# Sodium tungstate mimics insulin effect on nuclear translocation of FBPase in rat liver

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**Abstract**

Sodium tungstate (NaW) is an inorganic salt that has proven to be a potent insulin-mimetic agent, although the molecular events seems to differ. Inhibition of hepatic gluconeogenesis is one significant physiological action of insulin, therefore studying the effect of NaW on gluconeogenic enzymes will contribute to understand its elusive mechanism of action. Here, we show that NaW has no inhibitory effect over the gluconeogenic enzyme fructose 1,6-bisphosphatase (FBPase) *in vitro*, but mimics insulin in the induction of the nuclear translocation of FBPase in rat liver, which may have a negative impact on its activity. Then, at least in part, NaW may inhibit hepatic gluconeogenesis by inducing the proper subcellular distribution of FBPase, without directly interfering with its phosphatase activity.

**Keywords:** sodium tungstate, FBPase, gluconeogenesis, liver, diabetes

## Introduction

Insulin participates in a wide spectrum of metabolic responses in order to conserve homeostasis.[1-2] One key effect of insulin is its ability to suppress endogenous glucose production in liver by down-regulating the expression of rate-limiting enzymes of hepatic gluconeogenesis, *i.e*. phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBPase) and glucose 6-phosphatase (G6Pase), constituting a pivotal step in maintenance of glucose homeostasis.[1-2] By contrast, deregulation of gluconeogenesis in insulin resistant states leads to enhanced endogenous glucose production, which is one major pathway involved in chronic hyperglycemia of diabetic patients.[1-2] Thus, anti-diabetic drugs that mimic insulin anti-gluconeogenic action are good candidates for treatment of diabetes.

Sodium tungstate (NaW) is an inorganic salt that exerts potent insulin-mimetic and anti-diabetic actions in animal models of diabetes by normalizing hepatic glucose metabolism and blood glucose levels without producing hypoglycemic episodes.[3] Tungstate is a phosphate analogue and has been used as a phosphatase inhibitor,[4-6] although this property is not enough to account for its anti-diabetic actions.[7] NaW treatment normalizes expression of PEPCK, FBPase and G6Pase in the liver from diabetic rats,[8] and also acts as a potent inhibitor of G6Pase activity, which has been suggested to be an important part of its insulin-mimetic effect.[9]

FBPase (EC 3.1.3.11) is a phosphatase in the gluconeogenic pathway that catalyzes the hydrolysis of fructose 1,6-bisphosphate into fructose 6-phosphate and inorganic phosphate.[10] Although FBPase mRNA expression is increased in the liver from diabetic rats, which has been correlated with the increased flux through the gluconeogenic pathway that contributes to hyperglycemia,[8] protein levels are not significantly different from that of control rats,[11] which is in agreement with the fact that FBPase is mainly regulated at the level of activity by the physiological inhibitors fructose 2,6-bisphosphate and AMP rather than at the expression level[10,12] and also that other mechanism/s may modulate its activity, such as protein-protein interaction and subcellular

compartmentalization.[11,12] Indeed, diabetes has shown to impair nuclear translocation of FBPase in rat liver, which is proposed as an insulin-induced sequestration mechanism to inhibit the cytosolic gluconeogenic activity.[11] Due to its strategic position in the gluconeogenic pathway, hepatic FBPase is an interesting target for development of new anti-diabetic drugs.[13] However, the effect of NaW on hepatic FBPase activity and subcellular localization is still unknown.

Despite the evidence suggests that NaW exerts beneficial effects at the physiological level, the mechanism of action of NaW is more elusive. Although most of insulin effects are mimicked by NaW, the molecular events seems to differ. Therefore, given that inhibition of hepatic gluconeogenesis is one of the most significant physiological consequences of insulin, studying the action of NaW on gluconeogenic enzymes will greatly contribute to understand its mechanism of action. Here, we show that contrary to G6Pase, NaW is not an inhibitor of FBPase activity *in vitro*, but rather it stimulates a rapid translocation of FBPase into the nucleus and the cell periphery during feeding that could explain in part the acute normoglycemic action.

**Experimental**

**Animals.** 250 g male Sprague-Dawley rats were kept in a conditioned house and fed *ad libitum*. To induce diabetes, rats were injected with a single i.v. dose of 65 mg/kg streptozotocin (STZ; Merck) and the glycemia was measured periodically with a glucometer since it reached 400 mg/dl in average (one week later). At this moment, rats were separated in two groups of five animals each, they were all fasted for 12 h and then 1 group was fed for 12 h. After that, both groups were injected i.p. with 200 mg/Kg filtered NaW and sacrificed 6h later. All experiments were approved by the Institutional Animal Care and Use Committee of Universidad Austral de Chile.

**FBPase activity.** FBPase activity was estimated spectrophotometrically by changes in the absorbance at 340 nm due to reduction of $NAD^+$ in a coupled enzyme assay as previously described.[10] The reaction was carried out at 30°C in 0.5 ml final volume containing 50 mM Tris/HCl buffer, pH 7.5, 0.1 mM EDTA, 5 mM $MgSO_4$, 50 μM fructose 1,6-bisphosphate, 0.3 mM $NAD^+$, 1.2 U of each auxiliary enzyme phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (Sigma), and NaW (Sigma) at final concentration of 0, 2.5, 5.0, 7.5, 10, 20, 40, 60, 80 and 100 μM. The reaction was initiated by adding pure enzyme obtained as previously described.[10] Also, FBPase activity was estimated in homogenized liver samples from NaW-treated and untreated STZ-diabetic rats. One unit of FBPase activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of $NAD^+$ per minute under the conditions described.

**Immunofluorescence.** 7-μm tissue samples were deparaffinized in xylene and rehydrated in graded ethanol. Tissue was blocked with 3% BSA in PBS, primary antibody (rabbit anti-serum against FBPase was prepared in our laboratory[11]) was incubated in 1% BSA-PBS overnight at 4°C, washed in PBS. Secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG, Molecular probes, Eugene, OR) was incubated in 1% BSA-PBS for 1h at RT followed by washing in PBS.

**Statistical analysis.** Data are expressed as mean ± SD. Statistical analysis was performed with unpaired Student's t test using GraphPad Prism, version 6.01. Data were considered statistically significant for $p<0.05$.

**Results**

**Phosphatase inhibitory action of NaW over FBPase activity.** Given that NaW is a phosphatase inhibitor that has shown to potently inhibit G6Pase activity,[9] we assessed its effect on the activity of the other gluconeogenic phosphatase, *i.e*. FBPase. We used a wide range of NaW concentrations but a poor inhibitory effect, even at the highest concentration tested, was observed (Figure 1).

No significant differences on FBPase activity in liver from NaW-treated and untreated STZ-diabetic rats were observed (Figure 1).

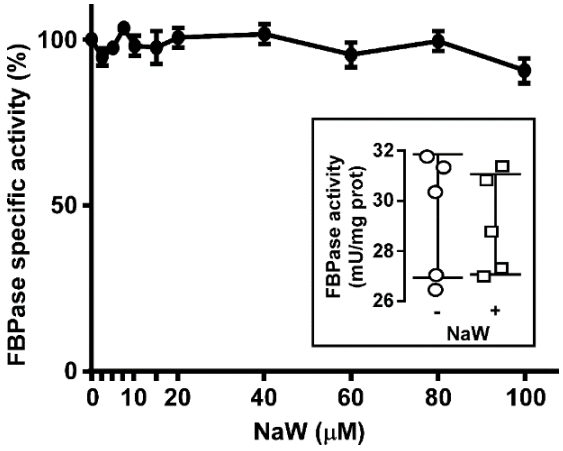


**Figure 1. Effect of NaW on FBPase activity.** Activity of pure FBPase was assessed in the presence of 0, 2.5, 5, 7.5, 10, 15, 20, 40, 60, 80 and 100 μM NaW. Inset: Activity of FBPase in total liver extract from NaW-treated and untreated STZ-diabetic rats.

**Effect of NaW on FBPase subcellular distribution in rat liver.** Confocal microscopy analysis have previously revealed that FBPase subcellular distribution in the liver is altered in response to the metabolic conditions of healthy rats (Figure 2). FBPase is mainly and uniformly localized to the cytoplasm of hepatocytes during fasting (Figure 2A, from [11]). Refeeding induces its translocation to the nucleus and the cell periphery (Figure 2B[11]). Notably, refeeding was unable to induce the characteristic pattern of subcellular redistribution in the liver from this type 1 diabetic rat model (Figure 2C-D[11]), suggesting that insulin is the main stimulus controlling FBPase subcellular localization. Indeed, insulin supplementation to fasted and refed diabetic rats probed to acutely restore nuclear translocation but only partially affected peripheral distribution of FBPase (Figure 2E-F[11]). 6h NaW treatment significantly restored blood glucose levels in fasted (427 ± 23 vs 162 ± 50 md/dl; p<0.0001) and fed (612 ± 30 vs 208 ± 25 md/dl; p<0.0001) STZ-diabetic rats. Interestingly, NaW was not as effective as insulin to induce nuclear translocation of FBPase in the fasted state (Figure 2G) but stimulated subcellular redistribution of the enzyme in the fed state (Figure 2H) that was similar to that observed in healthy fed rats (Figure 2B). A semi-quantitative analysis of FBPase subcellular distribution was performed by analyzing the fluorescence intensity after drawing a line across hepatocytes in the different conditions (Figure 3), comparing FBPase signal (green) with that of the nuclear marker propidium iodide (red) to assess for the different degrees of nuclear accumulation of FBPase after each treatment.

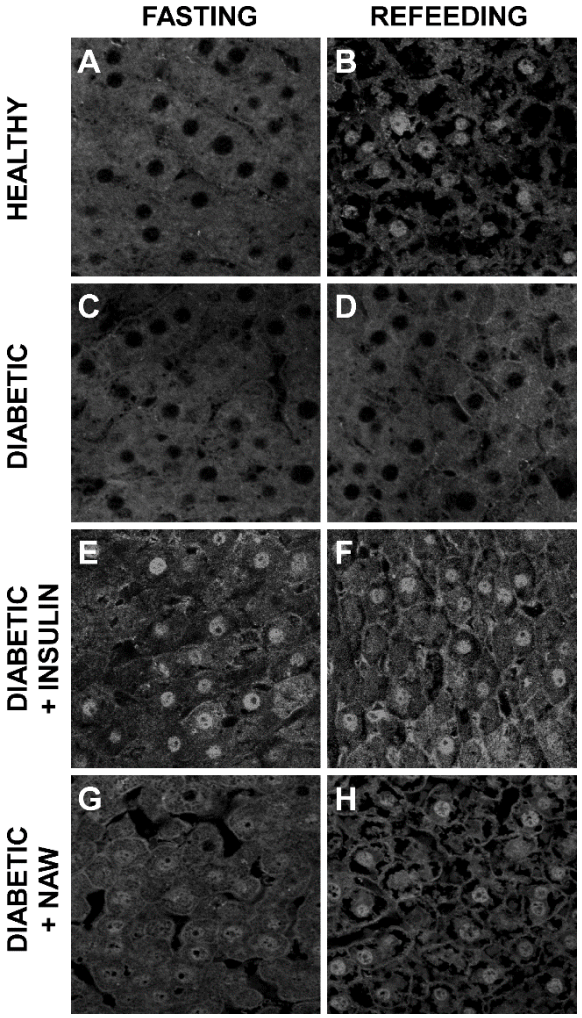


**Figure 2. Effect of NaW on FBPase subcellular distribution in diabetic rat liver.** Rats were fasted for 12 h and then re-fed for 12 h. **A-B**) healthy rats; **C-D**) untreated diabetic rats; **E-F**)

insulin-treated diabetic rats; **G-H**) NaW-treated diabetic rats. Images A-F are from [11] for direct comparison.

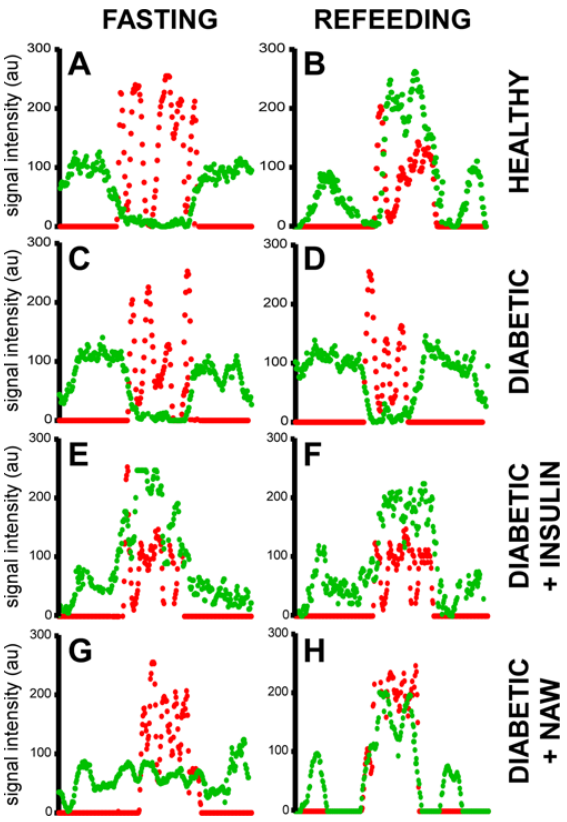


**Figure 3. Semi-quantitation of FBPase subcellular distribution.** Average fluorescence intensity across hepatocytes in the different conditions is presented for FBPase (green) and the nuclear marker propidium iodide (red). au: arbitrary units.

**Discussion**

Insulin and different oral hypoglycemic drugs are commercially available; however diabetes mellitus still remains a major health concern. Although NaW may offer a potent therapy for treatment of obesity and both type 1 and type 2 diabetic patients, only one human trial with NaW has been performed to date.[14] In that study, the effect of NaW on obese normoglycemic subjects was addressed, with neither negative nor positive results.[14] Given that NaW toxicity is not well defined yet, the use of higher doses in human patients is still not recommended and this is why the human trial could have failed, as we used a 100-times higher dose administered through a more direct way (intraperitoneal vs oral). However, we cannot directly compare both studies, as subjects in the trial where normoglycemic[14] and NaW does not efficiently reduce glycemia when it is within the normal range.[3,8] Nevertheless, the great benefits reported in experimental animal models[3] suggest that NaW deserve more attention to reach a better understanding of the molecular mechanisms involved, in order to reproduce the anti-diabetic and anti-obesity effects in humans.

Subcellular localization of enzymes and their interaction with cellular structures and other proteins or enzymes is now widely recognized as a regulatory mechanism in several metabolic pathways, including gluconeogenesis.[12] It is expected that this regulation is altered during metabolic distress, such that occurring in diabetes. We have previously shown that the subcellular localization of FBPase, one key gluconeogenic enzyme, is altered in the liver from diabetic rats, which could be one regulatory mechanism of gluconeogenesis that is impaired, contributing to increase glucose production and chronic hyperglycemia.[11] Indeed, in healthy fasted rats, FBPase is concentrated in the cytoplasm, where gluconeogenesis takes place, whereas FBPase accumulates in the nucleus and cell periphery after refeeding.[11] Despite the severe hyperglycemia, FBPase was unable to translocate to the hepatocyte nucleus in type 1 diabetic rats, but insulin supplementation was enough to recover nuclear accumulation of the enzyme at both fasted and refed states, suggesting that insulin is the main physiological stimulus for FBPase subcellular redistribution after feeding.[11] Given that insulin inhibits gluconeogenesis, insulin-dependent nuclear translocation of FBPase is considered a way to isolate its activity from the cytoplasm when no gluconeogenesis is needed. Here, we observe that, on the contrary, the insulin-mimetic agent NaW is not as effective as insulin to induce nuclear translocation of FBPase. Given that

NaW signaling mechanism has probed to by-pass insulin receptor,[7] we speculate that the nuclear import mechanism of FBPase is controlled by different steps of the insulin cascade, some of which may not be activated by NaW. For instance, NaW strongly activates ERK1/2 but shows an inconsistent effect on Akt phosphorylation in hepatocytes.[8] Besides, phosphorylation has been proposed to mediate cytoplasmic retention of rat liver FBPase,[11] and NaW might be inhibiting a protein phosphatase involved in this process. By contrast, peripheral redistribution of the cytosolic pool of enzyme may occur by a pushing effect after reorganization of the cytosol during refeeding. Stimulation of hepatic glycogen synthesis and storage is one of the most important effects of insulin.[1-2] In healthy non-diabetic conditions, feeding is accompanied by insulin secretion to stimulate glucose uptake, consumption and storage.[1-2] Hence, glycogen accumulation is a good candidate to explain the severe change in the hepatocyte cytoplasm observed after refeeding, and it may also explain the effect of NaW on peripheral redistribution of FBPase after replenishment of glycogen, if we consider that NaW is a strong inducer of hepatic glycogen synthesis.[7-8]

We probed different concentrations of NaW that has shown to exert insulin-mimetic effect on cell culture[3] and phosphatase inhibitory actions on G6Pase.[9] However, the inhibitory action of NaW only affected 10% of FBPase activity *in vitro*, even at concentrations as high as 100 μM. We cannot rule out that the mild inhibitory action of NaW on pure FBPase could be an indirect effect over the auxiliary enzymes used for the coupled reaction. Moreover, although no significant effect of NaW over FBPase activity in total liver extracts was observed that could explain its potent normoglycemic action, an indirect inhibitory action of NaW over FBPase activity *in vivo* may be present, which is lost after homogenization of liver extracts.

**Conclusions**

NaW does not act as a direct FBPase inhibitor *in vitro* but partially stimulates its nuclear translocation and peripheral redistribution in the short-term. This incomplete effect of NaW to induce the nuclear sequestration of FBPase may account, at least in part, for its low hypoglycemic action, compared to insulin, as nuclear FBPase is proposed to be inactive. Thus, restoration of the proper subcellular distribution of FBPase in the liver by NaW may be one of its several normoglycemic mechanism.

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