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COMMENTARY

THE USE OF POST-BINDING AGENTS IN STUDYING INSULIN ACTION AND ITS RELATION TO EXPERIMENTAL DIABETES

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Impressive achievements have been made during the last decade with respect to the structure, basic properties, and behavior of the insulin receptor (reviewed in Refs. 1 and 2). This is substantiated by the recent elucidation of the amino acid sequence of the insulin receptor [3] and its intrinsic enzymatic activity as an insulin-activated tyrosine-specific protein kinase [1, 4–6]. In contrast, research concerning the post-receptor events involved in insulin action has progressed more slowly due to the fact that insulin, upon binding to its cellular receptor, initiates a multitude of cellular events (reviewed in Refs. 7–9) that do not appear to share a common mechanistic pathway. Many of the enzymes which are acutely modulated by insulin action undergo either phosphorylation or dephosphorylation in response to insulin action. Enzymes which may be phosphorylated as a result of insulin action include acetyl-Co A carboxylase [10], ATP-citrate lyase [11], and the S-6 ribosomal protein [12]. Examples of insulin-mediated dephosphorylation include the enzymes glycogen synthase [13], pyruvate dehydrogenase [14], and hormone-sensitive lipase [15]. All these modulations of enzyme activity, however, were made in intact cellular systems; in broken cell preparations insulin cannot modulate either most or all of these diverse biological effects [7]. This may imply then that the cellular machinery involved in insulin action is constructed of both integral plasma membrane and cytoplasmic components. The lack of a cell-free system for measuring the effects of insulin has been the major reason for the slow progress in elucidating the post-receptor events occurring after hormone binding. Studies on hormone-sensitive adenylate cyclase systems were more fortunate in the sense that all the essential components of the system are restricted to the plasma membrane [16], thus allowing for the direct measurement of hormone-stimulated enzyme activity in the cell-free state.

With respect to insulin enhancement of glucose uptake, two conceptual working hypotheses have been developed in recent years. The first hypothesis assumes that insulin modulates certain physiochemical and/or enzymatic properties in the plasma membrane which, in turn, activate dormant membranal glucose transporters. This hypothesis has to assume that any putative alteration in glucose transport is reversible and that the activated transporters are returned to their dormant state upon the removal of insulin [7, 8]. An alternative hypothesis assumes the existence of an intracellular pool of glucose transporters that can be recruited to the plasma membrane as a result of insulin stimulation. The first hypothesis encouraged studies in cell-free systems in order to determine which physiochemical or enzymatic parameters are involved in the enhancement of glucose transport activity. At present, the studies of Kono *et al.* [17, 18] and of Cushman *et al.* [19, 20] seem to validate the second hypothesis of insulin action. It appears that insulin binding initiates a cascade of events which lead to the recruitment of hexose transporters from an intracellular pool to the plasma membrane [17–20]. This phenomenon, which seems to involve both temperature- and energy-dependent exocytotic and endocytotic processes [17–20], cannot be studied currently in the cell-free state.

In accordance with the above conceptual approach, it appears likely that agents which affect insulin action in a post-binding manner, in intact cellular systems, may be powerful tools for future research. From an operational point of view, we refer to agents that modulate intramembranal and/or intracellular insulin-dependent mechanisms, while not altering certain insulin receptor properties, such as induced conformational changes, receptor aggregation, receptor internalization, intramolecular autophosphorylation or activation of the receptor kinase activity (reviewed in Refs. 2 and 9). Many of these processes have been well documented, although the knowledge linking these events to activated cellular processes is lacking. Such post-receptor agents reviewed here include vanadium, which mimics insulin action [21–24], and polymyxin B (PMXB), which specifically blocks activated glucose uptake [25–27].

It is the purpose of this review to summarize the current studies on vanadium and PMXB as post-binding modulators of insulin action and to encourage prospective research in this direction. As vanadium and PMXB are also active in the whole animal model, their potential use in the pathophysiology of experimental diabetes is evaluated as well.

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Chemical and biochemical properties of vanadium

Vanadium is now recognized as an essential nutritional element in higher animals but its function still remains unclear [28, 29]. The chemistry of vanadium is complex as the metal can exist in a variety of oxidation states, ranging from −1 to +5, and can be found in a multitude of polymeric forms. At physiological concentrations in mammals and avians, free vanadium exists in a hydrated monomeric state. In body fluids, at pH 4–8, the predominant species found is metavanadate (VO_3^-), which exists in the +5 oxidation state (reviewed in Refs. 30–33). Vanadium is an endogenous constituent of all or most mammalian tissues (reviewed in Ref. 31). The probable mode of VO_3^- entry into the cell is by means of an anion carrier system and, once entered, it is reduced nonenzymatically, by glutathione, to vanadyl (VO^{2+}: +4 oxidation state). VO_3^- is a structural analog of phosphate [33], and this may account for its inhibitory effect on enzymes involved in phosphate transfer and release mechanisms [31, 33–35]. VO_3^- can easily adopt a stable trigonal bipyramidal structure which resembles the transition state of phosphate during reaction [33]. The list of enzymes that are inhibited by vanadium includes ATP phosphohydrolases, ribonuclease, glyceraldehyde 3-phosphate dehydrogenase, alkaline phosphatase, Ca^{2+}/Mg^{2+} ATPase, Na^+/K^+ ATPase, and phosphotyrosyl protein phosphatase (reviewed in Ref. 30).

Perhaps the best described effect of VO_3^- is its inhibitory action on Na^+/K^+ ATPase activity [30–33]. It appears that VO_3^- binds to both the low and high affinity binding sites for ATP of the enzyme. The high affinity site of VO_3^- action ($K_{app} = 4 \times 10^{-9}$ M) corresponds to the low affinity ATP binding site, and vice versa. VO_3^- binding to the E_2 (K^+-dependent) state of the enzyme results in stabilization of the E_2 state and thereby prevents transition towards the E_1 (Na^+-dependent) state. It has been shown that Mg^{2+}, K^+, ouabain, and dimethyl sulfoxide favor the formation of the E_2 state, resulting in increased VO_3^- binding. Conversely, ATP, Na^+, and oligomycin, which favor formation of the E_1 state, result in a decrease in VO_3^- binding. A physiological role for VO_3^- regulation of Na^+ pump activity has been postulated [36]. VO_3^-, however, should interact with the cytoplasmic surface of the Na^+/K^+ ATPase. Intracellular vanadium is found primarily in the form of VO^{2+} [30–33], which is a relatively ineffective inhibitor of Na^+/K^+ ATPase *in vitro* [36]. Indeed, as will be discussed later, exogenously-added VO_3^-, up to a concentration of 1 mM, does not inhibit the Na^+/K^+ ATPase of intact adipocytes [21, 22]. This appears to be true for other cellular forms of Na^+/K^+ ATPase from various sources [30].

The high affinity Ca^{2+}/Mg^{2+} ATPase, from both human erythrocytes and the sarcoplasmic reticulum source is several times more insensitive to VO_3^- than the Na^+/K^+ ATPase [37]. A possible exception, however, is the cardiac sarcolemma Ca^{2+}/Mg^{2+} ATPase of canines, which is equally sensitive [38]. Several other related ATPases, such as the H^+/K^+ ATPase, dynein ATPase, and the myosin-actomyosin ATPases are also inhibited by VO_3^-. In all cases, none of these cellular forms are inhibited at VO_3^- concentrations lower than 1–10 mM [30].

Insulin-like actions of vanadium

In vitro *studies.* Shechter and Karlish [22] and Dubyak and Kleinzeller [21] previously demonstrated that vanadate ions mimic the actions of insulin in rat adipocytes. Exogenously-added VO_3^- mimicked insulin in stimulating hexose uptake [21], glucose oxidation [22], lipogenesis [39], and the inhibition of lipolysis [23]. The half-maximally effective concentrations (EC_{50}) of VO_3^- or VO_4^{3-} for mediating the various insulin-like effects range between 0.1 and 0.2 mM [22, 23, 39, 40]. Earlier, it was shown that ouabain partially mimicks insulin by inhibiting adipocytic Na^+/K^+ ATPase activity [41]. Therefore, it was evaluated whether VO_3^- resembles ouabain in this respect. Exogenously-added VO_3^-, up to a concentration of 1 mM, did not change significantly the rate of rubidium influx into rat adipocytes [21]. Also, VO_3^- was equipotent in stimulating glucose oxidation in adipocytes maintained in K^+-depleted Krebs–Ringer bicarbonate buffer [22]. Thus, VO_3^- does not seem to inhibit Na^+/K^+ ATPase activity in intact adipocytes.

The fate of the added VO_3^-, after entering the intact adipocyte, was evaluated using electron spin resonance spectroscopy [23]. This study revealed that VO_3^- is reduced intracellularly to VO^{2+} and that in the reduced state it was found to complex exclusively to reduced glutathione. The major vanadyl complex found has a VO^{2+}:GSH stoichiometry of 1:1. At least two such VO^{2+}–GSH complexes could be detected [23], whereas no complexes of VO^{2+} to other ligands (i.e. to ATP) could be observed. It is conceivable that VO^{2+} binding to GSH prevents the reoxidation to VO_3^-, which would tend to occur at the intracellular pH. This, together with the lack of inhibition of Na^+/K^+ ATPase, suggests, but does not confirm, that intracellular VO^{2+} (rather than VO_3^-) alone or complexed to reduced glutathione is the relevant chemical species responsible for mimicking the actions of insulin.

Further studies have indicated that insulin and vanadate share a common mechanistic pathway. VO_3^-, at sufficiently high concentrations, maximally stimulates hexose uptake [21, 40], glucose oxidation [22], and lipogenesis [39]. Furthermore, no increment in stimulation could be achieved by the addition of insulin to vanadate-stimulated cells, and vice versa (unpublished data). In addition, both agents show the same concentration dependency on extracellular glucose [22]. Agents or conditions which suppress the effects of insulin, such as anti-calmodulin drugs [42], polymyxin B, bicarbonate-depleted buffers [39, 43], and exogenously-added ATP [22, 44] are equipotent in suppressing vanadate-mediated effects. Also, similar rates in the termination of lipogenesis ($T_{\frac{1}{2}} = 14 \pm 3$ min) were observed after removing either insulin or VO_3^- from stimulated adipocytes [45].

VO_3^- was also shown to stimulate glycogen synthase in rat adipocytes [24]. Thus, VO_3^- resembles insulin also in its ability to potentiate an endogenous activity that is irrelevant to increased hexose influx

[24]. VO_3^- was also found to mimic insulin in stimulating K^+ uptake in cultured cardiac muscle cells [46] and to inhibit Ca^{2+} transport activity in plasma membranes derived from rat adipocytes [47]. Both vanadate and vanadyl are effective in inhibiting this activity [47]. In addition, stimulation of DNA synthesis, by either VO_3^- alone or in synergism with insulin [48] or epidermal growth factor [49], in cultured cells has been documented.

In vivo *studies*. Heyliger *et al.* [50] first demonstrated that the inclusion of sodium orthovanadate (Na_3VO_4; 0.8 mg/ml) and 80 mM NaCl in the drinking water of streptozotocin-treated diabetic rats (ST-rats) alleviated some symptoms of diabetes. This treatment resulted in the normalization of blood glucose levels and the elimination of depressed cardiac performance [50]. These effects were shown not to result from increased levels of endogenous insulin; therefore, insulin target tissues have been implicated as the site(s) of vanadate action. This study was further extended by Meyerovitch *et al.* [51], who found that a lower concentration of vanadate ($NaVO_3$, 0.2 mg/ml) in the drinking water was optimal for achieving stable normoglycemia in ST-rats over a period of several weeks [51]. Blood glucose levels dropped to nearly normal values within 3–4 days after the inclusion of VO_3^- in the drinking water. Following the removal of VO_3^- from the drinking water, normoglycemia persisted for another 3–4 days before the onset of hyperglycemia, indicating that vanadate-induced normoglycemia is reversible. ST-rats, which are known to be in a catabolic state [52], became anabolic (gaining 1.3 g/day) several days after receiving the lower vanadate dosages [51]. The treatment also repaired various tissue alterations known to develop in ST-rats [52–54]. For example, VO_3^- therapy reduced the elevated insulin binding capacity of liver to normal capacity and partially restored the responsiveness of adipocytes to insulin [51].

It has been shown that vanadate therapy doubles the rate of 3-*O*-methyl glucose uptake in muscle and liver tissues in ST-rats [51]. Thus, the normoglycemia observed in vanadate-treated ST-rats seems to result from VO_3^- stimulation of glucose uptake and its metabolism *in vivo*, in agreement with the known action of VO_3^- in *in vitro* systems.

No signs of VO_3^- toxicity could be detected in ST-rats receiving lower doses of VO_3^-. The level of VO_3^- in the serum of treated rats did not exceed 0.7 to 0.9 μg/ml [51], a concentration about 1/100 of that required to inhibit a variety of Na^+/K^+ ATPases of intact cellular systems.

Considerations for future research

As there are multiple theories concerning how insulin mediates its biological action, determination of the overlapping sites of action for both vanadate and insulin may assist in focusing insulin research in the right direction. As VO_3^- does not influence insulin binding, it seems that an extracellular site for VO_3^- action can be excluded, although more work is necessary to verify this assumption. Most studies support the notion that the site of VO_3^- action in mimicking insulin-dependent actions is distal to the insulin receptor. For example, VO_3^- increases the rate of hexose transport in the liver [51], whereas insulin itself does not. Also, VO_3^- has an identical dose–response curve for the stimulation of glucose uptake in both control adipocytes and in adipocytes in which 60% of the insulin receptor sites were down-regulated [40]. On the other hand, VO_3^- was shown to stimulate the autophosphorylation (and, therefore, the activation) of insulin-receptor kinase in a cell-free state [24, 55]. Although this stimulating effect could not be reproduced by Kadota *et al.* [56], a direct action of VO_3^- on the insulin-receptor kinase cannot yet be excluded.

Attention should also be given to earlier documentation of the cellular effects of VO_3^-, both in insulin target tissues and in other cell types as well. It has been reported that VO_3^- increases Ca^{2+} influx in both adipose tissue and skeletal muscle [57], and raises the intracellular pH in human A431 cells [58]. Both phenomena were previously associated with the actions of insulin [7]; however, insufficient evidence is currently available to conclusively link them. On the contrary, the actions of insulin [42] or vanadate are not significantly reduced in Ca^{2+}-free (EGTA-supplemented) buffers, as previously shown in rat adipocytes [42]. Thus, Ca^{2+} influx would not seem to be required for either insulin- or VO_3^--dependent effects in rat adipocytes.

At present, it seems likely that the insulin-like effects of VO_3^- occur distally to the activation of insulin-receptor kinase. As VO_3^- can augment tyrosine phosphorylation in cells [59], presumably by inhibiting phosphotyrosine phosphatase [60], it may alter the phosphorylation states of the putative substrates that may be involved in insulin action. Recently, it was shown that VO_3^- can esterify (non-enzymatically) the phenyl moieties of tyrosines. The equilibrium constant for this reaction is 4 orders of magnitude larger than the equilibrium constant for phosphate-mediated esterification of phenol [61]. It is tempting, therefore, to speculate that such a modification, if induced intracellularly by exogenously-added VO_3^-, may alter the enzyme activity that is relevant to the biological actions of insulin.

Polymyxin B, a specific inhibitor of stimulated glucose uptake in vivo *and* in vitro

Polymyxin B (PMXB) is a cyclic polycationic decapeptide antibiotic produced in *Bacillus polymyxa* and has a structure as illustrated in Fig. 1. This peptide was shown to effectively disrupt gram-negative bacteria, and it is used infrequently in human medicine for treatment of conditions of gross contamination with such bacteria (i.e. salmonelosis).

Amir and Shechter [25] first observed that PMXB effectively inhibits the effects of exogenously-administered insulin in mice. The peptide blocked insulin-induced hypoglycemia and prevented mortality in mice challenged with a lethal dose of insulin [25]. In rats, insulin-dependent hypoglycemia was efficiently blocked at a 12–13 : 1 molar ratio of PMXB to insulin [26]. These effects were found to be highly specific to PMXB. Out of several dozens of different substances tested, including basic molecules and various types of antibiotics, none could mimic the anti-insulin action of PMXB [25]. Also, colistin A (polymyxin E), which differs from PMXB by a single con-

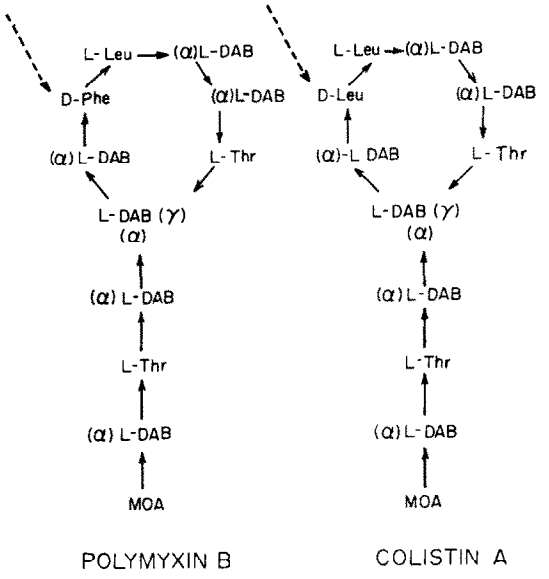

Fig. 1. Peptide structures of polymyxin B and colistin A. Abbreviations: DAB, diaminobutyric acid; and MOA, 5-methyloctanoic acid. The arrows indicate the sole amino acid moiety which differentiates polymyxin B from colistin A.

servative amino acid substitution in the ring structure (Fig. 1), was completely ineffective in inhibiting insulin-mediated hypoglycemia [25, 26]. The possibilities that PMXB may interact with insulin, alter the rate of insulin absorption and/or degradation, or interfere with the ability of insulin to bind to target tissues have so far been excluded.

Further studies using chemical modifications of PMXB have revealed that the ring structure, rather than the tail structure, is important for the anti-insulin activity of PMXB [26]. Also, a PMXB-derivative devoid of the antibacterial action of PMXB had about half of the original anti-hypoglycemic activity of PMXB. Therefore, it appears that different structural domains of the PMXB seem to participate in the antibacterial and anti-insulin activities of PMXB.

Subsequent studies in *in vitro* systems (summarized in Refs. 26 and 27) have revealed that: (a) PMXB does not interfere with insulin binding to its target tissues; (b) the peptide specifically inhibits insulin-stimulated glucose uptake in isolated muscle tissue and adipocytes, whereas basal glucose uptake is not affected; (c) other insulin-dependent activities, such as inhibition of lipolysis in adipocytes, synthesis of DNA in muscle cells, and the activation of glycogen synthase in mouse skeletal muscle, are not affected; (d) PMXB does not alter the ability of insulin to induce insulin receptor autophosphorylation and to activate the receptor kinase activity in the cell-free state; (e) PMXB also blocks vanadate-stimulated glucose transport activity; and (f) PMXB inhibits the effect of insulin in stimulating α-amino-isobutyric acid uptake.

These *in vivo* and *in vitro* observations, in both mice and rats, seem to indicate that PMXB exerts its inhibitory action at a site which is distal to the insulin-receptor kinase and is most likely to be specific to the activation of glucose and amino acid uptake, and not to other insulin-dependent activities. Polymyxin B is the first reported compound capable of specifically blocking these insulin-dependent uptake mechanisms in both *in vitro* and *in vivo* systems.

There is a lack of sufficient information at present to speculate on how PMXB inhibits the activated state of hexose or amino acid transport. In gram-negative bacteria, PMXB, which consists of a lipophylic and lipophobic group, is inserted between the lipid and protein layers, causing disorientation of the lipoprotein membrane, impairment in the cellular osmotic barrier, and the subsequent escape of the intracellular content [62]. Polymyxin B-sensitive bacteria bind more PMXB and have a higher ratio of lipid-P/lipid-N [62]. In mammalian cells, PMXB binding does not lead to irreversible cellular damage. In fact, rat adipocytes remained viable subsequent to their incubation for 1 hr at 37° with 1 mg/ml PMXB (unpublished observation). Also, they were fully responsive to insulin after the washing out and removal of the peptide.

Earlier works have demonstrated that PMXB binds to a variety of mammalian tissue homogenates [63]. It appears that PMXB binds electrostatically to acidic phospholipids (e.g. phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol), as PMXB does not bind to chloroform-extracted defatted liver tissue. Also, the binding of PMXB to various tissue preparations is inhibited by the addition of certain phospholipids [64]. There is no evidence for specific and saturable receptor sites for PMXB, or for the existence of enzymes that degrade or metabolize PMXB, either in bacteria or in a variety of mammalian tissues [64].

PMXB has been shown to inhibit phospholipid-dependent Ca^{2+}-activated protein kinase activity (PKC) (for a general review on PKC see Ref. 65), perhaps by the direct binding of PMXB to phospholipids that are required to activate the enzyme [66–68]. Protein kinase C has been suggested to be involved in the action of insulin. For example, in some cultured cell lines, the activation of protein kinase C by phorbol esters enhances hexose transport [69, 70]. Also, the insulin receptor itself is a substrate for protein kinase C [71]. In some cell lines, activation of protein-kinase C by phorbol esters decreased insulin binding and/or insulin-dependent action of biological processes [72]. This, together with the known binding affinity of PMXB toward the acidic phospholipids of mammalian tissues [64], may serve as initial clues for studying the site(s) of PMXB inhibition.

Summary

This review includes data related to two substances that modulate insulin mechanisms, both *in vitro* and in the whole animal model. It seems to us that these agents (vanadate and PMXB) will be of potential use in the next decade for basic and applied research. They may assist in characterizing the essential post-binding events involved in insulin action, which cannot presently be identified. As vanadate and PMXB modulate the effects of insulin both *in vivo* and *in vitro*, they may be of use in clinical and pathophysiological research as well. VO_3^- is a low mol-

ecular weight substance which permeates the intestinal tract and mimics the actions of insulin in target tissues [51]. Studies that were summarized here may even suggest that VO_3^- is superior to insulin in stimulating its effects in tissues that are down-regulated or desensitized to the hormone itself. Both VO_3^- and PMXB may be useful in the treatment of diabetes in the future if long-range toxicity studies prove these agents to be clinically safe. PMXB has already been in use in medicine for several decades now.

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