# Involvement of Insulin-like Growth Factor Type 1 Receptor and Protein Kinase C$\delta$ in Bis(maltolato)oxovanadium(IV)-Induced Phosphorylation of Protein Kinase B in HepG2 Cells[†]

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ABSTRACT: Vanadium(IV) oxo-bis(maltolato) (BMOV), an organovanadium compound, is a potent insulinomimetic agent and improves glucose homeostasis in various models of diabetes. We have shown previously that BMOV stimulates the phosphorylation of PKB which may contribute as one of the mechanisms for the insulinomimetic effect of this compound. However, the upstream mechanism of BMOV-induced PKB phosphorylation remains elusive. Therefore, in this study, we examine the upstream events leading to BMOV-induced PKB phosphorylation in HepG2 cells. Since BMOV is an inhibitor of protein tyrosine phosphatases and through enhanced tyrosine phosphorylation may activate various protein tyrosine kinases (PTK), we have investigated the potential role of different receptor or nonreceptor PTK in mediating BMOV-induced PKB phosphorylation. Among several pharmacological inhibitors that were tested, only AG1024, a selective inhibitor of IGF-1R-PTK, almost completely blocked BMOV-stimulated phosphorylation of PKB. In contrast, AG1295 and AG1478, specific inhibitors of PDGFR and EGFR, respectively, were unable to block the BMOV response. Moreover, efficient reduction of the level of IGF-1R protein expression by antisense oligonucleotides (ASO) attenuated BMOV-induced PKB phosphorylation. BMOV-induced PKB phosphorylation was associated with an increased level of tyrosine phosphorylation of the IR$\beta$ subunit, IGF-1R$\beta$ subunit, IRS-1, and $p^{85\alpha}$ subunit of PI3-kinase. However, this response was independent of IR-PTK activity because in cells overexpressing a PTK-inactive form of IR, insulin response was attenuated while the effect of BMOV remained intact. A role of PKC in BMOV-induced response was also tested. Pharmacological inhibition with chelerythrine, a nonselective PKC inhibitor, or rottlerin, a PKC$\delta$ inhibitor, as well as chronic treatment with PMA attenuated BMOV-induced PKB phosphorylation. In contrast, GÖ6976 and RO31-8220 PKC$\alpha/\beta$ selective inhibitors failed to alter the BMOV effect. Taken together, these data suggest that IGF-1R and PKC$\delta$ are required to stimulate PKB phosphorylation in response to BMOV in HepG2 cells and provide new insights into the molecular mechanism by which this compound exerts its insulinomimetic effects.

Vanadium is a transition metal, and its compounds have been shown to exert insulin-like properties in both in vivo and in vitro systems (reviewed in ref *1*). Oral administration of vanadium leads to improved insulin resistance and lowers the incidence of hyperglycemia in rodent models of diabetes mellitus as well as in limited studies with human subjects (reviewed in ref *1*). Vanadium mimics many physiological effects of insulin, including stimulation of glucose uptake, glycogen synthesis, and lipid synthesis in muscle, adipose, and hepatic tissues as well as inhibition of gluconeogenesis in the liver and kidneys and lipolysis in fat cells (*1*). In cultured cells, vanadium activates several keys components of the insulin signaling cascade implicated in mediating the physiological responses of insulin, including the tyrosine phosphorylation of the insulin receptor substrate (IRS-1)[1] (*2*, *3*) and activation of extracellular signal-regulated kinase (ERK1/2) (*4*) and the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) signaling cascade (*2*−*4*).

PKB, also known as Akt, is a 57 kDa serine/threonine kinase which has been implicated in the regulation of many physiological processes such as glucose transport, glycolysis,

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[1] Abbreviations: BMOV, vanadium(IV) oxo-bis(maltolato); CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GSK-3, glycogen synthase kinase-3; IGF-1R, insulin-like growth factor type 1 receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PDGFR, platelet-derived growth factor receptor; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC$\delta$, protein kinase C$\delta$; PMA, phorbol-12-myristate-13-acetate; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PY99, monoclonal antiphosphotyrosine antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VS, vanadyl sulfate.

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protein synthesis, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size, and cell-cycle progression (reviewed in ref *5*). In most cell types, PKB is activated via a PI3-K-dependent mechanism through dual phosphorylation of serine 473 in the C-terminal regulatory region and threonine 308 within the catalytic loop. Phosphorylation of PKB at Thr 308 is catalyzed by phosphatidylinositol 3,4,5-triphosphate (PIP3)-dependent protein kinase 1 (PDK-1), but the kinase responsible for phosphorylation at Ser 473, the putative PDK-2, remains elusive (*5*, *6*). Activated PKB exerts its effect through the phosphorylation of several downstream targets, such as glycogen synthase kinase-3 $\beta$ (GSK-3$\beta$), forkhead transcription factor (FKHR), Bcl-2-associated death promoter (BAD), IkB kinase, Mdm2, caspase 9, and endothelial nitric oxide synthase (eNOS) (reviewed in refs *5* and *6*).

Recently, we have demonstrated that organovanadium compounds are more potent than inorganic vanadium salts in inhibiting the total protein tyrosine phosphatase (PTPase) activity and in increasing the level of total protein tyrosine phosphorylation which was associated with a robust activation of the PKB pathway (*3*). A much higher potency of organovanadium compounds, compared to that of inorganic vanadium salts, in enhancing the phosphorylation of PKB and its downstream substrates has been suggested as one of the mechanisms to explain their greater antidiabetic effects (*7*−*9*). We have also shown that vanadium(IV) oxo-bis-(maltolato) (BMOV), a well-established antidiabetic and insulinmimetic organovanadium compound (*10*−*13*), enhanced the tyrosine phosphorylation of the $\beta$-subunit of the insulin receptor (IR$\beta$) in Chinese hamster ovary cells overexpressing IR (CHO-IR) (*3*), suggesting the involvement of IR-protein tyrosine kinase (PTK) activity in provoking the BMOV response. However, the clear involvement of IR-PTK as an upstream inducer of vanadium action has not been established, and a role of both IR-PTK-dependent (*14*−*16*) and -independent (*2*, *17*−*20*) events has been suggested to contribute to the insulin-like effects of vanadium. Therefore, in these studies, we have investigated a potential role of receptor PTKs in BMOV-induced PKB phosphorylation in human hepatoma (HepG2) cells.

## MATERIALS AND METHODS

### *Materials*

Insulin was from Eli Lilly Co. (Indianapolis, IN). Vanadium(IV) oxo-bis(maltolato) (BMOV) was a kind gift from D. Crans (Colorado State University, Fort Collins, CO). Phorbol-12-myristate-13-acetate (PMA) and luciferase/luciferin were from Sigma Aldrich (St. Louis, MO). HepG2 cells (HB-8065) were obtained from American Type Culture Collection (Rockville, MD). Human IGF-1 was from PeproTech Inc. Epidermal growth factor (EGF), platelet growth factor receptor (PDGF), and all pharmacological inhibitors were from Calbiochem (La Jolla, CA). Polyclonal insulin receptor-$\beta$ subunit antibody, polyclonal insulin-like growth factor type 1 receptor-$\beta$ subunit antibody, monoclonal antiphosphotyrosine antibody (PY99), polyclonal phospho-GSK-3$\beta$ ($Ser^{9}$) antibody, polyclonal PKC$\delta$ antibody, and polyclonal GSK-3$\beta$ antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal phospho-PKB ($Ser^{473}$), polyclonal PKB, and monoclonal phospho-insulin-like growth factor type 1 receptor ($Tyr^{1131}$)/phospho-insulin receptor ($Tyr^{1146}$) antibodies were from Cell Signaling (Beverly, MA). Polyclonal insulin receptor substrate and polyclonal $p^{85}$ antibodies were from Upstate (Lake Placid, NY). Phospho-PKC$\delta$ (Tyr 311) antibody and phospho-insulin-like growth factor type 1 receptor (Tyr 1131/1135/1136)/phospho-insulin receptor (Tyr 1158/1162/1163) were from Biosource (Camarillo, CA). Protein A-Sepharose beads and the enhanced chemiluminescense (ECL) detection system kit were from Amersham Pharmacia Biotech (Baie d'Urfé, PQ). Lipofectamine and all cell culture materials were from Invitrogen Corp. (Grand Island, NY).

### *Methods*

*Cell Culture.* HepG2 cells were maintained in DMEM containing 10% fetal bovine serum. Chinese hamster ovary cells overexpressing either wild-type human insulin receptor (CHO-IR) or the PTK mutant form (CHO-1018), a gift from M. F. White (Boston Children's Hospital, Boston, MA), were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to 80−90% confluence in 100 mm plates or 60 mm plates at 37 °C in a humidified atmosphere of 5% $CO_2$. Prior to the experiment, cells were incubated in serum-free medium for 20 h (*3*).

*Transfection with Oligonucleotides.* HepG2 cells were cultured in a collagen-coated Petri dish overnight. The following day, cells were transfected with either IGF-1R antisense oligonucleotides (CGGCTTCTCCTCCATGGTCC) or scrambled oligonucleotides (TCTTCCGCGACTTGCTCCGC) using lipofectamine according to the manufacturer's instructions. Forty-eight hours after post-transfection, cells were serum starved overnight followed by stimulation with BMOV. Incubation was terminated by quickly washing the cells in cold PBS. Phosphorothioate oligonucleotides (*21*) were synthesized by Invitrogen Corp.

*Immunoblotting.* Cells subjected to various experimental treatments were lysed in buffer A [25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 $\mu$g/mL aprotinin, and 1% Triton X-100], and the lysates were clarified by centrifugation to remove insoluble material. The clarified cell lysates normalized to contain equal amounts of protein were electrophoresed via 7.5 or 10% SDS−PAGE under reducing conditions, transferred to PVDF membranes, and incubated with the indicated antibody. Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (*3*). In some cases, cells were directly lysed in sample buffer containing $\beta$-mercaptoethanol followed by Western blot analysis. The immunoblots were quantified by densitometric scanning using NIH ImageJ.

*Immunoprecipitation.* The clarified cell lysates, normalized to contain equal amounts of protein (500 $\mu$g), were subjected to immunoprecipitation with 2 $\mu$g of various antibodies overnight at 4 °C, followed by incubation with protein A-Sepharose for 2 h. Immunoprecipitated proteins were collected by centrifugation and washed two times with buffer A and once with phosphate-buffered saline (PBS) containing PTPase and protease inhibitors. The immunoprecipitates were

electrophoresed via 7.5% SDS−PAGE under reducing conditions, transferred to PVDF membranes, and incubated with the respective primary antibodies. Proteins were detected with a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (*3*).

*Measurement of the Level of Intracellular ATP.* The ATP levels was measured according to the procedure described previously (*22*) with minor modifications. HepG2 cells cultured in six-well plates were treated with increasing concentrations of rottlerin for 30 min. The cells were washed twice with cold PBS and lysed in a 0.1 M NaOH/0.5 mM EDTA mixture. Cell lysates were incubated at 60 °C for 20 min and frozen at −20 °C. The ATP levels was measured by adding 10 $\mu$L of the lysates to 50 $\mu$L of the luciferin/luciferase assay mix and measuring the light output for 15 s in a luminometer (Turner Designs). A standard curve was also constructed by using serial dilutions of ATP (from 10 nM to 1 $\mu$M).

*Statistics.* Statistical analysis was performed by one-way, repeated measures analysis of variance (ANOVA), followed by a Newman-Keuls post-test. All data are reported as means ± the standard error. The differences between means were considered significant when $P < 0.05$.

## RESULTS

*Effect of BMOV on PKB and GSK-3β Phosphorylation in HepG2 Cells.* Our previous studies have demonstrated that organovanadium compounds such as BMOV are more potent activators of the PKB pathway than inorganic vanadium compounds in CHO-IR cells (*3*). Therefore, in these studies, we have first evaluated whether BMOV alters the activation of PKB and its downstream targets such as GSK-3$\beta$ in HepG2 cells which have been used as a model to investigate insulin action in liver (*23*). Since enhanced phosphorylation of specific serine/threonine residues of both PKB and GSK-3$\beta$ is critical for their activation state, we have utilized phospho-specific antibodies to monitor their activity. As shown in Figure 1A, treatment of HepG2 cells for 15 min with escalating concentrations of BMOV enhanced PKB and GSK-3$\beta$ phosphorylation in a dose-dependent manner. BMOV elicited a robust phosphorylation of these two kinases at 1 mM. Next, we assessed the time dependence of the 1 mM BMOV response; as shown in Figure 1B, 1 mM BMOV enhanced the phosphorylation of PKB and GSK-3$\beta$ within 10 min which reached a maximum in 30 min.

*Effect of BMOV on Tyrosine Phosphorylation of Total Protein, Insulin Receptor (IR), IRS-1, and $p^{85}$ in HepG2 Cells.* Since BMOV is a potent inhibitor of PTPases, and an increase in the level of tyrosyl phosphorylation of several key proteins such as IR and IRS-1 is an early step in triggering the insulin signaling cascade, we determined the effect of BMOV on tyrosine phosphorylation of the total proteins as well as on the phosphorylation of IR$\beta$, IRS-1, and $p^{85}$, the regulatory subunit of PI3-K in HepG2 cells. As illustrated in panels A and B of Figure 2, BMOV enhanced the tyrosine phosphorylation of several proteins in a dose- and time-dependent fashion. The molecular size of these proteins ranged between 35 and 200 kDa, and the increase in the level of phosphorylation caused by BMOV could be detected within 10 min of treatment (Figure 2B). To further analyze if some of these proteins were IR and its phospho-

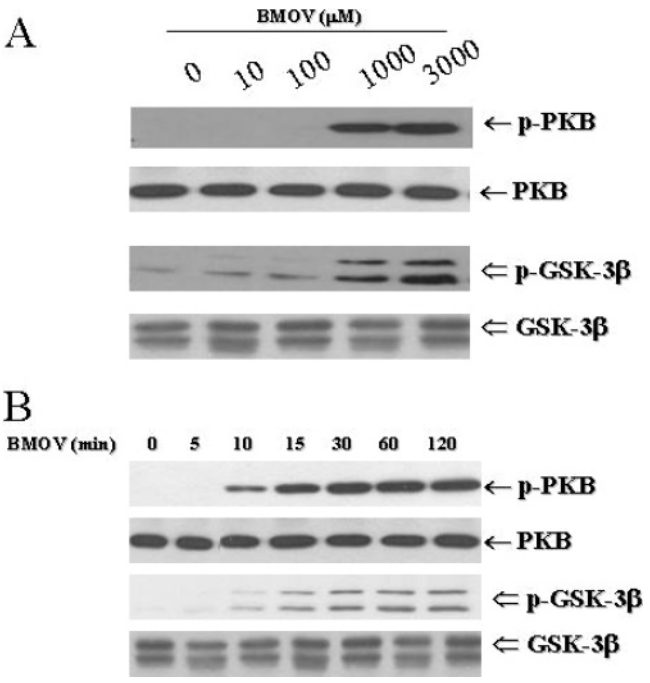


FIGURE 1: BMOV-induced PKB and GSK-3$\beta$ phosphorylation is time- and dose-dependent in HepG2 cells. Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (A) or with 1 mM BMOV for the indicated time periods (B). The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies (top immunoblot in panels A and B) and phospho-specific (Ser 9) GSK-3$\beta$ antibodies (bottom immunoblot in panels A and B). The results are representative of three independent experiments.

tyrosyl-protein substrates, immunoprecipitates from BMOV-treated cells were immunoblotted with antibodies to IR, IRS-1, and $p^{85}$. As shown in Figure 2C, BMOV treatment resulted in a significant increase in the level of phosphorylation of IR$\beta$, IRS-1, and $p^{85}$. To further confirm the identity of these proteins, IR$\beta$ or IRS-1 was immunoprecipitated from the lysates of the cells stimulated with or without BMOV followed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Figure 2D, a similar enhancement of IR$\beta$ and IRS-1 tyrosine phosphorylation was observed under these conditions.

*Role of IR-PTK on BMOV-Induced PKB Phosphorylation.* BMOV-induced enhanced tyrosine phosphorylation of IR$\beta$ suggested a possible role of IR-PTK in BMOV-induced effects on PKB phosphorylation. This possibility was probed further by utilizing CHO cells that overexpress an inactive form of IR-PTK (CHO-1018). The inactivation of IR-PTK in these cells was achieved by the mutation of lysine 1018 to alanine in the ATP-binding domain of IR$\beta$ (*24*). As illustrated in Figure 3, both insulin and BMOV enhanced the phosphorylation of PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells overexpressing PTK-inactive IR, the level of insulin-induced phosphorylation of PKB was significantly reduced, whereas the BMOV-evoked increase was not affected. These data suggested that in contrast to insulin, BMOV-induced phosphorylation of PKB was independent of IR-PTK activity.

*Effect of Receptor Tyrosine Kinase Inhibitors on BMOV-Induced PKB.* A potential role of epidermal growth factor receptor (EGFR) transactivation in vanadyl sulfate (VS)-induced signaling has been demonstrated (*25*, *26*), and

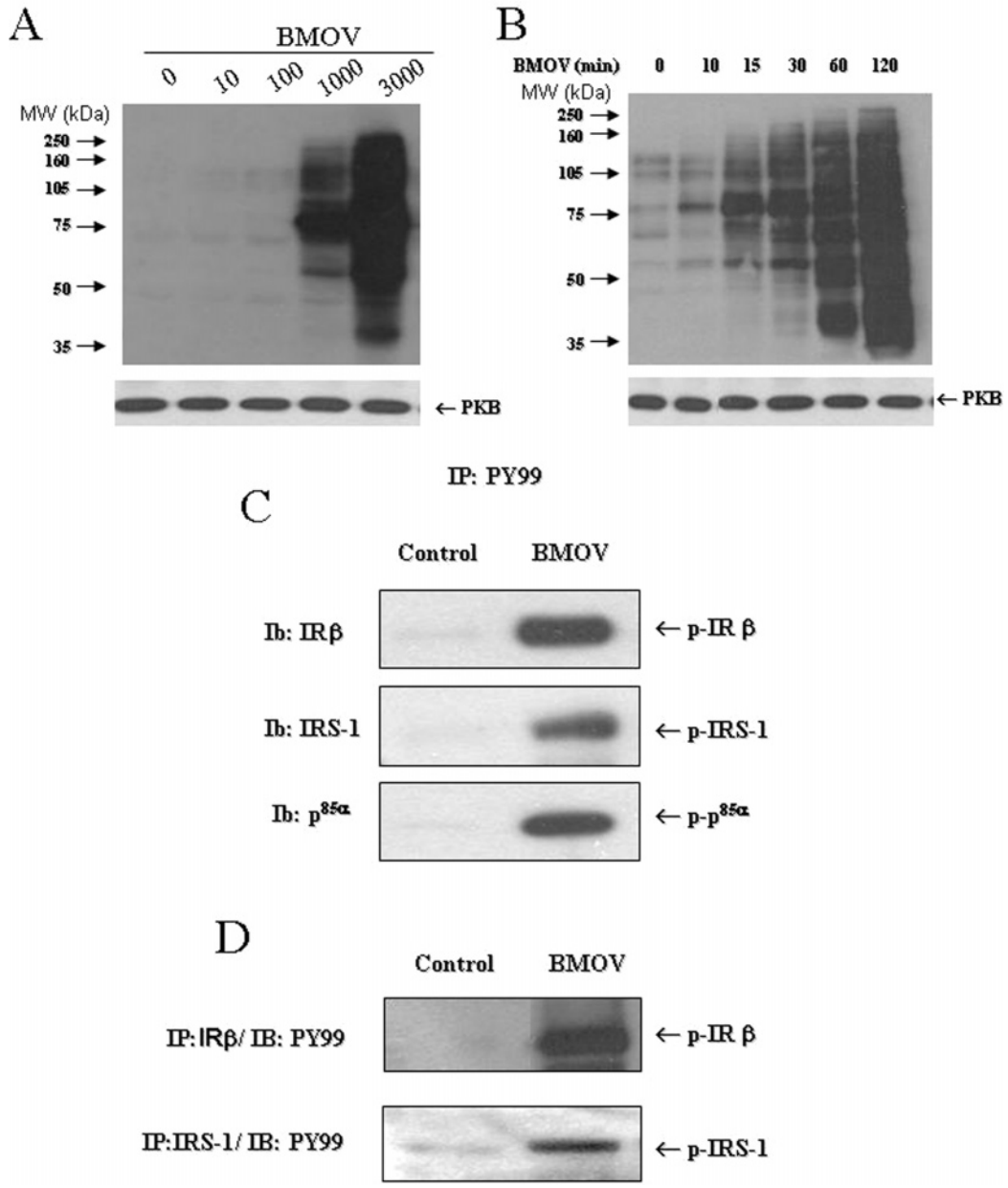


FIGURE 2: BMOV-enhanced tyrosine phosphorylation of total proteins and of IR$\beta$, IRS-1, and $p^{85}$. Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (A) or with 1 mM BMOV for the indicated time periodes (B). The cells were lysed, and the lysates were subjected to immunoblotting, using antiphosphotyrosine antibodies (PY99) (A and B). The blots shown in panels A and B were also probed with total PKB antibodies to control for equal loading. In panels C and D, cells treated without or with 1 mM BMOV for 15 min were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99) (C) or with anti-IR$\beta$ or -IRS-1 antibodies (D). The immunoprecipitates were immunoblotted (IB) with anti-IR$\beta$, -IRS-1, or -$p^{85}$ antibodies (C) or with antiphosphotyrosine antibodies (PY99) (D). A representative immunoblot from three independent experiments is shown.

insulin-like growth factor type 1 receptor (IGF-1R) activation has also been thought to play a role in vasoactive peptide-induced signaling (*27*, *28*). Therefore, we determined if IGF-1R, EGFR, or platelet growth factor receptor (PGDFR) was the putative PTK mediating BMOV-induced PKB phosphorylation. As depicted in Figure 4A, AG1024, a highly specific inhibitor of IGF-1R-PTK (*29*), prevented the phosphorylation of PKB induced by BMOV as well as IGF-1. In contrast, while AG1478, an EGFR-PTK inhibitor (*30*), completely blocked EGF-induced PKB phosphorylation, it failed to change the stimulatory effect of BMOV on this event (Figure 4B). Similarly, AG1295, an inhibitor of PDGFR-PTK (*31*), also had no effect on PKB phosphorylation induced by BMOV (Figure 4C). In fact, PKB phosphorylation was not detected in HepG2 cells in response to PDGF treatment, suggesting that PDGFR may be expressed at extremely low levels in these cells (*32*).

*Effect of Different Doses of AG1024 on BMOV-Induced Signaling.* Since BMOV-induced PKB phosphorylation was altered solely by AG1024, we analyzed the effect of this inhibitor in more detail. As shown in Figure 5A, treatment of cells with AG1024 inhibited the phosphorylation of PKB and GSK-3$\beta$ in a dose-dependent fashion with almost complete attenuation observed at 1 $\mu$M. Since tyrosine phosphorylation of IGF-1R $\beta$-subunit (IGF-1R$\beta$) is primordial in increasing its PTK activity, we investigated the effect of BMOV on the tyrosine phosphorylation of IGF-1R$\beta$. As shown in Figure 5B, the level of tyrosine phosphorylation

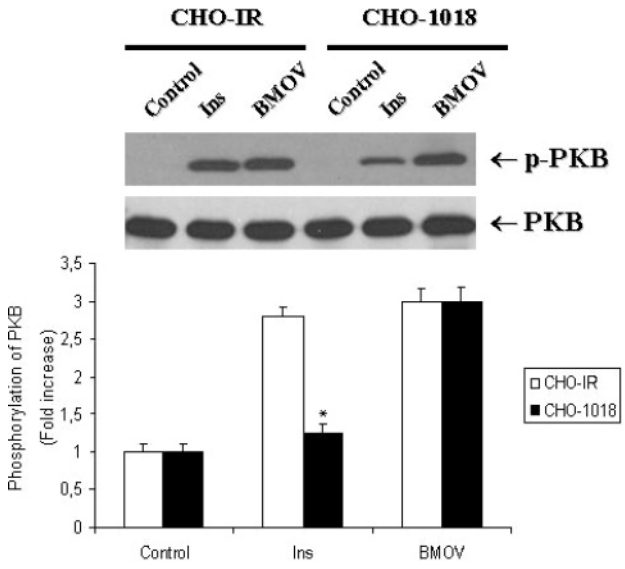


FIGURE 3: BMOV-induced PKB phosphorylation is independent of IR-PTK activity in CHO cells. Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 nM insulin (Ins) or with 1 mM BMOV for 15 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies. The phosphorylation level of PKB was quantified by densitometric scanning using NIH ImageJ and expressed as the fold increase over control cells. Values are means ± the standard error of at least three independent experiments. The asterisk indicates $P < 0.001$ vs CHO-IR cells stimulated with insulin (Ins).

of IGF-1 $\beta$ was increased by BMOV and pretreatment of cells with AG1024 prior to stimulation with BMOV resulted in a significant reduction in this response. IGF-1R$\beta$ has three critical tyrosines at positions 1131, 1135, and 1136 in the kinase domain crucial for maintaining its activity and for eliciting all IGF-1R-dependent functions (*33*). Therefore, by using two phospho-specific antibodies, one which recognizes phosphorylation of IGF-1R$\beta$ at Tyr 1131, Tyr 1135, and Tyr 1136, and the other that recognizes phosphorylation only at Tyr 1131, we investigated the effect of BMOV on the phosphorylation of these sites. As depicted in Figure 5C, BMOV increased the level of phosphorylation of IGF-1R$\beta$ on all three sites. Furthermore, like its effect on the total tyrosine phosphorylation of IGF-1R$\beta$, AG1024 markedly blocked this response (Figure 5C). These results suggested that BMOV-induced PKB phosphorylation was associated with an increased level of tyrosine phosphorylation of IGF-1R$\beta$.

*Effect of AG1024 on the Phosphorylation of IRS-1 and $p^{85}$ Induced by BMOV.* Increased levels of phosphorylation of IRS-1 and $p^{85}$ regulatory subunit of PI3-K are critical intermediary steps to signal the activation of PKB signaling in response to IGF-1R activation. Therefore, we next assessed if BMOV-enhanced tyrosine phosphorylation of IRS-1 and $p^{85}$ in HepG2 cells (Figure 2C) was dependent on IGF-1R-PTK activity. The results shown in Figure 5D demonstrate that BMOV induced tyrosine phosphorylation of both IRS-1 and $p^{85}$ which was significantly attenuated by AG1024.

*Effect of IGF-1R$\beta$ Antisense Oligonucleotides (ASO) on BMOV-Indcued PKB Phosphorylation.* To further confirm the role of IGF-1R in BMOV-induced PKB phosphorylation, we utilized IGF-1R$\beta$-specific antisense oligonucleotides (ASO) which reduced the level of expression of IGF-1R$\beta$ (*21*). Transfection of HepG2 cells was carried out with either scrambled (SCR) or increasing doses of IGF-1R$\beta$ ASO. ASO treatment dose-dependently decreased the level of IGF-1R$\beta$ expression (Figure 6A). When IGF-1R$\beta$ ASO or SCR-treated cells were incubated with BMOV, the enhanced PKB phosphorylation was significantly diminished in the IGF-1R$\beta$ ASO-treated group and not in the SCR group (Figure 6B). These data provide additional evidence to support a potential role of IGF-1R in mediating the BMOV response.

*Requirement of PKC$\delta$ in BMOV-Induced PKB Phosphorylation.* A potential role of PKC in mediating the responses of different stimuli such as PDGF (*34*), EGF (*35*, *36*), vascular endothelial growth factor (VEGF) (*37*, *38*), angiotensin II (*39*), and $H_2O_2$ (*40*) has been suggested. Therefore, we sought to verify if PKC-dependent pathways were involved in BMOV-induced phosphorylation of PKB in HepG2 cells. PKCs are composed of three groups. Members of the classical (cPKC) subtype ($\alpha$, $\beta$I, $\beta$II, and $\gamma$) are activated by calcium, diacylglycerol (DAG), phosphatidylserine (PS), and phorbol esters; members of the the novel (nPKCs) subtype ($\delta$, $\epsilon$, $\eta$, and $\theta$) are activated by DAG, PS, phorbol esters, and unsaturated fatty acids, and members of the atypical (aPKCs) subtype ($\zeta$ and $\lambda/\iota$) are insensitive to

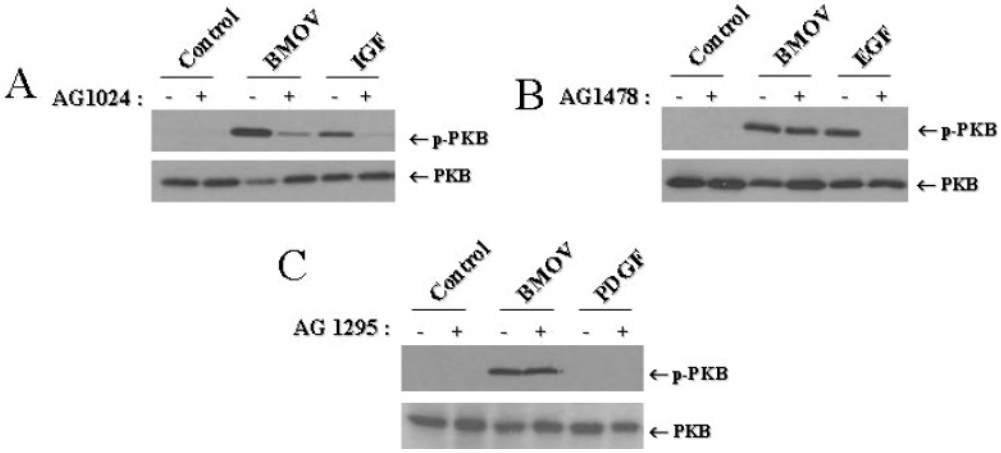


FIGURE 4: BMOV-induced PKB phosphorylation is inhibited by AG1024 but not by AG1478 or AG1295. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) 10 $\mu$M AG1024 (A), AG1478 (B), or AG1295 (C) for 30 min followed by stimulation with 1 mM BMOV for 15 min or 10 ng/mL IGF-1, 10 nM EGF, or 10 ng/mL PDGF for 10 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies. The results are representative of three independent experiments.

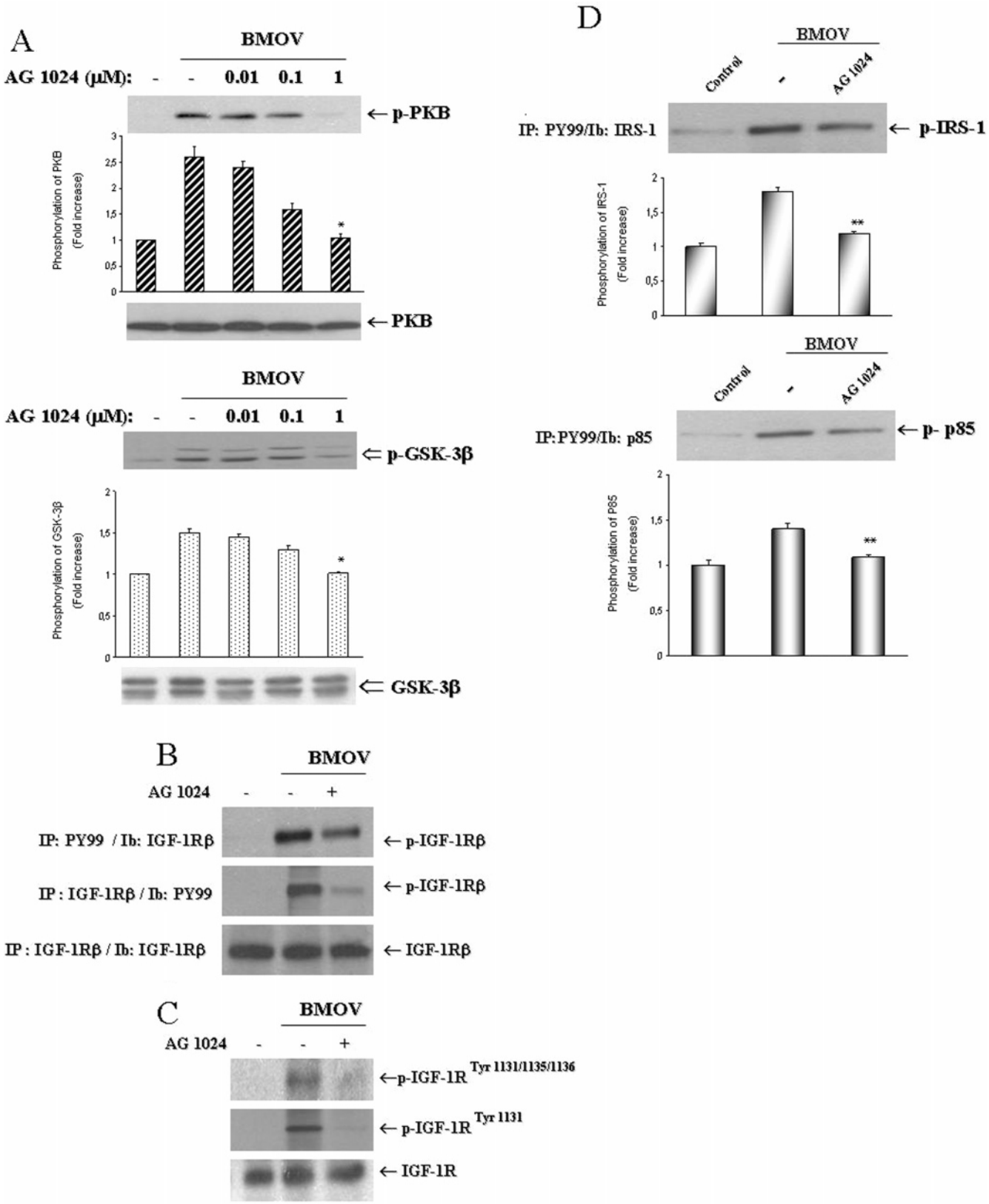


FIGURE 5: AG1024 decreased the level of BMOV-induced PKB phosphorylation in a dose-dependent manner and reduced the level of tyrosine phosphorylation of IGF-1R$\beta$, IRS-1, and $p^{85}$ subunit of PI3-K. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) different doses of AG1024 (A) or 1 $\mu$M AG1024 (B−D) for 30 min followed by stimulation with 1 mM BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies and phospho-specific (Ser 9)-GSK-3$\beta$ antibodies (A) or phospho-IGF-1R ($Tyr^{1131/1135/1136}$)/phospho-IR ($Tyr^{1158/1162/1163}$) and phospho-IGF-1R ($Tyr^{1131}$)/phospho-IR ($Tyr^{1146}$) (C) or to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99) followed by immunoblotting with the indicated antibodies (B and D). The phosphorylation levels of the protein shown in panels A and D were quantified by densitometric scanning using NIH ImageJ software and expressed as fold increase over control cells. Values are means ± the standard error of at least three independent experiments. One asterisk indicates $P < 0.001$ vs BMOV alone, and two asterisks indicate $P < 0.01$ vs BMOV alone.

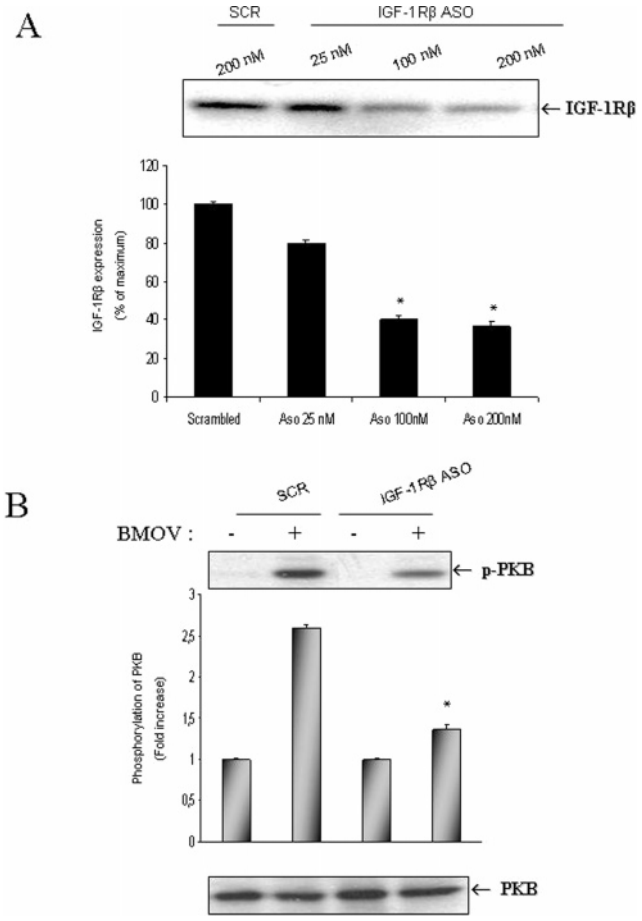


FIGURE 6: IGF-1R$\beta$ ASO decreased BMOV-induced PKB phosphorylation. HepG2 cells were transfected with 25, 100, and 200 nM antisense oligonucleotides (ASO) to IGF-1R$\beta$ or 200 nM scrambled oligonucleotides (SCR) (A) or with 100 nM ASO to IGF-1R$\beta$ or 100 nM SCR (B). Unstimulated (A) or cells stimulated in the absence (−) or presence (+) of BMOV (B) were lysed, and the lysates were subjected to immunoblotting using total IGF-1R$\beta$ antibodies (A) or phospho-specific (Ser 473)-PKB antibodies (B). Blot shown in top panel of (B) was reprobed for total PKB for equal loading and is shown in bottom. The results are representative of three independent experiments. The immunoblots were quantified by densitometric scanning using NIH ImageJ software. Values are means ± the standard error of at least three independent experiments. The asterisk indicates $P < 0.001$ vs scrambled.

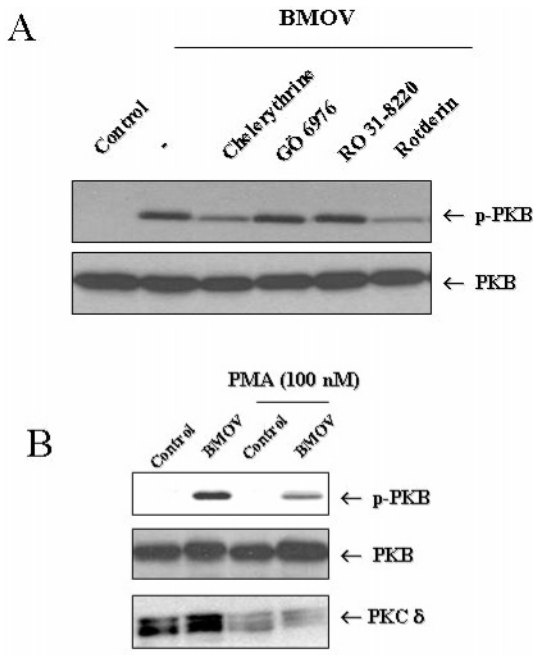


FIGURE 7: Chelerythine chloride, rottlerin, and long-term PMA pretreatment inhibits BMOV-induced PKB phosphorylation. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) 5 $\mu$M chelerythine chloride, GÖ6976, RO31-8220, or rottlerin for 30 min (A) or with 100 nM PMA for 24 h (B) followed by stimulation with 1 mM BMOV for 15 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies (top immunoblot in panels A and B) or total PKC$\delta$ antibodies (bottom immunoblot in panel B). The results are representative of three independent experiments.

DAG but are activated by PS and phosphatidylinositides (reviewed in ref *41*). The results shown in Figure 7A demonstrate that GÖ6976 and RO31-8220, selective chemical inhibitors of cPKC and aPKC isoforms, respectively, failed to inhibit BMOV-induced phosphorylation of PKB, whereas chelerythrine chloride, an isoform-nonselective inhibitor, and rottlerin, a highly selective PKC$\delta$ inhibitor, treatment significantly reduced the level of PKB phosphorylation induced by BMOV. The involvement of PKC was further confirmed by downregulating the PKC activity in HepG2 cells by a 24 h treatment with phorbol-12-myristate-13-acetate (PMA). As shown in Figure 7B, PMA-treated cells exhibited significantly attenuated PKB phosphorylation in response to BMOV as compared to untreated cells. Immunoblotting of parallel samples showed that PMA treatment significantly reduced the amount of PKC$\delta$ protein in these cells, whereas the total PKB levels remained unaltered after this treatment (Figure 7B). Rottlerin has been suggested to exert inhibitory effects through depletion of ATP levels (*22*, *42*); in view of this, we determined if the rottlerin-induced decrease in ATP levels was contributing to its inhibitory effect on BMOV-stimulated PKB phosphorylation. It was found that up to 10 $\mu$M rottlerin was unable to lower the ATP levels in these cells (data not shown), suggesting that ATP depletion was not responsible for attenuating the BMOV effect in HepG2 cells.

*Effect of BMOV on Tyrosine Phosphorylation of PKC$\delta$.* Recent studies have identified tyrosine phosphorylation as a potential mechanism through which PKC$\delta$ activation is regulated (*41*). Therefore, we investigated the effect of BMOV on tyrosine phosphorylation of PKC$\delta$ by immunoprecipitating PKC$\delta$ from lysates of cells treated with BMOV followed by immunoblot analysis with antiphosphotyrosine antibody. As depicted in Figure 8, BMOV treatment of HepG2 cells caused a significant increase in the level of tyrosine phosphorylation of PKC$\delta$. We also assessed the effect of BMOV on the tyrosine phosphorylation of PKC$\delta$ at tyrosine 311 (Tyr 311). This tyrosine residue is flanked by the regulatory and catalytic domains and is critical for generating the active form of PKC$\delta$ in response to $H_2O_2$ and PMA (*43*, *44*). As shown in Figure 8A, BMOV induced the Tyr 311 phosphorylation of PKC$\delta$. In addition, both total tyrosine and Tyr 311 phosphorylation of PKC$\delta$ enhanced by BMOV were blocked by rottlerin (Figure 8B). Taken together, these data indicated that BMOV-induced phosphorylation of PKB is associated with an activation of PKC$\delta$ as judged by an increase in the level of phosphorylation of key tyrosine residues in its catalytic domain.

*Effect of AG1024 on BMOV-Induced Tyrosine Phosphorylation of PKC$\delta$.* Since PKC$\delta$ has been shown to be

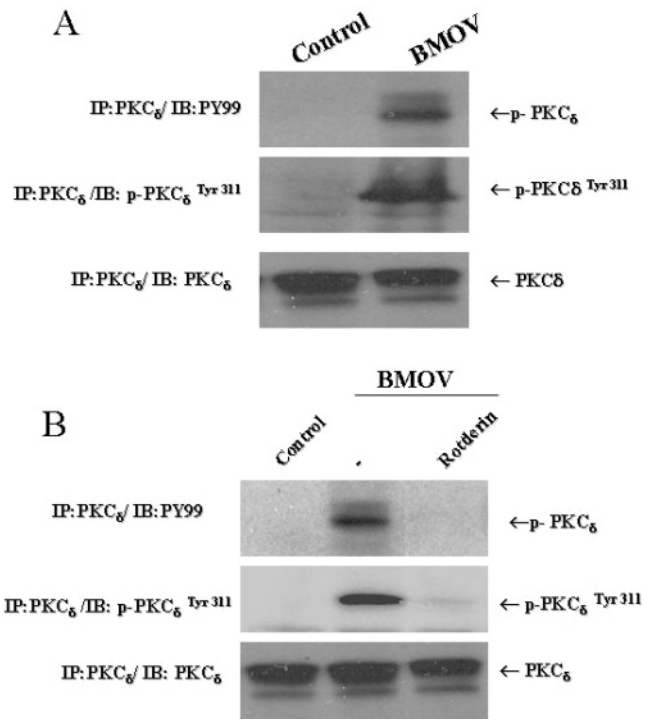


FIGURE 8: Rottlerin decreased the level of BMOV-induced total tyrosine phosphorylation at tyrosine 311 of PKC$\delta$. Confluent, serum-starved HepG2 cells were pretreated with or without 5 $\mu$M rottlerin for 30 min followed by stimulation in the absence or presence of 1 mM BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKC$\delta$ antibodies followed by immunoblotting with antiphosphotyrosine antibodies (PY99) or phospho-specific (Tyr 311) PKC$\delta$ antibodies. A representative immunoblot from three independent experiments is shown.

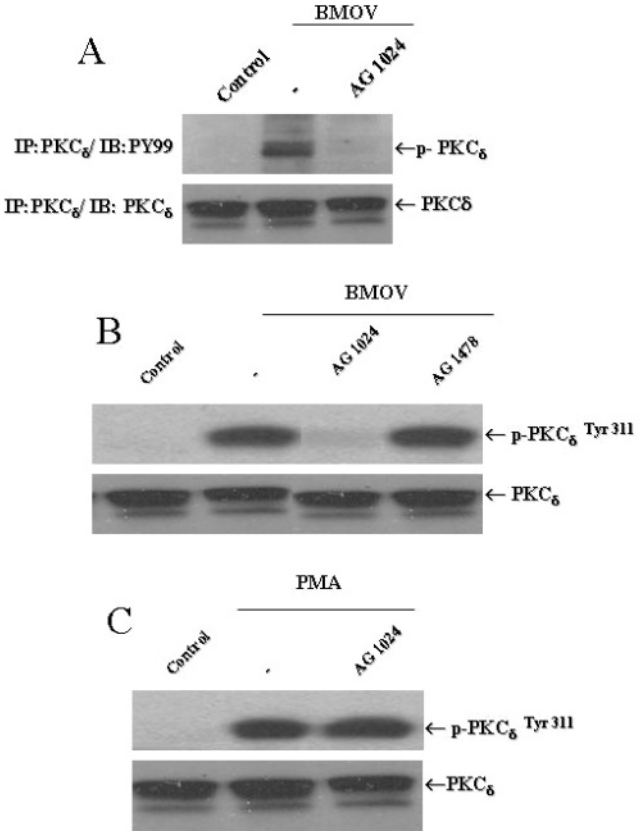


FIGURE 9: AG1024 decreased the level of BMOV- but not PMA-induced tyrosine phosphorylation of PKC$\delta$ at tyrosine 311. Confluent, serum-starved HepG2 cells were pretreated with or without 10 $\mu$M AG1024 or AG1478 (B) for 30 min followed by stimulation with 1 mM BMOV or 100 nM PMA for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKC$\delta$ antibodies followed by immunoblotting with antiphosphotyrosine antibodies (PY99) (A) or were immunoblotted using phospho-specific (Tyr 311) PKC$\delta$ antibodies (B and C). A representative immunoblot from three independent experiments is shown.

tyrosine-phosphorylated by various protein tyrosine kinases, including IGF-1R (*45*, *46*), we investigated if IGF-1R-PTK was responsible for triggering the phosphorylation of PKC$\delta$ in response to BMOV. As shown in Figure 9A, treatment of HepG2 cells with AG1024 almost completely abolished BMOV-stimulated total tyrosine phosphorylation of PKC $\delta$. In addition, AG1024 also inhibited phosphorylation of PKC$\delta$ at Tyr 311 while AG1478, an EGFR inhibitor, failed to alter the phosphorylation of PKC$\delta$ at this site (Figure 9B). Since PKC$\delta$ has been shown to be phosphorylated at Tyr 311 in response to various agonists, including PMA, we determined if like those of BMOV, PMA-induced effects are also dependent on IGF-1R-PTK. As shown in Figure 9C, in contrast to its inhibitory effect on PKC$\delta$ phosphorylation stimulated by BMOV, AG1024 was unable to block the effect of PMA, suggesting that IGF-1R is an upstream regulator of PKC$\delta$ phosphorylation in response to BMOV and not PMA.

## DISCUSSION

BMOV has been shown to exert glucoregulatory effects in many rodent models of diabetes (*10*−*13*, *47*); however, the precise mechanism through which this response is mediated remains largely unclear. We have shown previously that BMOV activates PKB (*3*), a central player involved in carbohydrate metabolism (*5*). In the study presented here, we demonstrate that BMOV-enhanced phosphorylation of PKB in HepG2 cells is associated with an increased level of tyrosine phosphorylation of the total cellular proteins, IR$\beta$, IGF-1R$\beta$, IRS-1, and $p^{85}$ subunit of PI3K. We have also provided evidence that the PTK activity of the IGF-1R$\beta$ is responsible for BMOV-induced activation of the PKB pathways. This conclusion is based on the use of highly selective inhibitors of IGF-1R, EGFR, and PDGFR-PTKs which showed that only the inhibition of IGF-1R-PTK by AG1024 blocked BMOV-induced PKB phosphorylation. Our results showing that BMOV treatment increased the level of total tyrosyl phosphorylation as well as Tyr 1131, 1135, and 1136 phosphorylation of IGF-1R$\beta$ which was specifically inhibited by AG1024 suggested that the PTK activity of IGF-1R$\beta$ was indeed being stimulated in response to BMOV. In addition, the data showing that antisense-induced reduction of IGF-1R$\beta$ decreased the level of BMOV-stimulated PKB phosphorylation provided further proof for the participation of IGF-1R in mediating the effect of BMOV. A role of IGF-1R$\beta$ in angiotensin II and $H_2O_2$ (*48*, *49*) induced ERK1/2 phosphorylation and in angiotensin II and purinergic receptor $P_2Y_{12}$-induced PI3-K/PKB signaling pathway has been reported previously (*28*, *50*). Transactivation of other growth factor receptor PTKs has also been suggested to play a critical intermediary role in response to agonists coupled to G-protein receptors (GPCR) (reviewed in ref *51*). However, the data presented here are the first to demonstrate that

BMOV, a non-GPCR agent, also signals through IGF-1R-PTK transactivation to activate PKB.

IR and IGF-1R exhibit a high degree of homology and share several common signaling features (reviewed in ref *52*), and despite having a higher $IC_{50}$ for IGF-1R-PTK, AG1024 can also inhibit IR-PTK activity at high concentrations (*29*). Furthermore, our results showing that BMOV treatment increased the level of tyrosine phosphorylation of IR$\beta$ might support the idea of a role for IR-PTK in BMOV-induced responses. However, our observations that in CHO-1018 cells the extent of the insulin response was significantly decreased while the BMOV effect was intact suggested an IR-PTK-independent mechanism of BMOV action. Earlier studies using inorganic vanadium compounds have also documented the lack of a role of IR-PTK in inducing the vanadium response (*17, 53*). In addition, several investigators have also reported that the insulin-like effects of vanadium were not associated with an increase in the level of tyrosine phosphorylation of IR$\beta$ in many systems (*2*, *17*−*20*). Thus, despite the fact that some studies have demonstrated an increase in the level of IR tyrosine phosphorylation in vivo in response to vanadium treatment in diabetic animal models (*10*, *54*), our data support the notion that IGF-1R-PTK activation by BMOV may serve as an alternate mechanism to mediate the insulin-like effect of this compound.

PKC$\delta$ is a serine/threonine kinase that plays a key role in many physiological responses such as growth regulation (*55*), tissue remodeling (*56*), migration (*57*), and transformation (*45*). In response to insulin, PKC$\delta$ and PKC$\theta$, two novel PKC isoforms, have been shown to convey an insulin signal toward glucose transport (*58*, *59*), glycogen synthesis (*60*), and cell proliferation (*61*). In contrast, several studies have reported that PKC$\delta$ may downregulate insulin signaling in response to high glucose concentrations (*62*) and other stimuli (*63*). Therefore, it appears that novel PKC isoforms may both mediate stimulatory effects of insulin on glucose metabolism and inhibit the intracellular insulin signaling pathway. Our data showing that PKB phosphorylation was blocked by chelerythrine and rottlerin and not by GÖ6976 or RO31-8220 suggested a role for PKC$\delta$ in BMOV action. Since tyrosine phosphorylation of PKC$\delta$ has been shown to activate its catalytic activity (*43*, *44*), the results showing that both total tyrosine phosphorylation and phosphorylation of Tyr 311 in PKC$\delta$ were enhanced by BMOV provide additional support for the involvement of this isoform of PKC in mediating the BMOV response in HepG2 cells. Interestingly, like its effect on PKB phosphorylation, rottlerin pretreatment blocked the PKC$\delta$ tyrosine phosphorylation induced by BMOV. Rottlerin has been extensively used as a selective inhibitor of PKC$\delta$; however, recent reports have suggested that it may have additional, non-PKC$\delta$-dependent responses such as depletion of the intracellular ATP level (*22*, *42*, *64*). However, our data showing that rottlerin was unable to alter the ATP concentration in HepG2 cells suggested that rottlerin was exerting its effect via an ATP-independent mechanism in these cells.

Furthermore, our results showing that in cells chronically treated with PMA, the attenuated response of BMOV on PKB phosphorylation was associated with a decreased level of expression of PKC$\delta$ provided additional evidence of a role for PKC$\delta$ in BMOV action. To the best of our knowledge, these data are the first to demonstrate the involvement of PKC in vanadium-mediated PKB activation. This is consistent with previous reports where an involvement of PKCs in mediating PKB activation was documented (*37*, *66*). In contrast, however, a negative regulatory role of PKC$\delta$ in PKB activation has also been suggested (*67*, *68*). Because PKC expression and its action have been reported to be tissue-specific (*69*), it is possible that PKC$\delta$ may elicit different responses depending on cell type. The attenuation of BMOV-induced PKC$\delta$ phosphorylation by AG1024 observed in our studies indicates that IGF-1R may be an upstream mediator of this event. A requirement for PKC$\delta$ in IGF-1R-induced signaling has also been described (*45*, *70*, *71*). However, the precise mechanism and intermediary steps responsible for PKC$\delta$-mediated PKB activation in response to BMOV remain to be explored. Moreover, additional studies using genetic approaches to silence or knock down PKC$\delta$ by siRNA and/or oligonucleotides will be needed to provide unequivocal evidence of a role for PKC$\delta$ in vanadium-induced responses on PKB in HepG2 cells.

In summary, our studies have provided experimental evidence of a role for IGF-1R-PTK in BMOV-induced activation of the PKB signaling pathway in HepG2 cells. We have also demonstrated that PKC$\delta$ activation plays an intermediary role in transducing IGF-1R signaling leading to PKB phosphorylation in response to BMOV. These data provide novel insights into the cellular mechanism of the insulinomimetic and glucoregulatory action of BMOV.

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