Molecular mechanism of bis(maltolato)oxovanadium(IV)-induced insulin signaling in 3T3-L1 and IM9 cells: impact of dexamethasone

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Abstract

The organovanadium compound bis(maltolato)oxovanadium(IV) (BMOV) enhanced the tyr-phosphorylation of major upstream insulin signaling proteins including the vital site-specific phosphorylation of insulin receptor β (IRβ) in IM9 and 3T3-L1 cells in dose- and time-dependent manners more efficiently than insulin. Nevertheless, insulin in general had a synergistic impact on those phosphorylations in both cell lines, while its presence was obligatory to induce Tyr972-phosphorylation of IRβ in IM9 cells at 18-h treatment with BMOV. However, prolonged exposure of cells to BMOV caused depletion in IR level and using IM9 cells we found that this event was counteracted by insulin, where monensin, a monocarboxylic acid ionophore made an additive impact, suggesting that a novel mechanism is being involved in the recycling of internalized IR in BMOV-treated cells. On the other hand, dexamethasone elevated the IR level in both cell lines. However, no correlation was found between the cellular content and the degree of phosphorylation of IRβ in cells receiving combined treatment of BMOV, and dexamethasone with short insulin post-exposure. BMOV also induced the phosphorylation of Thr308 and Ser473 of Akt in both cell lines receiving insulin post-treatment, while dexamethasone decreased those phosphorylations. However, this activation/deactivation of Akt did not correlate with the phosphorylation status of Ser² of glycojen synthase kinase (GSK)-β and Raf respectively. Taken together, it is conceivable that BMOV and/or dexamethasone modulate insulin signaling by acting differentially on the components of the insulin signaling network. We also consider that the observed dexamethasone-mediated modulation of insulin receptor kinase in BMOV-treated 3T3-L1 cells probably occurs through the activation/deactivation of some mechanism which needs further studies for proper characterization.

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Introduction

Initiation of insulin signaling occurs through the binding of ligand to insulin receptor (IR), leading to the activation of the kinase domain of the latter in association with autophosphorylation of specific tyrosine residues (White & Kahn 1994, Saltiel & Pessin 2002). This in turn results in recruitment and phosphorylation of a number of cytosolic insulin receptor substrates (IRSs and SHc), which then mediate the cellular responses important for insulin action by docking src homology 2 (SH-2) domain-containing signaling molecules (White 2003), ultimately leading to the activation of two major signaling cascades. In one cascade, the interaction of IRS-1 with Grb-2-SOS complex triggers the activation of the mitogen-activated protein kinase (MAPK) pathway composing of Ras–Raf–MEK(mitogen-activated protein kinase (MAPK) kinase)–ERK(extracellular signal-regulated protein kinase) protein network that finally accounts for mediating the mitogenic and growth-promoting effects of insulin (White & Kahn 1994, White 2002). The second cascade which initiates with the activation of PI3-kinase by IRS complex follows a series of events along with the stimulation of Akt. This in turn activates several effector molecules that finally contribute to the metabolic effects of insulin at the glucose transport, GLUT-4 translocation, and the synthesis of glycogen and protein (White 2002).

Accordingly, the major sites of autophosphorylation in the protein tyrosine kinase (PTK) domain of the β-subunit of IR were found to be Tyr¹¹³⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ residues (Ebina et al. 1985) located within the PTK activation segment (White et al. 1988). Autophosphorylation of all three sites is necessary for maximal activation of IR by insulin (White et al. 1988) and is thought to be mediated via stabilization of the active conformation of kinase domain of IR. Following this activation, phosphorylation occurs on a tyrosine (Tyr⁹⁷²) located in the Asn–Pro–Glu–Tyr (NPET) motif of the juxtamembrane region of IRβ. Phosphorylation of the above motif plays an important role in binding of IRβ to IRS-1 and recruitment of p85 subunit of PI-3 kinase or in mediating insulin’s
metabolic and mitogenic effect, and was found to be necessary for Shc phosphorylation (Berhanu et al. 1997). On the other hand, it has been reported that the termination of insulin signaling mediates through inactivation of the IR by dephosphorylation of the Tyr1158/Tyr1162/Tyr1163 residues (Tonks 2003) in which protein tyrosine phosphatase (PTPase) 1B (PTP 1B) plays an important role (Boute et al. 2003). Additionally, several lines of evidence have also shown that PTPases including PTP 1B negatively regulates insulin signaling and plays an important role in conferring insulin resistance in different models (Ahmad et al. 1995, Elchebly et al. 1999, Klamann et al. 2000, Tonks 2003). Accordingly, the cellular distribution of PTP 1B has been described in rat adipocytes (Calera et al. 2000) and overexpression of this PTPase in 3T3-L1 adipocytes was found to attenuate insulin-induced IR, IRS-1 phosphorylation as well as PI3-kinase and MAPK activation (Venable et al. 2000). Owing to the above facts, inhibition of PTP 1B has been proposed to be a potent therapeutic target for type II diabetes.

The insulinomimetic activity of vanadium compounds has been well documented (reviewed by Srivastava & Mehdi 2005, Mehdi et al. 2006) and is believed to be mediated via inhibition of PTPases (Posner et al. 1994, Tsiani & Fantus 1997, Srivastava & Mehdi 2005, Mehdi et al. 2006). Organovanadate has been shown to initiate insulin signaling in 3T3-L1 adipocytes by potentiating tyrosine phosphorylation of IR (Ou et al. 2005, Basuki et al. 2006), while autophosphorylation of insulin receptor kinase (IRK) by pervanadate in rodent adipocytes was found to be mediated via PTPases inhibition (Shisheva & Shechter 1993). Moreover, organovanadium compounds like bis(maltolato)oxovanadium(IV) (BMOV) have been described as being more potent than inorganic vanadate in inhibiting PTPase activity and enhancing cellular tyrosine phosphorylation of IR (Mehdi & Srivastava 2005). However, the insulinomimetic effect of BMOV has not been investigated in detail at molecular level on insulin-target cells especially under a prolonged exposure to this vanadate. Besides, no attempt has so far been made to address the impact of this compound on human IM9 lymphocytes that are nontarget cells of insulin but express IR on their membrane surfaces. The above evaluations might have significant contribution in identifying the targets of BMOV in the network of major insulin signaling proteins of cells. Furthermore, at the molecular level, there is a lack of information addressing whether exposure to BMOV can cause the activation of kinase domain in association with autophosphorylation of Tyr1158, Tyr1162, and Tyr1163 residues of IRβ in IM9 cells. Besides, the impact of BMOV on the phosphorylation of NPEY motif of IRβ which plays a vital role in insulin signaling has not yet been elucidated in both insulin target cells and IM9 lymphocytes. Additionally, in depth studies have not been conducted so far to correlate the extent of phosphorylation of major insulin signaling proteins at different time intervals of BMOV treatment in order to properly understand the mode of action of this compound. Finally, detailed information is unavailable on the effect of insulin on BMOV-induced phosphorylation of insulin signaling proteins.

Like insulin, BMOV has been found to cause downregulation of cell surface IR in IM9 cells (Torossian et al. 1988), although the underlying molecular mechanism is poorly understood and the impact of insulin on this particular molecular event has not yet been evaluated. In order to focus on this issue more deeply, we have examined the effect of monocarboxylic ionophore monensin, an inhibitor of receptor recycling (Whittaker et al. 1986), on the soluble IR pool of IM9 cells receiving an exposure to BMOV for 18 h and a brief insulin treatment for 15 min. Consequently, we have also checked whether treatment of dexamethasone which has been shown to increase the cellular content of IR in 3T3 fibroblasts and IM9 cells (Fantus et al. 1982, Knutson et al. 1982, McDonald & Goldfine 1988) can encounter the above negative impact of BMOV on IR level in both 3T3-L1 and IM9 cells and thereby resulting in an augmentation in the phosphorylation of IR with subsequent modulation in the downstream insulin signaling molecules, including Akt, GSK, and Raf.

In order to understand the molecular mechanism underlying insulinomimetic activity of BMOV more precisely, a thorough investigation has been carried out in the present study to evaluate the impact of this compound on the phosphorylation of major insulin signaling proteins including vital site-specific tyr-phosphorylation of IRβ in 3T3-L1 and IM9 cells. Effort has also been made to address the probable molecular mechanism of BMOV-induced downregulation of IR, and the role of insulin in this process. Finally, the impact of dexamethasone on the BMOV-modulated phosphorylation of major insulin signaling proteins has been evaluated in both cell lines. On the basis of our findings, it appears that BMOV and/or dexamethasone-mediated modulation in insulin signaling occurs through the differential action of these two compounds on the components of insulin signaling pathway. Our data also suggest that an increase in BMOV-induced phosphorylation of IRβ in 3T3-L1 cells by dexamethasone probably mediates through some mechanism which requires further studies for proper identification.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS),
antibiotic/antimycotic agent, and penicillin/streptomycin were purchased from Gibco. BMOV was kindly provided by Prof. Cheong-Soo Hwang (Dankook University, Korea). 3-Isobutylole-1-methylxanthine (IBMX), N-hydroxy-succinimide (NHS), 1-ethyl-3-(dimethylaminopropyl)carbodiime (EDAC), dexamethasone, insulin, monensin, protease inhibitor cocktail, dithiothreitol (DTT), sodium orthovanadate (Na3VO4), phenylmethanesulfonyl fluoride (PMSF), p-nitrophenyl phosphate (pNPP) and the most other chemicals were procured from Sigma, while AGL-2263 and Protein G plus/Protein A agarose suspension were obtained from Calbiochem (Darmstadt, Germany). Polyvinyl difluoride (PVDF), nitrocellulose blotting membranes, and western blotting kit were purchased from Amersham Biosciences, whereas Laemmli sample buffer and other reagents for electrophoresis were from Bio-Rad.

Antibodies

The anti-phosphotyrosine (clone 4G10), anti-phospho-IR/IGFR (Tyr1158/Tyr1162/Tyr1163), and anti-IRS-1 antibodies were procured from Upstate (Lake Placid, NY, USA), whereas anti-phospho-IR (pTyr1172) and anti-PTP 1B antibodies were from Sigma and Calbiochem respectively. Anti-IRβ (C-19) and anti-IRS-3 (S-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt (Thr308 and Ser273), anti-Akt, anti-phospho-GSK-3β (Ser21), and anti-phospho-Raf (Ser259) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). CY3-conjugated anti-mouse or anti-rabbit monoclonal immunoglobulin G (IgG) secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell cultures and treatments

3T3-L1 mouse fibroblasts and human B lymphoblast IM9 cells were obtained from American Type Cell Culture (ATCC, Manassas, VA, USA). The former cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin in a completely humidified atmosphere with 95% air and 5% CO2 at 37 °C. While the latter cells were cultured under a similar condition in suspension in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic/antimycotic agent. After being reached to confluency, differentiation of the fibroblasts was induced by treating the cells with DMEM containing 0.5 mM IBMX, 0.25 μM dexamethasone, 0.86 μM insulin, and 10% FBS for 48 h, followed by another 48-h incubation of cells in DMEM supplemented with insulin (0.86 μM) and 10% FBS. Cells were then fed with DMEM containing 10% FBS every alternate day for the next 4–10 days and finally used for the experiments when around 90% of fibroblasts were transformed into matured adipocytes except for insulin-binding studies where a large fraction of undifferentiated cells was employed.

The cells were exposed accordingly to BMOV, dexamethasone, monensin, and AGL-2263 either alone or in combination, with or without receiving insulin post-treatment, depending on the nature of experiment as described in the figure legends. Appropriate controls were also maintained in each experiment for comparison.

Immunoprecipitation and western blotting

Following the desired treatments, cells were washed thoroughly with PBS (pH 7–4) and lysed in a buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7–4) containing 1 mM DTT, 30 mM Na2P2O7, 10 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 20 μl/ml protease inhibitor cocktail, and 1% Triton X-100 (v/v)) for 20 min at 4 °C followed by ultrasonication. The homogenate was centrifuged at 14,000 g at 4 °C for 20 min to remove the insoluble materials and the supernatant was stored in aliquots at −80 °C until used. After being denatured at 95 °C in Laemmli sample buffer containing 100 mM DTT, the lysate proteins were resolved by SDS–PAGE. While the samples being subjected to immunoprecipitation prior to electrophoresis were incubated with the specific antibodies for 2 h at 4 °C with constant rotation. Following this, 30 μl protein G plus/protein A agarose beads were added, mixed thoroughly, and incubated for another 2 h at 4 °C with constant rotation. The beads were sedimented by centrifugation at 14,000 g at 4 °C for 5 min, and washed with cold lysis buffer. The proteins bound to the beads were eluted by boiling for 5 min in Laemmli sample buffer containing 100 mM DTT and were subjected to SDS–PAGE. Eventually, the gels were electrophoretically transferred to either PVDF or nitrocellulose blotting membranes using semidry apparatus (Bio-Rad). Following blocking with the appropriate agents of Amersham, blots were incubated with specific primary antibodies as mentioned in the figure legends. Immunodetection was performed according to the enhanced chemiluminescence (ECL) protocol and reagents of Amersham, using horseradish peroxidase-conjugated appropriate anti-IgG as secondary antibody. Band intensities were quantified by densitometric analysis using ChemiDoc system (Bio-Rad) with the help of Quantity One 1-D analysis software (Bio-Rad). For most of the cases, the membranes were stripped in buffer (62.5 mM Tris–HCl (pH 6.7) containing 2% SDS and 100 mM β-mercaptoethanol) and reprobed several times to analyze the binding of multiple antibodies sequentially.
Determination of impact of BMOV on PTP 1B-related phosphatase activity of IM9 cells

Suspensions of IM9 cells were divided into groups and subjected to vanadate treatment by exposing to 100 μM BMOV for either 2 or 18 h. The corresponding controls received vehicle only, while the cells of untreated group were devoid of any treatment. Following desired treatments, cells were washed thoroughly with PBS (pH 7.4) and lysed in buffer A (50 mM HEPES (pH 7.4) containing 1 mM DTT, 1 mM EDTA, 30 mM Na4P2O7, 10 mM NaF, 1 mM PMSE, 20 μl/ml protease inhibitor cocktail, and 1% Triton X-100 (v/v)) for 20 min at 4°C followed by ultrasonication. The homogenate was centrifuged at 14000 g at 4°C for 20 min and the resultant supernatant was used as the source of PTPases.

Aliquots containing equal amount of supernatant protein (1-5 mg) were incubated with anti-PTP 1B antibody at 4°C overnight under constant rotation and then subjected to immunoprecipitation using protein G plus/protein A agarose beads as previously described. The immunoprecipitates of control and BMOV-treated cells were washed three times with buffer B (20 mM Tris (pH 7.5) containing 10 mM DTT) and used directly for the determination of PTPase activity. While after washing, the immunoprecipitates of untreated cells were incubated with 200 μl buffer B supplemented with 1 nM–1 μM BMOV for 30 min at 37°C under constant rotation. Following this, the beads were collected by centrifugation and subjected to PTPase assay. The measurement of PTPase activity was performed by employing pNPP as substrate (Shimizu et al. 2002). The beads were incubated with 100 μl buffer B containing 10 mM pNPP for 2 h at 37°C. The enzymatic reaction was terminated by the addition of 1 M NaOH and the PTPase activity was determined by absorbance at 405 nm. The autohydrolysis of pNPP was corrected by measuring the increase in absorbance at 405 nm in the absence of enzyme. Statistical analysis was performed by Student’s t-test in order to determine the level of significance in the effect of BMOV on the PTPase activity of intact cells.

Immunocytochemistry

After desired treatments, IM9 cells were fixed with 4% paraformaldehyde at room temperature for 20 min followed by permeabilization of cells with 0.1% Triton X-100, wherever required. After repeatedly washing with PBS, the cells were blocked by incubating with either 10% goat serum (Jackson ImmunoResearch) or 10% blocking agent (Amersham) for 1 h at room temperature. The cells were then incubated with either anti-phosphotyrosine or anti-IRβ antibodies at room temperature for 2 h in order to determine the degree of cellular tyr-phosphorylation and level of IRβ respectively. After thorough washing, cells were incubated with the appropriate Cy3-conjugated secondary antibodies for 2 h at room temperature. Following repeated washing, the cells were observed by confocal laser scanning microscopy (Leica, Heidelberg, Germany) using argon laser and 514 and 525–610 nm filter settings for excitation and emission respectively.

Cellular insulin binding studies using insulin-tagged fluospheres

Binding of insulin being immobilized on fluorescently labeled carbonate-modified microspheres (Fluospheres, 0.2 μm, excitation and emission maxima at 505 and 515 nm respectively; Molecular Probes, Eugene, OR, USA) to the cell surface was analyzed by microscopy in order to get a rough estimation of plasma membrane IR level. Immobilization of insulin on the fluospheres was performed by following a standard amine coupling protocol as previously described for 3-aminophenylboronic acid binding of carbonyl acid-terminated magnetic beads (Farah et al. 2005) using NHS and EDC. After coupling reaction, fluospheres were incubated with 50 mM ethanolamine for 1 h at 4°C in order to quench the non-reacted groups. Insulin immobilization was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis of the fluospheres yielding an insulin-specific peak in the mass spectra (data not shown). The resultant insulin-fluosphere complex was used to analyze the impact of BMOV on the IR pool of plasma membrane in terms of changes in the cell surface binding of this complex in response to the treatment of above vanadate. IM9 cells and culture of 3T3-L1 cells with a large fraction of undifferentiated fibroblasts were treated with 100 μM BMOV for 18 h. The cells serving as control received vehicle only. Following desired treatments, 3T3-L1 cells were trypsinized, detached from the plate, and suspended in serum-free medium. Those and IM9 cell suspensions were washed repeatedly and subsequently incubated with insulin-fluosphere complex in serum-free medium for 20 min at 4°C with gentle vortexing. The cells were then washed extensively and observed in an inverted fluorescence microscope (Olympus, Tokyo, Japan) using the appropriate filter settings for green fluorescence.

Results

Characterization of BMOV-induced gross tyr-phosphorylation of the major key proteins in the upstream insulin signaling cascade

To further understand the major molecular events by which BMOV initiates its insulinomimetic action on cells, we first evaluated the impact of this compound on
the gross tyr-phosphorylation of IR and IRSs which have important functional roles in the upstream insulin signaling pathway. The study was conducted in both 3T3-L1 and IM9 cells upon exposing them to BMOV in dose- and time-dependent manners, with or without providing insulin post-treatment.

The present study has revealed a pronounced BMOV-mediated induction in the tyr-phosphorylation of IRβ and IRS-1 in 3T3-L1 (Fig. 1A) and IRβ in IM9 cells (Fig. 1B) as analyzed by immunoblotting. The above findings are also in keeping with our immunofluorescence study demonstrating enhanced phosphorylation of total tyrosines in IM9 cells in response to BMOV treatment regardless of the exposure of cells to insulin (Supplementary Fig. 1). Notably, unlike in 3T3-L1 adipocytes, the western blot analysis did not reveal any sign of BMOV-induced phosphorylation of a protein in IM9 cells that can correspond to IRS-1. Additionally, in both cell lines BMOV also induced the net tyr-phosphorylation of a ~60 kDa protein, which was further identified as IRS-3 in 3T3-L1 cells upon immunoprecipitation and immunoblotting of samples using anti-IRS-3 antibody as a probe (Fig. 1A). This is in relation to the earlier reports describing the existence of IRS-3 in 3T3-L1 cells (Giovannone et al. 2000) and rodent adipocytes as a 60 kDa protein (Lavan et al. 1997, Smith-Hall et al. 1997), which in its tyr-phosphorylation state can bind and activate P13-kinase (Smith-Hall et al. 1997). While by employing the above techniques of protein detection, we could not trace IRS-3 in the lysates of IM9 cells (data not shown). IRS-3 has been described to be non-determined in IM9 cells (Giovannone et al. 2000) and the gene encoding this protein was found to be lacking in human genome (Björnholm et al. 2002). In keeping with the previous reports (Lavan et al. 1997, Smith-Hall et al. 1997), we termed this particular protein of IM9 cells as pp60 and at this juncture opine that it probably has some role in BMOV-induced signaling that may or may not be linked with insulin signaling network.

Although BMOV exerted its effect almost in a dose-dependent manner, the kinetics in the net tyr-phosphorylation of the above-mentioned proteins varied between the two cell lines, with the maximum shown by the highest dose of the compound (100 μM) for IRβ (6.2-fold), IRS-1 (5.2-fold), and IRS-3 (6.9-fold) at 4 h in 3T3-L1 cells, and IRβ (87.5-fold) and pp60 (345.0-fold) at 18 h in IM9 cells. These results are in agreement with earlier reports where exposure of cells to inorganic- or organic-based vanadium compounds was found to increase the tyr-phosphorylation of IRK and IRS-1 in dose- and time-dependent manners (Band et al. 1997, Cuncic et al. 1999, Tardif et al. 2003), and the effect of vanadate on tyr-phosphorylation of IRK was cell-specific (Band et al. 1997).

**Impact of BMOV on the NPEY972-phosphorylation of IRβ and its equivalent of IRSs**

While causing an elevation in the gross tyr-phosphorylation of major upstream insulin signaling proteins, BMOV also augmented the phosphorylation of Tyr972 in the NPEY motif of IRβ in 3T3-L1 cells almost in dose- and time-dependent manners. The level of this phosphorylation was increased by 5.5-fold at 18-h treatment with 100 μM BMOV (Fig. 2A). In contrast, in keeping with an average of 1.3-fold increase in gross tyr-phosphorylation of IRβ, insulin alone augmented the level of Tyr972-phosphorylation of this receptor approximately by 1.4-fold. Taken together, it is conceivable that the insulin mimicking action of BMOV in 3T3-L1 cells is mediated via the modulation of the core component of insulin signaling machinery and, in this context, BMOV appeared to be far more effective than insulin when the latter is used at its conventional dose of 100 nM. Notably, no Tyr972-specific phosphorylation of IRβ was detected in IM9 cells either in basal or in those treated with insulin alone (Fig. 2B). BMOV treatment on the other hand, although it could not make any marked impact on Tyr972-phosphorylation in IM9 cells at 2 and 18 h in the absence of insulin post-exposure, it induced this phosphorylation alone only at a moderate level at 6 h in a dose-dependent manner.

Quite interestingly, upon probing the western blot membranes with anti-phospho-IR (Tyr972) antibody, apart from IRβ the bands corresponding exactly to the expected position of IRS-1 and IRS-3 proteins were found in the immunoblots of the lysates of 3T3-L1 cells, especially those receiving BMOV or insulin treatment. This indicates an immunoreactivity of those IRS proteins towards the above antibody under the prevailed experimental condition (Fig. 2A). The intensities of those bands increased in response to BMOV treatment more or less in a similar fashion as the Tyr972-specific phosphorylation of IRβ itself was augmented by this vanadate. Taken together, we consider that the above two bands in the immunoblot most likely represent the BMOV-sensitive phosphorylation of tyrosine-containing regions of IRS-1 and IRS-3 respectively, which probably have immense stoichiometrical resemblance to the NPEY972 locus of IR and hence we referred the term Tyr972-equivalent phosphorylation to denote this particular phosphorylation of proteins. Notably, no sign of this phosphorylation was seen for pp60 of IM9 cells, either in basal or in those being treated with insulin or BMOV or both (Fig. 2B).

**Effect of insulin on the BMOV-induced phosphorylation of major upstream insulin signaling proteins**

In the present experimental conditions, BMOV appeared overall to be far more potent than insulin in

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Figure 1 Effect of BMOV on the gross tyr-phosphorylation of IRβ, IRS-1, and IRS-3 in 3T3-L1 (A), and IRβ and pp60 in IM9 cells (B), with or without receiving insulin post-exposure. The cells were treated with the indicated BMOV concentrations at 37°C for varying time periods as mentioned which also covers the 15-min insulin post-treatment time, wherever applicable. The cells used for determining basal tyr-phosphorylation level were treated with BMOV vehicle only, while the cells serving as insulin control were exposed to 100 nM insulin for 15 min. After sampling, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblotting using anti-phosphotyrosine antibody. Densitometric scanning of the immunoblots was performed to quantitate the level of tyr-phosphorylation of those proteins, which for the treated groups of 3T3-L1 cells is expressed as a percentage of basal (A), while the tyr-phosphorylation of IRβ and pp60 of IM9 cells are expressed in absolute values and are shown as mean ± S.E.M. of three independent determinants (B). Following the above measurement, IRβ and IRS-1 proteins were traced in the blots by stripping and re-probing the membranes with anti-IRβ and anti-IRS-1 antibodies respectively. While the presence of IRS-3 in 3T3-L1 cells as a 60 kDa protein which showed marked tyr-phosphorylation in response to BMOV treatment was confirmed separately by immunoprecipitation and immunoblotting of lysates using anti-IRS-3 antibody as a probe (box in the upper right corner, A). The data shown for 3T3-L1 cells are the representatives of two separate experiments.
Figure 2 Effect of BMOV on the Tyr<sup>972</sup>-specific phosphorylation of IRβ and its equivalent of IRS-1 and IRS-3 in 3T3-L1 (A), and IRβ in IM9 cells (B), with or without receiving insulin post-exposure. The cells were treated with the indicated BMOV concentrations at 37 °C for varying time periods as mentioned which also covers the 15-min insulin post-treatment time, wherever applicable. The cells used for determining basal Tyr<sup>972</sup>-phosphorylation level were treated with BMOV vehicle only, while the cells serving as insulin control were exposed to 100 nM insulin for 15 min. After sampling, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblotting using anti-phospho-IR (Tyr<sup>972</sup>) and anti-IRβ antibodies. Densitometric scanning of the immunoblots was performed to quantitate the level of Tyr<sup>972</sup>-phosphorylation of those proteins, which for the treated groups of 3T3-L1 cells is expressed as a percentage of basal, while the Tyr<sup>972</sup>-phosphorylation of IRβ in IM9 cells is expressed in absolute values. The data shown are the independents of two individual determinants.
promoting the phosphorylation of the major upstream insulin signaling proteins in both cell lines. However, insulin post-treatment in general made a synergistic impact on the BMOV-induced phosphorylation of those proteins, the degree of which varied with the types of cells used, dose and the duration of BMOV treatment. More specifically, this effect of insulin was found to be exerted on gross tyr-phosphorylation of IRβ, IRS-1 and IRS-3, and Tyr972-specific and its equivalent phosphorylation of IRβ and IRS-1 respectively, in most of the BMOV-treated groups of 3T3-L1 cells (Figs 1A and 2A). In similar conditions, Tyr972-equivalent phosphorylation of IRS-3 was increased by insulin only at 18-h treatment of BMOV. An additive effect of insulin was also observed to some extent on the BMOV-induced phosphorylation of IRβ in IM9 cells although this was restricted mainly up to the initial 2-h exposure to the vanadate (Fig. 1B). The above fact is in parallel with our findings in the immunofluorescence study where practically no synergistic effect of insulin was revealed on the gross tyr-phosphorylation of IM9 cells being treated for as long as 18 h with 50 or 100 μM BMOV (Supplementary Fig. 1). On the other hand, BMOV-induced phosphorylation of pp60 protein in IM9 cells showed a differential response to insulin where a synergistic effect of this hormone was demonstrated by the entire BMOV-treated groups up to the initial 2-h exposure to this vanadate (Fig. 1B). Moreover, despite making no marked impact on the Tyr972-phosphorylation of IRβ in IM9 cells at 2- and 6-h BMOV treatment, insulin was found to be essential for BMOV at 18 h in modulating the above phosphorylation during which a dramatic increase in this phosphorylation was shown by 100 μM BMOV (Fig. 2B).

Effect of BMOV on the Tyr1158/Tyr1162/Tyr1163-phosphorylation of IRβ in IM9 cells receiving insulin post-treatment

As we have found a profound impact of BMOV on the net-tyrosine as well as Tyr972-specific phosphorylation of IRβ in both experimental cell lines, our next experimental approach was to address whether the above compound can exert any effect on the kinase domain of IRβ by modulating the autophosphorylation of tyrosine residues 1158, 1162, and 1163. We did not perform this experiment on 3T3-L1 adipocytes as organovanadate is already known to augment autophosphorylation of IRK in rodent adipocytes (Shisheva & Shechter 1993). Accordingly, the effect of BMOV on this particular phosphorylation was assessed in IM9 cells by exposing them to 50 and 100 μM BMOV for 18 h. The cells were also subjected to insulin post-exposure as insulin was found to be obligatory for those cells in inducing Tyr972-specific phosphorylation of IRβ at 18-h treatment with BMOV.

No appreciable signal for Tyr1158/Tyr1162/Tyr1163 autophosphorylation of IR was seen in IM9 cells treated with insulin alone (Fig. 3), in parallel with our findings revealing the ineffectiveness of insulin in inducing Tyr972-phosphorylation of the above receptor (Fig. 2B). While the treatment of cells with BMOV for 18 h before insulin exposure increased this autophosphorylation dramatically in a dose-dependent manner (Fig. 3), quite comparable with the effect of above vanadate on Tyr972-phosphorylation of IRβ under a similar experimental condition (Fig. 2B).

Effect of inhibition of IRK on the insulin- and BMOV-mediated NPEY972-phosphorylation of IRβ in 3T3-L1 cells

In order to assess whether BMOV-induced NPEY972-phosphorylation is mediated through a direct interaction of the above compound with IR, we have conducted a short experiment using AGL-2263, a commercially available cell-permeable benzoazolone-containing bioisostere of tyrphostin AG 538 that acts as an inhibitor of IR (Blum et al. 2003). Accordingly, exposure of 3T3-L1 cells to 5 μM of this agent caused a 22% decrease in insulin-induced NPEY972-phosphorylation, in keeping with insulin binding of IR as the...
prerequisite factor for the activation of the above motif (Fig. 4). However, we could not find much more depletion of this particular phosphorylation by further increasing the dose of AGL-2263 up to 50 μM (data not shown), indicating a saturation in inhibition. In an earlier study, the IC₅₀ values of three tyrphostins for IR-phosphorylation inhibition were reported to be varied between 42 and 57 μM in insulin-stimulated intact WT2 cells (Pa´rrizas et al. 1997). However, the above estimation was based on the measurement of phosphorylation of total tyrosines of IR without reflecting the status of inhibition of Tyr₉⁷²-phosphorylation individually. Accordingly, as we did not quantitate the gross tyr-phosphorylation of IR separately in this study, no definite conclusion can be made on the extent of inhibition of IR per se by AGL-2263. Moreover, in order to ensure maximal effectiveness of AGL-2263 in our experiment, the dose of this tyrphostin was kept over ten times higher than the reported IC₅₀ value of this compound for partially purified IRK (0.4 μM; Blum et al. 2003). Besides, it has been found that while causing around 40% depletion in the IGF-I-induced gross tyr-phosphorylation of IGF-IR, both 10 and 50 μM doses of AGL-2263 decreased the tyr-phosphorylation of IRS-1 over 80% in NIH-3T3 cells (Blum et al. 2003). This might be considered as indirect evidence about the potent effectiveness of above tyrphostin in inhibiting NPEY motif, since Tyr₉⁷²-phosphorylation plays a vital role in binding of IR to IRS-1 (Berhanu et al. 1997), which in turn triggers phosphorylation of the latter protein. Taken together, it can be opined that although a comparatively lower degree of inhibition of Tyr₉⁷²-phosphorylation was shown by AGL-2263 in our study, it might be potent enough to inhibit further downstream insulin signaling. However, future elaborate studies are needed to fully understand the mode of action of tyrphostin on Tyr₉⁷²- and other vital site-specific tyr-phosphorylations of IR.

In contrast, a 5 μM dose of AGL-2263 did not produce any negative impact on Tyr₉⁷²-phosphorylation in cells receiving both BMOV and insulin. This does imply that BMOV can exert its insulinomimetic effect consistently despite the imposition of a functional barrier on IR. Accordingly, our results suggest that an induction in Tyr₉⁷²-phosphorylation by BMOV does not involve IR directly and mediates through a mechanism which is quite different from that by which insulin promotes this particular phosphorylation.

**Identification of PTP 1B-related proteins in IM9 cells and their inhibition by BMOV**

As BMOV in general caused a remarkable increase in gross- as well as site-specific tyr-phosphorylation of major upstream insulin signaling proteins in both cell lines, our next approach was to judge whether BMOV can inhibit intracellular PTPases like PTP 1B which is known to dephosphorylate the key tyrosine kinases of insulin signaling network (Kenner et al. 1996, Seely et al. 1996, Calera et al. 2000). However, we did not perform this study on 3T3-L1 adipocytes since the cellular distribution of PTP 1B has been reported in rodent adipocytes (Calera et al. 2000) and the negative role of PTP 1B in insulin signaling has already been demonstrated in 3T3-L1 cells overexpressing the above PTPase (Venable et al. 2000, Shimizu et al. 2002). Organovanade triggers insulin signaling in 3T3-L1 cells by potentiating IR tyr-phosphorylation (Ou et al. 2005, Basuki et al. 2006) and pervanadate induces autophosphorylation of IRK in rodent adipocytes through PTPases inhibition (Shisheva & Shechter 1993).
Accordingly, three major bands with approximate molecular weight of 48, 38, and 26 kDa were identified in the immunoblot of IM9 cell lysates following their immunoprecipitation and western blotting using anti-IRS-3 antibody as a probe (Fig. 5A). Difference in molecular weight of PTP 1B has been noticed between the studies employing different techniques of purification of this enzyme from various sources. This is also in keeping with the fact that alternate mRNA splicing generates structural and functional diversity among several PTP gene products (reviewed by Tonks 2003). The approximate molecular weight of this protein was reported to be 24 kDa (Jones et al. 1989), 37 kDa (Charbonneau et al. 1989, Pedersen et al. 2004), and 50 kDa (predicted by cDNA cloning; Chernoff et al. 1990). Besides, a major placental membrane PTPase (PTP-I, 45 kDa estimated by SDS/PAGE, 50 kDa determined by gel filtration and at least 46 kDa calculated by amino acid analysis) was identified as a larger form of PTP 1B (Pallen et al. 1991). Taken together, it is conceivable that the appearance of the above three major bands in the immunoblot of IM9 cell lysates might be the indication of possible existence of a heterogeneous population of PTP 1B-related proteins in those cells. To the best of our knowledge, no information is available on the existence and functional role of PTP 1B in IM9 cells, and accordingly we termed those proteins as PTP 1B-related proteins or phosphatases. Further extensive studies are needed to exactly characterize the nature and properties of those proteins.

As can be seen, exposure to 100 μM BMOV caused a significant reduction in the activity of PTP 1B-related phosphatases in intact IM9 cells. However, the extent of inhibition was more pronounced at 18 h (65%, P<0.001) than that noticed at 2-h BMOV treatment (17%, P<0.05, Fig. 5B). This is in parallel with the observed time-dependent increment in the net tyr-phosphorylation of IRβ and pp60 of IM9 cells in response to the treatment of 100 μM BMOV (Fig. 1B), suggesting that these two phosphorylations are under the negative control of PTP 1B-related phosphatases. Moreover, a direct exposure to BMOV resulted in the inhibition of those phosphatases being isolated from untreated IM9 cells in a dose-dependent manner (Fig. 5C), in keeping with an earlier report (Mohammad et al. 2002),

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**Figure 5** Identification of PTP 1B-related proteins in IM9 cells and determination of their inhibition by BMOV. Suspensions of cells were divided into groups and treated with 100 μM BMOV for either 2 or 18 h. The corresponding controls received vehicle only, while the cells of untreated group were kept alone. Following desired treatments, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoprecipitation. The immunoprecipitates of untreated cells were either processed for western blotting to identify PTP 1B-related proteins using anti-PTP 1B antibody as a probe (A) or subjected to of PTPase assay after being exposed to the indicated concentrations of BMOV for 30 min at 37 °C where the enzymatic activity of the BMOV-treated samples is expressed as a percentage of that of untreated one (C), while the immunoprecipitates of control and BMOV-treated cells were used directly for PTPase assay. The enzymatic activity of treated cells is expressed in percentage of control cells (B). *P<0.05 and **P<0.001.
supporting further the negative regulation of the above enzymes by BMOV.

**Impact of BMOV on the cellular level of IR and IRS-1: counter effect of insulin on IR pool**

While maintaining a higher degree of phosphorylation of IRβ, a prolonged treatment of BMOV showed a trend in declining the level of the above receptor in both experimental cell lines, more predominantly in IM9 cells (Fig. 6), which was reflected consistently throughout our experiments. This is also in parallel with our microscopic studies where binding of insulin-tagged fluorospheres to cells was found to decrease in response to treatment of 100 μM BMOV for 18 h (Fig. 7). With immunoblotting techniques, 25% depletion of IRβ was recorded in 3T3-L1 cells being exposed to 100 μM BMOV for 18 h (Fig. 6A). On the other hand, in IM9 cells, IRβ content decreased by 48% at 18 h exposure to 50 μM BMOV, while the level of this protein declined by 32 and 71% at 6- and 18-h treatment with 100 μM of the above vanadate respectively (Fig. 6B). Interestingly, post-BMOV treatment of insulin was found to play an important role in compensating the above loss of IR protein, more predominantly in IM9 cells. The hormone increased the level of IRβ by 26% in 3T3-L1 cells being treated for 18 h with 100 μM BMOV (Fig. 6C). While the content of this receptor was enhanced by 22, 27, and 89% by insulin in IM9 cells receiving the treatment of 100 μM BMOV for 2, 6, and 18 h respectively (Fig. 6D). In contrast, BMOV treatment did not cause any change in the IRS-1 content of 3T3-L1 cells with or without having insulin post-exposure (Fig. 6E).

**Effect of monensin on the IR pool of IM9 cells receiving insulin treatment following BMOV exposure**

Since the insulin-mediated replenishing of IR in BMOV-treated cells was a very rapid process, it is quite unlikely to be conceivable that the above event can be attributed to an increase in the biosynthesis of IR. Rather, we considered it as a consequence of alteration in one or multiple steps of cellular processes that are associated with the packaging, release, trafficking, or the membrane flow of the receptor protein. In order to judge this more appropriately, we have exposed the BMOV-treated or -untreated IM9 cells for 2 h before insulin administration to monocarboxylic Na+/H+ antiport monensin, an antibiotic inhibitor of intracellular protein transport, which is also known to block the recycling of insulin receptor in hepatocytes (Whittaker et al. 1986).

![Figure 6](https://www.endocrinology-journals.org)

**Figure 6** Negative impact of BMOV on the level of IR in 3T3-L1 (A) and IM9 cells (B). Insulin-mediated recovery of BMOV-depleted IR is shown in 3T3-L1 (C) and IM9 cells (D). While the effect of 18-h treatment of BMOV on IRS-1 level of 3T3-L1 cells with or without receiving insulin post-exposure is shown in E. The cells were exposed to the indicated BMOV concentrations at 37 °C for varying time periods as mentioned which also covers the 15-min insulin post-treatment time, wherever applicable. After sampling, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblotting using anti-IRβ or anti-IRS-1 antibodies. Densitometric scanning of the immunoblots was performed to quantitate the level of IRβ and IRS-1 with the former protein being expressed in absolute values in A and B. The effect of insulin on the cellular IRβ content of BMOV-untreated or -treated group is reflected in terms of percentage changes relative to the corresponding controls that were devoid of insulin post-treatment (C and D), while the level of IRS-1 in the BMOV-treated 3T3-L1 cells is expressed as a percentage of corresponding BMOV-untreated cells. Data are mean ± S.E.M. of three independent determinants.
As can be seen, treatment of 25 or 50 μM monensin alone in the prevailed experimental condition did not cause any change in the cellular IR content (Fig. 8). Notably, in our assay, the level of IR is represented by the total Triton X-100-soluble cellular content of this protein without reflecting its plasma membrane and intracellular pools separately. Accordingly, no definite statements can be made in our study confirming the incidence of monensin-mediated inhibition of IR recycling that would result in an increase of IR in cytosolic compartment. On the other hand, in a similar experimental condition, this ionophore caused an augmentation in the level of soluble IR in dose-dependent manner in cells receiving BMOV treatment. Taken the above effect of monensin into consideration, one can find a possible link between the immediate responses of cells to insulin in recovering the BMOV-depleted IR and the pathway regulating intracellular transport and processing of the above receptor.

**Effect of dexamethasone on BMOV-modulated upstream insulin signaling cascade**

Since the positive impact of BMOV on the upstream insulin signaling pathway is supposed to be largely negated by its adverse effect on the cellular IR content, one can consider the possibility that a condition which can increase the cellular level of IR might have significant relevance in amplifying the degree of net tyr-phosphorylation of IRβ in the above vanadate-treated cells. In order to judge this probability, dexamethasone was chosen in our experiment to be co-administered with BMOV because the former drug is known to augment the transcription of IR gene and synthesis of IR in IM9 cells (Fantus *et al.* 1982, McDonald & Goldfine 1988), and result in time- and dose-dependent increase in IR protein in mouse 3T3-C2 fibroblasts (Knutson *et al.* 1982). This is also further confirmed by our study where the total cellular IRβ content was increased in response to dexamethasone treatment, almost in a dose-dependent manner in 3T3-L1 cells revealing 41% augmentation at 10 μM drug (Fig. 9A). On the other hand, between 40 and 47% increment in IRβ was noticed in IM9 cells when treated with 1.25, 2.5 and 5 μM doses of the above glucocorticoid, while 79% augmentation of this receptor was found at 10 μM drug (Fig. 9B), in parallel with our findings in immunofluorescence study (Supplementary Fig. 2).

Strikingly, despite its capability to enhance the IRβ level in normal cells, dexamethasone at both 5 and 10 μM doses failed to augment the content of this receptor in 3T3-L1 cells being co-treated with 100 μM BMOV for 18 h and receiving post-exposure to 100 nM insulin for 15 min (Fig. 10). However, under the prevailing experimental condition, the above doses of dexamethasone increased the gross tyr-phosphorylation level of IRβ by 23 and 31% respectively. In a
similar condition, only a marginal 5% increase in Tyr972-phosphorylation of IRβ was shown by 5 μM dexamethasone, while at 10 μM dose the drug increased this phosphorylation by 11%. On the other hand, a negative impact of dexamethasone was seen on the level of IRS-1 in the above BMOV-treated cells, with 11 and 20% depletion in this protein at 5 and 10 μM doses of the glucocorticoid respectively, in agreement with dexamethasone-mediated downregulation of IRS-1 (Turnbow et al. 1994, Buren et al. 2002). However, despite causing this sort of adverse effect, dexamethasone increased both the gross tyr- and Tyr972-equivalent phosphorylation of IRS-1. Accordingly, the former phosphorylation was enhanced by 20 and 30%, and the latter one by 8 and 30% in response to 5 and 10 μM dexamethasone respectively.

In sharp contrast, exposure to 2.5, 5, or 10 μM doses of dexamethasone caused a remarkable increase in the content of IRβ in IM9 cells receiving co-treatment of 100 μM BMOV for 18 h and post-exposure to 100 nM insulin for 15 min (Fig. 11A). However, despite promoting this augmentation, no modulation in the net tyr-phosphorylation of IRβ was shown by any of above-selected doses of dexamethasone. Moreover, under the prevailing experimental condition, the glucocorticoid at 10 μM dose almost completely inhibited the Tyr972-phosphorylation of IRβ to be induced by BMOV and insulin together (Fig. 11B).

Effect of dexamethasone on BMOV-modulated downstream insulin signaling cascade

Next, we have analyzed the impact of 18-h treatment of 100 μM BMOV or 10 μM dexamethasone either alone
or in combination on the activation/deactivation of insulin downstream signaling proteins namely Akt and GSK-3β as the representative of PI3-kinase pathway and Raf as the member of MAPK cascade, in both 3T3-L1 and IM9 cells receiving post-exposure to 100 nM insulin for 15 min.

As can be seen, exposure to BMOV elevated Thr<sup>308</sup>-phosphorylation of Akt by 67% and Ser<sup>473</sup>-phosphorylation of the same protein by 44% in 3T3-L1 cells, indicating an effective induction of Akt under the prevailed condition (Fig. 12). On the other hand, dexamethasone decreased Thr<sup>308</sup> and Ser<sup>473</sup> phosphorylations by 66 and 56% respectively, thus inhibiting Akt. While in comparison to control, net 38 and 29% reduction in the phosphorylation of Thr<sup>308</sup> and Ser<sup>473</sup> respectively, was noticed in cells receiving combined treatment of BMOV and dexamethasone, suggesting prevailed antagonism of these two compounds on these phosphorylations. However, no change in the cellular Akt level was seen due to any of the above treatments. Surprisingly, despite triggering Akt activation, BMOV treatment resulted in 25% reduction in Ser<sup>9</sup>-phosphorylation of GSK-3β, thus favoring activation of the latter kinase. On the other hand, dexamethasone decreased this phosphorylation by 53%. While in comparison to control, 62% reduction in Ser<sup>9</sup>-phosphorylation was noticed in cells receiving combined treatment of dexamethasone and BMOV, suggesting some sort of additive impact of these two compounds in facilitating GSK-3β activation. On the other hand, 28% decrease in Ser<sup>259</sup>-phosphorylation of Raf was noticed due to BMOV treatment. While dexamethasone increased this phosphorylation by 26%, and thereby inactivating Raf kinase. In comparison to control, around 11% decrease in Ser<sup>259</sup>-phosphorylation was noticed in cells receiving combined treatment of BMOV and dexamethasone, suggesting the prevailed antagonism of these two compounds on this particular phosphorylation.

The response of Akt phosphorylation to BMOV and dexamethasone in IM9 cells was more or less similar to that found in 3T3-L1 cells. BMOV increased the phosphorylation of Thr<sup>308</sup> by 79% and Ser<sup>473</sup> by 49%, and dexamethasone decreased the above two phosphorylations by 42 and 52% respectively. However, in comparison to control, 87% increase in Thr<sup>308</sup> and 37% augmentation in Ser<sup>473</sup>-phosphorylation was noticed in cells receiving combined treatment of the above two compounds, suggesting nil and weak antagonistic effect of dexamethasone on the BMOV-induced former and latter phosphorylations respectively. Like 3T3-L1 cells, no alteration in the cellular content of Akt was also seen in all of the treated groups of IM9 cells. Quite interestingly, despite of making changes in Akt phosphorylation, BMOV or dexamethasone either alone or in combination could not affect the phosphorylation of both Ser<sup>9</sup> of GSK-3β and Ser<sup>259</sup> of Raf in IM9 cells.

Figure 9 Effect of dexamethasone on the level of IR in 3T3-L1 (A), and IM9 cells (B). The cells were incubated with dexamethasone at the indicated concentrations at 37 °C for 18 h. The cells serving as vehicle control for dexamethasone were exposed to 0.1% (v/v) ethanol for 18 h. After sampling, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblotting using anti-IRβ antibody. Densitometric scanning of the immunoblots was performed to determine the level of IRβ. Data are mean ± S.E.M. of three independent determinants.
Figure 10 Effect of dexamethasone on the tyr-phosphorylation and cellular content of IRβ and IRS-1 in BMOV-treated 3T3-L1 cells receiving insulin post-treatment. The cells were incubated with 5 or 10 μM dexamethasone together with 100 μM BMOV at 37 °C for 18 h, while the cells exposed to 100 μM BMOV plus 0.1% ethanol for 18 h served as the dexamethasone-untreated control. The cells under all experimental groups were exposed to 100 nM insulin for 15 min prior to sampling. After desired treatments, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblotting using anti-phosphotyrosine, anti-phospho-IR (Tyr972), anti-IRβ, and anti-IRS-1 antibodies. Densitometric scanning of the immunoblots was performed to quantitate the cellular content and the tyr-phosphorylation level of IRβ and IRS-1 which for each dexamethasone-treated group are expressed in percentage of the dexamethasone-untreated control. The data shown are the representatives of two separate experiments.
Discussion

The insulinomimetic effects of vanadium compounds have been demonstrated both in vitro and in vivo on various physiological functions, like stimulation of glucose transport, glucose oxidation, glycogen synthesis, and lipogenesis in addition to the inhibition of lipolysis and gluconeogenesis (reviewed by Srivastava & Mehdi 2005). Cumulative reports suggest that vanadium can trigger the activation of many components of insulin signaling pathway including PI3-kinase/Akt and Ras/Raf/MEK/ERK1/2 cascades (reviewed by Mehdi et al. 2006). On the other hand, several lines of evidence indicate that BMOV acts as a non-selective competitive inhibitor of PTPases (Mohammad et al. 2002, Peters et al. 2003, Winter et al. 2005), some of which like PTP 1B plays a crucial role in the negative regulation of insulin signaling (Elchebly et al. 1999), through dephosphorylation of the key tyrosine kinases, including IRK, IRS-1, and IRS-2 (Kenner et al. 1996, Seely et al. 1996, Calera et al. 2000). This is also in keeping with the BMOV-mediated enhancement in tyr-phosphorylation of IRβ and IRS-1 proteins (Peters et al. 2003, Mehdi & Srivastava 2005, Winter et al. 2005).

Accordingly, an increase in the gross tyr-phosphorylation was observed in our study for IRβ, IRS-1 and IRS-2 of 3T3-L1 and IRβ, and pp60 of IM9 cells in response to BMOV treatment. It has been reported that organovanadate triggers insulin signaling in 3T3-L1 cells by directly potentiating tyrosine phosphorylation of IR (Ou et al. 2005, Basuki et al. 2006), while pervanadate modulates autophosphorylation of IRK in rodent adipocytes through PTPases inhibition (Shisheva &

Figure 11 Effect of dexamethasone on the tyr-phosphorylation and cellular content of IRb in BMOV-treated IM9 cells receiving insulin post-treatment. In order to assess the impact of dexamethasone on the BMOV-induced gross tyr-phosphorylation of IRb, the cells were incubated with the indicated doses of this glucocorticoid with 100 μM BMOV at 37 °C for 18 h (A), while the cells receiving co-treatment of 100 μM BMOV and 10 μM dexamethasone for 18 h were used to analyze the effect of the latter drug on the BMOV-stimulated Tyr972-phosphorylation of IRb (B). In both cases, cells being exposed to 100 μM BMOV plus 0.1% ethanol served as dexamethasone-untreated control. The cells under all experimental groups were exposed to 100 nM insulin for 15 min prior to sampling. After desired treatments, the cells were lysed as described in Materials and methods and the lysates were subjected to immunoblot using anti-phosphotyrosine, anti-phospho-IR (Tyr972), and anti-IRb antibodies. Densitometric scanning of the immunoblots was performed to quantitate the cellular content, gross tyrosine, and Tyr972-phosphorylation level of IRb. The first two parameters for each dexamethasone-treated group are shown as a percentage of dexamethasone-untreated control and are expressed as mean ± S.E.M. of three independent determinants (A), while the level of last parameter is expressed in absolute values (B), and the data shown are the representatives of two individual experiments.
In parallel, we have shown the existence of PTP 1B-related phosphatases in IM9 cells and their inhibition by BMOV. We also found a close correlation between the degree of above inhibition and the extent of net tyr-phosphorylation of IR\(\beta\) and pp60, suggesting that the latter process is under the negative control of PTP 1B-related phosphatases in IM9 cells.

However, in the prevalent experimental condition, whether the net tyr-phosphorylation of IRS-1, IRS-3, and probably of pp60 is mediated either through their direct interaction with the BMOV-induced IRK or through the inhibition of specific PTPases regulating their own phosphorylation or by both is yet to be clarified. In 3T3-L1 cells receiving BMOV treatment, the overall profile of gross tyr-phosphorylation among IRK, IRS-1, and IRS-3 was found to be nearly identical.

Likewise, the extent of insulin-induced phosphorylation of gross tyrosines among the above three proteins appeared to be almost uniform. These two evidences collectively at least in part suggest that the phosphorylation of the last two proteins under BMOV treatment is triggered by the first one following its activation by vanadate, a situation which is quite comparable with the effect of insulin on its own signaling pathway (White & Kahn 1994). Besides, our data, which to the best of our knowledge the first to show a profound enhancement of Tyr972-phosphorylation of IR\(\beta\) in 3T3-L1 cells in response to BMOV treatment, is in keeping with the above view. However, the phosphorylation of this specific tyrosine of IR\(\beta\) by BMOV was found to be insensitive to the pre-treatment of AGL-2263, a compound which blocks the insulin-mediated signaling
by inhibiting IRK (Blum et al. 2003). This does imply that the insulin-like action of BMOV in those cells is not mediated through a direct interaction with IR and further speaks for the involvement of IRK-associated PTPases in this context.

Interestingly, a correlation was also found among the Tyr972-equivalent phosphorylation of IRS-1 and to some extent that of IRS-3 and Tyr972-specific phosphorylation of IRβ in BMOV-treated 3T3-L1 cells. Likewise, similarities have also been observed to some extent between the pattern of above phosphorylations of IRβ and IRS-1 in cells being exposed to insulin alone or insulin plus BMOV. Based on these, we speculate that Tyr972-like phosphorylation of the above might be the indication of phosphorylation on a specific region of IRS-1 and IRS-3 protein representing any of their 21 and 9 potential tyrosine phosphorylation sites respectively (White & Kahn 1994, Lavan et al. 1997) that could have immense stoichiometric homology with the NPEY locus of IRβ. However, further detailed studies are needed for precise characterization of the above phosphorylation emphasizing the exact molecular basis for its striking resemblance with the Tyr972-phosphorylation of IRβ along with the elucidation of its specific role in the insulin signaling pathway.

Notably, the pattern of gross tyr-phosphorylation of IRβ and pp60 in IM9 cells being treated with BMOV alone also exhibited an overall correlation with each other, suggesting the possible involvement of the latter protein in BMOV-induced insulin signaling. Except for some discrete reports (Kasuga et al. 1982, Khartonenko et al. 1995), no detailed information are available on the physiological relevance of IRK and the validity of conventional insulin signaling system in IM9 cells. Accordingly, on the basis of our findings, no definite conclusion can be made whether pp60 belongs to an insulin signaling network, and whether BMOV-induced gross tyr-phosphorylation of this protein is mediated via activated IRK or through a direct inhibition of PTP 1B-related phosphatases controlling its own phosphorylation or by both. Besides, although the treatment of 100 μM BMOV alone showed the highest induction in net tyr-phosphorylation of IR at 18 h, an appreciable stimulation of Tyr972-phosphorylation by this dose of BMOV was noticed only at 6 h. The exact molecular basis for the differential response of these two phosphorylations of IR to BMOV is yet to be clarified.

Strikingly, in the present experimental condition, BMOV overall, particularly at its higher doses, appeared to be far more potent than insulin in phosphorylating the gross tyrosines of key insulin upstream signaling proteins in both cell lines. Such stronger effects of vanadium over insulin on tyr-phosphorylation of IRβ and many endogenous proteins of the cells have also been demonstrated in an earlier study (Band et al. 1997). In keeping with previous findings (Kenner et al. 1996, Seely et al. 1996, Calera et al. 2000), it is therefore conceivable that in 3T3-L1, and most probably also IM9 cells, it is the status of activation of PTP 1B or related phosphatases which largely determine the extent of phosphorylation of the ligand-induced IRK.

However, for most of the cases in our study with 3T3-L1 cells, insulin post-treatment resulted in an enhancement in the BMOV-induced gross tyr-phosphorylation of IRβ, IRS-1, and IRS-3 and Tyr972-phosphorylation of IRβ, in agreement with the synergistic effect of insulin on peroxovanadium-mediated phosphorylation of IRβ (Band et al. 1997). This is also consistent with BMOV and insulin acting through different mechanisms to induce the phosphorylation of IRK. Besides, in many cases an additive effect of insulin was also found to be exerted on the BMOV-induced Tyr972-equivalent phosphorylation of IRS-1 and to some extent to that of IRS-3 protein, further speaking for the possible physiological relevance of those phosphorylations in insulin signaling cascades. Moreover, in many instances a correlation was also observed among the above insulin-modulated phosphorylation of proteins. Taking all into consideration, it is conceivable that in 3T3-L1 cells, like insulin, BMOV also involves IRK for the initiation of insulin signaling, while the subsequent transduction of the signal mediates through the recruitment of IRS-1 and IRS-3.

In contrast, BMOV-induced gross tyr-phosphorylation of both IRβ and pp60 of IM9 cells showed differential response to insulin, being induced by it in many cases while showing negligible or even negative response to the hormone in some instances. However, insulin post-treatment was found to be essential for BMOV at 18 h of its exposure in order to promote Tyr972-phosphorylation of IRβ. In this condition, a profound enhancement in this phosphorylation was shown by 100 μM of the above vanadate. This is also in keeping with the marked induction in the phosphorylation of Tyr1158/Tyr1162/Tyr1163 residues by 18-h treatment of BMOV in a dose-dependent manner in cells receiving insulin post-exposure. The role of autophosphorylation of the above three tyrosines in the activation of IR by insulin has been described (White et al. 1988) and is believed to be mediated via stabilization of the active conformation of kinase domain of IR. The above activation process eventually leads to the phosphorylation of Tyr972 in the NPEY motif of IRβ. In contrast, the termination of insulin signaling mediates via inactivation of the IR through dephosphorylation of Tyr1158/Tyr1162/Tyr1163 residues (Tonks 2005) in which PTP 1B has been described as playing an important role (Boute et al. 2003). Taking all of the above into account, it can be generalized that IRK of IM9 cells in normal conditions remains under the tight control of PTP 1B-related phosphatases and upon removal of this restriction by BMOV, insulin can
modulate the IRK in a similar way as it does for its effector cells. However, further detailed studies are needed to address the insulin signaling pathway of IM9 cells and its possible physiological relevance.

A fall in the level of IRβ was seen in Triton X-100 solubilized fraction of IM9 cells in response to BMOV treatment in time- and dose-dependent manners, with 71% depletion caused by 100 μM of the above vanadate at 18 h. Under the prevailing experimental condition, a similar kind of negative effect of BMOV on IR was also seen in 3T3-L1 cells at 18-h treatment of the compound. This is in agreement with the vanadate-induced down-regulation of cell surface IR in IM9 cells due to internalization (Torossian et al. 1988) and a decrease in the insulin binding activity of IR in the solubilized extracts of vanadate treated FAO cells as a consequence of an inhibition in IR gene expression (Bortoli et al. 1997). Several lines of evidence have suggested that internalization of the insulin-receptor complex utilizes both coated pits and caveolae (McClain & Olefsky 1988, Backer et al. 1991, Moss & Ward 1991), which are enriched with cholesterol and insoluble in Triton X-100 at 4 °C (Bickel 2002). Our results also suggest that an intense induction of insulin signaling in IM9 cells by BMOV may result in internalization of IR in association with caveolae that could account for an apparent loss in the IR content in the solubilized extract of cell lysates used in our experiment. It is also conceivable that the above internalization of IR might solely be responsible for the decrease in the plasma membrane content of this protein in IM9 cells during the early hours of BMOV treatment. This factor along with the impaired IR gene expression might account for the depletion of IR in cells receiving a prolong exposure to BMOV. However, further detailed studies are needed to elucidate the intrinsic molecular mechanism being involved in the BMOV-mediated internalization of IR.

Strikingly, a brief insulin treatment showed a trend in recovering the BMOV-mediated loss of IRβ in the solubilized fraction, especially in IM9 cells receiving a long exposure to this vanadate. Moreover, in a similar experimental condition, Na+/K+-antiport agent monensin produced the insulin-like effect in IM9 cells in terms of making an enhancement in IRβ content. This is in relation to an earlier study where arrest of endosome acidification by bafilomycin A1 was found to mimic insulin action on GLUT4 translocation in 3T3-L1 adipocytes (Chinni & Shisheva 1999). Accordingly, our study at least in part suggests that the mechanism for recycling of internalized IR in BMOV-treated cells, which can be modulated by insulin, is different from that by which insulin-IR complex is normally recycled in insulin-treated cells. However, future studies are needed for a detailed understanding of the above processes.

Consistent with earlier reports (Fantus et al. 1982, Knutson et al. 1982, McDonald & Goldfine 1988), we have found an increase in the content of IRβ in both cell lines in response to dexamethasone treatment. This prompted us to extend our studies in order to check whether the above glucocorticoid may have any impact on insulin signaling in BMOV-treated cells with depleted IRβ level. So far, contradictory reports are available on the effect of this drug on the insulin signaling cascades of normal cells. Treatment with dexamethasone, especially at high doses, is shown to induce insulin resistance (Ruzzin et al. 2005) and deplete the level of IRS-1, PI3-kinase, and Akt (Buren et al. 2002), while the above drug strongly enhanced the insulin-activated glycogen synthesis and promoted insulin-stimulated association of PI3-kinase with IRS-1 and IRS-2 (Klein et al. 2002).

To address the above issue, we first analyzed the effect of dexamethasone on the BMOV-modulated upstream insulin signaling cascade in both experimental cell lines receiving insulin post-treatment. Accordingly, despite its positive impact on IR pool in normal cells, dexamethasone did not make any augmentation in the level of IRβ in BMOV-treated 3T3-L1 cells, the exact molecular basis of which is yet to be resolved. Strikingly, in the prevailed experimental condition, a modulation in the insulin signaling by this glucocorticoid was reflected in terms of an overall increase in the tyr-phosphorylation of IRβ and IRS-1. In contrast, despite making an appreciable increase in the IR content of IM9 cells under BMOV treatment, dexamethasone failed to augment the gross tyr-phosphorylation of IRβ. Moreover, in this condition, the drug almost completely impaired the Tyr972-phosphorylation of the above receptor at 10 μM dose, in accordance to the dexamethasone-induced resistance in insulin signaling (Ruzzin et al. 2005). This does imply the cell-specific effect of dexamethasone on BMOV-induced upstream insulin signaling cascade where no correlation exists between the net protein content and degree of phosphorylation of IRβ.

We further extended our study in order to deeply understand the mode of action of BMOV on the insulin downstream signaling pathway emphasizing the impact of dexamethasone on this particular event in 3T3-L1 and IM9 cells receiving insulin post-treatment. Accordingly, an appreciable induction in the phosphorylation of Thr308 and Ser473 of Akt was seen in both cell lines due to 18-h treatment of BMOV, indicating activation of this kinase by the above vanadate. Augmentation in those phosphorylations of Akt by vanadate either in the presence or absence of insulin has been demonstrated in different cell lines including 3T3-L1 adipocytes (Tardif et al. 2003, Li et al. 2004, Zhang et al. 2004, Mehdi & Srivastava 2005, Ou et al. 2005, Basuki et al. 2006). Based on earlier reports
(Tardif et al. 2003, Mehdi & Srivastava 2005, Mehdi et al. 2006), we opine that such activation process of Akt in BMOV-treated 3T3-L1 cells may be mediated via PI3-kinase pathways as those cells showed increased phosphorylation of IRS-1. However, at this juncture, no definite conclusion can be made on the exact molecular mechanism of BMOV-mediated induction of Akt in IM9 cells, which needs further elaborate studies elucidating the insulin signaling network of IM9 cells.

On the other hand, treatment with dexamethasone for 18 h inactivated Akt in both cell lines by reducing Thr308 and Ser473-phosphorylation, in agreement with earlier reports (Ruzzin & Jensen 2005, Ruzzin et al. 2005). We speculate that this negative impact of dexamethasone may be mediated via a PI3-kinase pathway at least in 3T3-L1 cells used in our study, since dexamethasone-induced insulin resistance is known to be linked with the inhibition of insulin-stimulated IRS-1 associated PI-3 kinase activity (Saad et al. 1995). However, further studies are needed to verify this hypothesis. On the other hand, dexamethasone acts differentially on BMOV-induced Akt phosphorylation in a cell-specific manner. More specifically, despite causing an enhancement in tyr-phosphorylation of IRS-1 in BMOV-treated 3T3-L1 cells, dexamethasone produced a remarkable negative impact on the induced phosphorylation of both Thr308 and Ser473 of Akt. It is plausible that the observed depletion in IRS-1 protein by dexamethasone in those cells at least in part might account for the above fact. However, it may also be possible that dexamethasone can exert this effect without involving PI3-kinase. On the other hand, despite decreasing Tyr972-phosphorylation of IRβ abruptly in BMOV-treated IM9 cells, dexamethasone did not antagonize the induced phosphorylation of Thr308 and inhibited that of Ser473 very slightly in those cells. This does further suggest that the negative regulation of Akt by dexamethasone might not be mediated solely via an insulin signaling pathway.

Quite surprisingly, we found around 25% reduction in Ser9-phosphorylation of GSK-3β by BMOV treatment in 3T3-L1 cells receiving insulin post-exposure, although in a similar condition this vanadate caused an effective induction of Akt activity. This is also in contrast to previous studies where vanadate-mediated enhancement in the phosphorylation GSK-3β has been noted (Mehdi & Srivastava 2005, Ou et al. 2005, Basuki et al. 2006). However, in those investigations, the duration of vanadate treatment was very short, ranging from 5 to 10 min, whereas in our experiment the cells were subjected to a prolonged exposure to BMOV for 18 h. It has been demonstrated that serine phosphorylation of GSK-3 inhibits its catalytic activity, whereas tyrosine phosphorylation exerts the opposite effect (Wang et al. 1994, Doble & Woodgett 2003). Besides, a marked enhancement in tyr-phosphorylation of GSK-3 by pervanadate treatment (Sayas et al. 2006) supports the fact that PTP 1B is able to dephosphorylate GSK-3β at tyrosine residues and thereby reduce the GSK-3 enzymatic activity (Wang et al. 1994). Taking these into consideration, we postulate that BMOV can stimulate GSK-3β through inhibition of PTP 1B and this process may be closely associated with the dephosphorylation of Ser9, which is prerequisite for GSK-3β activation. However, at this juncture, the expected negative regulation of GSK-3β by BMOV-induced Akt should also be considered. In our opinion, under the prevailed experimental condition, the extent of induction of tyr-phosphorylation of GSK-3β by BMOV is strong enough to overcome the negative regulation imposed by BMOV-induced Akt, maintaining the enzyme in an active state. Our results are also in contrast to the findings revealing stimulation of glycogen synthesis by vanadate in different in vitro models (reviewed by Srivastava & Mehdi 2005). However, we did not check the status of glycogen synthesis in BMOV-treated cells. It has been reported that vanadate treatment in rat adipocytes increases glycogen synthase content, and elevates glucose-6-phosphate (Sekar et al. 1999), which in turn promotes activation of glycogen synthase in a reversible fashion. We speculate that the above effects, which are principally in opposition to the consequences of BMOV-mediated activation of GSK-3β, may be potent enough to account for the more likely possibilities of increased glycogen synthesis in BMOV-treated 3T3-L1 cells in our study. However, further detailed investigations are needed to judge this hypothesis. On the other hand, BMOV treatment did not show any change in Ser9-phosphorylation of IM9 cells despite causing a remarkable stimulation of Akt, the exact reason for which is yet to be identified.

In keeping with a negative impact on Akt activity, treatment of dexamethasone decreased Ser9-phosphorylation of GSK 3β remarkably in 3T3-L1 cells receiving insulin post-exposure. This is in agreement with earlier reports demonstrating negative regulation of GSK-3β phosphorylation in rat muscles by this glucocorticoid (Ruzzin & Jensen 2005, Ruzzin et al. 2005). Besides, dexamethasone produced some sort of additive impact on BMOV-mediated inhibition of GSK-3β phosphorylation, more likely suggesting the differential mode of action of these two compounds in this particular phosphorylation. While IM9 cells did not show any change in Ser9-phosphorylation by dexamethasone either alone or in combination with BMOV despite demonstrating an effective inhibition of Akt by the above glucocorticoid, suggesting a lack of cross-talking between Akt and GSK-3β at least for this particular event.

In parallel with a reduction in Ser9-phosphorylation of GSK-3β, we also found a decrease (28%) in Ser259-phosphorylation of Raf kinase by BMOV treatment in
3T3-L1 cells receiving insulin post-exposure. It has been reported that dephosphorylation of Ser\textsuperscript{259} is an essential part of the Raf-1 activation (Dhillon \textit{et al.} 2002\textit{a}). Such induction in Raf kinase by BMOV is in keeping with the stimulatory effect of vanadate on MAPK pathway (reviewed by Mehdi \textit{et al.} 2006). We speculate that in the prevailed experimental condition BMOV promotes the activation of Raf to an extent which would be effective enough to overcome the negative regulation of this kinase to be expected from BMOV-induced Akt. It has been stated that the activity of Raf-1 correlates with its phosphotyrosine content and tyrosine dephosphorylation inactivates this protein (Jelinek \textit{et al.} 1996). In the above study, it has been shown that treatment with PTP 1B diminishes the ability of Raf-1 to phosphorylate and activate MEK, while orthovanadate opposed Raf-1 inactivation. Based on this, it appears that BMOV-induced activation of Raf may be mediated through inhibition of PTP 1B and we speculate that this event can be associated with dephosphorylation of Ser\textsuperscript{259}. While, despite causing an elevation in Akt phosphorylation, BMOV treatment did not change the Raf activity in IM9 cells, the exact reason for which is yet to be identified.

On the other hand, dexamethasone treatment in 3T3-L1 cells increased Ser\textsuperscript{259}-phosphorylation of Raf despite causing an effective inhibition of Akt. This is in keeping with the attenuation of IGF-I-induced MAPK activation in 3T3 fibroblasts by long-term treatment with dexamethasone (Hansson \textit{et al.} 1996). We postulate that the inactivation of Raf by dexamethasone is mediated via PKA because this glucocorticoid stimulates PKA (Verrière \textit{et al.} 2005) and the activated PKA in turn can inhibit Raf-1 through phosphorylation of Ser\textsuperscript{259} (Dhillon \textit{et al.} 2002\textit{b}). Antagonism on Ser\textsuperscript{259} phosphorylation was shown by dexamethasone and BMOV in their combined treatment, suggesting the differential mode of action of these compounds in modulating Raf kinase activity. On the other hand, similar to GSK-3β phosphorylation, Ser\textsuperscript{259}-phosphorylation of Raf in IM9 cells did not respond to either dexamethasone or dexamethasone plus BMOV, suggesting a lack of coordination between Akt and Raf-kinase pathway.

In the present study, we have evaluated the insulinomimetic effect of BMOV on the phosphorylation of major components of the insulin signaling pathway including NPEY motif of IRβ, in both 3T3-L1, the effector cells of insulin and IM9 that are nontarget cells of insulin but express IR on their surfaces. We have also addressed the probable molecular mechanism underlying BMOV-mediated downregulation of IR in IM9 cells and evaluated the possibilities whether dexamethasone can modulate the BMOV-induced insulin signaling in both cell lines by elevating the depleted IR level. However, further studies are needed to completely understand the insulin signaling cascades of IM9 cells with elucidation of the exact molecular events involved in BMOV-induced downregulation of IR.

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