

Vanadyl acetylacetonate upregulates PPAR γ and adiponectin expression in differentiated rat adipocytes

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Abstract Vanadium compounds are promising agents in the therapeutic treatment of diabetes mellitus, but their mechanism of action has not been fully elucidated. The current work investigated the effects of vanadyl acetylacetonate, VO(acac)₂, on peroxisome-proliferator-activated receptor γ (PPAR γ) and adiponectin, which are important targets of antidiabetic drugs. The experimental results revealed that vanadyl complexes increased the expression and multimerization of adiponectin in differentiated rat adipocytes. VO(acac)₂ caused activation of p38 mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) and elevation of PPAR γ levels. The specific inhibitors SB203580 (p38 MAPK inhibitor) and T0070907 (PPAR γ inhibitor) decreased the expression of adiponectin; however, compound C (AMPK inhibitor) did not significantly reduce the expression of adiponectin. In addition, vanadyl complexes induced protein–protein interaction between PPAR γ and a vanadium-binding chaperone, heat shock protein 60 kDa. Overall, our results suggest that vanadyl complexes may upregulate PPAR γ by suppressing PPAR γ degradation, and thus stimulate adiponectin expression and multimerization. The present work has provided new insights into the mechanism of the antidiabetic actions of vanadium compounds.

Keywords Vanadium · Adiponectin · Adipocytes · Peroxisome-proliferator-activated receptor γ · p38 mitogen-activated protein kinase

Introduction

In metal-based drug research, vanadium compounds have long been recognized as potent hypoglycemic agents that are effective for treatment of both type 1 and type 2 diabetes mellitus [1, 2] and are potential chemoprevention agents for cancer [3]. Vanadium compounds exhibit antidiabetic global effects in vivo and in vitro [4, 5] and normalize/improve a variety of diabetes-altered gene expressions [6–8]. However, a full understanding of the molecular mechanisms of vanadium compounds is still limited to the success in clinical applications of novel vanadium drugs [9].

Various mechanisms of the actions of vanadium compounds, including the interactions of vanadium with cellular redox chemistry, have been proposed [4–13]. The most recognized mechanism occurs through inhibition of protein tyrosine phosphatase 1B (PTP1B) [10], which enhances the phosphorylation of insulin receptor substrate 1 and activation of phosphatidylinositol 3-kinase (PI3K)–Akt signal transduction. This mechanism for vanadium compounds may explain the enhancement of the insulin signaling pathway and promotion of glucose metabolism. Unfortunately, the clinical development of all known PTP1B inhibitors has been troubled by their limited selectivity and adverse pharmacological properties [14].

Some research has indicated other mechanisms for vanadium compounds. Studies on the genomic effects [6–8] and biodistributions [11, 12] of vanadium compounds have suggested that vanadium may act as a general

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transcription modulator. One recent noteworthy finding was that vanadyl sulfate increased adiponectin production in 3T3-L1 adipocytes [13].

Adiponectin (also known as Acrp30, AdipoQ, apM1, or GBP28) is a novel protein hormone primarily synthesized by adipocytes [15]. This hormone plays a role in the suppression of the metabolic derangements that may result in type 2 diabetes, obesity, and atherosclerosis [16]. Adiponectin automatically self-associates into multimers that have been considered improved biologically active forms for glucose homeostasis [17]. Adiponectin in combination with leptin has been shown to reverse completely insulin resistance in mice [18]. Therefore, the discovery of novel molecules enhancing adiponectin expression and multimerization led to these being thought of as an effective therapeutic approach for the treatment of insulin resistance and its associated metabolic and cardiovascular diseases [19].

Peroxisome-proliferator-activated receptor γ (PPAR γ) is the major player regulating adiponectin expression, self-assembly, and secretion [20]. Activation of PPAR γ can upregulate adiponectin levels and secretion by promoting adiponectin gene transcription or via a posttranslational mechanism. PPAR γ activation also induces expression of some endoplasmic reticulum chaperones involved in adiponectin multimerization. In addition, the cellular levels and multimerization of adiponectin can be regulated by posttranslational mechanisms via AMP-activated protein kinase (AMPK) signaling pathways. We hypothesized that vanadium compounds may regulate PPAR γ and adiponectin, thus playing an important role in antidiabetic actions.

In this work, we have studied the effects of vanadyl acetylacetonate, VO(acac)₂, on the regulation of adiponectin in differentiated rat adipocytes. Our work has provided the first evidence that vanadyl complexes may upregulate PPAR γ and thus stimulate adiponectin expression and multimerization, a process also involving p38 mitogen-activated protein kinase (MAPK).

Materials and methods

Materials

VO(acac)₂ was from Fluka (Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM)/F-12 was from HyClone (Logan, UT, USA). Antibodies for PPAR γ and glyceraldehyde 3-phosphate dehydrogenase, and protein A Sepharose CL-4B beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for adiponectin, total p38, phospho-Tyr182-p38, total AMPK, and phospho-Tyr172-AMPK were from Cell Signaling

Technology (Beverly, MA, USA). Antibody for heat shock protein 60 kDa (Hsp60), enzyme inhibitors, including SB203580 (p38 MAPK inhibitor), T0070907 (PPAR γ inhibitor), and compound C (AMPK inhibitor), bovine serum albumin (fraction V), and 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (Milwaukee, WI, USA). Rosiglitazone was from Cayman Chemical (Ann Arbor, MI, USA). Lactate dehydrogenase (LDH) assays kits were from Applygen Technologies (Beijing, China).

Cell culture

Primary adipocytes were isolated from epididymal fat pads of normal male Sprague–Dawley rats (160–180 g) [21]. Visible blood vessels were removed, and the fat pads were minced into millimeter-sized pieces, which were then digested in Hanks solution containing 1 mg/ml type I collagenase. After 1 h, the digested fat pads were filtered through 250- μ m nylon mesh, and then rat preadipocytes in the digestion mixture were collected by centrifugation at 800g for 5 min. Finally, rat preadipocytes were plated and differentiated into adipocytes for 3 days in serum-free DMEM/F-12 (1:1) supplemented with 5 μ g/ml insulin, 33 μ M biotin, and 200 pM triiodothyronine as described in [21]. On day 4, the differentiated adipocytes were transferred to serum-free DMEM and incubated for 24 h before the experiments.

Cell viability assay

The cells were seeded in a 96-well plate. Viability was estimated by the MTT assay and LDH leakage. For the MTT assay, the tetrazolium salt (MTT) was added to each well to a final concentration of 0.5 mg/ml and kept for 4 h. After removal of the medium, 100 μ l of dimethyl sulfoxide was added and the mixture was incubated at 37 °C for 20 min to dissolve the formazan product. Then, the absorbance at 490 nm was measured with a microplate reader (Multiskan Mk3; Thermo Labsystems, Vantaa, Finland). Cell viability rates were calculated from the absorbance at 490 nm and were presented as a percentage of control values. For LDH leakage, LDH activity in the culture medium after treatment was measured with a commercial kit (Applygen Technologies, Beijing) by the absorbance at 440 nm according to the protocol provided by the manufacturer.

Total RNA isolation and reverse transcription PCR

Total RNA was isolated from cells using a TRIzol kit (Invitrogen, Grand Island, NY, USA) according to the protocol provided by manufacturer. Complementary DNA

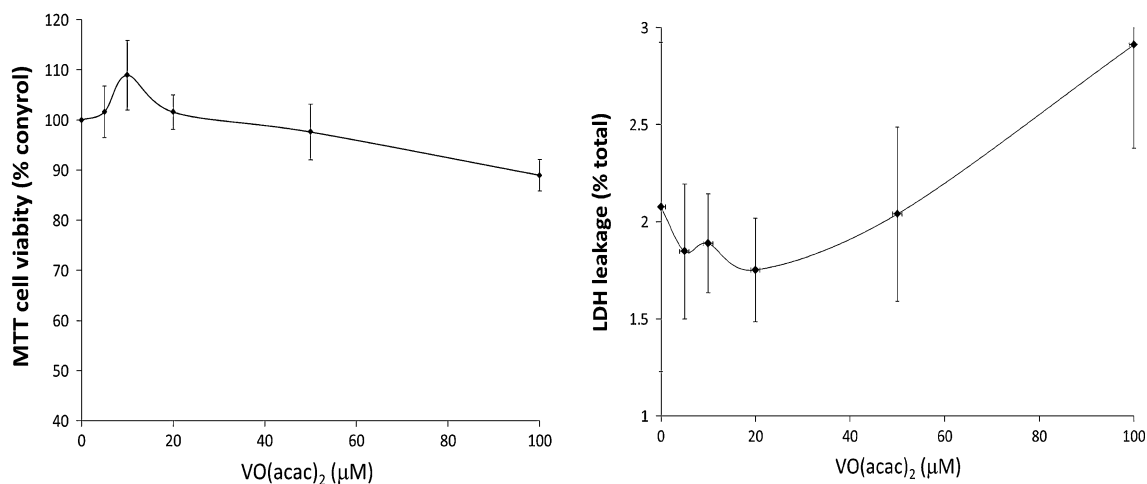


Fig. 1 Effects of vanadyl acetylacetonate, $VO(acac)_2$, on the viability of adipocytes. Differentiated rat adipocytes cells were treated with $VO(acac)_2$ for 24 h and then cell viability were estimated by 3-(4,5-

dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (*MTT*) assay and lactate dehydrogenase (*LDH*) leakage. Data are presented as the mean \pm the standard deviation of three independent measurements

(cDNA) was synthesized from the total RNA by reverse transcription using a RevertAid first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). For PCR amplification, 1 μ l of cDNA was added to 20 μ l of reaction mixture containing 0.2 μ M primer, 0.2 μ M dNTP mix, and 0.6 U *Taq* polymerase. PCR products (10 μ l) were analyzed on 2 % agarose gel, stained with ethidium bromide, and analyzed with a GK-330c Plus Transil-Luminator 2020D gel imaging analysis system from United Bio (Tokyo, Japan). PCR was performed with a DNA thermal cycler (Bioer, Japan) by preincubation at 94 °C for 5 min followed by 26 cycles according to the manufacturer's instructions. The primers for reverse transcription PCR were 5'-GTTTGCTGTGAAGTTC AATGC-3' and 3'-GTCTGTCTCCGCTCTTGGAT-5' for *PPAR* γ , and 5'-GCACGAGGCGAGAAA GGA-3' and 3'-CTACGCTGAATGCTGAGTGAT-5' for adiponectin. The images were analyzed and quantified by Scion Image (Scion, Frederick, MD, USA).

Western blotting analysis

$VO(acac)_2$ -treated rat differentiated adipocytes cells were rinsed twice with ice-cold phosphate-buffered saline and harvested from six-well plates. Then cells were lysed in 80 μ l of ice-cold lysis buffer. The supernatant of cell lysate was collected by centrifugation (12,000g for 20 min) at 4 °C. Then, the samples (40 μ g total protein) were separated by 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrically transferred to poly(vinylidene difluoride) membranes (Immobilon; Millipore, Bedford, MA, USA). The membrane was blocked with tris(hydroxymethyl)aminomethane-buffered saline containing 0.1 % Tween 20 and 5 % bovine serum albumin. The blocked membrane was incubated overnight with

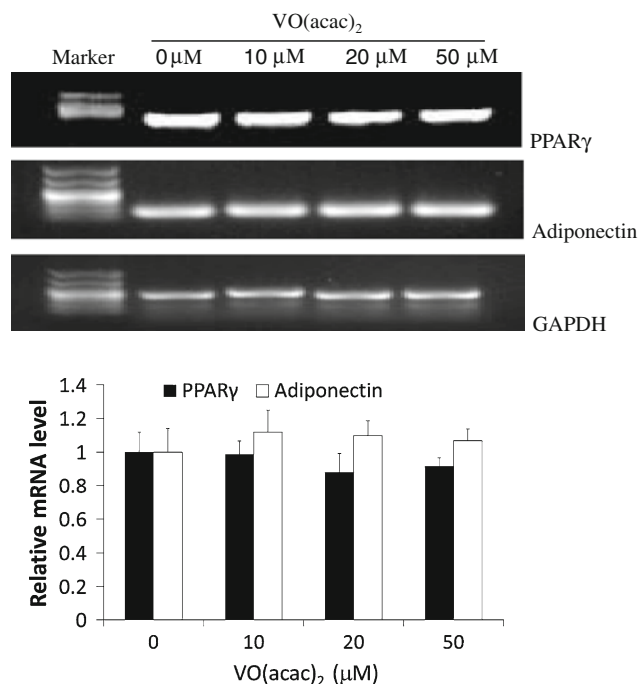


Fig. 2 Effects of $VO(acac)_2$ on messenger RNA (*mRNA*) levels of peroxisome-proliferator-activated receptor γ (*PPAR* γ) and adiponectin in differentiated rat adipocytes. After treatment of the cells with the indicated concentrations of $VO(acac)_2$ at 37 °C for 24 h, the mRNA was extracted and assayed as described in “Materials and methods.” Quantitative data are the average of three independent experiments. *GADPH* glyceraldehyde 3-phosphate dehydrogenase

primary antibodies at 4 °C and visualized with horseradish peroxidase conjugated secondary antibody (1:5,000) at room temperature for 1 h. Finally, the image was developed with an ECL Western blotting substrate kit (Pierce, Rockford, IL, USA) on an X-ray film (Kodak, Fujian,

China) according to the manufacturer's instructions. The images were analyzed and quantified by Scion Image (Scion, Frederick, MD, USA).

For analysis of isoforms of adiponectin, adipocyte samples after VO(acac)₂ treatments were subjected to 7.5 % nondenaturing polyacrylamide gel electrophoresis before Western blotting as described above.

Immunoprecipitation

Cells were lysed in fresh Roth lysis buffer on ice. After centrifugation (12,000g for 10 min, 4 °C), the cell lysate

samples (250–500 µg total protein) were precleaned with 20 µl of protein A Sepharose CL-4B beads. Then the primary antibodies (anti-PPARγ or anti-Hsp60 antibodies, 2 µg/10 µl) were added and incubated for 2 h at 4 °C. Then, 40 µl of protein A beads was added and incubated overnight at 4 °C. The pellets were obtained by centrifugation (12,000g for 30 s, 4 °C) and thoroughly washed with 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid buffers and buffered saline containing 1.0 % Triton X-100 and 0.1 % sodium dodecyl sulfate, pH 7.8. Then samples were reduced with 0.1 mM dithiothreitol (final) and subjected to Western blotting assays.

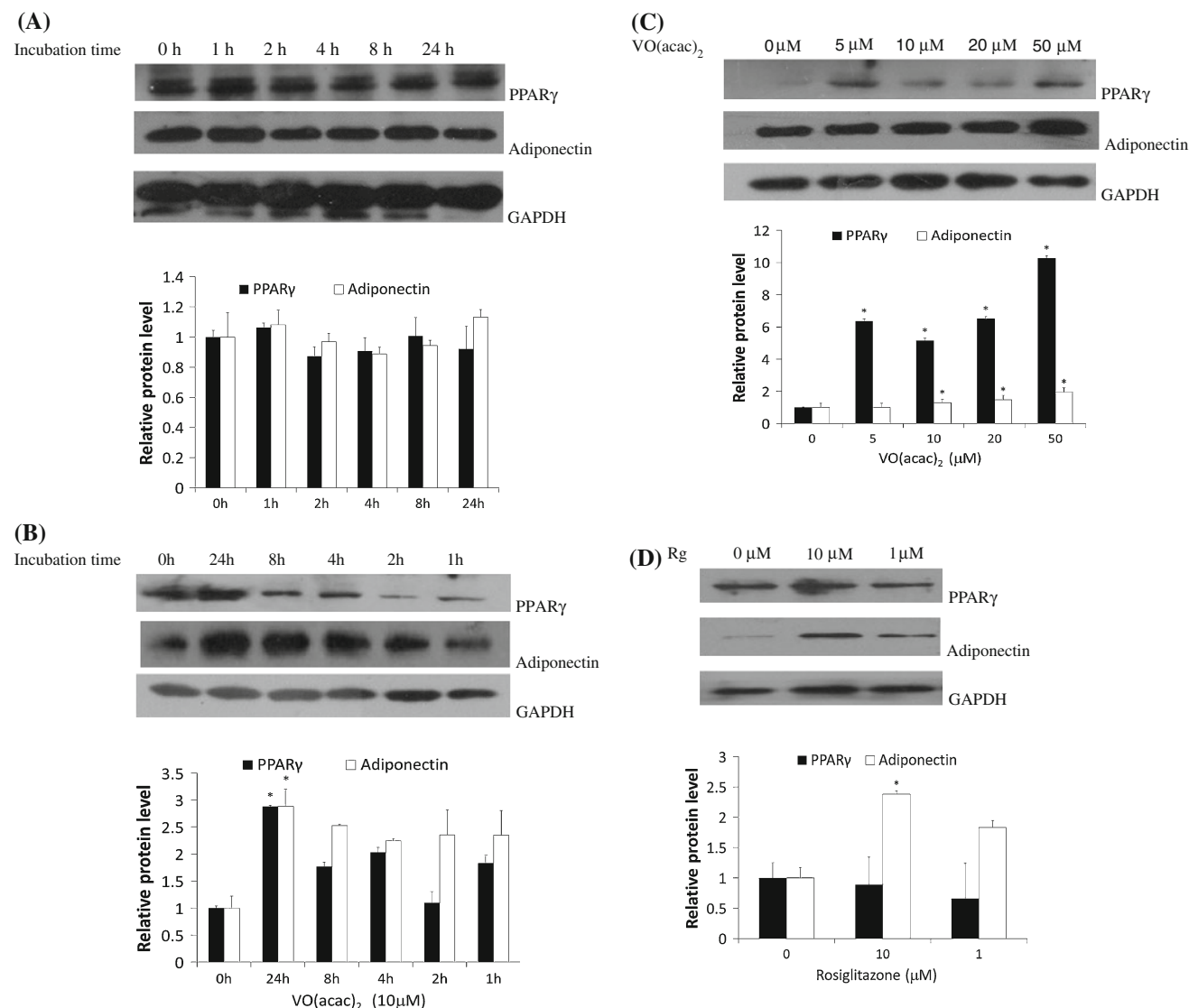


Fig. 3 Effects of VO(acac)₂ on protein levels of PPARγ and adiponectin in differentiated rat adipocytes. **a** The basal expression of PPARγ and adiponectin during 24 h incubation. **b** Time dependence of expression of PPARγ and adiponectin on incubation with 10 µM VO(acac)₂. **c** Concentration dependence of expression of PPARγ and adiponectin on incubation with VO(acac)₂ for 24 h. **d** The

expression of PPARγ and adiponectin with rosiglitazone (*Rg*) treatment for 24 h. Total proteins were extracted after treatment of the cells under the conditions indicated and the samples were assayed by Western blotting as described in “Materials and methods”. Quantitative data are the average of three independent experiments. Asterisk *P* < 0.05 versus controls

Statistical analysis

Differences between values were tested using Student's *t* test; $P < 0.05$ was considered statistically significant.

Results

Effect of VO(acac)₂ on cell viability of differentiated rat adipocytes

The cell viability of differentiated rat adipocytes on incubation for 24 h with various concentrations of VO(acac)₂ was estimated by both MTT assay and LDH leakage (Fig. 1). In the concentration range from 0 to 50 μM, VO(acac)₂ did not exhibit any signs of cytotoxicity. The microscopic observations of cell morphology agreed with the cell viability assays; therefore, VO(acac)₂ in the 0–50 μM range was used in the following experiments to determine the effects of vanadyl complexes on cellular functions.

The effects of VO(acac)₂ on PPAR γ and adiponectin in differentiated rat adipocytes

Treatment of differentiated rat adipocytes with VO(acac)₂ did not significantly affect messenger RNA (mRNA) levels of PPAR γ and adiponectin (Fig. 2), indicating that VO(acac)₂ did not alter PPAR γ or adiponectin transcription.

The protein levels of PPAR γ and adiponectin were analyzed by Western blotting (Fig. 3). As basal protein expression in differentiated adipocytes from primary cultures may change between batches, Western blot images were quantified by image analysis as described in “[Materials and methods](#).” The differentiated adipocytes produced a stable basal expression of PPAR γ and adiponectin during 24 h incubation (Fig. 3a). Treatment with VO(acac)₂ resulted in a dose- and time-dependent increase of PPAR γ and adiponectin levels (Fig. 3b, c). For comparison, the PPAR γ agonist rosiglitazone did not cause an increase of PPAR γ levels (Fig. 3d), although treatment of adipocytes with rosiglitazone *in vitro* is known to increase adiponectin levels and multimerization [22]. In this experiment, rosiglitazone also increased the adiponectin levels (Fig. 3d). In addition, VO(acac)₂ promoted multimerization of adiponectin by increasing the amounts of higher molecular weight forms of adiponectin (Fig. 4).

Effects of VO(acac)₂ on p38 MAPK, PPAR γ , and AMPK signaling

To clarify the role of signaling transductions related to PPAR γ and adiponectin regulation, we investigated the activation of p38 MAPK, PPAR γ , and AMPK on VO(acac)₂

treatment as well as the effects of specific inhibitors. The results (Fig. 5) were that (1) VO(acac)₂ increased p38 and AMPK phosphorylation (Fig. 5a) and (2) T0070907 (PPAR γ inhibitor) significantly suppressed VO(acac)₂-stimulated expression of adiponectin and SB203580 (p38 MAPK inhibitor) also decreased adiponectin expression, but compound C (AMPK inhibitor) did not (Fig. 5b–d). Vanadium compounds at high concentrations have been reported to activate extracellular-signal-regulated kinase [23]; however, we did not observe activation of extracellular-signal-regulated kinase in the concentration range from 0 to 50 μM in differentiated adipocytes.

Vanadium-induced Hsp60–PPAR γ interaction

The protein–protein interaction between Hsp60 and PPAR γ was analyzed by co-immunoprecipitation (Fig. 6). In the control samples, only one protein band corresponding to the same capture antibody was observed; however, after treatment of adipocytes with 10 μM VO(acac)₂, both Hsp60 and PPAR γ protein bands were observed, indicating that vanadium induced protein–protein interaction between Hsp60 and PPAR γ in adipocytes.

Discussion

In the treatment of diabetes and associated metabolic diseases, the enhancement of adiponectin expression and

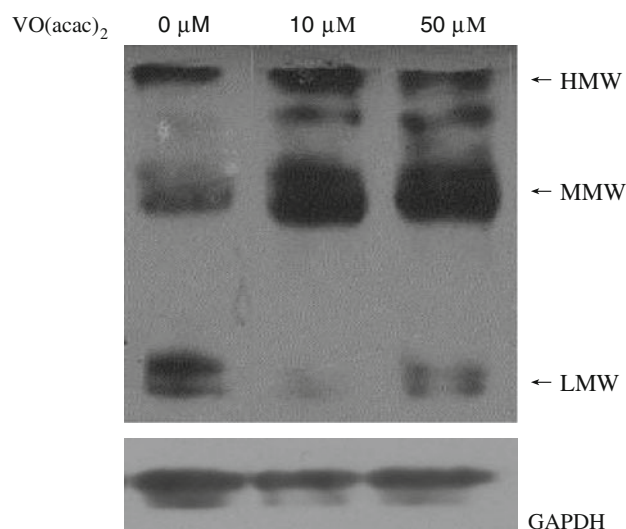


Fig. 4 Effects of VO(acac)₂ on multimerization of adiponectin. Differentiated rat adipocytes were stimulated with 10 μM VO(acac)₂ for 24 h, and then protein samples were subjected to 7.5 % nondenaturing polyacrylamide gel electrophoresis and Western blotting as described in “[Materials and methods](#).” *HMW* high molecular weight, *LMW* low molecular weight, *MMW* medium molecular weight

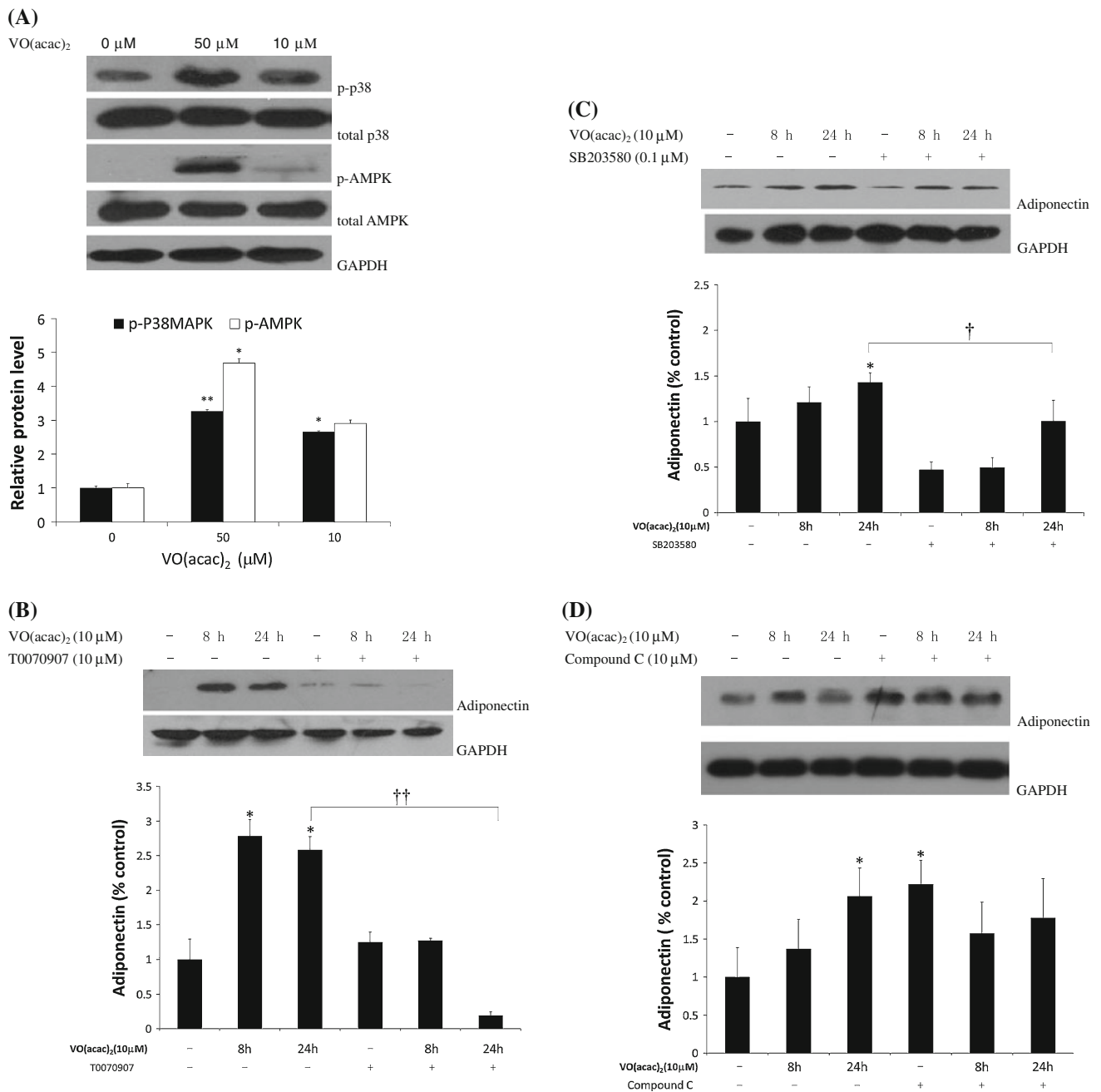


Fig. 5 Effects of VO(acac)₂ on p38 mitogen-activated protein kinase (MAPK), PPAR γ , and AMP-activated protein kinase (AMPK) signaling related to adiponectin. **a** Effects of VO(acac)₂ on the p38 MAPK and AMPK phosphorylation. **b** Effects of T0070907 on vanadium-stimulated expression of adiponectin. **c** Effects of SB203580 on vanadium-stimulated expression of adiponectin. **d** Effects of compound C on vanadium-stimulated expression of

adiponectin. Differentiated rat adipocytes were stimulated with 10 μ M VO(acac)₂ and/or specific inhibitors for 8 or 24 h. Then total protein extracts were analyzed by Western blotting. Quantitative data are the average of three independent experiments. *Two asterisks* $P < 0.01$, *single asterisk* $P < 0.05$ versus controls; *single dagger* $P < 0.05$, *two daggers* $P < 0.01$ versus corresponding treatments in the absence of inhibitors

multimerization has been regarded as a promising therapeutic approach [24]. Previously, vanadyl sulfate was reported to stimulate adiponectin production and long-term release in 3T3-L1 adipocytes [13]; however, there was a remaining question in this study that PI3K activation (the

direct effect of PTP1B inhibition by the vanadium compound) was not related to stimulation of adiponectin by vanadyl sulfate, but Akt (the downstream target of PI3K) inhibition reduced the expression of adiponectin. Because of the observation of the activation of PPAR γ in pancreatic

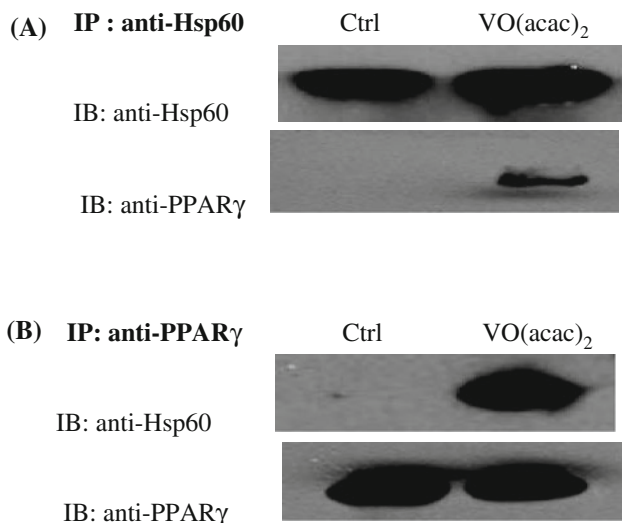


Fig. 6 VO(acac)₂ induced interaction between heat shock protein 60 kDa (*Hsp60*) and PPARγ in differentiated adipocytes. Cells were incubated with 10 μM VO(acac)₂ for 24 h. Then, the total protein extracts were analyzed by immunoprecipitation (IP) using anti-Hsp60 (a) or anti-PPARγ (b) capture antibodies. *Ctrl* control

β cells [25], we investigated the effects of vanadyl complexes, VO(acac)₂, on PPARγ and adiponectin in differentiated rat adipocytes.

The results indicate that vanadyl complexes upregulated PPARγ and adiponectin for the following reasons.

First, in the nontoxic concentration range of VO(acac)₂ (Fig. 1), the complexes increased the protein levels of PPARγ and adiponectin (Fig. 3) and enhanced adiponectin multimerization (Fig. 4) of adipocytes. This was further confirmed by a preliminary in vivo test (Fig. S1) showing that vanadyl complexes significantly increased the expression of adiponectin and PPARγ in white adipose tissue of the *db/db* mouse, a recognized model of type 2 diabetes mellitus, at a dosage of 0.04 mmol/kg/day.

Adiponectin is known to be synthesized by adipocytes as a 30-kDa monomer and then automatically self-associates into larger structures [26]. Recent studies have shown that the high molecular weight isoforms may be the most biologically active form [27–29]. Many of the pharmacological effects of adiponectin (e.g., insulin sensitivity,

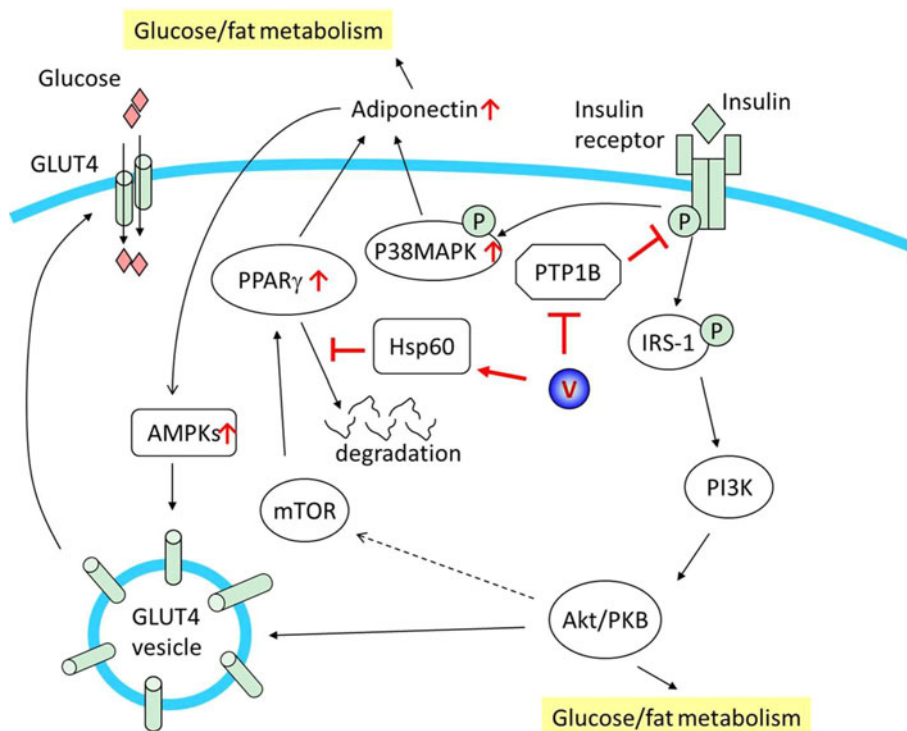


Fig. 7 The hypothesis of the mechanism of VO(acac)₂ upregulating PPARγ and adiponectin expression in differentiated rat adipocytes. The proposed new pathway is that vanadyl ions bind to Hsp60 and induce PPARγ–Hsp60 interaction, leading to inhibition of PPARγ degradation and an increase of the PPARγ level. Subsequently, the increase of PPARγ activity results in adiponectin expression and downstream events, i.e., activation of AMPK and glucose transporter 4 (*GLUT4*) translocation. The related pathways include inhibition of protein tyrosine phosphatase 1B (*PTP1B*) by vanadium species

[VO(acac)₂ or vanadate, the oxidized product via the cellular redox system], which leads to increased phosphorylation of insulin receptor substrate 1 (*IRS-1*) and activation of the phosphatidylinositol 3-kinase (*PI3K*)–Akt/protein kinase B (*PKB*) cascade, and/or enhanced p38 MAPK activation by phosphorylation. The effects of vanadium are in red. Red arrow activation, red T inhibition, solid black arrow proposed pathway, dashed black arrow speculative pathway, *mTOR* mammalian target of rapamycin

glucose uptake, lipid catabolism, triglyceride clearance, weight loss, and cardiovascular protection) are the same as the antidiabetic effects of vanadium compounds and explain one previous observation of stimulation of AMPK activation in L6 myotubes [30].

Second, as shown in Fig. 5b–d, VO(acac)₂-induced expression of adiponectin was significantly inhibited by T0070907 (PPAR γ inhibitor) or SB203580 (p38 MAPK inhibitor), but was not affected by compound C (AMPK inhibitor). Since p38 kinase, a putative regulator of PPAR γ activation [31], was activated by vanadium treatment (Fig. 5a), this suggests that stimulation of adiponectin by vanadyl complexes was through the PPAR γ pathway involving p38 MAPK. PPAR γ was shown to increase the adiponectin level by transactivating adiponectin gene expression [28] and/or posttranscriptional regulations [32]. Since VO(acac)₂ did not change PPAR γ or adiponectin mRNA levels (Fig. 2), vanadyl complexes likely to modulate adiponectin through PPAR γ via a posttranscriptional mechanism, which needs further investigation.

Third, in addition to ligand-dependent activation, PPAR γ activity was regulated by transactivation of gene expression and posttranscriptional modifications. The p38 kinase has been proposed to modulate PPAR γ activity [31]; however, we did not observe p38 influencing PPAR γ expression. Further, Akt/mammalian target of rapamycin (mTOR) signaling was recently found to regulate PPAR γ levels [33, 34], possibly through the Akt–tuberous sclerosis 1/2–Rheb–mTOR cascade, and thus the inhibition of Akt may decrease PPAR γ levels and subsequently adiponectin levels as observed by Seale et al. [13].

As shown in Figs. 2 and 3, VO(acac)₂ increased PPAR γ protein levels, but the PPAR γ mRNA levels were not changed. These results suggest that vanadyl complexes modulated PPAR γ levels by decreasing PPAR γ degradation while promoting the posttranscriptional modifications of PPAR γ . To investigate this possible mechanism, we explored vanadium-induced protein interactions of PPAR γ by co-immunoprecipitation. The results (Fig. 6) indicated that with vanadyl complex treatment, PPAR γ was bound to Hsp60. Hsp60 is a major molecular chaperone protein regulating protein folding and stability [35] and was found to bind tightly to vanadyl ions [36]. It is possible that the Hsp60–PPAR γ interaction induced by VO²⁺ may prevent PPAR γ protein degradation. Nevertheless, further experiments elucidating the mechanism of vanadyl ion–Hsp60–PPAR γ interactions are required.

In conclusion, we have provided the first evidence that vanadyl complexes may upregulate PPAR γ and thus simulate adiponectin expression and multimerization. On the basis of our results and the relevant literature, a hypothetical mechanism for regulation of PPAR γ and adiponectin by vanadyl complexes has been proposed (Fig. 7).

The signal transductions due to PTP1B inhibition by vanadium species cross-talk with the PPAR γ and adiponectin regulation and possibly cooperate in the action of insulin enhancement. The results provide new insights into the mechanisms of the antidiabetic action of vanadium compounds.

Additionally, it is worthwhile noting that vanadyl complexes modulate expression and activity of PPAR γ and adiponectin in a way different from that of thiazolidinedione drugs, e.g., rosiglitazone. Rosiglitazone is a potent PPAR γ agonist, but did not upregulate PPAR γ protein (Fig. 3d). This observation is in agreement with studies that demonstrated vanadium compounds cause weight loss rather than weight gain, as occurs as an important side effect of rosiglitazone [37, 38]. Therefore, further studies on vanadium–thiazolidinedione interactions in the action of stimulating PPAR γ and adiponectin could lead to the discovery of novel antidiabetic vanadium compounds and/or organic PPAR γ ligands.

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