# Differential Acute Effects of Oxovanadiums and Insulin on Glucose and Lactate Metabolism Under In Vivo and In Vitro Conditions

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**Oxovanadium compounds such as vanadate and peroxovanadiums have been shown to have insulin-mimetic effects on various metabolic pathways, including glucose metabolism. A differential effect of various oxovanadium species on glucose metabolism in different tissues has been reported. The results from our present in vivo studies using rats show that peroxovanadiums and insulin have similar acute effects on decreasing blood glucose levels, but dissimilar effects on blood lactate levels. Furthermore, when bisperoxovanadate (BPV) was administered acutely to intact animals immediately before a bolus insulin challenge, it blunted the effectiveness of insulin in decreasing the blood lactate level, but at the same time demonstrated a synergistic effect on the hypoglycemic action of insulin. It was also observed in in vitro studies using normal 3T3-L1 adipocytes (not serum-deprived) that 1,10-phenanthroline bisperoxovanadate (PHEN-BPV) attenuates the incorporation of carbon from lactate but not from glucose, into lipid in both the absence and presence of insulin. Additionally, it was observed that PHEN-BPV had no effect on lactate dehydrogenase (LDH) activity. Thus, one may speculate that PHEN-BPV interferes with carrier-mediated lactate transport. These observations demonstrate that insulin and oxovanadiums differ in the handling of different metabolic substrates. Thus, even though oxovanadiums mimic many of the metabolic actions of insulin, their metabolic effects are by no means identical. Moreover, since vanadate had no acute effect on glucose metabolism under in vivo conditions, this may suggest that to be effective as a hypoglycemic agent vanadate needs to be converted to some other biologically active oxovanadium species. Finally, the observed interference by PHEN-BPV in the metabolism of lactate may predispose subjects using oral vanadate, as a part of the therapeutic regimen for management of diabetic hyperglycemia, to lactic acidosis.**



SEVERAL OXOVANADIUM SPECIES have been shown to mimic a significant number of the metabolic actions of insulin under both in vivo and in vitro conditions.[1] This insulin-mimetic activity of oxovanadiums in cells and intact animals has been associated with increased levels of phosphorylated tyrosine proteins, which appear to result from their inhibitory effect on phosphotyrosine phosphatases.[2-5] It has been reported that some peroxovanadium compounds are more powerful phosphotyrosine phosphatase inhibitors and insulin mimetics than vanadate,[4] although the underlying mechanism(s) responsible for the increased inhibitory potency has not been delineated. Vanadium is a widely distributed element and forms a variety of complexes in oxidation states ranging from $-1$ to $+5$.[6] Most tissues of higher animals contain intracellular vanadium at concentrations less than 0.2 μmol/L. The only biologically important oxidation states for vanadium are IV (vanadyl) and V (vanadate).[6] Intracellular vanadate can easily be reduced to vanadyl ($VO^{2+}$) by various physiological reducing moieties such as glutathione (GSH), catechols, and other cellular constituents.[7] However, it has recently been shown that, at least under in vitro conditions, the interaction between vanadate and GSH has a relatively low rate constant.[8] Of course, the apparent rate constant for interactions between these two species under in vivo conditions may be significantly different. Vanadyl ions form extremely strong complexes with proteins and other organic and inorganic ligands and have been shown to have insulin-mimetic activities under both in vivo and in vitro conditions.[8,9]

Oxovanadiums have been shown to influence a wide range of metabolic activities under both in vitro and in vivo conditions, primarily through their effects on phosphorylation/dephosphorylation systems inside the cells.[6,7] In general, increased levels of tyrosine-phosphorylated proteins are associated with the hormone-mimetic effects of oxovanadiums on cells.[10] Oxovanadium species are known to be potent inhibitors of tyrosine phosphatases, albeit with differing potencies. A stimulatory effect of oxovanadiums on both receptor tyrosine kinase and cytosolic protein tyrosine kinase activities has been demonstrated.[8,11,12] Monomeric vanadate is structurally analogous to phosphate, and this has been speculated to be an important factor that accounts for its inhibitory effects on enzymes involved in phosphate transfer or release reactions. However, the exact mechanism(s) of the biological actions of oxovanadium compounds remains unclear. Peroxovanadates, which are more potent inhibitors of tyrosine phosphatase than vanadate, do not bear structural similarities to ground-state phosphate, but are structurally similar to the trigonal-bipyramidal transition state of phosphate. Addition of vanadate or peroxovanadate to isolated adipocytes increases glucose transport and metabolism.[11,13] Long-term treatment with vanadate or other oxovanadium species has been found to be effective in normalizing blood glucose concentrations in diabetic animal models.[1,14] This hypoglycemic effect of vanadate is achieved through stimulation of glucose transport and metabolism in both peripheral tissues and the liver. However, the specific mechanisms involved in vanadate-mediated restoration of glucose uptake and utilization in diabetic animals are not well defined.

Vanadate, when present in high concentrations, has the potential to disrupt normal metabolism by inhibiting many key enzymes that are important for normal life processes.[15] The

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toxic effects of vanadate include loss of body weight, kidney failure, morbidity, and even death.[16] It has not been established whether there is a safe window for the extended use of vanadate in the treatment of diabetes. Studies with young female rats of reproductive age have demonstrated that oral vanadate treatment reduces the rate of conception and the ability to carry pregnancy to term in both diabetic and nondiabetic animals.[17] It also has been observed that vanadate is only partially effective in normalizing blood glucose levels in pregnant diabetic rats[18] and causes morbidity and a significant number of mortalities in this group. The specific mechanism responsible for the high degree of vanadate toxicity in the diabetic pregnant group is not known; however, since lactic acidosis is a frequent occurrence among diabetics under stressful situations such as pregnancy, it is possible that vanadate may aggravate lactic acidemia in diabetic pregnant animals.

It has been demonstrated that, unlike vanadate, peroxovanadium species administered acutely to intact animals produce insulin-like effects on the uptake and metabolism of glucose.[4,19] In the present study, we compared and contrasted the effects of an acute intravenous (IV) challenge with insulin and peroxovanadium species on blood glucose and lactate levels in intact anesthetized rats when administered both separately and in tandem. We further examined the impact of these two agents on the stimulation of lipid synthesis in 3T3-L1 cells and compared their relative efficiency in using glucose and/or lactate substrates as the source of carbon in this process.

## MATERIALS AND METHODS

Virgin female Sprague-Dawley rats weighing 210 to 230 g were purchased from Harlen Laboratories (Indianapolis, IN). Routine chemicals were obtained from either Sigma Chemical (St Louis, MO), Bio-Rad (Hercules, CA), or Fisher Scientific (St Louis, MO). Insulin (Lente 100 U/mL; Eli Lilly, Indianapolis, IN) was obtained from a pharmacy. Bicinchoninic acid (BCA) protein assay kits were obtained from Pierce (Rockford, IL). Polyvinylidemedifluoride (PVDF) filter paper for immunoblots was obtained from Millipore (Milford, MA). ECL chemiluminescence kits were purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase–conjugated anti–rabbit immunoglobulin G antibody, cell culture media, and newborn and fetal bovine calf serum were obtained from GIBCO BRL Life Technologies (Grand Island, NY). U-$^{14}$C-lactate and 6-$^{3}$H-glucose were obtained from ICN (Costa Mesa, CA). Nembutal solution was purchased from Abbott Laboratories (Chicago, IL).

### Experimental Design

#### *In Vivo Studies*

Mature female Sprague-Dawley rats weighing 230 to 300 g were used in this study. Each experimental rat was fasted for 10 to 12 hours before cannulation. On the day of the experiment, the rat was anesthetized by injection of nembutal sodium in saline solution at a dose of 50 mg/kg body weight (BW), and both the femoral vein and carotid artery were cannulated. Each cannula then was filled with heparin solution (50 U/mL) to prevent blood from clotting in the cannula. After a 30-minute stabilization period, a 0.2-mL blood sample was drawn from the carotid artery. A 0.2-mL bolus infusion of test agent, either 0.6 μmol various oxovanadium species/0.10 kg BW, 1.5 μg insulin/0.10 kg BW, or normal saline solution, was introduced into the femoral vein. Blood samples of 0.2 mL were collected through the cannula in the carotid artery at 5, 15, 30, 60, 120, and 180 minutes following bolus infusion of the test agent. Half of each blood sample (0.1 mL) was immediately mixed with 0.2 mL 8% cold perchloric acid and vortexed for 30 seconds. The blood-precipitate mixture was kept cold for an additional 5 minutes to ensure complete protein precipitation and then centrifuged for 10 minutes at approximately 1,500 × *g*. This clear perchloric acid supernate was stored for lactate analysis. The other half of the blood sample was centrifuged at 1,500 × *g* for 10 minutes, and this plasma was stored for glucose and vanadium analyses. During each experiment, 0.5 mL normal saline was infused slowly through the femoral vein at 60-minute intervals to compensate for loss of blood volume from multiple sampling.

An additional group of normal female rats was used to determine the effect of the combination of peroxovanadate and insulin on blood glucose and lactate levels. Following an overnight fast, each rat was surgically prepared as already described. All animals in this group received a bolus of 1,10-phenanthroline bisperoxovanadate (PHEN-BPV) 0.6 μmol/0.10 kg BW in normal saline at time −30 minutes through the femoral vein. After a 30-minute period following administration of PHEN-BPV, a bolus dose of insulin 1.5 μg/0.10 kg BW was injected through the vein at time 0. Blood samples were collected at the same intervals described earlier.

#### *In Vitro Studies*

*3T3-L1 cell culture system.* 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (Rockville, MD) and maintained in six-well culture plates in Dulbecco's modified Eagles (DME) medium with 450 mg/dL glucose supplemented with 5% fetal calf serum and 5% newborn calf serum at 37°C in a 5% $CO_2$ environment. The cells were transformed by previously reported methods[20,21] with minor modifications. Briefly, 2 days following confluency, cells were treated for 3 days with differentiation medium consisting of DME medium with high glucose (450 mg/dL) containing 0.5 mmol/L isobutylmethylxanthine, 0.25 μmol/L dexamethasone, and 5 μg/mL insulin in 10% fetal calf serum. The medium then was changed to one containing only 5 μg/mL insulin in 10% fetal calf serum in a high-glucose DME medium, and the cells were cultured in this medium for an additional 3 days. The converted cells, adipocytes, were maintained for an additional 4 days in high-glucose DME medium supplemented with 10% fetal calf serum before the experiments. Depending on the experimental protocol, cells were placed either overnight or for 2 hours in a serum-free Hanks buffer, pH 7.4, with 5 mmol/L glucose and supplemented with 20 mmol/L HEPES and 1 mmol/L sodium pyruvate containing 0.5% bovine serum albumin (BSA). Before experiments on the incorporation of glucose and/or lactate carbon into lipid, cells were washed in Hanks buffer and placed in fresh prewarmed Hanks buffer at 37°C supplemented with HEPES, glucose, and pyruvate as already described. The test agents, oxovanadiums or insulin, and radioactively labeled glucose and lactate were added to the cultured cells to assess the stimulation of lipid synthesis.

*Incorporation of $^{3}$H-glucose and $^{14}$C-lactate carbon into lipid.* Transformed 3T3-L1 adipocytes in six culture plates received exactly 2 mL Hanks buffer with 20 mmol/L HEPES, were challenged with the test agent, PHEN-BPV and/or insulin, and were incubated for an additional 30 minutes before addition of the radioactively labeled substrates to the cell culture wells. Radioactive labeled 6-$^{3}$H-glucose (2 μCi/well) and/or U-$^{14}$C-lactate (0.5 μCi/well) were then added to the cells, which were incubated for an additional 1 hour under conditions already described. Immediately following incubation, the culture plates were placed on ice, the medium was discarded by aspiration, and the cells were rinsed twice with ice-cold phosphate-buffered saline (PBS). The cells were scraped from the plate in 1 mL distilled water, transferred to 15-mL culture tubes, and frozen rapidly in liquid nitrogen. The cells were then thawed, and 6 mL 2:1 mixture of chloroform/methanol was added to each tube. The tubes were capped, vortexed vigorously for 1 minute, and placed on a shaker at 40 cycles per minute overnight. The tubes were centrifuged at 3,000 rpm for 10 minutes, and the aqueous layer

was discarded. The chloroform phase was then transferred to scintillation vials and evaporated to dryness. A nonaqueous scintillation cocktail (10 mL) was added to each tube, and the tubes were vortexed vigorously, stored overnight, and counted in a dual-channel liquid scintillation counter.

### *Lactate Dehydrogenase Assays*

Levels of lactate dehydrogenase (LDH) in the test samples were assessed in both intact cells and cell homogenates by measuring the kinetics of NADH oxidation using an assay kit (Sigma). For measurement of LDH levels in intact transformed 3T3-L1 cells, 4 days following transformation, the cells in culture plates were placed in serum-free Hanks medium supplemented with 20 mmol/L HEPES, pH 7.4, for 1 hour. The culture plates were treated with various concentrations of PHEN-BPV for 1.5 hours. The cells were then washed twice in PBS and scraped off the plate with a rubber spatula. The cells were placed in test tubes on ice, sonicated at three-fourths speed, and centrifuged at 14,000 rpm for 15 minutes. A 50-μL aliquot of supernate from each sample was added to 1 mL prewarmed (37°C) reaction buffer with NADH, and absorbance was monitored at 340 nm at exactly 30 and 60 seconds following addition of the test sample. Three separate culture plates were used for each PHEN-BPV dose, and the supernate from each culture plate was assayed in duplicate.

To determine the effect of PHEN-BPV on LDH activity in cell homogenate, 4-day posttransformed cells were used. The culture plates were washed in PBS, and the cells were scraped, pooled, and sonicated in PBS. Sonicated cells were centrifuged in a microfuge at 14,000 rpm for 15 minutes. The supernate was removed and separated into 1-mL portions, and PHEN-BPV was added to the tubes at final concentrations of 6, 12, 24, and 48 μmol/L. The tubes were vortexed and incubated in a 37°C water bath for 90 minutes. LDH activities were determined for each dose of PHEN-BPV in triplicate using a protocol similar to that described earlier.

### *Protein Determination*

Protein concentrations in cellular lysates were determined with BCA dye-binding assay kits using bovine albumin as a standard.

### *Blood Vanadium Assay*

Blood vanadium levels were determined by graphite-furnace atomic absorption spectrometry. The AA-spectrometer is a Varian Spectra AA-10 (Sunnyvale, CA) fitted with a GTA-96 graphite-tube atomizer and a programable sample dispenser. Serum samples or solubilized cell samples were stored frozen immediately following collection as described earlier. Samples were thawed, microfuged at high speed, diluted with a 0.5% Triton X-100 solution, and vortexed before analysis.

### *Blood Glucose Assay*

Blood glucose was determined by a glucose-specific enzymatic reaction using the glucose-6-phosphate dehydrogenase (G-6-PDH) method. Glucose assay kits were obtained from Sigma. The analysis was performed according to the protocol provided by the manufacturer, using glucose as a standard.

### *Blood Lactate Assay*

Blood lactate levels were determined by the pyruvate reduction method. Lactate assay kits were obtained from Sigma. The assays were performed according to the manufacturer's instructions, using L-lactate as a standard. The increased absorbance at 340 nm due to NADH formation from $NAD^+$ in the presence of LDH is proportional to the lactate levels present in the sample. All assays were performed in triplicate.

### *Synthesis of Peroxovanadium Complexes*

Crystalline peroxovanadium complexes of formulas $M[VO(O_2)_2LL] \cdot nH_2O$, where M is Na or K, LL represents some ancillary ligand such as 1,10-phenanthroline or 4,7-dimethyl-1,10-phenanthroline, and n is 0 to 5, were prepared according to procedures described previously.[22] The purity of peroxovanadium complexes was determined by UV/Vis and infrared spectroscopy.

### *Statistical Analyses*

A one-factor ANOVA was performed on each of the variables of interest (ie, glucose, lactate, or vanadium levels). If a significant difference (at the .05 level) for treatment effects was observed, additional multiple comparison testing was performed to assess the significance of the difference between the two treatments. Dunnett's method was chosen to compare each treatment group with the control, since it is specifically designed for such a comparison. For any two treatments (not involving a control), Tukey's method of comparison was chosen based on its conservative nature. All tests were conducted at a .05 level of significance. Wherever indicated, errors associated with data are shown as standard deviations.

## RESULTS

Figure 1 shows the effects of various oxovanadium species and insulin treatments on blood glucose levels at different time points during 180 minutes following initiation of the treatments. Only bolus administrations of PHEN-BPV and of insulin were effective in precipitating a marked and significant reduction in blood glucose for all times through 60 minutes. This observation was first noted by Posner et al.[4] However, it has been reported by Bevan et al[19] that a challenge with 4,7-dimethyl-1,10-phenanthroline bisperoxovanadate (DPV) at doses one order of magnitude higher than that used in this study does yield measurable hypoglycemic action. As expected, a bolus IV insulin challenge caused a precipitous decrease in blood glucose that reached a minimum value, approximately 52% of the original level, by 15 minutes. A PHEN-BPV challenge also produced a rapid decline in blood glucose and reached a minimum value, approximately 70% of the original level, 15 to 30 minutes after IV bolus infusion. Thus, for the doses used, insulin demonstrated a significantly greater acute effect in decreasing blood glucose both quantitatively and temporally compared with PHEN-BPV. However, it is also obvious from the figure that the PHEN-BPV effect on blood glucose is markedly more sustained compared with that of insulin. Blood glucose in the PHEN-BPV–treated group, but not in the insulin-treated group, was significantly lower than that in the control group at both 120 and 180 minutes following initiation of the treatments. Furthermore, blood glucose of the PHEN-BPV–treated group was significantly lower than that of the insulin-treated group at 180 minutes. Interestingly, statistical analysis of the total integrated area under the glucose curves from 0 to 180 minutes showed no significant difference for the insulin and the PHEN-BPV groups, even though the initial reduction in blood glucose levels of the insulin-treated group was significantly greater than that of the PHEN-BPV–treated group. In contrast to PHEN-BPV treatment, the other two oxovanadium species, vanadate and DPV, caused no significant reduction in blood glucose levels at any point over the 180-minute period compared with blood glucose levels of the control group during the same period. Thus, among these

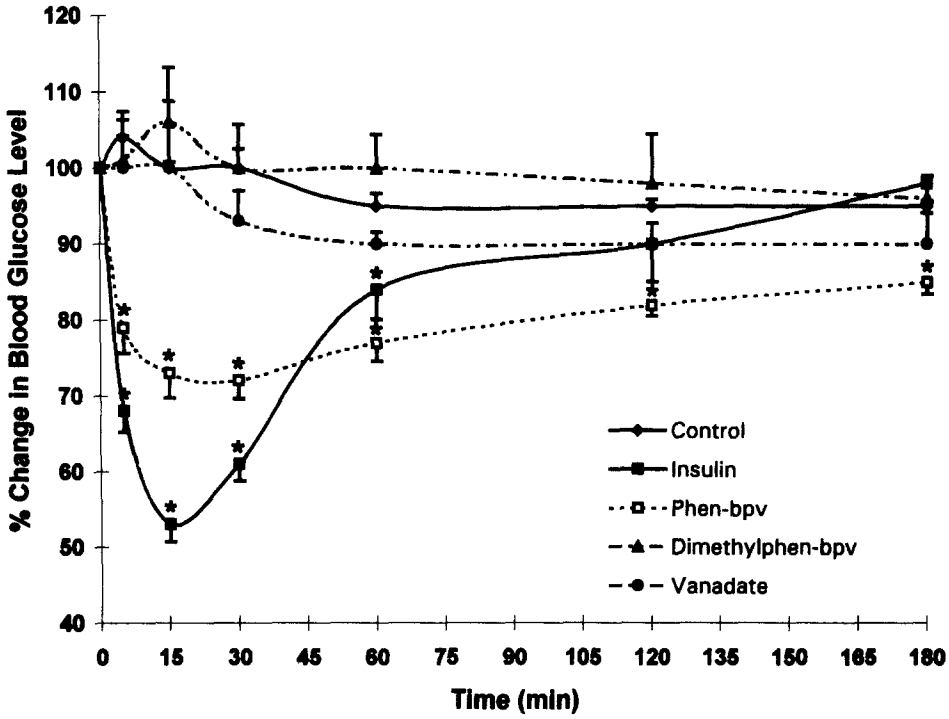


**Fig 1. Effect of bolus IV injections of vanadate, PHEN-BPV, DPV, and insulin on blood glucose levels of overnight-fasted normal female rats. The y-axis shows the % change (mean ± SD) from basal values in response to various treatments. Bolus injections (0.6 μmol/0.10 kg BW for each oxovanadium species and 1.5 μg/0.10 kg BW for insulin) were administered at time 0. Basal values for the various treatment groups were 155 ± 21 mg/dL for insulin, 166 ± 19 mg/dL for PHEN-BPV, 191 ± 8 mg/dL for vanadate, and 208 ± 23 mg/dL for DPV.**

oxovanadiums, only PHEN-BPV is effective as a hypoglycemic agent when administered acutely at the dose levels used in this study.

Figure 2 shows the effect of an acute IV bolus infusion of various oxovanadium species and insulin on blood lactate levels in normal young female rats, the identical groups described in Fig 1. It is evident from this figure that only an insulin challenge caused a marked and significant reduction in blood lactate compared with that of the control group. Following an insulin challenge, blood lactate decreased significantly below the lactate level in the control group by 30 minutes, and remained significantly lower through the duration of the study. It should be noted that the PHEN-BPV challenge that produced a marked reduction in blood glucose failed to produce any significant effect on blood lactate levels in this same group of animals. Thus, insulin and PHEN-BPV have qualitatively similar hypoglycemic effects when administered acutely under in vivo conditions, but have markedly different effects on blood lactate levels.

To determine if the observed differences in the efficacy of various oxovanadiums in decreasing blood glucose when administered acutely to intact animals is due to any difference in the clearance rate of the administered oxovanadium, we examined blood vanadium levels following bolus administration of the three different oxovanadiums. These levels of blood vanadium as a function of time following acute IV administration (0.6 μmol/0.10 kg BW) of various oxovanadium species are shown in Fig 3. There were no statistically significant differences in blood vanadium levels at any point during the 180-minute period for the three different oxovanadium species. Following an IV bolus challenge, blood vanadium levels for all three oxovanadium species reached a maximum value within 5 minutes and declined rapidly thereafter, reaching approximately half of the maximum measured blood level (value at 5 minutes) during the first 30 minutes. A plateau at approximately 800 ng/mL was reached by 60 minutes and was sustained through the next 120 minutes. These observations suggest that the distribution space, the rapidly mixing pool, and the clearance rate for all three different oxovanadium species are similar. The decay kinetics for the oxovanadiums indicate that an equilibrium is reached quickly, within 60 minutes, between blood and tissue pools. However, the equilibrium plateau value for blood vanadium, 800 ng/mL, is two to three times greater than the value for blood vanadium levels reported by our group and others for animals under long-term oral vanadate treatment. Thus, the overall clearance of oxovanadiums from the tissues is much slower than the rate of disappearance from the plasma. The data in Fig 3 also point to the fact that the difference in biological activity for different oxovanadiums, when administered acutely, is not based on their biological half-life.

In light of the observed differential acute effects of insulin and PHEN-BPV on blood glucose, and particularly blood lactate levels, experiments were performed to determine the acute effect of these two agents administered in tandem in normal female rats. The effect of this treatment on blood glucose levels is shown in Fig 4. Also included in this figure for comparison purposes are the previously described effects of saline infusion (controls) and of PHEN-BPV and insulin when administered separately. An insulin challenge following a PHEN-BPV challenge precipitated a marked and sustained

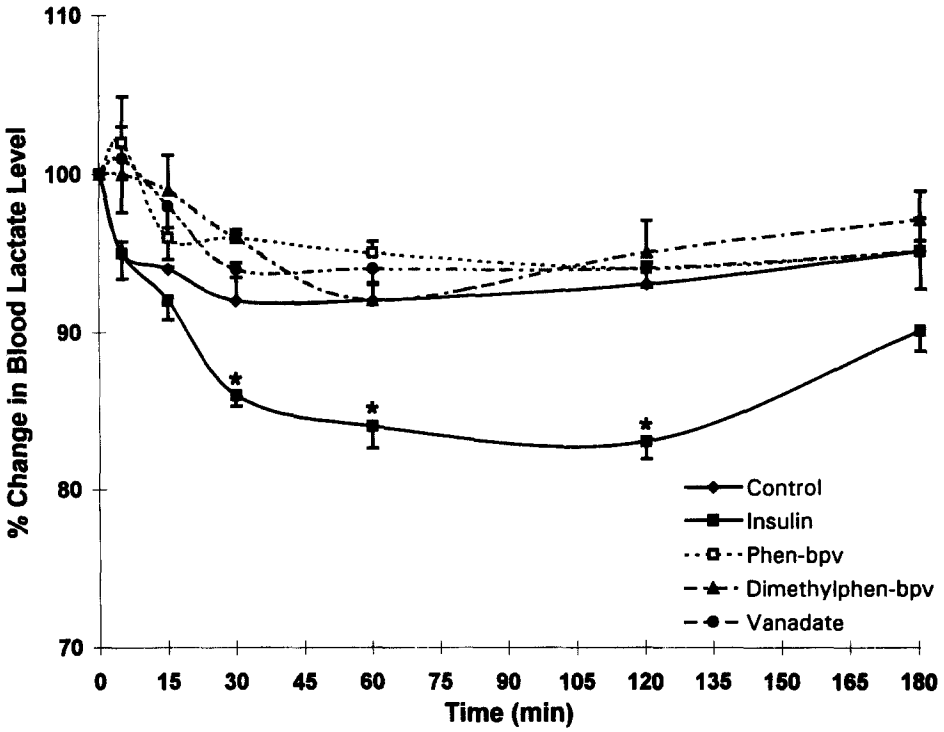


**Fig 2. Effect of bolus IV injections of vanadate, PHEN-BPV, DPV, and insulin on blood lactate levels of overnight-fasted normal female rats. The y-axis shows the % change (mean ± SD) from basal values in response to various treatments. Treatment was the same as in Fig 1 (identical animals used). Basal values for the various treatment groups were 1.011 ± 0.25 mmol/L for insulin, 1.22 ± 0.20 mmol/L for PHEN-BPV, 1.17 ± 0.05 mmol/L for vanadate, and 1.12 ± 0.10 mmol/L for DPV.**

reduction in blood glucose that was significantly greater than that attained by either of these two hypoglycemic agents administered separately. However, the time for maximum reduction of blood glucose was delayed about 30 to 45 minutes. This observation suggests that the prior presence of PHEN-BPV may interfere with the insulin-mediated hypoglycemic actions of insulin. Statistical analysis using the area under the glucose curve and glucose levels at different sampling points confirms

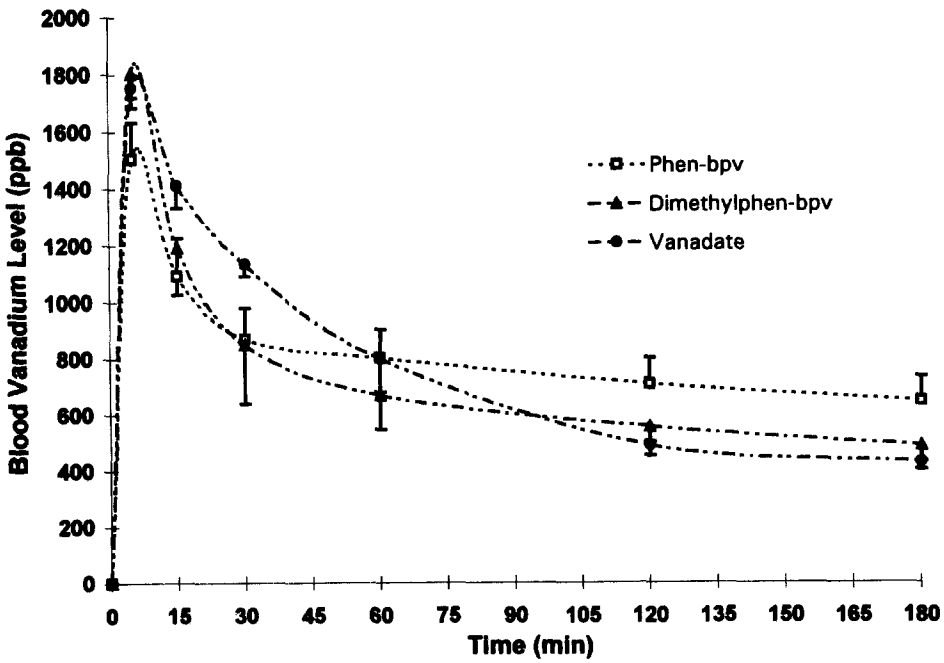


**Fig 3. Effect of bolus IV injections of vanadate, PHEN-BPV, and DPV on blood vanadium levels of overnight-fasted normal female rats. The y-axis shows the absolute values (mean ± SD) of blood vanadium levels. Bolus injections (0.6 μmol/0.10 kg BW) for each oxovanadium species were administered at time 0.**

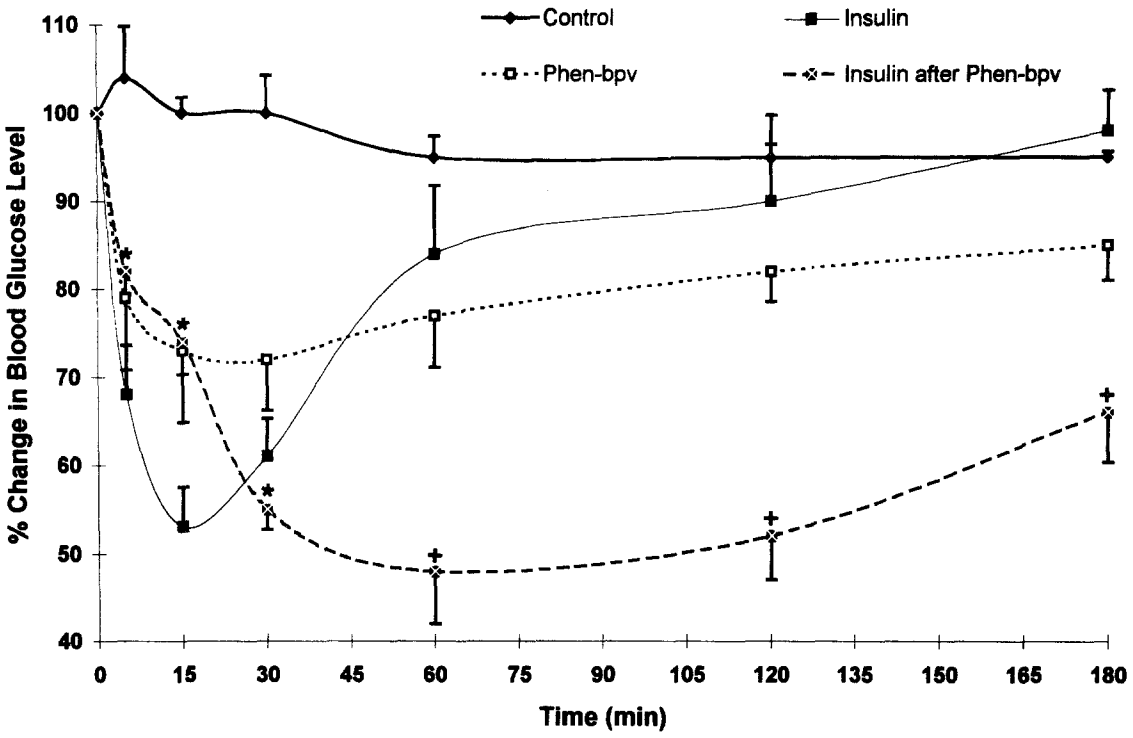


**Fig 4. Effect of bolus injections of PHEN-BPV and insulin in tandem on blood glucose levels. PHEN-BPV (0.6 μmol/0.10 kg BW) was administered 30 minutes before the insulin bolus (1.5 μg/0.10 kg BW). The y-axis shows the % change in blood glucose values (mean ± SD) from the basal values. The controls illustrate the effects on blood glucose levels of bolus injections of PHEN-BPV and insulin (administered separately at the same dosage) and of saline, as shown in Fig 1. Basal values for the controls are shown in Fig 1. The absolute value for blood glucose of the treatment group receiving a PHEN-BPV pulse followed by insulin challenge was 116 ± 15 mg/dL just before insulin challenge.**

that the combined effects of PHEN-BPV and insulin on blood glucose levels are synergistic. At all sampling times following the 30-minute sampling point, the combined effect of PHEN-BPV and insulin on blood glucose was significantly greater than the sum of the individual effects.

Depicted in Figure 5 are the effects of PHEN-BPV and insulin on blood lactate levels when administered in tandem (identical animals as in Fig 4). Also included in this figure are levels of blood lactate for the saline (control) and PHEN-BPV– and insulin-treated groups. It is evident from this figure that a prior PHEN-BPV challenge blunted the effect of insulin on blood lactate levels. It is interesting that 30 minutes after an insulin challenge following a prior PHEN-BPV challenge, the blood lactate level was significantly higher than lactate levels in all three groups: control, insulin-treated, and PHEN-BPV–treated. The blood lactate level in the group receiving the tandem treatment gradually declined after 30 minutes, and was never again significantly different from that of the control group throughout the remainder of the study. Furthermore, the blood lactate level in this tandem-treatment group also remained significantly higher than that of the group treated with insulin alone for the time points 15 to 120 minutes following insulin challenge.

Since insulin and PHEN-BPV demonstrated similar effects on blood glucose metabolism but markedly different effects on blood lactate metabolism under in vivo conditions, the effects of these two hypoglycemic agents on glucose and lactate metabolism were critically examined under in vitro conditions using 3T3-L1 adipocytes. The effectiveness of insulin and PHEN-BPV administered separately or in tandem in the stimulation of lipid synthesis using two different radioactively labeled substrates, 6-$^{3}$H-glucose and U-$^{14}$C-lactate, is depicted in Figs 6, 7, and 8. For this set of experiments, 3T3-L1 adipocytes were maintained in a high-glucose (25 mmol/L) DME medium with 10% fetal calf serum and were switched to Hanks buffer with 5 mmol/L glucose, pH 7.4, and supplemented with 20 mmol/L HEPES, 1 mmol/L pyruvate, and 0.5% BSA for 2 hours before challenge with insulin or oxovanadium separately and together. Thus, in this culture system, all the insulin-inducible enzymes such as pyruvate dehydrogenase complex (PDH), glycerol-3-phosphate dehydrogenase, fatty acid synthase, and acetyl coenzyme carboxylase involved in the conversion of glucose and/or lactate carbon into lipid are fully active. This model enables one to interpret whether the test agent is interfering with the activity of the enzyme and not the induction of the enzyme. However, in separate studies, overnight serum-starved 3T3-L1 adipocytes were used, and these cells demonstrated an increase in both glucose (6-$^{3}$H-glucose) and lactate (U-$^{14}$C-lactate) carbon incorporation into lipid in response both to an insulin challenge and to a PHEN-BPV challenge in a dose-dependent manner. The dose-response curve for glucose carbon incorporation was similar for both treatments, reaching a fivefold increase above the basal value. The dose-response curve for lactate carbon incorporation into lipid was significantly different. PHEN-BPV treatment was found to be 40% to 45% as effective as insulin in converting lactate into lipid. Furthermore, when PHEN-BPV was added before an insulin challenge, PHEN-BPV blunted the insulin-mediated effect on lactate carbon incorporation into

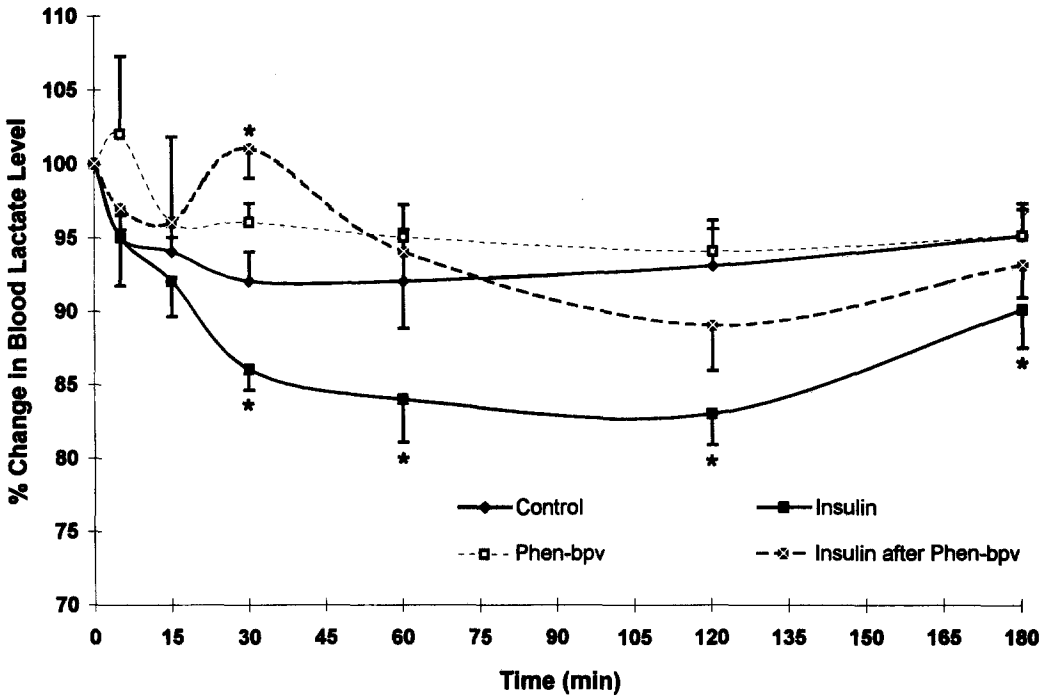


**Fig 5. Effect of bolus injections of PHEN-BPV and insulin in tandem on blood lactate levels. Treatment was the same as described in Fig 4 (identical animals were used). The y-axis shows the % change in blood lactate values (mean ± SD) from the basal values. The controls illustrate the effects on blood lactate levels of bolus injections of PHEN-BPV and insulin (administered separately at the same dosage) and of saline, as shown in Fig 2. Basal values for the controls are shown in Fig 2. The absolute value for blood lactate of the treatment group receiving a PHEN-BPV pulse followed by insulin challenge was 1.12 ± 0.05 mmol/L just before insulin challenge.**

lipid, yielding a dose-response curve similar to that of PHEN-BPV alone (data not shown). In contrast, in normal fully stimulated 3T3-L1 adipocytes, insulin had no significant effect on lipid synthesis over and above the high basal level (fivefold higher than that of serum-starved 3T3-L1 adipocytes) when either glucose or lactate was used as a substrate (Fig 6). Dose-responses for PHEN-BPV on lipid synthesis using glucose and lactate are shown in Fig 7. Like insulin (Fig 6), PHEN-BPV had no significant effect on the incorporation of glucose carbon into lipid, but in contrast, PHEN-BPV in a dose-dependent manner caused inhibition of lactate carbon incorporation into lipid. The inhibitory effects of PHEN-BPV were significant at all doses above and including 12 μmol/L.

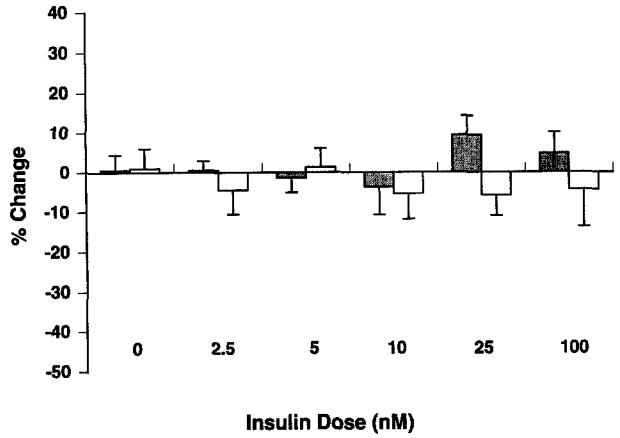


**Fig 6. Dose-response curve of insulin on incorporation of glucose (■) and lactate (□) carbons into lipid in 3T3-L1 adipocytes. Nonstarved (fully stimulated) 3T3-L1 adipocytes were challenged with insulin for 30 minutes before addition of 6-$^{3}$H-glucose and U-$^{14}$C-acetate. Incorporation of the label into extracted lipid was measured 1 hour after addition of labeled glucose and lactate. The dose is indicated on the x-axis, and the % change from basal values (mean ± SD) on the y-axis.**

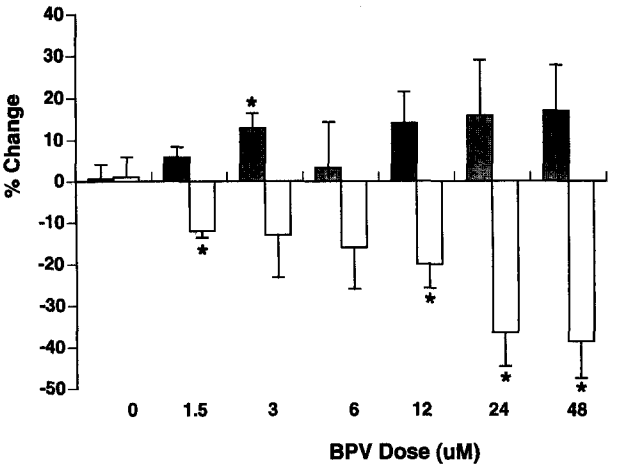


**Fig 7. Dose-response curve of PHEN-BPV on incorporation of glucose (■) and lactate (□) carbons into lipid in 3T3-L1 adipocytes. Nonstarved (fully stimulated) 3T3-L1 adipocytes were challenged with PHEN-BPV for 30 minutes before addition of 6-$^{3}$H-glucose and U-$^{14}$C-lactate. Incorporation of the label into extracted lipid was measured 1 hour after addition of labeled glucose and lactate. The dose is indicated on the x-axis, and the % change from basal values on the y-axis. Values represent the mean ± SD.**

This is not a toxic effect of PHEN-BPV, since the same dose of PHEN-BPV had no significant effect on the incorporation of glucose carbon into lipid. Finally, when a constant dose of PHEN-BPV 25 μmol/L was present along with insulin, lactate carbon incorporation into lipid was markedly attenuated at all doses of insulin (Fig 8). It is important to note that the adipocytes used were preincubated with fetal calf serum in a high-glucose medium and were in a stimulated state even at the 0 dose level of insulin. Interestingly, except at the two highest dose levels of insulin, the presence of PHEN-BPV along with insulin had no measurable effect on glucose carbon incorporation into lipid (Fig 8).

## DISCUSSION

Oxovanadiums demonstrate a wide range of metabolic effects under both in vivo and in vitro conditions.[1-5] Specifically, the insulin-mimetic effect of oxovanadiums has been of much interest during the past decade[1,4,6-9,13-16,19] for possible therapeutic use in the treatment of diabetes. However, oxovanadium compounds demonstrate significant variations both qualitatively and quantitatively in the ability to affect various metabolic pathways involved in the uptake and utilization of glucose and other nutrients.[4,12,19,23,24] An understanding of the basis for this observed selectivity, specificity, and variable biopotency of various oxovanadium species has been difficult. One of the major confounding problems in understanding the mechanisms of action of various oxovanadium species is based on the fact that the chemistry of oxovanadiums is extremely complex in biological systems, both in terms of their oxidation states and in their association with various ligands.[4,6-8] Either or both of these factors are likely responsible for the observed variability in the

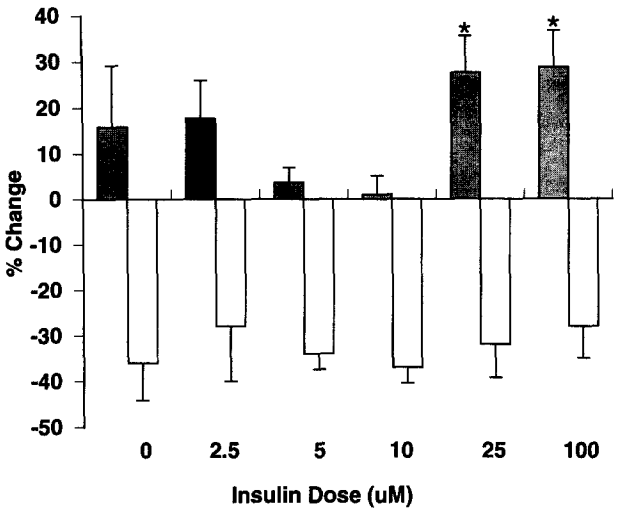


**Fig 8. Dose-response curve on incorporation of glucose (■) and lactate (□) carbons into lipid in 3T3-L1 adipocytes for insulin in the presence of a constant level of PHEN-BPV (25 μmol/L). Nonstarved (fully stimulated) 3T3-L1 adipocytes were treated with 25 μmol/L PHEN-BPV for 30 minutes before insulin challenge. The cells were then incubated for an additional 30 minutes before addition of 6-$^{3}$H-glucose and U-$^{14}$C-lactate. Incorporation of the label into extracted lipid was measured 1 hour after addition of labeled glucose and lactate. The insulin dose is indicated on the x-axis, and the % change from basal values on the y-axis. Values represent the mean ± SD.**

actions of oxovanadium compounds on biological systems. At this time, there is no clear consensus as to whether the variable oxidation states or the structural attributes of various oxovanadium species are the major factor that determines the biological effectiveness of this class of compounds.

The data in the present study demonstrate both the selectivity and the complexity associated with the biological actions of various oxovanadium species. Qualitatively, a similar effect of PHEN-BPV and insulin on blood glucose metabolism is evident from the data in Fig 1, but at the same time, a markedly different effect of these agents on blood lactate metabolism is also evident from Fig 2. This observation suggests that the steps involved in insulin's action on glucose and lactate metabolism, at least in part, may not share identical pathway(s), and that PHEN-BPV action can partition these two signaling axes. The data in the present study also demonstrate that the association with different ligands can influence the effectiveness of oxovanadium compounds as modulators of metabolic activities, since only PHEN-BPV, not vanadate or DPV, had any effect on decreasing blood glucose when administered acutely at these dose levels.

Short-term administration of vanadate, in contrast to long-term administration, had no significant effect on decreasing blood glucose (Fig 1). These data indicate that vanadate needs to be converted to some other species, perhaps to a BPV by endogenous $H_2O_2$ or to vanadyl through the actions of intracellular reducing moieties including GSH. In a recent study, it has been suggested that vanadyl (vanadium IV) and not vanadate is the oxovanadium species that is more effective in producing insulin-like actions, since vanadyl was found to be more effective in activating a cytosolic protein tyrosine kinase in 3T3-L1 cells and in cell lysates.[8] However, it is well documented in a number of studies that at higher concentrations vanadate can produce acute insulin-like effects in various biological systems.[23,25,26] These different biopotencies may be explained by different cellular uptake kinetics for these two oxovanadium species, as we have determined that the cellular uptake of vanadate is much slower than that of PHEN-BPV (Gangali S, Reuland DJ, submitted, Nov 1996).

A critical upstream event in insulin action is the activation of the insulin receptor tyrosine kinase and increased tyrosine phosphorylation of key cytosolic proteins that are involved in downstream signaling. It is well documented that various oxovanadium compounds also enhance tyrosine phosphorylation of key cytosolic proteins. Some of these oxovanadium compounds have vanadium in the same oxidation state, are similar in structure and spatial orientation, and consequently should have almost identical reduction potentials. For instance, PHEN-BPV and DPV differ only in two additional methyl groups on the ancillary ligand of DPV and yet have different biological effectiveness. Thus, the observed differences in biological effectiveness would indicate that the associated ligands may bestow specificity in terms of their interaction with various key proteins. Furthermore, following a bolus IV challenge, vanadium levels in the blood and clearance rates for these two oxovanadium species were statistically indistinguishable (Fig 3). Again, at our laboratory using a cell culture system, we have observed significantly different uptake kinetics for these three oxovanadium species. The cellular uptake for PHEN-BPV

was markedly faster compared with the uptake of either vanadate or DPV, which were close to each other. This observation may explain, in part, the observed differences in the effectiveness of these oxovanadium compounds in biological systems.

It is evident from Fig 2 that an insulin challenge precipitated a marked and sustained reduction in the blood lactate level. Following an insulin challenge, blood lactate decreased significantly below the blood lactate level in the control group by 30 minutes and remained significantly lower through 120 minutes. It is well documented that, parallel with its effect on glucose uptake and utilization, insulin also causes an increase in pyruvate/lactate production. Following an insulin challenge, glucose uptake and lactate production are increased in all insulin-sensitive peripheral tissues, including fat tissues.[23,27-29] However, following an in vivo insulin challenge, the level of lactate uptake and utilization by peripheral tissues plus the hepatic extraction of lactate exceeds total lactate production, and the blood lactate level actually decreases below the basal level. In contrast to an insulin challenge, an acute PHEN-BPV challenge, like insulin, caused a significant reduction in blood glucose levels, but failed to demonstrate any measurable effect on blood lactate levels (Fig 2). This observation suggests that a PHEN-BPV challenge, unlike insulin, fails to stimulate lactate uptake and utilization that exceed lactate production. These data also suggest that insulin-stimulated glucose and lactate metabolism do not share identical metabolic pathways and that PHEN-BPV differs from insulin in its effect on the metabolism of these different substrates. For example, insulin may phosphorylate a key protein on tyrosine, either directly or indirectly, that is not accessible to PHEN-BPV. Alternatively, in addition to tyrosine phosphorylation, other signaling steps not accessible to PHEN-BPV may be a prerequisite for the insulin action on lactate metabolism.

The data presented in Figs 4 and 5 further magnify differences in the actions of these two hypoglycemic agents on glucose and lactate metabolism. A prior challenge with PHEN-BPV magnified the hypoglycemic action of insulin (Fig 4). This synergistic relationship suggests, at least in part, that separate signaling axes are used by these two agents in enhancing glucose uptake and/or utilization. A recent study on the use of oral vanadate treatment to control diabetes-associated hyperglycemia in humans also has indicated similar synergistic phenomena on glucose metabolism.[30] In contrast to this synergistic effect of these two agents on glucose metabolism, PHEN-BPV administered before insulin had a blunting effect on the blood lactate–decreasing action of insulin. In contrast to the effect of an insulin challenge on the blood lactate level in the absence of a prior PHEN-BPV challenge, there was actually a spurious and significant increase in the blood lactate level in the presence of a prior PHEN-BPV challenge at early times when insulin-mediated glucose utilization, and consequently, lactate production, is at its maximum (Fig 5). Afterward, the blood lactate level of this group gradually declined and was never significantly different from that of the control group. However, it still remained significantly above the blood lactate level in the group treated with insulin alone until 180 minutes. These data indicate that a prior PHEN-BPV challenge blunts the insulin action on blood lactate metabolism, and that this effect is sustained as long as the blood vanadium level remains elevated. Thus, PHEN-BPV affects the insulin-mediated metabolism of lactate in a manner opposite to that of its action on the insulin-mediated metabolism of glucose. This observation also reemphasizes the fact that insulin actions on glucose and lactate metabolism do not share an identical signal transduction pathway.

To critically assess the relationship between insulin and PHEN-BPV on glucose and lactate metabolism, the effectiveness of these two agents for incorporation of glucose and lactate carbons into lipid was studied on 3T3-L1 adipocytes. In fully active 3T3-L1 adipocytes that were maintained in a high-glucose (450 mg/dL) medium supplemented with 10% fetal calf serum, neither insulin nor PHEN-BPV (at all but one dose) had any significant stimulatory effect on glucose carbon incorporation above and beyond the prevailing high basal level of glucose carbon incorporation into lipid (approximately fivefold higher than the basal level of glucose incorporation in identical cells that were maintained overnight in a serum-free, low-glucose medium). Similarly, in these fully stimulated cells, insulin had no additional stimulatory effect on lactate carbon incorporation into lipid above the existing high basal level (greater than threefold higher than the basal rate observed in serum-starved cells in experiments performed simultaneously). In contrast to insulin, PHEN-BPV caused a dose-dependent inhibition of lactate carbon incorporation into lipid that was significant at a dose of 12 μmol/L and increased to its maximum inhibition, a 40% reduction from the original level, at a dose of 24 μmol/L (Fig 7). Thus PHEN-BPV specifically inhibited lactate carbon incorporation but had no significant effect on glucose carbon incorporation into lipid, again except at one dose, under identical experimental conditions. The data presented in Fig 8 demonstrate that prior treatment with PHEN-BPV 25 μmol/L inhibited insulin-mediated lactate carbon incorporation into lipid irreversibly even at an insulin dose as high as 100 nmol/L. However, at the higher insulin doses, there was a statistically significant increase in glucose carbon incorporation into lipid. This observation emphasizes the point that a PHEN-BPV challenge interferes to a much greater extent with lactate carbon incorporation into lipid than with glucose carbon incorporation into lipid. Finally, as stated earlier, qualitatively similar results were obtained with non-starved 3T3-L1 adipocytes.

The data presented here clearly demonstrate that PHEN-BPV interferes with the metabolism of lactate under both in vivo and in vitro conditions. However, this does not identify the specific step(s) in lactate metabolism that may be the site of interference by PHEN-BPV. As mentioned before, lactate metabolism involves a number of pathways. Lactate can be taken up by the peripheral tissues, converted to pyruvate by the action of LDH, and then used through irreversible decarboxylation via a PDH-complex pathway. It can be taken up by the liver and metabolized irreversibly through the PDH pathway, or it can be channeled into a gluconeogenic/glycogenic pathway following its conversion to pyruvate and then to oxaloacetate by the action of pyruvate carboxylase and phosphoenolpyruvate carboxykinase.[31]

Additionally, lactate/pyruvate can be converted to alanine

through participation in a transamination reaction.[32,33] Although our data suggest that a site proximal to that of the PDH complex is involved in limiting insulin-mediated lactate carbon incorporation into lipid by PHEN-BPV, an effect on the PDH complex by PHEN-BPV cannot be ruled out, since the higher levels of glucose necessary to saturate the PDH complex with pyruvate were not used in this study. This study showed no significant difference in LDH activity in response to a direct application of PHEN-BPV for 90 minutes to intact 3T3-L1 cells or to the cell lysate. Thus, at least part of the action of PHEN-BPV on lactate metabolism does occur at or near the site of lactate uptake and/or its transport within the cell. It is well documented that there are stereospecific $H^+$/L-lactate and lactate/bicarbonate cotransporters (carriers) in the cell membrane that can transport lactate bidirectionally, and that alterations in the carrier properties or the presence of specific inhibitors can impede lactate transport.[33,34] It is thus possible that PHEN-BPV either directly or indirectly interferes with the intrinsic activities, the movement of lactate transporters, or the binding of lactate to the transporter proteins. It appears that the PHEN-BPV effect on lactate transport is more obvious at a metabolic state when lactate production by cells/tissues is high, ie, when glucose uptake and utilization is at a high level. In this metabolic state, both recruitment of additional lactate carriers and enhancement of the intrinsic activity of existing carriers may be necessary to handle the additional lactate load. In this study under both in vivo and in vitro conditions, the presence of PHEN-BPV before an insulin challenge interfered with lactate metabolism. Although it is speculative at this point, it is possible that PHEN-BPV interferes directly or indirectly with recruitment and/or activation of lactate carriers.

The effectiveness of oral vanadate treatment has already been tested in clinical trials and this compound has been identified as one that may have the potential to be used in the management of diabetes.[30] On the basis of our observation of the acute effects of PHEN-BPV on lactate metabolism, a more careful and cautious approach must be taken prior to any long term use of oxovanadium compounds as a part of a therapeutic strategy.

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