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Synergism in insulin-like effects of molybdate plus H_2O_2 or tungstate plus H_2O_2 on glucose transport by isolated rat adipocytes

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Abstract—The effect of molybdate, tungstate, molybdate plus H_2O_2 or tungstate plus H_2O_2 on 3-*O*-methylglucose (3-*O*-MG) uptake was studied in isolated rat adipocytes to investigate whether these agents possess an insulin-like action. High concentrations (10–30 mM) of molybdate or tungstate significantly stimulated the uptake of 3-*O*-MG while 1 mM of the metaloxides did not. The combination of 1 mM molybdate and 1 mM H_2O_2, or 1 mM tungstate and 1 mM H_2O_2 induced striking stimulation of the uptake of 3-*O*-MG in a synergistic manner, whereas 1 mM H_2O_2 alone showed only a small effect. The effect of metaloxides plus H_2O_2 (1 mM) and the effect of insulin (20 nM) were not additive, and both effects were ATP or energy dependent based on experiments using KCN. These results indicate that a weak insulin-like effect of molybdate or tungstate is potentiated synergistically with H_2O_2, presumably by producing peroxocompounds. Based on the present findings, these new agents may be useful for investigating the mechanism of insulin action and may indicate a new class of drugs for diabetes mellitus.

It is well established that vanadate [1–3], peroxovanadate [4–8] and selenate [9] have insulin-like actions. We found recently that molybdate and tungstate, which resemble vanadate and selenate, also had a weak insulin-like effect on glucose transport. This observation led us to investigate how this action could be potentiated at low concentrations and we performed further experiments based on the working hypothesis that the peroxocompounds possess more insulinomimetic activity than the parent compounds.

Materials and Methods

Materials. 3-*O*-[^{3}H]Methyl-D-glucose and L-[1-^{14}C]glucose were purchased from New England Nuclear (Boston, MA, U.S.A.); sodium molybdate from Nacalai tesque (Kyoto, Japan); sodium tungstate, sodium orthovanadate and H_2O_2 from Wako Pure Chemical Industries (Osaka, Japan); porcine insulin, phloretin and bovine serum albumin (RIA grade) from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and collagenase (CLS 1) from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.).

Glucose transport assay. Epididymal and perirenal adipose tissues were removed from male Wistar rats weighing 160–200 g under anesthesia induced by the intraperitoneal injection of 100 mg/kg sodium pentobarbital and isolated adipocytes were prepared by the collagenase method [10]. The cell suspension in Krebs–Henseleit HEPES buffer [11] supplemented with 20 mg/mL bovine serum albumin and 3 mM sodium pyruvate, pH 7.4, was adjusted to a cytocrit value of 21.3% (20.0% in the net cell volume as 6% of the packed cell volume is occupied by extracellular water [12]). The glucose transport activity was assessed by measuring the rate of specific uptake of 3-*O*-methylglucose(3-*O*-MG) for 3 sec at 37°, which was corrected for the non-specific uptake estimated using L-glucose. This method was based on the method of Toyoda *et al.* [13] and provided 52.3 ± 3.3 (N = 4) as the per cent of intracellular water space filled after 3 sec in the presence of 20 nM insulin. Unless otherwise stated, the metaloxides (sodium molybdate, sodium tungstate and sodium orthovanadate) and H_2O_2 were mixed immediately before use

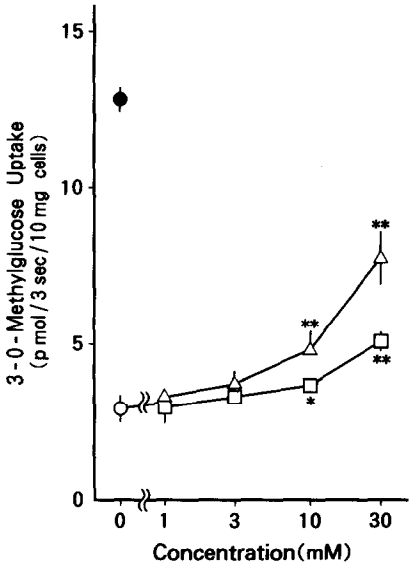

Fig. 1. Effect of molybdate or tungstate on 3-*O*-MG uptake. Aliquots of cells were exposed to 20 nM insulin (●) or to the indicated concentrations of molybdate (□) or tungstate (△) for 10 min at 37° and 3-*O*-methylglucose uptake was measured. Values are means ± SD, N = 3. The bar (SD) is included in the symbol when it is not evident. * $P < 0.05$, ** $P < 0.01$ vs basal values (○).

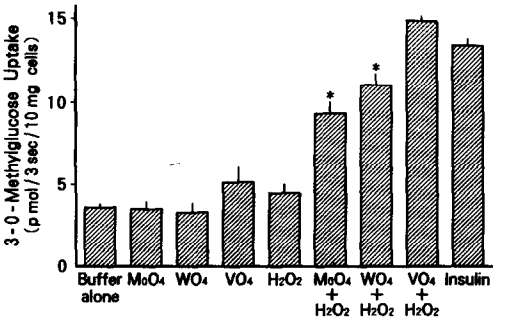

Fig. 2. Effect of insulinomimetic agents on 3-*O*-MG uptake. Aliquots of cells were incubated with 20 nM insulin or 1 mM of the indicated agents for 10 min at 37° and 3-*O*-MG uptake was measured. Values are means ± SD, N = 6. * $P < 0.01$ vs H_2O_2.

and the mixed solutions, at a concentration 16 times as high as the final concentration in the experimental incubations, were added within 5 min of mixing. Aliquots of cells were kept at 37° for at least 15 min and were further incubated with the indicated concentrations of each agent for 10 min at 37°. At the end of the incubation, the glucose transport assay was initiated by adding 0.1 mM 3-*O*-[^{3}H]-methyl-D-glucose (1.6 μCi/tube) and 0.1 mM L-[^{14}C]glucose (0.4 μCi/tube). Both the agent-stimulated cells and the non-stimulated (basal) cells were incubated with 3-*O*-MG for 3 sec. The uptake reaction was terminated by adding 1 mM phloretin and the cell suspension was transferred into a centrifuge tube containing dinonyl phthalate. The cells in the suspension were collected by the oil-flotation method and the radioactivities of ^{3}H and ^{14}C were counted in a Tri-Carb 300C liquid scintillation counter (Packard Instrument Co., Downers Grove, IL, U.S.A.). The uptake values were not corrected to give initial uptake rates but were simply expressed as picomoles of 3-*O*-MG taken up specifically per 3 sec per 10 mg of adipocytes. In the experiments using KCN, aliquots of cells were preincubated with 2 mM KCN for 5 min at 37° and the rate of 3-*O*-MG uptake was measured following the indicated additions as in the experiments by Kono *et al.* [14, 15]. In their studies, the insulin effect was not observed in adipocytes depleted of intracellular ATP following exposure to 1–2 mM KCN for 5–10 min. In experiments examining how long the activities of the metaloxides plus H_2O_2 are retained after mixing, the mixed solutions were left at room temperature for the indicated times before adding them to cell suspensions for assay as described above. The concentration of insulin (20 nM) used in this study provided the maximal effect.

Statistical analysis. All results were expressed as means ± SD. The two-tailed and one-tailed unpaired *t*-tests were applied as appropriate.

Results

As shown in Fig. 1, high concentrations of molybdate or tungstate showed a significant stimulatory effect on 3-*O*-MG uptake. The effect was 21.6% and 52.8% of the maximal effect of insulin in 30 mM molybdate-treated cells and in 30 mM tungstate-treated cells, respectively. But 1 mM of the metaloxides showed no significant effect on 3-*O*-MG uptake.

Figure 2 shows synergistic effects of metaloxides plus H_2O_2 on 3-*O*-MG uptake. The stimulatory effect on 3-*O*-MG uptake was not observed as above in cells treated with 1 mM molybdate or 1 mM tungstate and the effect of 1 mM H_2O_2 was small. On the other hand, the effect was increased synergistically on combining molybdate, tungstate or vanadate (1 mM) with 1 mM H_2O_2 with stimulation of uptake to 59.2%, 76.2% and 115.6% of the maximal effect of insulin, respectively.

Next, we examined whether the combination of insulin (20 nM) and metaloxide plus H_2O_2 (1 mM) produced a larger effect. As shown in Table 1, the maximal effect of insulin could not be increased by the metaloxides.

To test whether the effects of metaloxides plus H_2O_2 were ATP or energy dependent, the cells were preincubated with 2 mM KCN and intracellular ATP was depleted. In such cells the effects of 1 mM molybdate plus 1 mM H_2O_2, 1 mM tungstate plus 1 mM H_2O_2 or 20 nM insulin were not observed (Table 2).

Table 1. Effect of the combination of insulin and molybdate plus H_2O_2 or insulin and tungstate plus H_2O_2 on 3-*O*-MG uptake

Addition	3-*O*-MG uptake (pmol/3 sec/10 mg cells)
None	3.647 ± 0.330
1 mM MoO_4 + 1 mM H_2O_2	10.019 ± 1.173
1 mM WO_4 + 1 mM H_2O_2	12.424 ± 1.124
20 nM Insulin	14.912 ± 0.458
Insulin & MoO_4 + H_2O_2	14.484 ± 0.256
Insulin & WO_4 + H_2O_2	14.628 ± 0.472

Aliquots of cells were incubated with the indicated agents for 10 min at 37° and 3-*O*-MG uptake was measured. Values are means ± SD, N = 6.

Table 2. Effect of KCN on actions of insulin, molybdate plus H_2O_2 or tungstate plus H_2O_2

Addition	3-*O*-MG uptake (pmol/3 sec/10 mg cells)	Effect
None	3.998 ± 0.254	1.00
1 mM MoO_4 + 1 mM H_2O_2	12.871 ± 0.194*	×3.22
1 mM WO_4 + 1 mM H_2O_2	13.392 ± 0.866*	×3.35
20 nM Insulin	14.231 ± 0.274*	×3.56
2 mM KCN alone	3.511 ± 0.344	1.00
2 mM KCN & MoO_4 + H_2O_2	3.322 ± 0.335	×0.95
2 mM KCN & WO_4 + H_2O_2	3.612 ± 0.393	×1.03
2 mM KCN & Insulin	3.393 ± 0.252	×0.97

Aliquots of cells were preincubated with or without 2 mM KCN for 5 min at 37° and were further incubated with the indicated agents for 10 min prior to determining 3-*O*-MG uptake.

Values are means ± SD, N = 6.

* $P < 0.01$ vs corresponding controls (None).

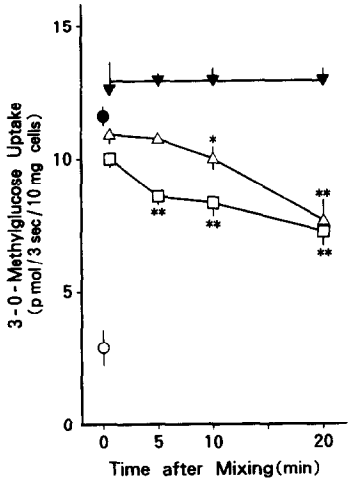

Fig. 3. The change in insulinomimetic activity with time after mixing the metaloxides with H_2O_2. The solutions were left at room temperature for the indicated periods after mixing, and were added to cell suspensions at the noted times for assay. Aliquots of cells were incubated with 1 mM molybdate plus H_2O_2 (□), tungstate plus H_2O_2 (△) or vanadate plus H_2O_2 (▼) for 10 min at 37° and 3-*O*-MG uptake was measured. Basal uptake values (○) and 20 nM insulin-stimulated uptake values (●) are also depicted. Values are means ± SD, N = 3. The bar (SD) is included in the symbol when not evident. * $P < 0.05$, ** $P < 0.01$ vs corresponding initial values.

We noticed that the activity of molybdate plus H_2O_2 or tungstate plus H_2O_2 to stimulate 3-*O*-MG uptake declined as time passed following the mixing of the reagents. Figure 3 depicts how long the activities of metaloxides plus H_2O_2 were retained after mixing. The activity of 1 mM vanadate plus 1 mM H_2O_2 persisted fully for at least 20 min after mixing. But the activity of 1 mM molybdate plus 1 mM H_2O_2 or 1 mM tungstate plus 1 mM H_2O_2 declined as time passed and their activities at 20 min after mixing were 61.5% or 60.0%, respectively, of the initial activity.

Discussion

The present study shows that high concentrations of molybdate and tungstate had a stimulatory effect on glucose transport in rat adipocytes. We tried to potentiate this action at low concentrations of the metaloxides. When 1 mM molybdate or 1 mM tungstate, which showed no stimulatory effect on glucose transport, were combined with 1 mM H_2O_2, which by itself showed a small effect, the stimulatory effect was more than additive. Posner's group [4] had reported that vanadate plus H_2O_2 exerted powerful insulin-like effects in a synergistic manner. They suggested, based on experiments using catalase [5], that this was due to the newly produced compound, peroxovanadate, and they further demonstrated that peroxovanadate was a potent inhibitor of phosphotyrosyl phosphatase [6, 7] and provoked marked activation of the insulin receptor tyrosine kinase as the major mechanism of its action [4–7]. Although such investigations were not performed in the present study, we showed that the maximal effect of insulin and the effect of metaloxides plus H_2O_2 were not additive and that both effects were ATP or energy dependent. According to these findings, peroxocompounds generated from molybdate or tungstate are considered to be responsible for the enhanced effects and the effects may be exerted through a mechanism similar to that of insulin. That the effects seen in the absence of H_2O_2 are due to traces of peroxocompounds present in the reagents is a possibility that cannot be ruled out.

A difference with respect to stability of the insulinomimetic activities was observed between the activity of vanadate plus H_2O_2 and the activity of molybdate plus H_2O_2 or tungstate plus H_2O_2. The activity of vanadate plus H_2O_2 to stimulate glucose transport persisted at a high level for at least 20 min after the compound and H_2O_2 were mixed, but the activity of molybdate plus H_2O_2 or tungstate plus H_2O_2 declined in this time interval. This difference may reflect a reduced stability of the peroxocompounds generated from the two metaloxides.

Although we have not yet examined the effects of these new agents *in vivo* using diabetic animals, the present *in vitro* findings suggest new agents for managing diabetes mellitus. Vanadate has been administered to diabetic rats and Heyliger *et al.* [16] demonstrated that orthovanadate added to the drinking water of streptozotocin-diabetic rats normalized blood glucose levels. The successful use of vanadate [16, 17] and vanadyl sulfate [18, 19] in animal models of diabetes mellitus has raised the possibility that

vanadate and the related compounds including molybdate, tungstate and peroxocompounds may be useful drugs for diabetes mellitus in man. Nevertheless, further studies are required to elucidate the detailed mechanism of these agents and to establish a new class of drugs for managing diabetes mellitus.

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Inhibition of transketolase and pyruvate decarboxylase by omeprazole

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Abstract—Omeprazole inhibited two thiamin diphosphate-dependent enzymes, pyruvate decarboxylase (EC 4.1.1.1, PDC) from *Zymomonas mobilis* and transketolase (EC 2.2.1.1, TK) from human erythrocytes. Inhibition of PDC was competitive with the coenzyme with a K_i value of $42 \pm 3\ \mu M$, whereas inhibition of TK was complex.