Troglitazone acutely inhibits protein synthesis in endothelial cells via a novel mechanism involving protein phosphatase 2A-dependent p70 S6 kinase inhibition

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Troglitazone acutely inhibits protein synthesis in endothelial cells via a novel mechanism involving protein phosphatase 2A-dependent p70 S6 kinase inhibition. Am J Physiol Cell Physiol 291: C317–C326, 2006; doi:10.1152/ajpcell.00491.2005.—Thiazolidinediones (TZDs), synthetic peroxisome proliferator-activated receptor γ (PPARγ) ligands, have been implicated in the inhibition of protein synthesis in a variety of cells, but the underlying mechanisms remain obscure. We report that troglitazone, the first TZD drug, acutely inhibited protein synthesis by decreasing p70 S6 kinase (p70S6K) activity in bovine aortic endothelial cells (BAEC). This inhibition was not accompanied by decreased phosphorylation status or in vitro kinase activity of mammalian target of rapamycin (mTOR). Furthermore, cotreatment with rapamycin, a specific mTOR inhibitor, and troglitazone additively inhibited both p70S6K activity and protein synthesis, suggesting that the inhibitory effects of troglitazone are not mediated by mTOR. Overexpression of the wild-type p70S6K gene significantly reversed the troglitazone-induced inhibition of protein synthesis, indicating an important role of p70S6K. Okadaic acid, a protein phosphatase 2A (PP2A) inhibitor, partially reversed the troglitazone-induced inhibition of p70S6K activity and protein synthesis. Although troglitazone did not alter total cellular PP2A activity, it increased the physical association between p70S6K and PP2A, suggesting an underlying molecular mechanism. GW9662, a PPARγ antagonist, did not alter any of the observed inhibitory effects. Finally, we also found that the mTOR-independent inhibitory mechanism of troglitazone holds for the TZDs ciglitazone, pioglitazone, and rosiglitazone, in BAEC and other types of endothelial cells tested. In conclusion, our data demonstrate for the first time that troglitazone (and perhaps other TZDs) acutely decreases p70S6K activity through a PP2A-dependent mechanism that is independent of mTOR and PPARγ, leading to the inhibition of protein synthesis in endothelial cells.

THIAZOLIDINEDIONES (TZDs) are synthetic ligands that activate the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ; see Ref. 36). These compounds are widely used in the treatment of type 2 diabetes mellitus (DM; see Refs. 32 and 33). In addition to their beneficial effects on insulin resistance, growing evidence indicates that TZDs have an array of anti-inflammatory effects in endothelial cells and macrophages, either by decreasing nuclear factor-kB activity (7, 9) or by increasing endothelial nitric oxide synthase activity (8), which may attenuate atherogenesis in patients with type 2 DM (4, 13, 14). Furthermore, the TZDs inhibit both ANG II-induced cardiac hypertrophy in vitro and pressure overload-induced cardiac hypertrophy in vivo and improve cardiac function in these patients (2, 39). TZDs also have antitumor activity in a wide variety of experimental cancer models, both in vitro and in vivo (26, 34), by modulating the cell cycle, inducing cell differentiation and apoptosis, and inhibiting tumor angiogenesis. All of these effects are mediated by either PPARγ-dependent or -independent signaling pathways, according to the TZD concentration and target cell type.

Protein synthesis has a pivotal role in various pathological states, including atherosclerosis, cardiac hypertrophy, and cancer (11, 12, 15). The process of mRNA translation in mammalian cells is a major step in protein synthesis, and its control involves the regulation of multiple translation factors in the translation machinery. This is affected primarily by alterations in phosphorylation, which modulates their activities or capacities to interact with one another (29). The protein kinase called “the mammalian target of rapamycin” (mTOR) is a convergence point for mRNA translation signaling pathways. It plays a critical role in regulating mRNA translation initiation by modulating its downstream effectors p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1; see Refs. 29 and 37). The activities of these translation regulators dramatically increase as a consequence of multisite phosphorylation during active cell growth. Furthermore, it has also been reported that genetic defects causing constitutive mTOR activation lead to the generation of tumors, and, in this context, inhibitors targeting mTOR have been tested as cancer therapy (6, 31), suggesting a critical role for the mTOR signaling pathway in protein synthesis under pathological conditions. Besides the mTOR signaling pathway, the phosphorylation states of eIF2α and eukaryotic elongation factor 2 (eEF2) play important roles in mRNA translation initiation and elongation, respectively (21, 29). However, the phosphorylation of these two translation factors is downregulated in active cell proliferation (5, 21).

It seems very likely that TZDs inhibit protein synthesis by modulating the activity of multiple translation factors. However, the molecular mechanism for these inhibitory effects in

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endothelial cells remains unclear. In this study, we demonstrated that troglitazone, the first TZD synthesized, inhibited p70S6K activity via a protein phosphatase 2A (PP2A)-dependent mechanism, which led to the inhibition of protein synthesis in endothelial cells. Furthermore, we found that neither mTOR nor PPARγ was involved in this mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials.** Troglitazone was obtained as a gift from Sankyo (Tokyo, Japan). Ciglitazone, pioglitazone, and rosiglitazone were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against mTOR, p-mTOR-Ser2448, p70S6K, and hemagglutinin (HA) were purchased from Cell Systems (Walkersville, MD), and Cell Systems Corporation (Kirkland, WA), respectively. Okadaic acid and rapamycin were obtained from Sigma Chemical (St. Louis, MO). GW-9662 was purchased from Tocris (Ellisville, MO), l-[3,4,5-3H(N)]leucine and [γ-32P]ATP were obtained from Perkin-Elmer Life Sciences (Ann Arbor, MI), respectively. Purified histone H1 protein was purchased from Calbiochem, Protein G-Sepharose was obtained from Amersham Biosciences (Uppsala, Sweden). Antibodies against mTOR, p-mTOR-Ser2448, p70S6K, p-p70S6K-Thr389, p-p70S6K-Thr421/Ser424, 4E-BP1, and p-4E-BP1-Thr70 were from Cell Signaling Technology (Beverly, MA). Antibodies against the catalytic subunit of PP2A (PP2Ac) and hemagglutinin (HA) were purchased from Upstate Biotechnology (Lake Placid, NY) and Covance (Berkeley, CA), respectively. Collagenase (type 2) was purchased from Worthington Biochemical (Freehold, NJ). Minimal essential medium (MEM), DMEM, Dulbecco’s PBS (DPBS), newborn calf serum (NCS), penicillin, streptomycin, l-glutamine, trypsin, EDTA solution, and the plastic labware used for cell culture were purchased from Gibco-BRL (Gaithersburg, MD). Endothelial growth media-2 (EGM-2) and CS-C complete media were obtained fromCambrex Bio Science (Walkersville, MD) and Cell Systems Corporation (Kirkland, WA), respectively. All other chemicals were of the purest analytical grade.

**Cell culture, drug treatment, and transfection.** Bovine aortic endothelial cells (BAEC) were isolated and maintained in MEM supplemented with 5% NCS at 37°C under 5% CO2 in air, as described previously (18). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Bio Science and cultured in EGM-2 according to the manufacturer’s instructions. Human brain microvascular endothelial cells (HBMEC) were obtained from Cell Systems and cultured in CS-C complete medium according to the supplier’s instructions. Rat vascular smooth muscle cells (RVSMC) were a generous gift from Dr. Hyun-Young Park (National Institute of Health, Seoul, Korea), and Madin-Darby canine kidney (MDCK) cells and HeLa cells were kind gifts from Dr. Jae-Hwan Nam (Catholic University, Seoul, Korea). RVSMC, MDCK cells, and HeLa cells were maintained in DMEM supplemented with 10% FBS at 37°C under 5% CO2 in air. When the cells had grown to between 60 and 80% confluence, they were maintained for the indicated times in either 0.5% NCS-MEM (for BAEC, HUVEC, and HBMEC) or serum-free DMEM (for RVSMC, MDCK cells, and HeLa cells) containing various concentrations of troglitazone or vehicle. In some experiments, BAEC were pretreated with the indicated chemicals for 1 h at 4°C. The precipitates were washed two times with 95% ethanol, dissolved in 1 M NaOH, and then neutralized with 1 M HCl. The radioactivity of the incorporated l-[3,4,5-3H(N)]leucine was measured using liquid scintillation counting, normalized to the protein concentration, and used as an index of protein synthesis.

**Lactate dehydrogenase release assay.** Lactate dehydrogenase (LDH) was used as an index of cellular injury: the activity in the cultured medium represented LDH release from the BAEC treated with troglitazone or vehicle (DMSO) for the indicated times. For a negative control, cells were treated with only MEM supplemented with 0.5% NCS. A positive control was prepared by treating cells with 0.5% Tween 20. Cultured medium was collected, and the LDH release assay was performed using an LDH-LQ assay kit (ASAN Pharmaceutica, Seoul, Korea) according to the manufacturer’s instructions.

**Western blot analysis.** For Western blot analysis, cells treated with TZDs in the absence or presence of various chemicals were washed with ice-cold DPBS and lysed in lysis buffer A [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and 1X Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. Protein concentrations were determined using the BCA protein assay kit (Sigma). Equal quantities of protein (20 μg) were separated on SDS-polyacrylamide gels under reducing conditions and then transferred to nitrocellulose membranes electrophoretically. The blots were then probed with the appropriate antibody directed against mTOR, p-mTOR-Ser2448, p70S6K, p-p70S6K-Thr389, p-p70S6K-Thr421/Ser424, 4E-BP1, p-4E-BP1-Thr70, HA, or PP2Ac, followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence reagents (Amersham Biosciences).

In vitro mTOR kinase assay. BAEC were treated with 20 μM troglitazone for vehicle for 30 min, lysed in lysis buffer B [20 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 137 mM NaCl, 50 μM EDTA, 1 mM PMSF, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na3VO4, and 1X Protease Inhibitor Cocktail], and then centrifuged at 12,000 g for 10 min. The supernatant (450 μg protein) was immunoprecipitated using 2 μl of mTOR antibody. As a control experiment, 2 μl of nonimmune serum (normal rabbit IgG) were used instead of mTOR antibody. The immunoprecipitates were washed three times with lysis buffer B and two times with kinase assay buffer [50 mM Tris-Cl (pH 7.5), 5 mM MgCl2, 1 mM PMSF, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na3VO4, and 1X Protease Inhibitor Cocktail]. The immunoprecipitated mTOR was then resuspended in 25 μl of kinase assay buffer, and the kinase assay was started by adding 1 μCi of [γ-32P]ATP and 2 μg of histone H1 as substrate. After a 20-min incubation at 30°C, the reaction was terminated by adding Laemmli sample buffer. The samples were boiled for 5 min and subjected to 12% SDS-PAGE, and the dried gel was exposed to X-ray films at −80°C. The mTOR kinase activity was quantified by measuring the densitometry of bands obtained using an image analysis program (ImageJ; NIH, Bethesda, MD).

**Coimmunoprecipitation.** After treatment with 20 μM troglitazone or vehicle for 30 min, the BAEC were lysed with lysis buffer A and centrifuged at 12,000 g for 10 min. The supernatant (250 μg protein) was precleared with 50 μl of a 50% slurry of precleared protein G-Sepharose at 4°C for 2 h. The precleared supernatant was incubated for 16 h at 4°C with 2 μl of p70S6K antibody and then for 2 h at 4°C with 30 μl of a 50% slurry of preclequired protein G-Sepharose. In a separate experiment, the precleared supernatant was also incubated with 2 μl of PP2Ac antibody. The immunoprecipitates were washed thoroughly five times with lysis buffer A. The bound proteins were eluted with Laemmli sample buffer and subjected to Western blot analysis using the appropriate antibodies.

**PP2A activity assay.** The PP2A activity assay was carried out using the Serine/Threonine Phosphatase Assay System (Promega, Madison, WI) with minor modifications. BAEC were treated with 20 μM TCA for 1 h at 4°C. The precipitates were washed two times with 95% ethanol, dissolved in 1 M NaOH, and then neutralized with 1 M HCl. The radioactivity of the incorporated l-[3,4,5-3H(N)]leucine was measured using liquid scintillation counting, normalized to the protein concentration, and used as an index of protein synthesis.
troglitazone or vehicle for 30 min, lysed in lysis buffer B lacking serine/threonine phosphatase inhibitors such as NaF and β-glycerophosphate, and then centrifuged at 12,000 g for 10 min. The supernatant (500 μg protein) was immunoprecipitated using 2 μl of PP2Ac antibody. As a control experiment, 2 μl of nonimmune serum (normal rabbit IgG) were used instead of PP2Ac antibody. The immunoprecipitates were washed three times with lysis buffer B lacking serine/threonine phosphatase inhibitors and two times with PP2A assay buffer [50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% β-mercaptoethanol, 1 mM PMSF, and 1× Protease Inhibitor Cocktail]. The PP2A enzyme reactions were started by adding PP2A assay buffer containing 100 μM okadaic acid was used for the enzyme reaction. After a 20-min incubation at 30°C, the reaction was terminated by adding 50 μl of Molysbdate Dye/Additive mixture. The cellular PP2A activity was quantified by measuring the optical density at 630 nm and normalized to the optical density obtained from the control experiment.

Statistical analysis. All results are expressed as means ± SD, with n indicating the number of experiments. Statistical significance was determined using Student’s t-test for two points. P < 0.05 was assumed statistically significant.

RESULTS

Troglitazone acutely inhibits protein synthesis in BAEC. First, we tested whether troglitazone inhibited protein synthesis in BAEC. As shown in Fig. 1, A and B, troglitazone decreased protein synthesis in a time- and dose-dependent manner. In kinetic experiments, troglitazone (20 μM) acutely (within 20 min) decreased protein synthesis (Fig. 1A), and this inhibitory effect of troglitazone was prolonged for up to 3 h (data not shown). Protein synthesis was reduced ~60% with troglitazone treatment at 20 μM for 30 min compared with the vehicle (DMSO)-treated control (Fig. 1B). To test whether this troglitazone-induced inhibition of protein synthesis was the consequence of troglitazone-mediated cellular toxicity, we performed an LDH release assay. As shown in Fig. 1C, no alterations in cellular viability or integrity were found under our experimental conditions (20 μM troglitazone treatment for 30 min). This was also true for longer (3h) troglitazone exposures (data not shown).

Troglitazone inhibits the activity of p70S6K independently of mTOR. Because the process of mRNA translation in mammalian cells is a major step in protein synthesis, and mTOR serves as a convergence point for mRNA translation, we next investigated whether troglitazone modulates the activity of multiple translation factors involved in the mTOR signaling pathway. As shown in Fig. 2A, we found a significant decrease in p70S6K phosphorylation at the earliest (10 min) time point, and this inhibitory effect was time dependent. Although we used antibodies raised against p-p70S6K-Thr389 and p-p70S6K-Thr421/Ser424 to measure p70S6K activity, the same results were obtained; therefore, only the data using antibody against p-p70S6K-Thr421/Ser424 are presented here. In contrast with p70S6K phosphorylation, however, no alteration in the phosphorylation of mTOR, a well-known upstream molecule of p70S6K, was found under the same conditions. Similarly, we also found no alteration in the phosphorylation of 4E-BP1, a downstream substrate of mTOR related to mRNA translation initiation. These incompatible decreasing patterns were con-
firmed (Fig. 2B), suggesting that the mTOR-signaling pathway is not responsible for the acute inhibition of p70S6K activity by troglitazone. No alteration in mTOR activity under this experimental conditions was found in vitro mTOR kinase assay, as shown in Fig. 2C. To define the mechanism by which troglitazone acutely inhibits protein synthesis via the mTOR-independent inhibition of p70S6K activity, all the subsequent experiments, unless otherwise stated, were performed with 20 μM troglitazone treatment for 30 min. To further clarify the mTOR-independent modulation of p70S6K activity by troglitazone, we used rapamycin, a well-known mTOR inhibitor. As shown in Fig. 2D, pretreatment with rapamycin alone (500 nM) significantly decreased the phosphorylation of mTOR, 4E-BP1, and p70S6K. Furthermore, when cells were cotreated...
with rapamycin and troglitazone, the inhibitory effect on p70S6K was additive. However, under this experimental condition, no further alterations in the phosphorylation of mTOR and 4E-BP1 were observed. These results indicate that rapamycin and troglitazone decrease p70S6K phosphorylation via separate signaling pathways, further showing that the inhibition of p70S6K phosphorylation by troglitazone is mediated by an mTOR-independent signaling pathway. Under our experimental condition, we also found that rapamycin and troglitazone each decreased protein synthesis (Fig. 2E). Furthermore, when rapamycin and troglitazone were added together, the inhibitory effect on protein synthesis was almost additive, suggesting that there are two separate mechanisms for the rapamycin- and troglitazone-induced inhibition of protein synthesis in BAEC.

Ectopic expression of the p70S6K gene reverses the troglitazone-mediated inhibition of protein synthesis. Because the time- and dose-dependent decreases in p70S6K phosphorylation by troglitazone (Fig. 2) are fairly consistent with those of protein synthesis (Fig. 1), we next examined whether the mTOR-independent inhibition of p70S6K activity by troglitazone plays a role in the troglitazone-mediated inhibition of protein synthesis. To this end, we transfected a HA-tagged wild-type p70S6K (WT-p70S6K) gene construct into BAEC. This was successful, as shown by the high expression of p70S6K protein and HA-tagged p70S6K in transfected cells (Fig. 3A). Furthermore, overexpression of the WT-p70S6K gene significantly reversed the troglitazone-induced inhibition of protein synthesis (Fig. 3B), suggesting a physiological role of p70S6K in this inhibitory signaling pathway. Our data shown in Figs. 1–3 demonstrate that troglitazone acutely inhibits protein synthesis at least in part by inhibiting p70S6K activity, which is mTOR independent.

PP2A mediates the troglitazone-induced inhibition of p70S6K phosphorylation and protein synthesis. PP2A is not only involved in regulating mTOR phosphorylation but also in the direct dephosphorylation or inactivation of p70S6K (17, 27). Therefore, we examined whether PP2A plays roles in the troglitazone-induced inhibition of p70S6K phosphorylation and protein synthesis. As shown in Fig. 4A, the inhibition of PP2A activity by the addition of okadaic acid (100 nM) at a concentration specific for PP2A (10, 24) significantly reversed (~80%) the repressed p70S6K phosphorylation by troglitazone, suggesting an important role of PP2A in this pathway. Although okadaic acid increased mTOR phosphorylation in the troglitazone-stimulated cells, the increased phosphorylation was also seen in basal cells, suggesting the involvement of PP2A in basal mTOR phosphorylation. Okadaic acid did not alter the phosphorylation of 4E-BP1. All these data suggest that the troglitazone-induced acute inhibition of p70S6K was mediated by PP2A, but not by mTOR. Next, we tested whether okadaic acid also reverses the inhibition of protein synthesis by troglitazone. As shown in Fig. 4B, okadaic acid significantly restored the inhibitory effect of troglitazone on protein synthesis, suggesting that the troglitazone-induced inhibition of p70S6K and protein synthesis is mediated at least in part by PP2A. Next, we investigated how PP2A exerts its role in mediating the troglitazone-induced inhibition of p70S6K activity. First, we tested whether troglitazone alters total cellular PP2A activity, but we failed to observe a significant alteration (Fig. 4C). Because the PP2Ac has been shown to dephosphorylate and inactivate p70S6K via a direct interaction between PP2Ac and p70S6K (28), we tested whether troglitazone increases binding of PP2Ac with p70S6K without altering the total cellular PP2A activity. As shown in Fig. 4D, troglitazone significantly (~2-fold) increased the interaction between PP2Ac and p70S6K as evidenced in communoprecipitation experiments, suggesting that troglitazone inhibits p70S6K activity by inducing or stabilizing this PP2Ac/p70S6K complex.

Inhibition of p70S6K and protein synthesis by troglitazone does not require PPARγ. It is still controversial whether the inhibitory effect of troglitazone on protein synthesis and cell proliferation is dependent on PPARγ (20). In this study, we observed the inhibitory effect of p70S6K activity and protein synthesis at an earlier time point (10–20 min). Therefore, we hypothesized that a PPARγ-mediated genomic action may not be responsible for the troglitazone-mediated inhibitory effects. To test this hypothesis, we used GW-9662 (5 μM), a specific, irreversible PPARγ antagonist. Our results clearly showed that this antagonist had no effect on either the level of p70S6K
phosphorylation (Fig. 5A) or protein synthesis (Fig. 5B), suggesting that the acute inhibition of protein synthesis by the troglitazone-induced p70S6K inhibition is mediated via a PPAR\(^\gamma\)-independent signaling pathway.

Other TZDs have similar effects on the inhibition of protein synthesis and mTOR-independent p70S6K activity in BAEC. Next, we examined whether several different TZDs have the same effects as troglitazone. As shown in Fig. 6A, all the TZDs used in this study significantly and acutely (30 min) inhibited protein synthesis in BAEC. The order of efficacy for this inhibition was found to be troglitazone > ciglitazone > pioglitazone > rosiglitazone. Furthermore, at the concentration that causes 50% inhibition of protein synthesis, the TZDs tested in this study all significantly inhibited the phosphorylation of p70S6K without altering phosphorylation in mTOR and 4E-BP1 (Fig. 6B), suggesting that all of the TZDs tested share a common signaling pathway responsible for the acute inhibition of protein synthesis in BAEC.

The mTOR-independent inhibition of p70S6K phosphorylation by troglitazone is cell type-specific. We also tested whether the inhibitory effects of troglitazone are true for other cells. To this end, we used five more cell lines: two endothelial cell lines (HUVEC and HBMEC), two epithelial cell lines (MDCK and HeLa cells), and one vascular smooth muscle cell line (RVSMC). As shown in Fig. 7A, troglitazone (at 20 \(\mu\)M for 30 min) inhibited protein synthesis in all of these cells, with different efficacies. Furthermore, at the dose at which troglitazone significantly inhibited protein synthesis, it also significantly inhibited mTOR-independent p70S6K phosphorylation in the two endothelial cell lines and MDCK cells (Fig. 7B). In contrast, under the same experimental conditions, there was no inhibition of p70S6K phosphorylation in RVSMC or HeLa cells, suggesting that troglitazone mediates the mTOR-independent inhibition of p70S6K phosphorylation in a cell type-dependent manner.

DISCUSSION

One important finding of this study is evidence that the inhibition of protein synthesis in response to acute troglitazone treatment is mediated, at least in part, by inhibiting p70S6K activity in endothelial cells. Furthermore, this inhibitory effect appears to be mTOR independent, as evidenced by our kinetic analysis and in vitro mTOR kinase assay. There are many reports that mTOR and its downstream effectors 4E-BP1, p70S6K, and eEF2 kinase play a key role in protein synthesis
(21, 29). Although mTOR tightly regulates all these effectors in most cases, there is an exception: the phosphorylation of eEF2 by energy depletion is mTOR independent (23) and occurs only at earlier times. Furthermore, it was also reported that tuberous sclerosis complex tumor suppressor genes TSC1 and TSC2 exert their effects on p70S6K inhibition via an mTOR-independent pathway (16, 30). Here, we provide another case in which the acute inhibition of p70S6K phosphorylation by troglitazone is also mediated by an mTOR-independent pathway.

Next, we found that acute treatment with troglitazone modulates PP2A activity toward p70S6K by inducing its interaction with p70S6K and vice versa, without altering the intracellular PP2A activity (Fig. 4D). The interaction between PP2A and p70S6K has been observed previously (27). Although the detailed mechanism is unknown, we speculate that troglitazone acutely induces endoplasmic reticulum (ER) stress, which causes PP2A to release from the ER and translocate to p70S6K and other unidentified components. This idea is supported by previous findings that PP2A functions as an ER stress-responsive phosphatase (35) and that troglitazone exerts ER stress (26). Furthermore, our results showed that a PP2A inhibitor, okadaic acid, reversed p70S6K phosphorylation and protein synthesis only in troglitazone-treated cells, and not in basal cells (Fig. 4, A and B). Therefore, the modulation of PP2A activity by troglitazone does not require peroxisome proliferator-activated receptor γ (PPARγ). BAEC were prepared as described in Fig. 1. Cells were pretreated with 5 μM GW-9662 or vehicle (0.05% ethanol) for 1 h before treatment with 20 μM troglitazone for 30 min (A). In some experiments, the cells were cotreated with L-[3,4,5-3H(N)]leucine to measure protein synthesis (B). Western blotting and protein synthesis assay were performed (n = 4–6), and the data are presented as described in Figs. 2 and 1, respectively; n = 4 experiments.

![Fig. 5. Inhibition of p70S6K activity and protein synthesis by troglitazone](image)

![Fig. 6. Other TZDs have similar effects on the inhibition of protein synthesis and mTOR-independent p70S6K activity in BAEC. BAEC were prepared as described in Fig. 1. Cells were treated with various TZDs for 30 min (A). Protein synthesis was measured after cotreatment with L-[3,4,5-3H(N)]leucine (n = 4), as described in Fig. 1 (A). In some experiments, the cells were also treated for 30 min with TZDs at a concentration that causes 50% inhibition. The amount of phosphorylation of multiple translation factors was measured using Western blotting, as described in Fig. 2, and the blots are representative of at least four separate experiments (B).](image)
activity on p70S6K may be one of the most important mechanisms underlying the inhibition of protein synthesis by acute troglitazone treatment. In this regard, it was previously reported that transforming growth factor-β (TGF-β) induced G1 arrest by inhibiting p70S6K through the interaction with PP2A, which is released from the TGF-β receptor on receptor activation (28).

Our data also demonstrate that okadaic acid partially, but not completely, reversed the inhibition of p70S6K phosphorylation and protein synthesis by troglitazone (Fig. 4), suggesting that the troglitazone-induced inhibition of protein synthesis is mediated by at least two independent pathways: the PP2A-dependent inhibition of p70S6K phosphorylation and the PP2A-independent inhibition of other translation factors. This is
supported by the observation that overexpression of p70S6K in cells did not completely reverse the inhibition of protein synthesis by troglitazone (Fig. 3B). In this regard, eIF2α and eEF2 are potential candidates. Previously, it was reported that ATP depletion acutely increased eEF2 phosphorylation (23) and that troglitazone acutely decreased intracellular ATP levels (38). However, further studies are needed to clarify this issue, which is beyond the scope of this study.

It is still controversial whether the observed beneficial effects of troglitazone on atherosclerosis and cancer in vitro and in vivo necessarily require PPARγ. Our data imply that the troglitazone-induced inhibition of p70S6K and protein synthesis is mediated by a PPARγ-independent pathway because all the troglitazone-induced inhibitory effects were observed within 10 min (Figs. 1 and 2). Our results obtained using GW-9662 also clearly show that the inhibitory effects of troglitazone on p70S6K phosphorylation and protein synthesis were not mediated by a genomic action of PPARγ as a transcription factor (Fig. 5). These results support the previous report (26) showing that the antitumor activity of TZDs is not mediated via PPARγ and that the PPARγ gene itself is not a tumor-suppressor gene. Therefore, it is likely that other important PPARγ-independent actions of TZDs will emerge.

We also found that the TZDs ciglitazone, pioglitazone, and rosiglitazone inhibited p70S6K activity and protein synthesis. However, their efficacies differed dramatically (Fig. 6). Although these drugs have the same structural backbone, several reports show that they have different effects on cellular proliferation and apoptosis, and in clinical use (3, 22). At present, it is speculated that these different effects result from the structural differences in their side chains. However, further studies are needed to clarify the relationship between TZD structure and activity. Moreover, troglitazone inhibited mTOR-independent p70S6K phosphorylation only in HUVEC, HBMEC, and MDCK cells in addition to BAEC, whereas it increased p70S6K phosphorylation in RVSMC and HeLa cells (Fig. 7B). Although these incompatible findings are not fully understood, they suggest that the p70S6K-mediated signaling pathway does not provide a general mechanism in all cell types for the acute inhibition of protein synthesis by troglitazone. Nonetheless, our data are the first to demonstrate that troglitazone (and perhaps other TZDs) inhibits protein synthesis via mTOR-independent p70S6K inhibition, at least in endothelial cells. Recently, two different effects of troglitazone on apoptosis were also reported that depended on cell type: it induced apoptosis in vascular smooth muscle cells (25) and inhibited apoptosis in endothelial cells (1).

In conclusion, we demonstrated that a synthetic PPARγ ligand, troglitazone, acutely inhibits protein synthesis at least in part by inhibiting P2A-dependent p70S6K phosphorylation in endothelial cells and that mTOR and PPARγ are not involved in this acute inhibitory effect (Fig. 8). Our results are the first to demonstrate a new mechanism by which troglitazone acutely regulates translation factors and protein synthesis in endothelial cells.

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