Vol. 147, No. 1, 1987
August 31, 1987

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 259-266

PEROXIDE(S) OF VANADIUM : A NOVEL AND POTENT INSULIN-MIMETIC AGENT WHICH ACTIVATES THE INSULIN RECEPTOR KINASE

Satoru Kadota, I. George Fantus, Guy Deragon, Harvey J. Guyda, Bonnie Hersh, and Barry I. Posner

From the Protein and Polypeptide Hormone Laboratory, Department of Medicine, Royal Victoria Hospital and McGill University, Montreal, Quebec

Received July 1, 1987

Summary: The actions of insulin, vanadate (V) and hydrogen peroxide (H_2O_2) on IGF-II binding and insulin receptor tyrosine kinase activity were studied in rat adipocytes. Incubating adipocytes with a combination of V plus H_2O_2 resulted in a potent synergistic effect on both the increase in IGF-II binding and the activation of the insulin receptor kinase. Catalase, which removes H_2O_2, abolished this synergism if added at the time of mixing of V plus H_2O_2 but not if added 10 min. later, suggesting that the formation of peroxide(s) of vanadate generated a potent insulin mimicker. The data support a critical role for the insulin receptor kinase in insulin action. The novel insulin-mimetic compound, a presumed peroxide of vanadate, could prove useful for investigating insulin action and may be valuable for treating insulin resistance. © 1987 Academic Press, Inc.

In recent years there has been increasing interest in the biological significance of vanadium (1-3). It appears to be an essential nutrient for optimal growth of chicks and rats (4), a potent mitogen in several cell types (5-8) and capable of altering various enzyme activities (9-13). Its insulin-like effects were clearly documented in 1980 when it was shown that vanadate stimulated glucose uptake and oxidation in rat adipocytes (14,15). Subsequently vanadate was shown to stimulate glycogen synthase in rat adipocytes (16) and skeletal muscle (17) and to augment Type II insulin-like growth factor (IGF) receptor translocation in the former (18), a well established rapid insulin effect. Of great interest was the recent demonstration by McNeill et al that vanadate, in the drinking water of diabetic rats, normalized their blood sugar and cardiac performance (19).

Recent studies have established that the cytoplasmic domain of the β subunit of the insulin receptor is a tyrosine kinase (20,21) whose activity, augmented by insulin binding to the subunit, seems to be essential for realizing insulin action (22-25). One approach to understanding the latter is to evaluate the effect of

0006-291X/87 $1.50
Copyright © 1987 by Academic Press, Inc.
All rights of reproduction in any form reserved.

insulin mimickers on the relationship between receptor kinase activation and biologic response. We have shown that the insulin mimickers, vanadate (14-19) and H_2O_2 (26), augment the insulin receptor kinase while stimulating IGF receptor translocation (27). In the course of these studies we observed that the combination of vanadate and H_2O_2 is strongly insulin-mimetic and, in this communication, report the discovery that this synergism is due to the formation of peroxide(s) of vanadate, a novel and very potent activator of the insulin receptor kinase.

Materials and Methods

Materials: Porcine insulin was a gift from Connaught-Novo Laboratories (Willowdale Ont.). IGF-II was purified by HPLC from outdated human plasma and iodinated to a specific activity of 150-170 Ci/g as previously noted (18). Bovine serum albumin (Fraction V), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), catalase and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (H_2O_2) and dinonylphthalate were from BDH Chemicals Ltd. (Ville St. Laurent, Que.). Collagenase (Type I) was from Worthington Biochemical Corp. (Freehold, NJ).

Adipocyte preparation: Male Sprague-Dawley rats (160-200 g b wt.) were killed by cervical dislocation, and adipocytes were isolated from the epididymal fat pads by the method of Rodbell as described in detail previously (18).

^{125}I-IGF-II binding: Isolated adipocytes (3.5 x 10^5 cells) were incubated at 37°C in 0.5 ml Krebs-Ringer bicarbonate/HEPES buffer, pH 7.4, containing 20 mg/ml bovine serum albumin with or without insulin, vanadate (V) or vanadate plus H_2O_2 (H) as noted in the text. After 15 min the incubation temperature was reduced to 15°C and ^{125}I-IGF-II binding was determined as described in detail previously (18).

Insulin receptor purification by WGA-Agarose chromatography: Adipocytes were incubated with vanadate (V), H_2O_2(H), insulin or V + H as described in the legend to Fig. 3. The reaction was terminated by the addition of 10 ml of ice cold solubilization buffer (50 mM HEPES, 1% Triton X-100, 4 mM EDTA, 2 mM NaF, 1 TIU/ml aprotinin, 1 mM PMSF, pH 7.6) and the cells were immediately frozen at -80°C. After 60 min the cells were thawed, homogenized and allowed to stand for 60 min at 4°C to continue solubilization. The solubilized cells were centrifuged at 1800 x g at 4°C and the fat cake was removed. The cell extract was centrifuged at 100,000 x g for 15 min at 4°C and the supernatant applied to a wheat germ agglutinin (WGA)-agarose column (0.6 x 3.0 cm) and recycled 6 times. After washing the columns with 100 ml of 50 mM HEPES buffer (pH 7.6) containing 150 mM NaCl, 10 mM MgSO4 and 0.1% Triton X-100 followed by 10 ul of above buffer without 10 mM Mg SO4, the bound material was eluted with 50 mM HEPES buffer (pH 7.6) supplemented with 0.3 M N-acetyl-D-glucosamine, containing 150 mM NaCl, 0.1% Triton X-100, 1 TIU/ml aprotinin and 1 mM PMSF.

Assay of insulin receptor kinase using poly Glu:Tyr as substrate: Aliquots of WGA-purified insulin receptor were incubated with or without 10^{-7}M insulin for 30 min at 22°C in 50 mM HEPES buffer pH 7.6. The phosphorylation reaction was initiated by the addition of 2 mM $MnCl_2$, 15 mM $MgSO_4$, 2.5 mg/ml of poly (Glu, Tyr) (4:1) and 5 uM [γ-^{32}P] ATP (5 uCi/tube) in a total volume of 160 ul as described previously (27). After 10 min at 22°C the reaction was terminated by spotting 80 ul of the reaction mixture onto Whatman No. 3 filter paper which was placed into 10% TCA, 10 mM sodium pyrophosphate solution. After extensive washing in the above

solution, the radioactivity of the paper in 20 ml Aquasol-2 was determined in an LKB (Model 1272) beta counter.

Results

As previously reported (27) the incubation of isolated rat adipocytes for 15 min at 37°C with insulin or vanadate alone (10^{-5} to 10^{-3}M) augmented IGF-II binding in a dose-dependent manner (Fig. 1). Hydrogen peroxide also augmented IGF-II binding to a maximum of 52% over control at 10^{-4}M. The combination of H_2O_2 (10^{-3}M) with different doses of vanadate (10^{-7} to 10^{-3}M) synergistically augmented IGF-II binding (Fig. 1). Thus, the effect of vanadate (10^{-4}M) plus H_2O_2 (10^{-3}M) was maximal and almost twice that of the added individual effects of vanadate and H_2O_2 or of the maximum effect achieved by insulin (Fig. 1). A perusal of the dose-response curves indicates that the combination of vanadate plus H_2O_2 was of the order of one thousand-fold more potent than vanadate alone. In previous work we showed that the increase in IGF-II binding induced by all these agents reflected an increase in the number of surface receptors (27) presumably due to the stimulation of receptor translocation from an intracellular compartment (28).

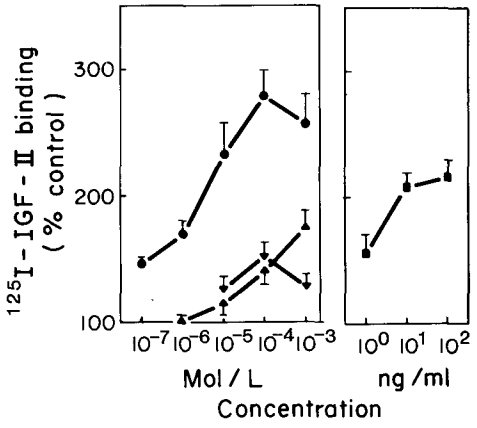

Figure 1. Binding of ^{125}I-IGF-II to rat adipocytes as a function of the dose of vanadate plus H_2O_2. Adipocytes (3 x 10^5 cells) were incubated with vanadate (V, ▲), H_2O_2 (H, ▼), vanadate plus H_2O_2 (●) or insulin (■) at 37°C in 0.5 ml Krebs-Ringer bicarbonate/HEPES buffer (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH_2PO_4, 1.2 mM MgSO4, 24.9 mM $NaHCO_3$, 30 mM HEPES, pH 7.4) containing 20 mg/ml bovine serum albumin (BSA) and 5 mM glucose. V + H were mixed and preincubated for 15 min at 22°C. The compounds or insulin were added to the cells and, after 15 min, the incubation temperature was reduced to 15°C and ^{125}I-IGF-II (0.2 ng, 6.6 x 10^4 dpm) was added with or without excess (500 ng) unlabeled IGF-II for an additional 60 min. Binding in the presence of excess unlabeled IGF-II was subtracted from total binding to yield specific binding. Each value is the mean ± S.E. of 3 separate experiments. The specific binding of ^{125}I-IGF-II to control cells was 8.6 ± 1.1%.

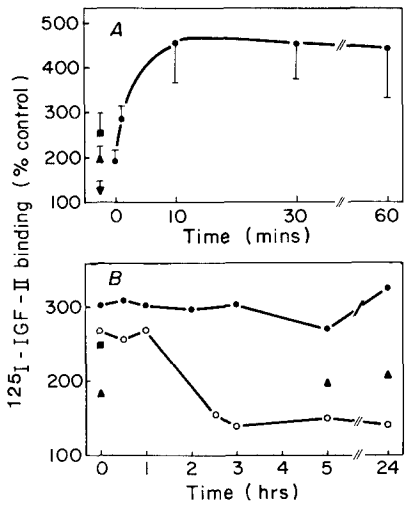

Figure 2. The effect of catalase, added to the combination of vanadate plus H_2O_2, on IGF-II binding to isolated rat adipocytes. (A) Vanadate and H_2O_2 were preincubated at 22°C and at the times indicated catalase was added. After a further 15 min at 22°C, adipocytes were added and the incubation was continued for 15 min at 37°C followed by cooling and measurement of IGF-II binding as described in the legend to Figure 1. Each value is the mean ± S.E. of 3 separate experiments. (●) vanadate (10^{-3}M) + H_2O_2(10^{-3}M) + catalase (200 ug/ml); (▲) vanadate (10^{-3}M); (▼) H_2O_2(10^{-3}M); (■) insulin (10 ng/ml). (B) Vanadate and H_2O_2 were preincubated for 15 min at 22°C followed by the addition of catalase or buffer for the duration of time indicated. Isolated adipocytes were then added for 15 min at 37°C followed by cooling to 15°C and measurement of ^{125}I-IGF-II binding as above. The data presented is one of two separate experiments with similar results. (●) vanadate (10^{-4}M) + H_2O_2(10^{-3}M); (○) vanadate (10^{-4}M) + H_2O_2(10^{-3}M) + catalase (200 ug/ml); (▲) vanadate (10^{-4}M); (■) insulin (10 ng/ml).

To determine if the synergism seen on combining vanadate and H_2O_2 required the independent cellular action of each compound we evaluated the effect of catalase treatment on the potency of the mixture. The addition of catalase (200 ug/ml) simultaneously with the mixing of vanadate (10^{-3}M) and H_2O_2(10^{-3}M) resulted in a mixture whose potency was the same as vanadate (10^{-3}M) alone. However when catalase was added $>$ 10 min. after mixing vanadate and H_2O_2 the full synergism of the combination was maintained (Fig. 2A). Since, under the conditions employed, H_2O_2 readily combines with vanadate to form peroxides of vanadate (pervanadate, pV) (29) our data indicate that the augmented biologic potency is the result of the action of pV rather than the independent cellular action of each agent. In Fig. 2B one can see that a mixture of vanadate (10^{-4}M) and H_2O_2 (10^{-3}M) was still biologically fully active on IGF-II binding at times up to 24 hours. However, 2 to 3 hours after adding catalase, to remove H_2O_2, the augmented potency was gone (Fig. 2B),

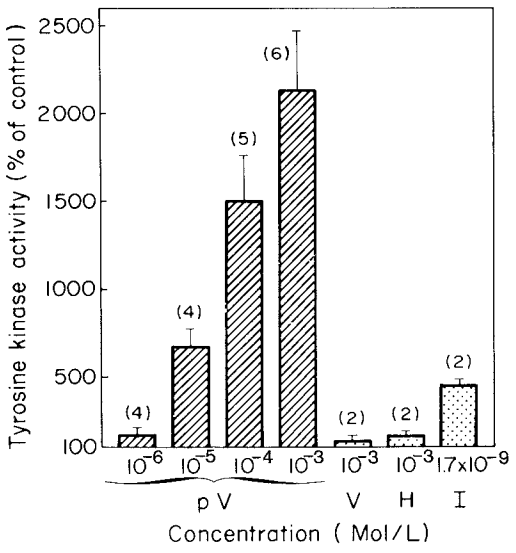

Figure 3. Activation of the insulin receptor tyrosine kinase of rat adipocytes by peroxide of vanadate (pervanadate, pV). Adipocytes (2 x 10^7 cells) were incubated with vanadate (V), H_2O_2 (H), insulin (I) or pV (H_2O_2 concentration was always 10^{-3}M) in 15 ml KRBH buffer, pH 7.4, containing 2% BSA and 5 mM glucose in a 50 ml plastic tube for 15 min at 37°C. Pervanadate (pV) was generated by the premixing of V + H for 15 min at 22°C followed by the addition of catalase (200 ug/ml) for a further 15 min before addition of the adipocytes. The reaction was terminated, the cells solubilized, insulin receptors partially purified by WGA-agarose chromatography, and receptor kinase activity assayed as described in Materials and Methods. Results were normalized to identical amounts of receptor. The number of experiments are indicated in parentheses. Values are mean ± S.E.

indicating instability of the active compound in the absence of an appropriate oxidizing environment.

We next evaluated the effect of pervanadate (pV) added to adipocytes on insulin receptor tyrosine kinase activation. The combination of different concentrations of vanadate with H_2O_2 (10^{-3}M) for 15 min prior to catalase addition produced a powerful dose-dependent activation of wheat germ agglutinin - purified tyrosine kinase as measured by ^{32}P incorporation into the synthetic substrate poly (Glu, Tyr) (4:1) (Fig. 3). At the vanadate (10^{-4}M) plus H_2O_2 (10^{-3}M) concentration which maximally stimulated IGF-II binding tyrosine kinase activity was 1400% above control, whereas vanadate (10^{-3}M) alone, H_2O_2 (10^{-3}M) alone and insulin (10 ng/ml) augmented kinase activity by only 32,58 and 349% over control respectively. We previously have shown that all the augmented tyrosine kinase activity was due to the insulin receptor since it was fully immunoprecipitated by insulin receptor antibody (27).

Table 1

Effect of Pervanadate on the tyrosine kinase activity of WGA-purified insulin receptors

Pervanadate (M)	Tyrosine Kinase Activity (% of control)	
	-Ins	+Ins
0	100	326 ± 80
10^{-7}	121 ± 21	277 ± 40
10^{-6}	93 ± 1	327 ± 87
10^{-5}	86 ± 9	301 ± 58
10^{-4}	99 ± 16	357 ± 7

Adipocytes from male Sprague-Dawley rats were isolated and washed 5 times in Krebs-Ringer bicarbonate/HEPES buffer as described in the legend to Fig. 1. After incubation in buffer alone for 15 min at 37°C 10 ml of ice cold solubilization buffer was added and the cells were frozen at -80°C. The partial purification of insulin receptors was carried out as described in the legend to Fig. 3. Aliquots were incubated for 30 min at 22°C with or without various concentrations of vanadate + H_2O_2 (10^{-3}M) (premixed) and exposed to catalase as described, with and without insulin (Ins, 10^{-7}M). Tyrosine kinase activity was assessed with poly (Glu, Tyr) (4:1) as substrate as described in Fig. 3. The same amount of receptor (13.3 fmol) was present in all the assays. The mean ^{32}P incorporation into poly (Glu, Tyr) in the control receptor was 60.5 fmol ^{32}P/fmol Ins binding / 10 min. Values are the mean ± 1/2 range of 2 separate experiments.

To probe the mechanism of pervanadate action solubilized wheat germ agglutinin - purified insulin receptors were incubated *in vitro* at 24°C with various concentrations of vanadate plus H_2O_2 (10^{-3}M) in the presence or absence of insulin (10^{-7}M). In contrast to insulin, pervanadate did not stimulate tyrosine kinase activity directly *in vitro* (Table 1); nor did it prevent insulin-induced augmentation of kinase activity. We also failed to see a significant *in vitro* effect of vanadate alone in contradistinction to the observation of Tamura et al (16). Of interest is preliminary evidence suggesting that pervanadate, unlike insulin, does not activate the insulin receptor with respect to autophosphorylation after incubation with intact cells or solubilized receptor preparations (manuscript in preparation).

Discussion

Recent studies have shown that pervanadate stimulates lipogenesis and inhibits lipolysis in adipocytes with the same relative potency compared to vanadate (unpublished data) as seen here for the augmentation of IGF-II binding. The fact that the more potent insulin-mimetic effect of pervanadate is associated with

greater potency in regard to receptor kinase activation is compatible with a functional role for the kinase in insulin action. This supports previous observations in which anti-receptor kinase antibodies (25) and site-directed mutagenesis of the insulin receptor kinase (22-24) have demonstrated parallel loss of insulin action and receptor kinase activation.

Vanadate has been previously found to augment tyrosine phosphorylation in cells (8) presumably by inhibiting phosphotyrosine phosphatase (11). The mechanism of pervanadate action is presently unclear since unlike vanadate it does not appear to be a potent phosphatase inhibitor (unpublished data). Since vanadate can stimulate NADH oxidase (13) and promote H_2O_2 production (2) it is possible that some of its effect on intact cells may actually be mediated by pervanadate. The failure to see a stimulatory effect of pervanadate on partially-purified insulin receptors raises the possibility that the active entity is different from pervanadate or that its action is indirect. Either possibility could provide deeper insight into tyrosine kinase regulation. In respect to the former possibility it has been shown that vanadate readily enters cells where it is rapidly converted to vanadyl which is largely bound to phosphates intracellularly (1). It will be important to see if pervanadate is handled by cells in a similar fashion or rather differently.

Whatever the mechanism of pervanadate action it promises to be a novel tool for exploring insulin action and may even prove useful clinically in managing insulin resistance and deficiency states.

Acknowledgements: The authors appreciate the technical assistance of Ms Celyne Brule and Ms Jeanne Parodo in preparing and iodinating IGF-II. This work was supported by grants from the MRC of Canada.

References

1. Nechay, B.R., Nanninga, L.B., Nechay, P.S., Post, R.L., Grantham, J.J., Macara, I.G., Kubena, L.F., Phillips, T.D., and Nielsen, F.H. (1986) Federation Proc. 45, 123-132.
2. Ramasarma, T., and Crane, F.L. (1981) Curr. Top. Cellul. Regul. 20, 247-301.
3. Macara, I.G. (1980) Trends Biochem. Sci. 5, 92-94.
4. Nechay, B.R. (1984) Ann. Rev. Pharmacol. Toxicol. 24, 501-524.
5. Hori, C. and Oka, T. (1980) Biochim. Biophys. Acta. 610, 235-240.
6. Smith, J.B. (1983) Proc. Natl. Acad. Sci. (USA) 80, 6162-6166.
7. Canalis, E. (1985) Endocrinology 116, 855-862.
8. Klarlund, J.K. (1985) Cell 41, 707-717.
9. Cantley, L.C. Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C. and Guidotti, G. (1977) J. Biol. Chem. 252, 7421-7423.

10. Walderhaug, M.O., Post, R.L., Saccomani, G., Leonard, R.T. and Briskin, D.P. (1985) J. Biol. Chem. 260, 3852-3859.
11. Swarup, G., Speeg, K.V., Jr., Cohen, S., and Garbers, D.L. (1982) J. Biol. Chem. 257, 7298-7301.
12. Schwabe, U., Puchstein, C., Hannemann, H., and Sochtig, E. (1979) Nature 277, 143-145.
13. Crane, F.L., MacKellar, W.C., Morre, D.J., Ramasarma, T., Goldenberg, H., Grebing, C., and Low, H. (1980) Biochem. Biophys. Res. Commun. 93, 746-754.
14. Dubyak, G.R., and Kleinzeller, A. (1980) J. Biol. Chem. 255, 5306-5312.
15. Shechter, Y., and Karlish, S.J.D. (1980) Nature 284, 556-558.
16. Tamura, S., Brown, T.A., Whipple, J.H., Fujita-Yamaguchi, Y., Dubler, R.E., Cheng, K., and Larner, J. (1984) J. Biol. Chem. 259, 6650-6658.
17. Clark, A.S., Fagan, J.M., and Mitch, W.E. (1985) Biochem. J. 232, 273-276.
18. Kadota, S., Fantus, I.G., Hersh, B., and Posner, B.I. (1986) Biochem. Biophys. Res. Comm. 138, 174-178.
19. Heyliger, C.E., Tahiliani, A.G., and McNeill, J.H. (1985) Science 227, 1474-1477.
20. Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Haring, H.U., and Kahn, C.R. (1982) J. Biol. Chem. 257, 9891-9893.
21. Roth, R.A., and Cassell, D.J. (1983) Science 219, 299-301.
22. Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A., and Rutter, W.J. (1986) Cell 45, 721-732.
23. Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O.M. (1987) J. Biol. Chem. 262, 1842-1847.
24. Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A., and Rutter, W.J. (1987) Proc. Natl. Acad. Sci. (USA) 84, 704-708.
25. Morgan, D.O., and Roth, R.A. (1987) Proc. Natl. Acad. Sci. (USA) 84, 41-45.
26. Czech, M.P., Lawrence, J.C., Jr., and Lynn, W.S. (1974) J. Biol. Chem. 249, 1001-1006.
27. Kadota, S., Fantus, I.G., Deragon, G., Guyda, H.J., and Posner, B.I. J. Biol. Chem. (in press).
28. Wardzala, L.J., Simpson, I.A., Rechler, M.M., and Cushman, S.W. (1984) J. Biol. Chem. 259, 8378-8383.
29. Howarth, O.W., and Hunt, J.R. (1979) J. Chem. Soc. Dalton 1388-1391.