced PKR expression. T3 effect on Poly IC-induced PKR expression was dose dependent, and at SPCs (25–75 μM), T3 significantly amplified Poly IC-mediated PKR expression (Fig. 1A). These results indicate that T3 at SPCs has an amplifying effect on PKR expression induced by dsRNA viral mimic Poly IC. The effect of SPCs of T3 on the viability of HeLa cells was tested using the MTT assay. As shown in Figure 1B, T3 did not have any cytotoxicity effects on the viability of these cells when treated with various concentrations for 24 hours.

To investigate if T3 induced PKR activation (phosphorylation), HeLa cells were transfected with Poly IC and treated with 75 μM T3 or DMSO. Cells were harvested at different time points (two and four hours) at which Poly IC alone had only minor effect on PKR phosphorylation. Western blot analysis showed that T3 not only amplified the Poly IC-induced expression of total PKR but also Poly IC-induced phosphorylation (activation) of PKR (Fig. 1C and D). We also studied the effect of T3 alone on the levels of phospho- and total PKR. Cells were treated with 75 μM T3 for different time periods (8, 16, and 24 hours); Western blot analysis revealed that although T3 alone did not induce phosphorylation of PKR, it significantly induced the total PKR (Fig. 1E and F). Together, these results show that T3 at SPCs amplifies Poly IC-mediated expression and activation (phosphorylation) of PKR. In addition, T3 alone induces PKR expression but not PKR phosphorylation.

T3 induces eIF2α phosphorylation, SG formation, and activation of ER stress pathways. Activation of PKR is known to phosphorylate eIF2α, resulting in the translational shutdown and activation of stress pathways.\(^2,22,23,36\) To explore whether T3 affects the phosphorylation of eIF2α, HeLa cells were treated with DMSO or T3 or transfected with Poly IC in the presence or absence of T3 and analyzed by immunofluorescence (IF) using antibodies to phospho-eIF2α. T3 alone, like Poly IC, induced the phosphorylation of eIF2α and its translocation to nuclear compartment (Fig. 2A). A combination of T3 and Poly IC increased the intensity of nuclear phospho-eIF2α at basal as well as T3-mediated XBP1 splicing, whereas it had no effect on the expression of the unspliced XBP1. These results confirm that T3-induced splicing of XBP1 is mediated by IRE1 pathway.
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Figure 1. T3 hormone at SPCs amplifies Poly IC-mediated expression and activation of PKR. (A) HeLa cells were transfected with 5 μg Poly IC and treated with DMSO or various concentrations of T3 for 16 hours. RNA was isolated from the cell pellets, and the expression of PKR and GAPDH mRNAs was monitored by real-time PCR. Statistical significance between DMSO treated mock and Poly IC transfected cells is shown. Also shown is the statistical significance between DMSO- and T3-treated Poly IC transfected cells. (B) HeLa cells (2 × 10^4) were seeded in 96-well plates in 100 μl medium for 24 hours. Medium was removed and the cells were either treated with DMSO or with indicated concentrations of T3 for 24 hours. Cell proliferation was estimated using CellTiter 96® Non-Radioactive Cell Proliferation Assay kit from Promega Corporation. Statistical significance between DMSO treated and T3 treated cells is shown. NS, not significant. (C) HeLa cells were mock transfected or transfected with 5 μg of Poly IC and treated with either T3 or DMSO. Cells were harvested at indicated time periods for protein extraction. Western blot analysis was performed using antibodies to total PKR, phospho-PKR, and GAPDH. (D) Quantitation of band intensities of GAPDH-normalized p-PKR and total PKR from Figure 1C. (E) HeLa cells were treated with DMSO or 75 μM T3. Cells were harvested at indicated time periods for protein extraction. Western blot analysis was performed using antibodies to total PKR, phospho-PKR, and GAPDH. (F) Quantitation of band intensities of GAPDH-normalized total PKR from Figure 1E.
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T3 inhibits the replication of VSV and amplifies host response to VSV-mediated stress. In this study, we have shown that T3 induces activation of PKR, eIF2α phosphorylation, SG formation, and induction of ER stress pathways. Because all these T3-induced pathways are also the hallmarks of a strong host-mediated antiviral response,12,18,22,23,41 we tested the ability of T3 to inhibit the replication of an RNA virus, VSV, in HeLa cells. For VSV infection, cells were pretreated with DMSO or different concentrations of T3 and infected with VSV-GFP in the presence of DMSO or T3, and viral replication was analyzed by fluorescent microscopy, RT-PCR, and Western blotting. Fluorescent microscopy revealed that T3 inhibited viral replication dose dependently at concentrations between 25 and 75 μM, whereas lower concentration (1–10 μM) had no significant effect on the replication (Fig. 4A and B). RT-PCR of VSV-G and -M mRNAs (Fig. 4C) and Western blot analysis using antibodies to VSV-G protein (Fig. 4D) also showed that T3 inhibited the VSV replication dose dependently between 25 and 75 μM, confirming the IF data.

The ability of host to restrict viral replication by the induction of SG formation is known.18,41 VSV was also recently
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To explore the underlying mechanisms of antiviral effects of T3, we studied the role of T3-induced SG formation in the inhibition of VSV replication. HeLa cells were pretreated with T3 (75 μM) or DMSO for four hours, and then infected with VSV-GFP in the presence of T3 or DMSO. Cells were then stained with anti-TIAR antibodies and SG formation studied using fluorescent microscopy. The results (Fig. 5A and B, SGs marked by arrows) showed that SG-positive cells did not exhibit strong viral replication and supported only low levels of replication. Cells that did not form SGs and were not GFP positive may represent the cells that were not infected with the virus. Although a significant number of cells containing SGs were observed in DMSO-treated cells, the number of SG-positive cells in T3-treated cells was markedly higher (Fig. 5C), implicating the ability of T3 to induce SG formation as the possible mechanism of its inhibition of VSV replication.

In this study, we have shown that T3 induces ER stress markers XBP1, CHOP, and GADD34. To explore if VSV infection induces ER stress response and if T3 amplifies this response, we pretreated HeLa cells with T3 or DMSO followed by infection with VSV-GFP in the presence of T3 or DMSO. RT-PCR for XBP1, CHOP, and GADD34 mRNAs

Table 1. Induction of ER stress response genes by T3.

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<th>GENE SYMBOL</th>
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Notes: HeLa cells were treated with DMSO or 75.0 μM T3 for 16 hours and the expression of a panel of ER stress response-specific genes was analyzed by real-time RT-PCR. The results of 15 genes that were induced by 1.5-fold or more following treatment with T3 are shown.

Figure 3. T3 induces phosphorylation of ER stress sensor IRE1α. (A) HeLa cells were transfected with 2.5 μg IRE1α plasmid and after 24 hours treated with DMSO, T3 (75 μM), TG (5 μM), or a combination of T3 and TG for 6 hours. Western blot analysis was performed with protein extracts using antibodies to total and phospho-IRE1α. Antibody to total PERK was used for loading control. (B) Quantitation of the band intensity ratios of normalized p-IRE1α and total IRE1α from (A). For comparison, DMSO-treated cells were assigned a ratio of 1. (C) HeLa cells were treated for 16 hours with DMSO or T3 (75 μM) in the presence or absence of 5.0 μM 4μ8C. RNA was isolated from the cell pellets. Real-time PCR was used to quantitate the expression of XBP1U, XBP1S, and GAPDH mRNAs. Statistical significance between DMSO and various treatments is shown.
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indicated an induction of low-level expression of these ER stress markers by infection with VSV-GFP (Fig. 5D). However, treatment of VSV-infected cells with T3 markedly induced the expression of these markers, indicating that T3 amplifies host response to VSV infection by synergistically enhancing UPR response.

GBZ induces SG formation, inhibits VSV replication, and enhances T3-mediated stress response and antiviral activity. GBZ was recently identified as an inhibitor of stress-induced dephosphorylation of eIF2α by its ability to inhibit the activity of eIF2α phosphatase GADD34, resulting in the accumulation of phospho-eIF2α. Our results demonstrated that both T3 and GBZ induce eIF2α phosphorylation (Fig. 2A and 6A and B). In addition, additive effects on the levels of eIF2α phosphorylation were observed when HeLa cells were treated with a combination of T3 and GBZ (Fig. 6A and B). We next explored the effect of GBZ on the levels of stress markers XBP1, CHOP, and GADD34. The results (Fig. 6C) show that GBZ induced the expression of these genes, indicating that GBZ activates stress response pathways in these cells. Next, the effect of T3 on GBZ-induced expression of these markers was explored. The results revealed that a combination of T3 and GBZ had an additive effect on the induction of XBP1, CHOP, and GADD34 expression (Fig. 6C). Taken together, these results indicate that eIF2α phosphatase inhibitor GBZ induces ER-mediated stress response and a combination of GBZ and T3 amplifies this response.

We next studied the effect of GBZ and a combination of GBZ and T3 on the VSV replication. HeLa cells were pretreated with 10 or 50 μM of GBZ or a combination of 50 μM GBZ and 75 μM T3. Cells were then infected with VSV-GFP in the presence of GBZ or T3 or both. Fluorescent microscopy (Fig. 6D and E), RT-PCR for VSV-G

Figure 4. T3 inhibits the replication of VSV. (A–C) HeLa cells were pretreated with DMSO or indicated concentrations of T3 for four hours and infected with one TCID50 of VSV-GFP for four hours in the presence of DMSO or T3. The cells were washed with PBS and incubated in the medium containing DMSO or T3 for additional 16 hours. Viral replication was assayed by fluorescent microscopy (A) and the quantitation of GFP-positive cells (B) (statistical significance between the number of GFP-positive cells in DMSO and T3 treated VSV-GFP infected cells is shown), quantitative RT-PCR using VSV-G and VSV-M primers (C) (statistical significance between DMSO and T3 treated VSV-GFP infected cells are shown), and by Western blot analysis for VSV-G and cellular GAPDH proteins (D) (the numbers represent quantitation of the VSV-G band intensity).
and -M mRNAs (Fig. 6F), and Western blot analysis using anti-VSV-G antibodies (Fig. 6G) showed that GBZ inhibited VSV replication. Although a significant inhibition of viral replication was seen at 10 μM GBZ, more than 90% inhibition of viral replication was observed at 50 μM GBZ. In addition, a combination of 75 μM T3 and 50 μM GBZ had a robust additive effect on the inhibition of VSV replication. The additive effect of T3 and GBZ was further evaluated using lower concentrations of T3 during infections. As shown in Figure 4, lower concentrations of T3 (1–10 μM) alone did not inhibit VSV replication. However, when combined with GBZ, lower concentration of T3 had a dramatic effect on
Figure 6. GBZ inhibits VSV replication and amplifies T3-mediated stress response and antiviral activity. (A) HeLa cells were treated with T3 (75 μM) or GBZ (50 μM) or a combination of T3 and GBZ for 16 hours. Cell extracts were analyzed by Western blotting using total- and phospho-eIF2α antibodies. (B) Quantitation of the band intensity ratios of p-eIF2α and total eIF2α from (A). (C) HeLa cells were treated with DMSO or 75 μM T3 either alone or in combination with 50 μM GBZ for 16 hours. RNA was isolated from the cell pellets, and the expression of DDIT3/CHOP, GADD34, XBP1S, and GAPDH mRNAs was quantitated by real-time PCR. Statistical significance between different treatments is shown by connecting horizontal bars. (D–G) HeLa cells were pretreated for four hours with 10 and 50 μM GBZ or a combination of 50 μM GBZ and T3 (75 μM). Cells were then infected with one TCID50 of VSV-GFP for four hours in the presence of GBZ or T3. The cells were washed with PBS and incubated in the medium containing T3, GBZ, a combination of T3 and GBZ or DMSO for 16 hours. Viral replication was assayed by fluorescent microscopy (D) and GFP-positive cells were quantitated (E) (statistical significance between the number of GFP-positive cells in mock treated and GBZ-treated VSV-GFP-infected cells is shown), quantitation of VSV-G and VSV-M mRNAs by RT-PCR (F) (statistical significance between DMSO- and T3-treated VSV-GFP-infected cells are shown), and by Western blot analysis of VSV-G and cellular GAPDH proteins (G) (the numbers represent quantitation of the VSV-G intensity).
viral replication (Fig. 7A). To assess the effect of GBZ and a combination of GBZ and T3 on VSV-induced SG formation and host stress response, HeLa cells were infected as above in the presence and absence of GBZ or a combination of GBZ and T3. SG formation was studied using fluorescence microscopy, and the levels of XBP1, CHOP, and GADD34 mRNA levels were estimated by RT-PCR. These data indicate that like T3, GBZ also amplified VSV-induced SG formation.

**Figure 7. GBZ-mediated stress response and SG formation.** (A) HeLa cells were pretreated with 10 μM of GBZ in the presence of DMSO or indicated concentrations of T3 for 4 h. Cells were then infected with one TCID50 of VSV-GFP for 4 h in the presence of 10 μM GBZ and DMSO or 10 μM GBZ and indicated concentrations of T3 as above. Viral replication was assayed by RT-PCR for VSV-G mRNA (statistical significance between the DMSO treated and GBZ+DMSO, and GBZ+T3 infected cells is shown. (B) HeLa cells were pretreated for 4 h with DMSO, GBZ (50 μM), or combination of GBZ and 75 μM T3 followed by infection with one TCID50 of VSV-GFP for 4 h in the presence of DMSO, GBZ, or combination of GBZ and T3 as above. The cells were stained with antibodies to TIAR and Alexa Fluor-conjugated secondary antibodies and analyzed by fluorescent microscopy. Arrows or arrowheads indicate the presence of Sgs. (C) Enlarged view of TIAR staining with GBZ+T3-treated VSV-GFP infected cells (arrows indicate the presence of Sgs). (D) Number of SG positive cells from each treatment were counted and plotted. Statistical significance between DMSO and GBZ+DMSO and between GBZ+DMSO and GBZ+T3 treated VSV-GFP infected cells is shown. (E) HeLa cells were pretreated for 4 h with DMSO, GBZ (50 μM), T3 (75 μM) or combination of GBZ and T3 followed by infection with one TCID50 of VSV-GFP for 4 h in the presence of DMSO, GBZ, T3 or combination of GBZ and T3 as above. rna was isolated from the cell pellets and the expression of DDIT3/CHOP, XBP1S, GADD34, and GAPDH mRNA quantitated by real-time PCR. Statistical significance between different treatments is shown by connecting horizontal bars.
The ability of T3 to inhibit the replication of VSV was confirmed by IFN-γ-mediated inhibition of VSV replication by IFN-γ. However, they did not observe any inhibition of VSV replication by T3 alone. Therefore, the effect of higher concentrations of T3 on VSV replication was studied. It was observed that T3 augmented VSV-induced SG formation, indicating that inhibition of eIF2α phosphorylation by T3 is sufficient to stall the VSV replication. Although the activation of ISR pathways by SPCs of T3 seems to be the hallmark of T3-mediated antiviral response, the mechanism of T3 action at concentrations that are nontoxic to the cells can effectively inhibit VSV replication.

We provide evidence that T3 at SPCs induces the activation of PKR and ER-mediated UPR pathways. T3 modulates gene expression either by genomic mechanisms involving direct binding to THR on the promoters of target genes or via nongenomic mechanisms that may or may not involve the binding of T3 to THR and is mostly nonnuclear. Analysis of the promoters of PKR- and ER-mediated stress genes modulated by T3 did not find the presence of classical THR binding sequences, indicating that genomic mechanisms may not be involved in their induction by T3. In addition, induction of these pathways at nonphysiological concentrations of T3 and the expression of nonfunctional THR in HeLa cells provide additional evidence that T3 modulates gene expression by nongenomic mechanisms that do not involve the binding of T3 to THR.

The effect of T3 on the activation of PKR, a central player that links innate immune response with stress response pathways, is as striking as its ability to induce the expression of ER-response genes, phosphorylation of IRE1α, and induction of XBPI splicing. These effects are consistent with T3-induced ISR pathways that lead to PKR activation, eIF2α phosphorylation, SG formation, and activation of ER stress pathways. It is important to note that although T3 alone induced PKR protein expression, phosphorylation of PKR by T3 required the presence of Poly IC, indicating that binding of dsRNA is important for the autophosphorylation of PKR. However, T3 was able to induce phosphorylation of eIF2α without Poly IC, indicating that PKR protein induction by T3 is sufficient to induce phosphorylation of eIF2α. Presence of Poly IC, however, amplified T3-mediated eIF2α phosphorylation. Our studies do not rule out the role of other ER-resident proteins PERK and ATF6 in T3-mediated responses. Significant induction of CHOP and GADD34 expression by T3 seen in this study may also be due to the activation of PERK and ATF6 in addition to the activation of PKR-eIF2α and IRE1-XBP1S pathways.

PKR activation, SG formation, and activation of ER stress markers are all the hallmarks of host-mediated antiviral responses. Previous studies have shown that a number of viruses induce SG formation in host cells, VSV, a negative-sense single-stranded RNA virus, was recently shown to induce SGs. The ability of T3 to inhibit the replication of VSV in our study confirmed the antiviral and functional stress-mediated responses of T3. HeLa cells that exhibited SG formation were unable to sustain the replication of VSV. We provide evidence that T3 at SPCs activates ISR pathways that are hallmarks of host antiviral response.

Discussion

Although SPCs of T3 have been used in the treatment of chronic fatigue syndrome, prophylaxis-resistant psychiatric illnesses, and obesity, the mechanism of T3 action at such concentrations is not known. Here, we focused our study on the role of SPCs of T3 in modulating cellular stress signaling pathways and stress-mediated antiviral responses. We have shown that T3 at SPCs activates ISR pathways that are hallmarks of host antiviral response.

A number of studies have linked the transcriptional programs activated by innate immune responses to ER stress and UPR responses. PKR plays a central role as an ISG and coordinates a number of cellular stress and inflammatory responses including ER stress response by modulating a number of cellular kinases and phosphatases. We provide evidence that T3 at SPCs induces the activation of PKR and ER-mediated UPR pathways.

T3 modulates gene expression either by genomic mechanisms involving direct binding to THR on the promoters of target genes or via nongenomic mechanisms that may or may not involve the binding of T3 to THR and is mostly nonnuclear. Analysis of the promoters of PKR- and ER-mediated stress genes modulated by T3 did not find the presence of classical THR binding sequences, indicating that genomic mechanisms may not be involved in their induction by T3. In addition, induction of these pathways at nonphysiological concentrations of T3 and the expression of nonfunctional THR in HeLa cells provide additional evidence that T3 modulates gene expression by nongenomic mechanisms that do not involve the binding of T3 to THR. The nongenomic effects of T3 have been defined in a number of previous reports.

The effect of T3 on the activation of PKR, a central player that links innate immune response with stress response pathways, is as striking as its ability to induce the expression of ER-response genes, phosphorylation of IRE1α, and induction of XBPI splicing. These effects are consistent with T3-induced ISR pathways that lead to PKR activation, eIF2α phosphorylation, SG formation, and activation of ER stress pathways. It is important to note that although T3 alone induced PKR protein expression, phosphorylation of PKR by T3 required the presence of Poly IC, indicating that binding of dsRNA is important for the autophosphorylation of PKR. However, T3 was able to induce phosphorylation of eIF2α without Poly IC, indicating that PKR protein induction by T3 is sufficient to induce phosphorylation of eIF2α. Presence of Poly IC, however, amplified T3-mediated eIF2α phosphorylation. Our studies do not rule out the role of other ER-resident proteins PERK and ATF6 in T3-mediated responses. Significant induction of CHOP and GADD34 expression by T3 seen in this study may also be due to the activation of PERK and ATF6 in addition to the activation of PKR-eIF2α and IRE1-XBP1S pathways.

PKR activation, SG formation, and activation of ER stress markers are all the hallmarks of host-mediated antiviral responses. Previous studies have shown that a number of viruses induce SG formation in host cells, VSV, a negative-sense single-stranded RNA virus, was recently shown to induce SGs. The ability of T3 to inhibit the replication of VSV in our study confirmed the antiviral and functional stress-mediated responses of T3. HeLa cells that exhibited SG formation were unable to sustain the replication of VSV. We provide evidence that T3 at nonphysiological concentrations that are nontoxic to the cells can effectively inhibit VSV replication.

We have shown that GBZ, an inhibitor of eIF2α phosphatase GADD34, amplified VSV-induced SG formation, inhibited VSV replication, and amplified T3-mediated stress response and antiviral activity. An earlier study has shown that GADD34-knockout MEF cells support enhanced VSV replication as compared to wild-type cells, implicating the role of GADD34 in restricting viral replication. Our results are consistent with this observation because T3 amplifies the expression of GADD34 following infection and markedly inhibits viral replication. GBZ-mediated inhibition of VSV replication was associated with a significant induction of ER stress response genes CHOP, GADD34, and XBPI and the formation of SGs, indicating that inhibition of eIF2α dephosphorylation and subsequent shut down of host protein translation is sufficient to stall the VSV replication. Although the activation of ISR pathways by SPCs of T3 seems to be the hallmark of T3-mediated antiviral response in this study, the modulation of other antiviral pathways by T3 cannot be ruled out and are currently being actively investigated.

GBZ, marketed as a clinically licensed antihypertensive drug (Wytensin), has also recently been found to restore the glucose and lipid homeostasis in mice developing symptoms of metabolic syndrome. Other studies have indicated the potential role of GBZ in neurodegenerative diseases. Our results demonstrated that GBZ can also function as an antiviral agent. Previous reports have identified salubrinal, another eIF2α phosphatase GADD34 inhibitor, to inhibit the replication of a number of viruses that include HSV, HCV, EBV, and RSV, indicating the potential use of GADD34 inhibitors as antiviral agents. A combination of T3 and GBZ...
had a strong additive effect on the induction of ER stress markers, SG formation, and anti-VSV replication, indicating commonality in the mechanism of action between the two compounds. This finding is of significant importance when antiviral activity of T3 is considered because T3 concentrations of 1–25 μM that were not effective in inhibiting VSV replication alone were, however, highly effective in inhibiting VSV replication when combined with lower concentration (10 μM) of GBZ. This finding may be clinically relevant considering possible in vivo toxicity of high doses of T3 and effectiveness of lower concentrations of T3 as an antiviral agent if combined with GBZ. It is important to note that future studies are needed to design structural analogs of T3 that can be effective at physiological concentrations. Nevertheless, this study also offers a proof of principle of the concept that ISR activating agents like T3 at SPCs and GBZ can be potential antiviral agents.

Conclusions
To summarize, we have uncovered novel principles of T3 function in this study and shown that T3 at SPCs activates ISR signal transduction pathways. Activation of these pathways induces PKR activation, SG formation, induction of ER stress pathways, and anti-VSV response. We also found that GBZ, an inhibitor of eIF2α phosphatase GADD34, induces stress pathways, exhibits anti-VSV activity, and enhances the effects of T3 to exhibit ER stress and anti-VSV response. These results have implications not only in our basic understanding of the effects of nonphysiological concentrations of T3 in the regulation of stress pathways but also the possible clinical importance of T3 and GBZ in the treatment of viral diseases. Much research is needed to understand the intricate complexities of stress-mediated signal transduction pathways and the mechanisms that link them to antiviral responses (Fig. 8). Our study offers a proof of principle of the concept that ISR activating agents like SPC of T3 and GBZ can be potential antiviral agents. Designing structural analogs of T3 active at physiological concentrations, together with GBZ, hold promise in drug discovery studies in targeting viral diseases.

Abbreviations
IFN, interferon; ISGs, interferon-stimulated genes; ER, endoplasmic reticulum; SGs, stress granules; UPR, unfolded protein response; GBZ, guanabenz; VSV, vesicular stomatitis virus; Poly IC, polyinosinic-polycytidylic acid. T3, triido-L-thyronine; ISR, integrated stress response.

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Author Contributions
Conceived and designed the experiments: MI, VN. Analyzed the data: MI, VN. Wrote the first draft of the manuscript: MI. Contributed to the writing of the manuscript: MI, VN. Agree with the manuscript and results: MI, VN. Jointly developed the structure and arguments for the paper: MI, VN. Made critical revisions and approved final version: MI, VN. All authors reviewed and approved of the final manuscript.

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