

Adipogenic action of vanadium: a new dimension in treating diabetes

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Abstract Vanadium is a well known anti-diabetic agent which mimics most of the actions of insulin on mature adipocytes. We report here the effect of vanadium on proliferation and differentiation of 3T3-L1 preadipocytes. Like insulin, vanadium treatment leads to increased proliferation as evidenced by H^3 thymidine uptake studies and differentiation of 3T3-L1 cells into adipocytes as evidenced by oil-red-O staining. Adipogenic potential of vanadium can be attributed to CREB activation, as documented by phospho-CREB antibody staining. This adipogenic potential is of significance in an in vivo scenario as the new adipocytes are likely to be insulin sensitive as against resistant existing mature adipocytes and thus indirectly may help in reduction of insulin resistance. Till today decrease in insulin resistance by vanadium treatment has been mainly attributed to its potential to inhibit PTP-1B, however the present study opens a new dimension in vanadium treatment for diabetes due to its novel role in adipogenesis.

Keywords Vanadium · 3T3-L1 cells · Adipogenesis · CREB activation

Abbreviations

DMEM	Dulbecco's modified eagle medium
NBCS	New born calf serum
CREB	cAMP response element binding protein
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

Introduction

Vanadium is a well-known antidiabetic metal under investigation since 1980s when its insulin-mimetic effects had been demonstrated on isolated adipocytes (Dubyak and Kleinzeller 1980). Since then several researchers have demonstrated its usefulness in treatment of type 1 as well as type 2 diabetes using various animal models as well as limited human trials (Beliaeva et al. 2000). Srivastava and Mehdi (2005) have very well summarized various actions of vanadium in both in-vivo and in-vitro models. Like insulin, vanadium has been shown to increase lipogenesis and glycogenesis as well as inhibit lipolysis in adipocytes. Moreover it has been shown to activate glucose uptake in primary as well as 3T3-L1 differentiated adipocytes indicating its insulin enhancing and/or sensitization potential. These metabolic effects of vanadium are attributed to its ability to inhibit tyrosine phosphatases and activation of several key components of insulin-signaling pathways that include the mitogen-activated-

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protein kinases (MAPKs) extracellular signal-regulated kinase 1/2 (ERK1/2) and p38MAPK, and phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) (Mehdi et al. 2006).

Insulin is known to play a key role in carbohydrate and lipid metabolism. Moreover insulin also acts as a mitogenic agent for a number of cells and is responsible for differentiation of preadipocytes into mature adipocytes (Guller et al. 1988). Adipogenesis is a complex process and is initiated by activation of specific initial adipogenic transcription factors like peroxisome proliferator-activated receptor- γ (PPAR γ 2), CCAAT/enhancer binding proteins (CEBP α , β and δ), adipocyte determination and differentiation factor-1 or sterol regulatory element binding protein-c (ADD1/SREBP-1c), GATA-binding transcription factors (GATA-2 and GATA-3) and cAMP response element binding protein (CREB), which in turn triggers a cascade of activation of several other transcription factors and expression of adipogenic genes like fatty acid-binding protein (FABP), aP2, fatty acid synthase (FAS) etc. (Gregoire 2001). Composition of standard adipogenic cocktail is serum-containing medium supplemented with supraphysiological concentrations of insulin, dexamethasone, and isobutylmethylxanthine (IBMX) thus highlighting the involvement of the insulin/IGF-1, glucocorticoid, and cAMP signaling pathways in the adipocyte differentiation process (Gregoire et al. 1998). The transcription factor CREB is constitutively expressed prior to and during adipogenesis, and is upregulated by conventional differentiation-inducing agents such as insulin and IBMX (Klemm et al. 2001). Overexpression of a constitutively active CREB in 3T3-L1 preadipocytes is necessary and sufficient to initiate adipogenesis, whereas overexpression of a dominant-negative CREB alone blocks adipogenesis in cells treated with conventional differentiation-inducing agents (Reusch et al. 2000).

There are many studies reporting the effect of antidiabetic agents on the process of insulin-induced adipogenesis. Mostly these agents like troglitazones (Tchoukalova et al. 2000; Tafuri 1996) are known to accelerate the process. So far, effect of vanadium on mature adipocytes has been demonstrated however its potential to bring about adipogenesis has not been examined. Since vanadium has insulin-mimetic activity we hypothesized that it might be able to differentiate 3T3-L1 preadipocytes into mature

adipocytes. In this study we demonstrate for the first time potential of VOSO₄ to induce differentiation of 3T3-L1 preadipocytes to adipocytes independent of insulin. Moreover it is able to activate CREB in 3T3-L1 cells indicating the similarity of signaling pathway between VOSO₄ and insulin.

Materials and methods

Materials

Tissue culture grade plastic wares from Falcon, DMEM and NBCS from Gibco, Insulin and Oil-red-O from Sigma, rabbit phospho-CREB antibody and FITC-conjugated anti-rabbit antibody from Santacruz, H³thymidine from BRIT, India and VOSO₄ was a kind gift from Dr S B Padhye, Professor and Head, Department of Chemistry, University of Pune, Pune, India.

Thymidine uptake assay

3T3-L1 cells were obtained from Cell Repository of National Centre for Cell Science, Pune, India and were regularly maintained in DMEM with 10% NBCS and incubated at 37°C with 5% CO₂ atmosphere. 5×10^3 cells/well were seeded in 96-well-plate and allowed to attach and grow for 24 h. The cells were then stimulated with different concentrations of vanadyl sulfate (1–50 μ M) and 5 μ g/ml insulin for next 48 h. About 1 μ Ci H³thymidine was added to each well 12 h before the end of the 48 h incubation. The cells were then harvested by Filtermate harvester, Packard, Bioscience. The incorporation of H³thymidine into DNA was determined by using microplate scintillation and luminescence counter, Topcount. NXT (Packard, Bioscience).

Differentiation assay

5×10^3 cells/well were seeded in 96-well-plate and allowed to attach and grow till confluency in DMEM supplemented with 10% NBCS. The confluent cells were then stimulated with DMEM supplemented with 10% FCS and 10 μ M vanadyl sulfate or 5 μ g/ml

insulin and cells were observed every 24 h under light microscope to check the fat droplet formation, unstimulated cells were kept as a negative control and cells stimulated with standard adipogenesis cocktail (0.5 mM IBMX + 1 μ M dexamethasone + 5 μ g/ml insulin) were used as a positive control. Media was changed every 48 h during the differentiation assay and each time the agents were added, in the positive control set only the insulin treatment was repeated. Finally, after 6 days the cells were stained with Oil-red-O to verify the extent of adipogenesis. Briefly, stock solution of oil-red-O stain was prepared by dissolving 300 mg stain in 100 ml of 99% isopropanol. To prepare working dilution, three parts of stain solution was mixed with two parts of deionized water. Working solution was filtered before use. For staining cells were gently rinsed with PBS twice, fixed with 10% formalin for one hour at room temperature. After fixation, cells were given two gentle washes with PBS and incubated in working solution of oil-red-O for one hour at 37°C. Stain solution was removed and cells were washed with PBS before microscopic observation.

CREB activation

3T3-L1 cells were grown on coverslips so as to use them for immunostaining. The cells were serum starved for 16 h and then stimulated with 10 μ M vanadyl sulfate or 5 μ g/ml insulin for different time intervals (10, 30, 60 and 120 min) and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.1% triton-X-100 and then stained with rabbit-anti-phospho-CREB antibody (Santacruz). The cells were counter stained with FITC conjugated anti-rabbit antibody (Santacruz) and visualized under confocal microscope.

Results

Cell proliferation

It is seen from Fig. 1, that VOSO₄ increases 3T3-L1 cell proliferation as evidenced by H³thymidine incorporation in a dose dependent manner. At 10 μ M concentration cell proliferation rate reaches the maximum which was comparable to insulin.

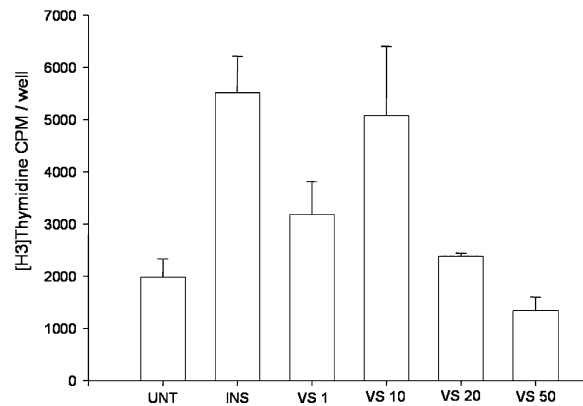


Fig. 1 H³thymidine uptake in 3T3-L1 cells by VOSO₄. The graph compares H³thymidine incorporation in 3T3-L1 cells upon stimulation by 5 μ g/ml insulin (INS) and various concentrations (1–50 μ M) of vanadyl sulfate (VS1-VS50) with unstimulated cells (UNT). Values represent mean \pm SD

Further rise in concentration led to drop in H³thymidine uptake which could be due to toxic effect of the agent.

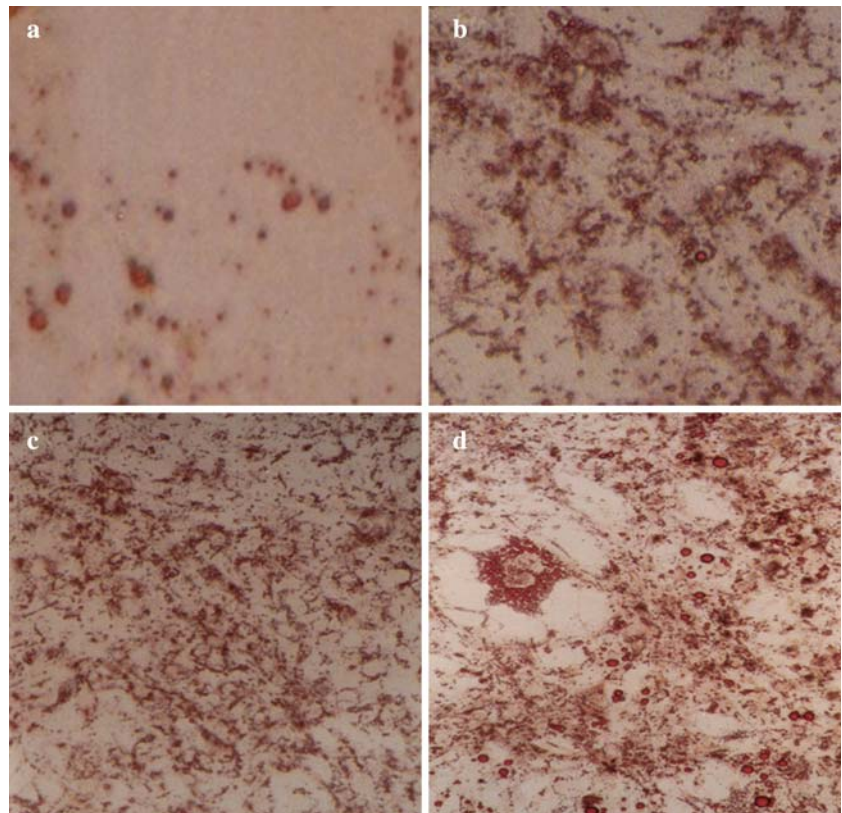
Adipogenesis of 3T3-L1

The rate and extent of differentiation of 3T3-L1 fibroblasts into adipocytes depends upon a number of factors like cell confluency, passage number and the signal from the differentiating agent. The differentiation can be easily monitored under light microscope as cell morphology changes from spindle shape to spherical and cells initiate accumulating several fat-droplets which can be stained by Oil-red-O. As depicted in Fig. 2, extent of differentiation of 3T3-L1 fibroblasts into adipocytes upon treatment with 10 μ M VOSO₄ for 6 days was found to be comparable to the cells treated with 5 μ g/ml insulin as evidenced by oil-red-O staining. Unstimulated cells exhibited very little oil droplet formation, while cells treated with the standard cocktail appear to be fully differentiated.

CREB activation

CREB is one of the initial transcription factors of adipogenesis. Figure 3 shows immunostaining of insulin (5 μ g/ml) and VOSO₄ (10 μ M) stimulated 3T3-L1 cells with phospho-CREB antibody. VOSO₄

Fig. 2 Adipogenesis in 3T3-L1 cells by VOSO_4 3T3-L1 cells were kept as untreated control (a) and were treated with standard adipogenic cocktail (b), 10 μM VOSO_4 (c) and 5 $\mu\text{g/ml}$ insulin (d) for 6 days. The cells were then fixed and stained with oil-red-O to see the extent of adipogenesis (300 \times magnification)



led to activation and translocation of CREB into nucleus which was comparable to insulin.

Discussion

Present study deals with the effect of VOSO_4 on proliferation and differentiation of 3T3-L1 cells. Smith BM (1983) has reported that vanadium ion stimulates DNA synthesis in a concentration dependent manner likewise we have earlier demonstrated dose-dependent mitogenic action of vanadium on CHO cells (Shukla et al. 2004). Similarly, in the present study we have obtained maximum proliferation of 3T3-L1 cells at 10 μM concentration of VOSO_4 after which there seems to be toxic effect of the agent (Fig 1). Treatment of 3T3-L1 cells with 10 μM VOSO_4 for 6 days resulted in differentiation of the fibroblasts into adipocytes to an extent comparable to insulin. Differentiation of 3T3-L1 cells into adipocytes by VOSO_4 can be attributed to its potential to activate CREB similar to insulin

(Klemm et al. 1998) however the possibility of activation of other related transcription factors can not be excluded. Immunofluorescence staining demonstrated that the extent of CREB activation by vanadium was comparable to insulin however the signal lasted longer (Fig 3). This extended signal is in corroboration with another study wherein authors demonstrated prolongation of insulin signal by vanadium (Theberge et al. 2003). Our data clearly shows for the first time that vanadium has adipogenic potential in addition to known insulin-mimetic effects.

Insulin and other agents like troglitazone, which have been proved to be adipogenic in vitro have been shown to cause weight gain in an in vivo scenario (Fonseca 2003). On the contrary, upon in vivo treatment no weight gain has been observed in case of VOSO_4 or other vanadium compounds. This paradox between our in vitro observation and in vivo findings can be explained on the basis of the fact that there is a decrease in food intake upon vanadium treatment (Wang et al. 2001) whereas $\text{PPAR}\gamma$

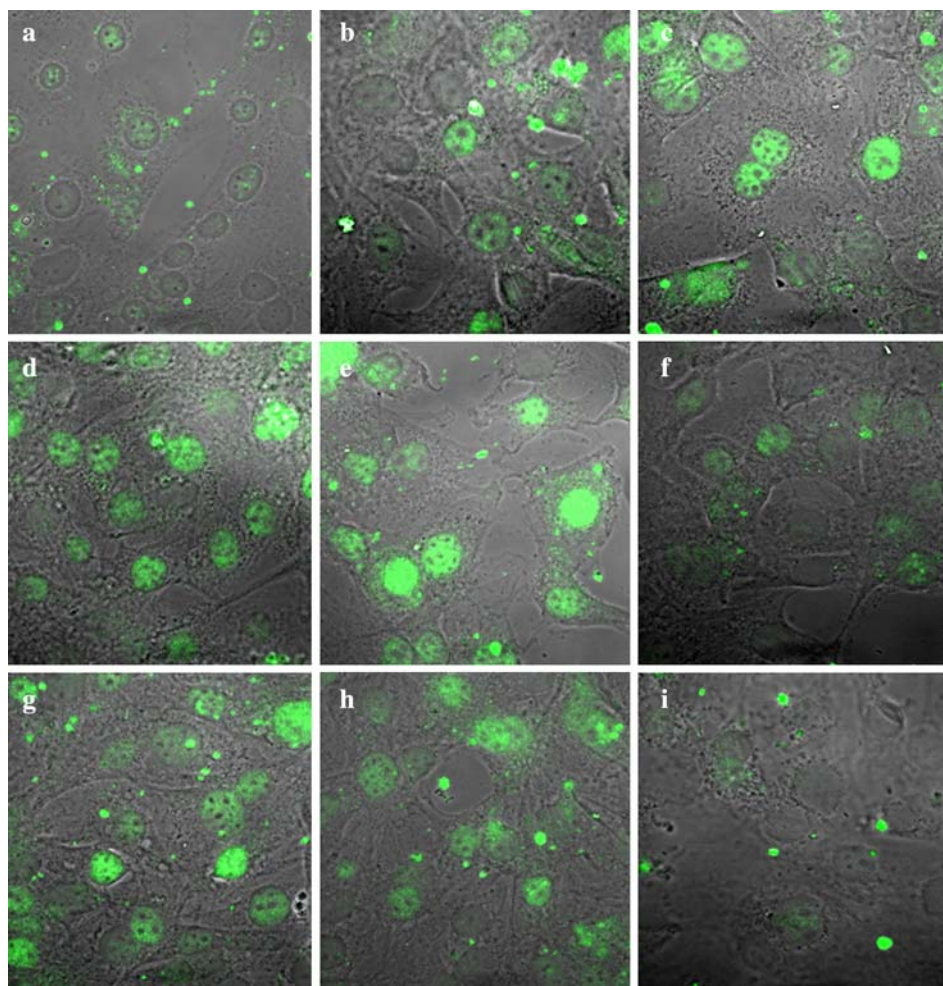


Fig. 3 CREB activation by VOSO_4 Serum starved 3T3-L1 cells were kept as untreated control (**a**) and were stimulated with $10 \mu\text{M}$ VOSO_4 (**b–e**) or $5 \mu\text{g/ml}$ insulin (**f–i**) for 10, 30, 60 and 120 min respectively. The cells were then fixed and

stained with p-CREB antibody and counter stained with FITC-conjugated secondary antibody as described in *material and methods*. The cells were imaged in confocal microscope

agonists are known to cause increased food consumption probably via suppression of ob gene (Sinha et al. 1999). This unique and exclusive feature, induction of adipogenesis without weight gain, makes vanadium a drug of choice for treating obese diabetic subjects as it serves dual purpose of new adipocytes formation without weight gain which is much desired. However, vanadium compounds are known to be associated with various side effects especially gastrointestinal disorders (Boden et al. 1996; Goldfine et al. 2000). Organic salts of vanadium are much less toxic than the parental inorganic salts (Srivastava 2000) and we have previously demonstrated the

safety of vanadium-quercetin conjugate (BQOV) (Shukla et al. 2006). The adipogenic potential of vanadium is not just restricted to the salt VOSO_4 , since BQOV was also able to activate CREB and induce adipogenesis in 3T3-L1 preadipocytes (data not shown). Thus less toxic organic vanadium derivatives can be further explored for their adipogenic potential.

Our present finding clearly demonstrates the adipogenic action of vanadium (VOSO_4) which has not been reported previously. The in vitro data obtained on the role of VOSO_4 in 3T3-L1 cell proliferation by H^3 thymidine uptake studies indicate

dose dependent increase in cell number. Similarly VOSO₄ treatment continuously for 6 days resulted in conversion of almost 70% of fibroblastic spindle shaped preadipocytes into spherical adipocytes loaded with fat droplets in the cytoplasm as confirmed by Oil-red-O positive staining; following CREB activation pathway. In this respect VOSO₄ mimics insulin, however VOSO₄ differs from insulin as far as weight gain is concerned. Taken together our study suggests a novel and desirable way of employing vanadium treatment for diabetes due to its role in adipogenesis.

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